The Role of Tumour Necrosis Factor Receptor Family Members CD137 (4-1BB) and CD30 in Antiviral T Cell Responses

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Department of Immunology
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2018

Abstract

The tumour necrosis factor receptor (TNFR) superfamily is a collection of immunomodulatory molecules that aid in the fine-tuning and regulation of immunity through stimulatory or inhibitory signalling functions at various stages of the T cell response. In this thesis, I investigate the roles of TNFR family members CD137 (4-1BB) and CD30 in antiviral T cell responses, most notably during influenza virus infection. Using a prime-boost vaccine immunization regimen in which mice are infected with mild influenza and then boosted intranasally with a replication defective adenovirus containing the influenza nucleoprotein (NP) and CD137L (4-1BBL) (Ad-NP-4-1BBL), I find that this supraphysiological administration of 4-1BB signals induces a robust long-lived circulating effector memory (Tem) population and a persistent lung tissue-resident memory (Trm) population, providing protection against lethal heterotypic influenza challenge. Ad-NP-4-1BBL acts directly on 4-1BB expressed by CD8 T cells to induce this expansion, and accumulation of memory CD8 T cells in the lung is dependent on local immunization with both antigen and 4-1BBL, recruitment of circulating effector cells into the tissue, and mTOR signalling. Moreover, we find endogenous 4-1BB to be a critical regulator of effector CD8 T cell accumulation in the lung tissue after influenza infection, which correlates with Trm establishment. This implicates 4-1BB as a potent regulator of both local and systemic T cell responses to influenza and may be a potential therapeutic target in future vaccine design. On the other hand, CD30 has no discernable roles in CD4 or CD8 T cell responses during acute influenza or chronic lymphocytic choriomeningitis virus (LCMV) clone 13 infections. Rather, CD30 regulates the age-dependent expansion of the T memory compartment, which may have
implications for the role of CD30 in human lymphoproliferative diseases. Taken together, we have identified novel functions of two TNFR members in regulating T cell responses during infection and at steady state. A better understanding of the post-priming mechanisms of these molecules can provide insight into future clinical interventions for viral infection and cancer malignancies.
Acknowledgments

First and foremost, I would like to thank my supervisor Dr. Tania Watts for her support and guidance over the course of my degree. Your passion for science is motivating, and I thank you for all the time and effort that you tirelessly dedicate to your students. I also appreciate the opportunities that you have given me to go abroad to conferences—the chance to present my findings to the field and the scientific community has been an integral part of my development as a doctoral candidate and has been rewarding both personally and scientifically.

I would also like to thank my supervisor committee, Dr. Michele Anderson and Dr. Juan-Carlos Zúñiga-Pflücker, for making yourselves available to meet with me and for all of the advice and guidance you have provided me over the past five years. I have always very much enjoyed (and dare I say, looked forward to) my committee meetings, and I thank the both of you for stimulating scientific discussions that were so important to the progression of this project, as well as my own personal development as a researcher.

I am grateful to all colleagues and collaborators that I have worked with over the past few years—I have learned so much from all of you. I would like to thank all members of the Watts lab, in particular two extension members, Dionne White and Joanna Warzyszynska, who keep the Flow Cytometry Facility operational and have been an integral source of technical support over the years. The two of you are always great company on those long experiment days of endless samples by the machine. I would like to extend my gratitude to the Department of Immunology and the wonderful people that have supported me over the years. To the IGSA, SciChat, and IMMpress teams that I have had the privilege to work with, I am in awe of your talents and I thank you for making my experience in this department so much more fulfilling.

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Ad-NP-4-1BBL induces greater IL-7Rα (CD127) expression on memory CD8 T cells, which may contribute to the longevity of the response

Delivery of 4-1BBL directly into the airways is required for the lung-resident memory response

Expansion of Trm during boosting is dependent on recruitment of T cells into the lung

The endogenous 4-1BB/4-1BBL pathway is critical for the formation or maintenance of the lung-resident CD8 population during primary influenza infection

3.4 Discussion

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4.2 Introduction

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Intrinsic 4-1BB signals are important for effector CD8 T cell accumulation in the lung tissue following influenza infection

The signalling adaptor TRAF1 contributes to systemic T cell effector numbers and lung Trm formation

Local antigen is necessary for 4-1BB-mediated secondary CD8 T cell effector accumulation in the lung tissue

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<tr>
<td>Ad</td>
<td>Replication-defective adenovirus vector</td>
</tr>
<tr>
<td>ALCL</td>
<td>Anaplastic large cell lymphoma</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presentation cell</td>
</tr>
<tr>
<td>B6</td>
<td>C56Bl/6</td>
</tr>
<tr>
<td>Bcl</td>
<td>B cell lymphoma (i.e. Bcl-2, Bcl-XL)</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>ConA</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CCR</td>
<td>C-C chemokine receptor (i.e. CCR7)</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation (i.e. CD137, CD8, CD3)</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>cIAP</td>
<td>Cellular inhibitors of apoptosis protein (i.e. cIAP1, cIAP2)</td>
</tr>
<tr>
<td>CXCR</td>
<td>C-X-C chemokine receptor (i.e. CXCR3, CXCR5)</td>
</tr>
<tr>
<td>CX3CR</td>
<td>C-X-3-C chemokine receptor (i.e. CX3CR1)</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>dLN</td>
<td>Draining lymph node</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ds</td>
<td>Double-stranded (i.e. dsDNA, dsRNA)</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>Eomes</td>
<td>Eomesodermin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>GP</td>
<td>Glycoprotein (i.e. GP33-41)</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>ID</td>
<td>DNA-binding protein inhibitor (i.e. ID2, ID3)</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon (i.e. IFNγ)</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin (i.e. IL-7, IL-15, IL-2)</td>
</tr>
<tr>
<td>ILC</td>
<td>Innate lymphoid cell</td>
</tr>
<tr>
<td>i.n.</td>
<td>Intranasal</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor (i.e. IRF3, IRF7)</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KLF</td>
<td>Krüppel-like Factor (i.e. KLF2)</td>
</tr>
<tr>
<td>KLRG</td>
<td>Killer cell lectin-like receptor (i.e. KLRG-1)</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LCMV cl 13</td>
<td>Lymphocytic choriomeningitis virus clone 13</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph node</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCMV</td>
<td>Murine cytomegalovirus</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby Canine Kidney</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescent intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex (i.e. MHC class I, MHC class II)</td>
</tr>
<tr>
<td>mLN</td>
<td>Mediastinal lymph node</td>
</tr>
<tr>
<td>MPEC</td>
<td>Memory precursor effector cell</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>mTORC</td>
<td>Mammalian target of rapamycin complex (i.e. mTORC1)</td>
</tr>
<tr>
<td>NA</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NIK</td>
<td>NF-κB-inducing kinase</td>
</tr>
<tr>
<td>NK</td>
<td>Natural kill cell</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T cell</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide-binding oligomerization domain (NOD)-like receptors</td>
</tr>
<tr>
<td>NLRP</td>
<td>NOD-like receptor family pyrin domain</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed cell death protein 1</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-4,5-biphosphate 3-kinase</td>
</tr>
<tr>
<td>PR8</td>
<td>Influenza type A/Puerto Rico/8/34</td>
</tr>
<tr>
<td>RAMD</td>
<td>Repair-associated memory depot</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RDC</td>
<td>Respiratory dendritic cell</td>
</tr>
<tr>
<td>RIG</td>
<td>Retinoic acid-inducible gene (i.e. RIG-I)</td>
</tr>
<tr>
<td>RING</td>
<td>Really interesting new gene finger domain</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>S1PR</td>
<td>Sphingosine-1-phosphate receptor (i.e. S1PR1)</td>
</tr>
<tr>
<td>sCD30</td>
<td>soluble CD30</td>
</tr>
<tr>
<td>SLEC</td>
<td>Short-lived effector cell</td>
</tr>
<tr>
<td>SLO</td>
<td>Secondary lymphoid organ</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SPF</td>
<td>Specific pathogen free</td>
</tr>
<tr>
<td>ss</td>
<td>Single-stranded (i.e. ssDNA, ssRNA)</td>
</tr>
<tr>
<td>Tcm</td>
<td>Central memory T cell</td>
</tr>
<tr>
<td>TCF</td>
<td>T cell factor (i.e. TCF-1)</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tem</td>
<td>Effector memory T cell</td>
</tr>
<tr>
<td>Tfh</td>
<td>T follicular helper cell</td>
</tr>
<tr>
<td>Tfr</td>
<td>T follicular regulatory cell</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor (i.e TGFβ)</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell (i.e. Th1, Th2, Th17)</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Description</td>
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<tr>
<td>--------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor (i.e. TNFα)</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumour necrosis family receptor</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cell</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor-associated receptor (i.e. TRAF1, TRAF2)</td>
</tr>
<tr>
<td>Trm</td>
<td>Tissue-resident memory T cell</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion protein (i.e. VCAM-1)</td>
</tr>
<tr>
<td>VLA</td>
<td>Very late antigen (i.e. VLA-1)</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
</tr>
<tr>
<td>X31</td>
<td>Influenza type A/Hong Kong/X31</td>
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</tbody>
</table>
Chapter 1
Introduction
1.1 Overview

The induction of robust and enduring memory CD8 T cell responses against influenza virus infection has been a challenge in vaccine design. Unlike antibody responses to the virus, which appear to be lifelong (1), even a natural infection in humans results only in a transient boost of influenza-specific CD8 T cells in the circulation for a few months post-infection (2). Furthermore, older individuals, who make up a majority of influenza-related deaths, show signs of immune senescence in their CD8 T cells responses characterized by an enrichment for a terminally differentiated late-effector KLRG-1hiCD57hi population (3, 4). Moreover, work in mouse models has shown a precipitous decline in influenza-specific CD8 T cells resident to the lungs shortly after infection that correlates with a loss in protection against lethal challenge (5-7). This presents obstacles for the development of more effective immunizations since CD8 T cells are a critical mediator of anti-influenza immunity and have been shown to provide cross-protection against heterotypic strains of the virus (8).

The ability to generate and sustain populations of memory CD8 T cells for long-term protection is still unchartered territory for many infection and immunization contexts. Recently, the discovery of tissue-resident memory that act as a frontline defense has put an emphasis on the importance of the local tissue microenvironment to the longevity and maintenance of memory cells (9, 10). The inflammatory milieu along with early signals of T cell activation presented within the tissue are immensely influential to the differentiation and programming of memory responses (9-12). Of particular interest is the tumour necrosis factor receptor (TNFR) superfamily, which contain receptors that have potent costimulatory effects on the survival and effector function of CD8 T cells during viral infection and cancer (13, 14). In this thesis, I will discuss the regulation of CD8 T cell responses, the molecular networks that govern circulating and tissue-resident memory, and the role of two TNFR family receptors—CD137 (4-1BB) and CD30—in antiviral effector and memory responses against influenza. I also extensively investigate 4-1BB as a factor critical to the establishment of lung-resident CD8 T cells and its implications for vaccine design.
1.2 Memory CD8 T cells

Immunological memory is a key component of adaptive immunity. Although almost all licensed vaccines are based upon the induction of antibody responses, CD8 T cells play a key role in limiting viral infection by cytotoxic killing of infected host cells (15). Memory CD8 T cells are antigen-experienced cells that can become reactivated upon secondary challenge to undergo a rapid proliferative burst and enact effector capabilities that facilitate more immediate pathogen clearance than naïve CD8 T cells undergoing a primary expansion (16) (Figure 1.1; Table 1.1). Naïve CD8 T cells, which express the lymph node (LN) homing receptors CD62L and CCR7, circulate between the blood and secondary lymphoid organs (SLOs) to survey and patrol for antigens presented on major histocompatibility complex (MHC) class I molecules by dendritic cells (DCs) (17). The infection or immunization induces innate immune responses that result in maturation of DCs, whereby they upregulate costimulatory molecules, produce cytokines, and home into SLOs for engagement with T cells (17). CD4 T cells, which are necessary for optimal induction of primary CD8 T cell responses, aide in this process by licensing DCs through CD40-CD40 ligand (CD40L) interactions, as well as facilitating DC production of the necessary chemokines for migration into SLOs (18-21). CD4 T cells have also been known to directly provide CD40 ligation to CD8 T cells as well (22) and to provide interleukin-2 (IL-2) for CD8 T cell expansion (23-25). Upon receiving a full context of activation signals—consisting of signals 1 (antigen), 2 (costimulation), and 3 (pro-inflammatory cytokines) (26-28)—antigen-specific CD8 T cells proliferate extensively and differentiate into short-lived effector cells that can rapidly clear infection through cytolytic killing of infected cells and the production of interferon gamma (IFNγ) and tumour necrosis factor alpha (TNFα) (29, 30). Upon clearance of the pathogen, contraction occurs in which 90-95% of the expanded CD8 T cell population will die (11, 16). The remaining CD8 T cells become memory cells that are maintained for a much longer period by an active, dynamic, antigen-independent and cytokine-driven process, notably through IL-7 and IL-15 which are important for survival and homeostatic proliferation, respectively (31-34). It has been suggested that CD4 T help is necessary for either the programming or the maintenance of memory CD8 T cells (18). “Unhelped” memory CD8 T cells upregulate TNF-related apoptosis-inducing ligand (TRAIL) upon re-activation and subsequently undergo activation-induced cell death, although TRAIL-deficiency does not always rescue the secondary
Figure 1.1. Response of CD8 T cells upon infection or immunization. Activation of naïve CD8 T cells by antigen, along with other necessary costimulatory and cytokine signals, results in expansion of the antigen-specific population, making up an effector CD8 T cell response. Upon clearance of the pathogen, 90-95% of these cells die during a contraction phase, and the cells remaining make up the memory pool. Memory CD8 T cells found in the circulation are made up of central memory (Tcm), effector memory (Tem), and peripheral memory (Tpm) T cells. The tissue-resident memory (Trm) population, can be found to reside permanently in peripheral tissue after local immunization. Re-exposure to the antigen results in secondary expansion of effector cells which undergo a proliferative burst and are more effective at clearing the pathogen.
Table 1.1. Phenotypic markers for identifying murine CD8 T cell subsets. Typical surface markers (CD44, CD62L, CCR7, KLRG-1, CD127, CD69, CD103, CX3CR1) and transcription factors (Eomes, T-bet, Blimp-1, and Hobit) for identifying and characterizing different subsets of murine CD8 T cells. The profile of tissue-resident memory (Trm) cells differ depending on what tissue they localize in. While the conventional markers for Trm are CD103 and CD69, there have been resident populations identified as CD103 CD69\(^{-}\) or double negative.
response (18, 35). IL-2 is critical early in the CD8 T cell priming to program full secondary memory expansion, but its source appears to be autocrine (36) although paracrine production from CD4 T cells or DCs may compensate in certain settings (23). Furthermore, competition from CD4 Tregs may control exposure of CD8 T cells to IL-2 following infection or immunization (37, 38). It should also be noted that one study from Sun et al. showed a requirement for CD4 T cells in the maintenance of memory CD8 T cell responses to lymphocytic choriomeningitis virus (LCMV) Armstrong after the initial priming stage, independent of programming (39).

The potential to become a memory cell is not inherently identical between all expanded effector precursors; CD8 T cells that commit to the memory pool—or memory precursor effector cells (MPEC)—are distinguished by a KLRG-1loCD127hi phenotype, while terminally-differentiated short-lived effector cells (SLEC) are KLRG-1hiCD127lo (28, 31, 40-42). The context and input signals during T cell activation can affect the ability to differentiate into memory with shortened duration of antigen exposure, increased intraclonal competition, and dampened inflammation increasing the capacity for memory formation (43-47), while pro-inflammatory cytokines like IL-12 can drive terminal differentiation of effector cells (12, 42). Furthermore, the immunoregulatory cytokine transforming growth factor beta (TGFβ) has also been shown to promote memory formation by restraining inflammatory signals, as well as maintain memory identity as memory cells lacking the TGFβ receptor, TGFβR2, upregulate KLRG-1 in the absence of antigen and exhibit defective recall responses (48). There is also evidence showing asymmetric cell division in which effector and memory cells both arise from the same precursor with the daughter cell that inherits the immunological synapse receiving stronger TCR signalling and costimulation for commitment into the effector fate (49).

The T-box transcription factors T-bet and Eomesodermin (Eomes) play a role in regulating T cell differentiation. T-bet and Eomes cooperate to induce granzyme B, IFNγ, perforin, CXCR3, and CXCR4 expression in cytotoxic effector cells, as well as maintain memory homeostasis through upregulation of CD122, the IL-2Rβ subunit that mediates IL-15 sensitivity (12, 42, 50-52). Despite this, T-bet and Eomes have independent roles in the establishment of effector and memory cells, respectively (12). T-bet is induced by T cell receptor (TCR) signalling and its expression is amplified by IL-12 and the mammalian target of rapamycin (mTOR) pathway (42,
53, 54). Deficiency in T-bet results in decreased SLEC formation, while forced expression can induce effector cell formation (42). Furthermore, the transcription factor Zeb2 works in conjunction with T-bet to drive terminal cytotoxic effector T differentiation by restricting memory potential (55). Alternatively, deficiency of Eomes, whose expression is RUNX3-dependent, amplified by IL-2 and repressed by mTOR (54), results in both decreased MPEC and dysfunctional memory generation (50, 56, 57). Other counter-regulating pairs of transcription factors such as Blimp-1 and Bcl6 or ID2 and ID3 have also been identified as influencing the effector (Blimp-1 and ID2) versus memory (Bcl6 and ID3) axis (12, 58-61). Thus, a better understanding of the signals and axes involved in T cell differentiation is imperative in the context of vaccine design for the induction of protective memory responses against infection and disease.

There is heterogeneity, both in phenotype and in function, of memory cells and their maintenance and longevity is heavily influenced by their migration capabilities and the tissue microenvironment in which they reside (Figure 1.2; Table 1.1). Early analysis of migration and surface markers distinguished two subsets of recirculating memory cells: central memory (Tcm) which are CD62L+CCR7+ and home to SLOs, and effector memory (Tem) which lack those homing receptors and were understood to survey the blood and peripheral tissue (62-65). Tcm are more quiescent, have greater proliferative potential, and produce abundant IL-2, while Tem are constitutively able to produce cytotoxic functions and produce IFNγ and TNFα (63, 64). However, there is little consensus regarding which circulating subset produced optimal protective immunity in various infections, prompting some groups to turn to more functionally relevant markers of memory classification, such as CXCR3, CD27, CD43, and more recently the fractalkine receptor CX3CR1, which was shown to correlate with the degree of differentiation (66-68). Furthermore, the recirculation properties of Tem came into question when it was discovered that CCR7 was also required for egression out of LN (69, 70), therefore renewing interest in resolving whether memory T cells found in peripheral tissues actually re-enter the bloodstream. This was clarified when it was shown that memory CD8 T cells resided in the dorsal root ganglia and skin permanently after herpes simplex virus (HSV) infection (71, 72). This was further supported by a separate group shortly after that found a similar memory population resident to the small intestines after LCMV infection (73); since then, tissue-resident
Figure 1.2. Migration properties of memory CD8 T cell subsets. Memory CD8 T cells found in the circulation are made up of central memory (Tcm) cells that migrate to secondary lymphoid organs, effector memory (Tem) that patrol the vasculature, and peripheral memory (Tpm) T cells that circulate between the vasculature and peripheral tissues. A fourth subset, the tissue-resident memory (Trm) population, can be found to reside permanently in peripheral tissue after local immunization/infection, and do not re-enter circulation. It should be noted that Trm have been identified in both lymphoid and non-lymphoid tissues.
memory T cells (Trm) have been discovered in various other tissues following different mucosal infections and shown to correlate with protection against certain infections (9, 10). While Trm remain permanently in tissues, Gerlach et. al. recently identified a CX3CR1\(^{\text{int}}\) peripheral memory (Tpm) subset that patrol non-lymphoid tissues and can re-enter circulation, unlike classical CX3CR\(^{\text{hi}}\) Tem that remain completely in the bloodstream (68). Furthermore, this CX3CR1\(^{\text{int}}\) Tpm subset has self-renewing properties and can also replenish the CX3CR1\(^{-}\)Tcm population (68). The distinction between Tem and Tpm is still unclear, and made complicated as experimental methods, such as intravascular staining, only provide a “snapshot” of where cells are localized and does not inform upon whether they recirculate. Thus, the migration and functional properties of memory CD8 T cells are still being investigated and elucidated.

**Tissue-resident Memory Cells (Trm)**

Memory T cells have long been characterized in the peripheral tissue, but were assumed to be Tem populations that migrated through, passing in and out of the infected tissue and the bloodstream. This was supported at the time by the CD44\(^{+}\)CD62L\(^{-}\)CCR7\(^{\text{lo}}\) phenotype that these cells exhibited, much akin to the Tem profile. Since then, it has been shown that Trm populations from various tissues exhibit a core transcriptional signature distinct from Tcm and Tem subsets, suggesting differential commitment and programming characterized by upregulation of chemokine receptors and adhesion molecules, and downregulation of tissue egression signals (74, 75). Trm are defined functionally as memory cells permanently present *in situ* that do not recirculate into the bloodstream (9, 10), and have been identified using mouse models in the skin, genital tract, brain, small intestine, lung, and even SLOs, just to name a few (71-73, 76-80). In humans, Trm have been identified in the skin, spleen, LN, lung, and intestines (10, 81, 82). A study by Sathaliyawala *et. al.* of 24 organ donors showed a predominantly memory CD45RO\(^{+}\) population of T cells within non-lymphoid tissue—including lung, colon, and small intestines—which accumulated with age, while blood and lymphoid compartments contained large populations of both memory and naïve T cells (81).

While most studies have focused on CD8 T cells, which are far better characterized, CD4 Trm have also been described in the skin, lung, and genital tract and also shown to mediate protection.
to various infections in murine models (83-85). CD8 Trm have conventionally been identified as CD103⁺CD69⁺ and often express the integrin CD49a (VLA-1), although recent work has shown far more heterogeneity in these populations (72, 74, 79, 86). CD103⁺CD69⁺ Trm subsets have been identified in the small intestines, liver, and SLO (78, 80, 87), while subsets of Trm in the pancreas, salivary glands, and female reproductive tract have been found to be CD69⁻ (88). As such, it is important to evaluate migration properties, rather than simply relying on the phenotype of these cells. Assays for migration have become a standard in the field to extensively evaluate the in vivo recirculation of Trm populations. Parabiosis is the gold standard for studying memory T cell recirculation. In a parabiosis experiment, pairs of mice are surgically attached through the abdominal skin, which allows their vasculature to join, and the equilibration of cell populations between the parabionts is assessed (76, 77, 80, 83, 85, 88, 89). Adoptive transfer models have incorporated labelled or photoreactive transgenic T cells for tracking of migrant and non-migrant populations (73, 90-92). Pharmacological agents, such as FTY720 which binds and internalizes the sphingosine-1-phosphate receptor (S1PR), can be used to prevent lymphocyte egression out of lymph nodes and sequester circulating lymphocytes (93-95). Graft rejection models, commonly using male T cells transferred into female hosts, allow investigators to assess whether donor cells that seed the Trm pool, and therefore protected within the tissue, will become exposed to circulating host cells that mediate donor rejection (71, 73, 96, 97). However, these techniques are often not practical, especially when studying human Trm where characterization has mostly been based on our understanding of CD69, CD103, and CD49a described in animal models (10, 81, 82). The human memory T cell populations identified in non-lymphoid tissues by Sathaliyawala et. al. mostly expressed CD69, a marker for Trm, and were CD127⁺, indicating a resting memory phenotype (81). Furthermore, CD103 appears to define mucosal CD8 Trm as all memory CD8 T cells in the intestines and colon and a significant fraction of Trm in the lung, intestinal-draining LN are CD103⁺ (81), whereas memory CD8 T cells in lymphoid compartments are CD103⁻.

Another technical struggle in the study of Trm in animal models is the ability to differentiate resident populations within the tissue from those in the vasculature traveling through the tissue. This can be tackled by an in vivo intravascular (i.v.) staining technique whereby all vascular CD8 T cells are labelled by a fluorescent antibody through i.v. injection immediately prior to
euthanasia (78, 98). The evaluation of cell populations within different tissues using single-cell based analysis like flow cytometry can also be incomplete because of inefficiencies in the tissue processing step that can reduce cell yield (10, 88). Thus, imaging approaches need to be incorporated into analyses for a more complete evaluation, that also reveal the spatial localization, of resident T cells.

As these technical hurdles are addressed and overcome, more is starting to be understood about the development and ontogeny of Trm cells. It is thought that full commitment and differentiation occurs in situ and a potential KLRG-1lo precursor, common to both Tcm and Trm, homes into the tissue microenvironment to receive local cues (74, 99). The Tcm-Trm precursor is supported by TCR sequencing analysis showing an identical clonal repertoire between Trm in the skin and Tcm in the LN following poxvirus infection (100). However, some groups have also shown continual effector T cell and Tem recruitment into the tissue for maintenance of the Trm pool, notably in the lung (101-103) where the issue is still controversial. The requirement for persistent antigen for longevity of the population is also another controversial topic. This will be discussed further in the context of lung Trm in section 1.3.

