# Chemokine-mediated control of immunity to tumours and infectious pathogens

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A thesis submitted to the University of Adelaide in fulfilment of the requirements for the degree of Doctor of Philosophy

2018

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Signed,

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## Acknowledgements

Firstly I must thank my principal supervisor Shaun, for allowing me into the lab in the first place and for sparking my love with immunology. I really appreciate the independence you have always given me to lead my own research and follow my scientific interests, with all of the achievements and challenges that comes along with it. Thank you for all of your feedback and advice over the years, I've learnt so much from you about science, grant-writing, collaborations, English grammar and even a bit of French, and I'm incredibly grateful.

To my co-supervisor Iain, you have been an invaluable help to me over my years in the lab and I really appreciate all of your feedback on experimental design, analysis of results and for your careful examination of my drafts. The lab would be lost without your expertise with flow cytometers and animal ethics, and I want to thank you for all of your patience and insights over the years.

To my external supervisor Mark Smyth, thank you for your intellectual input in guiding this work and for performing key experiments. I look forward to more collaborations in the future.

I must also thank Richard Harvey and James Paton for being instrumental in setting up the *S. pneumoniae* work, as well as Mohammed Alsharifi for helping with the influenza work, as well as all of the collaborators who have sent us reagents.

I'm incredibly grateful that I was able to go through this PhD with such a fun and intelligent group of lab members. I've learnt from each of you, and you've all helped me so much, whether it has been with late experiments, experimental advice or making me laugh. Adriana, you're such an instrumental part of the lab and I hate to think what it would be like without you. Thanks for making me laugh, all the food chats and for cutting me some slack the past few months. The lads: Ervin, I will never be able to thank you enough for all your guidance, experimental advice, dumb jokes and fire music. Kev, thanks for challenging me about my experiments, showing me weird food and the ESL lessons. Duncan, for lifting the calibre of the lads and for the civilised

discussions. Cam, for the laughs and terrible puns. The rest of the lab: Jade, thanks for always lifting my spirits, you're one of the most thoughtful people I've met. Kerrie, for being the best roomie on countless retreats. Maleika and Caitlin, I really appreciate all of your help with mouse work etc. while writing my thesis and working elsewhere, thank you for your patience, cakes and the lols. Jaz, Tim and Todd, the banter would be poor without you guys. I also thank past members of the lab, especially Yuka, Michelle, Manuela, Sarah and Marina for teaching me foundations and introducing me to new models. I feel really lucky to have shared this experience with you all.

I could not have made it through these last few years without the support of my family and friends. To the girls – thank you for asking me how my cells are going and knowing my mood depends on the outcome, for all of the dinners and lunches and between meal snacks and for all the laughter and love.

Mum and Dad – thank you for your never-ending support and love, for always encouraging me to do what I wanted with my life and for being a constant source of guidance. To Matt and Rachel – thank you for always asking me, and not asking me, how my thesis is going, and for inspiring me and supporting me. Nona – thanks for all the cooked meals when I was busy, you are the best. Jessie, thanks for all the cuddles and for always being excited to see me in the middle of the night.

Benjamin – I don't know what I would do without your endless encouragement, patience and love. Thank you for helping me believe in myself, and for being fun. I couldn't have done this without you.

# Table of Contents

# List of Figures

# List of Tables

# Abbreviations

| | |
|---|---|
| ACKR | atypical chemokine receptor |
| APC | antigen-presenting cell |
| ATP | adenosine triphosphate |
| B6 | C57Bl/6J |
| BAL | bronchoalveolar lavage |
| BM | bone marrow |
| CAF | cancer-associated fibroblast |
| cDC | conventional dendritic cell |
| cDNA | complementary deoxyribonucleic acid |
| CFA | complete Freund's adjuvant |
| CFU | colony-forming unit |
| DAMP | danger-associated molecular pattern |
| DC | dendritic cell |
| dLN | draining lymph node |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DRG | dorsal root ganglia |
| EAE | experimental autoimmune encephalomyelitis |
| ELISA | enzyme-linked immunosorbent assay |
| EV | extravascular |
| FACS | fluorescence-activated cell sorting |
| FBS | foetal bovine serum |
| FDA | Food and Drug Administration |
| GFP | green fluorescent protein |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| GPCR | G-protein coupled receptor |
| GzmB | granzyme B |
| HEV | high endothelial venule |
| HRP | horseradish peroxidase |
| HSV | Herpes Simplex Virus |
| ICOS | inducible T-cell costimulator |
| IFN | interferon |
| IL | interleukin |
| IMDM | Iscove's Modified Dulbecco's Medium |
| iTreg | induced regulatory T cell |
| IV | intravascular |
| KLRG1 | killer cell lectin-like receptor G1 |
| LEC | lymphatic endothelial cell |
| LN | lymph node |
| MCA | 3-methylcholanthrene |
| MDSC | myeloid-derived suppressor cell |
| medLN | mediastinal lymph node |
| MHC | major histocompatibility complex |
| MMTV | mouse mammary tumour virus |
| MOG | myelin oligodendrocyte glycoprotein |
| MRCLB | mouse red cell lysis buffer |
| NK | natural killer |
| NKT | natural killer T |
| Nrp1 | neuropilin-1 |
| NSCLC | non-small cell lung cancer |
| NT | nasal tissue |

| | |
|---|---|
| nTreg | natural regulatory T cell |
| NW | nasal wash |
| OVA | ovalbumin |
| PAMP | pathogen-associated molecular pattern |
| PB | peripheral blood |
| PBS | phosphate buffered saline |
| PD-1 | programmed cell death protein 1 |
| PFA | paraformaldehyde |
| PFU | plaque-forming unit |
| $PGE_2$ | prostaglandin E2 |
| PMA | phorbol 12-myristate 13-acetate |
| PP | Peyer's patch |
| PRR | pattern recognition receptor |
| PspA | Pneumoccocal surface protein A |
| PyMT | Polyoma middle T antigen |
| qPCR | quantitative polymerase chain reaction |
| RA | retinoic acid |
| RORγt | retinoic acid receptor-related orphan receptor gamma t |
| RPMI | Roswell Park Memorial Institute medium |
| RT | room temperature |
| SLO | secondary lymphoid organ |
| STAT | signal transducer and activator of transcription |
| $TCID_{50}$ | tissue culture infectious dose 50 |
| $T_{CM}$ | central memory T cell |
| TCR | T cell receptor |
| $T_{EM}$ | effector memory T cell |
| $T_{FH}$ | T follicular helper cell |
| $T_{FR}$ | T follicular regulatory cell |
| TGFβ | transforming growth factor beta |
| $T_H$ | T helper cell |
| TIGIT | T cell immunoreceptor with Ig and ITIM domains |
| TIL | tumour infiltrating lymphocyte |
| TMB | 3,3',5,5'-tetramethylbenzidine |
| TME | tumour microenvironment |
| TNF | tumour necrosis factor |
| TRAIL | TNF-related apoptosis-inducing ligand |
| Treg | regulatory T cell |
| $T_{RM}$ | tissue resident memory T cell |
| VEGF | vascular endothelial growth factor |
| WT | wildtype |
| YFP | yellow fluorescent protein |

# Publications arising during the course of this PhD

## Manuscripts

*Note: Carly Whyte previously known as Carly Gregor*

**Gregor CE,** Foeng J, Comerford I, McColl SR. Chemokine-driven CD4+ T cell homing: new concepts and recent advances. *Adv Immunol*. 2017;135:119-181. doi: 10.1016/bs.ai.2017.03.001.

Kara EE, Bastow CR, McKenzie DR, **Gregor CE**, Fenix KA, Babb R, Norton TS, Zotos D, Rodda LB, Hermes JR, Bourne K, Gilchrist DS, Nibbs RJ, Alsharifi M, Vinuesa CG, Tarlinton DM, Brink R, Hill GR, Cyster JG, Comerford I, McColl SR. Atypical chemokine receptor 4 shapes activated B cell fate. *J Exp Med.* 2018 Mar 5;215(3):801-813. doi: 10.1084/jem.20171067.

McKenzie DR, Kara EE, Bastow CR, Tyllis TS, Fenix KA, **Gregor CE**, Wilson JJ, Babb R, Paton JC, Kallies A, Nutt SL, Brüstle A, Mack M, Comerford I, McColl SR. IL-17-producing γδ T cells switch migratory patterns between resting and activated states. *Nat Commun.* 2017 Jun 5;8:15632. doi: 10.1038/ncomms15632.

Boyle ST, Gieniec KA, **Gregor CE**, Faulkner JW, McColl SR, Kochetkova M. Interplay between CCR7 and Notch1 axes promotes stemness in MMTV-PyMT mammary cancer cells. *Mol Cancer.* 2017 Jan 31;16(1):19. doi: 10.1186/s12943-017-0592-0.

Kara EE, McKenzie DR, Bastow CR, **Gregor CE**, Fenix KA, Ogunniyi AD, Paton JC, Mack M, Pombal DR, Seillet C, Dubois B, Liston A, MacDonald KP, Belz GT, Smyth MJ, Hill GR, Comerford I, McColl SR. CCR2 defines in vivo development and homing of IL-23-driven GM-CSF-producing Th17 cells. *Nat Commun.* 2015 Oct 29;6:8644. doi: 10.1038/ncomms9644.

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Kara EE, Comerford I, Fenix KA, Bastow CR, **Gregor CE**, McKenzie DR, McColl SR. Tailored immune responses: novel effector helper T cell subsets in protective immunity. *PLoS Pathog.* 2014 Feb 20;10(2):e1003905. doi: 10.1371/journal.ppat.1003905.

Comerford I, Harata-Lee Y, Bunting MD, **Gregor C**, Kara EE, McColl SR. A myriad of functions and complex regulation of the CCR7/CCL19/CCL21 chemokine axis in the adaptive immune system. *Cytokine Growth Factor Rev*. 2013 Jun;24(3):269-83. doi: 10.1016/j.cytogfr.2013.03.001.

## Conference Proceedings

46th Annual Scientific Meeting of the Australasian Society for Immunology, Brisbane (2017); Oral presentation: Atypical chemokine receptor 4 restrains anti-tumor $CD8^+$ T cell recruitment in breast cancer.

Third International Cancer Immunotherapy Conference, Mainz, Germany (2017); Poster presentation: Atypical chemokine receptor 4 restrains anti-tumor $CD8^+$ T cell recruitment into solid tumors.

13th Adelaide Immunology Retreat, Adelaide (2017); Oral presentation: Atypical chemokine receptor 4 restrains anti-tumour $CD8^+$ T cell recruitment into solid tumours.

12th Adelaide Immunology Retreat, Adelaide (2016); Oral presentation: The role of stromal ACKR4 in restraining anti-tumour immunity in breast cancer.

International Congress of Immunology, Melbourne (2016); Poster presentation: ACKR4 promotes breast cancer progression through suppression of anti-tumour immunity.

10th Adelaide Immunology Retreat, Adelaide (2014): Oral presentation: The role of ACKR4 in regulating anti-tumour immunity and lymphoid neogenesis.

# Abstract

The ability of immune cells to migrate to distinct niches and peripheral sites is critical for their appropriate differentiation and for execution of their effector functions. This migration is facilitated to a large degree by the expression of chemokine receptors, which allow for migration in a spatiotemporally-controlled manner. The work presented in this thesis addresses two distinct issues regarding how regulation of immune cell migration affects development of anti-tumour immunity and infectious immunity.

In the first part of this thesis, a novel role for the atypical chemokine receptor ACKR4 in controlling anti-tumour immune responses was identified. As a scavenging receptor, ACKR4 regulates the bioavailability of the CCR7 ligands, CCL19 and CCL21, and the CCR9 ligand, CCL25. These ligands have previously been shown to be critical for many aspects of immune homeostasis, as well as contributing to tumour cell growth and metastasis. However, the contribution of ACKR4 in regulating tumour-specific responses has been unclear. Using multiple orthotopic, transgenic and chemically-induced models of cancer, loss of ACKR4 resulted in inhibited tumour growth. In the absence of ACKR4, enhanced CCL21 levels were associated with enhanced tumour infiltration of IFN$\gamma^+$ CD8$^+$ T cells. The reduced tumour growth seen was dependent on the enhanced CD8$^+$ response, with depletion of CD8$^+$ T cells restoring growth of *Ackr4*$^{-/-}$ tumours to wildtype levels. The enhanced CD8$^+$ T cell response was not a result of altered priming in draining lymph nodes, although there was increased intratumoural proliferation of CD8$^+$ T cells. Furthermore, ACKR4-deficient tumours showed increased retention of CD103$^+$ DCs, with these cells previously being shown to be critical for effective recruitment of CD8$^+$ T cells to tumours. Moreover, intratumoural administration of CCL21 into wildtype tumours also enhanced the accumulation of DCs, suggesting a direct role for the scavenging ability of ACKR4. These data support the notion that ACKR4, through its regulation of CCL21 bioavailability, controls DC migration in tumours thus regulating the development of anti-tumour immune responses. Furthermore, multiple immunotherapies show increased efficacy in the absence of ACKR4, suggesting ACKR4 may be useful as a potential novel target for immunotherapy.

In the second part of this thesis, the role of CCR2 on memory CD4$^+$ T cells was explored. Relatively little is understood about the generation, maintenance and effector functions

of memory $CD4^+$ T cells, despite correlations with improved disease outcomes. Furthermore, how these cells migrate to inflammatory sites is still largely unknown. In this project, CCR2 was identified as being enriched on antigen-specific memory $CD4^+$ T cells in response to infection with the extracellular bacteria *Streptococcus pneumoniae* and infection with influenza A virus. Competitive co-transfer of wildtype and CCR2-deficient TCR-transgenic $CD4^+$ T cells showed enhanced contraction of *Ccr2*$^{-/-}$ cells, suggesting a cell-intrinsic role for CCR2 in $CD4^+$ T cell maintenance. CCR2-deficient effector cells were unaffected in their ability to secrete cytokines or enter into effector sites. Moreover, despite being numerically reduced at memory timepoints compared with CCR2-sufficent cells, they were equally capable of expanding upon secondary challenge. These data highlight CCR2 as an important regulator of $CD4^+$ T cell memory maintenance.

Taken together, this project has furthered our understanding of the complexity of cell migration in dictating immune responses. The identification of CCR2 as a mediator of memory $CD4^+$ T cell generation may allow further investigation into how these cells are induced and maintained. In ACKR4, a novel level of post-transcriptional regulation of intratumoural DC trafficking has been identified, with this having the potential to be a tractable target for therapeutic manipulation in malignant disease.

# CHAPTER 1

# Introduction

# Chapter 1 – Introduction

## 1.1 Chemokine-mediated control of immune responses

### 1.1.1 Cell migration in the immune system

The immune system is an integral component of our body's ability to protect itself from invading pathogens, destroy mutated or non-functioning cells and repair damaged tissues. It achieves these functions through coordination of multiple cell types with specific roles, with each response tailored to the specific threat being faced. Classically, cells of the immune system have been divided into two arms: innate cells, which recognise pathogens relatively non-specifically and provide an immediate source of protection; and adaptive cells, which possess antigen-specific receptors and enable targeted destruction of the threat. However, interplay between these groups of cells is critical for effective immune-mediated control.

A key feature of cells of the immune system is their ability to migrate to different sites, and this is critical for their development and function. During homeostasis, cells recirculate throughout blood, lymphatics and tissues to survey for signs of damage or foreign insult. When a threat is identified, immune responses require controlled migration of cells to distinct niches to facilitate priming, as well as migration of effector cells from secondary lymphoid organs (SLOs) to peripheral sites to eliminate the danger and initiate repair. This migration is tightly controlled both spatially and temporally, and is facilitated to a large degree by the chemokine system.

### 1.1.2 Chemokine system overview

Chemokines are a family of structurally-related low molecular weight cytokines that guide the migration of cells through binding to cognate chemokine receptors. These chemokine receptors are differentially expressed in immune cells (as well as on many cells of non-haematopoietic origin), which enables selective migration of cells expressing a receptor towards areas of production of the specific chemokines to which the receptor binds. Chemokine receptors are a family of seven transmembrane-domain G protein-coupled receptors (GPCRs), with the N-terminus and extracellular loops responsible for ligand binding. In typical chemokine receptors, the third intracellular domain contains a

conserved DRY motif, which enables coupling to G proteins, initiating downstream signalling cascades that induce cell polarisation and remodelling of the cytoskeleton that ultimately result in directional cell migration. Furthermore, receptors can become desensitised or internalised in response to chemokine signalling, which acts to regulate the magnitude of the response, with receptors being either degraded or recycled back to the surface for subsequent reengagement.

Chemokine receptors can be subdivided depending on the structural class of chemokines to which they bind, with these classes being determined by the N-terminal cysteine motif present in the chemokine. On this basis, there are four subfamilies of chemokine receptors: CC, CXC, XC, and $CX_3C$, where X is a non-conserved residue adjacent to the cysteine (C) residue (**Table 1.1**). However, there is promiscuity in the chemokine system, with multiple chemokines capable of binding to the same receptor, and an individual chemokine capable of binding multiple receptors. In addition to these subfamilies are a family of atypical chemokine receptors (ACKRs), which are defined by the absence of the canonical DRY motif and consequently lack classical G-protein mediated signalling[1]. Thus, these atypical chemokine receptors do not induce cell migration, but upon ligand binding can internalise, transcytose or degrade the chemokines, providing a further level of regulation of this system[2]. In Chapter 3, the role of an atypical chemokine receptor in regulating anti-tumour responses will be described, whilst Chapter 4 assesses the chemokine-mediated regulation of $CD4^+$ T cell memory in infectious immunity.

## 1.2 Immune responses to tumours

### 1.2.1 Tumour initiation and progression

Tumours are masses of cells that have arisen from uncontrolled cell proliferation. In healthy tissues, cell growth and division is tightly regulated by a number of different signals and checkpoints, with cells displaying defects being eliminated through apoptosis. However, cancerous cells can develop from an accumulation of mutations in genes involved in regulating these processes, so called oncogenes, which promote cell growth and division, and tumour suppressor genes, which regulate cell division and promote DNA repair. There are a number of features that define a successful tumour (**Figure 1.1**), with these aspects being required to overcome the many hurdles to developing and

sustaining an energy-intensive cellular mass. Furthermore, tumour cells can gain the ability to migrate from their original site, which can range from invasion of surrounding tissues without further dissemination, through to cells breaking away from the primary tumour and entering the bloodstream or lymphatics, where they can metastasize to distant sites. Metastatic disease requires both the initial ability to escape the primary tumour, often through an epithelial-mesenchymal transition, as well as the ability to adapt to and colonise the secondary site, without which metastatic cells may remain dormant and not form macrometastases[3]. Once tumours have successfully metastasized, treatment can be much more difficult, with patient prognosis generally poorer.

### 1.2.2 The tumour microenvironment (TME)

Whilst the initial focus in cancer research was on tumour cell-intrinsic characteristics, over the last decade, there has been increased recognition that the TME is a key contributor to the development of malignant tumours. In carcinomas, which are neoplastic lesions derived from epithelial cells, the tumour parenchyma consists of the epithelial cell mass, with the surrounding stroma including fibroblasts, blood and lymphatic endothelial cells, pericytes, mesenchymal stem cells and infiltrating immune cells. These components can each contribute to tumour progression, and have been the focus of intense research to determine novel potential therapeutic targets. In particular, much attention has been placed on cells of the immune system, which have been identified in most if not all solid tumours and can drive an inflammatory environment. Tumour-infiltrating immune cells are a heterogeneous population of both myeloid and lymphoid-lineage cells, which can have either anti-tumourigenic or pro-tumourigenic roles.

Pro-tumourigenic responses typically involve creation of an immunosuppressive or tolerogenic environment, mediated in part by cytokines such as transforming growth factor β (TGFβ), IL-4 and IL-10, among others[4]. This environment invokes the development of M2-polarised macrophages, myeloid-derived suppressor cells (MDSCs) and $Foxp3^+$ $CD4^+$ Treg cells, which can all act to suppress T cell responses to the tumour, as well as promoting angiogenesis and tissue-remodelling to support tumour growth and survival. The presence of Treg cells in tumours is often associated with poor prognosis, as these cells can suppress anti-tumour responses through production of IL-10,

sequestration of IL-2 from effector T cells, or competition for antigen-presenting cell (APC) costimulation through CTLA-4 expression[5-9]. Recently, neutrophils have been identified as a key facilitator of metastatic spread, by inducing leaky vasculature, supporting a premetastatic niche and limiting anti-tumour responses through production of soluble mediators such as matrix metalloproteinase 9 and inducible nitric oxide synthesis[10-13].

On the other hand, anti-tumourigenic responses are generally Type 1 responses, with $CD8^+$ cytotoxic T cells and natural killer (NK) cells both capable of targeting and destroying tumour cells. $CD8^+$ T cells act in an antigen-specific manner, predominantly recognising neoantigens created through tumour mutations or oncogenic viral epitopes, although tissue-specific differentiation antigens and cancer-testis antigens can also act as targets[14]. $CD8^+$ T cells become activated upon cognate recognition of their antigen presented in the context of MHC class I by APCs, and can differentiate into cytotoxic T cells capable of inducing tumour cell death through provision of IFNγ, Fas ligand (FasL), TRAIL and cytolytic effector proteins, perforins and granzymes. The presence of $CD8^+$ T cells and their effector proteins is correlated with improved patient prognoses in a range of different cancers[15-17]. NK cells are part of the innate immune response, and so their recognition of tumours is not antigen-specific. Rather, activation of NK cells is mediated through a balance of activating and inhibitory receptors, which can detect perturbations in normal cell health[18,19]. A major target for their inhibitory receptors are MHC class I molecules, which are expressed on normal cells, but can be downregulated on tumours cells to avoid antigen-presentation to $CD8^+$ T cells. Other important NK receptors are TIGIT, CD96 and CD226, which regulate a number of ligands altered in response to cellular stress. NK cell cytotoxic mechanisms are similar to those of $CD8^+$ T cells, with perforin/granzyme cytolytic complexes and IFNγ being the predominant mediators. These anti-tumour responses are also orchestrated through provision of cytokines such as IFNγ from $T_H1$-polarised $CD4^+$ T cells, with $CD4^+$ T cells also purported to have direct cytotoxicity in certain scenarios[20]. In addition, a wide range of other cells have been reported to be antitumourigenic in certain scenarios, including dendritic cells and M1 macrophages[21-23].

### 1.2.3 Cancer immunoediting

There is now great evidence for the immune system actively shaping tumour progression, with initial crucial experiments showing that tumours grown in immunodeficient animals were more immunogenic upon subsequent transfer into immunocompetent hosts[24,25]. This influence of the immune system is referred to as the cancer immunoediting hypothesis, in which there are three distinct phases of tumour-immune interactions; elimination, equilibrium and escape **(Figure 1.2)**. The elimination phase consists of the immune system detecting and destroying cancerous cells, prior to the development of malignancy or clinical disease. Evidence for this phase comes from early experiments demonstrating increased incidence of tumours in immunodeficient animals compared with wildtype controls, and has been further supported by models lacking specific cellular or molecular immune components[26,27]. The elimination phase relies on appropriate recognition of early tumours through mechanisms which are not fully understood, but requires integration of signals from both the innate and adaptive arms of the immune system. The next phase is known as equilibrium, in which tumour growth is controlled, but not entirely eliminated by the immune system. This stable coexistence of tumour cells and immune cells relies on the adaptive immune response, and was first demonstrated in a fibrosarcoma model induced by the chemical carcinogen 3-methylcholanthrene (MCA). Deletion of $CD4^+$ and $CD8^+$ T cells approximately 200 days post-MCA injection induced stable tumours to grow progressively, indicating a constant underlying immune response that controls tumour growth without eradicating the tumour[28]. This active immune response towards the tumour can also promote emergence of antigen-loss tumour variants, thus providing selective pressure for tumours to evolve towards a less immunogenic phenotype[29]. A loss in adaptive control can lead to the last phase of escape, in which tumours can successfully evade immune detection and progressively develop into malignant and often metastatic disease.

### 1.2.4 Mechanisms of immune evasion by tumours

There are a number of mechanisms by which tumours can escape immune control; one major issue is lack of sufficient quantity or quality of antigen able to be recognised as foreign by $CD8^+$ T cells, which is perhaps unsurprising given tumours arise from host cells. The frequency of predicted neoantigens is positively correlated with cytolytic gene signatures in a range of human tumour types[30]. However, even in tumours with strong mutational burden, the vast majority of mutations do not give rise to immunogenic

epitopes which can be presented on class I or II MHC, and thus cannot be recognised by $CD4^+$ or $CD8^+$ T cells[31,32]. Another hindrance to effective recognition is the downregulation of MHC class I molecules on some tumour cells, preventing antigen presentation to $CD8^+$ T cells[33]. However, this can be circumvented by NK cells as lack of engagement with MHC class I molecules induces their cytolytic activity. As eluded to above, lack of immunogenic antigen can also be a result of Darwinian selection from antigen-loss during an ongoing immune response, with variants that have lost this antigen, perhaps due to the inherent genomic instability of the tumour, able to become dominant and cause progressive outgrowth[34].

Another barrier to developing effective anti-tumour immunity is through sequestration of $CD8^+$ T cells from the tumour parenchyma. A pioneering study by Naito and colleagues found that the presence of $CD8^+$ T cells in the tumour bed was significantly associated with improved survival in human colorectal cancer, compared with $CD8^+$ localisation in the stroma or at the parenchyma-stroma interface, and this has since been supported in a range of other cancers and tumour models[15,35,36]. The TME has been shown to selectively favour entry of immune cells such as MDSCs and Treg cells, whilst preventing $CD8^+$ T cell entry[37]. The vascular endothelium of certain tumours can induce apoptosis in $CD8^+$ T cells through high expression of FasL, whereas Treg cells are protected through higher endogenous expression of the anti-apoptotic protein c-FLIP. FasL expression on the vasculature can be induced by tumour-derived VEGF, $PGE_2$ and IL-10, although these cytokines can also be expressed by infiltrating immunosuppressive immune cells[38]. Similarly, expression of the endothelin B receptor on tumour vasculature can prevent T cell adhesion to the endothelium, inhibiting T cell entry and correlating with poor patient prognoses[39]. Cancer-associated fibroblasts (CAFs) can also exclude T cell entry into the parenchyma, in part through deposition of extracellular matrix that acts as a physical barrier[40]. Furthermore, CAFs can produce the chemokine CXCL12 which binds to tumour cells and prevents T cell colocalisation, through an as yet undescribed mechanism[41].

In addition to T cell entry to the tumour being prevented, T cell effector function in tumour settings is often dampened. The expression of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) on T cells correlates with reduced anti-tumour activity, with CTLA-4 initially reported to antagonise CD28-mediated costimulation of T cells by

outcompeting CD28 for binding to their mutual ligands CD80 and CD86[42-44]. Intracellular stores of CTLA-4 can accumulate at the immunological synapse in response to TCR stimulation and act to inhibit signalling and induce anergy[45,46]. Furthermore, cells expressing CTLA-4 can strip CD80/CD86 expression from cells in trans through trans-endocytosis, resulting in degradation of these molecules and thus impaired costimulation[7].

PD-1 expression can also affect T cell effector function, and can be upregulated on $CD8^+$ T cells after prolonged antigen stimulation. Although initially believed to inhibit TCR signalling, recent evidence indicates PD-1 ligation actually induces dephosphorylation of CD28, thus attenuating downstream signalling and hence $CD8^+$ T cell function[47,48]. Both PD-L1 and PD-L2 act as ligands for PD-1, and can be expressed by cancer cells as well as infiltrating immune cells such as DCs and monocytes[49-51]. PD-L1 expression is also upregulated in response to IFNγ produced by infiltrating $CD4^+$ and $CD8^+$ T cells, and thus acts as a homeostatic negative feedback loop to induce T cell exhaustion and diminished function in cells for which persistent antigen exists, presumably as a safeguard to prevent autoimmunity[52,53]. However, this mechanism can be exploited by tumour cells to avoid cytolytic destruction, with PD-1 expression correlating with poorer prognoses in many human cancers[54,55]. Furthermore, PD-L1 has also been shown to promote the differentiation and suppressive function of inducible Treg (iTreg) cells through dampening of the Akt-mTOR pathway[56]. In addition to PD-1, other markers of T cell exhaustion have been identified including TIM-3 and LAG-3[57,58].

The TME itself can act as a potent site for T cell proliferation, with Batf3-dependent DCs capable of cross-presenting antigen to $CD8^+$ T cells and inducing clonal expansion in situ[21,59,60]. However, production of indole 2,3-dioxygenase (IDO) by cancer cells, DCs and myeloid cells, which catabolises tryptophan to generate kynurenine, can inhibit this proliferation as well as promoting the generation of Treg cells[61,62]. The TME can also promote T cell apoptosis, with production of the pro-death molecules FasL, TRAIL and TNFα by infiltrating myeloid cells. Furthermore, accumulation of adenosine through breakdown of ATP by ectonucleotidases CD39 and CD73 can both promote tumour cell proliferation, as well as promoting T cell immunosuppression[63,64,65].

Given the multitude of different mechanisms mediating tumour escape from immune control, the lack of effective immune responses to tumours is perhaps not surprising. However, these mechanisms have provided potential targets for therapeutic treatment of cancers, through enhancing the immune system's capability to target tumour cells.

### 1.2.5 Immunotherapy

For many years, the gold standards in cancer treatment have consisted of targeting the hyperproliferative nature of the tumour through non-specific destruction using chemotherapy or radiotherapy. However, as greater understanding of the role of the immune system in regulating cancer was gained, more specific targets have emerged, including several of the immune checkpoint inhibitors. The first of these drugs targeting immune control of tumours to be FDA-approved was ipilimumab (Bristol Myers Squibb), a monoclonal antibody targeting CTLA-4. Clinical trials in patients with previously treated advanced metastatic melanoma demonstrated increased survival in patients receiving anti-CTLA-4 plus a peptide vaccine or anti-CTLA-4 alone, compared with only the peptide vaccine[66]. Since then, pooled analysis of multiple clinical trials using ipilimumab in metastatic melanoma have demonstrated an overall three-year survival rate of 22% of all patients, after which survival curves plateau, indicating durable responses in these surviving patients[67].

It is proposed that anti-CTLA-4 treatment prevents CTLA-4 engagement with CD80/CD86, thus allowing CD28-mediated costimulation of effector T cells. Detailed analysis of TILs in mouse tumour models using mass cytometry showed that anti-CTLA-4 treatment predominantly induces the expansion of $ICOS^+$ $Tbet^+$ $PD\text{-}1^+$ $T_H1$-like CD4, as well as distinct $CD8^+$ populations[68]. However, there are also controversial reports regarding effects of anti-CTLA4 on the Treg compartment, which express increased levels of CTLA-4 compared with effector T cells[69]. Treg-specific deletion of CTLA-4 reduces the suppressive capacity of these cells, resulting in impaired control of anti-tumour T cell responses and hence reduced tumour growth[8]. Conversely, there is evidence that anti-CTLA-4 treatment expands the Treg compartment through an increase in proliferation[70,71]. More recently, it was reported that despite the expansion of Treg cells in the lymph nodes (LNs) in response to anti-CTLA-4 treatment, there is a selective depletion of Treg cells in the TME, mediated through FcγR-expressing macrophages[72].

This, coupled with anti-CTLA-4-induced expansion of effector T cells, resulted in an increased Teffector/Treg ratio, driving tumour rejection. Furthermore, optimal enhancement of effector T cell function is only seen when CTLA-4 is blocked in both the Treg and non-Treg populations, demonstrating CTLA-4 acts on both compartments to suppress T cell activity[73].

The success of anti-CTLA-4 treatment has encouraged clinical trials targeting other checkpoint inhibitors, with monoclonal antibodies targeting PD-1 (pembrolizumab, Merck; nivolumab, Bristol Myers Squibb) being approved for advanced melanoma in 2014. Pembrolizumab treatment showed significantly increased overall survival rates at 12 and 24 months compared with ipilimumab in metastatic melanoma, as well as reduced toxicities and adverse events[74]. Anti-PD-1 treatment has been shown to induce the expansion of PD-$1^+$ TIM-$3^+$ $CD8^+$ T cell subsets specifically, leading to enhanced proliferation and cytokine production by these cells[68]. Antibodies targeting PD-L1 (atezolizumab, Genentech) have also been recently approved for treatment of NSCLC and urothelial carcinoma after promising results in clinical trials[75,76].

Given that CTLA-4 and PD-1 work with distinct mechanisms to impair tumour-directed immune responses, it is perhaps not surprising that combination of anti-CTLA-4 and anti-PD-1 treatments leads to increased patient survival[77-79]. In a phase III trial, combination of nivolumab and ipilimumab in previously untreated melanoma resulted in median progression-free survival of 11.5 months, compared with 6.9 months and 2.9 months for nivolumab or ipilimumab alone, respectively[80]. However, combination immunotherapy also leads to significant increases in toxicity, with significantly more patients experiencing adverse events when treated with both nivolumab and ipilimumab, compared with either therapy alone.

Despite these remarkable advances, the percentage of patients with durable clinical responses is still limited, and large proportions of patients remain unresponsive to immunotherapy, or develop resistance. Studies comparing responsive and unresponsive patients have shed some light on factors that dictate whether anti-tumour responses will be induced in response to therapy. Mutational load is a significant prognostic factor for responsiveness to anti-PD-1 and anti-CTLA-4 therapy, with high mutational burden correlated with improved survival[81,82]. Mutations in beta-2-microglobulin, a critical

constituent of antigen presentation through MHC class I, are also significantly increased in metastatic melanoma patients non-responsive to anti-CTLA-4 or anti-PD-1 treatment and are correlated with lower survival, highlighting the importance of $CD8^+$ T cell responses in these therapies[83]. Another major prognostic factor for anti-PD-1/PD-L1 responsiveness is the extent of immune infiltration in the tumours prior to therapy. On this basis, tumours can be subdivided into three categories: an immune-inflamed phenotype, in which $CD4^+$ and $CD8^+$ T cells can be found within the tumour parenchyma; an immune-excluded tumour, in which T cells are found within the stroma surrounding the tumour but are physically separated from contact with tumour cells; and the immune-desert tumour, which lack T cells within the entire TME[14,75]. Patients with an immune-inflamed phenotype display the greatest proportion of clinical responses to anti-PD-1/PD-L1 therapy, suggesting that T cell exhaustion is a major contributor to tumour escape in these patients[84]. However, not all patients with a parenchymal infiltrate are responsive, suggesting the existence of alternate mechanisms of immunosuppression. In patients with an immune-excluded phenotype, anti-PD-1 treatment can induce T cell activation and proliferation, however these T cells still cannot enter into the tumour parenchyma and so tumour regression is rare[85]. In these patients, therapies targeted at overcoming this exclusion and enhancing T cell migration are likely to be beneficial. Tumours that completely lack a T cell infiltrate may be a result of a paucity in priming, perhaps due to defective antigen presentation or lack of tumour antigen able to be recognised as foreign. In these scenarios, tumour vaccination strategies or adoptive transfer of *ex vivo*-expanded lymphocytes may be of interest therapeutically.

## 1.3 Chemokine-mediated control of anti-tumour immune responses

### 1.3.1 The chemokine system in the TME

Tumour-directed immune responses, whether protumourigenic or antitumourigenic, rely on coordinated migration of immune cells into the TME and this is mediated to a large extent by the chemokines expressed within the tumour milieu. A prominent chemokine receptor mediating intratumoural recruitment of $CD8^+$ T cells, $T_H1$ cells and NK cells is CXCR3, which induces migration towards its ligands CXCL9 and CXCL10[86]. Expression of CXCR3 ligands is correlated with enhanced T cell infiltration and improved survival in several human tumour types[87-90]. In mice, intratumoural injection

of CXCL10 stimulated $CD8^+$ T cell infiltration and enhanced tumour regression[91,92]. Moreover, CXCR3 was essential for $CD8^+$ T cell intratumoural migration *in vivo*, with loss of this receptor abrogating migration to the same extent as pertussis toxin pretreatment, an inhibitor of most chemokine signalling through $G_{\alpha i}$ protein blockade[93]. CXCL9 and CXCL10 can be secreted by tumour cells and infiltrating macrophages[94]. More recently, it was shown that the predominant source of CXCL9 and CXCL10 in tumours was $CD103^+$ DCs, which were both sufficient and essential for intratumoural recruitment of adoptive transferred $CD8^+$ T cells[95]. These $CCR5^+$ DCs are recruited into the tumour through expression of CCL4 by transformed cells[96].

CCR5 and CCR2 have also been linked with intratumoural T cell accumulation and enhanced survival, suggesting potential redundancies in $CD8^+$ T cell trafficking mechanisms[97]. Transgenic expression of CCL5, a ligand for CCR5, in xenografted tumours can overcome collagen-mediated exclusion of T cells in an *ex vivo* assay[40]. However, despite CCR5 and CCR2 being functional on $CD8^+$ T cells and their ligands being expressed within the tumour parenchyma, deletion of these receptors had no effect on intratumoural homing or extravasation from blood vessels in B16-OVA bearing mice[93]. This may be due in part to post-translational modification of chemokines in the TME, as CCL2 can be nitrated in response to reactive nitrogen species produced by tumour cells. Nitrated-CCL2 can induce recruitment of myeloid cells such as MDSCs but not effector T cells, perhaps owing to the higher expression of CCR2 on myeloid cells, providing another means of immune evasion by tumour cells[98]. Indeed, recruitment of myeloid cells through CCL2 is a major contributor to immunosuppression, with neutralising antibodies or small molecule inhibitors to CCL2 showing anti-tumour activity in preclinical models of prostrate and breast cancer[99,100]. CCR2 is also used by Treg cells to traffic from draining LNs into mouse tumour models and $CCR2^+$ Treg cells are enriched in human oral squamous cell carcinoma patients[101]. Moreover, Treg cells can also traffic through CXCR3, CCR4 and CCR10, thus showing diverse mechanisms of recruitment into different tumour types[102-104]. Other chemokine receptors have also been implicated in regulation of both tumour-directed immune responses as well as tumour cells themselves, and three receptors of key interest to this study are discussed below in more detail.

### 1.3.2 CCR7

CCR7 is a key facilitator of homeostatic immune trafficking, and binds to the chemokines CCL19 and CCL21. CCR7 is highly expressed by naïve and central memory T cells and allows their entry into LNs and Peyer's patches (PP) through interaction with CCL21, which is constitutively expressed on the luminal surface of high endothelial venules (HEV), specialised vasculature controlling immune cell entry into these SLO[105]. Inside LNs, CCL19 and CCL21 are expressed by follicular reticular cells, which promotes naïve T cell motility and guides their ability to scan for cognate antigen presented by dendritic cells[106,107]. CCR7 is also essential for dendritic cell migration, with CCR7 upregulated concomitantly with DC maturation in response to antigen processing and required for homing to lymphatic vessels and subsequently LN entry[108,109]. CCR7 is also required for a number of other homeostatic functions of immune cells[110].

In tumour settings, the presence of CCR7-expressing T cells is associated with favourable prognosis in colorectal carcinoma and prostate cancer[111,112]. In mice, overexpression of CCL19 in a breast cancer cell line was shown to induce an immune response dependent on $CD4^+$ T cells and NK cells, resulting in inhibition of tumour growth[113]. Similarly, intratumoural injection of CCL21 into two murine lung cancer models led to enhanced recruitment of $CD8^+$ T cells and DCs and inhibition of tumour growth, with 40% of mice exhibiting complete tumour regression[114]. Enhanced $CD8^+$ T cell responses and inhibited tumour growth were also identified in other murine cancer models in response to CCL21 delivery[115-117]. Mechanistically in the L1C2 lung carcinoma model, the anti-tumour effects of CCL21 were partially dependent on IFNγ, CXCL9 and CXCL10, with neutralisation of these proteins reversing tumour inhibition[118]. Conversely, it has been reported that CCL21 can promote tumour growth in the B16 melanoma model, with overexpression of CCL21 inducing a tolerogenic environment and increasing infiltration of Treg cells, along with inducing development of lymphatic vessels reminiscent to that found in LNs[119]. In the same study, knockdown of CCL21 by B16 cells reduced tumour growth and was associated with enhanced infiltration of T cells and higher IFNγ levels. The reasons for these conflicting reports on the role of CCL21 in tumours are unclear and require more research, but emphasise the importance of the TME context in dictating anti-tumour immune responses.

The CCR7 axis can also regulate tumour growth independently of its effects on the immune system. Activation of PI3K/Akt signalling downstream of CCR7 promotes survival of cancer cells in head and neck tumours[120]. CCR7 contributes to the maintenance of stem-like cells in breast cancer, with deletion of this receptor resulting in reduced tumour growth in the MMTV-PyMT transgenic model of breast cancer[121]. CCR7 is also important for metastatic spread, with its expression on tumour cells promoting metastasis to regional LNs due to the high levels of CCL19 and CCL21 expressed in these sites[122-125]. CCR7 can promote lymphangiogenesis or the development of new lymphatic vessels, in part through induction of VEGF-C, which also helps to facilitate metastatic dissemination of tumour cells from the primary tumour[126]. Furthermore, signalling through CCR7 can inhibit anoikis or detachment-induced cell death, which can prevent metastatic spread[127]. Thus, the CCR7 axis has clear tumour-promoting roles but also predominantly promotes anti-tumourigenic immune responses, and the overarching balance of these effects in cancer is still not fully understood.

### 1.3.3 CCR9

CCR9 is expressed by a subset of T cells, plasmablasts and plasmacytoid dendritic cells (pDCs) and is essential for homing of these cells from the peripheral blood to the small intestine, the predominant site of expression of its ligand, CCL25[128-130]. Neutralisation or ablation of CCR9 on $CD8^+$ T cells inhibited their ability to migrate to the small intestinal mucosa but did not affect migration to lung, liver or LNs[131,132]. Thus, CCR9 plays a pivotal role in controlling migration of leukocytes to the intestine, which is an essential form of immune surveillance needed to counteract potential threats posed by commensals and ingested pathogens.

CCR9 has been reported to be overexpressed in many different cancer models[133-135]. CCR9-bearing tumour cells preferentially migrate to the intestine in mouse tumour models, and CCR9 was strongly expressed in human melanoma metastases found in the small intestine, but not in other locations[136,137]. It was recently shown that CCR9/CCL25 interaction could promote survival of lung carcinoma cells by inhibiting apoptosis through activation of Akt signalling[138]. However, the functions of CCR9 in tumour settings outside of guiding metastasis are still largely unexplored.

### 1.3.4 ACKR4

The atypical chemokine receptor ACKR4 (formerly known as CCX-CKR, CCRL1) acts as a scavenging receptor for the ligands CCL19, CCL21, and CCL25 both *in vitro* and *in vivo*[139,140]. Upon ligand binding, ACKR4 rapidly internalises and subsequently degrades chemokines and thus acts to regulate the bioavailability of its ligands. ACKR4 is highly expressed by lymphatic endothelial cells (LECs) and cortical thymic epithelial cells but has been shown to be widely expressed in many tissues, including the heart, lungs and intestine[141-144]. ACKR4 has been shown to regulate many CCR7-dependent processes such as DC migration and T cell priming, likely through its scavenging of CCR7 ligands. ACKR4-deficient mice display reduced trafficking of DCs from skin to draining LNs at both steady state and in response to inflammation[141]. Further analysis showed that ACKR4 was specifically expressed by lymphatic endothelial cells (LECs)s on the ceiling of the subcapsular sinus of LNs, where it was required for establishment of a chemokine gradient across the sinus, thus promoting DC entry into the LN through the floor of the sinus[144]. Furthermore, ACKR4-deficient mice immunized with $MOG_{35-55}$/CFA in a mouse model of multiple sclerosis showed enhanced $CD4^+$ T cell priming in the spleen and a $T_H17$-biased response, which correlated with earlier disease onset and enhanced disease severity[140].

In tumour settings, little is known about the function of ACKR4. In a breast cancer xenograft model, which thus lacks an intact adaptive immune system, overexpression of ACKR4 on human breast cancer cell lines reduced tumour growth and metastasis to the lung[145]. This was also accompanied by reduced proliferation and invasion capability *in vitro*, although the mechanism behind these phenotypes remains unclear. Furthermore, we have shown that overexpression of ACKR4 in an orthotopic mouse breast cancer model also reduced tumour growth *in vivo* but conversely enhanced spontaneous and haematogenous metastasis to the lung[146]. Interestingly, this was not correlated with altered abundance of chemokine ligands but instead was associated with enhanced epithelial-to-mesenchymal transition, potentially mediated through altered regulation of TGF-β1 as a result of transgenic expression of ACKR4 in these cell lines. In analyses of human breast and cervical cancer specimens, ACKR4 expression has been correlated with improved survival and reduced LN metastasis[145,147,148]. These correlations were determined from immunohistochemical staining of tissue samples using the same

polyclonal antibody against ACKR4, with one report showing that 50-70% of this staining was found in the cytoplasm[147]. Given that the known function of ACKR4 is to scavenge its chemokine ligands from extracellular spaces, the specificity of this antibody and the functional significance of cytoplasmic sources of ACKR4 is uncertain. A separate study found that ACKR4 expression in hepatocellular carcinoma was inversely correlated with tumour stage and overall survival[149]. Manipulation of ACKR4 expression in hepatocellular carcinoma cell lines showed that ACKR4 was inversely correlated with tumour growth *in vivo* in nude mice, as well as being inversely correlated with CCL19 and CCL21 levels in the tumour, indicating a potential chemokine scavenging role in tumour settings. Furthermore, *in vitro* analysis indicated that when ACKR4 expression was reduced in these cell lines, there was greater Akt phosphorylation and nuclear accumulation of β-catenin.

While the above reports provide some evidence for a role of ACKR4 in cancer, the studies to date are very limited in number and the mechanism of action of this receptor in regulating tumour growth is unclear. Furthermore, only one mechanistic study was performed in mice with a fully-intact immune system, and this was studying the effect of overexpression of ACKR4 on cancer cells, with unaltered ACKR4 in the host[146]. Since ACKR4 that is endogenously expressed by the host is known to markedly influence immune responses at homeostasis as well as in non-tumour settings, it is highly likely that modulation of host ACKR4 would also impact on anti-tumour responses, however investigation of the role of host ACKR4 has not yet been reported to date. Thus, the role of ACKR4 in regulating tumour progression and mediating anti-tumour immune responses will be analysed in Chapter 3.

## 1.4 Immune responses to infectious diseases

### 1.4.1 Tailored immune responses to infectious pathogens

Over the course of a lifetime, a human body will be exposed to a diverse array of infectious pathogens, including bacteria, viruses and fungi. Successful protective immunity to these pathogens requires coordination of elements of both the innate and adaptive immune response. Typically, a pathogen threat will be rapidly recognised by pattern recognition receptors (PRRs) present on antigen-presenting cells such as DCs,

which recognise conserved features of pathogens, known as pathogen-associated molecular patterns (PAMPs), although danger-associated molecular patterns (DAMPs) and other disruptions to tissue homeostasis can also stimulate immunity[150,151,152]. This leads to DC maturation, where cells express high levels of MHC class II presenting antigen, and distinct cytokines depending on the PRR-induced signalling pathway engaged. Given that pathogens have a number of different modes of infection, there is a subsequent requirement for diversity in the immune response needed to eliminate the pathogen **(Table 1.2).** Infection with pathogens which are predominantly intracellular, including viruses such as influenza and some bacteria, induces a Type 1 response, with IFNγ production by $CD4^+$ T helper 1 ($T_H1$) cells coordinating clearance of infected cells through cytolytic destruction by $CD8^+$ T cells. Type 2 responses are generated towards macroscopic parasites as well as both toxic and inert compounds, including allergens. Removal of these insults involve tissue-wide changes, with $T_H2$ cells, mast cells and eosinophils often required, along with mucus secretion and muscle contractility[153]. Type 17 responses are induced in response to infection with extracellular pathogens, including bacteria such as *Streptococcus pneumoniae* and many fungi[154,155]. Production of cytokines such as IL-17A and IL-17F by $T_H17$ cells drives recruitment of neutrophils which can phagocytose these pathogens, as well as inducing the release of anti-microbial compounds such as β-defensins and S100 peptides[156,157]. Furthermore, in each of these responses, antibody production by B cells is tailored to the secretion of specific isotypes with the help of $CD4^+$ T follicular helper ($T_{FH}$) cells[158]. Other $CD4^+$ T cells such as $T_H9$ and $T_H22$ cells can also contribute to distinct facets of these responses. Thus, considerable diversity is required in the immune response in order to successfully eliminate infectious pathogens, with $CD4^+$ $T_H$ cells playing key roles in coordinating these responses.

### 1.4.2 Memory response to infectious pathogens

A defining feature of the adaptive immune response is the ability to mount responses with enhanced quality and efficiency upon secondary exposure, known as memory. Following a primary infection, the large pools of effector T and B lymphocytes generated in the response contract, leaving behind a small population of memory lymphocytes[159,160]. These memory lymphocytes can reside at the site of the infection or in SLOs, or circulate through tissues and blood. Upon re-exposure to the same antigenic stimulus, these

differentiated lymphocytes can reactivate and expand more readily, leading to enhanced elimination of the pathogen and improved protection.

Memory B cells can provide long-lasting protection against pathogens, with different subsets of memory B cells contributing different functions[161]. Long-lived plasma cells can secrete antibody constitutively, which may provide protection if the neutralising antibody is present at sufficiently high concentrations. Furthermore, reactivation of memory B cells in response to antigen allows more efficient production of isotype-switched, high affinity antibody compared with that from naïve B cells.

In response to viral and other intracellular pathogens, $CD8^+$ T cell memory is critical for improved protection. In addition to mounting cell-mediated cytotoxicity with enhanced efficiency, populations of memory $CD8^+$ T cells can also act as innate-like sensors of pathogens. IL-12 and IL-18, released in response to pathogen invasion, can stimulate these memory $CD8^+$ T cells, leading to the release of IFNγ in an antigen-independent manner[162]. Both of these functions of memory $CD8^+$ T cells result in improved protection against infectious challenges, with pre-existing $CD8^+$ T cells providing protection against otherwise lethal challenges[163-165].

$CD4^+$ T cell memory is not as well understood as for $CD8^+$ T cell memory, however pre-existing memory $CD4^+$ T cells are correlated with reduced viral load and improved protection in some models[166-169]. Memory $CD4^+$ T cells specific for influenza infection promote production of inflammatory cytokines upon heterosubtypic infection with an independent influenza strain, which is associated with improved viral control[170]. Additionally, vaccination-induced protection against a mouse model of Herpes Simplex Virus (HSV)-2 infection was entirely dependent on viral-specific $CD4^+$ T cells[168]. However, the mechanisms promoting generation and maintenance of memory $CD4^+$ T cells are still mostly undefined.

## 1.5 $CD4^+$ T cells and their chemokine-mediated control

*The following sections are adapted from Gregor et al., 2017.*

Each $T_H$ subset is defined by their expression of distinct lineage-specific transcription factors, as well as the production of cytokines which shape the outcome of the immune response. The diversity of $CD4^+$ T cells generated in response to a pathogen stems from the signals received during their priming in SLO. Depending on the affinity of the interaction, naïve T cells expressing a T cell receptor specific for an antigen-MHC class II complex expressed on a DC (signal 1) will reduce their motility and form prolonged interactions with the DC. Subsequently, costimulation by DC-expressed molecules such as CD80 and CD86 (signal 2) and cytokines to promote polarisation of the naïve T cell (signal 3) are also required for differentiation into an effector $T_H$ cell subset.

Central to the role of $CD4^+$ T cells in shaping the outcome of the immune response is their ability to migrate in a spatiotemporally controlled manner. The potentially large surface areas of the body exposed to antigen, combined with the rare clonal frequency of antigen-specific $CD4^+$ T cells, necessitate this efficiency in migration. Precise control of cell migration ensures that $CD4^+$ T cells encounter antigen when it is present, permits appropriate cross-talk with other immune cells, regulates homing to sites of peripheral inflammation, and is also critical for immune surveillance and memory maintenance. It is well recognised that the various functional states of $CD4^+$ T cells (e.g. naïve, effector, memory) have profoundly distinct migratory patterns and that the effector subsets of $CD4^+$ T cells that shape adaptive immune responses all have distinguishable and in some cases defining homing characteristics **(Figure 1.3)**. The profiles of $T_H$ subsets relevant to this project are discussed in more detail below.

### 1.5.1 $T_H1$ cells

$T_H1$ cells promote cell-mediated immunity and are critical for protection against intracellular pathogens. They are also important for clearance of cancerous cells, but can contribute to pathology in cases of transplantation or autoimmunity. These roles are predominantly mediated through $T_H1$ production of the cytokine IFNγ, as well as TNFα and IL-2. IFNγ is essential for control against numerous pathogens, through both direct effects on the pathogen itself as well as more broadly stimulating a concerted immune response by numerous cell types[171]. $T_H1$ cell differentiation is first induced by TCR engagement in the presence of IFNγ or type I IFNs, which leads to expression of the master transcriptional regulator T-bet and consequently the high affinity IL-12Rβ2 chain.

IL-12 then acts via STAT4 to further stimulate expression of T-bet, which drives IFNγ production to create a feed-forward loop which stabilises the $T_H1$ differentiation program.

$T_H1$ cells can assist in many facets of type 1 immunity against infectious pathogens. A critical role for these cells is in promoting the priming and expansion of antiviral $CD8^+$ T cells. In the absence of $CD4^+$ T cells, $CD8^+$ T cells fail to expand or persist as effectively in response to HSV or vaccinia virus, although robust type I IFN signalling bypasses this requirement for $CD4^+$ help[172,173]. Furthermore, $CD4^+$ T cells are critical for effective generation of $CD8^+$ T cell memory in response to infections, in part through reducing susceptibility of $CD8^+$ T cells to TRAIL-mediated apoptosis and through provision of CD40L[174-177]. $T_H1$ cells are also critical for promoting formation of $CD8^+$ tissue resident memory ($T_{RM}$) cells, with $CD4^+$-derived IFNγ required for localisation of $CD8^+$ T cells to the airways after influenza infection, where they receive signals promoting their retention and memory formation[178]. IFNγ produced by $T_H1$ cells can also induce class switching of B cells to produce IgG2a antibody, a potent neutralising antibody for viruses, although this role is largely fulfilled by the specialised $T_{FH}$ subset of $CD4^+$ T cells[158,179].

The canonical chemokine receptor expressed by $T_H1$ cells is CXCR3. The CXCR3 axis is a prototypical inflammatory axis, with CXCR3 absent on naïve T cells and the ligands CXCL9 and CXCL10 (and CXCL11 in humans) only induced upon exposure of cells to inflammatory stimuli[180]. These ligands can be produced by a broad range of cell types, with fibroblasts, leukocytes and keratinocytes all reported sources of expression. CXCR3 is essential for the peripheral localisation of $T_H1$ cells in a multitude of diseases, with deletion of CXCR3 broadly inhibiting $T_H1$ migration in infections, autoimmune diseases and many cancers[181]. Furthermore, the CXCR3 axis is important for amplifying the effector response, with IFNγ inducing the expression of the CXCR3 ligands, which in turn recruits in more $T_H1$ cells and $CD8^+$ T cells expressing IFNγ[181]. Indeed, in HSV infection, $CD4^+$ T cells are essential for $CD8^+$ migration to the vaginal mucosa and $T_H1$-derived IFNγ is critically required for this[182]. $T_H1$ cells at the infection site produce IFNγ which upregulates expression of CXCL9 and CXCL10 by local epithelial cells, thereby allowing recruitment of CTLs via CXCR3 where they can clear infected cells.

Another chemokine receptor classically associated with $T_H1$ cells is CCR5, which binds to CCL3, CCL4 and CCL5. CCR5 is also used by effector $T_H1$ cells to migrate to peripheral sites to exert their function[183-185]. CCR5 expression on OT-II cells, which express a transgenic TCR specific for the ovalbumin peptide $OVA_{323-339}$, is also required for optimal upregulation of CD40L on DCs and hence development of $IFN\gamma^+$ $CD8^+$ cells, thus pointing to a role for CCR5 in promoting cross-presentation by DCs to induce CTL responses[184].

Although CXCR3 and CCR5 are often coexpressed by $T_H1$ cells, they do not appear to have redundant functions. In chronic hepatitis C infection, CCR5 ligands are expressed in vessels within the portal triad, whereas CXCR3 ligands are expressed on the sinusoidal epithelium, suggesting these receptors may instead control different aspects of $T_H1$ trafficking[186]. Furthermore, during malarial infection with blood-stage *Plasmodium yoelii*, CXCR3 was equivalently expressed in $T_H1$ cells that were IL-$10^+$ or IL-$10^-$, however CCR5 was preferentially expressed on IL-$10^+$ $T_H1$ cells[187].

### 1.5.2 $T_H17$ cells

$T_H17$ cells were first described in 2005 as a lineage independent to $T_H1$ and $T_H2$, that were capable of producing the proinflammatory cytokines, IL-17A and IL-17F[188,189]. These cells are characterised by their expression of the master transcription factor, retinoic acid receptor-related orphan receptor-γt (RORγt) and have since been described to secrete a diverse repertoire of cytokines, including IFNγ, GM-CSF, IL-10 and IL-22[190]. $T_H17$ cells are critical for host defence against extracellular microorganisms and this protective role against both bacterial and fungal pathogens is primarily mediated through IL-17A and IL-17F, which bind to the heterodimeric IL-17 receptor, composed of IL-17RA in complex with IL-17RC[191]. IL-17 can induce expression of a number of proteins with antimicrobial roles, including CXCL1 and CXCL2, which can induce the recruitment of neutrophils to phagocytose microbes[192].

The characteristic chemokine receptor expressed by $T_H17$ cells is CCR6, which binds to the sole known chemokine ligand, CCL20. CCL20 can be upregulated in response to various inflammatory signals, but is also expressed under resting conditions in a diverse range of tissues, including the skin, gut and airways, attracting $T_H17$ cells to these sites

through CCR6[193,194]. Expression of CCR6 appears to be tightly coupled to the initial $T_H17$ differentiation program, as forced expression of RORγt in naïve T cells is sufficient to upregulate CCR6 and in mice with transgenic expression of RORγt and GFP under the CD4 promoter, the vast majority of $GFP^+$ IL-$17^+$ cells were also $CCR6^+$ [195,196].

$T_H17$ cells are highly enriched in the intestinal tract, which given the abundance of microbes at this site, is in accordance with the demonstrated role of $T_H17$ cells in protection against extracellular bacterial infection. CCR6 has been strongly implicated in $T_H17$ cell recruitment to the intestine and as a consequence is suggested to be a driver of intestinal inflammation. CCL20 is abundantly expressed in the subepithelial dome of PP, as well as in isolated lymphoid follicles. Accordingly, transferred *Ccr6*$^{-/-}$ $T_H17$ cells displayed impaired migration to PP, the small intestinal lamina propria and the peritoneal cavity[195]. Aside from CCR6, other chemokine receptors may contribute to intestinal homing of $T_H17$ cells. $T_H17$ cells from the large intestine have been shown to express CCR4 and CXCR5 and, although it is not clear if these are functional in mouse $T_H17$ cells, CCR4 is also expressed on human memory $T_H17$ cells[195,197]. CCR9 is also enriched in $T_H17$ cells in the small intestine, and along with $\alpha_4\beta_7$ can be induced on *in vitro*-generated $T_H17$ cells by addition of retinoic acid (RA)[198]. RA-induced $T_H17$ cells use these receptors to migrate to the small intestinal lamina propria, where epithelial cells produce the CCR9 ligand, CCL25.

