Signaling Mechanisms in Adaptive Immunity

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Signaling Mechanisms in Adaptive Immunity
by
Saravanan Raju

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ABSTRACT OF THE DISSERTATION

Signaling Mechanisms in Adaptive Immunity

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Professor Andrey Shaw, Mentor

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The adaptive immune response consists of interplay between CD4 T cells, CD8 T cells, and B cells which function in control of pathogen in the host. T cells responding via their TCR express cytokines and costimulatory molecules that support direct effector activity and also promote high-affinity antibody generation through augmenting of B cell responses. However, the molecular components that contribute T cell function to balance viral control and the potential for host damage are incompletely understood. In this work, we establish a role for the adaptor molecule CD2AP in modulation of CD4 T cell responses to chronic LCMV infection in mice though the generation of T Follicular Helper cells. We find that CD2AP modulates TCR signaling during viral infection and its deletion augments the germinal center response and generation of neutralizing antibodies. CD8 T cells are subject to negative regulation via inhibitory receptors such as PD-1 which is correlated with T cell “exhaustion”. Here we
determine the functions of PD-1 during different stages of chronic viral infection which reveals an important role in maintenance of durable immunity. Treating mice infected by a chronic strain of LCMV with PD-1 blockade at the peak of viremia does not cause fatal immunopathology, but eventually resulted in disruption of lymphoid architecture and a reduction in the CD8 T cell response. Furthermore, transient blockade of type-I interferon completely prevented death of LCMV-infected mice treated with PD-1 blockade at the time of infection, intriguingly a combination of the transient blocking IFNAR signaling and delayed PD-1 blockade, which alone would compromise antiviral immunity, accelerated viral clearance without causing immunopathology. These results indicate that harnessing CD8 T cells at early stages of chronic viral infection in paradoxically benefits long-term immunity and eventually results in enhanced control of viral infection.
CHAPTER 1:

Introduction
1.1 General Aspects of Immunity to Pathogens

A central feature of all life is the potential for invasion by parasitic entities including but not limited to viruses and bacteria. Evidence of constant attack exists in genomes which harbor active and inactive selfish genetic elements and the evolved genetic machinery to restrict their spread. These conflicts have persisted through biological time and have resulted in so-called “evolutionary” arms races to aid in survival and fecundity of the parasite or host. In higher order metazoans, highly unique and novel molecular, cellular, and physiological entities have evolved to provide protection to the host from microbial entities whose presence has the potential to cause survival disadvantages. This physiologic process of protection is known as an organisms’ “immune system”.

We now focus on features of the immune system present in animals phylogenetically above jawed fish. This distinction is made as a highly anticipatory repertoire to foreign entities that is not germline encoded exists – the adaptive immune system. We thus make a broad categorization of the immune system between an “innate” and an “adaptive” component. The innate immune system can be loosely defined as defense mechanisms that are germline encoded. The innate immune system which is responsible for control of >95% of encountered pathogens is inclusive of physical barriers (skin, mucous layers of the gut), the complement pathway, and the more well-studied cells of hematopoietic origin including those of the myeloid lineage (Macrophage, Monocyte, Dendritic Cells, and granulocytes). These cells form significant part of innate immune system by recognizing common pathogenic molecular patterns and or damage associated molecular patterns and inducing specific responses. These responses can be broadly summarized as the induction of cytokines, chemokines, and other
effector products to both directly inhibit and facilitate the recruitment of other cells in response to pathogenic stimuli.

While the innate immune system recognizes relatively non-specific moieties present in microbes, the adaptive immune system is much more specific. Specificity is achieved through germline rearrangements at specific loci whose gene products are uniquely expressed on individual cells. The cells that engage in this process during their development include B and T lymphocytes; each cell expresses a unique B cell receptor (BCR) or T cell receptor (TCR) reflecting this germline rearrangement, known as V(D)J recombination. The germline recombination generates a repertoire of lymphocytes within a host to recognize pathogens with virtually indefinite breath and incredible specificity. The BCR of B cells can be secreted in the form of an antibody which carries a unique binding site for antigen allowing for recognition any molecular moiety. However, the TCR of T cells can only recognize antigen in the form of peptide or lipid complexes presented on the cell surface by MHC or MHC-like molecules. Together, one can think of the B cells as providing recognition and effector capabilities in the extracellular compartments, while T cells recognize intracellular components.

1.2 Components of the Adaptive Immune Response

The ultimate products of the adaptive immune system are the generation of high-affinity potentially neutralizing antibodies, and the generation of effector antigen-specific CD4+ and CD8+ T cells. The generation of high titers of antibody in the serum bind to antigen which facilitates elimination from the host by a variety of mechanisms described elsewhere. Effector T cells migrate to peripheral tissues, recognize cells presenting antigenic peptide on their surface
and can either directly mediate cytotoxicity, and secrete soluble mediators such as cytokines or chemokines which promote clearance of pathogen. The next section will describe how the adaptive immune response is initiated.

Following V(D)J recombination and development in the bone marrow, B cells reside in secondary lymphoid organs in areas known as B cell follicles or in an adjacent structure known as the marginal zone. B cells remain in a naïve state until encounter with antigen where they become partially activated and then migrate to the T-B border and receive CD4+ T cell help. Following these initial activation events, antigen specific B cells can form germinal centers, a specialized structure within the B cell follicle where B cells undergo somatic hypermutation and affinity maturation. A key player in this process is selection mediated by a specialized subset of CD4 T cells, known as T Follicular Helper cells, which form the rate-limiting step in the GC reaction. The result of this process generates memory B cells and plasma cells which actively secrete antibody in the bone marrow.

T cells have a more specialized developmental process that is initiated by precursors in the bone marrow, and then is completed in a specialized organ, known as the thymus. This process occurs as T cells can only recognize antigen in the context of MHC, a highly polymorphic locus. Given that the TCR and MHC loci are unlinked, this poses an interestingly problem. In the thymus, committed progenitors undergo V(D)J recombination at the TCR locus, a sequential steps of positive selection, and negative selection to remove potentially auto-reactive cells to MHC complexes, effectively generating and pruning potential TCRs.

Following their development in the thymus, naïve T cells continuously traffick between secondary lymphoid organs and reside in T cell zones. Upon antigen encounter in the form of
peptide:MHC complexes presented on antigen presenting cells (APCs), T cells become activated undergo clonal expansion and effector differentiation commensurate the cytokine milieu present in the microenvironment.

A key distinction is made between CD4+ T cells which recognize MHCII and CD8 T cells which recognize MHC I. MHCII is expressed on professional antigen presenting cells, including B cells, and thus forms the molecular basis for specificity in antigen specific B cell mediated help. MHC1 however is expressed on all nucleated cells, and are thus potential targets for CD8 T cells. In the context of infection, local chemokines recruit T cells into the tissue non-specifically, when effector T cells recognize cognate peptide:MHC either on the surface of APCs for CD4 T cells or infected non-hematopoietic epithelial, or mesenchymal cells for CD8 T cells they release additional cytokines such as IFN-γ and TNF-α.

1.3 Signaling Modalities and Mechanisms Governing T cell Function

An overview of T cells has been given in the previous sections, however, the molecular underpinnings the regulate and modulate their function in specific contexts will be described herein. Specific focus will be placed upon the cellular basis T cell activation in lymphoid organs and the interplay between APCs, antigen, and cytokine milieu. In addition, the biochemical signal transduction pathways occurring within T cells upon activation and the ultimate global transcriptional changes that “program” differentiation will be discussed.