The tissue-dependency of Trm commitment means that the heterogeneity in local microenvironments will produce phenotypically heterogeneous Trm populations at different sites. However, despite this, there appears to be a universal transcriptional signature of tissue-residency that encapsulates not only Trm, but other tissue-resident lymphocyte populations as well, including innate lymphoid cells (ILCs) and natural killer T (NKT) cells (75) in mice. This program is regulated by two transcription factors Hobit and Blimp-1 (75). Deficiency in either of these factors results in a decrease of the Trm pool, while deficiency in both completely ablates Trm populations in the skin, gut, liver, and kidney. Hobit and Blimp-1 share substantial homology in the DNA-binding zinc-finger domains and bind to some of the same target sequences of the same genes, and may share some functional redundancy. However, there may be a division of labour as different tissue Trm populations preferentially rely more on one or the other. Furthermore, Blimp-1 expression is most highly induced on effector T cells by IL-2 and IL-12, independently of T-bet, and is maintained in memory, while Hobit is upregulated specifically in Trm in a T-bet and IL-15-dependent manner; therefore, there may be temporal and anatomical distinctions in their regulation of Trm commitment (75, 104). The expression of
Hobit and Blimp-1 together induce a transcriptional signature that suppresses lymphocyte egression through downregulation of sphingosine-1-phosphate receptor 1 (S1PR1) and Krüppel-Like Factor 2 (KLF2), as well as repressing genes like TCF-1 and CCR7 that are associated with Tcm differentiation (75, 104). Interestingly, the Blimp-1 and Hobit signature is not a defining characteristic of human lung Trm, as both Trm and Tem populations are found to express these transcription factors (105). However, human lung Trm do similarly exhibit a distinct profile of upregulated adhesion molecules, chemokine receptors, and effector molecules. Moreover, both human and murine lung Trm exhibit a strong Notch signature. This study will be discussed more extensively in section 1.3. Given the tissue-specific niche of Trm development and the lack of research in the gene expression profile of other tissues, it remains to be seen whether a similar “universal” regulator of the Trm program also exists in humans.

The mechanism of Trm retention in different tissues is an area that is still being explored. CD69 is an activation marker that is also known to block S1PR1, a receptor that mediates the egression of T cells out of the lymph node through binding with the sphingosine-1-phosphate (S1P) chemokine (106). Deficiency for CD69 results in defective Trm generation in the skin and lung (74, 107), while forced expression of S1PR1 ablates Trm in the salivary glands and lamina propria (108). IL-33 and TNFα have been shown to upregulate CD69 and contribute to Trm formation (108). The other Trm marker CD103, also known as αE integrin, binds E-cadherin and is thought to allow direct adherence of Trm to the tissue epithelium (74, 92). Without CD103, T cells migrate into the tissue but cannot be retained (79, 107). TGFβ has been shown to induce CD103 expression and Trm formation in a dose-dependent manner (79, 107), while CD103-Trm in the lamina propria are unaffected by TGFβ, after Yersinia pseudotuberculosis infection (87). TGFβ has also been shown to negatively regulate KLF2 (108), which is an upstream positive regulator of S1PR1, and T-bet (109). Unlike conventional memory cells, Trm are Eomes- and T-betlo (74, 110). T-bet negatively regulates CD103 expression and its forced expression decreases Trm formation. However, unlike Eomes, which is completely ablated in Trm, T-bet expression is maintained at very low levels in Trm, possibly to maintain IL-15 sensitivity. As previously mentioned, not all Trm express CD103 and CD69; therefore, the mechanism of retention of many populations is still unclear. It does, however, appear that the specific inflammatory
environment of the tissue, as induced by infection or immunization, determines the establishment and maintenance of various Trm populations.

The induction of Trm has received much attention since their initial discovery, due to the implications for vaccine design as well as the potential role of Trm in immunopathology and autoimmunity. Trm can induce protective immunity against infection at mucosal surfaces, serving as a front-line defence against pathogens, while circulating Tcm populations take time to respond to inflammation and traffic into tissue. Trm can exhibit cytotoxic and direct killing capabilities (72, 79, 111, 112) and increased motility after antigen re-exposure (96, 113).

Furthermore, they function as innate sensors and facilitate recruitment of bystander immune cells through IFNγ production and upregulation of VCAM-1 on endothelial cells. Trm also induce a tissue-wide antiviral state through upregulation of innate immune genes such as IFITM3, as observed in the skin (112, 114). Recently, Trm in humans have been identified in the skin, spleen, LN, lung, and intestines, largely relying on the markers CD69, CD103, and CD49a described in animal models (10, 81, 82). Trm may also contribute to human disease, as skin Trm have been identified in psoriasis patients (115), while CD4 Trm can be found in the lung tissue of murine house dust-mite models of allergy (116). As the memory field evolves and we shift our focus to tissue-specific local immunity, our understanding of Trm can better inform correlates of protection against localized disease, such as influenza infection, and potentially identify targets for future therapeutic design through the elucidation of the critical cues and signals required for T cell memory differentiation.

1.3 Influenza

Influenza is a negative sense, single-stranded (ss) RNA member of the orthomyxoviridae family of enveloped viruses (117). The virus is composed of eight segments of viral RNA tightly surrounded by nucleoprotein, and is known to infect humans, pigs, birds, and marine mammals (117, 118). Influenza type A can cause acute respiratory infection responsible for not only seasonal illness, but also pandemic outbreaks such as the 1918 Spanish flu and the more recent 2009 H1N1 swine flu that caused the World Health Organization (WHO) to declare its first ever
“public health emergency of international concern” (117). An important finding from studies conducted during the 2009 pandemic was that patients with higher numbers of pre-existing circulating CD8 T cells experienced less severe pandemic H1N1 influenza illness (119). This corresponds with earlier studies in mouse models showing that CD8 T cell immunity can provide cross-reactive protection against heterotypic strains of the virus and can transfer protection (5, 7, 29, 120), as well as human studies correlating CD8 T cell immunity to reduced viral shedding in the absence of neutralizing antibodies (121). The seasonal trivalent inactivated vaccine (TIV) is formulated using viral coat protein subunits hemagglutinin (HA) and neuraminidase (NA) from three influenza strains to elicit neutralizing antibody responses, but these viral components undergo rapid mutations in antigenic drifts and shifts and show limited efficacy in older adults (1). CD8 T cells immunity, which can target more conserved regions of the virus, may provide a potential strategy for the elusive “universal” vaccine that can potentially protect the population against various strains of both seasonal and pandemic outbreaks (8). Live attenuated influenza vaccines (LAIV), but not inactivated influenza vaccines (IIV), have been shown to at least transiently boost heterotypic CD8 T cell immunity to influenza (122). The LAIV “FluMist” (AstraZeneca) was licensed for use in 2003 in the United States and 2010 in Canada, and has since faced ongoing debate surrounding its efficacy above the existing seasonal TIV. Although still used in Canada, FluMist is no longer recommended by the Centre for Disease Control and Prevention in the US (123), further exemplifying the complexities of inducing protective immunity against influenza virus infection.

**Viral Immunity against Influenza**

Influenza is typically transmitted in airborne droplets and travels through the oral or nasal cavity to the airways where viral attachment is facilitated by influenza hemagglutinin (HA) binding to sialic acids expressed on the respiratory epithelium. Viral replication occurs productively in airway epithelial cells, but can also spread to neighbouring non-immune such as type I and II alveolar cells, and immune cells such as dendritic cells (DCs) and macrophages (124, 125). Host innate immune defenses depend on the activation of both cell-intrinsic and extrinsic pattern recognition receptors (126). Cell-intrinsic recognition events, many of which are dependent on
direct infection of cells, include the cytosolic sensor retinoic acid-inducible gene I (RIG-I), nod-like receptors (NLRs) and toll-like receptor (TLR) 3 activation (126). RIG-I in infected epithelial cells, macrophages, and DCs recognizes 5’ triphosphates on both intact and shorter subgenomic ssRNA after viral replication (127, 128), and activates interferon regulatory factor (IRF) 3 to induce type I interferon and nuclear factor kappa-B (NF-κB) to induce proinflammatory cytokine (such as IL-6, TNFα, IL-1β). The NLR-family NLRP3, which is expressed by many myeloid cell types as well as in human bronchial epithelial cells (129, 130), is also activated by influenza infection and promotes inflammasome activation, shown to be important for antibody responses and activation of T cells (131, 132). TLR3, which is constitutively expressed on human respiratory epithelium (133), recognizes endosomal double-stranded (ds) RNA in virus-infected cells, and likewise activates IRF3 (133, 134). The identity of this RNA ligand is still unidentified as influenza does not express dsRNA, and recognition may instead be due to structures present in phagocytosed dying influenza-infected cells (134) and therefore not dependent on direct infection itself. Although important for viral restriction, TLR3 appears dispensable for T and B responses but may play a role in the recruitment of CD8 T cells into the lung tissue (135). Cell-extrinsic viral recognition is mediated by TLR7 and TLR8, which recognize endosomal ssRNA in sentinel cells (136, 137). Plasmacytoid DCs (pDCs), for example, expose RNA ligands to TLR7 when viral capsids get degraded in the acidified endosomes (137). TLR7 activates downstream IRF7 and NF-κB for type I interferon and proinflammatory cytokine production (138), respectively, and is required for optimal antibody responses, but largely dispensable for T cell immunity (138). TLR8, which is functional in human but not in mouse, is expressed on human monocytes and macrophages and can induce IL-12 production upon stimulation (139); however, its role during influenza infection is unclear. Although functional redundancy exists between some of these pathways, distinct downstream effectors and differential roles in activating adaptive immune responses present a molecular signature that needs to be better understood in the context of vaccine design for optimal priming of T cell responses.

The release of cytokines and chemokines from the infected respiratory epithelium results in the recruitment of neutrophils, monocytes, NK cells, and various subsets of DCs to the site of infection (140). Migratory respiratory tissue-resident MHCIIhiCD11cibi DCs (RDCs) sample viral antigen in the airways and travel back to the draining mediastinal lymph node (LN) where
antigen presentation to naïve CD8 T cells takes place (141, 142). Although transfer of antigen from these RDCs to LN-resident CD8α+ DCs does occur (143), it has recently been shown that the RDCs themselves are more efficient presenters to both CD4 and CD8 T cells (141, 142, 144). CD103+ RDCs (CD103+CD11b<sup>neg</sup>-hi), which are intraepithelial as well as in the surrounding respiratory vasculature, are the most potent antigen-presenters to CD8 T cells due to their superior ability to process and load viral antigen onto MHC I (145, 146). They are also efficient cross-presenters, and have a unique ability to present apoptotic-derived antigen (141, 147, 148), although it is still not clear which is the more effective method for T cell activation. On the other hand, CD11b<sup>+</sup> RDCs (CD103<sup>-</sup>CD11b<sup>med</sup>-hi) are found in the lung interstitium and are major producers of chemokines in response to infection (141). It is thought that, while CD103<sup>+</sup> RDCs reach maximum numbers in the lung early during infection, CD11b<sup>+</sup> RDCs arrive later to dominate antigen presentation at the peak of infection and act to expand the pool of activated effector T cells while expressing much higher levels of CD70—the ligand for the costimulatory receptor CD27—compared to CD103+ RDCs (141, 144, 146). The two RDC subsets also seem to differ in how they dictate CD8 T cell fate differentiation, as CD103<sup>+</sup> RDCs induce a more effector-like phenotype, while CD11b<sup>+</sup> promotes central memory differentiation (149). Both subsets are involved in the continual presentation of residual antigen in the lung tissue for the activation of memory CD8 T cells (101), and have been shown to present effectively to CD4 T cells although preferential skewing to different Th subtypes have been largely context-dependent on the infection model studied (150-153). The role of a third subset, monocyte-derived DCs (MHCII<sup>lo</sup>CD11c<sup>lo</sup>) in T cell activation is less clear. Although regarded to be poor T cell activators, they are localized within the lung interstitium and their deficiency does result in fewer numbers of CD8 T cells in the lung tissue after influenza infection (141). Recent work from our laboratory show that it is these inflammatory monocyte-derived antigen presentation cells (APCs), including inflammatory DC and macrophages that present TNF family ligands 4-1BBL, OX40L, CD70 and GITRL during viral infection (Chang <i>et al.</i>, <i>Immunity</i>, in press).

CD8 T cells play a dominant role in restricting viremia by the cytolytic killing of influenza-infected epithelial cells in the lung and airways through perforin/granzyme-dependent cytolysis and CD90-CD90L (Fas-FasL) or TRAIL-mediated induction apoptotic cell death (29, 154). Production of proinflammatory cytokines, such as IFNγ and TNFα, by CD8 T cells can also
recruit bystander T cells as well as innate immune cells such as inflammatory CD11c\textsuperscript{hi} DCs that continue to present antigen for optimal T cell activation and survival (140, 155). On the other hand, CD4 T cells only play a modest cytolytic role in viral clearance and seem to predominate to provide help to support B cell responses and antibody production (156). Antibody-mediated immunity to influenza can be T-dependent and T-independent, and neutralizing immunity includes local IgA as well as systemic IgG, typically specific for HA and NA glycoproteins, while early IgM production is important for viral clearance and survival in mouse models (140, 157).

The primary function of the lung is gas exchange, and it is imperative that the tissue is preserved to uphold that task; therefore, immunoregulation is crucial to prevent tissue injury and pathology. TNFR-deficient mice have shown reduced influenza illness when infected with H5N1, without any difference in their ability to clear the virus (158). Similarly, IL-17 produced by γδ T cells has been shown to contribute to immunopathology after influenza as IL-17R-deficient mice exhibit less weight loss and reduced tissue damage (159). Furthermore, endogenous CD8 T cells produce IL-10 to coincide with proinflammatory mediators at initial influx into the lung tissue (160). Immunosuppression is also mediated by Tregs, CD200-expressing macrophages (such as alveolar macrophages), and pDCs, through IL-10 and TGFβ production (140, 155, 160-163). Innate lymphoid cells (ILCs) that are resident in the lung can produce the growth factor amphiregulin to facilitate tissue repair in response to IL-33 produced by the infected lung epithelium, although the depletion of ILCs does not affect viral clearance (164). Tregs are also known producers of amphiregulin, independent of their suppressor function, and this is important for tissue repair during influenza infection (165). In many cases, the preservation of lung capacity for respiration may come at a price of continued protection and immune surveillance within the tissue, as exemplified by the lack of persisting lung parenchymal memory CD8 T cell populations to be addressed in the next section.

**Lung-resident memory CD8 T cells**

Memory CD8 T cells have been shown to reside in lung tissue following respiratory infections as a first line of defense that can rapidly respond to antigen re-exposure and mediate protection (5,
These lung memory T cells have been characterized as expressing the conventional Trm markers CD103 and CD69 (5, 74, 97, 102), and studies in the parabiosis model have confirmed their residency in the tissue as they do not exit and re-enter circulation (93, 102). Deficiency in either CD103 or CD69 results in defective Trm retention in the lung tissue after infection (107). Histological studies have shown them clustering in the walls of the airways and surrounding blood vessels within the lung parenchyma (5), as well as close to sites of slow tissue repair (93), gradually waning as the injury heals. Unlike Trm identified in the other tissue, lung-resident influenza-specific memory CD8 T cells appear to be transient, with the population dropping quickly after viral clearance until virtually undetectable at seven months post-influenza infection (5-7, 97) despite steady numbers of splenic and circulating memory CD8 T cells that can traffic into the lung tissue (166). Furthermore, this loss of lung Trm is closely correlated with decreased heterotypic protection to secondary challenge (5, 7). As such, there is much interest in understanding the cellular interactions and molecular signals required for the induction and maintenance of long-lived protective CD8 lung Trm to better inform vaccine design against influenza.

A recent paper published by Slütter et. al. identified cell death, rather than lymphocyte egression, as a possible cause of the diminishing lung Trm pool, with influenza-specific Trm exhibiting an elevated pro-apoptotic signature (97). This includes low expression of pro-survival Bcl-2, which stands in contrast to the upregulation of Bcl-2 previously suggested as a crucial step in skin Trm lineage commitment following cutaneous infection (74, 77). These findings highlight the lung as a potentially unique environment that may prove less hospitable for Trm persistence. Slütter et. al. noted that the rate of cell death exceeded the rate of Trm diminishment in the lung, and continual recruitment from the circulation, although inefficient, is required to replenish the Trm pool (97). They elegantly demonstrated de novo Trm formation using the P14 model, a transgenic mouse line carrying TCR specific for the LCMV glycoprotein 33 (GP33) epitope, by systemically transferring antigen-experienced P14 cells into naïve or infection-matched hosts (with or without antigen) 21 days post-infection. This process was antigen-independent and instead relied on infection history and the local inflammatory cytokine milieu within the lung tissue. Notably, this process was dependent on TNFα and IL-33, which have been shown to upregulate CD69 and downregulate KLF2 for retention within the tissue (79, 108). The recruited
Trm precursors were from the Tem pool, and over time, circulating cells become more Tcm-like and lost the capacity for migration into peripheral tissue, causing the lung Trm population to diminish. This contradicts previous reports suggesting that antigen persistence in the lung and/or MLN for at least two months post-influenza infection is driving Trm formation. This was thought to occur through continuous waves of stimulation and recruitment of circulating memory CD8 T cells into the lung tissue, since the timing of antigen persistence conveniently corresponds with the gradual waning of lung Trm (101-103). Consistent with an antigen dependent process, Lee et. al. showed that antibody-mediated blocking of MHC I resulted in a loss of CD69 and CD103 expression that also corresponded with loss of antigen-specific CD8 T cells in the lung tissue (107). Thus, the process of Trm establishment appears to be dependent on both infection history and antigen, which is further supported by the highly-activated PD-1+ phenotype of these cells (5). It has also been shown that without antigen, inflammation in the lung and respiratory tract alone is insufficient to mediate Trm establishment (102, 103), but it is possible that early Trm precursors require different cues for commitment compared to later immigrants. It should be noted that work in parabiosis models, as well as experiments using FTY720 to sequester circulating lymphocyte populations, have failed to show contribution of circulating memory CD8 T cells in continuously seeding the Trm population (93, 167). Slütter et. al. speculate this to be a result of parabiotic and adoptive transfer experiments, or temporary pharmaceutical treatments, only accounting for a snapshot analysis of these populations and a matter of interpretation of the data as most parabiosis studies focus on equilibration—rather than complete lack of migration—of host and partner parabionts in the lung compared to the spleen (168). The other limitation of parabiosis is that not all cells are able to cross the surgical site as there is not a complete overlap in how the circulation is stitched together. However, Takamura et. al. found that the small population of cells that did migrate into the partner parabiont did not acquire a Trm profile and therefore may instead be peripheral circulating Tem cells travelling through the lung rather than acquiring and maintaining residency (93). It should also be noted that although dynamic imaging is informative and has contributed immensely to our knowledge of mucosal immunity, long-term studies are just not feasible for continued monitoring of cellular migration.
The mechanism of commitment and maintenance (or lack thereof) of lung Trm is still unclear. It has been shown that lung Trm have reduced responsiveness to IL-7 and IL-15 due to downregulation of CD127 (IL-7Rα) and CD122 (IL-2Rβ), respectively (31, 74, 78, 169). Although important for homeostatic maintenance and survival of circulating memory CD8 T cells (31-34), the importance of classical cytokines like IL-7 and IL-15 in Trm establishment is still up for debate as Trm from the brain are maintained independently of IL-7 and IL-15, but requires persistent antigen (72). Likewise, Trms from secondary lymphoid organs also do not require IL-15 (80), while skin Trm do need IL-15 in order to persist after HSV infection (74).

Lung Trm are, however, dependent on TGFβ which regulates CD103 expression and Trm formation in a dose-dependent manner, independent of Smad4-signalling (107, 170). Influenza neuraminidase (NA) can activate TGFβ to promote immunosuppression during infection (171, 172). TGFβ is synthesized in a latent form and the active peptide is highly-charged with a very short half-life (173, 174). The activation of TGFβ may be a much more localized and regulated process in the lung tissue since it is associated with lung diseases like pulmonary fibrosis (175), and thus may act as a limiting factor for CD8 Trm development within the lung parenchyma (176). Localization of effector T cells to the lung epithelium requires the homing molecule CXCR3 (177). CXCR3 expression on CD8 T cells is dependent on CD4 T cell help (178). Consistently, CD4 T cells are required for CD8 Trm formation in the lung, a process that is dependent on CD4 T cell-derived IFNγ production but CD8 T cell-extrinsic (178).

CD4 Trm have also been characterized in the lung tissue following influenza infection; they are CD103+ but express CD69 and CD11a, and have been shown to produce cytokines and transfer protection against weight loss and mortality upon lethal challenge (83, 167). Lung CD69+ CD11a+ memory CD4 T cells are non-circulating and persist without continual waves of recruitment from the circulation, as exemplified in short-term experiments in which lymphocyte egress from lymph nodes into circulation is blocked (83, 167). However, influenza-specific CD4 Trm have not been extensively studied and their mechanisms of maintenance and protection remain unclear.

A recent study on human lung Trm was conducted in which memory CD45RA- T cells were collected from the non-involved lung tissue of either non-small cell lung carcinoma or chronic obstructive pulmonary disease patients (105). Microarray analysis determined a transcriptional
profile of CD103+ and CD103− T cells in the lung which were more related to one another but dramatically distinct from circulating populations of memory T cells in the blood. Consistently with studies in murine models, these lung Trm populations showed lower expression for genes encoding S1PR1, CD62L, and KLRG-1 compared to blood memory populations. Moreover, human lung Trm showed upregulation of genes encoding transcription factors EGR2, FOSB, ATF3, and RBPJ; chemokines XCL1, CCL3, CCL4, and CCL20; chemokine receptors CXCR6, CXCR3, CCR5, and CCR6; effector molecules IFNγ, granzyme B, TNFα; and members of the tumour necrosis factor receptor signalling family TRAF1 and TANK (105). Interestingly, lung CD103+ Trm were also associated with an active Notch signature. Consistently, T cell-specific deletion of Notch1 and Notch2 in mice resulted in fewer antigen-specific CD8 T cells after influenza infection (105). Furthermore, treatment with γ-secretase inhibitors, which prevents the final cleavage step of the Notch precursor form (179), in influenza-experienced wildtype mice with already established Trm cells resulted in a loss of the CD103+ lung-resident population as well as transcriptional changes notably in genes involved in mTORC1 and glycolysis, suggesting a role for the Notch pathway in the persistence of CD103+ lung Trm perhaps through the control of metabolic programs (105).

Given the importance of the lung microenvironment in Trm commitment, it is no surprise that local intranasal immunization is crucial for the accumulation of CD8 T cells in the lung (5, 93, 103). Systemic intraperitoneal (i.p.) infection mounts effective circulating Tem but a poor lung Trm response (103) particularly since infection is not productive, thereby producing suboptimal priming of DCs for antigen presentation. Moreover, i.p. infected mice succumbed to secondary intranasal lethal influenza challenge (103). Interestingly, i.p. immunization does result in residual antigen in the draining LN after influenza infection, suggesting that it may be antigen persistence in the lung itself that is required for the maintenance of Trm as RDC populations traffic from the lung to the LN for continual activation of naïve CD8 T cells (101, 103). Thus, the induction of local environment and inflammatory cues within the lung tissue itself critically contributes to the fate-determination of CD8 T cells, as we have addressed in this section. It should also be noted that CD8 Trm have been identified in the airways as well following influenza infection with the ability to protect against challenge (180-182), but they have a short half-life of 10-14 days and it is widely regarded that continual antigen-independent recruitment is the mechanism of their
persistence (180). Since commonly used inactivated influenza vaccine are given intramuscularly, a better understanding of the importance of the local milieu for priming for optimal protection is imperative.

1.4 T cell Exhaustion and Lymphocytic Choriomeningitis Virus Clone 13 (LCMV cl 13)

During a chronic infection when viral load remains high and antigen is persistent, CD8 T cells become functionally unresponsive and undergo a stepwise loss of effector responses (183, 184). CD8 T cells first lose the ability to produce IL-2, followed by TNFα, and finally IFNγ (184). This exhaustion phenotype has been characterized in a number of infections, such as lymphocytic choriomeningitis virus (LCMV), human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), and many malignancies (183). Unlike acute infections where virus is cleared and memory cells establish, exhausted CD8 T cells maintain a CD62L-CD127- phenotype and do not develop into stable memory cells (185). Rather, they maintain high expression of activation markers like CD69 and CD43, sustain expression of inhibitory molecules like PD-1, Tim-3, and LAG-3, and become unresponsive to homeostatic cytokines such as IL-7 and IL-15 (185, 186). Severe, high-grade exhaustion can also result in deletion of epitope-specific CD8 T cells, although not all epitope specificities experience the same degree of exhaustion to a given infection in the same host (184). CD4 T cells have also been shown to experience exhaustion and epitope deletion, thus further impairing CD8 T cells activation during the infection (183, 187). Exhaustion is regulated by a unique molecular signature, distinct from T cell anergy or senescence (183, 185, 186), and is an important mechanism to protect from immune pathology when the inability to clear antigen is causing continuous T cell activation. In this regard, while transient blocking of the PD-1-PD-L1 pathway helps viral clearance, the absence of the pathway in PD-L1-deficient mice results in death from immunopathology (188).