$T_H17$ cells are also critical for controlling bacterial infection at other peripheral sites, particularly at other mucosal barrier sites such as the lung. Intranasal challenge with an array of extracellular bacteria including *Klebsiella pneumoniae* and *Streptococcus pneumoniae* induces $T_H17$ cells, with IL-17 being a key mediator of bacterial clearance through induction of a neutrophilic response[155,199,200]. Similarly, fungal pulmonary infections such as with *Aspergillus fumigatus* or *Pneumocystic carinii* also induce potent $T_H17$ responses. The trafficking receptors used by $T_H17$ cells for migration to the lung are not well understood, although given that CCL20 is upregulated in response to pulmonary infection, it is likely that CCR6 plays a role here as well[201]. This is supported by studies of human $T_H17$ cells, in which $T_H17$ memory subsets with a $CCR6^+CCR4^+$ profile responded vigorously upon incubation with *Candida albicans* and *Staphylococcus*

*aureus*. However, functional evidence identifying chemokine receptors involved in $T_H17$ homing to the lung is currently lacking.

It is now clear that a spectrum of cellular phenotypes exist within the $T_H17$ subset with differing capacities to promote inflammation. Populations of $T_H17$ cells can co-express the anti-inflammatory cytokine IL-10 in addition to IL-17, with these cells having a less pathogenic profile and generated in response to mild insults and transient infection[202]. Conversely, $T_H17$ cells can co-express proinflammatory cytokines such as IFNγ and GM-CSF, with these being termed pathogenic $T_H17$ cells and being main contributors to autoimmune-associated pathology[203]. Furthermore, $T_H17$ cells can lose IL-17 expression completely and become ex$T_H17$ cells. Pioneering work by the Stockinger laboratory used an IL-17 fate-mapper mouse that permanently marks cells and their progeny with eYFP if IL-17A is expressed. This showed a sequential change in cytokine production by differentiated $T_H17$ cells in experimental autoimmune encephalomyelitis (EAE) that transitions from IL-17$^+$ IFNγ$^-$ to IL-17$^+$ IFNγ$^+$ and then to a IL-17$^-$ IFNγ$^+$ profile [204]. Loss of IL-17 expression by $T_H17$ cells in EAE was concomitant with downregulation of CCR6 and CCR6$^-$ eYFP$^+$ cells had substantially higher levels of *Ifng* mRNA than CCR6$^+$ eYFP$^+$ cells, suggesting pathogenic $T_H17$ cells may not use CCR6 for their trafficking. Indeed, we have recently demonstrated that there is a temporal switch in $T_H17$ trafficking programs during EAE, with CCR6 only required for migration of $T_H17$ cells during the initial stages of pathogenesis[205]. As disease progresses, CCR2-expressing $T_H17$ cells emerge from the SLO, with this being the critical migratory receptor for homing of encephalitogenic $T_H17$ to the CNS. Furthermore, production of the pathogenic cytokines GM-CSF and IFNγ by $T_H17$ cells primarily emanated from cells with a CCR6$^-$CCR2$^+$ phenotype. Expression of CCR2 by $T_H17$ cells does not appear to be limited to autoimmune disease, as $T_H17$ cells generated in response to chronic *S. pneumoniae* nasopharyngeal infection displayed similar temporal upregulation of CCR2, with CCR6$^-$ CCR2$^+$ cells again being the predominant source of GM-CSF and IFNγ. Whether CCR2 is important for $T_H17$ trafficking in situations of transient infection remains to be determined.

### 1.5.3 CD4$^+$ memory response

Upon antigen clearance, $CD4^+$ effector T cells rapidly contract, with approximately 90% of cells dying within 1-2 weeks. However, memory $CD4^+$ T cells persist long-term and are critical for the rapid response to subsequent antigen encounter. The precise mechanisms by which $CD4^+$ memory T cells develop and function is still not well described, however significant advances to improve our understanding have been made in recent years. $CD4^+$ memory T cells are a heterogenous population and have been divided into at least 3 categories based on their migratory patterns. The first division of function of memory T cells was first described by Sallusto and colleagues in 1999 on the basis of CCR7 expression[206]. Central memory ($T_{CM}$) cells are defined as $CCR7^+$ $CD62L^{hi}$ $CD4^+$ T cells that preferentially produce IL-2 upon restimulation and cross HEVs to recirculate from blood through SLOs **(Figure 1.4)**[207]. Conversely, effector memory ($T_{EM}$) cells were described as $CCR7^-CD62L^{lo}$ and migrate to inflamed peripheral tissues for immunosurveillance and enter LNs via afferent lymph. Upon antigen engagement, these cells preferentially produce effector cytokines such as IFNγ and IL-4. More recently, $CD11a^{hi}$ $CD69^+$ resident memory ($T_{RM}$) that permanently reside in previously-infected peripheral tissues and are restricted from accessing the circulation were described, with these cells being critical for protective immunity upon rechallenge at the site of the primary infection[208,209]. These subsets of $CD4^+$ T cells, and the chemokine receptors that control their functions, will be described here.

### *1.5.3.1 $T_{CM}$*

$T_{CM}$ cells are mainly restricted to recirculating through the blood and lymphoid organs, where they rely on Ag-presenting APCs in SLOs for their reactivation. Because of this, $T_{CM}$ cells are the slowest of the memory cell subsets to respond to antigen and take days to expand. However, $T_{CM}$ cells have a unique role in that they produce high levels of IL-2 and display enhanced proliferative capacity, giving rise to multiple effector subsets that can then emigrate from SLO to traffic to peripheral sites. The expression of CCR7 by $T_{CM}$ is critical for this recirculation, and along with CD62L expression allows trafficking patterns akin to naïve T cells.

$T_{CM}$ can also express CXCR5, with Ag-specific $CD4^+$ $T_{CM}$ cells ($CCR7^+CD62L^{hi}$) at 60 days post infection with *L. monocytogenes* displaying a characteristic $CXCR5^+$ $T\text{-}bet^{lo}$ phenotype, while $T_{EM}$ were $CXCR5^-CCR7^-$ $T\text{-}bet^{hi}$ [210]. These $CXCR5^+$ memory cells

efficiently produced IL-2 but secreted less IFNγ than $T_{EM}$, supporting their status as $T_{CM}$ cells. Perhaps surprisingly given their expression of CXCR5, these cells were excluded from follicles and localised to the paracortex upon adoptive transfer, which suggests that other migratory cues, possibly including CCR7, confine them to T cell areas. However, $CXCR5^+$ $T_{FH}$ cells can also persist into memory in the absence of continued antigen, where they drive enhanced secondary responses and can provide more efficient B cell help than $T_{FH}$ cells in the primary response[211,212].

Other chemokine receptors have also been reported to be expressed by human $CD4^+$ $T_{CM}$ cells and correlate to the differentiation capability of those $T_{CM}$ cells, suggesting reduced multipotency and perhaps partial differentiation[213]. $CXCR3^+$ $T_{CM}$ cells secrete low levels of IFNγ and were able to generate fully differentiated $T_H1$ cells *in vitro*, while $CCR4^+$ $T_{CM}$ cells secreted low levels of IL-4 but not IL-5 and gave rise to $T_H2$ cells. In contrast, $CXCR5^+$ $T_{CM}$ that were $CXCR3^-$ and $CCR4^-$ were not polarised and relied on exogenous cytokines to divert them to a $T_H1$ or $T_H2$ phenotype. Evidently, there is much to learn about the further classification of diversity within $T_{CM}$ cells based on their chemokine receptor expression. Furthermore, the precise role of these chemokine receptors in guiding $T_{CM}$ cells to specific SLO niches supportive of their function is also largely unexplored.

*1.5.3.2 $T_{EM}$*

Under steady state conditions, $T_{EM}$ circulate through the blood and peripheral tissues. $CD4^+$ $T_{EM}$ are generally thought to be excluded from entering HEV due to low expression of CCR7 and CD62L, but can migrate into reactive LN through CD62P where they can enhance naïve T cell priming through provision of CD40L to DCs[214]. Due to their efficient expression of effector cytokines, $T_{EM}$ provide an earlier response to secondary infections than $T_{CM}$ and can be recruited to the site of infection to respond within hours to days. $T_{EM}$ are thought to arise from T effector cells that have survived the contraction phase, although it is not clear at which point memory fate determination is conferred. Certainly, the markers used to define $CD8^+$ memory precursors such as CD127 and KLRG1 do not apply to $CD4^+$ memory[215]. It is also not clear if all $T_H$ subsets equally form memory, with most reports describing $T_H1$-like memory cells, although $T_H2$ and $T_H17$ memory cells have been described[205,216].

Very little is known about the migration patterns and cues used by $CD4^+$ $T_{EM}$ to migrate through the periphery. However, it is clear there are substantial differences compared to memory $CD8^+$ T cells. For example, in skin following cutaneous HSV infection, $CD4^+$ memory T cells are confined to the dermis, while $CD8^+$ memory T cells populate the epidermis[208]. Similarly, intravaginal HSV infection permits localisation of memory $CD4^+$ T cells to the lamina propria and memory $CD8^+$ T cells to the epithelium of the genital tract. In HSV infection, memory $CD4^+$ T cell trafficking to the skin is transient and these cells exit the tissue and recirculate. Chemokine receptor expression on $T_{EM}$ has been reported. In response to EAE immunisation, memory $CD4^+$ T cells express higher levels of CXCR3 and CCR5 than effector T cells[217]. Transfer of these $T_{EM}$ into $Tcr\alpha\beta^{-/-}$ mice given EAE induced greater disease severity than transfer of effector T cells, which was associated with enhanced trafficking to the CNS, suggesting $T_{EM}$ migration was mediated through these axes. We have recently shown that IL-17-producing $CD4^+$ memory cells are present in the lung after S. pneumoniae infection and these cells express high levels of both CCR6 and CCR2, with the proportion of $CCR2^+$ $CCR6^-$ $T_H17$ cells preferentially increasing upon rechallenge[205]. Furthermore, $T_{EM}$ from human PBMCs have also been reported to express CCR10, CCR5, CXCR3, CCR6 and CCR4, suggesting diverse trafficking within the $T_{EM}$ population that may follow similar classification to $T_H$ cells[218]. This was partially supported by analysis of $T_{EM}$ from human cord blood, in which $CXCR3^+$ $T_{EM}$ cells preferentially produced IFNγ, and $CCR6^+$ $T_{EM}$ cells displayed a transcriptional profile biased to a $T_H17$ phenotype, although they could not robustly secrete IL-17[219]. Taken together, it is clear that the signals controlling tissue niche occupancy and recirculation for memory $CD4^+$ T cells are not well understood and there is much still to be learned in this area.

#### *1.5.3.3 $T_{RM}$*

$T_{RM}$ are the most recently discovered subset of memory cells with parabiosis experiments first definitively revealing $CD8^+$ $T_{RM}$ in 2009 and $CD4^+$ $T_{RM}$ in 2011[208,209]. The essential defining property of these cells is that they are resident in peripheral tissues and do not recirculate through the blood or lymph, although the factors promoting their residence are still incompletely understood. $T_{RM}$ cells reported to date display enhanced protective

responses upon reencounter of antigen and due to their peripheral location can have the shortest response time of all memory subsets. $CD4^+$ $T_{RM}$ generated in response to *L. major* infection in the skin are essential for optimal protection against secondary parasite challenge as they enhance recruitment of circulating memory cells through production of CXCL9 and CXCL10[220]. A recent study also demonstrated that $CD4^+$ $T_{RM}$ resident in the spinal cord and dorsal root ganglia (DRG) in response to HSV-2 infection facilitate protection through production of IFNγ which enhances vascular permeability thus allowing access of antiviral antibodies to the brain[169]. Again, $CD4^+$ $T_{RM}$ are much less well described than their $CD8^+$ counterparts, although they generally express a $CD44^{hi}$ $CD62L^{lo}$ $CD11a^{hi}$ $CD69^+$ phenotype[168,209,221]. In contrast to $CD8^+$ $T_{RM}$, CD103 is not considered a marker of $CD4^+$ $T_{RM}$ in neither mice nor humans [222-225].

$CD4^+$ $T_{RM}$ generated in the genital tract in response to intravaginal HSV-2 immunisation do not express CCR7, in support of their restriction from entering lymphatic circulation[168]. However, chemokine signalling is required to maintain their residency in the vaginal parenchyma, as treatment with pertussis toxin results in expulsion into the lumen. Furthermore, neutralisation of CCL5 reduces the numbers of $T_{RM}$ in the vagina and accordingly reduces the protection observed in the presence of these cells. $T_{RM}$ in this model express the CCL5 receptors CCR1 and CCR5 as well as CXCR3, although neutralisation of the CXCR3 ligand CXCL9 does not affect $CD4^+$ $T_{RM}$ residency. $CD4^+$ $T_{RM}$ are also generated in the spinal cord and dorsal root ganglia (DRG) in response to HSV-1 infection, with the ligands for CXCR3, CCR5 and CCR2 expressed in DRG[169]. However, the migratory cues required for entry into these regions remain to be determined.

### 1.5.4 CCR2

CCR2 is defined as an inflammatory chemokine receptor, with its ligands generally being absent at homeostasis but induced upon inflammation[226]. CCR2 binds to the ligands CCL2, CCL7, CCL8 (in humans), CCL12 (in mice), CCL13 and CCL16 (in humans). CCR2 is known mostly for its roles in guiding monocyte trafficking in response to infection and is used to subclassify monocytes, with high expression of CCR2 found on inflammatory monocytes and low CCR2 expression on resident monocytes[227]. Functionally, CCR2 is required for control of monocyte egress from the bone marrow,

with *Ccr2*$^{-/-}$ mice displaying reduced monocyte frequencies in the blood but enhanced levels in the bone marrow[228]. Furthermore, CCR2 is used by monocytes to migrate into inflamed tissues in infection and autoimmune models, with loss of CCR2 in infection models resulting in impaired pathogen control[229-233].

On T cells, CCR2 has been reported to be expressed within subpopulations of Treg cells, with this axis being required for their migration into tumours and arthritic joints[101,234]. In an islet allograft model, CCR2 was expressed on Treg cells and used for their migration between the allograft and the draining lymph nodes, with loss of CCR2 resulting in impaired migration and suboptimal suppression[235]. As mentioned earlier, CCR2 has also been shown to be induced on $T_H17$ cells in EAE, with this receptor being critical for migration of the pathogenic GM-CSF-secreting $T_H17$ subset into the CNS[205]. Indeed, T cell-intrinsic loss of CCR2 reduced disease severity, highlighting the importance of this axis in mediating disease. CCR2 was also shown to be expressed on IL-17-secreting cells in the lung during persistent infection with *S. pneumoniae*, although whether this receptor was required functionally for $T_H17$ cells in infection was not determined[205]. Expression of CCR2 on other T cell subsets has been reported, although the functional significance of this receptor remains unclear[236,237].

## 1.6 The research project

The ability of immune cells to migrate to peripheral sites as well as within distinct lymphoid niches is critical for execution of their effector functions. The chemokine system plays a critical role in this migration, with spatiotemporal regulation of chemokine receptor expression allowing exquisite specificity and control. However, many open questions remain about how expression of certain chemokine receptors affects overall immune function.

### 1.6.1 ACKR4 in solid tumours

Recently approved immunotherapies have emphasised the potency of the immune system's ability to eradicate tumours, but many patients remain unresponsive or develop resistance to current drugs and novel targets are needed to improve efficacy. The CCR7/CCL19/CCL21 axis is critical for many homeostatic functions of the immune system, as well as for the generation of an immune response to tumours. However, the

CCR7 and CCR9 axes have also been implicated to have pro-tumourigenic tumour-intrinsic functions. ACKR4, an atypical chemokine receptor, regulates the bioavailability of the ligands of CCR7 and CCR9, and has been shown to influence multiple CCR7-mediated processes. However, the function of ACKR4 in tumour settings is largely unstudied, particularly regarding how it may influence the immune response to cancer. This has led to the following hypothesis, tested in Chapter 3:

*Hypothesis: ACKR4 regulates the immune response to solid tumours.*

This will be addressed with the following aims:
Aim 1.1: To investigate the effect of ACKR4 deletion in solid tumour models.
Aim 1.2: To investigate if ACKR4 influences the immune response to solid tumours.

### 1.6.2 CCR2 in CD4$^+$ T cells

Memory CD4$^+$ T cells are critical for protection in a number of infectious models as well as correlating with protection in human disease. However, relatively little is known about the establishment and maintenance of memory CD4$^+$ T cells. Furthermore, the migratory cues that guide memory CD4$^+$ T cells to and within effector sites are not well understood. Previous work from our laboratory has identified CCR2 as being enriched on T$_H$17 cells at memory timepoints after *S. pneumoniae* infection, although its function on these cells is unknown. Additionally, CCR2 has been reported to be expressed on other CD4$^+$ T cell subsets but it is unclear if it contributes to their migration and function in inflammatory sites. This has led to the following hypothesis, tested in Chapter 4:

*Hypothesis: CCR2 regulates migration of memory CD4$^+$ T cells in infection.*

This will be addressed with the following aims:
Aim 2.1: To determine the spatiotemporal expression of CCR2 on T$_H$17 cells in response to *S. pneumoniae* infection.
Aim 2.2: To determine the function of CCR2 on T$_H$17 cells in *S. pneumoniae* infection.
Aim 2.3: To investigate the function of CCR2 on other CD4$^+$ T cell subsets in infectious settings.

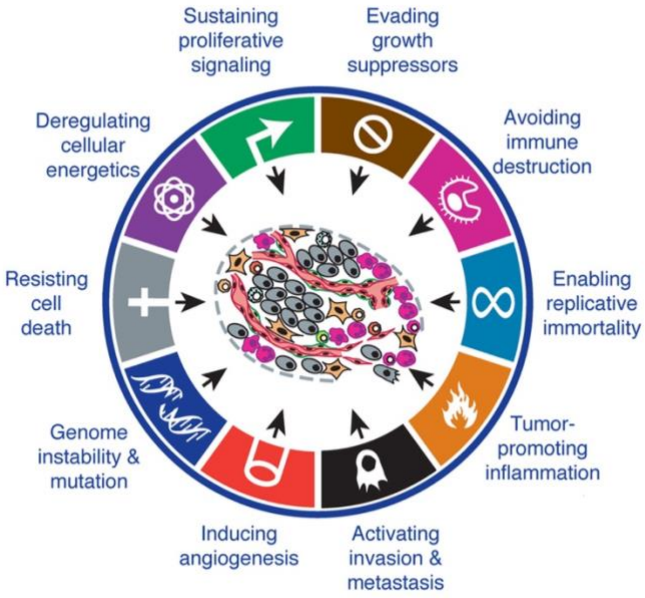



**Figure 1.1: Hallmarks of cancer.**

In order for an aberrant cellular mass to successfully form a malignant tumour, it must acquire certain characteristics. Although these functional characteristics are common amongst multiple distinct tumour types, the mutations and mechanisms by which they are gained are diverse. The uncontrolled proliferation that defines tumour cells is accompanied by dysregulation of growth suppressors, cell cycle checkpoints, and cell death pathways. This proliferative cell mass requires high energy input, with dysregulation of metabolic pathways and an enhanced reliance on glycolysis, as well as induction of new blood vessels to support the growing mass. Successful tumours are also able to evade immune-mediated killing, either through exclusion of cytotoxic cells from the tumour microenvironment or through recruitment of tolerogenic cells. Furthermore, the inflammation generated in response to tumours can also support tumour progression through induction of growth factors and enhancing mutagenesis. Lastly, tumour cells can acquire metastatic capability and migrate to secondary sites, where they may successfully colonise and form metastases. Adapted from [238].

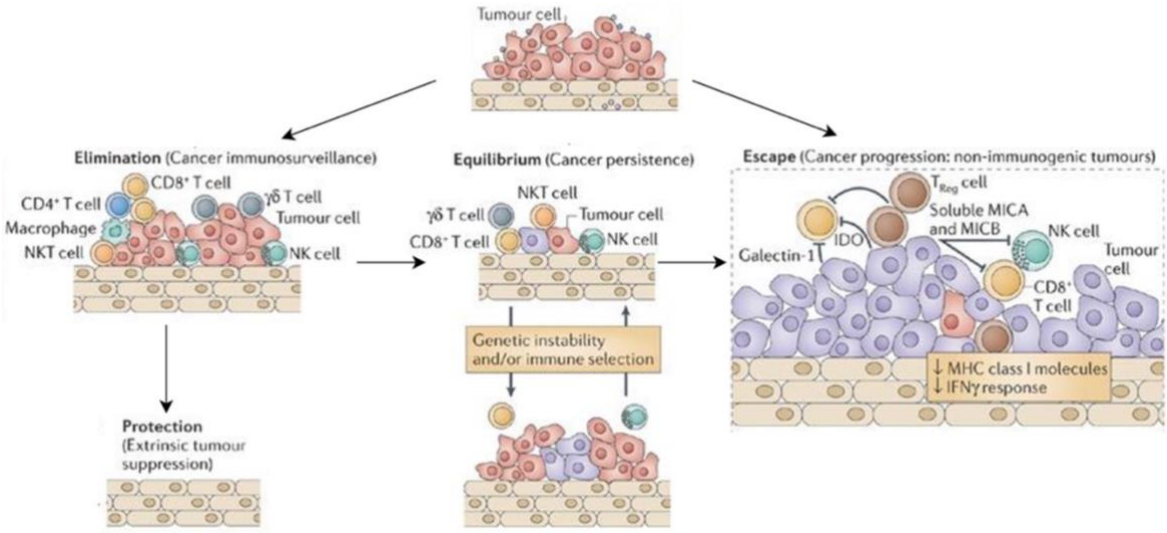



**Figure 1.2: Cancer immunoediting.**

The cancer immunoediting hypothesis consists of three phases of tumour cell–immune cell interactions. The elimination phase is when tumour cells are recognised and destroyed by a range of innate and adaptive lymphocytes, leading to host protection. In the equilibrium phase, an ongoing immune response is able to control but not completely eliminate the tumour. In the escape phase, tumours are able to overcome immune-mediated control and progress into malignant and in some cases metastatic tumours. In all cases, the presence of a tumour-directed immune response exerts a selective pressure, favouring the emergence of tumour antigen-loss variants that may arise as a result of genomic instability. Adapted from [239].

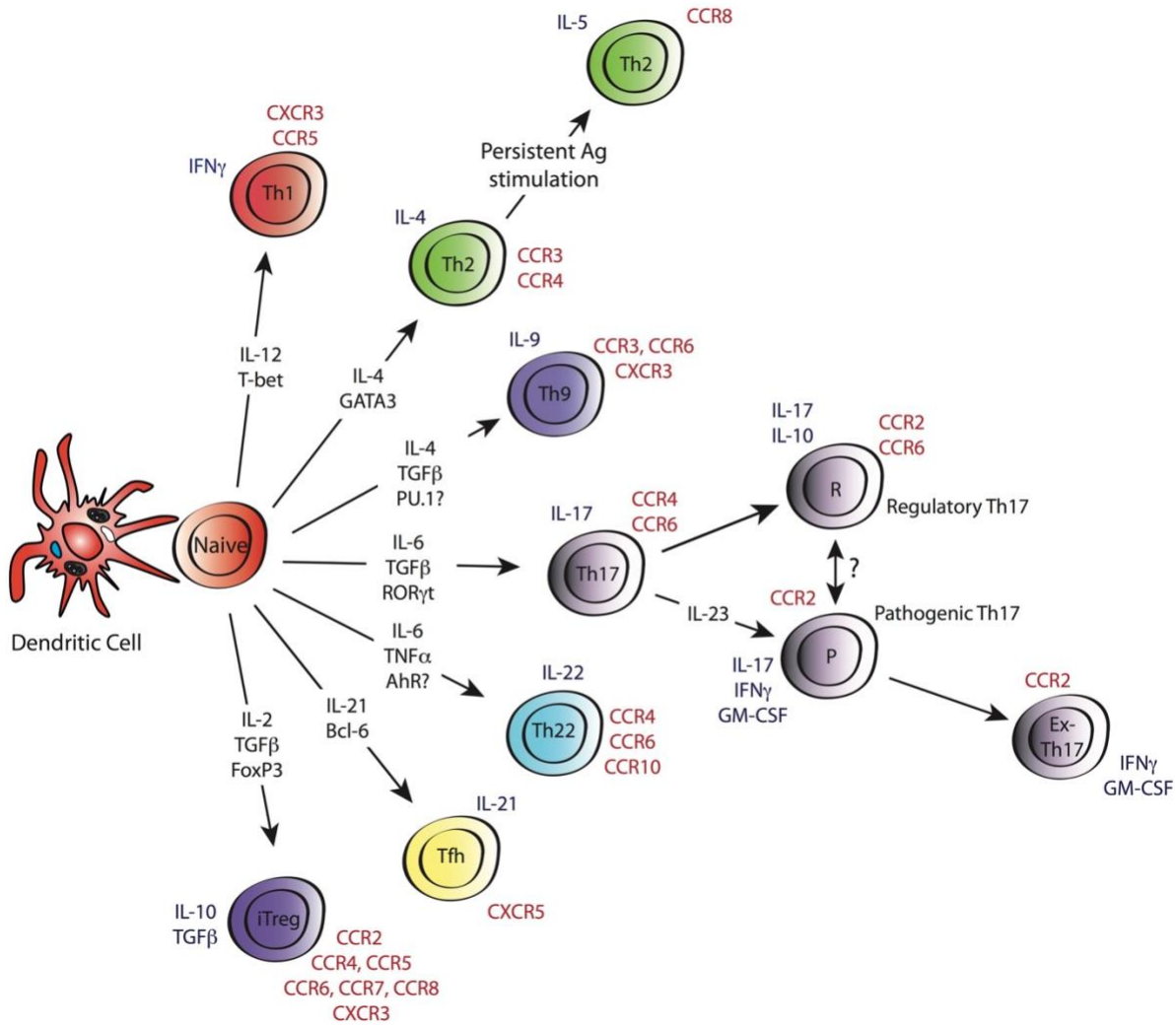



**Figure 1.3: CD4$^+$ T cell subsets are distinguished by distinct chemokine receptor expression and differentiation cues.**

Upon cognate interactions with peptide:MHC II complexes presented by DCs, priming of naïve CD4$^+$ T cells is influenced by the local cytokine milieu. This leads to expression of distinct transcription factors that can induce subset-specific transcriptional profiles, which encompass expression of effector cytokines as well as chemokine receptors that are tailored to enable optimal responses to the initial antigen. The key subsets of $T_H$ cells that have been described are shown, along with the cytokines and transcription factors that drive/maintain their development (black text); characteristic cytokines that they produce (blue text); and the chemokine receptors expressed (red text). From [240].

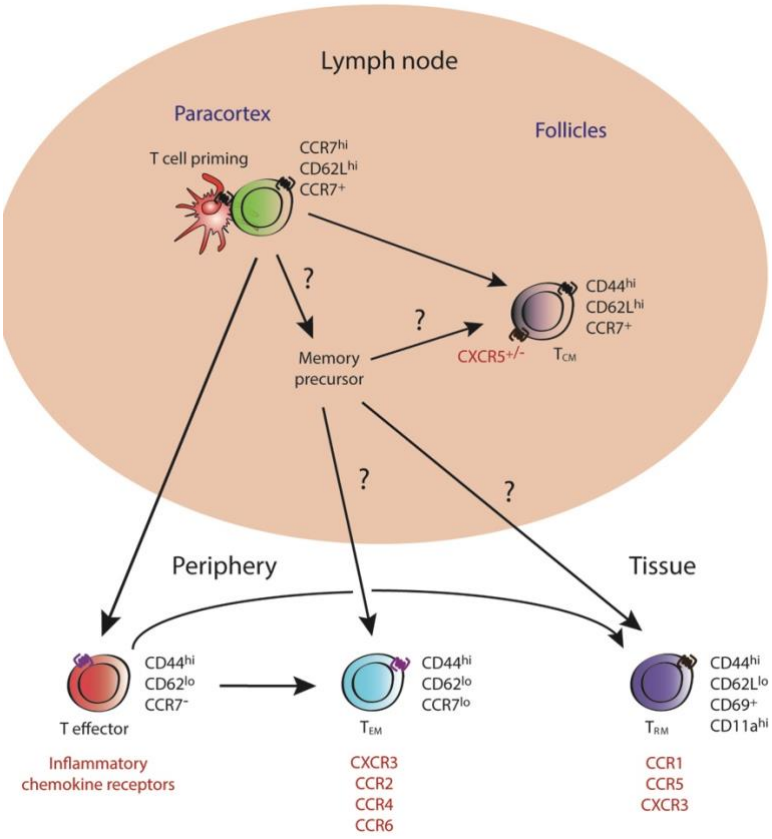



**Figure 1.4: Trafficking of CD4$^+$ T memory cells.**

Long-lived CD4$^+$ memory T cells are classified into three subsets based on their patterns of recirculation. $T_{CM}$ cells are mainly found in the blood and SLO, with expression of CCR7 and CD62L enabling their migration into SLO through HEVs. $T_{EM}$ recirculate through peripheral tissues and can reenter LNs through the afferent lymph. $T_{RM}$ permanently reside in peripheral tissues after infection and are restricted from reentering the circulation. The ontogeny of the different memory subsets is still unclear and markers of memory precursors in the effector phase of the response has not yet been reported, unlike that of CD8$^+$ T cells. At which stage memory fate is conferred remains to be determined. Chemokine receptor alterations between these memory subsets are depicted. Arrows indicate differentiation/transdifferentiation of T cell subsets, ? indicates an as yet unproven differentiation pathway. Adapted from [240].

**Table 1.1 Chemokine receptors, their ligands and atypical receptor-mediated regulation**

| Chemokine receptor | Chemokine ligands | Atypical receptor(s) that regulate axis |
|---|---|---|
| **CCR1** | CCL3, CCL3L1†, CCL5, CCL8†, CCL14†, CCL15, CCL16 CCL23 | ACKR1, ACKR2 |
| **CCR2** | CCL2, CCL7, CCL8†, CCL11, CCL16† | ACKR1, ACKR2 |
| **CCR3** | CCL3L1, CCL5, CCL7, CCL11, CCL13†, CCL14†, CCL15, CCL24, CCL28 | ACKR1, ACKR2 |
| **CCR4** | CCL17, CCL22 | ACKR1, ACKR2 |
| **CCR5** | CCL3, CCL3L1†, CCL4, CCL4L1†, CCL5, CCL8, CCL11, CCL16 | ACKR1, ACKR2 |
| **CCR6** | CCL20 | ? |
| **CCR7** | CCL19, CCL21 | ACKR4 |
| **CCR8** | CCL1, CCL18† | ? |
| **CCR9** | CCL25 | ACKR4 |
| **CCR10** | CCL27, CCL28 | ? |
| **CXCR1** | CXCL6, CXCL7, CXCL8 | ACKR1 |
| **CXCR2** | CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8† | ACKR1 |
| **CXCR3** | CXCL4*, CXCL9, CXCL10, CXCL11, CXCL13 | ACKR3 |
| **CXCR4** | CXCL12 | ACKR3 |
| **CXCR5** | CXCL13 | ACKR4† |
| **CXCR6** | CXCL16 | ? |
| **XCR1** | XCL1, XCL2† | ? |
| **CX3CR1** | CX3CL1, CCL26† | ? |

† Indicates in humans but not mice
* CXCL4 binds to the CXCR3-B isoform of CXCR3

Adapted from [240]

**Table 1.2 Diversity in types of immune responses.**

| Response | Targets | Cell subsets involved | Effector cytokines |
|---|---|---|---|
| **Type 1** | Intracellular pathogens e.g. viruses, bacteria Tumours | $T_H1$ cells, $CD8^+$ T cells, NK cells, NKT cells, ILC1, $T_{FH}$ | IFNγ, TNFα |
| **Type 2** | Parasites Allergens | $T_H2$ cells, mast cells, eosinophils, $T_H9$ cells, ILC2, $T_{FH}$ | IL-4, IL-5, IL-13, IL-9 |
| **Type 17** | Extracellular pathogens | $T_H17$ cells, neutrophils, $T_H22$, $T_{FH}$ | IL-17A, IL-17F, IL-22, GM-CSF |
| **Regulatory** | Inflammation, autoreactive cells | nTreg, iTreg, $T_{FR}$ | TGF-β, IL-10 |

NK: natural killer; NKT: natural killer T; ILC1: innate lymphoid cell; $T_{FH}$: T follicular helper; nTreg: natural regulatory T cell; iTreg: inducible regulatory T cell; $T_{FR}$: T follicular regulatory cell; IFN: interferon; TNF: tumour necrosis factor; IL: interleukin; GM-CSF: granulocyte-macrophage colony stimulating factor; TGF: transforming growth factor.

# CHAPTER 2

# Materials and Methods

# Chapter 2 – Materials and Methods

## 2.1 Mice

C57Bl/6J and B6.SJL Ptprca (Ly5.1) mice were purchased from the Animal Resource Centre (Western Australia), bred at the University of Adelaide animal house or bred at QIMR Berghofer animal house (Queensland). OT-I mice were purchased from the Walter and Eliza Hall Institute (Victoria). *Ccr2*$^{-/-}$, OT-II, MMTV-PyMT and *Ackr4*$^{-/-}$ mice were sourced from Prof. Christian Engwerda (QIMR Berghofer), Dr Kerrilyn Diener (University of Adelaide), Dr Marina Kochetkova (University of Adelaide) and Prof. Rob Nibbs (University of Glasgow), respectively, with these mice bred at the University of Adelaide animal house. In addition, *Ackr4*$^{-/-}$ mice were bred at QIMR Berghofer animal house. *Ccr2*$^{-/-}$ OT-II mice were generated by crossing *Ccr2*$^{-/-}$ mice to OT-II mice, and were bred at the University of Adelaide animal house. OT-II x Ly5.1 mice were generated by crossing OT-II mice to Ly5.1 mice, and were bred at the University of Adelaide animal house. OT-I x Ly5.1 mice were generated by crossing OT-I mice to Ly5.1 mice, and were bred at the University of Adelaide animal house. MMTV-PyMT x *Ackr4*$^{-/-}$ mice were generated by crossing MMTV-PyMT males to *Ackr4*$^{-/-}$ females, and were bred at the University of Adelaide animal house. Mice were housed under specific pathogen-free conditions. Experiments used age-matched and gender-matched mice between 6 and 15 weeks of age. Mice were humanely euthanized by $CO_2$ asphyxiation. All experiments were conducted with the approval of the University of Adelaide Animal Ethics Committee or QIMR Animal Ethics Committee.

## 2.2 *In vivo* techniques

### 2.2.1 Primary tumour growth

Cell lines were harvested, washed twice and resuspended in PBS. Cells were kept on ice until injection. C57Bl/6J mice were anaesthetised by isofluorane (Henry Schein Animal Health) inhalation and 10-20µl was injected into the fourth mammary gland (E0771) or subcutaneously in the ventral flank (B16F10, MC38) using a 50µl glass syringe (Hamilton Company, NV, USA). Tumour sizes were measured every 2 days from day 7 using digital callipers (Mitutoyo, Japan), with tumour size calculated as the multiple of the longest tumour diameter with its perpendicular diameter. Mice were culled if the

tumour diameter exceeded 15mm or tumours became ulcerated. For antibody treatments, mice were injected intraperitoneally with antibodies listed in **Table 2.3**.

### 2.2.2 Haematogenous metastasis

Cell lines were harvested, washed twice and resuspended in PBS. Cells were kept on ice until injection. C57Bl/6J mice were restrained and injected with 100-200µl of cells into the tail vein. Mice were monitored for signs of distress and weight loss, and lung metastases visually counted with use of a dissection microscope. *These experiments were performed by Prof. Mark Smyth.*

### 2.2.3 MCA induction

Male C57Bl/6J mice were injected subcutaneously in the hind flank with the indicated dose of 3-methylcholanthrene (MCA) dissolved in 100µl of sterile corn oil. Mice were monitored for the development of fibrosarcoma for over 200 days. *These experiments were performed by Prof. Mark Smyth.*

### 2.2.4 CCL21 administration

Female C57Bl/6J mice were injected into contralateral sides of the fourth mammary gland with $10^5$ E0771 cells. Beginning on day 7, mice were anesthetised and tumours were injected every 2 days. The left hand side tumour was injected with 3µg CCL21 or $MCP_{ala}$ [241,242], with the right hand side tumour injected with PBS.

### 2.2.5 Adoptive transfers

Splenocytes and inguinal LN were harvested from naïve congenic OT-I or OT-II mice. For naïve OT-I transfers, the percentage of naïve $CD8^+$ T cells in single cell suspensions was determined by flow cytometry and $5x10^6$ unpurified naïve $CD8^+$ T cells were transferred. For naïve OT-II cell transfers, $CD4^+$ T cells were purified using EasySep™ Mouse Naïve $CD4^+$ T cell Isolation Kit (Stem Cell Technologies) as per the manufacturer's instructions. Purity was determined to be at least 85% pure by flow cytometry prior to transfer, with the number of cells transferred indicated in text. Mice were placed under a heat lamp, restrained and injected with 100-200µl of cell suspension via the tail vein.

### 2.2.6 Intravascular labelling

3μg of fluorescently labelled antibody was diluted in sterile PBS (**Table 2.3**). Mice were placed under a heat lamp, restrained and 200μl of diluted antibody injected into the tail vein. Mice were culled by $CO_2$ asphyxiation 5 min after injection.

### 2.2.7 Streptococcus pneumoniae infection

D39 stocks were concentrated to $3.33x10^8$ cfu/ml by centrifugation at 13,000rpm for 5 min. Female C57Bl6/J mice were anesthetised with pentobarbitone (Ilium) and 30μl of bacteria was administered intranasally. Mice were allowed to recover on a 37°C heatpad. Mice were monitored every 4 hours for the first 72 hours, and subsequently once daily for signs of distress, and culled according to ethical guidelines. To measure bacterial load, the nasal wash and lung were collected as described (**Sections 2.3.4, 2.3.6**). Nasal tissue was harvested by cutting the upper palate from nose to below the eye, removing the nose and skin. Lung and nasal tissue were homogenised in 1ml PBS in a ceramic bead tube on a Precellys 24 homogeniser (Bertin Technologies). Lysates and nasal wash were subsequently serially diluted in serum broth, plated in duplicate on blood agar plus gentamycin and incubated for at least 16 hours at 37°C, 5% $CO_2$. *Some experiments performed in collaboration with Dr. Richard Harvey.*

### 2.2.8 Influenza infection

Influenza viral stocks were diluted in sterile PBS to 313 $TCID_{50}$/ml (X31-$OVA_{323-339}$; PR/8-$OVA_{323-339}$) or 228 $TCID_{50}$/ml (X31). Female C57Bl6/J mice were anesthetised with pentobarbitone (Ilium) and 32μl of virus was administered intranasally. Mice were allowed to recover on a 37°C heat pad and given wet food and soft bedding. Mice were weighed daily and monitored for signs of distress, with mice culled if weight loss exceeded 20% of their original weight.

### 2.2.9 Bone marrow chimeras

Ly5.1 mice were lethally irradiated with 1000 Rads. The next morning, bone marrow was isolated from the femur and tibia of 8-16 week old donor mice of the indicated genotypes and red blood cells were lysed with sterile MRCLB (**Section 2.7.2**). $4\text{-}5x10^6$ total bone marrow cells were injected into the tail vein of irradiated mice and allowed to reconstitute for at least 8 weeks. Mice were weighed and monitored daily for signs of distress during

the acute phase of reconstitution. *Irradiation was performed in collaboration with Dr. Josef Nguyen.*

## 2.3 Cell isolation

### 2.3.1 Lymphoid organs

Spleens were prepared by mechanical disruption through a 70µm filter (BD Biosciences), incubated in MRCLB for 5 min at 37°C and washed in PBS. Inguinal and mediastinal LN were digested for 15 min in 200µl digestion medium (**Section 2.7.3**) at 37°C, passed through a 70µm filter (BD Biosciences) and washed in PBS. All cells were centrifuged at 300 x g for 4-10 min.

### 2.3.2 Tumours

Tumours were excised, manually minced into small pieces and incubated in digestion medium for 40-120 min at 37°C, with mixing every 20 min. Tumour homogenates were passed through a 70µm filter (BD Biosciences), washed in PBS, incubated in MRCLB for 5 min at 37°C and washed again in PBS.

### 2.3.3 Mammary glands

The fourth (inguinal) pair of mammary glands were manually minced into small pieces and digested in mammary gland digestion medium (**Section 2.7.4**) for 3.5 hr at 37°C with agitation. Suspensions were then washed in DMEM, resuspended in a solution of 6U/ml Dispase II (Sigma-Aldrich) and 300U/ml DNase I (Sigma-Aldrich) and incubated for 15 min at 37°C. After vigorous pipetting, cells were washed with DMEM supplemented with 2% FBS, incubated in MRCLB for 5 min at 37°C and washed again in PBS.

### 2.3.4 Lungs

Immediately following asphyxiation, the chest cavity was opened and mice were perfused with PBS through the left ventricle. Lung lobes were harvested, manually minced into small pieces and incubated in digestion medium for 40-60 min at 37°C, with mixing every 20 min. Lung homogenates were passed through a 70µm filter (BD Biosciences), washed in PBS, incubated in MRCLB for 5 min at 37°C and washed again in PBS.

### 2.3.5 Peripheral blood

Immediately following asphyxiation, the chest cavity was opened through the ribs without cutting into the peritoneum. The right ventricle was cut and blood was collected into heparin-coated Vacutainer tubes (BD Biosciences). Blood was incubated in MRCLB for 10 min at 37°C and washed twice in PBS.

### 2.3.6 Nasal wash

The chest cavity was opened and the trachea exposed, with a small incision made part way into the trachea. A syringe fitted with an Insyte Autoguard Catheter (BD Biosciences) was inserted into the tracheal opening directed away from the lung, with 500µl of PBS injected and collected through the nostrils.

### 2.3.7 Bronchiolar alveolar lavage

The chest cavity was opened and the trachea exposed, with a small incision made part way into the trachea. A syringe fitted with an Insyte Autoguard Catheter (BD Biosciences) was inserted into the tracheal opening directed towards the lung, with three sequential washes of 1ml PBS collected.

## 2.4 *Ex vivo* techniques

### 2.3.1 Flow cytometry

Single cell suspensions were plated into round-bottom 96-well trays (Corning) at $2x10^6$ cells per well, with the exception of tumour suspensions in which $8x10^5$-$1x10^6$ cells were plated. For intracellular cytokine staining, cells were first incubated in 50µl of restimulation medium for 4 hr at 37°C, 5% $CO_2$. After centrifugation at 400 x g for 2 min, cells were resuspended in 50µl of FACS buffer containing a 1:1000 dilution of LIVE/DEAD™ Fixable Near-IR Stain (ThermoFisher Scientific) plus 10µg mouse gamma globulin (mγg) (Rockland) and incubated for 5 min at room temperature in the dark, to exclude dead cells and block Fc receptors. To this, 10µl of a cocktail of antibodies was added (**Table 2.1**). If an unlabelled antibody was included in the stain, this was incubated for 20-60 min at 4°C, washed once in FACS buffer, stained with secondary antibody preadsorbed with mγg and normal mouse serum (NMS) for 20 min at 4°C, washed once in FACS buffer and blocked with rat gamma globulin for 15 min at 4°C. Cells were stained with directly conjugated and biotinylated antibodies for 20 min at 4°C

and washed with FACS buffer. For biotinylated antibodies, this was followed by incubation with fluorescently-conjugated streptavidin for 15 min at 4°C. For intracellular cytokine staining or transcription factor staining, the BD Cytofix/CytoPerm™ kit (BD Biosciences) or the Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher Scientific) were used respectively according to the manufacturer's instructions. All stains were washed and resuspended in 1% PFA (**Section 2.7.7**) and stored at 4°C in the dark. Flow cytometry data was acquired on a LSR II, FACSAria, or LSRFortessa (all BD Biosciences) and analysed using FlowJo software (BD Biosciences).

### 2.3.2 RNA isolation and qPCR

Mammary gland cells were isolated and stained as described above and sorted on a FACSAria. Total RNA was extracted from sorted cells using the RNeasy Micro Kit (Qiagen) with on-column DNase treatment, and reverse-transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). qPCR was conducted using LightCycler480 SYBR Green I Master mix (Roche Applied Science) on a LightCycler480 instrument (Roche Applied Science). All procedures were carried out as per the manufacturer's instructions. Cycle threshold (CT) values were determined by the second derivative method and relative gene expression was calculated using the formula $2^{-[CT(target)–CT(reference)],}$ where the reference gene was *Rplp0*. The melting curve of each product was also analysed to confirm the specificity of the product. Primer sequences used for qPCR are listed in **Table 2.5**.

### 2.3.3 ELISA

Supernatants from digested tumours were collected, protease inhibitors (Sigma) added and stored at -80°C. Capture antibodies were diluted in ELISA coating buffer (**Section 2.7.8**), applied to 96-well high binding plates (Corning) and incubated overnight at 4°C. All further incubations were at RT and all washes performed in PBS/Tween (**Section 2.7.11**). The next day, wells were washed 4 times and blocked with 200µl ELISA blocking buffer (**Section 2.7.9**) for 2 hr. Plates were washed 4 times and protein standards and samples diluted in ELISA diluent (**Section 2.7.10**) were added and incubated for 2 hr. Plates were washed 4 times and detection antibodies diluted in ELISA diluent were added and incubated for 1 hr. Plates were washed 4 times and incubated with streptavidin-HRP (Rockland) diluted in ELISA diluent for 30 min. Plates were

washed 6 times, developed with TMB (ThermoFisher Scientific), stopped with 1M orthophosphoric acid and read at 450nm on a Biotrak II spectrophotometer (Amersham Biosciences).

### 2.3.4 Genotyping

Tail tips from weaned pups were digested overnight at 55°C in tail tip lysis buffer, heat shocked at 95°C for 5 min, centrifuged and stored at 4°C. PCR was conducted using MyTaq (Bioline) according to the manufacturer's instructions, using 0.5µl of tail tip DNA per reaction. Annealing temperatures for CCR2, PyMT and ACKR4 were 60°C. Primers used for PCR are listed in **Table 2.5**. PCR products were separated on 1.5% agarose gel and visualised using GelRed (Life Technologies) in a ChemiDoc imager (Bio-Rad).

## 2.5 Tissue culture

### 2.5.1 E0771 cells and derivatives

The murine mammary carcinoma cell line E0771 and its derivate of E0771-OVA were kindly provided by Prof. Mark Smyth and A/Prof. Phil Darcy (Peter MacCallum Cancer Centre), respectively. Cells were maintained in DMEM (Gibco) supplemented with 10% foetal bovine serum (FBS), 1mM sodium pyruvate (Gibco) and 1x penicillin/streptomycin (Gibco) and cultured at 37°C in 5% $CO_2$. Cells were passaged every 2-3 days by rinsing the flasks with sterile PBS and dissociating cells with trypsin/EDTA in PBS for approximately 3 min at 37°C.

### 2.5.2 B16F10 cells

The murine melanoma cell line B16F10 was kindly provided by Prof. Mark Smyth. Cells were maintained in RPMI 1640 (Gibco) supplemented with 10% FBS and 1x penicillin/streptomycin (Gibco) and cultured at 37°C in 5% $CO_2$. Cells were passaged every 2-3 days by rinsing the flasks with sterile PBS and dissociating cells with trypsin/EDTA in PBS for approximately 5 min at 37°C.

### 2.5.3 Other cell lines

The murine prostrate carcinoma cell line RM-1, murine carcinoma cell line MC38 and murine lung carcinoma cell line 3LL were kindly provided and maintained by Prof. Mark

Smyth. Cells were maintained in complete DMEM or RPMI 1640 and cultured at 37°C in 5% $CO_2$. Cells were passaged every 2-3 days by rinsing the flasks with sterile PBS and dissociating cells with trypsin/EDTA in PBS for approximately 5 min at 37°C.

## 2.6. In vitro techniques

### 2.6.1 Generation of genetically modified Streptococcus pneumoniae strains

*S. pneumoniae* strains expressing $OVA_{SIINFEKL}$ and $OVA_{323-339}$ were constructed by inserting these sequences into the surface protein, pneumococcal surface protein A (PspA), analogous to that described previously[243]. Mutants were constructed in the D39 background using the complete transformation medium (CTM) method and the Janus cassette[244-246]. The Janus cassette was inserted in *pspA* of a D39 derivative with a *rpsL* mutation to generate D39*rpsL-pspA*::Janus. Insertion of the Janus cassette suppresses the *rpsL* $Strep^R$ phenotype and confers kanamycin resistance. Primers for generation of these mutants are listed in **Table 2.5**. The construct to insert the Janus into *pspA* was generated by overlap-extension PCR using primers TTM051(D39)F and RHPspA(J)R for the upper flanking product, primers RHPspA(J)F and TTM059 for the lower flanking product, and primers JanusF and JanusR for the Janus cassette. The $Kan^R/Strep^S$ D39*rpsLΔpspA*::Janus strain was then transformed with a *pspA* construct containing either the $OVA_{SIINFEKL}$ or $OVA_{323-339}$ epitope nucleotide sequence. These constructs were generated by overlap-extension PCR using primers TM051(D39)F and OTIR, and OTIF and TTM059 for $OVA_{SIINFEKL}$ and primers TM051(D39)F and OTIIR, and TTM059 for $OVA_{323-339}$. Successful transformants were selected by plating on blood agar with streptomycin. The inserted sequence was confirmed by DNA sequencing, and PspA expression confirmed by Western blot analysis. *Generation of these strains were performed by Dr. Richard Harvey.*

### 2.6.2 Generation of Streptococcus pneumoniae stocks

Stocks of the genetically modified *S. pneumoniae* strains were generated from a streak plate and inoculated into 10ml of serum broth for 3-6 hr at 37°C, 5% $CO_2$ without agitation. Once the $OD_{600}$ reached 0.20-0.22, bacteria was aliquoted and stored in -80°C without additives. A frozen aliquot was thawed to determine bacterial titre, with serial dilutions in serum broth plated in duplicate on blood agar with gentamycin and incubated for at least 16 hours at 37°C, 5% $CO_2$.

### 2.6.3 Generation of influenza A virus stocks

X31 stocks were kindly provided by Dr. Mohammed Alsharifi (University of Adelaide). X31-OVA$_{323-339}$ and PR/8-OVA$_{323-339}$ were generated by Dr. Paul Thomas (St. Jude Children's Research Hospital, USA) and an aliquot was kindly provided by Dr Katherine Kedzierska (Doherty Institute, Victoria). Stocks of influenza A virus were made by inoculating approximately $10^5$ PFU in the allantoic cavity of 10-day old embryonated chicken eggs, which were subsequently incubated for 48 hours at 37°C. The allantoic fluid was collected, clarified through centrifugation and aliquoted for storage at -80°C. The absence of bacterial contamination was confirmed by plating virus stocks on to LB agar plates in the absence of antibiotic selection.

### 2.6.4 Quantification of influenza A viral titre

The concentration of influenza A virus stocks were determined by analysing the 50% tissue culture infectious dose (TCID$_{50}$) on Madin-Darby Canine Kidney (MDCK) cells. 5x$10^4$ MDCK cells were plated into a round-bottom 96-well tray in DMEM supplemented with 1% FBS, 1x penicillin/streptomycin (Gibco) and 2mM L-glutamine (Gibco) and incubated overnight at 37°C, 5% $CO_2$. The following day, medium was replaced with 100µl of DMEM supplemented with 1% FBS, 1x penicillin/streptomycin, 2mM L-glutamine and 0.08% trypsin. 100µl of serial dilutions of viral stocks were made across the plate in replicates of 7 and incubated for 72 hr at 37°C, 5% $CO_2$. 50µl of 0.6% packed chicken red blood cells in saline were added to each well and allowed to agglutinate for 30 min at room temperature. TCID$_{50}$ was determined using the Spearman and Karber algorithm (Hierholzer & Killington, Virol Methods Manual 96). Viral concentrations are listed in **Table 2.6**.

## 2.7 General reagents

### 2.7.1 PBS

1x PBS was either purchased from University of Adelaide Technical Services Unit (TSU) or prepared by diluting 20x PBS (TSU) with MilliQ water.

### 2.7.2 MRCLB

255mM $NH_4Cl$ (AnalaR) solution and 170mM TRIS (pH 7.65) were mixed at a 9:1 ratio and pH was adjusted to 7.2.

### 2.7.3 Digestion medium

DMEM (Gibco) was supplemented with 5% FBS, 2.5mM $CaCl_2$, 10mM HEPES (Gibco), 1x penicillin/streptomycin (Gibco), 30U/ml DNase I (Sigma-Aldrich) and 1mg/ml collagenase IA (Sigma-Aldrich).

### 2.7.4 Mammary gland digestion medium

DMEM (Gibco) was supplemented with 2% FBS, 1x penicillin/streptomycin (Gibco) and 1x collagenase/hyaluronidase (StemCell Technologies).

### 2.7.5 Restimulation medium

1x Incomplete IMDM powder (Gibco) was reconstituted according to the manufacturer's instructions and supplemented with 10% FCS, 200mM L-glutamine, 1x penicillin/streptomycin and 54pM β-mercaptoethanol (Sigma). For restimulation, 20ng/ml phorbol-12-myristate 13-acetate (PMA; Sigma-Alrich), 1nM ionomycin (Life Technologies) and GolgiStop (1:1500 dilution; BD Biosciences) were added.

### 2.7.6 FACS buffer

PBS was supplemented with 2% FBS and 0.04% $NaN_3$ and stored at 4°C.

### 2.7.7 Paraformaldehyde

4% (w/v) paraformaldehyde (PFA) was prepared by dissolving PFA in PBS at 55°C with stirring overnight, and diluted to 1% PFA in PBS. These solutions were stored at 4°C for short-term storage or -20°C for long-term storage.

### 2.7.8 ELISA Coating buffer

3.03g of $Na_2CO_3$ and 6.0g of $NaHCO_3$ were resuspended in 1L of MilliQ water and pH was adjusted to 9.6.

### 2.7.9 ELISA Blocking buffer

PBS was supplemented with 3% bovine serum albumin (BSA).

### 2.7.10 ELISA Diluent

PBS was supplemented with 1% BSA.

### 2.7.11 PBS/Tween

PBS was supplemented with 0.05% (v/v) polyoxyethylene-sorbitan monolaurate (Tween 20; Sigma-Aldrich) and mixed thoroughly at RT.

### 2.7.12 Tail Tip Lysis Buffer (TTLB)

100mM Tris-HCl pH 8.5, 5mM EDTA, 0.2% SDS, 200nM NaCl and 100ug/ml proteinase K (Roche).

### 2.7.13 Serum broth

Nutrient broth was obtained from TSU and supplemented with 5% horse serum and 5μg/ml gentamycin.

### 2.7.14 Blood agar

Oxoid blood agar base (TSU) with 5% horse blood and 5μg/ml gentamycin.

### 2.7.15 Antibodies and staining reagents

Antibodies, streptavidin conjugates and tetramers used in flow cytometry, ELISA and *in vivo* studies are listed in **Tables 2.1-2.4**.

### 2.7.16 Oligonucleotides

Primers used for genotyping and quantitative PCR (qPCR) are listed in **Table 2.5**. All primers were purchased from Sigma-Aldrich and stocks were reconstituted with nuclease-free water to 100μM, and further diluted to a working concentration of 20μM.

## 2.8 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 7.0 for Mac OS X or version 7.02 for Windows. An unpaired t-test was used when comparing two sets of unpaired, continuous data with a normal distribution and similar standard deviations. An unpaired t-test with Welch's correction was used when comparing two sets of unpaired, continuous data with a normal distribution and unequal variances. A paired t-test was

used when comparing two sets of paired, continuous data with a normal distribution. A Wilcoxon matched-pairs signed rank test was used when comparing two sets of paired, continuous data with a non-normal distribution. A Mann-Whitney test was used when comparing two sets of discrete quantitative data with a non-normal distribution. A one-way ANOVA was used when comparing three or more groups of continuous data with approximately normal distribution, with one dependent and one independent variable. A two-way ANOVA was used when comparing three or more groups of continuous data with approximately normal distribution, with one dependent and two independent variables. A Mantel-Cox logrank test was used to compare survival distributions of two samples.

