CYTOKINE AND MICROBIAL REGULATORS OF T CELL DEVELOPMENT,
DIFFERENTIATION AND FUNCTION.

by

Adam Plumb

B. Sc., University of Alberta, 2008

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(Microbiology & Immunology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

January 2016

© Adam Plumb, 2016
Abstract

The cytokine Interleukin-7 (IL-7) is critical for T cell development and function. Mutations that block IL-7 signaling in humans cause severe combined immunodeficiency syndrome due to the failure of T cells to develop. Conversely, mutations that result in constitutive signaling through the IL-7 receptor can drive human leukemias, including Early Thymic Progenitor (ETP) acute lymphoblast leukemia. How IL-7 influences ETP development is still unclear since ETPs do not normally express IL-7Rα. We found that ETPs are highly dependent on and sensitive to changes in IL-7 signaling however, IL-7Rα expressing bone marrow progenitors are not. IL-7 does not regulate the survival or proliferation of ETPs, although it does at subsequent stages of T cell development. We hypothesize an instructive role of IL-7 signaling in the differentiation or migration of bone marrow precursors of ETPs that is distinct from its classic mechanisms regulating survival and proliferation.

Adjuvant IL-7 has been shown to increase the quantity and quality of T cell responses to clear chronic viral infections, however, its physiological role in anti-viral T cells is unclear. We found that IL-7 signaling was required for efficient clearance of Influenza A virus (IAV) and protection from viral induced pathology. T cells require cell intrinsic IL-7 signaling for efficient primary CD4 and CD8 T cell responses to IAV. The requirement for IL-7 signaling in the anti-viral response, and the ability of exogenous IL-7 to boost T cell responses suggests that it may be clinically useful in therapy or vaccination against IAV.
IAV remains an important global health challenge, with up to 10% of the global population infected annually. Current IAV vaccines do not generate universal cross-serotype immunity. T cell memory responses to IAV correlate with cross-serotype protection. Transcutaneous immunization has shown potential to induce memory T cell responses that can protect against infections at other mucosal sites, including the lung. We found that transcutaneous immunization generates a strong memory CD8 T cell response that can protect from challenge with IAV. Therefore, transcutaneous immunization may be a useful to produce cross-serotype IAV immunity.
Preface

Chapter 2

For the work presented in Chapter 2, I was responsible for design, execution and data analysis of all experiments. Dr. Ninan Abraham supervised the project and aided in experimental design. Dr. Jung Hee Seo and Dr. Daniel T Patton provided technical assistance.

Chapter 3

A version of Chapter 3 is published:

Adam W Plumb, Daniel T Patton, Jung Hee Seo, Emma-Kate Loveday, François Jean, Steven F Ziegler, Ninan Abraham. (2012) Interleukin-7, but not thymic stromal lymphopoietin, plays a key role in the T cell response to influenza A virus. PLoS One. e50199

I was responsible for experimental design, execution and data analysis with the following exceptions. Dr. Emma-Kate Loveday performed rtPCR for IAV M1 viral RNA. Dr. Daniel T Patton and Dr. Jung Hee Seo provided technical support. Dr. Steven F Ziegler and Dr. François Jean provided reagents. Dr. Ninan Abraham supervised the project and aided in experimental design. I was responsible for writing the manuscript that was revised with the help of Dr. Ninan Abraham.
Chapter 4

All experimental design, execution and data analysis was done in equal collaboration between myself and Dr. Wing Ki Cheung. Dr. Jan Dutz and Dr. Ninan Abraham supervised the project.

Animal studies were conducted in the Westbrook Animal Unit and the Centre for Disease Modeling at the University of British Columbia. All animal work was performed under strict accordance with the recommendations of the Canadian Council for Animal Care. Animal protocols were approved by the Animal Care Committee of the University of British Columbia (A13-0062).
Table of contents

Abstract ........................................................................................................................................... ii
Preface ............................................................................................................................................... iv
Table of contents ............................................................................................................................... vi
List of figures ...................................................................................................................................... ix
List of abbreviations ......................................................................................................................... xi
Acknowledgements ............................................................................................................................ xiv
Chapter 1: Introduction and background ......................................................................................... 1
  1.1 T cells and the adaptive immune response .............................................................................. 1
    1.1.1 Early lymphocyte development ......................................................................................... 2
    1.1.2 Progenitor egress from the bone marrow ........................................................................ 6
    1.1.3 Progenitor entry into the thymus ...................................................................................... 8
    1.1.4 T cell development in the thymus .................................................................................... 10
  1.2 Interleukin-7 ........................................................................................................................... 12
    1.2.1 IL-7 signaling .................................................................................................................. 13
    1.2.2 IL-7 in T cell development ............................................................................................. 15
    1.2.3 IL-7 and mature T cells .................................................................................................. 17
  1.3 Influenza A virus ....................................................................................................................... 18
    1.3.1 Influenza A pathology and burden .................................................................................. 18
    1.3.2 Influenza A ecology ......................................................................................................... 20
    1.3.3 Influenza A host specificity ............................................................................................. 22
    1.3.4 The Influenza A lifecycle ................................................................................................ 23
    1.3.5 Innate immune response to Influenza A ......................................................................... 24
    1.3.6 Primary adaptive immune response to Influenza A ....................................................... 27
    1.3.7 Antibody responses to Influenza A .................................................................................. 30
  1.4 Protection from Influenza A and vaccines ................................................................................. 31
    1.4.1 Novel skin-delivered vaccination strategies ................................................................... 34
    1.4.2 Inflammation and initiation of adaptive immune responses in the skin ....................... 34
    1.4.3 The potential of transcutaneous immunization ............................................................... 36
    1.4.4 TLR9 and CpG oligodeoxynucleotides ........................................................................ 38
    1.4.5 CpG ODN as a vaccine adjuvant .................................................................................... 39
  1.5 Research aims and rationale ....................................................................................................... 40
Chapter 2: IL-7 signaling controls T cell development at the transition from the bone marrow to the thymus

2.1 Introduction ............................................................................................................. 44

2.2 Results ..................................................................................................................... 47

2.2.1 IL-7Rα signaling is not required in early hematopoiesis .................................... 47

2.2.2 Early Thymic Progenitors are highly dependent on IL-7Rα, but not TSLPR signaling ............................................................................................................. 49

2.2.3 The bone marrow to ETP transition requires cell intrinsic signaling through IL-7Rα Tyr449 ......................................................................................................... 51

2.2.4 IL-7Rα Tyr449 signaling does not regulate survival of thymocyte progenitors ......................................................................................................................... 54

2.2.5 IL-7Rα Tyr449 signaling does not regulate proliferation of thymocyte progenitors ....................................................................................................................... 57

2.3 Discussion ............................................................................................................... 59

2.4 Materials and methods .......................................................................................... 63

Chapter 3: Interleukin-7, but not thymic stromal lymphopoietin, plays a key role in the T cell response to Influenza A virus

3.1 Introduction ............................................................................................................. 65

3.2 Results ..................................................................................................................... 68

3.2.1 IL-7 is critical for the control of and protection from influenza A virus infection ..................................................................................................................... 68

3.2.2 IL-7 signaling is necessary for a robust specific T cell response against influenza A virus ........................................................................................................... 71

3.2.3 IL-7 and TSLP regulate CXCR3 expression on virus specific T cells .............. 75

3.2.4 Generation of influenza A specific T cells requires cell intrinsic IL-7 signaling ......................................................................................................................... 77

3.3 Discussion ............................................................................................................... 80

3.4 Materials and methods .......................................................................................... 83

Chapter 4: Topical CpG adjuvant enhances protection against influenza A infections

4.1 Introduction ............................................................................................................. 87

4.2 Results ..................................................................................................................... 90

4.2.1 Adjuvant epicutaneous CpG (epCpG) ODN induced short and long term protection from IAV ................................................................................................. 90

4.2.2 Adjuvant epCpG ODN immunization generated strong systemic and lung memory CD8 T cell responses to IAV challenge .................................................................... 93

4.2.3 Adjuvant epCpG immunization induced a strong IAV specific antibody response ..............................................................
4.3 Discussion .......................................................................................................................... 97
4.4 Materials and methods ..................................................................................................... 101

Chapter 5: Conclusions and future directions .................................................................. 104

5.1 Discussion .......................................................................................................................... 104
5.1.1 IL-7 controls the development of the ETP population ............................................. 106
5.1.2 IL-7 signaling is necessary for robust and protective T cell responses to IAV infection .................................................................................................................. 108
5.1.3 Topical CpG ODN adjuvant enhances protection against influenza A infections .................................................................................................................. 109

5.2 Future directions ............................................................................................................... 110
5.2.1 Investigating the role of IL-7 and T cell development ............................................. 110
5.2.2 Further investigation of the role of IL-7 in IAV infection .................................... 113
5.2.3 Transcutaneous vaccination and IAV vaccines ....................................................... 115

5.3 Conclusions ...................................................................................................................... 116

References .......................................................................................................................... 118
List of figures

Figure 1: T cell development in bone marrow and thymus.........................................................3
Figure 2: Migration of progenitors from the BM to the thymus. .................................................7
Figure 3: Bone marrow progenitor populations are independent of IL-7Rα signaling. ..............48
Figure 4: IL-7 signaling is essential for the development of ETPs. ...........................................50
Figure 5: Direct IL-7 signaling is required for the transition between bone marrow and thymic T cell progenitors. ........................................................................................................53
Figure 6: IL-7Rα Tyr449 signaling does not regulate survival of ETPs. .................................55
Figure 7: IL-7Rα Tyr449 signaling does not regulate proliferation of ETPs..............................58
Figure 8: IL-7Rα signaling is required for control of Influenza A virus.................................69
Figure 9: Quantification of M1 viral RNA in the lungs.................................................................70
Figure 10: Total CD4 and CD8 T cell response in the lung and BAL.................................72
Figure 11: Baseline CD4 and CD8 T cell in the lung and spleen. ..............................................73
Figure 12: CD4 and CD8 T cell response to Influenza A requires IL-7Rα, but not TSLPR, signaling.................................................................74
Figure 13: Expression of CD11a, CD29 and CXCR3 by Influenza A specific T cells.....76
Figure 14: Cell-intrinsic requirement for IL-7Rα, but not TSLPR, signaling in CD8 T cell response to Influenza A.................................................................78
Figure 15: Cell-intrinsic requirement for IL-7Rα, but not TSLPR, signaling in CD4 T cell response to Influenza A.................................................................79
Figure 16: Adjuvant epicutaneous CpG generated optimal protection against Influenza infection. ........................................................................................................92
Figure 17: Adjuvant epicutaneous CpG generated a strong memory CD8 T cell response during Influenza infection..........................................................94

Figure 18: Normal antibody response from epCpG immunized mice.................................96

Figure 19: Novel roles for IL-7 in ETP development and primary T cell responses. ....107
## List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute Lymphoblastic Leukemia</td>
</tr>
<tr>
<td>Alum</td>
<td>Aluminum Hydroxide</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B cell lymphoma 2</td>
</tr>
<tr>
<td>Cas</td>
<td>Caspase</td>
</tr>
<tr>
<td>CLP</td>
<td>Common Lymphoid Progenitor</td>
</tr>
<tr>
<td>CpG ODN</td>
<td>CpG oligodeoxynucleotides</td>
</tr>
<tr>
<td>Cre</td>
<td>Cre recombinase</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>DN</td>
<td>Double Negative</td>
</tr>
<tr>
<td>DP</td>
<td>Double Positive</td>
</tr>
<tr>
<td>EdU</td>
<td>Ethynyl deoxyuridine</td>
</tr>
<tr>
<td>ep</td>
<td>Epicutaneous</td>
</tr>
<tr>
<td>ETP</td>
<td>Early Thymic Progenitor</td>
</tr>
<tr>
<td>Flt3</td>
<td>Fms-like tyrosine kinase</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HAU</td>
<td>Hemagglutinin Unit</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic Stem Cell</td>
</tr>
<tr>
<td>IAV</td>
<td>Influenza A Virus</td>
</tr>
<tr>
<td>IIIV</td>
<td>Inactivated Influenza Vaccine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>LAIV</td>
<td>Live Attenuated Influenza Vaccine</td>
</tr>
<tr>
<td>LC</td>
<td>Langerhans cell</td>
</tr>
<tr>
<td>Lin</td>
<td>Lineage</td>
</tr>
<tr>
<td>LMPP</td>
<td>Lymphoid primed multipotent precursor</td>
</tr>
<tr>
<td>LSK</td>
<td>Lineage- Sca(^+) c-kit(^+)</td>
</tr>
<tr>
<td>M1</td>
<td>Matrix protein 1</td>
</tr>
<tr>
<td>M2</td>
<td>Matrix protein 2</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MPP</td>
<td>Multipotent precursor</td>
</tr>
<tr>
<td>NA</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>NEP</td>
<td>Nuclear export protein</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand-1</td>
</tr>
<tr>
<td>Rag1</td>
<td>Recombination activating gene 1</td>
</tr>
<tr>
<td>RIG-1</td>
<td>Retinoic acid-inducible gene 1</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>S1P</td>
<td>Sphingosine-1-phosphate</td>
</tr>
<tr>
<td>sc</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>Sca-1</td>
<td>Stem cell antigen 1</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficiency</td>
</tr>
<tr>
<td>Sialic acids</td>
<td>N-acetylneuraminic acids</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signaling</td>
</tr>
<tr>
<td>SP</td>
<td>Single positive</td>
</tr>
<tr>
<td>TC</td>
<td>Transcutaneous</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tfh</td>
<td>T follicular helper cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic Stromal Lymphopoietin</td>
</tr>
<tr>
<td>TSP</td>
<td>Thymic Seeding Progenitor</td>
</tr>
</tbody>
</table>
Acknowledgements

First, thank you to my supervisor, Dr. Ninan Abraham, for your constant, consistent, scientific and personal support during this degree. Thank you for the right amount of high-fives for successes, and for pushing for me to do better.

To all current and past members of the Abraham Lab, thanks for your help both in and out of the lab, and in particular, thanks to Drs. Lisa Osborne and Daniel Patton for innumerable scientific discussions and for trying to teach me to think a little clearer. Special thanks to Dr. Jung Hee Seo, for her irreplaceable technical aid, laughs in the office and random chocolate.

Thanks to members of my committee, Drs. Marc Horwitz, Kenneth Harder and Colby Zaph, for their time and effort, insight and guidance, and especially, for offering a fresh view on data and proposals. Thanks also to Drs. Michael Gold, Linda Matsuuchi and Georgia Perona-Wright for their help and advice.

Thanks to my friends from the Horwitz, Harder, Johnson, Perona-Wright, Gold and Matsuuchi labs, for help with protocols, mice and reagents, and for grabbing a beer to either talk about science or to avoid it.

Thank you to my entire family, and especially my parents, for their support and unwavering belief in me.

Most importantly, to Sonja Christian - going home with you is always the best part of the day.
Chapter 1: Introduction and background

The development of the mature immune cells from hematopoietic stem cells (HSCs) and the ability of these cells to properly respond are essential for protection from infections. Lymphocytes are a family of immune cells that contains innate immune cells as well as the two adaptive arms of the immune systems. Both innate and adaptive lymphocytes play important roles in the immune response [1].

1.1 T cells and the adaptive immune response

As one of the arms of the adaptive immune response, T cells play a central role in the immune response wherein the impairment of T cell development or function can cause severe immunodeficiency [2]. Although T cell differentiation begins in the bone marrow, progenitors must migrate to the thymus, the unique environment that allows for their development [3]. In the thymus, the T cell receptor (TCR) is rearranged and selected for its affinity to the Major Histocompatibility Complex (MHC), and against strong affinity for self peptide-MHC complexes.

After maturation and selection in the thymus, mature, naïve T cells exit for the periphery where they patrol the body until an infection [4]. During an infection, T cells that recognize peptides from the invading organism presented on MHC will respond [1]. These T cells specific for the invading organism will expand, differentiate into effector T cells and help clear the infection. A small number of the T cells in the primary response
will develop into long lived memory cells that are more efficient in responding to subsequent infections [5]. Vaccines that elicit a strong, memory T cell response, such as the Vaccinia vaccine, are highly effective at protecting from disease [6].

1.1.1 Early lymphocyte development

HSCs are the multipotent precursors to all hematopoietic cells. They are located in the bone marrow and are long lived with a high capacity for self-renewal [7-9]. As HSCs differentiate into mature immune cells, they become more fate restricted to specific lineages. Although previous models proposed a hierarchical step-wise pathway for early lymphocyte development from HSCs, it is now clear that the model is more nuanced. Some developmental steps do correspond with restriction from entry into a different lineage pathway. However many lymphoid progenitor cell populations may not be restricted from a lineage, but are biased towards one of several lineages [3].

The earliest lymphoid progenitors in the bone marrow, including HSCs, can be identified through surface markers (Figure 1). In mice, these progenitors do not express any markers of more mature linages (Lin-) and express high levels of stem cell antigen-1 (Sca-1) and c-kit (or CD117). This population, termed LSK cells, can be broken down into several progenitor subsets. HSCs can be identified as cells that do not express CD34 or fms-like tyrosine kinase receptor-3 (Flt3). This population contains the least differentiated cells with the potential for long term self-renewal [10, 11]. HSCs progressively differentiate through progenitor stages that are increasingly lineage
Figure 1: T cell development in bone marrow and thymus. Early lymphoid development begins in the bone marrow from HSCs. They differentiate through MPPs into LMPP and CLP stages. These populations migrate through the blood to the thymus and become ETPs. ETPs commit to the T cell lineage, rearrange the TCR and undergo positive and negative selection as they differentiate through the DP and into SP CD4 and CD8 stages. After selection, cells leave the thymus as mature, naïve, T cells. IL-7R expression is shown. IL-7R is first expressed at the CLP stage in the bone marrow. It is not expressed at the ETP stage in the thymus but is expressed at other DN stages during TCR rearrangement.
restricted and have reduced self-renewal capacity. HSCs differentiate first into multipotent progenitors (MPPs) and then into lymphoid primed multipotent progenitors (LMPPs), and common lymphoid progenitors (CLPs) [7, 12]. Although these progenitors are increasingly lineage restricted, there is no clear delineation between more mature progenitor compared to its precursors. Instead, more mature subsets usually have reduced potential for a specific lineage instead of abolished potential. HSCs differentiation into MPPs is accompanied by the loss of their self-renewing capability while retaining lineage potential [10, 11, 13]. Further development into LMPPs partially restricts the myeloid potential of the cells while retaining all lymphocyte potential, whereas CLPs have lost all myeloid potential [14, 15]. Thus, all myeloid lineages are derived from either MPPs or LMPPs, while lymphocytes develop from LMPPs and CLPs.

The LMPPs and CLPs are the branching point for all lymphoid lineages. While it was originally believed that LMPPs would first differentiate into CLPs before proceeding down different developmental pathways, this view has changed as it has become apparent that both the LMPP and CLP populations are heterogeneous and that development of more mature cells may arise from different progenitors with varying efficiencies. LMPPs can be divided into two populations based on the expression of recombination activating gene 1 (Rag1). LMPPs expressing Rag1, called early lymphoid progenitors are primed towards a lymphoid fate [16]. Similarly, CLPs can be divided into two distinct populations primed for either a T cell or B cell fate based on their expression
of Ly6D. Ly6D+ CLPs are thought to develop from Ly6D- CLPs, and are primed towards a B cell fate while the Ly6D- CLPs are less committed [17].

