2020

T-Bet Expressing B Cells With Distinct Residency And Functional Characteristics Give Rise To Plasma Cells

Rebecca Laura Rosenthal

*University of Pennsylvania*

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Abstract

An increasing body of work shows that T-bet+ B cells play important roles in immune responses to infections as well as in autoimmune diseases. These studies investigate the kinetics, tissue distribution, and functions of T-bet+ in a murine influenza infection model. Both T-bet+ and T-bet- HA-specific B cells emerge early after infection, acquire the memory markers CD73, PD-L2 and CD80, and persist indefinitely with limited interconversion between T-bet+ and T-bet- B cell pools. Moreover, T-bet+ B cells are required for HA stalk specific antibodies and sustained protective HAI titers. T-bet+ HA-specific B cells can be further divided into T-bethigh and T-betlow B cell pools. While T-bethigh, T-betlow, and T-bet- HA-specific B cells are initially found in the spleen, lungs, and draining mediastinal lymph nodes; at later timepoints T-bethigh HA-specific memory B cells are restricted to the spleen, despite the continued presence of T-bet- HA-specific B cells at other anatomical locations. Parabiotic studies show that HA-specific T-bethigh B cells are splenic residents, whereas T-betlow and T-bet- B cells recirculate. T-bethigh B cells give rise to T-betlow B cells but T-betlow B cells do not become T-bethigh. However, T-betlow B cells persist for a minimum of three weeks in the absence of T-bethigh B cells, indicating that they are likely distinct populations. CD138 levels increase with decreasing T-bet expression and T-betlow and T-bethigh B cells downregulate T-bet and give rise to plasma cells. Both B220+ and B220- plasma cells arise from T-bet+ B cells in the spleen and bone marrow. Together, these data suggest that T-bethigh memory B cells are a unique splenic resident population with stem cell like properties that sustains plasma cell numbers and protective antibody titers long term.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Immunology

First Advisor
Michael P. Cancro

Keywords
B cell memory, Humoral immunity, Influenza, Plasma cells, T-bet+ B cells, Tissue-resident

Subject Categories
Allergy and Immunology | Immunology and Infectious Disease | Medical Immunology

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T-BET EXPRESSING B CELLS WITH DISTINCT RESIDENCY AND FUNCTIONAL CHARACTERISTICS GIVE RISE TO PLASMA CELLS

Rebecca Rosenthal

A DISSERTATION

in

Immunology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2020

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I dedicate this dissertation to my family, without whom none of this would have been possible. To my mother, Sharon Rosenthal, for her unwavering love and support my entire life. To my father, Stanton Jay Rosenthal, for always encouraging my curiosity—you were my first and favorite teacher. I will always appreciate our dinnertime conversations of interesting medical cases even if they disgusted the rest of the family. To my sister, Anne Rosenthal, for helping me maintain my sanity and always encouraging me and believing in me, even when I doubted myself. To Adam Schroeder, you are like the brother I never had.

To the memory of my grandparents, Lillian and Avrom Lustgarten who were like a second set of parents to me. Without your love and support I would not be the person I am today. I will always miss our weekly Sunday dinners and your plethora of jokes.
ACKNOWLEDGMENT

I would like to thank Michael Cancro for agreeing to be my mentor and for his scientific support and advice. I would also like to acknowledge all the members of the Cancro lab for many helpful discussions and experimental aid and advice. I would especially like to thank John Johnson for his support and aid, both scientific and otherwise—I have greatly enjoyed all of our conversations. I would also like to thank Caleb Ng for his support and encouragement. You always believed in me and encouraged me even when I doubted myself. I would like to acknowledge my committee, Eline Luning Prak, David Allman, Michael Atchison, and Christopher Hunter for many helpful suggestions and discussions. I appreciate the support provided by the Immunology Graduate Group and the VMD/PhD program. I have greatly benefitted from being part of such an academic and supportive environment. Without all of you, much of this would not have been possible.
ABSTRACT

T-BET EXPRESSING B CELLS WITH DISTINCT RESIDENCY AND FUNCTIONAL CHARACTERISTICS GIVE RISE TO PLASMA CELLS

Rebecca L. Rosenthal
Michael P. Cancro

An increasing body of work shows that T-bet\(^+\) B cells play important roles in immune responses to infections as well as in autoimmune diseases. These studies investigate the kinetics, tissue distribution, and functions of T-bet\(^+\) B cells in a murine influenza infection model. Both T-bet\(^+\) and T-bet\(^-\) HA-specific B cells emerge early after infection, acquire the memory markers CD73, PD-L2 and CD80, and persist indefinitely with limited interconversion between T-bet\(^+\) and T-bet\(^-\) B cell pools. Moreover, T-bet\(^+\) B cells are required for HA stalk specific antibodies and sustained protective HAI titers. T-bet\(^+\) HA-specific B cells can be further divided into T-bet\(^{\text{high}}\) and T-bet\(^{\text{low}}\) B cell pools. While T-bet\(^{\text{high}}\), T-bet\(^{\text{low}}\), and T-bet\(^-\) HA-specific B cells are initially found in the spleen, lungs, and draining mediastinal lymph nodes; at later timepoints T-bet\(^{\text{high}}\) HA-specific memory B cells are restricted to the spleen, despite the continued presence of T-bet\(^-\) HA-specific B cells at other anatomical locations. Parabiotic studies show that HA-specific T-bet\(^{\text{high}}\) B cells are splenic residents, whereas T-bet\(^{\text{low}}\) and T-bet\(^-\) B cells recirculate. T-bet\(^{\text{high}}\) B cells give rise to T-bet\(^{\text{low}}\) B cells but T-bet\(^{\text{low}}\) B cells do not become T-bet\(^{\text{high}}\). However, T-bet\(^{\text{low}}\) B cells persist for a minimum of three weeks in the absence of T-bet\(^{\text{high}}\) B cells, indicating that they are likely distinct populations. CD138 levels increase with decreasing T-bet expression and T-
Bet^low and T-bet^high B cells downregulate T-bet and give rise to plasma cells. Both B220^+ and B220^− plasma cells arise from T-bet^+ B cells in the spleen and bone marrow. Together, these data suggest that T-bet^high memory B cells are a unique splenic resident population with stem cell like properties that sustains plasma cell numbers and protective antibody titers long term.
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<td>Age Associated B Cell</td>
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<tr>
<td>AD</td>
<td>Agglutination dose</td>
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<tr>
<td>AF647</td>
<td>Alexa Flour 647</td>
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<tr>
<td>ASC</td>
<td>Antibody Secreting Cell</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial Artificial Chromosome</td>
</tr>
<tr>
<td>BAFF</td>
<td>B Cell Activating Factor</td>
</tr>
<tr>
<td>BAFFr</td>
<td>B Cell Activating Factor Receptor</td>
</tr>
<tr>
<td>BCR</td>
<td>B Cell Receptor</td>
</tr>
<tr>
<td>BLyS</td>
<td>B Lymphocyte Stimulator</td>
</tr>
<tr>
<td>BPL</td>
<td>Betapropiolactone</td>
</tr>
<tr>
<td>BR3</td>
<td>B Lymphocyte Stimulator Receptor 3</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-Bromo-2’-Deoxyuridine</td>
</tr>
<tr>
<td>BV</td>
<td>Brilliant Violet</td>
</tr>
<tr>
<td>cKO</td>
<td>Conditional Knockout</td>
</tr>
<tr>
<td>CLP</td>
<td>Common Lymphoid Progenitor</td>
</tr>
<tr>
<td>D</td>
<td>Diversity Gene Segment</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage Associated Molecular Pattern</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment Crystallizable</td>
</tr>
<tr>
<td>GC</td>
<td>Germinal Center</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HAI</td>
<td>Hemagglutinin Inhibition</td>
</tr>
<tr>
<td>HAU</td>
<td>Hemagglutination Units</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon Gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>i.n.</td>
<td>Intranasal</td>
</tr>
<tr>
<td>JH</td>
<td>Joining Gene Segment (Heavy Chain)</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic Choriomeningitis Virus</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph Node</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MACs</td>
<td>Magnetic-Activated Cell Sorting</td>
</tr>
<tr>
<td>MBC</td>
<td>Memory B Cell</td>
</tr>
<tr>
<td>medLN</td>
<td>Mediastinal Lymph Node</td>
</tr>
<tr>
<td>MZ</td>
<td>Marginal Zone</td>
</tr>
<tr>
<td>NP-CGG</td>
<td>4-Hydroxy-3-Nitrophenylacetyl-Chicken Gamma Globulin</td>
</tr>
<tr>
<td>OAS</td>
<td>Original Antigenic Sin</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
</tr>
<tr>
<td>PC</td>
<td>Plasma Cell</td>
</tr>
<tr>
<td>PNA</td>
<td>Peanut Agglutinin</td>
</tr>
<tr>
<td>PR8</td>
<td>Influenza strain A/PR/8/34</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
</tr>
<tr>
<td>Abbr</td>
<td>Full Form</td>
</tr>
<tr>
<td>------</td>
<td>-----------</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SIP₁</td>
<td>Sphingosine 1-phosphate receptor 1</td>
</tr>
<tr>
<td>SLC</td>
<td>Surrogate Light Chain</td>
</tr>
<tr>
<td>TACI</td>
<td>Transmembrane Activator and Calcium-Modulating Cyclophilin Ligand Interactor</td>
</tr>
<tr>
<td>Tam</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>T-bet</td>
<td>T-box Expressed in T cells</td>
</tr>
<tr>
<td>Tbx21</td>
<td>T-Box Transcription Factor 21</td>
</tr>
<tr>
<td>TCID₅₀</td>
<td>50% Tissue Culture Infective Dose</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>Tₕₜ</td>
<td>T Follicular Helper</td>
</tr>
<tr>
<td>Th</td>
<td>T Helper</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll Like Receptor</td>
</tr>
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<td>Treg</td>
<td>T Regulatory Memory</td>
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<tr>
<td>Trm</td>
<td>Resident Memory T Cell</td>
</tr>
<tr>
<td>T₉M</td>
<td>Tissue Resident Memory</td>
</tr>
<tr>
<td>V₉H</td>
<td>Variable Gene Segment (Heavy Chain)</td>
</tr>
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</table>
CHAPTER 1: Introduction

1.1 Overview of Innate and Adaptive Immunity

The immune system provides protection from pathogens, but also underlies diseases such as autoimmunity and allergy. As such, the ability to mount protective immune responses while preventing the development of autoimmunity is essential for continued health. Four attributes of the immune response in vertebrates are key to achieving these goals. These are (1) recognition of an incipient infection, (2) control/clearance of the infection via immune effector functions, (3) regulation to prevent/limit immune mediated damage to the host, and (4) establishment of immunological memory that provides durable protection from reinfection.

When a pathogen breaches host defenses, including barriers such as the skin and mucus membranes, innate immune mechanisms provide a rapid first-line of defense. The innate immune system includes myeloid cells such as granulocytes, dendritic cells, macrophages, and mast cells, that rapidly sense the pathogen. This is mediated by germ-line encoded pattern recognition receptors (PRRs) that recognize evolutionarily conserved pathogen associated molecular patterns (PAMPs) expressed by microbes, as well as damage associated molecular patterns (DAMPs) that are released from cells following damage and death. Engagement of PRRs by PAMPs and/or DAMPs activates cells of the innate immune system. Moreover, this activation can result in a spectrum of
differentiative programs that in turn will instruct the effector choices undertaken by the cells participating in the adaptive immune response\textsuperscript{3}.

While the innate immune system generates a rapid response by recognizing broadly conserved molecular moieties, the adaptive immune system generates a response that is finely tailored to unique molecular determinants of the pathogen. Many key aspects of the adaptive response can be explained by Burnet’s clonal selection paradigm. Fundamental to Burnet’s theory is that each lymphocyte expresses antigen receptors of a single specificity, and that occupation of these receptors induces cells to respond and divide, thus producing daughter cells with the same specificity as the original responding cell.

\subsection*{1.2 B Lymphocyte Development}

In adults, B cell development begins in the bone marrow with pluripotent hematopoietic stem cells that generate multipotent progenitor cells, which have lost their potential as self-renewing stem cells, but can give rise to both lymphoid and myeloid cells. These multipotent progenitor cells express the cell surface receptor tyrosine kinase FLT3, and signaling through this receptor leads to differentiation into the common lymphoid progenitor (CLP)\textsuperscript{4, 5, 6, 7}. Some CLP progeny then differentiate into a pro-B cell, which is the first cell type firmly committed to the B cell lineage. These cells initiate the process of immunoglobulin (Ig) gene rearrangement, which ultimately leads to expression of a functional B cell receptor (BCR). The first stage of Ig gene rearrangement results in rearrangement of the germline heavy chain segment via joining of a
diversity (D) gene segment to a joining (J<sub>H</sub>) gene segment<sup>8</sup>. In the late pro-B cell, a variable (V<sub>H</sub>) gene segment is joined to the previously generated DJ<sub>H</sub> segment. This can generate a productive V<sub>H</sub>DJ<sub>H</sub> segment that is expressed as part of the pre-BCR, together with a surrogate light chain (SLC)<sup>9</sup>. Proper V<sub>H</sub>DJ<sub>H</sub> rearrangement and pairing with the SLC is required for transit to the next stage of B cell development, and roughly half of pro-B cells fail to successfully complete this process and are eliminated<sup>10</sup>. In the next stage of B cell development, the large pre-B cell undergoes extensive proliferation, expanding 30-60 fold. The developing B cell then becomes a resting small pre-B cell<sup>11, 12, 13</sup>. The small pre-B cell once again expresses RAG and rearranges the germline light chain sequence to generate a light chain VJ segment<sup>14, 15</sup>. After a productive light chain VJ rearrangement, the small pre-B cell becomes an immature B cell and expresses a full IgM BCR on its surface with both a rearranged heavy and light chain. The IgM-expressing B cell is subject to positive and negative selection based on BCR signal strength in order to eliminate autoreactive clones and identify B cells with signaling-competent receptors. Strikingly, as many as 75% of immature B cells are initially autoreactive<sup>16, 17</sup>. Given this stringent selection, only 10% of immature B cells exit the bone marrow into the peripheral lymphoid organs. Here, they undergo further selection and more than half of the remaining cells are eliminated prior to entry into the mature B cell pools<sup>18, 19, 20, 21, 22</sup>. 
1.3 Lymphocyte Effector Subsets

Following antigen encounter and activation, both T and B lymphocytes undergo further differentiation into distinct effector and memory subsets. For example, CD4 T cells differentiate into distinct effector lineages characterized by unique cytokine production profiles and lineage-specific transcription factors that are required for their differentiation\(^\text{23}\). These distinct effector lineages have differing functions, and their proper regulation is key to initiating and maintaining a protective immune response, as inappropriate effector lineage commitment leads to failed pathogen clearance and/or autoimmune and inflammatory pathologies\(^\text{24}\). The cytokine milieu, as well as T cell receptor (TCR) signaling and costimulation, determine commitment to a given effector lineage. Similarly, CD8 T cells include distinct effector subsets with specialized functions and distinct transcription factor profiles. These can differ in terms of tissue residency and recirculation, which is important for tissue surveillance and protection from reinfection\(^\text{25}\). Importantly, resident memory T cells (Trm) fail to recirculate and remain tissue resident, as demonstrated through parabiosis experiments\(^\text{26, 27, 28}\). Most of these Trm are not exposed to circulation as demonstrated by a failure to label following intravenous fluorescent antibody injection\(^\text{29}\). These Trm are important for protective immunity against local reinfection\(^\text{30}\). For example, in a local vaccinia virus re-challenge model, mice with skin resident Trm rapidly cleared reinfection whereas mice lacking Trm failed to control the virus despite similar levels of central memory T cells\(^\text{26}\).
While the importance of effector T cell subsets with distinct tissue residency and recirculation properties is appreciated, a similar understanding of the breadth of memory B cells is ongoing. One way to stratify B cells into various effector subsets is on the basis of heavy chain isotype. The BCR heavy chain has five main isotypes; delta, mu, gamma, alpha, and epsilon. These heavy chain isotypes pair with either a kappa or lambda light chain to form IgD, IgM, IgG, IgA, or IgE. Furthermore, the IgG isotype can be split into four subtypes; IgG1, IgG2a/c (with IgG2a present in BALB/c and IgG2c in C57BL/6\textsuperscript{31}), IgG2b, and IgG3 in mice and IgG1, IgG2, IgG3, and IgG4 in humans. Similar to T cell effector lineages, the cytokine milieu along with receptor signaling are key drivers of class switch recombination decisions with the various BCR isotypes serving differing roles in an immune response\textsuperscript{32, 33}. Isotype-specific Fc receptors are expressed on a range of hematopoietic origin cells. These Fc receptors can be either activating or inhibitory and as such the Ig isotype has important effects on cell activation and behavior. These Fc receptor interactions can regulate multiple effector responses including antibody-dependent cytotoxicity, phagocytosis, and inflammatory mediator release\textsuperscript{33}.

However, heavy chain isotype is not the only way in which mature B cells can be divided into distinct subsets. Following antigenic stimulation, B cells can be recruited into a germinal center (GC) response. These GC B cells can undergo robust proliferation, with the magnitude of T cell help being an important determinant of the extent of division. GC B cells acquire point mutations in their BCR and compete for survival signals, such that higher affinity clones are
selected for survival. These high affinity GC B cells go on to become memory B cells or plasma cells and can even participate in further rounds of GC selection\textsuperscript{34, 35}. These GC derived memory B cells may be primed for rapid differentiation into antibody secreting cells upon reexposure\textsuperscript{36, 37}.