LCMV clone 13 (cl 13) is a non-cytopathic arenavirus that causes chronic infection in mice wherein the antigen persists for several months and is eventually cleared by antibody-mediated immunity despite CD8 T cells being crucial for early viral control (189). Unlike the acute LCMV
Armstrong virus, which only differs from cl 13 by 3 amino acids, LCMV cl 13 is able to bind the viral receptor α-dystroglycan expressed on DCs with much higher affinity, leading to higher levels of viral replication, rendering the virus more difficult to clear by host immune responses (190). CD8 T cells undergo a hierarchical loss of function, as previously described, with certain epitopes undergoing deletion, causing a skewing of the immunodominance hierarchy (184). NP396-specific CD8 T cells start to diminish at day 8 post-infection and become almost undetectable by day 25, while GP33- and GP276-specific CD8 T cells populations are more stable with the latter normally subdominant epitope emerging as the dominant population late in the infection (184). Chronic LCMV infection is also associated with high production of the anti-inflammatory cytokines IL-10 and TGFβ (191, 192). Blocking the IL-10 receptor can reduce viral titres and improve CD8 T cell responses, with accompanying decreases in PD-1 expression (193). TGFβ appears to regulate the size of the CD8 T cell response but not its function (192). Blockade of the inhibitory molecule PD-1 can also improve CD8 T cell responses and reduce viral load (188, 194). PD-1 is expressed transiently by effector CD8 T cells upon activation and remains high on exhausted cells, but is not expressed on resting memory cells (188). Interestingly, a recent study from Im et. al. discovered that anti-PD-1 blockade selectively induced proliferation of a TCF-1-dependent CXCR5*PD-1+ CD8 T cell population in lymphoid tissues with self-renewing properties. These cells retained the ability to differentiate into, and therefore restore, terminally-differentiated effector cells found in both lymphoid and non-lymphoid tissues (194).

CD4 T cells are essential for viral control during chronic LCMV, as shown in CD4 T cell-depletion studies (195, 196). Notably, CD4 T cell production of IL-21 maintains CD8 T cell responses during the chronic phase of infection, and IL-21 treatment in CD4 T cell-depleted mice have reduced viral load and enhanced CD8 T cell responsiveness (197, 198). LCMV cl 13 also induces a shift in CD4 T cell responses from T helper 1 (Th1) to T follicular helper (Tfh), which provide help to B cell responses in the germinal centre for late antibody immunity that ultimately clears the infection (199).
1.5 Tumour Necrosis Factor Receptor (TNFR) Superfamily

As highlighted already in this introduction, a fine-tuned and well-regulated immune response is critical for defense against foreign invading pathogens without collateral tissue damage and immune pathology. The tumour necrosis factor receptor (TNFR) superfamily is one collection of immunomodulatory molecules that help strike a balance through its role in both co-stimulating and co-inhibiting T cell responses (13, 14). This modulation occurs at several stages of the T cell response, from the initial activation of DCs, through T cell expansion, contraction, and memory, affecting not only the size of the T cell pool but also effector function and the programming of the secondary response. The TNFR superfamily is defined by structural homology in the cysteine-rich ectodomain and generally form trimeric receptors for ligand-binding (200). There are three subsets of TNFRs, distinguished by their cytosolic signalling domains: 1) those that contain a death domain, 2) those that act as decoy receptors, and 3) those that lack death domains but recruit TNFR associated factors (TRAFs) through TRAF binding sites. (13). The TRAF-binding TNFRs contain members known to particulate in T cell regulation, and their functions are dependent on the context of the infection or immunization (13, 14). These receptors engage TRAF proteins through TRAF-binding motifs on their cytoplasmic tails, which then result in the recruitment of cellular inhibitors of apoptosis (cIAP) proteins that subsequently engage downstream activation of the classical NF-κB pathway for the transcription of pro-survival and pro-inflammatory genes. These receptors can also activate the alternative NF-κB and mitogen-activated protein kinase (MAPK) pathways, which can also promote proliferation and effector function (13, 14). In mice, the TNFR members GITR, OX40, and CD27 use TRAF2 and TRAF5, while 4-1BB binds TRAF1 and TRAF2, and CD30 has been shown to interact with TRAF1, TRAF2, TRAF3, and TRAF5 (13, 201). Despite some of these similarities, TNFR family members appear to have non-redundant roles in the immune responses at different stages of an infection or immunization. While CD28-family costimulation is important for the activation and priming of T cells, TNFR family members are expressed beyond the initial priming stage and thus respond to continued viral replication in the system. As such, their roles in post-priming survival of T cells allow for the modulation of immune responses to both acute and persistent viral infection, the latter of which often induces sustained expression of certain TNFR family
receptors. Thus, TNFR family members respond to the severity of infection and persistence of antigen, and the expression of their receptor and ligands are very tightly regulated (13, 14).

We will focus on two specific members of this family, 4-1BB and CD30, and discuss their roles in T cell immunity.

**4-1BB**

4-1BB, also known as CD137 or TNFRSF9, is one of the more well studied members of the TNFR superfamily. It is found on chromosome 1 in humans and chromosome 4 in mice, and in both cases clusters with TNFR2, CD30, OX40 and APO3 (202). 4-1BB is not expressed on resting T cells, but is induced by TCR stimulation (203) in an extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK)-dependent manner (204), prior to the first cell division and concomitant with CD69 expression on both CD4 and CD8 T cells (205). 4-1BB is also expressed on other hematopoietic-lineage cells, such as DCs, monocytes, granulocytes, NK cells, NKT cells, and mast cells, as well as endothelial and epithelial cells (206). On T cells, the expression of 4-1BB is very transient upon clearance of antigen, and can be prolonged if the antigen is persistent in the system (207-210). As such, it has been suggested that 4-1BB may be a useful surrogate marker for identifying human activated T cells (13, 211). In the absence of antigen, 4-1BB is induced on Treg by IL-2, while in vivo administration of IL-2/anti-IL-2 monoclonal antibody (mAb) can also upregulate 4-1BB expression on both adoptively transferred antigen-specific memory CD8 T cells and endogenous memory phenotype CD8 T cells (212). IL-15 has also been shown to induce 4-1BB on memory CD8, but not CD4, T cells in a MAPK p38 and ERK-dependent manner to maintain long-term survival of this population, although this was only demonstrated using human IL-15 on murine T cells ex vivo and has not been confirmed in vivo (213). Anti-4-1BB agonistic antibody can expand memory CD8 T cells in unimmunized mice in the collective absence of IL-15, IL-7 and IL-2 signaling (214). Fellow TRAF-binding TNFR member GITR can also regulate 4-1BB expression and this can contribute to pathological splenomegaly and liver inflammation caused by systemic administration of anti-4-1BB agonistic antibody (214). The regulation of 4-1BB signalling is also mediated through the tightly-controlled expression of its ligand 4-1BB ligand (4-1BBL, also known as CD137L or...
TNFSF9), which can be found on activated antigen presentation cells (APCs) and non-hematopoietic cells at sites of inflammation (206). Recent work from our laboratory shows that during influenza and LCMV clone 13 infection, 4-1BBL is primarily found on inflammatory monocyte-derived APC rather than classical DC (Chang et al., *Immunity*, in press). 4-1BBL is susceptible to metalloprotease cleavage and can be downregulated by 4-1BB-4-1BBL interaction (215, 216).

4-1BB (Figure 1.3) mediates CD28-independent co-stimulation by binding to TRAF1 and TRAF2 in mice, and TRAF1, TRAF2, and TRAF3 in humans (217-219). Unlike other TRAF molecules, TRAF1 does not contain an N-terminus RING finger domain that is required for classical NF-κB activation (220, 221). However, structural data indicates that the TRAF1-(TRAF2)$_2$ heterotrimer is a more effective recruiter of cIAP BIR domain than a TRAF2 homotrimer (222), thus supporting a role for TRAF1 in NF-κB activation. Furthermore, TRAF1 is known to stabilize TRAF2 and prevent its degradation (223), thus also contributing to the activation of downstream pathways. 4-1BB signalling results in classical NF-κB-dependent expression of pro-survival Bcl-2-family molecules Bcl-XL and Bfl-1 (224). Furthermore, the ERK pathway is stimulated in a TRAF1-dependent manner to induce downregulation of pro-apoptotic Bim (225-227), thus further inducing a pro-survival state in T cells. This also requires the F actin binding protein LSP-1, which is recruited to the 4-1BB signalosome by TRAF1 to mediate downstream ERK signalling (228). In resting cells, TRAF2, TRAF3, cIAP1, and cIAP2 form a complex that degrades the kinase NIK, an activator of the alternative NF-κB pathway (229, 230). The ligation of 4-1BB results in TRAF3 degradation that disrupts this immunoregulatory complex and allows accumulation of NIK (231). This activation of the alternative NF-κB is limited by TRAF1, perhaps through its stabilizing effect on TRAF2, as TRAF1$^-$/T cells experience hyperproliferation upon anti-CD3 stimulation which was reduced upon siRNA knockdown of NIK (231). 4-1BB signalling also induces activation of the MAPK p38 and c-jun pathways for T cell production of IL-2 and IFNγ (218, 232, 233). There is also evidence of ERK-dependent 4-1BB activation of the PI3K/AKT pathway, although this is with delayed kinetics and may be an indirect effect of intermediary cytokine signalling (234). The PI3K/AKT activation results in the induction of Wnt signalling and β-catenin for activation of
Figure 1.3. 4-1BB signalling promotes survival and cytokine production in T cells. Ligation of 4-1BB results in the recruitment of TRAF1 and TRAF2 in a heterotrimeric complex to the cytoplasmic tail of the receptor. TRAF1/2 then recruits a single cIAP 1 or 2 molecule that ubiquitinates RIP for downstream activation of the classical NF-κB pathway, resulting in upregulation of the pro-survival Bcl-2 family molecules Bcl-xL and Bfl-1. T cell survival is also contributed by TRAF1-dependent ERK signalling that leads to the phosphorylation and degradation of pro-apoptotic Bim. Activation of JNK and p38 MAPK pathways by the TRAF1/2 complex is important for cytokine production, with the latter being associated with 4-1BB-induced production of IL-2 and IFNγ. In the absence of 4-1BB stimulation, TRAF2, TRAF3, and cIAP1/2 form a complex that inhibits NIK accumulation. The absence of any of these components—as is in the case of 4-1BB stimulation, where TRAF3 becomes degraded—NIK activates the alternative NF-κB pathway to increase T cell production of cytokines.
the transcription factor T cell factor 1 (TCF-1) (234), which is an important component upstream of Eomes in CD8 T cell central memory differentiation (235).

The role of the 4-1BB-4-1BBL pathway in CD8 T cell immunity is dependent on the severity of the infection itself. As previously mentioned, 4-1BB expression is transiently induced by TCR signalling and maintained if the antigen is persistent (207-210). During infection with mild influenza A/HK/X31 (X31), where mice clear the infection within 8 days and only lose about 10% of their body weight before making a full recovery, 4-1BB is upregulated on CD8 T cells in the lung at day 6 but is undetectable by day 8 (207). Furthermore, 4-1BBL$^{-/-}$ mice have comparable CD8 T cell expansion to their wildtype controls, thus suggesting the pathway to be dispensable in the primary CD8 T cell response against this virus. However, in the context of a severe A/PR8 (PR8) infection, which can cause 30% loss in body weight and lethality, 4-1BB expression increases between days 6 and 8 in the lung, and 4-1BBL$^{-/-}$ have impaired viral control, fewer influenza-specific CD8 T cells in the lung, and greater mortality compared to their wildtype counterparts (207). It is likely that 4-1BB expression is tightly regulated to prevent excessive unnecessary immunity and immune-mediated pathology to the lung tissue when a pathogen is easily controlled, and is only allowed to persist if a prolonged CD8 T cell response is required. Interestingly, in the case of chronic infection where the virus cannot be controlled, 4-1BB signalling becomes desensitized. Work in the lymphocytic choriomeningitis virus (LCMV) clone 13 model, where antigen is cleared eventually between days 60 and 90 post-infection by CD8 T cell and antibody-mediated immunity, indicate that although 4-1BBL$^{-/-}$ exhibit higher viral load and lower frequency of the LCMV epitope NP396-specific CD8 T cells at day 8, there is no longer a difference in the response by day 21 between wildtype and knockout mice despite 20-60% of LCMV-specific CD8 T cells still expressing 4-1BB (208). This is due to a TGFβ-mediated loss of TRAF1 over time. Similarly, in HIV patient samples, TRAF1 expression in HIV-specific CD8 T cells also decreases over progression of the disease with controllers exhibiting higher TRAF1 expression than chronic progressors (208). Knocking down TRAF1 in T cells from these elite controllers reduce the ability of CD8 T cells to control HIV infection of CD4 T cells ex vivo. Thus, shutting down the 4-1BB-4-1BBL signalling pathway during persistent infections can potentially limit enduring tissue damage from chronic T cell activation but this may occur at the expense of protective immunity.
Studies in the influenza model have also implicated 4-1BB in memory CD8 T cell responses. Although 4-1BB is dispensable for primary CD8 T cell expansion during X31 infection, the late memory pool in 4-1BBL−/− mice assessed 21 days post-infection was significantly smaller, and subsequent secondary challenge with PR8 resulted in much reduced CD8 T cell expansion (236). It should be noted that this study only evaluated circulating influenza-specific memory CD8 T cell pools and the local tissue-resident responses within the lung were not investigated. Furthermore, 4-1BBL−/− mice infected with murine gamma herpes virus (MHV-68) exhibited normal numbers of primary CD8 T cells, although at reduced cytolytic effector function, but impaired secondary responses when challenged with vaccinia virus (209). Interestingly, unimmunized mice have higher 4-1BB expression on CD8+CD44hi T cells in the liver and bone marrow (BM) than the spleen, lung, and lung-draining LN (214), suggesting a 4-1BB-dependent niche for SLO-independent maintenance of memory or memory phenotype cells (14, 237, 238). 4-1BB can be induced by human recombinant IL-15 on OT-I memory T cells ex vivo (213); however, CD122-deficient T cells still express 4-1BB on memory T cells in the bone marrow (214), suggesting that IL-15 is dispensable for 4-1BB expression in vivo. 4-1BBL expression on a radioresistant cell type is responsible for the antigen-independent maintenance of this memory pool and VCAM-1+ stromal cells were shown to express 4-1BBL in the bone marrow (239). The 4-1BB-4-1BBL pathway likely maintains these cells through survival signals as adoptive transfer of carboxyfluorescein succinimidyl ester (CSFE)-labelled CD8 T cells show no difference in proliferative abilities in 4-1BBL-sufficient or deficient hosts, despite decreased recovery in the latter (213). Thus, 4-1BB-4-1BBL interactions affects various stages of the CD8 T cell response—from effector to memory responses.

It should also be noted that 4-1BB−/− and 4-1BBL−/− models do not always show complementary phenotypes, although rarely have both knockout models been tested in the same experiments. 4-1BB-deficiency results in hyperproliferation of T cells in a number of experimental settings (206, 240-244). In murine cytomegalovirus (MCMV) infection, antigen-specific CD8 T cells hyper-expand in 4-1BB−/− mice and this occurs independently of 4-1BBL as anti-4-1BBL blocking antibody and 4-1BBL−/− mice did not recapitulate this phenotype (240). Furthermore, 4-1BB−/− mice show enhanced splenic T cell proliferation to anti-CD3 or concanavalin A (ConA) stimulation, although with diminished effector function (241), and human PBMCs transfected
with 4-1BB exhibited reduced proliferation to anti-CD3 stimulation (244). Although 4-1BB is the only identified binding partner of 4-1BBL, 4-1BB is known to also bind to extracellular matrix proteins and galectin-9 (245, 246) and it may have a yet unidentified binding partner that mediates immunosuppression of T cell responses (206). It should be noted however, that 4-1BBL and 4-1BB deficient mice used in the above mentioned studies were made on the 129 background and in many cases littermate controls were not used.

The administration of systemic anti-4-1BB agonistic antibody in vivo has shown efficacy in expanding CD8 T cell (247-249) and improving anti-viral CD8 T cell responses in various mouse models of infection. When administered during influenza infection, anti-4-1BB mAb can increase the expansion of T cells specific for subdominant epitopes for improved diversity of responses, although the opposite is not seen in the 4-1BBL−/− model (250). Furthermore, systemic 4-1BB stimulation can replace CD28 for T cell priming responses in i.p. infection with influenza virus, and this in turn generates a full recall responses (251). Anti-4-1BB has also been able to enhance expansion and cytolytic effector function of antigen-specific CD8 T cells in herpes simplex virus type 1 (HSV-1), wherein that phenotype is maintained into the secondary response after challenge (252). In vaccinia infection, no defect has been shown in T cell responses using knockout models (210), but 4-1BB is expressed as anti-4-1BB antibody is still able to augment proliferation of CD8 T cell responses to the virus (253). Interestingly, in LCMV Armstrong acute infection, the timing of anti-4-1BB stimulation is crucial. When given 1 day post-infection, fewer CD8 T cells and a worst disease outcome arises, while delaying administration to 3 days post-infection results in enhanced CD8 T cell immunity (254). The highlights the importance of temporal regulation in TNFR family receptor and ligand expression in the outcome of disease. Paradoxically, while anti-4-1BB agonists enhance T cells during viral infection and cancer models in mice, the same antibodies can also ameliorate a number of autoimmune and inflammatory disease models (255). Overstimulation of 4-1BB by systemic agonistic antibodies have also been associated with toxicity both in mouse models and in the clinic. Splenomegaly, liver damage, B cell depletion, and hematopoietic perturbations have all been reported in mouse models (214, 256, 257). Anti-4-1BB agonist therapy in clinical trials for melanoma have also led to excessive inflammation and grade IV hepatitis in some individuals (258). Combination
therapies of anti-4-1BB with anti-PD-1 and anti-CTLA4 are currently being explored and immune-mediated side-effects carefully monitored (14).

For more targeted delivery of 4-1BB stimulation that can potentially avoid systemic pathological effects, vaccine vectors have been used where 4-1BB-4-1BBL interactions are co-opted to generate a strong adjuvant effect for CD8 T cell induction (14). The inclusion of 4-1BBL with the influenza nucleoprotein (NP) gene into a replication-defective adenovirus vector has been shown to induce greater proliferation and effector function of primary antigen-specific CD8 T cells that can protect mice with short-term protection from lethal challenge (259). Incorporating 4-1BBL into an HIV gag DNA vaccine was also able to enhance both CD4 and CD8 T cell as well as humoral responses, the latter of which was suppressed when anti-4-1BB agonistic antibodies were used instead (260). Another approach in the context of cancer immunotherapy has been to incorporate the 4-1BB signalling function into chimeric antigen receptor (CAR) expressing T cells, whereby the benefits of enhanced T cell survival is coupled to a specificity targeting only the tumor (261). This avoids the systemic effects of anti-4-1BB agonist antibodies, although these therapies also induce strong inflammation due to tumor lysis syndrome (262). As TNFRs increasingly become a therapeutic target in the clinic, a better understanding of the regulation and function of 4-1BBL, both in systemic as well as in localized immunity, is imperative for future immunotherapies and vaccine design.

**CD30**

One of the less studied members of the TNFR superfamily is CD30, otherwise known as TNFRSF8. CD30 was first characterized on Hodgkin’s lymphoma and Reed-Sternberg cells, and has since been associated with anaplastic large cell lymphoma (ALCL), multiple myeloma, adult T cell leukemia, and some solid tumours (263-266). The overexpression of CD30 can result in ligand-independent constitutive activation of the classical NF-κB pathway (267), thus potentially contributing to malignancies. CD30 is not expressed on resting T cells, but is induced upon TCR stimulation in a CD28-dependent manner upon activation (266, 268). Its expression can also be induced by Th2 cytokines, such as IL-4, through a STAT6-dependent mechanism (201, 268, 269). CD30 is also expressed on B cells, NK cells, eosinophils, and γδ T cells (201, 266, 270,
The CD30 ligand (CD30L, also known as CD153) is found on resting B cells and activated T cells, as well as non-hematopoietic cells at the site of inflammation (201, 272, 273). There have also been reports of CD30L and another TNFRSF ligand OX40L on a unique subset of CD4^+CD3^- accessory cell that supports primed and memory T cell survival at sites of T and B cell contact within B cell follicles (274, 275). Th2 cells were described as the recipients of these signals through their expression of CD30 and OX40, but it is possible that the subset described is the more recently-discovered T follicular helper (Tfh) cell that provide help to B cells in germinal centres to facilitate antibody responses (276).

The cytoplasmic tail of the CD30 signalling complex interacts with TRAF1, TRAF2, TRAF3, and TRAF5 to induce both the classical and alternative NF-κB, p38 MAPK, ERK, and PI3K/AKT signalling pathways (13, 277-284). Nuclear translocation of TRAF2 is associated with activation of both the classical and alterative NF-κB pathways (277). Dominant negative mutations in TRAF2 or TRAF5 result in aberrant NF-κB promoter activity in a reporter assay, while a separate study implicated TRAF1 and TRAF2 using similar reporter measures of NF-κB activation (285), suggesting a role for these molecules in the classical and/or alternative NF-κB pathways (282). Indeed, as previously discussed, loss of TRAF2—and thus the degradation of the TRAF2-TRAF3-cIAP1-cIAP2 immunoregulatory complex—results in the accumulation of NIK and constitutive activation of the alternative NF-κB pathway (229, 230). In vitro experiments have shown that engagement of CD30 in addition to TCR stimulation results in enhanced proliferation and cytokine production, such as IL-2, TNFα, and IFNγ, compared to TCR stimulation alone (201, 266, 268, 286-288). Furthermore, anti-CD30 treatment of already primed murine T cells results in TRAF2-dependent and p38-mediated production of IL-13 in the absence of TCR engagement (281). Paradoxically, CD30-CD30L engagement has also been shown to induce cell death or have suppressive functions in several lymphoma cell lines (286, 289). Moreover, TRAF2, along with the associated TRAF1 protein, can be degraded upon TRAF2 recruitment to the CD30 intracellular cytoplasmic tail, thus potentially limiting its own signal transduction in a negative feedback loop (290). This may contribute to the immunosuppressive function of the CD30-CD30L pathway.

Work using CD30^-/- or CD30L^-/- mouse models have implicated a role for the pathway in controlling certain infections. CD30-CD30L interactions were shown to be important for long-
term central memory CD8 T cell generation and protective CD4 memory phenotype T cells in *Listeria monocytogenes* infection (291), Th1 and IL-17A production by γδ T cells in *Mycobacterium bovis* (292, 293), lymphocyte recruitment into the lung, and IFNγ-producing CD4 T cells during *Mycobacterium avium* (294, 295), as well as in CD4 T cell differentiation into Th17 (296-298). Alternatively, no defect in T cell responses was reported in the vesicular stomatitis virus (VSV) model (299), while double knockouts of CD30 and OX40 resulted in defective antigen-specific CD8 T cell responses in MCMV infection (300). It should be noted that poxvirus encodes a structural homolog of the soluble CD30 (sCD30) molecule to bind and block CD30L interaction with its receptor (301, 302), thus suggesting an antiviral role of the pathway that needs to be subverted by invading pathogens. The role of CD30 in antiviral T cell immunity will be discussed more extensively in Chapter 5.

sCD30 has been measured in the clinic as a biomarker of immune activation (13), particularly in transplant patients where higher sCD30 has been predictive of reduced infection risk but also of rejection post-transplantation (303-305), while patients with chronic infection have a demonstrated association of higher sCD30 with increased pathogen load (306, 307). CD30 has also been associated with Th2-mediated autoimmunity (13), and there are conflicting data on whether the absence of CD30-CD30L signalling can ameliorate or exacerbate disease in diabetes (201, 308, 309). In the context of cancer, the US Food and Drug Administration (FDA) recently approved an anti-CD30 mAb conjugated to the chemotherapy drug MMAE called Brentuximab Vedotin to target CD30+ tumors (13). Despite the cytotoxic potential of CD30, as shown in its ability to induce apoptosis and cell cycle in ALCL cells *in vitro* (277), the goal of this drug is to use CD30 as a target marker for drug delivery (13). As CD30 is becoming increasingly relevant in the clinic, whether as a diagnostic biomarker or as a therapeutic target, more studies need to be done to examine the role of this pathway in various immunological contexts.

### 1.6 Thesis Objectives and Rationale

The role of TNFR family members in the establishment of Trm cells has not been thoroughly investigated, despite evidence implicating some of these molecules in systemic circulating
memory responses (13, 14, 236). As previously mentioned, a study looking at human lung tissue identified TRAF1 in the distinct gene profile signature of lung-resident CD103$^+$ and CD103$^+$ memory T cells (105). As TRAF1 is an NF-kB induced gene, it is plausible that TNFR family members that induce TRAF1 and also utilize it in their signaling pathways could influence the induction and/or maintenance of circulating and tissue-resident CD8 T cells during influenza infection. In Chapters 3 and 4 of this thesis, I identify a critical role for 4-1BB in the establishment of anti-influenza, lung-resident memory CD8 T cells, and explore a prime-boost immunization regimen to assess the biological implications of exploiting the 4-1BB-4-1BBL pathway in the context of a vaccine model. Chapter 5 is focused on CD30, in which I assess its role in effector and memory T cell responses during influenza infection, as well as its function on T cells in the more persistent LCMV cl 13 infection and at steady state. Taken together, I hope to inform on the context-dependent nature of TNFRs in the regulation of T cell responses and provide insight into the implications of co-opting these signals in future immunizations and immunotherapies.
Chapter 2
Materials and Methods
Mice. C57BL/6 mice were purchased from Charles River Laboratories (St. Constant, Quebec, Canada). Both male and female mice were used for experiments, but were age- and sex- matched within each experiment. For bone marrow chimera experiments, Thy1.1 (B6.PL-Thy1a/CyJ) and CD45.1 (C57Bl/6-Ly5.1) mice were purchased from Jackson Laboratories (Bar Harbor, USA), with the latter bred in-house at the University of Toronto. 4-1BB− mice, bred on the C57BL/6 background were obtained from B. Kwon (Tulane University, New Orleans, USA) (241) and used as source of donor cells for bone marrow chimeras. CD30− mice generated on the 129 background and extensively backcrossed to C57BL/6 (B6), mice were kindly provided by Tak W. Mak (Ontario Cancer Institute, Toronto) (299). These mice are now available from Jackson Laboratories (Bar Harbor, ME). We analyzed the CD30− mice by SNP analysis (performed by The Center for Phenogenomics, Toronto, Ontario) and found them to be 96% similar to Charles River B6 mice across 1200 SNPs. The mice were further backcrossed to B6 mice purchased from Charles River (Wilmington, MA) to generate F2 littermates for experiments. All mice were housed in sterile micro-isolator cages under specific pathogen-free conditions. Animal studies were approved by the University of Toronto Animal Care Committee in accordance with the regulations of the Canadian Council on Animal Care (animal protocol permit number 200111642).

