Homeostatic T Cell Receptor Interactions with Self-Peptide Tune CD4+ T Cell Function

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Homeostatic T Cell Receptor Interactions with Self-Peptide Tune CD4+ T Cell Function
by
Juliet Marie Bartleson

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

January 2021
St. Louis, Missouri
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I would like to thank my mentor, Paul Allen. Paul has proven to be incredibly supportive over the years, all while still pushing me to develop my scientific abilities beyond what I even thought was possible. Paul gave me the freedom to explore questions and techniques that I felt strongly about pursuing, and while he’s taught me to always be critical of science, he’s done so in a way that has fostered my enthusiasm for it. I am incredibly grateful for all he has taught me and for the confidence he has instilled in me over the years.

I would also like to thank the Immunology community at Washington University. This program is filled with intelligent, driven, gifted individuals, but what makes it so amazing is how everyone comes together to create a wonderfully collaborative environment. I have enjoyed training here immensely. I specifically want to thank my fellow Immunology graduate students for their support and friendship throughout the ups and downs of graduate school. I’d like to thank my thesis committee as well. Their advice and support have undoubtedly elevated my work and my scientific abilities. Celeste Morley, thank you for your wise advice that helped me navigate both my professional and personal worlds. Greg Wu, thank you for being so welcoming to both me and my husband. I will always appreciate the time you took to give me invaluable guidance during the writing of my F31. Ali Ellebedy, thank you for helping me with all of my B cell questions and for being such a joy to run into on the 8th floor. Haina Shin, thank you for not only mentoring me during my thesis project, but for also teaching me directly during my rotation with you. I carried many of the skills you taught me into my thesis lab, and I was a more productive, organized scientist because of it. Finally, thank you Megan Baldridge – I would not have been able to graduate without you!
My project would not have been possible without the help of all of the Allen Lab members. David Donermeyer was indispensable to this work. I thank him for teaching me many technical skills, giving me feedback on experimental design, and for being an all-around wonderful teammate along the way. I thank Stephen Horvath for not only contributing to my project as well, but for also being my computer savior and my candy-drawer enabler. Both David and Stephen were always willing to help me with anything that came up, and I will always appreciate them for that. I would also like to thank Darren Kraemalmeyer for his tireless work managing our mouse colonies. This project could not have been accomplished without him.

This work would also not have been possible without the help of our collaborators. I thank Takeshi Egawa for his critical advice on cell-sorting and his LCMV expertise. I thank Renee Wu and Ken Murphy for DC subset advice and the Xcr1-cre mouse. I thank Mike White and the Genome Engineering and iPSC Center for the generation of the $H2-Dma^{Df}$ mouse line. I thank Emma Walker and the Morley lab for help with the mitoROS and IHC protocols. I also thank WUCCI, especially Dennis Oakley and Michael Shih, for their microscopy assistance. I am also tremendously grateful to Emil Unanue for the freedom to use his BD FACS Canto. Furthermore, I would like to acknowledge the funding that has supported my graduate education: NIAID F31 AI138393 and NIAID R01 AI139540.

To the ‘Allen Lab Ladies’: thank you for bringing a smile to my face each and every day that I worked with you. Thank you to Ashley Milam for teaching me nearly everything I know. Ashley is an amazing mentor, and I was incredibly fortunate to have learned from such a patient, intelligent woman. Thank you to Donna Thompson, who treated my babies as if they were her own. Veronika Redmann, thank you for adopting my family into yours. Thank you to Cora Arthur for the many times you dropped everything to lend me a reagent I desperately needed. Thank you
to Sam Hsieh, Marta Everding, and Sharon Smith for being so kind and welcoming over the years. While our relationships may have begun on the 8th floor, I know they will continue on as life-long friendships.

I would also like to acknowledge the friends and family that initially encouraged me to attend Washington University. This has been a selfish pursuit. I have missed countless birthdays, anniversaries, births, weddings, graduations, and even the loss of Papa Tony and Grandad Emile. I am truly humbled by the support my friends and family continue to give me, and I thank each and every one of them.

Finally, I must acknowledge my husband, Tony. A little over five years ago, we loaded up our U-Haul trailer, hitched our car onto a tow dolly, and embarked on a 2,000-mile-journey across the nation with our two cats serenading us along the way. Since then, our life has been filled with more adventure than I could have ever possibly imagined. Through it all, Tony has never once expressed any doubt in me or shown any resentment for pulling him so far away from our family and friends. He has given me unrelenting support, and if it were not for his belief in me, this work would not have been possible. I thank him with every molecule of my being, and I look forward to making that 2,000-mile-journey yet again – only this time with the serenade of not only those two cats, but also one dog and one sweet baby girl: our eternal St. Louis-token, Josephine.

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January 2021
Dedicated to my daughter, Josephine Marie Bartleson
Mature CD4+ T cells circulate throughout peripheral secondary lymphoid organs using their T cell receptor (TCR) to surveil peptide presented on major histocompatibility complex class II molecules (pMHC) in search of cognate, antigenic peptide. In the absence of an immune challenge, however, the TCR is continuously interacting with self-pMHC, which induces a relatively weak TCR signal known as tonic signaling. These homeostatic TCR:self-pMHC interactions do not propagate canonical TCR activation pathways, but they do engage proximal TCR signaling molecules and affect basal gene expression patterns. Here, we question whether the strength of tonic signaling directly tunes CD4+ T cell function.

Utilizing a transgenic TCR system, we uncover a role for tonic signaling in predisposing a CD4+ T cell to either T effector (Teff) or T follicular helper (Tfh) lineage commitment early after activation. We then extend these findings to the polyclonal CD4+ T cell repertoire, and through a series of genetic mouse models, we confirm that direct manipulation of tonic signaling strength alters Tfh development. Ultimately, these data establish an inverse relationship between the strength of tonic TCR signaling and Tfh differentiation. Furthermore, we determine tonic signaling strength is also controlling the overall basal metabolic activity of CD4+ T cells, which corresponds
with the production of mitochondrial reactive oxygen species. This offers a potential mechanism for how tonic signaling strength influences TCR activation to skew CD4+ T cell fate decisions.

During the course of these studies we generated a novel mouse strain, $H2-DMa^{ff}$, to reduce the presentation of self-pMHC, thereby decreasing tonic signaling strength in polyclonal CD4+ T cells. Employing this mouse line, we also interrogated whether there is a specific subset of antigen presenting cells (APCs) responsible for maintaining CD4+ T cell tonic signaling. Our findings indicate a CD11c+ subset of APCs independent of the conventional DC1 lineage is responsible for CD4+ T cell tonic signaling maintenance. Collectively, this work elucidates the critical involvement of tonic signaling in early Teff versus Tfh lineage commitment and enhances our basic understanding of how tonic signaling is being maintained to regulate cellular metabolism during homeostasis.
Chapter 1: Introduction

1.1 The lifespan of a conventional CD4+ T cell

CD4+ T cells are a critical component of the adaptive immune response. They coordinate discrete responses to various types of pathogens, and they are essential to establishing long-lasting, antigen-specific immunity. Here, we discuss the typical life of a CD4+ T cell.

1.1.1 Thymic education

T cell precursors migrate from the bone marrow to the thymus where they receive the appropriate cues and signals to develop into the most immature thymocyte stage, generally defined by the absence of CD4 and CD8 on their cell surface (CD4 and CD8 double negative, or DN)\(^1\). Upon successful rearrangement of the T cell receptor (TCR) \(\beta\) chain, DN thymocytes proliferate and eventually express the CD4 and CD8 co-receptors\(^2\). These CD4/CD8 double positive (DP) thymocytes ultimately undergo the process of ‘thymic education’, whereby a DP thymocyte will successively rearrange its TCR\(\alpha\) chain until one of four possible events occurs: 1) death by neglect, 2) positive selection, 3) negative selection, or 4) agonist selection\(^3\). The affinity model of thymocyte selection postulates that these events are ruled by the affinity of the TCR for self-peptide bound to major histocompatibility complexes (self-pMHC) within the thymus, such that too weak of affinity (or no interaction) leads to death by neglect, too strong of affinity results in negative selection, and an intermediate or ‘just right’ affinity will allow for positive selection or possibly agonist selection to the Treg or iNKT cell fates if that ‘just right’ affinity is on the higher
end of the TCR:self-pMHC positively selecting affinity spectrum\textsuperscript{4, 5}. While the majority of DP thymocytes will die by neglect, a small pool of them will go on to successfully generate an $\alpha$:\(\beta\) TCR capable of interacting with self-pMHC to propagate the signaling events necessary to override their pre-programmed fate of cell death: i.e, positive selection\textsuperscript{6}. If the $\alpha$:\(\beta\) TCR uniquely expressed by the positively selected thymocyte recognizes self-pMHC bound to MHC class II, that cell will further go on to only express the CD4 co-receptor and it will egress from the thymus to enter the periphery as a mature CD4+ T cell\textsuperscript{3}.

\textbf{1.1.2 Clonal expansion and effector differentiation}

Mature naive CD4+ T cells circulate between secondary lymphoid organs and the blood, surveilling peptide presented on MHC class II by antigen presenting cells (APCs) in search of their cognate antigenic peptide. CD4+ T cells are responsible for coordinating discrete adaptive immune responses to a diverse array of pathogens. Classically, they accomplish this duty by differentiating into one of the major CD4+ T effector cell subsets in response to specific classes of pathogens: T helper (Th) 1, Th2, and Th17. Additionally, naive CD4+ T cells can differentiate into T follicular helper (Tfh) cells after activation in order to mediate T-dependent B cell responses. Tfh cells are critical to initiate and maintain germinal center (GC) reactions, supplying GC B cells with the cytokines and co-stimulatory molecules necessary for somatic hypermutation and affinity maturation of antibodies\textsuperscript{7-9}.

Once a naive CD4+ T cell engages its cognate antigenic-pMHC on a dendritic cell (DC), it will undergo a process of rapid proliferation known as clonal expansion. Generally, T cells will proliferate for 4-5 days following activation, allowing for the expansion and differentiation of antigen specific T effector cells that disseminate to the sites of the immune challenge and respond
immediately to their antigenic-pMHC without the need for co-stimulation. The differentiation of naive CD4+ T cells into distinct Th subsets is primarily driven by cytokine signaling during the time of activation\textsuperscript{10}. This cytokine signaling leads to the activation of specific STAT proteins, which ultimately regulates the expression of Th lineage defining transcription factors. For example, in the case of Th1 differentiation, IFN-$\gamma$ signaling activates STAT1, leading to the expression of T-bet. T-bet is the lineage defining transcription factor for Th1 cells, as it promotes IFN-$\gamma$ production and the upregulation of IL-12R$\beta$2. IL-12 signaling enforces commitment to the Th1 lineage via its activation of STAT4 (reviewed in\textsuperscript{11}). Traditionally, the Th subsets were thought to be distinct, terminally differentiated lineages, whereby commitment to one fate excluded plasticity to the other fates. This model has been challenged, however, and the most recent literature suggests there is some amount of plasticity among the Th subsets\textsuperscript{12, 13}.

\textbf{A brief focus on Tfh development}

Shortly after the initial T cell activation by DCs \textit{in vivo}, an early Tfh cell-like phenotype is induced in all primed T cells, as indicated by the uniform upregulation of Bcl6\textsuperscript{14}, the Tfh fate determining transcription factor\textsuperscript{15, 16}, within the first 24 hours post-TCR stimulation. It isn’t until 2 to 4 days later that the clear phenotypic divergence of a CXCR5$^{hi}$Bcl6$^{+}$BLIMP1$^{-}$ Tfh cell population and a CXCR5$^{lo}$Bcl6$^{-}$BLIMP1$^{-}$ non-Tfh T effector (Teff) cell population appears\textsuperscript{17-19}. The skewing of the Teff versus Tfh lineage commitment has been hypothesized to be due to the downregulation of Bcl6 in Teff-fated cells in response to IL-2 signaling\textsuperscript{17, 20-22}. Therefore, it has been suggested that activated T cells may escape IL-2 signaling in the T cell zones of a lymph node by unknown mechanisms or even purely by chance, and this escape from IL-2 signals is what maintains Bcl6 expression and eventually leads to Tfh fate commitment\textsuperscript{23}. In this sense, Tfh cell development is
not thought to be dependent on a set of “instruction” cytokines provided by APCs, as the Th1, Th2, and Th17 effector subsets are, but instead upon escape from those effector subset driving conditions to ultimately allow a preset default of Tfh cell development to persist and reinforce itself via re-localization of the T cell to the T and B cell border near the follicle\cite{23, 24}. Owing to the importance of Tfh cell development in the immune response, however, it seems unlikely that the driving force behind their differentiation would be left to “chance escape” alone.

This is supported by more recent data showing a transcriptional bifurcation of CD4\(^+\) T cells into Tfh and Teff cells as early as 8 hours post-activation \textit{in vitro}\cite{25}. While external cues are critical for sustaining Tfh-lineage development, the nearly immediate Tfh/Teff fate polarization post-stimulation of naive CD4\(^+\) T cells implies a strong role for a cell intrinsic factor as the initial determinant of Tfh outcome. One emerging hypothesis is that T cell antigen receptor (TCR) signal strength during activation dictates early Tfh commitment and is driven by TCR avidity for antigenic-pMHC. Many groups have studied the possibility that TCR avidity is instructing early Tfh/Teff fate decisions\cite{25-31}; however, the field has been contentious in its findings. Some groups have found evidence suggesting strong TCR avidity for antigenic-pMHC promotes Tfh development\cite{25-27}, others have concluded the opposite\cite{28, 29}, and still others have found no effect\cite{30, 31}.

Other factors indispensable to Tfh development include ICOS engagement via ICOSL on DCs during the initial priming. This leads to early Bcl6 expression that is necessary for the upregulation of CXCR5 and downregulation of non-follicular positioning molecules and the IL-2R, both of which repress Tfh cell development\cite{23}. CXCR5 expression results in homing of the activated T cells to the T and B cell border, where the T cells can now be identified as pre-Tfh cells and are CXCR5\(^+\)PD-1\(^+\). At this stage, the pre-Tfh cells have prolonged interactions with
cognate B cells in order to receive the necessary costimulatory signals that allow for ultimate
differentiation into the GC Tfh phase. This process further requires a variety of molecules,
including ICOS, CD40L, Bcl6, and SAP. Once a pre-Tfh cell has entered the final GC Tfh cell
fate, it is defined as CXCR5++PD-1+. Importantly, not all pre-Tfh cells will enter the GC though.
Why some pre-Tfh cells remain at the T and B border while others progress to the GC remains a
mystery. Furthermore, whether pre-Tfh cells and GC Tfh cells have unique functional differences
in the type of signals they provide B cells also remains unknown. As of now, the only
distinguishing factor between the two populations is location.

Interestingly, the Tcm precursor population is also phenotypically defined the same as the
pre-Tfh population (CXCR5+PD-1+)22. This has led to much speculation about whether Tfh cells
and Tcm cells develop from the same precursor or have distinct lineages33. A study by Pepper et
al. found that Tcm development was in fact independent from Tfh cell development; however, the
in this study they defined Tcm cells as CCR7+CXCR5+ 22. They contested that nearly all of the
CCR7+ cells were CXCR5+ in their system. Since then, it has been made clearer that circulating
memory Tfh cells exist, provide better B cell help upon a secondary challenge, and are defined as
CCR7+CXCR5+ 34. This suggests that perhaps the cells interrogated in the Pepper et al. study were
in fact memory T follicular helper cells, potentially generated from a pre-Tfh precursor. The Tcm
population actually consists of mostly CXCR5+ cells35, which are distinct from memory T follicular
helper cells in their ability to aid B cells36.

1.1.3 CD4+ T cell memory formation

Utilizing a peptide bound MHCII tetramer enrichment method to detect the presence of an epitope-
specific clonal repertoire within the diverse polyclonal B6 CD4+ T cell population, the Jenkins
group has observed a clonal expansion at the peak of the immune response of around 250 times the naive clonal amount. Shortly after this peak response, however, the effector cell population will contract by an order of magnitude, leaving behind a pool of memory CD4+ T cells that is roughly only 25 times larger than the naive frequency. The literature is riddled with conflicting reports about how memory CD4+ T cells are generated, and the functional differences in the heterogeneity of the memory CD4+ T cell population have remained relatively unexplored. Part of the confusion surrounding memory CD4+ T cells is the complex hierarchy of different signals that are most likely involved in the generation of these cells. Factors that play into memory cell formation involve TCR affinity for antigenic peptide, TCR signal strength upon activation, TCR affinity for self-peptide, co-stimulatory molecules, cytokine cues, location of priming, and even the timing of activation during the immune response (reviewed in [38-40]). Identifying markers that distinguish true memory T cell populations has been a challenge for the field, along with the low frequency of memory CD4+ T cells left to study following a primary response. This has made analyzing memory CD4+ T cells technically difficult.

Fortunately, researchers have begun to work through these challenges and slowly tease apart details of the memory CD4+ T cell response. It is now known that there is heterogeneity in the memory CD4+ T cell population, with the most studied and widely accepted subsets being the Tcm and Tem cells. When compared to Tem cells, Tcm cells have been shown to proliferate more robustly but secrete effector cytokines less quickly after activation. Tcm cells are also thought to circulate readily throughout the lymphoid tissue, as indicated by their definitive expression of CCR7 and CD62L, while Tem cells are primed to exit lymphoid tissue and quickly migrate to sites of infection. It is clear that Tem cells are generated from a T effector cell population (CXCR5+ PD-1+), although there may be a small amount of plasticity in the memory
However, the broad characterization of Tcm precursor cells (CXCR5$^+$PD-1$^+$) and Tcm differentiated cells (CCR7$^+$CD62L$^+$) has led to confusion in the literature regarding ‘true’ Tcm development and function, since these characterizations can include other various cell types, i.e. pre-Tfh cells and memory Tfh cells. The developmental connection between all of these populations remains unknown.

### 1.2 CD4+ T cells at homeostasis

Between positive selection and activation, mature CD4+ T cells circulate in the periphery. In the absence of their corresponding immune challenge, they circulate as quiescent, resting, naive T cells. Ultimately, it is highly likely that they will spend the majority of their lifetime in this homeostatic state. Importantly, cues and signals a T cell receives during this time have the potential to impact their eventual response to foreign antigen; therefore, it is critical that we gain a better understanding of homeostatic T cell biology.

#### 1.2.1 Homeostatic maintenance of CD4+ T cells

T cell homeostasis is a tightly controlled aspect of adaptive immunity and includes the regulation of T cell production, division, trafficking, and death$^{43}$. The overall number of peripheral T cells appears to remain relatively consistent$^{44-46}$, and naive T cells appear to occupy a separate homeostatic niche than memory T cells$^{47}$. The overall maintenance of the peripheral T cell pool is thought to be regulated through the loss of peripheral T cells rather than coordinated control over thymic generation and output of T cells$^{48}$, and even in the absence of recent thymic emigrants, the number of naive T cells in the periphery remains relatively unchanged$^{49, 50}$. The specific mechanisms underlying regulation of these phenomena, however, remain relatively unknown.
How naive T cells survive in the absence of their cognate antigenic-pMHC is still under investigation. Signals shown to be required for survival include bcl-2\textsuperscript{51}, the transcription factors LKLF and NFAT4\textsuperscript{52, 53}, cytokines\textsuperscript{54-59}, and JAK3\textsuperscript{60, 61}. Conversely, TGF-\(\beta\) may be responsible for limiting the size of the naive T cell pool\textsuperscript{62, 63}. TCR interactions with MHCII are also critical for the survival of naive CD4+ T cells\textsuperscript{64, 65}, and MHCII expression on dendritic cells (DCs) specifically has been shown to maintain CD4+ T cell survival during homeostasis\textsuperscript{66}. Homeostatic proliferation is primarily driven by the availability of IL-7 and IL-15\textsuperscript{58, 59, 67, 68}, but has also been shown to be dependent on TCR:self-pMHC interactions as well\textsuperscript{46, 69, 70}. It is important to note that delineating homeostatic T cell survival and homeostatic T cell proliferation is challenging, and whether or not homeostatic T cell proliferation occurs naturally at any significant amount is debated because these models involved either acute or chronic lymphopenic hosts; therefore, it’s truly unknown whether TCR:self-pMHC interactions are required for only one of these processes or whether these interactions are mandatory for both\textsuperscript{71}.

1.2.2 Tonic signaling

Homeostatic, peripheral interactions between the TCR and self-pMHC result in ‘tonic signaling’ that is critical for T cell maintenance and homeostasis\textsuperscript{72}. Tonic signaling consists of low-level interactions between the TCR and self-pMHC\textsuperscript{73}, and while it does not initiate T cell activation, it does impact the activation state of the T cell\textsuperscript{74, 75} and regulate gene expression levels\textsuperscript{76, 77}. Importantly, while tonic signaling strength is most obviously tied to affinity and/or avidity of the TCR for self-pMHC, the strength of signaling might also be impacted by other components, such as the availability of self-pMHC signals in a tissue dependent manner\textsuperscript{78}.  

8
The most reliable indicator of tonic signaling strength has traditionally been the amount of basal phosphorylation of the TCRζ chain\(^{79-83}\). Defining tonic signaling pathways, however, has generally stalled at the proximal TCR signaling molecules. While the mechanisms of downstream tonic signaling are unclear, a couple read-outs of TCR activation strength have proven sensitive enough to discern variations in the strength of tonic TCR signaling as well. CD5\(^{80}\), Ly6C\(^{84}\), and Nur77\(^{85, 86}\) have informed us of a broad spectrum of tonic signaling strengths experienced by the diverse repertoire of TCRs in a polyclonal population. CD5 especially has become an important tool in the field of tonic signaling because of its cell surface identification via flow cytometry and its confirmed positive correlation with basal phosphorylation of the TCRζ chain\(^{79-83}\).

CD5 is an immunomodulatory surface molecule that is a member of the Scavenger-Receptor Cysteine-Rich (SRCR) superfamily and clusters at the immune synapse upon TCR stimulation\(^{87-92}\). The role of CD5 during thymic development is to dampen TCR signaling\(^{93}\), and peripheral expression of CD5 has been shown to confer high tonic signaling T cells with an NF-κB-dependent survival advantage\(^{94}\). However, CD5 and the other TCR signaling strength markers, Ly6C and Nur77, have limitations in the study of tonic signaling. Imperatively, these markers do not distinguish tonic TCR signaling from TCR signaling in response to antigenic-pMHC; therefore, further elucidation of the tonic signaling networks connecting proximal TCR signaling molecules to the functional maintenance of CD4+ T cells will prove critical for the advancement of this field.