After development of these lymphoid primed progenitors, the next step in T cell development is for the progenitors to leave the marrow and migrate into the thymus. The progenitors that home to the thymus are termed thymic seeding progenitors (TSP), which quickly develop and expand into the readily detectable early thymic progenitor (ETPs) population after arrival in the thymus [18]. Many bone marrow populations retain T cell potential but transferring progenitor populations intravenously demonstrates that both CLPs and LMPPs generate T cells quickly and efficiently [18]. However, it remains unclear exactly which bone marrow progenitor subset or subsets are the main feeder populations for the TSP under normal physiological conditions. Indeed, what is known about the development potential, function, and lineage history of the bone marrow progenitors and the ETPs does little to clear up the mystery. As mentioned above, LMPPs retain both lymphoid and myeloid potential with a lymphoid bias, while CLPs are restricted to lymphoid lineages [7]. Surprisingly, the ETP population retains both lymphoid and myeloid potential and a small fraction of ETPs develop into myeloid cells in the thymus under physiological conditions [19-21]. This would appear to suggest the LMPPs are the physiological precursors to at least some TSPs and ETPs. However, lineage-tracing experiments using an Interleukin (IL)-7Rα driven Cre recombinase and a ROSA26 reporter reveal that although ETPs do not express IL-7Rα, their progenitors did [22]. This suggests that CLPs, or some other IL-7Rα expressing progenitor subset contributes substantially to the ETP population. Taken together, while the direct T cell...
progenitors in the bone marrow have not been conclusively identified, it is likely they reside within the LMPP or CLP populations.

1.1.2 Progenitor egress from the bone marrow

As the T cell progenitor that migrates out of the bone and into the thymus is not well defined, the mechanisms of egress and retention of these cells from and in the bone marrow are unclear. This is further complicated as the identity of the blood stage of the TSP is even less clear than in the bone marrow. Although cells resembling the phenotypic and functional characteristics of many bone marrow progenitor subsets have been found in the blood, they are extremely rare and their presence in the blood may be for reasons other than migration to the thymus. However, as it is likely that the TSPs reside within the LMPP or CLP populations, we can use what is known about the egress of these cells from the marrow for insight on how egress of the TSP may occur.

Progenitors are actively retained in the bone marrow and in close contact to supporting stromal cells by chemokine gradients and adhesion molecules [23, 24] (Figure 2). Progenitors express the chemokine receptor CXCR4, the receptor for CXCL12, and loss of the receptor or blockade of the pathway result in egress of progenitors from the marrow to the blood [25, 26].

Egress of progenitors is actively triggered by chemokines and sphingosine-1 phosphate (S1P) gradients. Several chemokines including CXCL2 and CXCL8 have
Figure 2: Migration of progenitors from the BM to the thymus. Progenitors are retained in the bone marrow by CXCL12 signaling. They are driven into the blood by S1P and CXCL8. Once in the blood they home to the thymus in a PSGL-1-dependent and CCL21/25-dependent manner.
been implicated in inducing progenitors to exit the marrow and may act in concert with reduced CXCR4 signaling [27, 28]. S1P plays a key role in the egress of immune cells into the periphery from the thymus and other peripheral lymphoid organs [29]. This also applies to the egress of hematopoietic progenitors from the bone marrow since egress is blocked by the inhibitor of S1P signaling, FTY720 [30].

Although these molecules, and others, are likely to play a role in controlling egress of TSPs to the blood, most also control retention and egress out of the marrow by many other cell types. How chemokines, integrins and S1P interact to specifically regulate TSP egress is unknown. Additionally, there is no good model for the kinetics of progenitor egress during homeostasis. It is unclear if there is a steady equilibrium between the marrow and the blood or if progenitors are triggered to exit from the marrow in waves. Overall, there is very little specific knowledge about this step in T cell development and many questions remain unanswered.

1.1.3 Progenitor entry into the thymus

Although details of T cell progenitor egress from the bone marrow and the blood borne progenitors are few, much more is known about entry of progenitors into the thymus and their subsequent commitment and development into mature T cells. Entry into the thymus requires a combination of signals similar to migration into other immune organs or tissues. Adhesion molecules and chemokine gradients coordinate in a multistep process to mediate the recruitment of T cell progenitors into the thymus. Initial
adhesion of progenitors is thought to involve binding of P-selectin glycoprotein ligand 1 (PSGL-1) to P-selectin expressed on thymic endothelial cells [31]. Progenitors lacking PSGL-1 are severely impaired in their ability to migrate into the thymus.

Entry of progenitors into the thymus is not constant, instead occurring in periodic waves [32]. The waves of entry are primarily regulated through expression of P-selectin by thymic endothelial cells and its binding to PSGL-1 on progenitors [33]. P-selectin expression is itself controlled by the size of the early thymocyte population in the thymus [31]. Mice deficient in ETPs, such as PSGL-1 knockout mice, have high, constitutive expression of P-selectin on thymic endothelial cells and thymi that are highly receptive to recruitment of normal T cell progenitors. In contrast, Rag1 knockout mice, that have normal levels of ETPs, but have a severe block at the DN3 developmental stage, are refractory to entry by progenitors. As ETPs develop and mature, P-selectin levels on endothelial cells rise and new progenitors enter to fill the niche.

Entry of progenitors into the thymus has similar phases to the extravasation of immune cells into other tissues. While PSGL-1 binding to P-selectin mediates the initial attachment, tight binding of migrating cells to endothelial cells is mediated by integrins [34]. Blockade of the integrins α4 or β2 on CLPs impairs their migration into the thymus. Similarly, blocking VCAM-1 or ICAM-1, which bind to the integrins α4β1 and αLβ2 expressed by CLPs, impairs the recruitment of progenitors to the thymus [34].
Several chemokines and chemokine receptors are implicated in the migration of progenitor cells to the thymus. The primary chemokine receptors that mediate recruitment are CCR7 and CCR9 [35-38]. These bind to the chemokines CCL19 and 21, and CCL25, respectively, which are produced by thymic epithelial cells [39-41]. Together, these molecules mediate the adhesion and migration of progenitors into the thymus.

Interestingly, what is known about the mechanism of entry into the thymus supports the current understanding of which cells are the proximal T cell progenitors in the bone marrow. The bone marrow populations that express high levels of PSGL-1 are LMPPs and CLPs, the likely T cell progenitor populations [42]. LMPPs and CLPs express the glycosylation enzymes necessary for maturation and proper function of PSGL-1. They also express the chemokine receptors CCR7 and CCR9, which detect chemokine gradients directing cells into the thymus [18]. This is consistent with the model that the proximal T cell progenitors in the bone marrow are likely within the LMPP and CLP population.

1.1.4 T cell development in the thymus

The thymus provides a unique environment that facilitates commitment to the T cell lineage and development. Progenitor cells that enter the thymus are uncommitted to the T cell fate, retaining both B cell and myeloid potential [19, 43]. One defining feature of the thymic environment, Notch activation, begins immediately following entry into the
thymus and helps to direct these precursors down the T cell developmental pathway. Notch, expressed on T cell progenitors, interacts with the Delta-like class of ligands expressed by stromal cells [3, 44, 45]. Notch activation, along with c-kit signaling, is essential for the initial, immediate expansion of the TSPs into ETPs, which are the earliest detectable T cell progenitors in the thymus [3, 46-49]. Notch signaling also induces a network of transcription factors, such as GATA3 and Tcf7 [50, 51]. These are necessary for early T cell commitment as ETPs transition into early double negative (DN) 2 cells [50, 51].

Transition to the DN2 stage is accompanied by the expression of IL-7Rα, which is not expressed at the ETP stage. Here, IL-7 signaling drives the survival and proliferation of DN2s while Notch signaling continues to drive the T cell development program [3]. Commitment to the T cell lineage is finalized in DN2s by the induction of the transcription factor Bcl11b [52, 53]. Bcl11b, in cooperation with Notch, induces expression of Rag1 and the pre-T cell receptor (TCR) α chain [54]. Cells then transition to DN3s where they attempt to rearrange the TCRβ chain [55]. Successful rearrangement triggers signaling through the TCRβ and pre-TCRα chains which drives another round of proliferation. Subsequent rearrangement of the TCRα chain drives differentiation into the double positive (DP) thymocyte stage characterized by expression of both CD4 and CD8. Here, thymocytes undergo positive and negative selection based on their TCR affinity for self-MHC/peptide complexes. Those that have a intermediate affinity for self-MHC, but do not recognize MHC or MHC/peptide complexes strongly enough to trigger a response in the periphery, receive positive
selection survival signals and avoid deletion by negative selection. These cells then differentiate into either CD4 or CD8 single positive (SP) thymocytes before exiting the thymus.

1.2 Interleukin-7

Cytokines play a central role in T cell development, homeostasis, response and memory. The γ common (γc) chain family of cytokines, IL-2, -4, -7, -9, -15, and -21 are all involved in T cell functions [5, 56]. IL-7 in particular regulates T cells throughout their lifespan and regulates T cell development, survival, responses and memory [57, 58]. IL-7 is essential for the development of murine B and T cells and of T cells in humans. Mutations in the IL-7 signaling pathway are the primary cause of T+B+NK+ severe combined immunodeficiency (SCID) [59].

In addition to its developmental role, IL-7 has important functions in the periphery. Naïve T cells depend on a combination of TCR and IL-7 signaling for homeostatic survival and proliferation, and memory T cells require signaling from IL-7 and IL-15 for long-term persistence [5, 58]. The central role of IL-7 in the development and homeostasis of T cell has led to phase II clinical trials examining IL-7 as a therapy to support immune reconstitution after chemotherapy and for AIDS induced lymphopenia [60].
1.2.1 IL-7 signaling

IL-7 has key roles in the development and homeostasis of the immune response, but overactive IL-7 signaling can drive lymphomas in many different settings [61]. Thus it is not surprising that IL-7 signaling is tightly controlled at many levels. Expression of IL-7Rα is quickly and dynamically regulated throughout lymphocyte development and function, signaling can be inhibited, and even basic downstream signaling events appear to be dependent on cell type and signaling context.

IL-7 signals through a heterodimeric receptor consisting of the γc chain and the IL-7Rα (or CD127) subunit. Binding of IL-7 to its receptor induces phosphorylation of Janus kinase (JAK) 1 and JAK3, which are associated with the Box1 domains of the IL-7Rα and the γc, respectively [62, 63]. JAK1 and 3 subsequently phosphorylate tyrosine residues on both IL-7Rα and the γc chain [64]. Disruption of any of these signaling components in both humans and mice renders IL-7 unable to signal effectively and results in severe immune deficiency due to a failure of T cells development [2]. Signaling downstream of IL-7Rα is largely dependent on phosphorylation of tyrosine (Tyr) 449, nested within a recruitment motif for signaling effectors. Phosphorylation of Tyr449 by JAK1 and 3 allows recruitment, phosphorylation and activation of the key signaling molecule, signal transducer and activator of transcription 5 (STAT5) [65-68]. Phosphorylation of Tyr449 has been shown to be essential for activation of STAT5 downstream of IL-7 in cell lines, as well as in a knock-in mouse model [65, 67]. Loss of STAT5 signaling in mice recapitulates many of the effects of loss of IL-7 signaling,
hence, it is thought that many, but not all, of IL-7 mediated effects is through STAT5 [69].

STAT5 is consistently activated downstream of IL-7 in multiple cell types by the same biochemical mechanism. However, other IL-7 signaling events are highly dependent on cell type and signaling context. Thymocytes and developing B-cells activate phosphatidylinositol-3 kinase (PI3K) and its downstream target AKT within 10 minutes after stimulation with IL-7 [70, 71]. Developing B cells appeared to activate PI3K by direct recruitment of the p85 subunit of PI3K to the Tyr449 motif on IL-7Rα [72, 73]. However, thymocytes were able to activate PI3K in the absence of the intracellular domain of IL-7Rα suggesting that the yc chain may mediate PI3K activation in this setting [68]. IL-7 signaling in other cell types may only weakly activate the PI3K signaling pathway or do so indirectly. Mature T cells require 18 hours of stimulation with IL-7 before weak activation of AKT is observed [74, 75]. It is thought that STAT5 induced genes may indirectly mediate AKT activation, as inhibiting STAT5 activation prevents activation of AKT in this setting [75].

Proximal IL-7 signaling events, including STAT5 and PI3K activation, drive changes in cellular function. A main function of IL-7 in many cell types is to mediate cell survival, which is often mediated by the Bcl-2 family of proteins [64]. IL-7 driven Bcl-2 expression plays a large role in early T cell development. Indeed, transgenic expression of Bcl-2 partially rescues T cell development in IL-7Rα deficient mice [76]. Regulation of Bcl-2 by IL-7 is complex, as it is only partially dependent on activation of STAT5 [65].
Other Bcl-2 family members are regulated by IL-7, including the expression of Mcl-1 and Bcl-xL, and the activity of the pro-apoptotic protein Bad [77].

In addition to cell survival, IL-7 signaling also mediates cell proliferation in many different settings. IL-7 drives proliferation and cell cycle progression in developing T and B cells as well as in mature T cells. IL-7 regulates the breakdown of the regulator of cell cycle entry, P27^{kip1}, and increases levels of the cyclin dependent kinase 2 [78].

1.2.2 IL-7 in T cell development

IL-7 signaling has roles throughout lymphoid development. Its receptor is dynamically regulated throughout T cell development and is known to play several critical roles. IL-7Rα is not expressed on HSC or their immediate progeny the MPPs or LMPPs [22, 79]. The first bone marrow progenitors to express IL-7Rα are the CLPs, and it is used as one of the markers that define the subset [3]. There is no known role for IL-7 at the CLP stage during T cell development. In contrast, in B cell development, IL-7 signaling in CLPs induces the expression of Ebf1 which is essential for differentiation past the pre-pro B cell stage [80].

The earliest thymocyte population, ETPs, do not express IL-7Rα. Interestingly, lineage-tracing experiments using an IL-7Rα-promoter driven Cre reveal that progenitor cells to the ETP did express the receptor [22]. How and why IL-7Rα is downregulated on ETPs is unknown. As thymocytes progress to the DN2 stage, IL-7Rα is re-expressed
and remains expressed until the DP stage [3]. IL-7 has several important functions during the DN stage of development. IL-7 driven Bcl-2 expression in DN2 thymocytes is essential for their survival [3]. IL-7 also cooperates with Notch signaling to maintain thymocyte proliferation [81]. After rearrangement of the TCRβ chain, IL-7 once again aids in mediating survival and proliferation [82]. Interestingly, IL-7 signaling at this stage delays rearrangement of the TCRα chain and progression to the DP stage. Interestingly, not only is IL-7Rα downregulated at the DP stage, but high expression of suppressor of cytokine signaling (SOCS)-1 prevents it from signaling even if it is expressed as a transgene [83]. Loss of SOCS-1 prevents differentiation of thymocytes from the DN4 to the DP stage and it is interesting to hypothesize that cessation of IL-7 signals by SOCS-1 might be necessary to allow for TCRα rearrangement and progression to the DP stage. In addition, IL-7 is present in limited amounts in the thymus and enforced expression of IL-7Rα on DP thymocytes results in greatly increased cell death in the DN populations due to IL-7 deprivation [84]. IL-7Rα is again re-expressed after positive selection on single positive (SP) CD4 and CD8 thymocytes. The role of IL-7 at this stage is controversial, with some evidence that it helps cells differentiate into the CD8 lineage [85, 86]. However, other studies suggest that IL-7 may just regulate the survival and proliferation of SP thymocytes, and the CD8 thymocytes are more sensitive to disruption in signaling [87].

The effects of IL-7 on T cell development are partially mediated through signaling from Tyr449 on IL-7Rα [5]. Mice with a loss of either IL-7 or IL-7Rα have a severe block in T cell development and less than a tenth of the normal complement of thymocytes
and mature T cells. While the specific *in vivo* loss of IL-7Rα Tyr449 signaling does result in a reduction of T cell numbers, the phenotype is far less severe, as the mice have approximately a quarter of the normal number of thymocytes and mature T cells. Interestingly, while both partial and total loss of IL-7 signaling result in a reduction in thymocyte numbers, CD4/8 thymic profiles are comparable [5, 82].

### 1.2.3 IL-7 and mature T cells

In addition to its role in T cell development, IL-7 signaling has several important roles in mature T cells. The size of the population of mature naïve T cells is regulated independently from the generation of new T cells and their output from the thymus. The number of naïve T cells and the survival of individual naïve T cells is controlled by a combination of IL-7 signaling and weak interactions between TCRs and MHC-self peptide complex [58, 88, 89]. IL-7 signaling induces both Bcl-2 and Mcl-1 that help protect from apoptosis [90-92]. IL-7 is produced at a relatively constant level and this restricts the size of the naïve T cell population. Relatively small amounts of IL-7 are necessary to drive Bcl-2 family member expression and survival; however, an increased amount is able to drive proliferation [93]. A smaller population of naïve T cells results in stronger IL-7 signaling per cell. This drives degradation of P27kip1 and allows the T cells to enter the cell cycle, proliferate and restore the size of the naïve T cell population to its homeostatic level [58]. The limited amount of IL-7 produced regulates the size of the mature T cell pool. Under homeostatic conditions, IL-7 is limiting and allows for T cell
survival, but not proliferation. Under lymphopenic conditions, the relative increase in IL-7 availability drives T cell proliferation to restore T cell numbers.

1.3 Influenza A virus

Influenza A (IAV) is one of six genera in the Orthomyxoviridae family of viruses, which are enveloped negative sense, segmented RNA viruses [94]. IAV is a highly contagious human respiratory pathogen with large impact on global health. IAV causes yearly epidemics and has both historical and ongoing potential to cause severe global pandemics. Immunity after infection or vaccination is not long lasting due to the high level of genetic change in viral epitopes that generate strong, neutralizing antibody responses. These changes occur by gradual genetic drift and by more abrupt genetic shifts. Genetic drift occurs by mutation of alleles within IAV strains and the expansion of these novel alleles within the strain. Genetic shifts occur with by reassortment of genomic sections of existing human influenza strains with other influenza strains. Genetic reassortment between IAV co-infecting a single host can give rise to new progeny that are substantially different from parental strains. These novel strains have high potential to generate pandemics due to the lack of pre-existing immunological memory [95].

1.3.1 Influenza A pathology and burden

Seasonal IAV virus infection causes an acute disease lasting approximately 10 days with viral replication peaking in the upper respiratory tract two days after infection
(reviewed in) [96]. Symptoms may include fever, cough, nasal mucus discharge and malaise, although not all symptoms may present. People of all ages can be infected but the disease can be particularly severe for the young, old and the immunocompromised. The virus is predominantly found in the upper respiratory tract but can also be recovered from the lower respiratory tract. More severe infections tend to have viral infection in the lower respiratory tract.

The yearly cycle that produces new, antigenically distinct viral strains ensures IAVs continuing, heavy burden on human health. Each year, up to 10% of the global population can be infected with seasonal strains of IAV [97]. In the United States, seasonal Influenza is responsible for over 200,000 hospitalizations and between 3,000 and 49,000 deaths each year [98, 99]. In addition to severe illness and mortality, influenza infections result in over 100 million lost workdays and cost $7 billion in lost productivity a year in the United States [100]. Children and adults over 65 years of age have higher susceptibility to IAV and account for the majority of influenza related hospitalizations [101].

In addition to seasonal epidemics, novel IAV strains resulting from antigenic reassortment can produce viruses capable of causing highly pathogenic pandemics. These strains often have increased disease severity and infectivity, at least partially due to the lack of any population level pre-existing immunity. During the most recent IAV pandemic in 2009, the swine hemagglutinin 1 neuraminidase 1 (H1N1) strain infected an estimated 24% of the world’s population and was responsible for nearly 300,000
deaths globally [102, 103]. Like many pandemic IAV strains, the 2009 H1N1 was severe in healthy adults as well as in children and the elderly [104]. While pandemic IAV outbreaks tend to cause more serious illness than seasonal strains, most still have a mortality rate of $<0.01\%$. However, the most severe pandemic, 1918 Spanish IAV pandemic had a mortality rate over 200x greater than normal seasonal IAV strains and was responsible for the death of 40-50 million people worldwide [105].

1.3.2 Influenza A ecology

The ecology and evolution of IAV strains is central to how new strains arise that are capable of causing severe disease in humans and worldwide pandemics. Interspecies transmission and antigenic assortment can result in novel IAV variants with enhanced infectivity or virulence. Although bats have been shown to be the natural reservoirs for two novel IAV serotypes [106, 107] waterfowl are the natural reservoir for nearly all serotypes of IAV virus [108].