A major driver of T cell activation is recognition of antigen by pMHC complexes by the TCR. The nature of what exactly is required for “activation” is subject to significant modulation by the environment; antigen dose, quantity, and duration can play a significant role in whether T cells become activated or not. Often this is co-integrated with additional signals such as
costimulatory signals and adhesion molecules that strengthen T cell-APC interactions \(^{10}\).
However, these do not dictate necessarily whether a response will occur or not; this is largely
determined by presence of antigen in lymphoid organs for sufficient periods of time\(^9\).

The TCR is composed of an alpha and beta chain which form a heterodimer in complex
with CD3 subunits which carry ITAM motifs required for signal transduction \(^{11}\). Upon TCR
binding to pMHC, the kinase Lck is recruited via the CD4 or CD8 co-receptors to phosphorylate
the CD3 ITAMs. This initiates a complex cascade of signal transduction events including ZAP-70
phosphorylation of LAT which recruits additional signaling and adaptor molecules such as
SLP-76, Grb2. A key focal point of these proximal signaling pathways is the activation of
PLC\(\gamma\)1 which converts PIP3 into IP3 and DAG. IP3 promote Calcium Flux and activation of
NFAT while DAG activates the MAPK, and NF-kB pathways. Ultimately the activation of these
pathways converges in the nucleus to drive transcriptional changes required for T cell clonal
expansion, and differentiation into effector T cells.

While the key molecular players leading from transduction of signal from outside the cell
to the nucleus are relatively well-defined how the TCR can discriminate affinity to functional
output, how the TCR signal is properly terminated, and the roles of a variety of other accessory
scaffold and pathways contribute to T cell function remain unclear. In addition, much of what
we know about TCR signal transduction is derived from \textit{in vitro} experiments, or administration
of model antigens to specific TCR-transgenes with many of end readouts for activity being initial
proliferation or cell-surface marker expression. In the context of a full immune response with T
cell activation in lymph node and repeated stimulation of CD4 T cells by B cells in GC reaction,
or in the context of persistent antigen in either chronic infection or tumors, how the TCR remains
active or inactive remain incompletely defined.
Broadly, the regulation of TCR signaling can be divided into two categories, intrinsic cell biological regulation of the TCR and its signaling components via post-translational modifications, degradation, endosomal sorting, recycling, and direct phosphatase activity or via extrinsic signaling from other cell surface molecules in trans\textsuperscript{12}. While these are somewhat related, an important distinction is that the T cell and consequently activity of the TCR can be regulated by factors present in the tissue microenvironment.

The TCR is well-known to undergo cycles of internalization and degradation, combined with recycling to both terminate the TCR signal\textsuperscript{13}. How this process is regulated is relatively unclear, and factors that modify this process could play a significant role in the immune response as aberrant TCR signaling has physiological consequences. In addition, a variety of direct gene products induced by the TCR include phosphatases, and phosphatase activity increases following TCR stimulation to terminate signal.

Following T cell activation numerous cell surface receptors are expressed that have both co-stimulatory and co-inhibitory functions\textsuperscript{10,14}. Namely, PD-1 has a strong negative effect on T cell signal transduction predominantly through CD28, but also directly through the TCR\textsuperscript{15}. PD-1 sits on top of the hierarchy of inhibitor receptors which also include CTLA-4, Tim-3, LAG-3, CD160, 2B4, and TIGIT, which effectively inhibit T cells via relatively undefined mechanisms. Conceptually, the ligation of these receptors allows for tuning of TCR signal according to microenvironmental cues, which is highlighted by several lines of evidence suggesting loss of these receptors result in autoimmune phenotypes\textsuperscript{16,17}. Thus, T cells are subject to strong negative feedback through a variety of mechanisms; these can inhibited, directly, or indirectly via supplementation of co-stimulatory signals from B7 or TNFR superfamily members. These
examples illustrate the potential for complexity in the execution of the T cell adaptive immune response and how certain factors can modulate the outcome of infection or tumors.

1.4 T cells in the Fray: Effector Subsets and Impact on Host Physiology

While naïve T cells each possess a unique TCR, functionally they can be considered the same, however, following activation, the resulting effector cells are well-known to exhibit significant heterogeneity. This heterogeneity is reflected primarily on differential cell surface receptor expression, transcription factor expression, and certain \textit{ex vivo} functional capabilities\textsuperscript{18}. Ultimately, one is interested in understanding how this heterogeneity contributes to a functional immune response and how this heterogeneity develops. The sections below will describe this phenomenon in some detail first for CD4+ T cells and then for CD8+ T cells.

The functional differentiation of CD4 T cells is primarily conceptualized in the T helper subset paradigm of TH1, TH2, and TH17 cells that are induced following exposure to so-called “lineage-specific” cytokines\textsuperscript{19}. These cytokines can be generalized to promote the differentiation of cells appropriate to the insulating pathogen, and consequently their effector functions are tailored to the needs of the host. It should be noted many of these studies are derived for \textit{in vitro} experiments and it appears there is considerable overlap in transcription factor expression and cytokine secretion between these subsets. Perhaps, a more conservative view, is that these transcription factors directly promote specific cytokine secretion.

Another paradigm which to view CD4 helper subsets is a functional one within the confines of a given infection. Taking a viral infection as an example, it is well-established the generation of an affinity matured antibody response requires CD4 T cells, specifically help provided to B cells, in the form of T\textsubscript{FH} cells. This form of help relies upon ICOS and CD40 co-
stimulatory signaling to B cells in addition to receiving soluble cytokine signals such as IL-21, and others for inducible class switching. In addition, CD4 T cells provide support in the form of cytokines such as IL-21 or IFN-γ to CD8 T cells via or indirectly via DC licensing, and to macrophages – increasing their bactericidal capacity. Although, the relevance of increased phagocytosis to viral infections is unclear. Thus, CD4 T cells can be divided into at least two subsets in some ways reflecting humoral and cellular immunity; T\textsubscript{FH} and non-T\textsubscript{FH} – more specifically T\textsubscript{H}1 (in viral infection). There is likely to be considerable plasticity between these two phenotypes, but at first approximation this distinction allows us to understand the function of CD4 T cells with some structure.

The CD8 T cell response is characterized by heterogeneity that mainly arises from an inverse correlation between the generation of long-lived “memory” cells and “short-lived” effector cells. This separation is rooted in both cell surface marker expression, and differential expression of transcription factors. It is currently thought that high levels of inflammatory cytokines “drive” cells to become short-lived effectors characterized by KLRG1 expression and lack of IL-7Ra (CD127) expression. Memory cells exhibit higher survival following transfer and have increased proliferative capacity compared to short lived effectors. These functions are likely programmed by transcription factors; T-bet, Blimp-1, Zeb2, and Id2 are characteristic of effector CD8 T cells while memory cells express higher levels of Eomes, TCF-1, Bcl6, and Bach2.

Our discussion so far has been generalized to “acute” infection in which the host clears the pathogen or succumbs to disease in 7-10 days. These types of infections are typically caused by cytopathic viruses such as VSV, Influenza. However, many viruses cause chronic infection
with persistent viral antigen such as HIV, Hepatitis B and C. In addition many viruses remain in a latent state such as herpesviruses which are under constant immune surveillance, likely providing constant antigenic stimulation. Under conditions of high antigen load, CD8 T cells exhibit altered *ex vivo* phenotypes including decreased cytokine production and cytolysis of targets. This phenomenon has been termed T cell “exhaustion”. This phenotype has been correlated with cell surface marker expression most notably PD-1, but also other inhibitory receptors including Tim-3, LAG-3, and 2B4. However, it must be noted that these molecules can be expressed in any activated T cell, and thus are not specific markers of an exhausted T cells. Importantly, their functional roles are highlighted in genetic inactivation of antibody-based blockade experiments which can in some cases “reverse” this exhausted state, and “reinvigorate” the T cell response.