### 1.2.3 Effects of tonic signaling on T cell responses to foreign antigen

There have been few studies conducted examining the role of tonic signaling in CD4\(^+\) T cell response to foreign antigen. What has been found, is that CD5\(^{\text{hi}}\) cells are more reactive to foreign
antigen, as read-out by quicker IL-2 production and phosphorylation of Erk following T cell activation \(^{81}\), as well as greater initial proliferative responses in vitro\(^{75}\). Using tetramer binding to define TCR affinity for foreign antigen, Mandl et al. found that CD5 expression positively correlated with antigenic-pMHC binding, and in a polyclonal population, CD5\(^{hi}\) cells dominated the primary response to a variety of foreign antigens\(^{82}\). Interestingly, our lab’s own findings contradict these results. In our LLO TCRtg system (detailed more extensively in section 1.5), we found that the cells experiencing high levels of tonic signaling did worse in a primary response when compared to their low tonic signaling counterparts\(^{81,95,96}\).

These contradictory results may be explained in part by the Mandl et al. finding that CD5\(^{hi}\) cells have stronger TCR affinity for their foreign antigen. Since they used a polyclonal transfer system, they could therefore not account for the variable of TCR affinity for antigenic-pMHC. However, there is also contention as to whether high or low affinity for foreign antigen dominates the immune response. Most studies in this field rely on sampling with pMHC tetramers, but when a limiting dilution assay was used to avoid the potential bias of pMHC tetramer staining, it was found that CD4\(^{+}\) T cells with lower affinity for foreign antigen actually dominated the primary immune response\(^{97}\). Regardless, our lab’s defined system utilizing two transgenic TCR T cells with identical affinities for foreign antigen allow us to eliminate that extra variable, perhaps offering clearer insight to the potential role of tonic signaling strength on CD4\(^{+}\) T cell function.

When the role of tonic signaling in CD8\(^{+}\) T cell responses was investigated, the Jameson group found that CD5 did not correlate with affinity for foreign antigen and that CD5\(^{hi}\) cells also dominated the primary immune response\(^{98}\). There could be substantial differences in how CD4\(^{+}\) and CD8\(^{+}\) T cells behave, however, especially regarding the effects early IL-2 production from CD5\(^{hi}\) cells may have on T cell function\(^{78}\).
1.2.4 APC subsets responsible for maintaining CD4+ T cell tonic signaling

A couple studies have begun to ask the question: is there a specific subset of APCs responsible for maintaining CD4+ T cell tonic signaling? One study found that the DCs control T cell tonic signaling, ultimately affecting T cell responsiveness to antigen\(^99\). They used a CD11c.DOG mouse that deleted CD11c+ cells upon treatment with diphtheria toxin (DT). After only 2 days of DT treatment, they found that CD4+ T cells displayed reduced proliferative capacity upon stimulation, owing to a defect in immune synapse maturation due to reduced TCR:self-pMHC engagement. While not required for their survival, MHCII has been shown to be imperative for maintaining proper function of memory CD4+ T cells\(^100\). The Stockinger group described that in the absence of MHCII, memory CD4+ T cells have reduced metabolic activity and impaired production of IL-2, which can be restored via the transfer of MHCII expressing B cells\(^101\). It is important to point out, however, that both of these studies utilized methods of depleting self-pMHC interactions in ways that impacted other homeostatic variables as well. For example, the complete depletion of the CD11c+ cellular subset\(^99\) and the utilization of a common \(\gamma\)-chain knockout mouse\(^101\) in their experimental approaches could additionally impact homeostatic cytokine production, which also affects CD4+ T cell function\(^55\). To date, there has been no study specifically interrogating just the role of self-pMHC presentation by various APC subsets on the maintenance of tonic signaling during homeostasis.

1.3 Metabolism

1.3.1 Naive CD4+ T cell metabolism

Naive T cells are in a state of quiescence, defined by their small cell size and lack of proliferation; therefore, they are primarily in a state of catabolism and rely almost entirely on oxidative
phosphorylation (OXPHOS) to generate the relatively low bioenergetic needs required by their resting state, while activated T cells shift their metabolism from OXPHOS to aerobic glycolysis in order to support the anabolic reactions demanded by clonal expansion and effector differentiation\textsuperscript{102, 103}. The mechanistic target of rapamycin (mTOR) has been extensively characterized as an integrating sensor of environmental and cellular cues in the regulation of cellular metabolism, growth, and survival\textsuperscript{104}. While the depletion of mTOR activity appears to have no effect on T cell homeostasis\textsuperscript{105}, the hyper-activation of mTORC1 has been shown to disrupt T cell quiescence\textsuperscript{106-110}; thus, the dampening of metabolic activity in quiescent T cells is required for proper T cell homeostasis.

Studies have unveiled a critical role for mTORC1 inhibitors (i.e., TSC1, PTEN, and LKB1) in the enforcement of the quiescent program in naive, peripheral T cells\textsuperscript{106, 111, 112}. These molecules have been shown to have distinct effects on the regulation of T cell homeostasis, and their deletion also uniquely modifies how T cells respond to stimulation. Loss of TSC1 in naive T cells impairs their subsequent \textit{in vivo} immune response to bacterial infection, PTEN-deficiency in mature T cells leads to a lymphoproliferative autoimmune condition, and LKB1-deficiency has negative effects on cell viability and proliferation post-TCR stimulation\textsuperscript{106, 111, 112}. This highlights the possibility that metabolic networks may prime naive T cells to tune their eventual response to antigenic-pMHC.

\textbf{1.3.2 Metabolic programming influences CD4+ T effector differentiation}

A multitude of signaling pathways essential for various T cell functions are closely interconnected with metabolic programming, either through shared signaling components or directly through metabolite regulation\textsuperscript{113}. While tremendous progress has been made over the past several years
showing metabolism is adjusted to fit T cell function, most of this work has focused primarily on CD8+ T cells\textsuperscript{114-129}. For CD4+ T cells, however, we are especially in the beginning stages of interrogating the role of metabolic programming on T effector differentiation. Studies have revealed distinct roles for mTORC1 and mTORC2 in Th1 and Th2 differentiation, and the glycolytic transcription factor HIF1a is critical in the development of Th17 cells\textsuperscript{130}. Most notably, however, metabolic programming appears to play critical roles in the generation of iTreg and Tfh cells. For example, repression of the AKT-mTOR pathway has been implicated in iTreg development\textsuperscript{131-133}, and it has been suggested that CD5 itself can block mTOR signaling in CD4+ T cells to promote Treg induction\textsuperscript{134}, although another study involving Th17 T cells suggests CD5 enhances mTOR activation\textsuperscript{135}. The role of metabolism in Tfh development is also conflicting. One study found that Tfh cells have decreased glucose metabolism\textsuperscript{136}, while another study found that glucose uptake enhanced Tfh cell formation\textsuperscript{137}. Bcl6 does repress genes underlying glycolysis\textsuperscript{23}; however, the ICOS-PI3K-AKT pathway promotes Tfh cell development, through the degradation of FOXO1 allowing for expression of Bcl6 and repression of Klf2, potentially activating mTOR and increasing the metabolic activity of Tfh cells\textsuperscript{9}. Despite all of the confusion surrounding the exact role of metabolism in specific T effector fates, it is not contended that metabolism in general does alter CD4+ T cell behavior.

1.3.3 Reactive oxygen species (ROS) and their role in TCR signaling

The major source of ROS in non-phagocytic cells is the mitochondria, which produces O$_2^-$ (superoxide) as electrons move through the electron transport chain\textsuperscript{138, 139}. Superoxide produced in the mitochondrial matrix is then mostly converted to the membrane permeable ROS, hydrogen peroxide (H$_2$O$_2$) through a reaction that is dependent on Mn-superoxide dismutase (MnSOD).
Prevailing thought is that this membrane permeable ROS is primarily responsible for the effects of ROS on TCR signaling in the cytoplasm\(^\text{140}\). There are three critical systems in all cell types to balance the reduction/oxidation (redox) state of the cell: NADPH/NADP\(^+\), thioredoxin\(_{\text{red}}\)/thioredoxin\(_{\text{ox}}\) (Trx) and GSH/GSSG (glutathione)\(^\text{141}\). Antioxidants are critical to reducing ROS, but a cell doesn’t always maintain a balanced redox level. Particularly during states of activation and proliferation, cells will display acute imbalances in their redox state. Although once thought to simply be a deleterious consequence of cell functions, recent studies have highlighted the importance of this ROS generation in the active regulation of cellular signaling pathways\(^\text{142}\). For example, ROS have been implicated in the regulation of T cell proliferation and death\(^\text{143-146}\), and ROS are required for expression of IL-2 after TCR stimulation\(^\text{147}\). Thus, ROS flux is essential for the proper activation of CD4+ T cells. Furthermore, ROS has been shown to influence the differentiation of Th1\(^\text{148, 149}\), Th2\(^\text{149, 150}\), Th17\(^\text{151}\), and iNKT\(^\text{152}\) cells. Continued disruption of redox states, however, can lead to aberrant T cell responses, as is most notably exemplified by the hyporesponsiveness of T cells during conditions of chronic oxidative stress, like that observed in rheumatoid arthritis\(^\text{141}\).

### 1.5 Literature review of the LLO system

The studies described in this dissertation involve two TCR transgenic mouse lines that the Allen laboratory has developed and extensively characterized\(^\text{81, 95, 153, 154}\). These two lines, LLO56 and LLO118, recognize the *Listeria monocytogenes* (Lm) immunodominant epitope LLO(190-205)/I-A\(^b\) with nearly identical affinities, as determined by identical *in vitro* proliferative responses to stimulation with peptide\(^\text{95}\) and surface plasmon resonance assessment of their TCR affinity for LLO(190-205)\(^\text{81}\). Both of these lines are on a B6 background and have been bred to the Rag1\(^{-/-}\).
background to eliminate any secondary TCR rearrangements. The LLO118 line also has the Ly5.1 congeneric marker, whereas the LLO56 line has the Thy1.1 congeneric marker\textsuperscript{95}. In the traditional adoptive transfer model established by our lab, 3,000 naive LLO T cells are injected into a naive B6 mouse, and the recipients are infected with Lm the following day. Following infection with Lm, the LLO118 T cells have been observed to expand more robustly than the LLO56 cells during a primary response, while the LLO56 cells respond better during a secondary response\textsuperscript{95, 96}. LLO56 show impaired proliferative responses \textit{in vivo}, as well as enhanced cellular death\textsuperscript{95}.

To further understand what could be causing the functional differences observed between the LLO cells during a primary and secondary response, our lab interrogated many facets of these two cells in their naive state. Analysis of cell surface phenotype by FACS revealed strikingly few differences: both cells had similar levels of CD28, CD25, CD3, CD4, ICOS, CD40L, PD-1, PD-L1, and CTLA-4, but the one striking difference between the LLO T cells was in CD5 expression\textsuperscript{95}. As CD5 expression has been shown to correlate with a TCR’s affinity for self-pMHC, our lab further investigated the possibility that the two LLO T cells varied in their affinity for self-pMHC, affecting the amount of tonic signaling they were receiving. Results indicated that LLO56 have higher basal phosphorylation of the TCR\(\zeta\) chain\textsuperscript{81}, confirming that LLO56 have greater tonic signaling through the TCR at homeostasis when compared to LLO118. The prior characterization of these two cells has provided me with an invaluable model to address the overarching question considered in this dissertation: does the strength of tonic signaling affect the quality of a CD4+ T cell response during an immune challenge?
1.6 References


Chapter 2: Strength of tonic T cell receptor signaling instructs T follicular helper cell fate

The contents of this chapter have been modified from the following previously published article:

Strength of tonic T cell receptor signaling instructs T follicular helper fate decisions

JM Bartleson, AAV Milam, DL Donermeyer, S Horvath, Y Xia, T Egawa, PM Allen.

*Nature Immunology*, 2020 (in press)

2.1 Abstract

T follicular helper (Tfh) cells are critical in adaptive immune responses to pathogens and vaccines; however, what drives initiation of their developmental program remains unclear. An accumulating body of evidence suggests a T cell antigen receptor (TCR)-dependent mechanism, although it has overlooked a critical aspect of the TCR: basal interactions with peripheral self-peptide. These interactions maintain a broad range of tonic signaling strength within the diverse TCR repertoire, which discretely tunes naive T cell responsiveness to antigenic-peptide. We hypothesized tonic signaling influences early Tfh cell development. Two murine TCR-transgenic CD4+ T cells,
LLO56 and LLO118, that recognize the same antigenic-pMHC but experience disparate strengths of tonic signaling, revealed low tonic signaling promotes Tfh cell differentiation. Polyclonal T cells paralleled these findings, with naive Nur77 expression distinguishing Tfh potential. Two mouse lines were then generated to increase and decrease tonic signaling strength, directly establishing an inverse relationship between tonic signaling and Tfh development. Our findings elucidate a central role for tonic signaling in early Tfh fate decisions.
2.2 Introduction

Naive CD4+ T cells are responsible for coordinating discrete adaptive immune responses to a diverse array of pathogens. Classically, they accomplish this duty by differentiating into one of the major CD4+ T effector cell subsets in response to specific classes of pathogens: T helper (Th) 1, Th2, and Th17. Additionally, naive CD4+ T cells differentiate into T follicular helper (Tfh) cells to mediate T-dependent B cell responses. Tfh cells are critical to initiate and maintain germinal center (GC) reactions, supplying GC B cells with the cytokines and co-stimulatory molecules necessary for somatic hypermutation and affinity maturation of antibodies1-3.

Complete Tfh cell differentiation requires a progression through multiple developmental stages that are dependent upon many factors, but initial commitment to the Tfh-lineage is determined during the naive T cell priming event4. A bifurcation of CD4+ T cells into Tfh and non-Tfh effectors (Teff) can be detected as soon as 48 hours post-activation in vivo5-7, and this divergence of cell fates has been observed as early as 8 hours post-activation in vitro8. While external cues are critical for sustaining Tfh-lineage development, the nearly immediate Tfh/Teff fate polarization post-stimulation of naive CD4+ T cells implies a strong role for a cell intrinsic factor as the initial determinant of Tfh outcome.

One emerging hypothesis is that T cell antigen receptor (TCR) signal strength during activation dictates early Tfh commitment and is driven by TCR avidity for antigenic-pMHC. Many groups have studied the possibility that TCR avidity is instructing early Tfh/Teff fate decisions8-14; however, the field has been contentious in its findings. Some groups have found evidence suggesting strong TCR avidity for antigenic-pMHC promotes Tfh development8-10, others have concluded the opposite11,12, and still others have found no effect13,14. As there is no clear consensus as to whether TCR avidity for foreign antigen can control Tfh-lineage commitment, we wondered
whether other factors could be responsible for intrinsically altering CD4+ T cells to instruct early Tfh/Teff commitment.

Another TCR-dependent element of T cells that has been theorized to tune TCR sensitivity to foreign antigen is basal, peripheral TCR signaling in response to self-pMHC (hereafter referred to as tonic signaling). After positive selection, mature CD4+ T cells exit the thymus and circulate in the periphery where they survey peptide bound to MHC class II (pMHC). In the absence of an immune challenge, homeostatic CD4+ T cell maintenance is dependent on tonic TCR interactions with peripheral self-pMHC15. These tonic TCR signals do not propagate complete T cell activation; yet, this weaker TCR signaling affects basal signaling, gene expression patterns, and metabolic activity of naive CD4+ T cells16-18, 19, 20, 21-24. Importantly, this does not occur as an all-or-none phenomenon. Multiple markers (CD525, Nur7726, 27, Ly6C28, phospho-TCRζ chain18) have informed us of a broad spectrum of tonic signaling strengths experienced by the diverse repertoire of polyclonal CD4+ T cells.

Recent work has begun to appreciate the possibility tonic signaling may act beyond a mechanism of population maintenance and may be responsible for discretely tuning individual CD4+ T cell responses to foreign antigen at the cellular level16, 29, 30. The focus of these studies, however, was on the Teff cell population. We questioned whether strength of tonic signaling experienced by an individual T cell prior to activation could influence early Tfh differentiation. Interestingly, there is evidence suggesting a possible connection between tonic signaling strength and the ability of T cells to support B cell responses in vivo19, 31, but there has been no direct interrogation of the role tonic signaling plays in Tfh development.

To address this question, we first utilized an established experimental model comprised of two TCRtg CD4+ T cells that respond to the same foreign antigen but experience disparate
strengths of tonic signaling. These two CD4+ T cells, LLO56 and LLO118, recognize the same immunodominant epitope from *Listeria monocytogenes* (Lm) with identical affinities; importantly, in steady states, LLO56 experiences strong tonic signaling while LLO118 experiences weak tonic signaling\(^{17,32}\). In this study, we assessed the ability of these two cells to generate a functional Tfh response, and we found LLO118 cells developed a robust Tfh population that was able to assist in the production of long-lived, class-switched, high-affinity antibodies, while LLO56 cells had a nearly absent Tfh subset and could not sustain GC formation, negatively impacting long-lived antibody production. The defect in LLO56 Tfh development could not be rescued by increasing TCR activation strength with a super agonist altered peptide ligand (APL). Furthermore, a genetically inducible voltage-gated sodium channel (Scn5a) expression mouse line\(^{30}\) allowed us to increase tonic signaling in LLO118 cells, which directly inhibited their Tfh response.

Interrogation of the polyclonal CD4+ T cell repertoire paralleled our TCRtg findings. FACS sorted Nur77-low cells generated a significantly greater population of Tfh cells during a primary immune challenge than their Nur77-high counterparts. We also generated a CD11c-cre/DM\(^{gt}\) mouse line to restrict the peripheral self-pMHC repertoire and facilitate an overall reduction in tonic signaling in polyclonal CD4+ T cells, which resulted in an enhanced Tfh response. Collectively, these data establish an inverse relationship between the strength of tonic TCR signaling and Tfh development.
2.3 Results

2.3.1 Tonic signaling strength distinguishes Tfh development in the LLO model

To explore how tonic signaling strength may affect Tfh development and function in vivo, we utilized our novel LLO CD4+ TCRtg system as a way to model the behavior of high and low tonic signaling cells responding to the same antigenic-peptide. This LLO system is comprised of two clones of CD4+ T cells, LLO56 and LLO118, that recognize the same immunodominant epitope (LLO190-205/I-A^b, hereafter referred to as LLOp) from Lm with essentially equal affinities, but LLO56 exhibits stronger tonic signaling in steady state than LLO118 cells. To assess Tfh outcome within the LLO T cell populations, we employed an attenuated actA mutant strain of Lm (actA-Lm) that drives Tfh responses to the immunodominant LLO epitope.

First, we characterized the kinetics of Tfh development within the two LLO T cell populations during a primary immune response. B6 mice received a co-injection of naive LLO56 and LLO118 cells the day prior to infection with actA-Lm, and splenocytes were harvested and analyzed by flow cytometry on days 4, 7, and 10 post-infection. Teff, pre-Tfh, and Tfh cell populations were analyzed within the activated (CD44^hi) CD4+ T cell population based on surface expression of PD-1 and CXCR5. Remarkably, we observed striking differences in the ability of the LLO T cells to generate a CXCR5^hi/PD-1^hi Tfh subset, with an apparent extreme deficiency by LLO56 (Fig. 2.1a,b). In the LLO118 population, a Tfh subset appeared as early as d4 post-infection, peaked at d7, and was drastically reduced by d10. In contrast, the LLO56 population had a barely-detectable Tfh subset at all time-points post-infection (Fig. 2.1b). Total LLO118 cell numbers were also significantly greater than LLO56 (Fig. 2.1c), consistent with our previous observations. The compounding effect of Tfh frequency coupled
with overall LLO cell numbers resulted in a drastic total Tfh number contrast between the two LLO T cell populations at all points during the primary immune response (Fig. 2.1d).

To validate the PD-1/CXCR5 gating strategy within our system, we analyzed Bcl6 expression in the Teff, pre-Tfh, and Tfh cellular subsets for the LLO and B6 CD4+ T cell populations. Bcl6 is the Tfh cell lineage-defining transcription factor\textsuperscript{34-36}, and pre-Tfh cells express an intermediate amount compared to Teff cells\textsuperscript{33}. We found that Bcl6 followed the expected pattern of expression within each genotype (Teff < pre-Tfh < Tfh), while expression in each distinct cellular subset did not differ across genotypes (LLO56 = LLO118 = B6) (Fig. 2.1e). These data confirm our Tfh gating strategy is a true representation of the Tfh cells present in the LLO populations.

Given the antagonistic nature of Tfh/Teff fate decisions, we wanted to know if LLO56 had a commensurate increase in the Teff cellular compartment compared to LLO118. Indeed, at days 4 and 7 post-infection there was a greater frequency of LLO56 Teff cells when compared to LLO118 (Fig.2.1f). Interestingly, while LLO118 did have a greater frequency of pre-Tfh cells than LLO56 early after infection, there was still a notable portion of LLO56 cells with a pre-Tfh phenotype at days 4 and 7 post-infection (Fig. 2.1f). Still, the reduced Tfh frequency in the LLO56 population was not accompanied by an increase in the pre-Tfh compartment, but rather by an increase in the Teff cell subset, suggesting differential skewing of Teff/Tfh fate decisions between the two LLO T cells. Thus, the LLO T cells can be distinguished by both their experienced strength of tonic signaling and their ability to make an early commitment to the Tfh-lineage.
2.3.2 Both LLO T cells generate a dominant Th1 effector response against actA-Lm

We wanted to further characterize the Teff subsets of the LLO T cells to determine whether tonic signaling was associated with differences in the entire T helper (Th) cell response or if it was selective to the Tfh response. We activated LLO T cells in vivo as before and analyzed them on day 7 post-infection for expression of the three classic Th subset master transcription factors: Tbet (Th1), GATA-3 (Th2), and ROR\(\gamma\)t (Th17)\(^{37}\). Roughly 40\% of both LLO T cell populations were expressing Tbet at this time-point (Fig. 2.1g), displaying an equally dominant Th1 effector phenotype in their response to actA-Lm. Flow cytometry analysis also revealed no differences in the frequency of IFN-\(\gamma\) producing cells between the two LLO populations (Fig. 2.1h). IFN-\(\gamma\) MFI was also equivalent at days 4 and 7 post-infection (Fig. 2.1i). By day 10 post-infection, LLO118 cells trended towards a slight advantage in IFN-\(\gamma\) production, although the LLO118/LLO56 MFI ratio was not significantly different than the earlier two time-points (Fig. 2.1i). In sum, although LLO56 had a slight advantage in the frequency of Teff cells, these data suggest that strength of tonic signaling does not affect the quality of Th1 effector responses.