IAV infection of birds causes no overt disease although some studies have shown reduced body mass in IAV hosts [109]. In birds the primary site of infection and replication is the intestinal tract [110]. As a result, IAV is shed at high concentration in the feces and new infections are acquired through contaminated water [110].

Waterfowl IAV can jump to other species, of which infection of domestic poultry and pigs pose the most relevance to human health [111]. In domestic poultry, H5 and
H7 viruses can mutate to become extremely pathogenic resulting in near certain mortality in infected hosts [112]. When these highly pathogenic viruses manage to infect humans, they retain their much of the pathogenicity seen in poultry and cause severe and often lethal disease [113, 114]. Fortunately, transmission of these viruses to humans and between humans is extremely difficult [115-117].

Pigs are host to many swine adapted strains of IAV [111]. IAV pathology in pigs can range from asymptomatic to severe and the illness presents similar to human infections. The virus primarily localizes to the respiratory tract and disease manifests with cough, mucosal discharge and fever. Swine are one of the most common species that facilitates the transition of avian IAV into a mammalian host [110]. In contrast to poultry, transmission of swine influenza to humans, and vice versa is very common while maintaining most of the infectivity in their new hosts with little adaptation needed [110]. This allows human, avian and swine IAV viruses to mix in a single co-infected host, which can result in highly novel virus strains. The 2009 H1N1 pandemic IAV strain is a perfect example of the role of swine as a generator for novel human IAV strains. The 2009 H1N1 IAV was a swine IAV that jumped into the human population and was able to spread and cause severe disease because of lack of pre-existing immunity.

Rodents are not natural hosts for IAV but there are several IAV strains that have been adapted to infect mice through serial infections. [118]. While these strains are valuable tools to investigate the pathogenicity and immune responses to IAV, there are several drawbacks to this model. IAV, including the murine adapted strains, do not
readily infect mice. Respiratory infection requires the virus to be delivered deep into the lungs and infected mice cannot pass on the infection to other mice. Furthermore, mice do not display many of the symptoms that are typical during human IAV infection including fever, coughing and nasal secretion [110]. In contrast to mice, ferrets are a natural host for IAV and are readily infected with human influenza strains. Ferrets infected with IAV reliably develop pathology that is indistinguishable from that in humans. They are also able transmit the virus to their cage mates. While ferrets are the best model to faithfully replicate the complete biology of IAV infection, they are not amenable to genetic manipulation and have more complicated husbandry and costs compared to mice. These factors limit the utility of ferrets to dissect the molecular details of the immune response to IAV.

1.3.3 Influenza A host specificity

While host specificity of IAV is multifactorial, the hemagglutinin (HA) protein plays a central role in determining the infectivity and pathogenicity of an IAV strain in different species. The main trait of HA that determines host specificity is its selectivity in binding to N-acetylneuraminic acids (sialic acids). Sialic acids can use either α2-3 or α2-6 linkages and HA selectively binds to one or the other [110]. Avian gastrointestinal tract epithelial cells predominantly express α2-3 sialic acid, and thus avian IAV HA has high affinity for α2-3 sialic acids [110]. In contrast human upper respiratory tract epithelial cells predominantly express α2-6 sialic acid and human IAV HA selectively binds to α2-6 sialic acid [119]. There is a small amount of α2-3 sialic acid in the human upper
respiratory tract but it is highly expressed in the lower respiratory tract [119]. This difference is central to the difficulty for humans to be infected with and transmit highly pathogenic avian IAV. The avian IAV cannot easily infect the human upper respiratory tract and must gain entry into the lower respiratory tract to establish an infection, which is less accessible. Swine express both \( \alpha_{2-3} \) and \( \alpha_{2-6} \) sialic acid throughout their respiratory tracts [120]. Their sialic acid expression is one of the primary reasons that they are susceptible to infection by both human and avian IAV viruses and makes them ideal for the generation of novel IAV strains that are capable of infecting humans.

### 1.3.4 The Influenza A lifecycle

Infection of a cell by IAV begins with binding of the HA to its host receptor, sialic acid, the terminal carbohydrate residues of many glycoproteins. Binding triggers endocytosis of IAV into the cell and endosome acidification facilitates fusion of the viral and endosomal membranes and uncoating of the viral genome. The low pH in the acidic endosome causes a conformational change in HA that mediates membrane fusion driven by the N-terminus of its H2 subunit [121, 122][123]. The Matrix-2 (M2) ion channel is activated and transports hydrogen ions across the viral membrane and into the capsid [124]. Low pH causes the dissociation of the viral ribonucleoproteins (vRNP) from the Matrix-1 (M1) protein, releasing the viral genome into the cytoplasm [125, 126]. The nuclear localization signals on the viral proteins direct the host cell to transport viral ribonucleoproteins into the nucleus via the nuclear pore complex [127-129].
Once in the nucleus, the viral RNA genome is transcribed by its cis-associated viral RNA-dependent RNA polymerase complex to produce mRNA. Normal cellular mechanisms export viral mRNA from the nucleus into the cytoplasm where they are translated by host ribosomes. Viral cRNA, stabilized by the nucleoprotein (NP) is generated and used as a template for new negative sense genomic vRNA [130]. The progeny viral ribonucleoproteins are bound by M1 and Nuclear export protein (NEP), that mediate their transport out of the nucleus by host factors [131].

Virus assembly takes place at the host cell membrane. vRNP associate with the MTOC after export from the nucleus [132]. The vRNP are carried to the apical membrane by host vesicles trafficking on microtubules [133, 134]. The transmembrane viral proteins, HA, NA and M2 also preferentially traffic to the apical membrane of epithelial cells [135-137]. NA and HA coordinate viral budding from lipid rafts [138-140] while the M1 protein directs virion assembly [141]. Budded virions may still be attached to the cell via binding to sialic acids and their release is by NA cleaving sialic acid from its associated glycoproteins and glycolipids [142, 143]. The released virions are then able to infect other susceptible cells.

1.3.5 **Innate immune response to Influenza A**

While the immune response against IAV is necessary to clear the virus, many aspects of the immune response contribute significantly to the pathology caused by the infection. Serious disease caused by IAV infection occurs with an overly strong immune
response, where immune cell infiltration into the lungs and very high levels of inflammatory cytokine production drive most of the pathology [144]. In the case of IAV infection, the immune response is a double edge sword, simultaneously protecting and damaging. Furthermore, nearly all arms of the IAV induced immune response have both a protective and pathogenic role. This makes efforts to find and block just the pathological immune responses very difficult.

The immune response to influenza infection begins as soon as the virus infects the respiratory epithelial cells. Epithelial cell pattern recognition receptors TLR3 and RIG-1 bind viral RNA to trigger inflammatory cytokine expression and activate intracellular antiviral defenses [145]. Intriguingly, while RIG-I is essential for protection from IAV infection, TLR3 increases pathogenicity by driving inflammatory cytokine expression [146]. This response includes a variety of proinflammatory cytokines and chemokines, which activate epithelial and resident immune cells and induce recruitment of innate immune cells from the blood. Type I interferon production by epithelial cells is essential for protection from IAV infection as it induces anti-viral responses in epithelial cells to limit viral spread [147]. In fact, one of the most prominent roles of the multifunctional IAV protein NS1 is to block both interferon production and signaling [148, 149]. Type 1 interferon (IFN) signaling in combination with viral recognition by intracellular pattern recognition receptors (PRR) induce IFN stimulated genes (ISG) that aid in the control of Influenza in infected cells. Several ISGs have demonstrated roles in IAV defense, including TRIM22, PKR, and Viperin, which target nucleocapsid for degradation, block translation and prevent budding, respectively. Despite it protective
role, type I interferon production also contributes to pathology by stimulating massive production of inflammatory cytokines [150].

The initial immune cells that respond to IAV infection are the resident alveolar macrophages and other resident monocytes and dendritic cells [145]. These are activated by Type 1 IFNs from epithelial cells and PRR signaling from phagocytosed virus. This triggers the production of more Type I IFNs from these cells, as well as other inflammatory molecules such as chemokines, that aid recruitment of early innate immune cells. Many monocytes are recruited from the blood and monocyte recruitment correlates with the severity of the disease. However, depletion of alveolar macrophages before infection results in increased mortality due to failure to control viral replication [151].

Neutrophils begin to be recruited in the first days of the immune response and, similar to monocytes, are both protective and pathologic [152]. While neutrophils are central to driving a pathological immune response, neutrophil depletion results in increased mortality due to failure to control the virus [151, 153]. However, partial depletion of neutrophils results in protection from immune induced pathology while still allowing for control of viral replication. This is a perfect example of the complexity of multifaceted innate response to influenza – the immune response is necessary to clear the virus and protect the host, yet failure to restrain the response results in severe pathology.
NK cells are critical for control of IAV infection early in the immune response and depletion of NK cells leads to increased mortality after IAV infection. In fact, they are one of the very few immune responses that is not known to cause pathology. NK cells fill a unique niche in the innate immune response, as they are the only cells that can directly recognize and destroy IAV infected cells. Cytotoxic NK cells in the lung kill IAV infected cells by direct recognition of viral HA with the NKp46 receptor [154]. NK cells are also a critical source of early IFN-γ production, which is necessary until the full development of the adaptive T cell response [146].

1.3.6 Primary adaptive immune response to Influenza A

The initiation of the adaptive immune response to IAV begins with antigen capture by lung resident dendritic cells (DCs) and their migration to the draining mediastinal lymph node [155]. Several lung resident DC subsets can by unproductively infected by IAV or can phagocytose infected or apoptosing cells [156]. Among these, CD103⁺ DCs have been found to be critical for the generation of a CD8 T cell response to IAV [157]. CD103⁺ DCs acquire IAV antigen through infection or phagocytosis and accumulate in the draining lymph node early in infection to present antigen to T cells [158].

Arriving at the lymph node, these DCs can either present IAV antigen directly to naïve T cells or transfer antigen to lymph node resident DCs to present to T cells [159]. Interestingly, although migratory CD103⁺ DCs are the best antigen presenting cells
early post-infection, this switches to lymph node resident DCs later in the immune response [160].

Naïve CD4 and CD8 T cells in the lymph node are continually scanning DCs for their cognate antigen [161, 162]. Presentation of antigen to T cells causes their activation and proliferation in the lymph node. The initial activation of T cells occurs within 3 days of infection [163]. Activation of T cells by mature DCs presenting their cognate antigen results in a large number of rounds of proliferation, greatly expanding IAV specific T cells [164, 165]. Fully activated T cells then migrate into the lungs. This is dependent on chemokine gradients and integrin/selectin binding. Although there appears to be a high level of redundancy in chemokines able to attract T cells to the lung, chemokine receptor CCR4 and CCR5 expressed on T cells have been shown to play important roles in their recruitment [166, 167]. Not only do chemokine gradients attract T cells into the lung, they also activate integrin to bind to blood endothelial cell-expressed selectins [168, 169]. The integrins CD11a and CD49d have been shown to be necessary for the recruitment and retention of effector T cells in the lung [170, 171]. Binding of integrins to their selectin receptors expressed on endothelial cells and chemokine signaling allow extravasation of T cells out of the blood and into the lung.

Effector T cell responses to IAV are central to control and clear the virus during the primary immune response. The arrival and action of CD4 and CD8 T cells in the lung causes a significant reduction in viral load and loss of all T cell responses renders mice unable to clear IAV [172]. The accumulation of effector T cells in the lung mediates
the rapid clearance of influenza from the peak viral load at day 7 post-infection to clearance at day 10 post-infection [173]. Mice unable to specifically mount a CD8 T cell response to IAV take significantly longer to clear the virus [173]. The CD8 T cell response against highly pathogenic viruses IAV viruses is even more critical, as mice lacking it have a much higher mortality rate [174]. Direct elimination of IAV infected cells is the main function of effector CD8 T cells in the adaptive immune response [175]. It has been elegantly demonstrated that either Granzyme B mediated or Fas mediated cytotoxic elimination of infected epithelial cells by effector CD8 T cells is required for efficient clearance of IAV from the lungs [172]. Elimination of either route of cytotoxicity did not impair effector CD8 T cell mediated viral clearance, however elimination of both pathways increased the duration of infection. Effector CD4 T cells play a supporting role in coordinating the anti-IAV immune response. They produce high levels of cytokines that orchestrate the immune response [176, 177] and provide help to both CD8 T cells and B cells to optimize their response to IAV [178-180].

Clearance of IAV and removal of cognate antigen stimulation causes most of the responding T cells to undergo apoptosis [181]. A small number of T cells develop into long-lived memory T cells. However, most of these cells are kept in a partially activated state, by residual deposits of IAV antigen in the lung [182, 183]. These deposits can persists for several months and provide a low level of TCR stimulation to IAV specific T cells in the draining lymph node. The final clearance of these antigen depots results in a large reduction in the number of IAV specific memory T cells. While antigen depots maintain early memory population in the lung by stimulating a low level of proliferation,
the lung population of IAV specific T cells after depot clearance is maintained by recruitment from the circulating memory population [184].

1.3.7 Antibody responses to Influenza A

B cells and the antibodies they produce are also required for efficient clearance of primary IAV infection [1]. The primary B cell response to IAV begins at the same time as the T cell response, after DCs carrying viral antigen have migrated to the draining lymph node [185, 186]. B cell differentiation and antibody production can occur through both the T cell-dependent in the germinal center and the T cell-independent pathway. The T independent pathway quickly generates mature lower affinity antibody-producing B cells [157]. In contrast, the slower T dependent pathway generates B cells producing high affinity antibodies. T cell help is provided by CD4 T follicular helper cells (Tfh) that recognize the B cells cognate antigen [187]. Tfh cells coordinate B cells activation with both cell-cell interactions, such as CD40L-CD40, and secreted cytokines, such as IFN-γ. This leads to class switching of the B cells to IgG or IgA and somatic hypermutation before differentiation into antibody producing cells [188]. Plasma cell numbers peak around day 7 post-infection and anti-IAV antibody titers peak around 2 weeks post-infection [189].

Similar to effector T cells, IAV specific antibody producing B cells undergo a dramatic reduction in number after viral clearance due to apoptosis. Two populations of long lived IAV-specific B cells are formed following viral clearance, antibody producing
plasma cells and memory B cells [190, 191]. Both populations originate from class switched germinal center B cells [192]. Plasma cells that produce IgG home to and reside in the bone marrow where they continually produce IAV specific antibodies. Plasma cells producing IgA home to the airways where they are able to secrete neutralizing antibody into the airways [193]. IAV memory B cells are found throughout the body, in tissue and secondary lymphoid organs [194]. Similar to memory T cells, this population is primed to quickly respond to reinfection and differentiate into antibody producing cells. This response forms the basis for protection by current IAV vaccines.

1.4 Protection from Influenza A and vaccines

Annual vaccination against seasonal IAV strains is recommended by many governmental health organizations[97]. These vaccinations are highly effective at eliciting a neutralizing antibody response against the IAV strains within that vaccine [97, 195, 196]. The vaccine driven antibodies are able to protect against the target virus for at least several years [197]. However, the primary epitopes targeted by the antibody response are on the highly variable HA and NA proteins. These proteins are highly diverse between IAV strains and immunization with one strain of virus will not protect against unrelated strains. Also, HA and NA can undergo significant genetic drift between seasonal influenza epidemics resulting in a loss of vaccine-conferred protection even to highly similar IAV strains [94]. Importantly, vaccine production must begin months before the start of the annual IAV epidemic. Strains included in the vaccine are those that are predicted to be the dominant strains during the upcoming IAV
season. When the vaccine strains are well matched to seasonal circulating strains that eventually arise, vaccination is highly effective but it is much less effective when vaccine strains are poorly matched to the circulating strains. Well-matched vaccines can reduce influenza-like illnesses by over 70%, while poorly matched vaccines are less than 50% effective [198]. The relatively low effectiveness of the seasonal IAV vaccines demonstrates the need for a universal IAV vaccine.

Generation of a universal IAV vaccine has focused on both a humoral and cellular immune response [199]. Unlike the highly variable epitopes targeted by the humoral immune response, T cells recognize highly conserved IAV epitopes. In humans, T cells primarily respond to epitopes within the M1 and NP IAV proteins [200-202]. These epitopes undergo little genetic drift and are highly conserved across IAV strains [203, 204]. The low variability of T cell epitopes against IAV make generation of a strong memory T cell response an attractive option for making a universal IAV vaccine. Unlike neutralizing antibodies, T cell memory cannot completely block IAV infection [1]. However, T cell memory has been shown to protect both humans and mice. Early work with mouse models demonstrated that transfer of IAV specific T cell clones to naïve mice would protect from lethal IAV challenge [205]. Memory CD8 T cells responses protect from IAV pathology and significantly reduce the duration of infection and levels of IAV virus in the lungs [206]. Similarly, in the absence of pre-existing antibodies, memory CD8 T cells mediate protection from IAV infection in humans [207]. Thus, there is good evidence that the induction of strong CD8 T cell memory to IAV can offer cross serotype immune protection.
The two major types of IAV vaccines in use today are either Inactivated Influenza Vaccines (IIV) or cold adapted Live Attenuated Influenza Vaccines (LAIV) [97]. While IIV are administered by intramuscular injection, LAIV are delivered by inhalation through the nose and result in non-replicative infection of the upper respiratory tract epithelial. While both vaccines are able to generate protection against the immunized strains, there are key differences between the humoral and cellular immune responses. Response to and protection with IIV is primarily determined by a systemic IgG response [97]. However, IIV administration does not generate a strong memory T cell response or a local IgA response in the lung. In contrast LAIV recipients often ‘fail’ to respond to the vaccination based on traditional serum IgG responses. Despite the lack of a systemic humoral response they are still protected from infection with the immunizing strains. Instead, protection is mediated primarily by a local IgA response in the lung [97]. In addition, the LAIV can induce a strong CD4 and CD8 T cell response to conserved viral peptides. In a small study, vaccination with the 2008 seasonal LAIV vaccine, unlike vaccination with IIV, induced a T cell response that was cross reactive to the pandemic 2009 H1N1 strain [208]. It is still unclear how much the memory T cell response contributes to protection in recipients of the LAIV vaccine.

The differences in the immune responses between the IIV and LAIV demonstrate the importance of the antigen, adjuvant and route of immunization to achieve protection. Research into other combinations of antigen, adjuvant and administration routes is
ongoing in an attempt to develop an IAV vaccine that provides long lasting cross-serotype protection.

1.4.1 Novel skin-delivered vaccination strategies

The skin is essential for protection from both environmental insults and infection from pathogens. In addition to presenting a formidable physical barrier to infection, it also contains a network of hematopoietic immune cells, ready to quickly respond to and defend against infections [209]. The skin is composed of the outer epidermal and the inner dermal layers. Each layer contains a unique set of immune cells with distinct functions in the generation of an immune response. This can be critical in determining the response to and efficiency of vaccines. This is clearly demonstrated in the case of Vaccinia immunization. The vaccine is delivered into the epidermis by scarification. When delivered by this route, it is highly effective at generating a protective immune response [210]. If delivered by intradermal administration the vaccine can generate an effective immune response, however, this is dependent on the development of a pox lesion. If the vaccination bypasses the skin by intramuscular administration, there is no effective immune response to the virus. This indicates that the involvement of the epidermis is critical for generation of a protective immune response.

1.4.2 Inflammation and initiation of adaptive immune responses in the skin
The epidermis, the outer layer of the skin, is primarily composed of keratinocytes. In addition to their barrier role, keratinocytes can recognize and respond to invading pathogens to induce and coordinate innate and adaptive immune responses [209]. Keratinocytes express an array of PRRs that recognize invading pathogens and signaling to activate the keratinocytes. Once activated, keratinocytes can secrete chemokines and inflammatory cytokines such as TNF-α and IL-1α [211-214]. The dermis is mainly composed of dermal fibroblasts. Similar to keratinocytes, these cells can be activated by either microbial products or inflammatory cytokines. Activated fibroblast can release cytokines and chemokines to mediate the immune response. These secreted products only partially overlap with those secreted by keratinocytes, and thus activation of dermal fibroblasts can both enhance and refine the immune responses initiated by keratinocytes [215].