Similar to T cells, mature B cells can be divided on the basis of key lineage commitment transcription factor expression\textsuperscript{38, 39}. The transcription factor T-bet is characteristic of Age Associated B Cells (ABCs) and will be the focus of the work discussed here.

1.4 T-bet\textsuperscript{+} Age Associated B Cells

While previous work demonstrated that T-bet expression in B cells is important for class switching to the IgG2a/c isotype\textsuperscript{40}, the discovery of a unique B cell subset characterized by T-bet expression can be credited to the Cancro and Marrack laboratories\textsuperscript{41, 42}. Despite using different criteria to define this novel B cell population, both observed an accumulation of these cells in mice with increasing age and termed them Age Associated B Cells (ABCs). Subsequent work has demonstrated that they are largely overlapping populations\textsuperscript{41, 42}. Furthermore, Rubtsov et al. observed that these cells accumulated earlier in females of various autoimmune prone mouse strains and that a similar cell population could be found in elderly women with autoimmune conditions\textsuperscript{42}.

ABCs display several unique characteristics. They are refractory to BCR crosslinking, thus distinguishing them from follicular B cells which robustly proliferate in response to BCR crosslinking, as well as from marginal zone (MZ)
and transitional B cells, which die upon BCR crosslinking. ABCs proliferate in response to endosomal Toll Like Receptor (TLR) stimulation. This proliferation can be enhanced by the addition of BCR stimulation, showing that while ABCs do not proliferate in response to BCR crosslinking alone, the BCR retains some signaling capabilities. However, ABCs fail to proliferate in response to TLR4 stimulation as provided by the ligand lipopolysaccharide (LPS), further distinguishing them from other mature B cell subsets such as MZ B cells which robustly expand following LPS treatment.

While ABCs accumulate with age, this does not reflect the emergence of a distinct precursor population in aged bone marrow. Sublethal irradiation with 500 cGy in aged mice results in the successful ablation of most peripheral B cell pools including ABCs, follicular B cells, and MZ B cells. Following irradiation, the ABC pool fails to rapidly autoreconstitute despite the rapid reestablishment of the follicular and MZ B cell pools. Additionally, adoptive transfer of splenic follicular B cells gives rise to ABCs within 30 days of transfer and donor or recipient age has no impact on the formation of these donor derived ABCs. Interestingly, cells with an ABC phenotype preferentially arise from the fraction of donor cells undergoing the most robust proliferation following adoptive transfer. While ABCs express the BAFF/BLyS receptors BAFFr (BR3) and TACI, they do not rely on BAFF signaling for their survival as ABC numbers are not impacted by treatment with doses of anti-BAFF antibody sufficient to deplete the follicular and MZ B cell pools.
A large portion of ABCs express the transcription factor T-bet\textsuperscript{42}. T-bet, or T-box containing protein expressed in T cells, is a transcription factor encoded by the \textit{Tbx21} gene. It was first described as a master regulator controlling the commitment of CD4 T cells to a Th1 effector lineage\textsuperscript{44}. Subsequently, it has been shown to be important for the development, survival, and function of various innate and adaptive immune cell types\textsuperscript{45}. Its role in B cells has gained increasing appreciation as T-bet expressing B cells are associated with a range of infections and autoimmune pathologies in both mice and humans\textsuperscript{42, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55}. T-bet\textsuperscript{+} B cells likely play an important role in protective immune responses as mice lacking T-bet expression in B cells show impaired viral control\textsuperscript{51, 55}.

**Requirements for T-bet Expressing B Cell Formation**

T-bet expression in B cells was first observed after in vitro treatment of splenic B cells with CD40, interleukin (IL) 12, and IL-18\textsuperscript{44}. Subsequent work demonstrated that T-bet promotes B cell isotype switching to IgG2a/c\textsuperscript{40}. T-bet expression is robustly induced in vitro after treatment of follicular B cells with either interferon gamma (IFN\textgamma) or IL-21 in the presence of TLR7 or TLR9 agonists\textsuperscript{60}. The addition of IL-4 has differential effects on T-bet induction depending on whether it is added to IFN\textgamma or IL-21 stimulated B cells: the addition of IL-4 to IL-21 stimulated follicular B cells blocks T-bet induction whereas addition of IL-4 to IFN\textgamma stimulated B cells preserves and possibly even enhances T-bet induction. The IL-4 mediated blockade of IL-21 induced T-bet induction is
STAT6 dependent and cell intrinsic. STAT6 knockout follicular B cells co-cultured with wildtype follicular B cells still upregulate T-bet in response to IL-21 and IL-4 treatment in the presence of a TLR9 agonist. However, cocultured wildtype cells failed to induce T-bet in response to this combination of stimuli. A large portion of IL-21 driven T-bet+ B cells express the integrin CD11c whereas IFNγ driven T-bet+ B cells are uniformly CD11c negative. Together, these results imply that IFNγ and IL-21 utilize different pathways to drive T-bet induction, and that T-bet expression alone is insufficient to drive CD11c expression. Thus, T-bet expression is unlikely to be a simple on/off switch but instead can be driven through multiple inductive pathways. A similar pattern of T-bet induction can be observed in vivo, as B6 mice, which show a strong TH1 skewing and resultant high levels of IFNγ, have much higher proportions of T-bet expression in GC and memory B cells than BALB/c mice, which have a TH2 bias and associated high IL-4 levels. Infection with the PR8 strain of influenza, which leads to a TH1 skewed response with robust IFNγ, IL-21, and IL-4 production by responding TFH cells, results in a strong T-bet+ GC B cell response in wildtype C57BL/6 but not IFNγ knockout mice, despite a similar magnitude in overall GC B cell response. The T-bet+ GC B cell response is rescued in IL-4/IFNγ double knockout mice, despite an overall decrease in the GC B cell response. Infection of C57BL/6 mice with the TH2 biased pathogen H. polygyrus, which leads to IL-21 and IL-4 production by TFH cells in the absence of IFNγ, fails to induce T-bet expression in the GC B cell response whereas IL-4 knockout mice display robust T-bet and
CD11c expression in GC B cells. This is not due to increased IFNγ in IL-4 knockout mice, as the T-bet⁺ CD11c⁺ GC B cell response is observed in IL-4/IFNγ double knockout mice⁶⁰.

**T-bet Expressing B Cells in Health and Disease**

The first demonstration of T-bet⁺ B cells in pathogen-driven immune responses was provided by the Winslow lab, who showed that IgM⁺ CD11c⁺ splenic plasmablasts⁶⁴ and memory B cells⁶⁵ emerge following *Ehrlichia muris* infection in mice. Later work showed that these cells express T-bet⁶⁶. T-bet⁺ B cells emerge following viral infections as first demonstrated by the identification of T-bet⁺ CD11c⁺ B cells following gamma herpes virus 68 infection. These cells produce virus specific IgG2a and are important for maintaining reduced virus titers⁵⁵. Furthermore, T-bet⁺ B cells are important in controlling lymphocytic choriomeningitis virus (LCMV) infection, as T-bet⁺ B cells are required for LCMV specific IgG2a production. While not required for control of acute LCMV infection, T-bet⁺ B cells are required for viral control during chronic infection. Serum transfer of LCMV specific IgG2a into infected mice with B cells lacking T-bet was unable to control chronic LCMV viral loads, indicating that T-bet⁺ B cells have important roles in viral control beyond IgG2a production⁵¹.

These results indicate that T-bet⁺ B cells are likely a memory B cell population. They persist long term following viral infection and clearance, as evidenced by the sustained presence of antigen specific T-bet⁺ B cells in mice following clearance of influenza infection⁴⁷. Studies in humans have
demonstrated the persistence of T-bet⁺ B cells in aviremic HIV patients after years of successful antiretroviral therapy⁵².

T-bet⁺ B cells are also important in autoimmunity. T-bet⁺ B cells or B cells with T-bet associated phenotypes can be observed at increased frequencies in patients with a wide range of autoimmune pathologies, such as lupus, multiple sclerosis, rheumatoid arthritis, Crohn’s disease, celiac disease, common variable immune deficiency, Sjogren’s syndrome, and hepatitis associated mixed cryoglobulinemia⁵⁶, ⁵⁹, ⁶⁷, ⁶⁸, ⁷⁰, ⁷¹, ⁷², ⁷³, ⁷⁴, ⁷⁵.

1.5 Scope

T-bet⁺ B cells are induced and persist in response to pathogen challenge, but outstanding questions regarding their kinetics, residency and function remain. In chapter 2, we examined the emergence and persistence of T-bet⁺ B cells following influenza infection and tested their contribution to the humoral response. As T-betʰ B cells display progressive splenic restriction, we tested their residency using parabiosis. In chapter 3, we further investigated the fate of T-bet⁺ B cells by examining their ability to differentiate into other cell types, including plasma cells. We found that spleen resident T-betʰ memory B cells likely differentiate into a circulating population that eventually gives rise to plasma cells in the spleen and bone marrow.
CHAPTER 2: The Emergence, Maintenance, and Residency of T-bet\(^+\) B Cells in an Influenza Response

2.1 Summary

In this section, we track the emergence and maintenance of influenza hemagglutinin (HA) specific B cells following influenza infection in mice. Using a T-bet ZsGreen reporter mouse strain, we show that a portion of HA-specific B cells express the transcription factor T-bet. While T-bet\(^-\) HA-specific B cells can be found in both the spleen and draining mediastinal lymph nodes (LNs) long term following infection, T-bet\(^+\) memory B cells are largely restricted to the spleen at memory time points and are absent from the lymphatics. Using parabiosis, we demonstrate that while T-bet\(^-\) and T-bet\(^{low}\) HA-specific B cells traffic and are found in the spleens of both partner mice, T-bet\(^{high}\) HA-specific B cells are restricted to the spleen of the previously infected partner, indicating that they are sessile splenic residents. T-bet\(^+\) B cells are important in generating influenza specific IgG2a/c titers as these are greatly reduced in mice lacking T-bet expressing B cells. Furthermore, HA stalk titers are nearly absent in mice without T-bet\(^+\) B cells. While HAI titers are similar between all genotypes at day 15 post infection, they are significantly reduced by day 40 in mice lacking T-bet expression in B cells, indicating that T-bet is likely required for sustained protective immune titers.
2.2 Introduction

The importance of T-bet in immune responses was first recognized in studies from the Glimcher lab, that showed T-bet is a key transcription factor in driving CD4 T cell commitment to a T helper 1 (Th1) cell fate\(^4^4\). Later work showed that T-bet is involved in the development and effector functions of various innate and adaptive immune cell types\(^4^5\). As such, there is an increasing appreciation of T-bet as a key feature of immune cells in type 1 immune responses.

T-bet plays an important role in protection, recovery, and/or pathogen burden in a range of infections. T-bet deficient mice show an increased incidence and severity of septic arthritis and increased renal bacterial counts following \textit{Staphylococcus aureus} infection\(^7^6\). Mice lacking T-bet are more susceptible to aerosol or IV \textit{Mycobacterium tuberculosis} infection as demonstrated by decreased survival and increased bacterial burdens in the lungs, liver, and spleen\(^7^7\). Furthermore, T-bet knockout mice have decreased survival and increased splenic bacterial counts after \textit{Salmonella typhimurium} infection\(^7^8\). In terms of \textit{Francisella tularensis} infection, T-bet knockout mice were more susceptible to death, even at lower doses than wildtype mice, and showed increased bacterial burdens in the spleen, liver, and lungs regardless of route of infection. They also displayed an increased susceptibility to re-challenge, as evidenced by increased death following high dose intra-nasal secondary infection following intra-dermal or intra-nasal vaccination\(^7^9\). This is further supported by
studies demonstrating a link between strength of *Francisella* vaccine engendered protection and magnitude of upregulation of T-bet expression\(^{80}\). However, not all immune protection following bacterial infection relies on T-bet. T-bet deficient mice do not show impaired *Listeria monocytogenes* bacterial control and clearance during the innate or adaptive phases of the response\(^{81}\). In terms of the intracellular protozoan *Leishmania major*, T-bet\(^{-/-}\) C57/BL6 mice were more susceptible than their wildtype littermate controls and showed a similar failure to resolve lesions and control pathogen burden as the susceptible Th2 biased BALB/c mouse strain\(^{82}\).

Furthermore, T-bet is important in controlling certain viral infections. Mice lacking T-bet had increased viral titers, accelerated disease onset, and impaired survival following genital Herpes Simplex Virus Type 2 infection. This impaired protection extended to secondary responses as T-bet\(^{-/-}\) mice receiving an attenuated virus vaccine had impaired control of viral replication and increased disease severity, viral titers, and death after secondary infection\(^{83}\). Mice lacking T-bet were more susceptible to *Vaccinia* virus infection as evidenced by decreased survival, increased weight loss, and greater persistence of viral titers\(^{84}\). While T-bet deficient mice did not display impaired viral clearance following rhinovirus infection, they failed to generate robust antiviral IgG2c titers and displayed exacerbated viral mediated airway inflammation as evidenced by increased airway eosinophilia and mucus hypersecretion\(^{85}\).

While T-bet is clearly a key mediator in the response to a variety of pathogens, the majority of studies have relied on global T-bet knockout mice. As
such, the observed effects may be related to a failure to generate Th1 polarized CD4 T cells and therefore decreased IFNγ production. The specific role of T-bet expressing B cells in immune protection remains less clear and may be complicated by B cell/T cell interactions such that T-bet deficiency in either T cells or B cells may have effects on other cell types. Research from the Wherry lab suggests that T-bet expressing B cells may be important in the control of chronic viral infections, as mice lacking T-bet in B cells are unable to control chronic LCMV infection. It remains unknown whether T-bet expression can be used to stratify memory B cells into subsets with distinct characteristics and functions.

In this chapter, we investigate the kinetics, anatomic residency, trafficking, and function of T-bet+ B cells in mice following influenza infection. We show that HA-specific B cells can be divided into T-bet+, T-betlow, and T-bethigh populations. Whereas all three populations are initially observed in the spleen, lungs, and draining LNs, the T-bethigh population is restricted to the spleen at memory timepoints and remains resident following parabiosis whereas the T-bet+ and T-betlow populations recirculate and can be found in the spleen of the partner mouse. We demonstrate that B cell specific T-bet expression is required for the development of HA-specific IgG2c titers and stalk specific antibody and durable neutralizing antibody titers. Together, these findings show that T-bet expression is an important attribute of the B cell response to influenza infection and can be
used to delineate memory B cell populations that differ in terms of residency, recirculation, and functional properties.

2.3 Results

2.3.1 Emergence and Maintenance of HA-Specific T-bet\(^+\) B Cells in the Spleen

While previous studies established the presence of T-bet\(^+\) HA-specific B cells in the spleen 100 days post PR8 influenza infection\(^{47}\), we wanted to undertake a detailed study of the kinetics of the emergence and maintenance of these cells. We utilized Tg(Tbx21-ZsGreen)E3ZJfz mice (hereafter referred to as T-bet ZsGreen) that are transgenic for a bacterial artificial chromosome (BAC) that reports T-bet expression using the fluorescent protein ZsGreen\(^{86}\). We infected T-bet ZsGreen reporter mice with the 30 TCID50 of influenza A/Puerto Rico/8/1934 (PR8) and verified successful infection via weight loss. As expected, mice initially lost weight following infection reaching the nadir at day 9 and then recovered over the following weeks (Figure 1A). We first wanted to determine the number and T-bet expression status of HA-specific B cells at various timepoints following infection. We used a double labelling technique in which biotinylated PR8 hemagglutinin was coupled to either Brilliant Violet 421 (BV421) or Alexa Flour 647 (AF647) fluorescently labelled streptavidin molecules in order to create a HA-biotin streptavidin “tetramer”. HA-specific B cells were identified as a double positive population binding to both of the HA-biotin streptavidin “tetramers”. This double labelling strategy greatly improved the specificity of detection for HA-specific B cells.
As expected, prior to infection HA-specific B cells are present at very low precursor frequencies. This is in line with prior estimates of \( \sim 1/50,000 \) splenic B cells (Figure 1B)\(^8^7\). For future analysis, we excluded this naïve primary B cell pool by focusing on IgD\(^-\) B cells (Figure 1C). Following infection, the HA-specific B cell population undergoes rapid expansion, reaching peak numbers at 15 days post infection. The HA-specific splenic B cell numbers then contract, but remain present at higher frequencies than observed prior to infection for at least 100 days (Figure 1E).