2.3.3 LLO56 and LLO118 have qualitatively distinct Tfh effector profiles

ICOS signaling during the initial naive CD4\(^+\) T cell priming event leads to early Bcl6 expression\(^7\). This allows for the upregulation of CXCR5 and downregulation of the IL-2R, both of which further support Tfh cell development\(^38\). CXCR5 expression homes activated T cells to the T and B cell border where T cells are now be identified as pre-Tfh cells. At this stage, pre-Tfh cells have prolonged interactions with cognate B cells in order to receive the necessary costimulatory signals that allow for ultimate differentiation into the Tfh phase within GCs\(^38\). This process requires a variety of molecules, including ICOS and CD40L\(^3\). It is known that not all pre-Tfh cells will
progress to become fully differentiated Tfh cells, but whether this represents a halting of the Tfh developmental program or possible lineage heterogeneity in the pre-Tfh cell population remains unclear. Although not as robust as in the LLO118 population, LLO56 still generated a pre-Tfh subset despite their terminal Tfh differentiation deficiency. We questioned whether the LLO pre-Tfh subsets were similar and whether both LLO genotypes were capable of performing Tfh effector functions.

As early as day 4 post-infection, the LLO56 population had a greatly reduced frequency of ICOS+ cells in their Teff and pre-Tfh compartments when compared to LLO118, although expression of ICOS in the Tfh subset remained consistent between the two LLO T cells (Fig. 2.2a). By day 7 post-infection, ICOS expression only differed between the two pre-Tfh subsets (Fig. 2.2a). Flow cytometry analysis of CD40L revealed an enhanced frequency of CD40L+ cells in the LLO56 pre-Tfh population at day 4 post-infection and in the LLO56 Teff cell population at day 7 post-infection (Fig. 2.2b). These data suggest the pre-Tfh populations of the LLO T cells appear phenotypically distinct. LLO56 may be unable to terminally differentiate into the Tfh population due to an early defect in ICOS signaling at the Teff and pre-Tfh phases; however, LLO56 may still support early B cell responses at the T-B border through CD40L co-stimulation.

We further analyzed the LLO T cell populations for production of two critical Tfh effector cytokines that promote optimal GC formation: IL-4 and IL-21. We found a greater percentage of the LLO56 population was capable of producing IL-4 at days 7 and 10 post-infection, although LLO118 cells had a higher per cell production of IL-4, as assessed by MFI (Fig. 2.2c). IL-21 results contrasted this, as LLO118 cells could produce more IL-21 as a population at day 7 post-infection with no differences in MFI expression between the two LLO T cells (Fig. 2.2d). These findings reveal differences in Tfh effector qualities between the two LLO T cells, but we were left to
speculate what this specifically implicated for B cell responses; therefore, we next sought to determine how the LLO T cells were directly affecting GCs and subsequent antibody outcome.

2.3.4 Development of NP-LLO^{LT}-N to utilize in a cognate LLO T cell:B cell-help model

To examine direct functional effects the LLO T cells have on the humoral immune response, we developed a hapten-carrier LLO T cell:B cell-help model. We set out to conjugate the LLO protein to 4-hydroxy-3-nitrophenyl acetyl (NP), which would allow us to assess the NP-specific B cell response as a readout of linked antigen recognition with the LLO T cells. To do this, we utilized a mutated version of LLO, named LLO^{LT}, that abrogates the cytotoxicity of the wildtype protein\textsuperscript{40}. Furthermore, we had to consider that while both TCRtg cells recognize LLOp, complete activation of LLO56 is dependent upon two flanking residues in the 190-205 epitope (P10 and P11) while LLO118 does not have the same dependency\textsuperscript{32} (Supplemental Fig. 2.2a), and one of these flanking residues (P11) is a lysine (203), which could be conjugated by the NP-OSu ester (Supplemental Fig. 2.2a). We hypothesized NP would conjugate to K\textsubscript{203} and selectively disrupt LLO56 activation.

\textit{In vitro} T cell stimulation assays confirmed NP-LLO^{LT} did not activate LLO56 cells optimally (Supplemental Fig. 2.2b), so we identified potential amino acid substitutions at residue 203 that could ablate NP conjugation while maintaining recognition by LLO56 T cells. Testing of these mutations \textit{in vitro} revealed one promising candidate: mutating the lysine to an asparagine recovered the activation effectivity of the haptenated protein to near wildtype LLO^{LT} levels (Supplemental Fig. 2.2b). Further testing of NP-LLO^{LT}-N in our adoptive transfer model confirmed LLO56 cells could be activated by the hapten conjugate \textit{in vivo}, and the immunization setup garnered the same Tfh outcome as the acute systemic infection had (Supplemental Fig. 2.2c
and Fig. 2.1a). Successful generation of this hapten-carrier system now allowed us to explore the direct effects of LLO T cell help on B cell and antibody outcomes.

2.3.5 Only low tonic signaling cells (LLO118) are able to support long-lived, high-affinity antibody production

Utilizing NP-LLO\textsuperscript{LT}-N, we implemented an immunization method to analyze direct effects the LLO T cells had on B cells and the resulting humoral response. We transferred either 3,000 LLO56 or 3,000 LLO118 cells into TCR\textsuperscript{α-}/- recipients one day before immunizing recipient mice with NP-LLO\textsuperscript{LT}-N in Alhydrogel: this method ensures the only CD4\textsuperscript{+} T cells able to assist B cells in the immune response are either LLO56 or LLO118, and the lower initial cell transfer amount demonstrates a physiologically relevant outcome. Immunization resulted in similar LLO Tfh differentiation patterns as the actA-Lm infection had, where LLO56 cells had a significantly lower Tfh frequency and total number of Tfh cells than LLO118, even though overall LLO expansion appeared to be similar in this model (Fig. 2.3a-c). At day 7 post-immunization, there were also able to detect fewer NP\textsuperscript{+}-GC B cells in recipient mice that had received transfers of LLO56 cells (Fig. 2.3d and Supplementary Fig. 2.3a for gating strategy).

To validate our GC flow cytometry results, we set out to visualize the GCs generated in this immunization model. We performed immunohistochemistry and fluorescence imaging of whole spleen sections from TCR\textsuperscript{α-} mice that had received either 3,000 LLO56 or 3,000 LLO118 cells prior to immunization with NP-LLO\textsuperscript{LT}-N, and as a control we also immunized TCR\textsuperscript{α-} mice that had received no T cells to determine the baseline effect of adjuvant plus protein (Supplementary Fig. 2.3b). We were able to detect GCs as early as d7 post-immunization (Fig. 2.3e), and when we quantified the total number of GCs/spleen volume, we found significant
The success of sustained GC reactions can be measured by the development of long-lived, isotype-switched, antigen-specific plasma cells\(^{41}\); therefore, we chose to further assess the effects LLO T cells had on B cell responses by analyzing serum anti-NP IgG endpoint titers calculated with control serum that was generated from TCR\(\alpha\)-\(\beta\)-mice that had received LLO T cell transfers but were immunized with the unconjugated LLO\(^{\text{L}}\)T-N protein\(^{42}\). At day 14 post-immunization, there was no observed difference in the ability of LLO56 or LLO118 to assist antibody production; however, by day 21 post-immunization, LLO118-assisted B cells were able to produce isotype-switched, long-lived, high- and low-affinity antibodies, while LLO56-assisted B cells had severely impaired antibody production with up to a 7-fold reduction in endpoint titers at days 21 and 100 post-immunization (Fig. 2.3g). Most notably, high-affinity antibody production at d100 post-immunization was apparent in the LLO118-assisted mice, but was nearly undetectable in the LLO56-assisted mice (Fig. 2.3g).

We wanted to determine if the functional effects observed in humoral outcome were dependent on the quality or quantity of the LLO Tfh cells; therefore, we transferred a supra-physiological amount of LLO T cells prior to immunization to see if we could overcome the GC defects in the LLO56-transferred mice. When 20,000 initial LLO T cells were transferred, we recovered nearly 10-fold as many LLO56 cells at day 7 post-immunization than we had with the 3,000 initial cell transfer amounts (Fig. 2.3h). This led to a near 10-fold increase in the total number of LLO56 Tfh cells as well, since LLO56 Tfh frequencies remained consistent at both cell transfer amounts (Fig. 2.3i,j). This increased the total number of LLO56 Tfh cells to a nearly equivalent number as the LLO118 Tfh cells in the 3,000 cell transfer model. Despite this substantial increase
in the total number of LLO56 Tfh cells, LLO56 were still essentially unable to assist B cells in isotype-switched, long-lived, high-affinity antibody production (Fig. 2.3g). Direct comparison of LLO56 and LLO118 B cell help in the higher initial cell transfer system also revealed a clear impairment in the ability of LLO56 cells to generate NP⁺-GC B cells at d7 post-immunization (Fig. 2.3k). Given these data, we conclude that not only do LLO56 and LLO118 differ in their ability to differentiate into Tfh cells, but the few Tfh cells that LLO56 do generate are qualitatively distinct from LLO118 Tfh cells.

Overall, these findings suggest that strength of tonic signaling primes naive CD4⁺ T cells in a way that influences Tfh-lineage decisions and impacts the resulting humoral immune response. These combined data informed our central hypothesis: strength of tonic TCR signaling predetermines plasticity to the Tfh fate.

### 2.3.6 Disparate Tfh outcome between the LLO T cells is independent of IL-2 signaling

Our laboratory has previously described a positive relationship between self-reactivity and IL-2 production. Specifically, we have shown increased IL-2 production in LLO56 cells compared to LLO118. It is well-established that IL-2 signaling inhibits Tfh development, prompting us to wonder if IL-2 signaling was inhibiting Tfh differentiation in LLO56 cells. A recent study, however, demonstrated the IL-2 producing population of CD4⁺ T cells shortly after activation actually had lower IL-2 signaling, as the IL-2 they generated acted in a paracrine fashion. In T cells, IL-2 signaling occurs via the high-affinity IL-2Rα (CD25); therefore, we analyzed CD25 expression in the LLO T cells.

Assessment by flow cytometry of very early time-points post-\textit{in vitro}-activation revealed a trend of increased CD25 expression in the LLO118 population at 2 and 4 hours post-activation,
but these differences were not sustained by 8 hours post-activation (Fig. 2.4a,b). We interrogated CD25 expression further by assessing the LLO T cells after in vivo stimulation. We found LLO118 Teff cells had an increased frequency of CD25+ cells as well as enhanced CD25 MFI early after activation, demonstrating greater potential for IL-2 signaling in vivo despite their increased Tfh cell development (Fig. 2.4c). Therefore, we concluded IL-2 signaling through CD25 is not responsible for the inability of LLO56 to generate Tfh cells.

Given the important role of IL-2 on T cell survival and previous findings demonstrating a stronger propensity for apoptosis in the LLO56 population when compared to LLO118, we questioned whether LLO56 pre-Tfh cells were dying at a greater rate than the LLO118 pre-Tfh cells. Annexin V staining of CXCR5+ LLO T cells at day 4 post-infection revealed a greater amount of early apoptosis present in the LLO56 pre-Tfh population (Fig. 2.4d); however, CXCR5- LLO56 cells also had greater Annexin V staining than LLO118 CXCR5- cells, and both LLO populations had increased apoptosis in CXCR5+ cells when compared to their CXCR5- counterparts (Fig. 2.4d). This suggests LLO56 cells are undergoing increased levels of apoptosis on a population-wide basis when compared to LLO118, and the defect in Tfh generation by LLO56 is not driven by a lack of pre-Tfh cell survival.

2.3.7 Increasing TCR activation strength does not rescue Tfh developmental defects in the LLO56 population

The above-mentioned study also found a correlation between the Tfh precursor population and strong TCR affinity for antigenic-pMHC, which is supportive of data from others as well. We have previously demonstrated LLO T cells’ TCRs have a nearly identical $K_D$ to LLOp. From this, we have concluded TCR affinity for LLOp does not vary between the two LLO T cells.
However, the binding kinetics between the two LLO TCRs and LLOp are different\textsuperscript{17}, and this could also have effects on T cell activation\textsuperscript{44}. This prompted us to further explore the strength of TCR activation during LLO T cell responses to antigenic-pMHC.

\textit{In vitro} T cell stimulation assays revealed enhanced activation of LLO118 T cells when compared to LLO56 cells at the lowest concentration of stimulating peptide (0.00316\textmu M), as determined by CD69 expression (Fig. 2.4e). LLO118 cells also had a trend of increased TCR\textbeta downregulation at the highest levels of stimulating peptide shortly after \textit{in vitro} activation (Fig. 2.4f). Taken together, these \textit{in vitro} findings suggest LLO118 T cells may be reacting to stimulating peptide more strongly than LLO56. \textit{In vivo} experiments also supported these findings, as LLO118 cells had a significantly greater PD-1 MFI in their Teff subset throughout the course of the primary immune response (Fig. 2.4g). Collectively, this led us to reason that LLO118 cells experience enhanced TCR activation \textit{in vivo} when compared to LLO56.

It is important to note that TCR activation strength is not necessarily independent of tonic signaling. Previous studies have concluded that tonic signaling affects TCR responsiveness to antigenic-pMHC, although there is no clear consensus as to how tonic signaling tunes TCR sensitivity\textsuperscript{16, 17, 23, 30, 32, 45}. Since LLO56 cells have weaker \textit{in vivo} TCR activation than LLO118 cells, we set out to find an APL that would result in enhanced LLO56 activation to determine if we could generate an LLO56 Tfh response similar to that of LLO118. We tested a series of APLs that had been previously generated by our laboratory and focused on one that was particularly promising: LLOp with a V\textrightarrow L point mutation at residue 200, named L200. For LLO56 cells stimulated \textit{in vitro}, L200 consistently generated a greater CD69 response than wildtype LLOp (WT), although this did not reach a level of significance (Fig. 2.4h). Nonetheless, \textit{in vivo} immunizations with the peptides revealed that LLO56 cells expanded much more robustly in
response to L200 than to WT, indicating L200 is a stronger agonist in vivo (Fig. 2.4i). Despite the increase in LLO56 expansion, however, Tfh development between WT- and L200-activated cells was equivalent (Fig. 2.4j). Interestingly, when compared to the actA-Lm infection and protein immunization methods, the peptide immunization model appeared to elicit a stronger Tfh response in the LLO56 cells, which we determined to be commensurate with an increase in the LLO118 Tfh response as well. Thus, increasing activation strength through the use of APLs does not affect Tfh outcome in high tonic signaling cells, further suggesting that the potential for Tfh differentiation is pre-programmed under the naive state prior to recognition of cognate antigenic-pMHC.

2.3.8 Increasing basal TCR signaling in LLO118 cells inhibits Tfh development

We hypothesized strength of tonic signaling instructs early Tfh fate decisions, where low tonic signaling facilitates the Tfh developmental program and high tonic signaling inhibits it. To test this, we took advantage of a knock-in mouse line with inducible expression of Scn5a, a pore forming component of a voltage-gated sodium channel which increases CD4+ TCR sensitivity to self-pMHC and has been previously characterized by our laboratory30,46. To eliminate any possible effect on thymic selection, we bred the Scn5a mouse line to a CD4-creERT2 strain (breeding scheme and Scn5a construct depicted in Fig. 2.5a). This endowed us with temporal control over the increase in TCR sensitivity to self-pMHC. Indeed, after tamoxifen treatment of LLO118/Scn5a/CD4-creERT2 mice, we could detect a GFP+ population of LLO118 cells indicating peripheral Scn5a expression (Fig. 2.5b). When compared to GFP- cells, GFP+ LLO118 cells had a significantly increased CD5 MFI and a significantly reduced Ly6C MFI, both of which indicate an increase in tonic signaling despite equivalent TCRβ expression (Fig. 2.5c-e).
To determine if increasing peripheral tonic signaling results in effects on Tfh development, we tamoxifen treated LLO118/Scn5a/CD4-creERT2 mice and then adoptively transferred the CD4+ T cells into recipients that were immunized with NP-LLO^LT-N the following day. 7 days post-immunization, there was a robustly expanded GFP+ population of LLO118/Scn5a/CD4-creERT2 cells (Fig. 2.5f) and their Tfh development was greatly impaired when compared to their GFP- counterparts (Fig. 2.5g). These data reveal that Tfh differentiation can be directly inhibited by increasing peripheral sensitivity to self-pMHC in naive CD4+ T cells.

2.3.9 Strength of tonic signaling determines Tfh outcome in the polyclonal repertoire

To extend our findings to polyclonal CD4+ T cell responses, we required a robust cell sorting method because of the low clonal frequency of epitope specific T cells in a naive population. It has been shown that a genetic Nur77-GFP reporter mouse offers the broadest range of tonic signaling detection in a polyclonal population^23, 27, which would better facilitate our extensive sorting experiments. We confirmed that Nur77 expression was different between the two LLO T cells by intracellular staining and flow cytometry analysis (Fig. 2.6a), and we then set out to utilize Nur77-GFP mice to test whether tonic signaling was deterministic of Tfh outcome in a polyclonal response. We FACS sorted naive CD4+ T cells from Nur77-GFP mice into two groups representing the top and bottom 25% of Nur77-GFP expression, Nur77-high and Nur77-low, and then we transferred the high- and low-Nur77 expressing cells into recipient mice to be activated in vivo (Fig. 2.6b). Because of the very limited number of LLO naive T cells in a polyclonal repertoire (less than 100, as described by Tubo et al.^47), we utilized the LCMV infection model to generate a stronger polyclonal primary response^48.
When Nur77-high and Nur77-low cells were sorted, transferred, and activated in vivo, we observed a reduction in the frequency of Tfh cells generated by the Nur77-high population in comparison to their Nur77-low counterparts despite there being no consistent differences in the expansion of the two populations (Fig. 2.6c,d). Although the Nur77-high cells did not have a significant increase in their Teff cell compartment (Fig. 2.6e), they did have an observable reduction in their population of pre-Tfh cells, suggesting early Tfh developmental differences from the Nur77-low cells (Fig. 2.6f). These data support our hypothesis and signify the generalizable effects tonic signaling has on early Tfh-lineage commitment in the polyclonal CD4+ T cell population.

2.3.10 Generation of a CD11c-cre/DM<sup>fl</sup> mouse line decreases tonic signaling in polyclonal CD4<sup>+</sup> T cells

Next, we sought a method to alter tonic signaling strength in polyclonal CD4<sup>+</sup> T cells to determine if we could also affect Tfh outcome in diverse TCR repertoires. As we have already demonstrated increased tonic signaling impairs Tfh development, we were particularly interested in utilizing an approach that decreased tonic signaling to test whether we could enhance Tfh differentiation. Broadly altering tonic signaling without also affecting TCR activation has presented a major challenge in the field of tonic signaling; therefore, we devised a new method that allowed us to restrict the self-pMHC repertoire and reduce TCR:self-pMHC interactions to affect tonic signaling strength.

DM is the protein responsible for facilitating the exchange of class II-associated invariant chain peptide (CLIP) for exogenous peptide on MHC-II molecules<sup>49</sup>. Previous groups have demonstrated mice deficient in DM (DMko) have a self-pMHC repertoire that is composed of
essentially only CLIP bound to MHC-II\textsuperscript{50, 51}. These complete DMko mice, however, also showed defects in presentation of antigenic-pMHC. We tested the ability of DMko APCs to present the LLOp \textit{in vitro}, and found when whole protein was the source of peptide, DMko APCs could not present LLOp to the LLO T cells; however, when exogenous peptide was the source, they were capable of presenting LLOp, although at a slightly reduced capacity (Fig. 2.7a). Therefore, we developed a DM deletion strategy that would ensure reduction of self-pMHC presentation without greatly altering TCR:antigenic-pMHC interactions.

First, we generated a conditional knockout allele of H2-DMa by using CRISPR/Cas9 technology to insert two loxP sites flanking the second exon of H2-DMa in B6 zygotes. We then crossed this DM\textsuperscript{loxp} mouse line to the CD11c-cre strain to delete DM selectively from CD11c\textsuperscript{+} APCs, a subset composed primarily of dendritic cells (DCs) (Fig. 2.7b). Our goal in deleting DM from DCs only was to allow for reduced DC-mediated TCR:self-pMHC interactions without affecting B cell-mediated TCR:antigenic-pMHC interactions, as these are critical for Tfh formation.

The CD11c-cre/DM\textsuperscript{loxp} mouse resulted in successful DM deletion from the CD11c\textsuperscript{+} cell subset while mostly unaffected DM expression in B cells (Fig. 2.7c). The deletion of DM was also associated with an expected increase in CLIP presentation within the CD11c\textsuperscript{+} population (Fig. 2.7d). Importantly, the restriction of self-pMHC presentation in the CD11c\textsuperscript{+} population was sufficient to cause polyclonal naive CD4\textsuperscript{+} T cells to reduce tonic signaling, as determined by lower expression of CD5 (Fig. 2.7e). Thus, we successfully developed a system that would allow us to decrease tonic signaling in polyclonal CD4\textsuperscript{+} T cell repertoires and interrogate the effects on Tfh outcome.
2.3.11 Reducing tonic signaling in polyclonal CD4+ T cells increases Tfh development

We employed our CD11c-cre/DM<sup>off</sup> mouse line to determine how decreasing tonic signaling affects Tfh development in diverse TCR repertoires. Since DMko APCs were not able to present peptide processed from whole protein but could present exogenous peptide (Fig. 2.7a), we directly immunized CD11c-cre/DM<sup>off</sup> mice and their littermate controls with LLOp in Alhydrogel. On day 7 post-immunization, we harvested splenocytes and analyzed them by flow cytometry for the presence of Tfh cells within the activated, CD4<sup>+</sup> T cell population. We observed nearly twice as many Tfh cells present in CD11c-cre/DM<sup>off</sup> mice when compared to their littermate controls (Fig. 2.7f). However, the increase in Tfh cell development was not accompanied by a commensurate increase in GC B cells by day 7 post-immunization (Fig. 2.7g). Nevertheless, these data demonstrate that decreasing tonic signaling in a diverse polyclonal T cell population enhances Tfh outcome. This further demonstrates the inverse relationship between tonic signaling strength and Tfh differentiation, and collectively, these findings establish a critical role for tonic signaling in early Tfh fate decisions.
2.4 Discussion

There is great interest in understanding the development of Tfh cells. Current evidence demonstrates a multistep process in the kinetics of canonical Tfh development with three general stages that are defined by distinct locations within secondary lymphoid organs and interactions with specific subsets of APCs. At each stage, a variety of factors affecting Tfh-lineage progression have been identified, including co-stimulatory molecules, cytokines, transcription factors, adhesion molecules, and homing cues. The accumulation of this body of evidence has led to a current model of Tfh development involving an intricate web of variables throughout the course of the immune response. Yet, a fundamental question remains elusive to researchers: why does a T cell initiate the Tfh developmental program rather than a Teff program?