The innate response from keratinocytes and fibroblasts mediates recruitment of additional immune cells to the skin and activation of skin-resident immune cells. This includes several populations of skin-resident dendritic cells. Langerhans Cells (LC) are professional APCs found on the basal side of the epidermis [216]. Once activated LCs can sample antigen throughout the epidermis by breaking tight junctions between keratinocytes [217]. Activated LCs can migrate to skin draining lymph nodes and efficiently present antigen to activate both CD4 and CD8 T cells [218]. Paradoxically, while LCs generate a strong cytotoxic CD8 T cell response, they preferentially generate Th2 CD4 cells that produce high levels of IL-4, however their activity may also depend on maturation signals they receive [218, 219].
Populations of resident DCs are also found in the dermis. Like LCs, these professional APCs are able to migrate to the lymph node and present antigen. However, they localize to germinal centers in draining lymph nodes and thus mediate the generation of humoral responses and are inefficient at generating cytotoxic T cell responses. Additionally, while LCs generate strong Th2 responses, dermal DCs tend to skew the immune response towards a Th1 phenotype [218]. The spatial and cellular separation of the ability to induce a strong Th1 or Th2 immune response can allow precise targeting of a vaccine to generate the desired protective immune response.

1.4.3 The potential of transcutaneous immunization

The generation of tissue specific memory responses is increasingly recognized as necessary to induce optimal protection from reinfection. This can be seen in the protection elicited by both the Vaccinia vaccine and the LAIV [97, 210]. However, vaccination at mucosal sites often produces only local immunity and fails to generate systemic responses that can contribute to protection, as is the case for LAIV [97]. Transcutaneous (TC) immunization, where part or all of a vaccine is topically applied to the skin, can generate strong global humoral and cellular memory responses. TC immunization elicits a strong systemic humoral response in humans and mice [220, 221]. Importantly, TC immunization can result in humoral immunity in distal mucosal sites. Vaccination with a membrane protein from Chlamydia results in secretion of chlamydia specific IgG and IgA into the vaginal tract [222]. Similarly, after TC
immunization with cholera toxin, not only is a strong sera IgG responses induced, but cholera specific IgG and IgA are detected in airways and stool as well [223]. This indicates that TC immunization can generate strong systemic immune responses that also can be directly protective at other barrier sites.

Strong cellular immunity can also be induced by TC immunization. Skin infection generates a population of resident memory CD8 T cell throughout the skin, and not just at the local site of infection [224]. These cells are long lived and are able to protect against reinfection anywhere on the skin. Strong CTL responses can also be detected in distal mucosal sites, including Peyer’s patches and in the lungs after immunization with an HIV peptide vaccine [225]. Similarly, exposure to Schistosoma mansoni eggs in the skin can induce a protective CD4 T cell response in the lungs [226]. These results suggest that TC immunization may be able to generate a strong CTL response that could protect against a broad range of IAV strains.

As described above, induction of systemic and mucosal immunity from skin infections and TC immunization is well documented. As such, it is a subject that is currently under intense study to try to migrate results from the lab into the clinic. Many clinical trials are underway to investigate the clinical utility of TC immunization against many diverse pathogens [220].

Several groups have evaluated the efficacy of transcutaneous immunization against IAV. Immunization with PR8 with a cholera toxin adjuvant induced strong and
protective IgG responses in mice and several groups have shown the induction of protection with DNA based IAV vaccines [227-229]. Importantly, a small human study showed that transdermal immunization generates both memory CD4 and CD8 T cell responses [230]. Thus transdermal immunization against IAV holds strong potential for strong cellular immune responses that may be protective across serotypes.

1.4.4 TLR9 and CpG oligodeoxynucleotides

The pattern recognition receptor TLR9 is responsible for recognition of unmethylated CpG oligodeoxynucleotide (CpG ODN) motifs in bacterial DNA [231, 232]. TLR9 is an endosomally located TLR and requires uptake of CpG ODN before binding and signaling are able to occur [233]. This uptake is independent of the CpG motifs in the CpG ODN. In the endosome, TLR9 is cross-linked by multiple CpG motifs within the same ODN and begins signaling [232]. TLR9 signals through both MyD88 dependent and independent mechanisms. MyD88 dependent signaling proceeds through IRAK1 and TRAF6, and is independent of TIRAP [234]. Interestingly, high levels of CpG ODN stimulation induces activation of TRIF, which induces a tolerogenic phenotype in DCs [235].

Expression of TLR9 is widespread in the immune system and is expressed by neutrophils, macrophages B cells and some DC subsets, including Langerhans cells, during steady state [236-239]. Interestingly, TLR9 is also widely expressed by epithelial cells including intestinal epithelial cells and keratinocytes [240-242]. In both of these cell
types, signaling through TLR9 can be critical in the induction of initial immune responses by these cells.

1.4.5 CpG ODN as a vaccine adjuvant

Effective vaccines need to stimulate the innate immune response to generate protective memory to the infectious agent. Activation of innate immune responses usually requires stimulation through PRR. Innate immune activation can be mediated directly by the immunizing agent or by an added adjuvant. Vaccines using inactivated or attenuated pathogens as immunizing antigen contain microbial products that can directly stimulate the innate immune system while subunit or conjugate vaccines do not and require an adjuvant to stimulate an innate immune response. The two adjuvants that are currently in use in approved vaccines are aluminum hydroxide (Alum) and monophosphoryl lipid A, a TLR4 agonist [243]. Interestingly, the mode of action of alum is not well understood, however it appears to be independent of PRR signaling. Adjuvants may also be useful to modulate the immune response to a vaccine to elicit the desired, protective response. Alum preferentially skews the immune response to a Th2 phenotype, while monophosphoryl lipid A generates a Th1 response [244-246]. CpG ODN signaling through TLR9 is currently being investigated for its utility as an adjuvant and an immune modulator [220]. CpG ODN treatment tends to stimulate a strong Th1 response, which can involve generation of CD4, CD8 and humoral immune responses. This can form long lasting and protective memory when administered by a variety of treatment routes [243, 247].
CpG ODN therapy has also proved effective in diverse disease settings including cancer, asthma, and viral infections. In cancer settings, CpG ODN therapy on its own may have therapeutic potential. CpG ODN treatment in addition to normal therapies can increase their anti-tumorigenic efficacy [248-251]. Addition of CpG ODN to monoclonal antibody treatments can boost efficacy by enhanced activation of NK cells, including increased cytotoxic potential and IFN-γ production [252, 253]. CpG ODN treatment is also able to increase the efficacy of chemo- and radiotherapy against cancer [251]. Although the exact mechanism of action in humans is not established, it is believed that CpG ODN effectively activates DCs to produce cytotoxic CD8 T cells that can kill tumour cells [254]. In keeping with the ability of CpG ODN to induce a Th1 response, it is a promising therapy in the treatment of allergies and asthma. CpG ODN coupled to antigen can reverse established asthma and prevent allergic responses in mice and humans [255-258]. While this is in part due to its Th1 skewing effect on CD4 T cell responses, CpG ODN can directly inhibit B cell class switching and production of IgE via multiple molecular mechanisms [256, 259].

1.5 Research aims and rationale

The proper development and response of the immune system is critical to protect against infections. IL-7 plays a central role in T cell development and homeostasis. While disruptions in IL-7 signaling result in SCID, overactivation of IL-7 signaling can drive leukemias and lymphomas. As IL-7 therapy is currently in clinical trials as a treatment for lymphopenia and to boost immune responses to chronic infections, it is
important to understand when, where, and how IL-7 may act in T cell development and responses.

IAV infection causes yearly epidemics with a large human and economic impact. Additionally, novel IAV strains can arise through genetic shift and cause widespread, pandemic infection with higher pathology than normal seasonal IAV strains. This pathology is due to both the viral infection and the immune response to that infection. Understanding how the immune system responds to IAV may help develop better preventative vaccines or therapies for those infected.

**Aim 1: IL-7 signaling controls T cell development at the transition from the bone marrow to the thymus**

Although there are several reported roles for IL-7 in thymocyte development it is still unclear where the earliest role of IL-7 occurs. To address this, and other questions, we used a previously established mouse model of hypomorphic IL-7Rα function by knock-in mutagenesis of the conserved IL-7Rα Y449xxM motif (IL-7Rα<sup>449F</sup>) [260]. In Chapter 2, we hypothesized that IL-7 has previously unappreciated roles in the development of the ETP population. We sought to identify the earliest role for IL-7 signaling in T cell development and the mechanisms by which it regulates such development. We showed that IL-7 is not necessary for early lymphoid development in the bone marrow, but was essential for the development of ETPs in the thymus. IL-7 did not regulate the ETP by influencing either survival or proliferation of ETPs or their
progenitors. Interestingly, although there was a requirement for direct IL-7 signaling in ETP development, ETPs do not express IL-7Rα and thus it is likely that IL-7 signaling at earlier developmental stages influences ETP development.

**Aim 2: Interleukin-7, but not thymic stromal lymphopoietin, plays a key role in the T cell response to Influenza A virus**

The immune response to viral infection is ideally rapid and specific, resulting in viral clearance and establishment of immune memory. While IL-7 has a critical role in T and B cell development, treatment with IL-7 has recently been shown to aid the adaptive T cell response in clearance of chronic viral infection. In contrast, the IL-7-related cytokine thymic stromal lymphopoietin (TSLP) has a limited role in lymphocyte development but is important in the immune response to parasitic worms and allergens. The role for these cytokines in the immune response to an acute viral infection is unclear. In Chapter 3, we investigated the role for IL-7Rα signaling in the primary T cell response to IAV infection. We found that IL-7, but not TSLP, played an important role in control of influenza A virus. We also showed that IL-7 signaling was necessary for the generation of a robust influenza A-specific CD4 and CD8 T cell response and that this requirement was intrinsic to CD8 T cells. This implied that IL-7 plays a critical role in the generation of CD8 effector T cell responses.
Aim 3: Topical CpG adjuvant enhances protection against influenza A infections

Although current IAV vaccines can induce protection against the specific strains in the vaccine, they are not generally protective against the broader range of IAV strains. This is due to diversity in the antigens that the adaptive humoral immune system responds to. In contrast, the epitopes that T cells recognize and respond to on IAV are highly conserved. Thus, a strong memory T cell response to IAV may be able to protect against a broad range of IAV strains. Transcutaneous immunization with adjuvant CpG ODN has shown promise in not only generating local immune protection, but protection at other barrier sites as well. In chapter 4, we hypothesized that CpG ODN may be an effective adjuvant when delivered topically on the skin. We tested if TC immunization with CpG ODN can induce a strong and protective T cell response against IAV infection in the lung. We found that epicutaneous application of CpG ODN generates a stronger and longer-lived memory T cell response to the vaccinating antigen than subcutaneous injection of CpG ODN. These T cells were able to respond to and protect from IAV infection in the lungs. This demonstrates that transcutaneous immunization against IAV with adjuvant CpG ODN may be effective at generating protective immune memory against a broad range of IAV strains.
Chapter 2: IL-7 signaling controls T cell development at the transition from the bone marrow to the thymus

2.1 Introduction

IL-7 signaling is essential for early T cell development in both humans and mice where loss of function of essential proteins in the IL-7 signaling pathway results in SCID in humans [2]. Several specific roles for IL-7 have been identified during T cell development. For example, IL-7 in cooperation with Notch signaling, mediates survival and proliferation in DN2 thymocytes [81]. IL-7 also mediates survival of DN3 thymocytes and delays reorganization of the TCRα chain in DN4 thymocytes [82]. IL-7 continues to play important roles later in T cell development and homeostasis; however, these are the earliest characterized roles for IL-7 during T cell development.

IL-7Rα expression is dynamically regulated throughout T cell development. In the bone marrow, IL-7Rα is not expressed on HSC, MPPs, or LMPPs and is first expressed by CLPs [3]. The LMPP and CLP populations are the likely source of bone marrow populations that migrate to and seed the thymus [18]. IL-7Rα is not expressed on the earliest identifiable thymocyte population, the ETPs, whereas it is re-expressed from the DN2 stage until the DN4 stage of thymocyte development [3]. Interestingly, lineage-tracing studies demonstrated that over 90% of ETPs in the thymus had once expressed IL-7Rα although they no longer do at that stage [22]. Although IL-7Rα is expressed on
some T cell progenitors before the DN2 stage, the role for IL-7 signaling in these progenitors is unknown.

The central role of IL-7 in the development and homeostasis of T cells has led to clinical trials examining IL-7 as a therapy to recover T cell numbers and support immune reconstitution after chemotherapy and for HIV induced lymphopenia [61]. These studies demonstrated that IL-7 therapy was clinically useful to boost T cell function or restore numbers after a period of lymphopenia to re-establish immunocompetence. IL-7 was also required for effective T cell responses to pathogens and tumours. We found that IL-7 signaling was necessary for primary T cell response to acute viral and bacterial infections [260, 261]. Treatment with exogenous IL-7 boosted the magnitude and effectiveness of chronic T cell response to both viral infections and tumours [262, 263]. IL-7 therapy is currently being evaluated in phase I and II clinical trials for treatment of tumours and chronic viral infections including renal cancer, HCV and HIV [264-267]. Understanding where and how IL-7 influences T cell development is critical for optimization of the efficacy of these therapies and to determine and mitigate any potential side effects.

IL-7 signaling is not only important for normal T cell development but also plays a role driving T cell leukemias and lymphomas. Gain of function mutations resulting in overexpression of the IL-7 cytokine or activation of IL-7Rα signaling are found in a significant proportion of leukemia and lymphomas, and are correlated with poor prognosis [268-271]. Uncontrolled IL-7Rα signaling causes increased expression of IL-7
regulated survival and proliferative factors, including Bcl-2 [272]. These gain of function mutations include Cys insertions in the IL-7Rα extracellular or transmembrane domain, which cause constitutive, ligand independent signaling [273]. These mutations can drive progression of ETP acute lymphoblastic leukemia (ETP-ALL), T cell ALL and B cell ALL in humans [268, 273]. Enforced expression of the gain-of-function IL-7Rα mutants in mice also results in the formation of ETP-ALL [274]. This role of IL-7 signaling in ETP-ALL formation is unexpected, as ETPs do not normally express IL-7Rα. As there is no known role for IL-7 signaling in the development of pre-thymic T cell progenitors, this could indicate that there is an unknown role for IL-7 signaling early in T cell development. Alternatively, it suggests that therapeutic use of IL-7 may influence early T cell development in the bone marrow.

We hypothesized that IL-7 influences early T cell development prior to its established roles mediating proliferation and survival in DN2-4 cells. How IL-7 controls the ETP and earlier T cell progenitor populations is directly relevant to the clinical application of IL-7 to aid T cell reconstitution. It may also inform the use of IL-7 therapy to boost T cell function and provide mechanistic insight to tumorigenesis driven by aberrant IL-7Rα signaling.

We investigated the role of IL-7Rα signaling in early T cell development using genetic mouse models that lack IL-7Rα completely (IL-7Rα−/−) or express a mutant hypomorphic allele IL-7Rα (IL-7Rα449F) that cannot activate STAT5 in response to IL-7 [260]. We examined early hematopoiesis populations in these mice directly \textit{ex vivo} and
in competition with WT cells in mixed bone marrow chimeras. We also investigate the mechanism of IL-7 action on these cells by examining progenitor populations for their ability to survive and proliferate.

2.2 Results

2.2.1 IL-7Rα signaling is not required in early hematopoiesis

To investigate the earliest role for IL-7 in T cell development, we examined the lymphoid progenitor populations in the bone marrow in WT, hypomorphic IL-7Rα449F and IL-7Rα−/− mice. All lymphoid progenitors in the bone marrow are within the Lin− population. The most immature progenitors also express high levels of c-kit and Sca, and are referred to as LSK cells [7]. The LSK population includes HSCs and their progeny, the LMPPs. These two cell types can be distinguished by high the expression of Flt3 on LMPPs. LMPPs are one candidate population for the bone marrow cells that seed the thymus under homeostatic conditions. LMPPs can develop into CLPs, another candidate population for bone marrow cells that seed the thymus [18]. CLPs are found in the Lin− c-kitlo Scalo population and express high levels of both Flt3 and IL-7Rα. We found that the frequency of both LSK cells and LMPPs was indistinguishable between WT, IL-7Rα449F and IL-7Rα−/− mice (Figure 3A). Similarly, the number of LMPPs per femur was identical between WT, IL-7Rα449F and IL-7Rα−/− mice (Figure 3B). We also examined the frequency and number of CLPs in
Figure 3: Bone marrow progenitor populations are independent of IL-7Rα signaling.
Bone marrow from WT, IL-7Rα^449F and IL-7Rα^-/- mice was harvested and progenitor cells were stained for flow cytometry. (A) Representative flow cytometry plots and frequencies of LMPPs and CLPs in WT, IL-7Rα^449F and IL-7Rα^-/- mice. (B) Quantification of total number of LMPPs and CLPs per femur in WT, IL-7Rα^449F and IL-7Rα^-/- mice. Representative of three independent repeats, n=3-5.
WT and IL-7R$\alpha^{449F}$ bone marrow. As with the earlier progenitor populations, there was no difference in the frequency or number of these cells between WT and IL-7R$\alpha^{449F}$ mice (Figure 3A, B). As IL-7R$\alpha$ is a defining marker of CLPs, it is difficult to evaluate this population in IL-7R$\alpha^{-/-}$ mice. However, when we examine the Lin$^{-}$ c-kit$^{lo}$ Sca$^{lo}$ Flt3$^{+}$ population, which includes the IL-7R$\alpha^{+}$ CLPs, we found no difference in the number of these cells in IL-7R$\alpha^{-/-}$ mice indicating that CLP development was not impaired. Overall, partial or total loss of IL-7R$\alpha$ signaling did not result in a diminished number of lymphoid progenitors in the bone marrow. This suggests that IL-7R$\alpha$ signaling was not required for the development of T cell progenitors at this stage.

2.2.2 Early Thymic Progenitors are highly dependent on IL-7R$\alpha$, but not TSLPR signaling

Since IL-7R$\alpha$ signaling was not required for development of bone progenitors, we examined thymocyte development for the earliest stage that requires IL-7R$\alpha$ signaling. We assessed T cell progenitor populations in WT, hypomorphic IL-7R$\alpha^{449F}$ and IL-7R$\alpha^{-/-}$ mice. Within the DN thymocytes, the earliest progenitors can be identified by the expression of high levels of CD44 and c-kit. Within this population, the first identifiable T cell precursors in the thymus, the ETPs, are distinguished from DN2 thymocytes by the lack of expression of CD25. We found that the far fewer ETPs in IL-7R$\alpha^{449F}$ mice and a near total loss of ETPs IL-7R$\alpha^{-/-}$ mice compared to WT ETP populations (Figure 4A). In comparison to WT mice, the frequency of ETPs in IL-7R$\alpha^{449F}$ and IL-7R$\alpha^{-/-}$ mice was reduced by 4-fold and 10-fold, respectively (Figure 4B). However, as total thymic
Figure 4: IL-7 signaling is essential for the development of ETPs.
Flow cytometry plots of ETP populations from WT, IL-7Rα449F and IL-7Rα/- mice (A). Quantification of frequency and total number of ETPs in the thymii of WT, IL-7Rα449F and IL-7Rα/- mice (B). Representative flow cytometry plots of ETP populations from WT and TSLPR/- mice (C). Frequency and number of ETPs in WT and TSLPR/- mice (D). Representative of at least three independent repeats. n=3-5 per group. *p<0.05.
cellularity was also reduced in these mice, the total number of ETPs was reduced 20- and 100-fold in IL-7Rα^{449F} and IL-7Rα^{-/-} mice, respectively (Figure 4B).

As IL-7Rα is shared as part of the heterodimeric receptors for both IL-7 and TSLP, impaired signaling of either cytokine could be responsible for the observed reduction in numbers of ETPs in IL-7Rα^{449F} and IL-7Rα^{-/-} mice. To determine which cytokine was necessary for ETPs, we compared the ETP population in WT and TSLPR^{-/-} mice (Figure 4C). TSLPR^{-/-} mice have normal IL-7 signaling but are unable to bind TSLP and initiate signaling. In the absence of TSLPR, no reduction in the frequency or number of ETPs was observed (Figure 4D), which suggests that IL-7 but not TSLP signaling was essential for development of the ETP population.