The ability to improve the immune response in the context of persistent antigen begs the question of exactly what beneficial role do these inhibitor receptors play in the host *in natura*. One answer comes from the partial role these molecules play in mediating tolerance and the prevention of autoimmunity which occurs to some extent in PD-1 deficient animals and as a side effect of anti-PD-1 based therapies in the clinic. However, an additional answer may lie in the ability of T cells to cause damage to the host as a byproduct of their function in pathogen clearance. This phenomenon is known as immunopathology, and exists on a typical spectrum of altered host physiology caused by immune activation. The relevance of these inhibitory receptors is they effectively “tune down” the immune response such that lethal or detrimental tissue damage is averted. A salient example of this concept is lethal disease following infection PD-1 deficient mice with certain strains of the noncytopathic virus LCMV which does not occur in sufficient animals. Thus, the tuning of the T cell response, and the development of T cell
“exhaustion” can be considered a protective mechanism for the host, but viewed from another lens, a virulence strategy by the pathogen to persist. These issues will be tacked in the following chapters and form the basis behind this work.
1.5 REFERENCES


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CHAPTER 2:

The adaptor molecule CD2AP in CD4 T cells modulates differentiation of follicular helper T cells during chronic LCMV infection

The contents of this chapter have been previously published in *Plos Pathogens*.

2.1 ABSTRACT

CD4 T cell-mediated help to CD8 T cells and B cells is a critical arm of the adaptive immune system required for control of pathogen infection. CD4 T cells express cytokines and co-stimulatory molecules that support a sustained CD8 T cell response and also enhance generation of protective antibody by germinal center B cells. However, the molecular components that modulate CD4 T cell functions in response to viral infection or vaccine is incompletely understood. Here we demonstrate that inactivation of the signaling adaptor CD2AP promotes CD4 T cell differentiation towards the follicular helper lineage, leading to enhanced control of viral infection by augmented germinal center response in chronic lymphocytic choriomeningitis virus (LCMV) infection. The enhanced follicular helper differentiation is associated with extended duration of TCR signaling and enhanced cytokine production of CD2AP-deficient CD4 T cells specifically under T\textsubscript{H1} conditions, while neither prolonged TCR signaling nor enhanced follicular helper differentiation was observed under conditions that induce other helper effector subsets. Despite the structural similarity between CD2AP and the closely related adaptor protein CIN85, we observed defective antibody-mediated control of chronic LCMV infection in mice lacking CIN85 in T cells, suggesting non-overlapping and potentially antagonistic roles for CD2AP and CIN85. These results suggest that tuning of TCR signaling by targeting CD2AP improves protective antibody responses in viral infection.
2.2 INTRODUCTION

CD4 T lymphocytes are critical mediators of the adaptive immune response to infection \(^1\). Upon encountering cognate antigen as peptide-MHC (pMHC) complexes on antigen presenting cells in the lymphoid organs, they initiate clonal expansion and undergo differentiation into effector cells, depending on the cytokine milieu initially established by innate immune cells \(^2\). In response to viral infection, differentiation of activated CD4 T cells is directed towards an IFN-\(\gamma\)-producing subset, refer to as T\(_{H1}\), that also produces IL-2 and TNF-\(\alpha\) and enhances cellular immune responses by CD8 T cells and macrophages. Activated CD4 T cells also differentiate into the follicular helper (T\(_{FH}\)) cells that migrate to germinal centers (GCs) \(^3\). During viral infection, T\(_{FH}\) cells provide help signals to B cells for antibody affinity maturation through their expression of co-stimulatory ligands and cytokines, such as CD40L and IL-21, and direct Ig class switching to the antiviral IgG2a/c subclass by secreting IFN-\(\gamma\) \(^4\). Thus, understanding the molecular mechanisms governing CD4 T cell responses through either TCR-dependent or cytokine-dependent signals can yield insight into host protection against viral infection or protective antibodies induced by vaccines.

Following ligation of the TCR by cognate pMHC complexes, re-organization of membrane proteins and post-translational modification of signaling components, ultimately result in activation of genes necessary for their proliferation and effector differentiation \(^5\). At the TCR-juxta-membrane regions, the TCR itself and its proximal kinases are densely packed and surrounded by adhesion molecules, such as LFA1 integrin, which together comprise the immunological synapse (IS) and facilitate quality control of TCR signals \(^6\). A number of previous studies have demonstrated that repeated cycles of this brief activation of TCR fine-tune the quality and magnitude of the activation program \(^7\). However, it remains unknown whether such fine-tuning
mechanisms are common across different effector subsets, or whether the altered regulation may have impact on CD4 T cell immune responses \textit{in vivo}.

The closely related proteins, CD2AP and CIN85, are scaffolding molecules that possess three tandem SH3 domains, a proline rich domain, and a C-terminal coiled-coil domain that mediates their hetero/homodimerization \cite{8}. CD2AP was originally identified via a yeast two-hybrid screen as an interacting partner of the adhesion molecule CD2 and a dominant negative form expressed in Jurkat T cells prevented formation of the immunological synapse \cite{9}. However, in naive T cells from AND TCR transgenic mice lacking CD2AP, formation of the immunological synapse was intact, suggesting that CD2AP functions independently of CD2 in primary T cells \cite{10}. Interestingly, following stimulation with pMHC, activation of proximal kinases and degradation of TCR are delayed, leading to prolonged TCR activation and enhanced cytokine production \textit{in vitro} \cite{10}. However, the role of CD2AP in tuning of TCR signaling in T cell immune responses or the impact of altered TCR signaling quality \textit{in vivo} caused by \textit{Cd2ap} deficiency has not been defined due to a fatal kidney disease that develops around 3 weeks of age in \textit{Cd2ap}–/– mice \cite{11}.

Here, we generated T cell-specific \textit{Cd2ap}–/– deficient mice and show that CD2AP deficiency enhances control of chronic viral infection by augmenting antiviral T\textsubscript{FH} and GC responses. In contrast, T\textsubscript{FH} and GC responses were minimally altered when the mice were immunized with sheep red blood cells, which are prone to elicit type 2 responses \cite{12}. TCR signaling in \textit{Cd2ap}–/– CD4 T cells was prolonged specifically under T\textsubscript{H1} conditions \textit{in vitro}, resulting in increased production of IFN-\textgreek{g}, while CD2AP was dispensable for temporal tuning of TCR signals in T\textsubscript{H2} or T\textsubscript{H17} cells. These results demonstrate that CD2AP-dependent tuning of TCR signaling in CD4 T cells is T\textsubscript{H} subset-specific and that CD2AP may be an effective target to accelerate the development of protective antibodies in antiviral immune responses.
2.3 RESULTS

**T<sub>FH</sub> differentiation is enhanced in CD2AP-deficient mice late in acute viral infection**

To conduct analyses of function of CD2AP in T cells in vivo, we generated the *Cd2ap*-flox allele, in which expression of cre recombinase results in deletion of exon 2 (Fig 1A). This allele was bred to *Cd4*-cre to inactivate *Cd2ap* in T cells. Numbers of mature CD4 and CD8 single positive thymocytes and CD4 and CD8 T cells in the spleen were comparable between *Cd4*-cre<sup>+</sup> *Cd2ap*<sup>F/F</sup> and control *Cd2ap*<sup>F/F</sup> mice (Fig 1, B and C).