We next assessed the T-bet expression status of these HA-specific B cells. Prior to infection, HA-specific B cells were uniformly negative for the transcription factor T-bet (Figure 1B). By day 7 post-infection, about 20% of HA-specific B cells are positive for T-bet, and a portion of the highest T-bet expressing cells express the integrin CD11c. We stratified these T-bet\(^+\) B cells into T-bet\(^{\text{high}}\) and T-bet\(^{\text{low}}\) populations. Using qPCR for Tbx21 expression, we showed that both T-bet\(^{\text{high}}\) and T-bet\(^{\text{low}}\) populations express increased T-bet levels as compared to T-bet\(^-\) or CD19\(^{\text{Cre/WT}}\) T-bet\(^{\text{fl/fl}}\) B cells (Figure 1D). This indicates that both T-bet\(^{\text{high}}\) and T-bet\(^{\text{low}}\) B cells represent bona fide T-bet expressing B cell populations. Furthermore, we compared the T-bet ZsGreen expression levels observed in these populations to that seen in canonical T-bet\(^+\) CD21\(^-\) CD23\(^-\) ABCs (Figure 1C). At all timepoints analyzed following PR8 influenza infection, a similar fraction of HA-specific B cells were in the T-bet\(^{\text{high}}\),
T-bet$^{\text{low}}$, and T-bet$^+$ populations, indicating that antigen specific T-bet$^+$ B cells persist in the spleen following PR8 influenza infection (Figure 1E).
Figure 1: Emergence and Maintenance of HA-Specific T-bet⁺ B Cells in the Spleen
T-bet-ZsGreen reporters were intranasally infected with 30 TCID\textsubscript{50} influenza A/Puerto Rico/8/1934 (PR8) (A) Weight loss and recovery from PR8 influenza infection in T-bet-ZsGreen mice compared to PBS-treated controls. (B) Fluorescently-conjugated PR8 hemagglutinin (HA) detects the precursor frequency of HA-specific (HA\textsuperscript{+}) B cells in naïve T-bet-ZsGreen mice, which are uniformly T-bet\textsuperscript{+}. The naïve precursor frequency per 100,000 B cells is plotted. (C) Gating scheme for the identification of HA-specific B cells in T-bet-ZsGreen mice and subsetting into T-bet\textsuperscript{+}, T-bet\textsuperscript{low}, and T-bet\textsuperscript{high} populations via flow cytometry. C57Bl/6 mice are included in the T-bet-ZsGreen expression plot as a control. (D) Tbx21 expression in sorted T-bet\textsuperscript{+}, T-bet\textsuperscript{low}, and T-bet\textsuperscript{high} B cell subsets via qPCR. CD19\textsuperscript{+} B cells were sorted into the corresponding subsets according to ZsGreen expression (C), and RNA was isolated and cDNA prepared for qRT-PCR analysis. (E) Fluorescently-conjugated PR8 hemagglutinin (HA) detects HA-specific B cells, and T-bet-ZsGreen expression in HA-specific B cells resolves T-bet\textsuperscript{+}, T-bet\textsuperscript{low}, and T-bet\textsuperscript{high} subsets in the spleen at acute (day 15) and memory (day 100) timepoints. Number of HA-specific B cells in the spleen at various time points after infection (top), and proportions of HA-specific B cells that are T-bet\textsuperscript{+}, T-bet\textsuperscript{low}, and T-bet\textsuperscript{high} (bottom). Data in (B), (D), and (E) are plotted as mean ± SEM. **p<0.01 Data in (E) are representative of or compiled from 2 independent experiments with at least 3 mice per experiment. HA-specific B cells were identified as live, singlet, DUMP\textsuperscript{+}, B220\textsuperscript{+}, CD19\textsuperscript{+}, IgD\textsuperscript{−} cells, HA-BV421\textsuperscript{+}, HA-AF647\textsuperscript{+} cells. DUMP gate includes CD4, CD8, Gr-1, and F4/80.
2.3.2 Germinal Center and Memory Marker Expression in Splenic HA-Specific B Cells

We wanted to further define HA-specific T-bet\textsuperscript{+} B cells to determine if they differed from T-bet\textsuperscript{-} HA-specific B cells in terms of GC or memory marker expression. We performed surface staining for GL7, CD95 (FAS), CD38, CD80, PD-L2, and CD73 at various timepoints following infection. GC B cells are positive for peanut agglutinin (PNA) binding and typically express increased levels of GL7 and CD95, and decreased levels of CD38 when compared to naïve follicular B cells\textsuperscript{88, 89, 90}. For our analyses, we classified GL7\textsuperscript{+} CD38\textsuperscript{-} B cells as GC B cells and GL7\textsuperscript{-} CD38\textsuperscript{+} B cells as memory B cells and analyzed both the absolute numbers and relative frequencies of these populations within HA-specific B cells (Figure 2A). To further demonstrate the validity of this gating strategy, we demonstrated that there was good correspondence between GL7 and other GC markers such as CD95 at all measured timepoints (Figure 2B). By day 7 post PR8 infection, a fraction of HA-specific B cells are beginning to express a GC phenotype as evidenced by increased GL7 expression; however a large portion of HA-specific B cells have not yet assumed this phenotype (Figure 2A). The T-bet\textsuperscript{+} HA-specific B cell fraction likely enters the GC response earlier than the T-bet\textsuperscript{-} subset, as a higher portion of T-bet\textsuperscript{+} B cells have upregulated GL7 by day 7 post infection (Figure 2A). Regardless, a large fraction of these cells have not yet downregulated CD38, consistent with a pre-GC phenotype\textsuperscript{89, 91} (Figure 2A). By day 15, the vast majority of T-bet\textsuperscript{+} and T-bet\textsuperscript{-} B cells have assumed a GC phenotype, as evidenced by increased GL7 decreased CD38
levels (Figure 2A). This same phenotype is observed at day 22, but by day 40 a large portion of HA-specific B cells are exiting the GC as they have begun downregulating GL7 while CD38 is beginning to increase (Figure 2A). The T-bet\(^+\) HA-specific B cells likely exit the GC earlier than the T-bet\(^-\) B cells as a higher portion of the T-bet\(^+\) B cells no longer express the GC phenotype at day 40 (Figure 2A). By day 100, virtually all T-bet\(^+\) and T-bet\(^-\) HA-specific B cells fail to express increased levels of GL7, indicating that the GC response has subsided (Figure 2A). At later timepoints, a large portion of T-bet\(^+\) and T-bet\(^-\) HA-specific B cells have upregulated CD73, and this is most pronounced at day 100 (Figure 2C). Increased CD73 expression is consistent with a GC origin for most T-bet\(^+\) and T-bet\(^-\) HA-specific memory B cells\(^{91,92}\).

In a broad sense, memory B cells can be defined as B cells that have responded to antigen, returned to a resting state, and remain present in expanded numbers indefinitely post exposure. These cells should be able to participate in the context of a re-challenge; contributing to a rapid memory response\(^{93,94}\). Beyond this, memory B cells express a defined pattern of surface markers as described by Shlomchick and others\(^{36}\). By day 40, most T-bet\(^+\) B cells are post GC and express memory markers whereas a large portion of T-bet\(^-\) B cells still show a GC phenotype and have not yet upregulated memory markers. The memory marker CD80 first rises during acute infection and undergoes a further increase starting at day 40 post infection (Figure 2C). CD73 and PD-L2 stay at low levels on HA-specific B cells until days 15-22 when they start to increase concomitant with commitment to a memory B cell fate (Figure 2C).
CD80, CD73, and PD-L2 can be seen at their highest levels in both T-bet\textsuperscript{+} and T-bet\textsuperscript{−} HA-specific B cells at day 100 post infection, consistent with establishment of a stable memory B cell pool (Figure 2C). Overall, T-bet\textsuperscript{+} and T-bet\textsuperscript{−} HA-specific B cells have a shared GC and memory marker expression pattern, although T-bet\textsuperscript{+} B cells may have somewhat accelerated kinetics.
Figure 2: Germinal Center and Memory Marker Expression of Splenic HA-Specific B Cells

T-bet-ZsGreen reporters were intranasally infected with 30 TCID\textsubscript{50} influenza A/Puerto Rico/8/1934 (PR8) (A) Gating scheme identifies splenic HA-specific
(HA\textsuperscript{+}) germinal center (GC) B cell (GL7\textsuperscript{+}CD38\textsuperscript{-}), memory B cell (MBC) (GL7\textsuperscript{-} CD38\textsuperscript{+}), and pre-GC B cell (CD38\textsuperscript{+}GL7\textsuperscript{+}) subsets; concatenated flow plots (bottom) depict CD38 and GL7 expression of T-bet\textsuperscript{+} (pooled T-bet\textsuperscript{low} and T-bet\textsuperscript{high}; green) and T-bet\textsuperscript{-} (purple) HA-specific B cells at each time point (bottom). Line plots (top) depict number of HA-specific GC B cells and MBCs separated by T-bet expression phenotype over time (B) Expression of GL7 and CD95 on T-bet\textsuperscript{-} and T-bet\textsuperscript{+} splenic HA-specific B cells at the indicated time points post PR8 infection. (C) Expression of memory markers (CD80, PD-L2, CD73) in T-bet\textsuperscript{+} (green) and T-bet\textsuperscript{-} (purple) splenic HA-specific MBCs (GL7\textsuperscript{-}CD38\textsuperscript{+}) and naive follicular B cells (IgD\textsuperscript{+}; grey). Data in (A), (B), and (C) are representative of 2 independent experiments with at least 3 mice per experiment. Data in (A) are plotted as mean ± SEM. HA-specific B cells were identified as live, singlet, DUMP\textsuperscript{-}, B220\textsuperscript{+}, CD19\textsuperscript{+}, IgD\textsuperscript{-} cells, HA-BV421\textsuperscript{+}, HA-AF647\textsuperscript{+} cells. DUMP gate includes CD4, CD8, Gr-1, and F4/80.
2.3.3 Tissue Localization of HA-Specific B Cells

In addition to investigating the dynamics of splenic T-bet\textsuperscript{+} and T-bet\textsuperscript{-} HA-specific B cells following influenza infection, we wanted to track the appearance of these cells in other tissues. As expected, following influenza infection we observed a rapid expansion of HA-specific B cells in the mediastinal LNs, lungs, and blood (Figure 3A). HA-specific B cells were especially prevalent in the mediastinal LNs, often reaching frequencies as high as 5% of total IgD- B cells (Figure 3A). As seen in the spleen (Figure 1D), after resolution of influenza infection, these HA-specific B cells underwent contraction, but remained present long term at numbers and frequencies higher than those seen prior to infection (Figure 3A). The kinetics showed some variance among tissues, with HA-specific B cell numbers in the mediastinal LNs and blood peaking at day 22, whereas in the spleen and lungs these numbers reached their highest point at day 15 post infection (Figures 1D; 3A). Strikingly, while T-bet\textsuperscript{-} and T-bet\textsuperscript{+} B cells remained relatively constant in their proportions throughout the immune response in the spleen, comprising about a quarter of HA-specific B cells (Figure 1D), a different pattern was observed in the mediastinal LNs and lungs. While early on in the response T-bet\textsuperscript{+} B cells were evident, their contribution waned with time, a trend particularly obvious with the T-bet\textsuperscript{high} HA-specific B cells (Figure 3A). Despite very low HA-specific B cell numbers in the lungs at day 7 post infection, nearly all these cells expressed high levels of T-bet (Figure 3A). By day 15, the number of HA-specific B cells in the lungs had greatly increased and roughly half of these cells were T-bet\textsuperscript{high}. By day 40 post infection, HA-specific B cells in the lungs had
undergone extensive contraction and very few of these cells expressed high levels of T-bet (Figure 3A). In the mediastinal LNs, at day 7 post infection roughly 75% of HA-specific B cells expressed T-bet and slightly more than a third of these could be classed as T-bet\textsuperscript{high} (Figure 3A). By day 15, none of the HA-specific B cells were T-bet\textsuperscript{high} and the T-bet\textsuperscript{low} population had also significantly waned (Figure 3A). A similar pattern was observed in the blood, albeit with somewhat delayed kinetics (Figure 3A). While HA-specific B cells are observed long term at increased frequencies in the spleens, mediastinal LNs, lungs, and blood of infected mice, the T-bet\textsuperscript{high} population persists long term in the spleen and not in other tissues. Low numbers of HA-specific B cells were observed in pooled peripheral (superficial cervical, axillary, brachial, and inguinal) and mesenteric LNs, but this contribution to the response was much smaller than that observed in the spleen and mediastinal LNs with the T-bet\textsuperscript{high} fraction nearly absent by day 100 (Figure 3B).
Figure 3: Tissue Restriction of T-bet⁺ HA-Specific B Cells

T-bet-ZsGreen reporters were intranasally infected with 30 TCID₅₀ influenza A/Puerto Rico/8/1934 (PR8) (A) Fluorescently-conjugated PR8 hemagglutinin (HA) detects HA-specific (HA⁺) B cells, and T-bet-ZsGreen expression in HA-specific B cells resolves T-bet⁺, T-bet⁻low, and T-bet⁻high subsets in the mediastinal lymph nodes (medLN) and lungs at acute (day 15) and memory (day 100) timepoints. Number of HA-specific B cells in the mediastinal lymph nodes, lungs,
and blood at different time points after infection (left), and proportions of HA-specific B cells that are T-bet\(^{-}\), T-bet\(^{\text{low}}\), and T-bet\(^{\text{high}}\) in each tissue (right). The number of HA-specific B cells in blood was estimated by calculating their frequency per 100,000 B cells, and proportions of T-bet-defined subsets in blood were calculated after concatenation due to low cell number. (B) Number of HA-specific B cells in peripheral and mesenteric lymph nodes (LN) by T-bet expression phenotype at different time points after infection. Data in (A) and (B) are representative of or compiled from 2 independent experiments with at least 3 mice per experiment and are plotted as mean ± SEM. HA-specific B cells were identified as live, singlet, DUMP\(^{-}\), B220\(^{+}\), CD19\(^{+}\), IgD\(^{-}\) cells, HA-BV421\(^{+}\), HA-AF647\(^{+}\) cells. DUMP gate includes CD4, CD8, Gr-1, and F4/80.
2.3.4 Tissue Residence of HA-Specific B Cells is Stratified by T-bet Expression

The splenic restriction of T-bet⁺ HA-specific B cells at memory timepoints raised the question of whether this population might be splenic resident. In order to investigate this question, we undertook a parabiosis based approach. A T-bet ZsGreen reporter mouse infected 40+ days prior with PR8 influenza was surgically joined to an uninfected congenic partner. These mice established a shared circulatory system after which they were euthanized and the presence of T-bet⁺ and T-bet⁻ HA-specific B cells was analyzed in each partner. As virtually all HA-specific memory B cells arise in previously infected T-bet ZsGreen partner, we reasoned that the presence of these cells in the B6.SJL partner suggests that they are a recirculating population. As a confirmation of successful anastomosis and establishment of a shared circulatory system, sequential tail bleeds were performed on both partner mice. By day 7 post-surgery, we observed mixing of CD45.1 and CD45.2 B cells in the blood of both partner mice, with these populations achieving stable proportions between partners by day 14 (Figure 4A). This is in accordance with previous studies calculating complete blood exchange occurring between parabiotic partners within 12 days of surgery. As such, parabiosed pairs were euthanized at ≥ 17 days post-surgery. Similar frequencies of CD45.2⁺ IgD⁺ B cells, which we expect to recirculate freely between partners, were observed in the spleen, lungs, and mediastinal LNs of each partner, suggesting equilibration of recirculating B cells within 17 days of surgery (Figure 4B).
HA-specific IgD⁺ memory B cells were identified in the spleen of both partners, and nearly all of these cells were CD45.2⁺, consistent with their origin in the previously infected T-bet ZsGreen partner (Figure 4C). Within the HA-specific IgD⁺ memory B cell population, T-bet⁺ and T-betlow B cells were identified in the spleens of both partners. While the number of cells in both of these populations was significantly lower in the uninfected B6.SJL partner (Figure 4D), this difference was no longer apparent after normalizing for differing numbers of CD45.2⁺ B cells in the spleens of the two partner mice (Figure 4E). This indicates that T-bet⁺ and T-betlow HA-specific B cells are likely a freely recirculating population. Importantly, T-bethigh HA-specific B cells were virtually absent from the spleen of the uninfected B6.SJL partner, despite being present in the spleen of the previously infected T-bet ZsGreen partner (Figure 4D; 4E). A similar trend showing a selective failure of HA-specific T-bethigh B cells to recirculate to the partner spleen can be appreciated when looking at the portion of HA-specific B cells that belong to the T-bethigh, T-bet⁺, or T-betlow population (Figure 4F). This near absence of T-bethigh HA-specific B cells in the B6. SJL partner spleen was apparent even when data was concatenated from 7 parabiotic pairs (Figure 4G).

To confirm that ZsGreen-expressing cells were not being rejected in the B6.SJL mice, we measured frequencies of CXCR3⁺CD8⁺ lymphocytes, which highly express T-bet-ZsGreen, and found similar frequencies of these cells in both partners (Figure 4H). T-betlow HA-specific B cells were present in the spleens of both mice (Figure 4D; 4E; 4F), together suggesting that broad rejection of ZsGreen⁺ cells is not occurring. These observations confirm previous
studies that failed to detect an immunogenic response to ZsGreen protein in C57Bl/6 mice\textsuperscript{96}. Taken together, these findings identify splenic T-bet\textsuperscript{high} HA-specific B cells as a tissue-resident memory pool.