2.2.3 The bone marrow to ETP transition requires cell intrinsic signaling through IL-7Rα Tyr449

Although ETPs do not express IL-7Rα, they appear to be the earliest cell T cell progenitor population that requires IL-7 signaling. It remained unclear if ETPs or their progenitors required direct IL-7 signaling or if IL-7 signaling in another cell type was necessary to facilitate ETP development. We set up mixed bone marrow chimeras to test if direct or indirect IL-7 signaling was required. Whole bone marrow from CD45.1 BoyJ mice was mixed in a 1:1 ratio with either CD45.2 WT or CD45.2 IL-7Rα^{449F} bone marrow and injected intravenously into lethally irradiated WT hosts. Bone marrow and
thymii from these mice were harvested 6-8 weeks later and T cell progenitor populations were analyzed for the relative contribution from each donor.

In mice that received a 1:1 mixture of CD45.1 BoyJ: CD45.2 WT bone marrow, there was equal contribution of each genotype to T cell progenitor populations including HSCs, LMPPs, CLPs, ETPs and DN2s (Figure 5). Similarly, in mice that received CD45.1 BoyJ: CD45.2 IL-7Rα^{449F} bone marrow, both donor populations contributed equally to the HSC and LMPP populations (Figure 5). This was expected as neither of these populations express IL-7Rα, or had ever expressed IL-7Rα, making any direct effects of IL-7 on these populations very unlikely. As IL-7Rα is a defining marker of CLPs, we hypothesized that IL-7Rα^{449F} cells may show a failure to compete with WT cells. Interestingly, IL-7Rα^{449F} and WT cells contributed equally to the CLP population and thus IL-7Rα Tyr449 signaling does not appear to be required for the development of CLPs.

In contrast to the bone marrow populations, IL-7Rα^{449F} cells failed to compete with WT cells at the ETP stage, with IL-7Rα^{449F} cells making up less than 10% of the ETP population (Figure 5). This demonstrated a cell intrinsic requirement for IL-7Rα Tyr449 signaling for the formation of the ETP population despite the absence of IL-7Rα expression on these cells. The competitive disadvantage of the cells at the ETP stage is remains at DN2 stage, and IL-7Rα^{449F} cells also make up less than 10% to this population (Figure 5). Interestingly, although DN2 cells express IL-7Rα and require IL-7 signaling for survival, IL-7Rα^{449F} cells are not further outcompeted by WT cells at the
Figure 5: Direct IL-7 signaling is required for the transition between bone marrow and thymic T cell progenitors.

Mixed bone marrow chimeras were generated by injecting a 1:1 ratio of either WT:BoyJ or IL-7Rα<sup>449F</sup>:BoyJ bone marrow into lethally irradiated WT recipients. Bone marrow and thymii were harvested 6-8 weeks later and analyzed for the relative donor contribution to T cell progenitor populations. Data is displayed as the Log2 transformation of the ratio of CD45.2/CD45.1 cells in each population. Representative of three independent experiments. n=5-7 per group. *p<0.05.
DN2 stage than at the ETP stage. These results demonstrated that hypomorphic IL-7Rα^{449F} bone marrow progenitors can efficiently compete with WT cells during early development in the bone marrow. However, IL-7Rα^{449F} cells fail to compete with WT cells to develop into ETPs. This shows a cell intrinsic requirement for IL-7 signaling for development between the bone marrow progenitors and the ETPs.

2.2.4 IL-7Rα Tyr449 signaling does not regulate survival of thymocyte progenitors

IL-7 signaling mediates cell survival in different cell types, including in pre- and pro-B cells, DN2 and DN3 thymocytes and mature T cells [3, 81, 82]. Signaling through IL-7Rα Tyr449 has also been shown to partially regulate expression of the key IL-7 regulated pro-survival protein Bcl-2 [260]. We hypothesized that IL-7 signaling in IL-7Rα expressing progenitors may mediate survival of ETPs. To test this, we examined the expression of IL-7 regulated survival factor Bcl-2 in ETPs and CLPs in IL-7Rα^{449F} mice. As IL-7 driven Bcl-2 expression protects DN2 thymocytes from apoptosis, DN2 cells were used as internal positive controls for detection of IL-7 dependent Bcl-2 expression and survival.

Interestingly, there was no difference in expression of the IL-7 driven survival factor Bcl-2 in the CLP or ETP populations between WT and IL-7Rα^{449F} mice (Figure 6A). In contrast at the DN2 stage there was a significant decrease in Bcl-2 expression in
Figure 6: IL-7Rα Tyr449 signaling does not regulate survival of ETPs.
(A) Expression of Bcl-2 in T cell progenitors from WT and IL-7Rα^{449F} mice. Representative flow cytometry plots and MFI quantification of Bcl-2 expression in CLPs, ETPs, and DN2s. WT – solid line, IL-7Rα^{449F} – Dashed line, Isotype – filled histogram.
(B) Frequency of T cell progenitors from WT and IL-7Rα^{449F} with active Caspase3/7. Representative of three independent experiments. n=3-5. *p<0.05.
IL-7Rα^{449F} DN2 cells (Figure 6B). This suggests that although IL-7Rα Tyr449 signaling can drive Bcl-2 expression in some cell types, it is not required for Bcl-2 expression in bone marrow progenitors or ETPs.

We also directly assessed the frequency of ETPs and CLPs that were undergoing apoptosis. We examined the activity of Caspase 3 and 7, which are activated in early apoptotic cells. We incubated cells with a substrate for Cas3/7 that would cause cells to fluoresce when cleaved. We examined the frequency of cells with Cas3/7 activity ex vivo from WT and IL-7Rα^{449F} mice. There was no difference in the frequency of cells with Cas3/7 activity in either the CLP or the ETP population between WT and IL-7Rα^{449F} mice (Figure 6B). As expected, IL-7Rα^{449F} DN2 cells had an increase frequency of cells with active Cas3/7 compared to WT. Consistent with of our findings on Bcl-2 regulation, these results demonstrated that while IL-7Rα Tyr449 signaling is important for protection from apoptosis in some cell types it is not required for the survival of ETPs or bone marrow progenitors. Thus, the requirement of IL-7Rα Tyr449 signaling for ETP development is not due to survival driven by this signaling motif and suggests that IL-7Rα Tyr449-dependent signaling regulate ETP population by another mechanism.
2.2.5 IL-7Rα Tyr449 signaling does not regulate proliferation of thymocyte progenitors

Upon entering the thymus, progenitor cells immediately receive Notch signaling which stimulates proliferation to expand the ETP population. IL-7 signaling can induce proliferation in developing lymphocytes and mature T cells. Furthermore, IL-7 and Notch signaling cooperate to control proliferation at the DN2 stage of thymocyte development \[81\]. We asked whether the requirement for IL-7Rα Tyr449 signaling in ETP development was due to proliferative signals mediated by this motif. To determine whether IL-7Rα Tyr449 signals were necessary for proliferation of T cell progenitor populations, we used an in vivo EdU incorporation assay to compare the rate of proliferation in the ETP and CLP populations in WT and IL-7Rα\(^{449F}\) mice. Bone marrow and thymii from WT and IL-7Rα\(^{449F}\) mice were harvested 24 hours after intraperitoneal injection of 2mg of EdU. Cells were stained for surface markers and EdU incorporation into the DNA. There was no difference in EdU incorporation in either the CLP or ETP populations between WT and IL-7Rα\(^{449F}\) mice (Figure 7A, B). Consistent with previous studies that demonstrated IL-7 signaling mediates proliferation in DN2 cells, there was reduced proliferation in IL-7Rα\(^{449F}\) DN2 cells compared to WT (Figure 7C).

This demonstrated that IL-7Rα Tyr449 signaling was not required proliferation of CLP and ETP cells and therefore the requirement of IL-7Rα Tyr449 signaling for ETP development was not due to proliferative signals downstream of this motif.
Figure 7: IL-7Rα Tyr449 signaling does not regulate proliferation of ETPs.
WT and IL-7Rα^{449F} mice were injected with 2mg EdU and bone marrow and thymocytes were analyzed for EdU incorporation 24 hours later. EdU incorporation in (A) CLPs, (B) ETPs, and (C) DN2 thymocytes from WT and IL-7Rα^{449F} mice. Representative of three independent experiments. n=4. *p<0.05.
2.3 Discussion

Although it is known that IL-7 signaling is essential for T cell development in both mice and humans, it has been unclear where the earliest role for IL-7 occurs. IL-7Rα is expressed on some bone marrow progenitor populations, including CLPs, and nearly all ETPs in the thymus have historical expression of the receptor [3, 22]. Therefore, we hypothesized that IL-7Rα signaling may play a role at these stages of thymocyte development.

Our results showed that IL-7 signaling was not required for the development of T cell progenitor populations in the bone marrow. Normal numbers of HSCs, LMPPs and CLPs were present in the bone marrow of mice with impaired or blocked IL-7 signaling. No developmental impairment was detected in IL-7Rα^{449F} cells even when placed in competition with WT cells. This clearly demonstrated the lack of a requirement for either direct or indirect IL-7Rα Tyr449 signaling for the development of these bone marrow progenitor populations.

Interestingly, although there appears to be no role for IL-7Rα Tyr449 signaling in regulating the total size of the CLP population, we have previously shown that IL-7Rα^{449F} mice have a lower frequency of CLPs that express Ly6D [275]. Ly6D expression divides CLPs into two functionally distinct populations, where Ly6D^- CLPs have near equal potential for all lymphoid lineages but Ly6D^+ CLPs are primed to differentiate into B cells [3]. This is likely due to the IL-7 driven induction of Ebf-1 in
CLPs, which is essential for early B cell development [80]. Interestingly, the relative increase in Ly6D- CLPs compared to Ly6D+ CLPs in IL-7Rα<sup>449F</sup> mice may suggest that this population may be more likely to differentiate down the T cell development pathway [275].

We have demonstrated that the ETP population is highly sensitive to changes in IL-7 signaling despite not expressing IL-7Rα. While total loss of IL-7 signaling resulted in 100-fold fewer ETPs, even the selective loss of IL-7Rα Tyr449 signaling caused the ETP population to shrink 20-fold compared to the ETP population in WT mice. TSLP signaling through IL-7Rα was not necessary for ETP development as TSLPR<sup>-/-</sup> mice had normal numbers of ETPs. Therefore, it was the loss of IL-7 signaling, and not TSLP signaling, in IL-7Rα<sup>-/-</sup> and IL-7Rα<sup>449F</sup> mice that resulted in the reduction of ETPs.

The difference in ETP numbers between IL-7Rα<sup>-/-</sup> and IL-7Rα<sup>449F</sup> mice suggests that IL-7 signaling may regulate the ETP population through more than one molecular mechanism. Phosphorylation of IL-7Rα Tyr449 was essential for IL-7 dependent activation of STAT5, and the reduction in ETPs in IL-7Rα<sup>449F</sup> mice may be due to reduced STAT5 activation. However, the further impairment of the ETP population size in IL-7Rα<sup>-/-</sup> mice compared to IL-7Rα<sup>449F</sup> mice suggest that IL-7 signaling also regulates the ETP population via a STAT5 independent mechanism.

Since ETPs do not express IL-7Rα but appear to be the earliest lymphoid subset to require IL-7 signaling, it was unclear how IL-7 signaling regulated the ETP population.
One model holds that IL-7 signaling in direct progenitors to ETPs is either permissive or instructive for their differentiation into ETPs. Alternatively, it was possible that the ETP population was mediated indirectly through another IL-7 responsive cell type. IL-7 acts directly to promote the survival and proliferation of DN2 thymocytes and mice with impaired IL-7 signaling have far fewer of these cells. An third alternative model is that the paucity of DN2 cells, or other thymocytes, caused ETPs to progress to the DN2 stage quicker, resulting in fewer ETPs in the thymus and an apparent block in development at the ETP stage.

We examined mixed bone marrow chimeras with IL-7Rα449F in competition with WT cells to test the three models described above. These mixed bone marrow chimeras demonstrated that there was a requirement for intrinsic IL-7 signaling for the transition between the bone marrow progenitors and ETPs in the thymus. When placed in competition with WT cells in mixed bone marrow chimeras, IL-7Rα449F cells contributed to all bone marrow progenitor populations equally with WT. However, IL-7Rα449F cells failed to compete with WT at the ETP stage. If IL-7 were acting indirectly on another cell type to regulate the ETP population, both WT and IL-7Rα449F cells would have developed in the same environment and contributed equally to the ETP population. Thus, these results clearly demonstrated that IL-7 signaling on ETPs or their direct precursors was essential for the formation of the ETP population.

Although these experiments demonstrated that direct IL-7 signaling was required for the development of ETPs, it remained unclear during which stage of development
that signaling was necessary. ETPs do not express IL-7Rα, and so do not directly recognize, respond and signal downstream of IL-7. However, most ETPs derive from a progenitor that once expressed IL-7Rα [22]. Thus it appears likely that IL-7 signaling on a progenitor to ETPs may be necessary for those cells to develop into ETPs or that signaling in those progenitors is required to program ETPs to fill the niche or survive. This had previously been shown in B cell development, where IL-7 signaling in the progenitor cells, CLPs, induced expression of transcription factor Ebf-1 [80]. This was not necessary for B cell development at the CLP stage or for the development into pre-pro B cells but it was required for the survival of pre-pro B cells and their subsequent development toward mature B cells. Thus, although ETPs do not express IL-7Rα, it is possible that IL-7 signaling in their IL-7Rα expressing progenitors have a lasting effect on their survival or proliferation. IL-7 signaling can drive survival and proliferation in different cell types, including many within the T cell lineage. IL-7Rα Tyr449 signaling was partially responsible for Bcl-2 expression in T cells and thymocytes, including DN2 thymocytes [260]. However, this did not appear to be the case in ETP development. ETPs from IL-7Rα<sup>449F</sup> mice were not impaired in their ability to survive or expand. Thus, our results demonstrate that it is likely that IL-7 signaling is required for the development of ETPs, rather than the maintenance of the population.

Interestingly, IL-7 signaling has been implicated in survival of DN1 thymocytes in other studies. Loss of the transcription factor Miz1 resulted in a large reduction in all subsets of early thymocytes [276]. The authors demonstrated that this was due to impaired survival, which was correlated with decreased expression of both IL-7Rα and
Bcl-2. This could indicate that other, IL-7Rα dependent but Tyr449 independent IL-7 signaling events are important for ETP expression of Bcl-2 and survival. It is also possible that Miz1 mediated regulation of IL-7Rα is coincidental and not related to its role in thymocyte survival. Importantly however, all ETPs are DN1 thymocytes, but the entire DN1 population is heterogeneous and contains cells at other differentiation steps. Taken together, we have demonstrated a cell intrinsic requirement for IL-7 signaling in the development of the ETP population, despite its lack of IL-7 receptor expression. Interestingly, this was not due to IL-7 mediated survival or proliferation and therefore it is likely that IL-7 signaling is necessary for development of progenitors into ETPs. We hypothesize that IL-7 signaling was required for bone marrow progenitors fail to migrate to the thymus to seed the ETP population. We are currently testing if progenitors to migrate out of the bone marrow, fail to enter the thymus, and if the defect in ETP development can be bypassed by placing bone marrow progenitors directly into the thymus. In summary, our results demonstrated a previously uncharacterized role for IL-7 in early T cell development.

2.4 Materials and methods

Mice: Mice were housed at the UBC Centre for Disease Modelling facility, and all work was carried out according to University of British Columbia Animal Care and Biosafety Committee guidelines. C57BL/6 and BoyJ (B6.SJL-Ptprc<sup>a</sup> Pepc<sup>b</sup>/BoyJ) mice were obtained from the Jackson Laboratory (Bar Harbour, MA). IL-7Rα<sup>449F</sup> mice have a
mutant form of the IL-7Rα expressing a single amino acid mutation from Tyr to Phe at position 449 as previously described [260]. TSLPR−/− mice [277] were a gift of Dr. James Ihle (St. Jude Children’s Research Hospital, Memphis, TN). All mouse strains have been backcrossed at least ten times with C57BL/6 mice. Age- and sex-matched mice between 6 and 10 weeks of age were used for all experiments.

**Flow cytometry:** Cells were stained on ice with antibodies purchased from BD Bioscience (San Diego, CA), eBioscience (San Diego, CA) or BioLegend (San Diego, CA). Cells were fixed and permeabilized with the FoxP3 fix/perm kit (eBioscience) before staining for intracellular antigens. Analysis of flow cytometry data was done using FlowJo software (Tree Star).

**Mixed bone marrow chimeras:** Wild-type recipient mice were irradiated with two doses of 6.5Gy four hours apart and then injected 24 hours later with a 1:1 mixture of IL-7Rα449F:BoyJ or WT:BoyJ whole bone-marrow. Bone marrow and thymii were harvested and cell populations analyzed by flow cytometry 6-8 weeks later.

**Caspase-3/7 activity:** Cells from bone marrow and thymus were incubated in suspension with CellEvent Casapse-3/7 (Invitrogen) for 30 minutes at 37°C. Cells were then stained prepared for and analyzed by flow cytometry, as above.

**In vivo EdU labeling:** Mice were intraperitoneally injected with 2mg/kg EdU (5-ethynyl-2’-deoxyuridine, Sigma Aldrich). Bone marrow and thymii were harvested 24 hours later and cell suspensions were labelled with the Click-iT EdU Flow Cytometry Assay kit (Life Technologies) before antibody staining and analysis by flow cytometry.
Chapter 3: Interleukin-7, but not thymic stromal lymphopoietin, plays a key role in the T cell response to Influenza A virus

3.1 Introduction

Influenza A virus is a common human pathogen, which causes significant morbidity and mortality worldwide [278]. Seasonal strains that cause annual epidemics can cause severe disease in immunocompromised individuals, including the young and the elderly [279, 280]. Novel reassortments of viral genes can occasionally result in highly pathogenic strains with the potential to cause severe disease in healthy adults [281, 282]. Although a robust immune response is necessary to clear the virus, highly pathogenic strains can induce an overactive response that can significantly contribute to disease. Thus, a more detailed understanding of the determinants of the outcome of response to IAV infection might assist in preventative or therapeutic approaches.

Influenza A/PR/8/34 (H1N1) (PR8) was derived from a human influenza strain and subsequently adapted to transmit from ferrets to mice [283]. This IAV strain infects lung epithelial cells, forming a local infection that resolves in 10 days in wild-type mice [284]. Resolution of the primary infection is dependent on development of a well-defined IAV specific T cell response. As a result, it is a clinically relevant model of a local respiratory virus infection.
IL-7 has a central role in the development of the adaptive immune system and its response [285, 286]. While IL-7 has been primarily characterized for its role in lymphocyte development, it has also been recently shown to boost the T cell response against chronic viral infections and tumours [262, 263]. The role of the IL-7-related cytokine thymic stromal lymphopoeitin (TSLP) has been more recently appreciated in the development of allergic and anti-parasite responses [287]. However, the role of these cytokines in acute viral infection is not clear.