To define the role of CD2AP in T cells in vivo, we infected *Cd4*-cre<sup>+</sup> *Cd2ap*<sup>F/F</sup> and control *Cd2ap*<sup>F/F</sup> littermate control mice with the Armstrong strain of lymphocytic choriomeningitis virus (LCMV), which elicits acute antiviral responses and is cleared by CD8 T cells. At the peak of the T cell response on day 8 after infection, no difference was seen in numbers of total CD8 T cells, KLRG1<sup>+</sup> short-lived effector CD8 T cells or that of LCMV glycoprotein (gp)-specific CD8 T cells between *Cd4*-cre<sup>+</sup> *Cd2ap*<sup>F/F</sup> and *Cd2ap*<sup>F/F</sup> mice (Fig 2A, 2B, and 2D), suggesting that CD2AP is not required for normal CD8 T cell responses to acute LCMV infection. Comparable numbers of LCMV-gp-specific CD4 T cells also were detected in both genotypes eight days after infection (Fig 2C and 2D), suggesting that CD2AP is dispensable for priming and initial expansion of LCMV-specific CD4 T cells. Both *Cd2ap*<sup>−/−</sup> and control LCMV-specific CD4 T cells differentiated into Ly6C<sup>+</sup> Th1 effector cells or T<sub>FH</sub> cells as defined by CXCR5 and PD-1 expression on day 8 after infection (Fig 2E and 2F). Accordingly, we did not observe a difference in either frequency or absolute number of Fas<sup>+</sup> GL7<sup>+</sup> GC B cells at this time point (Fig 2E and 2F). At a later time-point (day 22), however, the frequency and absolute number of total and LCMV-specific CXCR5<sup>+</sup> PD-1<sup>+</sup> T<sub>FH</sub> cells as well as GL7<sup>+</sup> Fas<sup>+</sup> GC B cells was significantly increased in *Cd4*-cre<sup>+</sup> *Cd2ap*<sup>F/F</sup>
mice (Fig 2G and 2H). These results indicate that Cd2ap-deficiency enhances CD4 T cell differentiation into the T\textsubscript{FH} subset following initial bifurcation between T\textsubscript{H}1 effector and T\textsubscript{FH} fates early in infection, and augments GC B cell responses.

To determine whether T\textsubscript{FH} differentiation of Cd2ap\textsuperscript{-/-} CD4 T cells is also enhanced by immunization that induces other types of CD4 T cell responses, we analyzed the GC response after immunization of Cd4-cre\textsuperscript{+} Cd2ap\textsuperscript{F/F} and Cd2ap\textsuperscript{F/F} mice with SRBCs, which predominantly induces development of IL-4 producing CD4 T cells\textsuperscript{13,14}. While in the absence of CD2AP, a statistically insignificant trend in increased T\textsubscript{FH} and GC responses was seen at day 12 following immunization, a relatively late time point when antigen levels are declining\textsuperscript{15} (Fig 3A-3H), numbers of T\textsubscript{FH} and GC B cells were comparable between Cd4-cre\textsuperscript{+} Cd2ap\textsuperscript{F/F} and Cd2ap\textsuperscript{F/F} mice both at a relatively early time point, day 6, and a later time point day 22 (Fig 4, A to D). In addition, we found no significant differences in T\textsubscript{FH} and GC B cell numbers between Cd4-cre\textsuperscript{+} Cd2ap\textsuperscript{F/F} and Cd2ap\textsuperscript{F/F} mice following immunization with NP-CGG precipitated in aluminum salts (Fig 4, E and F), which also induces a preferential T\textsubscript{H}2 biased CD4 T cell response\textsuperscript{16}. These results suggested that CD2AP suppresses CD4 T cell differentiation towards the follicular helper subset dependent on the context in which the response is induced.

**Enhanced control of chronic LCMV infection in T cell-specific Cd2ap-deficient mice**

While we observed enhanced T\textsubscript{FH} differentiation in Cd2ap-deficient mice to LCMV-Armstrong, CD8 CTLs rather than LCMV-specific antibodies are required for control of acute LCMV infection\textsuperscript{17}. To determine whether enhanced T\textsubscript{FH} differentiation seen in CD2AP deficiency had an impact on antiviral antibody-mediated immunity, we utilized the LCMV-clone13 (LCMV-
c13) model of chronic viral infection since T<sub>FH</sub>-dependent high affinity antibody responses in the GCs and class-switch recombination to IgG2a/2c subclasses are required for control of the infection<sup>18-20</sup>. After infection, LCMV replicated and established viremia to a similar extent in Cd4-cre<sup>+</sup> Cd2ap<sup>F/F</sup> and control cre<sup>-</sup> Cd2ap<sup>F/F</sup> mice on day 8 (Fig 5A). In control cre<sup>-</sup> Cd2ap<sup>F/F</sup> mice, viral abundance, as determined by the quantities of the LCMV gp transcript in the plasma of mice, started to gradually decline around day 30 (Fig 3A), coinciding with expansion of T<sub>FH</sub> and GC B cells in response to a surge of IL-6 production by follicular dendritic cells<sup>18</sup>. The decline in LCMV abundance was significantly accelerated in Cd4-cre<sup>+</sup> Cd2ap<sup>F/F</sup> mice, with the viral titers below the limit of detection in some Cd4-cre<sup>+</sup> Cd2ap<sup>F/F</sup> mice by day 45 (Fig 5A and 6A). Consistent with the accelerated clearance of LCMV, the frequencies and numbers of total and LCMV gp-specific T<sub>FH</sub> cells in the spleen 22 days after infection were significantly increased (Fig 5, B and D; 6 B and C), although total numbers of LCMV-specific CD4 T cells were not changed in Cd4-cre<sup>+</sup> Cd2ap<sup>F/F</sup> mice compared to control mice (Fig 5D). Accordingly, frequencies and numbers of GC B cells were significantly increased in Cd4-cre<sup>+</sup> Cd2ap<sup>F/F</sup> mice compared to control mice (Fig 5C and 5E; Fig 6D). It has been demonstrated that T follicular regulatory (T<sub>FR</sub>) cells can suppress the GC response<sup>21</sup>; thus we analyzed the frequency of Foxp3<sup>+</sup> cells within the T<sub>FH</sub> compartment, however, we did not find differences between genotypes indicating T<sub>FR</sub> differentiation was unimpaired (Fig 6, E and F). Moreover, plasma from Cd4-cre<sup>+</sup> Cd2ap<sup>F/F</sup> mice exhibited elevated neutralizing activity against LCMV in vitro, while titers of total anti-LCMV antibodies with dominant IgG2c and sub-dominant IgG1 subclasses were comparable between Cd4-cre<sup>+</sup> Cd2ap<sup>F/F</sup> and control Cd2ap<sup>F/F</sup> mice (Fig 5F; 6G). As a measure of GC output we also analyzed the number of memory B cells and plasma cells in the bone marrow at 60 days following LCMV-c13 infection and observed no difference in overall number between Cd4-cre<sup>+</sup> Cd2ap<sup>F/F</sup> and control Cd2ap<sup>F/F</sup>,
suggesting enhanced acquisition of mutations rather than total output contributed to the phenotype (Fig 7). Given the importance of CD4 T cell help in promoting long-lived polyfunctional CD8 T cells we also analyzed the CD8 T cell response at day 22. Importantly, we did not detect differences in overall CD8 T cell numbers or LCMV-specific H-2D\(^b\)(gp33-41) tetramer binding T cells in \(Cd4-\text{cre}\^+ \, Cd2ap^{F/F}\) mice compared to control mice (Fig 8A and B). In addition, we did not observe differences in the polyfunctionality of LCMV-specific CD8 T cells as determined by IFN-\(\gamma\) and TNF-\(\alpha\) production (Fig 8C). These results indicate that while inactivation of CD2AP only minimally affects the CD8 T cell response, it enhances \(T_{FH}\) differentiation and generation neutralizing antiviral antibodies by augmenting B cell help.