There is speculation that a T cell intrinsic factor may control the earliest Tfh/Teff fate decisions. A growing body of evidence has suggested initiation of the Tfh-developmental program may be set by the TCR; specifically, many groups have investigated how strength of TCR:antigenic-pMHC interactions affects Tfh differentiation. However, their results have been contentious. In this study, we interrogated another TCR-dependent factor for its contribution to Tfh fate decisions: tonic TCR signaling via self-pMHC interactions in the steady state. Our findings reveal that tonic signaling instructs the Tfh differentiation program, where strong tonic signaling impairs Tfh development and weak tonic signaling facilitates it.

We utilized two murine TCRtg CD4+ T cells, LLO56 and LLO118, that recognize the same immunodominant antigenic-pMHC but experience disparate strengths of tonic signaling to reveal that low tonic signaling cells, LLO118, generate a Tfh response, while high tonic signaling cells, LLO56, do not. Polyclonal T cells paralleled these findings, with naive Nur77 expression distinguishing Tfh potential. We also generated two mouse lines to directly increase or decrease
tonic signaling, LLO118/Scn5a/CD4-creERT2 and CD11c-cre/DM\textsuperscript{f/f}, respectively. Both approaches affected T\textsubscript{fh} development in support of our initial findings: strong tonic signaling impairs T\textsubscript{fh} development while weak tonic signaling promotes it. These data establish a direct link between tonic signaling and T\textsubscript{fh} fate decisions.

Overall, these results offer insight into the conflicting studies that interrogated the role of TCR affinity for antigenic-pMHC in T\textsubscript{fh} development. The Sciammas group concluded that increasing TCR signal strength biases a Teff response\textsuperscript{11}. To show this, they used the 5cc.7 TCRtg system with a combination of APLs to demonstrate that cellular concentrations of Irf4 are determined by TCR signal strength. They then induced Irf4 expression in OT-II cells to further show that Irf4 instructs T\textsubscript{fh}/Teff fates by coordinating the Bcl6-Blimp-1 counter-antagonistic gene regulatory module. Interestingly, the magnitude of Irf4 expression is also a proposed mechanism of basal tonic signaling gene regulation \textsuperscript{21}, and the inducible expression of Irf4 in this study occurred many weeks prior to activation of the CD4\textsuperscript{+} T cells, potentially increasing their basal tonic signaling programming. The Williams group also used an experimental approach that increased tonic signaling strength as well as TCR activation strength\textsuperscript{12}. Taking this into account, the findings of these two groups are in support of our data, as strong tonic signaling led to a biased Teff over T\textsubscript{fh} response in their studies and in ours.

Groups that found increasing TCR affinity for antigenic-pMHC promoted T\textsubscript{fh} development utilized methods that relied on either TCRtg models\textsuperscript{9,10} or tetramer-pMHC binding\textsuperscript{8,10} to generate their conclusions. However, in light of this study, these techniques may have inadvertently biased the T\textsubscript{fh} response. For example, the 5cc.7 TCRtg T cells used by the McHeyzer-Williams group have a reported low tonic signaling profile\textsuperscript{45}, which our results suggest would facilitate T\textsubscript{fh} development. Perhaps in low tonic signaling, T\textsubscript{fh}-capable cells, increasing
TCR signal strength plays a complementary role in the promotion of Tfh differentiation. This hypothesis would also explain their observation of increased tetramer-pMHC binding in the polyclonal Tfh compartment, as our data suggests low tonic signaling cells would be the primary contributors to the Tfh subset.

Further delineation of the interplay between TCR activation strength and tonic signaling strength on Teff/Tfh decisions will need to be described in future studies. This will be technically challenging, as many ways of alter tonic signaling strength may also subtly effect TCR activation strength and vice versa. Even in our current study, it is possible that expression of Scn5a is altering TCR activation in a way that is not observable through standard T cell activation read-outs. Similarly, despite utilizing peptide immunization, the absence of DM in the CD11c+ population may still be impacting antigen presentation at the initial priming step. This is why our current approach involved varied manipulations of tonic signaling strength as well as the sorting of unaltered cells based on tonic signaling strength: it is the accumulation of this data that suggests tonic signaling strength influences T cell plasticity into the Tfh developmental program. It will be highly advantageous if future techniques were developed to more definitively separate the effects of tonic signaling and TCR activation strength.

Indeed, tonic signaling strength is most likely not the only determining factor in early Tfh/Teff fate decisions; however, we propose that it does distinguish a naive CD4+ T cell’s Tfh potential. LLO56 appear to have an inhibition of the Tfh developmental program that is independent of the well-established IL-2/Blimp-1 axis of Bcl6 antagonism and TCR activation strength. Conversely, LLO118 appear endowed with Tfh potential such that CD25 must be upregulated in some daughter clones to override commitment to the Tfh lineage and produce Teff cells, a process that could involve input from other signals, such as TCR activation strength,
cytokine signaling, or engagement of costimulatory molecules. This supposes a model of early Teff/Tfh decisions whereby strong tonic signaling either directly obstructs Tfh or promotes Teff development, or weak tonic signaling either directly promotes Tfh or inhibits Teff development. One can speculate that impairment of Tfh development in strong tonic signaling cells could be a mechanism of immune tolerance, as stronger TCR:self-pMHC interactions may facilitate unwanted autoantibody production through GC processes. It is also possible that strong TCR:self-pMHC interactions may somehow disrupt the highly regulated process of pMHC-sensing and high-affinity clonal selection within the GCs; thus, eliminating the formation of these highly self-reactive Tfh cells may be critical for proper adaptive immune responses. Further studies will be needed, however, to determine whether high or low tonic signaling is responsible for Teff/Tfh skewing.

Since tonic signaling likely instructs Tfh fate through early Tfh/Teff decisions, possible mechanisms of this control could be through known early Tfh developmental factors. This study rules out IL-2 signaling and TCR signal strength as primary mechanisms, but in addition to the previously mentioned possible connection between tonic signaling and the magnitude of Irf4 expression, this study opens up two further avenues for potential molecular control of Tfh development: ICOS and IL-6 signaling. While we did not interrogate IL-6 signaling directly, we did see a marginal increase in RORγt expression in the LLO118 population. IL-6 is critical for both Tfh and Th17 differentiation, so this may warrant future studies into the IL-6 signaling pathway in high and low tonic signaling cells. LLO56 cells also had a substantial reduction of ICOS+ cells in Teff and pre-Tfh subsets early after infection in vivo. Owing to the critical role ICOS signaling plays in Tfh formation, it is possible that increased tonic TCR signaling may control Tfh outcome by dampening early ICOS expression. It is important to note, however, that
while the effects of Irf4 expression, ICOS engagement, and IL-6 signaling would all result in downstream destabilization and reduction of Bcl6 expression, the LLO56 cells had comparable Bcl6 expression to the LLO118 cells in the Teff, pre-Tfh, and Tfh subsets. Therefore, there may be previously unidentified mechanisms underlying the Tfh impairment in strong tonic signaling cells.

One possible mechanism is tonic signaling tunes T cell fate through metabolic programming. Our laboratory has recently shown an inverse relationship between the strength of tonic signaling and metabolic activity in CD4+ T cells\textsuperscript{24}. This is further supported by evidence that two primary indicators of tonic signaling strength, CD5 and Nur77, act as regulators of T cell metabolism to alter Teff function \textit{in vivo}\textsuperscript{52-54}. Furthermore, another group has described a tonic LAT-Rasgrp1-mTORC1 pathway that when perturbed to increase metabolic activity in CD4+ T cells drives a Th2 autoimmune condition with spontaneous Tfh formation\textsuperscript{19}. Thus, future studies to elucidate a tonic signaling-metabolic programming-Tfh axis are highly justified.

The findings of this study have a significant impact on our basic understanding of Tfh cell development. As Tfh cells play a central role in the adaptive arm of immunity, this offers tonic signaling as a potential new therapeutic target in the manipulation of Tfh cells for many human-related concerns, including vaccine design, allergic responses, commensal microbiota homeostasis, autoimmunity, and cancer immunity\textsuperscript{2}. For example, in the case of subunit vaccines, the challenge now will be whether you can preferentially target low tonic signaling cells to better design an efficacious vaccine. There are also potential therapeutic methods that could impact tonic signaling strength to tune CD4+ T cell behavior. For example, our inducible Scn5a mouse reveals the possibility that selective voltage-gated channel inhibitors could be used to increase tonic signaling strength and inhibit Tfh development, which could prove beneficial for certain
autoimmune conditions. Additionally, this study may also offer insights into the mechanism underlying the current use of FTY720 to treat autoimmune conditions, since FTY720 administration blocks egress of CD4⁺ T cells from self-pMHC rich areas and effectively increases tonic signaling. If a therapeutic method were designed to decrease tonic signaling in CD4⁺ T cells, it could perhaps be administered prior to a vaccine injection to increase Tfh development and positively alter the humoral outcome. This could prove especially useful for people that would otherwise not generate a protective humoral response to the vaccine alone. Indeed, our results highlight the necessity of a consideration for tonic signaling in previous studies as well as future experimental designs.
2.5 Methods

Mice

All mice were bred and housed in specific pathogen-free conditions of the animal facility at Washington University Medical Center. All use of laboratory animals was approved and carried out in accordance with the Washington University Division of Comparative Medicine guidelines. Initial generation of the LLO TCR transgenic mouse lines has been previously described by our laboratory\textsuperscript{32}. LLO56 and LLO118 are maintained by breeding to a Rag1\textsuperscript{-/-}, homozygous congenic marker (LLO56-Thy1.1; LLO118-Ly5.1) background. B6 (C57BL/6J, Stock No. 000664), TCR\textsuperscript{\alpha-/-} (B6.129S2-Tcra\textsuperscript{tm1Mom/J, Stock No. 002116}), CD4-creERT2 [B6(129X1)-Tg(Cd4-cre/ERT2)11Gnri/J, Stock No. 022356], and CD11c-cre [B6.Cg-Tg(Itgax-cre01-1Reiz/J, Stock No. 008068] mice were all initially purchased from The Jackson Laboratory (Bar Harbor, ME) and subsequently maintained in our animal facility. To generate the LLO118/Scn5a/CD4-creERT2 line, LLO118 and CD4-creERT2 were crossed to the FSF-Scn5a mouse line also previously detailed by our laboratory\textsuperscript{30}. To generate the DM\textsuperscript{\textalpha f} mouse line, CRISPR/Cas9 technology was used to insert two loxP sites flanking exon 2 of H2-DMa in B6 zygotes. The Nur77-GFP mouse strain [B6.FVB(Cg)-Tg(Nr4a1-EGFP)GY139Gsat/WeisMmucd, RRID:MMRRC_036737-UCD] was obtained from the Mutant Mouse Resource and Research Center at University of California, Davis, an NIH-funded strain repository, and was donated to the MMRRC by Arthur Weiss, M.D., Ph.D., University of California, San Francisco\textsuperscript{26}. All mice were aged 6-12 weeks at the start of every experiment, and both sexes were used throughout the experiments. Sex and age matching and co-housing were done within experiments to the greatest extent possible.
**Cell transfers, mouse infections, protein and peptide immunizations**

For LLO56, LLO118, and LLO118/Scn5a/CD4-creERT2 transfer experiments, CD4⁺ T cells were enriched from spleen by negative selection using a CD4⁺ T Cell Isolation Kit (Miltenyi Biotec). Cells were then transferred iv into recipient mice, and 12-24 hours later the mice were either infected iv with 10⁷ cfu of actA-deficient *Listeria monocytogenes* (strain DPL1942₅₅) or immunized as indicated. NP-conjugated protein immunizations were performed by ip injection of mice with 100µg of the conjugated protein in equal parts adjuvant aluminum hydroxide gel, Alhydrogel (InvivoGen). For peptide immunizations, mice were injected ip with 10µM peptide in 200µl of a 1:1 PBS:Alhydrogel mixture. For the Nur77-high/low and CD5-high/low transfers, CD4⁺ T cells were first negatively selected from spleen using a CD4⁺ T Cell Isolation Kit (Miltenyi Biotec) and then stained for CD4, CD44, CD62L, CD25, and CD5. Naive CD4⁺ T cells (CD4⁺/CD44lo/CD62L⁺/CD25⁻) in the top and bottom 25% of Nur77-GFP or CD5 expression were sorted using a BD FACSAlirII cytometer. 7.5-10x10⁶ cells were injected iv into recipient mice (transferred amounts varied across experiments, not within). One day later, recipient mice were infected ip with 2x10⁵ PFU Armstrong strain LCMV₅₆.

**Flow cytometry**

All samples were analyzed on BD FACS CantoII or BD LSR Fortessa cytometers (BD Biosciences), and data were analyzed using FlowJo software 10.5.3 (Treestar). Gating strategies are described in figure legends. For all Tfh and GC B cell analysis experiments, 2.5 million lymphocyte events were acquired to ensure proper detection of the rare populations. The following antibodies and reagents were used for flow cytometry analysis: anti-CD44 [(FITC; clone IM7; Biolegend; cat. no. 103006), (PE; clone IM7; Biolegend; cat. no. 103008), (AlexaFluor 700; clone
IM7; Biolegend; cat. no. 103026), anti-CD62L [(FITC; clone MEL-14; Fisher Scientific; cat. no. BDB553150), (PE; clone MEL-14; Biolegend; cat. no. 104408), (PerCP-Cy5.5; clone MEL-14; Biolegend; cat. no. 104432), (APC; clone MEL-14; Biolegend; cat. no. 104412)], anti-CD4 [(FITC; clone RM4-5; Biolegend; cat. no. 100510), (PE; clone RM4-4; Biolegend; cat. no. 116006), (APC; clone RM4-5; Biolegend; cat. no. 116014), (eFluor 450; clone RM4-5; Fisher Scientific; cat. no. 501129511), (APC-Cy7; clone RM4-5; Biolegend; cat. no. 100526), (AlexaFluor 700; clone GK1.5; Biolegend; cat. no. 100430), (PE-Cy7; clone GK1.5; Fisher Scientific; cat. no. 501129609), (PerCP-Cy5.5; clone RM4-5; Fisher Scientific; cat. no. 5015746)], anti-TCRβ (PerCP-Cy5.5; clone H57-597; Biolegend; cat. no. 109228), anti-CD3ε [(FITC; clone 145-2C11; Biolegend; cat. no. 100305), (PE-Cy7; clone 145-2C11; Biolegend; cat. no. 100320), (PE; 145-2C11; Biolegend; cat. no. 100308)], live/dead stain (violet; Life Technologies; cat. no. L34955), anti-CD45.1 [(PE-Cy7; clone A20; Fisher Scientific; cat. no. 501129620), (FITC; clone A20; Biolegend; cat. no. 110706), (PerCP-Cy5.5; clone A20; Fisher Scientific; cat. no. BDB560580)], anti-CD90.1 [(FITC; clone HIS51; Fisher Scientific; cat. no. BDB554894), (PerCP-Cy5.5; clone HIS51; Fisher Scientific; cat. no. 501123735), (eFluor 450; clone HIS51; eBioscience; cat. no. 48-0900-82)], anti-CXCR5 (APC; clone 2G8; Fisher Scientific; cat. no. BDB560615), anti-PD-1 (eFluor 450; clone J43; Fisher Scientific; cat. no. 501129091), anti-ICOS (Biotin; clone 7E.17G9; BD Biosciences; cat. no. 552145), anti-CD40L (PE; clone MR1; Fisher Scientific; cat. no. 12-1541-81), anti-CD69 [(FITC; clone H1.2F3; Biolegend; cat. no. 104506), (PE; clone H1.2F3; Biolegend; cat. no. 104508), (APC; clone H1.2F3; Biolegend; cat. no. 104514), (PE-Cy7; clone H1.2F3; Biolegend; cat. no. 104512)], anti-CD19 (APC-Cy7; clone 6D5; Biolegend; cat. no. 115530), anti-B220 [(eFluor 450; clone RA3-6B2; Fisher Scientific; cat. no. 501129551), (APC; clone RA3-6B2; Fisher Scientific; cat. no. 50-149-73), (PE; clone RA3-6B2;
Biolegend; cat. no. 103208)], anti-Fas (PE-Cy7; clone JO2; Fisher Scientific; cat. no. BDB557653), anti-GL7 (FITC; clone GL7; Biolegend; cat. no. 144604), anti-IgD ((PE; clone 11-26c.2a; Biolegend; cat. no. 405705), (PerCP-Cy5.5; clone 11-26c.2a; Biolegend; cat. no. 405709)), NP-PE (Fisher Scientific; cat. no. NC1316967), anti-CD25 [(FITC; clone PC61; Biolegend; cat. no. 102006), (eFluor 450; clone 3C7; Affymetrix; cat. no. 48-0253-82)], anti-CD5 [(PE-Cy7; clone 53-7.3; Biolegend; cat. no. 100622), (PE; clone 53-7.3; Fisher Scientific; cat. no. BDB553022)], anti-Ly6C (PacBlue; clone HK1.4; Biolegend; cat. no. 128013), anti-CLIP (FITC; clone 15G4; Santa Cruz Biotechnology; cat. no. sc-53946 FITC), anti-CD11c (PE-Cy7; clone N418; Biolegend; cat. no. 117318), anti-CD16/CD32 Fc block (clone 93; Biolegend; cat. no. 101330), anti-I-A/I-E (PE; clone M5/114.15.2; Biolegend; cat. no. 107629), and Streptavidin [(PE-Cy7; Biolegend; cat. no. 405206), (FITC; Fisher Scientific; cat. no. BDB554060)], Annexin V (PE; Biolegend; cat. no. 640908), and 7-AAD (BD Pharmingen; cat. no. 5168981E).

**Intracellular FACS staining**

For intracellular cytokine analysis, splenocytes were first stimulated with 1ng/ml PMA (Sigma-Aldrich) plus 1μg/ml ionomycin (Sigma-Aldrich) at 37° C for 30 min, followed by a 4 hr incubation with 2 μg/ml brefeldin A (Sigma-Aldrich). Splenocytes were then washed and stained for surface proteins, followed by fixation and permeabilization in accordance with the Foxp3/Transcription Factor Staining Buffer Kit (eBioscience). The Foxp3/Transcription Factor Staining Buffer Kit was also used after surface staining for all transcription factor staining and for intracellular staining of DM. The following antibodies were used for intracellular flow cytometry analysis: anti-Bcl6 (PE; clone BCL-DWN; Fisher Scientific; cat. no. 501122326), anti-Tbet (PE; clone eBio4B10; Fisher Scientific; cat. no. 5010893), anti-GATA-3 (PE-Cy7; clone TWAJ; Fisher
Scientific; cat. no. 501129305), anti-RORγt (BV421; clone Q31-378; BD Biosciences; cat. no. 562894), anti-IFN-γ (FITC; clone XMG1.2; Biolegend; cat. no. 505806), anti-IL-4 (Biotin; clone BVD6-24G2; BD Biosciences; cat. no. 554390), anti-IL-21 (PE; clone FFA21; Fisher Scientific; cat. no. 501172), anti-H2-DM (clone 2E5a; Fisher Scientific; cat. no. 552405), anti-rat IgG1 (FITC; clone RG11/39.4; Fisher Scientific; cat. no. BDB553892), and anti-Nur77 (PE; clone 12.14; Fisher Scientific; cat. no. 5011028).

**Protein and peptide generation**

All peptides used in this study were purchased from Peptide 2.0 Inc., purified by reverse-phase high pressure liquid chromatography, and analyzed by mass-spectroscopy. A pET29b expression vector containing the His-tagged LLO<sub>LT</sub> sequence was provided by Dr. Emil Unanue (Washington University, St. Louis, MO). For generation of LLO<sub>LT</sub>-N, site-directed mutagenesis was used to change the lysine at position 203 to an asparagine (N). Mutated clones were confirmed by sequencing. LLO<sub>LT</sub> and LLO<sub>LT</sub>-N were expressed in BL21 (DE3) competent cells (ThermoFisher) and purified as previously described. Protein purity was confirmed by SDS-PAGE. For conjugation of NP to LLO<sub>LT</sub> and LLO<sub>LT</sub>-N, 0.5mg of NP-OSu (LGC Biosearch Technologies) was added to 5mg of either LLO<sub>LT</sub> or LLO<sub>LT</sub>-N in 10 equal fractions over 20 minutes. The solution was then incubated at room temperature with rotations for 2 hours before being dialyzed into 0.1M NaHCO<sub>3</sub>, 145mM NaCl, pH 8.5. NP:LLO<sub>LT</sub>(-N) ratio was determined with the following extinction coefficients: LLO<sub>LT</sub>(-N) = 75750 M<sup>-1</sup>cm<sup>-1</sup> and NP = 4230 M<sup>-1</sup>cm<sup>-1</sup>. Ratios of NP:LLO<sub>LT</sub>(-N) ranged from 5:1-10:1, and batches of NP-LLO<sub>LT</sub>(-N) were aliquoted and stored at -20°C to allow for continuity across experiments.
**T cell stimulation assays**

Bone marrow-derived macrophages (BMDM) were plated in a 96-well plate at 1x10^5 cells per well in I-10 media (IMDM, 10% FBS, pen/strep, gentamycin, and 2-ME). BMDM were stimulated for the first 24 hr with IFN-γ (2000 U/ml) and then were washed with PBS and rested for another 24 hr in fresh I-10 media before T cell and antigen plating. To isolate CD4^+ T cells from the spleen of LLO transgenic mice, a negative CD4^+ selection kit (Miltenyi) was used, and 5x10^5 CD4^+ T cells were subsequently plated on top of the BMDMs along with the protein or peptide of interest. T cells were then harvested from the wells at various time-points post-plating for FACS analysis. Generally, cells were harvested 18-24 hr post-plating unless otherwise specified.