IL-7 exerts its effects via a heterodimeric receptor of IL-7Rα paired with the gamma common chain (γc) [288]. IL-7Rα also serves as a receptor for TSLP when it is paired with TSLPR [289]. IL-7Rα and TSLPR are expressed on T cells and dendritic cells, as well as other innate immune cells with most of these cells co-expressing the γc. IL-7 is currently in clinical trials to enhance the response of and expand T cells in patients infected with HIV as well as in cancer patients and after bone marrow transplants [61]

TSLP acts primarily in the generation of immune response against parasites, however, in contrast to IL-7, it has little role in the development of the immune system [277]. TSLP has effects on the adaptive immune system both directly on T cells and via innate immune cells, such as dendritic cells [290-292]. TSLP is over expressed in many atopic diseases in humans including asthma and dermatitis [293]. Its expression can also be induced in bronchial epithelial cells by viral molecular motifs, such as CpG, suggesting it may play a role in defense against viral infections [294].
Signaling downstream of IL-7Rα is largely dependent on phosphorylation of tyrosine 449, nested within a YxxM recruitment motif. Mice bearing a mutated receptor with homozygous knock-in replacement of this tyrosine with phenylalanine (IL-7Rα^{449F}) have impaired signaling in response to IL-7 [260]. Although IL-7Rα^{449F} mice have fewer mature T cells compared to WT mice, the lymphocyte compartment is significantly restored compared to IL-7Rα^{-/-} mice. This allowed functional studies to be performed on these mice to investigate the role of IL-7Rα Tyr449 signaling. We showed that IL-7Rα^{449F} mice have a defective CD4 T cell response to Listeria monocytogenes infection, which suggested an unexpected role for IL-7-related cytokines in the primary T cell response [260]. Although IL-7 can boost T cell function during chronic inflammation, its role in the acute primary T cell response is unknown. Similarly, while TSLP has defined roles in the immune response to parasites, its role in viral infections has not been characterized.

We have investigated the role of IL-7 family cytokines during acute respiratory infection in mice infected with IAV PR8. We found that IL-7, but not TSLP, played an important role in viral clearance and protection from weight loss. We showed that IL-7 signaling in CD4 and CD8 T cells was critical for the development of the antigen-specific anti-IAV response. These results highlight the role for IL-7 signaling in the antiviral effector T cell response and its potential to modulate the immune response to respiratory viruses.
3.2 Results

3.2.1 IL-7 is critical for the control of and protection from influenza A virus infection

To assess the role of IL-7Rα signaling in IAV pathogenicity, WT, IL-7Rα\(^{449F}\), TSLPR\(^{-/-}\) and IL-7Rα\(^{449F}\) TSLPR\(^{-/-}\) double-mutant (Dmu) mice were infected with 5 Hemagglutinin units (HAU) of IAV PR8 by intranasal inoculation. This complement of genetic strains allowed us to distinguish the requirement for IL-7 or TSLP. We monitored mice for weight loss as an indicator of the severity of disease. While TSLPR\(^{-/-}\) mice showed similar weight loss compared to WT mice, both IL-7Rα\(^{449F}\) and IL-7Rα\(^{449F}\);TSLPR\(^{-/-}\) mice lost significantly more weight from day 7 post-infection onwards compared to WT (Figure 8A) suggesting that IL-7, not TSLP, was critical for protection from IAV. We then determined whether the weight loss seen was due to a failure to clear the virus by examining the levels of infectious virus in the lungs of mice nine days after infection. While WT lung homogenates did not develop detectable plaques on MDCK cells and TSLPR\(^{-/-}\) homogenates had low numbers of plaques, the lung homogenates from IL-7Rα\(^{449F}\) and IL-7Rα\(^{449F}\);TSLPR\(^{-/-}\) mice had elevated levels of infectious virus (Figure 8B). These results suggested that while TSLP had no role in control of IAV virus or protection from virus induced weight loss, IL-7 signaling through IL-7Rα\(^{Y449}\) was critical for both. Interestingly, viral M1 transcript levels were only elevated in IL-7Rα\(^{449F}\);TSLPR\(^{-/-}\) mice (Figure 9), showing a discrepancy between the
Figure 8: IL-7Rα signaling is required for control of Influenza A virus. C57BL/6, IL-7Rα449F, TSLPR-/- and Dmu mice were infected intranasally with 5 HAU of Influenza A PR8. Mice were weighed daily, and the mean change in body mass plotted. Weight is expressed as a percentage of the weight on day 0 (A). Lungs from mice at day 9 post infection were homogenized and Influenza A virus was measured by plaque assay (B). Data shown is from one representative experiment of three experiments, n=3-5 *p<0.05 by ANOVA with Tukey’s post-test.
Figure 9: Quantification of M1 viral RNA in the lungs. C57BL/6, IL-7Rα449F, TSLPR−/− and Dmu mice were infected intranasally with 5HAU of influenza A PR8. At day 9 post-infection lungs were harvested, homogenized in Trizol and absolute amount of influenza A M1 mRNA was determined by qPCR. Data shown is pooled from three experiments. *p<0.05 by ANOVA with Tukey’s post-test.
level of detectable infectious virus and viral transcripts in IL-7Ra$^{449F}$ mice for unknown reasons.

### 3.2.2 IL-7 signaling is necessary for a robust specific T cell response against influenza A virus

As IL-7Ra signaling is necessary for the formation of an effective primary CD4 T cell response and influences the response to TCR stimulation [260], we examined whether IL-7Ra was important for the formation of the T cell response to IAV. We evaluated the CD4 and CD8 T cell response in WT, IL-7Ra$^{449F}$, TSLPR$^{-/-}$ and IL-7Ra$^{449F}$;TSLPR$^{-/-}$ mice infected with IAV virus. The number of CD4 and CD8 T cells in the lung and BAL of IL-7Ra$^{449F}$ and IL-7Ra$^{449F}$;TSLPR$^{-/-}$ mice was reduced compared to WT after infection, while TSLPR$^{-/-}$ mice had normal numbers of CD4 and CD8 T cells (Figure 10). However, as both IL-7Ra$^{449F}$ and IL-7Ra$^{449F}$;TSLPR$^{-/-}$ mice have reduced number of baseline CD4 and CD8 T cells in the spleen and lung prior to infection (Figure 11) we examined the frequency of CD8 T cells that recognize the virus with MHC class I tetramers containing immunodominant viral epitopes NP$^{366-378}$ and PA$^{224-233}$ [295, 296]. At day 9 post-infection, the proportion of CD8 T cells that recognized both the NP$^{366-378}$ and PA$^{224-233}$ tetramers was significantly reduced in IL-7Ra$^{449F}$ compared to WT in both the BAL and the lung (Figure 12A). IL-7Ra$^{449F}$ mice also had reduced frequency of CD4 T cells that recognize the NP$^{311-325}$ epitope (Figure 12B). In contrast, TSLPR$^{-/-}$ mice had similar levels of tetramer-binding CD4 and CD8 T cells compared to
Figure 10: Total CD4 and CD8 T cell response in the lung and BAL.
Total number of (A) CD8 and (B) CD4 T cells in the lung and BAL in day 9 post-infection from C57BL/6, IL-7Ra^{449F}, TSLPR^{-/-} and Dmu mice. Data shown is from one representative experiment of three experiments, n=3-5. *p<0.05 by ANOVA with Tukey’s post-test.
Figure 11: Baseline CD4 and CD8 T cell in the lung and spleen. 
Total number of (A) CD8 and (B) CD4 T cells in the lung and spleen from uninfected C57BL/6, IL-7Rα^{449F}, TSLPR^{-/-} and Dmu mice. Data show from one representative experiment of three experiments, n=3-4. *p<0.05 by ANOVA with Tukey’s post-test.
Figure 12: CD4 and CD8 T cell response to Influenza A requires IL-7Rα, but not TSLPR, signaling.
Representative FACS plots and bar graphs of percent of total CD8 T cells specific for \( \text{NP}_{366-376} \) (top) or \( \text{PA}_{224-233} \) tetramer (bottom) in the BAL and lung (A). Representative FACS plots and bar graph of percent of total CD4 T cells specific for \( \text{NP}_{311-325} \) tetramer in the BAL and lung (B). Data shown is from one representative experiment of three experiments, \( n=3-5 \). *\( p<0.05 \) by ANOVA with Tukey’s post-test.
WT mice and IL-7Ra^{449F};TSLPR^{-/} mice had a comparable T cell response to IL-7Ra^{449F} mice. These results indicate that IL-7, but not TSLP, was important for the IAV specific T cell response.

3.2.3 IL-7 and TSLP regulate CXCR3 expression on virus specific T cells

We then asked if the reduction in T cells seen in the lung of infected IL-7Ra^{449F} mice was due to a failure of cells to migrate from the lymph nodes into the infected areas of the lung and airways. Several integrins and chemokine receptors, in particular CD11a [171], CD29, and CXCR3 [297] have been implicated in migration of T cells to the lung during IAV infection. Although IL-7 has been reported to influence expression of the integrins CD11a and CD29 [298], we found no difference in the expression of these proteins on influenza specific T cells from IL-7Ra^{449F}, TSLPR^{-/} or IL-7Ra^{449F};TSLPR^{-/} mice (Figure 13A). However, there was a significant reduction in the expression of the chemokine receptor CXCR3 on IAV specific CD8 T cells from the lungs of infected IL-7Ra^{449F}, TSLPR^{-/} and IL-7Ra^{449F};TSLPR^{-/} mice (Figure 13B). This suggested that IL-7 and TSLP both regulate the expression of CXCR3 in mice infected with IAV.
Figure 13: Expression of CD11a, CD29 and CXCR3 by Influenza A specific T cells. Lungs from mice were taken nine days after infection and stained for CD11a, CD29 and CXCR3. Events shown were gated on NP\textsubscript{366-376} tetramer\textsuperscript{+} CD8 T cells. Grey histograms are a representative WT control, black lines are from the labeled mutant strain. The mean fluorescence intensity of each marker is shown on the representative FACS plots and bar graph. One representative experiment of two experiments with n=3-5. *p<0.05 by ANOVA with Tukey’s post-test.
3.2.4 Generation of influenza A specific T cells requires cell intrinsic IL-7 signaling

To investigate whether the requirement for IL-7Rα signaling was due to signaling within T cells, we made a series of mixed bone marrow chimeras to examine the response of congenically marked WT and mutant lymphoid cells within the same mouse. Irradiated RAG1−/− mice were reconstituted with a mixture of BoyJ (WT, CD45.1+) and IL-7Rα^{449F} (CD45.2+) bone marrow. As IL-7Rα^{449F} T cells performed poorly in competitive repopulation against WT during development [260] we transferred bone marrow at a ratio of 9:1 IL-7Rα^{449F}:WT to obtain mice with a sufficiently large IL-7Rα^{449F} CD8+ T cell compartment to accurately evaluate their response. Reconstituted mice were then infected with IAV six weeks after irradiation and transplantation and the frequency of IAV specific CD8 T cells within either the WT or IL-7Rα^{449F} derived cells was examined at day 9 post-infection. As expected, IL-7Rα^{449F} T cells were heavily outcompeted by WT T cells during development (Figure 14A). As a result, IL-7Rα^{449F} T cells make up only 20% of the total T cell population in these mice, despite having been reconstituted at a 9:1 advantage. This output ratio is consistent with chimerism we have observed in uninfected mice (data not shown). Our data showed that within the same host, the frequency of NP_{366-374} specific cells among CD8 T cells in the lung was significantly reduced in the IL-7Rα^{449F} population compared to the WT population (Figure 14A). The NP_{311-325} CD4 T cell response in IL-7Rα^{449F} cells also showed a trend towards impairment, but this was not a significant difference (Figure 15A), perhaps due
Figure 14: Cell-intrinsic requirement for IL-7Rα, but not TSLPR, signaling in CD8 T cell response to Influenza A.

Lethally irradiated CD45.1 BoyJ mice were reconstituted with (A) 9:1 IL-7Rα<sup>449F</sup>:BoyJ or (B) 1:1 TSLPR<sup>−/−</sup>:BoyJ bone-marrow and allowed to recover for 6-8 weeks. Mice were infected with 5HAU of PR8 and the T cell response was analyzed at day 9 post-infection. CD8 T cells were stained for CD45.1 and CD45.2 to identify WT or mutant derived cells. Each population was analyzed for the percent of total CD8 T cells that recognize the NP<sub>366-374</sub> tetramer. Data shown is from one representative experiment of two experiments, n=3-7. *p<0.05 by Student’s t-test
Figure 15: Cell-intrinsic requirement for IL-7Rα, but not TSLPR, signaling in CD4 T cell response to Influenza A.

Lethally irradiated CD45.1 BoyJ mice were reconstituted with (A) 9:1 IL-7Rα^{449F}:BoyJ or (B) 1:1 TSLPR^{−/−}:BoyJ bone-marrow and allowed to recover for 6-8 weeks. Mice were infected with 5HAU of PR8 and the CD4 T cell response was analyzed at day 9 post-infection. CD4 T cells were stained for CD45.1 and CD45.2 to identify WT or mutant derived cells. Each population was analyzed for the percent of CD4 T cells that recognize the NP_{311-325} tetramer. Irrelevant tetramer staining on CD4 T cells is shown below NP_{311-325} staining in each panel. Data shown is from one representative experiment of two experiments, n=3-7. *p<0.05 by Student’s t-test.
to the lack of a robust response in WT CD4 T cells in these chimeric mice. Taken together, our data clearly indicated a cell-intrinsic requirement for IL-7Rα signaling in the development of the specific CD8 T cell response to IAV.

TSLP has been shown to influence CD8 T cell homeostasis and survival in competition with WT cells although no defect was observed in TSLPR<sup>-/-</sup> mice [299]. To determine if a role for TSLP in the generation of IAV specific T cells could be observed in competition with WT cells, we transferred a 1:1 mixture of TSLPR<sup>-/-</sup>:WT bone marrow into irradiated recipients. Reconstituted mice were then infected with IAV six weeks after irradiation and transplantation, and the frequency of IAV specific CD8 T cells within either the WT or TSLPR<sup>-/-</sup> derived cells was examined at day 9 post-infection. WT and TSLPR<sup>-/-</sup> T cells contributed equally to the IAV specific CD8 T cell populations in the lung (Figure 14B) and BAL (data not shown). Furthermore, the NP<sub>311-325</sub> specific CD4 T cell response in the TSLPR<sup>-/-</sup> compartment also showed no impairment, consistent with direct infection of TSLPR<sup>-/-</sup> mice (Figure 15B). Our data refute a role for cell intrinsic TSLP signaling in the T cell response to IAV, suggesting that the observed defects in IL-7Rα<sup>449F</sup> cells in chimeric animals were due to the loss of IL-7 signals.

3.3 Discussion

IL-7 and TSLP have well described roles as modulators of T cell immunity. IL-7 has been shown to enhance the T cell response against chronic viral infections and tumours [262, 263] and we have previously reported its role in the T cell response to
systemic challenge by *Listeria monocytogenes*. TSLP has primarily been characterized for its role in Th2-mediated immune responses, however it can be strongly induced by a variety of viral ligands and has been shown to influence the T cell response to BCG [294, 300]. The role of IL-7 in mature lymphocytes is difficult to study due to the severity of B and T cell defects in IL-7Rα−/− mice [301]. We previously generated IL-7Rα449F mice that are hypomorphic for IL-7Rα function. Unlike IL-7Rα−/− mice, these mice develop mature B and T cells allowing evaluation of the role of IL-7Rα signaling in mature lymphocyte function. By comparing IL-7Rα449F mice to WT and TSLPR−/− mice we are able to identify discrete roles for IL-7 or TSLP for an observed phenotype.

We showed that IL-7-mediated signaling was necessary for the generation of the primary CD4 and CD8 T cell responses against a physiologically relevant, acute IAV mucosal challenge. The impaired CD4 T cell response to IAV in IL-7Rα449F mice is consistent with our previous findings with a systemic *Listeria monocytogenes* challenge [260]. However, the defect in the primary CD8 T cell response in IL-7Rα449F mice to IAV was surprising as no such defect was observed in the *Listeria* study. As both CD4 and CD8 IL-7Rα449F T cells showed a dose dependent defect in anti-CD3 induced division [260], we postulate the route of infection and antigenicity of pathogen challenge can determine the requirement for IL-7R signals. Our current model is that TCR engagement by sub-optimal antigenic stimuli is enhanced by IL-7R signals, is impaired in IL-7Rα449F T cells and that this can be negated by strong T cell receptor signaling.
Our data showed no role for TSLP in the CD8 T cell response to IAV PR8 in contrast to recent findings [302]. As alterations in the gut normal flora can influence the immune response to IAV [303], it is possible that differences in the flora of the mice alter the contributions of the TSLP dependent response in these mice. Alternatively, although both studies examined low dose challenges of IAV, the virus dose may still contribute to the differences seen between the studies.

Previous studies have shown that depletion of lymphoid subsets could raise the susceptibility of mice to IAV infection [284]. However, these studies were performed in mice that lacked one or more lymphoid subsets. Models of partial lymphopenia have not been studied and thus it is unclear whether the reduced number of CD4 and CD8 T cells in IL-7Rα<sup>449F</sup> mice contribute to the increased severity of the IAV infection in these mice. Nonetheless, our bone marrow chimeras with IL-7Rα<sup>449F</sup> T cells clearly showed in lymphoreplete mice that IL-7Rα signals are necessary for an effective T cell response. Thus, while lymphopenia may contribute to disease severity in IL-7Rα<sup>449F</sup> mice, it is by no means the sole reason for the loss of viral control observed.

Migration of cells to the site of influenza infection is dependent on expression of integrins and chemokine receptors. We found no defect in the expression of the integrin subunits CD11a or CD29. IL-7Rα<sup>449F</sup> and TSLPR<sup>−/−</sup> mice both showed reduced expression of CXCR3. However due to the lack of a defect in both the proportion of tetramer positive cells or disease in TSLPR<sup>−/−</sup> mice, we would argue that although interesting, this chemokine receptor is not required for the T cell response to IAV. The
contribution of a migratory defect to the T cell deficiency observed in the lung could be determined by comparing the number of IAV specific T cells in the lung compared to the draining lymph nodes. If the response is normal in the mediastinal lymph node in IL-7Rα449F mice, this would imply that defective migration of the cells to the lung, and not initiation and amplification of the T cell response is responsible for the reduction in IAV specific T cells observed in these mice.

IL-7 has a clear role in survival and homeostasis of T cells, which is in part mediated by signaling through STAT5. STAT5 also has a critical role in maintaining the T cell response to LCMV as expression of dominant-negative STAT5 in T cells results in a reduction in cellular proliferation and survival [304]. IL-7Rα449F cells do not phosphorylate STAT5 in response to IL-7, suggesting that the defect seen in this mouse may mirror that seen in dominant-negative STAT5 mutant cells.

We show that IL-7, and not TSLP, plays an important role in the control of IAV virus and in the generation of the IAV specific T cell response. These findings have implications for shaping therapies directed at use of IL-7 in immune reconstitution and in agonist treatment of TSLP in atopic pathologies.

### 3.4 Materials and methods

**Ethics Statement:** Mice were housed at the UBC Centre for Disease Modelling facility, and all work was carried out according to University of British Columbia Animal Care
and Biosafety Committee guidelines. Breeding and project protocols (A07-0415 and A07-0417) were approved by the institutional committee for this work. All efforts were made to minimize suffering, with minimally invasive procedures, and where warranted (viral infection), isoflurane anaesthesia was used.

**Mice**: C57BL/6, BoyJ (B6.SJL-Ptprc<sup>a</sup> Peps<sup>b</sup>/BoyJ) and Rag1<sup>−/−</sup> (B6.129S7-Rag1<sup>tm1Mom/J</sup>) mice were obtained from the Jackson Laboratory (Bar Harbour, MA). IL-7Rα<sup>449F</sup> mice have a mutant form of the IL-7Rα expressing a single amino acid mutation from Tyr to Phe at position 449 as previously described [260]. TSLPR<sup>−/−</sup> mice [277] were a gift of Dr. James Ihle (St. Jude Children’s Research Hospital, Memphis, TN). All mouse strains have been backcrossed at least ten times with C57BL/6 mice. Age- and sex-matched mice between 6 and 9 weeks of age were used for all experiments.

**Infection**: Influenza A/PR/8/34 (PR8) was obtained from Charles River Laboratories (Wilmington, MA). Mice were anaesthetized using isoflurane and infected intranasally with 5 hemagglutinin units (HAU) of PR8 in 12.5µL of sterile PBS.

**Cell Preparation and flow cytometry**: Bronchoalveolar lavage (BAL) fluid was obtained by inserting a tracheal catheter and washing the bronchoalveolar space four times with 1mL of PBS with 10% FBS. Lymphocytes were extracted from the lungs of mice by mincing with scissors, digesting with 100 units/mL Collagenase IV for 1 hour at 37°C before filtering with a 70µm filter to remove debris. Spleens were collected and forced through a 70µm filter to obtain a single cell suspension.