Viruses. Influenza A/HK/X31 and A/PR8 viruses were grown in eggs and their tissue culture infectious dose determined by infection of Madin-Darby Canine Kidney (MDCK) cells (310). Mice (5 to 6 weeks of age) were immunized with 30 μL of diluted virus at the indicated doses by i.n. infection while anaesthetized with isofluorane. For PR8 infections, mice were monitored closely with daily weights taken and were euthanized when moribund. Replication-defective adenovirus 5 expressing influenza NP in the E1 region were kindly provided by J. Bramson (McMaster University, Hamilton, Canada). The NP gene was derived from influenza A/PR8 GenBank: J02147.1. Adenovirus expressing NP in E3 and 4-1BBL in E1 were generated as previously published (259). Immunizations of mice with adenovirus were performed at the indicated doses and schedule by i.n. or i.p. injections as indicated. For the chronic infection model, female CD30+/+ and CD30− littermate mice were infected intravenously with 2x10^6 focus-forming units (ffu) of LCMV clone 13, provided by Michael B.A. Oldstone (Scripps Research Institute, San Diego, CA).
**Tissue harvest and processing.** Mice were injected intravenously with 3 μg of anti-mouse CD8α (clone: 53-6.7) antibody in PE (eBioscience, San Diego, USA) or BV605 (BioLegend, San Diego, USA) and euthanized 10 minutes later (78). Lung tissue was perfused with 10 mL of PBS to remove cells in the pulmonary vasculature, and then minced and digested in 100 units/mL of collagenase IV (Invitrogen, Carlsbad, USA) for one hour at 37 degrees in a shaker. Lung tissue were then mechanically disrupted and filtered through a 70 μM to create single cell suspensions, and treated with RBC lysis buffer prior to staining. For CD30 experiments where tissue-resident (Trm) populations were not specifically assessed—i.e. vascular and parenchymal populations were not differentiated—the i.v. *in vivo* staining technique was not performed prior to perfusion and digestion of lung tissue. Spleen (after RBC lysis) and dLN were mechanically disrupted and filtered through a 70 μM to create single cell suspensions. Blood was collected from the saphenous vein and treated with RBC lysis buffer.

**In vitro T cell stimulation.** Splenocytes from CD30+/+ and CD30−/− B6 mice were stimulated *in vitro* with 1µg/ml of plate-bound anti-CD3 (145-2C11) and 10 µg/ml of soluble anti-CD28 (37.51), and expression of CD30 was assessed by flow cytometry after 24, 48, and 72 hours of treatment.

**Flow cytometry and intracellular cytokine staining.** Influenza NP_{366-374} and LCMV GP_{33-41} and NP_{396-404}-specific CD8 T cells were analyzed using MHC class I tetramers obtained from the National Institute for Allergy and Infectious Disease tetramer facility (Emory University, Atlanta, USA) and conjugated to streptavidin-allophycocyanin (Prozyme, purchased through Cedarlane, Ontario, Canada). For intracellular cytokine staining, lung and spleen samples were restimulated *ex vivo* with 1 μM of the MHC I-restricted NP_{366-74} peptide for 6 hours with GolgiStop (BD Pharmingen, San Jose, USA) at 37°C. Cells were surfaced stained, fixed, permeabilized, and stained intracellularly for the appropriate cytokines. Unstimulated samples (no peptide) were used as negative controls. For LCMV experiments, splenocytes were restimulated in the same way with
either MHC I-restricted GP33-41 or NP396-404 peptides, or MHC II-restricted GP60-81 peptide. Samples were analyzed using LSRFortessa (BD Biosciences), and FlowJo (TreeStar Inc., Ashland, USA) software.

**Antibodies.** The antibodies used in this study are as follows: anti-mouse CD8α (clone: 53-6.7) (eBioscience), anti-mouse CD3ε (clone: 145-2C11) (eBioscience, BioLegend), anti-mouse Thy1.1 (clone: HIS51) (eBioscience), anti-mouse CD45.1 (clone: A20) (eBioscience), anti-mouse CD45.2 (clone: 104) (eBioscience), anti-mouse CD62L (clone: MEL-14) (eBioscience), anti-mouse CD127 (clone: A7R34) (eBioscience), anti-mouse IFNγ (clone: XMG1.2) (eBioscience), anti-mouse TNFα (clone: MP6-XT22) (BD Pharmingen), anti-mouse IL-2 (clone: JES6-5H4) (eBioscience), anti-mouse CD107a (clone: 1D4B) (eBioscience), anti-mouse T-bet (clone: 4B10) (eBioscience), anti-mouse Eomesodermin (clone: Dan11mag) (eBioscience), rabbit anti-mouse TCF-1 (clone: MA5-14965) (Thermo Fisher, Waltham, USA) with labelled secondary donkey anti-rabbit (Thermo Fisher), anti-mouse CD69 (clone: H1.2F3) (eBioscience), anti-mouse CD103 (clone: 2E7) (eBioscience), anti-mouse CD49a (clone: Ha31/8) (BD Pharmingen), anti-mouse CD30 (clone: mCD30.1), anti-mouse CD44 (clone: IM7) (eBioscience), anti-mouse Foxp3 (clone: J43) (eBioscience), anti-mouse CXCR5 (clone: SPRCL5) (eBioscience), anti-mouse Tim3 (clone: RMT3-23) (eBioscience), anti-mouse CD25 (clone: PC61.5), and fixable viability dye (eBioscience). In vivo IL-7R blockade was performed with i.p. injections of 400 µg/dose/mouse of anti-CD127 monoclonal antibody (clone: A7R34) at two doses, two days apart in the indicated immunization schedule. The hybridoma was kindly provided by C. Paige (University Health Network, Toronto, Canada).

**Mixed bone marrow chimeras.** Bone marrow was obtained from the femur and tibia of the indicated donor mice and transferred at a 1:1 ratio into the indicated host mice that were lethally-irradiated with two doses of 550 cGy using the Nordion GammaCell 40 Exactor. A mix of 2.5×10⁶ cells from each donor type (for a total of 5.0×10⁶ cells) were transferred in a total of 200 µL by intravenous tail vein injection. Reconstituted chimeric mice received water supplemented with 2
mg/mL neomycin sulfate (Bio-Shop, Burlington, Canada) during the first two weeks, and were rested for a total of 90 days before chimerism was checked. They were then immunized according to the indicated schedules.

**FTY720 treatment.** Mice were daily given 200 μL of FTY720 (Sigma-Aldrich, St Louis, USA) by i.p. injection at a dose of 1 mg/kg diluted in PBS, according to the indicated treatment regimen.

**Rapamycin treatment.** Mice were daily given 200 μL of rapamycin (LC Laboratories, Woburn, MA, USA) by i.p. injection at a dose of 0.75 μg/kg. Rapamycin was initially reconstituted in dimethyl sulfoxide (DMSO) and then diluted in PBS, for a final working concentration of ~1% DMSO.

**Focus forming assay for LCMV viral load.** Organs were immediately frozen at -80°C upon harvest. For viral load, organs were thawed, homogenized, and the supernatant collected to perform dilutions (10² to 10⁵-fold) for infection of an MC57 cell monolayer under a 2% methylcellulose-MEM overlay. MC57 monolayers were fixed with 4% paraformaldehyde 48 hours later, permeabilized with 1% Triton X-100, and stained with Rat anti-LCMV mAb (VL-4). Secondary staining with Goat anti-Rat HRP and o-Phenylenediamine (Sigma-Aldrich, Oakville, ON, Canada) was used to induce a colorimetric reaction to label LCMV-infected foci.

**Statistics.** Statics were performed using GraphPad Prism 6 (San Diego, USA), with the specific test performed as indicated in the figure legends.
Chapter 3

Intrinsic 4-1BB signals are indispensable for the establishment of an influenza-specific tissue-resident memory CD8 T cell population in the lung

The results in this chapter have been published in *Mucosal Immunology*:


**Author Contributions**

A.C.Z. and T.H.W. designed the study, analyzed the data, and wrote the manuscript. A.C.Z. performed all experiments with technical assistance from L.E.W. and M.E.W. on the long-term blood-tracking cohorts.
3.1 Abstract

The induction of long-lived heterotypic T cell protection against influenza virus remains elusive, despite the conservation of T cell epitopes. T cell protection against influenza is critically dependent on lung-resident memory T cells (Trm). Here we show that intranasal administration of 4-1BBL along with influenza nucleoprotein (NP) in a replication-defective adenovirus vector to influenza pre-immune mice induces a remarkably stable circulating effector memory (Tem) CD8 T cell population characterized by higher IL-7Rα expression than control-boosted T cells, as well as a substantial lung parenchymal CD69+ CD8 Trm population including both CD103+ and CD103− cells. These T cell responses persist to greater than 200 days post-boost and protect against lethal influenza challenge in aged (year old) mice. The expansion of the NP-specific CD8 Trm population during boosting involves recruitment of circulating antigen-specific cells and is critically dependent on local rather than systemic administration of 4-1BBL as well as on 4-1BB on the CD8 T cells. Moreover, during primary influenza infection of mixed bone marrow chimeras, 4-1BB-deficient T cells fail to contribute to the lung-resident Trm population. These findings establish both endogenous and supraphysiological 4-1BBL as a critical regulator of lung-resident memory CD8 T cells during influenza infection.
3.2 Introduction

A universal vaccine against influenza virus remains elusive. Although the induction of broadly neutralizing antibodies to influenza virus shows promise (122), cross-protective T cell immunity will likely be an important component of any broadly protective long-lasting vaccine. T cell epitopes are highly conserved in diverse influenza strains (8). However, even a live influenza infection only transiently boosts circulating T cell responses in previously immune adults (2, 311). Moreover, older people have impaired immunity to influenza and are highly vulnerable to influenza-induced morbidity and mortality (3). Therefore, we need a means of inducing long-lived protective immunity that could be administered in midlife to allow protection late in life.

Tissue-resident memory T cells (Trm) reside in tissue to provide a first line of defense against recurrent infection (9, 10). Trm are induced when T cells enter the infected tissue during primary infection and differentiate in situ to form a non-circulating population that can persist to rapidly respond upon re-exposure to antigen (9, 10). Within the lung, CD8 Trm have been shown to mediate protective heterotypic immunity to influenza infection (5, 182). However, longevity is a problem as lung CD8 Trm disappear within months after influenza infection, and heterotypic immunity is lost despite steady numbers of memory CD8 T cells in the spleen and circulation that can still traffic into the lung tissue (5-7).

CD137 (4-1BB) signals are particularly potent in enhancing the expansion and maintenance of CD8 effector and memory T cells (312). Moreover, lack of 4-1BBL impairs the recall response to influenza virus (313), attributed to a loss of effector memory T cells at the late stage of the primary response (236). However, to date, the effect of 4-1BBL on tissue-resident memory T cells has not been examined.

Most individuals receive influenza vaccines in adulthood after previous exposure to natural influenza infection. Therefore, to mimic the effect of vaccination of a previously immune adult, we asked whether boosting with influenza nucleoprotein (NP) together with 4-1BBL improves the duration of protection to influenza virus. We show that intranasal (i.n.) immunization of influenza-immune mice with a replication-defective adenovirus containing influenza NP and 4-1BBL results in induction of a long-lived circulating memory population of effector memory T
cells (Tem) as well as a substantial Trm population in the lung that protects mice against lethal heterotypic influenza challenge into old age (one-year-old). These effects require 4-1BB on the CD8 T cells. Remarkably, in mixed bone marrow chimeras 4-1BB-deficient T cells are almost completely absent from the lung parenchymal pool of memory CD8 T cells established during primary influenza infection. These findings establish an essential role for 4-1BB/4-1BBL interactions in the formation of persistent CD8 Trm within the lung during influenza infection.

3.3 Results

Local stimulation of 4-1BB in a secondary immunization augments the number of circulating memory CD8 T cells and provides long-lived protection against lethal influenza challenge

Intranasal (i.n.) administration of a replication-defective adenovirus containing the influenza NP gene and 4-1BBL (Ad-NP-4-1BBL) can lower the required vaccine dose for induction of NP366-374-specific CD8 T cells that is protective against lethal influenza challenge compared to a vaccine containing NP (Ad-NP) alone (259). However, this effect is short-lived, providing protection for only an additional three-months over NP alone (259). Moreover, studies of T cell responses during the 2009 pandemic showed that even a live infection induces only transient boosting of circulating pre-existing CD8 memory populations in humans (2, 311). Thus, there is a need to improve upon even the natural infection to obtain long-term robust T cell immunity to influenza virus. As 4-1BB stimulation is more effective in a secondary as compared to a primary CD8 T cell response (251, 314, 315), we asked whether delivery of 4-1BBL in a replication-defective adenovirus vaccine to influenza-immune mice would induce longer-lived protection against lethal influenza infection. Accordingly, mice were infected with a low dose of influenza virus A/HK/X31 (X31) (316) and then either left unboosted or boosted one-month later with replication-defective adenovirus alone (control Ad), Ad-NP, or Ad-NP-4-1BBL (Figure 3.1A). We chose a vector dose of 10^6 pfu/mouse, which is 100-fold lower than the optimum dose required for maximal NP366-374-specific T cell expansion with Ad-NP alone during priming (259). Circulating memory CD8 T cells were followed in the blood for nine to eleven months in
Figure 3.1. Intranasal (i.n.) delivery of 4-1BBL in a replication-defective adenovirus vector with influenza NP induces a long-lived circulating memory CD8 T cell population and protects against lethal flu challenge. (A) Mice were infected i.n. with A/HK/X31 (5 HAU/mouse) and then left unboosted or boosted i.n. one-month later with a control replication-deficient adenovirus (control Ad), adenovirus containing the influenza NP gene (Ad-NP),...
or adenovirus containing NP and 4-1BBL (Ad-NP-4-1BBL) at $10^6$ pfu/mouse. (B) Influenza NP<sub>366-374</sub>-specific CD8 T cells were tracked in the blood using the D<sup>3</sup>/NP<sub>366-374</sub> tetramer, with (C) representative gating at ten-months (day 300) post-boost. Control here and in subsequent figures refers to pooled data from unboosted and control Ad-boosted, which gave indistinguishable results. Statistical analysis is shown between Ad-NP and Ad-NP-4-1BBL-boosted groups. (D) Frequency (of total CD8 T cells) and absolute numbers of D<sup>3</sup>/NP<sub>366-374</sub> tetramer+ CD8 T cells quantified in the spleen at seven-months (day 210) post-boost. Mice were challenged at nine-to-eleven-months (day 270 – 330) post-boost with a lethal dose of A/PR8 influenza ($5\times10^7$ TCID<sub>50</sub>/mouse). (E) Weight loss and (F) survival curve are shown post-challenge. Data in (B, C, F) are pooled from two separate experiments with n=6-8 per group per experiment, while (E) shows a representative of the two experiments (mean ± SEM). (D) was pooled from two separate experiments with n=4 per group per experiment. ** P < 0.005, *** P < 0.0005, **** P < 0.00005 (2way ANOVA test (B, E)), Kruskal-Wallis test (D), or Mantel-Cox test (F)).

Two separate cohorts of mice, followed by influenza challenge. Inclusion of 4-1BBL in the vector dramatically enhanced the expansion of circulating NP<sub>366-374</sub>-specific CD8 T cells, which reached a maximum of 30% of circulating CD8 T cells by day 7, before declining over the next few weeks, and stabilizing at approximately 10% of the CD8 T cell population between day 60 and 120 (Figure 3.1B, C). Remarkably, this stable population of circulating CD8 memory cells persisted to at least 300 days. Similar results were recapitulated in the spleen for at least seven-months, where there was not only a higher frequency of NP<sub>366-374</sub>-specific memory cells in the circulating CD8 T cell pool, but also an increase in the absolute number in Ad-NP-4-1BBL compared to Ad-NP or control boosted groups (Figure 3.1D). At this low vector dose, the response to Ad-NP was only marginally higher than that of control Ad-boosted or unboosted mice (Figure 3.1B).

To test the protective efficacy of Ad-NP-4-1BBL treatment, mice were challenged with a lethal dose of influenza A/PR8 (PR8) at nine to eleven-months post-boost. PR8 shares the same NP epitope as the initial priming X31 strain but contains different HA and NA proteins (PR8 is H1N1 while X31 is H3N2), thereby allowing us to assess protection from CD8 T cell immunity without the interference of neutralizing antibody responses (316). Mice pre-treated with Ad-NP-4-1BBL were protected from influenza-mediated weight loss and the majority survived challenge, whereas Ad-NP boosting offered a much smaller survival advantage over controls (Figure 3.1E, F). There were no signs of splenomegaly or liver toxicity as measured by alanine transaminase levels in the serum (data not shown), suggesting that the localized 4-1BBL administration avoids the pathological effects that have been associated with systemic administration of anti-4-1BB agonistic antibody (257).
**4-1BBL-induced circulating memory cells have characteristics of long-lived effectors**

Circulating memory T cells can be characterized as central memory (Tcm) or effector memory (Tem) based on their migration patterns and functional properties. Tcm are identified by high expression of CD62L and CCR7, and are more quiescent with greater proliferative potential upon reactivation. In contrast, Tem, which lack CD62L and CCR7, patrol the circulation and rapidly respond to antigen re-encounter through IFNγ and TNFα production (11, 62). We therefore evaluated the circulating memory T cells for CD62L as well as for CD127, the IL-7Rα chain, to distinguish between memory T cells from terminally-differentiated effectors (41, 317). NP366-374-specific CD8 T cells from Ad-NP-4-1BBL-boosted mice showed delayed acquisition of CD62L compared to Ad-NP or control Ad-boosted or unboosted mice (Figure 3.2A, B). This difference was first apparent at two-months post-boost in the blood and persisted until at least ten-months. This decrease in Tcm and increase in Tem compartments was similarly observed in lymphoid organs, including the spleen (Figure 3.2 C, D) and dLN (data not shown). The delayed acquisition of CD62L by the Ad-NP-4-1BBL-boosted T cells was not accompanied by lower CD127 expression (Figure 3.2A, D) and therefore not a result of expanded terminally-differentiated effectors.

The T-box transcription factors T-bet and Eomesodermin (Eomes) are key drivers of CD8 T cell lineage differentiation. The higher expression of T-bet or Eomes is associated with effector-like or memory-like phenotypes, respectively (12). Consistent with the effector-like nature of the memory CD8 T cells, the Ad-NP-4-1BBL boost induced higher T-bet expression early (day 7 post-boost, Figure 3.2E), and lower Eomes between day 30 and 60 (Figure 3.2F) compared to Ad-NP in the spleen. The transcription factor T cell factor 1 (TCF-1) is induced downstream of the canonical Wnt pathway, and regulates memory CD8 T cell differentiation and longevity, partially through the induction of Eomes (235). Splenic memory T cells from Ad-NP-4-1BBL-boosted mice have lower TCF-1 expression early post-boost (day 7, Figure 3.2G). This effector-like transcriptional profile after 4-1BB stimulation appears to be a characteristic of secondary immunization, as T cells induced during primary immunization with Ad-NP or Ad-NP-4-1BBL
Figure 3.2. Ad-NP-4-1BBL i.n. boost induces an effector-like circulating memory CD8 T cell population. (A) Tracking of central memory (Tcm; NP tetramer⁺CD127⁺CD62L⁺) and effector memory (Tem; NP tetramer⁺CD127⁻CD62L⁻) populations in the blood of mice left unboosted or boosted with control Ad, Ad-NP, or Ad-NP-4-1BBL, as described in Figure 3.2A. (B) Frequencies of Tcm and Tem were quantified at six-months (day 180) post-boost in the blood. (C) Representative gating and (D) frequencies of Tcm and Tem at seven-months (day 210) post-boost in the spleen of Ad-NP and Ad-NP-4-1BBL-boosted mice. Mean fluorescence intensity (MFI) and representative histograms of transcription factors (E) T-bet, (F) Eomesodermin (Eomes), and (G) TCF-1 in NP.
were similar with respect to T-bet and Eomes profiles (data not shown). Differences in TCF-1 and T-bet were noted early (day 7) post-boost and thus are likely associated with memory programming rather than differences in the proportions of Tcm and Tem, which were not different at day 7 in the spleen when CD62L expression was very low in both Ad-NP and Ad-NP-4-1BBL-boosted groups (data not shown). By six-months post-boost, there is no longer a measureable difference in expression of these transcription factors between Ad-NP and Ad-NP-4-1BBL boosted NP-specific CD8 T cells, whereas Tcm and Tem frequency differences persist (Figure 3.2C, E-G).

Consistent with the more effector-like memory phenotype, Ad-NP-4-1BBL boosting induced a significantly higher frequency, but not MFI, of IFNγ-producing CD8 T cells, as determined by ex vivo restimulation with the NP\_366-374 peptide, even at ten-months post-boost when Ad-NP no longer differed substantially from controls (Figure 3.3A, B). Similar results were seen with TNFα and CD107a, with the latter showing a significantly higher MFI in Ad-NP-4-1BBL-boosted CD8 T cells. Ad-NP-4-1BBL-boosted mice also had more polyfunctional memory CD8 T cells, with a higher frequency of triple and quadruple producers of IFNγ, TNFα, CD107a, and IL-2 in the blood (Figure 3.3C), lung (Figure 3.3D), and spleen (3E). This polyfunctionality was not recapitulated with increasing doses of Ad-NP, which resulted in loss of functionality when titrated up 100-fold (Figure 3.3D, E), consistent with previously reported effects of high dose Ad immunization (318). This loss of functionality at high Ad-NP dose occurs despite a greater number of NP tetramer+ CD8 T cells in the lung and a fairly constant number in the spleen with increasing vector dose (Figure 3.3F). Thus 4-1BBL in the boost phase induces a long-lived effector memory population that correlates with long-lived protection against influenza challenge.
Figure 3.3. 4-1BBL-boosted memory CD8 T cells are more polyfunctional at a lower dose of the adenovirus vector than NP only boosted T cells. Samples were stimulated ex vivo with the NP$_{366-374}$ peptide and intracellular staining was performed to assess (A) Frequency and MFI of IFNγ, TNFα, and CD107a-producing CD8 T cells in the blood at ten-months (day 300) post-boost with (B) representative gating. Frequency of polyfunctional CD8 T cells (C) in the blood at ten-months post-boost producing IFNγ, TNFα, and CD107a; and at day 30 post-boost in the (D) lung and (E) spleen producing any two, three, or four combinations of IFNγ, TNFα, CD107a, and IL-2 with Ad-NP dosage titrated up 10- and 100-fold. (F) Absolute numbers of D$^b$/NP$_{366-374}$ tetramer+ CD8 T cells in lung and spleen at 30 days post-
boost with Ad-NP titration and Ad-NP-4-1BBL. Data in (A-C) are pooled from two separate experiments with n=6-8 per group per experiment, while (D-F) was performed once at day 30 and repeated independently with similar results at day 60 post-boost, all with n=4 mice per group per experiment (mean ± SEM). NS not significant, * P < 0.05, ** P < 0.005, *** P < 0.0005, **** P < 0.00005 (Kruskal-Wallis test).