**Table 2.1. Primary antibodies and tetramers used for flow cytometry.**

| Specificity | Conjugate | Clone | Concentration | Source |
|---|---|---|---|---|
| **B220** | BV421 | RA3-6B2 | 0.67 µg/ml | BD Biosciences |
| **B220** | PE-CF594 | RA3-6B2 | 0.67 µg/ml | BD Biosciences |
| **CCR2** | - | MC21 | 3.64 µg/ml | Prof. M. Mack |
| **CD103** | PE | 2E7 | 0.67 µg/ml | eBioscience |
| **CD11a** | biotin | M17/4 | 1.39 µg/ml | BD Biosciences |
| **CD11b** | PE-Cy7 | M1/70 | 0.56 µg/ml | BD Biosciences |
| **CD11b** | BV510 | M1/70 | 0.56 µg/ml | Biolegend |
| **CD11c** | PerCP-Cy5.5 | N418 | 1.11 µg/ml | Biolegend |
| **CD11c** | BV786 | HL3 | 1.11 µg/ml | BD Biosciences |
| **CD140α** | BV421 | APA5 | 0.67 µg/ml | BD Biosciences |
| **CD172a** | PE/Dazzle594 | P84 | 0.67 µg/ml | Biolegend |
| **CD1d-αGalCer** | V421 | - | 1:1000 | Prof. Dale Godfrey |
| **CD24** | PE | M1/69 | 0.67 µg/ml | BD Biosciences |
| **CD31** | FITC | MEC 13.3 | 2.78 µg/ml | BD Biosciences |
| **CD3ε** | biotin | 145-2C11 | 1.67 µg/ml | eBioscience |
| **CD3ε** | FITC | 145-2C11 | 2.08 µg/ml | BD Biosciences |
| **CD3ε** | PE-Cy7 | 145-2C11 | 0.83 µg/ml | eBioscience |
| **CD4** | PE | H129.19 | 0.67 µg/ml | BD Biosciences |
| **CD4** | PE | GK1.5 | 0.67 µg/ml | BD Biosciences |
| **CD4** | BUV395 | GK1.5 | 0.67 µg/ml | BD Biosciences |
| **CD4** | PerCP-Cy5.5 | RM4-5 | 0.67 µg/ml | BD Biosciences |
| **CD4** | Alexa Fluor 647 | RM4-5 | 0.67 µg/ml | BD Biosciences |
| **CD4** | V450 | RM4-5 | 0.67 µg/ml | BD Biosciences |
| **CD4** | BV786 | RM4-5 | 0.67 µg/ml | BD Biosciences |
| **CD8α** | PE | 53-6.7 | 0.67 µg/ml | BD Biosciences |
| **CD8α** | PE-Cy7 | 53-6.7 | 0.67 µg/ml | BD Biosciences |
| **CD8α** | Alexa Fluor 647 | 53-6.7 | 0.67 µg/ml | BD Biosciences |
| **CD8α** | BV510 | 53-6.7 | 0.67 µg/ml | BD Biosciences |
| **CD8β** | FITC | 554973 | 1.67 µg/ml | BD Biosciences |
| **CD44** | FITC | IM7 | 1.67 µg/ml | BD Biosciences |
| **CD44** | V450 | IM7 | 0.67 µg/ml | BD Biosciences |
| **CD44** | BV711 | IM7 | 0.67 µg/ml | BD Biosciences |
| **CD45** | PE | 30-F11 | 0.67 µg/ml | BD Biosciences |
| **CD45** | FITC | 30-F11 | 1.67 µg/ml | BD Biosciences |
| **CD45.1** | biotin | A20 | 1.67 µg/ml | Biolegend |
| **CD45.1** | PE | A20 | 0.67 µg/ml | BD Biosciences |
| **CD45.1** | Alexa Fluor 647 | A20 | 1.67 µg/ml | Biolegend |
| **CD45.1** | FITC | A20 | 1.67 µg/ml | BD Biosciences |
| **CD45.1** | BV421 | A20 | 0.67 µg/ml | BD Biosciences |
| **CD45.1** | PerCP-Cy5.5 | A20 | 0.67 µg/ml | eBioscience |

| | | | | |
|---|---|---|---|---|
| **CD45.2** | APC | 104 | 0.67 µg/ml | BD Biosciences |
| **CD45.2** | PE | 104 | 0.67 µg/ml | BD Biosciences |
| **CD45.2** | V450 | 104 | 0.67 µg/ml | BD Biosciences |
| **CD45.2** | FITC | 104 | 1.67 µg/ml | BD Biosciences |
| **CD49f** | APC | GoH3 | 0.83 µg/ml | eBioscience |
| **CD62L** | PE | MEL-14 | 0.67 µg/ml | BD Biosciences |
| **CD62L** | APC | MEL-14 | 0.67 µg/ml | BD Biosciences |
| **CD69** | PECy7 | H1.2F3 | 0.67 µg/ml | BD Biosciences |
| **CTLA-4** | APC | UC10-4F10-11 | 1.11 µg/ml | BD Biosciences |
| **CXCR5** | purified | 2G8 | 2.78 µg/ml | BD Biosciences |
| **CXCR5** | Biotin | 2G8 | 4.17 µg/ml | BD Biosciences |
| **F4/80** | FITC | BM8 | 2.78 µg/ml | eBioscience |
| **Foxp3** | Alexa Fluor 488 | MF23 | 1.33 µg/ml | BD Biosciences |
| **Foxp3** | PerCPCy5.5 | FJK-16s | 1.33 µg/ml | eBioscience |
| **Foxp3** | Alexa Fluor 647 | MF23 | 1.33 µg/ml | BD Biosciences |
| **gp38** | biotin | 8.1.1 | 2.08 µg/ml | eBioscience |
| **GzmB** | Alexa Fluor 647 | GB11 | 4µl/sample | Biolegend |
| **H-2K$^b$ SIINFEKL** | APC | - | 1:400 | Dr. Stephen Turner; conjugated in-house |
| **IFNγ** | FITC | XMG1.2 | 3.33 µg/ml | BD Biosciences |
| **IFNγ** | PE | XMG1.2 | 1.33 µg/ml | BD Biosciences |
| **IFNγ** | PECy7 | XMG1.2 | 1.33 µg/ml | BD Biosciences |
| **IL-17** | PE | TC11-18H10 | 1.33 µg/ml | BD Biosciences |
| **IL-17** | V450 | TC11-18H10 | 1.33 µg/ml | BD Biosciences |
| **Ki67** | PE-Cy7 | SolA15 | 1.11 µg/ml | eBioscience |
| **Lag3** | BB515 | C9B7W | 1.11 µg/ml | BD Biosciences |
| **Ly6C** | PE | AL-21 | 0.56 µg/ml | BD Biosciences |
| **Ly6G** | - | 1A8 | 1.39 µg/ml | BD Biosciences |
| **MHC-II I-A$^b$** | APC | AF6-120.1 | 0.83 µg/ml | BD Biosciences |
| **MHC-II I-A/I-E** | biotin | 2G9 | 2.08 µg/ml | BD Biosciences |
| **NK1.1** | PE | PK136 | 1.11 µg/ml | BD Biosciences |
| **Nrp1** | BV421 | 3E12 | 0.10 µg/ml | Biolegend |
| **PD-1** | PE-Cy7 | J43 | 0.83 µg/ml | eBioscience |
| **T-bet** | PE | 4B10 | 1.33 µg/ml | eBioscience |
| **Tim3** | PE | 5D12 | 0.83 µg/ml | BD Biosciences |
| **TNFα** | FITC | MP6-XT22 | 3.33 µg/ml | eBioscience |
| **Vα2 TCR** | APC | B20.1 | 0.67 µg/ml | BD Biosciences |
| **XCR1** | BV421 | ZET | 0.67 µg/ml | Biolegend |

**Table 2.2. Secondary antibodies and streptavidin conjugates used for flow cytometry.**

| Specificity | Conjugate | Concentration | Source |
|---|---|---|---|
| **Rat IgG** | Alexa Fluor 647 | 10 µg/ml | Molecular Probes |
| **Streptavidin** | Alexa Fluor 647 | 2.5 µg/ml | Biolegend |
| **Streptavidin** | BV421 | 2.5 µg/ml | BD Biosciences |
| **Streptavidin** | BV510 | 2.5 µg/ml | BD Biosciences |
| **Streptavidin** | PerCPCy5.5 | 2.5 µg/ml | BD Biosciences |

**Table 2.3. Reagents for *in vivo* use.**

| Specificity | Conjugate | Clone | Dose | Source |
|---|---|---|---|---|
| **CD4** | PE | GK1.5 | 3µg | BD Biosciences |
| **CD4** | Alexa Fluor 647 | RM4-5 | 3µg | BD Biosciences |
| **CD4** | FITC | GK1.5 | 3µg | BD Biosciences |
| **CD8α** | PE | 53.6-7 | 3µg | BD Biosciences |
| **CD8β** | – | 53.6-7 | 100µg | BioXCell |
| **CD137** | – | 3H3 | 100µg | BioXCell |
| **CTLA4** | – | UC10-4F10 | 250µg | BioXCell |
| **PD-1** | – | RMP1-14 | 250µg | BioXCell |

**Table 2.4. Reagents used for ELISA.**

| Reagent | Conjugate | Final concentration (µg/ml) | Source |
|---|---|---|---|
| **anti-CCL19** | purified | 2 | R&D Systems |
| **anti-CCL19** | biotin | 0.2 | R&D Systems |
| **anti-CCL21** | purified | 2 | R&D Systems |
| **anti-CCL21** | biotin | 0.05 | R&D Systems |
| **anti-CCL25** | purified | 2 | R&D Systems |
| **anti-CCL25** | biotin | 0.2 | R&D Systems |
| **Streptavidin** | HRP | 0.1 | Rockland |

**Table 2.5. Sequences of primers.**

| Primer name | Sequence (5′-3′) |
|---|---|
| **qPCR primers** | |
| ***Rplp0* F** | TGC AGA TCG GGT ACC CAA CT |
| ***Rplp0* R** | ACG CGC TTG TAC CCA TTG A |
| ***Ackr4* F** | AAT GCT AGG TGC ACT CCC ATC T |
| ***Ackr4* R** | GCC GAT TTC CAG CAT CTG A |
| **Genotyping primers** | |
| ***Ccr2* F** | CCA CAG AAT CAA AGG AAA TGG |
| ***Ccr2* R** | CCA ATG TGA TAG AGC CCT GTG |
| ***Ackr4* p1 [Set 1]** | TTT CTT CCA CGT TCT GTC TCT G |
| ***Ackr4* p2 [Set 1]** | GCT CAT CAA GAT GCC CAA CA |
| ***Ackr4* p3 [Set 1]** | TTT GGC AAA GAA TTC ACT CCT C |
| ***Ackr4* p1 [Set 2]** | TGC TGG TGA GCT CTG GGT TC |
| ***Ackr4* p2 [Set 2]** | CCC TAG ATG CAT GCT CGA CG |
| ***Ackr4* p1 [Set 3]** | AAT CGC CAC AAC TAC GGA GTT C |
| **MMTV-PyMT F** | GGA AGC AAG TAC TTC ACA AGG G |
| **MMTV-PyMT R** | GGA AAG TCA CTA GGA GCA GGG |
| ***S. pneumoniae* mutagenesis primers** | |
| **TTM051(D39) F** | CTT TGG GCA GTA GTG AGA AC |
| **RHPspA(J) R** | CAT TAT CCA TTA AAA ATC AAA CGG AGT TTT TTC TAA TTC AGC |
| **RHPspA(J) F** | AGA GAC CTG GGC CCC TTT CCG AAG CTG ACC TTA AGA AA |
| **TTM059** | GGTCTG ATC CTT GCC ATT GTC |
| **Janus F** | CCG TTT GAT TTT TAA TGG ATA ATG |
| **Janus R** | AGA GAC CTG GGC CCC TTT CC |
| **OTI R** | CAA TTT TTC AAA ATT AAT AAT AGA AGT TTT TTC TAA TC AGC |
| **OTI F** | TCT ATT ATT AAT TTT GAA AAA TTG GAA GCT GAC CTT AAG |
| **OTII R** | TAA TTT CAG CAT GAG CAG CAT GAA CAG CTT GAG AAA TAG TTT TTT CTA ATT CAG C |

**Table 2.6. Influenza stock titres.**

| Strain | Stock concentration ($TCID_{50}$/ml) |
|---|---|
| **X31** | $2.28x10^5$ |
| **X31-$OVA_{323-339}$** | $3.13x10^5$ |
| **PR/8-$OVA_{323-339}$** | $8.48x10^7$ |

# CHAPTER 3

# The role of ACKR4 in regulating anti-tumour immune responses

# Chapter 3: The role of ACKR4 in regulating anti-tumour immune responses.

## 3.1 Introduction

The atypical chemokine receptor ACKR4 has previously been shown to regulate the bioavailability of the CCR7 ligands, CCL19 and CCL21, as well as the CCR9 ligand, CCL25[139,193]. The CCR7 and CCR9 axes have critical roles in directing many homeostatic immune functions, and accordingly ACKR4 has been implicated as a regulator of some of these pathways[140,141,144]. In tumour settings, CCR7 has emerged as an important axis controlling the anti-tumour immune response, as well as intrinsically in tumour cell metastasis and survival. However, there have been very limited reports on how ACKR4 may influence tumour progression, and its role in regulating tumour-directed immune responses is unknown. In this chapter, the importance of ACKR4 in regulating anti-tumour immune responses was assessed in multiple murine tumour models.

## 3.2 ACKR4 promotes tumour growth in a range of in vivo tumour models

To begin to dissect a potential role for ACKR4 in tumour biology, ACKR4-deficient mice were utilised[140]. Previous reports have highlighted the importance of CCR7 and its ligands in breast cancer[121,122,247], and we and others have reported that over-expression of ACKR4 in orthotopic or xenograft breast cancer models results in reduced tumour growth [145-147]. Thus, the role of ACKR4 in regulating the immune response to breast cancer was studied. For this, the E0771 mammary carcinoma cell line, syngeneic to C57Bl/6 (B6) mice, was injected orthotopically into wildtype (WT) and ACKR4-deficient mice. The E0771 model displays a triple negative, basal-like phenotype, which is associated with poor prognosis and reduced treatment options in human patients[248,249]. In this model, ACKR4-deficiency is restricted to the host. E0771 tumour growth was significantly reduced in ACKR4-deficient animals (mean size at day 21 42.8±5.1mm$^2$ in *Ackr4*$^{-/-}$ versus 63.2±8.1mm$^2$ in WT mice), as measured by tumour size over time **(Fig. 3.1 A).** Similarly, the weight of tumours in *Ackr4*$^{-/-}$ mice at day 21 was significantly reduced (236.2±30.2 mg) compared with tumours grown in WT mice (444.9±90.5 mg) (**Fig. 3.1 B)**, the ethical endpoint of the experiment. To support this finding, the MMTV-PyMT

transgenic model of breast cancer was used, in which the Polyoma virus middle T antigen is driven by the mouse mammary tumour virus long terminal repeat[250]. This model more faithfully recapitulates the stepwise progression of human breast cancer, as well as many morphological features and expression of biomarkers[251,252]. MMTV-PyMT mice on a B6 background were crossed with *Ackr4*$^{-/-}$ mice to generate PyMT$^{+}$ *Ackr4*$^{-/-}$ mice and the development of tumours was assessed. Palpable tumours were detected with median onset of 81 days in PyMT$^{+}$ B6 mice, but were significantly delayed in PyMT$^{+}$ *Ackr4*$^{-/-}$ mice with median onset of 88 days **(Fig. 3.1 C)**. Furthermore, the total combined weight of mammary glands at twenty weeks of age was significantly reduced in PyMT$^{+}$ *Ackr4*$^{-/-}$ mice (882.6 ± 58.0mg) compared with PyMT$^{+}$ B6 mice (1095 ± 58.7mg), indicative of reduced tumour burden (**Fig. 3.1 D)**. ACKR4 expression has also been reported in the skin[253], and so to determine if ACKR4 may also play a role in regulation of tumour growth in other cancer types, a fibrosarcoma model was used, in which tumours are induced with 3-methylcholanthrene (MCA). With both high (300μg) and medium (25μg) dose MCA treatments, *Ackr4*$^{-/-}$ mice showed significantly increased survival compared with WT mice (**Fig. 3.1 E, F**).

To assess a potential role for ACKR4 in regulating metastatic disease, lung colonisation capability was measured in haematogenous metastases experiments. In these experiments, B16F10 melanoma cells, 3LL lung carcinoma cells and RM1 prostrate cells were used, which are all syngeneic to C57Bl/6 mice. Two different doses of each cell line were injected intravenously into the tail vein of WT and *Ackr4*$^{-/-}$ mice **(Fig. 3.2)**. In both doses of each cell line, lung colonisation was significantly reduced in *Ackr4*$^{-/-}$ mice. Thus, in multiple models of primary tumour growth and lung metastasis, loss of host ACKR4 reduces tumour progression.

## 3.3 ACKR4 controls chemokine abundance within the mammary gland and TME

Given that a known biological role for ACKR4 is to regulate the bioavailability of its ligands CCL19, CCL21 and CCL25, the reduced tumour growth in ACKR4-deficient mice may be a result of dysregulation of these chemokines. To test if chemokine abundance at the site of tumour inoculation was altered by deletion of ACKR4, naïve mammary glands from mature virgin female mice were analysed **(Fig. 3.3 A)**. The levels

of CCL19 and CCL25 in mammary glands were below the limits of detection in both WT and *Ackr4*$^{-/-}$ mice. However, the level of CCL21 protein was significantly increased in *Ackr4*$^{-/-}$ glands (11.5±1.0 pg/mg) compared with WT glands (3.44±0.1 pg/mg), indicating that loss of ACKR4 leads to increased abundance of CCL21, consistent with its known role as a scavenger of CCL21[140]. To assess whether this difference was maintained or disrupted in a tumour setting, end-point E0771 tumours were harvested and similarly analysed **(Fig. 3.3 B)**. CCL19 levels in E0771 tumours were again below the limit of detection, whilst CCL25 levels were low and unaltered between tumours in WT and *Ackr4*$^{-/-}$ mice. The concentration of CCL21 was significantly increased in tumours from *Ackr4*$^{-/-}$ mice (30.5±8.1 pg/mg) compared with tumours from WT mice (5.52±2.2 pg/mg), again indicating that ACKR4 is important for normal regulation of CCL21 abundance. Furthermore, although the concentration of CCL21 in tumours from WT mice was similar to that of in naïve WT mammary glands, in *Ackr4*$^{-/-}$ mice CCL21 levels were further increased in tumours compared with naïve mammary glands, indicating that the tumour leads to increased abundance of CCL21, which is only apparent in the absence of ACKR4. These data suggest that the reduced tumour growth in *Ackr4*$^{-/-}$ mice may potentially be a result of altered CCL21 abundance in these animals.

The hyperabundance of CCL21 within the mammary gland of ACKR4-deficient mice implies that one or more cell types within the mammary gland may express ACKR4 and act to control CCL21 abundance at this site. Although ACKR4 transcript has been detected in a wide range of tissues[143], analysis of mammary glands has not been reported to date. Currently, no antibodies with high specificity against murine ACKR4 exist, with commercially available clones displaying reactivity to *Ackr4*$^{-/-}$ mice (Cameron Bastow, personal communication). As an alternate approach, major cell populations from mammary glands were sorted and ACKR4 transcript detected by quantitative PCR **(Fig. 3.4)**. Resident immune cells, including myeloid cells, $CD4^+$ and $CD8^+$ T cells, were sorted on the basis of CD45 expression. Of the $CD45^-$ non-immune compartment, the luminal and basal epithelial cell populations were distinguished by differential expression of CD24 and CD49f[254], while a subpopulation of the remaining $CD24^-$ $CD49f^-$ stromal cells expressed CD140α and gp38, indicative of a fibroblast phenotype. Abundant transcript of ACKR4 was found in luminal epithelial cells, which line the milk ducts in

mammary glands, with no or minimal expression detected within the other compartments **(Fig. 3.4)**.

## 3.4 ACKR4-deficient tumours have altered composition of tumour-infiltrating immune cells.

The increased CCL21 levels within *Ackr4*$^{-/-}$ tumours led to the hypothesis that CCL21 dysregulation may enhance the tumour-directed immune response. This is because one of the main functions of CCL21 is to recruit CCR7-expressing immune cells and thus altered CCL21 abundance may influence the extent of immune cell infiltration of tumours. It was hypothesised that these changes in immune cell infiltration may lead to more effective anti-tumour immunity, which would explain the reduced tumour growth in *Ackr4*$^{-/-}$ mice. Furthermore, unpublished data taken from melanoma tumours with artificially reduced expression of ACKR4 suggested that when ACKR4 expression is reduced, enhanced CD8$^{+}$ T cell-dependent anti-tumour immune responses result (C.E. Whyte et al., data not shown). To begin to address this, E0771 tumours from WT and *Ackr4*$^{-/-}$ mice were harvested and infiltrating immune cells were analysed by flow cytometry. A large proportion of the immune infiltrate in E0771 tumours from WT mice were myeloid cells **(Fig. 3.5)**, with monocytic CD11b$^{+}$ Ly6C$^{+}$ Ly6G$^{-}$ cells making up approximately 30% of the tumour infiltrate, and being marginally but significantly increased in number in tumours from *Ackr4*$^{-/-}$ mice **(Fig. 3.5 C)**. These cells have a heterogeneous phenotype, likely consisting of monocytes at various stages of maturation, as well as monocytic MDSCs, a class of immature monocytes that develop in tumour settings with the capacity to suppress T cell responses[255]. Granulocytic CD11b$^{+}$ Ly6C$^{+}$ Ly6G$^{+}$ myeloid cells, which may encompass granulocytic MDSCs as well as anti-tumourigenic neutrophils, were unaltered in *Ackr4*$^{-/-}$ mice, as were CD11b$^{+}$ Ly6C$^{lo}$ F4/80$^{+}$ macrophages **(Fig. 3.5 A, B)**. A range of innate lymphocytes have been reported to influence tumour growth, including NK cells and CD1d-restricted natural killer T (NKT) cells, with subsets of these cells capable of expressing CCR7[256,257]. Intratumoural accumulation of NK1.1$^{+}$ CD3$^{-}$ NK cells was unaltered in *Ackr4*$^{-/-}$ mice, as were NK1.1$^{+}$ CD3$^{+}$ NKT cells **(Fig. 3.6 A-C)**. Only a proportion of NKT cells express NK1.1 and type I iNKT cells are more reliably detected by their capacity to bind CD1d-αGalCer tetramers[258], although frequency of CD3$^{+}$ tetramer$^{+}$ type I NKT cells were also unaffected in *Ackr4*$^{-/-}$ mice **(Fig. 3.6 D, E)**.

Although ACKR4 expression is generally restricted to the non-haematopoietic compartment, we have recently reported the expression of ACKR4 on recently activated B cells, with this receptor important in controlling their correct localisation and proliferation[259]. In line with this, an increased frequency of B cells in tumours from *Ackr4*$^{-/-}$ mice was detected compared with WT tumours, although the overall frequency within the tumour remained relatively low **(Fig. 3.7 B)**. Foxp3$^{+}$ CD4$^{+}$ Treg cells are a major contributor to suppression of anti-tumour responses and are enriched within most mouse and human tumours[260]. However, frequencies of intratumoral Treg cells were unaltered between WT and *Ackr4*$^{-/-}$ mice. Foxp3$^{-}$ effector CD4$^{+}$ cells were also equivalent in number between WT and *Ackr4*$^{-/-}$ mice, however there was a significant increase in percentage of activated CD44$^{hi}$ CD8$^{+}$ T cells in tumours from *Ackr4*$^{-/-}$ mice.

## 3.5 *Ackr4*$^{-/-}$ develop an enhanced anti-tumour CD8$^{+}$ T cell response

CD8$^{+}$ T cells are one of the most critical cell types for executing anti-tumour immunity, and are capable of specifically recognising and killing tumour cells. Thus, to test if the increased number of CD8$^{+}$ T cells in tumours from *Ackr4*$^{-/-}$ mice were responsible for the reduced tumour growth, CD8$^{+}$ depletion experiments were performed **(Fig. 3.8 A)**. Depleting antibody towards CD8β was administered intraperitoneally on two consecutive days, with E0771 inoculation into the fourth mammary fat pad on the second day. Depleting antibody was subsequently administered every seven days to eliminate newly generated CD8$^{+}$ T cells. Depletion of CD8$^{+}$ T cells in WT mice did not affect tumour growth, suggesting that although CD8$^{+}$ T cells are present within WT tumours, they are ineffective at controlling tumour growth **(Fig. 3.8 B, C)**. However, depletion of CD8$^{+}$ T cells in *Ackr4*$^{-/-}$ mice significantly increased tumour burden to a similar size as tumours in WT mice, suggesting that enhanced CD8$^{+}$ responses in *Ackr4*$^{-/-}$ mice are responsible for the reduced tumour growth in these mice.

Intratumoural CD8$^{+}$ T cells were characterised further to see if there were any differences in the quality or phenotype of CD8$^{+}$ T cells generated, in addition to the increased quantity seen in *Ackr4*$^{-/-}$ mice. Upon *ex vivo* PMA restimulation, there was a higher frequency of IFNγ-production from CD8$^{+}$ T cells taken from *Ackr4*$^{-/-}$ mice compared with WT mice, consistent with increased anti-tumour activity **(Fig. 3.9 A)**. The proportion of granzyme B-expressing CD8$^{+}$ T cells was unaltered between WT and

*Ackr4*$^{-/-}$ mice, indicating a similar ability to secrete cytotoxic granules **(Fig. 3.9 B).** Expression of PD-1 was significantly enhanced in CD8$^+$ T cells from *Ackr4*$^{-/-}$ mice, indicative of enhanced exhaustion of these cells **(Fig. 3.9 C)**. However, expression of other exhaustion markers such as TIM-3 and LAG-3 were unaltered **(Fig. 3.9 D, F)**. Furthermore, PD-1$^+$ TIM-3$^+$ and PD-1$^+$ LAG-3$^+$ CD8$^+$ T cell frequencies were also unaltered **(Fig. 3.9 E, G)**, with these cells having been described as more highly exhausted subsets, compared with cells positive for single markers[261,262]. CTLA-4, another checkpoint inhibitor and target for immunotherapy, was not detected on CD8$^+$ T cells in this model **(Fig. 3.9 H)**.

CD8$^+$ T cells are reported to lack expression of ACKR4, and the altered CD8 response in ACKR4-deficient mice is almost certainly cell-extrinsic. To assess whether the enhanced response generated in *Ackr4*$^{-/-}$ mice was a result of increased CD8$^+$ T cell proliferation, expression of Ki67, a marker of proliferating or recently divided cells, was measured **(Fig. 3.10)**[263]. There was a significant increase in geometric MFI of Ki67 among tumour-infiltrating CD8$^+$ T cells, and a trend towards increased frequency of Ki67$^+$ CD8$^+$ T cells (p=0.077). Conversely, there was a significant reduction in Ki67 expression by CD8$^+$ T cells in the draining lymph node (**Fig 3.10 D-F)**. This is consistent with previous reports indicating reduced T cell priming in *Ackr4*$^{-/-}$ LN[140,144]. Taken together, these data suggest that the enhanced anti-tumour CD8$^+$ T cell response in *Ackr4*$^{-/-}$ mice may be a result of enhanced intratumoural proliferation of CD8$^+$ T cells, rather than a result of enhanced priming in draining lymph nodes. However, these data were obtained from advanced tumours where there is already a robust CD8$^+$ T cell presence in *Ackr4*$^{-/-}$ mice, rather from an earlier time-point in which the response is being generated. Furthermore, the bulk of the CD8$^+$ T cells present in the dLN are irrelevant for the tumour antigen, making conclusions about tumour-specific responses difficult in this model.

## 3.6 Priming of tumour-specific CD8$^+$ T cells is unaltered in *Ackr4*$^{-/-}$ mice.

To address these issues, a variant of the E0771 cell line expressing the model antigen ovalbumin (OVA) was used, with endogenous CD8$^+$ T cells specific for the immunodominant SIINFEKL peptide analysed with the use of a class I MHC H-2K$^b$-tetramer. Tumours were harvested at day 10, which is prior to a significant difference in

tumour size between WT and *Ackr4*$^{-/-}$ mice becoming apparent. At this time, there was already a substantial increase in OVA-specific CD8$^+$ T cells in tumours from *Ackr4*$^{-/-}$ mice, however there was no difference in cell frequency or number of these cells in the dLN **(Fig. 3.11)**. This indicates that the alterations leading to the enhanced CD8$^+$ T cell response in *Ackr4*$^{-/-}$ mice may occur outside of priming in the draining lymph nodes.

Although priming of anti-tumour CD8$^+$ T cells is thought to predominantly occur within SLO, priming of naïve CD8$^+$ T cells within tumours has also been reported. Tumours were able to support activation of naïve tumour-specific CD8$^+$ T cells in splenectomised mice with *in utero*-ablated LNs, with these CD8$^+$ T cells acquiring normal effector functions such as IFNγ production and cytotoxic capacity[59]. Furthermore, naïve CD8$^+$ T cell entry into tumours has been reported to occur in a CCR7-dependent manner with adhesion and intravasation through CCL21-coated PNAd$^+$ LN-like vasculature[264]. Thus, whether the increased levels of intratumoural CCL21 in *Ackr4*$^{-/-}$ mice could support naïve T cell recruitment and thus potentially *in situ* priming was tested. Congenically-labelled OT-I cells, which are specific for the OVA$_{\text{SIINFEKL}}$ peptide in the context of H-2K$^b$, were adoptively transferred into WT or *Ackr4*$^{-/-}$ recipients 10 days after inoculation with the parental E0771 cell line. In this model, the transferred CD8$^+$ T cells are not specific for tumour-derived antigen and so entry into the tumour only occurs in an antigen-independent manner. 24 hours after transfer, mice were injected intravenously with fluorescently labelled antibody against CD8β in order to label cells within the vasculature. Although naïve OT-I cells were found to be present both within tumour vasculature as well as in the tumour parenchyma by this time, there was no difference in total number of naïve OT-I cells or parenchymal naïve OT-I cells between tumors from WT and *Ackr4*$^{-/-}$ mice **(Fig. 3.12 C, D)**. Furthermore, there was also no difference in the ratio of naïve OT-I cells present within the parenchyma versus the vasculature of WT or *Ackr4*$^{-/-}$ tumours. One possibility for this lack of difference is that the specialised vasculature required to support naïve T cell entry had not yet developed at this early stage of tumour growth, and so in a separate experiment naïve OT-I cells were transferred into mice with tumours inoculated 16 days prior. However, there was again no difference in total number or ratio of naïve OT-I recruitment in these mice **(Fig. 3.12 F-H)**, indicating the increased abundance of CCL21 in tumours from *Ackr4*$^{-/-}$ mice does not mediate

enhanced intratumoural recruitment of naïve $CD8^+$ T cells, at least under the conditions investigated.

## 3.7 DCs are enhanced within tumours of *Ackr4*$^{-/-}$ mice.

Given that priming in the dLN as well as intratumoural priming of naïve T cells did not appear to account for the enhanced $CD8^+$ T cell response in *Ackr4*$^{-/-}$ mice, the loss of ACKR4 appears to be affecting $CD8^+$ T cells after they have been activated. Although naïve $CD8^+$ T cells express high levels of CCR7, upon activation they downregulate this receptor[265], making a direct role for CCL21 in recruiting activated $CD8^+$ T cells unlikely. However, DCs also rely on CCR7-mediated signalling and play critical roles in controlling the magnitude of anti-tumour $CD8^+$ T cell responses. In their canonical role, DCs take up tumour-derived antigen and upregulate CCR7 to migrate to CCL21-expressing lymphatic vessels, where they can egress from tissue and migrate to dLN to prime T cells. However, recent reports have demonstrated that subsets of DCs also have important roles within the tumour microenvironment, with intratumoural $CD103^+$ DCs being critical for the cytotoxic activity of transferred tumour-specific $CD8^+$ T cells[21]. Furthermore, $CD103^+$ DCs have been reported to be the predominant source of CXCL9 and CXCL10 in tumours, thus mediating recruitment of activated $CD8^+$ T cells into the tumour[95]. The classification of DCs based on their functional capacity, transcriptional ontogeny and surface marker expression has recently been updated with unsupervised analysis of high-parameter flow and mass cytometric data, allowing more clear delineations between subsets to become apparent[266]. Within conventional DCs (cDCs), cDC1s are marked by their expression of XCR1 and their potent ability to cross-present antigen to prime $CD8^+$ T cell responses, while $CD172a^+$ cDC2s are considered to be more important for the generation of $CD4^+$ T cell help[266,267]. $CD103^+$ DCs in the skin have been described as a sub-population of cDC1s, consistent with their ability to cross-present antigen and prime $CD8^+$ T cell responses, although it is not entirely clear from previous reports whether the $CD103^+$ DCs described in tumours are synonymous with $XCR1^+$ cDC1s. Indeed, some cDC2s have been reported to express low levels of XCR1[268]. To assess whether disrupted CCL21 gradients within *Ackr4*$^{-/-}$ tumours would affect DC migration, the presence of $Ly6C^-$ MHC-$II^+$ $CD11c^+$ DCs were analysed within E0771 tumours. Total DCs were significantly increased in both number and frequency within day 15 tumours from *Ackr4*$^{-/-}$ mice. Within this population, cDC2s were much more

abundant than cDC1s, although both subsets were significantly increased within tumours from *Ackr4*$^{-/-}$ mice **(Fig. 3.13)**. Furthermore, CD103$^{+}$ DCs were also augmented in tumours from *Ackr4*$^{-/-}$ mice compared with WT mice. A recent report has indicated that CD103$^{+}$ Ly6C$^{+}$ myeloid-derived cells can also potently activate anti-tumour CD8$^{+}$ T cells[269]. However, these cells were unaltered between tumours from WT and *Ackr4*$^{-/-}$ mice.

The frequencies of DC subsets were also analysed in the dLN, where migratory DCs can be distinguished from resident DCs based on their increased expression of MHC-II, which is upregulated upon their activation in tissues **(Fig. 3.14)**. Total migratory DCs or resident DCs were not altered in number or frequency in *Ackr4*$^{-/-}$ dLN, with migratory cDC1s and cDC2s also being unchanged. However, CD103$^{+}$ migratory DCs were reduced in *Ackr4*$^{-/-}$ dLNs, which along with their enhanced presence in tumours from *Ackr4*$^{-/-}$ mice is suggestive of decreased egress from tumours.

## 3.8 CCL21 promotes intratumoural DC accumulation

To assess whether the enhanced retention of DCs within the tumour was a direct result of excess CCL21 seen in *Ackr4*$^{-/-}$ tumours, which presumably disrupts homeostatic CCL21 distribution, it was tested whether exogenous administration of CCL21 into tumours in WT mice would phenocopy this result. To test this, E0771 tumours were established in contralateral sides of the fourth mammary gland. From day 7, at which point tumours had just become palpable, 3ug of CCL21 or MCP$_{ala}$, a non-functional control peptide, were injected intratumourally into the left tumour, with the PBS vehicle control injected into the right tumour[241,242]. In CCL21-treated tumours, levels of CCL21 were similar or slightly enhanced compared with tumours taken from *Ackr4*$^{-/-}$ mice **(Fig 3.15 B, Fig. 3.3 B)**. Intratumoural administration of CCL21 did not lead to a systemic increase in chemokine abundance, as measured in the serum **(Fig. 3.15 C)**. Paired analysis of tumours weights at day 17 showed no difference between CCL21-treated tumours and PBS-treated tumours, nor the corresponding MCP$_{ala}$ controls. However, in tumours where sufficient numbers of cells could be obtained to perform robust flow cytometric analysis, the frequency of DCs within CCL21-treated tumours was significantly increased over the PBS control, but not in mice treated with MCP$_{ala}$ **(Fig. 3.15 D)**. This shows that disrupted CCL21 levels in tumours are sufficient to induce an enhanced DC presence within

tumours, but this alone did not lead a reduction in tumour growth. This may be due to other alterations in *Ackr4*$^{-/-}$ mice that were not mimicked by intratumoural CCL21 administration in WT mice, or due to the altered kinetics of CCL21 disruption, with CCL21 levels and DC retention unaltered until tumours were already established.

## 3.9 ACKR4 deficiency improves responsiveness to immunotherapies

The presence of CD8$^+$ T cells within the tumour parenchyma has been reported to be conducive to responsiveness to immunotherapies that enhance the quality of CD8$^+$ T cell responses. Given that there is an increase in CD8$^+$ T cells within tumours from *Ackr4*$^{-/-}$ mice, it was tested whether loss of ACKR4 would increase the efficacy of these immunotherapies. To assess this, WT or *Ackr4*$^{-/-}$ mice given E0771 tumours were treated with an agonistic antibody targeting CD137. CD137, also known as 4-1BB or TNFRS9, is a potent costimulatory molecule for T cells and NK cells, with stimulation of this receptor leading to inhibited tumour growth in a variety of models[270,271]. The efficacy of this therapy in the E0771 model has not been previously reported, and WT mice treated with anti-CD137 showed significantly reduced tumour growth (mean tumour size 82.04mm$^2$ ± 18.34 at day 26) compared with the control group (156.89mm$^2$ ± 7.8). However, tumour growth was even more strongly inhibited in *Ackr4*$^{-/-}$ mice (103.08mm$^2$ ± 4.95 in control vs 17.25mm$^2$ ± 7.65 in anti-CD137), indicating enhanced efficacy in the absence of ACKR4.

To extend these findings, responsiveness to anti-PD-1 and anti-CTLA-4 combination therapy was tested in the B16F10 melanoma model, a widely-used tumour model for immunotherapy. As previously reported, combination of anti-PD-1 and anti-CTLA-4 reduced B16F10 tumour growth in WT mice **(Fig 3.17 A)**. There was a modest reduction in tumour growth in *Ackr4*$^{-/-}$ mice treated with control antibody, but this was further reduced when treated with the dual therapy, with the extent of inhibition induced by the therapy significantly increased in *Ackr4*$^{-/-}$ animals compared with their WT counterparts. Similar results were seen with the immunogenic MC38 colon carcinoma model, with *Ackr4*$^{-/-}$ animals displaying minimal tumour burden when treated with anti-PD-1 **(Fig. 3.17 B)**. Indeed, 4 out of 5 mice in this group displayed complete tumour regression, with 2 of these mice remaining tumour-free up to 63 days post-inoculation.

## 3.10 Conclusion

The findings in this chapter establish ACKR4 as an important regulator of anti-tumour immunity. In the mammary gland, ACKR4 is expressed by luminal epithelial cells where it acts to control local bioavailability of CCL21. Loss of ACKR4 is protective, with reduced tumour growth seen in multiple orthotopic and transgenic models of cancer. This reduction in growth is the result of an increased tumour-directed $CD8^+$ T cell response, with enhanced accumulation of $CD8^+$ T cells and increased production of IFNγ. Priming of $CD8^+$ T cells in *Ackr4*$^{-/-}$ mice is unaffected, suggesting the enhanced response is a result of increased recruitment or proliferation of activated $CD8^+$ T cells within the TME. Furthermore, ACKR4 regulates intratumoural DC numbers, with loss of ACKR4 promoting DC retention within tumours due to dysregulation of the CCL21/CCR7 axis. Loss of ACKR4 also enhances the efficacy of immunotherapies against CD137, PD-1 and CTLA-4. This work reveals a previously unappreciated level of regulation of tumour-directed immune responses, and provides a possible novel target for enhancing the efficacy of current immunotherapies.

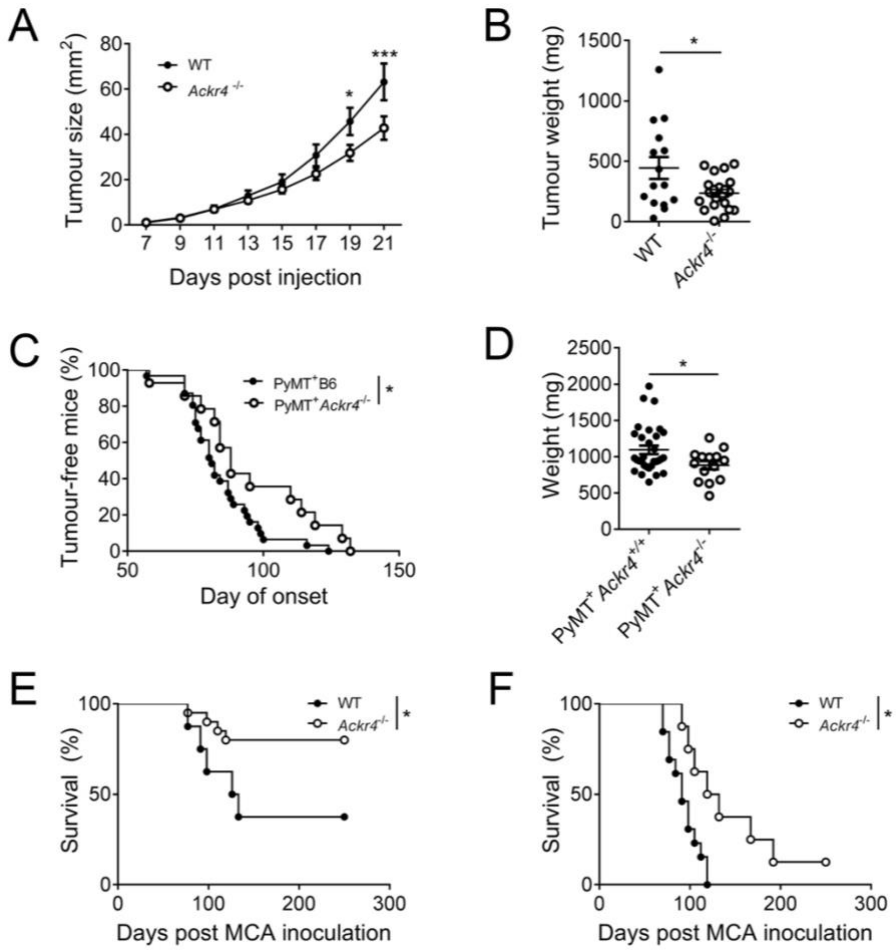



**Figure 3.1: Loss of ACKR4 inhibits tumour growth *in vivo*.**

**(A)** Growth curves and **(B)** weights of E0771 mammary tumours injected into WT or *Ackr4*$^{-/-}$ mice in the fourth mammary fat pad. Data are pooled from three independent experiments; n=16 WT mice, 21 *Ackr4*$^{-/-}$mice, (A) two-way ANOVA, (B) unpaired t-test. **(C)** Day of tumour onset for MMTV-PyMT B6 (Ackr4$^{+/+}$) and *Ackr4*$^{-/-}$mice; Mantel-Cox test. **(D)** Total weight of mammary glands in MMTV-PyMT B6 and *Ackr4*$^{-/-}$at 20 weeks of age; unpaired t-test. **(E-F)** Survival curve of WT and *Ackr4*$^{-/-}$mice inoculated with (E) 25µg or (F) 300µg methylcholanthrine (MCA) in the hind flank; Mantel-Cox test. Mean ± SEM. * p≤0.05, *** p≤0.001. *(E-F) Experiment designed and executed by Prof. Mark Smyth.*

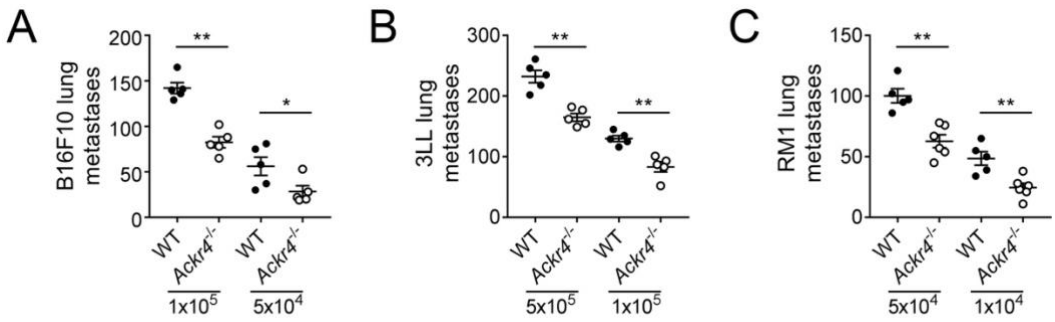



**Figure 3.2: Loss of ACKR4 inhibits haematogenous metastasis *in vivo*.**

WT or *Ackr4*$^{-/-}$ mice were injected with **(A)** B16F10 melanoma, **(B)** 3LL lung carcinoma or **(C)** RM1 prostrate cells intravenously at the indicated doses and the number of lung metastases were counted; n=5-6 mice, Mann-Whitney test. Mean ± SEM. * $p \leq 0.05$, ** $p \leq 0.01$. *Experiment designed and executed by Prof. Mark Smyth.*

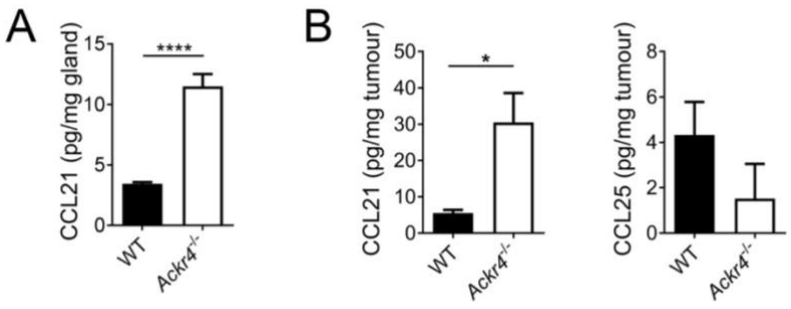



**Figure 3.3: Loss of ACKR4 alters chemokine abundance in the mammary gland.** Concentrations of CCL19, CCL21 and CCL25 as detected by sequential ELISA. **(A)** Chemokine abundance in naïve mammary glands of virgin 12-week old female mice. n=7. **(B)** Chemokine abundance in endpoint E0771 tumours. n=6. Data representative of two independent experiments, unpaired t-test. Mean ± SEM. * $p \leq 0.05$, **** $p \leq 0.0001$.

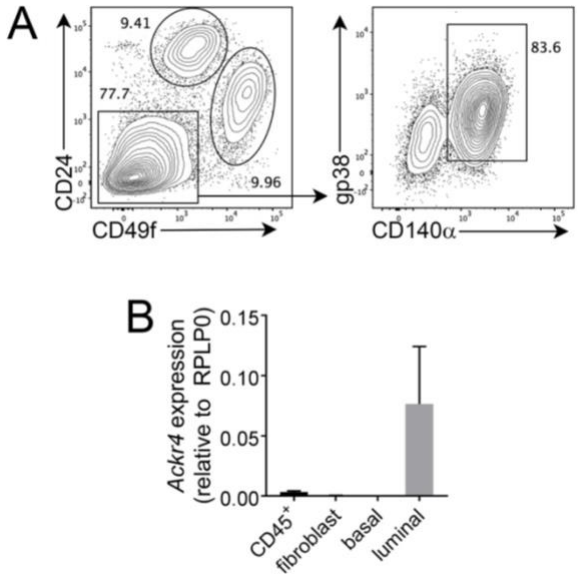



**Figure 3.4: ACKR4 is expressed by luminal epithelial cells in the mouse mammary gland.**

Immune, stromal and epithelial cell populations were sorted from the fourth mammary glands from naïve virgin 12-week old female mice. **(A)** Representative gating strategy of luminal epithelial cells ($CD45^{-}$ $CD24^{hi}$ $CD49f^{int}$), basal epithelial cells ($CD45^{-}$ $CD24^{int}$ $CD49f^{hi}$) and fibroblasts ($CD45^{-}$ $CD24^{lo}$ $CD49f^{lo}$ $CD140\alpha^{+}$), pre-gated $CD45^{-}$. **(B)** Relative expression of *Ackr4* in sorted cell populations. n=7, pooled from two independent experiments. Mean ± SEM.

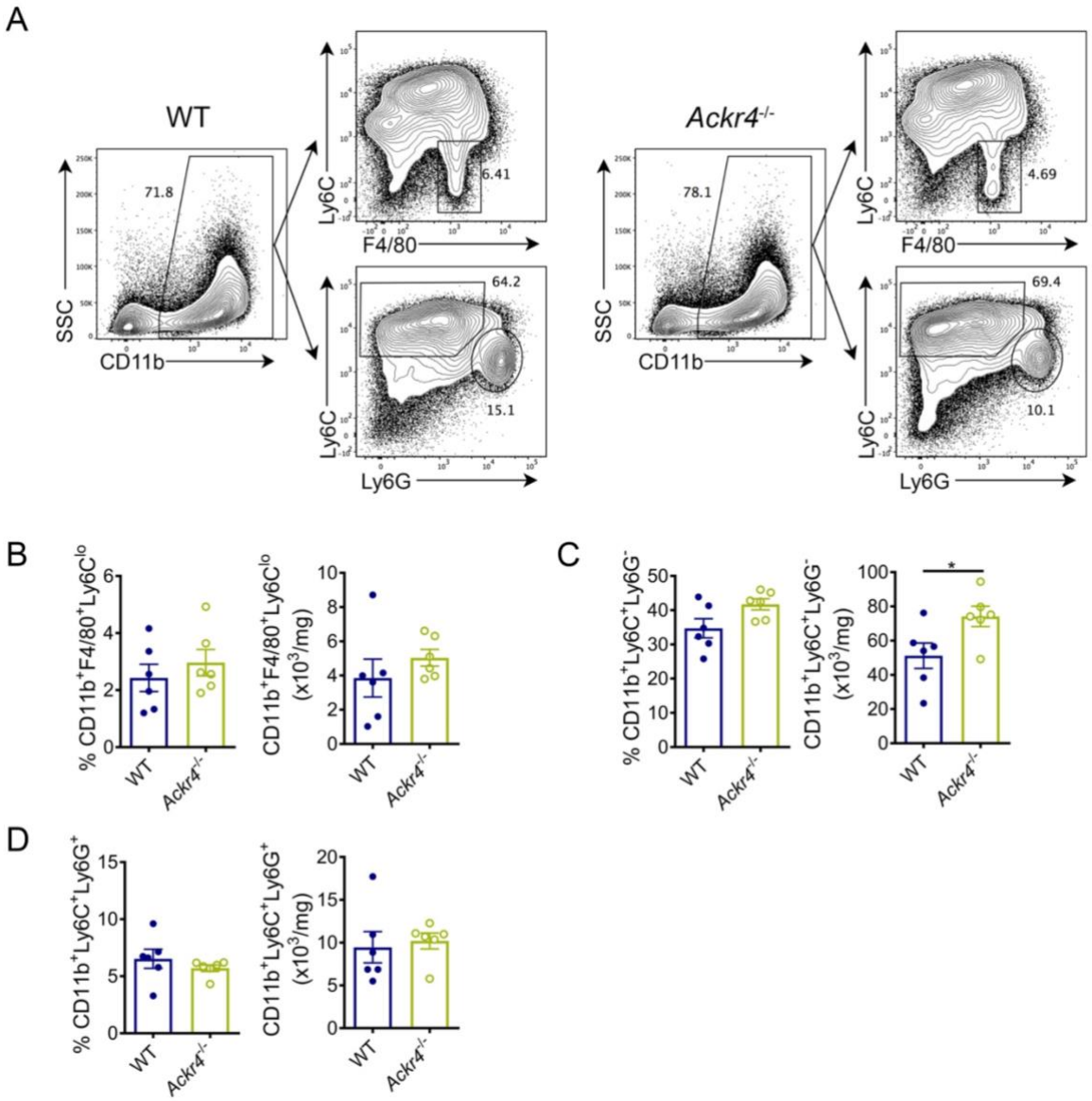



**Figure 3.5: Myeloid cell accumulation in tumours of WT and *Ackr4*$^{-/-}$ mice.**

WT or *Ackr4*$^{-/-}$ mice were injected with E0771 mammary carcinoma cells into the fourth mammary gland, with tumours analysed 21 days post-inoculation. **(A)** Representative flow cytometry of myeloid cells in tumours, pre-gated on live CD45$^{+}$ cells. **(B)** Macrophage-like (CD45$^{+}$CD11b$^{+}$F4/80$^{+}$Ly6C$^{lo}$) cell frequency and number. **(C)** Monocyte (CD45$^{+}$CD11b$^{+}$Ly6C$^{+}$Ly6G$^{-}$) cell frequency and number. **(D)** Neutrophil-like (CD45$^{+}$CD11b$^{+}$Ly6C$^{+}$Ly6G$^{+}$) cell frequency and number. Mean ± SEM. n= 5-6 mice, unpaired t-test. Data is representative of two similar experiments. * p≤0.05.

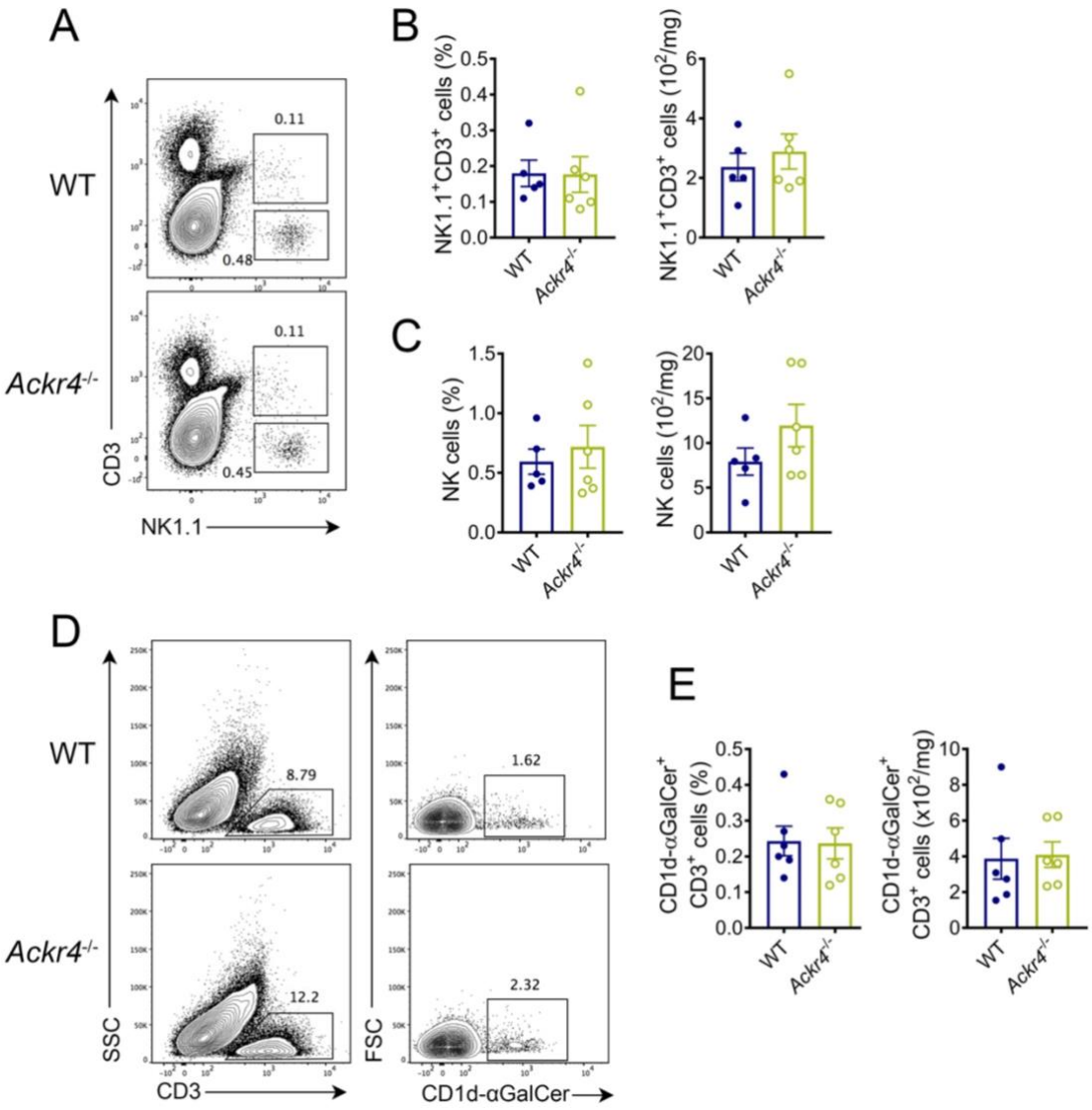
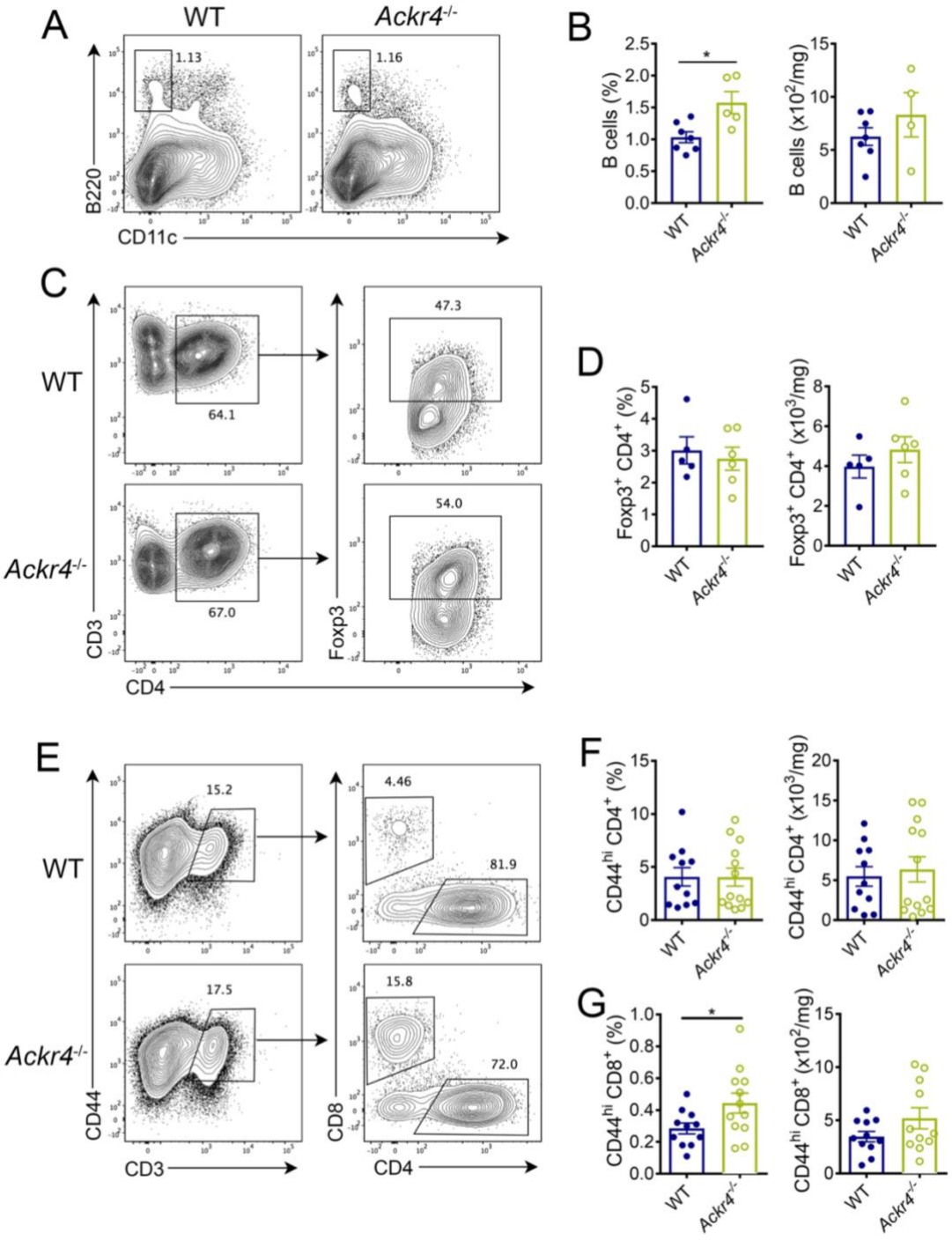



**Figure 3.6: NK cell and NKT cell accumulation in tumours is unaltered in *Ackr4*$^{-/-}$ mice.**

WT or *Ackr4*$^{-/-}$ mice were injected with E0771 mammary carcinoma cells into the fourth mammary gland, with tumours analysed 21 days post-inoculation. **(A)** Representative flow cytometry of NK cells and NK1.1$^{+}$ CD3$^{+}$ cells in tumours, pre-gated on live CD45$^{+}$ cells. **(B)** CD45$^{+}$ NK1.1$^{+}$ CD3$^{+}$ cell frequency and number. **(C)** NK cell (CD45$^{+}$ NK1.1$^{+}$ CD3$^{-}$) frequency and number. **(D)** Representative flow cytometry of CD1d-restricted iNKT cells in tumours, pre-gated on live CD45$^{+}$ cells. **(E)** CD1d-restricted iNKT cell (CD45$^{+}$ CD3$^{+}$ CD1d-αGalCer$^{+}$) frequency and number. n= 5-6 mice, representative of two similar experiments. Mean ± SEM.

**Figure 3.7: Adaptive lymphocyte accumulation in tumours is altered in *Ackr4*$^{-/-}$ mice.**

WT or *Ackr4*$^{-/-}$ mice were injected with E0771 mammary carcinoma cells into the fourth mammary gland, with tumours analysed 21 days post-inoculation. **(A)** Representative flow cytometry of B cells in tumours, pre-gated on live CD45$^{+}$ cells. **(B)** B cell frequency and number. **(C)** Representative flow cytometry of Treg cells in tumours, pre-gated on live CD45$^{+}$ cells. **(D)** Treg cell frequency and number. **(E)** Representative flow cytometry of activated CD4$^{+}$ and CD8$^{+}$ T cells in tumours, pre-gated on live CD45$^{+}$ cells. **(F)** CD44$^{hi}$ CD4$^{+}$ T cell frequency and number. **(G)** CD44$^{hi}$ CD8$^{+}$ T cell frequency and number. n=11-13 mice, unpaired t-test. Data is pooled from two independent experiments. Mean ± SEM. * $p \leq 0.05$.