**CD2AP deficiency promotes \(T_{FH}\) differentiation in a cell-intrinsic manner**

To determine whether the increased \(T_{FH}\) differentiation in \(Cd4-\text{cre}\^+ \, Cd2ap^{F/F}\) mice was cell-intrinsic, we generated BM chimeras by transferring a mixture of BM cells from \(Cd4-\text{cre}\^+ \, Cd2ap^{F/F}\) or \(\text{cre}^- \, Cd2ap^{F/F}\) mice (CD45.2) and CD45.1 WT mice into lethally irradiated CD45.1 WT mice. Following reconstitution of donor-derived hematopoiesis, the recipient mice were infected with LCMV-c13 and contribution of \(Cd2ap\)-deficient and -sufficient cells to the \(T_{FH}\) compartment was examined 22 days after infection (Fig 9A). While the percentages of CD45.2 cells in the B cell and CD44\(^lo\) PD-1\(^-\) naive CD4 T cell compartments were equivalent between chimeras reconstituted with \(Cd4-\text{cre}\^+ \, Cd2ap^{F/F}\) or \(\text{cre}^- \, Cd2ap^{F/F}\) BM cells, the contribution of CD45.2 cells to the CXCR5\(^+\) PD-1\(^+\) \(T_{FH}\) pool was significantly greater in chimeras receiving \(Cd4-\text{cre}\^+ \, Cd2ap^{F/F}\) compared to control chimeras receiving \(\text{cre}^- \, Cd2ap^{F/F}\) BM cells (Fig 9B). In addition to the quantitative increase in \(T_{FH}\) numbers in \(Cd4-\text{cre}\^+ \, Cd2ap^{F/F}\) mice, \(Cd2ap\)-deficient \(T_{FH}\) cells
expressed elevated levels of genes that are associated with T\(_{FH}\) functions, including \(Il21\), \(Il21r\) and \(Il17ra\), and also those associating with active cell cycling (Fig 9C)\(^{2}\). We confirmed these results by analysis of frequencies of Ki67\(^+\) cells within T\(_{FH}\) cells and found them to be increased in KO cells compared to WT (Fig 6H). In contrast these changes, we did not observe changes in expression of T\(_{FH}\) associated molecules such as ICOS and OX-40, suggesting these costimulatory pathways might not contribute to the observed phenotype (Fig 6I). These results suggest that Cd2ap-deficient CD4 T cells are maintaining highly activated states, potentially through enhanced TCR-dependent signaling, and establish a stable T\(_{FH}\) program potentially by the IL-21-IL-21R feed-forward loop and thus better support GC B cell responses against viral infection.

**CD2AP-deficiency causes sustained TCR activation specifically in T\(_{H1}\) cells**

Our data demonstrated that T\(_{FH}\) differentiation was enhanced by Cd2ap-deficiency specifically in viral infection, but not in immunization with SRBCs or NP-CGG in alum. These results suggest that CD2AP regulates TCR signaling in a T\(_{H}\)-subset-specific manner. To dissect the subset-specific role of CD2AP in CD4 T cells, we activated naive CD4 T cells from \(Cd4-cre^+\) Cd2ap\(^{F/F}\) and control Cd2ap\(^{F/F}\) cells under polarizing conditions that promote generation of T\(_{H1}\) or T\(_{H2}\) cells and compared their responses to antigen receptor stimulation (Fig 10A). While production of IL-4 under T\(_{H2}\) conditions was comparable between Cd2ap-deficient and -sufficient CD4 T cells, the production of IFN-\(\gamma\) was increased by 3-fold in Cd2ap-deficient T\(_{H1}\) cells compared to control cells (Fig 10B). Consistently, activation of the MAPK pathway was prolonged specifically in Cd2ap-deficient T\(_{H1}\) compared to control T\(_{H1}\) cells, whereas we did not observe the difference in T\(_{H2}\) cells (Fig 10C). Previous studies of CD4 T cells using germline Cd2ap-
deficient mice showed that \textit{Cd2ap}^{-/-} TCR-transgenic naive T cells are defective in TCR downregulation \textsuperscript{10}. In polarized T\textsubscript{H}1 cells, downmodulation of surface TCRs was significantly impaired in the absence of CD2AP, while the difference was not observed under T\textsubscript{H}0, T\textsubscript{H}2 or T\textsubscript{H}17 conditions between \textit{Cd2ap}-deficient and -sufficient CD4 T cells (\textbf{Fig 10D}). Furthermore, when \textit{Cd2ap}-deficient and control T\textsubscript{H}1 cells were re-stimulated with plate-bound anti-CD3 and anti-CD28 antibodies or with PMA and Ionomycin for 4 hours, similar frequencies of cells expressed IFN-γ and TNF-α (\textbf{Fig. 10,E and F}). However, when they were stimulated with plate-bound anti-CD3 and anti-CD28 antibodies for 24 hours, the frequencies of cells that expressed IFN-γ or TNF-α during the last 2 hours of the stimulation were significantly increased in \textit{Cd2ap}-deficient T\textsubscript{H}1 cells relative to WT T\textsubscript{H}1 cells presumably due to prolonged retention of TCR on the cell surface, which was crosslinked by the plate-bound antibodies (\textbf{Fig. 10, E and F}). These results indicate that CD2AP deficiency prolongs TCR signaling specifically in CD4 T cells under T\textsubscript{H}1 conditions.

\textbf{CIN85 plays a non-redundant role in T cell-dependent control of LCMV infection}

CIN85 and CD2AP are closely related and may have redundant functions \textsuperscript{8,12}. To test this, we also generated mice in which T cells are deficient for CIN85 by breeding a \textit{Cin85}−flox allele to both \textit{Cd4-cre} \textsuperscript{25}. \textit{Cd4-cre}\textsuperscript{+} \textit{Cin85}\textsuperscript{F/F} mice exhibited no differences in CD4 T and CD8 T cell response to acute LCMV infection (\textbf{Fig 11}). However, in contrast to \textit{Cd4-cre}\textsuperscript{-} \textit{Cd2ap}\textsuperscript{F/F} mice, \textit{Cd4-cre}\textsuperscript{+} \textit{Cin85}\textsuperscript{F/F} mice were unable to control chronic LCMV infection with significantly higher viral burden in the peripheral blood at days 45 and 60 after infection compared to \textit{cre}\textsuperscript{-} littermate controls (\textbf{Fig 13A}). In addition, while all WT mice had cleared virus from the plasma by day 80, approximately half of \textit{Cd4-cre}\textsuperscript{+} \textit{Cin85}\textsuperscript{F/F} still exhibited high viral load (\textbf{Fig 13B}). The
clearance phenotype was more pronounced when we infected Vav1-icre+ Cin85F/F mice, in which all hematopoietic cells, including B cells, were deficient for Cin85 (Fig 12A), suggesting CIN85 plays additional roles in other hematopoietic cells in the context of LCMV-c13 infection, potentially through B cells as previously reported. However, when we analyzed mice at day 30 following infection we did not find any significant differences in either frequency or absolute number of CD8 T cell, T_FH, or GC response (Fig 12, B to D). Consistently, when we analyzed Cd4-cre+ Cin85F/F mice, the frequency of Fas+ GL7+ GC B cells was only marginally, but insignificantly, decreased in Cd4-cre+ Cin85F/F mice compared to control Cin85F/F mice (Fig 13C) with anti-LCMV IgG titers unchanged, suggesting no severe defects in the GC response (Fig 13D). However, plasma from infected Cd4-cre+ Cin85F/F mice exhibited a significantly reduced neutralizing activity compared to plasma from cre− littermate controls (Fig 13E). These results indicate that CIN85 is required for protective antibody responses in a T cell-dependent manner presumably through controlling T_FH function and potentially the quality of help provided to GC B cells with respect to generation of high-affinity neutralizing antibodies required for clearance of chronic LCMV. These results suggest that although CD2AP and CIN85 share significant homology, their functions in antiviral CD4 T cell responses are non-redundant, and potentially antagonistic.
2.4 DISCUSSION

This study demonstrated that deletion of CD2AP in T cells results in skewing of CD4 T cell differentiation towards T<sub>FH</sub> cells in response to viral infection, leading to enhanced control of LCMV that requires GC-derived high affinity antibody responses<sup>18,20,27</sup>. T<sub>FH</sub> differentiation was correlated with sustained TCR signaling under T<sub>H1</sub> conditions, while TCR signaling <i>in vitro</i> under non-T<sub>H1</sub> conditions was not altered. Thus, our work revealed a specific role of CD2AP in subset-specific CD4 T cell responses.