We next investigated whether HA-specific memory B cells show evidence of residency in the mediastinal LNs and lungs, the other primary locations of influenza memory B cells, as others recently demonstrated\textsuperscript{97}. We identified significant IgD\textsuperscript{-} HA-specific memory B cell populations in the mediastinal LNs and lungs of the previously infected partner, but these cells were absent in the uninfected partner, suggesting they are tissue resident populations (Figure 4I; 4J). Interestingly, nearly all mediastinal LN and lung-localized HA-specific B cells were negative for T-bet (Figure 4K; 4L). However, this residency phenotype may reflect persistent GCs as GL7\textsuperscript{+} CD38\textsuperscript{-} HA-specific B cells are observed in the mediastinal LNs as much as 100 days post infection (Figure 4M). Together, these results demonstrate that tissue-resident HA-specific B cell memory is anatomically compartmentalized, comprised of T-bet\textsuperscript{high} B cells in the spleen and T-bet\textsuperscript{-} B cell populations in mediastinal LNs and lungs.
Figure 4: T-bet expression resolves spleen resident versus recirculating memory B cell pools

(A) T-bet-ZsGreen reporters (CD45.2⁺; ≥ 40 days post infection) and uninfected B6.SJL (CD45.1⁺) were surgically conjoined and showed evidence of blood sharing by day 7, with equilibrium reached by day 14. Parabionts were
euthanized at ≥ 17 days post-surgery for analysis. **(B)** Frequencies of naïve follicular (IgD⁺) B cells expressing CD45.2 in lymphoid and non-lymphoid tissues from each parabiosis pair. **(C)** Identification of HA-specific IgD⁻ B cells expressing either CD45.1 or CD45.2 in parabiosis partners. **(D)** Numbers of T-bet⁺, T-betₜₜₜ, and T-betₚₚₚ HA-specific (HA⁺) splenic memory B cells (MBCs) in T-bet-ZsGreen (red) and B6.SJL (black) partners. **(E)** Numbers of T-bet⁻, T-betₜₜₜ, and T-betₚₚₚ HA-specific splenic memory B cells per 1₀⁶ CD45.2⁺ B cells in T-bet-ZsGreen (red) and B6.SJL (black) partners. **(F)** Percentage of splenic HA-specific memory B cells that are T-bet⁻, T-betₜₜₜ, or T-betₚₚₚ in parabiosis partners. **(G)** Identification of T-bet-ZsGreen reporter-derived (CD45.2⁺) T-bet⁻, T-betₜₜₜ, and T-betₚₚₚ HA-specific memory B cells in spleens of T-bet-ZsGreen and B6.SJL partners; data concatenated from 7 pairs. **(H)** The percent of splenic CD45.2⁺ CD8⁺ T cells that are T-bet-ZsGreen⁺ CXCR3⁺ in each partner after ≥17 days of parabiosis from 7 parabiotic pairs. **(I and J)** Number of HA-specific memory B cells in mediastinal lymph nodes (medLN) (I) and lungs (J) of parabiosis partners. **(K and L)** T-bet-ZsGreen expression in HA-specific memory B cells from mediastinal lymph nodes (K) and lungs (L) of T-bet-ZsGreen partner. HA-specific memory B cells were not detected in the mediastinal lymph nodes or lung of the B6.SJL partner. Data displayed are from 8 pairs across three independent experiments for spleen and 4 pairs across two-independent experiments for mediastinal lymph nodes and lungs. HA-specific memory B cells were identified as live, singlet, DUMP⁻, B220⁺, CD19⁺, CD45.2⁺, IgD⁻, HA-BV421⁺, HA-AF647⁺ cells. **(M)** The percentage of HA-specific memory B cells
that are GL7⁺CD38⁻ in the mediastinal lymph nodes and lungs of T-bet-ZsGreen mice 100 days post PR8 infection, and the percentage that are T-bet⁺ in each tissue (top). HA-specific memory B cells cells were identified as live, singlet, DUMP⁻, B220⁺, CD19⁺, IgD⁻, HA-AF647⁺, HA-BV421⁺ cells. DUMP gate includes CD4, CD8, Gr-1, and F4/80. Data in (D), (E), (F), (I), (J), and (M) show individual points with the mean ± SEM indicated. Statistical comparisons performed using paired two-tailed t-test. ns = not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.001
2.3.5 Influence of B Cell Specific T-bet Expression on Antibody Isotype, Titers, and Specificity Following Influenza Infection

T-bet promotes class switching to the IgG2a/c isotype in mice and this isotype is important for multiple anti-viral and anti-bacterial responses, including influenza\textsuperscript{40, 55, 98, 99, 100, 101, 102, 103}. As such, we compared the betapropiolactone (BPL) inactivated PR8 (PR8) and PR8 hemagglutinin (HA) specific IgG2c and IgG1 titers at various timepoints post PR8 influenza infection. We observed that titers peaked around day 21 and PR8-specific IgG2c and HA-specific IgG2c titers showed a greater increase than IgG1, evident by days 12 and 15 post infection, respectively (Figure 5A; 5B). This is consistent with prior studies\textsuperscript{104} and implies that T-bet likely plays a key role in antibody production following influenza infection.

We wanted to explore the role of B cell specific T-bet expression on the PR8 influenza response. In order to investigate this, we infected B cell-specific T-bet conditional knockout mice obtained by crossing \textit{Cd19}\textsuperscript{tm1(cre)Cgn} mice with \textit{Tbx21}\textsuperscript{Flox/Flox} mice (referred to as cKOs), heterozygous \textit{Cd19}\textsuperscript{tm1(cre)Cgn} cre controls (CD19 Cre), and wild type C57Bl/6 mice with PR8 influenza and interrogated the response in the three groups. All three groups showed similar weight loss kinetics, although the CD19 Cre controls had less overall weight loss and recovered lost weight faster (Figure 5C). The three groups had similar numbers of total HA-specific B cells at both day 15 and day 40 post infection (Figure 5D) along with an intact HA-specific GC response at day 15 (Figure 5E). As such, CD19 heterozygosity does not significantly impair the response to influenza.
infection. Furthermore, an HA-specific B cell response can be initiated and maintained in the absence of T-bet expression in B cells.

We next explored the effect of B lineage T-bet deletion on antibody functionality as measured by hemagglutination inhibition (HAI) assay. At day 15 post infection, the majority of mice in all three groups had HAI titers of at least 40, a level associated with protection in human studies\textsuperscript{105, 106} although a subset of cKO and wildtype mice had titers of 20 and below (Figure 5F). By day 40 post infection, HAI titers had decreased in all three groups, likely reflecting a decline in acute infection related IgM titers\textsuperscript{107} (Figure 5F). This decrease in titers was particularly notable in the cKO group, as by day 40 these mice had significantly lower HAI titers as compared to the wildtype or CD19-Cre controls and the majority of cKOs at day 40 had sub-protective levels of titers (Figure 5F). This implies that B cell specific T-bet expression may be required for sustained protective titers following influenza infection.

We proceeded to determine if the lower HAI titers in cKOs may be related to loss of a specific component of the antibody response. As expected, cKOs had significantly lower total BPL-inactivated PR8 IgG2c titers at both day 15 and 40 post infection (Figure 5G). At day 15, cKOs had low IgG2c titers but these were nearly absent by day 40 post infection (Figure 5G), implying that T-bet-independent mechanisms may initiate limited IgG2c switching during acute infection but B cell specific expression of T-bet is required for sustained IgG2c PR8-specific titers. We measured PR8 HA-specific titers as HA is a key antigenic target for protective humoral immunity to influenza. At both day 15 and 40 post
infection, wildtype and CD19-Cre control mice had robust anti-PR8-HA IgG2c titers while these were largely absent in the cKO mice (Figure 5H), implying that B cell intrinsic T-bet is important for both an acute and sustained IgG2c response to PR8 HA. Furthermore, we measured IgG1 titers to both BPL-inactivated PR8 and PR8 HA as this is a T-bet independent isotype. These titers were similar in all three groups at both days 15 and 40 post infection (Figure 5G; 5H) and did not undergo a compensatory increase in response to the lower IgG2c titers in cKO mice. While the majority of the influenza specific IgG2c response requires B cell specific T-bet expression; the IgG2c independent components of this response are preserved in the absence of T-bet expression in B cells.

Finally, we explored whether B cell specific T-bet expression is required for the influenza antibody response to defined specificities. Specifically, we explored the HA stalk specific antibody response as recent studies have shown a key role of HA-specific IgG2a/c antibodies for in vivo influenza protection, which are largely skewed toward stalk recognition\textsuperscript{108, 109}. We measured IgG2c and IgG1 titers to a chimeric construct comprised of the PR8-related H1 stalk and an unrelated H6 head\textsuperscript{110, 111}. This allows us to selectively identify stalk specific antibodies as most PR8-generated HA head-binding antibodies are strain-specific and do not recognize the unrelated H6 head. In wildtype mice, this stalk specific antibody response is dominated by IgG2c at both early and late timepoints and IgG1 stalk titers are negligible at both timepoints (Figure 5I). Furthermore, these IgG2c stalk-reactive titers are largely lost in cKOs with no compensatory increase in stalk-reactive IgG1 titers (Figure 5I). This indicates that
the bulk of the HA stalk-specific antibody response requires T-bet-expressing B cells.
Figure 5: Antibody Isotype, Titers, and Specificity Associated with B Cell Specific T-bet Expression

(A and B) Total betapropriolactone (BPL)-inactivated PR8-specific IgG1 and IgG2c (A) and PR8 hemagglutinin (HA)-specific IgG1 and IgG2c (B) in sera from infected T-bet-ZsGreen mice over time. (C). Weight loss and recovery from influenza infection in wild type C57Bl/6, CD19<sup>cre/WT</sup>T-bet<sup>fl/fl</sup>, and CD19<sup>cre/WT</sup> mice compared to PBS-treated controls. (D) Number of HA-specific splenic B cells at day 15 and 40 post infection. (E) Number of HA-specific splenic germinal center B (GCB) cells at 15 days post infection. (F) Hemagglutination inhibition (HAI) titers at 15 and 40 days post infection. (G-I) Antibody titers to BPL-inactivated
PR8 (G), full-length PR8-HA (H), or chimeric construct comprised of H1 stalk and H6 head (I). Wild type C57Bl/6 were used for naïve controls in (F-I). Data are represented as mean ± SEM from 3 independent experiments with at least 3-5 mice in each group. Statistical comparisons performed using two-sided t-test (G-I) and Wilcoxon rank-sum test (F). *p<0.05, **p<0.01, ***p<0.001. Cells in (D, E) were identified as DUMP⁻, CD19⁺, B220⁺, CD138⁻, IgD⁻, HA-PE⁺, with the additional definition of germinal center B cells in (E) as PNA⁺CD95⁺. DUMP gate includes CD4, CD8, Gr-1, and F4/80.
2.4 Discussion

There are multiple outstanding questions regarding the role, establishment, and characteristics of T-bet$^+$ B cells in immune responses. In order to address this, we undertook a detailed study of T-bet expressing B cells following PR8 influenza infection. We found that T-bet expression can be used to separate PR8 HA-specific B cells into three subsets. These populations differ in multiple ways, including residency, effector function, and epitope specificity. At early timepoints, both T-bet$^+$ and T-bet$^-$ HA-specific B cells display a GC phenotype and at later timepoints they assume a similar memory B cell fate. At acute timepoints post-infection, HA-specific T-bet$^-$, T-bet$^{\text{low}}$, and T-bet$^{\text{high}}$ B cells can be observed in the responding secondary lymphoid tissues and the lungs. While all three populations persist at similar relative frequencies in the spleen for at least 100 days post infection, T-bet$^{\text{high}}$ B cells largely disappear from the lymphatics and lungs. Using parabiosis, we have demonstrated that HA-specific T-bet$^-$ and T-bet$^{\text{low}}$ B cells recirculate between the spleens of the two partner mice, but T-bet$^{\text{high}}$ B cells are splenic residents that remain restricted to the spleen of the previously infected partner. Furthermore, B cell specific T-bet expression is required for most HA-specific IgG2c and HA stalk specific titers, as mice lacking T-bet in B cells display a loss of these titers along with a failure to maintain protective neutralizing HAI titers at memory timepoints. Together, this shows that T-bet expression in B cells is an important determinant of populations that differ in terms of recirculation, anatomic niche, isotype and specificity.
We observed that HA-specific B cells appear in the spleen early following PR8 influenza infection and undergo expansion followed by contraction and maintenance regardless of T-bet expression. A fraction of these cells begin displaying a GC phenotype as early as day 7 post infection as evidenced by increased expression of GL7 and CD95 along with decreased CD38. This GC phenotype peaks at days 15 and 22, as a large majority of T-bet$^+$ and T-bet$^-$ HA-specific B cells express markers consistent with GC fate. Interestingly, T-bet expression may be related to the timing of entry and exit from the GC as HA-specific T-bet$^+$ B cells acquire and then lose a GC phenotype with faster kinetics than their T-bet$^-$ counterparts. The significance of possible accelerated GC kinetics in T-bet$^+$ B cells remains unclear.

At later timepoints, a large portion of T-bet$^+$ and T-bet$^-$ HA-specific B cells express increased CD73. Higher levels of CD73 are suggestive of a GC origin for most T-bet$^+$ and T-bet$^-$ HA-specific memory B cells$^{91, 92}$. However, BrdU pulse labelling studies in an NP-CGG response have shown that a portion of memory B cells arising prior to day 5 and before the establishment of a robust GC response can also express elevated CD73. As such, expression of CD73 does not necessarily indicate a GC origin$^{112}$. Nevertheless, the CD73$^+$ B cell population is enriched for somatically mutated B cells as about 80% of CD73$^+$ memory B cells are somatically mutated as compared to only 30% of the CD73$^-$ population$^{92}$. A GC origin of T-bet$^+$ memory B cells is consistent with prior findings showing that T-bet$^+$ B cell formation requires CD40/CD40L interactions and is associated with a high incidence of somatic mutation$^{47}$. 43
Outstanding questions regarding the role of HA-specific B cells in recall responses remain. Zuccarino-Catania et al. have demonstrated that memory B cells can be stratified into populations with different recall functions based on surface marker expression. In recall responses CD80⁺ PDL2⁺ memory B cells are biased towards rapid plasma cell differentiation and limited GC recruitment whereas CD80⁻ PDL2⁻ memory B cells participate in a robust GC response and fail to generate many early plasma cells. A series of elegant experiments by Mesin et al. utilized a variety of approaches including clonal diversity and somatic hypermutation analysis, fate-mapping, and parabiosis to demonstrate that there is limited re-entry of germinal-center derived memory B cells into newly generated GCs following a CGG-Alum boost. Rather, memory B cells that participated in a GC following primary immunization are biased towards plasmablast differentiation in the recall response. Taken together, the fact that the majority of HA-specific memory B cells in our studies expressed elevated levels of CD80 and PDL2 along with a GC history, it seems likely that these HA-specific memory B cells would differentiate into antibody-secreting cells and fail to re-enter the GC during secondary exposure to influenza. Additional studies are required to confirm this hypothesis and to determine whether T-bet⁺ and T-bet⁻ HA-specific B cells play differing roles in recall responses to influenza.

Our studies demonstrated unique anatomic compartmentalization corresponding to T-bet expression, with both T-bet⁺ and T-bet⁻ HA-specific B cells present in the spleen, mediastinal LNs, lungs, and blood at early timepoints post-influenza infection. While T-bet⁻ B cells persisted in all these tissue
compartments for at least 100 days post infection, T-bet\(^+\) B cells displayed increasing splenic restriction with time. While the precise function and mechanism of this tissue compartmentalization remains unclear, it may be tied to differences in recirculation properties of T-bet\(^{\text{high}}\), T-bet\(^{\text{low}}\), and T-bet\(^-\) HA-specific B cells. In parabiotic studies, T-bet\(^-\) and T-bet\(^{\text{low}}\) B cells are observed to recirculate between the spleens of the two partner mice whereas T-bet\(^{\text{high}}\) B cells remain restricted to the spleen of the previously infected partner. We posit that the transient appearance of T-bet\(^{\text{high}}\) HA-specific B cells in the mediastinal LNs and lungs early after infection may reflect extra-splenic generation of these cells. Prior work from our lab has shown that intracellular nucleic acid sensing Toll-like receptor stimulation together with Th1 type cytokines such as IFN\(_{\gamma}\) can induce T-bet expression in B cells\(^{60}\). An inflammatory environment in the draining LNs or lungs shortly after infection may be sufficient to transiently drive T-bet expression in B cells at these sites. Consistent with this hypothesis, Barnett et al. observed a strong enrichment of T-bet\(^+\) memory B cells in the small intestinal mucosa following LCMV infection; a phenotype that was especially pronounced in the intraepithelial lymphocytes with more than 65% of these B cells expressing T-bet\(^{51}\). The small intestinal mucosa, and in particular subepithelial CD11c\(^+\) dendritic cells, which reside in close proximity to intraepithelial lymphocytes, represents a major site of Clone 13 LCMV viral infection\(^{113, 114}\). Chronic viremia, immune activation, and inflammation may be sufficient to drive persistent T-bet expression at extra-splenic sites. Pathogens such as HIV and Toxoplasma gondii
which lead to persistent lymphoid tissue infections are associated with T-bet$^{\text{high}}$ B cells residing in the infected LNs$^{115,116}$.