Local 4-1BB stimulation induces a long-lived lung-resident memory population consisting of both CD103+ and CD103- subsets

Trm are important mediators of protective immunity to influenza virus (5, 6). Therefore, we investigated whether the local introduction of 4-1BBL with antigen through i.n. immunization impacted T cells in the lung. Intravascular infusion of labelled anti-CD8 antibody prior to euthanasia was used to distinguish vascular from parenchymal T cells that reside within the lung tissue (Figure 3.4A) (78). Much like the circulating response characterized in the blood and spleen, there was a dramatic augmentation in the initial expansion of NP366-374-specific CD8 T cells in the lung tissue parenchyma that peaked at day 7, and declined by day 60 to form a stable population that persisted to at least seven-months post-Ad-NP-4-1BBL-boost (Figure 3.4A, B). This occurred at a dose where the response induced by Ad-NP alone diminished within the first 60 days. Whereas NP366-374-specific cells made up the majority of the CD8 T cells within the lung tissue at their peak (day 7 post-boost) in both Ad-NP and Ad-NP-4-1BBL treated mice, the low numbers of CD8 T cells that remained in the lung at seven-months after Ad-NP-boost contained few that were NP366-374-specific (Figure 3.4C). This was in contrast to Ad-NP-4-1BBL-boosted mice, where the stable population of Trm was largely NP366-374-specific. These persistent lung parenchymal NP-specific T cells from the Ad-NP-4-1BBL-boosted mice consisted of CD103+CD69+, CD103-CD69+, and CD103-CD69- cells (Figure 3.4A, D). CD103+CD69+ Trm have previously been identified in the lung (107), while CD103-CD69+ Trm have been reported in the lamina propria, secondary lymphoid organs, and thymus (9). Both these populations expressed low T-bet, Eomes, and CD127, as well as high lung-homing CD49a compared to lung vascular memory populations (Figure 3.4E), as previously reported for lung Trm (78, 86, 169, 181, 319). We also observed lower TCF-1 expression in the lung NP-specific Trm. The CD103-CD69- population had an intermediate phenotype between the vascular and the CD69+ parenchymal populations for many of these markers. Thus, stimulating 4-1BB directly in
Figure 3.4. Ad-NP-4-1BBL i.n. boost induces a persistent population of CD103⁺ and CD103⁻ lung-resident populations of memory CD8 T cells. (A) Gating strategy for intravascular antibody staining to differentiate vascular and parenchymal lung populations at seven-months (day 210) post-boost in the lung. (B) Long-term tracking of NP³⁶⁶-³⁷⁴-specific CD8 T cells in the lung tissue parenchyma. (C) Frequencies of D³⁹/NP³⁶⁶-³⁷⁴ tetramer⁺ within lung parenchymal CD8 T cells at day 7 and seven-months post-Ad-NP or Ad-NP-4-1BBL boost. (D) Absolute numbers of total NP³⁶⁶-³⁷⁴-specific memory CD8 T cells, and subpopulations of CD103⁺CD69⁺, CD103⁻CD69⁺, CD103⁺CD69⁻, and CD103⁻CD69⁻.
CD69+, and CD103 CD69+ memory CD8 T cells in the lung tissue at seven-months post-boost. (E) Flow cytometry analysis of T-bet, Eomes, TCF-1, CD127, and CD49a MFI between total lung parenchymal, parenchymal CD103+CD69+, parenchymal CD103 CD69+, parenchymal CD103 CD69−, and lung vascular memory CD8 T cells. Data were pooled from two separate experiments per time-point with n=3 per group per experiment (mean ± SEM). NS not significant, * P < 0.05, ** P < 0.005, *** P < 0.0005, **** P < 0.00005 (2-way ANOVA test).

the respiratory tract induces a robust enlargement of the Trm population over control Ad-NP immunization, and this effect persists to at least 210 days post-boost.

**Ad-NP-4-1BBL acts directly on the CD8 T cells to induce expansion in local and systemic tissue compartments**

To determine if 4-1BB was acting directly on the CD8 T cells or through another cell type, we used mixed bone marrow (BM) chimeras in which lethally irradiated mice were reconstituted with a 1:1 mix of 4-1BB+/+ and 4-1BB−/− bone marrow. Blood was monitored for degree of chimerism at three-months post-reconstitution, followed by priming with influenza A/X31. One-month later, mice were given Ad-NP-4-1BBL by the i.n. route (Figure 3.5A). Prior to infection, the ratio of 4-1BB+/+ to 4-1BB−/− CD8 T cells was approximately 1:1 (Figure 3.5B, C). At 27 days after infection with X31, there was already a 3 to 4-fold advantage of 4-1BB+/+ over 4-1BB−/− NP366-374-specific CD8 T cells in the blood, while the non-NP-specific CD8 T cell compartment remained at the 1:1 ratio of the initial reconstitution (Figure 3.5C, D). These data recapitulate previous studies on the role of 4-1BB/4-1BBL in maintaining the circulating CD8 T cell memory pool (236). At day 7 post-Ad-NP-4-1BBL boost (the peak response), the NP366-374-specific CD8 T cells were almost exclusively from the 4-1BB+/+ donor in the lung parenchyma, lung vasculature, dLN, and spleen (Figure 3.5E, F), and this dichotomy persisted out to day 50 post-boost (Figure 3.5G). Thus the effects of 4-1BBL on the circulating and the lung-resident NP-specific memory CD8 T cell populations are completely dependent on direct (intrinsic) effects of 4-1BB on the CD8 T cells.
Figure 3.5. Ad-NP-4-1BBL acts directly on the CD8 T cell compartment to induce enhanced localized and circulating memory responses. (A) Mixed bone marrow (BM) chimeras were generated by reconstituting a 1:1 ratio of 4-1BB+/+ (Thy1.1 CD45.2) and 4-1BB−/− (Thy1.2 CD45.2) BM into lethally irradiated 4-1BB+/+ (Thy1.2 CD45.1) host mice. Chimeric mice were rested for 90 days and then infected i.n. with A/HK/X31 infection followed by Ad-NP-4-1BBL one-month later. (B) Ratios of 4-1BB+/+ to 4-1BB−/− of CD8 T cells, CD4 T cells, and Treg compartments in the blood of chimeric mice after reconstitution and before immunization. (C) Representative flow plot for donor CD8 T cells in blood pre- and 27 days post-infection. (D) Ratios of CD45.2 donor 4-1BB+/+ to 4-1BB−/− of NP366-374 tetramer-negative and NP366-374 tetramer-positive CD8 T cells in the blood at 27 days post-X31 infection, with dotted line indicating pre-infection reconstitution ratios in the blood. (E-G) Ratios of CD45.2 donor 4-1BB+/+ to 4-1BB−/− of NP366-374 tetramer-negative and NP366-374 tetramer-positive CD8 T cells in the lung parenchyma, lung vasculature, dLN, and spleen at (E) 7 days with (F) representative flow plots, and (G) day 50 post-Ad-NP-4-1BBL-boost. Statistical analysis is shown between tetramer positive and negative CD8 T cells within each organ. Data from (B, D, E) pooled from two separate experiments with n=8 per experiment, while (G) is pooled from two experiments with n=3 per experiment—chimeras were independently generated for each replicate set of experiments (mean ± SEM). * P<0.05, ** P < 0.005 (Wilcoxon test).

Ad-NP-4-1BBL induces greater IL-7Rα (CD127) expression on memory CD8 T cells, which may contribute to the longevity of the response

We next asked if 4-1BB stimulation leads to long-lived memory T cells through effects on cytokine receptors associated with T cell survival, memory commitment and maintenance, including CD25 (IL-2Rα) and CD122 (IL-2Rβ) and CD127 (the IL-7Rα subunit). CD25 and CD122, although present, did not show consistent differences between the Ad-NP and Ad-NP-4-1BBL groups (data not shown). However, Ad-NP-4-1BBL boosted mice exhibited a higher frequency of CD127+ NP366-374-specific memory CD8 T cells in the lung parenchyma but not lung vasculature compared to Ad-NP boosted mice as early as day 7 post-boost, with similar results in the dLN. Modest frequency differences persisted in the spleen at day 30 (Figure 3.6A, B), but were no longer apparent by day 60 in any of the tissues examined (data not shown). Of note, the NP-specific CD8 T cells from the 4-1BBL-boosted group expressed higher CD127 at the per cell level from day 30 (Figure 3.6C) to at least six-months post-boost (Figure 3.6D, E). MFI of CD127 was examined on total lung tissue at this time-point instead of separated into lung parenchymal and vascular compartments because there were very few memory CD8 T cells left in the lung tissue in the Ad-NP group late in the boost response. The higher frequency and per cell expression of CD127 was specific to 4-1BB stimulation in the secondary response as mice given Ad-NP or Ad-NP-4-1BBL i.n. as a primary immunization did not exhibit this difference (data not shown).
Figure 3.6. Ad-NP-4-1BBL-boosted memory cells have increased CD127 (IL-7Rα) expression. (A) Frequency of CD127 expression on NP\textsubscript{366-374}-specific memory CD8 T cells in the lung parenchyma, lung vasculature, and dLN.
at day 7; and the spleen at day 30 post-Ad-NP or Ad-NP-4-1BBL-boost, with (B) representative gating of the lung tissue and spleen. MFI of CD127 in the lung and spleen at (C) day 30 and (D) six-months (day 180) post-boost, with (E) representative histograms. (F) Mice were infected i.n. with X31 and then boosted 30 days later with either Ad-NP or Ad-NP-4-1BBL along with an i.p. injection of Ratlg or anti-CD127 (A7R34) blocking antibody at day 0 and day 2 post-boost (400 μg/dose). (G) NP366-374-specific CD8 T cells were enumerated in the spleen at day 30 post-boost. Data are pooled from two (A (day 7), D) or three (A (day 30)-C separate experiments with n=4 per group per experiment (mean ± SEM). NS not significant, * P < 0.05, ** P < 0.005, *** P < 0.0005 (Mann-Whitney test).

To test the importance of IL7Rα induction in the boost phase, we used an anti-CD127 blocking antibody (Figure 3.6F). IL-7R blockade at day 0 and 2 post-boost decreased the number of NP-specific memory T cells in the spleen by a similar proportion, regardless of whether the boost contained Ad-NP or Ad-NP-4-1BBL (Figure 3.6G), consistent with a critical role for IL-7Rα in general. However, the lung Trm population was not affected by IL-7R blockade (data not shown). Whether this is because the systemic antibody does not effectively penetrate the lung parenchyma to block IL-7R or whether IL-7R is dispensable for the Trm cannot be distinguished in this experiment. Thus 4-1BBL in the boost phase induces a sustained elevation of IL-7Rα levels on the NP-specific CD8 memory T cells, which may contribute to their increased persistence; however, it is difficult to ascertain the significance of this increase as IL-7R is critical for the memory response regardless of inclusion of 4-1BBL in the boost.

**Delivery of 4-1BBL directly into the airways is required for the lung-resident memory response**

We next asked whether existing CD8 Trm populations in the lung can be boosted by a systemic administration of 4-1BBL. Primary infection with low dose X31 i.n. induced a small number of lung parenchymal NP366-374-specific memory CD8 T cells, which remained stable between day 30 and 70 post-infection in the tissue (Figure 3.7A, B). Mice were boosted at 30 days after the i.n. prime with Ad-NP or Ad-NP-4-1BBL by either the i.n. or the i.p. route (Figure 3.7C). As expected, by day 7 the systemic i.p. boost had resulted in a much greater peak NP366-374-specific memory CD8 T cell response in the spleen compared to i.n. administration (Figure 3.7D).

However, i.p. boost did not result in efficient accumulation of memory CD8 T cells in the lung, despite i.n. priming immunization. Importantly, systemic delivery of Ad-NP-4-1BBL did not significantly increase the number of NP366-374-specific CD8 T cells in the lung compared to Ad-
Figure 3.7. Delivery of 4-1BB stimulation directly into the airways by i.n. immunization is required for lung parenchymal secondary memory expansion, which relies predominantly on recruitment of antigen-specific CD8 T cells from the circulation. (A) Representative gating and (B) absolute number of lung parenchymal memory CD8 T cells at 30 and 70 days after i.n. X31 infection. In order to assess whether systemic 4-1BBL-boost can expand local Trm, (C) mice were infected i.n. with A/HK/X31 (5 HAU/mouse) and one-month later boosted with Ad-NP or Ad-NP-4-1BBL either by i.n. or i.p. immunization (10⁶ pfu/mouse). (D) Expansion of NP₃₆₆-₃₇₄specific memory CD8 T cells at day 7 post-boost peak response is quantified in the lung and spleen. (E) Mice were infected with Influenza A X31 and then one-month later boosted i.n. with either Ad-NP or Ad-NP-4-1BBLand given an i.p. injection of either PBS control or FTY720 (1mg/kg) daily, until sacrificed at day 7 post-boost. (F)
Representative flow plots confirm sequestration of CD8 T cells from the circulation. (G) Absolute number and frequency of NP$_{366-374}$-specific memory CD8 T cells were quantified in the lung. Data from (D, G) pooled from two separate experiments with n=4 per group per experiment (mean ± SEM). For (A), day 30 was pooled from two experiments while day 70 is only one experiment, all n=4 per experiment. NS not significant, * P < 0.05, *** P < 0.0005, **** P<0.00005 (2way ANOVA test).

NP alone. Thus, local delivery of the 4-1BBL-containing vector is required for lung-resident Trm accumulation in response to 4-1BBL during the boost phase.

**Expansion of Trm during boosting is dependent on recruitment of T cells into the lung**

The increase in Trm following intranasal boost with Ad-NP-4-1BBL could be due to expansion of existing memory T cells in the lung or recruitment of new effector T cells into the Trm pool. To distinguish these possibilities, we treated influenza-primed mice daily by i.p. injection of either PBS or the immunomodulatory drug FTY720 during the boost phase (Figure 3.7E). FTY720 causes internalization of the sphingosine 1-phosphate receptor (S1PR) resulting in the sequestration of lymphocytes within lymph nodes, thereby preventing lymphocytes from entering the circulation (109). Thus, any CD8 memory T cell expansion within the lung would be a result of 4-1BBL acting on populations already present in the lung prior to adenoviral boost. As expected, FTY720 treatment caused an increase of NP$_{366-374}$-specific memory CD8 T cells within the dLN (data not shown), and a decrease in the blood (Figure 3.7F) and spleen (data not shown). Without T cells trafficking into the lungs, there was a significant reduction of NP-specific cells in the tissue parenchyma after Ad-NP-4-1BBL-boost (Figure 3.7G), reducing the size of the Trm pool to a level comparable to the Ad-NP control boost. Interestingly, Ad-NP boost was not affected by FTY720 treatment. Furthermore, FTY720 did not affect the frequency of NP-specific CD8 T cells in the lung. These data suggest that the enhancement of Trm cells in the lung through local 4-1BB stimulation relies on recruitment of additional antigen-specific cells from the circulation.
The endogenous 4-1BB/4-1BBL pathway is critical for the formation or maintenance of the lung-resident CD8 population during primary influenza infection

Having established that a supra-physiological dose of 4-1BBL can locally augment the pre-existing NP-specific Trm population in the lung, we next asked whether the endogenous 4-1BB costimulatory pathway also plays a role in the establishment of the lung Trm population during primary influenza infection. To test the role of endogenous 4-1BB signalling in Trm induction, we again used 4-1BB+/−:4-1BB−/− mixed BM chimeras (Figure 3.8A). After reconstitution, mice were infected i.n. with a low dose of X31 and sacrificed one-month later to study lung-resident populations. Late after primary infection, the NP366-374-specific CD8 T cells were almost exclusively from the 4-1BB+/− donor. This dichotomy was not nearly as striking in the dLN or spleen (Figure 3.8B-D). Similar results were obtained for the immunodominant influenza PA224-233 epitope (data not shown). This endogenous 4-1BB+/− lung-resident memory pool was comprised of the conventional Trm-associated CD103+CD69+ as well as CD103−CD69+ T cells (Figure 3.8E). The lack of 4-1BB−/− memory CD8 T cells specifically in the lung tissue suggests that the 4-1BB/4-1BBL pathway acts directly on Trm and is critical for the establishment or maintenance of Trm during influenza infection.

3.4 Discussion

Tissue-resident memory T cells provide a vital defense against infection at barrier and mucosal surfaces (9, 10). Here we have shown that both supraphysiological as well as endogenous 4-1BB provide an indispensable signal for influenza virus-specific Trm. Using 4-1BB+/−: 4-1BB−/− mixed bone marrow chimeras we show that there is an almost complete failure of 4-1BB−/− T cells to contribute to the lung-resident Trm pool after primary i.n. influenza infection. Moreover, administering 4-1BBL in a local boost with antigen in the same vector can induce a highly stable population of memory CD8 T cells that persists for almost a year and protects mice from lethal influenza challenge. This long-lived memory T cell population includes a circulating effector
Figure 3.8. 4-1BB expression on CD8 T cells is required for their contribution to the tissue-resident memory (Trm) compartment following primary influenza infection. (A) Mixed bone marrow (BM) chimeras were generated as described in Figure 3.5. After 90 days of reconstitution, chimeric mice were monitored for degree of chimerism in the blood and then infected i.n. with A/HK/X31 (5 HAU/mouse) and tissues were harvested 30 days post-infection. (B) Representative gating for donor cells within the vascular and parenchymal memory CD8 T cell compartments. (C) Ratios of CD45.2 donor 4-1BB+/+ to 4-1BB−/− of NP366-374 tetramer-negative and NP366-374 tetramer-positive CD8 T cells in the lung parenchyma, lung vasculature, draining lymph node (dLN), and spleen with (D) representative gating of the tetramer+ population. Dotted line indicates pre-infection reconstitution ratio of CD8 T cell compartment. Statistical analysis is shown between tetramer-negative and tetramer-positive CD8 T cells. (E) Frequency and representative gating for CD103+/CD69+ and CD103−/CD69+ Trm populations within the lung parenchymal NP366-374-specific CD8 T cells from the 4-1BB++ compartment. Data are pooled from three separate experiments for a total of 7 chimeric mice, with 2-3 mice per experiment and each replicate set of chimeras generated independently (mean ± SEM). NS not significant, * P < 0.05 (Wilcoxon test for (C) or Mann-Whitney test for (E)).
memory subset that expresses higher levels of IL-7R compared to cells boosted without 4-1BBL as well as a robust Trm population, consisting of both CD103+ and CD103- subsets. The accumulation of Trm in response to 4-1BBL is completely dependent on 4-1BB expression on the CD8 T cells (Figure 3.5), local delivery of 4-1BBL into the airways of the mouse (Figure 3.7C-D), recruitment of antigen-specific CD8 T cells from the circulation (Figure 3.7E-G), and inclusion of Ag and 4-1BBL in the same vector (data not shown).

The effect of local 4-1BBL administration on the Trm pool requires that new effector cells are recruited to the lung (Fig 7 E-G). Previous work has shown that 4-1BB/4-1BBL interactions are dispensable for controlling the number of NP-specific CD8 T cells observed in the lung at day 9-10 of a primary infection with influenza A/X31 as used here (207). Thus, it seems unlikely that 4-1BBL is involved in recruitment of the effectors to the lung per se, but rather in the establishment or maintenance of the Trm once these cells reach the tissue. Effects of exogenous 4-1BB stimulation on T cell accumulation are largely due to effects on survival rather than on the initial rate of division (226). Consistently, we did not observe significant increases in bromodeoxyuridine uptake by CD8 T cells boosted with or without 4-1BBL (data not shown). 4-1BB stimulation has been shown to impact multiple survival pathways, through NF-κB mediated upregulation of the anti-apoptotic genes Bcl-xL and Bfl1 (224) and TRAF1-Erk-dependent downmodulation of the proapoptotic Bim (225, 226). Thus it is likely that 4-1BB mediates several prosurvival signals to enhance the Trm population. These survival signals may be important in keeping the antigen-specific T cells alive in the lung as they differentiate into Trm and/or they may be important in the maintenance of the already established Trm cells. Regardless, it is clear that without 4-1BB, the Trm are virtually absent by day 30 post priming.

The effect of endogenous 4-1BB was much more striking on the lung Trm population, than the circulating effector memory population observed in the spleen at day 30. Previous work showed that 4-1BBL has about a 2-fold effect on the Tem population observed in the spleen at day 38 post-prime (236). However, in the current studies, this small effect did not reach statistical significance (Figure 3.8C). In contrast, 4-1BB+/+ cells almost exclusively populated the lung Trm pool (Figure 3.8C). On the other hand, when supraphysiological 4-1BBL signals are given in the
boost using the Ad-NP-4-1BBL vector, effects on Tem and Trm were both dramatic. This is likely because 4-1BB is more highly expressed upon reactivation of primed T cells than on naïve T cells (314) and because the amount of endogenous 4-1BBL is much lower than the amount achieved with the adenovirus vector.

Adenoviruses can enter cells that express the coxsackievirus and adenovirus receptor (CAR), such as airway epithelial cells (320), but are also known to infect monocytes and dendritic cells by a CAR-independent pathway (321, 322). Previous work showed that Ad-4-1BBL induces 4-1BBL expression on both CD45− and CD45+ cells in the lung, with the former showing greater expression (207) and no detectable expression in the draining LN, suggesting a largely local effect. Given that the airway epithelial cells are by far the most abundant targets for adenoviral infection in the lung and the very low viral vector dose administered in our model, it is plausible that the NP and 4-1BBL delivered by the Ad vector are presented to the T cells by epithelial cells to induce local effects on the Trm or in the recruited Trm precursors. The ability of 4-1BBL to impact T cell responses through non-professional APC is perhaps not surprising given the CD28-independence of the 4-1BB signal (233). The source of endogenous 4-1BBL for driving expansion of the Trm population during primary influenza infection remains to be determined. However, 4-1BBL is expressed on CD11chiCD11bhi MHC II+ cells in the lung of influenza infected mice (323) and 4-1BBL mRNA is also present albeit at low levels in the lungs of unimmunized mice as measured by PCR (207).

Although anti-4-1BB agonist antibodies have been administered systemically to induce expansion of T cells (248, 249), this is associated with expansion of endogenous CD4 and CD8 effector memory cells independent of immunization (324) and leads to immunopathology(257). In contrast, by delivering 4-1BBL in the same vector as antigen straight into the airways, we did not observe splenomegaly or increased liver enzyme activity that is observed with systemic anti-4-1BB administration (257). This is likely because the low dose local delivery of 4-1BBL with antigen preferentially targets the antigen-specific response to the immunogen.

IL-7R has been identified as necessary but not sufficient for the formation of memory CD8 T cells in the context of viral infection (34). In the present study, we identified greater IL-7R expression, both in frequency at early time points and in per cell expression late in the response
in 4-1BBL-boosted circulating and lung-resident memory CD8 T cells. IL-7R expression is correlated with the pro-survival molecule Bcl-2 and identifies long-lived memory precursor cells (41). However, it is difficult to determine the importance of the relatively small increase in per cell level of expression of the IL-7 receptor for the 4-1BBL-mediated effects, given that IL-7R is necessary for memory CD8 T cell formation in general during viral infection (34) and blocking IL-7R during the boost similarly attenuated memory formation in both the Ad-NP and Ad-NP-4-1BBL groups.

Antigen presentation can be detected within draining lymph nodes for at least 40 days post-immunization with replication-defective adenoviruses, with CD8 T cells exhibiting signs of chronic activation even out to 60 days (318). Thus the prolonged expression of NP and 4-1BBL in the airways may be an important component of the dramatically enlarged Trm population and the long-lived protective response. Indeed, prolonged antigen stimulation has been associated with Tem differentiation (325). Furthermore, prolonged cognate antigen stimulation has been implicated in maintaining the expression of the Trm marker CD103 (5). Despite the reported persistence of Ad-induced antigen-presentation for ~two-months, 4-1BBL administration in the present study induces Trm for at least 210 days post-boost and protection out to at least a year. It is possible that durable changes induced by 4-1BBL are either programmed during the period that the adenovirus delivered genes are expressed, or potentially that the initial response is maintained by the lower level of endogenously expressed 4-1BBL (207). Indeed, in earlier adoptive transfer studies of circulating CD8 T cell memory, 4-1BBL was found to be dispensable for priming, but required for maintenance of CD8 memory T cells (251). It is also important to note that prolonged antigen-presentation by replication-defective adenovirus vectors at high doses can induce a CD8 T cell phenotype consistent with partially exhausted Tem cells (318). This was observed in our study as well. At higher dose of Ad-NP, we saw a loss in the ability of memory CD8 T cells to produce multiple cytokines (Figure 3.3D, E). However, boosting with 4-1BBL induced a polyfunctional influenza-specific CD8 T cell response at a much lower dose of Ad vector and antigen, potentially avoiding the exhaustive effect of high-dose persisting antigen.

In sum, we have shown that 4-1BB is critical on CD8 T cells for induction of a long-lived lung Trm population through endogenous as well as supra-physiological stimulation. This signal must be locally delivered and is critical in the induction of optimal T cell responses within the lung for
protective immunity against influenza infection. Although the inclusion of a self-molecule like 4-1BBL may not be a practical vaccine strategy, the upregulation of 4-1BBL and other TNFR-family ligands is an important factor to consider when developing adjuvants for future vaccines.
Chapter 4
The role of mTOR, TRAF1, and local antigen in lung CD8 tissue-resident memory establishment

The results in this chapter are unpublished.

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4.1 Abstract

The tumour necrosis factor receptor (TNFR) superfamily receptor 4-1BB has been shown to play a role in tissue-resident memory (Trm) establishment in the lung tissue following influenza infection, and supraphysiological stimulation of 4-1BB directly in the airways after initial mild influenza priming can induce long-lived Trm cells that protect against lethal heterotypic challenge. However, the mechanisms of Trm longevity in the lung is still under investigation as these memory populations appear to be transient and disappear after a few months post-infection. In this study, we further elaborate on the role of 4-1BB in the induction of long-lived memory CD8 T cell immunity resident to the lung tissue. We identify a role for 4-1BB in regulating the size of the initial effector pool within the lung after primary influenza infection, which may be dependent on the downstream adaptor molecule TRAF1. To further investigate the requirements for the establishment of Trm, we used a prime-boost model in which mice were given mild influenza and then boosted intranasally with a replication-defective adenovirus containing the influenza nucleoprotein gene and 4-1BBL. This model induces a robust Trm population under conditions where the time and location of the 4-1BBL signal is known. Through this model, we determined that 4-1BB-mediated Trm generation is dependent on local antigen and the mTOR pathway. We hypothesize that a signalling axis involving 4-1BB, TRAF1 and mTOR contributes to the establishment of Trm in the lung.
4.2 Introduction

The induction of tissue-resident memory CD8 T cells in the lung following respiratory infection is an integral component of front-line mucosal defense against future pathogen exposure (5, 7). However, the mechanism of lung tissue-resident memory (Trm) differentiation and commitment remains unclear and these populations are transient within the tissue (5-7, 97).