**Microscopic imaging of germinal centers**

Mice were sacrificed and perfused with ice-cold PBS. Spleens were harvested and snap frozen in OCT tissue-freezing solution and stored at -80°C. 8μm sections were cut, mounted to slides, and fixed with ice-cold acetone. Immediately prior to performing immunohistochemistry, sections were rehydrated and then blocked with 5% FCS. Reagents used to stain GCs were: IgD (PE, clone 11-26c.2a, Biolegend) and PNA (fluorescein labeled, Vector Labs). Hoechst staining was then performed and slides were mounted with Fluoromount G. Fluorescent images were taken of the entire spleen sections using a fully automated, brightfield/fluorescence slide scanning system (AxioScan.Z1, ZEISS). Spleens were imaged at 40X. Images were stitched in Zen Software (Blue Edition) v3.1 (ZEISS) and then uploaded to Imaris Software v8.4 (Bitplane) for quantification of GCs, which were manually counted for each image and defined as dense areas (>50μm in diameter) of PNA staining within IgD^+ follicles. Analysis was performed blinded, and two sections
were averaged for each individual spleen value. TCRαβ mice that received no T cell transfers but were immunized with NP-LLOLT-N were used as controls.

**Anti-NP ELISAs**

Serum collected from mice was aliquoted before storage at -70°C to ensure only one freeze-thaw cycle for all samples. The night before our ELISAs were performed, Immulon Two ELISA plates were coated with 5µg/ml of NP-BSA at either a 2:1 or 28:1 NP:BSA ratio, hereafter referred to as NP(2)-BSA or NP(28)-BSA (purchased from LGC Biosearch Technologies). To detect low-affinity antibodies, NP(28)-BSA was used, and to detect high-affinity antibodies, NP(2)-BSA was used. The next day, plates were washed and blocked with 0.5% BSA in PBS for 1 hr at RT. Plates were washed again and serum was added for 1 hr at RT. After another washing, an anti-mouse IgG-HRP antibody (Southern Biotech, cat. no. 1030-05) was added at 1:5000 for 45 min at RT. Final washes were performed, One-Step Ultra TMB Substrate was added to each well for 2 minutes before adding H2SO4 and measuring A450. Endpoint titers were determined as previously described42. Control serum was generated from TCRαβ mice that had received LLO cell transfers but were immunized with the unconjugated protein (LLOLT-N) instead of NP-LLOLT-N.

**Tamoxifen Treatment for CD4-creERT2 induction**

Tamoxifen (Sigma-Aldrich) was suspended in corn oil (Sigma-Aldrich) at a concentration of 100mg/ml. Mice were orally gavaged for three consecutive days with 50µl of the tamoxifen solution for a total treatment of 5mg of tamoxifen per day. Efficiency was determined by monitoring GFP expression in the CD4+ T cell population of LLO118/Scn5a/CD4-creERT2 mice.
Statistical Analysis

The only experiments in which investigators were blinded to sample identity was during the analysis of microscopy images. In immunization experiments involving LLO T cell transfers into TCRα−/− recipients, samples were excluded from analysis if an LLO T cell population did not expand beyond background antibody staining levels, as determined by control mice that were immunized but did not receive LLO T cell transfers. All statistical analysis was performed using Prism v8.2.1 (GraphPad Software, Inc.), and statistical significance is indicated as follows: ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05. Statistical tests used are stated in each figure legend and were two-tailed unless otherwise stated. Sample size and number of replicate experiments performed are also indicated in figure legends.
2.6 Author contributions

J.M.B. designed and performed experiments and wrote the manuscript; A.A.V.M, S.H., Y.X., and T.E. assisted in performing experiments; D.L.D. assisted in performing experiments and generated the \textit{in vitro} APL data; and P.M.A. designed experiments and wrote the manuscript.
Figure 2.1. High and low tonic signaling cells (LLO56 and LLO118, respectively) generate equivalent Th1 immune responses, but differ in their ability to produce a Tfh population. 20,000-100,000 naive LLO56 and LLO118 cells were co-transferred into recipient B6 mice and then infected with actA-Lm the following day. Spleens were harvested on the indicated days post-
infection for flow cytometry analysis of the activated LLO T cell populations (Supplemental Fig. 2.1). Data collected from each individual recipient mouse are paired. a, Representative flow plots depicting Teff, pre-Tfh, and Tfh PD-1/CXCR5 gating strategies. Numbers shown are the frequency of each subset within the activated LLO parent population. b, Quantification of the frequency of Tfh cells. Three independent experiments for day 4 (n=13), eight for day 7 (n=31), and two for day 10 (n=10). c, Total numbers of activated LLO T cells. Three independent experiments for days 4 (n=13) and 7 (n=14), two for day 10 (n=10). d, Total numbers of LLO Tfh cells from the same experiments as in (c). e, Percentage difference in Bcl6 MFI of the paired pre-Tfh and Tfh subsets for each genotype relative to the average B6 Teff subset at day 7 post-infection. Data are from the same experiments as in (c) and exclude mice with no LLO56 Tfh generation. f, The frequency of Teff and pre-Tfh cells from the same experiments as in (b). g, LLO T cells were assessed for Tbet, GATA-3, and RORγt expression via intracellular staining at day 7 post-infection. Three independent experiments (n=14) are shown. h, Splenocytes were stimulated with PMA and ionomycin before intracellular cytokine staining was performed to assess frequency and i, MFI of IFN-γ expression in the activated LLO populations. Three independent experiments for days 4 (n=15) and 7 (n=14) and two for day 10 (n=7). MFI data shows the mean ± SEM. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05. Paired t test or Wilcoxon matched-pairs signed rank test for nonnormally distributed data (b-d, f-h). Two-way ANOVA using tukey’s multiple comparisons test for comparison of subsets across genotypes and Sidak’s multiple comparisons test for comparisons among subsets within each genotype (e). One-way ANOVA analysis (i).
Figure 2.2. LLO56 and LLO118 have distinct Tfh effector qualities. 100,000 naive CD4+ T cells of each LLO genotype were co-transferred into recipient B6 mice and infected with actA-Lm the following day. Spleens were harvested post-infection for flow cytometry. a. Frequencies of
ICOS$^+$ cells and b, CD40L$^+$ cells in the Teff, pre-Tfh, and Tfh subsets of the LLO populations. Data points from individual recipient mice are paired. Three independent experiments for both days 4 (n=15) and 7 (n=14). c, On the indicated day post-infection, splenocytes were harvested and stimulated with PMA and ionomycin before intracellular cytokine staining was performed. The frequency of cytokine producing cells as well as cytokine MFI, given as a ratio of LLO118/LLO56 for each recipient mouse, are shown for IL-4 and d, IL-21. For both (c) and (d), three independent experiments were performed for day 4 (n=14) and two for days 7 (n=10) and 10 (n=7). MFI data show the mean ± SEM. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. Two-way ANOVA or tukey’s multiple comparisons test (a). Paired t test or Wilcoxon matched-pairs signed rank test for nonnormally distributed data (b-d). One-way ANOVA analysis (MFI data for c, d).
Figure 2.3. Low tonic signaling cells (LLO118) support long-lived, high-affinity antibody production, while high tonic signaling cells (LLO56) do not. 3,000 LLO56 or LLO118 cells
were transferred into recipient TCRα−/− mice and immunized one day later with NP-LLOLT-N. On day 7 post-immunization, splenocytes were analyzed by flow cytometry for a, total number of activated LLO T cells, b, Tfh cell frequency of the LLO T cell populations, c, total number of LLO Tfh cells, and d, frequency of GC, NP+-B cells (see Supplemental Fig. 2.3a for gating) Six independent experiments (n=16 for LLO56 and n=14 for LLO118). e, 3000 LLO56 or LLO118 cells were transferred and activated as in (a). Spleens were harvested post-immunization for immunohistochemistry analysis. GC staining: PNA (yellow), IgD (white), and Hoechst (blue). Representative images are shown for three independent experiments at day 7 (n=6 for LLO56 and LLO118, n=4 for controls) and two at d10 (n=3 for LLO56 and LLO118, n=4 for controls). Scale bars (bottom left corners) = 300μm. Controls are TCRα−/− mice that were immunized with NP-LLOLT-N but had received no prior T cell transfers (representative images in Supplemental Fig. 2.3b). f, For images obtained in (e), quantification of the number of GCs is shown. GCs were manually counted in a blinded manner and then normalized to spleen volume. Two sections per individual mouse were averaged. g, TCRα−/− mice receiving either 3,000 (data represented by the left Y axis) or 20,000 (data represented by the right Y axis) LLO56 or LLO118 cells were immunized with NP-LLOLT-N, and serum was collected on days 14, 21, and 100 post-immunization. Endpoint titers were determined with serum from TCRα−/− mice that had received LLO T cell transfers but were immunized with unconjugated protein, LLOLT-N. High- and low-affinity antibodies were determined by coating ELISA plates with NP(2)-BSA and NP(28)-BSA, respectively. For 3,000 cell transfers, data represent three independent experiments for all time-points [(d14: n=8 for LLO56, n=7 for LLO118), (d21: n=11 for LLO56, n=9 for LLO118), (d100: n=10 for both LLO genotypes)]. For 20,000 cell transfers, four independent experiments for day 14 (n=16 for LLO56, n=19 for LLO118), three for d21 (n=13 for both LLO genotypes), and two
for day 100 (n=8 for both LLO genotypes). h, 20,000 LLO56 or LLO118 cells were transferred and activated as in (a). On day 7 post-immunization, splenocytes were analyzed by flow cytometry for the total number of activated LLO T cells, i, frequency of Tfh cells within the LLO populations, j, total number of LLO Tfh cells, and k, frequency of NP+ GC B cells. Three independent experiments (n=9). All data represent mean ± SEM. ***p < 0.001, **p < 0.01, *p < 0.05. Unpaired t test or Mann-Whitney test for nonnormally distributed data (a-d, g-k). One-way ANOVA (f).
Figure 2.4. LLO56 Tfh impairment is independent of IL-2 signaling and cannot be rescued by increasing TCR activation strength. a. In vitro T cell stimulation assays with LLO T cells
and LLOp to determine the frequency of CD25+ cells within the activated LLO T cell populations [live/single/dump−(I-A/I-E)/CD4+/CD69+], as well as b, the MFI of CD25 at 2-8 hours post-activation. Assay was performed in duplicate; two independent experiments. c, 100,000 naive LLO T cells were co-transferred into recipient B6 mice and infected with actA-Lm the following day. Splenocytes were analyzed on day 4 post-infection for CD25+ frequencies and MFI in the Teff, pre-Tfh, and Tfh subsets of the activated LLO populations. Two independent experiments (n=10). 

d, Annexin V staining in the CXCR5− and CXCR5+ subsets of activated LLO T cells at day 4 post-infection. Three independent experiments (n=15). e, In vitro T cell stimulation assays with LLO T cells and LLOp. T cells were harvested 4-36 hours post-activation and analyzed for the frequency of CD69+ cells in the LLO populations (live/single/dump−/CD4+). The highest (left graph, 10µM) and lowest (right graph, 0.00316 µM) peptide concentrations used for stimulation are shown. Data points are the duplicate averages and the paired LLO values are shown for each independent experiment (three for the 16 hour time-point and two for all others). f, In vitro LLO T cell stimulation assays with LLOp to assess TCRβ expression. Assay was performed in duplicate, and data are from two independent experiments. g, 20,000-100,000 naive LLO56 and LLO118 cells were co-transferred into recipient B6 mice and infected with actA-Lm the following day. Splenocytes were analyzed for expression of PD-1. Percentage differences in PD-1 MFI are shown relative to the average LLO56 PD-1 MFI for each time-point. Data are paired points from individual recipient mice; two independent experiments for day 3 (n=8), three for day 4 (n=13), 8 for day 7 (n=31), and two for day 10 (n=10). h, In vitro LLO56 T cell stimulation with either LLOp (referred to as WT) or LLOp with a point mutation of V→L at residue 200 (referred to as L200). CD69+ frequency within the LLO56 population one day after stimulation. Assay was done in duplicate, and data points for LLO56 + WT and LLO56 + L200 are shown paired for each
independent experiment: two for $10^{-3}$ and $10^{-4}$ μM concentrations, three for all other concentrations. i, j, 100,000-200,000 naive LLO56 were transferred into recipient B6 mice and then immunized with 10μM of either WT or L200 peptide. On day 7 post-immunization, splenocytes were analyzed for (i) frequency of activated LLO56 cells and (j) frequency of Tfh cells in the activated LLO56 cell population. LLO118 cells were also transferred into recipient B6 mice and immunized with WT peptide for Tfh analysis. In (i), only experiments with transfers of 100,000 cells are shown to keep expansion frequencies consistent (three independent experiments, n=15), and in (j) data are shown from all transfer amounts (six independent experiments, n=22 for LLO118 and n=25 for LLO56). All data represent mean ± SEM. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05. Unpaired t tests with correction for multiple comparisons using the Holm-Sidak method (a, b, e, f, h). Tukey’s multiple comparisons test (c). Paired t test or Wilcoxon matched-pairs signed rank test for nonnormally distributed data (d, g). Unpaired t test (i). Dunn’s multiple comparisons test (j).
Figure 2.5. Increasing basal TCR signaling in LLO118 cells inhibits Tfh development. a, Outline of the Scn5a construct that was used to generate the F/S/F-Scn5a mouse, previously
described\textsuperscript{30}, and the breeding scheme to generate the LLO118/Scn5a/CD4-creERT2 line. Upon tamoxifen treatment of this line, ectopic Scn5a expression in peripheral CD4\textsuperscript{+} T cells can be detected by GFP expression. b, Representative FACS plots showing GFP detection in LLO118 cells analyzed 7 days after tamoxifen treatment. Both the LLO118/Scn5a/CD4-creERT2 mouse and its littermate control were treated with tamoxifen. Gating shows the parent LLO118 population: live/single/CD4\textsuperscript{+}/TCR\textbeta\textsuperscript{+}/CD44\textsuperscript{lo}. c-e, FACS analysis of CD5, Ly6C, and TCR\textbeta MFI of LLO118 GFP\textsuperscript{+} and GFP\textsuperscript{-} T cells from LLO118/Scn5a/CD4-creERT2 mice 7 days post-tamoxifen treatment. Gating on the LLO118 population was done as described in (b). Data points are the paired GFP\textsuperscript{+}/GFP\textsuperscript{-} populations from individually treated mice; data shown are from 3 independent experiments (n=7). f, Analysis of LLO118/Scn5a/CD4-creERT2 expansion during a primary immune response. To activate LLO118/Scn5a/CD4-creERT2 cells \textit{in vivo}, LLO118/Scn5a/CD4-creERT2 mice were treated with tamoxifen and 7 days later the LLO118/Scn5a/CD4-creERT2 CD4\textsuperscript{+} T cells were enriched and transferred into TCR\textalpha\textsuperscript{-} recipient mice. The following day, recipient mice were immunized with NP-LLO\textsubscript{LT}-N. Graphs show the frequency of GFP\textsuperscript{+} cells in the LLO118 populations immediately prior to the transfer (unstimulated, left graph) and 7 days post-immunization (d7, right graph). Graphs depict data from 3 independent experiments (n=7-9). Mean ± SEM are shown. g, In the same experiments as (f), \textit{in vivo}-activated LLO118/Scn5a/CD4-creERT2 cells were also analyzed by FACS at day 7 post-immunization for the frequency of Tfh cells within the GFP\textsuperscript{+} and GFP\textsuperscript{-} populations. \(*\ast\ast\ast p \textless 0.0001, \ast\ast p \textless 0.01, \ast p \textless 0.05\). Paired t test or Wilcoxon matched-pairs signed rank test for nonnormally distributed data (c-e, g). Unpaired t test or Mann-Whitney test for nonnormally distributed data (f).
Figure 2.6. Nur77 expression distinguishes Tfh outcome in the polyclonal repertoire. a, Naive LLO T cells and B6 T cells (defined as: live/single/CD4+/CD44lo/CD62L+/CD25-) were assessed.
by intracellular staining and flow cytometry for the expression of Nur77. Representative histograms are shown and Nur77 MFI is quantified in the LLO populations by determining the percentage differences relative to the average LLO56 Nur77 MFI for each experiment. Two independent experiments are shown (n=6-7). b, Depiction of the Nur77-sort experimental setup. Naive (live/single/CD44lo/CD62L+/CD25−, shown in Supplemental Fig. 2.4) CD4+ T cells were FACS sorted from Nur77-GFP donors into two populations: Nur77-high (top ~25% of GFP expression) and Nur77-low (lowest ~25% of GFP expression). 7.5-10x10^6 cells were transferred into individual TCRα−/− recipients for each sorted population, and one day later recipient mice were infected with LCMV-Armstrong. c, Following the experimental protocol in (a), infected recipient mice were harvested on day 7 post-infection and splenocytes were analyzed by FACS for the total number of activated T cells (live/single/CD4+/CD3+/GFP+/CD62Llo) within each Nur77-sorted population. d, The frequency of Tfh cells, e, frequency of Teff cells, and f, frequency of pre-Tfh cells within the activated Nur77-high/low populations from (c) are shown. Data points for (c-f) are the connected Nur77-high/low pairs from each independent experiment; 3 experiments total (n=3). All data represent mean ± SEM. ***p < 0.001, *p < 0.05. Unpaired t test (a). Paired t test (c-f).
Figure 2.7. Restricting the self-pMHC repertoire decreases tonic signaling and enhances Tfh development in polyclonal CD4+ T cells. a, In vitro T cell stimulation assays were performed
with LLO56 cells and either whole LLO protein or LLOp in the presence of BMDMs from control (B6) and DMko mice. LLO56 cells were harvested 18 hours post-activation and assessed by FACS for the frequency of CD69+ cells in the live/ single/ CD4+ population. Assays were performed in duplicate and representative graphs of three independent experiments are shown (n=3). b, Depiction of the generation of a CD11c-cre/DMlof/f mouse line. c, FACS histograms showing DM expression and d, CLIP bound to MHCII expression in the CD11c+ and B220+ cellular subsets of CD11c-cre/DMlof/f mice and their littermate controls. Histograms are representative of two independent experiments (n=5-6). e, For the same mice analyzed in (c, d), CD5 expression in the naive (live/ single/ TCRβ+/ CD44lo/ CD62Lhi/ CD25- ) CD4+ T cell population was also assessed by FACS and the percentage differences in MFI are shown relative to the average control MFI for each experiment. Data are from two independent experiments (n=5-6). f, CD11c-cre/DMlof/f mice and their littermate controls were immunized directly with LLOp and splenocytes were assessed day 7 post-immunization for the frequency of Tfh cells in the activated, polyclonal CD4+ T cell populations (activated T cells: live/single/CD4+/ TCRβ+/CD44hi/CD62Llo, Tfh cells: CXCR5hi/PD-1hi). Data are representative of two independent experiments (n=8). g, In the same experiments as (f), splenocytes were also analyzed for the frequency of GC B cells within the CD19+/B220+ population (GC B cells: live/single/CD19+/B220+/GL7+/IgDlo/Fas+). All data represent mean ± SEM. *p < 0.05. Unpaired t test or Mann-Whitney test for nonnormally distributed data (e-g).
Supplemental Figure 2.1. Gating on activated CD4⁺ T cells in the LLO co-transfer model.

20,000 LLO56 and 20,000 LLO118 cells were co-transferred into a B6 recipient and infected with actA-Lm the following day. Splenocytes were harvested on day 7 post-infection. Representative flow plots are shown depicting the complete gating strategy for activated CD4⁺ T cells (live/single/CD4⁺/CD44⁺) within the LLO56 (CD90.1⁺), LLO118 (CD45.1⁺), and B6 (CD90.1⁻/CD45.1⁻) populations. For all plots, the numbers shown represent the frequency of the population within the drawn gate.
Supplemental Figure 2.2. Generation of NP-LLOLT-N. a, A schematic depicting the LLOp epitope. The core residues are in purple, flanking residues in green, and the lysine that is hypothesized to bind NP and interfere with LLO56 activation is highlighted in yellow. b, In vitro T cell stimulation assays were performed with LLO56 cells in the presence of either LLOLT, NP-
LLO\textsuperscript{LT}, or NP-LLO\textsuperscript{LT}-N. T cells were harvested 24 hours post-stimulation and assessed by flow cytometry for upregulation of CD69. The graph shown is representative of 2 independent experiments. 

c, 20,000-500,000 naive LLO cells were co-transferred into naive B6 mice and then immunized with NP-LLO\textsuperscript{LT}-N to determine Tfh cell frequencies in the LLO populations at days 4, 7, and 10 post-immunization. Representative flow plots are shown for one experiment (n=5), numbers represent the cell frequencies in the Teff, pre-Tfh, and Tfh gates.
Supplemental Figure 2.3. Gating strategy for NP\textsuperscript{+}-GC B cell analysis by flow cytometry and control images for GC B cell fluorescence imaging. a, Representative flow plots depicting the gating strategy for NP\textsuperscript{+}-GC B cells (live/single/CD19\textsuperscript{+}/B220\textsuperscript{+}/GL7\textsuperscript{+}/IgD\textsuperscript{lo}/Fas\textsuperscript{+}/NP\textsuperscript{+}). Included is a control LLO\textsuperscript{LT-N} immunized mouse for NP-gating reference. For all plots, the numbers shown represent the frequency of the population within the drawn gate. b, Representative images from TCR\textalpha\textsuperscript{+} mice that received no T cell transfers but were immunized with NP-LLO\textsuperscript{LT-N} in
Alhydrogel. On days 7 and 10 post-immunization spleens were harvested and prepared for immunohistochemistry. Sections were stained with PNA (yellow), IgD (white), and Hoechst (blue). Fluorescent images were taken of entire spleen sections, and images are representative of n=4 for each time-point, 3 independent experiments for day 7 and 2 independent experiments for day 10. Scale bars (bottom left corners) = 500µm.
Supplemental Figure 2.4. Gating on naive CD4+ T cells post-CD4 enrichment. Spleens from naive Nur77-GFP mice were harvested and a CD4 enrichment was performed prior to antibody staining for flow cytometry analysis. Shown are representative flow plots depicting the complete gating strategy for a naive CD4+ T cell population (live/single/CD4+/CD62L+/CD25-/CD44lo) that will be further sorted by Nur77-GFP expression, as shown in Fig. 2.6b. For all plots, the numbers shown represent the frequency of the population within the drawn gate.
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Chapter 3: Tonic T cell receptor signaling inversely regulates the basal metabolism and mitochondrial reactive oxygen species production of CD4+ T cells

Some of the contents of this chapter have been modified from the following previously published article:

Tonic TCR signaling inversely regulates the basal metabolism of CD4+ T cells

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3.1 Abstract

Whether or not tonic TCR signaling can affect the metabolic programming of naive CD4+ T cells has not been definitively established. Here, we employed LLO118 and LLO56 to help address this question, as we have previously shown these two CD4+ TCR transgenic (TCRtg) T cells have disparate strengths of tonic signaling during homeostasis. We performed metabolic profiling on
the two LLO TCRtg cells, and we found that naive LLO118 (low tonic signaling cells) had a dramatically higher basal respiration rate, maximal respiration rate, and glycolytic rate relative to LLO56 (high tonic signaling cells). We extended the correlation of low self-reactivity (CD5lo) with increased basal metabolic activity to polyclonal CD4+ T cells through 2-NBD-glucose uptake analysis of the CD5hi and CD5lo naive T cell populations. We hypothesized the reduced overall metabolic activity in the high tonic signaling cells was a result of strong interactions with self-pMHC, as mediated through TCR signaling. To test this, we utilized an inducible knock-in mouse expressing the Scn5a voltage gated sodium channel: a channel that when expressed in peripheral CD4+ T cells, enhances basal TCR-mediated signaling. Indeed, Scn5a expression in peripheral, polyclonal CD4+ T cells resulted in an expected decrease of overall metabolic activity. Additionally, we also reduced the self-pMHC repertoire via the DMlo mouse line to decrease tonic signaling in naive polyclonal CD4+ T cells, which resulted in their enhanced metabolic activity and further supported our hypothesis. Genes and Metabolites analysis of LLO118 and LLO56 T cells identified a metabolic bifurcation point that was centered at glycerol 3-phosphate. In LLO118 T cells, this metabolite is directed towards the glycerol phosphate shuttle via the action of mitochondrial glycerol phosphate dehydrogenase (mGpd2), a process that has been implicated in the production of mitochondrial reactive oxygen species (ROS). Therefore, we interrogated the levels of ROS in CD5hi and CD5lo cells, unveiling increased production of ROS in CD5lo CD4+ T cells that was commensurate with their increased basal metabolic activity. Finally, we confirmed that the metabolic activity of naive CD4+ T cells is being actively maintained in the periphery through tonic TCR signaling via self-pMHC presentation by a subset of CD11c+ antigen presenting cells. Overall, these studies highlight the critical relationship between peripheral TCR:self-pMHC interaction, metabolism, and ROS production.
3.2 Introduction

Initial metabolic analyses of T cells focused on differences between resting and activated cells. These studies established that naive T cells mainly utilize oxidative phosphorylation (OXPHOS) to generate the relatively low bioenergetic needs required by their quiescent state, while activated T cells shift their metabolism from OXPHOS to aerobic glycolysis in order to support the anabolic reactions demanded by clonal expansion and effector differentiation \(^1,^2\). However, metabolic networks influence T cell function beyond simply meeting the energy demands of the T cell. A multitude of signaling pathways essential for various T cell functions are closely interconnected with metabolic programming, either through shared signaling components or directly through metabolite regulation \(^3\). In the last decade, complex roles for distinct metabolic programming in nearly every facet of the T cell response to antigen have been further elucidated, encompassing expansion, differentiation, effector function, and memory cell formation and maintenance \(^3\). Conversely, while there has been some work interrogating the metabolic intricacies of naive, quiescent T cells, this remains a relatively underappreciated area of T cell biology.