Integrin and chemokine receptor expression in T-bet$^+$ B cells may be an additional important factor in the localization and recirculation properties of this population. We have observed that a substantial portion of T-bet$^{\text{high}}$ B cells express the integrin CD11c. Work from the Wherry lab comparing gene expression between T-bet$^+$ and T-bet$^-$ B cells showed that CXCR3, a gene involved in cell migration, was upregulated in T-bet$^+$ B cells. They also confirmed this upregulation via flow cytometry$^{51}$. In human peripheral blood, T-bet$^+$ B cells express the integrin CD11c and the chemokine receptor CXCR3 along with low levels of CXCR4, CXCR5, and CCR7 which are chemokine receptors associated with homing to lymphoid organs$^{49,56,71}$. Together, these surface receptors may act to block lymphatic entry of T-bet$^{\text{high}}$ B cells and help establish their splenic residency. Intriguingly, we observed HA-specific T-bet$^{\text{high}}$ B cells in the blood and their peak incidence corresponded to the loss of T-bet$^{\text{high}}$ HA-specific B cells from the lungs and mediastinal LNs, consistent with their entry into the blood after activation or recent tissue exit. In accordance with this, an increase in T-bet$^{\text{high}}$ B cells in human blood can be observed following influenza vaccination$^{117}$.

In addition to their differences in localization and residency, T-bet$^+$ and T-bet$^-$ B cells differ in terms of function. B cell specific T-bet knockout mice fail to generate HA-specific IgG2c titers and show deficiencies in HA stalk specific antibody production and a failure to maintain protective HAI titers. We posit that T-bet$^+$ and T-bet$^-$ B cells may have distinct roles following challenge with
heterologous strains of influenza. More specifically, T-bet\(^+\) B cells may be important in sustaining sterilizing immunity against reinfection and in generating broadly neutralizing HA stalk specific antibodies that could provide lasting protection against a wide variety of strains. At both baseline and 28 days post-vaccination, higher absolute IgG stalk specific influenza titers in humans are associated with increasing age\(^\text{118}\). As T-bet\(^+\) ABCs accumulate with age, this increase in stalk specific antibodies could be dependent on T-bet\(^+\) B cells along with repeated exposure to divergent influenza strains. Additionally, T-bet\(^+\) B cells can be observed at increased frequencies following influenza vaccination in humans\(^\text{49, 117, 119}\). A major goal of current influenza research is the development of an HA stalk reactive vaccine which could provide protection against a broad range of divergent influenza strains and mitigate the need for annual revaccination and redesign. Given the likely importance of T-bet\(^+\) B cells in generating stalk specific antibodies and sustaining protective titers, developing vaccination strategies that promote the generation and maintenance of HA-specific T-bet\(^+\) B cells is potentially a promising approach in the quest for a universal influenza vaccine.
CHAPTER 3: T-bet⁺ Memory B Cells are a Stable Population That Contributes to Plasma Cell Pools

3.1 Summary

In this chapter, we focus on the stability and fate of T-bet⁺ B cells. We show that T-bet expression in memory B cells is a durable state with T-bet⁺ and T-bet⁻ memory B cells comprising stable, independent populations. We observed a trend of increasing CD138 expression with decreasing T-bet expression, suggesting that circulating Tbet<sub>low</sub> B cells may be losing T-bet on their way to becoming plasma cells. In fact, a significant fraction of the plasma cell pool in both the spleen and bone marrow is derived from T-bet expressing B cells, and plasma cells can come from either T-bet<sub>high</sub> or T-bet<sub>low</sub> precursors. Utilizing both fate mapper adoptive transfer and parabiosis followed by separation surgery techniques, we demonstrate that T-bet<sub>high</sub> B cells give rise to T-bet<sub>low</sub> B cells but not vice versa. Nonetheless, T-bet<sub>low</sub> B cells persist for a minimum of three weeks in the absence of T-bet<sub>high</sub> B cells.
3.2 Introduction

The findings in the previous chapter resolve three memory B cell subsets with differing anatomic distributions and specificities, but the relationships between them, if any, is unclear. Moreover, because B cell-specific T-bet expression is required for the bulk of HA-specific antibodies and sustained HAI titers, we reasoned that T-bet$^+$ subsets likely contribute to plasma cell pools. Accordingly, we wanted to address whether there is frequent conversion between T-bet$^+$ and T-bet$^-$ B cells, and to explore the relationship between spleen-resident T-bet$^{\text{high}}$ B cells, recirculating T-bet$^{\text{low}}$ B cells, and plasma cells. We were particularly interested in the ability of T-bet$^+$ B cells to give rise to plasma cells and to determine whether T-bet$^{\text{low}}$ B cells are a transition stage between T-bet$^+$ memory B cells and T-bet$^-$ plasma cells.

Our prior results demonstrate that T-bet$^{\text{high}}$ memory B cells are maintained long term at stable numbers in the spleen. In conjunction with the tissue residency of T-bet$^{\text{high}}$ memory B cells, this suggests T-bet$^-$ and T-bet$^{\text{low}}$ B cells do not continuously give rise to T-bet$^{\text{high}}$ B cells, as this would preclude T-bet$^{\text{high}}$ B cells from being a uniquely splenic resident population. If T-bet$^{\text{low}}$ and T-bet$^-$ precursors generated T-bet$^{\text{high}}$ B cells, then we would have expected to find T-bet$^{\text{high}}$ memory B cells in the spleens of both parabiotic partners. The maintenance of T-bet$^{\text{high}}$ populations at steady numbers suggests it is unlikely that T-bet$^{\text{high}}$ B cells simply lose T-bet expression as this would lead to decreasing levels of T-bet$^{\text{high}}$ B cells. However, it remains possible that T-bet$^{\text{high}}$
B cells continually cycle with daughter cells leaving the T-bet\textsuperscript{high} pool to become T-bet'.

In T cells, there is evidence that T-bet expression can be either a stable or dynamic state. In Th1 T cells, T-bet expression is required and acts to prevent the expression or function of transcription factors such as GATA-3 or RORγt which are associated with the differentiation of Th2 or Th17 T cells\textsuperscript{45, 82, 86, 120, 121, 122}. Studies in T-bet fate mapping mice have shown that T-bet expression in conventional CD4\textsuperscript{+} CD25\textsuperscript{−} T cells is stable, as the majority of these fate mapped cells maintain T-bet expression\textsuperscript{123}.

On the other hand, regulatory T cells can express either T-bet or GATA3\textsuperscript{124, 125, 126, 127}, but it remained unclear whether these were two separate subsets or if there was substantial interchange between these regulatory T cell populations. Using T-bet fate mapper mice, Yu et al. showed that T-bet expression in CD4\textsuperscript{+} CD25\textsuperscript{+} regulatory T cells is a dynamic state, as a large portion of these fate mapped cells rapidly lost T-bet expression. This was particularly pronounced in the LNs; over 60% of fate mapped CD4\textsuperscript{+} CD25\textsuperscript{+} regulatory T cells no longer expressed T-bet one week after tamoxifen treatment\textsuperscript{123}.

However, T-bet expression is not a simple on/off switch, as graded expression of T-bet has important implications on cell fate decisions. In CD8 T cells, the severity of inflammation and resultant IL-12 levels yield a gradient of T-bet expression, with higher IL-12 concentrations corresponding to increased T-
bet. The T-bet expression level influences cell fate decisions as low level T-bet expression promotes memory precursor effector cell development whereas high levels of T-bet drive the development of short lived effector cells\(^\text{128}\).

Dynamic control of transcription factors also drives cell fate decisions in B cells. For example, IRF4 contributes to both GC B cell development and plasma cell differentiation, despite these processes being regulated by mutually antagonistic gene expression programs\(^\text{129, 130}\). IRF4 is thought to act in a “kinetic control” fashion, as transient low level expression is required for GC B cell development, whereas sustained high level expression leads to plasma cell generation\(^\text{129}\).

Plasma cells and plasmablasts, collectively termed antibody secreting cells (ASCs), are terminally differentiated B cells that secrete antibody. The transition from B cell to antibody secreting cell involves extensive changes in gene expression, morphology, and cellular lifespan and entails the loss of B cell specific transcription factors and acquisition of plasma cell specific factors. These B cell and plasma cell specific transcription factors control gene expression networks that are mutually antagonistic resulting in a stable and irreversible B cell to plasma cell transition\(^\text{131, 132}\).

T-bet expressing B cells may be important plasma cell precursors. Following *Ehrlichia muris* infection, CD11c\(^+\) T-bet expressing IgM plasmablasts are observed and likely give rise to long lasting bone marrow IgM plasma cells\(^\text{66, 133, 134}\). Upon adoptive transfer followed by *Ehrlichia muris* reinfection, T-bet\(^+\) IgM memory B cells give rise to CD138\(^+\) ASCs\(^\text{53}\). Additionally, T-bet\(^+\) B cells are
required for the generation of long lived plasma cells and secondary ASCs following initial influenza infection or re-challenge with a heterologous strain\textsuperscript{54}.

In this chapter, we address the stability of T-bet\textsuperscript{+} B cells and examine their fate using genetic fate-mapping mice. We show that T-bet\textsuperscript{+} memory B cells are a stable population with limited interconversion into T-bet\textsuperscript{−} B cell pools. However, we observe an increase in plasma cell characteristics, specifically CD138 expression, that accompanies loss of T-bet expression, indicating that B cells downregulating T-bet may be on their way to becoming plasma cells. Indeed, both T-bet\textsuperscript{high} and T-bet\textsuperscript{low} B cells give rise to plasma cells and T-bet expressing B cells can generate both B220\textsuperscript{+} and B220\textsuperscript{−} plasma cells. Additionally, T-bet\textsuperscript{high} B cells give rise to T-bet\textsuperscript{low} B cells. However, T-bet\textsuperscript{low} B cells can be maintained independently from T-bet\textsuperscript{high} B cells for a minimum of three weeks.

3.3 Results

3.3.1 T-bet\textsuperscript{+} and Tbet\textsuperscript{−} memory B cells are stable, independent populations

We wanted to determine whether T-bet\textsuperscript{+} and T-bet\textsuperscript{−} memory B cells frequently interconvert or if they are maintained as separate B cell populations. In order to explore this question, we used a Tg (Tbx21-ZsGreen,-cre/ERT2)H3Jfz reporter/inducible cre transgenic mouse line\textsuperscript{123} bred to the Gt(Rosa)26Sor\textsuperscript{tm14(CAG-tdTomato)Hze conditional reporter allele background\textsuperscript{135}. This yielded a combined T-bet reporter/fate mapper mouse (hereafter referred to as fate mapper). This mouse is T-bet sufficient and expresses the ZsGreen construct fused to cre\textsuperscript{ERT2} under the control of the T-bet promoter on a bacterial
artificial chromosome. In these mice, treatment with tamoxifen during active T-bet transcription causes irreversible TD Tomato expression, and active T-bet expression is associated with ZsGreen florescence\textsuperscript{123}.

Together, these two fluorescent markers delineate four cell populations; [1] cells that are not currently expressing T-bet and did not express T-bet at the time of tamoxifen administration (ZsGreen\textsuperscript{-} TD Tomato\textsuperscript{-}), [2] cells that expressed T-bet at the time of tamoxifen administration and continue to express T-bet (ZsGreen\textsuperscript{+} TD Tomato\textsuperscript{-}), [3] cells that expressed T-bet at the time of tamoxifen administration but no longer express T-bet (ZsGreen\textsuperscript{-} TD Tomato\textsuperscript{+}), and [4] cells that currently express T-bet but did not express T-bet at time of tamoxifen administration (ZsGreen\textsuperscript{+} TD Tomato\textsuperscript{-}). By administering tamoxifen and performing longitudinal bleeds on the treated mice, we can track the persistence of T-bet expression with time by monitoring changes in the relative frequency of cells that retain T-bet expression (ZsGreen\textsuperscript{+} TD Tomato\textsuperscript{+}) and cells that expressed T-bet during the treatment period but have subsequently lost expression (ZsGreen\textsuperscript{-} TD Tomato\textsuperscript{+}). We reasoned that if substantial conversion from the T-bet\textsuperscript{+} to the T-bet\textsuperscript{-} B cell pool occurs, then there should be significant numbers of B cells that lose T-bet expression after being marked during tamoxifen treatment.

We treated ≥ 20-week-old fate mapper mice with tamoxifen on days 0, 2, and 4 and performed a series of longitudinal bleeds. All mice displayed TD Tomato labelled CD3\textsuperscript{-} CD19\textsuperscript{+} B cells by day 10 post tamoxifen treatment, and T-bet ZsGreen\textsuperscript{+} B cells outnumbered T-bet ZsGreen\textsuperscript{-} B cells in the TD Tomato\textsuperscript{+}
population, often by a ratio of greater than 10:1 (Figure 6B). Importantly, the ratio of T-bet ZsGreen+ to T-bet ZsGreen− cells in the TD Tomato+ B cell population was maintained steadily with time in all mice (Figure 6B), suggesting that most B cells expressing T-bet at day 0 maintained T-bet expression for at least 40 days.

We next wanted to explore T-bet persistence in memory B cells. In both humans and mice, most antigen-experienced B cells downregulate surface IgD expression upon transitioning towards memory B cell or plasma cell fate\textsuperscript{136, 137, 138, 139, 140, 141}. As such, we focused our analysis of T-bet persistence in tamoxifen treated fate mapper mice on IgD− B cells. Similar to total B cells, CD3− CD19+ IgD− B cells displayed evidence of TD Tomato fate mapping by day 10 post-tamoxifen treatment and the majority of these TD Tomato+ fate mapped IgD− B cells continued to express T-bet as evidenced by ZsGreen florescence (Figure 6C). Furthermore, there was no evidence of increasing T-bet loss with time in our sequential bleeds (Figure 6C), indicating that once T-bet expression is established it is stably maintained for a minimum of 40 days.

While the majority of fate mapped IgD− B cells maintain T-bet expression, there are low numbers of fate mapped cells that have lost T-bet. Plasma cells are a terminally differentiated B cell fate and as such we wanted to determine whether cells that have lost T-bet expression remain memory B cells or are transitioning towards a plasma cell fate. In fate mapped IgD− B cells that lost T-bet ZsGreen florescence, there is an increased level of CD138 expression as compared to fate mapped cells that maintained T-bet ZsGreen, indicating that these cells may be differentiating into plasma cells rather than being maintained
as T-bet− memory B cells (Figure 6D). This, along with sequencing data from our lab showing limited clonal overlap between T-bet+ and T-bet− HA-specific memory B cells in both mice and humans, suggests that T-bet expressing memory B cells represent a stable B cell pool with limited interconversion into the T-bet− B cell pool.
Figure 6: T-bet$^+$ and T-bet$^-$ memory B cells are a stable, independent populations
T-bet Fate Mapping mice$^{123}$ were treated with three doses of tamoxifen (Tam) to mark T-bet expressing cells with permanent, Rosa21-driven, tdTomato expression (A) and the status of T-bet expression of marked CD3$^-$ CD19$^+$ (B) or CD3$^-$ CD19$^+$ IgD$^-$ (C) B cells in the blood was tracked over 40 days. (D) Histogram of CD138 expression in TD Tomato$^+$ (fate mapped) IgD$^-$ T-bet ZsGreen$^-$ (purple) or T-bet ZsGreen$^+$ (blue) B cells a minimum of 40 days after tamoxifen treatment. Four independent experiments were carried out with an average of at least 3 mice per group; one representative experiment is shown here.
3.3.2 T-bet expressing B cells give rise to plasma cells

In the previous section, we established that T-bet\textsuperscript{+} B cells are a stable, independent population and that the limited number of fate mapped cells that have lost T-bet expression have increased levels of CD138 and may be becoming plasma cells. In order to further address the correlation between loss of T-bet and gain in plasma cell characteristics, specifically CD138, we treated fate mapper mice with three doses of tamoxifen and waited for 40+ days. We then gated on live, CD19\textsuperscript{+}, B220\textsuperscript{+}, dump\textsuperscript{−}, IgD\textsuperscript{−}, IgM\textsuperscript{−} switched splenocytes (Figure 7A). Within this population, we analyzed CD138 expression in TD Tomato\textsuperscript{+} cells expressing varying levels of T-bet ZsGreen (Figure 7B). As we gated on cells positive for TD Tomato expression, we analyzed cell populations expressing T-bet at the time of tamoxifen administration 40+ days prior. We observed a clear trend of increasing CD138 expression with decreasing levels of ZsGreen (Figure 7B). Thus, loss of T-bet expression is likely associated with a gain in plasma cell character.

As increasing CD138 correlates with loss of T-bet expression, we wanted to further explore the ability of T-bet\textsuperscript{+} B cells to give rise to plasma cells. Prior work has shown that T-bet\textsuperscript{+} B cells can give rise to various cell types upon transfer and re-challenge, including plasmablasts and memory B cells\textsuperscript{53}, but it remains unclear what happens to these T-bet expressing B cells in the absence of re-challenge. In order to address this question, we again utilized T-bet fate mapper mice.
Fate mapper mice were treated with tamoxifen every other day for a total of three doses and maintained for a minimum of 40 days post-treatment. Following euthanasia, the spleen and bone marrow were analyzed for the presence of CD138$^+$ plasma cells and the fraction of these cells expressing TD Tomato, indicating that they had been T-bet$^+$ at the time of tamoxifen administration, was analyzed (Figure 8 A-D). We observed that on average greater than 16% of total splenic plasma cells were TD Tomato$^+$, indicating that T-bet expressing B cells robustly give rise to CD138$^+$ plasma cells (Figure 8C). TD Tomato$^+$ CD138$^+$ plasma cells were also present in the bone marrow, albeit at lower proportions, averaging around 7% of total bone marrow plasma cells (Figure 8D).