In Chapter 3, we demonstrated that local 4-1BB stimulation generates robust, long-lived systemic and lung CD8 T cell immunity against influenza virus (326). We identified a role for CD8 T cell-intrinsic 4-1BB signalling in the establishment of Trm cells within the lung tissue. Moreover, exogenous 4-1BB stimulation with a replication defective adenovirus expressing the influenza nucleoprotein (NP) gene along with 4-1BBL dramatically boosted local immunity to protect long-term against lethal heterotypic challenge. This implicates 4-1BB as a regulator of Trm formation in a mucosal, non-lymphoid tissue. However, it was not clear whether the effects of 4-1BB were on survival of the effector T cell precursors that convert to Trm, or whether 4-1BB is important in maintaining Trm within the tissues.

Recently, a study of non-involved lung tissue of non-small cell lung carcinoma or chronic obstructive pulmonary disease patients identified TRAF1 as a part of the human lung Trm signature that was highly upregulated on CD103+ lung Trm cells compared to blood Tem (105). TRAF1 is an NF-κB-induced gene that is important in signalling downstream of a subset of TNFR family members, including 4-1BB. In particular, TRAF1 is essential for NF-κB as well as ERK activation downstream of 4-1BB in mouse CD8 T cells (225, 231). Here, we further investigate the mechanism by which 4-1BB induces long-lived Trm cells in the lung tissue and the downstream pathways implicated in this response. We identify 4-1BB as an early regulator of effector CD8 T cell accumulation in the lung tissue, suggesting that 4-1BB plays a role in the establishment of the lung-resident memory pool post-primary infection. This effect is in part dependent on TRAF1. To better understand the effects of 4-1BB, we used a prime-boost model in which mice were given a mild influenza infection and then boosted with a replication-defective adenovirus encoding influenza NP and 4-1BBL (Ad-NP-4-1BBL) for activation of 4-1BB in the airways and the generation robust lung CD8 Trm immunity. We found that this 4-1BB-mediated lung memory CD8 T cell accumulation is a result of local antigen-specific
recruitment of effector CD8 T cells into the lung tissue and mTOR signalling. Thus, this offers insights into mechanisms of Trm establishment and potential biological targets for improving protective mucosal immunity against infection.

4.3 Results

*Intrinsic 4-1BB signals are important for effector CD8 T cell accumulation in the lung tissue following influenza infection*

In Chapter 3, we determined that endogenous 4-1BB signals intrinsic to CD8 T cells are required for the establishment of Trm in the lung tissue, measured at 30 days following influenza A/X31 infection (Figure 3.8C). Here, we asked whether 4-1BB is acting early to regulate the size of the initial effector CD8 T cell pool in the lung, or potentially acting later to maintain the tissue-resident memory pool. To assess this, we set up mixed bone marrow (BM) chimeras in which 4-1BB+/+ CD45.1 and 4-1BB−/− CD45.2 BM cells were transferred in a 1:1 ratio to reconstitute lethally-irradiated wildtype 4-1BB+/+ CD45.1 host mice (Figure 4.1A). These mice were rested for 90 days, and then infected with influenza X31. As previously reported, there appeared to be no notable developmental disadvantage to 4-1BB-deficiency and reconstitution remained close to 1:1 (Figure 3.5B from previous chapter). At day 9, at the peak of the primary response to influenza, 4-1BB+/+ CD8 T cells already outcompeted 4-1BB−/− CD8 T cells for accumulation in the lung tissue, with wildtype cells making up 70% of the NP366-374-specific response (Figure 4.1B). This persisted into day 18, and by day 30, the defect of 4-1BB-deficiency was even greater as wildtype cells made up 80% of the lung-resident NP366-374-specific memory CD8 T cell response. It should be noted that when this experiment was performed at day 30 previously in Chapter 3 (Figure 3.8C, with slightly different congenic markers), we observed that more than 90% of the entire lung-resident memory pool consisted of 4-1BB+/+ cells. The reasons for the difference in magnitude of the 4-1BB effect in the current series of experiments versus the initial series are not clear and are being further investigated. Although the ratios of 4-1BB+/+ to 4-1BB−/− NP-specific CD8 T cells seems to increase over time in the lung parenchyma, this difference is not statistically significant (Figure 4.1C). The effect of CD8 T cell-intrinsic 4-1BB is also more
Figure 4.1. 4-1BB expression on CD8 T cells is required for the maintenance or survival of tissue-resident memory CD8 T cells in the lung after mild influenza X31 infection. (A) Mixed bone marrow (BM) chimeras were generated by reconstituting a 1:1 ratio of 4-1BB+/+ (CD45.1) and 4-1BB−/− (CD45.2) BM into lethally irradiated 4-1BB+/+ (CD45.1) host mice. Chimeric mice were rested for 90 days and then infected intranasally (i.n.) with influenza A/HK/X31. The lung parenchyma was assessed on days 9, 18, and 30 post-X31 infection. (B) Frequency of 4-1BB+/+ and 4-1BB−/− NP366-374 tetramer positive CD8 T cell populations, and (C) ratio of 4-1BB+/+ to 4-1BB−/− cells within NP366-374 tetramer positive CD8 T cells are presented in the lung tissue, along with the (D) frequencies and (E) ratios in the spleen. Data pooled from two to three separate experiments, using independently generated chimeras for each replicate set, for a total of 5 to 8 mice per time-point (mean ± SEM). NS not significant, * P<0.05, ** P < 0.005, *** P < 0.0005 (2way ANOVA test for (B), Kruskal-Wallis test for (C, E)).
dramatic in the lung tissue than in the spleen (Figure 4.1D, E). Taken together, it appears that CD8 T cell-intrinsic 4-1BB signalling regulates the size of the effector memory CD8 T cell pool in the lung-tissue early during influenza infection, correlating with its effect on the establishment of the Trm pool. There is a trend towards greater defects in the 4-1BB\(^{-}\) Trm cells at day 30, but further work will be required to establish whether 4-1BB has a role in the maintenance of the Trm population once established.

The signalling adaptor TRAF1 contributes to systemic T cell effector numbers and lung Trm formation

TRAF1, an adaptor molecule that contributes to signalling by TNFRs such as 4-1BB and TNFR2 (13, 14), was identified as part of the gene signature of human lung CD8 Trm (105). To investigate whether TRAF1 plays a role in the formation of lung Trm, we infected TRAF1\(^{+/+}\) and TRAF1\(^{-/-}\) littermates intranasally (i.n.) with influenza X31 and euthanized them to assess the tissues at day 30 post-infection (Figure 4.2A). TRAF1\(^{-/-}\) mice had fewer NP\(_{366-374}\)-specific CD8 T cells in the lung parenchyma compared to TRAF1\(^{+/+}\) littermates, while these numbers were comparable in the spleen and draining LN (dLN) (Figure 4.2B). Overall frequencies of NP tetramer\(^{+}\) cells were lower in the lung and dLN, although this was not statistically significant. Despite lower numbers of influenza-specific CD8 T cells in the lung tissue, the absolute number of CD103\(^{+}\)CD69\(^{+}\) NP-specific lung Trm was not different between wildtype and knock-out littermates (Figure 4.2C).

It was possible that the effects of TRAF1 would be more apparent when T cells compete for limited resources in the same mice. Therefore, we set up mixed BM chimeras to assess the effect of TRAF1 when wildtype and knockout CD8 T cells respond in the same environment. TRAF1\(^{+/+}\) CD45.1 and TRAF1\(^{-/-}\) CD45.2 BM cells were injected at a 1:1 ratio into lethally-irradiated wildtype CD45.1 TRAF1\(^{+/+}\) hosts (Figure 4.2D). After reconstitution, these mice were bled and T cell compartments were found to remain largely 1:1, indicating no discernable role of TRAF1 in their development within the irradiated hosts (Figure 4.2E). These mice were infected with X31 and at day 45 post-infection, it was found that TRAF1\(^{+/+}\) outnumbered TRAF1\(^{-/-}\).
Figure 4.2. TRAF1 signalling contributes to the establishment of lung-resident memory CD8 T cells after influenza infection as well as to T cell responses in the secondary lymphoid organs. (A) TRAF1<sup>+/+</sup> and TRAF1<sup>−/−</sup> littermates were infected with influenza X31 and tissues were harvested for analysis at day 30 post-infection. (B) Absolute numbers and frequencies of NP<sub>366-374</sub> tetramer<sup>+</sup> CD8 T cells in the lung parenchyma, spleen, and dLN. (C) Frequency and absolute number of CD103<sup>+</sup>CD69<sup>+</sup>NP<sub>366-374</sub>-specific Trm population in the lung tissue. (D) Mixed bone marrow (BM) chimeras were generated by reconstituting a 1:1 ratio of TRAF1<sup>+/+</sup> (CD45.1) and TRAF1<sup>−/−</sup> (CD45.2) BM into lethally irradiated TRAF1<sup>+/+</sup> (CD45.1) host mice. Chimeric mice were rested for 90 days and then infected intranasally (i.n.) with influenza A/HK/X31. (E) Reconstitution ratios of TRAF1<sup>+/+</sup> to TRAF1<sup>−/−</sup> in the blood pre-infection with X31. (F) Frequency of TRAF1<sup>+/+</sup> and TRAF1<sup>−/−</sup> compartments in the NP<sub>366-374</sub>-specific CD8 T cell population at day 45 post-X31 infection assessed in the lung parenchyma and vasculature, spleen, and dLN. Data pooled from two separate experiments, with chimeras generated independently for each replicate set, with n= 4 to 5 per group per experiment (mean ± SEM). NS not significant, *P<0.05, **P<0.0005, ****P<0.00005 (Mann-Whitney test for (B, C), 2way ANOVA test for (F)).
memory NP\textsubscript{366-374}-specific CD8 T cells, making up 70\% to 30\% of the response, in both local and systemic tissues assessed (Figure 4.2F). Thus, unlike 4-1BB which has a much greater effect in the lung than in the spleen (Figure 3.8B), TRAF1 in a competition model confers a similar advantage on memory establishment both locally and systemically.

**Local antigen is necessary for 4-1BB-mediated secondary CD8 T cell effector accumulation in the lung tissue**

To further assess the role of 4-1BB in Trm development, we adopted a prime-boost model that allows for generation of a robust Trm population in the lung and in which 4-1BB signals are delivered at a defined time, rendering it easier to manipulate the system. Mice were given a primary i.n. immunization of influenza X31 and then boosted i.n. 30 days later with a replication-defective adenovirus containing the influenza NP gene, with or without 4-1BBL (Ad-NP, Ad-NP-4-1BBL). This model mimics immunization of previously influenza-immune adults. This local secondary 4-1BB stimulation was previously shown to induce a robust long-lived circulating effector memory population (Figure 3.1B, 3.2A-D) as well as a substantial long-lived lung-resident memory CD8 T cell population (Figure 3.4A, B). Importantly, this prime-boost regimen protected against lethal heterotypic influenza challenge for a year post-boost (Figure 3.1E, F).

Given the importance of the tissue microenvironment to the development and commitment of Trm populations (9, 10), we next investigated the mechanisms by which local i.n. airway administration of Ad-NP-4-1BBL was mediating this robust CD8 T cell accumulation and long-lived response in the lung tissue. Previous work established that Ad-NP-4-1BBL is acting directly on CD8 T cells in our system (Figure 3.5E). We next wished to know whether local delivery of antigen is required for CD8 T cell recruitment into the lung parenchyma, or whether this accumulation was a result of inflammatory cues from the adenovirus infection that are attracting CD8 T cells into the tissue. It has been reported that antigen persistence in the lung and/or dLN is driving continual activation and recruitment of CD8 T cells into the lung tissue for Trm commitment (101-103), while a separate study indicated that recruitment of CD8 T cells
into the lung during influenza was mediated by the cytokine milieu independent of local antigen (97). To test this in our system, we primed mice with X31 and then boosted them 30 days later i.n. with Ad-NP-4-1BBL (local inflammation, local antigen), i.n. with Ad-4-1BBL (local inflammation, no antigen), i.p. with Ad-NP (no local inflammation, systemic antigen), or simultaneously i.n. with Ad-4-1BBL and i.p. with Ad-NP (local inflammation, systemic antigen) (Figure 4.3A). Strikingly, we found that without local i.n. delivery of antigen, CD8 T cells do not accumulate in the lung tissue at all (Figure 4.3B, C), while antigen, regardless of the route of administration, is sufficient to induce memory CD8 T cell responses in the spleen and dLN (Figure 4.3B). Thus, inflammation is insufficient, and trafficking of CD8 T cells into the lung upon Ad-NP-4-1BBL boost is antigen-dependent for the enhanced secondary effector response within the local tissue.

Critical role for mTOR signalling in generation of Trm

Mammalian target of rapamycin (mTOR) is a metabolic checkpoint kinase that critically regulates cell size and is important during T cell proliferation and CD4 T helper differentiation (327, 328). The mTOR inhibitor rapamycin, which directly inhibits the downstream mTOR complex 1 (mTORC1) and has been shown to interfere with mTORC2 upon chronic administration (329), was previously shown to augment and accelerate the formation of memory T cells systemically (54, 330). However, rapamycin treatment reduced CD8 Trm accumulation in the small intestines and female reproductive tract following vesicular stomatitis virus (VSV) infection (331). Whether mTOR is required for Trm accumulation in the lung after influenza infection has not been investigated.

Mice were primed with X31 and boosted with Ad-NP or Ad-NP-4-1BBL, and treated daily with rapamycin to block mTOR until day 7 (peak response) (Figure 4.4A). Consistent with literature (330), rapamycin treatment resulted in an decrease in the proportion of KLRG1+CD127- short-lived effector cells (SLECs) and an increase of KLRG1+CD127+ memory precursor effector cells (MPECs) in the circulating splenic CD8 T cell population, but only when 4-1BBL is present in the boost vector (Figure 4.4B). Furthermore, rapamycin treatment in Ad-NP-4-1BBL-boosted
Figure 4.3. The Ad-NP-4-1BBL boost response requires antigen and 4-1BBL to be co-delivered locally to
induce accumulation of memory CD8 T cells in the lung tissue. (A) Mice were infected i.n. with influenza A/HK/X31 and then boosted i.n. with Ad-NP-4-1BBL (as described in Figure 3.1), i.n. with Ad-4-1BBL (no antigen, but local inflammation, intraperitoneal (i.p.) with Ad-NP (systemic antigen, no local inflammation), or i.n. with Ad-4-1BBL and i.p. with Ad-NP (local inflammation, systemic antigen). (B) Absolute number of NP\textsubscript{366-374} tetramer\textsuperscript{+} CD8 T cells were quantified at day 7 peak response in the lung parenchyma, spleen, and draining mediastinal lymph node (dLN). (C) Representative flow plot of CD8 T cell accumulation in the lung parenchyma following \textit{in vivo} intravascular staining (gating strategy presented in Figure 3.5). Data pooled from two separate experiments, with n= 4 per group per experiment (mean ± SEM). NS not significant, ** P < 0.005, *** P<0.0005 (Kruskal-Wallis test).

Figure 4.4. The mTOR pathway contributes to 4-1BBL-dependent memory CD8 T cell accumulation in the lung tissue after local Ad-NP-4-1BBL secondary boost. (A) Mice were infected with influenza X31 and then one-month later boosted i.n. with either Ad-NP-4-1BBL and given an i.p. injection of either PBS control or rapamycin (0.75μg/kg) daily, until sacrificed at day 7 post-boost. (B) Frequency of short-lived effector cells (SLECs, KLRG-1\textsuperscript{+}CD127\textsuperscript{-}) and memory precursor effector cells (MPECs, KLRG-1\textsuperscript{-}CD127\textsuperscript{+}) in the spleen. (C) Frequency of central memory (Tcm, CD62L\textsuperscript{+}CD127\textsuperscript{+}) and effector memory (Tem, CD62L\textsuperscript{-}CD127\textsuperscript{+}) in the spleen and dLN. (D) Absolute number of NP\textsubscript{366-374} tetramer\textsuperscript{+} memory CD8 T cells and CD103\textsuperscript{+}CD69\textsuperscript{+} Trm in the lung tissue, and (E) NP\textsubscript{366-374} tetramer\textsuperscript{+} CD8 T cells in the spleen and dLN. Data pooled from two separate experiments, with n= 4 per group per experiment (mean ± SEM). NS not significant, *P<0.05, ** P<0.005, *** P < 0.0005 (2way ANOVA test).
mice resulted in a greater frequency of CD62L⁻CD127⁺ effector memory (Tem) cells in the spleen, and a greater frequency of CD62L⁺CD127⁺ central memory (Tcm) in the dLN, which once again was not observed in the Ad-NP control group (Figure 4.4C). Interestingly, rapamycin treatment in Ad-NP-4-1BBL-boosted mice prevented NP-specific CD8 T cell accumulation in the lung tissue and decreased lung memory and CD103⁺CD69⁺ populations to levels comparable to control Ad-NP-boosted (Figure 4.4D). Once again, no effect was seen in the Ad-NP control boost, where rapamycin treatment if anything showed a slight increase in CD8 T cell accumulation in the lung, although this was not statistically significant. The decrease in lung memory T cell numbers after mTOR blockade occurs despite the lack of a discernable difference in the splenic and dLN memory populations (Figure 4.4E). Thus, even though recruitment of circulating effectors is the mechanism of CD8 T cell accumulation in the lung tissue during peak response in this model (Figure 3.7E-G), the mTOR pathway appears to regulate local 4-1BB-mediated secondary CD8 effector T cell accumulation in the lung tissue, independent of circulating memory populations that feed the Trm population.

4.4 Discussion

The local cues from the tissue microenvironment that permit Trm commitment and persistence within the lung are not well understood and still an active area of investigation. Here, we have elaborated on the role of the TNFR superfamily member 4-1BB in inducing long-lived protective immunity against influenza infection. Through 4-1BB⁺/⁺:4-1BB⁻/⁻ mixed BM chimeras, we have identified a role for 4-1BB in regulating the size of the primary lung effector CD8 T cell pool during influenza infection, which in turn impacts the number of tissue-resident memory T cells formed (Figure 4.1). There may also be a role for 4-1BB in further maintaining the lung Trm pool after viral clearance, but this will require further investigation. The effect of 4-1BB may be in part TRAF1-dependent, as TRAF1⁻/⁻ mice have defective lung tetramer⁺ T cell accumulation after X31 infection compared to their littermate controls, and are outcompeted in both the systemic and the local memory CD8 T cell pools by TRAF1⁺/⁺ T cells in TRAF1⁺/⁺:TRAF1⁻/⁻ mixed BM chimeras (Figure 4.2). Using a prime-boost model in which local administration of 4-1BBL into the airways along with antigen induces robust lung Trm generation, we determined
that lung inflammation is insufficient for CD8 T cell accumulation in the lung tissue. We found that both 4-1BBL and antigen must be delivered locally to induce a robust, 4-1BBL-boosted CD8 T cell responses in the lung (Figure 4.3). We also show that this effect of 4-1BBL requires the activity of mTOR, as rapamycin abrogates the effect of 4-1BBL on boosting antigen-specific CD8 T cell accumulation in the lung parenchyma (Figure 4.4).

The costimulatory TNFR superfamily members regulate T cells post-priming, fine-tuning effector and/or memory responses in accordance with the severity of infection and the persistence of antigen (13, 14). Previous work from our lab revealed a role for 4-1BB in maintaining lung effector CD8 T cell populations in the context of a severe influenza A/PR8 infection, but not in a mild X31 that is rapidly cleared (207). Here, in a competition model, we identify 4-1BB as an important regulator of the primary effector CD8 T cell pool within the lung tissue after X31 infection. It is possible that 4-1BB may play an additional role in maintaining the lung-resident Trm pool, although further work will be required to determine whether 4-1BB has additional effects once the Trm pool is established. Conditional knockout of 4-1BB or 4-1BBL at late time points would clarify this issue.

The degree to which 4-1BB contributes in the induction or maintenance of Trm is still unclear since the bone marrow chimeras have shown varying magnitudes of effect. A first set of experiments, performed three times with a total of 7 mice, in Chapter 3 showed almost a complete reliance on 4-1BB for contribution into the lung Trm pool at day 30 (Figure 3.8C, D). In contrast, a second set of experiments done much later, showed a 4-fold survival advantage in 4-1BB-competent compared to 4-1BB-deficient antigen-specific CD8 T cells. The variability may be a result of the BM chimeric model itself where irradiation is known to cause lung damage and perhaps disrupts the niches in which Trm form in the host mice (332). The concept of a defined niche in which T cells compete for finite resources to allow establishment of Trm is supported by a recent study showing that, in contrast to effector T cells which are widely distributed, Trm are localized to specific sites in the lung that are involved in regeneration after tissue injury (93). Thus, we hypothesize that after irradiation, there may be variable levels of damage to these sites that affect how limiting the niche is, which in turn would affect the level of competition. Nonetheless, our studies clearly show an important role for 4-1BB in establishment
of the lung Trm population. The use of non-irradiated models in which the effect of 4-1BB is compared in conditions of competition and non-competition may help resolve this issue.

The adaptor molecule TRAF1 contributes to CD8 T cell accumulation in the lung. We used both an irradiated chimeric competition model as well as unirradiated whole-body knockout experiments to demonstrate decreased numbers of memory CD8 T cells in the lung tissue after influenza infection in the absence of TRAF1. This finding is complementary to a previous study that identified TRAF1 as a component of the human lung Trm signature (105). Of note, the effect of TRAF1 was greater under conditions of competition, again consistent with the concept of limited resources available for the establishment or maintenance of the Trm population. The effects of 4-1BB were much more striking on the lung CD8 T cell population than on the splenic T cells, whereas effects of TRAF1 were seen in both lung and secondary lymphoid organs. It is possible that this reflects a role for TRAF1 downstream of other TNFRs on T cells, such as TNFR2 (13, 14).

There have been conflicting data in the literature about the requirement for local persistent antigen for the establishment of Trm. Slütter et al. recently reported that continued recruitment of effector cells is a mechanism of Trm maintenance but that this is independent of antigen and relies instead on induction of cytokines locally, notably TNFα and IL-33. It is important to note that antigen presentation is still detectable in the lung and dLN for at least 2 months after influenza infection, with CFSE-labelled transgenic CD8 T cells showing the greatest amount of division in the dLN despite viral RNA being no longer detectable by PCR after 30 days (101, 102). Furthermore, Zammit et al. showed that there was retention of CD8 T cells within the dLN after infection, as parabiotic experiments show equilibration between X31-infected congenically-marked partners in the non-draining peripheral LNs, but not in the draining mediastinal LN (102). This was deduced to be a continual source of CD8 T cells that would replenish the Trm population as parabiotic experiments showed equilibration in the lung and airways slowly over time between two X31-infected partners. It should also be noted that i.p. infection of influenza, as well as Sendai virus, results in defective CD8 T cell recruitment into the lung despite maintaining residual antigen in the dLN (103). These CD8 T cells localize to the same regions within the dLN but do not have an activated CD69+ phenotype and also do not express lung retention markers like CD103 and CD49a (103). Thus, the initial priming of CD8 T cells by
respiratory dendritic cells (RDCs), determined by the route of immunization, is important for T cell recruitment and Trm commitment. Indeed, although antigen presentation persists in the dLN and RDCs in the dLN are more effective stimulators of transgenic CD8 T cells than lung populations ex vivo, antigen itself is deposited long-term in the lung tissue within radioresistant cells after PR8 infection (101). Migrant CD11c<sup>hi</sup> RDC subsets, CD103<sup>+</sup> and CD11b<sup>+</sup>, travel from these antigen depots in the lung to the dLN to facilitate continued presentation of CD8 T cells; it was demonstrated that lung-specific depletion of these populations by low dose diphtheria toxin (DTx) in a CD11c-DTR model results in reduced RDCs in the dLN and impaired CD8 T cell responses (101). Our data further reaffirms the importance of local antigen in the Ad-NP-4-1BBL prime-boost model and also shows that systemic antigen delivered as Ad-NP with i.n. Ad-4-1BBL can induce a strong memory CD8 T cell response in the spleen and dLN, but does not result in CD8 T cell accumulation in the lung tissue itself despite lung inflammation (Figure 4.4B). It has also been shown by Lee et. al. that antibody-mediated MHC I blockade results in a loss of CD69 and CD103 that corresponded with a loss of antigen-specific CD8 T cells in the lung tissue (107).