A
WT
Ackr4-/-
1.13
1.16
B220
CD11c
B
B cells (%)
B cells (x10²/mg)
*
WT
Ackr4-/-
C
WT
Ackr4-/-
64.1
47.3
67.0
54.0
CD3
CD4
Foxp3
D
Foxp3+ CD4+ (%)
Foxp3+ CD4+ (x10³/mg)
E
WT
Ackr4-/-
15.2
4.46
81.9
17.5
15.8
72.0
CD44
CD3
CD8
CD4
F
CD44hi CD4+ (%)
CD44hi CD4+ (x10³/mg)
G
CD44hi CD8+ (%)
CD44hi CD8+ (x10²/mg)

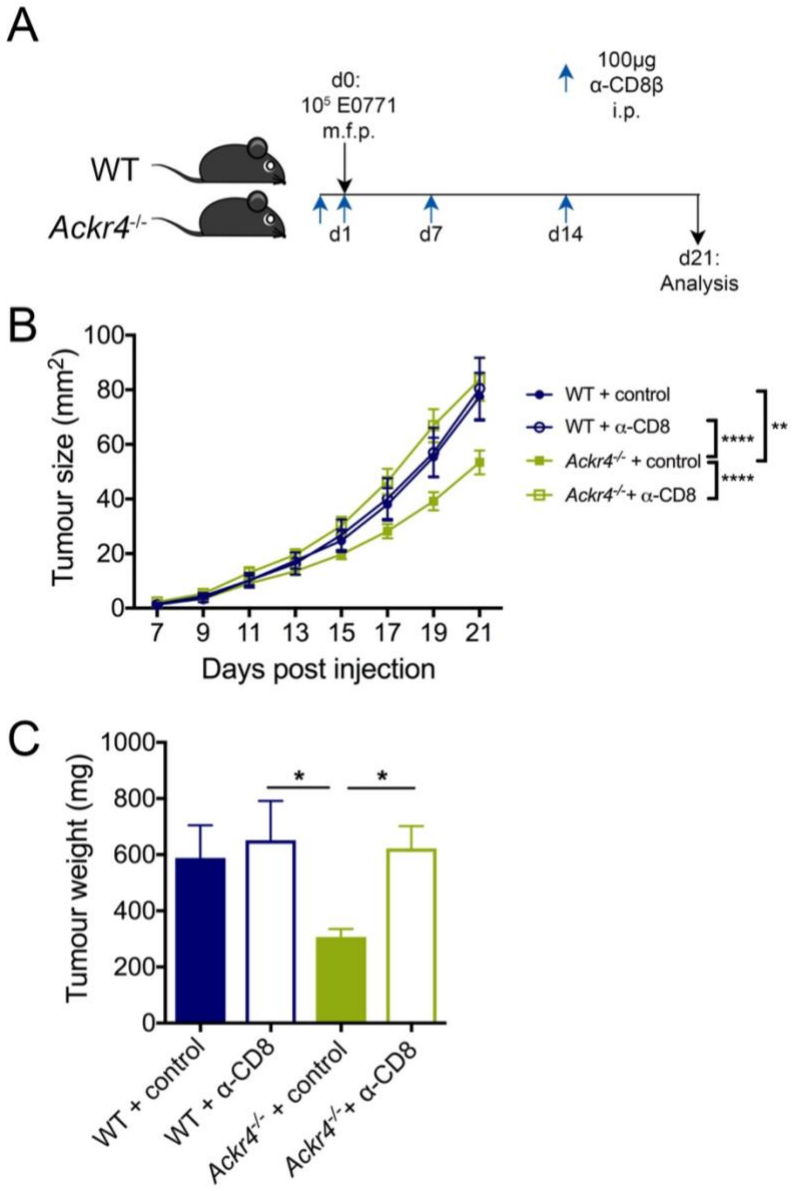
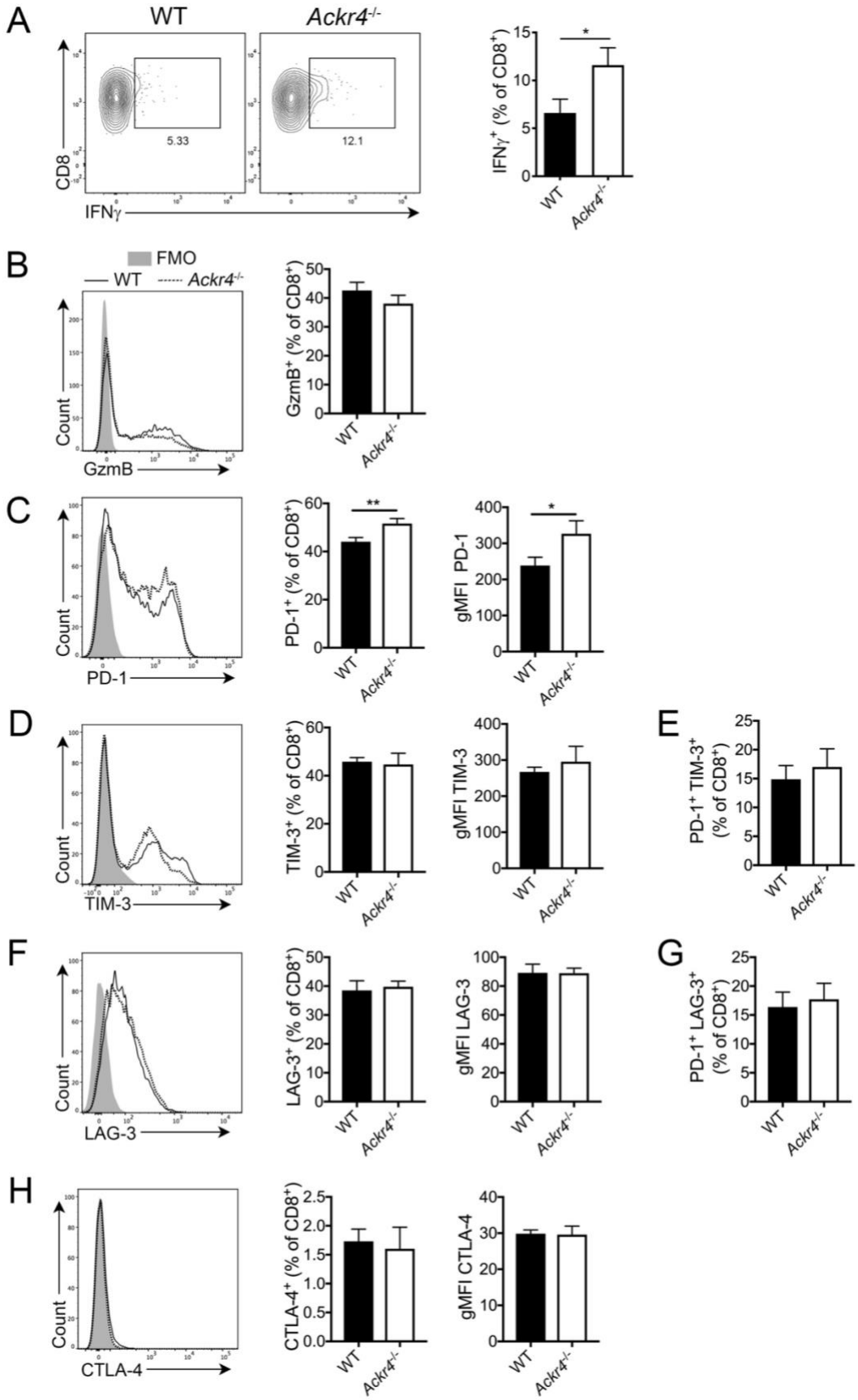



**Figure 3.8: CD8$^+$ T cells control tumour growth in ACKR4-deficient mice.**

WT or *Ackr4*$^{-/-}$ mice were injected with E0771 mammary carcinoma cells into the fourth mammary gland and treated with antibody to deplete CD8$^+$ T cells on days -1, 0, 7 and 14. **(A)** Schematic of experiment. **(B)** Tumour growth curves; two-way ANOVA. **(C)** Tumour weights at day 21; one-way ANOVA. n= 9 (WT + control), 10 (WT + α-CD8), 14 (*Ackr4*$^{-/-}$ + control; *Ackr4*$^{-/-}$ + α-CD8) mice, pooled from two independent experiments. Mean ± SEM. * p≤0.05, ** p≤0.01, **** p≤0.0001.

**Figure 3.9: Characterisation of CD8$^+$ T cell response in ACKR4-deficient mice.**
WT or *Ackr4*$^{-/-}$ mice were injected with E0771 mammary carcinoma cells into the fourth mammary gland, with tumours analysed 18 days post-inoculation. **(A)** Representative flow cytometry and frequency of IFNγ-producing CD8$^+$ T cells. **(B)** Representative flow cytometry and frequency of granzyme B (GzmB)-producing CD8$^+$ T cells. **(C)** Representative flow cytometry, frequency and geometric mean fluorescent intensity (gMFI) of PD-1 on CD8$^+$ T cells. **(D)** Representative flow cytometry, frequency and gMFI of TIM-3 on CD8$^+$ T cells. **(E)** Frequency of PD-1$^+$ TIM-3$^+$ CD8$^+$ T cells. **(F)** Representative flow cytometry, frequency and gMFI of LAG-3 on CD8$^+$ T cells. **(G)** Frequency of PD-1$^+$ LAG-3$^+$ CD8$^+$ T cells. **(F)** Representative flow cytometry, frequency and gMFI of CTLA-4 on CD8$^+$ T cells. (A-C) Representative of at least two experiments, n=6-10, unpaired t-test. (D-H) Data from a single experiment, n=10. Mean ± SEM. * $p \leq 0.05$, ** $p \leq 0.01$.


A
WT
Ackr4-/-
5.33
12.1
CD8
IFNγ
IFNγ+ (% of CD8+)
B
FMO
WT
Ackr4-/-
Count
GzmB
GzmB+ (% of CD8+)
C
PD-1
PD-1+ (% of CD8+)
gMFI PD-1
D
TIM-3
TIM-3+ (% of CD8+)
gMFI TIM-3
E
PD-1+ TIM-3+ (% of CD8+)
F
LAG-3
LAG-3+ (% of CD8+)
gMFI LAG-3
G
PD-1+ LAG-3+ (% of CD8+)
H
CTLA-4
CTLA-4+ (% of CD8+)
gMFI CTLA-4

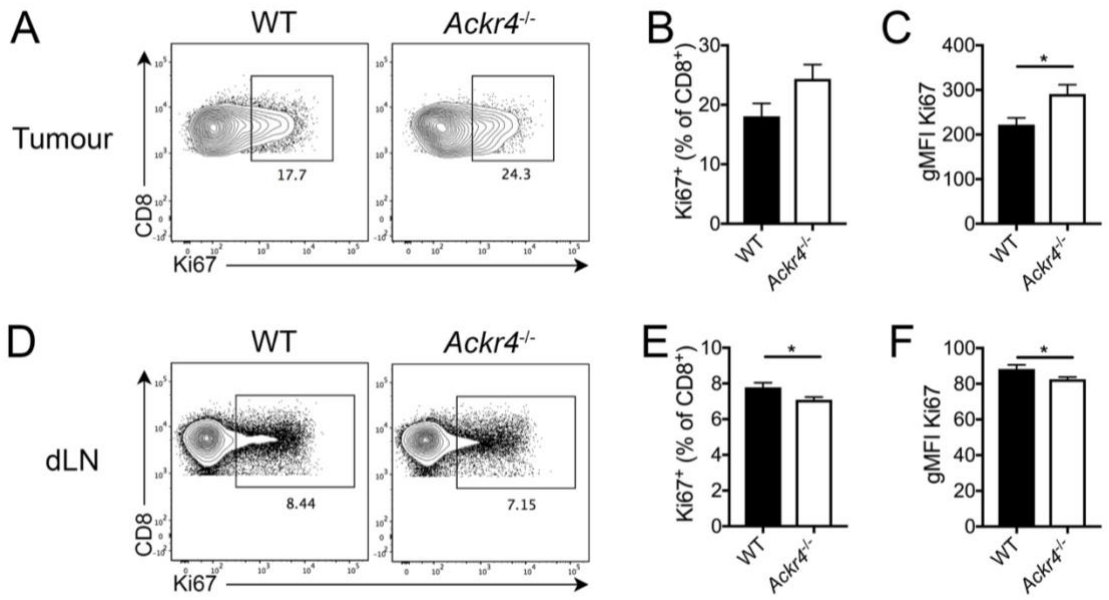



**Figure 3.10: Assessment of intratumoural CD8$^+$ T cell proliferation in ACKR4-deficient mice.**

WT or *Ackr4*$^{-/-}$ mice were injected with E0771 mammary carcinoma cells into the fourth mammary gland, with tumours analysed 18 days post-inoculation. **(A)** Representative flow cytometry of Ki67 expression on intratumoural CD8$^+$ T cells. **(B)** Frequency of Ki67$^+$ CD8$^+$ T cells in the tumour. **(C)** Geometric mean fluorescent intensity (gMFI) of Ki67 on intratumoural CD8$^+$ T cells. **(D)** Representative flow cytometry of Ki67 expression on CD8$^+$ T cells from the draining lymph node (dLN). **(E)** Frequency of Ki67$^+$ CD8$^+$ T cells in the dLN. **(C)** gMFI of Ki67 on CD8$^+$ T cells in dLN. n=10, unpaired t-test. Mean ± SEM. * p≤0.05.

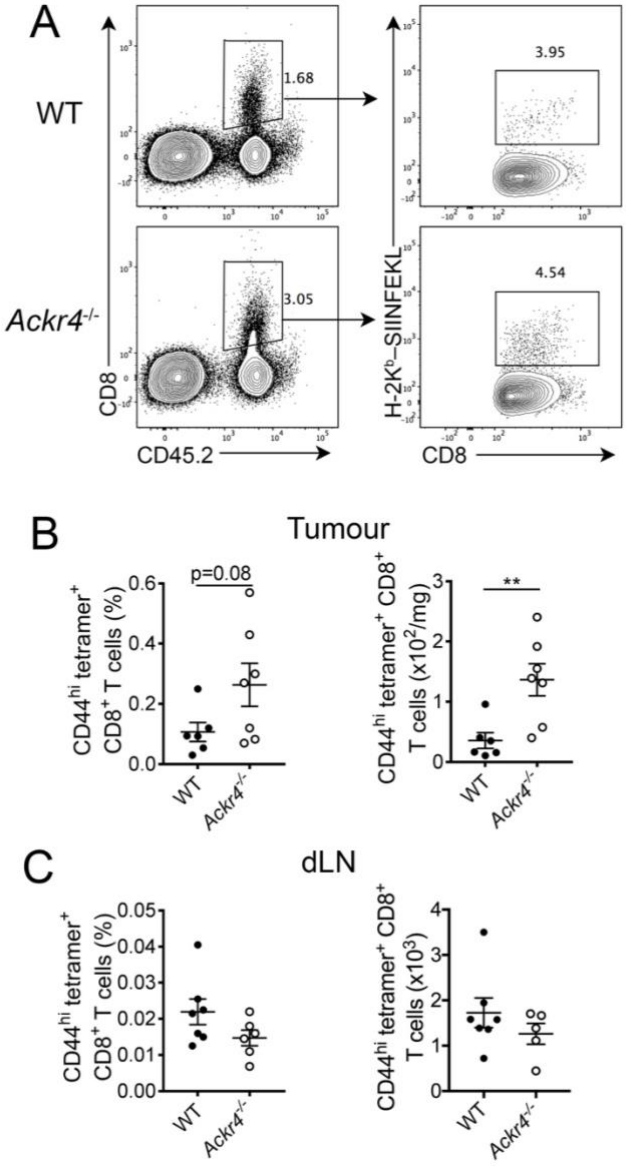



**Figure 3.11: ACKR4 deficiency does not affect tumour-specific CD8$^+$ T cell priming.** WT or *Ackr4*$^{-/-}$ mice were injected with 5x10$^5$ E0771-OVA mammary carcinoma cells into the fourth mammary gland with tumours harvested at day 11. **(A)** Representative gating strategy of endogenous OVA-specific CD8$^+$ T cells in the tumour, pre-gated on live, CD45$^+$ cells. **(B)** Frequency and number of OVA-specific CD8+ T cells in the tumour. **(C)** Frequency and number of OVA-specific CD8$^+$ T cells in the draining LN (dLN). n= 6-7 mice, unpaired t-test. Mean ± SEM. ** p≤0.01.

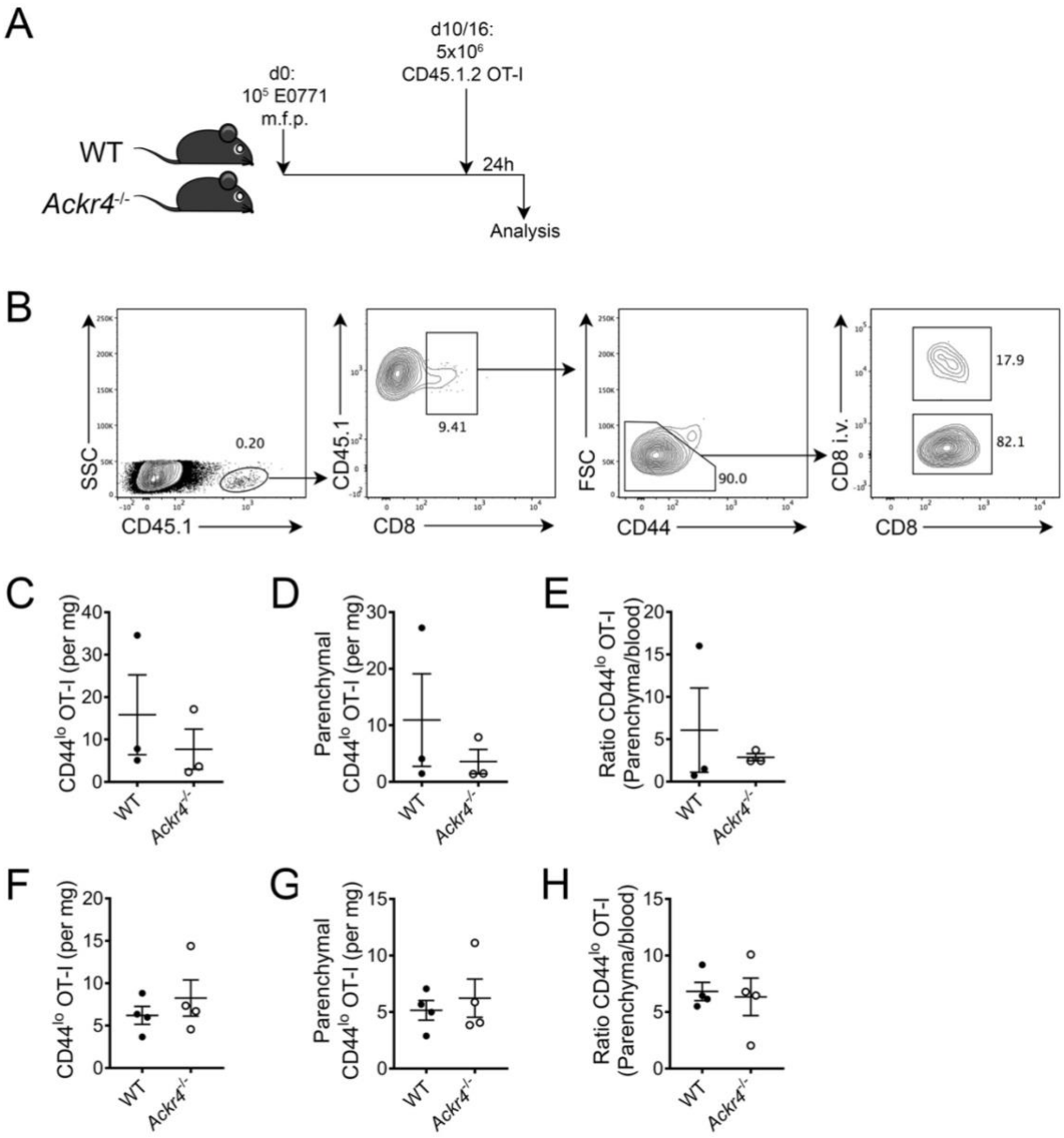
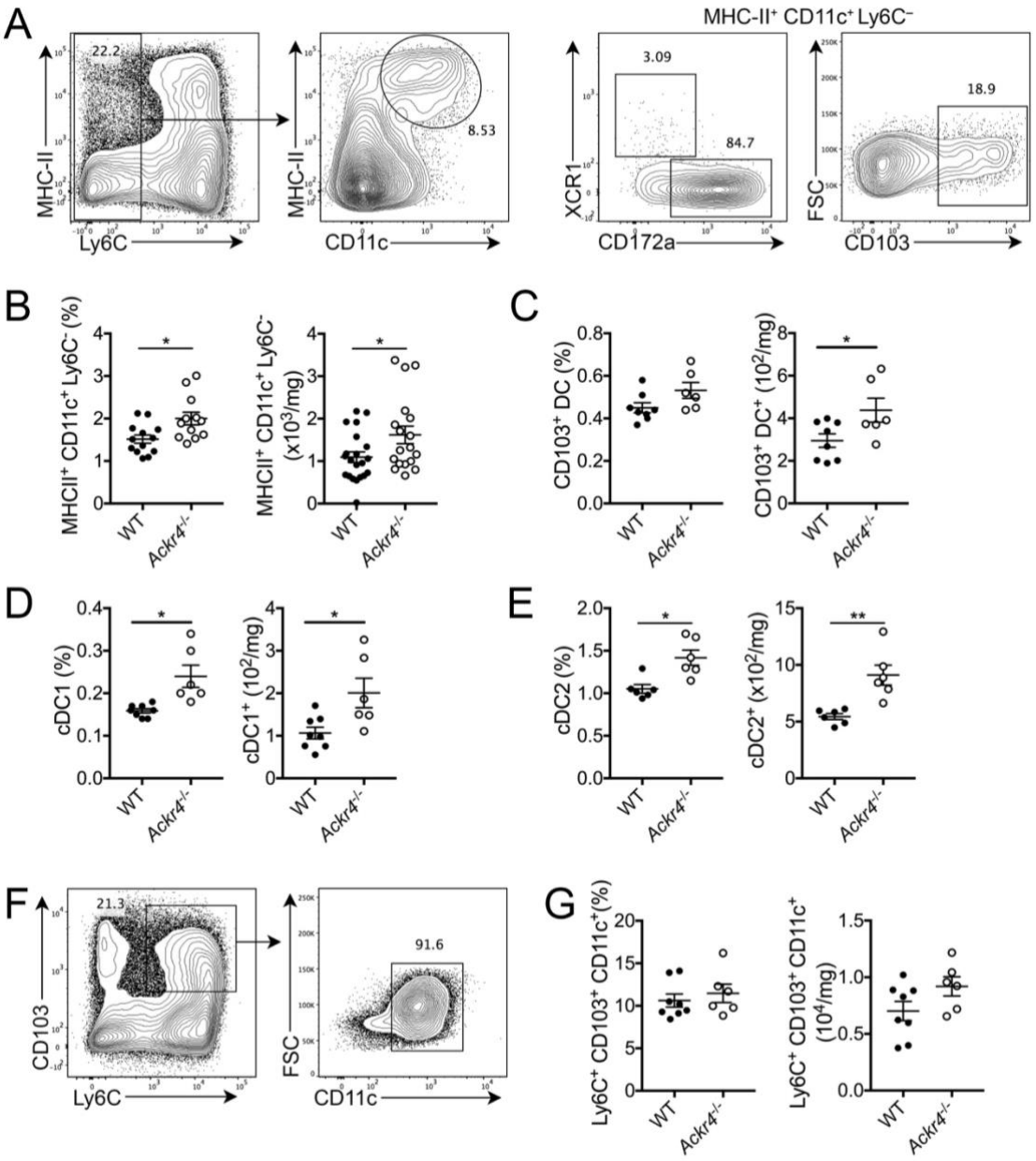



**Figure 3.12: ACKR4 deficiency does not affect intratumoural recruitment of naïve CD8$^+$ T cells.**

WT or *Ackr4*$^{-/-}$ mice were injected with E0771 mammary carcinoma cells into the fourth mammary gland. Congenic OT-I cells were adoptively transferred at d10 (C-E) or d16 (F-H), and tumours were harvested 24 hours later. Intravascular labelling of CD8$^+$ T cells was performed 5 minutes prior to harvest. **(A)** Schematic of experiment. **(B)** Representative gating strategy of intratumoural OT-I cells. **(C,F)** Number of total intratumoural naïve OT-I cells. **(D, G)** Number of parenchymal naïve OT-I cells. **(E, H)** Ratio of naïve OT-I cells in the parenchyma versus the blood. n= 3-4 mice. Mean ± SEM.

**Figure 3.13: Loss of ACKR4 promotes intratumoural DC accumulation.**

WT or *Ackr4*$^{-/-}$ mice were injected with E0771 mammary carcinoma cells into the fourth mammary gland, with tumours analysed 18 days post-inoculation. **(A)** Representative gating strategy of intratumoural DCs, pre-gated on live, CD45$^{+}$ cells. **(B)** Frequency and number of DCs (MHC-II$^{+}$ CD11c$^{+}$ Ly6C$^{-}$) in the tumour. **(C)** Frequency and number of CD103$^{+}$ DCs in the tumour. **(D)** Frequency and number of XCR1$^{+}$ cDC1s in the tumour. **(E)** Frequency and number of CD172a$^{+}$ cDC2s in the tumour **(F)** Representative gating strategy of CD103$^{+}$ myeloid cells, pre-gated on live, CD45$^{+}$ cells. **(G)** Frequency and number of Ly6C$^{+}$ CD103$^{+}$ CD11c$^{+}$ cells in the tumour. (B) Pooled from two independent experiments. (C-G) Representative of at least two independent experiments. n= 6-10 mice, unpaired t-test. Mean ± SEM. *$p \leq 0.05$, ** $p \leq 0.01$.


A
MHC-II+ CD11c+ Ly6C−
22.2
MHC-II
Ly6C
8.53
CD11c
3.09
84.7
XCR1
CD172a
18.9
FSC
CD103
B
MHCII+ CD11c+ Ly6C- (%)
MHCII+ CD11c+ Ly6C- (x10³/mg)
C
CD103+ DC (%)
CD103+ DC+ (10²/mg)
D
cDC1 (%)
cDC1+ (10²/mg)
E
cDC2 (%)
cDC2+ (x10²/mg)
F
21.3
CD103
Ly6C
91.6
FSC
CD11c
G
Ly6C+ CD103+ CD11c+(%)
Ly6C+ CD103+ CD11c+ (10⁴/mg)
WT
Ackr4-/-

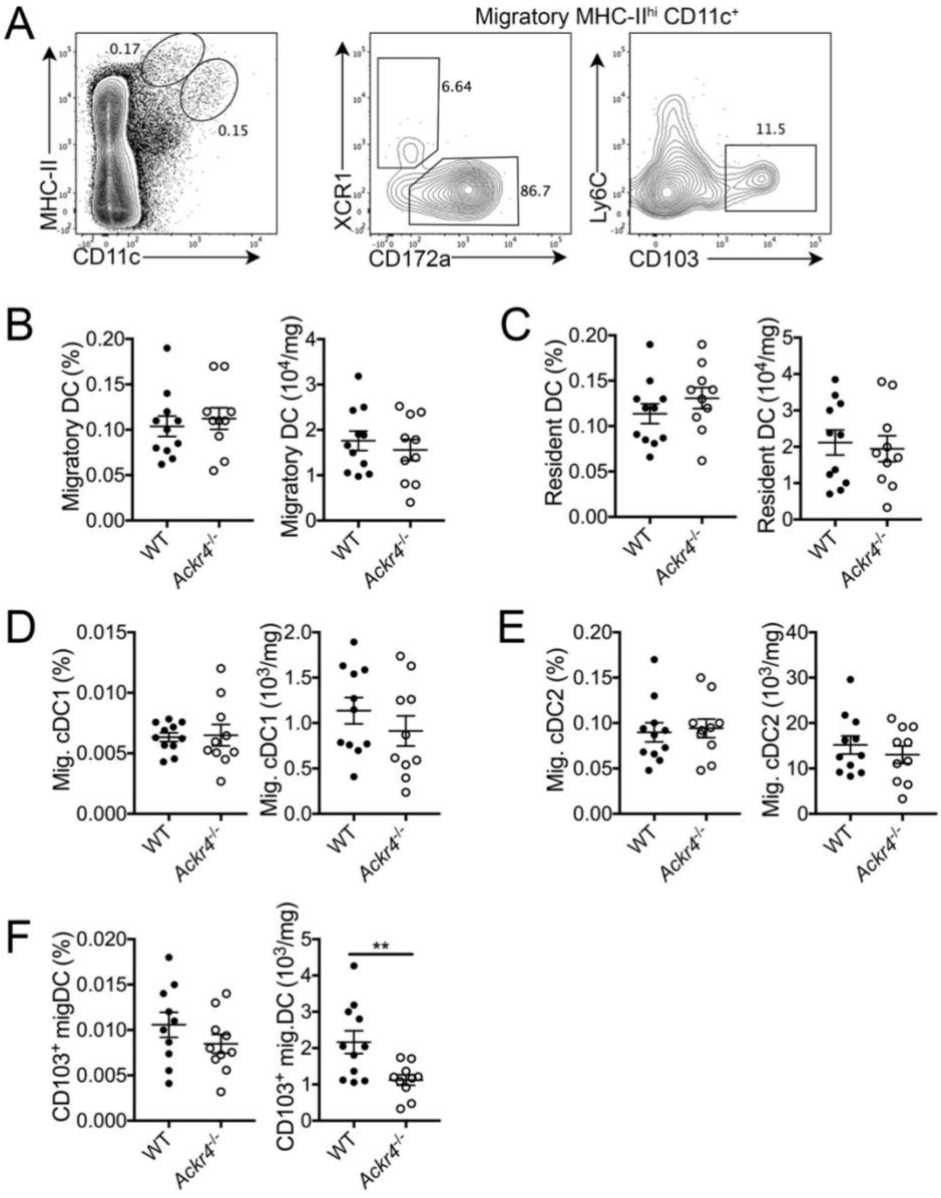



**Figure 3.14: CD103$^+$ migratory DCs are reduced in ACKR4-deficient lymph nodes.** WT or *Ackr4*$^{-/-}$ mice were injected with E0771 mammary carcinoma cells into the fourth mammary gland, with tumours analysed 18 days post-inoculation. **(A)** Representative gating strategy of resident and migratory DCs, pre-gated on live, CD45$^+$ cells. **(B)** Frequency and number of migratory DCs (MHC-II$^{hi}$ CD11c$^+$) in draining inguinal LNs (dLN). **(C)** Frequency and number of resident DCs (MHC-II$^+$ CD11c$^+$) in dLN. **(D)** Frequency and number of migratory XCR1$^+$ cDC1s in dLN. **(E)** Frequency and number of migratory CD172a$^+$ cDC2s in dLN. **(F)** Frequency and number of CD103$^+$ migratory DCs cells in dLN. Representative of two independent experiments. n= 6-10 mice, unpaired t-test. Mean ± SEM. ** p≤0.01.

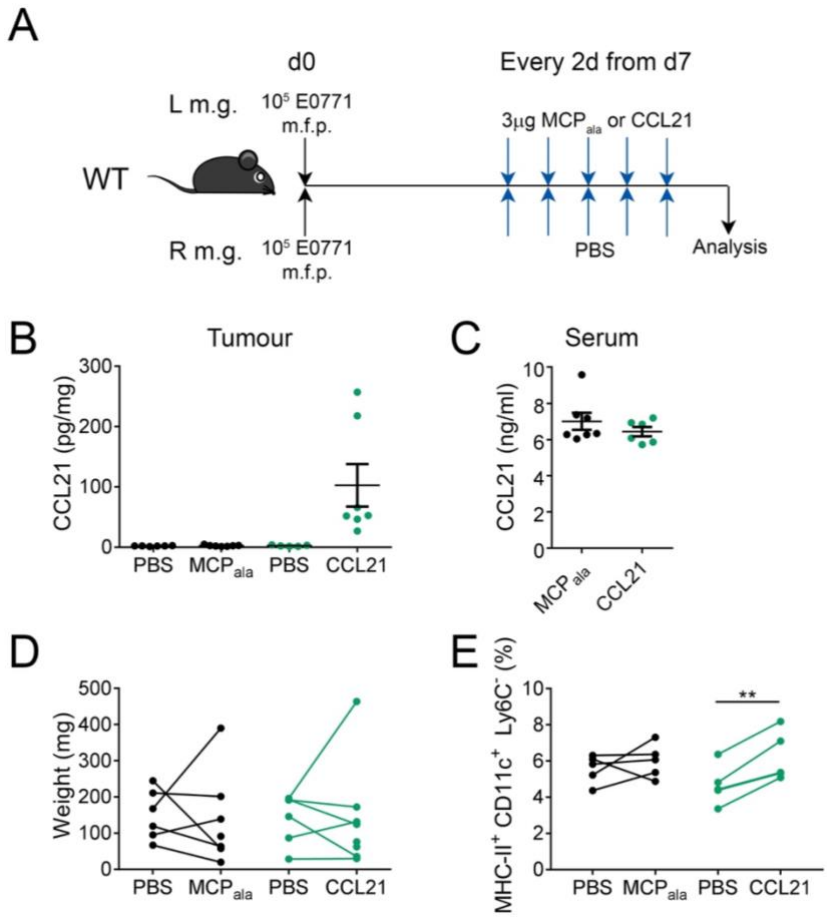



**Figure 3.15: Intratumoural administration of CCL21 promotes DC retention.**
WT mice were injected with E0771 mammary carcinoma cells in the left and right side of the fourth mammary gland (m.g.). From day 7, tumours in the left m.g. were injected with 3µg $MCP_{ala}$ or CCL21, while the right m.g. was injected with PBS. **(A)** Schematic of experiment. **(B)** CCL21 concentration in the tumour as measured by ELISA. **(C)** CCL21 concentration in serum of treated mice as measured by ELISA. **(D)** Tumour weight at day 21. **(E)** Frequency of intratumoural DCs. n= 6-7 mice; paired t-test. Data is representative of two similar experiments. Mean ± SEM. ** $p \leq 0.01$.

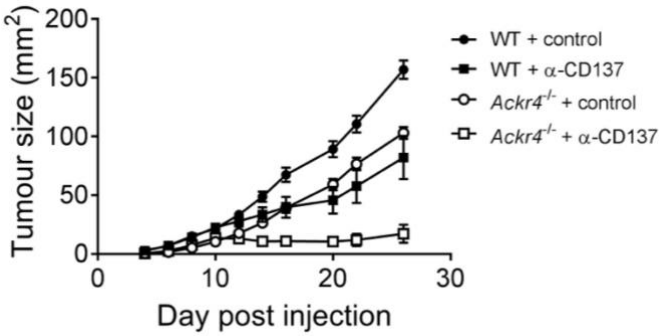



**Figure 3.16: ACKR4 deficiency improves response to agonistic anti-CD137 therapy.** Tumour growth in WT or *Ackr4*$^{-/-}$ mice injected with E0771 mammary carcinoma cells and administered 100μg anti-CD137 (clone 3H3) or rat IgG every 3 days from day 10. n=7-9. Mean ± SEM. *Experiment conceptualised in collaboration with and executed by Prof. Mark Smyth.*

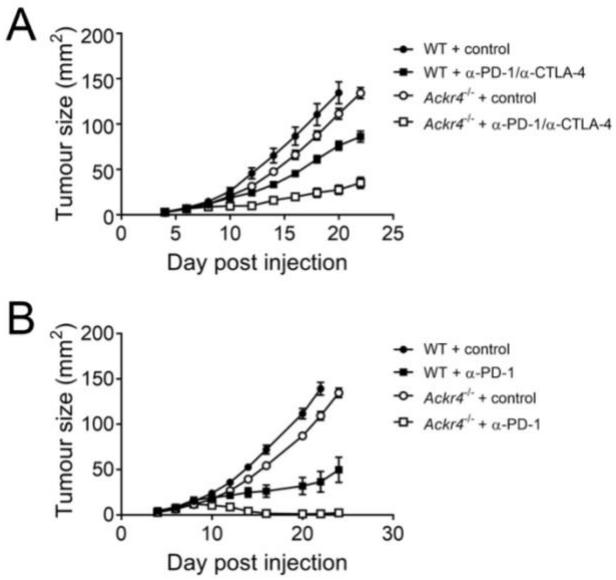



**Figure 3.17: ACKR4 deficiency improves response to anti-PD-1 and anti-CTLA4 therapy.**

**(A)** Tumour growth curves from WT or *Ackr4*$^{-/-}$ mice injected with B16F10 melanoma cells and administered 4 doses of 250µg anti-PD-1 (clone RMP1-14) and 250µg anti-CTLA-4 (clone UC10-4F10) or 500µg control hamster IgG every 3 days from day 6. Representative of two independent experiments, n=4-6 mice. **(B)** Tumour growth curves from WT or *Ackr4*$^{-/-}$ mice injected with MC38 colon adenocarcinoma cells and administered 4 doses of 250µg anti-PD-1 (clone RMP1-14) or 250µg rat IgG every 3 days from day 6. From a single experiment, n=5 mice. Mean ± SEM. *Experiment conceptualised in collaboration with and executed by Prof. Mark Smyth.*

# CHAPTER 4

# The role of CCR2 in regulating $CD4^+$ T cell memory

# Chapter 4: The role of CCR2 in regulating $CD4^+$ T cell memory

## 4.1 Introduction

One of the hallmark features of the adaptive immune system is its ability to mount enhanced responses upon secondary reencounter of an antigenic stimulus. The significance of memory $CD8^+$ T cells and B cells in protective immunity is well established, however relatively little is understood about the role of memory $CD4^+$ T cells, despite correlations with improved pathogen control[168,272]. The generation and maintenance of $CD4^+$ T cell memory is largely unknown, and despite early subclassification of memory $CD4^+$ T cells on the basis of their expression of the chemokine receptor CCR7, little progress has been made regarding how these subsets migrate. We have previously shown that the majority of memory $T_H17$ cells generated in response to infection with the extracellular bacteria *S. pneumoniae* express the inflammatory chemokine receptor CCR2, however the functional significance of this receptor on these cells is unclear. Thus, in this chapter the role of CCR2 on memory $CD4^+$ T cells in infectious immunity was studied.

## 4.2 Development of an antigen-specific *Streptococcus pneumoniae* infection model.

Our previous work has shown that IL-17-producing $CD4^+$ cells present in the lung up to 84 days post-primary infection with *S. pneumoniae* express high levels of CCR2[205]. In order to study the functional consequences of CCR2 expression, a transgenic *S. pneumoniae* infection model was first developed in which antigen-specific $CD4^+$ T cells could specifically be tracked. Because the immunodominant $CD4^+$ T cell epitopes of *S. pneumoniae* in mouse models are unknown, the OT-II TCR transgenic system was utilised, wherein $CD4^+$ T cells from the OT-II transgenic mouse are specific for the $OVA_{323-339}$ peptide presented in the context of the MHC-II molecule, $H\text{-}2^b$ [273]. The sequence for $OVA_{323-339}$ was inserted into the pneumococcal surface protein A (PspA) of D39 (D39-$OVA_{323-339}$), an encapsulated serotype 2 strain[274]. This approach has previously been used in a whole killed cell vaccine to induce robust immunity in DO11.10$Rag^{-/-}$ mice, which are similar to OT-II mice but recognise $OVA_{323-339}$ in the context of $H\text{-}2^d$ [243]. Independently, the $OVA_{SIINFEKL}$ peptide was inserted into PspA, with

the OVA$_{SIINFEKL}$ epitope being specifically recognised by transgenic CD8$^+$ OT-I cells, but in this case acting as an irrelevant genetically-modified control. To confirm that insertion of these sequences did not affect the virulence of the strains, CD45.2$^+$ C57Bl/6 (henceforth referred to as B6) mice were infected intranasally with D39-OVA$_{323-339}$, D39-OVA$_{SIINFEKL}$ or the unmodified parental D39 strain of *S. pneumoniae*. At 7 days post-infection, there was a significant reduction in bacterial burden in the nasal wash for both genetically-modified strains compared to the parental strain, however colonisation in the nasal tissue was unchanged, indicating the genetic manipulation reduced virulence **(Fig. 4.1 A)**. To confirm that the inserted peptide sequence was presented to T cells and could induce an antigen-specific response, congenically-marked OT-II cells were adoptively transferred into Ly5.1 hosts which were subsequently challenged intranasally with either D39-OVA$_{323-339}$ or D39-OVA$_{SIINFEKL}$ (**Fig. 4.1 B**). Challenge with D39-OVA$_{323-339}$ induced more than a 10-fold increase in OT-II cell frequency in the lung and spleen and more than 4-fold increase in the mediastinal LN, as well as a significant increase in CD44 expression compared with challenge using the control D39-OVA$_{SIINFEKL}$ **(Fig. 4.1 C, D).**

## 4.3 CCR2 is expressed on *S. pneumoniae*–specific memory CD4$^+$ T cells

To assess the kinetics of CCR2 expression on the antigen-specific CD4$^+$ T cells generated in response to *S. pneumoniae*, OT-II cells were transferred into Ly5.1 hosts and analysed at different time-points after infection. Bacterial burden in the nasal wash was highest 7 days post-infection and then decreased over time **(Fig. 4.2 B)**. CCR2 was not expressed by naïve CD4$^+$ T cells, but was upregulated on OT-II cells after infection. In the spleen, CCR2 expression on OT-II cells peaked on day 14, and was expressed on approximately 13% of OT-II cells 28 days post-infection. Conversely, expression of CCR2 increased over time on OT-II cells in the mediastinal LN (medLN) and lung, with approximately 21% of OT-II cells in the medLN and 36% of OT-II cells in the lung expressing CCR2 on day 28 **(Fig 4.2 C-F)**.

CD44$^{hi}$ CD4$^+$ T cells at memory time-points have classically been subdivided into circulating (T$_{CM}$) or effector (T$_{EM}$) memory T cells on the basis of their expression of CD62L (and CCR7)[206]. More recently, CD4$^+$ tissue-resident memory (T$_{RM}$) cells, which remain in tissues and are excluded from circulation, have been identified and shown to co-express CD11a and CD69, in addition being CD62$^{lo}$ as for T$_{EM}$ cells[209]. Thus, the

proportion of OT-II cells entering each memory compartment was assessed 28 days post-infection with *S. pneumoniae* (**Fig. 4.3**). In the spleen, a large proportion of OT-II cells formed $CD44^{hi}$ $CD62L^{hi}$ $T_{CM}$ cells, whilst a smaller proportion formed $CD44^{hi}$ $CD62L^{lo}$ $T_{EM}$ cells, and there were virtually no $CD11a^{hi}$ $CD69^{hi}$ $T_{RM}$-like cells. In the lung, OT-II cells predominantly had a $T_{EM}$ surface phenotype, although $T_{CM}$ cells were also apparent. Furthermore, approximately 20% of OT-II cells expressed $T_{RM}$-like markers, suggesting they may be resident in the tissue. In the spleen, CCR2 was expressed at low levels on $T_{CM}$ cells, but was greatly enriched on $T_{EM}$ cells (**Fig. 4.3 D**). This expression was mirrored on $T_{CM}$ and $T_{EM}$ cells in the lung, while $T_{RM}$-like OT-II cells expressed similar levels of CCR2 to $T_{EM}$ cells. To confirm that CCR2 expression was not solely a feature of this specific TCR transgenic system, endogenous memory $CD4^+$ T cells were also analysed, with these cells present in the lung as a result of exposure to various antigens over the course of the mouse's lifetime. Similar patterns of CCR2 expression were obtained, confirming that CCR2 is expressed by memory $CD4^+$ T cells regardless of the specificity of their TCR (**Fig. 4.3 F, G)**.

## 4.4 *Ccr2*$^{-/-}$ OT-II cells show enhanced contraction after *S. pneumoniae* infection

In order to assess the functional significance of CCR2 on $CD4^+$ T cells, a competitive cotransfer system was used. Congenically disparate WT ($CD45.1.2^+$) and *Ccr2*$^{-/-}$ ($CD45.2^+$) OT-II cells were mixed at a 1:1 ratio and transferred into Ly5.1 ($CD45.1^+$) hosts, which were then infected with *S. pneumoniae* (**Fig 4.4**). On days 7 and 14 post-infection, similar ratios of *Ccr2*$^{-/-}$ to WT OT-II cells were maintained, however by day 28 the WT OT-II cells outnumbered the *Ccr2*$^{-/-}$ OT-II cells in spleen, medLN and lungs, with at least a 3-fold increase in the spleen **(Fig 4.4 C-E**). This was also reflected in the ratio of *Ccr2*$^{-/-}$ to WT OT-II cells decreasing at the memory time-point (**Fig. 4.4 F-H**), suggesting that there is an accelerated contraction of $CD4^+$ T cells in the absence of CCR2 expression.

Exposure to inflammatory cues is critical for appropriate differentiation and cytokine production by T cells. To determine if the lack of ability to migrate through CCR2 by memory $CD4^+$ T cells also affected their functional output, the ability of WT and *Ccr2*$^{-/-}$ OT-II cells to secrete IL-17 and IFNγ upon PMA restimulation was measured **(Fig. 4.5**).

*S. pneumoniae* infection has been reported to induce both $T_H1$- and $T_H17$-responses, although $T_H17$ cells, through their secretion of IL-17, appear to be the critical $T_H$ subset required for protection against rechallenge[154,155,275]. A low proportion of OT-II cells in the spleen expressed IL-17 or IFNγ. This was somewhat enriched in the medLN and lung, with a higher frequency of IL-17-expressing cells than IFNγ-expressing cells. However, *Ccr2*$^{-/-}$ OT-II cells were unimpaired in their ability to secrete effector cytokines upon restimulation compared with WT OT-II cells.

Given that $T_{CM}$, $T_{EM}$ and $T_{RM}$-like cells expressed different levels of CCR2, it was assessed whether CCR2-deficiency differentially affected formation of these memory subsets. In the spleen, despite $T_{EM}$ expressing significantly more CCR2 than $T_{CM}$, the frequency of $T_{EM}$ and $T_{CM}$ subsets of *Ccr2*$^{-/-}$ OT-II cells were equivalent to that of WT OT-II cells, although the total numbers of *Ccr2*$^{-/-}$ cells were reduced **(Fig. 4.6 A, B)**. In the lung, there was a trend towards a reduction of $T_{CM}$ cells in the *Ccr2*$^{-/-}$ OT-II compartment compared with WT OT-II, and a significant increase in formation of $T_{EM}$ and $T_{RM}$-like cells **(Fig. 4.6 C-E)**. However, the total numbers of *Ccr2*$^{-/-}$ $T_{CM}$ and $T_{EM}$ OT-II cells were still significantly reduced in the lung **(Fig. 4.6 F, G)**. This suggests that although *Ccr2*$^{-/-}$ $T_{CM}$ and $T_{EM}$ OT-II cells both underwent enhanced contraction compared with WT OT-II memory cells, this was more pronounced in the $T_{CM}$ compartment. Furthermore, the *Ccr2*$^{-/-}$ $T_{RM}$-like OT-II cells appeared to be protected from this effect and were present at comparable numbers to their WT counterparts **(Fig. 4.6 H)**, although their frequency was significantly increased due to the contraction of the $T_{CM}$ and $T_{EM}$ compartments.

## 4.5 CCR2-deficiency does not affect effector responses by OT-II cells in *S. pneumoniae* infection

To assess whether the enhanced contraction of the *Ccr2*$^{-/-}$ OT-II cells might be explained by a difference in the effector phase of the response, cytokine production by OT-II cells was measured at early time-points post-infection. Similarly to the memory response, production of IL-17 and IFNγ at 7 days post-infection was unimpaired in *Ccr2*$^{-/-}$ compared to WT OT-II cells in the spleen, medLN and lung **(Fig. 4.7 A, C, E)**. On day 14, at which point there is minimal difference in the numbers of WT and *Ccr2*$^{-/-}$ OT-II cells, the production of IFNγ and IL-17 was also unchanged, although there was a trend

towards decreased production of IFNγ in the lung by *Ccr2*$^{-/-}$ OT-II cells (significance value of p=0.06) **(Fig. 4.7 B, D, F)**.

CCR2 has previously been shown to be required for entry of T cells to sites of inflammation[205,276]. The equal numbers of WT and *Ccr2*$^{-/-}$ OT-II cells present in the lung at 7 days post-infection suggest that CCR2 is not required for CD4$^{+}$ T cell entry into the lung in response to *S. pneumoniae* infection, but to test this more directly, the ratio of *Ccr2*$^{-/-}$ OT-II cells to WT OT-II cells was compared between the peripheral blood (PB) and the lung **(Fig. 4.8)**. If CCR2 was required for entry in the lung, then an increased ratio would be expected in the PB versus the lung. At 7 days post-infection, the ratio was comparable between PB and lung, suggesting entry into the lung is CCR2-independent. Furthermore, on day 14 the ratio of *Ccr2*$^{-/-}$ to WT OT-II cells was higher in the lung than in the PB. This indicates that the *Ccr2*$^{-/-}$ OT-II cells may contract more rapidly in the circulation than in the lung tissue, or are diverted elsewhere.

## 4.6 CCR2 is expressed on influenza-specific $T_H1$ cells

Previous publications have reported CCR2 expression on diverse subsets of CD4$^{+}$ T cells in both human and mouse[234,236,237,277]. As described above, CCR2 is expressed on CD4$^{+}$ T cells in response to *S. pneumoniae*, which induces a mixed type 1/type 17 response, although a greater proportion of OT-II cells expressed IL-17 at memory timepoints. To test if CCR2 is also important for CD4$^{+}$ T cells induced in a predominantly type 1 infection, the influenza A virus model was used. Influenza induces a type 1 response through intracellular infection of lung epithelial cells[278], with protection largely mediated through influenza-specific antibody and the cytolytic activity of CD8$^{+}$ T cells. In this model, $T_H1$ cells are critical for enabling optimal CD8$^{+}$ T cell priming and memory, with $T_{FH}$ cells mediating T-dependent humoural immune responses.

To assess the endogenous CD4$^{+}$ T cell response to influenza, the A/HKX31 (referred to henceforth as X31) was used. This is a recombinant influenza A virus expressing the H3N2 surface proteins and is considered to be of low pathogenicity in mice, despite inducing weight-loss following infection[279]. B6 mice were infected intranasally with 7.3 $TCID_{50}$ of X31, and analysed 7 to 10 days post-infection **(Fig. 4.9 A)**. To detect X31-specific CD4$^{+}$ T cells, cells were stimulated *ex vivo* with either $HA_{211-225}$ or $NP_{311-325}$,

peptides that have previously been shown to be immunodominant class II-restricted epitopes in B6 mice following influenza infection[280]. A small proportion of $CD4^+$ T cells in the lung and spleen of immunised mice expressed IFNγ in response to stimulation, with no IFNγ detected in the absence of these peptides **(Fig. 4.9 B)**. However, low cell yields prevented robust analysis of IFNγ production by $CD4^+$ T cells in the medLN, PB and bronchoalveolar lavage (BAL), and so $CD44^{hi}$ $CD4^+$ T cells were also measured as a surrogate, since the patterns of CCR2 expression on $CD44^{hi}$ and $IFN\gamma^+$ $CD4^+$ T cells were similar in the lung and spleen. CCR2 expression was induced on $T_H1$ cells and activated $CD4^+$ T cells on day 7 after influenza infection in spleen and lung, and further upregulated in these organs by day 10. The expression of CCR2 on $HA_{211-225}$– and $NP_{311-325}$–specific $CD4^+$ T cells was similar. On $CD44^{hi}$ $CD4^+$ T cells isolated from the medLN, PB and BAL similarly upregulated CCR2 expression by day 10. A similar analysis was performed at day 28 to assess CCR2 on memory $CD4^+$ T cells (data not shown), but IFNγ-producing $CD4^+$ T cells could not be reliably detected using this approach, likely due to the low frequency of these cells at memory timepoints.

To circumvent this issue, the OT-II TCR transgenic system was again utilised. Congenically labelled OT-II cells were transferred intravenously into Ly5.1 hosts, and infected the following day with X31 that has been modified to express $OVA_{323-339}$ (X31-$OVA_{323-339}$)[281] **(Fig. 4.10)**. The OT-II cells showed robust activation and expansion in this model. In the spleen, CCR2 was upregulated by day 7, but $CCR2^+$ cells remained at a consistent frequency on days 14 and 28 post-infection. Conversely, in the medLN and lung, CCR2-expressing OT-II cells were further enriched at later time-points. This may reflect that OT-II cells increasingly upregulated CCR2 after infection, or that CCR2-expressing cells had an increased propensity to survive the contraction phase.

The ability of OT-II cells to form different memory subsets in response to influenza was then assessed. In both spleen and lung, $T_{EM}$ cells outnumbered $T_{CM}$ cells, although both subsets were present **(Fig. 4.11 B-C)**. Similarly to *S. pneumoniae* infection, cells of a $T_{RM}$-like phenotype were identified in the lung, but were not reliably detected in the spleen. In contrast to that observed in response to *S. pneumoniae*, CCR2 was expressed at similar levels among different memory subsets **(Fig. 4.11 D-E)**.

## 4.7 CCR2 is expressed on regulatory T cells

Briefly, to determine whether CCR2 is expressed by regulatory $CD4^+$ T cells in this model, CCR2 expression on the endogenous repertoire of Treg cells was measured. Treg cells can be categorised based on their ontogeny, with thymic-derived Tregs referred to as natural Treg (nTregs) and those generated in the periphery as induced Tregs (iTregs)[282]. CCR2 expression was highly expressed on both Treg subsets in the lung, and expressed at reduced levels in the spleen and medLN **(Fig. 4.12 B)**. This is consistent with the higher expression of CCR2 on $T_H1$ and $T_H17$ cells in the lung, compared with in the secondary lymphoid organs. It was then assessed whether transferred OT-II cells could turn on Foxp3 and gain a regulatory phenotype in influenza infection. On day 7 post-infection, Foxp3 expression was low in all tested organs, however on day 14, approximately 10-15% of OT-II cells in the spleen and medLN, but not the lung, upregulated Foxp3 expression **(Fig. 4.12 D-E)**. Furthermore, when CCR2 expression was compared between OT-II cells that were $Foxp3^-$ and $Foxp3^+$, CCR2 was significantly upregulated on those that had gained Foxp3 expression **(Fig. 4.12 F-G)**.

## 4.8 *Ccr2*$^{-/-}$ OT-II cells show enhanced contraction after influenza A infection

To determine if, as observed in the response to *S. pneumoniae*, CCR2 on $T_H1$ cells in influenza promotes maintenance into the memory phase, WT and *Ccr2*$^{-/-}$ OT-II cells were co-transferred into congenic recipients and infected with X31-$OVA_{323-339}$. Similarly to *S. pneumoniae* infection, *Ccr2*$^{-/-}$ OT-II showed enhanced contraction compared with WT OT-II cells from day 14, despite being present at equivalent numbers on day 7 **(Figure 4.13)**. This was also reflected in the ratio of *Ccr2*$^{-/-}$/WT OT-II cells, with an approximately 1:1 ratio on day 7, followed by a sharp skew in favour of the WT cells. This suggests that although the initial expansion of these cells was similar, *Ccr2*$^{-/-}$ OT-II cells had a reduced ability to survive or proliferate during the contraction phase.

As above, the functional capacity of memory $T_H1$ cells was analysed. At 28 days after infection, memory *Ccr2*$^{-/-}$ OT-II cells in the spleen and medLN had unimpaired production of IFNγ **(Fig. 4.14 A, B)**. However, there was a significant decrease in IFNγ production by *Ccr2*$^{-/-}$ OT-II cells taken from the lung **(Fig. 4.14 C)**. Despite there being no significant difference in the frequency of WT or *Ccr2*$^{-/-}$ OT-II cells forming $T_{EM}$ cells,

overall *Ccr2*$^{-/-}$ $T_{EM}$ numbers were reduced compared with WT $T_{EM}$, in line with the enhanced contraction seen by *Ccr2*$^{-/-}$ OT-II cells **(Fig. 4.14 D)**. Although not statistically significant, the frequency of *Ccr2*$^{-/-}$ OT-II cells with a $T_{RM}$-like phenotype trended towards an increase (p=0.08) compared with that of WT OT-II cells, despite their numbers being slightly, but significantly reduced **(Fig. 4.14 E)**. These data are similar to those obtained in the *S. pneumoniae* model, in which $T_{RM}$-like cells appeared to be somewhat protected from the enhanced contraction seen in the circulating populations.

## 4.9 CCR2 is not required for initial $T_H1$ effector responses in influenza.

To assess whether CCR2 had a cell-intrinsic role in the initial effector phase of the $T_H1$ response to influenza, mixed bone marrow (BM) chimeras were generated. This was possible since the endogenous influenza-specific CD4$^+$ T cells could be analysed with peptide restimulation, unlike the *S. pneumoniae* model. BM from Ly5.1 mice and *Ccr2*$^{-/-}$ mice were mixed at a 1:1 ratio and transferred into lethally irradiated Ly5.1 hosts and the immune system was allowed to reconstitute for 8 weeks prior to experimentation **(Fig. 4.15**, green). To control for any possible congenic biases as has been previously reported for B cells[283], control chimeras were performed in which a 1:1 ratio of BM from Ly5.1 and B6 was transferred **(Fig. 4.15,** black). In all experiments, no differences were seen in the control chimeras, suggesting there is no congenic bias on CD4$^+$ T cells in this model. Seven days after infection with X31, the generation of influenza-specific $T_H1$ cells was assessed through *ex vivo* stimulation with the peptides $HA_{211-225}$ and $NP_{311-325}$. In the medLN and spleen, equal frequencies of IFNγ-producing CD4$^+$ T cells were detected in the *Ccr2*$^{-/-}$ and Ly5.1 compartments, regardless of which peptide was used for stimulation **(Fig. 4.15 B-E)**. The critical lineage-defining transcription factor for $T_H1$ cells is T-bet, with this being critical for their appropriate differentiation and production of IFNγ and other effector cytokines[284]. *Ccr2*$^{-/-}$ CD4$^+$ T cells displayed upregulation of T-bet equivalent to Ly5.1 CD4$^+$ T cells **(Fig. 4.15 F-G)**. Expression of TNFα by activated *Ccr2*$^{-/-}$ CD4$^+$ T cells was also unaltered compared with WT. Furthermore, the formation of $T_{FH}$ cells, which are robustly induced in influenza to promote the humoural immune response, was also unaffected by loss of CCR2.

In these mixed BM chimeras, mice were injected with fluorescently-labelled antibody prior to being culled in order to distinguish between cells in the lung vasculature (intravascular, IV) versus those in the parenchyma (extravascular, EV). This allowed comparison of the frequency of $CD45.2^+$ cells that were present in each compartment, which tested whether CCR2 had any role in $CD4^+$ T cell migration into the lung parenchyma at this timepoint. Both $HA_{211-225}$– and $NP_{311-325}$–specific *Ccr2*$^{-/-}$ $CD4^+$ T cells were equivalently represented in the IV and EV compartments, ruling out a role for CCR2 in lung entry at this time-point **(Fig. 4.16)**. Thus, CCR2 does not appear to play a role in the major effector functions or trafficking of $T_H1$ cells in the initial response to influenza, and its function seems to be restricted to a later time-point, coinciding with its increased expression.

## 4.10 *Ccr2*$^{-/-}$ OT-II cells display impaired cytokine production upon influenza rechallenge

To assess if *Ccr2*$^{-/-}$ $T_H1$ cells have deficient recall ability, WT and *Ccr2*$^{-/-}$ OT-II cells were co-transferred at a 1:1 ratio and infected with X31-$OVA_{323-339}$ (**Figure 4.17**). Twenty eight days after the primary infection, mice were rechallenged with the heterotypic influenza A strain PR/8-$OVA_{323-339}$. The internal proteins of X31 are derived from PR/8, meaning these residues will be recognised by memory $CD4^+$ and $CD8^+$ T cells, but PR/8 expresses surface H1N1 proteins instead of H3N2 in X31, limiting antibody-mediated secondary responses. In the lung, the number of $CD44^{hi}$ OT-II cells were increased seven days post-rechallenge, compared to mice that were not rechallenged **(Figure 4.17 B)**. However, there was no detectable expansion of OT-II cells in the spleen or medLN in rechallenged mice compared with the control, indicating only OT-II cells in the lung were responding to the secondary infection. In the lung, WT and *Ccr2*$^{-/-}$ OT-II cells showed no difference in their ability to expand upon secondary infection (**Figure 4.17 E**). However, *Ccr2*$^{-/-}$ OT-II cells were significantly impaired in their ability to secrete IFNγ compared with WT OT-II cells (**Figure 4.17 F**).