Since B220 expression can be used to stratify plasma cells into a primarily long lived B220$^-$ population and a largely short lived B220$^+$ pool$^{142}$, we decided to analyze the portion of plasma cells that were TD Tomato$^+$ in each population (Figure 8B). While TD Tomato$^+$ plasma cells were seen in both the B220$^+$ and B220$^-$ plasma cell pools, a higher portion of B220$^+$ plasma cells were TD Tomato$^+$ as compared to B220$^-$ plasma cells in both the spleen and bone marrow (Figure 8C-D). This disparity was particularly evident in the bone marrow where there was a nearly 10-fold enrichment in the proportion of plasma cells that expressed TD Tomato in the B220$^+$ versus B220$^-$ pools (Figure 8D). However, it remains to be seen whether fate mapped B cells give rise to higher proportions of B220$^-$ plasma cells at later timepoints following tamoxifen treatment.
We directly assessed the turnover of TD Tomato⁺ plasma cells using BrdU labelling in the tamoxifen-treated fate mappers. As before, fate mapper mice were administered tamoxifen every other day for a total of three doses. Forty days after tamoxifen treatment, mice were placed on continuous BrdU treatment for either 13 or 20 days and the portion of B220⁺ and B220⁻ plasma cells that were positive for BrdU was analyzed (Figure 8 E-F). In both TD Tomato⁺ and TD Tomato⁻ plasma cells, a greater portion of B220⁺ as compared to B220⁻ plasma cells had incorporated BrdU. In TD Tomato⁺ B220⁺ plasma cells, roughly 70% had incorporated BrdU whereas only around 20% of TD Tomato⁺ B220⁻ plasma cells were positive for BrdU (Figure 8 E-F). This is consistent with prior results showing that B220⁺ plasma cells are a largely short lived population whereas B220⁻ plasma cells represent a primarily long lived ASC pool142.

As T-bet\(^{\text{high}}\) and T-bet\(^{\text{low}}\) B cells have different tissue residency properties, we wanted to determine if T-bet\(^{\text{low}}\) B cells were the plasma cell progenitor population or if T-bet\(^{\text{high}}\) B cells could give rise to plasma cells. This will help determine whether plasma cells arise from a continuously recirculating T-bet\(^{\text{low}}\) population which could provide tissue surveillance for reinfection or if they are derived from a splenic resident T-bet\(^{\text{high}}\) population. In order to evaluate this, we utilized an adoptive transfer technique. We sorted splenic T-bet\(^{\text{high}}\) or T-bet\(^{\text{low}}\) IgD⁻ B cells following CD4, CD8, Ter119, F4/80, and Gr1 magnetic depletion from fate mapper mice treated 40+ days ago with tamoxifen. We then injected the sorted cells retro-orbitally into separate B6.SJL congenic donors. We waited a minimum of three weeks prior to sacrificing the recipient mice and analyzed live, dump⁻, TD
Tomato\(^+\), CD45.2\(^+\) adoptively transferred CD19\(^+\) B cells and CD138\(^+\) plasma cells derived from T-bet\(^{\text{high}}\) or T-bet\(^{\text{low}}\) transferred memory B cells in order to determine what populations give rise to plasma cells (Figure 9A-C). As TD Tomato\(^+\) B cells were expressing T-bet at the time of tamoxifen administration 40+ days prior to sorting and adoptive transfer, we used TD Tomato positivity as a surrogate marker for memory B cells.

Despite the low number of recovered cells, we observed donor derived CD138\(^+\) plasma cells in mice receiving either T-bet\(^{\text{high}}\) of T-bet\(^{\text{low}}\) B cells (Figure 9D). Thus, both T-bet\(^{\text{high}}\) and T-bet\(^{\text{low}}\) B cells can serve as progenitors for plasma cells with increasing plasma cell characteristics acquired with loss of T-bet in memory B cells.
Figure 7: Increasing CD138 with loss of T-bet ZsGreen in fate mapped B cells and plasma cells

(A) Pregating strategy for the analysis of CD138 expression in class switched B cells in tamoxifen treated fate mapper mice. (B) Overlay flow plots showing CD138 expression in TD Tomato+ fate mapped T-bet^{high} (purple), T-bet^{low} (blue), and T-bet^- (orange) B cells. DUMP gate includes CD4, CD8, Gr-1, and F4/80. Data displayed are representative of three independent experiments with a minimum of three mice each.
Figure 8: T-bet+ B cells give rise to B220+ and B220- plasma cells

(A) Pregating strategy for the identification of plasma cells (PCs) in tamoxifen treated fate mapper mice. (B) Stratification of plasma cell populations by B220 staining and identification of the TD Tomato+ fate mapped fraction. (C and D) Proportions of plasma cell populations in the spleen (C) and bone marrow (D) that are TD Tomato+ (fate mapped). (E) Representative histogram showing BrdU labelling in plasma cell populations. (F) Proportions of plasma cell populations
that are BrdU+ after various periods of BrdU administration. DUMP gate includes CD4, CD8, Gr-1, and F4/80. Data in (C) and (D) show individual points with the mean ± SD indicated. Data in (A), (B), (C), and (D) are representative of or compiled from a minimum of three independent experiments with a minimum of three mice each. Data in (E) and (F) are representative of or compiled from two independent experiments with two mice each (Day 13) or one experiment with 3 mice (Day 20). Statistical comparisons performed using paired two-tailed t-test. **p<0.01, ****p<0.001
Figure 9: T-bet\textsuperscript{high} and T-bet\textsuperscript{low} B cells give rise to plasma cells
(A) Experimental design for fate mapper T-bet$^{\text{high}}$ and T-bet$^{\text{low}}$ adoptive transfer studies. (B) Sorting strategy for T-bet$^{\text{high}}$ and T-bet$^{\text{low}}$ B cells from tamoxifen treated fate mapper donor mice. (C) Gating scheme for the identification of TD Tomato$^+$ fate mapped donor derived CD45.2$^+$ B cells and plasma cells in B6.SJL adoptive transfer recipients. (D) Overlay flow plots showing CD138 and T-bet ZsGreen expression in TD Tomato$^+$ fate mapped donor derived CD45.2$^+$ B cells and plasma cells recovered from B6.SJL recipients receiving either T-bet$^{\text{high}}$ (red) or T-bet$^{\text{low}}$ (blue) sorted donor cells. DUMP gate includes CD4, CD8, Gr-1, and F4/80. Data displayed are representative of 3 recipient groups across two independent experiments.
3.3.3 T-bet\textsuperscript{high} memory B cells give rise to T-bet\textsuperscript{low} memory B cells that persist for a minimum of three weeks in the absence of a T-bet\textsuperscript{high} progenitor pool.

Since both T-bet\textsuperscript{high} and T-bet\textsuperscript{low} B cells give rise to plasma cells, we wanted to further explore the relationship between these B cell populations. In order to evaluate this, we once again utilized an adoptive transfer approach. In brief, we treated fate mapper mice with three doses of tamoxifen and waited 40+ days after which we sorted splenic T-bet\textsuperscript{high} or T-bet\textsuperscript{low} IgD- B cells following CD4, CD8, Ter119, F4/80, and Gr1 magnetic depletion (Figure 9A-B). We then injected these cells retro-orbitally into separate B6.SJL congenic donors and waited for a minimum of three weeks prior to analyzing transferred live, dump\textsuperscript{-}, TD Tomato\textsuperscript{+}, CD45.2\textsuperscript{+} memory B cells in the spleen of the recipient mice (Figure 10A).

We observed that adoptively transferred TD Tomato\textsuperscript{+} T-bet\textsuperscript{high} B cells gave rise to T-bet\textsuperscript{low} B cells whereas TD Tomato\textsuperscript{+} T-bet\textsuperscript{low} B cells failed to become T-bet\textsuperscript{high} B cells (Figure 10B). This indicates that, unlike the total T-bet\textsuperscript{+} and T-bet\textsuperscript{-} memory B cell pools which are maintained as separate populations with limited interconversion, there is substantial transit from the spleen resident T-bet\textsuperscript{high} into the circulating T-bet\textsuperscript{low} memory B cell pool. This does not occur in the reverse direction, as T-bet\textsuperscript{low} memory B cells do not give rise to T-bet\textsuperscript{high} memory B cells.

Based on our data showing that T-bet\textsuperscript{high} memory B cells can give rise to T-bet\textsuperscript{low} B cells, we wanted to address whether T-bet\textsuperscript{low} B cells can be maintained as a stable population or if they represent a transient population continuously seeded from T-bet\textsuperscript{high} B cells. Utilizing our adoptive transfer results, we observed that TD Tomato\textsuperscript{+} T-bet\textsuperscript{low} B cells were still present three weeks after transfer.
(Figure 10B). This indicates that T-bet\textsuperscript{low} memory B cells can persist for a minimum of three weeks in the absence of a T-bet\textsuperscript{high} feeder pool and do not require T-bet\textsuperscript{high} B cells for their continued maintenance. Future studies will be required to evaluate the sustained presence of T-bet\textsuperscript{low} memory B cells in the absence of T-bet\textsuperscript{high} memory B cells at later timepoints.

As another means of assessing the persistence of T-bet\textsuperscript{low} memory B cells in the absence of a T-bet\textsuperscript{high} population, we separated parabiosied partners and tracked circulating memory B cell subsets in the uninfected B6.SJL partner (Figure 11A). In our earlier work, we demonstrated that by 17 days post-parabiosis the mice had established a shared circulatory system and that HA-specific T-bet\textsuperscript{+} and T-bet\textsuperscript{low} B cells recirculated and could be found in the spleens of both partner mice (Figure 4B-G). Importantly, T-bet\textsuperscript{high} HA-specific B cells were restricted to the spleen of the previously infected partner and were absent from the congenic B6.SJL partner (Figure 4D-G). While the previously infected partner has all three HA-specific memory B cell subsets, the B6.SJL congenic partner lacks the T-bet\textsuperscript{high} HA-specific B cell pool, allowing us to determine whether maintenance of the T-bet\textsuperscript{low} memory B cell pool requires a T-bet\textsuperscript{high} progenitor pool. If the T-bet\textsuperscript{low} B cell pool was short-lived and required continuous seeding from T-bet\textsuperscript{high} progenitors, T-bet\textsuperscript{low} B cells would not be sustained once isolated from T-bet\textsuperscript{high} B cells. Therefore, we euthanized the mice after being separated for a minimum of three weeks and analyzed the HA-specific memory B cell pools in both partner mice.
As expected, IgD⁺ HA-specific B cells were observed in both partner mice and the vast majority of these HA-specific B cells were CD45.2⁺ (Figure 11B), indicative of their origin in the previously infected T-bet ZsGreen partner. The HA-specific T-bet⁺ cell population was only observed in the previously infected ZsGreen partner and was absent in the uninfected B6.SJL partner while HA-specific T-bet⁺ B cells were observed in both partner mice (Figure 11C-E). Importantly, we were able to detect an appreciable HA-specific T-bet⁻ population in both the B6.SJL and T-bet ZsGreen partners (Figure 11C-E). This indicates that T-bet⁻ B cells are maintained independently from T-bet⁺ B cells for a minimum of three weeks, as HA-specific T-bet⁺ B cells were absent from the B6.SJL partner and unable to contribute to the maintenance of the T-bet⁻ B cell pool. It is possible that the frequency of HA-specific T-bet⁻ B cells may be decreasing in the absence of a T-bet⁺ seeder population, indicating that over a longer timeframe this T-bet⁻ population may be lost in the absence of a T-bet⁺ progenitor pool. These data together with our adoptive transfer results demonstrates that while T-bet⁻ memory B cells can be generated from T-bet⁺ precursors, T-bet⁻ memory B cells do not require T-bet⁺ progenitors for their continued maintenance for a minimum of three weeks. Thus, T-bet⁻ B cells may represent a self-renewing population which can be maintained in the absence of continued contribution from a T-bet⁺ B cell pool.
Figure 10: T-bet<sup>high</sup> B cells give rise to T-bet<sup>low</sup> B cells that persist in the absence of a T-bet<sup>high</sup> feeder pool for a minimum of three weeks

(A) Gating scheme for the identification of TD Tomato<sup>+</sup> fate mapped donor derived CD45.2<sup>+</sup> B cells in B6.SJL adoptive transfer recipients. (B) Overlay flow plots showing TD Tomato<sup>+</sup> fate mapped donor derived CD45.2<sup>+</sup> B cells recovered from B6.SJL recipients receiving either T-bet<sup>high</sup> (red) or T-bet<sup>low</sup> (blue) sorted donor cells. DUMP gate includes CD4, CD8, Gr-1, and F4/80. Data displayed are representative of 3 recipient groups across two independent experiments.
Figure 11: Independent maintenance of T-bet\textsuperscript{low} memory B cells as demonstrated by parabiont separation

(A) Experimental design for parabiosis followed by separation surgery studies.

(B) Gating scheme for the identification of T-bet-ZsGreen derived CD45.2\textsuperscript{+} HA\textsuperscript{+} B cells in T-bet ZsGreen (top) and B6.SJL (bottom) separated parabionts. (C) Subsetting of HA\textsuperscript{+} CD45.2\textsuperscript{+} B cells into T-bet\textsuperscript{-}, T-bet\textsuperscript{low}, and T-bet\textsuperscript{high} populations via flow cytometry in T-bet ZsGreen (left) and B6.SJL (right) separated parabionts. (D) Numbers of T-bet\textsuperscript{-}, T-bet\textsuperscript{low}, and T-bet\textsuperscript{high} HA\textsuperscript{+} splenic memory B
cells (MBCs) per $10^6$ CD45.2$^+$ B cells in T-bet-ZsGreen (left) and B6.SJL (right) separated parabionts. (E) Numbers of T-bet$, T$-bet$^{low}$, and T-bet$^{high}$ HA$^+$ splenic memory B cells in T-bet-ZsGreen (left) and B6.SJL (right) separated parabionts. DUMP gate includes CD4, CD8, Gr-1, and F4/80. Data in (D) and (E) show individual points with the mean ± SD indicated. Data displayed are representative of or compiled from 3 separated pairs across two independent experiments.
3.4 Discussion

These studies investigate the stability and fate of T-bet expressing B cells. Using T-bet fate mapper mice, we demonstrated that T-bet\(^+\) B cells are a stable population with limited conversion into the T-bet\(^-\) B cell pool. In fate mapped memory B cells, increasing CD138 expression corresponds with lower T-bet levels, indicating that a decrease in T-bet expression in B cells may be associated with a gain in plasma cell characteristics. Indeed, a substantial portion of both splenic and bone marrow plasma cells, especially in the B220\(^+\) plasma cell population, are derived from T-bet expressing B cells despite only a brief window of tamoxifen treatment, and both T-bet\(^{\text{high}}\) and T-bet\(^{\text{low}}\) B cells can give rise to plasma cells. Additionally, T-bet\(^{\text{high}}\) B cells give rise to T-bet\(^{\text{low}}\) B cells, but T-bet\(^{\text{low}}\) B cells do not upregulate T-bet in order to enter the T-bet\(^{\text{high}}\) B cell pool. Nevertheless, T-bet\(^{\text{low}}\) memory B cells can be maintained in the absence of a T-bet\(^{\text{high}}\) B cell pool for a minimum of three weeks, indicating that T-bet\(^{\text{low}}\) B cells may represent a self-renewing population that can be maintained in the absence of T-bet\(^{\text{high}}\) B cells. Taken together, these findings demonstrate that T-bet\(^+\) memory B cells give rise to plasma cells, and that T-bet\(^{\text{high}}\) B cells can contribute to both the T-bet\(^{\text{low}}\) B cell pool and plasma cell populations.

While our prior results demonstrated that both T-bet\(^+\) and T-bet\(^-\) memory B cells are generated and sustained long term in the spleen following PR8 influenza infection and that T-bet\(^+\) B cells are important for IgG2c and stalk-specific antibody responses as well as sustained protective HAI titers, it remained unclear whether there was substantial ongoing exchange between T-
bet\(^+\) and T-bet\(^-\) B cell pools. Our fate mapper results demonstrate the absence of sustained entry of T-bet\(^+\) B cells into the T-bet\(^-\) B cell pool, indicating that T-bet\(^+\) and T-bet\(^-\) B cells are maintained as separate populations.

There are remaining questions regarding the role of T-bet\(^+\) and T-bet\(^-\) B cells, particularly in recall responses to mutating pathogens as seen with immune imprinting or original antigenic sin (OAS). Infective pathogens, particularly rapidly mutating viruses, can undergo antigenic variation wherein mutations lead to alterations in surface antigens, subverting high affinity recognition by previously generated antibodies. In the case of influenza, antigenic drift can lead to frequent changes in antigenically important sites which can drastically impair antibody recognition and immune protection\(^\text{143}\). During subsequent exposure to the antigenically drifted pathogen, the antibody response is biased towards the previously encountered strain which impairs the response to the current infecting strain\(^\text{144}\). The concept of OAS has striking similarities to carrier-mediated hapten suppression wherein prior exposure to a carrier protein leads to suppression of the hapten antibody response during subsequent hapten-carrier immunization\(^\text{145}\). Additional work has shown that carrier-mediated hapten suppression has a particularly strong effect on blocking hapten specific IgG2a/c responses during the secondary hapten-carrier immunization\(^\text{146}\). As class switching to the IgG2a/c isotype is strongly associated with T-bet expression\(^\text{40,42,100}\), T-bet\(^+\) B cells may play an important role in carrier-mediated hapten suppression and OAS. Indeed, in carrier-mediated hapten suppression, the Th1 type response is largely absent\(^\text{147}\). As B cell specific T-bet expression requires a Th1 type cytokine
environment, lack of a Th1 type response likely impairs the T-bet+ B cell response. OAS can be subverted in a murine heterologous influenza challenge model if adjuvants that induce a Th1 type response are added to the vaccination regimen, implying that the lack of Th1 type cytokines and T-bet+ B cells may play a key role in OAS and carrier-mediated hapten suppression. As such, properly regulating the recruitment of T-bet+ memory B cells during vaccination and re-challenge may be an important way to avoid OAS and provide optimal sustained protection against rapidly mutating pathogens. Specifically, inducing a Th1 type environment may help recruit memory T-bet+ B cells or generate new T-bet+ B cells in the recall response and allow subversion of OAS.