Sustained TCR stimulation during chronic LCMV infection or in the cancer microenvironment causes deregulation of CD8 T cells, a phenomenon known as exhaustion<sup>1,28</sup>. Frequent interactions with cognate pMHC-I result in the persistent upregulation of several inhibitory receptors which act to dampen T cell proliferation and effector functions, a hallmark of the “exhausted state”<sup>2,28</sup>. However, the impact of sustained TCR stimulation on the function of CD4 T cells has been less clearly understood. In chronic LCMV infection, CD4 T cells exhibit less IL-2 production and increased IL-10 production, a phenomenon that is similar in nature to CD8 T cell “exhaustion”<sup>3,29-31</sup>. However, these CD4 T cells with the altered activation state acquire the capability of producing IL-21, a key cytokine that enhances the GC response and also supports the CD8 T cell response; both are required for control of the viral infection<sup>4,30-32</sup>. Thus, although sustained TCR signaling compromises CD8 T cell functions, CD4 T cells are able to tolerate sustained signaling through TCR to mediate pathogen control.

Several recent studies indicate that during chronic LCMV infection, CD4 T cells exhibit a relatively unique propensity to acquire T<sub>FH</sub> features, a process that is dependent on continuous antigen stimulation<sup>5,33</sup>. The acquisition of T<sub>FH</sub> phenotype in chronic infection appears to be
different compared to acute LCMV infection. Interestingly, in late phases >day 20 of LCMV-c13 infection B cells do not appear to be absolutely required for the development of CXCR5+ cells, suggesting other types of antigen presenting cells could contribute to the sustained TFH response as this does not occur in MHCII KO. Preferential Tfh accumulation has also been shown to be dependent on type-I Interferon which has not been explicitly observed in acute contexts, via a cell-extrinsic mechanism, suggesting other soluble or cell-associated factors could have a more direct influence. These results illustrate the complexity in direction of the CD4 T cell response, and illustrate the variety of mechanisms than influence the humoral response in response to the nature of the insulting pathogen. However, in several contexts it appears that modulation of TCR affinity and or signal duration impacts the differentiation of Tfh cells, supporting a more broadly applicable role for the TCR.

Our study with CD2AP-deficient T cells confirm and extend previous studies that suggest that sustained TCR signaling promotes Tfh differentiation. Specifically, it was shown following infection by Listeria monocytogenes that TCR:pMHCII dwell time correlated well with GC-Tfh differentiation. In addition, in antigen-specific cells elicited by immunization with pigeon cytochrome c, Tfh phenotype cells tended to have higher affinity for tetramer compared to non-Tfh cells, and transfer of TCR transgenic cells with higher affinity for pMHCII revealed a preference for Tfh differentiation. One potential explanation is that Tfh and Th1 cells may share a transitional stage, at which sustained TCR signaling may restrict T-bet upregulation to direct the activated CD4 T cells into the Tfh subset, which favors low-T-bet, while Tfh differentiation during a type-2 immune response may require a distinct mechanism. This may explain why Tfh differentiation is enhanced when CD4 T cells receive sustained TCR stimulation as antigen levels decline. Interestingly, enhanced Tfh differentiation and increased
IL-21 expression by Cd2ap-deficient CD4 T cells did not noticeably alter the CD8 T cell response, which is also dependent on CD4 T cell help. These results potentially indicate that the nature of CD4 T cell help to B cells and CD8 T cells may be independent.

While downregulation of the TCR and subsequent termination of TCR downstream signaling is broadly shared by T cells, our results indicate that CD2AP-dependent regulation of TCR signaling duration is Th1-specific in vitro, which correlates with enhanced Tfh differentiation in LCMV infection. However, it still remains unclear whether the in vivo functions of CD2AP are completely explained by TCR signaling via the immunological synapse, interactions with CD2, or potentially via signaling via other cytokines such as IL-21 or Type I interferon and is an area of further investigation. Notably, a recent study reported Th2-specific regulation of the duration of TCR signaling by the Rab-GTPase Dennd1b. Dennd1b-deficiency in CD4 T cells results in sustained TCR signaling and increased cytokine production specifically under Th2 conditions. Furthermore, a DENND1B SNP in human, causing reduced expression of its protein, is associated with asthma. These two studies, contrasting lineage-specific requirement for CD2AP and Dennd1b in controlling TCR signaling in Th1 and Th2 cells, respectively, imply that the control of TCR signaling may be uniquely regulated by different CD4 effector subsets. Despite strong correlations between sustained duration of TCR signaling in vitro and enhanced T cell polarization in vivo in Cd2ap-deficient mice and Debdb1b-deficient mice, however, there may be additional mechanisms, such as cell-cell contact or signaling through CD2, by which T cell polarization are enhanced in these genetically modified mice.

Our study highlights CD2AP as a potential target for immunotherapy to enhance B cell immune responses. Given the importance of high-affinity neutralizing antibodies in the clearance of both acute and chronic viral infections or vaccines, boosting the GC response is an attractive
therapeutic target. The development of small molecules that inhibit the function of CD2AP in the context of viral infection or in the context of vaccines, could enhance the antibody response. T$_{FH}$ responses, however, have also been associated with the development of autoantibodies and autoimmunity.

Collectively, our results demonstrate that the quality of TCR signaling is regulated by distinct mechanisms in CD4 effector T cells, and regulates differentiation of antigen-specific CD4 T cells towards distinct subsets.
2.5 MATERIALS AND METHODS

Ethics Statement

Overall care and use of the animals was consistent with *The guide for the Care and Use of Laboratory Animals* from the National Research Council and the USDA *Animal Care Resource Guide* and were performed according to a protocol approved by Washington University’s Animal Studies Committee under a protocol number of 20150187, which was approved on 10/26/2015 and expires 10/9/2018. Euthanasia procedures are consistent with the “AVMA guidelines for the Euthanasia of Animals 2013 edition.”

Mice

C57BL/6N and B6-CD45.1 mice were purchased from Charles River Laboratory. *Cd4*-cre mice in the C57BL/6 background were purchased from the Jackson Laboratory. A *Cin85*-floxed allele was described previously. To generate a *Cd2ap*-flox allele, a genomic fragment containing *Cd2ap* exon2 was flanked with two *loxP* sites and subcloned into a vector containing a *loxP*-flanked neomycin resistant gene, a diphtheria toxin A (DTA) gene, and a 6.0-kb 5’ and a 2.1-kb 3’ homology arm. The targeting vector was electroporated into C57BL/6-derived Bruce4 embryonic stem (ES) cells. After the selection with G418 (Thermofisher), correctly targeted clones were identified by PCR. The neomycin resistant gene was removed by a transient transfection of a *cre* expression vector. The targeted ES clones were injected into blastocysts of BALB/c mice. The chimeric mice were crossed with C57BL/6 mice to obtain germline transmission. To generate BM chimeras, B6-CD45.1 mice were lethally irradiated (10.5 Gy) and
reconstituted with donor BM cells for at least 8 weeks before experiments. All mice were housed in a specific pathogen-free facility at Washington University in St. Louis, and were analyzed at 8 to 10 weeks of age, unless stated otherwise. Both sexes were included without randomization or blinding.