## 4.11 Conclusion

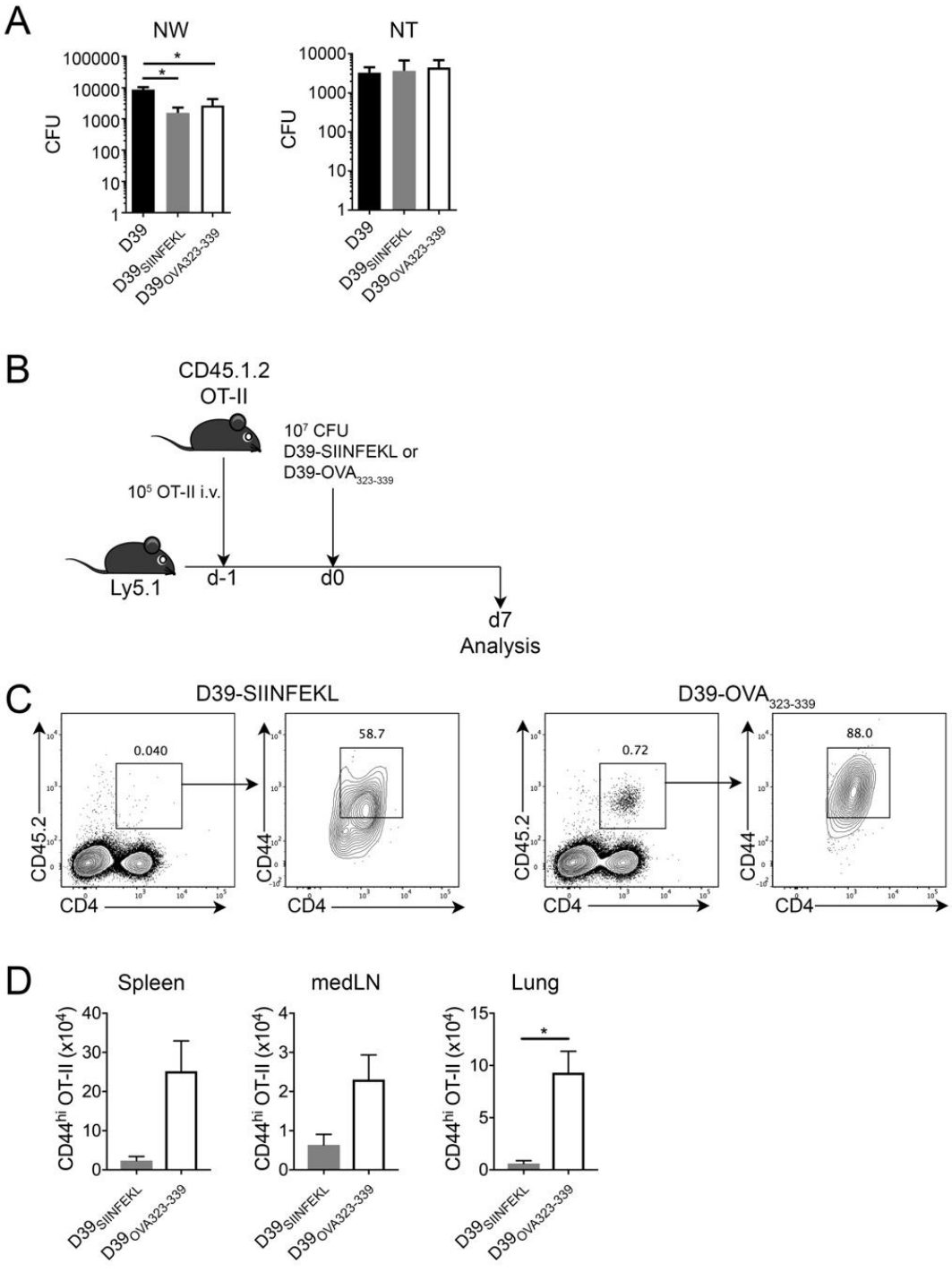
In this chapter, the role of CCR2 on $CD4^+$ T cells was studied in two disparate infectious models. In response to both *S. pneumoniae* and influenza A infection, CCR2 was expressed at an increased frequency on antigen-specific $CD4^+$ T cells at later time-points.

CCR2 was preferentially expressed on $T_{EM}$ and $T_{RM}$-like *S. pneumoniae*–specific memory cells, whilst being consistently expressed among $T_{CM}$, $T_{EM}$ and $T_{RM}$-like cells in influenza infection. Cell-intrinsic loss of CCR2 led to enhanced contraction of $CD4^+$ T cells after infection in both models, with $T_{RM}$-like cells present in the lung being somewhat protected from this effect. The absence of CCR2 did not appear to affect initial cytokine production or entry into the inflamed lung, however it did lead to reduced IFNγ production by memory $T_H1$ cells in influenza infection. Despite the reduced numbers of *Ccr2*$^{-/-}$ $CD4^+$ T cells in the memory response, these cells were equally capable of expanding upon secondary challenge in both models, although influenza-specific $CD4^+$ T cells displayed inhibited ability to secrete IFNγ. Thus, these data suggest that CCR2 is an important mediator of memory $CD4^+$ T cell maintenance in infectious immunity.

**Figure 4.1: Validation of antigen-specific *S. pneumoniae* model.**

Genetically modified D39 *S. pneumoniae* strains were generated through insertion of $OVA_{SIINFEKL}$ or $OVA_{323-339}$ peptides into the PspA protein. **(A)** WT B6 mice were infected with the unmodified parental strain D39, D39-$OVA_{SIINFEKL}$ or D39-$OVA_{323-339}$. Bacterial load in the nasal wash (left) and homogenised nasal tissue (right) was analysed seven days post-infection. n=5 mice, one-way ANOVA. Mean ± SEM **(B-D)** $CD45.1.2^{+}$ OT-II cells were adoptively transferred into Ly5.1 hosts. Mice were subsequently infected with D39-$OVA_{SIINFEKL}$ or D39-$OVA_{323-339}$. (B) Schematic of experiment. (C) Representative gating of transferred OT-II cells in lung of D39-$OVA_{SIINFEKL}$ challenged mice (left) or D39-$OVA_{323-339}$ challenged mice 7 days post-infection (right). (D) Number of $CD44^{hi}$ OT-II cells in the indicated organs. n=2-4 mice, Mean ± SEM. * $p \leq 0.05$.

*This experiment was conceptualized and executed in collaboration with Dr. Richard Harvey.*


A
NW
NT
CFU
100000
10000
1000
100
10
1
*
*
D39
D39SIINFEKL
D39OVA323-339
B
CD45.1.2
OT-II
10⁷ CFU
D39-SIINFEKL or
D39-OVA323-339
10⁵ OT-II i.v.
Ly5.1
d-1
d0
d7
Analysis
C
D39-SIINFEKL
D39-OVA323-339
0.040
58.7
0.72
88.0
CD45.2
CD44
CD4
D
Spleen
medLN
Lung
CD44hi OT-II (x10⁴)
*
D39SIINFEKL
D39OVA323-339

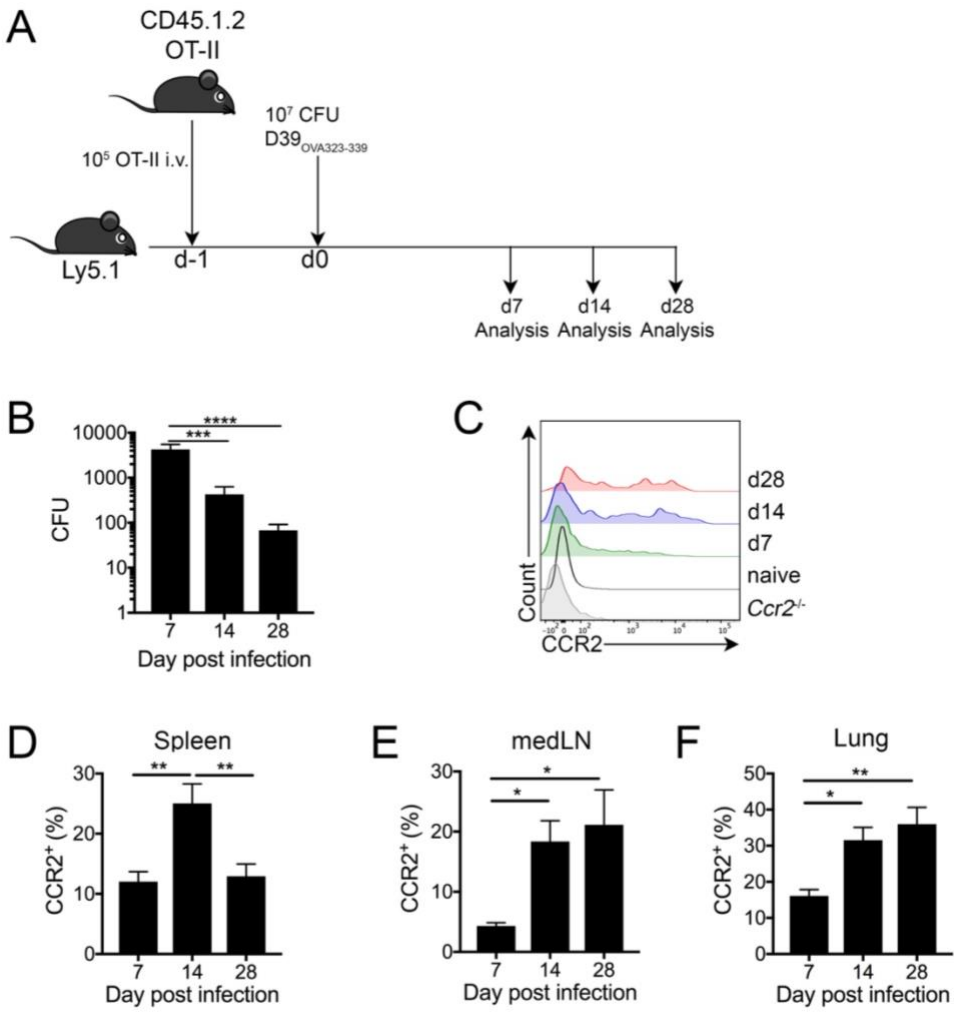



**Figure 4.2: CCR2 is expressed on antigen-specific CD4$^+$ T cells in *S. pneumoniae* infection.**

CD45.1.2$^+$ OT-II cells ($10^5$) were adoptively transferred into Ly5.1 hosts which were infected intranasally one day later with $10^7$ CFU of D39-OVA$_{323-339}$. **(A)** Schematic of experiment. **(B)** Bacterial load in the nasal wash. **(C)** Representative expression of CCR2 on CD44$^{hi}$ or naïve OT-II cells post infection. **(D-F)** Quantification of CCR2 expression on CD44$^{hi}$ OT-II cells in the indicated organs. n=10-12 mice, one-way ANOVA. Pooled from two independent experiments. Mean ± SEM. * $p \leq 0.05$, ** $p \leq 0.01$

**Figure 4.3: CCR2 is expressed on memory subsets after *S. pneumoniae* infection.** CD45.1.2$^{+}$ OT-II cells ($10^{5}$) were adoptively transferred into Ly5.1 hosts which were infected intranasally one day later with $10^{7}$ CFU of D39-OVA$_{323-339}$. Mice were analysed 28 days post infection. **(A)** Representative gating of CD62L$^{hi}$ CD44$^{hi}$ ($T_{CM}$), cells CD62L$^{lo}$ CD44$^{hi}$ ($T_{EM}$) cells and CD11a$^{hi}$ CD69$^{+}$ ($T_{RM}$-like) cells. Cells were pregated on live CD4$^{+}$ CD45.2$^{+}$ cells. **(B)** Frequency of transferred OT-II cells with different memory phenotypes in the spleen. **(C)** Frequency of transferred OT-II cells with different memory phenotypes in the lung. **(D)** Frequency of CCR2 expression on memory OT-II cells in the spleen, unpaired t-test. **(E)** Frequency of CCR2 expression on memory OT-II cells in the lung, one-way ANOVA. **(F)** Frequency of CCR2 expression on endogenous memory CD4$^{+}$ T cell subsets in the spleen, one-way ANOVA. **(G)** Frequency of CCR2 expression on endogenous memory CD4$^{+}$ T cell subsets in the lung. n=7-10 mice, one-way ANOVA. Pooled from 2 independent experiments. Mean ± SEM. * $p \leq 0.05$, ** $p \leq 0.01$.

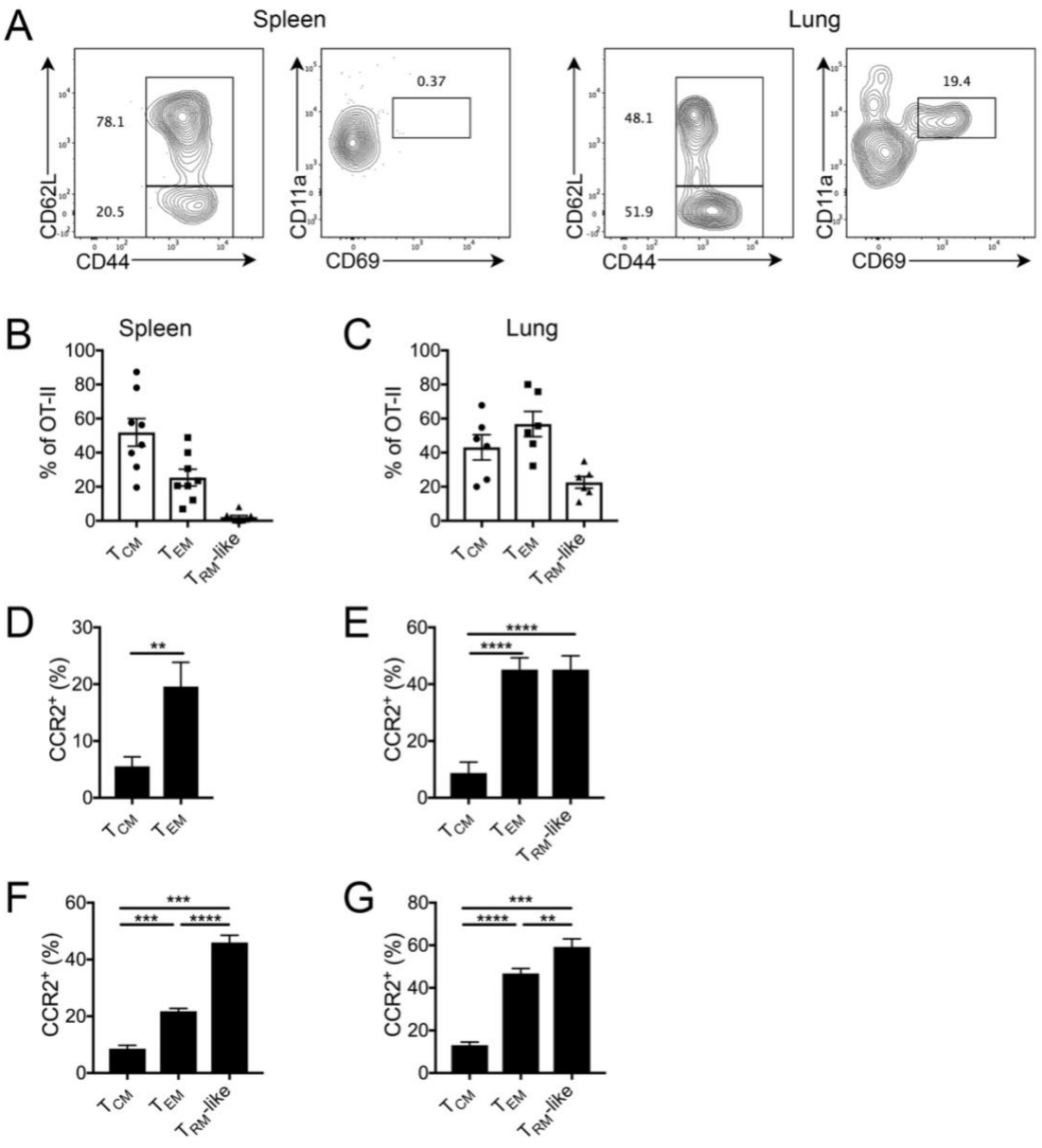
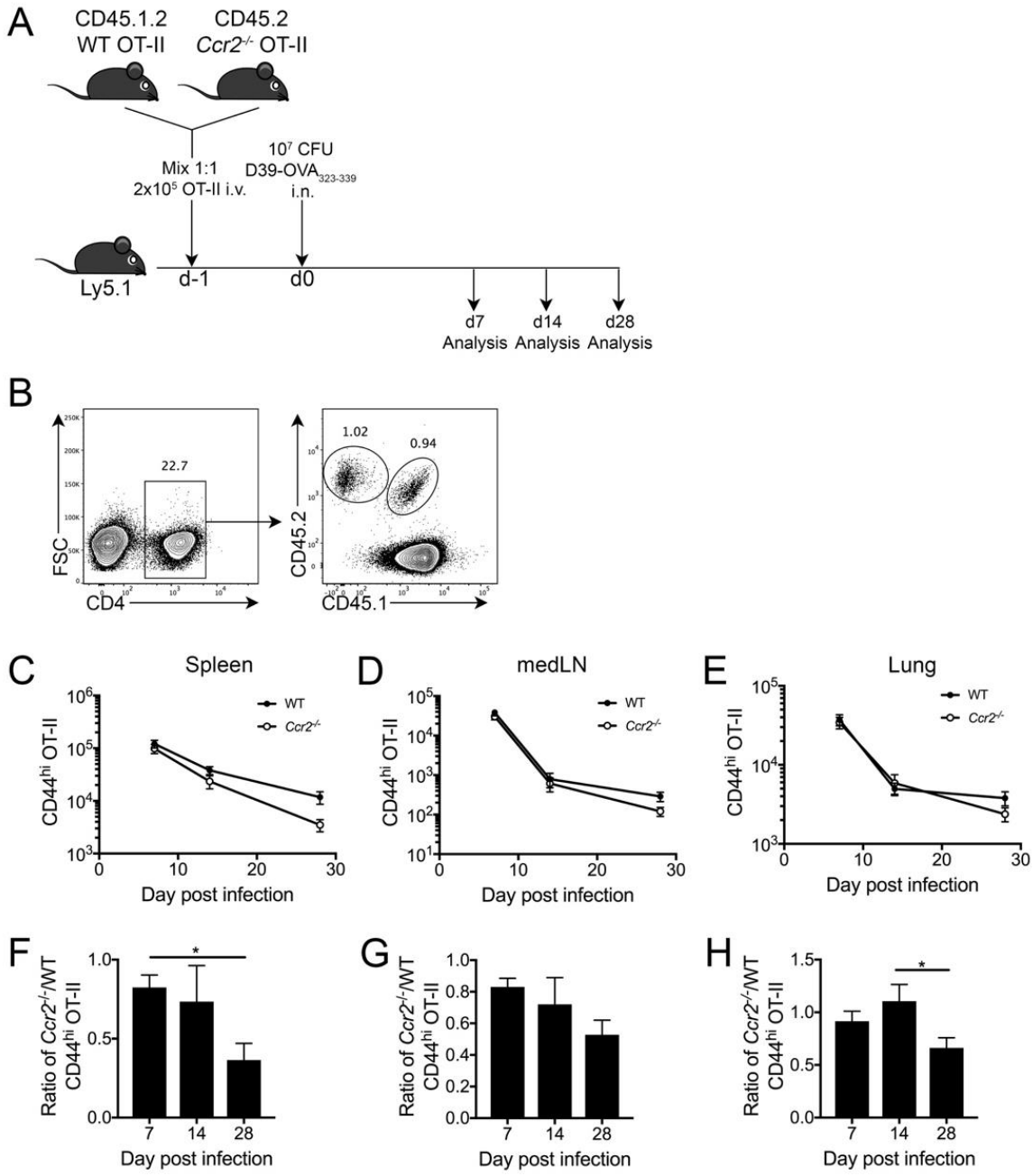

A
Spleen
Lung
78.1
20.5
0.37
48.1
51.9
19.4
CD62L
CD44
CD11a
CD69
B
Spleen
C
Lung
% of OT-II
TCM
TEM
TRM-like
D
E
F
G
CCR2+ (%)

**Figure 4.4: CCR2-deficient OT-II cells contract more rapidly after *S. pneumoniae* infection.**

WT (CD45.1.2$^{+}$) and *Ccr2*$^{-/-}$ (CD45.2$^{+}$) OT-II cells were mixed at a 1:1 ratio and 2x10$^{5}$ total cells were transferred into Ly5.1 hosts, which were subsequently infected with 10$^{7}$ CFU of D39-OVA$_{323-339}$. **(A)** Schematic of experiment. **(B)** Representative gating strategy of transferred OT-II cells, pre-gated on live cells. **(C-E)** Kinetic analysis of number of WT and *Ccr2*$^{-/-}$ OT-II cells in (C) spleen, (D) mediastinal LN and (E) lung after infection. **(F-H)** Ratio of *Ccr2*$^{-/-}$ OT-II to WT OT-II cells in the (F) spleen, (G) mediastinal LN and (H) lung after infection. n=12 mice, one-way ANOVA. Data pooled from two independent experiments. Mean ± SEM. * $p \leq 0.05$.


A
CD45.1.2
WT OT-II
CD45.2
Ccr2-/- OT-II
Mix 1:1
2x10^5 OT-II i.v.
10^7 CFU
D39-OVA323-339
i.n.
Ly5.1
d-1
d0
d7
Analysis
d14
Analysis
d28
Analysis
B
22.7
FSC
CD4
1.02
0.94
CD45.2
CD45.1
C
Spleen
D
medLN
E
Lung
WT
Ccr2-/-
CD44hi OT-II
Day post infection
F
G
H
Ratio of Ccr2-/-/WT CD44hi OT-II
Day post infection

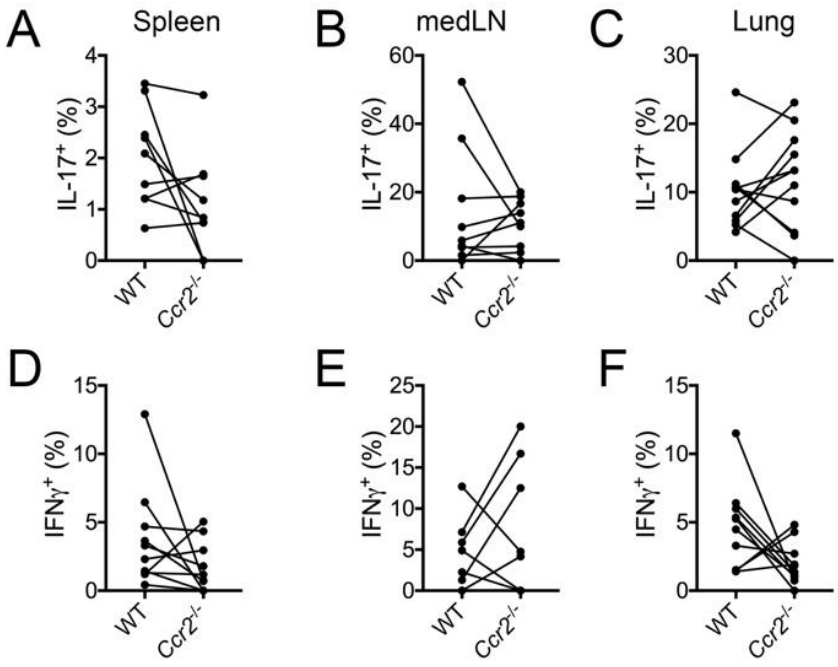



**Figure 4.5: CCR2 does not affect cytokine production by memory OT-II cells.**
WT (CD45.1.2$^+$) and *Ccr2*$^{-/-}$ (CD45.2$^+$) OT-II cells were mixed at a 1:1 ratio and 2x10$^5$ total cells were transferred into Ly5.1 hosts, which were subsequently infected with 10$^7$ CFU of D39-OVA$_{323-339}$ and analysed 28 days post-infection. **(A-C)** Frequency of IL-17$^+$ OT-II in the (A) spleen, (B) medLN and (C) lung. **(D-F)** Frequency of IFNγ$^+$ OT-II in the (D) spleen, (E) medLN and (F) lung. n=11 mice. Pooled from 2 independent experiments, Wilcoxon matched-pairs sign rank test. Mean ± SEM.

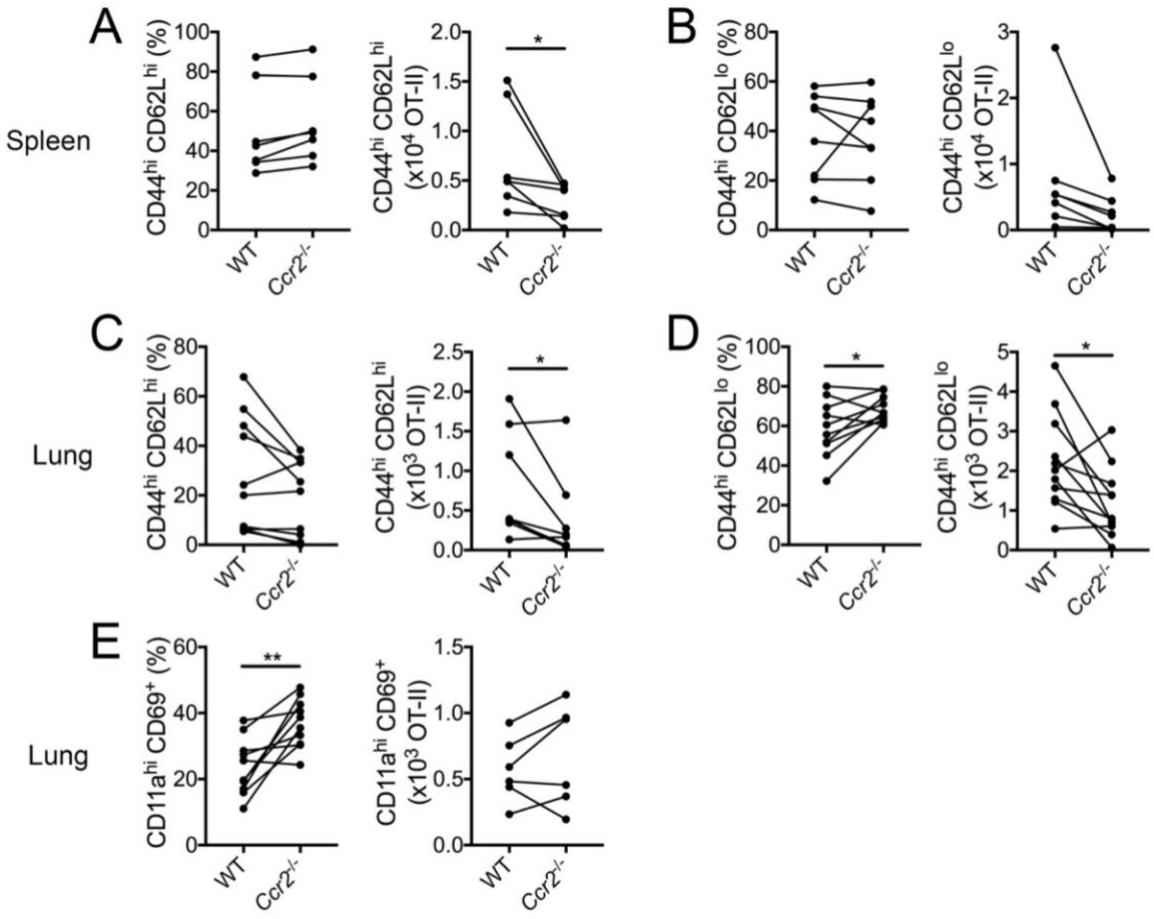



**Figure 4.6: *Ccr2*$^{-/-}$ $T_{EM}$ and $T_{CM}$, but not $T_{RM}$-like OT-II cells undergo enhanced contraction compared with WT OT-II cells.**

WT (CD45.1.2$^{+}$) and *Ccr2*$^{-/-}$ (CD45.2$^{+}$) OT-II cells were mixed at a 1:1 ratio and 2x10$^{5}$ total cells were transferred into Ly5.1 hosts, which were subsequently infected with 10$^{7}$ CFU of D39-OVA$_{323-339}$ and analysed 28 days post-infection. **(A)** Frequency and number of CD62L$^{hi}$ CD44$^{hi}$ ($T_{CM}$) cells in the spleen. **(B)** Frequency and number of CD62L$^{lo}$ CD44$^{hi}$ ($T_{EM}$) cells in the spleen. **(C)** Frequency and number of CD62L$^{hi}$ CD44$^{hi}$ ($T_{CM}$) cells in the lung. **(D)** Frequency and number of CD62L$^{lo}$ CD44$^{hi}$ ($T_{EM}$) cells in the lung. **(E)** Frequency and number of CD11a$^{hi}$ CD69$^{+}$ ($T_{RM}$-like) cells in the lung. n=7-10 mice, Wilcoxon matched-pairs signed rank test. Pooled from 2 independent experiments. Mean ± SEM. * p≤0.05, ** p≤0.01.

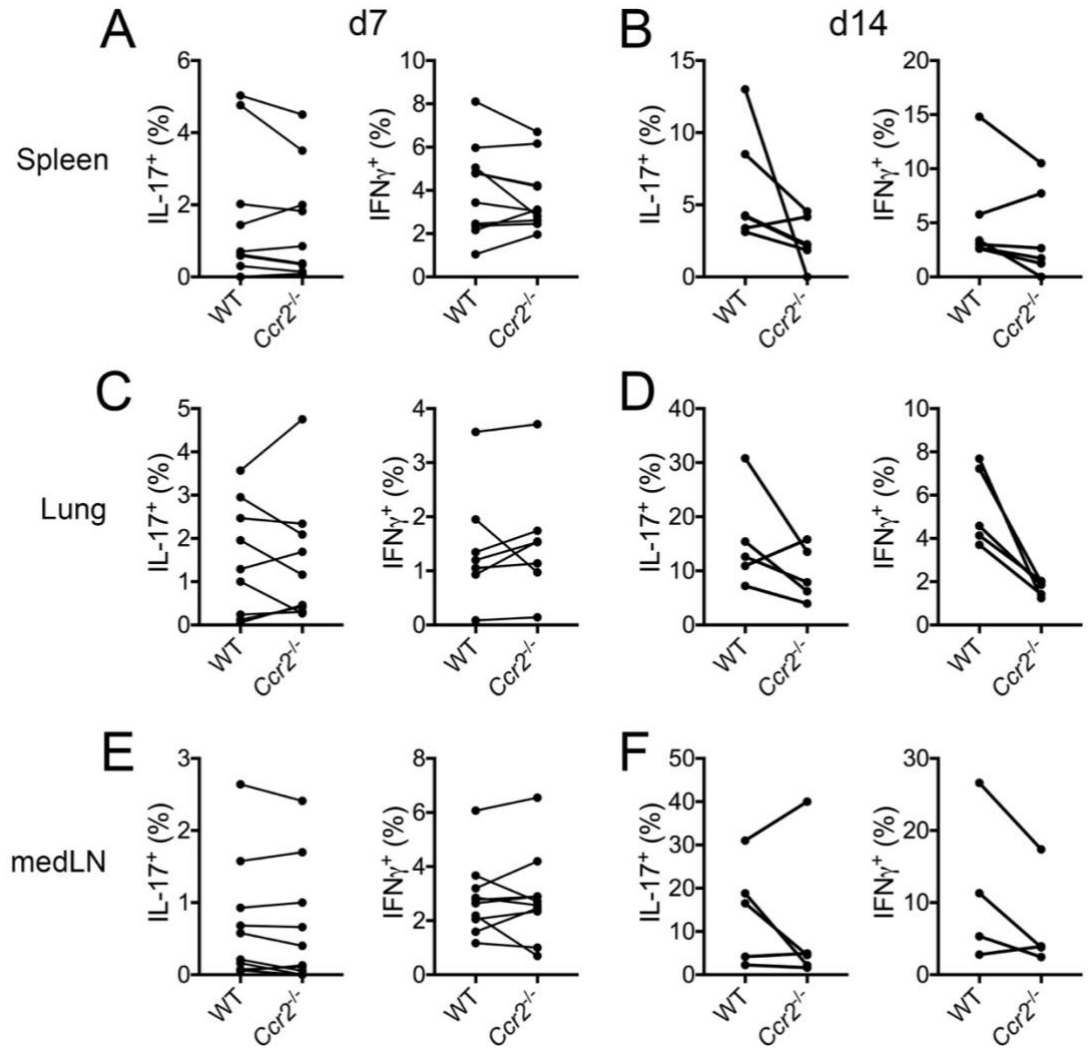



**Figure 4.7: CCR2 does not affect cytokine production by OT-II cells in the acute response to *S. pneumoniae* infection.**

WT (CD45.1.2$^+$) and *Ccr2*$^{-/-}$ (CD45.2$^+$) OT-II cells were mixed at a 1:1 ratio and 2x10$^5$ total cells were transferred into Ly5.1 hosts, which were subsequently infected with 10$^7$ CFU of D39-OVA$_{323-339}$. **(A-B)** Frequency of IL-17$^+$ and IFNγ$^+$ OT-II cells in the spleen at (A) 7 or (B) 14 days post-infection. **(C-D)** Frequency of IL-17$^+$ and IFNγ$^+$ OT-II cells in the lung at (C) 7 or (D) 14 days post-infection. **(E-F)** Frequency of IL-17$^+$ and IFNγ$^+$ OT-II cells in the medLN at (E) 7 or (F) 14 days post-infection. n=5-10 mice, Wilcoxon matched-pairs signed rank test. (A, C, E) Pooled from two similar experiments. (B, D, F) from a single experiment. Mean ± SEM. * p≤0.05.

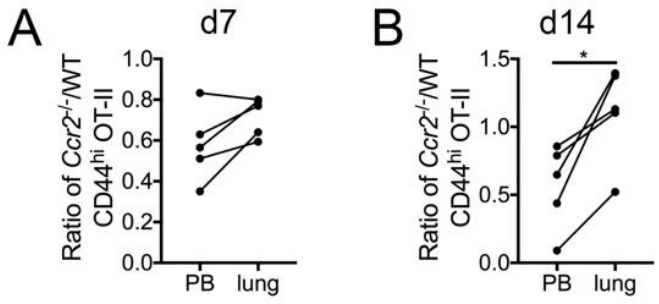



**Figure 4.8: CCR2 is not required for OT-II entry into the lungs.**

WT (CD45.1.2$^{+}$) and *Ccr2*$^{-/-}$ (CD45.2$^{+}$) OT-II cells were mixed at a 1:1 ratio and 2x10$^{5}$ total cells were transferred into Ly5.1 hosts, which were subsequently infected with 10$^{7}$ CFU of D39-OVA$_{323-339}$. Ratio of *Ccr2*$^{-/-}$ OT-II to WT OT-II cells in the peripheral blood versus the lung **(A)** 7 days or **(B)** 14 days post-infection. n=5 mice, paired t-test. Mean ± SEM. * p≤0.05.

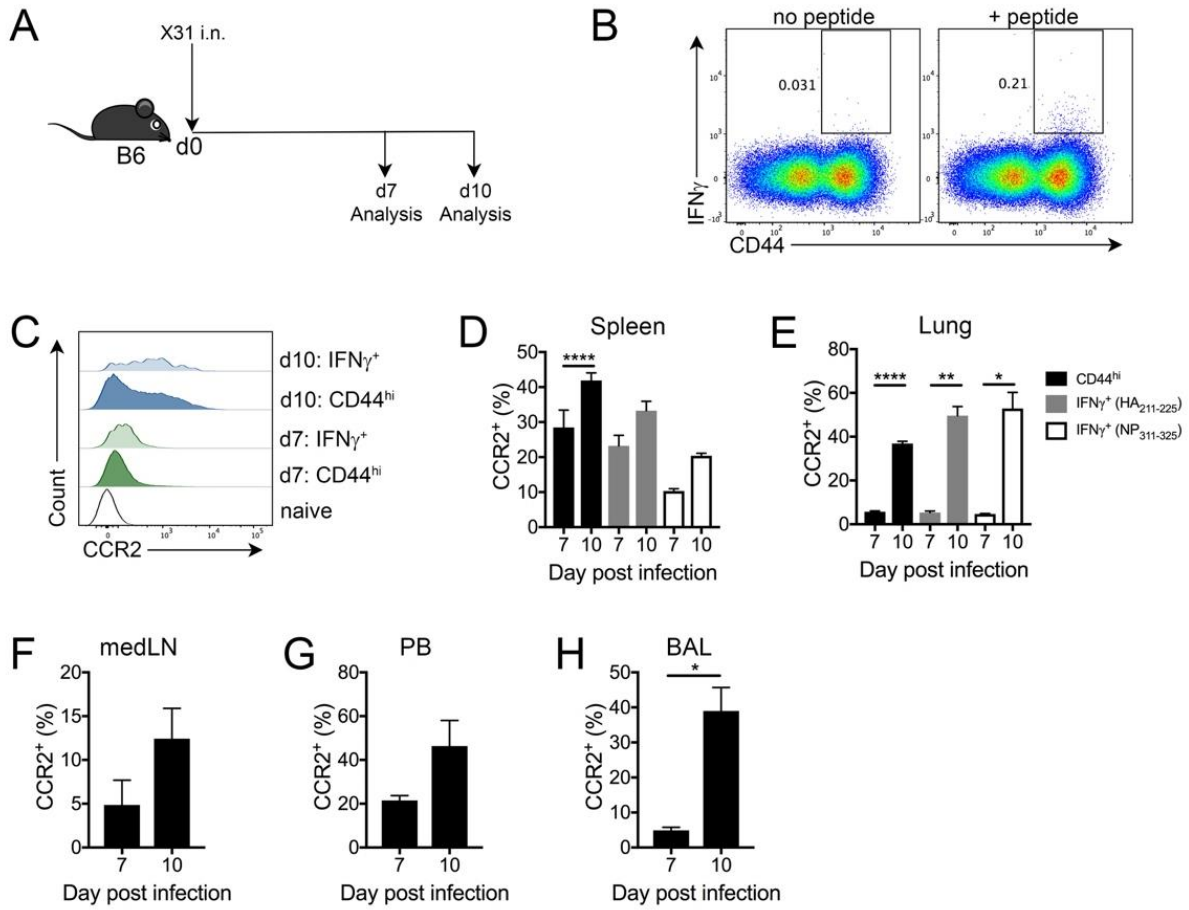



**Figure 4.9: CCR2 is expressed on influenza-specific CD4$^+$ T cells.**

WT mice were infected with 7.3 $TCID_{50}$ X31 intranasally and endogenous influenza-specific $T_H1$ cells were analysed by *ex vivo* restimulation with $HA_{211-225}$ or $NP_{311-325}$ peptide. **(A)** Schematic of experiment. **(B)** Representative gating strategy of IFNγ production by CD4$^+$ T cells in the absence or presence of peptide restimulation. **(C)** Representative expression of CCR2 on IFNγ$^+$ or CD44$^{hi}$ CD4$^+$ T cells from the lung on day 7 or 10 after influenza infection. **(D-E)** Frequency of CCR2 expression on CD44$^{hi}$ (black) or IFNγ$^+$ CD4$^+$ T cells from (D) spleen or (E) lung after restimulation with $HA_{211-225}$ (grey) or $NP_{311-325}$ (white) peptide. **(F-H)** Frequency of CCR2 expression on CD44$^{hi}$ CD4$^+$ T cells from (F) mediastinal LN, (G) peripheral blood and (H) bronchoalveolar lavage. n=5 mice, unpaired t-test. Data representative of two similar experiments. Mean ± SEM. * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$.

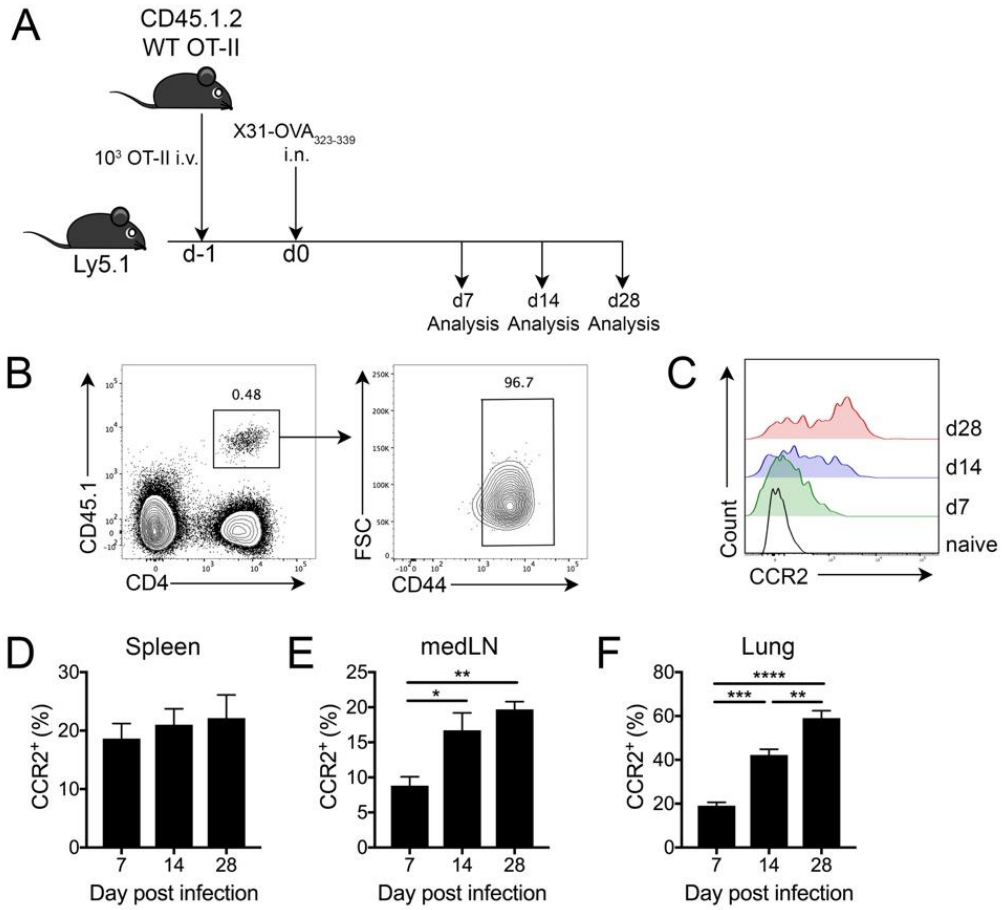



**Figure 4.10: CCR2 is expressed on OT-II cells in response to influenza infection.**
WT (CD45.1.2$^{+}$) OT-II cells ($10^3$) were transferred into Ly5.1 hosts, which were subsequently infected with 10 $TCID_{50}$ X31-$OVA_{323-339}$. **(A)** Schematic of experiment. **(B)** Representative gating strategy of transferred OT-II cells. **(C)** Representative expression of CCR2 on transferred OT-II cells or naïve $CD4^{+}$ T cells in the lung after infection. **(D-F)** Frequency of CCR2 expression on $CD44^{hi}$ OT-II cells from (D) spleen, (E) mediastinal LN and (F) lung after infection. n=5 mice, one-way ANOVA. Mean ± SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

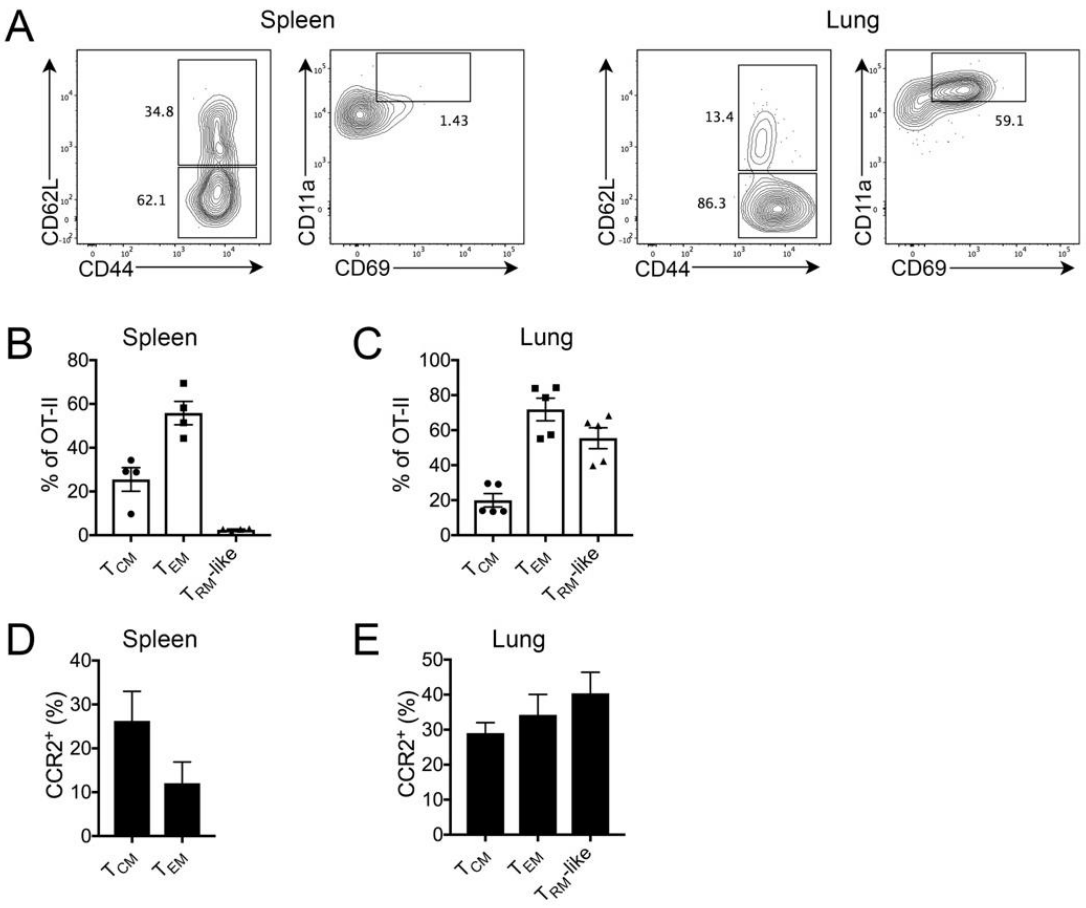



**Figure 4.11: CCR2 is expressed on memory CD4$^+$ subsets after influenza infection.** WT (CD45.1.2$^+$) OT-II cells ($10^3$) were transferred into Ly5.1 hosts, which were subsequently infected with 10 $TCID_{50}$ X31-$OVA_{323-339}$. Mice were analysed 28 days post-infection. **(A)** Representative gating strategy of CD62L$^{hi}$ CD44$^{hi}$ ($T_{CM}$), cells CD62L$^{lo}$ CD44$^{hi}$ ($T_{EM}$) cells and CD11a$^{hi}$ CD69$^+$ ($T_{RM}$-like) cells. **(B)** Memory phenotype of transferred OT-II cells in the spleen. **(C)** Memory phenotype of transferred OT-II cells in the lung. **(D)** Frequency of CCR2 expression on memory OT-II cells in the spleen. **(E)** Frequency of CCR2 expression on memory OT-II cells in the lung. n=5 mice. Mean ± SEM.

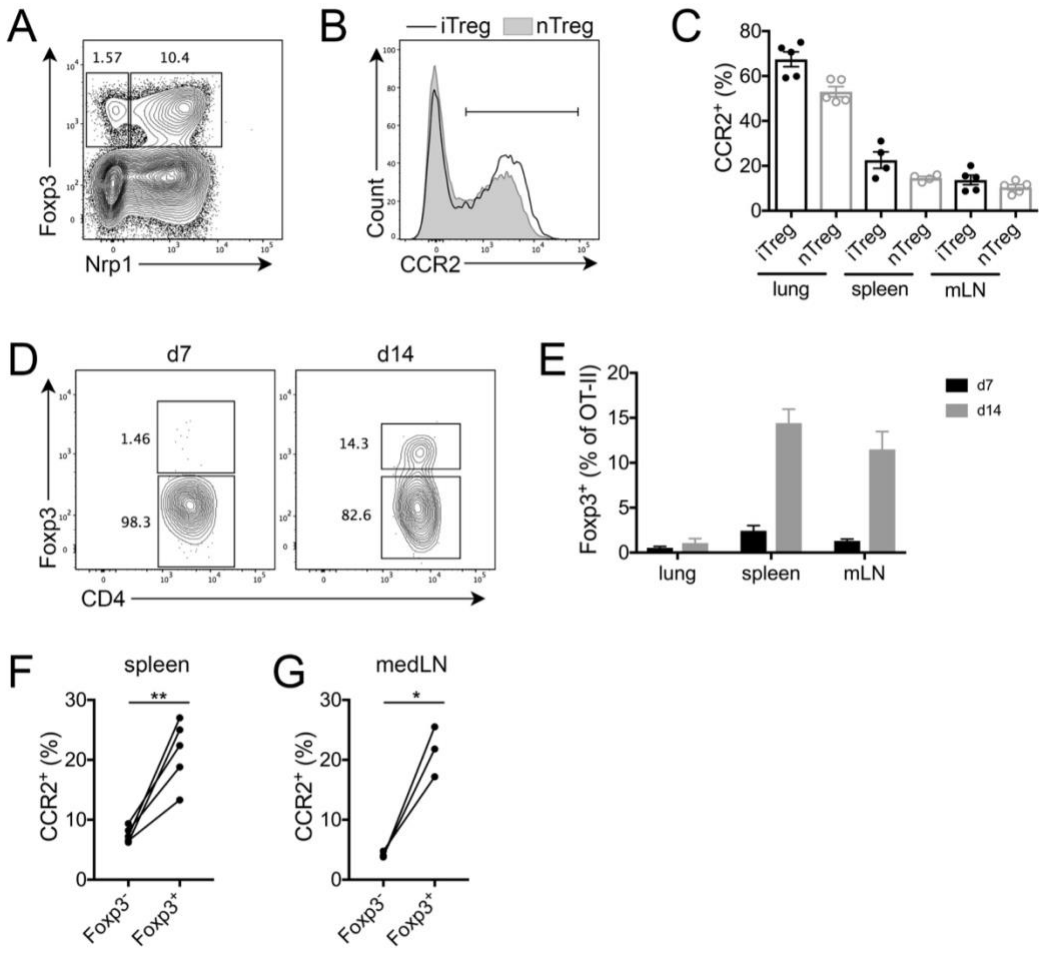



**Figure 4.12: Regulatory T cells express CCR2.**

WT (CD45.1.2$^+$) OT-II cells were transferred into Ly5.1 hosts, which were then infected with X31-OVA$_{323-339}$. **(A)** Representative gating strategy of host iTreg (CD4$^+$ Foxp3$^+$ Nrp1$^-$) and nTreg (CD4$^+$ Foxp3$^+$ Nrp1$^+$) cells from the lung 7 days post-infection, pregated CD4$^+$. **(B)** Representative expression of CCR2 on iTreg (black line) and nTreg (shaded grey) from the lung. **(C)** Frequency of CCR2 expression on iTreg and nTreg from the indicated organs, seven days post-infection. **(D)** Representative gating strategy of Foxp3 expression by transferred OT-II cells. **(E)** Frequency of Foxp3 upregulation on transferred OT-II cells. **(F)** Frequency of CCR2 expression on Foxp3$^-$ and Foxp3$^+$ OT-II cells in the spleen 14 days post-infection. **(G)** Frequency of CCR2 expression on Foxp3$^-$ and Foxp3$^+$ OT-II cells in the mediastinal LN 14 days post-infection. n=5 mice, paired t-test. Mean ± SEM. * $p \leq 0.05$, ** $p \leq 0.01$.

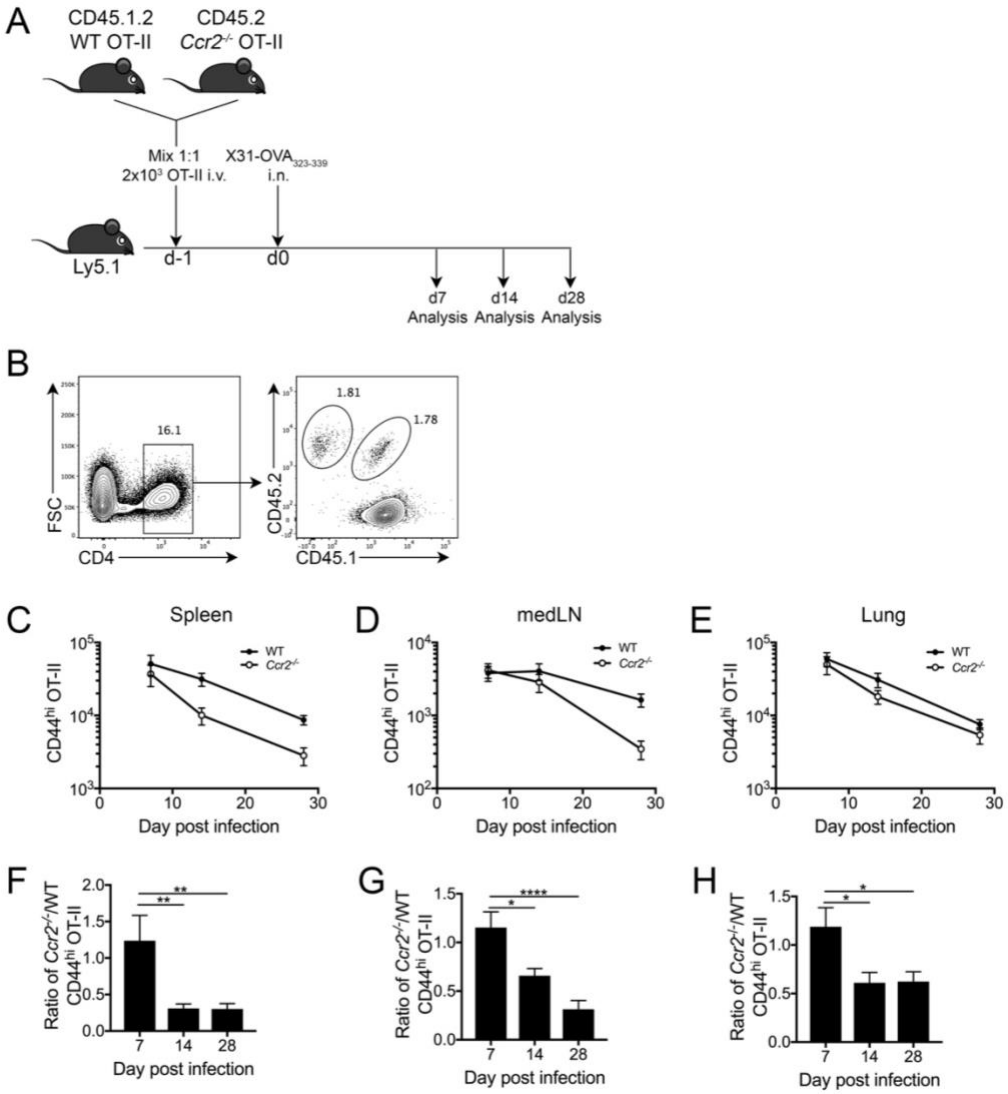



**Figure 4.13: CCR2-deficient OT-II cells contract more rapidly after influenza infection.**

WT (CD45.1.2$^+$) and *Ccr2*$^{-/-}$ (CD45.2$^+$) OT-II cells were mixed at a 1:1 ratio and 2x10$^3$ total cells were transferred into Ly5.1 hosts, which were subsequently infected with 10 $TCID_{50}$ X31-$OVA_{323-339}$. **(A)** Schematic of experiment. **(B)** Representative gating strategy of transferred OT-II cells, pre-gated on live cells. **(C-E)** Kinetic analysis of number of WT and *Ccr2*$^{-/-}$ OT-II cells in (C) spleen, (D) mediastinal LN and (E) lung after infection. **(F-H)** Ratio of *Ccr2*$^{-/-}$ OT-II to WT OT-II cells in the (F) spleen, (G) mediastinal LN and (H) lung after infection. n=10-15 mice (4-5 mice per experiment), one-way ANOVA. Pooled from three similar experiments. Mean ± SEM. * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$.