We have shown that loss of T-bet correlates with increasing CD138 levels and that T-bet+ B cells are able to generate plasma cells. This prompts questions regarding the potential role of T-bet expression in controlling antibody-secreting cell differentiation. Stone et al. have demonstrated that suppression of IFNγ mediated inflammatory effects on B cells via T-bet expression is likely important in promoting plasma cell differentiation, in particular in recall responses. Additionally, B cell intrinsic T-bet is required for IgG2a/c plasma cell differentiation, likely via STAT1. As rising levels of CD138 are observed with decreasing T-bet, it is possible that CD138 itself may function to help assure plasma cell differentiation and survival upon the progressive loss of T-bet. Notably, Blimp1 can antagonize T-bet expression and plasma cells with higher levels of Blimp1 had decreased expression of the majority of T-bet target genes.
assessed, implying a possible link between increasing plasma cell differentiation and decreasing T-bet levels. CD138 likely acts in a cell intrinsic manner to promote plasma cell accumulation and survival in vivo. Cell surface CD138 expression increases heparan sulfate levels on plasma cells and this leads to increased binding of pro-survival cytokines such as IL-6 and APRIL and increased plasma cell survival. T-bet mediated suppression of IFNγ induced inflammation may no longer be required for survival and plasma cell differentiation upon increased expression of CD138.

While both T-bet\textsuperscript{high} and T-bet\textsuperscript{low} B cells give rise to plasma cells, there are important differences between these cell populations warranting further investigation of the relationship between these B cell pools. Our prior results demonstrated that T-bet\textsuperscript{high} and T-bet\textsuperscript{low} B cells differ in their recirculation capacity, as T-bet\textsuperscript{high} HA-specific memory B cells are splenic resident whereas T-bet\textsuperscript{low} memory B cells recirculate between parabiotic partners. While it is tempting to speculate that circulating T-bet\textsuperscript{low} B cells are a transient population derived from stationary, self-renewing T-bet\textsuperscript{high} B cells on their way to becoming T-bet\textsuperscript{plasma} cells, the situation is likely more complex. T-bet\textsuperscript{low} B cells can be maintained in the absence of a T-bet\textsuperscript{high} population for a minimum of three weeks. Thus, a portion of T-bet\textsuperscript{low} B cells are a stable and persistent population that may have separate maintenance and induction requirements from the T-bet\textsuperscript{high} subset. These two possibilities are not mutually exclusive as the T-bet\textsuperscript{low} B cell population may contain both a stable and a transient component.
It will be of interest to determine the role of T-bet\textsuperscript{high} and T-bet\textsuperscript{low} B cells in a recall response, as both populations give rise to plasma cells. Future studies exploring whether T-bet\textsuperscript{high} and T-bet\textsuperscript{low} B cells have different requirements for their induction and maintenance are warranted. One possibility is that T-bet\textsuperscript{high} B cells may require a splenic niche for their generation whereas T-bet\textsuperscript{low} B cells may be able to be generated at alternative sites such as reactive LNs. Splenectomy followed by infection and subsequent evaluation of B cell populations at early time points may help in resolving this question.

As T-bet\textsuperscript{high} B cells are maintained long term at stable numbers while also giving rise to T-bet\textsuperscript{low} B cells and plasma cells, we posit that T-bet\textsuperscript{high} memory B cells may have a stem like nature. Indeed, Kenderes et al. have demonstrated that T-bet\textsuperscript{+} IgM memory B cells generated following Ehrlichia muris infection can both self-renew and generate multiple lineages of effector B cells following re-challenge, demonstrating their stem-like nature\textsuperscript{53}. Our studies extend these findings by establishing the presence of both T-bet\textsuperscript{high} and T-bet\textsuperscript{low} memory B cells and showing that T-bet\textsuperscript{+} B cells continue to generate plasma cells in the absence of re-challenge. Future studies such as rapamycin treatment or sublethal irradiation to further explore the stem-like nature of T-bet\textsuperscript{high} B cells are of interest.
CHAPTER 4: Conclusions, Implications, and Future Directions

We have undertaken a detailed investigation of T-bet\(^+\) B cells including their emergence, maintenance, and role in a murine influenza infection model, their stability, and their ability to give rise to antibody secreting cells. As T-bet expressing B cells are important in the control of a variety of infectious diseases and contribute to autoimmunity in both mice and humans, a better understanding of the requirements for the emergence and maintenance of this population along with their terminal differentiation fate could help inform more effective prophylactic vaccines and treatments for autoimmune pathologies.

In chapter two, we have shown that following PR8 influenza infection in mice, T-bet\(^+\) HA-specific B cells emerge in the spleen, lungs, and draining mediastinal LNs at acute timepoints. At memory timepoints, T-bet\(^+\) HA-specific B cells become progressively restricted to the spleen, despite the continued presence of T-bet\(^-\) HA-specific B cells at other anatomical sites. Both T-bet\(^+\) and T-bet\(^-\) HA-specific B cells first display a GC phenotype before transitioning to a CD73\(^+\) CD80\(^+\) PD-L2\(^+\) memory phenotype. T-bet\(^+\) B cells can be further subset into T-bet\(^{\text{high}}\) and T-bet\(^{\text{low}}\) B cells, with T-bet\(^{\text{high}}\) HA-specific B cells being a spleen resident population as evidenced by parabiosis studies. Furthermore, T-bet expression in B cells is required for HA-specific IgG2c titers and sustained protective HAI titers. Additionally, stalk specific influenza antibodies are largely ablated in the absence of T-bet expressing B cells (Figure 12).

In chapter 3, we utilized fate mappers to demonstrate that most T-bet expressing B cells maintain their T-bet positivity and are a stable, separate
population from T-bet- B cells. We also showed that T-bet+ B cells differentiate into plasma cells: downregulation of T-bet is associated with a gain in plasma cell characteristics, as evidenced by increasing CD138 expression, and a portion of plasma cells in both the bone marrow and spleen are derived from T-bet expressing B cells. Notably, T-bet+ B cells are especially apt at producing B220+ plasma cells. We further demonstrated that T-bethigh B cells can give rise to T-betlow B cells as well as plasma cells. However, T-betlow B cells can still be maintained independently of T-bethigh B cells for a minimum of three weeks (Figure 12).
Following PR8 influenza infection, T-bet*, T-bet\textsuperscript{low}, and T-bet\textsuperscript{high} memory B cells are established and maintained long term. While T-bet* and T-bet\textsuperscript{low} memory B cells recirculate, T-bet\textsuperscript{high} memory B cells are restricted to the spleen and are resident. T-bet* B cells are required to establish hemagglutinin stalk specific antibody titers. While T-bet\textsuperscript{high} B cells can give rise to T-bet\textsuperscript{low} B cells, T-bet\textsuperscript{low} B cells fail to become T-bet\textsuperscript{high}. Furthermore, the T-bet* B cell pool is maintained as a stable, separate population from the T-bet* B cell population. Both T-bet\textsuperscript{high} and T-bet\textsuperscript{low} B cells can give rise to B220* and B220* plasma cells with increasing CD138 expression corresponding with decreasing T-bet levels.
These studies suggest that the T-bet expressing B cell population constitutively generates plasma cells at a basal rate. While this may reflect antigenic stimulation from self-antigens or antigens endogenous to our colony, the magnitude of plasma cell differentiation observed raises alternative possibilities. We postulate that there may be continuous differentiation of T-bet+ B cells into plasma cells, potentially in an antigen-independent manner, functioning to support maintenance of neutralizing antibody titers at levels sufficient to provide sterilizing immunity.

While the classic view of immune memory emphasizes the effectiveness of antigen specific memory lymphocytes in mounting a rapid secondary response, protective immune memory relies heavily on maintenance of protective antibody titers. In fact, vaccination is often aimed at eliciting sufficient protective titers to maintain sterilizing immunity long term, even in the absence of persistent antigen\textsuperscript{151}. This is particularly important in providing protection against toxins, where pathology may be too rapid to allow the generation of a secondary response to be effective\textsuperscript{152}. In clinical veterinary medicine, the importance of protective antibody titers can be seen in the management of newborn animals. Assuring that newborn cattle rapidly get appropriate, high quality colostrum in order to allow passive transfer of maternal antibodies is considered the most important management factor in assuring calf survival\textsuperscript{153}. Up to 31% of calf mortality events in the first 21 days of life can be attributed to failure of passive transfer\textsuperscript{154}. Furthermore, passive immunization in the form of antibody transfer has been used clinically since the 19th century. The importance of passive
immunization and neutralizing antibodies can be seen in the treatment and prevention of diphtheria, where Behring's discovery of the use of immune serum massively reduced morbidity and mortality. In fact, it was hailed by the Lancet as the "most important advance of the century in the medical treatment of acute infective disease"\textsuperscript{155}. In Germany alone, diphtheria treatment via passive immunization is credited with having saved as many as 45,000 lives per year\textsuperscript{156}.

Appropriate maintenance of protective titers is essential for effective immune protection and optimal health. While these lasting titers have often been accredited to long-lived bone marrow plasma cells, recent work has shown that newly formed, rapidly turning over plasma cells likely play a major role in long term antibody titer maintenance\textsuperscript{142}. These plasma cell subsets can be distinguished on the basis of B220 expression as BrdU labelling studies have demonstrated that B220\textsuperscript{−} plasma cells are primarily long lived whereas B220\textsuperscript{+} plasma cells are largely short lived\textsuperscript{142}. While our studies show that T-bet expressing B cells can generate both B220\textsuperscript{+} and B220\textsuperscript{−} plasma cells, a higher portion of B220\textsuperscript{+} plasma cells are derived from T-bet expressing B cells as compared to B220\textsuperscript{−} plasma cells. Thus, we posit that T-bet\textsuperscript{+} memory B cells play a key role in the maintenance of lasting protective titers by continuously generating antibody secreting plasma cells.

Multiple lines of research support the importance of T-bet\textsuperscript{+} B cells in giving rise to plasma cells that maintain antibody titers. Our work showed that mice lacking B cell specific T-bet expression failed to sustain protective HAI titers long term following PR8 influenza infection. Additionally, Kenderes et al. propose that
T-bet\(^+\) IgM memory B cells generate ASCs following \textit{Ehrlichia muris} infection\textsuperscript{53}. Influenza vaccine-generated CD21\(^\text{low}\) B cells, which express increased levels of T-bet mRNA and protein, are similarly poised for plasma cell differentiation as they have elevated expression of plasma cell associated genes such as BLIMP-1\textsuperscript{49}. Further, T-bet\(^+\) B cells isolated from lupus patient blood are able to rapidly differentiate into plasma cells upon TLR7 and IL-21 stimulation in the absence of cell division\textsuperscript{67}, and lupus prone mice lacking T-bet\(^+\) B cells demonstrate reduced autoantibody titers and kidney pathology, leading to improved survival\textsuperscript{67}. Interestingly, the memory marker profile observed on our HA-specific T-bet\(^+\) B cells is also consistent with plasma cell differentiation as opposed to GC entry following re-stimulation\textsuperscript{36, 37}. A recent study by Stone et al. further demonstrates the role played by T-bet-expressing B cells in plasma cell differentiation during the influenza recall response to heterologous challenge, as inducible deletion of T-bet in memory B cells causes an up to 10-fold reduction in levels of induced total and IgG2c antigen-specific plasma cells. Interestingly, T-bet is not required for the survival of T-bet\(^+\) memory B cells, but rather for plasma cell differentiation, as memory B cell numbers are not impacted by inducible T-bet deletion. T-bet mediated repression of an IFN\(\gamma\) dependent inflammatory gene program may be important in promoting plasma cell differentiation\textsuperscript{54}.

Assuming that T-bet\(^+\) B cells are continuously generating plasma cells, regulation of this process is necessary to assure that appropriate numbers of plasma cells and levels of antibody titers are maintained. One possibility is that
this differentiation is regulated by circulating antibody levels. T-bet^+ B cells may detect a decrease in free circulating antibody, as could happen after re-exposure to a pathogen due to formation of antigen-antibody complexes. This could induce increased numbers of T-bet^+ B cells to undergo plasma cell differentiation. In support of such an idea, Kenderes et al. observed that T-bet^+ IgM memory B cells failed to differentiate into plasma cells in chronically infected mice that have high titers of protective antibodies but most of these cells became plasma cells in newly infected mice lacking pre-existing titers^{53}. Multiple myeloma patients have reduced levels of normal polyclonal serum immunoglobulins, likely due to a B cell extrinsic mechanism which prevents B cells from differentiating into plasma cells^{157}; such a mechanism could be dependent on the high levels of monoclonal antibodies arising from malignant plasma cells. Studies by Pape et al. further support such a mechanism, as immune serum transfer decreased memory B cell to plasma cell differentiation^{158}. If such a mechanism is in play, we would expect decreased levels of plasma cell generation from T-bet expressing B cells in the presence of increased antibody levels. Conversely, plasma cell genesis from T-bet^+ B cells would likely be increased in mice with lower total antibody titers. While the mechanism is unclear, increased Fc receptor binding is a possible candidate that may suppress this differentiation^{33, 159}. Future studies to compare the expression levels and types of Fc receptors on T-bet^+ and T-bet^- memory B cells will be necessary to investigate this possibility.

Conversely, T-bet^+ B cells may be induced to differentiate into plasma cells in response to polyclonal activation. Such a situation is seen in human
memory B cells that proliferate and differentiate into plasma cells following activation with polyclonal stimuli such as CpG DNA or bystander T cell help\textsuperscript{160}. ABCs, a large fraction of which are T-bet\textsuperscript{+}, show robust responses to innate stimuli: as compared to follicular B cells, ABCs fail to respond to BCR crosslinking and/or anti-CD40, but robustly proliferate in response to TLR9 stimulation as provided by CpG\textsuperscript{41}.

An alternative possibility is that T-bet\textsuperscript{+} B cells may express broadly reactive BCRs allowing them to be stimulated and differentiate into plasma cells in response to a range of conserved antigenic determinants. Such ideas are not unprecedented, as MZ B cells often express polyreactive BCRs that can bind to a range of microbial molecular patterns. In addition, MZ B cells have high levels of TLR expression and dual stimulation of the BCR and TLR can result in rapid differentiation of this subset into antibody secreting plasma cells\textsuperscript{161}. A similar situation may be occurring with T-bet\textsuperscript{+} B cells, and indeed these B cells are responsible for the antibody response to the conserved stalk epitopes in influenza infection. It would be interesting to see if T-bet\textsuperscript{+} B cells are responsible for the antibody response to other conserved antigenic determinants.

Importantly, these possibilities imply that T-bet\textsuperscript{+} B cell memory may have innate-like characteristics, and this may help to explain the purpose for splenic residency of T-bet\textsuperscript{high} memory B cells. Under this concept, T-bet\textsuperscript{+} B cells localized to the spleen would function to rapidly detect circulating factors associated with infection in order to quickly increase plasma cell genesis and protective antibody titers. Much of the previous research on resident lymphocytes has been done on
T cells, where parabiosis studies demonstrate that both CD4 and CD8 T cells establish tissue resident memory (T\textsubscript{RM}) populations in various organs and barrier sites\textsuperscript{26, 162, 163, 164}. These T\textsubscript{RM} populations provide important and rapid protection against a wide variety of local reinfections through various means including direct effector functions and recruitment and activation of other immune cells\textsuperscript{30}. However, T\textsubscript{RM} can also be maladaptive and contribute to allergic or autoimmune pathology\textsuperscript{165}.

In terms of B cells, tissue resident memory is less appreciated. B cells give rise to plasma cells, which secrete antibodies that freely recirculate, and as such the importance of residency is less clear. Studies from Young et al. comparing lymphocytes in the blood and lymph of sheep demonstrated that the majority of non-recirculating peripheral blood lymphocytes were B cells. These non-recirculating B cells lacked surface expression of CD21 and thus may correspond to the CD21\textsuperscript{−} CD23\textsuperscript{−} T-bet\textsuperscript{+} ABC population observed in mice and humans\textsuperscript{166}. Furthermore, it may be important for B cells to persist at sites of infection to allow rapid stimulation and differentiation into antibody secreting plasma cells upon local reinfection. In fact, research from the Randall lab has utilized parabiotic studies to demonstrate that lung resident memory B cells are established following intranasal influenza infection and require local antigen encounter for their generation. Upon local intranasal re-challenge, these lung resident memory B cells rapidly give rise to antibody secreting plasmablasts whereas recirculating memory B cells failed to contribute to this plasmablast response\textsuperscript{97}. 