The mTOR pathway was recently identified as a regulator of memory CD8 T cell differentiation (330, 331). In vivo rapamycin treatment during the LCMV Armstrong infection resulted in comparable effector CD8 T cell expansion but decreased contraction and greater generation of memory precursors (330). Memory cells derived from rapamycin-treated mice exhibited improved memory CD8 T cell longevity and secondary responses (330). mTOR signalling also influences trafficking of T cells as inhibition by rapamycin is also associated with increased expression of CCR7, CD62L, and the transcription factor KLRF2 (333), all of which are associated with the Tcm phenotype. Sowell et. al. recently reported that rapamycin treatment during the effector phase inhibits the accumulation of CD8 T cells within the small intestines and female reproductive tract (331), suggesting a role for the pathway in determining circulating versus tissue-resident memory commitment. Similarly, our data in the Ad-NP-4-1BBL prime-boost model shows a skewing of circulating splenic populations towards Tcm and a decline in the number of lung parenchymal cells in rapamycin-treated groups. It should be noted that rapamycin treatment has been associated with lung toxicity and interstitial pneumonitis in transplant patients, although the exact mechanism causing this damage is unknown (334-336).
Although it is possible that tissue damage is affecting our results, the reduction in lung parenchymal memory CD8 T cells is only seen in Ad-NP-4-1BBL-boosted groups, and not Ad-NP controls, suggesting that the observed role of the mTOR pathway in mucosal CD8 T cell accumulation is downstream of 4-1BB stimulation (Figure 4.3). Other members of the TNFR family, such as OX40 have been shown to activate AKT, an upstream regulator of mTORC1 (337). 4-1BB shows TRAF1-dependent ERK activation (225); ERK can phosphorylate TSC2, to relieve upstream inhibition of mTORC1 (338). How the 4-1BB-TRAF1 signalling axis is linked to mTOR activation requires further investigation. It is also possible that mTOR regulates CD8 T cell responsiveness to the cytokine milieu, as both mTORC1 and mTORC2 have been implicated in the ability of CD4 T cells to respond to cytokine and upregulate lineage transcription factor for differentiation in T helper subsets (328, 339). Furthermore, mTORC1 signalling, mediated by IL-12, is important for sustaining T-bet expression in CD8 T cell effector differentiation, while inhibition of mTORC1 is associated with increased Eomes expression (54, 340). A similar mechanism may be involved in Trm commitment as these cells are known to sustain T-bet expression at low levels but no longer express Eomes (74, 104).

In conclusion, the induction of lung Trm after influenza infection requires 4-1BB to regulate the size of the effector precursor CD8 T cell pool. This process is in part TRAF1-dependent, and relies on local antigen stimulation and activation of the mTOR pathway. These factors may be important regulators of tissue-residency and contribute to our understanding of long-term T cell persistence in the lung.
Chapter 5
CD30 is dispensable for T cell responses to influenza virus and LCMV clone 13 but contributes to age-associated T cell expansion in mice

The results in this chapter have been accepted for publication in *Frontiers in Immunology*.


**Author Contributions**

Conceived and designed experiments: ACZ LMS THW. Performed experiments: ACZ LMS. Analyzed the data: ACZ LMS. Wrote and edited the paper: ACZ LMS MEW THW. Figure panels 5.2A-B have appeared in LMS PhD thesis.
5.1 Abstract

CD30 is a tumor necrosis factor receptor (TNFR) family member whose expression is associated with Hodgkin’s disease, anaplastic large cell lymphomas, and other T and B lymphoproliferative disorders in humans. A limited number of studies have assessed the physiological role of CD30/CD30 ligand interactions in control of infection in mice. Here we assess the role of CD30 in T cell immunity to acute influenza and chronic lymphocytic choriomeningitis virus (LCMV) clone 13 infection, two viral infections in which other members of the TNFR superfamily are important for T cell responses. We show that CD30 is expressed on activated but not resting CD4 and CD8 T cells \textit{in vitro}, as well as on regulatory T cells and marginally on Th1 cells \textit{in vivo} during influenza infection. Despite this, CD4 and CD8 T cell expansion in response to influenza virus was comparable in CD30\textsuperscript{+/+} and CD30\textsuperscript{-/-} littermates, with no discernable role for the pathway in the outcome of influenza infection. Similarly, during persistent infection with LCMV clone 13, CD30 plays no obvious role in CD4 or CD8 T cell responses, the level of T cell exhaustion or viral control. On the other hand, in the steady state, we observed increased numbers of total CD4 and CD8 T cells as well as increased numbers of regulatory T cells in unimmunized older (~8 months) CD30\textsuperscript{+/+}, but not in CD30\textsuperscript{-/-} age-matched littermates. Naïve T cell numbers were unchanged in the aged CD30\textsuperscript{+/+} mice compared to their CD30\textsuperscript{-/-} littermate controls, rather the T cell expansions were explained by an increase in CD4\textsuperscript{+} and CD8\textsuperscript{+} CD4\textsuperscript{mid}CD62L\textsuperscript{-} effector memory cells, with a similar trend in the central memory T cell compartment. In contrast, CD30 did not impact the numbers of T cells in young mice. These data suggest a role for CD30 in the homeostatic regulation of T cells during aging, contributing to memory T cell expansions which may have relevance for CD30 expression in human T cell lymphoproliferative diseases.
5.2 Introduction

CD30, a member of the TNFR superfamily that is expressed on B cells, NK cells, eosinophils, macrophages, and activated T cells, is perhaps best studied for its overexpression on Reed Sternberg cells in lymphoma (201, 266). Its ligand, CD30L (CD153), can be detected on dendritic cells, macrophages, resting B cells, neutrophils, eosinophils, activated T cells, as well as on a CD4⁺CD3⁻CD11c⁻ accessory cell implicated in the survival of CD4 memory Th2 cells (201, 266, 275). Stimulation of CD30 on T cells via agonistic anti-CD30 antibody or recombinant CD30L in the presence of anti-CD3 or antigen-primed dendritic cells can enhance T cell activation, proliferation and cytokine production (288, 341-343). CD30 expression is a hallmark of various pathological lymphoproliferations, and has been associated with classical Hodgkin’s lymphoma, anaplastic large cell lymphomas (ALCL), and primary cutaneous CD30⁺ T cell proliferative disorders (263-265).

Much work has focused on the role of CD30 in CD4 T cell responses in vivo. CD30, in synergy with another TNFR family member OX40, was reported to be crucial for the survival of Th2 CD4 memory cells necessary to provide help to B cells for memory antibody responses (275, 344, 345). Indeed, CD30⁻/- mice were found to have defective memory antibody responses compared to wild-type C57BL/6 mice (345). Moreover, Th2 cells preferentially express CD30 and OX40, and interact with CD30L- and OX40L-expressing CD4⁺CD3⁻ accessory cells at the T-B cell border to help B cell responses (275, 344). It is possible that these Th2 cells identified are instead the more recently discovered T follicular helper cell subset that contribute to the formation and maintenance of germinal centers and B cell responses (276). CD30 has also been implicated in the polarization of CD4 Th17 cells (296, 297), and to play a role in several CD4 Th1 responses (346-348).

There is also evidence of a role for CD30 in CD8 T cell activation and the maintenance of CD8 T cell memory (291, 300, 349). CD30L⁻/- mice have defective generation of long-term memory CD8 T cells following *Listeria* infection, particularly affecting central memory (291). In contrast, studies of VSV and MCMV infection revealed no role for CD30 in either CD8 T cell or antibody responses (299, 300). Pox viruses of murine and bovine origin are noted to encode a soluble CD30 homologue which inhibits CD30L binding to its cellular receptor (301, 302). The finding that CD30 is a target for subversion by viruses (301, 302) suggests that CD30 signalling may be
important in antiviral immunity. In addition to viral immunity, the CD30-CD30L pathway is also important for the clearance of mycobacterial infections by mediating IL-17A production by γδT cells, as shown through studies with CD30−/− mice (292, 293).

Here we address the role of CD30 in T cell immunity to viral infection by assessing an acute localized infection with influenza A virus and a chronic systemic infection with LCMV clone 13. Several TNFR family members have previously been shown to have non-redundant and significant impact on T cell responses in these two infection models (14, 24, 207, 350-354). Surprisingly, however, by comparing CD30-deficient mice to their littermate wild-type controls, we found that CD30 appears to be completely dispensable for CD4 and CD8 T cell responses to these two viruses. As CD30 is highly expressed on regulatory Foxp3+ T cells (Tregs), we also examined whether CD30 affected the number of Tregs in aged mice. Remarkably, we found that CD30+/+, but not their CD30−/− littermates, exhibited age-dependent T cell increases in the number of CD4 and CD8 T cells as well as regulatory T cells. This increase in total T cell numbers was largely due to expansion of memory T cells, with significant effects on numbers of effector memory T cells and a similar trend in the central memory compartment. This may be relevant to the presence of CD30 on expanded T cells in human T cell lymphoproliferative diseases.

5.3 Results

**CD30 is expressed on subsets of T cells in vitro and in vivo**

To examine the expression of CD30 on T cells, we stimulated splenocytes from CD30+/+ and CD30−/− littermate mice with anti-CD3 and anti-CD28 antibodies *in vitro*. CD30 was undetectable on resting T cells but induced on CD8 T cells by 48 hrs of stimulation, and on both CD4 and CD8 T cells after 72 hours (Figure 5.1A), consistent with the literature (268). CD30−/− splenocytes were used as a staining control and showed only background levels of staining. Having demonstrated CD30 expression on activated T cells, we next asked whether CD30 could be detected *in vivo*, during a viral infection. Previous work has shown that the inducible TNFR family member 4-1BB
Figure 5.1. CD30 is expressed on CD4 and CD8 T cell subsets. (A) Splenocytes from CD30+/+ and CD30−/− littermate C57BL/6 mice were stimulated in vitro with 1 µg/ml anti-CD3 and 10 µg/ml anti-CD28, and expression of CD30 was assessed by flow cytometry after 24, 48, and 72 hours of treatment. (B) Wild-type CD30+/+ mice were infected intranasally with 10⁴ TCID₅₀/mouse (sublethal dose) of influenza A/PR8 (PR8) and CD30 expression was assessed on various T cell subsets in the lung and mediastinal lymph node (MLN) at day 9 post-infection. Infected CD30−/− littermate mice were used as a staining control. Representative gating strategy and histograms of CD30 expression are shown on Db/NP366-74 antigen-specific CD8 T cells, Th1 and Treg cells from the lung, with follicular helper T cells (Tfh) and follicular regulatory T cells (Tfr) shown from the MLN, with mean fluorescent intensity (MFI) of CD30 quantified and graphed for Th1 and Treg. Data from (A) are representative of two experiments, performed with one mouse per group each experiment; while data in (B) are representative of two to three mice per experiment, with two independent experiments performed at day 9 post-infection (median ± range). NS not significant, * P<0.05 (Mann-Whitney test).

is readily detectable on murine T cells in the lung at 6-8 days post-infection with a sublethal dose of influenza A/PR8 (PR8) (207). Therefore, we analyzed CD30 expression at similar time points post intranasal (i.n.) PR8 infection. CD30 was significantly expressed on Tregs and marginally on Th1 cells, but undetectable on antigen-specific CD8 T cells at day 9 pi (Figure 1B). CD30 was not detected at days 3, 5, or 7 in the lung and draining mediastinal lymph node (MLN) on antigen-specific CD8 T cells, Th1, T follicular helper (Tfh), T follicular regulatory (Tfr), or Treg cells (data not shown, n= 2-3 mice per time point). These results confirm that CD30 is expressed during influenza virus infection, albeit on a limited subset of cells, prompting us to examine the effect of CD30 on immunity to influenza virus.

**CD30 is dispensable for the primary expansion, memory conversion, and secondary response of influenza-specific CD8 T cell following acute respiratory influenza A infection**

In pilot experiments, CD30 was not required for mouse to survive influenza A/PR8 infection and the CD8 T cell responses to sublethal influenza A/PR8 at day 10 post-infection in CD30+/+ and CD30−/− littermates were comparable (data not shown). Therefore, we switched to a milder strain of influenza, Influenza A/HK/X31 (X31, an H3N2 virus) (316), with the idea that a weaker infection might be more costimulation-dependent. CD30+/+ and CD30−/− mice were infected i.n. with influenza X31 and, the antigen-specific CD8 T cell response to the immunodominant epitope NP366-74 was assessed using Db/NP366-74 MHC class I tetramers at day 10 (the peak of the primary CD8 T cell response). Assessment of the frequency and absolute number of NP366-74-specific CD8
T cells in the spleen, mediastinal lymph node (MLN), and lung showed comparable primary expansion of influenza-specific CD8 T cells in CD30^{+/+} and CD30^{-/-} mice (Figure 5.2A). The tetramer^{+} cells were CD62L^{lo} in both groups, indicating an effector phenotype (Figure 5.2A). Therefore, CD30 is dispensable for primary CD8 T cell expansion to influenza virus.

*In vitro* studies have shown that CD30 stimulation of T cells can enhance their production of IFN\(_{\gamma}\), among other cytokines (342). Therefore, we assessed IFN\(_{\gamma}\) production by *ex vivo* restimulation with NP\(_{366-74}\) peptide at day 10 and day 100 post-X31 infection in CD30^{+/+} and CD30^{-/-} mice and found no significant difference in the proportion of IFN\(_{\gamma}\) producing CD8 T cells at day 10 or 100 (Figure 5.2B). Thus, CD30 does not play a discernable role in the function of effector and memory CD8 T cells during influenza infection.

Despite the lack of an obvious role for CD30 in CD8 T cell responses to influenza A X31, it was possible that CD30 could influence protective memory against a more severe influenza infection, such as induced by influenza A/PR8. To this end, we infected mice with influenza X31 and allowed them to naturally clear the virus and develop a memory response, then challenged the mice at day 30 with a sublethal dosage of influenza A/PR8, which typically causes a 20-25% weight loss in naïve mice. PR8 shares the same NP epitope as the initial priming X31 strain but contains different HA and NA proteins (PR8 is H1N1, whereas X31 is H3N2), thereby allowing us to assess protection from CD8 T cell immunity without the interference of neutralizing antibody responses.

CD30^{+/+} and CD30^{-/-} mice both exhibited protection to the challenge, losing only about 5% of their body weight before making a full recovery, and exhibited comparable weight loss (Figure 5.2C). CD30^{+/+} and CD30^{-/-} mice also showed equivalent secondary expansion of NP-specific CD8 T cells in the spleen, lung, and MLN (Figure 5.2D). IFN\(_{\gamma}\) production by CD8 T cells upon *ex vivo* peptide restimulation was assessed and although MFIs were not different, CD30^{-/-} mice had a greater frequency of IFN\(_{\gamma}\)-producing CD8 T cells than their CD30^{+/+} littermates (Figure 5.2E), despite disease outcome being comparable. Taken together, these data show that CD30 is dispensable for the establishment and programming of secondary recall responses to influenza virus, and does not play a role in the disease outcome of a secondary infection.
Figure 5.2. CD30 is dispensable for the primary expansion, memory conversion, and secondary response of influenza-specific CD8 T cells following acute respiratory influenza A infection. CD30⁺/⁺ and CD30⁻/⁻ mice were infected intranasally with 5 HAU of influenza A/HK-X31 (X31) and mice were sacrificed at day 10 and day 100 post-
infection. The frequency and absolute numbers of Db/NP366-74 antigen-specific CD8 T cells was determined using MHC tetramers, from various organs at (A) day 10 with representative gating. (B) Splenocytes from day 10 and day 100 post-infection were restimulated ex vivo with NP366-74 peptide for 6 hours and stained for IFNγ expression, with representative intracellular staining of IFNγ-producing CD8 T cells at day 10 post-infection. (C-E) CD30+/+ and CD30-/- littermate mice were infected with 5 HAU of X31 and then challenged 30 days later with 10^5 TCID50 of PR8. (C) Weight loss was tracked for 7 days post-challenge. (D) The frequency and absolute numbers of Db/NP366-74 antigen-specific CD8 T cells were determined at day 7, with (E) frequency and MFI of IFNγ producing CD8 T cells quantified from ex vivo NP366-74 peptide restimulation of splenocytes. (F) The frequency and absolute numbers of Db/NP366-74 antigen-specific CD8 T cells from spleen and bone marrow was determined at day 100 after primary X31 infection. Panel A, B, were done with non-littermate mice but were repeated with littermate mice for PR8 model also with no effect of CD30. Panel (C, D, E) were done with littermate mice. Panel F was done with non-littermate mice. Data are representative of two (day 100) or three (day 10) independent experiments with n=4-5 per group (A, B, F), and PR8 challenge experiment pooled from two independent experiments with n=2-3 per group per experiment (median) (C, D, E). NS not significant, ** P<0.005 (Mann-Whitney test).

A previous study by Nishimura et al. reported that early antigen specific CD8 T cell responses to L. monocytogenes infection in CD30L-/- mice are intact but the memory pool assessed at 84 days post-infection was defective compared to wild-type controls (291). This implies a role for the CD30-CD30L pathway in the generation or maintenance of CD8 T cell memory. Therefore, we investigated whether CD30 is necessary for the establishment of a long-lived CD8 memory T cell pool following influenza infection. As the bone marrow is a known reservoir of memory T cells and a preferential organ for CD8 memory T cell homeostasis, we analyzed this organ as well. Mice were infected with influenza X31 and examined for NP366-374-specific CD8 T cells 100 days after infection. There were no significant differences observed between CD30+/+ and CD30-/- mice in the spleen and bone marrow at this much later time point (Figure 5.2F). Therefore, CD30 is dispensable for the generation and maintenance of CD8 memory T cells to influenza virus.

**CD30 is dispensable for CD4 T cell responses following acute respiratory influenza A infection**

Given the expression of CD30 on lung Tregs during peak influenza response (Figure 5.1B), we evaluated the frequency and absolute numbers of Tregs in CD30+/+ and CD30-/- mice at day 9 post-PR8 infection and found no difference in the lung (Figure 5.3A) despite detecting expression at this time point. Th1 cells, which have only marginal expression of CD30 in the lung (Figure 5.1B), were also evaluated and comparable populations found as well (Figure 5.3B). Similarly, no
Figure 5.3. CD30 is dispensable for CD4 T cell responses following acute respiratory influenza A infection. CD30+/+ and CD30-/- littermate mice were infected intranasally with 10^4 TCID_{50} of Influenza A/PR8 and mice were euthanized at day 9 post-infection. The frequency and number of (A) CD25+Foxp3+ Tregs and (B) CD44hiT-bet+ Th1 were evaluated in the lung, and (C) Bcl6^+PD-1^+CXCR5^+ Foxp3^+ Tfh and (D) Bcl6^+PD-1^+CXCR5^+ Foxp3^+ Tfr were evaluated in the mediastinal lymph node. Data are pooled from four experiments with n=2-3 per group per experiment (median). NS not significant (Mann-Whitney test).
differences in Tfh or Tfr frequencies and absolute numbers were found when the draining lymph node was examined at peak response (Figure 5.3C-D). Taken together, the data show that CD30 is dispensable for both CD8 and CD4 T cell expansion during acute influenza infection.

**CD30 is dispensable for CD4 and CD8 T cell responses during chronic LCMV clone 13 infection**

It was possible that CD30 is more important in a persistent as compared to an acute rapidly cleared infection. Therefore, we examined the response of CD30+/+ and CD30-/- littermates to LCMV clone 13, an infection in which viral clearance takes several months. This chronic infection is characterized by the hierarchical loss of effector functions and peripheral exhaustion of virus-specific T cells (186). We assessed T cell responses at day 21 post-infection, a time point at which T cells clearly exhibit signs of exhaustion. Frequencies and absolute numbers of antigen-specific CD8 T cells in the spleen, as measured by MHC tetramers containing GP33-41 and NP396-404 peptides, were comparable between CD30+/+ and CD30-/- littermate mice (Figure 5.4A). Furthermore, the absence of CD30 did not significantly affect the levels of Tim-3 (Figure 5.4B) and PD-1 (Figure 5.4C) on the antigen-specific CD8 T cells, two markers whose persistent expression is typically associated with T cell exhaustion. The overall frequency of PD-1-expressing tetramer+ cells was also comparable between CD30+/+ and CD30-/- littermates (Figure 5.4D). *Ex vivo* restimulation of splenic CD8 T cells with GP33-41 or NP396-404 peptides did not reveal functional differences in their ability to produce IFNγ, both in terms of total frequencies of IFNγ-producing CD8 T cells and per cell IFNγ production as measured by the mean fluorescence intensity (MFI) (Figure 5.4E). Viral loads assessed in the spleen, lung, and kidney at day 21 also did not differ between CD30+/+ and CD30-/- littermate mice (Figure 5.4F). These results suggest that CD30 is dispensable for the CD8 T cell response and control of LCMV cl 13 infection.

We next assessed the role of CD30 in the CD4 T cell response during LCMV cl 13 infection. It was previously reported that mice depleted of CD4 T cells fail to clear the virus, implicating CD4 T cells in control of LCMV clone 13 the infection (196, 355). Analysis of Tfh, Tfr and Treg populations revealed no difference in frequency or total number of cells (Figure 5.5A-D).
Figure 5.4. CD30 is dispensable for antigen-specific CD8 T cell responses and control of LCMV clone 13. CD30<sup>+/+</sup> and CD30<sup>-/-</sup> B6 littermate mice were infected with 2x10<sup>6</sup> focus-forming units (ffu) of lymphocytic choriomeningitis virus clone 13 (LCMV cl 13) and sacrificed at day 21 post-infection. (A) Splenocytes were isolated and LCMV-specific CD8 T cell responses were assessed for frequency and absolute number using H-2D<sup>b</sup>/GP<sub>33-41</sub> and H-2D<sup>b</sup>/NP<sub>396-404</sub> tetramers with representative staining for the GP<sub>33-41</sub> tetramer. Mean fluorescence intensity (MFI) of (B) Tim-3 and (C) PD-1 within tetramer-specific CD8 T cells were measured along with (D) overall frequency of PD-1-expressing tetramer<sup>+</sup> CD8 T cells (GP<sub>33-41</sub>-specific response is shown, with similar results obtained for NP<sub>396-404</sub> not shown). (E) Splenocytes were restimulated <i>ex vivo</i> with GP<sub>33-41</sub>Or NP<sub>396-404</sub> peptide for 6 hours and stained for IFNγ production, with representative staining shown for GP<sub>33-41</sub>. (F) Viral load was evaluated in CD30<sup>+/+</sup> and CD30<sup>-/-</sup> mice at day 21 post-infection in the spleen, liver, and kidney. Data are pooled from two independent experiments with n=2 to 3 per group per experiment (median). NS not significant (Mann-Whitney test).

**Figure 5.5.** CD30 is dispensable for CD4 T cell responses during chronic LCMV clone 13 infection. CD30<sup>+/+</sup> and CD30<sup>-/-</sup> B6 littermate mice were infected with 2x10<sup>6</sup> ffu of LCMV cl 13 and sacrificed at day 21 post-infection. Splenocytes were isolated and examined for CD4 T cell subsets. (A) Gating strategy is shown. Frequency and absolute
numbers of (B) T follicular helper cells (Tfh), (C) T follicular regulatory cells (Tfr), and (D) T regulatory cells (Treg) were assessed. (E) Splenocytes were restimulated ex vivo with the GP<sub>61-80</sub> peptide for 6 hours and stained for IFN<sub>γ</sub> production. Representative staining and frequency of IFN<sub>γ</sub>-producing CD4 T cells is shown. Data are pooled from two independent experiments with n=3 per group per experiment (median). NS not significant (Mann-Whitney test).

Moreover, ex vivo restimulation with the GP<sub>61-88</sub> peptide did not reveal differences in CD4 T cell production of IFN<sub>γ</sub>, thus suggesting comparable Th1 responses (Figure 5.5E). Therefore, CD30 is dispensable for CD4 and CD8 T cell responses in chronic LCMV cl 13 infection.

**CD30 contributes to the expansion of the T lymphocyte compartment in older mice**

As CD30 is expressed at its highest levels on Tregs during influenza infection and also expressed on T cells in human lymphoproliferative disorders, we asked whether CD30 contributed to the number of steady state T cells, including Tregs over time. Strikingly, by 8 months of age, wild-type unmanipulated CD30<sup>+/+</sup> mice had significantly more T cells than their young counterparts (age 4-6 weeks) (Figure 5.6A-C). While naïve young CD30<sup>+/+</sup> and CD30<sup>-/-</sup> littermate mice did not show differences in T cell populations in the spleen, aged CD30<sup>-/-</sup> mice exhibited significantly fewer CD8 T cells (Figure 5.6A), CD4 T cells (Figure 5.6B), and Treg cells (Figure 5.6C) compared to their wild-type littermate controls. The lower numbers of Tregs in older CD30<sup>-/-</sup> mice likely reflects the lower number of CD4 T cells overall, as the frequency of CD25<sup>-</sup>Foxp3<sup>+</sup> were comparable between CD30<sup>+/+</sup> and CD30<sup>-/-</sup> littermates (Figure 5.6D, E). The MFI for CD25 also did not differ between CD30<sup>+/+</sup> and CD30<sup>-/-</sup> littermates (Figure 5.6F). This age-associated expansion of the T cell compartment seen in wild-type mice was completely mitigated in CD30<sup>-/-</sup> littermates, independent of the size and cellularity of the spleens themselves, as the T cell numbers between young and aged CD30<sup>-/-</sup> mice were comparable.