**LCMV Infection and Immunization**

Mice were infected with $2 \times 10^5$ plaque-forming units (PFU) of LCMV-Armstrong strain via the intraperitoneal route or $2 \times 10^6$ (PFU) of LCMV-c13 by intravenous injection. For the quantification of plasma viral load, RNA was extracted from 10 μL of plasma using Trizol (Life Technologies). Before RNA extraction a spike-in of RNA extracted from 293T cells (American Type Culture Collection, ATCC) expressing $gfp$ mRNA was added to the plasma samples. The amounts of the LCMV GP transcript relative to that of ‘‘spiked- in’’ $gfp$ RNA were determined by real-time qRT-PCR as previously described $^{20,40}$. For plasma viral titers, plasma was frozen at -80°C before performing plaque assay on Vero cells as previously described. Briefly, $7.5 \times 10^5$ Vero cells (ATCC) were plated per well of a 6-well plate 24 hour prior to incubation with serial 10-fold dilutions of plasma in 200 μL of MEM/1%FBS (Thermofisher) for 1 hour at 37°C. Following the incubation Vero cells were overlaid with 0.5% Agarose (Thermofisher) solution in complete MEM and incubated for 5-6 days. Plaques were visualized following fixation in 1% PFA (Electron Microscopy Sciences) and staining with 0.1% crystal violet (Sigma).

*In vitro* neutralization assay was performed according to a published protocol with some modifications $^{26}$. Heat-inactivated (1 hr at 55°C) plasma were serially diluted with media and incubated with an equal volume of viral supernatant containing ~30-40 focus forming units
(FFU) of LCMV clone 13 at 37°C for 90 min. 4 x 10⁴ MC57G cells (ATCC) were added to the virus mixture and once the cells had adhered to the plate ~4 hours, were overlaid with a 1% methylcellulose solution in completed DMEM. 40 hours following infection, overlay was removed, and cells were fixed with 4% PFA for 1 hour at RT. Cells were permeabilized with 0.5% Triton X-100 in PBS and blocked with 10% FBS in PBS. Cells were stained with 1μg/mL of anti-LCMV NP (VL-4; BioXCell) for 1 hour at RT followed by anti-rat IgG HRP. Foci were developed using KPL TrueBlue Substrate.

Mice were immunized with 8-10 x 10⁸ SRBC (Lampire) in 200 μL via the intraperitoneal route or with 100μg of NP-CGG (Biosearch Technologies) precipitated Potassium Aluminum Sulfate (Sigma).

**Flow cytometry and Cell Sorting**

Single-cell suspensions were prepared by manual disruption of spleens with frosted glass slides. Absolute cell counts were determined using Vi-Cell (Becton-Dickson). The following monoclonal antibodies were used: anti-CD4 (GK1.5; Biolegend), anti-CD8a (53-6.7; Biolegend), anti-CD25 (PC61; Biolegend), anti-CD44 (IM7; Biolegend), anti-CD45.2 (104; Biolegend), anti-CD45R (B220) (RA3-6B2; Biolegend), anti-CD62L (MEL-14; Biolegend), anti-CD95 (Fas) (Jo2; BD Biosciences), anti-KLRG1 (2F1/KLRG1; Biolegend), anti-IFN-γ (XMG1.2; eBioscience), anti-TCRb (H57-597; eBioscience), anti-PD-1 (29F.1A12; Biolegend), anti-CXCR5 (2G8; BD Biosciences) or anti-CXCR5 (L138D7; Biolegend), anti-Ly6C (HK1.4; Biolegend), and anti-GL7 antigen (GL7; Biolegend).
For sorting of naive CD8 T cells, splenocyte samples were initially depleted of B220+ cells through the use of magnetic beads (Thermofisher). CD4 T cells were negatively selected using the Dynabeads FlowComp Mouse CD4 Kit (Thermofisher) and then were stained with monoclonal antibodies to purify CD62L+ CD44- CD25- cells on a FACSaria II or FACSariaII (BD Biosciences). For phenotypic analysis, cells were stained with monoclonal antibodies, phycoerythrin (PE)-conjugated H-2Db-gp(33-41) (MBL International) for 1 hour at RT or PE-I-Ab(gp66-77) (obtained from NIH Tetramer Core) for 90 minutes at 37°C. Dead cells were excluded by staining with DAPI (4,6-diamidino-2-phenylindole; Sigma). Data were analyzed with FlowJo software (TreeStar).

**In Vitro T cell Culture**

Sort purified naive (CD62L+ CD44lo CD25-) CD4 or CD8 T cells were cultured in RPMI 1640 medium supplemented with 10% FBS (Thermofisher) and Gluta-MAX in the presence of plate-bound anti-CD3 (145-2C11; Biolegend) and soluble anti-CD28 (37.51; Bio X Cell) at concentrations of 0.1 μg/ml and 0.5 μg/ml, respectively, unless specified otherwise, in multiwell tissue culture plates coated with rabbit antibody to hamster IgG (0855395; MP Biomedicals). For T_h1 polarization 10 ng/mL of IL-12 (R&D Systems) and 10 μg/mL of anti-IL-4 (11B11) were added to culture media. For T_h2 polarization 10 ng/mL IL-4 (eBioscience), 10 μg/mL anti-IL-12 (BioXCell), and 10 μg/mL anti-IFN-γ (Biolegend) were added to culture media. Following 3 days of anti-CD3 and anti-CD28 stimulation cells were removed from stimulation and rested for 3 days in conditioned medium at concentration of 0.5-1 x 10^6 cells/mL. For CD8 T cell cultures, cells were cultured in 40 U/mL IL-2 during the resting period.
Day 6 CD4 T cells were harvested, washed in fresh media, and restimulated using 50 ng/mL PMA (Sigma) and 1 μM Ionomycin (Sigma) or plate-bound anti-CD3 (Biolegend) and soluble anti-CD28 (BioXcell) as described above for the indicated times. For intracellular cytokine analysis, Brefeldin A (Biolegend) was added to the cultures 2 hours before harvest. Supernatant was collected 24 hours post-stimulation.

**ELISA**

ELISA for cytokines was performed on Nunc Maxisorp plates using ELISA MAX IFN-γ and IL-4 kits (Biolegend) and developed using TMB substrate (Dako). OD$_{450}$ values were read on a spectrophotometer. Standard curves for these ELISAs were generated with purified cytokines.

Anti-LCMV ELISA was performed as previously described $^{41}$. Nunc Polysorp plates were coated with 10ug/mL sonicated cell lysate from LCMV-infected BHK-21 cells (gift from Marco Colonna, Washington University) as capture antigen or uninfected BHK-21 cell lysate overnight followed by UV irradiation (300 mJ in Stratalinker 1800; Stratagene). Plasma antibody was detected with biotinylated anti-mouse IgG1 antibody (BD) and anti-mouse IgG2c antibody (1077-08; Southern Biotech). Endpoint titers were calculated by a sigmoidal-dose response curve using a Graphpad Prism 7 software.