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While tissue residency in both B and T cells may be important in providing surveillance and protection against local reinfection, this explanation seems less likely for spleen resident HA-specific T-bet\(^\text{high}\) B cells. Influenza is a respiratory infection and does not replicate in the spleen, making this an unlikely site for reinfection\(^\text{167}\). However, the idea of resident memory cells in secondary lymphoid organs is not unprecedented: resident CD4 T cells can be found in the cervical and mesenteric LNs and peyers patches\(^\text{168}\), and parabiosis studies identify antigen-specific resident CD8 T in the draining mediastinal LN more than a month after influenza infection. This is likely related to the persistence of processed viral antigens in the draining nodes, since CFSE labelled antigen-specific CD8 T cells show increased proliferation in the mediastinal LNs following transfer into previously infected mice as much as 60 days after infection\(^\text{169}\).

Antigen persistence in the draining LNs may also explain the maintenance of mediastinal LN-resident HA-specific T-bet\(^-\) B cells that we observed in parabiosis studies, as these displayed a GC phenotype as late as 100 days post influenza infection.

While it is possible that antigen persists long term in the spleen following influenza infection, this is unlikely to explain the splenic residency of T-bet\(^\text{high}\) memory B cells for several reasons. First, only T-bet\(^\text{high}\) memory B cells were splenic resident and T-bet\(^-\) and T-bet\(^\text{low}\) B cells were able to freely recirculate between parabiotic partners. Additionally, there was no noticeable expansion of HA-specific cells in the CD45.1 B cell fraction in the spleen of the previously infected partner as would be expected in the case of persistent antigen. Further,
spleen T-bet$^+$ B cells’ loss of GC phenotype and transition to memory phenotype by day 40 post infection suggests antigen is largely cleared. In support of antigen-independent maintenance, parabiosis studies show that lymphocytes can be resident in secondary lymphoid organs in the absence of persistent antigen: resident LCMV-specific memory CD8 T cells can be found in the spleen and LNs of LCMV Armstrong infected mice well after viral clearance, and CD69, which is associated with tissue residency, is not upregulated until after viral clearance$^{170}$.

Furthermore, the localization of resident memory lymphocytes within secondary lymphoid organs is not random, and this may have important functional implications for splenic resident T-bet$^{\text{high}}$ memory B cells. Using a combination of parabiosis and immunofluorescence microscopy, Schenkel et al. showed that antigen specific CD8 memory T cells in the LNs of the previously LCMV infected partner were largely localized to the LN sinus, whereas in the uninfected partner these cells were primarily found in the T cell zone. Likewise, these antigen specific CD8 T cells preferentially reside in the splenic MZ at the expense of the white pulp in the previously infected parabiont$^{170}$. Importantly, the splenic MZ and LN subcapsular sinus are prime sites for a wide range of initial microbial entry and antigen capture and presentation$^{171, 172, 173, 174, 175}$. In particular, the splenic MZ is specialized for blood borne antigen surveillance, with a unique microarchitecture resulting in the slowing of blood flow to allow efficient capture of antigens from the blood stream$^{176}$. MZ macrophage antigen capture is important for the proper control of certain infections, as mice lacking proper antigen uptake in the MZ are impaired in their ability to control infection by
pathogens such as *Listeria monocytogenes* or *Streptococcus pneumoniae* and are more susceptible to death\textsuperscript{174, 177}. MZ B cells are a largely resident population, as demonstrated by their limited migration in parabiosis studies and numerical persistence in the non-irradiated section of the partially irradiated spleen in rats\textsuperscript{178, 179}. This MZ retention is likely dependent on the interaction between Sphingosine 1-phosphate receptor 1 (SIP\textsubscript{1}) and CXCL13, since MZ B cells are displaced from the MZ into the splenic follicles in S1P\textsubscript{1} deficient mice. In S1P\textsubscript{1}/CXCL13 double deficient mice, normal MZ B cell localization is restored, indicating that S1P\textsubscript{1} functions to block CXCL13 mediated recruitment of MZ B cells to the splenic follicles\textsuperscript{180}.

In light of the importance of the MZ in antigen surveillance, it is tempting to speculate that the splenic resident T-bet\textsuperscript{high} population observed after influenza infection may be localized to the MZ. In such a case, these cells would be uniquely positioned to support broad immune surveillance and rapidly detect invading pathogens. Indeed, previous studies have shown that memory B cells can be found in the MZ and perifollicular regions of the spleen\textsuperscript{181, 182}, and much of this localization is dependent on CCR6. In mice lacking CCR6, the frequency of memory B cells in the perifollicular region is massively decreased, while their frequency in follicular regions is increased more than 6-fold. However, the frequency of memory B cells in the MZ is not significantly changed in CCR6 knockout mice. CCR6 may be important in recall responses as bone marrow and splenic plasma cell numbers, along with serum titers, were reduced in the recall response in CCR6 knockout mice. These CCR6 mediated effects on the
secondary response are B cell intrinsic, as demonstrated by mixed bone marrow chimera studies\textsuperscript{182}. Future studies involving immunohistochemistry staining of splenic sections will be required to determine the precise splenic localization of T-bet\textsuperscript{high} B cells relative to their T-bet\textsuperscript{low} and T-bet\textsuperscript{−} counterparts. It will also be important to determine differences in chemokine and cytokine receptor profiles between these subsets and to determine how these contribute to localization and recirculation properties. Assuming that splenic resident T-bet\textsuperscript{high} B cells are preferentially localized to the splenic MZ, it is tempting to postulate that they are poised to rapidly differentiate into antibody secreting cells and produce antibodies for systemic dissemination following reinfection.
CHAPTER 5: Materials and Methods

**Mice:** C57BL/6 and B6.SJL (10-12 weeks old, females, purchased from The Jackson Laboratory); Tbx21^{F/F} possessing loxP sites flanking exons 2-6 of Tbx21^{183} and Cd19^{tm1(cre)Cgn} containing a targeted cre cassette into exon 2 of Cd19^{184} (obtained from the laboratory of E. John Wherry, University of Pennsylvania); Tbx21-ZsGreen with a ZsGreen reporter gene inserted at the Tbx21 start site in BAC RP23-237M14^{86}; Tbx21-ZsGreen^{creERT2} possessing the BAC RP23-237M14 with the ZsGreen reporter gene, T2A self-cleaving peptide, and inducible creERT2 recombinase gene^{123}; and Rosa26^{LSL/tdTomato} with a Rosa26 targeted construct containing CAG-LSL-tdTomato-WPRE^{135} were maintained and used in accordance with the University of Pennsylvania Institutional Animal Care and Use Committee guidelines.

**Infections:** Mice were infected by intranasal infection with 30 tissue culture infectious dose50 (TCID50) of influenza strain A/Puerto Rico/8/1934 (PR8; American Type Culture Collection).

**Parabiosis and separation surgery:** Age-matched T-bet ZsGreen reporters and B6.SJL adult female mice were conjoined as described previously^{185}. Briefly, a skin incision was made from the olecranon to the knee of each of the mice to be joined. The elbows and knees of the two paired mice were then tied together with surgical suture, followed by connecting of the skin with surgical sutures and staples. For pain control, mice were given buprenorphine (0.1 mg/kg every 6
hours for 36 hours) and meloxicam (5 mg/kg every 12 hours for 72 hours) and provided with sulfamexathole (400mg/L) and trimethoprim (800mg/L) antibiotics in their drinking water to prevent infection. Mice were monitored for signs of pain, infection, or damage to sutures. Blood was periodically drawn from the tail to check for anastomoses, which appeared complete by day 14, therefore, mice were euthanized at day 17. The spleen was harvested from both partners for all pairs, and the lungs and mediastinal LNs were also collected from some pairs. Seperation surgery was performed similar to parabiosis surgery except an incision was made from the olecranon to the knee of the paired mice and the joining suture was severed after which sutures were used to appose the dorsal and ventral skin flaps in each partner. Mice were maintained for a minimum of three weeks following separation surgery.

**Flow cytometry:** Flow cytometry reagents were purchased from BioLegend (BL), BD Biosciences (BD), eBioscience (eBio), Southern Biotech (SB), or Invitrogen (Inv). The following antibodies were used: CD11c (N418; BL), IgM (R6-60.2; BD), CD38 (90; eBio), CD73 (TY/11.8; BL), CD80 (16-10A1; BD or BL), PD-L2 (TY25; BL), CD138 (281-2; BL), IgD (11–26c.2a; BL), B220 (RA3-6B2; BL or eBio), CD19 (1D3; BD or eBio), CD19 (6D5; BL) peanut agglutinin–FITC (Sigma), CD45.1 (A20; BL), CD45.2 (104; BL), CD183/CXCR3 (CXCR3-173; BL) and CD3 (17A2; BL). DUMP gate comprised CD8 (53-6.7; eBio), CD4 (H129.19; BL), F4/80 (BM8; eBio), Ly-6G/GR1 (RB6-8C5; eBio). For detection of murine influenza hemagglutinin binding B cells, recombinant HA PR8^186_ was obtained
from the laboratory of Dr. Barney Graham, National Institute of Allergy and Infectious Disease, biotinylated, and conjugated to streptavidin-fluorophores as previously described\textsuperscript{186}. Mouse samples were prepared for flow cytometry as follows: Mouse Fc fragment (Jackson ImmunoResearch; 015-000-008) was added to all staining cocktails at a final concentration of 1:200. Mouse spleens were homogenized, on ice, in staining buffer (PBS + 0.5%BSA + 2mM EDTA) and passed through nylon mesh (50\(\mu\)M) to obtain single cell suspension. Bone marrow was flushed from the femur and tibia with staining buffer following removal of muscles and and passed through nylon mesh (50\(\mu\)M). Red blood cells were lysed using ACK lysing buffer (Lonza, cat 10-548E) as per manufacturer’s instructions. Mouse LNs were homogenized, on ice, in staining buffer and passed through nylon mesh (50\(\mu\)M). Cells were washed with PBS and stained as described previously\textsuperscript{41, 60}. Live/dead discrimination was done using Zombie Aqua fixable viability kit (BL). Data were acquired on BD LSR II flow cytometer and FACS analyses were performed using FlowJo v9 and v10 (Becton Dickinson Co., Ashland, OR).

**Serum antibody titers:** Serum was harvested by spinning whole blood at 13000g for 10 minutes and stored at -20°C until use. Antibody titers were assessed using ELISA as previously described\textsuperscript{41, 60} with the following modifications: 96-well medium-binding plates were coated with either 20HAU/well of BPL-inactivated PR8, 2 \(\mu\)g/mL of PR8 HA, or 2 \(\mu\)g/mL of H6/H1 chimeric constructs (expressed in baculovirus system as previously described\textsuperscript{187}).
specific monoclonal antibodies (from Dr. Jonathan Yewdell, National Institute of Allergy and Infectious Diseases) were used as standards to determine concentration of IgG1 and IgG2a/c. Standards were used at a starting concentration of 100 ng/mL for IgG2a and 10 ng/mL for IgG1 and diluted 2-fold across.

**HAU (hemagglutination unit) and HAI assays:** Viral HAU titers were determined before every HAI assay. All dilutions were prepared in PBS. 50 μL diluted virus, 50 μL diluted heat-inactivated sera and 12.5 μL of 2% turkey erythrocytes were used per well for all assays, which were performed in round-bottom plates.

Starting with a 1:100 dilution of live virus, 2-fold dilutions were mixed with 2% turkey erythrocytes (Lampire biologicals) and incubated for 1 hour at room temperature. Agglutination dose (AD) was determined at the end of the incubation period, and confirmed by repeating the process with a 2-fold dilution series of virus, ranging from 4AD to 0.25 AD. This dose was subsequently used for the HAI assay.

Sera were heat-treated at 55°C for 30 minutes, diluted 2-fold in PBS (starting dilution 1:20), mixed with 4AD and 2% turkey erythrocytes, and incubated as for HAU assay. HAI titers are expressed as inverse of the highest dilution that inhibited agglutination.
**Tamoxifen Treatment:** Tamoxifen (Sigma; cat. T5648-1G) was dissolved in 100% ethanol to a concentration of 80 mg/ml and vortexed and heated at 42°C until completely dissolved. An equal volume of Kolliphor (Sigma, cat. C5135-500G) was added to bring the solution to a concentration of 40 mg/ml. Aliquots were then stored at -20°C. Immediately prior to administration, tamoxifen aliquots were thawed at 42°C and diluted with PBS to 8 mg/ml. Mice were administered three doses of 0.8 mg of tamoxifen via intraperitoneal injection every other day.

**Cell Sorting:** Murine splenocytes were prepared as described prior then negatively selected using a cocktail of biotinylated antibodies against CD4 (RM4-5, eBio), CD8a (53-6.7; eBio), F4/80 (BM8; eBio), Ly-6G/GR1 (RB6-8C5; BD), and Ter-119 (Ter-119; BL). After washing with staining buffer, cells were incubated with 100ul Streptavidin MicroBeads (Miltenyi Biotec) in 900ul staining buffer for 30 min. Cells were then washed and ran through a negative selection LD column (Miltenyi Biotec) according to manufacturer's instructions. Flow thru was collected and washed with staining buffer then stained for FACS. Samples were sorted on a BD FACS Aria into serum supplemented media and stored on ice. Sorted samples were centrifuged and resuspended in 100-200ul PBS for retroorbital injection into recipient B6.SJL mice.

**BrdU Administration and Staining:** Mice were injected IP with 200 ul of 3 mg/ml BrdU in PBS at 12 hour intervals for 3 days. Mice subsequently were
given drinking water with 1mg/ml sucrose and 0.5 mg/ml BrdU until takedown. Splenocytes were harvested and stained as described prior then prepared for intracellular staining with the Fix and Perm Cell Fixation and Permeabilization Kit (ThermoFisher) according to manufacturer's instructions. After ICS, cells were treated with DNase solution for 40 minutes, washed, and stained with anti-BrdU APC antibody (BD) for 40 minutes at 1:80 dilution in FACS buffer. Cells were washed and stored at 4 degrees in the dark until running on the cytometer.

**QUANTIFICATION AND STATISTICAL ANALYSIS:**

All p values were determined using one of the following as mentioned in figure legends: unpaired non-parametric t-test or paired t-test using GraphPad Prism version 7 or version 8 (GraphPad Software, La Jolla, CA 92037 USA). ns=not significant, *p < 0.05, **p < 0.01, ****p < 0.001. Data are represented as mean ± SEM or mean± SD. The number of mice used in each experiment, as well as the number of times an experiment was repeated, is mentioned in the figure legends.
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**Bacterial and Virus Strains**

- Influenza strain A/Puerto Rico/8/1934 (PR8) | American Type Culture Collection

**Biological Samples**

- Turkey Erythrocytes | Lampire Biologicals | Cat# 7209401

**Chemicals, Peptides, and Recombinant Proteins**
| Recombinant hemagglutinin PR8 (HA-PR8) | Laboratory of Dr. Barney Graham, National Institute of Allergy and Infectious Disease | Whittle et al., 2014 |
| HA-specific monoclonal antibodies (IgG1 and IgG2a/c) | Laboratory of Dr. Jonathan Yewdell, National Institute of Allergy and Infectious Diseases | Margine et al., 2013 |
| H6/H1 chimeric constructs | Laboratory of Scott E. Hensley, University of Pennsylvania | |
| Meloxicam | Boehringer Ingelheim | NDC 0010-6013-01 |
| Buprenorphine HCl | Reckitt Benckiser | NDC 12496-0757-1 |
| Sulfamethoxazole and Trimethoprim Oral Suspension | Aurobindo | NDC 65862-496-47 |
| Isoflurane | Phoenix | 0010250 |
| Tamoxifen | Sigma-Aldrich | T5648 |
| Kolliphor | Sigma-Aldrich | C5135 |

### Critical Commercial Assays

| Streptavidin MicroBeads | Miltenyi Biotec | 130-048-101 |
| LD Columns | Miltenyi Biotec | 130-042-901 |
| Fix and Perm Cell Fixation and Permeabilization Kit | ThermoFisher | GAS003 |
| APC BrdU Flow Kit | BD Biosciences | 552598 |

### Experimental Models: Organisms/Strains

| Mouse: C57BL/6 | The Jackson Laboratory | MGI:5656552 |
| Mouse: B6.SJL | The Jackson Laboratory | Jax Stock No: 002014 |
| Mouse: Tbx21<sup>floox</sup> | from the laboratory of E. John Wherry, University of Pennsylvania | Jax Stock No: 022741 |
| Mouse: Cd19<sup>cre</sup> | from the laboratory of E. John Wherry, University of Pennsylvania | MGI:1931143 |
| Mouse: Tbx21-ZsGreen | from the laboratory of Jinfang Zhu, National Institutes of Health | Zhu et al., 2012; MGI:5690118 |
| Mouse: Tbx21-ZsGreen<sup>creERT2<sup>-<sub>LSL/tdTomato</sub></sup></sub> | from the laboratory of Jinfang Zhu, National Institutes of Health | Yu et al., 2015; MGI:5690125; MGI:3809524 |

### Software and Algorithms

| FlowJo | v9 and v10 | Becton Dickinson Co., Ashland, OR |
| Prism | V7 and V8 | GraphPad, La Jolla, CA |

**Table 1: List of Key Resources**