The dampening of metabolic activity in quiescent T cells is an active process \(^4\). Studies have unveiled a critical role for mTOR1 inhibitors (i.e., TSC1, PTEN, and LKB1) in the enforcement of a quiescent program in naive, peripheral T cells. These molecules have been shown to have distinct effects on the regulation of T cell homeostasis; interestingly, their deletion also uniquely modifies how T cells respond to stimulation \(^5^7\). This highlights the possibility that metabolic networks may prime naive T cells to tune their eventual response to antigen, leading us to question whether there is metabolic heterogeneity within the naive CD4+ T cell population.

Activation of CD4+ T cells involves the integration of multiple variables: TCR signaling, co-stimulation, and cytokine instruction. All three of these activating components have been
shown to affect the metabolism of CD4+ T cells. In a naive state, cytokine signaling and TCR signaling, through tonic self-pMHC interactions, also occur. IL-7R signaling has been previously shown to affect naive T cell metabolism, but this was considered in an all-or-none manner where no IL-7R signaling lead to the death of naive T cells due to an inability to meet their quiescent bioenergetic needs 8; therefore, we wanted to determine whether subtle differences in tonic TCR signaling could generate a metabolically heterogenous pool of naive CD4+ T cells.

Peripheral TCR:self-pMHC interactions are distinct from thymic-self-pMHC interactions, as they do not induce the same signals required for positive selection. Instead, peripheral tonic TCR signaling involves low-level stimulation that doesn’t propagate canonical activation pathways, but rather generates nuanced effects on the activation state of the T cell and gene expression levels 9,10. There is a wide range of tonic signaling strengths in the naive polyclonal T cell population, as shown by Nur77 11,12 and CD5 13 expression. This implies that interactions of an individual TCR with a specific self-pMHC ligand controls the level of tonic signaling on a cellular basis.

Several studies involving blockade of TCR:self-pMHC interactions, either with anti-MHC class II antibodies or genetic deletion of MHC class II on APCs, have revealed the role tonic signaling plays in survival, homeostatic expansion, and antigen reactivity of CD4+ T cells 14-24. Interestingly, tonic signaling has also been linked to metabolic activity in memory CD4+ T cells. When deprived of class II interactions, memory T cells responded poorly and had indications of overall diminished metabolic activity relative to T cells that maintained TCR:self-pMHC interactions 14,16,19. The two primary indicators of tonic TCR signaling strength, CD5 and Nur77, have also been shown to act as regulators of T cell metabolism post-activation to alter Teff function in vivo 25-27.
To explore whether tonic TCR signaling could influence a naive T cell’s metabolic programming, we first employed LLO56 and LLO118. Here we show that naive LLO118 cells, which have low tonic signaling, exhibit heightened basal metabolic activity and increased levels of ROS production when compared to their high tonic signaling counterparts, LLO56. We then extend these findings to polyclonal high and low tonic signaling CD4+ T cells, and we confirm that the metabolic regulation is dependent upon peripheral TCR:self-pMHC interactions, as maintained by a CD11c+ antigen presenting cell subset. Collectively, these data establish a previously unidentified link between tonic TCR signaling and basal metabolic activity in CD4+ T cells.
3.3 Results

3.3.1 CD4+ T cells with low tonic signaling are metabolically more active than CD4+ T cells with high tonic signaling

To compare the basal metabolic profiles of LLO56 and LLO118, splenocytes of naive, age-and sex-matched matched mice were enriched for CD4+ T cells, and Seahorse platform analysis was performed on the enriched populations. The respiratory rate of LLO118 cells was higher than that of LLO56 cells, as evidenced by the higher oxygen consumption rate (OCR) of these cells (Fig. 3.1a). Both baseline and maximal respiration, reached after addition of the uncoupling reagent carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), was higher in naive LLO118 CD4+ T cells than in naive LLO56 cells (Fig. 3.1b-c). Spare respiratory capacity (SRC), which has been defined as a cell’s energy reserve capable of fueling cellular function above and beyond basic energy needs, was also higher in LLO118 than in LLO56 \(^{28}\) (Fig. 3.1d). Likewise, glycolytic function was greater in LLO118 than in LLO56, as evidenced by the higher basal extracellular acidification rate (ECAR) and the higher maximal ECAR of LLO118 cells (Fig. 3.1e-g). Interestingly, we found no difference in the ratio of OCR to ECAR in LLO56 and LLO118 cells, indicating they have similar relative reliance on respiration and glycolysis for meeting their energy needs (Fig. 3.1h). The same metabolic differences between LLO118 and LLO56 were observed when the LLO T cells were activated \textit{in vivo} with \textit{Listeria} and harvested on day 7 post-infection, where LLO118 T cells had a greater OCR and ECAR than LLO56 cells (Fig 3.1i-l). Overall, these findings indicate that the less self-reactive LLO118 CD4+ T cell is more metabolically active overall in both its naive and activated state than the more self-reactive, LLO56 cell.
3.3.2 Increased 2-NBD-glucose uptake correlates with higher basal metabolic activity, and low tonic signaling polyclonal T cells have significantly greater uptake of 2-NBD-glucose than their high tonic signaling counterparts

Since we observed enhanced glycolysis in LLO118 compared to LLO56, we also sought to determine if glucose uptake was enhanced in these cells. To test this, we measured in vitro uptake of 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG), a non-metabolize-able glucose analog, in LLO56 and LLO118 T cells. Complementing our observation of higher glycolysis in LLO118 cells, 2-NBDG uptake was approximately twice as high in LLO118 cells as it was in LLO56 cells (Fig. 3.2a,b). 2-NBDG uptake was also measured in vivo following intraperitoneal (IP) injection, and we observed higher 2-NBDG uptake in LLO118 cells, relative to LLO56 cells (Fig. 3.2c). We were further able to extend this finding into polyclonal T cells, where we observed an inverse correlation between CD5 levels and 2-NBDG uptake (Fig. 3.2d,e). In C57BL/6J mice, peripheral CD4+ T cells with the lowest 10% of CD5 expression took up significantly more 2-NBDG than peripheral CD4+ T cells with the highest 10% of CD5 expression in vitro (Fig. 3.2d,e). A similar distinction in 2-NBDG uptake between CD5lo and CD5hi cells was observed for CD8+ T cells as well (Fig. 3.2e). These findings show an inverse correlation between strength of TCR:self-pMHC interactions and metabolism exist for both polyclonal CD4+ and CD8+ T cells.

3.3.3 Peripheral CD4+ T cells with increased sensitivity to self-pMHC have decreased basal metabolism

To further elucidate the relationship between self-reactivity and metabolism in polyclonal CD4+ T cells, we sought a method that would allow us to genetically control metabolic activity through
manipulation of tonic TCR signaling. To this end, we employed a knock-in mouse line with inducible expression of Scn5a. Scn5a is the pore-forming component of a voltage-gated sodium channel (VGSC) typically expressed in cardiac myocytes. We previously reported that when Scn5a is ectopically expressed in DP thymocytes, they are endowed with enhanced signaling to weak self-pMHC ligands during positive selection. Scn5a expression in CD4+ T cell hybrids equips them with increased sensitivity to self-pMHC, to the level that they are capable of responding to their positive-selecting peptide. Recently, we demonstrated that expression of Scn5a in peripheral CD4+ T cells (using Scn5a+CD4-Cre+ mice) resulted in increased proximal TCR signaling and increased peripheral CD5 expression. This expression of Scn5a in the LLO118 T cells led to an impaired in vivo response to L. monocytogenes infection. To directly test the impact of increased sensitivity to self-pMHC on metabolism, we used Seahorse platform analysis to compare Scn5a+CD4-Cre+ mice to Scn5a+CD4-Cre-negative littermates. Mirroring the differences we observed in LLO56 and LLO118 basal metabolism, we found that increasing self-sensitivity in Scn5a+CD4-Cre+ mice led to a compensatory decrease in respiration and glycolysis, as determined by OCR and ECAR (Fig. 3.2f). These data indicate that increasing TCR signaling sensitivity in naive CD4+ T cells reduces their basal metabolic activity.

3.3.4 Reducing the self-peptide repertoire increases the basal metabolic activity of polyclonal naive CD4+ T cells

We previously described the generation of a DMff mouse, which when bred to a CD11c-cre strain resulted in a reduction of self-pMHC presentation to CD4+ T cells, effectively decreasing tonic signaling strength globally in the polyclonal CD4+ T cell repertoire (Fig. 2.7b,e). Here, we wanted to definitively show that reducing TCR:self-pMHC interactions in the periphery is sufficient to
affect the basal metabolic programming of naive CD4+ T cells, so we bred the DM°/° line to a UBC-creERT2 strain. The UBC-creERT2/DM°/° mouse line endows us with genetic, temporal control over the deletion of DM. Upon tamoxifen treatment, DM is deleted ubiquitously, although incompletely, from all antigen presenting cell (APC) subsets within these mice. We allowed the mice to mature to adult age, ensuring their circulating T cell repertoire was fully developed, and then we administered tamoxifen by gavage for 3 consecutive days. By 7 days after the last tamoxifen treatment, only ~20% of B cells are still expressing DM (Fig. 3.2g) which corresponds with ~75% of B cells expressing CLIP bound to MHCII (Fig. 3.2g). These frequencies are essentially maintained through day 35 post-tamoxifen treatment (Fig. 3.2g). Two months post-tamoxifen treatment, CD4+ T cells were harvested from the UBC-creERT2/DM°/° mice, along with their tamoxifen treated littermate cre-negative controls, and Seahorse analysis was performed. The results indicate that when CLIP bound to MHCII is the predominant self-pMHC in the repertoire, polyclonal CD4+ T cells have enhanced basal metabolic activity, as determined by increased OCR and ECAR rates (Fig. 3.2h). These data further support our hypothesis that the basal metabolic activity of CD4+ T cells is being inversely regulated by homeostatic TCR:self-pMHC interactions.

3.3.5 A CD11c+ APC subset independent of the cDC1 lineage is responsible for maintaining tonic signaling through the presentation of self-pMHC

Interestingly, little is known about the maintenance of tonic signaling in CD4+ T cells. Groups have shown that MHCII is necessary to sustain homeostatic TCR signaling through either the use of complete MHCII knockout mice or MHCII blocking antibodies23, 32. Other groups have interrogated whether a specific group of APCs is responsible for maintaining tonic signaling in CD4+ T cells, but their experimental approach utilized the complete abrogation of cellular subsets
This approach left ambiguity in their conclusions, as there remained the possibility that some other APC factor besides self-pMHC presentation could also be necessary for sustaining tonic signaling. Our DM$^{\text{eff}}$ mouse line offered us a novel experimental approach to definitively address the question of whether it is specifically self-pMHC presentation by a particular APC subset that is responsible for CD4+ T cell tonic signaling maintenance.

We chose to first cross the DM$^{\text{eff}}$ mouse line to the CD19-cre and CD11c-cre strains to determine whether B cells or CD11c+ cells could sufficiently maintain tonic signaling through self-pMHC presentation. Analysis of CD5 expression in naive CD4+ T cells from the CD19-cre/DM$^{\text{eff}}$ and CD11c-cre/DM$^{\text{eff}}$ mice revealed that self-pMHC presentation by CD11c+ but not CD19+ APCs is required to sustain tonic signaling in CD4+ T cells (Fig. 3.3a,b). These results are in alliance with the previous literature\textsuperscript{17}. The CD11c+ APC population is further comprised of various APC subsets, however, including the cDC1 and cDC2 lineages; therefore, we obtained the Xcr1-cre mouse line from the laboratory of Kenneth Murphy\textsuperscript{33} and crossed it to the DM$^{\text{eff}}$ mouse line in an effort to further delineate whether self-pMHC presentation by cDC1s is required for CD4+ T cell tonic signaling maintenance. We interrogated the naive CD4+ T cell populations from CD11c-cre/DM$^{\text{eff}}$ and Xcr1-cre/DM$^{\text{eff}}$ mice, and CD5 expression was not altered in Xcr1-cre/DM$^{\text{eff}}$ mice (Fig. 3.3c). This reveals that a specific subset of APCs may indeed be responsible for the presentation of self-pMHC, and that subset is independent of the cDC1 lineage.

### 3.3.6 FTY720 can be used therapeutically to modulate CD4+ T cell tonic signaling and metabolic activity \textit{in vivo}

Given the peripheral, transient nature of TCR:self-pMHC interactions, we wondered whether we could therapeutically manipulate tonic signaling to modulate the basal metabolic activity of CD4+
T cells. T cells isolated from blood display diminished tonic signaling, suggesting TCR:self-pMHC interactions occur primarily in lymphoid tissue\textsuperscript{23}. Mature naive T cells are continuously circulating between blood and secondary lymphoid organs, so they naturally experience occasional breaks of tonic TCR signaling; therefore, we speculated we could utilize the S1PR1 antagonist, FTY720 \textsuperscript{34, 35} to effectively increase tonic signaling by trapping T cells in lymphoid tissue. To test this hypothesis, we utilized the low tonic signaling LLO118 cells to see if we could reverse their tonic signaling and metabolic profiles with FTY720. After administration of FTY720 (as previously described \textsuperscript{36}) to naive LLO118 mice, we did indeed see enhanced tonic signaling, as determined by an increase in CD5 expression (Fig. 3.4a). Furthermore, this enhanced tonic signaling was accompanied by an overall decrease in metabolic activity, as indicated by Seahorse analysis of OCR and ECAR rates (Fig. 3.4b). These data highlight the therapeutic potential of manipulating CD4+ T cell metabolic activity \textit{in vivo} via modulation of tonic TCR signaling. Since FTY720 is a drug commonly used to treat autoimmune disorders, these experiments also have deeper implications on the basic mechanisms underlying the effectivity of this drug.

3.3.7 Identification of metabolic pathway differences between the LLO T cells in their activated states

To gain unbiased insight into differences between LLO118 and LLO56 CD4+ T cells, we performed transcriptional profiling of naive LLO118 and LLO56 T cells and those isolated at day 7 post-activation \textit{in vivo}. The naive cells had few transcriptional differences, with no obvious candidates to explain their distinct characteristics; therefore, we chose to analyze the activated cell data in the context of metabolic networks by utilizing Genes and Metabolites (GAM) analysis \textsuperscript{37}. Using this innovative approach, we found coordinated changes in a number of metabolic pathways
(Fig. 3.5). Overall, LLO118 cells appeared to be metabolically much more active than LLO56 cells, consistent with our Seahorse analysis (Fig. 3.1). We found a metabolic bifurcation point that discriminated between LLO118-like metabolism and LLO56-like metabolism, centered at glycerol 3-phosphate (Fig 3.5 inset). In LLO118 T cells, this metabolite can be directed towards the glycerol phosphate shuttle via the action of mitochondrial glycerol phosphate dehydrogenase (mGpd2). In LLO56 T cells, however, glycerol 3-phosphate is directed towards glycerolipid metabolism via the enzyme glycerophosphocholine phosphodiesterase 1 (Gpcpd1). The glycerol phosphate shuttle is a secondary mechanism that allows NADH generated in the cytosol by glycolysis to contribute to oxidative phosphorylation in the mitochondria, thereby sustaining ATP production (reviewed in 38). The rate-limiting enzyme for the glycerol phosphate shuttle is mGPD2, and this enzyme has been linked to effects on TCR signaling via the production of mitochondrial ROS (mitoROS) 39. Thus, we next sought to determine whether the differences in metabolic activity between low and high tonic signaling T cells was also connected to production of mitoROS.

3.3.8 ROS production is inversely regulated by the strength of tonic TCR signaling, correlating with metabolic activity

To ascertain if ROS production varied in high and low tonic signaling cells, we began with an investigation of cellular ROS in the LLO TCRtg T cells. First, we stained naive LLO T cells with 2’-7’-Dichlorodihydrofluorescein diacetate (DCFDA) to assess the redox state of the cells 40. In their naive, resting state, LLO CD4+ T cells displayed significant differences in their cellular production of ROS, as LLO118 T cells had increased fluorescence of the DCFDA probe when compared to LLO56 (Fig. 3.6a). Interestingly, the ROS production of LLO56 was at a level consistent with the population average of polyclonal B6 CD4+ T cells (Fig. 3.6.a). We sought to
determine whether the ROS production differences were unique to the naive T cell state, or whether there were also differences after activation; therefore, we stimulated CD4+ T cells with PMA and ionomycin, and 24 hours post-stimulation, we harvested the cells and assayed their redox state again using DCFDA. LLO118 T cells displayed a greater level of ROS production even after stimulation (Fig. 3.6b); however, LLO56 cells had a steeper increase in ROS production from their basal to their activated state relative to both the LLO118 and B6 CD4+ T cells (Fig. 3.6c).

Curious as to whether the differences in ROS production could be extended to polyclonal high and low tonic signaling CD4+ T cells, we assessed the level of mitochondrial ROS (mitoROS) production in high and low tonic signaling cells from naive B6 mice. We defined the high and low tonic signaling populations as cells within the top and bottom 20% of CD5 expression within the naive CD4+ T cell population, respectively. Strikingly, the CD5lo population exhibited a much greater production of mitoROS at a resting, basal timepoint when compared to their CD5hi counterparts (Fig. 3.6d). This parallels our LLO TCRtg findings and further suggests that the strength of tonic TCR signaling inversely regulates not only basal metabolic activity, but ROS production as well.

Previously, we showed that deleting DM from CD11c+ cells independent of the cDC1 lineage reduces tonic signaling in naive, polyclonal CD4+ T cells (Fig. 3.3c). Therefore, we set out to determine whether ROS production could also be modulated through this method of restricting the self-pMHC repertoire. MitoSOX staining revealed that mitoROS production is increased in naive CD4+ T cells harvested from CD11c-cre/DMff mice when compared to their WT littermate controls (Fig. 3.6e), correlating with their reduced tonic signaling strength (Fig. 3.3c). Furthermore, analysis of mitoROS in naive CD4+ T cells from Xcr1-cre/DMff mice displayed no differences from their WT littermate controls (Fig. 3.6e). Thus, mitoROS production is inversely
regulated by the strength of tonic signaling, as set by TCR:self-pMHC interactions between the CD4+ T cells and CD11c+ APCs independent of the cDC1 lineage.
3.4 Discussion

TCR interactions with self-pMHC are critical for the development and maintenance of the adaptive immune system. While the developmental role of TCR:self-pMHC interactions during thymic education of T cells is well-established (reviewed in 41), how TCR:self-pMHC interactions maintain and influence the behavior of mature CD4+ T cells in the periphery remains a relatively unexplored area of T cell biology. In a polyclonal T cell repertoire, there is a spectrum of homeostatic TCR signaling strengths set by self-pMHC interactions. In this present study, we describe a role for tonic signaling in regulating the overall basal metabolic activity and ROS production of naive CD4+ T cells. We show that cells on opposite ends of that tonic signaling strength spectrum display unique metabolic characteristics; whereby, there is an inverse relationship between the strength of tonic signaling and basal metabolic activity.

This study elucidates coordinated, controlled metabolic heterogeneity within the naive CD4+ T cell repertoire. Given the emerging evidence of functional differences in CD4+ T cells with different strengths of self-pMHC reactivity, one can postulate that the metabolic tuning of naive CD4+ T cells via the TCR may contribute to the diversity of CD4+ T cell responses to foreign antigen12, 13, 30, 42, 43. Interestingly, anergic self-reactive T cells and Tregs have also been shown to have altered metabolism relative to naive cells 44, and the development of both of these populations is heavily influenced by the strength of their TCR:self-pMHC interactions. Other factors besides the level of TCR affinity for self-pMHC may also be involved in regulating metabolism, however, such as cytokine receptor signaling and even the availability of self-pMHC in a tissue-dependent manner. Future investigation will need to establish how signaling through the TCR via self-pMHC controls basal metabolism. Roose and colleagues have made the important finding that tonic mTORC1 signals in naive CD4+ T cells influence T cell fate decisions, and the
Ras exchange factor Rasgrp1 is necessary to generate tonic mTORC1 signals. Additionally, since quiescent T cells actively dampen metabolic activity through the engagement of mTOR1 inhibitors (i.e., TSC1, PTEN, and LKB1), the well-established involvement of TORC1 in controlling cellular metabolism offers a highly plausible potential mechanism by which tonic TCR signaling may inversely regulate basal metabolism.