Antibodies were purchased from BD Bioscience (San Diego, CA), eBioscience (San Diego, CA) or BioLegend (San Diego, CA). H2-K<sup>b</sup> and I-A<sup>b</sup> tetramers loaded with immunodominant NP<sub>366-376</sub>, PA<sub>224-233</sub> and NP<sub>311-325</sub> peptides from influenza A and labeled
with PE or APC were made by the NIH Tetramer Core Facility (Atlanta, GA). Tetramer staining was carried out at RT for 10 minutes before antibody staining at 4°C for 30 min. Data was acquired on a LSRII using FacsDiva software (BD Bioscience) and analysed using FlowJo software (TreeStar, Stanford, CA)

**Plaque assays:** Lungs from infected mice were homogenized using a Fisher Tissuemiser, diluted and incubated on confluent MDCK cells for 1 hour at RT. The cells were then washed and a solution of 0.7% agarose, 0.1% trypsin in α-MEM was applied. Samples were incubated at 37°C for 4 days before staining with crystal violet and counting of plaques.

**M1 RNA detection:** Lung from infected mice were homogenized in Trizol (Invitrogen), RNA was extracted as per manufacturer’s instructions and cDNA was prepared. Levels of viral Matrix gene transcript were then detected using M1F AGATGAGTCTTCTAAACGAGGTG, M1R TGCAAAAACATCTTCAAAGTCTCTG and an internal probe TCAGGCACCGCAGAAAGCGGA in duplicate using a Mx3005p PCR multiplex quantitative PCR instrument (Stratagene) with the Qiagen Quantitect qRT-PCR Kit (Qiagen). Total M1 copy numbers were determined by comparison to *in vitro* transcribed standards of known concentration [305].

**Mixed bone marrow chimeras:** CD45.1 mice were irradiated with two doses of 6.5Gy four hours apart and then injected 24 hours later with a 9:1 mixture of IL-7Ra449F:BoyJ or 1:1 mixture of TSLPR−/−:BoyJ whole bone-marrow. Mice were infected with influenza A virus as above 6-8 weeks later.
**Statistical analysis:** Data are presented as mean ± SEM and analyzed by Student’s t test or one-way ANOVA with Tukey’s post-test as appropriate. Results giving a p-value of less than 0.05 were considered to be significant.
Chapter 4: Topical CpG adjuvant enhances protection against influenza A infections

4.1 Introduction

Vaccination is a broadly effective strategy at reducing morbidity and mortality caused by infectious disease. However, while some vaccines, such as the Vaccinia vaccine, can induce strong, life long immunity, many vaccines do not generate long-lasting protection [6, 97]. Vaccine efficacy may be reduced due to the generation of a sub-optimal response or by changes in the protective epitopes of the target organism [97]. Current IAV vaccines target viral strains that are predicted to circulate prominently in the upcoming year [197]. These vaccines generate a strong antibody response that is protective against the targeted IAV strains [195]. However, they do not generate heterotypic immunity that would be protective against a wide range of IAV strains, and only protect against the strains in the vaccine [97]. Although current IAV vaccines can induce a strong humoral immune response, this response targets highly variable and rapidly changing epitopes on the HA and NA proteins [97]. Thus, vaccination may offer little protection if the predominant IAV strains for the upcoming year are not well matched to the strains used in the vaccine [198]. Furthermore, protection will wane over time as the targeted IAV strains undergo genetic drift in the epitopes targeted by the humoral immune response [94]. A vaccine that targets highly conserved IAV epitopes could generate long lasting protection against a broad range of IAV strains.
In contrast to the humoral immune response, the T cell response to IAV targets epitopes that are highly conserved across IAV strains [202]. IAV specific memory T cells, and especially memory CD8 T cells, are protective against IAV infection in both mice and humans [204, 205, 207]. Current IAV vaccines do not induce an effective T cell memory response and, thus, development of an IAV vaccine that can induce T cell memory may greatly increase the efficacy of vaccination by inducing protection against diverse IAV strains [97].

Vaccine efficacy is highly dependent on route of delivery and its ability to properly stimulate the immune system [210]. Optimizing route of delivery and choice of adjuvant are essential for proper quality and strength of the response. Recently, the TLR9 agonist CpG ODN has shown promising results as a novel adjuvant to boost immune responses and is currently in clinical trails as an adjuvant and immune modulator [306-308].

Treatment with adjuvant CpG ODN during vaccination has yielded promising results. Adjuvant CpG ODN induced stronger immune responses when co-administered with vaccinating agents. This was demonstrated for several clinically relevant viruses including Hepatitis B and anthrax [306-308]. Importantly, CpG ODN stimulated a strong Th1 response including both T and B cell responses, which was similar to the immune response to IAV [309].
Delivering a vaccine at the site of natural infection is effective at generating protective immunity in many cases, however, lung delivery of LAIV vaccines is not sufficient to generate a protective T cell response [208]. Thus, research into novel delivery routes to generate a protective anti-IAV T cell response is warranted. Delivery of vaccines in the skin is a promising approach to generating protective immunity in many vaccination settings. Interestingly, although a primary immune response is initiated in the skin, migratory and resident memory T cell populations are observed at diverse mucosal sites [224]. Transcutaneous immunization against Chlamydia and Schistosoma mansoni elicited protective responses when challenged intravaginally or in the airways, respectively [223, 225, 226]. Importantly, these protective responses involve contribution from both humoral and cell mediated immunity [223, 225].

The ability of adjuvant CpG ODN to help induce protective immune responses to vaccination on the skin is an active area of research. Addition of CpG ODN to Leishmania major vaccination induced strong and protective Th1 and Th17 responses [310]. Adjuvant CpG ODN generated a robust CD8 cytotoxic T cell response when administered with protein antigens [311]. These responses can protect against subsequent Listeria monocytogenes infection [312]. Importantly, route of application was critical for CpG ODN to optimally boost T cell responses. While intramuscular and subcutaneous administration induced some antigen specific CD8 T cells, administration of CpG ODN by the transcutaneous route resulted in an enhanced CD8 T cell response that mediated protection.
We investigated whether CpG ODN applied topically to the skin could help drive a protective T cell response against IAV. We found that topical CpG ODN drove both a CD8 T cell and antibody response against IAV. This response was sufficient to protect against subsequent IAV challenge in the lungs. Our work demonstrated the potential efficacy of immunization against IAV with adjuvant CpG ODN in the skin to drive a cross protective T cell response to diverse IAV strains.

4.2 Results

4.2.1 Adjuvant epicutaneous CpG (epCpG) ODN induced short and long term protection from IAV

We sought to determine if the combination of cutaneous immunization with adjuvant epCpG ODN was able to induce a protective memory CD8 T cell response against IAV. Mice were injected subcutaneously (sc) with OVA protein and epCpG ODN or vehicle control was applied topically over the injection site. Mice were rested for 7 days before being boosted with identical treatments. 5 days after boosting, immunized or naïve mice were infected intranasally with the H1N1 IAV strain PR8, which had been engineered to express the MHC-I restricted OVA peptide, SIINFEKL (PR8-OVA). As SIINFEKL is the only common antigen between the immunization and the rechallenge, this experimental set up allows us to specifically the contribution of the CD8 T cell response to infection.
To determine if immunization with epCpG ODN adjuvant could induce a protective immune response lungs were harvested three days after infection with PR8-OVA and plaque assays were performed on lung homogenates to determine viral titre. Mice immunized with scOVA alone had similar levels of IAV PR8-OVA in their lungs as naïve mice. However, there was a significant reduction in the viral burden in mice that received adjuvant epCpG ODN (Figure 16A). This demonstrated that scOVA+epCpG ODN generated an immune response that can control PR8-OVA infection.

To test if adjuvant epCpG ODN was able to induce a long-lived protective memory response, mice were primed and boosted, as above, but rested for 30 days until infection with IAV PR8-OVA. Plaque assays showed that scOVA alone had no effect on viral burden in the lungs compared to naïve controls (Figure 16A). However immunization with scOVA+epCpG ODN resulted in far lower lung viral titre, showing that adjuvant epCpG ODN induced protective memory responses.

To determine if epicutaneous delivery of CpG was the ideal route to induce protection from IAV, we primed and boosted mice either with scOVA+epCpG ODN or scOVA+scCpG ODN where the CpG ODN was injected along with the OVA protein. Mice were infected with PR8-OVA either 5 or 30 days after the boost and lungs were harvested 3 days post-infection. No difference was observed between mice treated with scOVA or ecOVA when infected at 5 days following the boost, indicating that scOVA was equally efficient at protecting from IAV at an early time point (Figure 16B). However, when mice were infected 30 days post-boost there was significantly improved
Figure 16: Adjuvant epicutaneous CpG generated optimal protection against Influenza infection.

Unimmunized mice or mice were immunized with scOVA or scOVA + epCpG were infected with PR8 5 days or 30 days after boosting (A). Plaque assays were performed 3 days after infection. (B) Mice were immunized with scOVA + scCpG or sc OVA + epCpG and challenged with PR8-OVA 5 or 30 days after boosting. Plaque assays were performed 3 days after infection. Representative of 3 independent experiments. n=4-6. *p<0.05.
protection from epCpG treated mice compared to scCpG ODN treated mice. This indicated that although both routes of adjuvant administration could induce an immune response, only administration via the epicutaneous route induced long term protection.

4.2.2 Adjuvant epCpG ODN immunization generated strong systemic and lung memory CD8 T cell responses to IAV challenge

We examined the memory CD8 T cell response to PR8-OVA challenge in mice immunized with scOVA+epCpG ODN by staining with an MHCI-D b tetramer containing the SIINFEKL peptide. Mice were primed and boosted as described and challenged with PR8-OVA 30 days after boost. Mice primed with the heterotypic H3N2 X31 IAV strain and challenged with PR8 were used as a negative control, while mice primed with X31-OVA and challenged with PR8-OVA were used as a positive control [313]. The bronchoalveolar lavage fluid (BALF), lung, spleen and blood were harvested 3 days after infection and CD8 T cells specific for OVA were examined (Figure 17A). As expected mice primed with X31 and challenged with PR8 had no significant induction of OVA specific CD8 T cells. Similarly, immunization with scOVA alone did not result in a robust T cell response after challenging with PR8-OVA. However, mice primed with scOVA+epCpG ODN or X31-OVA had a significant increase in both the frequency and total number of OVA specific cells CD8 T cells (Figure 17B, C). Interestingly, while both groups showed a strong OVA specific CD8 T cell response, the magnitude of the response across tissues was different. The frequency of OVA specific CD8 T cells was similar between the two groups in the BALF and the lung, although X31-OVA primed
**Figure 17: Adjuvant epicutaneous CpG generated a strong memory CD8 T cell response during Influenza infection.**

Mice were immunized with scOVA or scOVA + epCpG and challenged with PR8-OVA. Mice infected/challenged with X-31 and PR8 served as a negative control. Mice infected and challenged with X-31-OVA and PR8-OVA serve as a positive control. OVA specific CD8 T cells were analysed 3 days after infection. (A) Representative FACS plots from the BAL, lung and spleen. Gated on CD8 T cells (B) Frequency of OVA specific cells within CD8 T cells. (C) Total number of OVA specific CD8 T cells. Representative of 3 independent experiments. n=3-5. *p<0.05.
mice had higher numbers of CD8 T cells in the BALF. However, while there were significant responses in the spleen and blood of scOVA+epCpG ODN primed mice, the response from X31-OVA primed mice in these tissues was minimal.

4.2.3 Adjuvant epCpG immunization induced a strong IAV specific antibody response

Although memory CD8 T cells can mediate protection from IAV infection, they are more efficient in combination with a neutralizing antibody response. To examine the antibody response after immunization with adjuvant epCpG, we harvested serum from naïve mice or mice primed and boosted with scOVA alone, scOVA+scCpG ODN or scOVA+epCpg ODN 30 days after boosting. Mice treated with adjuvant epCpG generated a comparable total IgG and IgG2c response to mice treated with adjuvant scCpG (Figure 18A). Next, to determine if epCpG ODN can boost responses against IAV as well as protein antigens we also primed and boosted mice with heat-inactivated PR8 with either adjuvant scCpG ODN or epCpG ODN. Both scCpG and epCpG generated a strong IgG2b response compared to controls that were only immunized with heat-inactivated PR8, and no difference was observed between scCpG ODN and epCpG ODN treated groups (Figure 18B). These results indicated that vaccination with adjuvant epCpG can effectively generate a humoral response against the immunizing antigens.
Figure 18: Normal antibody response from epCpG immunized mice.
(A) Mice were immunized with scOVA, scOVA + scCpG or sc OVA + epCpG. Serum was collected 4 weeks after boosting and analyzed for OVA specific total IgG and IgG2c. (B) Mice were immunized with scHKI (Heat killed influenza), scHKI + scCpG or scHKI + epCpG. Serum was collected 4 weeks after boosting and analyzed for Influenza specific total IgG2b. Representative of 3 independent experiments. n=3-4.
4.3 Discussion

Current IAV vaccines are able to generate protective humoral immunity, but do not protect against serologically distinct strains that are not in the vaccine [97]. When the IAV strains in the seasonal vaccine are not well matched to the emergent IAV strains, the efficacy of the vaccines is reduced [198]. Well matched vaccines can reduce total influenza-like illnesses by over 70%, while poorly matched vaccines can be less than 50% effective at reducing influenza-like illnesses.

While humoral immunity naturally targets highly variable epitopes on IAV, T cells recognize and respond to epitopes that are highly conserved across IAV serotypes [202, 314]. Generation of a strong cytotoxic T cell memory response to IAV after vaccination may be able to provide cross serotype protection to IAV. Indeed, pre-existing memory CD8 T cells to IAV are the best correlates of cross-serotype protection in humans [201].

Immune responses to vaccination are affected by the route of vaccination and the adjuvant applied. Determining the optimal vaccine delivery system is critical to developing a universal IAV vaccine. Cutaneous immunization is able to generate protective resident memory T cell populations at distal mucosal sites in various infection and vaccination settings [209]. In addition, the TLR9 antigen CpG ODN preferentially induces a strong CD4 Th1 and CD8 immune response [220]. The induction of a strong, cytotoxic CD8 T cell response may confer broad protection from IAV infection.
We investigated if the combination of immunization on the skin with adjuvant CpG ODN could induce protective memory T cell responses to IAV. Our immunization and challenge system allowed us to specifically examine at protection driven by memory CD8 T cells. This was accomplished since we immunized and boosted with ovalbumin plus adjuvant epCpG followed by challenge with PR8-OVA, with only the MHC-I restricted ovalbumin epitope, SIINFEKL, as a common antigen between the immunization and challenge is. We showed that immunization with adjuvant CpG ODN not only generated a protective CD8 T cell response to IAV challenge, but that the route of delivery was critical for optimal response and protection. While CpG ODN delivered subcutaneously was able to generate some memory CD8 T cell response, only epicutaneous CpG ODN generated a robust CD8 T cell response which mediated protection from IAV infection.

After an infection, memory T cells form distinct populations of tissue resident and migratory cells. Tissue resident memory (Trm) T cells permanently reside in their home tissue, since their populations are not depleted by blocking migration into other tissues and since parabiotic animals do not gain donor tissue resident cells even after long periods of time [315]. Trm are conditioned by the tissue environment in which they arise [316]. Transferring Trm cells to a naïve animal will result in nearly all of them returning to the imprinted tissue [317]. Interestingly, memory cells generated in the skin are able to home to multiple tissues [224]. The mechanism by which the skin can program T cells to home to other sites has not been determined. In contrast, migratory memory T cells patrol lymph nodes and tissues for antigen but do not permanently reside in the tissues.
or have any tissue specific conditioning [318]. While both of these populations confer protection against reinfection, tissue resident T cells tend to be more effective. Their location proximal to the site of infection enables them to respond rapidly to reinfection and they are able to efficiently recruit help from the innate immune system and circulating memory T cell populations. Our results demonstrated that immunization with ovalbumin and adjuvant epicutaneous CpG ODN was effective at generating a protective secondary CD8 T cell response to PR8-OVA infection in the lung and BALF. Whether the vaccination generated memory CD8 population in the lung were a resident or migratory population was unclear, however, they respond equally well to IAV reinfection as memory CD8 T cells generated by primary IAV infection.

The peripheral response to rechallenge with PR8-OVA was quantitatively different between mice vaccinated with scOVA+epCpG and infected with X31-OVA. The secondary T cell response in X-31-OVA primed mice was confined to the BALF and lung. This is typical for memory T cell response to IAV. However, mice vaccinated with scOVA+epCpG had an increased CD8 T cell response in the periphery. This indicated that skin vaccination might have induced a larger peripheral memory CD8 T cell population than primary IAV infection, which led to an enhanced secondary response. The clinical implications of an enhanced peripheral CD8 T cell response to IAV rechallenge are unclear.

In addition to generating a protective CD8 T cell response, adjuvant epCpG stimulated production of antigen specific antibodies. This has utility since although
memory T cell responses have potential to provide cross-serotype immunity against IAV, they cannot completely prevent reinfection like protective humoral immunity can. Therefore, it remains beneficial to elicit a humoral response against the strains within the vaccine in addition to the induction of CD8 T cell memory.

The development of an immune response is influenced by innate immune cells and the context of their activation. The skin is spatially segregated into layers with different stromal and immune cell types in each layer. Thus, in addition to functional separation of the immune response between cells, they are also separated spatially. This is useful in the skin as it eases delivery of treatments to different layers, modulating the local immune response. The difference in responses between mice treated with adjuvant scCpG versus epCpG demonstrated the importance of the spatial separation of functions in immune priming in the skin. In this setting, stimulation of the epidermis was critical for generation of protective CD8 T cell memory. The main cell types in the epidermis that express TLR9 and recognize CpG are keratinocytes and LCs. LCs activated by CpG are able to migrate to draining lymph nodes and cross-present antigen to CD8 T cells [319]. LC mediated cross-presentation is more effective than when antigen and CpG are systemically injected. Stimulation of keratinocytes through TLR9 triggers the production and release of cytokines and chemokines such as TNF-α, IL-1α and type I Interferons [320]. Furthermore, cytokines and chemokines released by keratinocytes after stimulation by CpG enhance the ability of LCs to cross-present and activate CD8 T cells [240]. It is likely that CpG stimulation of keratinocytes,
LCs or both was essential for priming of CD8 T cells in the skin to a memory population that protected from IAV challenge in the lung.

Vaccination is one of the most effective public health interventions in use today. Transcutaneous immunization is a promising delivery route for the development of new and improved vaccines. A universal IAV vaccine could increase the efficacy and longevity of protection of immunity against IAV compared to current seasonal vaccines. We have demonstrated that vaccination with adjuvant epCpG generated a strong memory CD8 T cell response that protected from IAV challenge in the lung. The combination of delivery route and adjuvant were important for an effective memory CD8 T cell response and may be applicable to other vaccine settings.

4.4 Materials and methods

Mice: C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were housed at the Centre for Disease Modeling (Vancouver, Canada) and the Child and Family Research Institute (Vancouver, Canada) or at. All experiments were performed according to University of British Columbia Animal Care and Biosafety Committee guidelines under an approved animal protocol (A13-0062). Immunization: Mice were anesthetised by intraperitoneal injection of 75 mg/kg ketamine (ketalean, Bimeda-MTC Animal Health Inc., Cambridge, ON) and 7.5 mg/kg xylazine (Rompun, Bayer Health Care Inc., Toronto, ON). The back of the mice were then shaved, tape-stripped 15 times using cellophane tape. The skin was cleaned with
acetone. 100 µg of ovalbumin antigen (OVA, Sigma) in or PBS was injected subcutaneously. CpG ODN 1826 (5'-TCCATGACGTTCCGTAGTT-3', Sigma) in PBS/DMSO (1:1 v/v) was applied topically to the skin over the injection site (250 µg) or co-injected with OVA antigen subcutaneously (50 µg). The immunization area was covered in tape for 3 days to prevent disruption. An identical boost immunization was performed one week after priming. To measure antibody titres to IAV, heat-killed PR8 in PBS was injected instead of OVA.