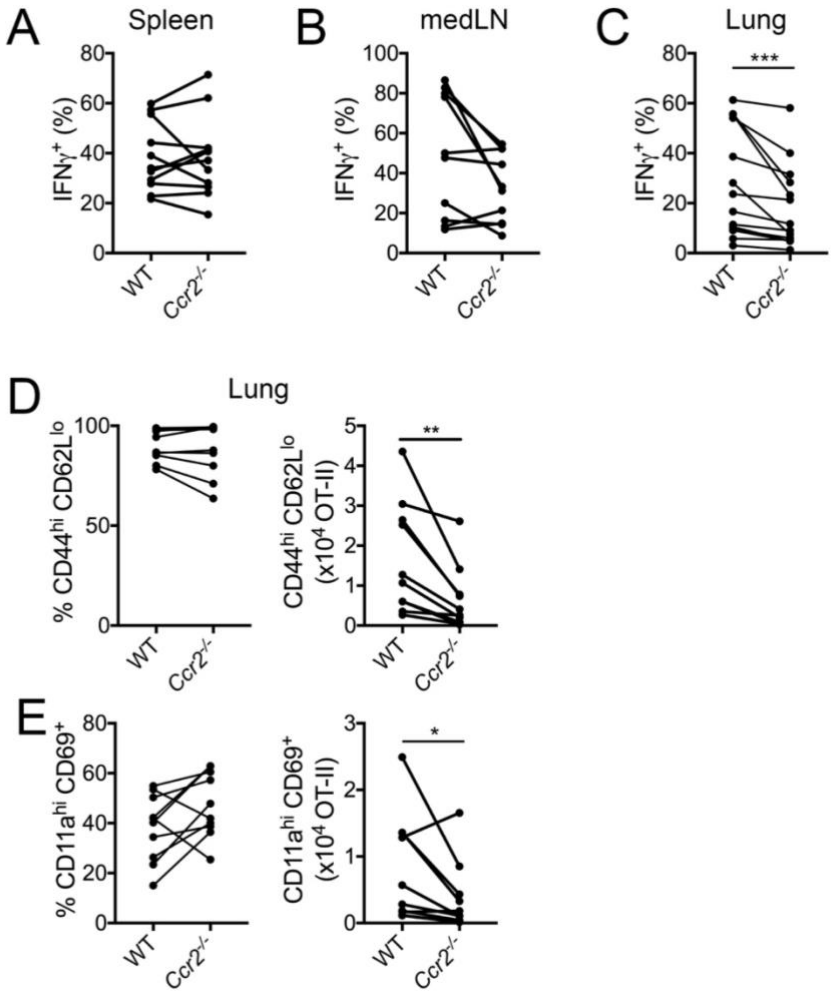
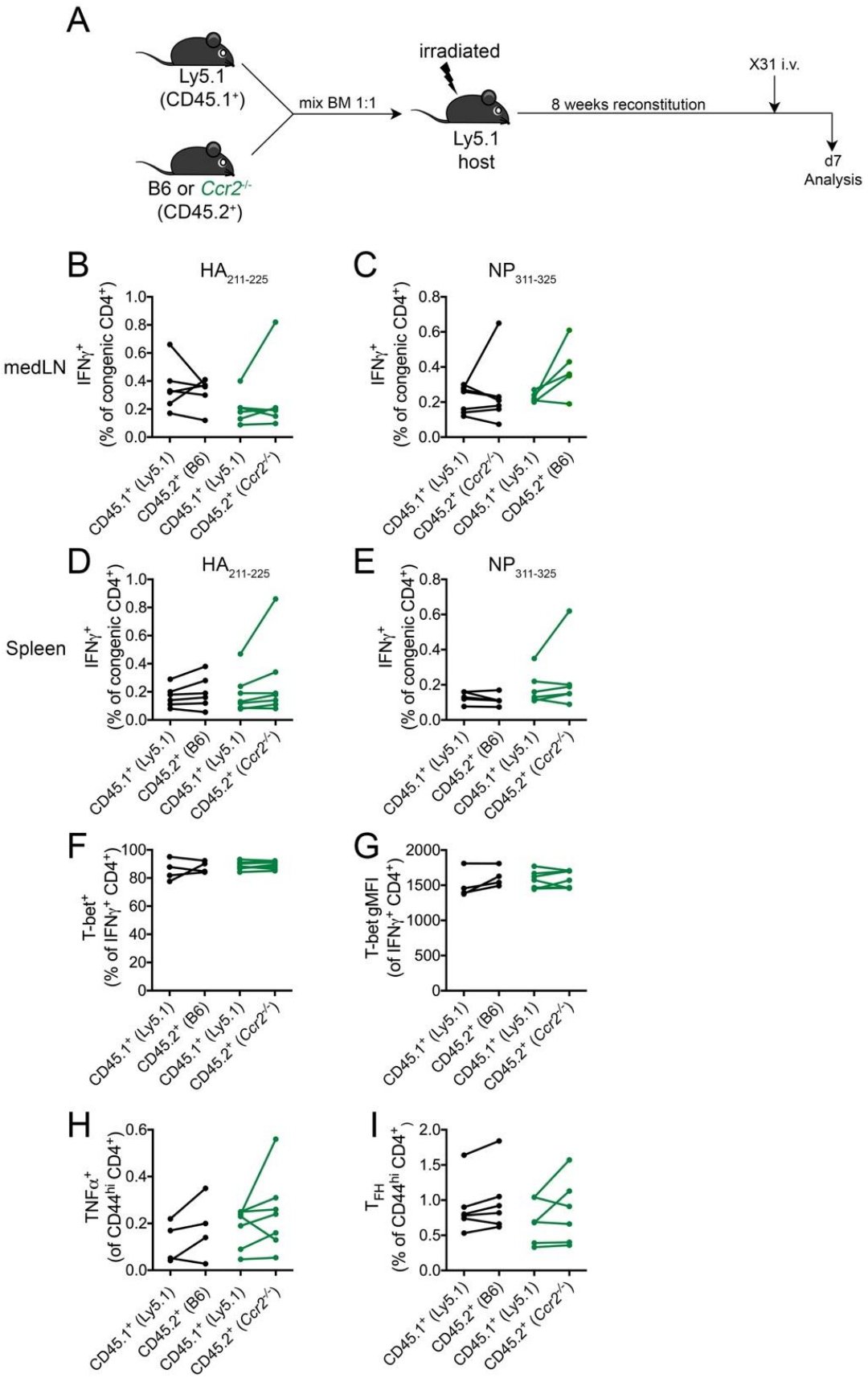



**Figure 4.14: CCR2 deficiency affects the memory phenotype of $T_H1$ cells in response to influenza infection.**

WT (CD45.1.2$^+$) and *Ccr2*$^{-/-}$ (CD45.2$^+$) OT-II cells were mixed at a 1:1 ratio and 2x10$^3$ total cells were transferred into Ly5.1 hosts, which were subsequently infected with 10 $TCID_{50}$ X31-$OVA_{323-339}$. Mice were analysed at 28 days post-infection. **(A-C)** IFNγ production by WT and *Ccr2*$^{-/-}$ CD44$^{hi}$ OT-II cells in (A) spleen, (B) mediastinal LN and (C) lung after infection. **(D)** Frequency and number of CD44$^{hi}$ CD62L$^{lo}$ $T_{EM}$ cells among OT-II cells from the lung. **(E)** Frequency and number of CD11a$^{hi}$ CD69$^+$ $T_{RM}$-like cells among OT-II cells from the lung. n=10 mice, Wilcoxon matched-pairs signed rank test. Data pooled from two independent experiments. Mean ± SEM. * p≤0.05, ** p≤0.01.

**Figure 4.15: CCR2 deficiency has no effect on the initial effector response to influenza infection.**

Bone marrow from Ly5.1 ($CD45.1^+$) was mixed at a 1:1 ratio with either B6 (control chimera; black) or *Ccr2*$^{-/-}$ (experimental chimera; green) $CD45.2^+$ bone marrow and transferred into irradiated Ly5.1 recipients. After 8 weeks to allow reconstitution, chimeric mice were infected with X31 and analysed 7 days post-infection. **(A)** Schematic of experiment. **(B-E)** Frequency of $IFN\gamma^+$ cells among congenic $CD4^+$ T cells in the (B, C) mediastinal LN or (D, E) spleen after restimulation with (B, D) $HA_{211-225}$ peptide or (C, E) $NP_{311-325}$ peptide. **(F)** Frequency of T-bet expression among congenic $IFN\gamma^+$ $CD4^+$ T cells in the spleen. **(G)** Geometric MFI of T-bet in congenic $IFN\gamma^+$ $CD4^+$ T cells from the spleen. **(H)** Frequency of $TNF\alpha^+$ cells among congenic $CD44^{hi}$ $CD4^+$ T cells in the spleen. **(I)** Frequency of $PD1^{hi}$ $CXCR5^{hi}$ $T_{FH}$ cells from congenic $CD44^{hi}$ $CD4^+$ T cells in the spleen. n=6-7 mice, paired t-test. Mean ± SEM.


A
Ly5.1
(CD45.1+)
B6 or Ccr2-/-
(CD45.2+)
mix BM 1:1
irradiated
Ly5.1
host
8 weeks reconstitution
X31 i.v.
d7
Analysis
B
HA211-225
C
NP311-325
medLN
IFNγ+
(% of congenic CD4+)
D
HA211-225
E
NP311-325
Spleen
F
T-bet+
(% of IFNγ+ CD4+)
G
T-bet gMFI
(of IFNγ+ CD4+)
H
TNFα+
(of CD44hi CD4+)
I
TFH
(% of CD44hi CD4+)
CD45.1+ (Ly5.1)
CD45.2+ (B6)
CD45.2+ (Ccr2-/-)

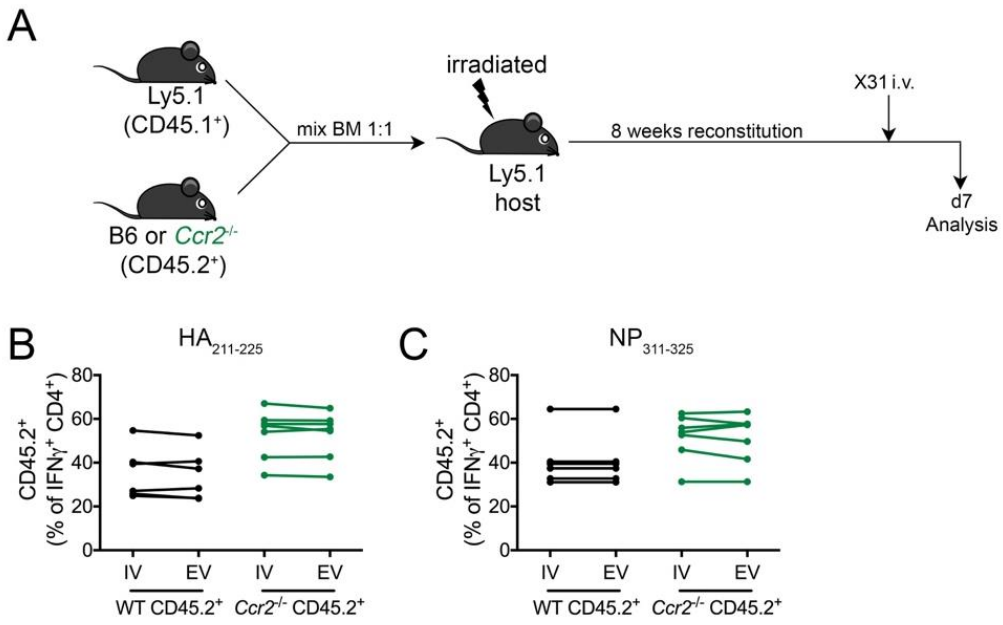



**Figure 4.16: CCR2 deficiency does not affect $T_H1$ cell entry into the lung parenchyma after influenza infection.**

Bone marrow from Ly5.1 (CD45.1$^+$) was mixed at a 1:1 ratio with either B6 (control chimera; black) or *Ccr2*$^{-/-}$ (experimental chimera; green) CD45.2$^+$ bone marrow and transferred into irradiated Ly5.1 recipients. After 8 weeks to allow reconstitution, chimera mice were infected with X31 and analysed 7 days post-infection. Intravascular CD4$^+$ T cells were labelled five minutes prior to harvest. **(A)** Schematic of experiment. **(B)** Frequency of CD45.2$^+$ cells among intravascular (IV) and extravascular (EV) IFN$\gamma^+$ CD4$^+$ T cells in the lung in response to restimulation with $HA_{211-225}$ peptide. **(C)** Frequency of CD45.2$^+$ cells among intravascular (IV) and extravascular (EV) IFN$\gamma^+$ CD4$^+$ T cells in the lung in response to restimulation with $NP_{311-325}$ peptide. n=6-7 mice, paired t-test. Mean ± SEM.

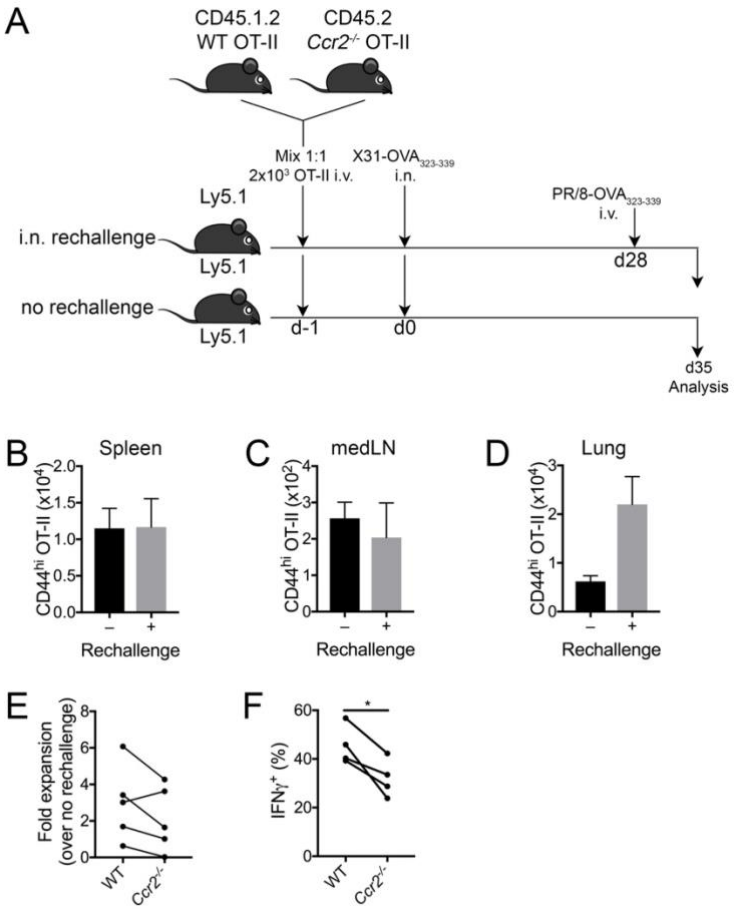



**Figure 4.17: CCR2 deficiency does not affect the recall response of OT-II cells upon influenza rechallenge.**

WT (CD45.1.2$^{+}$) and *Ccr2*$^{-/-}$ (CD45.2$^{+}$) OT-II cells were mixed at a 1:1 ratio and 2x10$^{3}$ total cells were transferred into Ly5.1 hosts, which were subsequently infected with 10 $TCID_{50}$ X31-$OVA_{323-339}$. At 28 days post-infection, mice were rechallenged with 10 $TCID_{50}$ PR/8-$OVA_{323-339}$ and analysed seven days post-rechallenge. **(A)** Schematic of experiment. **(B-D)** Number of CD44$^{hi}$ OT-II cells in (B) spleen, (C) medLN or (D) lung in mice with or without rechallenge with PR/8-$OVA_{323-339}$. **(E)** Fold expansion of CD44$^{hi}$ OT-II cells in rechallenged mice over CD44$^{hi}$ OT-II cells from non-rechallenged mice in the lung. **(F)** IFNγ production by CD44$^{hi}$ OT-II cells after rechallenge in the lung. n=5 mice, paired t-test. Mean ± SEM. * $p \leq 0.05$.

# CHAPTER 5

# Discussion

# Chapter 5 – Discussion

## 5.1 ACKR4 regulates anti-tumour immune responses

### 5.1.1 Loss of ACKR4 enhances tumour-directed immune responses

The data presented in Chapter 3 demonstrated a novel role for ACKR4 in restraining anti-tumour immune responses. ACKR4 deficiency led to inhibition of tumour growth in numerous orthotopic, transgenic and chemically-induced models of cancer. This was dependent on the enhanced intratumoural recruitment of $CD8^+$ T cells that occurred in the absence of ACKR4. Alterations in $CD8^+$ T cell priming were not apparent, suggesting that loss of ACKR4 affected activated $CD8^+$ T cells. This was associated with increased retention of $CD103^+$ DCs at the tumour site, with these cells previously being shown to critically regulate the magnitude of anti-tumour $CD8^+$ T cell responses. Furthermore, direct intratumoural administration of CCL21 into ACKR4-sufficient hosts also led to increased accumulation of DCs at the tumour site, implying that chemokine scavenging by ACKR4 is mechanistically involved in the control of intratumoural DC egress. Haematogenous metastatic colonisation in multiple tumour models was also significantly curtailed in ACKR4-deficient hosts, although due to time constraints, this was not investigated in more detail. Finally, in keeping with the observed effects on the anti-tumour immune response, loss of ACKR4 enhanced responsiveness to multiple immunotherapies, suggesting targeting intratumoural DCs through ACKR4 may potentially be a novel therapeutic target.

The findings presented here demonstrating that loss of ACKR4 is protective in cancer are somewhat in contrast to previously published reports. Although reports into ACKR4 in tumours are limited, ACKR4 expression has been inversely correlated with LN metastasis and positively correlated with improved survival in breast cancer, cervical squamous cell carcinoma and hepatocellular carcinoma[145,148,149]. Transgenic expression of ACKR4 in a human breast cancer cell line attenuated tumour growth and metastasis in mouse models *in vivo*[145]. Similarly, with transgenic expression or shRNA-mediated knockdown of ACKR4 in human HCC cell lines, ACKR4 expression was inversely correlated with tumour growth[149]. In those studies, CCL19, CCL21 or CCL25 levels were altered, suggesting that the effects of ACKR4 were mediated via chemokine scavenging, although the mechanism of how tumour growth was altered was not described. Given that previous data has shown that the CCR7/CCL21 axis can increase tumour cell survival and

proliferation[120,126,127], it is likely that when ACKR4 expression is low, the lack of chemokine scavenging leads to higher levels of CCL19 and CCL21, which can promote tumour growth. However, in both of those studies, all mouse experiments were performed in nude mice, which lack T cells and thus the ability to mount T cell-dependent tumour-directed immune responses. Thus, the relative contributions of the tumour-promoting functions of CCL21 and the immunomodulatory functions is undetermined. Given that in the data described in this thesis, loss of ACKR4 enhanced $CD8^+$ T cell-mediated control of tumour growth, studying the role of ACKR4 in systems with an intact immune system is crucial for accurate assessment of the translatability of targeting ACKR4 in human disease. The only published report of ACKR4 in tumour models with an intact immune system came previously from our laboratory, in which transgenic expression of ACKR4 on 4T1.2 murine breast cancer cells was shown to reduce primary tumour growth, whilst enhancing both spontaneous and haematogenous metastasis to the lung[146]. However, this effect appeared to be independent of the host immune system, as inoculation of these cell lines into NK cell-depleted SCID mice, which lack both T and B cells, still led to accelerated growth of the ACKR4-overexpressing line. In that model, enhanced metastasis was determined to be mediated through TGFβ-mediated enhanced epithelial-mesenchymal transition. These data emphasise that ACKR4 may have different roles depending on whether it is expressed by the host or on tumour cells. In most of the work described in Chapter 3, ACKR4 deletion is restricted to the host, with orthotopic injection of ACKR4-sufficient cells. However, inhibited tumour growth was still seen in the transgenic MMTV-PyMT model and the MCA-induced fibrosarcoma model in which tumours arise from *Ackr4*$^{-/-}$ cells. This suggests that at least in these models, any potential tumour-suppressive effects of tumour expression of ACKR4 as a result of the subsequent decreases in local chemokines are outweighed by its immunomodulatory role. Thus, these data are the first to establish a role for ACKR4 in regulating immune responses to cancer.

### 5.1.2 ACKR4 is expressed by luminal epithelial cells

This work also identified a novel source of expression of ACKR4 in the luminal epithelial cells of the murine mammary gland. In the absence of a highly specific antibody for mouse ACKR4, transcript of *Ackr4* was identified by cell sorting of mammary gland populations followed by quantitative PCR. It remains to be determined if functional

ACKR4 protein is expressed by these cells, but protein expression could be assessed through binding of fluorescently-labelled CCL19 to these cells, combined with the appropriate *Ackr4*$^{-/-}$ and *Ccr7*$^{-/-}$ controls as previously described[259]. Furthermore, assuming sufficient cells can be isolated, *ex vivo* scavenging assays may assess whether luminal expression of ACKR4 is directly capable of scavenging its ligands, although the enhanced levels of CCL21 in the mammary gland reported here are highly suggestive that it is. Lastly, it would be of great interest to track ACKR4 expression over tumour development, to determine if ACKR4 is dysregulated as malignancy progresses. This could be performed in orthotopic models, in which the effect of the tumour milieu on luminal ACKR4 expression could be assessed, as well as in the MMTV-PyMT model, which appears to derive from oncogenic mutations to the luminal cells themselves[285]. The analysis of CCL21 concentration in the naïve mammary gland compared with E0771 tumours suggests that it is likely that ACKR4 expression changes over the course of tumour progression. The results of the present study showed that in *Ackr4*$^{-/-}$ mice, CCL21 was significantly increased in E0771 tumours compared to naïve mammary glands, suggesting CCL21 is upregulated in response to the tumour. However, no substantial difference was seen between CCL21 in naïve mammary glands and tumours in WT mice, indicating ACKR4 may be upregulated by host cells in the tumour setting.

### 5.1.3 ACKR4 deficiency enhances anti-tumour CD8$^+$ T cell responses

The ligands of ACKR4 are critical in controlling numerous aspects of homeostatic lymphocyte trafficking. Therefore, a thorough investigation of tumour-infiltrating immune cells in WT and *Ackr4*$^{-/-}$ mice was conducted. The proportions of infiltrating cell subsets were largely unaltered, although CD8$^+$ T cells, B cells and Ly6G$^-$ monocytic cells were increased in *Ackr4*$^{-/-}$ tumours. The role of B cells in modulating anti-tumour responses is controversial, with both pro-tumourigenic and anti-tumourigenic responses being reported. Intratumoural B cell infiltrates have been shown to correlate with improved patient prognosis and greater survival in a range of cancers[286-288], with mouse models suggesting that protection may largely be mediated through antibody responses[289]. Conversely, B cell depletion in multiple mouse tumour models reduced tumour growth and led to greater therapeutic responses, suggestive of a regulatory function of B cells in those models[290-292]. Similarly, the function of Ly6G$^-$ monocytic cells is also difficult to address without further characterisation as these cells are a highly heterogenous population with varied function, ranging from MDSCs which promote

angiogenesis and tumour growth to CD103$^+$ Ly6C$^+$ cells which have been shown to cross-prime CD8$^+$ T cells in *Batf3*$^{-/-}$ mice[269,293]. Regardless, given that depletion of CD8$^+$ T cells in *Ackr4*$^{-/-}$ mice reverted tumour growth to WT levels, the enhanced CD8$^+$ T cell response appears to be the predominant mechanism of tumour inhibition in *Ackr4*$^{-/-}$ mice. Interestingly, depletion of CD8$^+$ T cells did not alter the tumour course in WT mice, despite CD44$^{hi}$ CD8$^+$ T cells being detected within the tumours by flow cytometry. Thus, although present, these cells are not contributing to a strong anti-tumour response, suggesting that in addition to CD8$^+$ T cell numbers being enhanced in *Ackr4*$^{-/-}$ mice, CD8$^+$ T cells in these mice are more capable of mediating anti-tumour responses. This may be a consequence of a number different factors, which will now be discussed in turn.

One possibility is that CD8$^+$ T cells from *Ackr4*$^{-/-}$ mice are more effective at inducing tumour cell death. A major mechanism of CD8$^+$ T cell mediated destruction is through the release of cytolytic granules containing the serine protease granzyme B, which can induce apoptosis in the target cell[294], although the proportion of CD8$^+$ T cells expressing granzyme B was not significantly altered between WT and *Ackr4*$^{-/-}$ mice. The cytokine IFNγ is also critical for tumour control[24,295,296], and this was significantly upregulated in CD8$^+$ T cells in the tumours of *Ackr4*$^{-/-}$ mice compared with WT mice, indicating this may contribute to the enhanced control in these mice.

Another hypothesis to explain why WT CD8$^+$ T cells could not control tumour growth is due to their expression of immunosuppressive checkpoint molecules such as PD-1, TIM-3 and LAG-3. These molecules are induced on subsets of exhausted T cells, and act to inhibit CD8$^+$ T cell effector function[297,298]. Indeed, approximately 40% of all CD8$^+$ T cells in WT tumours expressed these markers individually, with approximately 15% of cells being PD-1$^+$ TIM-3$^+$ or PD-1$^+$ LAG-3$^+$, reported to be the most highly exhausted subsets[261,262]. However, expression of these markers was comparable with CD8$^+$ T cells taken from *Ackr4*$^{-/-}$ tumours with the exception of PD-1, which was more highly expressed in *Ackr4*$^{-/-}$ mice. Together, these data indicate that the enhanced CD8$^+$ T cell response in *Ackr4*$^{-/-}$ mice is unlikely to be the result of reduced exhaustion, and in fact a higher proportion of *Ackr4*$^{-/-}$ CD8$^+$ T cell may display functional exhaustion as indicated by their increased PD-1 expression, perhaps owing to an enhanced activation state. This also suggests that E0771 tumours grown in *Ackr4*$^{-/-}$ mice may display a greater

response to anti-PD-1 or anti-PD-L1 therapy, which is a key future priority of this project, but was not performed due to time constraints.

Another factor that may contribute to the inability of WT intratumoural $CD8^+$ T cells to induce effective anti-tumour immunity is their localisation within the tumour. The presence of $CD8^+$ T cells within the tumour parenchyma has been shown to correlate with greater immune responses compared with cells that accumulate peritumourally or in the surrounding stroma, likely since many of the cytotoxic functions of $CD8^+$ T cells require direct cell contact with their target[15,35,36]. Thus, if $CD8^+$ T cells in WT tumours are excluded from the tumour bed, they may not be effective at inducing tumour cell apoptosis. Determining whether dysregulation of chemokines in *Ackr4*$^{-/-}$ tumours also alters the spatial distribution of $CD8^+$ T cells within tumours would also be important to establish in future experiments.

Interestingly, the enhanced $CD8^+$ T cell response in *Ackr4*$^{-/-}$ mice did not appear to be a result of enhanced priming of these cells. Expression of Ki67, a marker of recently-proliferated cells, by $CD8^+$ T cells in *Ackr4*$^{-/-}$ LN was significantly reduced compared with those in WT LN. This is consistent with previous reports demonstrating reduced intranodal priming of T cells in *Ackr4*$^{-/-}$ mice, and is likely to be a result of altered migration of DCs, as will be discussed later[140,144]. Analysis of the endogenous repertoire of $CD8^+$ T cells with TCRs specific for the immunodominant SIINFEKL epitope of the OVA protein, expressed by E0771-OVA tumour cells, revealed no difference in the numbers or frequencies of these tumour-specific cells within the draining LN, despite increased numbers being present in the tumour. To gain a more precise understanding of the early proliferation of these cells, future experiments could measure incorporation of the thymidine analog BrdU into these cells after their activation, to determine if there are more subtle differences in their priming which were not detected in the present study.

Another possible explanation for the enhanced $CD8^+$ T cell presence in *Ackr4*$^{-/-}$ tumours that was investigated was whether there was enhanced recruitment of naïve $CD8^+$ T cells into tumours. Naïve T cells abundantly express CCR7, and may be preferentially recruited in *Ackr4*$^{-/-}$ tumours due to the increased abundance of CCL21. Although intratumoural recruitment of naïve T cells may be surprising given their homeostatic recirculation pattern, multiple reports have indicated that naïve T cells are capable of

entering peripheral tissues, including tumours[59,299,300]. Furthermore, once inside tumours, naïve $CD8^+$ T cells can be primed *in situ* and contribute to an effective anti-tumour response, with activated intratumoural tumour-specific $CD8^+$ T cells reportedly generated even in splenectomised mice with *in utero*-ablated LNs[264]. In a subsequent study, naïve T cell entry into tumours was found to be dependent on CCR7 and occurred through PNAd-expressing vasculature, similar to the PNAd-expressing HEVs required for LN entry. HEV-like structures have been reported to spontaneously develop in many mouse and human tumours, suggesting this may be more common than previously considered[264,301,302]. Furthermore, the presence of HEVs in tumours have been correlated with increased expression of CCL19 and CCL21, as well as with improved patient survival[301]. Despite this evidence suggesting that increased CCL21 in E0771 tumours from *Ackr4*$^{-/-}$ mice may lead to enhanced recruitment of naïve $CD8^+$ T cells, this was not apparent when naïve OT-I cells were adoptively-transferred into established E0771 tumours at early or late stages of growth in this study. Although naïve T cells were detected within the tumour parenchyma of both WT and *Ackr4*$^{-/-}$ mice, there was no significant difference in naïve cell number. This experiment may have been hampered by the large number of transferred cells used, which potentially could have saturated the system through consumption of the CCL21 gradient through CCR7-mediated ligand internalisation. However, a large number of cells were required to be transferred in order to detect any transferred cells within the tumour, as the large majority of naïve cells home to the secondary lymphoid organs after transfer.

Collectively, the data discussed above suggest that the defining changes in the $CD8^+$ T cell response in *Ackr4*$^{-/-}$ mice occur after these cells have been activated. Although this work has not definitively clarified the precise mechanisms, there are a number of possibilities that will be explored in future work, including enhanced recruitment of activated $CD8^+$ T cells into the tumour, enhanced survival in the tumour, or enhanced proliferation in the tumour. For the latter, the data presented here suggests this may at least be one mechanism, as there was an increase in Ki67 expression among tumour-infiltrating $CD8^+$ T cells in *Ackr4*$^{-/-}$ mice. This may be further assessed in future experiments through a short-term administration of BrdU to detect proliferating cells within the tumour.

### 5.1.4 ACKR4 deficiency enhances intratumoural DC retention

Many aspects of the generation of anti-tumour $CD8^+$ T cell responses are controlled through DCs, including their recruitment and proliferation in tumours. Thus, the accumulation of DCs seen within *Ackr4*$^{-/-}$ tumours is highly likely to influence the extent of $CD8^+$ T cell infiltration. The Batf3-dependent lineage of DCs, which encompasses the migratory $CD103^+$ DCs and $CD8\alpha^+$ LN-resident DCs appear to be the most prominent DC subsets influencing $CD8^+$ T cell responses[21,60]. $CD103^+$ DCs have been shown to be the predominant transporter of tumour-associated antigen to the draining lymph nodes, where they can cross-prime $CD8^+$ T cells in addition to handing antigen to other DC subsets[21]. Depletion of this lineage using Zbtb46-DTR mice abrogated the tumour inhibition mediated by transfer of tumour-specific CTLs. Cross-presenting DCs, likely $CD103^+$ DCs, have also been shown to prime $CD8^+$ T cells directly within the tumour. In the B16-F1 model, the Tyr369 epitope is unable to be directly presented by tumour cells due to the absence of the appropriate class I MHC molecule[59]. However, in mice treated with FTY720 to prevent cell egress from LN, transferred Tyr369-specific $CD8^+$ T cells were still capable of being activated in the tumour, indicating that intratumoral APCs could cross-present antigen to these cells.

$CD103^+$ DCs have also been recently shown to mediate $CD8^+$ T cell recruitment to tumours. Despite $CD8^+$ T cells expressing multiple functional chemokine receptors, recruitment of $CD8^+$ T cells is predominantly mediated through CXCR3, the receptor for CXCL9 and CXCL10 (and CXCL11 in humans and some mouse strains)[93]. In a genetically-engineered melanoma model, intratumoural $CD103^+$ DCs were shown to be the predominant source of CXCL10 in the tumour, with these cells being critical for intratumoural recruitment of transferred antigen-specific cells[95]. Similar results were seen in the MMTV-PyMT breast cancer model, although $CD103^+$ DCs produced CXCL9 more abundantly than CXCL10[303]. Collectively, it is clear that $CD103^+$ DCs are critical in controlling the magnitude of the $CD8^+$ T cell response. Thus, it is possible that the enhanced number of $CD103^+$ DCs detected in *Ackr4*$^{-/-}$ tumours may be promoting increased recruitment of activated $CD8^+$ T cells through secretion of CXCL9 and CXCL10, or increasing intratumoural $CD8^+$ T cell proliferation through enhanced cross-presentation.

The increase in the number of CD103$^+$ DCs in tumours from *Ackr4*$^{-/-}$ mice is coupled with a decrease in migratory CD103$^+$ DC numbers in the LN. Given that CD103$^+$ DCs are critical for antigen trafficking and cross-priming in the LN, the enhanced CD8$^+$ T cell response in *Ackr4*$^{-/-}$ mice is perhaps surprising. Tumour-specific CD8$^+$ T cells are still clearly being primed despite the decrease in migratory CD103$^+$ DC at later time-points, and it would be of interest to examine the CD103$^+$ DC population in the tumour and LN to determine whether this deficit is also apparent at earlier time-points. In addition to priming by CD103$^+$ DCs, tumour-specific CD8$^+$ T cells have been shown to be cross-primed by CD169$^+$ macrophages in LN through passive draining of antigen from dead tumour cells, suggesting DCs egressing from tumours may not be the sole determinant of CD8$^+$ T cell priming in the LN[304]. It is also possible that there is a threshold of DC trafficking required to initiate CD8$^+$ T cell priming, and the migration observed in ACKR4-deficient mice is sufficient to achieve optimal priming. The increased number of these highly potent antigen-presenting cells would then contribute to the enhanced activation of the primed CD8$^+$ T cells at the tumour site.

ACKR4 appears to be regulating DC egress from tumours to LNs, as well as altering chemokine distribution at the tumour site. The altered CCL21 abundance in the tumour was mimicked experimentally through CCL21 administration directly into tumours of WT mice, which also led to increased accumulation of DCs in the tumour. It is well established that migratory DCs can use CCR7 to migrate to lymphatic vessels to egress from tissues[108,305-307]. Although all migratory DCs can express CCR7, in tumour models CD103$^+$ DCs have been reported to express the highest levels of CCR7[268,308]. Thus, in *Ackr4*$^{-/-}$ mice, the disruption of a CCL21 gradient towards afferent lymphatics would impair CCR7-mediated DC egress. This is consistent with a previous report identifying a key role for ACKR4 in regulating DC egress from the skin[253]. In that study, ACKR4 expressed by skin keratinocytes scavenged CCL19 and CCL21, and in mice lacking ACKR4 there was impaired egress of DC from skin to LN under both homeostatic and inflammatory conditions. As shown in this study, ACKR4 is also expressed by luminal epithelial cells in the mammary gland, and it is proposed that one of its main functions here is to scavenge CCL21 in order to maintain a functional chemokine gradient required for DC entry into the lymphatics through CCR7 **(Figure 5.1)**. Lymphatic vessels in the mammary gland have been reported to express CCL21, and the distal location of ACKR4 on luminal epithelial cells could regulate the CCL21 gradient[309]. However, in previous

work in *Ccr7*$^{-/-}$ mice, migratory CD103$^{+}$ and CD11b$^{+}$ DCs in the LN were drastically reduced, although intriguingly there was no concomitant increase within B16 tumours[268]. The reasons for the discrepancy between the previous study and the data presented in this study is unclear, but one possible explanation is that active signalling through CCR7 on DCs may be required for both recruitment and retention in *Ackr4*$^{-/-}$ tumours, rather than simply a loss of CCR7-mediated migration to lymphatic vessels as a result of a disrupted chemotactic gradient.

Another question posed by the data presented in this thesis is whether disruption of CCL21 alone in the mammary gland is sufficient to mimic the enhanced anti-tumour response seen in ACKR4-deficient mice. In tumours treated with exogenous CCL21, there was a significant enhancement in intratumoural DCs. This supports the idea that ACKR4 is controlling egress of intratumoural DC via scavenging of CCL21. However, CCL21 administration alone did not lead to inhibited tumour growth, suggesting dysregulation of this chemokine in the mammary gland alone was not sufficient for an enhanced anti-tumour response and inhibited growth. However, this experiment did not completely replicate loss of ACKR4 in the mammary gland, as CCL21 was not administered until tumours were palpable at day 7. Thus, the initial priming of CD8$^{+}$ T cells would not have been disturbed. Furthermore, although CCL21 appears to be the predominant chemokine that is altered in *Ackr4*$^{-/-}$ glands, the other ligands may also contribute to the defects seen, although this may be unlikely given the undetectable levels of CCL19 and equivalent levels of CCL25. Clearly, the experimental parameters tested with respect to administration of exogenous CCL21 should be expanded in future and further work will need to address the contribution of ACKR4 specifically in the mammary gland to the phenotype observed.

Lastly, the enhanced CD8$^{+}$ T cell response in *Ackr4*$^{-/-}$ mice was essential for control of E0771 tumours, but it remains to be seen whether CD8$^{+}$ T cells are also responsible for the reduced tumour growth in the other tumour models used in this study. Particularly for haematogenous metastasis experiments, previous reports have demonstrated a high dependence on NK cells for immunity, whereas CD8$^{+}$ T cells have varied contributions[310-312]. Although ACKR4 did not appear to influence NK cells in primary mammary gland responses, whether it may modulate NK cell-mediated metastasis responses should be investigated in future experiments. Additionally, any differences in

chemokine levels and immune cell recruitment into the lung tumours should also be assessed.

### 5.1.5 ACKR4 deficiency enhances immunotherapy responsiveness

Since the loss of ACKR4 enhanced the magnitude of the anti-tumour $CD8^+$ T cell response, it was assessed whether combining this with immunotherapies that enhance the activity of $CD8^+$ T cells may lead to greater therapeutic responses. Monoclonal antibodies (mAb) directed towards PD-1 and CTLA-4 are FDA-approved and thus analysis of these immunotherapies in *Ackr4*$^{-/-}$ mice is of great interest. However, the E0771 model is reportedly not responsive to anti-PD-1/anti-CTLA-4 treatment (Mark Smyth, personal communication) and so instead, a CD137 agonist was initially used in the E0771 model. Anti-CD137 treatment in tumours has been shown to protect $CD8^+$ T cells from activation-induced cell death, as well as promoting their effector function through enhanced production of IFNγ, granzyme B and perforin[313]. Administration of the agonistic anti-CD137 mAb in *Ackr4*$^{-/-}$ mice led to significantly reduced E0771 tumour growth compared with either ACKR4 deletion or anti-CD137 treatment alone. Thus, combining deletion of ACKR4 to enhance $CD8^+$ T cell recruitment with anti-CD137 treatment to enhance their survival and effector function had a synergistic effect. The B16F10 melanoma and MC38 colon carcinoma model were also utilized, since these are commonly used mouse models for assessing the efficacy of immunotherapy, and are known to be responsive to PD-1 and CTLA-4 treatment[314-316]. As observed with anti-CD137 in the E0771 model, the response to anti-PD-1 and anti-CTLA-4 treatment was significantly enhanced in *Ackr4*$^{-/-}$ mice compared with WT mice. Anti-PD-1 and anti-CTLA-4 are thought to affect distinct populations of cells, with anti-PD-1 treatment predominantly increasing exhausted-like $CD8^+$ T cell subsets, while anti-CTLA-4 promotes expansion of $CD4^+$ effectors, particularly $T_H1$-like cells, in addition to exhausted-like $CD8^+$ subsets[68]. In the absence of immunotherapy treatment, ACKR4 deficiency led to a reduction in B16F10 growth, although this was not as pronounced as in the E0771 model. This may reflect that there is not as significant an increase in intratumoural $CD8^+$ T cells in B16F10 tumours relative to E0771 tumours, with the B16F10 model generally considered to be a poorly immunogenic cell line[317]. Alternatively, it may suggest that the $CD8^+$ T cells in this model are more exhausted or suppressed than in E0771 tumours. In the MC38 model, only a modest inhibition of

tumour growth was observed in *Ackr4*$^{-/-}$ mice treated with control antibody. In contrast to the B16F10 line, this is a highly immunogenic line which is particularly sensitive to checkpoint blockade, as evidenced by the robust inhibition of tumour growth in WT mice treated with anti-PD-1[317]. However, ACKR4 deficiency greatly enhanced the therapeutic response to anti-PD-1 treatment, with minimal tumour burden in endpoint tumours observed.

These data suggest that inhibiting ACKR4 in cancer may be a promising therapeutic target. Given the data presented here is with mice with global ACKR4 knockout, which display normal growth, normal rates of reproduction and appear otherwise healthy, it is likely that systemic administration of neutralising antibodies or other antagonists would be possible without severe clinical consequences. However, there are a few potential major limitations which need to be addressed with future work. One key issue is that using ACKR4-deficient mice is akin to a prophylactic treatment regime, with disruption of this axis occurring prior to tumour implantation or initiation. Thus, if neutralising antibodies to ACKR4 are generated, it will be critical to determine whether inhibition after tumour onset will still inhibit tumour growth. Alternatively, if the effect of ACKR4 is entirely due to its scavenging ability, therapeutic administration of CCL21 or its other ligands may also be a useful approach. This is supported by previous studies, which have shown that exogenous injection of CCL19 or CCL21 into tumours can induce tumour rejection[113,114,318,319]. Although the mechanism for this was undescribed, one report indicated it was CXCL9–, CXCL10– and IFNγ–dependent[318]. However, this contrasts with the data described here, with exogenous administration of CCL21 into established E0771 tumours not affecting tumour growth. Thus, future experiments will need to address the timing of administration and tumour types used, to investigate if CCL21 may represent a plausible target. Lastly, the translatability to human cancer also remains to be seen, with analysis of ACKR4 in tumour exome datasets a priority for the future.

## 5.2 CCR2 regulates maintenance of CD4$^+$ T cell memory

In Chapter 4, CCR2 was identified as an important regulator of memory CD4$^+$ T cell responses. In response to infection with the extracellular bacteria *S. pneumoniae* or the intracellular influenza virus, CCR2 was upregulated on antigen-specific CD4$^+$ T cells at late time-points after infection. In competitive co-transfer systems, cell-intrinsic loss of

CCR2 on $CD4^+$ T cells had no discernible effect on priming of these cells, nor their ability to migrate to the lung during the effector phase of the response. However, during the resolution of the immune response, CCR2-deficient cells did not persist to the same extent as WT cells and contributed less to the memory compartment. CCR2-deficient $CD4^+$ cells in influenza also had reduced production of IFNγ compared to WT. Despite this, the proliferative response of CCR2-deficient $CD4^+$ T cells upon secondary rechallenge was unimpaired. Thus, the role of CCR2 appears to predominantly involve control of $CD4^+$ memory T cell proliferation or survival during the contraction phase. While a precise understanding of the mechanisms behind this remain unclear, these data provide the foundation for future investigation into the molecular control of $CD4^+$ T cell memory.

### 5.2.1 Antigen-specific infection models

The data described in this study were obtained from two independent infection models. In the *S. pneumoniae* infection model, a novel transgenic bacterial strain was generated to study antigen-specific $CD4^+$ T cell responses with the transgenic OT-II system. Infection with the $OVA_{323-339}$-expressing *S. pneumoniae* was shown to induce both IFNγ and IL-17 production by $CD4^+$ T cells, indicative of $T_H1$ and $T_H17$ responses, which is consistent with published reports of human and mouse *S. pneumoniae* infection[154,275,320,321]. In a second approach, the $CD4^+$ T cell response to influenza A virus was utilised. Similarly to the natural course of infection, OT-II cells predominantly formed $T_H1$ cells as evidenced by their expression of IFNγ. Furthermore, OT-II cells in influenza infection displayed much more robust expansion than in response to *S. pneumoniae* infection. This was evidenced during the optimisation experiments for OT-II cell numbers for transfer, as transfer of $10^5$ OT-II cells was required for robust analysis in *S. pneumoniae* infection, while transfer of only $10^3$ OT-II cells was sufficient for detection in influenza infection. Even with transfer of $10^5$ OT-II cells in *S. pneumoniae* infection, the frequency of OT-II cells recovered was very low, particularly at later timepoints after infection. This made analysis of the properties of these cells difficult, and likely contributed to with wide variation in cytokine responses often seen between mice. This was less of an issue in the influenza model due to the higher cell recovery as a result of increased expansion, but was still apparent.

The extent of proliferation of $CD4^+$ T cells in *S. pneumoniae* and influenza infections may have been influenced by a number of factors, including antigen quantity, which would be dependent on the replication of virus or bacteria during infection, as well as the expression of the PspA protein in which the epitope is inserted in the bacteria, and antigen accessibility, which may be more or less able to be processed and presented by APCs depending on its location within the pathogen structure. The number of transferred OT-II cells may also have influenced the outcome of response, with previous studies demonstrating that transfer of high precursor frequencies can reduce proliferative potential, possibly through increased competition for long-lived DC interactions[322,323]. Frequencies of endogenous $CD4^+$ T cell precursors have been estimated to range from 100-3000 cells per mouse depending on the TCR specificity[324-326], suggesting that the enhanced naïve T cell frequency in the *S. pneumoniae* OT-II model may affect their subsequent expansion, and potentially their transition into memory.

### 5.2.2 CCR2 is expressed by antigen-specific $CD4^+$ T cells after infection

Expression of CCR2 was absent on naïve $CD4^+$ T cells, but induced in response to infection with both *S. pneumoniae* and influenza. Upregulation of the receptor on this population coincided with the resolution of the response and was significantly enhanced on antigen-specific $CD4^+$ T cells in the lung, compared with those in the LN and spleen. In *S. pneumoniae* infection, CCR2 expression was more highly enriched on $T_{EM}$ and $T_{RM}$-like cells than on $T_{CM}$ in both spleen and lung. This is consistent with mass cytometry analysis of human $CD4^+$ T cell subsets, which found CCR2 was enriched on $CD69^+$ populations compared with $CD69^-$ populations on $CD4^+$ T cells isolated from the colon, skin and liver of human patients with a variety of conditions, although expression of CCR2 in the lung was low[237]. This suggests that CCR2 may be more important for $T_{RM}$-like populations, as will be discussed later. Furthermore, in addition to expression on $T_H1$ and $T_H17$ subsets, CCR2 was highly expressed by Treg cells, indicating it potentially plays a role in the trafficking of additional $CD4^+$ T cell subsets. This is also consistent with published reports showing that CCR2 is required for trafficking of Tregs in tumour and allograft models[101,235]. Intriguingly, comparison of the low frequency of OT-II cells that induced Foxp3 after influenza infection with $Foxp3^-$ OT-II cells revealed that CCR2 was significantly upregulated in those that had upregulated Foxp3, suggesting it may be preferentially expressed by cells with a regulatory phenotype.

Expression of CCR2 on $CD4^+$ T cell subsets increased over time in each of the models examined. This suggests that the cytokines and transcription factors driving CCR2 expression are likely those altered late in the response, and act to influence already differentiated cells. Previous data from our laboratory investigated the transcriptional regulation of CCR2 and CCR6 on $T_H17$ cells in EAE, specifically in the formation of $CCR2^+$ $CCR6^+$ and $CCR2^+$ $CCR6^-$ $T_H17$ cells[205]. Despite increasing the frequency of $CCR2^+$ $CCR6^+$ cells at the expense of $CCR2^+$ $CCR6^-$ cells, cell-intrinsic loss of T-bet did not affect overall expression of CCR2 on $T_H17$ cells, whereas loss of Eomesodermin promoted a significant but minor reduction in $CCR2^+$ $T_H17$ cells. Further insights can also be gained from transcriptional analysis of influenza-specific $CD8^+$ T cells, which also upregulate CCR2 after infection (Kevin Fenix, personal communication). Loss of Blimp1, but not T-bet, has been shown to abrogate expression of CCR2 on $CD8^+$ T cells in LCMV infection[327]. A candidate cytokine that may induce CCR2 is IL-2, which has been reported to induce CCR2 expression on human $CD4^+$ T cells stimulated *in vitro*[328]. IL-2 expression has been reported to peak on day 10 after influenza infection, whilst still remaining elevated during the contraction phase[329]. IL-2 also promotes Foxp3 expression and is indispensable for Treg function, while also acting to suppress $T_{FH}$ differentiation, consistent with the low expression of CCR2 on $T_{FH}$ in LCMV[212,330-333]. Experimental evidence to support this hypothesis, as well as analyzing other potential candidate signals, remain a priority for future work.

### 5.2.3 CCR2 promotes maintenance of $CD4^+$ T cells during the contraction phase

Loss of CCR2 on antigen-specific $CD4^+$ T cells in both *S. pneumoniae* and influenza infection did not appear to affect priming or production of effector cytokines during the peak of the response. This is perhaps unsurprising given the low level of expression of CCR2 at early time-points. The most prominent defect in CCR2-deficient OT-II cells identified from this study was their enhanced contraction after infection compared to WT. This suggests that CCR2 promotes the survival or proliferation of $CD4^+$ T cells during the resolution of the immune response and potentially promotes their transition into memory, which is evidenced by the increased expression of CCR2 on memory $CD4^+$ T cells. In future experiments, analysis of apoptosis rates and cellular turnover of WT and *Ccr2*$^{-/-}$ OT-II cells should be compared. If *Ccr2*$^{-/-}$ OT-II cells have a lower rate of cellular proliferation this may suggest reduced access to residual antigen presented by

APCs, whereas if they undergo a higher rate of cell death, this would suggest lack of access to survival signals required for their maintenance.

The signals promoting survival and maintenance of $CD4^+$ T cell memory are not well described, although a critical role for IL-7 has been identified. IL-7 has been shown to promote the survival of both $T_{EM}$ and $T_{CM}$ through induction of the anti-apoptotic protein Bcl2, although is not required for the generation of effector $CD4^+$ T cells[334,335]. IL-7 can be produced by fibroblastic reticular cells (FRCs) in LN, as well as stromal cells present in peripheral sites[336-338]. Furthermore, IL-15 can also contribute to $CD4^+$ T cell survival, although this appears to be less critical than IL-7, perhaps owing to their reduced expression of CD122 (IL-15Rβ)[339-342]. In contrast to the stromal expression of IL-7, IL-15 is predominantly produced by macrophages and DCs[343,344]. Thus, it is plausible that CCR2 may be required for migration towards cells that produce survival cytokines, in order for $CD4^+$ T cells to receive IL-7- or IL-15-mediated signals that promote their survival **(Figure 5.2)**. If this is the case, then in the absence of CCR2, $CD4^+$ T cells would have a reduced ability to colocalize with IL-7/15-expressing cells, leading to the enhanced contraction observed. This could be tested in future experiments by performing immunofluorescence to test if there are any apparent differences in localization of WT and *Ccr2*$^{-/-}$ OT-II in SLO or lungs. In SLO, WT OT-II cells would be predicted to localize with IL-$7^+$ FRCs. In the lung, Thy$1^+$ IL-$7^+$ lymphatic endothelial cells generated in inducible bronchus-associated lymphoid tissue structures have been shown to maintain pathogenic $T_H2$ cells in allergic inflammation, and may also be important for maintenance of $CD4^+$ memory T cells more generally[338]. Analysis of the expression of CCR2 ligands by cells in these tissues may also assist with this understanding.

An alternative possibility to CCR2 being required for direct migration towards cells expressing pro-survival cytokines is that it plays more subtle roles in niche positioning during the primary response, which affects formation of memory cells through reduced exposure to memory-promoting cytokines. A contrary scenario has been described for CXCR3 expression on $CD8^+$ T cells, where loss of CXCR3 reduces $CD8^+$ T cell migration to the marginal zone of the spleen early after infection, thus reducing exposure of these cells to the inflammatory milieu required for full effector differentiation and instead promotes increased formation of memory precursor cells[345,346]. Thus, it is

conceivable that in the absence of CCR2, $CD4^+$ T cells localize to different niches during the effector phase of the response and consequently receive reduced access to cytokines promoting development into memory cells. One possible candidate that influences formation of $CD4^+$ T cell memory is IL-2, which is required for efficient upregulation of IL-7Rα, necessary for responsiveness to IL-7 which maintains their survival[347,348]. Differential exposure to IL-2 between WT and *Ccr2*$^{-/-}$ OT-II cells during the effector phase of the response may lead to differential upregulation of IL-7Rα, thus affecting their ability to survive during the contraction phase. IL-2 is also an interesting candidate given its potential ability to upregulate CCR2, as discussed above. This creates an interesting scenario where if CCR2 enhances migration towards IL-2-expressing cells, and IL-2 then further induces CCR2 expression, it creates a feed-forward loop to promote survival. Alternatively, if CCR2 is found to be required for migration toward IL-7-expressing cells, IL-2 may be the initiating signal to promote both upregulation of CCR2 and IL-7Rα, thus allowing $CD4^+$ T cells to become responsive to memory signals as well as promoting their migration towards these signals. However, this currently remains hypothetical and future experiments will need to determine empirically whether this holds true.

Analysis of the subsets of WT and *Ccr2*$^{-/-}$ memory $CD4^+$ T cells formed after *S. pneumoniae* infection revealed no difference in the frequency of $T_{CM}$ or $T_{EM}$ produced, although both subsets displayed contraction. This suggests that CCR2 does not influence the formation of different memory subsets and acts more globally to promote their maintenance. However, in the lung, the $T_{CM}$ population appear to undergo more severe contraction than the $T_{EM}$ or $T_{RM}$-like populations, as reflected by the increase in frequency of $T_{EM}$ and $T_{RM}$ in *Ccr2*$^{-/-}$ OT-II cells, compared with WT OT-II cells. Despite this change in frequency, the number of *Ccr2*$^{-/-}$ $T_{EM}$ still significantly contracted, although the number of $T_{RM}$-like cells remained relatively unchanged. These results are perhaps surprising given the increased expression of CCR2 on $T_{EM}$ and $T_{RM}$-like cells relative to $T_{CM}$ in *S. pneumoniae* infection. However, these data do suggest that $T_{RM}$-like cells may rely on different signals for their maintenance than $T_{EM}$ and $T_{CM}$ cells, with CCR2 not required for their survival. This is also reflected in the ratio of *Ccr2*$^{-/-}$ OT-II to WT OT-II cells increasing from the peripheral blood to the lung, indicating circulating cells are more dependent on CCR2-mediated signals for their survival. Alternatively, this result may reflect *Ccr2*$^{-/-}$ OT-II T cells are diverted from the blood to another organ,

although given their contraction in the spleen and LN, the lymphoid organs are not likely to be a possible destination.

The above discussion raises the question of why CCR2 is so highly expressed on $T_{RM}$-like cells if it is not required for their maintenance. CCR2 does not appear to be required for migration of effector $CD4^+$ T cells into their effector site of the lung, which differs from the reported requirement for CCR2 in entry of encephalitogenic $T_H17$ cells into the CNS[205]. However, CCR2 may still be required for more subtle migration of $T_{RM}$ cells within resident tissues. $T_{RM}$ cells are motile within tissues, which has been proposed to facilitate rapid detection of nearby pathogenic insults[349,350]. The molecular cues dictating this local migration are not well understood, but at least for skin $CD8^+$ $T_{RM}$ have been reported to be pertussis toxin-sensitive, but independent of the highly-expressed chemokine receptors CXCR3, CXCR6, CCR8 and CCR10[351]. Furthermore, the ligands for CCR2 are induced in the lung and mucosa in the first 2 to 3 days after infection with influenza[170] or *S. pneumoniae* (Duncan McKenzie, PhD thesis) as well as upon other inflammatory stimuli[226], and thus CCR2 may play a role in $T_{RM}$ migration within tissues to scan for nearby pathogenic insults.

The relatively reduced contraction of *Ccr2*$^{-/-}$ $T_{RM}$-like cells also suggests that these cells are seeded early in the response. If they were derived from $T_{EM}$ or T effector cells from the periphery after antigen had been cleared, then the enhanced contraction of *Ccr2*$^{-/-}$ OT-II cells at these time-points should be mirrored in the $T_{RM}$-like compartment. Thus, it is likely that these cells seed the lung prior to the contraction phase, and are maintained independently of CCR2-mediated signals. This early seeding of $T_{RM}$ cells from the effector T cell pool is consistent with previous reports on $CD8^+$ $T_{RM}$ demonstrating that only transfer of early effector cells can traffic to the gut epithelium to give rise to $T_{RM}$ precursors, whilst effector cells taken from day 7 onwards of LCMV infection cannot[352].

Although loss of CCR2 significantly affected the ability of influenza-specific $CD4^+$ T cells to persist into the memory phase, the remaining *Ccr2*$^{-/-}$ OT-II cells had no apparent defect in their expansion upon antigenic rechallenge. This suggests that CCR2 is not required for migration to, or engagement with, APCs that drive memory $CD4^+$ T cell proliferation, but is still consistent with the possibility that CCR2 is primarily required for colocalisation with survival signals. This also raises the question of whether the

enhanced contraction seen for *Ccr2*$^{-/-}$ CD4$^+$ T cells would affect their ability to protect against rechallenge, given they show unimpaired secondary expansion. This could be assessed in *S. pneumoniae* infection by transfer of WT and *Ccr2*$^{-/-}$ OT-II cells into separate hosts with bacterial burden measured after secondary challenge with *S. pneumoniae*, since the protection seen in secondary challenges is known to be CD4$^+$ T cell-dependent[243,353]. Interestingly, although cytokine production by *S. pneumoniae*-specific memory CD4$^+$ T cells was unimpaired by CCR2 deficiency, influenza-specific $T_H1$ cells had reduced IFNγ production both upon restimulation at memory time-points, and after secondary challenge. IFNγ production by memory CD4$^+$ T cells is regulated through expression of T-bet and NFκB, suggesting these pathways may be impaired at later time-points, although this remains to be determined[354,355].

Another scenario to be explored in future experiments is whether there is a role for CCR2 in CD4$^+$ T cell trafficking from the bone marrow. As well as its role in haematopoiesis, the bone marrow is an important site of maintenance of CD4$^+$ T cell memory cells, with some studies reporting it as the predominant site for resting CD4$^+$ memory T cells, although this is controversial[337,356,357]. Entry of CD4$^+$ T cells into the bone marrow has been shown to be regulated through expression of CD49b and CD69, although the requirements for egress of these cells are as yet unknown[337,358,359]. Monocytes can also express high levels of CCR2, and this receptor is critical for their egress from bone marrow. As a result, CCR2-deficient monocytes pool in the bone marrow and are almost absent from the circulation[228]. Activation of CCR2 on monocytes has been shown to desensitise these cells to CXCR4-mediated signals that normally promote their retention in the bone marrow, providing a possible mechanism for CCR2-mediated emigration[360]. Thus, future experiments should assess WT and *Ccr2*$^{-/-}$ OT-II cell frequencies in the bone marrow, as an increase in *Ccr2*$^{-/-}$ OT-II cells would be predicted if this receptor is required for egress. Interestingly, this hypothesis is partially supported by a recent analysis of antigen-experienced CD4$^+$ T cells after LCMV infection, as CCR2 is specifically enriched on CD4$^+$ T cells in the bone marrow, compared with splenic CD4$^+$ T cells[361]. Similarly, analysis of total T cells in CCR2-RFP reporter mice showed highest expression of CCR2 in the bone marrow compared with spleen, LN or peripheral blood[362]. Thus, future experiments should aim to address the possibility that CD4$^+$ T cells require expression of CCR2 for their egress from bone marrow.

## 5.3 Concluding remarks

In summary, the data described in this thesis have revealed novel roles for chemokine receptors in regulating diverse aspects of immune cell function. In tumour settings, ACKR4 has been identified as a novel regulator of intratumoural DC trafficking and affects the magnitude of the tumour-directed $CD8^+$ T cell response. Preliminary evidence indicates that this receptor may potentially be a useful therapeutic target, with ACKR4-deficient mice showing enhanced responsiveness to immunotherapy treatment. In models of infection, a previously unappreciated role for CCR2 on $CD4^+$ T cells was described, with this receptor required for the maintenance of antigen-specific $CD4^+$ T cell populations. Thus, this study has highlighted the complexity of the chemokine system and enhanced our understanding of its modulation of immune function. However, many questions remain and as outlined in this discussion, further studies in each of the two main areas of focus will be required, particularly to dissect the molecular mechanisms that underlie these phenomena.