There are some limitations of our current experiments. Presently, it is very difficult to enhance tonic signaling in T cells. We have developed the Scn5a+ model system, which does show an increase in tonic signaling, but we do not know how the Scn5a+ voltage-gated sodium channel is enhancing tonic signaling or how it relates to the signaling pathways normally involved in tonic signaling. This is why our approach involved a variation of methods to manipulate tonic signaling (FTY720 and Scn5a expression to enhance it, utilization of the DMf/f mouse to limit the peripheral self-pMHC repertoire and reduce it) as well as an interrogation of the unmanipulated T cell repertoire through assessment of the uptake of the glucose analog, 2-NBD-glucose. While we found increased uptake of 2-NBD-glucose in naive B6 polyclonal T cells with low tonic signaling, future studies of glucose metabolism in naive CD4+ T cells will also be difficult given current techniques, as naive T cells are relatively metabolically inactive and have too low of an uptake of the labelled compounds needed to trace metabolic pathways and products. These limitations will make uncovering the connection between tonic signaling strength and basal metabolism challenging, although not altogether impossible.

Ultimately, future studies will also be needed to determine whether the tuning of metabolic activity via tonic signaling strength is directly responsible for the post-activation functional differences observed between low and high tonic signaling CD4+ T cells. Through the analysis of transcriptional data by the Genes and Metabolites program, we identified the glycerol
phosphate shuttle as one potential key metabolic pathway difference between T cells with high and low basal metabolisms. Thus, cells with higher basal metabolism require an increased glycerol phosphate shuttle function to provide NADH generated in the cytosol by glycolysis to contribute to oxidative phosphorylation in the mitochondria. Flavell and colleagues have recently shown that the malate aspartate shuttle, another mechanism through which cytoplasmic NADH is shuttled to the mitochondria, was important in T cell differentiation \(^{46}\). The malate aspartate shuttle was necessary for the proliferation of T helper cells, whereas, succinate dehydrogenase subsequently antagonized differentiation and enforced terminal effector function. This study identified the enzyme mitochondrial glycerol phosphate dehydrogenase (mGPD2) as a potential key enzyme in the enhanced metabolism of T cells with weak self-pMHC interactions. mGPD2 has been recently shown by Horng and colleagues to be a key regulator of glucose-oxidation in LPS-stimulated macrophages in the optimal control of inflammatory gene induction and suppression \(^{47}\). mGPD2 has also been previously shown to play a role in mitochondrial ROS production\(^{39}\). In support of this, we observed a higher amount of basal mitoROS in the more metabolically active, low tonic signaling cells when compared to their high tonic signaling counterparts. ROS produced shortly after T cell stimulation are essential for the activation of the transcription factors NF-κB and AP-1, and TCR activation pathways can be affected by ROS through the oxidation of protein thiols\(^{48, 49}\). Thus, it would be of great interest to further explore whether the ROS differences between low and high tonic signaling cells can affect their behavior during an immune response.

Overall, our findings reveal an inverse relationship between the strength of tonic signaling in naive CD4+ T cells and their basal metabolic activity and ROS production, with important implications for T cell activation, differentiation, and function. For example, in this study, we utilized FTY720 to exemplify the therapeutic potential of modulating tonic signaling as a means
to influence the metabolism of CD4+ T cells. Generally, metabolic pathways are conserved across cellular subsets, so exclusively targeting the metabolism of T cells in an effort to control their function presents a challenge; thus, our results offer tonic signaling as a potential novel way of altering the metabolic activity and function of T cells specifically, which could ultimately be exploited to benefit a range of human-related diseases and disorders.
3.5 Methods

Mice
The LLO56 and LLO118 TCR transgenic lines, specific for listeriolysin O (190-205) (LLO_{190-205}/I-A^b), have been previously described^{22,30,43}. These mice were maintained on a Rag1-knockout background with homozygous congenic marker expression (LLO118-Ly5.1; LLO56-Thy1.1). B6 mice were acquired from The Jackson Laboratory and subsequently maintained in our animal housing facilities. Derivation of the polyclonal Scn5a-transgenic mouse (and its subsequent crossing to a CD4-Cre line) has also been previously described^{30}. To generate the \( H2-DMa^{ff} \) mouse line, CRISPR/Cas9 technology was used to insert two loxP sites flanking exon 2 of \( H2-DMa \) in B6 zygotes. \( Itgax-cre \), herein referred to as CD11c-cre [B6.Cg-Tg(Itgax-cre01-1Reiz/J, Stock No. 008068] and CD19-cre [B6.129P2©-Cd19^{tm1(cre)Cgn}/J, Stock No. 006785] mice were also initially purchased from The Jackson Laboratory and subsequently maintained in our animal facility. The Xcr1-cre mouse was generously given to us from the laboratory of Kenneth Murphy^{33}. All mice were bred and housed in a specific pathogen-free facility at Washington University, according to guidelines established by the Washington University Division of Comparative Medicine.

Seahorse analysis
OCR and ECAR were measured using a 96-well XF extracellular flux analyzer (Seahorse Bioscience) and Extracellular Flux Assay Kit (Agilent Seahorse XFe96). Assay setup has been previously described^{50-53}. Briefly, cells were plated in XF media supplemented with glucose (25mM), L-glutamine (2mM), and sodium pyruvate (1mM). Measurements were taken at basal state, and after the step-wise addition of oligomycin (1μM), FCCP (1.5μM), and rotenone (100μM)
plus antimycin A (1µM). The mGPD2 specific inhibitor, iGP-1 (Calbiochem/EMD Millipore #5.30655.0001) was dissolved in DMSO and added to the extracellular flux analyses at either 2.5µM or 5.0µM final concentration.

2-NBD Glucose uptake

To measure in vitro 2-NBD-glucose uptake in T cells, red blood cell lysis was performed on naive splenocytes. TCR transgenic cells were further negatively bead enriched for CD4+ T cells (Mouse CD4+ T cell Kit, Miltenyi Biotec), while polyclonal cells were not enriched. 10⁶ CD4+ T cells or polyclonal splenocytes were then plated in duplicate in media (RPMI 1640 with 10% FBS and Gibco Glutamax) supplemented with 50µg/ml 2-NBDG (Cayman Chemical), for 20 minutes at 37°C. Cells were then washed twice in PBS and stained for FACS as described above. Only CD4+TCRβ+ T cells were included in analysis. For in vivo uptake, 2-NBD glucose was prepared at 200mg/ml in PBS and injected i.p. at a dose of 1000mg/kg. The mice were sacrificed after 15 minutes, the lymphoid organs harvested and analyzed as described above.

Flow cytometry

All samples were analyzed on BD FACSCanto II or BD LSRFortessa cytometers, and data were analyzed using FlowJo software (FlowJo, LLC). For intracellular DM staining, fixation and permeabilization was done in accordance with the Foxp3/Transcription Factor Staining Buffer Kit (eBioscience). The following antibodies/clones were used for cell analysis: CD3ε (clone 145-2C11, FITC, BioLegend; clone 145-2C11, APC, BioLegend), CD4 (clone RM4.5, FITC, BioLegend; clone RM4.5, eFluor 450, eBioscience; clone RM4.5, PerCP-Cy5.5, eBioscience), CD5 (clone 53-7.3, FITC, BD Biosciences), CD8α (clone 53-6.7, APC, BD Biosciences),

*mitoSOX analysis by flow cytometry*

Splenocytes were harvested and stained for cell surface proteins as usual, although staining time was limited to 15 minutes at room temperature. Cells were then washed with PBS prior to a 15 minute incubation with 2.5μM of mitoSOX at room temperature. Cells were then washed again and immediately analyzed by flow cytometry.

*Tamoxifen Treatment for UBC-creErt2 induction*

Tamoxifen (Sigma-Aldrich) was suspended in corn oil (Sigma-Aldrich) at a concentration of 100mg/ml. Mice were orally gavaged for three consecutive days with 50μl of the tamoxifen solution for a total treatment of 5mg of tamoxifen per day.

*FTY720 treatment*

Mice were injected i.p. at roughly the same time each day for 10 consecutive days with 1mg/kg of FTY720 (Sigma) dissolved in 200μl of PBS. Control mice were given PBS-only injections.

*Transcriptional profiling and Genes and Metabolites Analysis*

Transcriptional profiling of naive and D7 *in vivo* activated LLO118 and LLO56 was performed using Affymetrix microarrays (Affymetrix Mouse Gene ST 1.0) using standard Affymetric
protocols. Naive LLO118 and LLO56 T cells were purified from the spleens of individual mice (n=4) from each of the TCRtg lines using a Miltenyi CD4+ isolation selection kit (#130-104-454) following the manufacturer’s instructions. For the D7 in vivo activated T cell isolation, $10^4$ of either LLO118 or LLO56 T cells were injected into a wildtype C57BL/6J mouse on Day-1. On D0, they were infected with Listeria monocytogenes and the spleens from individual mice (LLO118 n=4; LLO56 n=3) were harvested on D7. The T cells were purified by FACS on a BD FACSARia by sorting on CD4+ and the appropriate congenic marker (Thy1.1 for LLO118 and Ly5.1 for LLO56). A dump gate of CD8, CD11b, CD11c, CD19, NK1.1, and MHC class II was used. RNA was purified from $1.5 - 2 \times 10^6$ T cells using an RNeasy Mini Kit (Qiagen #74104), the cDNA prepared using a NuGen Pico SL kit, and 5.5µg of cDNA was hybridized to the microarrays using standard Affymetric protocols. All data were normalized using RMA in Arraystar 12. Microarray data have been deposited in the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE146069. Genes and Metabolite analysis was performed comparing the naive LLO118 and LLO56 T cells and D7 in vivo activated LLO118 and LLO56 T cells using Shiny GAM (https://artyomovlab.wustl.edu/shiny/gam/) on an atom-based network$^{37,54}$. 

**Bacterial Infections**

The Listeria monocytogenes strain 1043S used in this study was generously provided by D. Portnoy (University of California, Berkeley, CA), and L. monocytogenes infections of LLO56 and LLO118 mice were performed as previously described$^{30}$. 

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**Statistical Analysis**

Prism (versions 7 and 8) software for Mac OS X was used for all statistical analysis. Statistical significance was determined using the either a paired t-test (for Seahorse runs), an unpaired t-test, or an ANOVA test and a $p$ value of $<0.05$ was designated as the criterion for significance. All statistical tests were two-tailed unless otherwise noted in the figure legends.
3.6 Author contributions

AVM and JMB designed and performed experiments, analyzed data, and wrote the manuscript, where AVM initially characterized the metabolic differences in the LLO and Scn5a systems, as well as performed 2-NBDG uptake analysis of the polyclonal T cell populations; MDB, CHC, DLD, and WYL performed experiments, analyzed data, and generated figures, specifically in regards to the activated LLO Seahorse analyses and 2-NBDG uptake analysis of the CD8 T cell population; MNA and AS performed the GAM analysis; ELP designed experiments; and PMA designed experiments and wrote the manuscript.
Figure 3.1. Naive LLO CD4+ T cells with low tonic signaling (LLO118) exhibit greater metabolic activity than those with high tonic signaling (LLO56). a, Enriched CD4+ T cells
from spleens of LLO56 and LLO118 TCRtg mice were analyzed using a standard Seahorse protocol, with stepwise injections of Oligomycin, FCCP and Rotenone plus Antimycin A. 2x10^5-3x10^5 cells per well were used, with the cell counts always matched between mice on individual runs, and a minimum of three wells per mouse were plated. The Oxygen Consumption Rate (OCR), a readout of cellular respiration, was measured, and a representative OCR curve is shown. b, Basal OCR, the first OCR data point collected, was plotted for LLO56 and LLO118 pairs from individual Seahorse runs (n=10). Plot points represent averages of a minimum of three wells; p=0.0009 by a paired t-test. c, Maximal OCR, the first OCR data point collected after the injection of FCCP, was plotted from pairs from individual Seahorse runs (n=10). Plot points represent averages of a minimum of three wells; p=0.0004 by a paired t-test. d, Spare respiratory capacity (SRC) was plotted from pairs from individual Seahorse runs (n=10). SRC is calculated by subtracting the basal OCR from the maximal OCR; p=0.0176 by a paired t-test. e, Enriched CD4+ T cells from spleens of LLO56 and LLO118 TCRtg mice were run using a standard Seahorse protocol, as described in (a). The Extracellular Acidification Rate (ECAR), a readout of cellular glycolysis, was measured, and a representative ECAR curve is shown. f, Basal ECAR, the first ECAR data point collected, was plotted for LLO56 and LLO118 pairs from individual Seahorse runs (n=10). Plot points represent averages of a minimum of three wells; p=0.0176 by a paired t-test. g, Maximal ECAR, the first ECAR data point collected after the injection of FCCP, was plotted for LLO56 and LLO118 pairs from individual Seahorse runs (n=10). Plot points represent averages of a minimum of three wells; p=0.0012 by a paired t-test. h, The ratio of OCR:ECAR was calculated by dividing the maximal OCR by the maximal ECAR for each pair of LLO56 and LLO118; p=0.5313 by a paired t-test. For i-l, LLO56 and LLO118 were activated in vivo by L. monocytogenes infection, and on day 7 post-infection LLO T cells were sorted and assessed by Seahorse analysis to
determine (i) basal OCR, (j) maximal OCR, (k) basal ECAR, and (l) maximal ECAR. The values represent the average of a minimum of 3 individual wells from individual mice from two independent experiments; for (i), p =0.0048; for (j), p =0.0266; for (k), p =0.0065; and for (l), p =0.0164; all statistical values determined by an unpaired t-test.
Figure 3.2. Metabolic activity is inversely regulated by the strength of tonic signaling, as determined by TCR:self-pMHC interactions. a, 2-NBDG uptake in naive splenocytes from...
either LLO56 (n=3) or LLO118 (n=4) mice was measured by FACS after a 20-minute in vitro incubation. Live/dead gating was used on single cell suspensions, followed by doublet discrimination. Cells were then gated on the CD3+CD4+ population, and a representative histogram is shown. b, Quantification of in vitro 2-NBDG uptake from (a) is shown, and data are representative of 3 independent experiments. c, In vivo 2-NBDG uptake was measured by FACS 15 minutes after an IP injection of 2-NBDG in LLO56 (n=4) and LLO118 (n=4) mice. Live/dead gating was used on single cell suspensions, followed by doublet discrimination. Cells were then gated on the CD3+CD4+ population, and quantified uptake is shown. Data are representative of 3 independent experiments. d, Naive B6 splenocytes were incubated with 2-NBDG in vitro and then assayed via flow cytometry. Live/dead gating was used on single cell suspensions, followed by doublet discrimination. Cells were then gated on the CD3+CD4+ population, and then further gated on CD5 expression (highest 10% and lowest 10%). A representative histogram is shown for 5 individual mice over the course of two independent experiments. e, Quantification of the 2-NBDG uptake in (d), including analysis of high (CD5hi) and low (CD5lo) tonic signaling cells from the CD8+ T cell population (CD3+CD8+ gate). f, Enriched CD4+ T cells from spleens of Scn5a+CD4-cre+ mice and Scn5+CD4-cre-negative littermate controls were analyzed using the standard Seahorse protocol as described in Figure 3.1. Shown are representative Seahorse graphs of the OCR and ECAR from 3 independent experiments. g, UBC-creERT2/DMff mice plus various control mice (cre-negative littermates, DMko, and B6) were all treated with tamoxifen by gavage for 3 consecutive days. Blood was then collected on various days following the start of tamoxifen treatment and the B220+ cellular population was analyzed by flow cytometry for DM expression and expression of CLIP bound to MHCII. Graphs are representative of 2 independent experiments. h, 60 days post-tamoxifen treatment, polyclonal CD4+ T cells were enriched from either UBC-
cre\textsuperscript{ERT2}/DM\textsuperscript{ff} mice or their cre-negative littermate controls and analyzed by Seahorse to determine OCR and ECAR levels. Shown are data from individual UBC-cre\textsuperscript{ERT2}/DM\textsuperscript{ff} mice (n=3) alongside data of pooled control mice (n=2). Control mice were pooled to ensure enough cells were available for plating.
Figure 3.3. CD11c+ cells independent of the cDC1 lineage are required to present self-pMHC to maintain tonic signaling in CD4+ T cells. a, Analysis of CD5 expression by flow cytometry for the naive CD4+ T cell population (gating: live/single/CD4+/TCRβ+ or CD3+/CD44lo) of CD11c-cre/DMff mice (n=3) and their WT littermate controls (n=3). Data are mean ± SEM for one experiment and represent 4 independent repeats. By unpaired t test, p=0.010. b, Analysis of CD5 expression by flow cytometry for the naive CD4+ T cell population of CD19-cre/DMff mice (n=5) and their WT littermate controls (n=4). Data are mean ± SEM for one experiment and
represent 2 independent repeats. By unpaired t test, p=0.8719. c, Analysis of CD5 expression by flow cytometry for the naive CD4+ T cell population of CD11c-cre/DM<sup>fl</sup> mice (n=3) and their WT littermate controls (n=3), along with Xcr1-cre/DM<sup>fl</sup> mice (n=4) and their WT littermate controls (n=4). Data are mean ± SEM for one experiment. By Tukey’s multiple comparisons test, p=0.7708 for Xcr1-cre/control versus Xcr1-cre/DM<sup>fl</sup>, and p<0.0001 for CD11c-cre/control versus CD11c-cre/DM<sup>fl</sup>.
Figure 3.4. *In vivo* treatments can modulate tonic signaling, altering the basal metabolism of CD4\(^+\) T cells. **a**, LLO118 transgenic mice were injected i.p. with either 1mg/kg of FTY720 or PBS daily for a total of nine days. On day 8 of treatment, blood was taken to ensure efficacy of FTY720 treatment (determined by the percentage of CD4\(^+\) T cells circulating in the blood), then on day 9, lymph nodes were harvested and CD4\(^+\) T cells were enriched. Flow cytometry analysis shows CD5 expression in the PBS control versus FTY720 treated group. Data points are of individual mice (n=6) from 2 independent experiments. By unpaired student t test, p<0.0001. **b**, Standard Seahorse analysis of the enriched LLO118 T cells from (a) was performed to determine ECAR and OCR of the unstimulated LLO118 T cells. Representative Seahorse graphs are shown of two independent
experiments, and data points are from each experiment were collected from the pooled samples of 4 individual PBS or FTY720-treated mice.
Figure 3.5. Metabolic pathway differences between LLO118 and LLO56 identifies glycerol 3-phosphate as a metabolic bifurcation point between the two LLO T cells. Microarray data for LLO118 and LLO56 T cells activated in vivo by *L. monocytogenes* infection were analyzed on day 7 post infection for metabolic pathway differences using the program Genes and Metabolites (GAM), revealing significant differences between the two T cells. The pathways upregulated in LLO118 are shown in blue, and those in LLO56 shown in red, with the intensity of the lines corresponding to the level of transcription. The enzymes involved in each step of the pathways are shown. For clarity, the substrates and products have been omitted. The inset highlights a key branch point between LLO118 and LLO56 T cells.
Figure 3.6. Low tonic signaling, highly metabolically active CD4+ T cells have enhanced ROS production. a, Naive B6 and LLO splenocytes were incubated with DCFDA and then assessed by
flow cytometry for the level of DCFDA fluorescence within the naive CD4+ T cell populations (gating: live/single/CD4+/TCRβ+/CD44lo). Data are from individual mice (B6, n=2; LLO56, n=2; LLO118, n=3) and represent two independent experiments. By Tukey’s multiple comparisons test, p=0.0012 for LLO56 versus LLO118, p=0.0009 for LLO118 versus B6, and p=0.762 for LLO56 versus B6. 

b, CD4+ T cells were enriched from splenocytes of naive B6 and LLO mice and then either stimulated in vitro with PMA and ionomycin or left unstimulated for 24 hours in complete media. After stimulation, T cells were harvested and incubated with DCFDA immediately prior to flow cytometry analysis. Data are from individual mice (B6, n=2; LLO56, n=2; LLO118, n=3) and represent two independent experiments. 

c, Data from (b) was used to generate ratios of the stimulated DCFDA MFI divided by the unstimulated DCFDA MFI. By Tukey’s multiple comparisons test, p=0.0119 for LLO56 versus LLO118, p=0.0295 for LLO56 versus B6, and p=0.6864 for LLO118 versus B6. 

d, Naive B6 splenocytes were incubated with mitoSOX immediately prior to flow cytometry analysis. The mitoSOX fluorescence is shown (as a percentage relative to the average CD5hi value) for both the CD5hi and CD5lo populations of naive CD4+ T cells, where gating is set on the top and bottom 10% of CD5 expression, respectively. Data points are from individual mice (n=14). By paired t test, p<0.0001. 

e, Analysis of mitoSOX fluorescence by flow cytometry within the naive CD4+ T cell populations from CD11c-cre/DMff mice (n=3) and their WT littermate controls (n=3), along with Xcr1-cre/DMff mice (n=4) and their WT littermate controls (n=4). MFI values are shown as a percentage relative to the average control value for each genotype. Data are mean ± SEM for one experiment. By Tukey’s multiple comparisons test, p=0.975 for Xcr1-cre/control versus Xcr1-cre/DMff, and p=0.0002 for CD11c-cre/control versus CD11c-cre/DMff.
3.7 References


Chapter 4: Conclusions and Future Directions

4.1 Introduction

The research focus of this dissertation has centered on the effects of tonic signaling on CD4+ T cell function, during homeostasis and an immune response. In this study, we conclude that the strength of tonic signaling during homeostasis not only controls the basal metabolic activity of naive T cells, but also influences the skewing of Teff versus Tfh lineage commitment early after activation. While these findings enhance our understanding of tonic signaling, Tfh development, and naive CD4+ T cell biology, they also provoke a multitude of important and yet-to-be-answered questions, which are further addressed here.