**Infections:** Influenza A/PR/8/34 H1N1 (PR8) and Influenza A/X-31, A/Aichi/68 (H3N2) (X31) were purchased from Charles River Laboratories (Wilmington, MA). PR8 and X31 strains engineered to express the OT-I epitope SIINFEKL were a kind gift from Dr. Paul Thomas and grown in house. Mice were anaesthetized using isoflurane and infected intranasally with 5 HAU of PR8, 10HAU of PR8-OVA, 10HAU of X-31, or 20 HAU of X-31-OVA.

**Plaque assays:** Lungs from infected mice were homogenized using a Fisher Tissuemiser, diluted and incubated on confluent MDCK cells for 1 hour at RT. The cells were then washed and a solution of 0.7% agarose, 0.1% trypsin in α-MEM was applied. Samples were incubated at 37°C for 4 days before staining with crystal violet and counting of plaques.

**Tissue preparation for flow cytometry:** Bronchoalveolar lavage (BAL) fluid was obtained by inserting a tracheal catheter and washing the bronchoalveolar space four times with 1mL of PBS with 10% FBS. Lymphocytes were extracted from the lungs of mice by mincing with scissors, digesting with 100 units/mL Collagenase IV for 1 hour at 37°C before filtering with a 70µm filter to remove debris. Spleens were collected and
forced through a 70µm filter to obtain a single cell suspension.

Antibodies were purchased from BD Bioscience (San Diego, CA), eBioscience (San Diego, CA) or BioLegend (San Diego, CA). H2-Kb and I-Ab tetramers loaded with immunodominant NP$_{366-376}$, PA$_{224-233}$ and NP$_{311-325}$ peptides from influenza A and labeled with PE or APC were made by the NIH Tetramer Core Facility (Atlanta, GA).

Tetramer staining was carried out at RT for 10 minutes before antibody staining at 4°C for 30 min. Data was acquired on a LSRII using FacsDiva software (BD Bioscience) and analysed using FlowJo software (TreeStar, Stanford, CA)

**Detection of IAV specific antibodies:** Flat bottom 96-well plates were coated overnight at 4°C with 500HAU/mL of heat inactivated PR8 per well. Serial dilutions of serum samples were incubated in individual wells for two hours before washing and detected with a SBA Clonotyping System-B6/C57J-HRP kit (Southern Biotech) and ABTS reagent (Sigma-Aldrich).

**Statistical analysis:** Data are presented as mean ± SEM and analyzed by Student’s t test or one-way ANOVA with Tukey’s post-test as appropriate. Results giving a p-value of less than 0.05 were considered to be significant.
Chapter 5: Conclusions and future directions

5.1 Discussion

The aim of this work was to characterize the role of IL-7 in T cell development and function, against IAV infection in particular, and to investigate generation of T cell responses to IAV. IL-7 has long been known to have a non-redundant role in T cell development and homeostasis [5, 58]. However, recent work has found it also plays an important role in T cell responses to pathogens and tumors [260, 262, 263]. The dual role for IL-7 in T cell development and function has led to clinical trials studying the efficacy of IL-7 therapy for lymphopenia and chronic viral infections [60]. Despite this, there are still gaps in our knowledge of the role of IL-7 in early T cell development. This is highlighted by the role of activating IL-7Rα mutations as drivers of ALL from multiple early T cell developmental stages, including ETPs, a stage in T cell development where the role for IL-7 has not been characterized [61]. We investigated the role of IL-7 in the development of the ETP population in Chapter 2. We found that although ETPs do not express IL-7Rα, they require cell intrinsic IL-7Rα signaling for their development. Increasing our understanding of how IL-7 signaling influences T cell development could lead to better application of IL-7 based therapies and an understanding of how activating IL-7Rα mutations drive T cell lymphomas.

IAV infection causes yearly epidemics with a large human and economic impact [97]. Additionally, novel IAV strains can arise through genetic shift and cause
widespread, pandemic infection with higher pathology than normal seasonal IAV strains [95]. This pathology is due to both the viral infection and the immune response to that infection. Understanding how the immune system responds to IAV in both protective and harmful ways may help develop better therapies for those infected. Exogenous IL-7 has been shown to enhance T cell responses to chronic viral infections, but the role of IL-7 in an acute T cell response to IAV is unknown [262, 263]. We studied the role of IL-7 in the T cell response to IAV in Chapter 3 and show that it is necessary for a robust T cell response to IAV infection.

Current IAV vaccines can induce protection against the specific strains in the vaccine, but are not generally protective against the broader range of IAV strains [97]. This is due to diversity in the antigens that the adaptive humoral immune system responds to. In contrast, the epitopes that T cells recognize and respond to on IAV are highly conserved [199]. Thus, a strong memory T cell response to IAV may be able to protect against a broad range of IAV strains. Transcutaneous immunization with adjuvant CpG ODN has shown promise not only in generating local immune protection, but protection at other barrier sites as well [224]. In Chapter 4, we investigated the efficacy of transcutaneous vaccination against IAV with adjuvant CpG ODN. We found that transcutaneous CpG ODN generated a strong, protective T cell response in the lung. This demonstrated that this route of vaccination may be able to generate broad protective immunity against diverse IAV strains.
5.1.1 IL-7 controls the development of the ETP population

In chapter 2, we investigated the role of IL-7 signaling in early T cell development. We used mutant mice with impaired IL-7Rα signaling to dissect the earliest roles for IL-7 in T cell development in the bone marrow and thymus. We found that IL-7 signaling played no role in the generation of T cell progenitors in the bone marrow, as there were normal numbers of LMPPs and CLPs in hypomorphic IL-7Rα^{449F} and IL-7Rα^{−/−} mice (Figure 1). In contrast, the first thymic progenitor population, the ETPs, are highly dependent on IL-7Rα signaling with 20- and 100-fold reduction in numbers in IL-7Rα^{449F} and IL-7Rα^{−/−} mice, respectively (Figure 2).

As IL-7Rα is part of the heterodimeric receptors for both IL-7 and TSLP, either cytokine could be required for ETP development. To determine which cytokine is required, we examined T cell development in TSLPR^{−/−} mice. Similar to previous studies, we found that TSLP is dispensable for ETP development, as TSLPR^{−/−} mice showed no impairment in the ETP population (Figure 2) [277]. This demonstrates that IL-7 signaling through IL-7Rα is necessary for ETP development (Figure 19).

Mixed bone marrow chimeric mice that competed WT and IL-7Rα^{449F} cells demonstrated a clear, cell intrinsic requirement for IL-7Rα signaling in the development of ETPs (Figure 3). Interestingly, no developmental impairment in bone marrow progenitors was detected in IL-7Rα^{449F} cells even when in competition with WT cells. This clearly demonstrates a lack of requirement for either direct or indirect IL-7Rα
Figure 19: Novel roles for IL-7 in ETP development and primary T cell responses. Depiction of the roles for IL-7 characterized in Chapter 2 and Chapter 3. IL-7 is essential for the development of the ETP population and is necessary for robust effector CD4 and CD8 T cell responses to IAV infection.
Tyr449 signaling for the development of these bone marrow progenitor populations. As ETPs do not express IL-7Rα, IL-7 cannot act on them directly [22]. This shows that IL-7 signaling is essential for the transition of T cell progenitors from the bone marrow to the thymus.

Although we had identified the initial role of IL-7 in T cell progenitor development, the mechanism by which it acts is unclear. We found that IL-7 signaling is not required for the survival or proliferation of either ETPs or their bone marrow progenitors (Figure 6 and 7). This suggests that IL-7 influences the development of ETPs by a mechanism other than its cannonical roles of mediating survival and proliferation.

5.1.2 IL-7 signaling is necessary for robust and protective T cell responses to IAV infection

We interrogated the role of IL-7 signaling in the primary T cell response to infection with IAV in Chapter 3. Using mice that express the hypomorphic IL-7Rα449F, we demonstrated that IL-7Rα signaling is necessary for protection from and clearance of IAV (Figure 8 and 9). Signaling through IL-7Rα was also necessary for generation of both CD4 and CD8 T cells that are specific to IAV (Figure 10 and 12). Protection from IAV and generation of a virus specific T cell response were dependent on IL-7 signaling and not TSLP, as there was no defect in IAV responses in TSLPR+/− mice compared to WT and no increased defect in double mutant IL-7Rα449F;TSLPR+/− mice compared to IL-7Rα449F (Figure 10, 11, 12, 13 and 14). We demonstrated a cell intrinsic requirement of
IL-7 signaling in CD8 T cells, and confirmed no role for direct TSLP signaling in T cell responses to IAV infection (Figure 14 and 15). We also observe a defect in the expression of the chemokine receptor CXCR3 in IL-7Rα<sup>449F</sup> mice, which may suggest that IL-7 signaling influences the migration of T cells to the site of infection (Figure 13). Overall, this demonstrated that IL-7 signaling was essential for effective T cell responses in response to IAV infection (Figure 19).

This work adds to the growing number of studies that support a key role for IL-7 in primary CD4 and CD8 T cell responses. Interestingly, our previous work suggested a requirement for IL-7Rα signaling in the primary response to *Listeria monocytogenes* infection in CD4 T cell, but not CD8 T cell, [260]. The discrepancy between the two studies may indicate that the requirement for IL-7Rα signaling in the primary CD8 T cell response is location or challenge dependent. Furthermore, treatment with exogenous IL-7 can boost T cell responses against tumours and chronic viral infections [262, 263].

### 5.1.3 Topical CpG ODN adjuvant enhances protection against influenza A infections

Current IAV vaccines are able to generate protective humoral immunity, but do not protect against serologically distinct strains that are not in the vaccine [97]. While humoral immunity naturally targets highly variable epitopes on IAV, T cells recognize and respond to epitopes that are highly conserved across IAV serotypes [95]. Generation of a strong cytotoxic T cell memory response to IAV after vaccination may
be able to provide cross serotype protection to IAV [207]. In Chapter 4, we investigated if transcutaneous immunization against IAV with topical CpG ODN adjuvant could generate a protective T cell response that could protect against IAV infection. We showed that immunization with adjuvant CpG ODN not only generates a protective CD8 T cell response to IAV challenge, but that the route of delivery was critical for optimal response and protection (Figure 16). We further demonstrated that immunization against IAV with adjuvant epicutaneous CpG ODN was as effective at generating a protective secondary CD8 T cell response to IAV reinfection (Figure 17).

A universal IAV vaccine could increase the efficacy and longevity of protection of immunity against IAV compared to current seasonal vaccines. We have demonstrated that vaccination with adjuvant epCpG generated a strong memory CD8 T cell response that protected from IAV challenge in the lung. The combination of delivery route and adjuvant were important for an effective memory CD8 T cell response and may be applicable to other vaccine settings.

5.2 Future directions

5.2.1 Investigating the role of IL-7 and T cell development

In Chapter 2, we demonstrated a cell intrinsic requirement for IL-7Rα in the development of ETPs. However, it is unclear how IL-7 signaling is regulating the development of ETPs as ETPs and their progenitors are not impaired in their ability to
survive or proliferate in IL-7Rα^{449F} mice. Thus, we hypothesize that IL-7 signaling is necessary for progenitor cells to migrate from the bone marrow into the thymus.

Current work is in progress examining if IL-7 signaling is necessary for migration of bone marrow progenitor populations to the thymus. PSGL1 is the high affinity receptor for P-selectin expressed on thymic epithelial cells. Consequently, PSGL1^{-/-} mice have defective homing of progenitors to the thymus. The deficiency of ETPs in PSGL1^{-/-} mice results in increased receptivity of their thymii to seeding by WT progenitor cells [31, 33]. Lineage negative bone marrow or sorted CLPs from CD45.2 IL-7Rα^{449F} and CD45.1/2 heterozygotic WT mice could be competitively transferred intravenously into CD45.1 PSGL1^{-/-} mice. Thymii will be analyzed by flow cytometry for seeding by the transferred cells. If IL-7Rα^{449F} progenitors are defective in migrating to the thymus, they will be out competed by the WT cells. If a migration defect is observed, bone marrow progenitors and ETPs can be analyzed for the expression of molecules with known roles in migration of progenitors to the thymus including PSGL1 and the chemokine receptors CCR7 and CCR9.

Conversely, if no defect in thymic entry is found, it is possible that egress from the bone marrow is dependent on IL-7 signaling. Although this is difficult to test directly, it can be indirectly analyzed by forcing bone marrow progenitors to exit the bone marrow and examining the thymus to see if the ETP population is rescued in IL-7Rα mutant mice. Specifically, CXCR4 signaling is necessary for retention of progenitors in the bone marrow [26]. The drug AMD3100 is a selective antagonist to CXCR4 signaling.
Treatment with this drug triggers immediate egress for a wide range of progenitor cells from the bone marrow. If these cells are able to migrate to the thymus and rescue the ETP population in IL-7Rα mutant mice, this indicates that their exit from the bone marrow may be impaired.

Alternatively, there may be a very brief intrathymic stage immediately following recruitment that expresses IL-7Rα and requires IL-7 signaling. Notch signaling in the thymus causes immediate transcriptional changes as soon as progenitors enter the thymus [3]. Another approach to test whether migration or differentiation from thymic seeding progenitors to ETPs is the impaired step, lineage negative bone marrow or sorted CLPs from CD45.2 IL-7Rα^{449F} and CD45.1/2 heterozygotic WT mice could be competitively transferred intrathymically into CD45.1 mice. The frequency of WT or IL-7Rα^{449F} cells within the ETP population could be monitored over the next 1-2 weeks. If there is a failure of IL-7Rα^{449F} cells to differentiate into ETPs, they would be under-represented in that population indicating a cell-intrinsic defect in development. However, if IL-7 signaling is required for migration to the thymus, IL-7Rα^{449F} cells would be equally competitive to WT since we bypassed the migration step.

Early T cell development in humans is similar to that in mice. Although surface markers on each cell type are different, human HSCs differentiate to LMPPs and CLPs. Recent work has shown that development of these populations is independent of γc, and therefore IL-7, signaling [321]. The earliest progenitors in the thymus are DN cells that are CD34^{+} and CD7^{-} [322]. However, this population is heterogeneous in
expression of many other markers of lymphoid and T cell development including CD25, γc, and importantly, IL-7Rα where about half of these progenitors express it [322]. The role of IL-7 signaling at this stage is unknown as is whether the human IL-7Rα− progenitor cells are equivalent to murine ETPs. Overall, further characterization of the role of IL-7 in ETP development could aid in the use of IL-7 as a therapy to restore T cell numbers and in understanding the role of activating IL-7Rα mutations in driving early T cell lymphomas. Whether ETPs from ETP-ALL patients with activating IL-7Rα mutations first express IL-7Rα at the ETP stage, or at a prior stage is not known and warrants examination.

5.2.2 Further investigation of the role of IL-7 in IAV infection

In chapter 3 we found that IL-7 signaling is required for a robust primary T cell response to acute IAV infection in the lung and BAL. Although we found that direct IL-7Rα signaling on CD8 T cells (and a statistically insignificant trend in CD4 T cells) was essential for their response, it is unclear when this signaling is required and how it influences the T cell response. IL-7 is well known for its role in regulating the survival and proliferation of T cells in many settings including during development, and naïve and memory T cell homeostasis [5, 58]. Thus, the role of IL-7 in T cell survival and proliferation during the response to IAV infection could be tested.

Recently, exogenous IL-7 has been shown to enhance the effector function of T cells responding to chronic viral infections and tumors through repression of SOCS-3
and Cbl-b [262, 263]. Whether IL-7 acts in a similar manner in acute T cell responses is unclear. The role of IL-7 differentiation of CD4 T cells into Th1 effector cells could be examined by measuring their expression of the transcription factor Tbet and production of cytokines such as IFN-γ, TNF-α and IL-2. Similarly the ability of CD8 T cells to differentiate into effective cytotoxic cells could be tested by their expression of IFN-γ and their ability to lyse IAV infected target cells.

It is unclear when IL-7 is required during the acute T cell response. While IL-7 is primarily thought to be expressed in lymphoid organs, it can also be induced in other organs during infections. Although we determined there is a requirement of IL-7 in the lung and BAL T cell response to IAV, we did not evaluate IAV specific T cell response in the draining mediastinal lymph nodes in IL-7Rα449F mice. If T cell responses in the lymph node are also impaired, it would imply that activation and proliferation of T cells requires IL-7 signaling. However, if the lymph node has a normal T cell response, that would imply that T cell migration to, expansion in or survival in the lung may require IL-7. Whether IL-7 signaling is required during T cell priming in the lymph node or later in the resolution of the infection could be tested using inducible, conditional IL-7Rα knock-out mice. IL-7Rαfl/fl mice crossed with estrogen receptor linked cre (ERT2-Cre) transgenic mice would allow for temporal deletion of IL-7Rα upon treatment with tamoxifen [323]. Deletion of IL-7Rα immediately before infection versus several days into the infection could help to identify if IL-7 signaling is necessary during T cell priming in the lymph node or clearance of virus in the lung.
Inducible, conditional IL-7Rα knock-out mice could also be used to test if IL-7 signaling influences memory responses to viral infection. As even partial abrogation of IL-7 signaling results in impaired T cell responses, it is difficult to then investigate its role in the memory response. To test the role of IL-7 specifically in the memory response, IL-7Rα\textsuperscript{fl/fl} ERT2-Cre mice could be infected with the IAV strain H3N2 X-31 virus. After viral clearance and establishment of stable memory T cell populations, the mice would be treated with tamoxifen to induce deletion of IL-7Rα and reinfected with IAV H1N1 PR8. This would prevent antibodies generated during the first infection from confounding the assessment of the protection from the memory T cell response. The memory T cell response in mice that had, or had not, deleted IL-7Rα would be compared. A reduced memory response in mice without IL-7Rα would indicate that acute IL-7 signaling is necessary for T cell recall responses as well as for primary responses.

5.2.3 Transcutaneous vaccination and IAV vaccines

In chapter 4 we demonstrated that transcutaneous vaccination with epCpG ODN has potential to generate a protective T cell response against IAV infection in the lungs. However it is unclear how this response is modulated by epCpG ODN. The discrepancy in lung T cell responses to epCpG and scCpG may be due to differences in the innate immune cells that can be activated by different routes of administration. Key candidates as targets for CpG ODN mediated activation in the skin are the TLR9 expressing LCs and dermal DCs. The role of LCs could be tested with the use of mice that express the human diphtheria toxin receptor (DTR) under the control of langerin. Treatment of these
mice with DTR results in the specific ablation of langerin expressing LC in the skin. Ablation of LCs followed by immunization with epCpG would determine if these cells are necessary for generation of T cell responses after vaccination. Vaccinating mice with conditional TLR9 knockout mice could directly test the role of TLR9 signaling on these cells with langerin driven Cre. If LCs are not required for generation of T cell responses with adjuvant epCpG, the role of dermal DCs could be tested in similar systems with CD11c driven DTR and Cre.

5.3 Conclusions

This thesis investigated the mechanisms that regulate T cell development and responses focusing on the influence of the cytokine IL-7 on these processes. While IL-7 has previously been identified as a non-redundent cytokine in T cell development, we described the earliest requirement for IL-7 signaling in T cell development, at the ETP stage. We also demonstrated that IL-7 was essential for protection from, and T cell responses to, acute IAV infection in the lungs. These results directly inform the current use of therapeutic IL-7 to treat T cell lymphopenia and to enhance immune responses to chronic infections. An IAV vaccine that generates a strong memory T cell response could increase the efficacy of protection against diverse IAV strains, compared to current seasonal vaccines. Vaccination with adjuvant epCpG generated a strong memory CD8 T cell response that protected from IAV challenge in the lung. The combination of delivery route and adjuvant were important for an effective memory CD8 T cell response and may be applicable to other vaccine settings. Overall, this work
advances the understanding of T cell biology in homeostasis and infection and informs future therapeutic developments.
References