To determine which T cells were expanded in aged CD30<sup>+/+</sup> compared to CD30<sup>-/-</sup> mice, we conducted additional analysis of aged littermate mice to differentiate naïve and memory T cell subsets. We assessed CD62L<sup>-</sup>CD44<sup>lo</sup>, CD62L<sup>+</sup>CD44<sup>mid</sup>, CD62L<sup>+</sup>CD44<sup>hi</sup>, CD62L<sup>-</sup>CD44<sup>lo</sup>, CD62L<sup>-</sup>CD44<sup>mid-hi</sup> subsets of CD4 and CD8 T cells by flow cytometry (Figure 5.7A-D). CD30<sup>+/+</sup> and CD30<sup>-/-</sup> mice had comparable numbers of naïve CD62L<sup>-</sup>CD44<sup>lo</sup> CD8 and CD4 T cells. In
Figure 5.6. CD30 contributes to expansion of T cell populations in older mice. Spleens were harvested from young (4-6 weeks) and aged (~8 months) CD30+/+ and CD30-/- naïve (uninfected) littermate mice and quantified for absolute numbers of (A) CD8 T cells, (B) CD4 T cells, and (C) CD25+Foxp3+ Tregs. (D) Representative gating and (E) frequency are also shown for Tregs, with (F) CD25 MFI quantified. Data shown are pooled from three experiments for aged mice and two experiments for young mice, with n=2 to 4 per group per experiment (median). * P<0.05, ** P<0.005, *** P<0.0005, NS not significant (two-way ANOVA).
contrast, the CD62L⁺CD44hi antigen-experienced effector or effector memory cells were significantly reduced in the knockout mice compared to wildtype littermate counterparts. As KLRG-1 was absent from the majority of these T cells (data not shown), the increased T cell expansions are largely due to memory T cells. This trend was also seen in the CD62L⁺CD44hi central memory T cell compartment, although it did not reach statistical significance. Thus, CD30 contributes to the expansion of memory T cells in older mice.

5.4 Discussion

Several members of the TNFR family, including OX40, 4-1BB and GITR have non-redundant roles in both influenza and LCMV clone 13 infection in mice (24, 207, 208, 351, 354, 356). In contrast, here we show that the absence of CD30 alone does not appear to impact CD4, CD8 or Treg responses in these two infection models.

We found that CD30 was induced on CD4 and CD8 T cells upon antigen receptor stimulation in vitro, consistent with the literature (268). However, in vivo, only regulatory T cells expressed high levels of CD30 after influenza virus infection. Th1 cells showed only marginal CD30 expression at day 9 in the lung after infection with Influenza A/PR8, whereas pilot experiments failed to detect CD30 on effector T cells at earlier time points. As TNFR family members are normally transiently upregulated by TCR signalling, it is possible that CD30 is transiently upregulated upon priming of the T cells, but at those time points there are too few responding T cells for us to detect expression immediately ex vivo. Nonetheless, based on the evidence that CD30 is expressed on activated T cells, we investigated its role in anti-viral immunity. However, we found no role for CD30 in the T cell response to influenza or LCMV clone 13. Although we did not exhaustively look at different time points or effector cytokines, the finding that LCMV clone 13 viral load did not change and that lack of CD30 did not affect protective immunity to
influenza challenge, as evidenced by mouse survival and ability of CD8 T cells to persist and re-expand, makes it unlikely that CD30 plays a role in the immune response to these viruses.

Of note, a previous study by Nishimura suggested that CD30 was important for the establishment of a long-term central memory T cell response to *Listeria monocytogenes*. The differences between our results and those seen by Nishimura *et al.* may be attributed to the differences in the infections studied. Nishimura *et al.* showed that CD30L was specifically required for the generation of central memory T cells during infection with *L. monocytogenes*. Even at 100 days post-influenza infection, however, a large proportion of antigen-specific CD8 T cells in WT mice were still CD62L<sup>lo</sup> (data not shown), and this was particularly evident in the bone marrow. Therefore, the effect of CD30 on CD8 T cells may depend on the type of memory response generated by specific pathogens. It should also be noted that Nishimura *et al.* (291) did not indicate the use of littermate controls, so it is conceivable that CD30-independent effects of the microbiome on long-term memory could have impacted the results.

Our data showing that CD30 does not play a significant role in the CD8 T cell responses to influenza and LCMV cl 13 are consistent with that seen with VSV virus infection. In that study, CD30<sup>−/−</sup> memory CD8 CTL responses were unimpaired and corresponded with similar protection from VSV challenge in both CD30-sufficient and -deficient mice (299). In contrast, another report showed that OX40<sup>−/−</sup>CD30<sup>−/−</sup> mice had significant defects in antigen-specific CD8 T cell responses seven days following murine cytomegalovirus (MCMV) infection (300). It should be noted however, that CD30 single knockout mice were not compared in that model and littermate controls were not mentioned. Moreover, OX40<sup>−/−</sup> CD30<sup>−/−</sup> mice did not exhibit any defect in the generation and persistence of CD8 memory T cells. The authors of this manuscript also point out that this may be epitope-specific, as has been observed in studies of MCMV in OX40<sup>−/−</sup> mice (357).

It was previously shown that CD4<sup>+</sup>CD3<sup>+</sup> accessory cells express high levels of CD30L and OX40L and interact with helper T cells at the T-B border and within B cell follicles (275, 344). It was therefore possible the Th2 cells studied at that time, were actually the more recently identified Tfh cells. However, we were unable to detect any differences in Tfh or Tfr populations in the two infections studied here. It is possible that during both influenza and LCMV cl 13 infection, CD30 is dispensable and other signals direct follicular helper T cell differentiation. In this regard, Gaspal
et al. reported that the defect in germinal centers and antibody production in the absence of CD30 was found only during the memory phase, in sustaining but not forming the germinal centers and in producing memory, but not primary, antibodies (345).

CD30 was initially discovered on neoplastic Reed-Sternberg cells in Hodgkin’s disease, and its overexpression has since been associated with constitutive NF-κB signalling that may promote cell growth and malignancy (267). Its expression has also been characterized in non-Hodgkin’s lymphoma, as well as various cutaneous lymphoproliferative disorders of both T and B cells (358). Here we found evidence for a role for CD30 in T cell expansion with aging. CD30−/− mice had lower numbers of total CD4 and CD8 T cells as well as Tregs at 8 months compared to their wildtype littermates. In contrast, young mice (aged 5-6 weeks) showed no such differences. This difference was due to CD44mid-hiCD62L− effector memory T cells, with central memory T cells showing a similar trend. In contrast, naïve T cell numbers were indistinguishable between aged CD30+/+ and CD30−/− littermates. CD30, like other members of the TNFR family induces nuclear factor NF-κB signalling, which may contribute to survival of CD30+/+ compared to CD30−/− memory T cells (279, 359).

It is still unclear if the expanded cells are antigen-experienced memory or antigen-inexperienced memory phenotype cells previously characterized in unimmunized mice and humans (360, 361). Furthermore, whether this effect of CD30 is due to T cell intrinsic or extrinsic effects remains to be determined. The finding that CD30 plays a role in regulating the size of the memory T cell pool in aged unimmunized specific pathogen free mice suggests that in addition to being a marker of T cell lymphoproliferative diseases, CD30 may contribute to the persistence of T cells over time.
Chapter 6
Concluding Remarks
6.1 Summary of Findings

The studies presented in this thesis investigated the roles of the TNFR superfamily members 4-1BB and CD30 in T cell responses during influenza infection. We have identified 4-1BB as an important regulator of lung CD8 T cell responses, required for optimal expansion of primary effector cells (Figure 6.1). This local accumulation of CD8 T cells in the lung may be in part mediated by TRAF1, a downstream adaptor molecule responsible for 4-1BB-induced pro-survival effects through ERK-dependent phosphorylation and degradation of pro-apoptotic Bim (225-227), as well as its contribution to classical NF-κB-dependent upregulation of pro-survival Bcl-2-family molecules Bcl-XL and Bfl-1 (224). On the other hand, CD30 appears to be dispensable for T cell responses to acute influenza infection, as well as to LCMV cl 13 where the infection is stronger and more persistent. However, CD30 contributes to the expansion of memory T cells in aged specific-pathogen free (SPF) mice, suggesting a role for the molecule in homeostatic regulation during aging (Figure 6.1).

Given the potency of 4-1BB in inducing CD8 T cell responses, particularly as a co-stimulator of secondary CD8 T cells, and its role in maintaining memory populations during influenza infection, we decided to exogenously stimulate this receptor in a vaccination context to boost pre-existing immunity in a prime-boost regimen. The intranasal inoculation of 4-1BBL in a replication-defective adenovirus along with the influenza nucleoprotein antigen (Ad-NP-4-1BBL) 30 days after initial i.n. mild influenza X31 infection induced a long-lived, remarkably stable circulating as well as a persistent local CD8 T cell memory response, and protected against lethal heterotypic challenge for a year post-boost (Figure 6.2A, B). The circulating memory CD8 T cell population exhibited a more effector-like profile of surface and transcriptional markers compared to the mice boosted with Ad-NP alone, and were more polyfunctional in cytokine production upon ex vivo restimulation with NP peptide. Strikingly, the lung tissue parenchyma harboured an enhanced population of effector CD8 T cells post-boost, which contracted after antigen clearance but remained a stable and robust population of memory T cells that did not decline in number between days 60 and 210 post-infection. The accumulation of CD8 T cells within the lung tissue is dependent on local administration of antigen into the airways and acts
Figure 6.1. Contribution of TNFR superfamily members 4-1BB and CD30 to T cell responses. 4-1BB is required for optimal CD8 effector T cell accumulation in the lung tissue during influenza infection, which correlates with the size of the long-lived lung-resident memory pool. Whether 4-1BB also maintains the tissue-resident (Trm) population is still under investigation. CD30 does not play a discernable role in CD4 or CD8 T cell responses to acute influenza and chronic lymphocytic choriomeningitis virus (LCMV) clone 13, but regulates age-dependent expansion of the antigen-experienced memory and/or antigen-inexperienced memory phenotype T cell pool in unimmunized, specific pathogen free (SPF) mice.
Figure 6.2. Intranasal administration of replication-defective adenovirus containing influenza nucleoprotein (NP) gene and 4-1BBL (Ad-NP-4-1BBL) to boost pre-existing T cell immunity to influenza induces long-lived circulating effector memory and lung tissue-resident memory CD8 T cell responses that protective against lethal heterotypic challenge. (A) Upon intranasal inoculation, adenovirus is known to infect lung epithelial cells or antigen presentation cells in the airways. Infected host cells present antigen and 4-1BBL directly to CD8 T cells, which then expand in a secondary response characterized by high CD127 (II-7α subunit) and increased production of cytokines. (B) Specifically, Ad-NP-4-1BBL-boost induces a robust and long-lived circulating effector memory (Tem) population, characterized by a lack of CD62L expression, as well as high T-bet, low Eomes, and low TCF-1. Locally, Ad-NP-4-1BBL-boost results in an enhanced accumulation of memory CD8 T cells in the lung tissue to establish tissue-resident memory (Trm) that persist for over 200 days. The accumulation of CD8 T cells in the lung depends on local administration of both antigen and 4-1BBL and mTOR signalling.
directly on 4-1BB expressed by CD8 T cells; thus, the 4-1BBL-boost may be acting directly on lung Trm generated by the initial X31 primary infection. However, rather than locally expanding this pre-existent population, the increase in the Trm population during the boost phase is dependent on recruitment of additional circulating antigen-specific CD8 T cells into the lung tissue for establishment of the Trm pool. Lastly, 4-1BB-stimulation induced CD8 T cell accumulation in the lung tissue in an mTOR-dependent manner, likely contributing to the expansion of the effector cell precursors of Trm in the lung tissue. The effect of 4-1BBL in inducing a long-lived circulating and Trm population that protects to >1 year, represents a dramatic improvement on existing influenza vaccination strategies in terms of magnitude of the response, but whether a self-molecule would be taken up by the vaccine industry remains to be determined.

In contrast to 4-1BB, CD30 does not seem to have any discernable role in CD8 T cell effector or memory responses to influenza infection. Despite the presence of CD30 being reported on Tfh cells (276) and reports suggesting its function, along with OX40, in supporting memory CD4 T cell survival at zones of T and B contact in germinal centres (275, 344, 345), we found no contribution of CD30 in CD4 T cell responses or antibody production (data not shown) during influenza infection. Given the context-dependent roles of TNFRs and their varying responses to the severity of infection, we decided to investigate the LCMV cl 13 model where antigen persists for months and T cells are known to experience hierarchal loss of effector function and peripheral exhaustion. In this model, we found that CD30 was once again dispensable for antiviral CD8 and CD4 T cell responses, with comparable viral clearance exhibited between CD30+/+ and CD30−/− littermates. Interestingly, CD30−/− mice do not seem to exhibit the age-dependent expansion in splenic T cells compartments seen in their CD30+/+ littermate counterparts. Although, CD30−/− mice have a comparable population of naïve CD44loCD62L+ population, have reduced numbers of CD44mid-hiCD62L− effector memory cells, with a similar trend in the CD44mid-hiCD62L+ central memory compartment. This suggests a role for CD30 in the homeostatic regulation of memory T cells during aging and may have implications for human lymphoproliferative diseases, such as Hodgkin’s lymphoma and ALCL (265, 267, 358), with which CD30 expression has been associated.
6.2 4-1BB and Tissue-Resident Memory – Implications and Future Directions

The persistence of lung Trm and the mechanisms through which they become established and maintained has been an ongoing topic of investigation. Despite there being a core universal transcriptional signature of tissue-residency (75), it is clear that specific local microenvironments produce heterogeneity in Trm populations assessed from tissue to tissue. Perhaps the need to prevent immune-mediated tissue injury and pathology in preservation of the lung tissue for gas exchange produces an immunoregulatory environment less hospitable for long-term T cell residency. Indeed, CD8 Trm have been shown to localize in specific lung niches of tissue remodelling within peribronchiolar foci, termed repair-associated memory depots (RAMDs), after influenza infection where these cells are retained independently of CD69 (93). The gradual decline in CD8 Trm numbers may thus be associated with the waning of RAMD areas as tissue repair progresses to completion. Interestingly, the mere presence of RAMDs is insufficient, and local i.n. administered cognate antigen is required for the development of Trm niches in the lung. This is in congruence with our data as well as others that have indicated a need for persistent antigen within the lung tissue itself in order to maintain Trm. Replication-defective adenovirus has been shown to persist in antigen presentation for up to 40 days post-immunization (318); thus, the prolonged delivery of antigen along with 4-1BBL in our Ad-NP-4-1BBL model may be contributing to the enlarged and long-lived Trm population that we observed. Furthermore, since 4-1BB expression is transient upon TCR activation and sustained by persistent antigen (207-210), our model may also be allowing prolonged 4-1BB-4-1BBL interactions for further supraphysiological enhancement of T cell responses through this costimulatory pathway.

It is still unclear what 4-1BB is doing mechanistically to induce this CD8 T cell response. The persistence of Trm for over 200 days in the lung tissue suggests an antigen-independent mechanism of maintenance. Slütter et. al. showed that apoptosis contributes to the decline in lung Trm as these CD103+ cells have decreased Bcl-2 expression and higher caspase 3/7 activity compared to lung CD103– and lung vascular populations (97). The increased IL-7R expression, whether directly or indirectly induced by 4-1BB, seen in our Ad-NP-4-1BBL-model may be contributing to the longevity of the lung response as IL-7R signals Bcl-2 expression downstream...
Furthermore, lung Trm are known to be unresponsive to IL-7 and IL-15 through downregulation of CD127 and CD122. 4-1BB could also be contributing to the survival of these cells through Bcl-XL expression and the downregulation of Bim (224-227). Those factors were not thoroughly investigated in our study.

A practical alternative to sustaining long-lived lung-resident T cells may be a “prime and pull” vaccination, which consists of two steps: the induction of systemic T cell responses (prime), followed by recruitment of these cells into the targeted tissue (pull) for establishment of a protective mucosal Trm niche (111, 362). Recent studies showing multiple waves of effector T cell recruitment into the lung tissue provide support for targeting this continued replenishment as a method of sustaining Trm. Takamura et. al. showed that while i.p.-primed memory CD8 T cells can be recruited into the lung tissue and airways by local inflammation through CpG ODN, they disappear after the resolution of inflammation (93). In contrast, CpG ODN delivered locally with the NP366-374 peptide enables conversion into Trm and sustains this de novo niche. Slütter et. al. argues for an antigen-independent, cytokine-mediated replenishment of lung Trm by systemic Tem, which becomes less efficient over time as circulating memory populations become dominated by Tcm cells (97). Indeed, PR8-GP33-immune CD62L− P14 cells are more efficient at Trm commitment when transferred into PR8 infection-matched wildtype hosts compared to the CD62L+ fraction. Systemic antigen-dependent boosting to enhance Tem populations resulted in an increase of lung CD8 Trm in a P14 transgenic adoptive transfer model that persisted 30 days post-boost (97). In our model, where we see a Tem-predominating systemic memory phenotype concomitant with the enlarged Trm population, it is possible that these circulating Tem cells are the source of continued replenishment for the lung-resident pool. It would be interesting to assess whether multiple waves of circulating memory cells enter the tissue after the initial peak response through a series of experiments using FTY720 to sequester circulating cells at much later time-points post boost, particularly when antigen presentation is no longer occurring in the system. This would allow us to determine whether continued T cell immigration into the lung still occurs after antigen is gone and whether replenishment is a mechanism through which Trm pools are maintained long term.

It should also be noted that the recent publication from Gerlach et. al. has further redefined the classical characterization of circulating memory CD8 T cell populations. This group found that
the chemokine receptor CX3CR1 defined three populations of circulating memory CD8 T cells through its correlation with the degree of effector differentiation (68). Classical Tcm and Tem are CX3CR1⁻ and CX3CR1hi, respectively, while a CX3CR1int peripheral memory (Tph) population has emerged with homeostatic properties that contribute to its own self-renewal as well as the replenishment of the Tcm population. Interestingly, Gerlach et. al. found that CX3CR1hi Tem cells were largely restricted from the peripheral tissue, while CX3CR1int Tph cells can migrate between peripheral tissues and the circulation (68). Thus, what may be described as CD62L⁻ Tem cells previously in the literature may instead be two distinct populations. Here, in our study, we identified three populations within the lung tissue at 7 months post-Ad-NP-4-1BBL-boost: CD103⁻CD69⁻, CD103⁻CD69⁺, CD103⁺CD69⁺. Having not assessed the migration properties of these lung parenchymal cells, it is plausible that one or more of these populations—and perhaps components of the lung vascular and systemic circulating memory CD8 T cells as well—is a Tph subset, also enhanced by Ad-NP-4-1BBL, that does not reside permanently, but rather traverse in and out of the tissue. In addition to migration, we also did not assess the localization of the cells within the lung tissue. It would be interesting to adopt immunofluorescent microscopy techniques into our study to better visualize the tissue niches that are occupied by the different persisting populations of lung parenchymal memory cells, notably to determine whether the induced Trm are also localizing to the peribronchiolar RAMDS that were previously discussed in this section (93). Furthermore, it has been a well-identified problem in the field that flow cytometry analysis does not produce a complete picture of Trm populations as the recovery of cells is very much compromised during tissue digestion and processing (88). Thus, microscopic imaging provides a companion analysis that produces better recovery of the cells resident within the tissue.

This study, along with others, have further confirmed the importance of local immunization in the establishment of mucosal Trm cells (5, 93, 103). In addition to the previously described lung antigen depots, the need for i.n. immunization is important for the proper priming of respiratory DC subsets (RDC). Indeed, CD11chi RDC subsets appear to mediate continued antigen presentation by travelling from antigen depots in the lung to the dLN where new waves of CD8 T cells become activated and recruited into the tissue (101). Previous papers identifying and characterizing these CD103⁺ and CD11b⁺ RDC populations did not assess markers associated
with monocytes and it is possible that the CD11b+ RDC population may include or be comprised of monocyte-derived DC populations (363). In this regard, recent work in our lab has identified monocyte-derived APCs as the providers of 4-1BBL within the lung during influenza infection (Chang et al., Immunity, in press). Given the role of endogenous 4-1BB in regulating effector and memory CD8 T cell populations in the lung, it is important to investigate what cell types are providing the signals to support this Trm development. We suspect that in our prime-boost model, the adenoviral vector is infecting primarily epithelial cells for the induction of NP and 4-1BBL expression due to the relative abundance of these cells and the low dosage of the vector administered. An Ad-Cre virus (364) was recently acquired in the lab and would be able to distinguish adenovirus infected cell types more effectively, as the vector dose employed in our model is below the limit of detection by current flow cytometry-based assays (207). Furthermore, the lab has commissioned conditional 4-1BBL knockouts that also contain a fluorescent reporter for 4-1BBL expression that will allow us to conduct time deletions to better assess when and where these signals are needed, as well as potentially allow visualization of 4-1BBL by microscopy. As a self-molecule like 4-1BBL is unlikely to be incorporated into future human vaccines, it is imperative that we understand how it becomes induced—that is, what cells provide TNFR superfamily costimulatory and coinhibitory signals and how can they be manipulated and incorporated into adjuvant design. We have shown a potent effect of 4-1BB stimulation on both circulating and local CD8 T cell immunity, achieved at a relatively low vector dose without notable pathology, thus demonstrating the clinical relevance of 4-1BBL for future therapies.

6.3 CD30 and T Cell Regulation – Implications and Future Directions

There are still many questions as to how CD30 is regulating T cell homeostasis and memory expansion in aged unimmunized specific pathogen free (SPF) mice. It should be noted that although our mice are housed in sterile micro-isolator units, they are not germ free and are exposed to environmental and commensal pathogens that are not routinely screened or flagged by the animal facility. As a result, it is difficult to determine whether the CD44hi populations of
cells that we have characterized are antigen-experienced effector and memory populations or antigen-inexperienced memory phenotype T cells that are spontaneously generated at steady state and have been described in humans to expand with age (360). Memory phenotype CD8 T cells have been characterized as CD62L+CD44hiEomes+T-bet+CD122+ (361), which are all markers associated with antigen-experienced Tcm cells. CD44hiCD25lo CD4 memory phenotype T cells have also been identified (365-367). It would be interesting to determine whether the effect on homeostatic T cell regulation by CD30 is intrinsic to the T cell compartment. This can be explored by using mixed BM chimeras where CD30+/+ and CD30−/− BM can be used to reconstitute wildtype CD30+/+ mice at a 1:1 ratio. These mice can then be aged to 6 to 8 months after reconstitution. This allows CD30+/+ and CD30−/− CD4 and CD8 T cells to develop and be assessed in the same environment where the CD30-competent donor BM can provide all other cell types expressing CD30 and thus providing a model to study the direct effect of CD30 expression on CD8 and CD4 T cells. Since wildtype and knockout cells are compared within the same host, this also eliminates any confounding effects produced by microbiota differences. It is critical that we emphasize the importance of normalizing for these microbial factors—we observed robust phenotypic differences between separately bred strains of wildtype and knockout mice that disappeared completely once we adopted littermate controls for our CD30 experiments. This may be a result of insufficient backcrossing of the mice or due to variations in the microbiota affecting the inflammatory environment within these mice that produced the initial artefact that we were studying and wrongfully attributing to CD30. Notably, much of the previous literature on CD30 does not indicate the use of littermate controls, and studies observing the synergistic effects of CD30 and OX40 together using double knockouts often did not even use single knockout controls, let alone littermates (300, 347). It is possible that this is a confounding factor in the conflicting data regarding the role of CD30 during viral infection reported in the literature.

Due to the association of CD30 with lymphoproliferative diseases such as Hodgkin’s lymphoma and anaplastic large cell lymphomas (ALCL) (267, 358), it would be noteworthy to investigate whether the molecule has a role in the etiology of T cell malignancies. CD30+/+ and CD30−/− can be bred on to a spontaneous transgenic T cell lymphoma mouse model, such as the NPM-ALK transgenic mouse which has a translocation of the NPM/B23 to ALK that results in constitutive
activation of the ALK tyrosine kinase for enhanced T cell proliferation and survival (368). The NPM/B23 gene is one of the most common translocations in ALCL (369) and would therefore be a model of that disease. It would be interesting to assess whether CD30+/− NPM-ALK mice would have the same propensity to develop spontaneous lymphomas as their CD30+/+ littermates. However, it should be noted that spontaneous models of T cell lymphomas do not recapitulate human disease and although there are also genetically engineered mouse models that transform cells by prolonged expressions of certain genes involved in T cell development, it is not well understood whether the oncogenic events occur in the T cell precursor stage or in the periphery once matured (370). Interestingly, there have been variants of cutaneous T cell lymphomas identified to have malignancies of Trm or Tcm populations (371-373). Thus, the role of CD30 in regulating age-related expansions of memory T cells may be an critical mechanism in T cell malignancies that needs to be further investigated.

6.4 Conclusion

Taken together, the studies presented in this thesis have identified novel functions of two TNFR superfamily receptors in regulating local and systemic T cell responses. These studies inform on the fine-tuning mechanisms of costimulatory receptors both at steady state and post-priming in the context of viral infection, and provide insight into potential targets for future therapeutic intervention of viral infections and cancer malignancies.
Chapter 7
Appendix
7.1 List of Publications

1. **Zhou, A.C.** and T.H. Watts. 2017. The role of mTOR, TRAF1, and local antigen in lung CD8 tissue-resident memory establishment. (manuscript in preparation).


References


47. Badovinac, V. P., J. S. Haring, and J. T. Harty. 2007. Initial T cell receptor transgenic cell precursor frequency dictates critical aspects of the CD8(+) T cell response to infection. *Immunity* 26: 827-841.


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