4.2 Future directions

4.2.1 Elucidate the mechanism underlying the influence of tonic signaling on early Teff/Tfh fate decisions

The results of this study demonstrate an inverse relationship between the strength of tonic TCR signaling and early Tfh fate commitment; whereby, low tonic signaling cells have a propensity to generate a greater frequency of Tfh cells during the primary immune response, and high tonic signaling cells skew towards a non-Tfh, Teff phenotype instead. Although we were able to establish a direct relationship between tonic signaling strength and Tfh development, we did not
uncover the mechanism underlying the control of developmental fates by tonic signaling. Elucidating this mechanism could offer further therapeutic targets in the race to control Teff/Tfh differentiation in settings of vaccine design and drug development for autoimmune disorders.

**A potential role for metabolism**

Given our other finding that tonic signaling strength also inversely regulates the basal metabolic activity of naive CD4+ T cells, one must consider the possibility that these two events are directly related. As such, we hypothesize that the strength of tonic signaling is responsible for endowing a naive CD4+ T cell with a unique metabolic program that predisposes it with a propensity to enter either the Teff or Tfh developmental program almost immediately after TCR stimulation. If that is the case, then a critical question remains: how can metabolic programming control T effector differentiation? Future directions aimed at answering these two questions would be tightly intertwined.

Metabolic networks can influence T cell function beyond simply meeting the energy demands of the T cell. A multitude of signaling pathways essential for various T cell functions are closely interconnected with metabolic programming, either through shared signaling components or directly through metabolite regulation. Since we found no difference in the OCR:ECAR ratio between the two LLO T cells, it’s highly unlikely that their functional differences would be a result of a metabolic skewing towards glycolysis or OXPHOS. Therefore, future experiments would have to utilize approaches that decreased or increased the overall metabolic activity of high and low tonic signaling cells to determine the effects on Tfh differentiation.

For example, a recent paper described the effects of utilizing a combinatorial inhibitory approach of both OXPHOS and glycolysis. By administering both metformin (an OXPHOS
inhibitor) and 2-deoxyglucose (2-DG, an inhibitor of glycolysis), one group was able to show that reducing the overall metabolic activity of T cells dampened their cytokine production and proliferation post-stimulation much more effectively than treating with just one of the drugs alone\textsuperscript{2}. Furthermore, they cleverly enhanced mTORC1 signaling by deleting its upstream inhibitors, TSC1 and TSC2, and then administered the combination therapy to tease apart whether the effects of reducing metabolic activity via the drug treatment were mTORC1 signaling dependent\textsuperscript{2}. Interestingly, their results indicated that the dampened functional effects observed in the T cells with reduced metabolic activity were independent of mTORC1 signaling\textsuperscript{2}. This suggests a regulatory role for metabolic programming exists in CD4+ T cells that stems downstream of the shared signaling networks between metabolism control and cellular function. This study perfectly highlights the complexities of understanding metabolic programming in CD4+ T cells.

For our future studies, it will be imperative that we consider all avenues of possible influences metabolic programming can have on Teff/Tfh differentiation. I think the first experimental steps we should take are to utilize a method, like the combinatorial metformin plus 2-DG approach, to decrease both OXPHOS and glycolysis in the LLO118 T cells and then read-out the effects on their function post-activation. This will prove challenging, as current methods used to manipulate metabolism are most effectively administered \textit{in vitro}, as they are generally nonspecific at the cellular level. Unfortunately, there is no great \textit{in vitro} culture method to generate Tfh cells; however, a recent study has shown a transcriptional bifurcation of CD4+ T cells into Tfh and Teff cells as early as 8 hours post-activation \textit{in vitro}\textsuperscript{3}. Therefore, we could utilize the combinatorial metformin plus 2-DG treatment to reduce the overall metabolic activity of LLO118 T cells \textit{in vitro}, then stimulate them and isolate the RNA from treated versus untreated groups at 8 hours post-stimulation. Through analysis of \textit{Il2}, \textit{Bcl6}, and \textit{Prdm1} (although this gene isn’t
upregulated until much later at 16-24 hours post-stimulation) by qPCR, we should be able to detect any changes in the Tfh-potential of the LLO118 T cells. If we do detect changes, we can conclude that the metabolic programming is most likely contributing to the early Tfh/Teff fate skewing observed in high and low tonic signaling cells. More studies could then be done to sort high and low tonic signaling polyclonal CD4+ T cells to extend the findings to the polyclonal repertoire, and if deemed appropriate, long-term experiments could further be designed to distinguish whether the metabolic control of Teff/Tfh fates is independent of mTOR signaling.

A potential role for ROS production

Our findings also elucidated the correlation between metabolic activity and ROS production in naive CD4+ T cells. If distinct metabolic programming is shown to be required for the Teff/Tfh skewing observed in high and low tonic signaling cells, and if this metabolic control is independent of mTOR signaling, a further interrogation into the role of ROS in high and low tonic signaling cells would be highly warranted. While it is possible that the basal differences in ROS production between the low and high tonic signaling cells could be responsible for influencing the observed Teff/Tfh skewing, it is also possible that the difference in the unstimulated/stimulated ratio of ROS production is the critical component of any effects ROS has on T cell fate decisions. We found that LLO56 (high tonic signaling cells) had a much greater unstimulated:stimulated ROS ratio than both LLO118 (low tonic signaling cells) and B6 polyclonal CD4+ T cells. I think this ratio may be more likely to play a role (if any) in the cellular functional outcomes, as LLO56 display many qualities characteristic of pathways influenced by ROS. For example, LLO56 have increased IL-2 production, Erk phosphorylation, and Ca^{2+} flux upon stimulation⁴, and some part of all of these signaling pathways are either dependent on or
enhanced by ROS production shortly after TCR signaling\textsuperscript{5}. However, the enhanced ROS production upon stimulation in the LLO56 cells could also simply be a consequence of the increased Ca\textsuperscript{2+} flux observed in high tonic signaling cells. Although counterintuitive, we may therefore want to explore future experiments where we increase the basal ROS level in LLO56 to determine if decreasing the unstimulated/stimulated ratio has any effect on LLO56 function. As the most common methods utilized to manipulate the redox state of cells are again best used \textit{in vitro}, we could again use a transcriptional analysis approach to determine if ROS has any effects on the early Teff/Tfh skewing of high and low tonic signaling cells.

Through the use of the Genes and Metabolites program to analyze transcriptional data from activated LLO T cells\textsuperscript{6}, we were able to identify mGPD2 as a potential key enzyme in the enhanced metabolism of T cells with weak self-pMHC interactions. Previously, mGPD2 has been shown to play a pivotal role in mitochondrial ROS production\textsuperscript{7}. Our lab has generated a mGPD2\textsuperscript{fl/fl} mouse and crossed it to the CD4-cre mouse strain, ensuring deletion of mGPD2 in cells that express CD4, including CD4+ T cells, CD8+ T cells, and some subsets of DCs. Future work characterizing this mouse should focus on analysis of mitoROS in the CD4+ T cell compartment. It will be interesting to see if other metabolic pathways will compensate for the deletion of mGPD2, as this is often the case with manipulations of metabolic programming. To overcome this, however, it could be useful to take advantage of a recently described protocol for the efficient CRISPR/Cas9 gene editing of uncultured naive T cells\textsuperscript{8}. In this study, the Parish group describes a method by which uncultured, naive CD8+ T cells enriched from a mouse can be electroporated with recombinant Cas9/sgRNA ribonucleoprotein and then transferred \textit{in vivo}, left to rest in the absence of stimulation, and still effectively delete the target gene without noticeably affecting the naive state of the cell. This method could be used to complement the mGPD2 deficient T cells by deleting any genes we
potentially identify as being upregulated in a compensatory fashion, while still allowing for interrogation of basal metabolic properties as well as determining effects on naive differentiation to the Teff/Tfh lineages post-activation in vivo.

*Establish a hierarchy of the strength of signals between tonic signaling and TCR activation in the influencing of early Teff/Tfh fate decisions*

LLO56 and LLO118 lie on opposite ends of the spectrum of tonic signaling strengths, and they both recognize their antigenic-pMHC at equal affinities. While these facets of the LLO model make it an ideal tool for investigating the role of tonic signaling on CD4+ T cell function, future work will need to be done to further integrate our findings from this model into the polyclonal CD4+ T cell repertoire. Since a diverse TCR repertoire does not have fixed affinities for antigenic-pMHC, it will be important to delineate the interplay between TCR activation strength and tonic signaling strength on Teff versus Tfh decisions. Groups have found that increasing TCR affinity for antigenic-pMHC promotes Tfh development in certain TCRtg models⁹,¹⁰ and when tetramer-pMHC binding is used to generate their conclusions³,⁹. The 5cc.7 TCRtg T cells used by the McHeyzer-Williams group have a reported low tonic signaling profile¹¹, which our results suggest would facilitate Tfh development. Therefore, in low tonic signaling, Tfh-capable cells, increasing TCR signal strength appears to play a complementary role in the promotion of Tfh differentiation. This hypothesis would also explain observations of increased tetramer-pMHC binding in the polyclonal Tfh compartment, as our data suggests low tonic signaling cells would be the primary contributors to the Tfh subset. It would be interesting to further explore what the thresholds are for tonic signaling versus TCR activation strengths in the control of Tfh development, and to establish a hierarchy of the strengths of the two signaling events on Teff/Tfh fate decisions. These studies
will be technically challenging, as many ways of altering tonic signaling strength also affect TCR activation strength. Without the generation of novel tools to distinctly manipulate tonic signaling versus TCR signaling upon activation, the best methods to approach this question would be through the use of APLs for both antigenic-peptides and self-peptides. This approach has its own hurdles, however, as no current TCRtg model exists that has defined APLs affecting TCR affinities for both an antigenic-peptide and self-peptide.

4.2.2 Connect tonic TCR signaling networks to basal metabolic programming

The results of this study reveal an inverse relationship between the strength of tonic TCR signaling and basal metabolic activity. Future studies aimed at uncovering the mechanisms underlying the control tonic signaling has on basal metabolic programming will be necessary to gain better insight into this critical component of T cell homeostasis.

High tonic signaling cells could be in a heightened state of quiescent metabolic regulation

Since T cell quiescence involves the active engagement of metabolic inhibition, it is easy to imagine that the increased strength of tonic signaling may simply be engaging metabolic repressors to a greater extent. Studies have unveiled a critical role for mTORC1 inhibitors (i.e., TSC1, PTEN, and LKB1) in the enforcement of the quiescent program in naive, peripheral T cells\textsuperscript{12-14}; therefore, future work should focus on investigating the level of mTORC1 and mTORC2 activity in naive CD4+ T cells experiencing high and low tonic signaling.
Clues from a paper describing a role for Rasgrp1 in basal metabolic programming

Rasgrp1 has been shown to be indispensable for TCR signaling, and thymocytes from mice deficient in *Rasgrp1* have a significant defect in their ability to be positively selected\(^{15}\). Rasgrp1 is a guanine exchange factor required to activate Ras, a GTPase that initiates the Ras-Mek-Erk signaling cascade to activate AP-1 following TCR stimulation\(^{16,17}\). One study, however, has described an additional role for Rasgrp1 in connecting TCR signaling to metabolic programming in naive CD4+ T cells\(^{18}\). Daley et al. have shown that an ENU-induced missense variant of Rasgrp1 (*Rasgrp1\(^{Anaef}\]*) leads to an accumulation of metabolically hyperactive, Tfh-prone, peripheral mature CD4+ T cells independent of altered thymic development. Rasgrp1\(^{Anaef}\) CD4+ T cells display a partial loss of function in the ability to activate the Ras-Mek-Erk pathway after stimulation\(^{18}\). Despite this decrease in phosphorylation of Erk, however, there is a greater frequency of activated CD4+ T cells in the periphery of naive Rasgrp1\(^{Anaef}\) mice when compared to WT counterparts\(^{18}\). Most intriguingly, Rasgrp1\(^{Anaef}\) mice also display a greater frequency of activated CD4+ T cells with a Tfh phenotype, directly resulting in the generation of autoantibodies. Furthermore, the phenotypes of the increased amount of activated T cells, enhanced Tfh development, and subsequent generation of autoantibodies in the Rasgrp1\(^{Anaef}\) mice can all be reversed through a genetic manipulation to mTOR that decreases the basal metabolic activity of the Rasgrp1\(^{Anaef}\) CD4+ T cells\(^{18}\).

The parallels between the Daley et al. finding and our own are striking. In our studies, the low tonic signaling cells are more metabolically active and are also prone to a Tfh phenotype. Furthermore, the LLO118 low tonic signaling cells have previously been shown to have less phosphorylation of Erk following TCR stimulation when compared to the high tonic signaling, LLO56 cells\(^{4}\). Therefore, there should be future studies aimed at interrogating the activity of
Rasgrp1 in high and low tonic signaling cells, and as Rasgrp1 is connected with TCR signaling, these studies could offer necessary insight into the connection between tonic TCR signaling and basal metabolic programming.

4.2.3 Further delineate which APC subset is responsible for maintaining tonic signaling through the presentation of self-pMHC

Through the use of our DM\textsuperscript{f/f} mouse line, we were able to show that CD11c+ APCs are responsible for presenting self-pMHC to maintain tonic signaling in CD4+ T cells. Furthermore, utilizing the Xcr1-cre mouse strain, we were able to narrow down that the subset of CD11c+ APCs responsible for tonic signaling maintenance is independent of the cDC1 lineage. It would be of great interest to continue attempting to identify whether a particular subset of APCs is controlling the presentation of self-pMHC to CD4+ T cells. To do this, further crossing of the DM\textsuperscript{f/f} mouse line with a LysM-cre line could further narrow the field down by revealing whether monocyte-CD11c+ lineages are responsible. If tonic signaling is not altered in the LysM-cre/DM\textsuperscript{f/f} mouse, this still leaves the possibility that cDC2s, pDCs, Langerhans cells, or red pulp macrophages are responsible (see Fig. 4.1 for a schematic overview of pertinent gene expression profiling among the various immunological subsets). Complicating the matter, however, is the possibility that a specific subset is not responsible and it is simply a matter of the overall amount of self-pMHC that is available in the periphery. It will be challenging to separate these issues, but utilizing mixed bone marrow chimeras of various ratios of WT versus global DMko bone marrow may be a good first approach at addressing this dilemma.
4.2.4 Generate novel tools to better study tonic signaling

Currently, the field of tonic signaling is severely limited by the challenge of separating tonic TCR signaling from TCR signaling at activation. All of the markers we use to identify tonic signaling strength are also markers of TCR activation strength, and all of the downstream tonic signaling molecules we know of are shared with TCR activation signaling. This makes discretely manipulating tonic signaling and TCR activation strength a challenge, as the two cannot easily be separated. Therefore, it would be highly beneficial to the field of tonic signaling to identify any possible distinguishing genes or proteins involved in TCR recognition of self-pMHC versus antigenic-pMHC.

If we are going to accomplish this, it will be imperative that we better define the transcriptional, epigenetic, and proteomic profiles of high and low tonic signaling cells. This could reveal unique molecular markers regulated by tonic signaling. Of course, any markers determined to be of interest utilizing this approach would then have to be cross-referenced with an activated CD4+ T cell to determine whether we can further identify a pathway or molecule discretely involved in tonic signaling. Unfortunately, it’s likely that this attempt could prove futile. We have tried microarray analysis of the naive LLO T cells, and it revealed very few transcriptional differences between the two cells with no obvious candidates. A recent promising report, however, utilized a method of pulsed stable isotope labeling by amino acids in cell culture (SILAC) to show that naive T cells utilize fine-tuned regulation of protein turnover to rapidly respond to stimuli, rather than relying on transcriptional control19. Therefore, further interrogation of protein turnover in high and low tonic signaling cells might prove most promising in the identification of tonic signaling networks.
4.2.5 Explore the implications of tonic signaling strength on memory cell development

One of our findings in the LLO system stood out to me as particularly confusing. Although it is significantly less than that of the LLO118 cells, at day 4 post-infection, LLO56 cells do produce a substantial pre-Tfh population. While it was possible that some of these LLO56 pre-Tfh cells were truly Tfh-fated and simply becoming developmentally arrested at the pre-Tfh stage, increasing the number of LLO56 Tfh cells was never able to overcome the Tfh-associated defects in the humoral immune response. The combination of these data suggests that high tonic signaling pre-Tfh/Tfh cells may have developed independently of the canonical Tfh lineage. Interestingly, T central memory (Tcm) precursors are also phenotypically defined the same as the pre-Tfh population (CXCR5⁺PD-1⁺)²⁰, and it is currently unknown whether Tfh cells and Tcm cells develop from the same precursor or have distinct lineages²¹. Preliminary evidence from my own studies would suggest that high tonic signaling cells are fated to become memory cells with a Tcm phenotype, as determined in the LLO system and then extended to polyclonal CD4+ T cells (Fig. 4.1). Future studies will be needed to definitively assess the memory potential of high and low tonic signaling cells in a polyclonal repertoire. Furthermore, characterizing any possible heterogeneity in pre-Tfh population, as currently phenotypically defined, could reveal insights into both Tfh and memory cell development. Performing single cell RNAseq on the pre-Tfh population could be a highly informative initial approach.
4.3 Concluding thoughts

Immunologists have often overlooked the possibility that homeostatic cues and signals experienced by a naive T cell can play a significant role in the downstream behavior and function of that cell post-activation, instead assuming that stimulation overrides any prior homeostatic programming. Our results challenge this idea, however, and highlight the importance of that time period in a CD4+ T cell’s life when they are circulating in the periphery as mature, naive T cell in search of their cognate antigen. It is imperative that we gain a better understanding of the factors that may influence a naive CD4+ T cell prior to its activation. For example, while tonic signaling strength is generally considered to be set by the TCR affinity for self-pMHC, this has yet to be shown experimentally. Tonic signaling is also influenced by the availability of self-pMHC, as T cells isolated from the blood, where there is an absence of TCR:self-pMHC interactions, display reduced tonic signaling qualities. Therefore, if a TCR is positively selected by a self-peptide that is simply less prevalent in the periphery, even though it has a high affinity for that self-pMHC, would that cell appear to have weak tonic signaling? Furthermore, would the effects of engaging in less frequent, but stronger affinity of TCR:self-pMHC interactions alternatively influence the priming of a naive CD4+ T cell compared to one that engaged more frequently with their self-pMHC at a lesser affinity? Additionally, it is not known whether other factors can influence the perceived strength of tonic signaling, just as factors besides TCR affinity for antigenic-pMHC can influence TCR activation strength (like the role of costimulatory molecules). Unravelling the mechanisms and signaling networks influenced by tonic signaling will help answer these questions in the future.

Interestingly, T cells isolated from the blood still express CD5, our reliable read-out of tonic signaling strength, albeit at the low end of CD5 expression for naive CD4+ T cells. This also
raises important questions. Are T cells at the low end of the tonic signaling spectrum actually engaging in TCR:self-pMHC interactions in the periphery? If not, could it simply be interactions between CD4 and MHCII that are allowing them to survive? Teleologically, this could make sense as another system in the process of tolerance to self. Having circulating CD4+ T cells that do not encounter their positively selecting self-pMHC in the periphery may offer advantages to diversifying the immune response. These cells could be primed to behave more functionally ‘actively’ than their CD4+ T cell counterparts that do engage with self-pMHC in the periphery. This could prove especially important in Tfh and Tmemory cell differentiation, as Tfh cells could lead to destructive autoantibody production and Tmemory cells require less stimulation for reactivation.

Peripheral expression of CD5 has been shown to confer high tonic signaling naive T cells with an NF-κB-dependent survival advantage. Interestingly, naive CD5hi T cells accumulate with age, a phenomenon that correlates with decreased thymic output of naive CD4+ T cells. Given the tight regulation of the homeostatic naive T cell pool, one can speculate that the decline in newly generated naive T cells may be compensated for by an enhancement in the lifespan of CD5hi T cells. Alternatively, and more intriguingly, is it possible that CD4+ T cells can accumulate enhanced CD5 expression over time? This would suggest that tonic signaling may be influenced by factors other than TCR affinity for self-pMHC.

While many studies have been focused on how long T cells simply survive in the absence of tonic signaling, another important question is how long do T cells function in the absence of tonic signaling? This question has important implications for CD4+ T cell function both in vivo and in vitro. Spatiotemporally, does the circulation of a CD4+ T cell impact its function? Would a T cell that has just entered the lymph node from the blood behave differently if it encountered its
antigenic-pMHC immediately versus if it encountered its antigenic-pMHC after residing in the lymph node for multiple days, having interacted extensively with self-pMHC? It will be important to also develop an awareness for how in vitro culturing methods alter tonic signaling in CD4+ T cells. For example, the Tfh field has not been able to develop a successful, highly reproducible culture method for the in vitro generation of Tfh cells. It would be interesting to investigate whether current culturing methods are artificially enhancing the tonic signaling of ex vivo CD4+ T cells, possibly from too high of an APC:T cell ratio, thereby inhibiting their ability to develop into Tfh cells.

Clearly, there are many remaining questions in the field of tonic signaling. Our work uncovering the influence of tonic signaling strength on basal metabolic programming and Tfh development highlight the importance of gaining a better understanding of this facet of CD4+ T cell biology.
Figure 4.1. Immunologic Genome Project’s Gene Skyline micro-array analysis (Immgen.org)
of the expression of the indicated genes in various immunological cellular subsets. Genes assessed, in the order that they are displayed: H2-DMa, Itgax, Xcr1, Lyz2. Dark boxes distinguish the cellular subsets that express both CD11c (encoded by Itgax) and H2-DMa, representing the subsets potentially responsible for presenting self-pMHC to CD4+ T cells. Boxes shaded in red represent the cellular subsets eliminated by the Xcr1-cre breeding. Boxes shaded in green are subsets that could be eliminated or potentially identified as responsible for maintaining tonic signaling by crossing the DM"f" mouse to a LysM-cre (LysM is encoded by Lyz2) strain.
Figure 4.2. High tonic signaling cells display a Tcm phenotype, while low tonic signaling cells have a Tem phenotype. 

**a.** 20,000 Naive LLO CD4+ T cells were transferred into B6 mice on and infected with $10^7$ cfu ActA-Lm the following day. Analysis was performed 35 days post-infection to determine the phenotype of the resting memory cell populations. FACS plots shown are gated on CD4+CD44hi cells, and are representative of two independent experiments. 

**b.** B6 mice were infected with $10^7$ cfu ActA-Lm, and 35 days later the polyclonal resting memory CD4+ T cell population (CD3+CD4+CD44hi) was assessed for CD5 expression in the following subsets: Tcm (CCR7+CD62L+) and Tem (CCR7-CD62Llo).
4.4 References