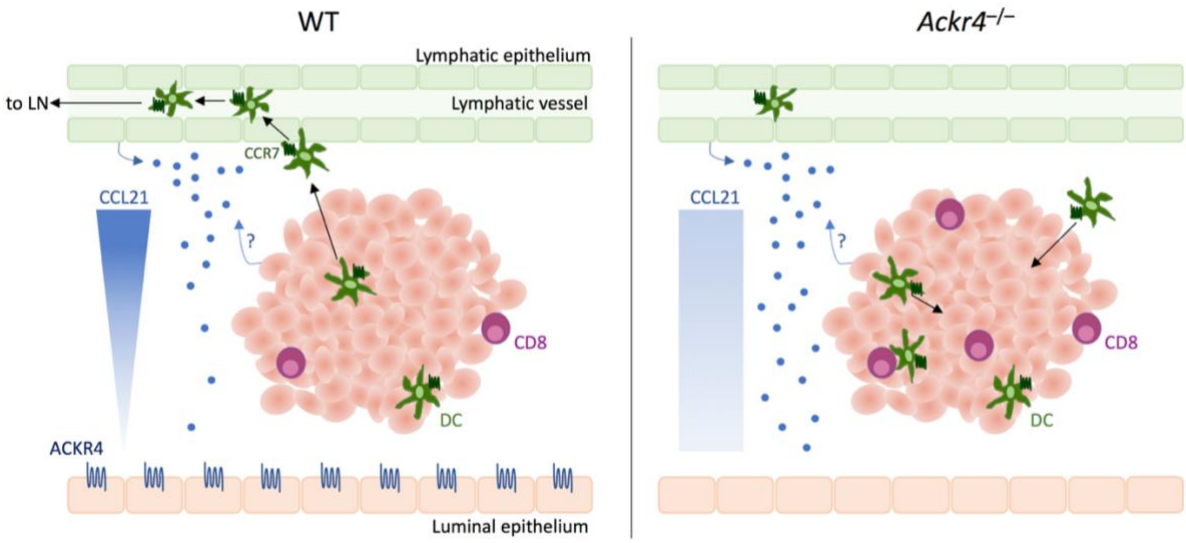
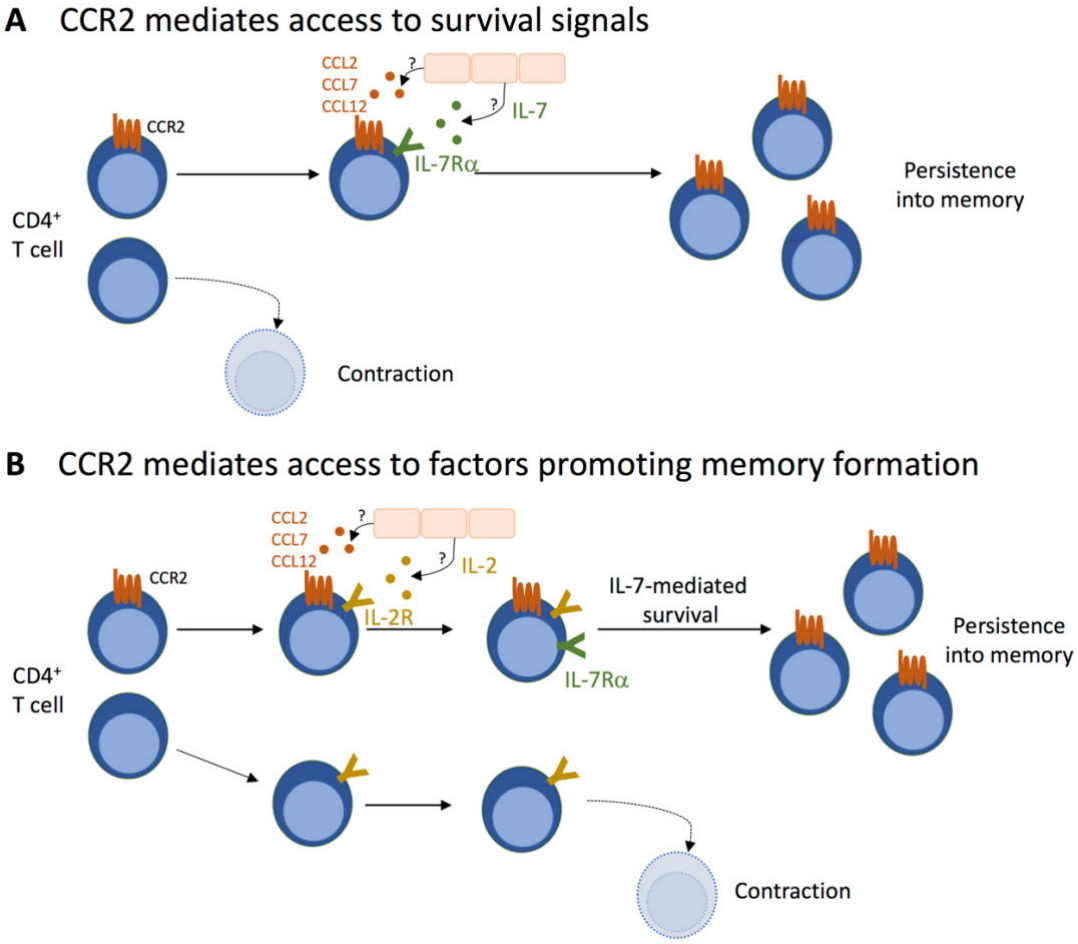



**Figure 5.1: Proposed model of ACKR4-mediated control of tumour immunity**

CCL21 is expressed by lymphatic endothelial cells[309] and potentially by the tumour itself. In wildtype (WT) mice, ACKR4 expressed by the luminal epithelium in the mammary gland scavenges CCL21, thus creating a chemotactic gradient towards the lymphatic vessels. This allows dendritic cells (DCs) present within E0771 tumours to migrate in a CCR7-dependent manner to the lymphatic vessels, promoting their egress from tumours and migration to the draining lymph node (LN). In *Ackr4*$^{-/-}$ mice, the absence of ACKR4 abrogates the development of a CCL21 gradient, and leads to enhanced levels of CCL21 within the tumour. Thus, DCs show reduced migration into lymphatic vessels and subsequently the LNs and are instead retained at higher frequencies in the tumour itself. Intratumoural DC accumulation can then induce a greater $CD8^+$ T cell response, potentially through enhancing recruitment of $CD8^+$ T cells or enhancing their proliferation, thus leading to reduced tumour growth.

**Figure 5.2: Potential models of CCR2-mediated control of $CD4^+$ T cell contraction**
CCR2 is expressed by a relatively low frequency of $CD4^+$ T cells during the effector phase of the response, but is increased at later time-points after infection. Furthermore, CCR2 is required for maintenance of $CD4^+$ T cell memory cells. **(A)** CCR2 may potentially regulate access to factors required for survival of $CD4^+$ T cells. CCR2 expression on $CD4^+$ T cells may promote localisation to cells expressing the CCR2 ligands CCL2, CCL7 and CCL12, with these cells also potentially producing cytokines promoting $CD4^+$ T cell survival, such as IL-7 or IL-15. Thus, *Ccr2*$^{-/-}$ cells or cells lacking CCR2 expression may have reduced access to these factors and undergo enhanced contraction. **(B)** Alternatively, CCR2 may drive differential localisation of $CD4^+$ T cells during the effector phase of the response, thus allowing different exposure to cytokines promoting formation of memory cells. One candidate is IL-2, which is required for upregulation of IL-7Rα, and thus responsiveness to IL-7 and survival[348]. Cells lacking CCR2 expression through transcriptional regulation or genetic deletion may have reduced exposure to IL-2, leading to reduced expression of IL-7Rα and hence an inability to receive survival signals, leading to their enhanced contraction.


A CCR2 mediates access to survival signals
CCL2
CCL7
CCL12
?
?
IL-7
CCR2
IL-7Rα
CD4+
T cell
Persistence
into memory
Contraction
B CCR2 mediates access to factors promoting memory formation
CCL2
CCL7
CCL12
?
?
IL-2
CCR2
IL-2R
IL-7-mediated
survival
IL-7Rα
CD4+
T cell
Persistence
into memory
Contraction

# CHAPTER 6

# References

# Chapter 6 - References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# Appendix

# Appendix

**Gregor CE,** Foeng J, Comerford I, McColl SR. Chemokine-driven CD4+ T cell homing: new concepts and recent advances. *Adv Immunol*. 2017;135:119-181. doi: 10.1016/bs.ai.2017.03.001.

CHAPTER FOUR

# Chemokine-Driven CD4$^+$ T Cell Homing: New Concepts and Recent Advances

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## Contents



## Abstract

CD4$^+$ T cells are critical regulators of the adaptive immune system and have diverse roles in regulating responses to the broad array of microbes encountered. Appropriate execution of their effector function requires precise and coordinated migration of these cells to specific lymphoid niches and peripheral sites. This migration is largely controlled by dynamic expression of chemokine receptors and the discrete functions

*Advances in Immunology*, Volume 135
ISSN 0065-2776
http://dx.doi.org/10.1016/bs.ai.2017.03.001

of distinct subsets of $CD4^+$ T cells can often be determined from their expression of specific chemokine receptors. In this chapter, we discuss recent advances in the subset-specific homing of distinct T helper populations, focusing on new insights stemming from the increased diversity and plasticity now observed among $CD4^+$ T cells as well as how chemokine receptors can govern T cell-fate decisions. We also discuss current understanding of $CD4^+$ memory T cells with reference to their diversification based on chemokine receptor expression.

## 1. INTRODUCTION

$CD4^+$ T cells play a pivotal role in the adaptive immune system by dictating the quality and magnitude of humoral and cellular immune responses. Central to this role is the ability of $CD4^+$ T cells to migrate in a spatiotemporally controlled manner. The rare clonal frequency of antigen specific $CD4^+$ T cells and the large surface areas of the body exposed to antigen necessitate this efficiency in migration. Precise control of cell migration ensures that $CD4^+$ T cells encounter antigen when it is present, permits appropriate crosstalk with other immune cells, regulates homing to sites of peripheral inflammation, and is also critical for immune surveillance and memory maintenance. It is well recognized that the various functional states of $CD4^+$ T cells (e.g., naïve, effector, memory) have profoundly distinct migratory patterns and that the effector subsets of $CD4^+$ T cells that shape adaptive immune responses (e.g., $T_H1$, $T_H2$, $T_H9$, $T_H17$, $T_H22$, Treg) all have distinguishable and in some cases defining homing characteristics. The major molecular determinants that confer these various cell homing patterns on $CD4^+$ T cells are chemokine receptors, a subfamily of G protein coupled receptors (GPCRs) that recognize secreted chemokine ligands, which are produced in niche specific and context dependent circumstances to attract appropriate chemokine receptor bearing cell types. The chemokine system is further regulated through atypical chemokine receptors, which control the bioavailability of their corresponding chemokine ligands (Nibbs & Graham, 2013) (Table 1). In this chapter, we will summarize the migration patterns of mature $CD4^+$ T cells both during homeostasis and during immune responses with a particular focus on the role of chemokines and chemokine receptors and highlight recent findings that advance our understanding of this intricate and critical process.

**Table 1** The Chemokine Receptors, Their Ligands, and CD4+ T Cell Chemokine Receptor Expression

| | Chemokine Ligands | CD4+ T Cell Subsets That Express Receptor | Atypical Receptor(s) That Regulate Axis |
|---|---|---|---|
| CC Chemokine Receptor | | | |
| CCR1 | CCL3, CCL3L1,[a] CCL5, CCL8,[a] CCL14,[a] CCL15, CCL16, CCL23 | $T_H2$, $T_H9$, $T_H17$, $T_{RM}$ | ACKR1, ACKR2 |
| CCR2 | CCL2, CCL7, CCL8,[a] CCL11, CCL16[a] | $T_H1$, $T_H17$, Treg | ACKR1, ACKR2 |
| CCR3 | CCL3L1, CCL5, CCL7, CCL11, CCL13,[a] CCL14,[a] CCL15, CCL24, CCL28 | $T_H2$, $T_H9$, Treg | ACKR1, ACKR2 |
| CCR4 | CCL17, CCL22 | $T_H2$, $T_H17$, $T_H22$, Treg | ACKR1, ACKR2 |
| CCR5 | CCL3, CCL3L1,[a] CCL4, CCL4L1,[a] CCL5, CCL8, CCL11, CCL16 | $T_H1$, $T_H9$, $T_H17$, Treg, $T_{EM}$, $T_{RM}$ | ACKR1, ACKR2 |
| CCR6 | CCL20 | $T_H9$, $T_H17$, $T_H22$, Treg | ? |
| CCR7 | CCL19, CCL21 | Naïve, $T_{CM}$, Treg | ACKR4 |
| CCR8 | CCL1, CCL18[a] | $T_H2$, Treg | ? |
| CCR9 | CCL25 | $T_H17$, $T_H22$, gut homing | ACKR4 |
| CCR10 | CCL27, CCL28 | $T_H17$, $T_H22$, skin homing | ? |
| CXC Chemokine Receptor | | | |
| CXCR1 | CXCL6, CXCL7, CXCL8 | — | ACKR1 |
| CXCR2 | CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8[a] | — | ACKR1 |
| CXCR3 | CXCL4,[b] CXCL9, CXCL10, CXCL11, CXCL13 | $T_H1$, $T_H9$, Treg, $T_{CM}$, $T_{RM}$ | ACKR3 |
| CXCR4 | CXCL12 | Most | ACKR3 |

*Continued*

**Table 1** The Chemokine Receptors, Their Ligands, and $CD4^+$ T Cell Chemokine Receptor Expression—cont'd

| | Chemokine Ligands | $CD4^+$ T Cell Subsets That Express Receptor | Atypical Receptor(s) That Regulate Axis |
|---|---|---|---|
| CXCR5 | CXCL13 | $T_{FH}$, $T_{FR}$, $T_H17$, $T_{CM}$ | ACKR4[a] |
| CXCR6 | CXCL16 | $T_H17$, $T_H22$ | ? |
| XC Chemokine Receptor | | | |
| XCR1 | XCL1, XCL2[a] | — | ? |
| $CX_3C$ Chemokine Receptor | | | |
| CX3CR1 | CX3CL1, CCL26[a] | $T_H2$ | ? |

[a]Indicates in humans but not mice.
[b]CXCL4 binds to the CXCR3-B isoform of CXCR3.

## 2. NAÏVE $CD4^+$ T CELL TRAFFICKING

Naïve $CD4^+$ T cells constantly recirculate through the lymphoid organs, scanning for cognate antigen presented in the context of MHC II by antigen presenting cells (APCs). Blood borne naïve $CD4^+$ T cells enter the T cell areas of secondary lymphoid organs (SLOs), scan APCs for antigen, then exit and ultimately reenter the bloodstream where the recirculation through SLOs continues. Homeostatic trafficking relies on shear flow through the blood and lymph, but requires additional cues to initiate rolling, arrest, and extravasation in lymph nodes (LN) and Peyer's patches (PP). Migration within secondary lymphoid organs is also tightly regulated to enhance the likelihood of interactions between very rare antigen reactive and antigen bearing cells. The most important chemokine receptor for naïve $CD4^+$ T cell trafficking is CCR7, which is highly expressed by all naïve T cells. Naïve T cells enter LNs and PP through specialized postcapillary vessels known as high endothelial venules (HEVs). Entry via HEVs is tightly restricted and requires the sequential steps of rolling, adhesion, and finally extravasation of cells into the LN. Naïve T cells that enter HEVs express the homing receptor L selectin (CD62L), which binds to a family of sialomucins collectively known as peripheral node addressins expressed by the HEV endothelium. This attachment facilitates rolling of the cell along the luminal surface, followed by firm adhesion of

the cell initiated by CCR7 induced signaling which triggers integrin activation. CCR7 on the naïve T cell is primarily activated via CCL21, a chemokine highly expressed by the HEV endothelium and presented on the luminal surface via heparin sulfate (Bao et al., 2010). Additional CCR7 activation can be mediated by CCL19, which has been reported to be transcytosed to the HEV luminal surface from the perivascular space (Baekkevold et al., 2001). However, *Ccl19*$^{-/-}$ mice display similar short term homing of naïve lymphocytes to SLO, suggesting it is not required for LN entry but instead mediates survival of naïve T cells within the LN (Link et al., 2007). Activation of CCR7 on rolling T cells induces activation of the integrin $\alpha_L\beta_2$ (LFA 1), allowing firm adhesion to ICAM 1/2 expressed on the luminal endothelium (Gunn et al., 1998; Stein et al., 2000). In PP and mesenteric LNs, CCL21 triggers high affinity binding of $\alpha_4\beta_7$ to MAdCAM 1 (Pachynski, Wu, Gunn, & Erle, 1998). In the absence of functional CCR7 or in *plt/plt* mice which lack CCL19 and CCL21ser, T cell arrest and migration into LNs is severely abrogated (Stein et al., 2000; Warnock et al., 2000). In addition to CCR7, relatively few other chemokine signals make significant contributions to naïve T cell trafficking. Indeed, naïve CD4$^+$ T cells do not express high levels of any other chemokine receptor with the exception of CXCR4. The sole CXCR4 ligand CXCL12 is also found on the luminal surface of HEVs, however, this axis seems to play only a minor role in naïve CD4$^+$ T cell recruitment, with CXCR4 dependent arrest only apparent in mice lacking CCR7 signals (Okada et al., 2002). Subsequent to integrin mediated arrest on HEVs, naïve CD4$^+$ T cells crawl on the HEV surface toward preferential sites of T cell entry where fibroblastic reticular cells (FRCs) in the LN form a cellular sheath around the HEV and the T cells can there transmigrate across the endothelium to enter the LN (Bajenoff et al., 2006).

Naïve T cells also enter LNs via the afferent lymphatics once they have entered the flow of lymph at an upstream LN. Experiments utilizing intralymphatic injection of naïve T cells demonstrated that these cells enter the parenchyma of the LN from the subcapsular sinus (SCS) region via peripheral medullary sinuses independently of CCR7 (Braun et al., 2011). However, subsequent migration from the medullary sinuses to the deeper paracortex strictly required expression of CCR7.

Upon reaching the LN paracortex, naïve CD4$^+$ T cells scan for APCs presenting their cognate antigen in the context of MHC II. To increase the likelihood of these encounters, naïve T cells appear to migrate through the LN with a random walk so that each T cell is equally likely to interact

with a given APC. However, this stochastic migration is guided by FRCs, which wrap around lymph transporting conduits to form a stromal network (Roozendaal, Mebius, & Kraal, 2008). Indeed, the exit ramps used by naïve T cells to leave the HEV and enter the LN are positioned such that they lead the T cell directly onto the FRC network (Bajenoff et al., 2006). These FRCs express high levels of adhesion molecules as well as CCL19, CCL21, and CXCL12 and are essential for dictating the migration of T cells within the paracortex. Imaging studies have revealed that T cells in the LN are always in close proximity to FRCs and their migration closely follows the path of the FRC fibers (Bajenoff et al., 2006). Thus, it appears that FRC bound chemokines provide a haptotactic signal for intranodal T cell migration. Indeed, T cells in *plt* mice or *Ccr7*$^{-/-}$ T cells show reduced velocity and motility in the paracortex, demonstrating that CCR7 signaling is essential for basal motility of naïve T cells (Worbs, Mempel, Bolter, von Andrian, & Forster, 2007). Additionally, depletion of FRCs in fibroblast activation protein α (FAP α) DTR mice resulted in abrogated CCL19 and CCL21 within the T cell zones, as well as disrupted T cell responses to subsequent influenza infection (Denton, Roberts, Linterman, & Fearon, 2014).

Egress of naïve T cells from lymph nodes occurs via the cortical sinuses into the efferent lymphatics (Cyster & Schwab, 2012). Both entry to and egress from lymph nodes is allowed to occur through reciprocal desensiti zation of CCR7 and the sphingolipid binding GPCR, sphingosine 1 phosphate receptor 1 (S1PR1) (Pham, Okada, Matloubian, Lo, & Cyster, 2008). In the blood and lymph, S1P is abundantly expressed, leading to S1PR1 internalization and thus desensitization to the ligand. Therefore, naïve T cells present in the blood express low levels of S1PR1 and can respond more effectively to CCR7 signals to promote their entry into LNs. CCR7 acts as a retention signal for T cells within the LNs, which regain responsiveness to S1P signals during LN transit. Gradual desensitization toward CCL21 and CCL19 occurs due to ligand induced internalization of CCR7, thus shifting the balance toward promoting S1PR1 mediated LN egress. Once entering the efferent lymph, T cells can migrate to downstream LNs or return to circulation in the blood.

Naïve T cell trafficking within the spleen has some distinct differences to that within LNs and PPs, due to the distinct architecture of the spleen. Blood enters the spleen via terminal arterioles that drain into cords in the red pulp without an endothelial lining, forming an open vascular system. T cells pre dominantly localize in the periarteriolar sheath (PALS) in the white pulp

which surrounds the arterioles. The marginal zone is situated at the interface between the red pulp and the marginal sinus of the white pulp and acts as a major transit route for T cells traveling between the blood and the white pulp.

T cell entry to splenic white pulp cords is dependent on GPCR signaling, as T cells in mice treated with pertussis toxin (PT) fail to localize to the white pulp and instead pool in the red pulp (Cyster & Goodnow, 1995). Entry to the white pulp is also partially facilitated by the integrin LFA 1 and to a lesser extent, $\alpha_4\beta_1$. However, the precise sequence of events and chemokines involved in initiating T cell entry into the marginal zone remain unclear. Trafficking from the marginal zone to the PALS is more well understood, and similar to LNs, is guided by an FRC network (Bajenoff, Glaichenhaus, & Germain, 2008; Katakai et al., 2008). Naïve lymphocytes traffic into the PALS at preferential gaps in the marginal sinus, termed mar ginal zone bridging channels (MZBCs). FRCs line these channels upon which naïve T cells migrate, presumably following haptotactic signals to the T cell zones as seen in LNs. Indeed, CCR7 is essential for effective local ization to the PALS as displayed by the impaired positioning of T cells in CCR7 deficient mice or *plt/plt* mice (Forster et al., 1999; Nakano et al., 1998). FRCs secrete CCL19 and CCL21 as well as CXCL12, providing fur ther support for a haptotactic model of chemokine driven cell navigation through the spleen (Luther, Tang, Hyman, Farr, & Cyster, 2000; Umemoto et al., 2012). Splenic egress of naïve T cells is not well under stood, but is believed to rely on similar signals to the lymph node. The red pulp provides a high concentration of S1P, so it is likely that gradual CCR7 desensitization in the white pulp promotes S1P mediated migration through the MZBCs to the marginal zone, and subsequent exit into the cir culation. In support of this, S1PR1 deficient T cells transferred to congenic hosts accumulated in the spleen as they were unable to exit (Matloubian et al., 2004). However, experimental evidence for the role of chemokines in T cell egress from the spleen is lacking.

Transcriptional control of the naïve T cell homing phenotype is critically regulated by Foxo1, which promotes expression of CD62L and CCR7, as well as the transcription factor KLF2, which has previously been reported to control expression of these surface proteins in addition to S1P1 (Carlson et al., 2006; Kerdiles et al., 2009; Ouyang, Beckett, Flavell, & Li, 2009). Fur thermore, Foxo1 is required for Bcl 2 expression and T cells deficient in Foxo1 show impaired survival. Expression of KLF2 also either directly or indirectly represses expression of chemokine receptors such as CXCR3,

CCR3, and CCR5, preventing distraction of naïve T cells toward inflammatory signals (Sebzda, Zou, Lee, Wang, & Kahn, 2008; Weinreich et al., 2009). Thus, concerted transcriptional control of migratory receptors is essential for optimal efficiency of the process of naïve T cell trafficking.

## 3. CHEMOKINE CONTROL OF CD4$^+$ T CELL PRIMING

Once naïve CD4$^+$ T cells interact with DCs presenting cognate antigen, a sequence of events known as T cell priming begins. This leads to a series of distinct changes in T cell motility that have been well described (Mempel, Henrickson, & Von Andrian, 2004; Miller, Safrina, Parker, & Cahalan, 2004; Miller, Wei, Parker, & Cahalan, 2002). Chemokine receptor expression patterns are also radically altered during T cell priming and this largely accounts for these changes in T cell motility. Thus, chemokines play critical roles in governing T cell priming, by altering CD4$^+$ T cell positioning within the SLO microenvironment as well as enhancing interaction time with APCs.

### 3.1 Early Stages of CD4$^+$ T Cell Activation

Within the first few hours after initial recognition, CD4$^+$ T cells significantly decrease their velocity and undergo multiple short interactions with DCs. This is partly mediated by DC surface bound CCL21, which forms tethers with CCR7 on naïve CD4$^+$ T cells, potentially enhancing the interaction time to enable the naïve T cell to scan for the peptide–MHC II complex (Friedman, Jacobelli, & Krummel, 2006; Molon et al., 2005). Multiple interactions between naïve T cells with different peptide–MHC II complexes on either the same DC or on DCs in trans induces T cell upregulation of early activation markers such as CD69, which suppresses S1PR1 surface expression, thus promoting retention in the SLO (Shiow et al., 2006). In the next stage of activation, CD4$^+$ T cells maintain interactions with DCs for extended periods of time, (reportedly from 2 to 12 h) (Mempel et al., 2004), where very close synapse like interactions form. During these encounters, T cell priming is influenced by TCR signal strength, costimulatory signals on the APC, and the local cytokine milieu which governs their subsequent differentiation, discussed in further detail below. Approximately 24–48 h after initial antigen recognition, the CD4$^+$ T cells begin to increase their motility again and proliferate (Hor et al., 2015; Miller et al., 2004). Constant sporadic engagements of the daughter cells with DCs seems to be required for full effector differentiation, as

repeated encounters of $CD4^+$ T cells with Ag bearing DCs is required for optimal cytokine secretion (Celli, Garcia, & Bousso, 2005).

Chemokine receptors are rapidly upregulated in response to activation, prior to cell division. Transfer of LPS and poly(I:C) activated DCs pulsed with OVA induced upregulation of CXCR3 on cognate OT II cells within 24 h of transfer (Groom et al., 2012). In this model, CXCR3 expression was required for optimal $T_H1$ differentiation, as *Cxcr3* $^{-/-}$ OT II cells showed impaired activation and IFNγ production. This was associated with reduced interactions with CXCL10 producing DCs, suggesting CXCR3–CXCL10 tethering enhances dwell time of the $CD4^+$ T cells on engaged DCs, facilitating increased TCR engagement and thus increased differentiation. Similarly, upregulation of CXCR3 within 12 h of influenza vaccination facilitated clustering of $CD4^+$ T cells with $CXCL10^+$ DCs (Woodruff et al., 2014). As CXCR3 expression is intrinsically linked with $T_H1$ differentiation, it remains to be determined whether early upregulation of characteristic lineage specific chemokine receptors is common to all transcriptional programs, or whether other subset specific chemokine receptors play similar roles in DC clustering.

As will be discussed in more detail later, another chemokine receptor that is upregulated at the early stages of $CD4^+$ T cell activation is CXCR5. Expression of CXCR5 by both T cells and B cells drives migration to the follicles, where CXCL13 is expressed by follicular dendritic cells (FDCs). Coexpression of CXCR5 with PD1 is used to delineate $T_{FH}$ cells, but there are conflicting reports regarding the timing of when CXCR5 is first expressed. It was initially reported that OT II cells responding to OVA/alum immunization did not express CXCR5 until after they had begun to proliferate, although they expressed Bcl6, the defining transcriptional regulator of the $T_{FH}$ program (Baumjohann, Okada, & Ansel, 2011). Subsequent CXCR5 upregulation coincided with increased expression of Bcl6. In contrast, reports have shown that CXCR5 is already expressed 12 h after activation, preceding upregulation of Bcl6 and cell division (Chen, Ma, Zhang, Wu, & Qi, 2015; Hardtke, Ohl, & Forster, 2005). This expression of CXCR5 was dependent on IL 6 production by radioresistant cells, although the identity of the precise cell type is unknown. CXCR5 was required for OT II migration to the T B border within the first 24 h of activation, in accordance with the role of $T_{FH}$ cells in supporting humoral immune responses. $T_H2$ cells have also been shown to upregulate CXCR5 and migrate to the perifollicular region of mesenteric lymph nodes in response to nematode infection (Leon et al., 2012). Expression of

CXCR5 by both $T_H2$ and $T_{FH}$ in this model of *Heligmosomoides polygyrus* was required for optimal differentiation of these subsets, potentially neces sitated by allowing colocalization with $CXCR5^+$ DCs which are situated adjacent to the follicles.

## 3.2 Chemokine Receptor-Directed Control of Early Fate Decisions

There is accumulating evidence that there is considerable plasticity during the early phases of $CD4^+$ T cell differentiation toward specific $T_H$ lineages. It is now clear that a single T cell clone has the capability to differentiate into diverse fates, depending on T cell intrinsic factors such as TCR signal strength, as well as environmental factors such as antigen abundance and the local cytokine milieu (Tubo et al., 2013). In the first few days following T cell activation, cells retain the potential to differentiate into different $T_H$ subsets. This was first supported by the detection of cells with a $PD1^{hi}$ $CXCR5^{hi}$ $T_{FH}$ like profile at early stages of $T_H1$ differentiation in in vitro cultures with IL 12 (Nakayamada et al., 2011). By day 3 of culture, $CD4^+$ T cells display an uncommitted phenotype with coexpression of Bcl6 and the $T_H1$ master regulator T bet, and production of both IL 21 and IFNγ. However, continued induction of T bet through IL 12 signaling is ultimately required to polarize the culture to $T_H1$ with no cells expressing a $T_{FH}$ like phenotype at advanced stages. Similar results were seen in an in vivo infection model with *Toxoplasma gondii*, a potent inducer of IL 12, where in competitive mixed transfer systems *Tbx21* $^{-/-}$ $CD4^+$ cells were more likely to display a $T_{FH}$ phenotype. This suggests that during the early path of $T_H1$ differentiation, cells upregulate CXCR5 and have the potential to divert toward $T_{FH}$ or $T_H1$ development, but signaling which favors induction of T bet pushes them toward the $T_H1$ pathway.

An intriguing possibility is that migration of nascent $T_H$ cells through CXCR5 determines their fate (Qi, 2016). Cells that are able to exit the T cell zone and migrate to the follicles escape from the high levels of IL 2 present in the T cell zone. As IL 2 is a potent suppressor of $T_{FH}$ devel opment, this follicular localization allows the $T_{FH}$ program to be enforced (Ballesteros Tato et al., 2012; Oestreich, Mohn, & Weinmann, 2012). In contrast, $T_H$ cells that are unable to reach the follicles and remain in the T cell zone are exposed to high levels of IL 2, promoting proliferation and differentiation toward a $T_H1$ or $T_H2$ phenotype. This is partly supported by recent work from Cyster and colleagues who identified a novel role for the GPCR EBI2 (GPR183) in promoting $T_{FH}$ development (Li, Lu, Yi, &

Cyster, 2016). EBI2 was upregulated on T cells within 12 h after protein immunization and facilitated translocation to the outer T cell zone through its ligand 7α,25 dihydroxycholesterol, where T cells could interact with $CD4^+$ DCs. These DCs expressed high levels of CD25 and could quench IL 2 from the local area, promoting $T_{FH}$ development. Thus, coordinated migration of recently activated T cells through EBI2 and CXCR5 promotes $T_{FH}$ development. Given that upregulation of EBI2 and CXCR5 appears to be fairly uniform among recently activated cells, it remains to be determined if this GPCR mediated escape from the T cell zone is a stochastic process or if there are as yet unidentified cues that favor follicular homing.

## 3.3 Chemokine Receptor-Directed Migration Between DC Subsets During Priming

$CD4^+$ T cells can interact with multiple DC subsets in SLO and have recently been reported to track between different DCs at different stages of the response (Hor et al., 2015). In a model of herpes simplex virus (HSV) skin infection, antigen specific $CD4^+$ T cells were initially primed by migratory DCs in the paracortex, but at later stages were found to interact with $CD8\alpha^+XCR1^+$ crosspresenting DCs, forming the platform for $CD8^+$ T cells to become efficiently crossprimed. This late interaction of $CD4^+$ T cells with $CD8\alpha^+XCR1^+$ DCs was also simultaneously reported in models of vaccinia virus infection (Eickhoff et al., 2015). It is currently unclear if this interaction with $CD8\alpha^+XCR1^+$ DCs is required for optimal $T_H$ differentiation, or if it is simply the execution of a helper function of this subset. However, given the strict requirement for antigen presence in maintaining $T_H$ cells, it is possible these interactions are required for main tenance of the $T_H$ response (Obst, van Santen, Mathis, & Benoist, 2005). How and when $CD4^+$ T cells migrate between different DC populations within the LN is currently unclear, and the migratory cues governing this require further investigation. One likely candidate is CXCR3, which as mentioned earlier is upregulated soon after activation in response to protein immunization. Given that CXCR3 is required for optimal $T_H1$ differenti ation, and CXCR3 is not expressed on naïve T cells, it is plausible that upregulation of CXCR3 after initial priming by a migratory DC promotes attraction toward CXCL10 producing DCs, potentially the $XCR1^+$ DCs, which is required for full differentiation. Indeed, CXCL10 was reported to be expressed by $CD8\alpha^+$ DCs in response to influenza vaccination, although $CD11b^+$ resident DCs had higher production (Woodruff et al., 2014).

Clearly, further investigation into the precise trafficking events governing early T cell differentiation needs to be undertaken.

## 4. CHEMOKINE CONTROL OF CD4$^+$ EFFECTOR FUNCTION DURING THE ACUTE PHASE

Differentiation into distinct $T_H$ lineages allows CD4$^+$ T cells to deliver specific help tailored to the type of immune challenge (Kara et al., 2014). The execution of many $T_H$ effector functions primarily involves egress from SLO and migration to the challenged peripheral tissue, and this is coordinated through expression of subset specific as well as tissue specific chemokine receptors. Tissue specific chemokine receptors include CCR9, which allow trafficking of subsets to the intestinal tract where high levels of CCL25 are produced, as well as CCR10, which promotes homing to high levels of CCL27 in the skin. These receptors can be expressed by multiple $T_H$ subsets, as they allow for general trafficking to the desired organ. Subset specific chemokine receptors are often expressed more strictly and can be regulated in response to the differentiation cues received during priming (Fig. 1). These receptors have been useful tools in aiding identification of $T_H$ subsets based on their chemokine receptor profile, but are also critical for $T_H$ cells to execute their effector functions. In this section, we will discuss the chemokine receptors that facilitate the function of each described $T_H$ subset.

### 4.1 $T_H1$

$T_H1$ cells promote cell mediated immunity and are critical for protection against intracellular pathogens. They are also important for clearance of cancerous cells, but can contribute to pathology in cases of transplantation or autoimmunity. These roles are predominantly mediated through $T_H1$ production of the cytokine IFNγ, as well as TNFα and IL 2. IFNγ is essential for control against numerous pathogens, through both direct effects on the pathogen itself as well as more broadly stimulating a concerted immune response by numerous cell types (Schoenborn & Wilson, 2007). $T_H1$ cell differentiation is first induced by TCR engagement in the presence of IFNγ or type I IFNs, which leads to expression of the master transcriptional regulator T bet and consequently the high affinity IL 12Rβ2 chain. IL 12 then acts via STAT4 to further stimulate expression of T bet, which drives IFNγ production to create a feedforward loop which stabilizes the $T_H1$ differentiation program.

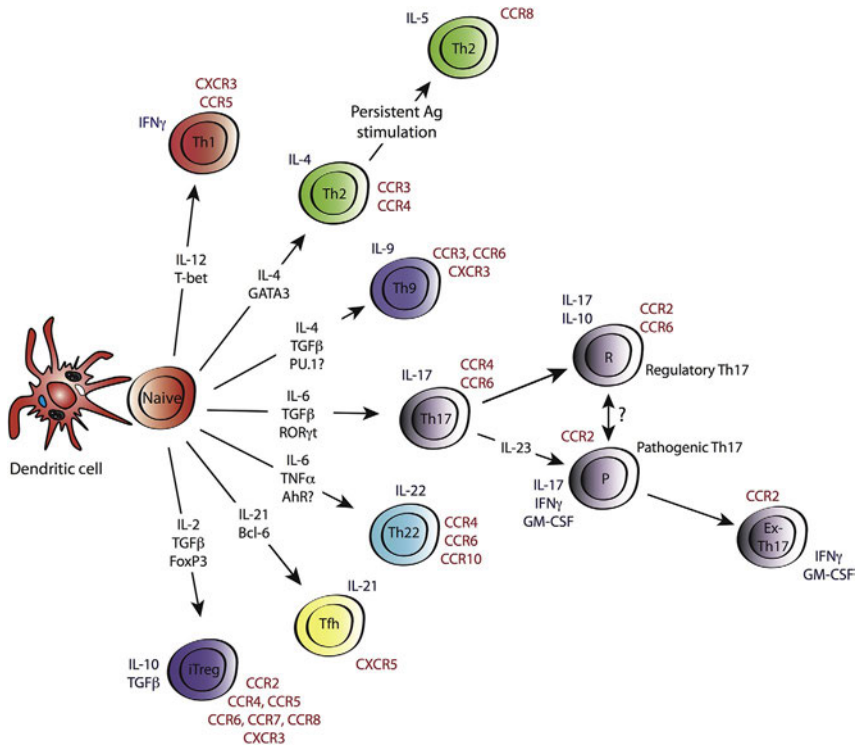


**Fig. 1** CD4+ T cell subsets are distinguished by distinct chemokine receptor expression and differentiation cues. Upon cognate interactions with peptide:MHC II complexes presented by DCs, priming of naïve CD4+ T cells is influenced by the local cytokine milieu. This leads to expression of distinct transcription factors that can induce subset-specific transcriptional profiles, which encompass expression of effector cytokines as well as chemokine receptors that are tailored to enable optimal responses to the initial antigen. The key subsets of $T_H$ cells that have been described are shown along with the cytokines and transcription factors that drive/maintain their development (*black text*); characteristic cytokines that they produce (*blue text*); and the chemokine receptors expressed (*red text*). *Arrows* indicate differentiation/transdifferentiation of T cell subsets, *?* indicates either uncertain transcriptional regulation or an as yet unproven transdifferentiation pathway.

The canonical chemokine receptor expressed by $T_H1$ cells is CXCR3. This receptor is directly bound and transactivated by T bet and as such appears to be highly coupled to the $T_H1$ program. The CXCR3 axis is a prototypical inflammatory axis, with CXCR3 absent on naïve T cells and the ligands only induced upon exposure of cells to inflammatory stimuli. Of the three ligands, CXCL11 is the most potent in humans, followed by CXCL10 and CXCL9 (Van Raemdonck, Van den Steen, Liekens,

Van Damme, & Struyf, 2015). These ligands can be produced by a broad range of cell types, with fibroblasts, leukocytes, and keratinocytes all reported sources of expression. CXCR3 is essential for the peripheral localization of $T_H1$ cells in a multitude of diseases, with deletion of CXCR3 broadly inhibiting $T_H1$ migration in infections, autoimmune diseases, and many cancers (Groom & Luster, 2011). In cases where pathology is driven by $T_H1$ cells, CXCR3 deletion or ligand neutralization is generally associated with favorable outcomes. In models of rheumatoid arthritis, CXCR3 is required for T cell recruitment to inflamed joints, and CXCR3 neutralization delayed and abrogated disease severity (Mohan & Issekutz, 2007; Tsubaki et al., 2005). In Rag deficient mice, transfer of CXCR3 deficient $CD4^+$ T cells fails to induce colitis (Kristensen et al., 2006). Recently, it was shown that IL 10 is protective in colitis models by downregulating CXCR3 expression on $T_H1$ cells, thus reducing their homing to inflammatory sites (Wadwa et al., 2016). The role of CXCR3 on $T_H1$ cells in experimental autoimmune encephalomyelitis (EAE) is more controversial, with disparate reports concerning the importance of this receptor. T cells found in lesions of multiple sclerosis (MS) patients as well as in mouse EAE CNS infiltrates show high levels of CXCR3 expression (Balashov, Rottman, Weiner, & Hancock, 1999; Sorensen et al., 1999; Sporici & Issekutz, 2010). In rats with EAE, CXCR3 neutralization inhibited migration of blood T cells to the CNS (Sporici & Issekutz, 2010). Clinical disease induced by passive transfer of myelin reactive T cells activated in vitro was abrogated with CXCR3 neutralization, and this corresponded to reduced trafficking of $CD4^+$ T cells to the CNS. This was in line with our finding that a peptide antagonist of CXCR3 inhibited the effector phase of EAE in SJL/J mice (Kohler et al., 2008). However, clinical disease in a model of active EAE immunization was unaffected by CXCR3 blockade and neutralization of CXCL10 or CXCR3 deletion has been reported to either exacerbate or have no effect on EAE severity (Byrne et al., 2009; Chung & Liao, 2016; Liu et al., 2006; Muller et al., 2007; Narumi et al., 2002; Sporici & Issekutz, 2010). These apparently conflicting findings may potentially be explained in part by the differential bias toward encephalitogenic $T_H1$ or $T_H17$ cells induced by different immunization routes, although even in EAE induced by transfer of encephalitogenic $T_H1$ cells, CXCR3 deletion did not affect prevent initiation of disease (Lalor & Segal, 2013). Furthermore, CXCR3 expression by glial cells in EAE further confounds interpretation of broad *Cxcr3* $^{-/-}$ studies and highlights the importance of utilization of systems that allow assessment of the impact of $CD4^+$ T cell subset specific deletion of chemokine

receptors when studying trafficking. To the best of our knowledge, these studies have yet to be performed with regard to CXCR3 in EAE.

The precise contributions of the different ligands to $T_H1$ homing is still unclear, although CXCL10 is more strongly induced by type I IFN, as opposed to CXCL9 which is more dependent on IFNγ (Groom & Luster, 2011). This dependence on inflammatory stimuli for CXCR3 ligand expression alludes to another key role of the CXCR3 axis, which is to amplify the effector response. Expression of IFNγ by $T_H1$ at periph eral sites stimulates upregulation of CXCR3 ligands, which in turn pro mote recruitment of more CXCR3 expressing $T_H1$ cells, leading to augmentation of the initial response. This is also essential for the ability of $T_H1$ cells to assist development of an effective CTL response, one of the major roles of $T_H1$ cells in viral and bacterial infections. In HSV infec tion, $CD4^+$ T cells are essential for $CD8^+$ migration to the vaginal mucosa and $T_H1$ derived IFNγ is critically required for this (Nakanishi, Lu, Gerard, & Iwasaki, 2009). $T_H1$ cells at the infection site produce IFNγ which upregulates expression of CXCL9 and CXCL10 by local epithelial cells, thereby allowing recruitment of CTLs via CXCR3 where they can clear infected cells. Given that the $T_H1$ cells required responsiveness to type I IFN, it is likely that TLR engagement stimulates innate cells to pro vide an initial source of type I IFN which initiates low levels of $T_H1$ migra tion into the tissue, presumably through CXCR3, which then through IFNγ production, promotes amplification of CXCL9 and CXCL10 pro duction and subsequent CTL entry.

Another chemokine receptor classically associated with $T_H1$ cells is CCR5, which binds to CCL3, CCL4, and CCL5. These chemokines are induced upon inflammation, with reported stimuli including IL 1β and TNFα (Kawka et al., 2014). CCR5 is also used by $T_H1$ cells to migrate to peripheral sites to exert their function. In patients with rheumatoid arthri tis, CCR5 expression is increased on $CD4^+$ T cells isolated from synovial fluid, and can be further upregulated upon culture with IL 15 (Wang & Liu, 2003). In tumor settings, CCR5 is critical for effective antitumor immunity, with CCR5 expression required on both OT I and OT II cells for efficient rejection of EG7 tumors (Gonzalez Martin, Gomez, Lustgarten, Mira, & Manes, 2011). This is at least partly mediated through intratumoral CCL5, as increased levels of CCL5 in the tumor lead to increased recruit ment of $CD4^+$ T cells and reduced tumor growth (Lavergne et al., 2004). CCR5 expression on OT II cells was also required for optimal upregulation of CD40L on DCs and hence development of $IFN\gamma^+$ OT I cells, thus

pointing to a role for CCR5 in promoting crosspresentation by DCs to induce CTL responses (Gonzalez Martin et al., 2011).

Although CXCR3 and CCR5 are often coexpressed by $T_H1$ cells, they do not appear to have redundant functions. In chronic hepatitis C infection, CCR5 ligands are expressed in vessels within the portal triad, whereas CXCR3 ligands are expressed on the sinusoidal epithelium, suggesting these receptors may instead control different aspects of $T_H1$ trafficking (Shields et al., 1999). Furthermore, during malarial infection with blood stage *Plasmodium yoelii*, CXCR3 was equivalently expressed in $T_H1$ cells that were IL $10^+$ or IL 10 , however CCR5 was preferentially expressed on IL $10^+$ $T_H1$ cells (Villegas Mendez et al., 2015). Given that IL 10 in malarial infections is critical for regulating inflammatory tissue damage and $T_H1$ cells are the dominant source of this cytokine, it suggests that the CCR5 ligands promote recruitment of $T_H1$ with a regulatory phenotype. This remains to be formally addressed using cells deficient in these receptors.

## 4.2 $T_H2$

$T_H2$ cells are induced in response to infection with extracellular parasites, such as helminths, where they promote expulsion or destruction of the parasite. $T_H2$ cells are also thought to be involved in protection from environmental toxins and irritants (Palm, Rosenstein, & Medzhitov, 2012). However, excessive activation of $T_H2$ cells can also mediate allergic pathologies such as asthma and atopic dermatitis. These roles of $T_H2$ cells are predominantly facilitated through production of IL 4, IL 5, and IL 13 (Islam & Luster, 2012; Licona Limon, Kim, Palm, & Flavell, 2013). IL 4 acts through STAT6 signaling to drive IgE driven allergic inflammation, in which IgE binds to the Fcε receptor on granulocytes to stimulate production of potent inflammatory molecules, such as cytokines and histamine. IL 5 production is more associated with eosinophilic inflammation, while IL 13 promotes airway hyperresponsiveness and mucus production. In addition to expression of these cytokines, $T_H2$ cells are characterized by expression of the lineage defining transcription factor GATA3. They are generally induced by pathogen or allergen exposure at epithelial barriers, where release of cytokines such as TSLP, IL 33, and IL 25 promote the maturation of DCs in a manner to prime $T_H2$ cells (Saenz, Taylor, & Artis, 2008). $T_H2$ cells can also be primed by basophils independently of DCs in response to haptens or peptide antigens, although this role has been controversial (Kara, McColl, & Comerford, 2013; Otsuka et al., 2013).

One of the major chemokine receptors expressed by $T_H2$ cells is CCR4, which binds to the ligands CCL17 and CCL22. CCR4 expression is enriched on IL 4 producing $T_H2$ cells from both mouse and human (D'Ambrosio et al., 1998; Sallusto, 2016). IL 4 also appears to drive a pos itive feedback loop by promoting differentiation of $T_H2$ cells and enhancing CCR4 expression on $T_H2$ cells (Kim, Nagata, & Butcher, 2003). CCL17 can be produced by endothelial cells, keratinocytes, and DCs in response to allergic or pathogen challenge (Lonsdorf, Hwang, & Enk, 2009). Conse quently, CCR4 drives the initial recruitment of $T_H2$ cells to the challenge site in a variety of models. In an atopic dermatitis model, epicutaneous chal lenge with OVA induced CCR4 expression on antigen specific OT II cells (Oyoshi et al., 2011). This was essential for their ability to initiate skin inflammation, as CCR4 deficient OT II T cells were unable to transfer allergic inflammation. CCR4 is also important for $T_H2$ trafficking in allergic asthma, and it is highly expressed on $CD4^+$ T cells recruited to asthmatic airways (Vijayanand et al., 2010). In an aerosolized OVA challenge, com petitive transfer of $CCR4^{-/-}$ OT II with $CCR4^{+/+}$ OT II showed that CCR4 is required for $T_H2$ migration to the lung and BAL (Mikhak et al., 2009). These sites can produce increased levels of both CCL17 and CCL22 in response to allergen challenge in atopic asthmatic patients (Pilette, Francis, Till, & Durham, 2004). These chemokines are produced by pulmonary dendritic cells in response to aerosolized OVA challenge in a STAT6 dependent manner, with either CD11b depletion or STAT6 dele tion leading to reduced $T_H2$ trafficking to the lung and reduced eosinophilic inflammation (Mathew et al., 2001; Medoff et al., 2009). Similarly, in a model of lung inflammation induced by *Nippostrongylus brasiliensis* infection, expression of STAT6 in a bone marrow derived myeloid population was required for $T_H2$ lung homing (Voehringer, Shinkai, & Locksley, 2004). However, in some cases of chronic allergic inflammation, $T_H2$ trafficking has been shown to be independent of CCR4. In a 7 week model of cuta neous OVA induced inflammation, CCR4 deletion failed to impair the development of atopic dermatitis, suggesting other chemotactic signals can drive $T_H2$ migration (Islam et al., 2011).

$T_H2$ cells in cases of chronic antigen are more highly differentiated and produce increased amounts of IL 5. Indeed, human $T_H2$ cells which were initially $IL\ 5^{ve}$ required multiple rounds of in vitro stimulation to induce IL 5 production (Upadhyaya, Yin, Hill, Douek, & Prussin, 2011). In accor dance with this, CCR4 expressing cells isolated from the peripheral blood

of healthy donors were enriched for IL 4 expression, but produced little IL 5 (Islam et al., 2011).

Unlike CCR4, CCR8 expression is enriched on IL 5 producing $T_H2$ cells in mouse and human, suggesting that there is a hierarchical chemokine receptor expression profile for $T_H2$ cells, with CCR4 expres sion predominating in the recruitment of $T_H2$ cells to barrier sites upon initial antigen exposure, but being downregulated upon subsequent encounters with antigen, in which trafficking occurs predominately through CCR8.

CCR8 binds to CCL1 and CCL8 in mouse, and CCL1 and CCL18 in humans (D'Ambrosio et al., 1998; Islam et al., 2011; Islam, Ling, Leung, Shreffler, & Luster, 2013; Zingoni et al., 1998). As well as being a key pro moter of eosinophilic responses, the CCR8 axis seems to be crucial for responses to recurring antigen (Islam et al., 2011). Indeed, CCR8 was only induced upon in vitro generated $T_H2$ cells after repeated rounds of polari zation. This fits with previous data showing that the *Ccr8* gene has both acti vation sites for the $T_H2$ associated transcription factor STAT6 as well as repressive sites for the $T_H1$ associated transcription factor STAT4 (Wei et al., 2010). Thus, it is possible that strong induction of $T_H2$ polarization made feasible through multiple rounds of antigen recognition is required for removal of STAT4 and binding of STAT6, to allow CCR8 expression. This CCR8 expression profile may help to explain conflicting results regard ing the importance of CCR8 in models of allergic asthma. CCR8 is highly expressed on $CD4^+$ T cells recruited to asthmatic airways, where increased levels of CCL1 are found (Gonzalo et al., 2007). In a model of OVA chal lenge beginning at day 40 after sensitization, CCL1 neutralization or dele tion of CCR8 reduced airway inflammation and hyperresponsiveness, and CCR8 deficient mice showed almost complete ablation of expression of IL 4, IL 5, and IL 13 expression in the lung, supporting the importance of CCR8 in this pathology (Gonzalo et al., 2007). Similarly, deletion of CCR8 in an asthmatic model induced by cockroach antigen showed impaired inflammation, associated with reduced $T_H2$ cytokine expression, and reduced accumulation of eosinophils (Chensue et al., 2001). However, other studies using CCR8 deficient mice with shorter sensitization and challenge periods report no differences in allergic asthmatic models, although CCR8 expression was induced upon aerosol challenge (Chung et al., 2003; Goya et al., 2003). Furthermore, competitive cotransfer of $Ccr8^{-/-}$ OT II cells with $Ccr8^{+/+}$ OT II cells in a 3 day aerosolized OVA challenge showed increased homing of $Ccr8^{-/-}$ OT II cells to the

lung and BAL (Mikhak et al., 2009). It is not clear whether CCR8 is expressed by OT II cells in this model, and further experiments to elucidate the mechanism for this enhanced homing are required.

CCR8 is also required for development of chronic allergic skin inflammation, with increased numbers of CCR8 expressing cells in skin from individuals with atopic dermatitis compared with healthy skin (Gombert et al., 2005; Islam et al., 2011). CCR8 deficient mice were protected from developing atopic dermatitis in a 7 week OVA sensitization model, but neutralization of CCL1 during the last 7 days of sensitization did not impair this inflammation, suggesting this response is instead dependent on CCL8 which, unlike CCL1, was induced in atopic skin. Cotransfer of WT and CCR8 $^{-/-}$ $T_H2$ cells showed an intrinsic defect in CCR8 deficient $CD4^+$ trafficking to the atopic skin and draining lymph node. This CCR8 mediated recruitment of IL 5 producing $T_H2$ cells is also required for production of IL 25, the eosinophil attracting chemokines CCL11 and CCL24, as well as IL 5 mediated class switching to IgG1. However, CCL1 can also mediate $T_H2$ trafficking via CCR8, with the chemokine being upregulated in response to atopic skin inflammation, allergen challenge, and microbial products, although not to as great an extent as mouse CCL8 or human CCL18 (Gombert et al., 2005). Intriguingly, CCL1 has been reported to have antiapoptotic activity and can promote survival of thymocytes (Ruckes, Saul, Van Snick, Hermine, & Grassmann, 2001). More research is required to determine the different contributions the CCR8 ligands play in $T_H2$ migration, which given the intraspecies differences in ligands between mouse and human, may pose some difficulties.

Another chemokine receptor that was one of the first reported to be expressed on $T_H2$ cells is CCR3 (Sallusto, Lenig, Mackay, & Lanzavecchia, 1998; Sallusto, Mackay, & Lanzavecchia, 1997). In cultures of $CD4^+$ T cells taken from human peripheral blood, $CCR3^+$ cells were enriched for expression of IL 4 and IL 5. Furthermore, $CCR3^+$ T cells were enriched in inflamed skin taken from patients with contact dermatitis as well as on $CD4^+$ T cells in cutaneous T cell lymphoma (Gerber et al., 1997; Miyagaki et al., 2010). $CCR3^+$ $CD4^+$ T cells were also increased in peripheral blood from allergic rhinitis patients, with less IL 5 production observed in cultures where $CCR3^+$ cells had been depleted (Francis, Lloyd, Sabroe, Durham, & Till, 2007). More recently, CCR3 was found to be expressed on approximately 50% of $CD4^+$ T cells from normal nasal mucosa suggesting a potential role for trafficking to mucosal sites, however functional evidence for this is still lacking (Danilova et al., 2015).

Recent evidence has implicated $CX_3CR1$ as another chemokine receptor critical for $T_H2$ trafficking in allergic inflammation. $CX_3CR1$ binds to $CX_3CL1$ in mice, as well as CCL26 in humans, with $CX_3CL1$ expression in the skin of atopic dermatitis patients positively correlating with disease severity (Echigo, Hasegawa, Shimada, Takehara, & Sato, 2004; Nakayama et al., 2010). Interestingly, $CX_3CR1$ expression is not required for migration into inflamed skin, but rather for retention at this site. Topical administration of a $CX_3CR1$ antagonist specifically promoted egress of $T_H2$ cells and consequently reduced skin inflammation. This receptor is also important in asthma models, with $CX_3CL1$ expression increased in lung and BAL in response to allergen challenge. $CX_3CR1$ expression on $CD4^+$ T cells is required for a robust asthmatic response, as $CX_3CR1$ deficient mice show impaired responses which can be restored by transfer of WT $CD4^+$ T cells. Again, $CX_3CR1$ does not appear to have a role in migration of $T_H2$ to the lung, but rather in promoting survival of these cells. $CX_3CR1$ deficient $T_H2$ cells showed increased apoptosis in vivo, which could be rescued by transduction with the antiapoptotic protein BCL 2 (Mionnet et al., 2010). However, $CX_3CR1$ is expressed at similar levels by $T_H1$ and $T_H2$ cells in humans and in mouse models of atopic dermatitis, thus not appearing to be restricted to the $T_H2$ lineage. Similarly, CCR10 is expressed at increased levels on $CD4^+$ T cells in a variety of skin pathologies, with its ligand CCL27 expressed by keratinocytes in response to the inflammatory cytokines TNFα and IL 1β (Hijnen et al., 2005; Homey et al., 2002). CCR10 is classified as a skin homing receptor rather than being specific to a $T_H$ subset, although it also is important for $T_H2$ trafficking under allergic conditions.

Another GPCR recently implicated in human $T_H2$ cells is the orphan receptor, GPR15 (Nguyen et al., 2015). GPR15 is enriched on colonic $T_H2$ cells from patients with ulcerative colitis, but is not expressed by $T_H2$ cells from mouse. Accordingly, GATA3 was found by CHIP Seq to bind to enhancer regions in GPR15 in humans but not mice. Given these species differences, it may be difficult to ascertain the functional role of this receptor in $T_H2$ trafficking in intestinal diseases.

In allergic inflammation, $T_H2$ recruitment is also mediated by GPCRs that do not bind to chemokines. CRTH2 is a characteristic GPCR expressed by $T_H2$ in humans but not mice, and binds to prostaglandin D2 ($PGD_2$), an acidic lipid. CRTH2 is expressed at increased levels on purified $CD4^+$ T cells isolated from patients with atopic dermatitis compared with those from healthy controls, and is likely to play a role in attracting $T_H2$ cells to sites of cutaneous inflammation (Hijnen et al., 2005). $PGD_2$

is also released by mast cells in cases of allergic asthma and polymorphisms in this gene are associated with altered susceptibilities to asthma in a range of studies (Islam & Luster, 2012). Similarly, leukotriene $B_4$ ($LTB_4$) is also pro duced by mast cells and binds to leukotriene $B_4$ receptor 1 (BLT1) which is expressed by T cells, suggesting these lipid chemoattractants are critical for the initial wave of $T_H2$ migration to the airways in allergic responses.

## 4.3 $T_H17$

$T_H17$ cells were first described in 2005 as a lineage independent to $T_H1$ and $T_H2$, that were capable of producing the proinflammatory cytokines, IL 17A and IL 17F (Harrington et al., 2005; Park et al., 2005). These cells are characterized by their expression of the master transcription factor, ret inoic acid receptor related orphan receptor γt (RORγt), and have since been described to secrete a diverse repertoire of cytokines, including IFNγ, GM CSF, IL 10, and IL 22 (Kara et al., 2014). $T_H17$ cells are critical for host defense against extracellular microorganisms and this protective role against both bacterial and fungal pathogens is primarily mediated through IL 17A and IL 17F, which bind to the heterodimeric IL 17 receptor, com posed of IL 17RA in complex with IL 17RC (Gaffen, 2016). This receptor is widely expressed on nonhematopoietic cells, and signaling promotes the induction of neutrophilic chemotactic factors, such as CXCL1, CXCL2, and CXCL8 resulting in recruitment of neutrophils to the site of pathogenic insult. In addition, $T_H17$ cells can induce stromal secretion of other inflam matory proteins such as matrix metalloproteases and the antimicrobial beta defensins and S100 peptides as well as promoting myelopoiesis through pro duction of G CSF and GM CSF (Liang et al., 2006; McGeachy, 2011). However, $T_H17$ cells are also implicated as key drivers of many autoimmune pathologies, as shown in mouse models of rheumatoid arthritis and MS where production of proinflammatory cytokines drives pathological destruction of host tissues (Hirota et al., 2007; Langrish et al., 2005).

$T_H17$ cell differentiation in vitro is triggered by IL 6 and TGF β1, with IL 21 and IL 23 reinforcing this program (Bettelli et al., 2006; Langrish et al., 2005; Mangan et al., 2006; Veldhoen, Hocking, Atkins, Locksley, & Stockinger, 2006). However, in vivo differentiation of $T_H17$ cells is more complex and it is now clear that a spectrum of $T_H17$ cellular phenotypes exist with differing capacities to promote inflam mation. $T_H17$ cells differentiated in the presence of TGF β1 and IL 6 alone express the antiinflammatory cytokine IL 10 in addition to IL 17, and these

cells have limited capacity to promote EAE following passive transfer (McGeachy et al., 2007). In contrast, when differentiated in the presence of IL 23 or TGFβ3, $T_H17$ cells did not express IL 10 and instead upregulated proinflammatory cytokines, specifically IFNγ and GM CSF (Lee et al., 2012). These IFNγ and GM CSF producing $T_H17$ cells have been described to have higher pathogenic potential than other $T_H17$ cells due to their production of these inflammatory cytokines. Indeed, studies using cytokine deficient transgenic $CD4^+$ T cells indicate that it is GM CSF and not IL 17A or IFNγ that is the key pathological cytokine pro duced by $T_H17$ cells in EAE (Ponomarev et al., 2007). Furthermore, in con ditions of persistent antigen and exposure to IL 23, such as in EAE, $T_H17$ cells can lose IL 17 expression while maintaining IFNγ and GM CSF production, giving rise to what have been termed ex $T_H17$ cells (Hirota et al., 2011). The relative contributions to disease of these differentiation states remain to be elucidated. Conversely, when antigen is transient, such as following resolution of bacterial infection, path ogenic $T_H17$ cells can convert to a more regulatory phenotype. Using a model in which an IL 17A fate mapping mouse was crossed to an IL $17A^{Katushka}IL\ 10^{eGFP}Foxp3^{RFP}$ triple reporter mice, $T_H17$ cells were shown to convert to IL $17\ ^{ve}Foxp3\ ^{ve}IL\ 10^+$ cells in model of self limiting inflammation or transient bacterial infection (Gagliani et al., 2015). Given the similarities of the transcriptional profile of these cells to $T_R1$ cells, these cells were termed $T_R1^{exTh17}$ cells. Furthermore, there are reports of $T_H17$ cells transdifferentiating into cells with a $T_{FH}$ like phenotype in PP (Hirota et al., 2013). Thus, differentiation of $CD4^+$ T cells to a $T_H17$ phenotype does not appear to be an endpoint state, with substantial plasticity being governed by antigenic load.

The characteristic chemokine receptor expressed by $T_H17$ cells is CCR6, which binds to the sole known chemokine ligand, CCL20. CCL20 can be upregulated in response to various inflammatory signals, but is also expressed under resting conditions in a diverse range of tissues, including the skin, gut, and airways, attracting $T_H17$ cells to these sites through CCR6 (Comerford et al., 2010; Lee, Eri, Lyons, Grimm, & Korner, 2013). Expression of CCR6 appears to be tightly coupled to the initial $T_H17$ differentiation program, as in in vitro generated $T_H17$ cells, TGF β1 but not IL 6 was sufficient to induce CCR6 expression. IL 2, which suppresses $T_H17$ differentiation in vitro, also reduces CCR6 expres sion on the IL $17^+\ CD4^+$ cells that are generated (Wang, Kang, Lee, Sun, & Kim, 2009). However, these cytokines may be influencing CCR6

expression indirectly through modulation of RORγt expression. Indeed, forced expression of RORγt in naïve T cells is sufficient to upregulate CCR6 and in mice with transgenic expression of RORγt and GFP under the CD4 promoter, the vast majority of $GFP^+$ IL $17^+$ cells were also $CCR6^+$ (Hirota et al., 2007; Wang et al., 2009). CCR6 expression can occur in the absence of RORγt expression but only in RORα sufficient cells, indicating redundancy in RORγt/RORα function in this regard (Yamazaki et al., 2008). RORγt also promotes CCR6 expression in human $T_H17$ cells, as retroviral transduction of cord blood $CD4^+$ T cells with RORγt induced CCR6 (Manel, Unutmaz, & Littman, 2008). Ablation of RORγt activity using the inverse agonist TMP778 also reduced CCR6 expression in human $T_H17$ cells from both healthy and psoriatic donors (Skepner et al., 2014). CCR6 is also directly regulated in human but not mouse $T_H17$ cells by the transcription factor PLZF, which works synergistically with RORγt and binds to the CCR6 promoter (Singh et al., 2015). Taken together, these data have led to the widespread notion that CCR6 is the defining chemokine receptor of $T_H17$ cells and highlighted the CCR6/CCL20 axis as a potential target for pharmacological blockade of inflammatory T cell trafficking in human pathologies. However, there are some important caveats to this that should be considered. These include the expression of CCR6 on regulatory T cells (Yamazaki et al., 2008) and our recent discovery that pathogenic GM CSF producing $T_H17$ cells lose expression of CCR6 and instead migrate through CCR2 (Kara et al., 2015).

$T_H17$ cells are highly enriched in the intestinal tract, which given the abundance of microbes at this site, is in accordance with the demonstrated role of $T_H17$ cells in protection against extracellular bacterial infection. CCR6 has been strongly implicated in $T_H17$ cell recruitment to the intestine and as a consequence is suggested to be a driver of intestinal inflammation. However, while a plethora of studies have demonstrated the importance of CCR6 in intestinal pathology, Tregs, and other cells also use CCR6 for their migration. Thus, many of these studies do not directly assess the role of CCR6 on $T_H17$ cells specifically, and disease outcomes in CCR6 deficient settings depend on the differing contributions of $T_H17$ and Tregs, or other CCR6 expressing cells, in those models. However, it is clear that CCR6 is required for efficient homing of $T_H17$ cells to the intestine. CCL20 is abundantly expressed in the subepithelial dome of PP, as well as in isolated lymphoid follicles. Accordingly, transferred *Ccr6*$^{-/-}$ $T_H17$ cells displayed impaired migration to PP, the small intestinal lamina

propria and the peritoneal cavity (Wang et al., 2009). Similarly, retroviral transduction of CCR6 in T cells enhanced migration to PP. The importance of CCR6 in intestinal $T_H17$ trafficking has also been demonstrated to have a major role in immune regulation following peripheral inflammation. In response to anti CD3 antibody injection, which transiently leads to strong TCR activation and systemic hyperabundance of IL 6 and TGF β1, there is massive recruitment of $T_H17$ cells to the small intestine, where they are expelled into the lumen or reprogrammed for immune regulation (Esplugues et al., 2011). In this model, frequencies of *Ccr6*$^{-/-}$ $T_H17$ cells were strongly reduced in the intestine but instead pooled in spleen and LN demonstrating that CCR6 was essential for this process. IL 17 secretion by intestinal $T_H17$ cells also upregulated CCL20 expression by epithelial cells in the duodenum, suggesting $T_H17$ cells promote a positive feedback loop to enhance recruitment of other $T_H17$ cells. Interestingly, CCL20 is also abundantly produced by $T_H17$ cells, suggesting autocrine amplification of CCR6 driven inflammation, although experimental data testing this pos sibility is lacking.

Aside from CCR6, other chemokine receptors may contribute to intes tinal homing of $T_H17$ cells. $T_H17$ cells from the large intestine have been shown to express CCR4 and CXCR5 and, although it is not clear if these are functional in mouse $T_H17$ cells, CCR4 is also expressed on human memory $T_H17$ cells (Ramesh et al., 2014; Wang et al., 2009). CCR9 is also enriched in $T_H17$ cells in the small intestine, and along with $\alpha_4\beta_7$ can be induced on in vitro generated $T_H17$ cells by addition of retinoic acid (RA) (Wang, Kang, HogenEsch, Love, & Kim, 2010). RA induced $T_H17$ cells use these receptors to migrate to the small intestinal lamina propria, where epithelial cells produce the CCR9 ligand, CCL25. CCR9 induction on T cells is dependent on BATF, as *Batf*$^{-/-}$ T cells fail to upregulate this receptor in response to RA and display impaired homing to the intestine (Wang et al., 2013). BATF binds with RARα in multiple sites upstream of the CCR9 gene, possibly regulating expression through histone acetylation. Given that BATF is also required for $T_H17$ differentiation, the coupling of BATF and CCR9 expression underlines the importance of homeostatic $T_H17$ trafficking to the gut.

$T_H17$ cells are also critical for controlling bacterial infection at other peripheral sites, particularly at other mucosal barrier sites such as the lung. Challenge with an array of extracellular bacteria including *Klebsiella pneumoniae* and *Streptococcus pneumoniae* induces $T_H17$ cells, with IL 17 being a key mediator of bacterial clearance through induction of a neutrophilic

response (Happel et al., 2003; Ye et al., 2001; Zhang, Clarke, & Weiser, 2009). Similarly, fungal pulmonary infections such as with *Aspergillus fumigatus* or *Pneumocystis carinii* also induce potent $T_H17$ responses. The trafficking receptors used by $T_H17$ cells for migration to the lung are not well understood, although given that CCL20 is upregulated in response to pulmonary infection, it is likely that CCR6 plays a role here as well (Khader, Gaffen, & Kolls, 2009). This is supported by studies of human $T_H17$ cells, in which $T_H17$ memory subsets with a $CCR6^+CCR4^+$ profile responded vigorously upon incubation with *Candida albicans* and *Staphylococcus aureus*. However, functional evidence identifying chemokine receptors involved in $T_H17$ homing to the lung is currently lacking.

In addition to control of extracellular microbes, $T_H17$ cells are also known to be drivers of many autoimmune diseases. Again, CCR6 has been demonstrated to play a critical role in the onset of these pathologies. CCR6 is required for trafficking of $T_H17$ to arthritic joints in SKG mice, and CCL20 is abundant in synovial fluid from patients with rheumatoid arthritis (Hirota et al., 2007; Matsui et al., 2001). $CCR6^+$ $T_H17$ cells are also enriched in human inflammatory bowel disease lesions (Kleinschek et al., 2009). In 2,4,6 trinitrobenzene sulfonic acid (TNBS) induced colitis, $CCR6^+$ $T_H17$ cells are increased in frequency in the gut, and neutralization of CCL20 blocks their recruitment (Katchar, Kelly, Keates, O'Brien, & Keates, 2007). Similarly, CCR6 is required for migration to the autoimmune kidney in the $T_H17$ driven pathology of crescentic glomerulonephritis (cGN). Intriguingly, a recent study in Kaede mice using photoconversion of intestinal $T_H17$ cells showed that $T_H17$ cells migrate from the small intestine to the kidney in response to cGN induction (Krebs et al., 2016). Egress from the small intestine lamina propria was dependent on S1PR1, while migration into the kidney was dependent on CCR6 expression. Furthermore, reducing intestinal $T_H17$ numbers through antibiotic treatment or germ free mice also reduced cGN pathology, while expanding intestinal $T_H17$ cells through infection with *Citrobacter rodentium* enhanced pathology. This suggests that the small intestine acts as a functional pool for $T_H17$ cells, that can then be recruited to sites of inflammation and/or autoimmunity using CCR6 and S1PR1. It is not clear if the $T_H17$ cells that enter the kidney from the intestinal pool are specific for kidney autoantigens, or whether they are simply recruited to the kidney through CCR6 and cause inflammation in a nonantigen specific manner. If the latter is true, this suggests $T_H17$ cells may constitutively traffic from the intestinal tract to other peripheral sites. Alternatively, it is possible that the autoimmune kidney produces

additional, as yet unidentified signal(s), to induce $T_H17$ egress from the gut. It will be of great interest to answer these questions, as well as determining if the intestine also harbors differentiated $T_H17$ cells that contribute to auto immunity in other tissues.

The contribution of CCR6 to $T_H17$ cell migration to the CNS in the mouse model of MS, EAE, has been tested by a number of investigators over the past decade. Transfer of MOG reactive $CD4^+$ T cells differentiated in the presence of IL 23 is sufficient to initiate disease; however, transfer of these cells from a CCR6 deficient donor did not induce clinical signs of EAE (Yamazaki et al., 2008). Initial studies indicated that CCR6 deficient mice were somewhat resistant to development of EAE (Liston et al., 2009; Reboldi et al., 2009; Yamazaki et al., 2008), but transfer of small numbers of CCR6 sufficient T cells made *Ccr6* $^{-/-}$ mice susceptible (Reboldi et al., 2009). Entry to the CNS of CCR6 expressing $T_H17$ cells was shown to be via the choroid plexus, where epithelial cells produce CCL20 prior to the onset of neuroinflammation. However, other investigators have questioned the importance of the choroid plexus as the entry point for $T_H17$ cells into the CNS in EAE and have identified an IL 6 driven induc tion of CCL20 by blood endothelial cells that vascularize the fifth lumbar spinal cord and show this to be the main entry point for $T_H17$ cells into the CNS (Arima et al., 2012). Regardless of the site of initial entry, once $T_H17$ cells enter the CNS a cascade of other inflammatory signals follow which results in further cell recruitment independently of CCR6. Interest ingly, the initial recruitment of $T_H17$ cells to the CNS that is driven by CCR6 is not an essential component of disease pathogenesis. This has been demonstrated by more recent reports that show that CCR6 deficient mice have only a delay in the onset of EAE, but ultimately develop exacerbated disease, likely due to a role for CCR6 in Treg recruitment (Elhofy, Depaolo, Lira, Lukacs, & Karpus, 2009; Villares et al., 2009). The signals that drive ongoing recruitment of $T_H17$ cells to the inflamed CNS remain to be fully revealed, however we recently demonstrated an essential role for CCR2 in this process (Kara et al., 2015), which is discussed below.

Conversion of $T_H17$ to pathogenic $T_H17$ and $exT_H17$ subsets has been reported in a number of models including EAE. Pioneering work by the Stockinger laboratory used an IL 17 fate mapper mouse that permanently marks cells and their progeny with eYFP if IL 17A is expressed. This showed a sequential change in cytokine production by differentiated $T_H17$ cells in EAE that transitions from IL $17^+$ IFN$\gamma$ $^{ve}$ to IL $17^+$ IFN$\gamma^+$ and then to a IL 17 $^{ve}$ IFN$\gamma^+$ profile (Hirota et al., 2011). Loss of IL 17

expression by $T_H17$ cells in EAE was concomitant with downregulation of CCR6 and CCR6 $^{ve}$ eYFP$^+$ cells had substantially higher levels of *Ifnγ* mRNA than CCR6$^+$ eYFP$^+$ cells, suggesting pathogenic $T_H17$ cells may not use CCR6 for their trafficking. Indeed, we have recently demonstrated that there is a temporal switch in $T_H17$ trafficking programs during EAE, with CCR6 only required for migration of $T_H17$ cells during the initial stages of pathogenesis (Kara et al., 2015). As disease progresses, CCR2 expressing $T_H17$ cells emerge from the SLO, with this being the critical migratory receptor for homing of encephalitogenic $T_H17$ to the CNS. In accordance with this, antagonism of CCR6 was only effective at reducing $T_H17$ migration during the preclinical phase of disease, but had no effect when administered during peak disease. Conversely, CCR2 antagonism did not reduce $T_H17$ recruitment during the preclinical phase but signifi cantly abrogated $T_H17$ accumulation in the CNS during peak disease, and was effective in inhibiting EAE relapse and blocking $T_H17$ entry to the CNS when administered during disease remission. Furthermore, GM CSF and IFNγ production by $T_H17$ cells primarily emanated from cells with a CCR6 $^{ve}$CCR2$^+$ phenotype. Given that GM CSF production by $T_H17$ cells is a critical requirement for neuroinflammation in EAE, this indi cates that CCR2 and its ligands may be tractable therapeutic targets to block recruitment of pathogenic GM CSF producing T cells. In support of this, GM CSF and IFNγ production in human $T_H17$ cells taken from healthy and MS patients was also confined to the CCR6 $^{ve}$CCR2$^+$ population. In the disease, targeting CCR2 has the added advantages of inhibiting mye loid cell recruitment, a major contributor to pathology in EAE, while not affecting Treg homing, unlike therapies directed at CCR6. Expression of CCR2 by $T_H17$ cells does not appear to be limited to autoimmune disease, as $T_H17$ cells generated in response to chronic *S. pneumoniae* nasopharyngeal infection displayed similar temporal upregulation of CCR2, with CCR6 $^{ve}$ CCR2$^+$ cells again being the predominant source of GM CSF and IFNγ. Whether CCR2 is important for $T_H17$ trafficking in situations of transient infection remains to be determined.

Altogether, it is clear that the canonical $T_H17$ chemokine receptor CCR6 is critical for a number of $T_H17$ migration events, particularly in homeostatic trafficking to the gut as well as in some autoimmune patholo gies. However, as we learn more about the complexities of the $T_H17$ subset, it is evident that plasticity of these cells is essential to regulate responses and either amplify or turn off inflammation. In situations of mild insult, such as for many transient bacterial infections, $T_H17$ cells can express a moderately

inflammatory cytokine profile (including IL 10), and do not promote chronic inflammation, whereas in cases of more persistent antigen and inflammation, IFNγ$^+$ GM CSF$^+$ $T_H17$ cells are generated, and these cells often contribute to pathological tissue damage. The progression of these cells to a more highly pathogenic state appears is accompanied by downregulation of the canonical $T_H17$ program, along with CCR6 expression, while con comitantly upregulating CCR2. Given that ex$T_H17$ cells share many aspects of a $T_H1$ transcriptional profile, it is likely that these cells may also utilize canonical $T_H1$ chemokine receptors such as CXCR3, although this remains to be tested. Thus, it will be crucial to further identify how chemokine recep tor usage changes with differing $T_H17$ states, in order to more accurately understand the homing of these cells and develop more targeted therapeutic approaches to diseases in which $T_H17$ cells play an important role.

## 4.4 $T_H22$

$T_H22$ cells were first identified in humans as IL 22$^+$ CD4$^+$ T cells present in the skin (Duhen, Geiger, Jarrossay, Lanzavecchia, & Sallusto, 2009; Eyerich et al., 2009; Nograles et al., 2009; Trifari, Kaplan, Tran, Crellin, & Spits, 2009). However, there has been some controversy over whether $T_H22$ cells genuinely represent a distinct T cell subset, in part because IL 22 production is also a fea ture of $T_H17$ cells in many scenarios (Ahlfors et al., 2014). Furthermore, the major transcriptional regulators of $T_H22$ cells reported so far are T bet and the aryl hydrocarbon receptor (AhR), which are not confined to the $T_H22$ lineage (Basu et al., 2012; Ramirez et al., 2010). However, at least in humans, CD4$^+$ T cells that express IL 22 without IL 17 or IFNγ production have been described, that also have minimal or absent expression of RORγt. This phenotype is also stable in prolonged culture, lending support to the idea these cells are an independent subset in humans.

Through their production of IL 22, $T_H22$ cells are believed to have important roles both in promoting repair of damaged epithelial barriers as well as in enhancing immune responses against some pathogens. IL 22 acts on nonhematopoetic cells including keratinocytes and epithelial cells to stimulate proliferation and differentiation, thus promoting wound healing and maintaining barrier integrity. Furthermore, IL 22 enhances stromal secretion of antimicrobial peptides like beta defensins and S100 peptides, as well as production of the chemokines CXCL9, CXCL10, CXCL11, CCL2, and CCL20, which promote recruitment of other immune cell types through CXCR3, CCR2, and CCR6, respectively (Aujla et al., 2008;

Eyerich et al., 2009; Jia & Wu, 2014; Zheng et al., 2008). Via these mechanisms, IL 22 has been shown to be protective against a number of bacterial and fungal pathogens (Aujla et al., 2008; De Luca et al., 2010; Gessner et al., 2012; Zheng et al., 2008). However, a direct contribution of $T_H22$ cells was only recently demonstrated for protection against infection, with IL $22^+$ IL $17^{ve}$ $CD4^+$ cells critical for protection in the late phase of *C. rodentium* infection (Basu et al., 2012). $T_H22$ cells have also been purported to play a role in pathological conditions in both mice and humans, with IL 22 and $T_H22$ cells linked to inflammatory diseases such as psoriasis and rheumatoid arthritis (Eyerich et al., 2009; Jia & Wu, 2014; Nograles et al., 2009). However, definitive evidence of a causative pathological role of IL 22 production by $T_H22$ cells in these diseases is not yet available. $T_H22$ generation from naïve human $CD4^+$ T cells in vitro is induced by IL 6 and TNFα, and enhanced further by IL 1β (Duhen et al., 2009). Similarly in mice, IL 6 in the absence of TGFβ promotes the development of IL $22^+$ IL $17^{ve}$ $CD4^+$ cells, with IL 23 also promoting IL 22 but dispensable for $T_H22$ development (Basu et al., 2012; Rutz et al., 2011). More recently, IL 21 was also shown to promote IL 22 production without inducing IL 17, with AhR and STAT3 cooperatively binding the IL 22 promoter in $CD4^+$ cells (Yeste et al., 2014). Furthermore, AhR agonists such as β napthoflavone and FICZ can further stimulate IL 22 production (Trifari et al., 2009).

Initial reports of human $T_H22$ cells identified these cells as expressing the chemokine receptors CCR10, CCR4, and CCR6 (Duhen et al., 2009; Trifari et al., 2009). Given that the CCR4 and CCR10 ligands are abundantly expressed in the skin, and $T_H22$ cells are enriched at this location, this implicates these two receptors as the homing receptors used by $T_H22$ cells to enter the skin. $T_H22$ cells are enriched in inflamed skin and blood from patients with psoriasis or atopic dermatitis and plasma levels of IL 22 are associated with increased disease severity, indicating that these cells may contribute to skin inflammation (Eyerich et al., 2009; Nograles et al., 2009). Although little work has been done to clarify how $T_H22$ cells traffic in these inflammatory skin conditions, we speculate that it is likely to involve CCR4 and CCR10, both well known skin homing chemokine receptors.

$T_H22$ trafficking into other organs, such as the intestine, is likely to be mediated through alternate receptors such as CCR6. In human colon cancer, IL 22 expression in $CD4^+$ T cells in the absence of IL 17 expression was restricted to $CCR6^+$ cells (Kryczek et al., 2014). These cells were the predominant source of IL 22 and acted to promote tumor growth, with CCR6

required for trafficking of IL 22$^+$ CD4$^+$ cells to human colon cancer tumors in NSG mice. However, although these cells lacked IL 17 expression they expressed RORγt, making it difficult to determine if these are bona fide $T_H22$ cells, which further clouds the distinction between $T_H17$ and $T_H22$ subsets. Recently, CyTOF analysis of human $T_H$ cells also identified expression of CXCR6, CCR2, and CCR5 in the IL 22$^+$ subset, although whether receptors are functional in $T_H22$ cells awaits further investigation (Wong et al., 2016). Taken together, there is much still to learn about the mechanisms used for homing of $T_H22$ cells and the significance of this subset of cells in human disease.

## 4.5 $T_H9$

$T_H9$ cells were first reported in 2008 as CD4$^+$ T cells that produce IL 9 and that developed independently of other $T_H$ lineage specific transcription factors (Dardalhon et al., 2008; Veldhoen et al., 2008). Although initially thought to be a $T_H2$ cytokine, IL 9 production in the absence of IL 4 production is the characteristic feature of the $T_H9$ profile. IL 9 has been described to have a multitude of functions, but is most often associated with type 2 responses like allergic inflammation and protective immunity against helminths (Faulkner, Renauld, Van Snick, & Grencis, 1998; McMillan, Bishop, Townsend, McKenzie, & Lloyd, 2002). IL 9 acts to promote survival and growth of mast cells, T cells, and ILCs, enhances airway mucus production, promotes IgE class switching, and generally modulates the activity and cytokine secretion of a variety of cell types (Kaplan, Hufford, & Olson, 2015). Unlike the other described $T_H$ subsets, no master transcription factor has yet been shown to define $T_H9$ lineage specification, although PU.1, IRF4, and BATF promote their development (Chang et al., 2010; Jabeen et al., 2013; Staudt et al., 2010). Differentiation of $T_H9$ cells in vitro is dependent on TGFβ and IL 4 (Chang et al., 2010; Veldhoen et al., 2008). Alternatively, under the iTreg polarizing conditions of TGFβ and IL 2, GITR ligation diverts naïve T cells from Treg differentiation and instead induces the $T_H9$ program (Xiao et al., 2015). There has been controversy over whether the $T_H9$ subset represents a distinct lineage due to the difficulty in identifying often transient IL 9 production and the fact that IL 9 is produced by other lineages (Tan et al., 2010). However, increasing evidence supports their discrete identity, particularly in humans where multiple reports of IL 9$^+$ IL 13 $^{ve}$ IL 4 $^{ve}$ IFNγ $^{ve}$ CD4$^+$ T cells have been described (Cortelazzi, Campanini, Ricci, & De Panfilis, 2013; Jones, Gregory, Causton, Campbell, & Lloyd, 2012; Purwar et al., 2012).

The contribution that $T_H9$ cells make to the adaptive immune response is not completely clear and experiments to fully address the dependence of IL 9 mediated effects on $T_H9$ cells are still required. Recent work using an IL 9 fate mapper mouse showed that in a model of papain induced asthma, the majority of IL 9 expression is not by $CD4^+$ T cells, but rather from ILC2s (Wilhelm et al., 2011). However, IL 9 expression was predominantly restricted to $CD4^+$ T cells in an OVA sensitization and challenge model. Upon infection with *N. brasiliensis*, transfer of $T_H9$ cells but not $T_H2$ cells induced worm expulsion in *Rag2* $^{-/-}$ hosts (Licona Limon et al., 2013). There is also increasing evidence for an antitumor role of $T_H9$ cells. Transfer of in vitro generated $T_H9$ cells inhibits melanoma growth and $T_H9$ cells are found in metastases from melanoma patients (Purwar et al., 2012). Similarly, in vitro generated OT II cells protected mice in hematogenous metastasis experiments with B16 OVA cells, and transfer of $T_H9$ cells drastically abrogated subcutaneous tumor growth (Lu et al., 2012). In these experiments, the antitumor effects of $T_H9$ cells were IL 9 dependent, and resulted from $T_H9$ induction of CCL20 in the tumor site, along with a strong CTL response which was CCR6 dependent.

Reports into chemokine receptor expression and trafficking of $T_H9$ cells are limited. We have shown that $T_H9$ cells express a broad chemokine receptor profile that includes receptors commonly associated with $T_H1$, $T_H2$, $T_H17$/Treg cells. Specifically, $T_H9$ cells displayed CCR3, CXCR3, and CCR6 both in vitro and in models of allergic inflammation and autoimmunity in vivo (Kara et al., 2013). In a type 2 OVA challenge, antagonism of CCR3 or CCR6, but not CXCR3 inhibited $T_H9$ migration into the peritoneal cavity. However, in EAE, antagonism of CXCR3 and CCR6 but not CCR3 prevented egress from draining LNs and migration into the CNS. This shows that different chemokine receptors are utilized by $T_H9$ cells under different circumstances, likely due to differences in chemokine expression in these scenarios, and suggests that $T_H9$ cells have versatile functions that contribute to distinct inflammatory environments. This broad chemokine receptor profile was also apparent in B16–F10 bearing mice, where $T_H9$ cells were shown to be enriched among $CCR3^+CXCR3^+CCR6^+CCR4^{ve}$ $CD4^+$ T cells (Vegran et al., 2014). Questions remain as to which receptors are used in different aspects of $T_H9$ homing, and if they may use other receptors in vivo. In addition, how expression of these receptors is transcriptionally controlled is also unknown, as T bet and RORγt, known transcriptional regulators of CXCR3 and CCR6, respectively, are apparently not expressed in $T_H9$ cells (Veldhoen et al., 2008).

## 4.6 $T_{FH}$

$T_{FH}$ cells specialize in providing help to B cells and are required for devel opment of high affinity antibodies in the germinal center (GC) as well as for long lived memory responses. The importance of these cells is clear, as their help is the limiting factor for affinity maturation of GC B cells and in the absence of $T_{FH}$ cells, GCs fail to develop (Johnston et al., 2009; Nurieva et al., 2009; Victora et al., 2010; Yu et al., 2009). $T_{FH}$ cells provide help through provision of CD40L, ICOS, and SAP signals to B cells as well as through production of cytokines such as IL 21 and IL 4 (Vinuesa, Linterman, Yu, & MacLennan, 2016). The lineage defining transcription factor for $T_{FH}$ cells is Bcl6, although other important regulators of $T_{FH}$ fate include Ascl2, c Maf, and Batf. $T_{FH}$ generation is promoted by IL 6 and IL 21 during priming in mice, with TGFβ, IL 12, and IL 23 important for $T_{FH}$ development in humans (Eto et al., 2011; Karnowski et al., 2012; Schmitt et al., 2014).

Initial reports of $T_{FH}$ cells described them as $CXCR5^+$ $CD4^+$ cells although now GC $T_{FH}$ cells are most commonly and accurately demarcated as PD $1^{hi}$ $CXCR5^{hi}$ $CD4^+$ T cells (Breitfeld et al., 2000; Haynes et al., 2007; Kim et al., 2001; Schaerli et al., 2000). CXCR5 is critical to their function as it allows T cells to migrate to the follicles, where CXCL13 is abundantly expressed by FDCs (Ansel, McHeyzer Williams, Ngo, McHeyzer Williams, & Cyster, 1999). CXCR5 is first upregulated on pre $T_{FH}$ cells that are generated upon priming by DCs in SLOs and enables migration to the T:B border, where $T_{FH}$ lineage commitment is conferred by interactions with Ag expressing B cells (Barnett et al., 2014). CXCR5 is further upregulated upon entry to the GC and is critical for optimal GC function, with CXCR5 deficient T cells failing to induce efficient forma tion of GCs (Arnold, Campbell, Lipp, & Butcher, 2007; Hardtke et al., 2005; Haynes et al., 2007; Junt et al., 2005). However, residual $T_{FH}$ cells can still reach the GC in the absence of CXCR5, suggesting alternate migra tory signals make a minor contribution to $T_{FH}$ homing toward the GC. CXCR5 is also critical for microanatomical localization within the GC, as CXCR5 deficient $T_{FH}$ cells able to access the follicle are unable to migrate to the light zone of the GC, the site of selection of high affinity B cell clones (Haynes et al., 2007). CXCR5 is also abundantly expressed on circulating $CD4^+$ T cells from human peripheral blood and these cells are capable of assisting B cell responses in vitro. However, the precise rela tionship of these cells to $T_{FH}$ cells requires further investigation (He et al., 2013; Locci et al., 2013; Morita et al., 2011).

As Bcl6 is a transcriptional repressor, it is unlikely to directly promote CXCR5 expression. However, one of the major targets of Bcl6 is the mutually antagonistic transcription factor Blimp1 (encoded by *Prdm1*), which is capable of repressing CXCR5 and other $T_{FH}$ like genes through direct binding to the promoter region (Oestreich et al., 2012). CXCR5 is also repressed by the Bcl 6 regulating miRNA miR 17–92 (Yu et al., 2009). However, monitoring induction of Bcl6 using Bcl6–IRES–GFP reporter mice concurrently with CXCR5 expression showed different kinetics, with CXCR5 rapidly increased at day 2 postimmunization and maintained at high levels, whereas Bcl6 was gradually upregulated beginning from days 2 to 7 (Liu et al., 2012). Indeed, it was recently reported that Ascl2 is the critical factor promoting CXCR5 expression prior to Bcl6 induction, with retroviral transduction of Ascl2, but not Bcl6, sufficient for CXCR5 induction and migration to the follicles (Liu et al., 2014). Ascl2 directly regulates CXCR5 expression and binds to multiple regions of the CXCR5 gene. The E proteins E2A and HEB also promote CXCR5 upregulation, and as they are expressed at earlier time points than Ascl2, have been hypothesized to be the preceding signal promoting $T_{FH}$ differentiation, although this remains to be determined (Shaw et al., 2016).

Although CXCR5 is required for migration to the follicle, overexpression of CXCR5 alone does not permit entry (Haynes et al., 2007). Pre $T_{FH}$ cells must also downregulate CCR7, in order to escape the CCL19 and CCL21 signals abundant in the T cell zones. Both Ascl2 and Bcl6 act to reduce CCR7 expression (Hatzi et al., 2015; Liu et al., 2014). Ascl2 also upregulates CXCR4, which may play roles in localization to the dark zone, as well as in extrafollicular responses, although this remains to be formally addressed (Elsner, Ernst, & Baumgarth, 2012). Non chemokine signaling cues are also important for $T_{FH}$ migration, with expression of S1PR2 required for retention of $T_{FH}$ cells in the GC (Moriyama et al., 2014). Interestingly, recruitment of activated T cells to the follicles requires ICOSL expression by bystander B cells, which promotes pseudopod formation and enhances motility of ICOS expressing $T_{FH}$ cells (Xu et al., 2013). These signals are essential for $T_{FH}$ migration from the T B border to the follicle, with the ICOS driven increase in motility allowing these cells to be influenced by other chemotactic signals including those through CXCR5 for optimal homing.

Recent analysis of $T_{FH}$ cells using two photon photoconversion with intravital microscopy has elucidated the differential kinetics of migration during primary and secondary responses (Suan et al., 2015). During the

primary response, $T_{FH}$ cells are generally confined to one GC, with limited trafficking between GCs. Although most $T_{FH}$ are confined to the center of the GC, there is a subpopulation of $T_{FH}$ that cross into the follicular mantle (FM) region, although the functional significance of these cells requires fur ther investigation. FM $T_{FH}$ cells have a distinct profile from GC $T_{FH}$ cells, with FM $T_{FH}$ cells expressing less CXCR5 and more CCR7 and the non chemokine GPCR, EBI2. EBI2 expression is repressed by Bcl6, with the receptor promoting localization to the subcapsular and interfollicular regions (Hatzi et al., 2015; Suan et al., 2015). EBI2 deficient $CD4^+$ T cells display increased GC localization during the primary response, but upon rechallenge are equally as able as WT $T_{FH}$ cells to localize to the GC, despite their high expression of this receptor. Also in contrast to the primary response, $T_{FH}$ cells in secondary responses readily migrate from the GC to the FM, as well as between neighboring GCs, with almost all $T_{FH}$ cells having done so 24 h after GC photoconversion (Shulman et al., 2013; Suan et al., 2015). This is believed to enhance the efficiency of T cell help, however the distinct migratory cues for this differential movement remain to be determined.

## 4.7 Treg

Treg cells play a critical role in negative regulation of immune responses and are essential both to maintain tolerance against self antigens and to limit pathogen induced inflammation to prevent excessive damage to host tissues. The two major subsets of Treg are thymic Treg (tTreg), which develop in the thymus, and induced Treg (iTreg) which are derived from naïve $CD4^+$ T cells following suboptimal antigenic stimulation, encompassing lack of inflammatory signals during priming as well as reduced TCR signal strength (Josefowicz, Lu, & Rudensky, 2012). Both of these cell types are defined by the expression of the transcription factor Foxp3 and the importance of these cells is clear from studies of Foxp3 deficient mice, which succumb to an early onset systemic inflammatory disorder (Brunkow et al., 2001). Treg can suppress immune responses through a variety of mechanisms, including through production of cytokines such as TGFβ and IL 10, through direct cell–cell contacts or through metabolic disruption of inflammatory T cells (Schmitt & Williams, 2013). The tTreg and iTreg populations share a similar surface profile which has made it difficult to study differences in their func tion and migration, although in some scenarios tTreg can now be distin guished from iTreg based on their higher expression of neuropilin 1

(Weiss et al., 2012; Yadav et al., 2012). This will facilitate more detailed analysis of the contribution of these subsets in the future. Other Foxp3 $^{ve}$ regulatory T cells exist, including $T_R1$ and $T_H3$ cells, but are not discussed here.

Given that Treg are responsible for suppressing T cell mediated inflammation in a diverse range of scenarios, it is perhaps not surprising that they appear to differentiate in a manner parallel to Foxp3 $^{ve}$ $CD4^+$ T subsets. Treg that develop in response to a particular challenge often express the same transcription factors and chemokine receptors as the inflammatory $T_H$ cells generated alongside them, which allows for similar trafficking to peripheral tissues and hence suppression. For instance, in $T_H17$ mediated colitis, Treg require expression of the $T_H17$ generating transcription factor STAT3 for optimal activity, as STAT3 deficient Treg fail to suppress colitis in vivo, although show similar suppressive capacity in vitro (Chaudhry et al., 2009). This was associated with reduced expression of CCR6 by Treg, which is required for $T_H17$ migration to the gut. Treg also use CCR6 to migrate to the CNS and kidney in EAE and glomerulonephritis models (Turner et al., 2010; Yamazaki et al., 2008). Similarly to $T_{FH}$ cells, there also exists a $CXCR5^+$ $Bcl6^+$ population of $Foxp3^+$ $CD4^+$ cells termed T follicular regulatory ($T_{FR}$) cells. These cells use CXCR5 to localize to the follicles where they can suppress GC responses (Chung et al., 2011; Linterman et al., 2011).

The $T_H2$ associated transcription factor IRF4 is also required for optimal Treg development, as IRF4 deficient Treg show impaired suppression of $T_H2$ responses (Zheng et al., 2009). IRF4 ablation also reduces expression of CCR8, which in models of graft vs host disease (GVHD) has been shown to be important for Treg maintenance (Coghill et al., 2013). CCR8 expression on transferred Tregs in this model allows colocalization with donor APCs to receive signals promoting survival, with CCR8 deficient Treg showing increased death. Treg also express the $T_H2$ receptor CCR4 in asthmatic cases of both mice and humans (Afshar et al., 2013). CCR4 expression on Treg is required for Treg suppression during the challenge phase of allergic asthmatic models but not during the sensitization phase. CCR4 is also more generally required for Treg recruitment to the skin and lung, as mice with a Treg specific deletion of CCR4 show impaired Treg homing to these sites and develop severe inflammatory pathology (Sather et al., 2007). This axis may be a useful therapeutic target in skin pathologies such as vitiligo, where overexpression of CCL22 in depigmented skin of vitiligo prone mice enhanced Treg migration to the site and reduced depigmentation (Eby et al., 2015).

Treg can also express CXCR3 in models of $T_H1$ mediated inflammation, such as *T. gondii* infection, autoimmune diabetes, and following anti CD40 treatment (Hall et al., 2012; Koch et al., 2009; Kornete et al., 2015). Akin to $T_H1$ cells, CXCR3 expression on Treg is dependent on T bet, STAT1, IL 27, and IFNγR signaling (Hall et al., 2012; Koch et al., 2009). T bet regulation of trafficking receptors is critical for optimal function of tTreg, as in an islet allograft model, T bet deficient Treg displayed impaired migration to dLN and accumulated in the graft (Xiong, Ahmad, Iwami, Brinkman, & Bromberg, 2016). This was associated with reduced expression of CXCR3 and increased expression of CCR4, inhibiting recruitment into lymphatic vessels and ultimately preventing graft survival.

In addition to exerting their suppressive effects in peripheral tissues, it is clear that localization of Treg to SLO is essential for multiple aspects of their function, including their activation and control of inflammatory T cell priming (Rudensky & Campbell, 2006). CCR7 is critical for Treg recirculation through LN as well as for exit from peripheral tissues, although some CCR7 independent trafficking into the medulla has been reported (Menning et al., 2007; Schneider, Meingassner, Lipp, Moore, & Rot, 2007; Ueha et al., 2007). In the LN, CCR7 allows localization of tTreg to the paracortex, where they can access the high levels of IL 2 required for their maintenance (Smigiel et al., 2014; Ueha et al., 2007). Alternatively, $CCR7^{lo}$ Treg can be maintained through continuous signaling through ICOS (Smigiel et al., 2014). LNs are not only important sites for generation of Treg, as sequential trafficking of Treg from peripheral sites to LN during an ongoing immune response is required for optimal immune suppression. In an islet allograft model, Treg first migrate from the blood to the graft using a variety of inflammatory receptors including CCR2, CCR4, and CCR5, as well as P and E selectin ligands (Zhang, Schroppel, et al., 2009). Treg then migrate from the graft to the dLN using a combination of CCR2, CCR5, and CCR7. The importance of this sequential migration is demonstrated by transfer of CCR7 deficient Treg, which migrate normally to inflamed grafts but show inhibited migration to dLNs, and consequently cannot suppress graft rejection.

The inflammatory chemokine receptors CCR2 and CCR5 are important for Treg trafficking to a broad range of responses. CCR2 is used for Treg trafficking to antigen in a model of delayed type hypersensitivity, as well as for entry into tumors in a range of experimental models (Hamano et al., 2014; Loyher et al., 2016). CCR5 is required for Treg recruitment to the site of *Leishmania major* infection, with CCR5 deficient

Treg unable to inhibit the antiparasitic response leading to protection (Yurchenko et al., 2006). CCR5 is also required for Treg trafficking in models of intestinal inflammation and *P. brasiliensis* infection (Kang et al., 2007; Moreira et al., 2008). In contrast to these broadly inflammatory chemokine receptors, Treg can also express more tissue specific receptors such as CCR9 which allows homing of Treg cells to the gut (Guo et al., 2008). Expression of CCR9 in Treg is regulated by BATF and RA (Benson, Pino Lagos, Rosemblatt, & Noelle, 2007; Wang et al., 2013).

One factor recently shown to critically regulate Treg migration to peripheral tissues is KLF2 (Pabbisetty et al., 2016). KLF2 deficient Treg express increased levels of CCR4, CCR6, CCR8, and CCR9, and reduced levels of CCR7. Mice with Treg specific KLF2 ablation displayed impaired LN migration of Treg and ultimately developed autoimmunity, highlighting the critical role of Treg LN trafficking for maintenance of peripheral tolerance. Transduction of CCR7 into KLF2 deficient Treg restored their ability to migrate to LN and prevented pathology in colitis models. Furthermore, therapeutic intervention to increase KLF2 activity in Treg using simvastatin or retroviral transduction promoted LN migration and reduced pathology in a GVHD model. This suggests that therapeutic targeting of Treg may be achieved through manipulation of their trafficking receptors. However, given that Treg and $T_H$ cells often use the same chemokine receptors for migration, selectively targeting Treg cells may present some difficulties, and identifying receptors that are differentially expressed will be useful. As mentioned earlier, we have recently shown in EAE that although $T_H17$ and Treg both express CCR6, during peak disease $T_H17$ trafficking is instead mediated predominantly through CCR2 (Kara et al., 2015). Thus, targeting of the CCR2 axis may be a more effective strategy to prevent recruitment of pathogenic $T_H17$ cells while allowing CCR6 dependent trafficking of Treg into the CNS to dampen existing inflammation. More detailed analysis of trafficking receptors used concomitantly by inflammatory and regulatory T cells in alternate models may assist in the development of other therapeutic strategies.

## 5. CHEMOKINE CONTROL OF CD4+ MEMORY FUNCTION

Upon antigen clearance, $CD4^+$ effector T cells rapidly contract, with approximately 90% of cells dying within 1–2 weeks. However, memory $CD4^+$ T cells persist long term and are critical for the rapid response to subsequent antigen encounter. The precise mechanisms by which $CD4^+$

memory T cells develop and function is still not well described, particularly compared to $CD8^+$ T memory, however significant advances to improve our understanding have been made in recent years. $CD4^+$ memory T cells are a heterogenous population and have been divided into at least three categories based on their migratory patterns (Fig. 2). The first division of function of memory T cells was first described by Sallusto and colleagues in 1999 on the basis of CCR7 expression (Sallusto, Lenig, Forster, Lipp, & Lanzavecchia, 1999). Central memory ($T_{CM}$) cells are defined as $CCR7^+$

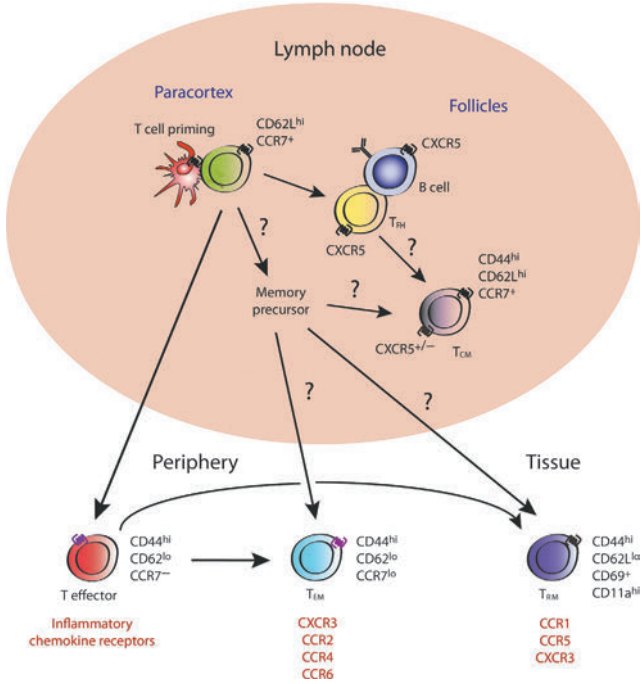


**Fig. 2** Formation and trafficking of $CD4^+$ T cell memory. Long-lived $CD4^+$ memory cells are classified into three subsets based on their patterns of recirculation. $T_{CM}$ cells are mainly found in the blood and SLO, with expression of CCR7 and CD62L enabling their migration into SLO through HEVs. $T_{EM}$ recirculate through peripheral tissues and can reenter LNs through the afferent lymph. $T_{RM}$ permanently reside in peripheral tissues after infection and are restricted from reentering the circulation. The ontogeny of the different memory subsets is still unclear and markers of memory precursors in the effector phase of the response has not yet been reported, unlike that of $CD8^+$ T cells. There is evidence that $CXCR5^+$ $T_{CM}$ cells derive from $T_{FH}$ cells, although $CXCR5^{-ve}$ $T_{CM}$ also exist. At which stage memory fate is conferred remains to be determined. Chemokine receptor alterations between these memory subsets are depicted. *Arrows* indicate differentiation/transdifferentiation of T cell subsets, *?* indicates an as yet unproven transdifferentiation pathway.

CD62L$^{hi}$ CD4$^{+}$ T cells that preferentially produce IL 2 upon restimulation and cross HEVs to recirculate from blood through SLOs (Reinhardt, Khoruts, Merica, Zell, & Jenkins, 2001). Conversely, effector memory ($T_{EM}$) cells were described as CCR7 $^{ve}$CD62L$^{lo}$ and migrate to inflamed peripheral tissues for immunosurveillance and enter LNs via afferent lymph. Upon antigen engagement, these cells preferentially produce effector cyto kines such as IFNγ and IL 4. More recently, resident memory ($T_{RM}$) that permanently reside in previously infected peripheral tissues and are restricted from accessing the circulation were described, with these cells being critical for protective immunity upon rechallenge at the site of the primary infection (Gebhardt & Carbone, 2009; Teijaro et al., 2011). These subsets of CD4$^{+}$ T cells, and the chemokine receptors that control their functions, will be described here.

## 5.1 $T_{CM}$

$T_{CM}$ cells are mainly restricted to recirculating through the blood and lym phoid organs, where they rely on APCs in SLOs for their reactivation. Because of this, $T_{CM}$ cells are the slowest of the memory cell subsets to respond to antigen and take days to expand. However, $T_{CM}$ cells have an unique role in that they produce high levels of IL 2 and display enhanced proliferative capacity, giving rise to multiple effector subsets that can then emigrate from SLO to traffic to peripheral sites. The expression of CCR7 by $T_{CM}$ is critical for this recirculation, and along with CD62L expression allows trafficking patterns akin to naïve T cells.

$T_{CM}$ can also express CXCR5, which has raised interesting questions regarding the relationship of these cells to $T_{FH}$ cells both in regard to their ontogeny and functional capabilities. For example, Ag specific CD4$^{+}$ $T_{CM}$ cells (CCR7$^{+}$CD62L$^{hi}$) at 60 days postinfection with *Listeria monocytogenes* displayed a characteristic CXCR5$^{+}$ T bet$^{lo}$ phenotype, while $T_{EM}$ were CXCR5 $^{ve}$CCR7 $^{ve}$ T bet$^{hi}$ (Pepper, Pagan, Igyarto, Taylor, & Jenkins, 2011). These CXCR5$^{+}$ memory cells efficiently produced IL 2 but secreted less IFNγ than $T_{EM}$, supporting their status as $T_{CM}$ cells. Perhaps surprisingly given their expression of CXCR5, these cells were excluded from follicles and localized to the paracortex upon adoptive transfer, which suggests that other migratory cues, possibly including CCR7, confine them to T cell areas. However, CXCR5$^{+}$ $T_{FH}$ cells can also persist into memory in the absence of continued antigen, where they drive enhanced secondary responses and can provide more efficient B cell help than $T_{FH}$ cells in the primary response

(Hale et al., 2013; Luthje et al., 2012). This potential relationship between $T_{FH}$ cells and $T_{CM}$ cells is currently controversial and the interdependence of these cells in memory formation remains to be established. Like $T_{FH}$ cells, $T_{CM}$ cells that develop in response to *L. monocytogenes* infection are also dependent on Bcl6 and ICOS signaling by B cells, suggesting $T_{CM}$ cells may actually arise exclusively from $T_{FH}$ cells in some scenarios (Pepper et al., 2011). However, during the acute phase of infection, two populations of $CXCR5^+$ $CD4^+$ T cells have been described: PD $1^{hi}$ $CXCR5^+$ $T_{FH}$ cells and PD $1^{ve}$ $CXCR5^+$ $CCR7^+$ T $bet^{lo}$ cells, which were termed $T_{CM}$ precursor cells given their similarity to the $T_{CM}$ cell phenotype described earlier. This posits a model by which during the effector phase of a response, a pool of $CXCR5^+$ $CD4^+$ cells exists in SLOs, some of which increase expression of CXCR5 and PD 1, and enter the follicle to become $T_{FH}$ cells. Upon cessation of the GC, some $T_{FH}$ cells then downregulate PD 1 expression and form $CXCR5^+$ memory cells, although entry into the GC has been shown to not be essential for memory formation (He et al., 2013; Tubo et al., 2016). In the memory phase, the $CXCR5^+$ $CD4^+$ population can be divided into $CCR7^{lo}$PD $1^{hi}$ $T_{FH}$ precursors, as well as a $CCR7^{hi}$PD $1^{lo}$ subset characteristic of resting memory cells (He et al., 2013). In response to LCMV infection, a bifurcation of these $CXCR5^+$ $CD4^+$ populations into $Ly6C^{lo}$ and $Ly6C^{int}$ subsets was reported, with the $Ly6C^{lo}$ cells displaying a transcriptional profile similar to that of $T_{FH}$ cells (Hale et al., 2013). The $Ly6C^{int}$ population has a less polarized gene signature with features of both $T_{FH}$ cells and $T_H1$ cells, and also has significantly higher proliferative potential than either the $CXCR5^+Ly6C^{lo}$ or CXCR5 $^{ve}$ memory populations and thus is more similar to the classical $T_{CM}$ phenotype. However, a $CD62L^{hi}$ CXCR5 $^{ve}$ population was also described in LCMV infection, suggesting some $T_{CM}$ cells may also arise from the CXCR5 $^{ve}$ population. Thus, further evidence is required to determine the precise ontogeny of $T_{CM}$ cells, whether they derive exclusively from $T_{FH}$ cells or from a common precursor to $T_{FH}$ cells, and whether they can derive from CXCR5 $^{ve}$ populations in other scenarios.

Further evidence for this complex interplay between $T_{CM}$ and $T_{FH}$ cells is found in human peripheral blood, where 20% of memory CD45RA $^{ve}$ $CD4^+$ T cells are $CXCR5^+$ (Morita et al., 2011). The majority of these cells coexpress CCR7, indicative of circulating memory cells. Circulating $CXCR5^+$ $CD4^+$ memory T cells retained the ability to differentiate into $T_H1$ or $T_H2$ subsets, as expected for $T_{CM}$ cells (Messi et al., 2003). However, $CXCR5^+$ memory cells were also capable of helping B cells in ex vivo culture, with CXCR3 $^{ve}CCR6^+$ and CXCR3 $^{ve}CCR6^{ve}$ but not

$CXCR3^{+}CCR6^{ve}$ cells able to promote class switching, suggesting the ability to differentiate into $T_{FH}$ cells was restricted to only a proportion of these $CXCR5^{+}$ memory cells (Morita et al., 2011). In other studies, $T_{FH}$ cells were preferentially confined to $CCR7^{lo}$ $CXCR5^{+}$ circulating memory cells (He et al., 2013). Clearly, many questions regarding the ontogeny of $T_{CM}$ cells as well as their differentiation potential remain unanswered.

Other chemokine receptors have also been reported to be expressed by human $CD4^{+}$ $T_{CM}$ cells and correlate to the differentiation capability of those $T_{CM}$ cells, suggesting reduced multipotency and perhaps partial differentiation (Rivino et al., 2004). $CXCR3^{+}$ $T_{CM}$ cells secrete low levels of IFNγ and are able to generate fully differentiated $T_H1$ cells in vitro, while $CCR4^{+}$ $T_{CM}$ cells secreted low levels of IL 4 but not IL 5 and gave rise to $T_H2$ cells. In contrast, $CXCR5^{+}$ $T_{CM}$ that were $CXCR3^{ve}$ and $CCR4^{ve}$ were not polarized and relied on exogenous cytokines to divert them to a $T_H1$ or $T_H2$ phenotype. Evidently, there is much to learn about the further classification of diversity within $T_{CM}$ cells based on their chemokine receptor expression. Furthermore, the precise role of these chemokine receptors in guiding $T_{CM}$ cells to specific SLO niches supportive of their function is also largely unexplored.

## 5.2 $T_{EM}$

Under steady state conditions, $T_{EM}$ circulate through the blood and peripheral tissues. $CD4^{+}$ $T_{EM}$ are generally thought to be excluded from entering HEV due to low expression of CCR7 and CD62L, but can migrate into reactive LN through CD62P where they can promote naïve T cell priming (Martin Fontecha et al., 2008). Owing to their efficient expression of effector cytokines, $T_{EM}$ provide an earlier response to secondary infections than $T_{CM}$ and can be recruited to the site of infection to respond within hours to days. $T_{EM}$ are thought to arise from T effector cells that have survived the contraction phase, although it is not clear at which point memory fate determination is conferred. Certainly, the markers used to define $CD8^{+}$ memory precursors such as CD127 and KLRG1 do not apply to $CD4^{+}$ memory (Marshall et al., 2011). It is also not clear if all $T_H$ subsets equally form memory, with most reports describing $T_H1$ like memory cells, although $T_H2$ and $T_H17$ memory cells have been described (Pepper & Jenkins, 2011). Indeed, recent data from our laboratory indicated that the highly inflammatory subset of $T_H17$ cells that coexpress GM CSF and IFNγ also clearly persist into memory (Kara et al., 2015).

Very little is known about the migration patterns and cues used by $CD4^+$ $T_{EM}$ to migrate through the periphery. However, it is clear there are substantial differences compared to memory $CD8^+$ T cells. For example, in skin following cutaneous HSV infection, $CD4^+$ memory T cells are confined to the dermis, while $CD8^+$ memory T cells populate the epidermis (Gebhardt & Carbone, 2009). Similarly, intravaginal HSV infection permits localization of memory $CD4^+$ T cells to the lamina propria and memory $CD8^+$ T cells to the epithelium of the genital tract. In HSV infection, memory $CD4^+$ T cell trafficking to the skin is transient and these cells exit the tissue and recirculate. Chemokine receptor expression on $T_{EM}$ has been reported. In response to EAE immunization, memory $CD4^+$ T cells express higher levels of CXCR3 and CCR5 than Teff (Elyaman et al., 2008). Transfer of these $T_{EM}$ into $Tcr\alpha\beta^{-/-}$ mice given EAE induced greater disease severity than transfer of effector T cells, which was associated with enhanced trafficking to the CNS, suggesting $T_{EM}$ migration was mediated through these axes. We have recently shown that IL 17 producing $CD4^+$ memory cells are present in the lung after *S. pneumoniae* infection and these cells express high levels of both CCR6 and CCR2, with the proportion of $CCR2^+$ $CCR6^{ve}$ $T_H17$ cells predominantly increasing upon rechallenge (Kara et al., 2015). Furthermore, $T_{EM}$ from human PBMCs have also been reported to express CCR10, CCR5, CXCR3, CCR6, and CCR4, suggesting diverse trafficking within the $T_{EM}$ population that may follow similar classification to $T_H$ cells (Brodie, Brenna, & Sallusto, 2013). This was partially supported by analysis of $T_{EM}$ from human cord blood, in which $CXCR3^+$ $T_{EM}$ cells preferentially produced IFNγ, and $CCR6^+$ $T_{EM}$ cells displayed a transcriptional profile biased to a $T_H17$ phenotype, although could not robustly secrete IL 17 (Zhang et al., 2014). In contrast, CCR4 expression was not restricted to $T_{EM}$ with a $T_H2$ signature and IL 4 production was detected in both $CCR4^+$ and $CXCR3^+$ $T_{EM}$ cells. Taken together, it is clear that the signals controlling tissue niche occupancy and recirculation for memory $CD4^+$ T cells is not well understood and there is still much to be learned in this area.

## 5.3 $T_{RM}$

$T_{RM}$ are the most recently discovered subset of memory cells with parabiosis experiments first definitively revealing $CD8^+$ $T_{RM}$ in 2009 and $CD4^+$ $T_{RM}$ in 2011 (Gebhardt & Carbone, 2009; Teijaro et al., 2011). The essential defining property of these cells is that they are resident in peripheral tissues

and do not recirculate through the blood or lymph, although the factors promoting their residence are still not well understood. $T_{RM}$ cells reported to date display enhanced protective responses upon reencounter of antigen and due to their peripheral location can have the shortest response time of all memory subsets. $CD4^+$ $T_{RM}$ generated in response to *L. major* infection in the skin are essential for optimal protection against secondary parasite challenge as they enhance recruitment of circulating memory cells through production of CXCL9 and CXCL10 (Glennie et al., 2015). A recent study also demonstrated that $CD4^+$ $T_{RM}$ resident in the spinal cord and dorsal root ganglia (DRG) in response to HSV 2 infection facilitate protection through production of IFNγ which enhances vascular permeability thus allowing access of antiviral antibodies to the brain (Iijima & Iwasaki, 2016). Again, $CD4^+$ $T_{RM}$ are much less well described than their $CD8^+$counterparts, although they generally express a $CD44^{hi}$ $CD62L^{lo}$ $CD11a^{hi}$ $CD69^+$ phenotype (Iijima & Iwasaki, 2014; Teijaro et al., 2011).

$CD4^+$ $T_{RM}$ generated in the genital tract in response to intravaginal HSV 2 immunization do not express CCR7, in support of their restriction from entering lymphatic circulation (Iijima & Iwasaki, 2014). However, chemokine signaling is required to maintain their residency in the vaginal parenchyma, as treatment with PT results in expulsion into the lumen. Furthermore, neutralization of CCL5 reduces the numbers of $T_{RM}$ in the vagina and accordingly reduces the protection observed in the presence of these cells. $T_{RM}$ in this model express the CCL5 receptors CCR1 and CCR5 as well as CXCR3, although neutralization of the CXCR3 ligand CXCL9 does not affect $CD4^+$ $T_{RM}$ residency. $CD4^+$ $T_{RM}$ are also generated in the spinal cord and DRG in response to HSV 1 infection, with the ligands for CXCR3, CCR5, and CCR2 expressed in DRG (Iijima & Iwasaki, 2016). However, the migratory cues required for entry into these regions remain to be determined.

Another potential subset of memory cells, referred to as recirculating memory ($T_{RCM}$) cells, has also been reported (Bromley, Yan, Tomura, Kanagawa, & Luster, 2013). Photoconversion of the skin of Kaede mice delineated two populations within homeostatic skin: $CCR7^{ve}$ $CD69^+$ $T_{RM}$ cells and $CCR7^{int/+}$ $CD62L^{int}$ $CD69^{ve}$ $T_{RCM}$ cells. Owing to their expression of CCR7 and lack of CD69, these $T_{RCM}$ were able to leave the skin and could also enter nondraining lymph nodes. The function of these cells is also intriguing, as they produce IL 2 but negligible IFNγ or IL 10 following restimulation. The relationship of these cells to $T_{EM}$, as well as their presence in other settings, remains to be investigated.

## 6. CONCLUDING REMARKS

More than 20 years of intensive research have shed much light on the significance and molecular mechanisms of $CD4^+$ T cell migration. As discussed above and summarized in Fig. 1 and Table 1, this is tightly controlled through regulation of chemokine receptor expression, which contribute importantly to almost all aspects of $CD4^+$ T cell function. Coordinated migration of the $CD4^+$ T cell is required for the optimal efficiency evident in priming, influences early fate decisions, and dictates the trafficking and effector functions of differentiated $T_H$ cells. Furthermore, differential migration mediated through specific chemokine axes is essential for the diversity seen in memory $CD4^+$ responses, enabling broad protection from the diverse range of pathogenic insults encountered during life (Fig. 2). Although our knowledge of $CD4^+$ T cell function and migration is steadily increasing, many unanswered questions remain. The timing and determinants involved in deciding lineage and memory formation are still not well understood, and continuing research is needed into whether expression of distinct chemokine receptors facilitates these decisions. Furthermore, it is increasingly apparent that vast diversity and plasticity exists within $T_H$ subsets, and more detailed analysis of how homing receptors change with transdifferentiation will enable more appropriate targeting of the specific T cells involved in disease. Lastly, the area of $CD4^+$ memory is still largely not well explored and understanding the migratory requirements for these cells may facilitate manipulation of their function, potentially enhancing vaccination strategies. Altogether, it is hoped that this more detailed understanding of molecular control of $CD4^+$ T cell migration will bring about the means for more effective therapeutic intervention in a wide range of human pathologies.

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