**Intracellular Cytokine Staining**
Cells were subject to LIVE/DEAD Aqua staining (Thermofisher) for 30 minutes at 4°C before being fixed with 4% PFA for 10 minutes at RT. Cells were washed twice with 0.03% saponin (Sigma) in 2% FBS/PBS before being stained with the indicated antibodies in 0.3% Saponin in 2% FBS/PBS for 20 min at 4°C.

**T cell stimulation and western blotting**

*In vitro* polarized T cells were rested for 1 hour in cytokine-free RPMI medium before being stained with 5 μg/mL anti-CD3 and 5 μg/mL anti-CD28 on ice for 20 minutes. TCR was then crosslinked by addition of 20 μg/mL of anti-Armenian Hamster IgG to the cells on ice. Cells were stimulated in 5 ml polystyrene tubes for the indicated times in a water bath at 37°C. Cells were lysed in NP-40 Lysis Buffer (1% NP-40, 50 mM Tris-HCl (pH7.5), 150 mM NaCl, 2 mM EDTA, phosphatase inhibitors) and cleared via centrifugation at 14,000 rpm for 10 minutes, then denatured in SDS sample buffer.

Lysates from equal numbers of cells were separated by 8% or 10% SDS PAGE and transferred to nitrocellulose membranes (Bio-Rad), which were incubated with primary antibodies (identified below), followed by detection with horseradish peroxidase–conjugated species-specific antibody to immunoglobulin light chain (115-035-174 or 211-032-171; Jackson ImmunoResearch) and a Luminata HRP substrate (Millipore). The following antibodies were used: anti-CD2AP^10^, anti-ERK2 (Santa Cruz, C-14), anti-p-MEK1/2 (Cell Signaling Technology, 9121).
RNA-sequencing

Total RNA was extracted from ~300,000 sorted cells using manufacturer’s instructions using the RNA XS Kit (Macherey Nagel). cDNA synthesis and amplification were performed with a Pico Input RNA Kit, according to the manufacturer's instructions (Clontech). Libraries were sequenced on a HiSeq3000 (Illumina) in single-read mode, with a read length of 50 nucleotides producing ~25 million reads per sample. Sequence tags were mapped onto the NCBI37 mm9 with TopHat\textsuperscript{42} followed by transcript assembly and RPKM estimation using Cufflinks\textsuperscript{43-45} on the Galaxy platform (https://usegalaxy.org/). The raw data has been deposited at NCBI GEO with an accession number GSE112778.

Statistical Analyses

Statistical analyses were performed with a two-tailed unpaired Student’s $t$-test or a Mann-Whitney test using GraphPad Prism software.
2.6 ACKNOWLEDGEMENTS

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2.8 REFERENCES


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Fig 2.1. **CD2AP is dispensable for T cell development.** (A) Generation of a *Cd2ap*-flox allele by gene targeting. Arrows indicate primer position for PCR. LA: long homology arm, SA: short homology arm, Neo: neomycin resistance cassette, DTA: diphtheria toxin A. (B, C) Flow cytometric analysis of expression of CD4, CD8α, TCRβ, CD24 and CD69 in thymocytes (B) and splenocytes (C) from *Cd4-cre* + *Cd2ap* F/F and control cre − *Cd2ap* F/F mice. Numbers indicates percentages of cells surrounded by rectangle or polygon gates. Data are representative of 3-6 mice in 2 independent experiments.
Fig 2.2. Enhanced T<sub>FH</sub> and GC B cell responses in T cell-specific <i>Cd2ap</i>-deficient mice in response to acute viral infection. (A) Flow cytometric analysis of expression of CD4, CD8 and CD44 and binding of H-2D<sup>b</sup>(gp33-41) tetramer in splenocytes 8 days after LCMV-Armstrong infection. Total CD8 T cells and LCMV-gp33-specific CD8 T cells are shown with rectangular gates. (B) Expression of CD8 and KLRG1 in splenocytes 8 days after LCMV-Armstrong infection. (C) Expression of CD44 and binding of I-A<sup>b</sup>(gp66-77) tetramer in CD4<sup>+</sup> splenic T cells 8 days after LCMV-Armstrong infection. LCMV-gp66-specific CD4 T cells are shown with rectangular gates. (E, G) Expression of PD-1, CXCR5 and Ly6C and binding of I-A<sup>b</sup> (gp66-77)<sup>+</sup> tetramer of CD4<sup>+</sup> T cells and Fas and GL7 expression in B cells in the spleens of Cd4-cre<sup>+</sup> Cd2ap<sup>F/F</sup> and control Cd2ap<sup>F/F</sup> mice 8 (E) and 22 (G) days after infection. Follicular helper CD4 T cells and GC B cells are shown with rectangular gates. Representative plots are shown with percentages of gated cells. Statistical analyses from 4-6 mice in 2 independent experiments are shown with means and standard deviation in (D, F, H).
Fig 2.3. Cdcap deficiency has a minimal impact on $T_{FH}$ differentiation and GC B cell responses following immunization with SRBCs. (A, B) Flow cytometric analysis of expression of PD-1 and CXCR5 on pre-gated CD4$^+$ B220$^-$ T cells (A) and GL7 and Fas expression on CD19$^+$ B220$^+$ B Cells (B) 12 days following SRBC immunization. (C-E) Numbers and frequencies of total CD4 T cells and CXCR5$^+$ PD-1$^+$ $T_{FH}$ in the spleen of Cdcap$^+$ Cdcap$^{+/}$ and control Cdcap$^{+/}$ mice 12 days after immunization with SRBCs. (F-H) Numbers and frequencies of total B cells and Fas$^+$ GL7$^+$ GC B cells in the spleen of Cdcap$^+$ Cdcap$^{+/}$ and control Cdcap$^{+/}$ mice 12 days after immunization with SRBCs. Data are pooled from 2 independent experiments shown as means and standard deviation.
Fig. 2.4 Time-course of GC B cell response following SRBC and NP-CGG Immunization in Cd2ap deficient mice. (A, C, E) Flow cytometric analysis of expression of PD-1 and CXCR5 on pre-gated CD4⁺ B220⁻ T cells and GL7 and Fas expression on CD19⁺ B220⁺ B Cells at 6 days (A), 22 days (C) following SRBC immunization, and (E) 10 days following Alum precipitated NP-CGG immunization. (B, D, F) Numbers and frequencies of CXCR5⁺ PD-1⁺ T_FH and Fas⁺ GL7⁺ GC B cells in the spleen of Cd4-cre⁺ Cd2ap^{FF} and control Cd2ap^{FF} mice at 6 days (B), 22 days (C) immunization with SRBC and and (F) 10 days following Alum precipitated NP-CGG immunization. Data are representative of 2 independent experiments shown as means and standard deviation.
Fig 2.5 Enhanced control of LCMV-c13 infection in Cd4-cre+ Cd2apFF is associated with elevated T\textsubscript{FH} response.

(A) Analysis of LCMV abundance in plasma in mice as determined by LCMV gp transcript levels. Horizontal bars indicate medians. The limit of detection is shown by a dashed line. Statistical significance was tested by Mann Whitney U-test.

(B-E) Expression of B220, GL7, Fas, CD4, CD44, PD-1 and CXCR5 and binding of I-A\textsuperscript{b} (gp66-77) tetramer of splenocytes from Cd2apFF and Cd4-cre+ Cd2apFF mice 22 days after LCMV-c13 infection. Representative plots are shown with percentages of gated cells. Statistical analyses from at least 7-10 mice per genotype in 2 independent experiments are shown with means and standard deviation in (D) and (E).

(F) (Left) Neutralizing activity and (Right) anti-LCMV IgG2c antibody titers of plasma from Cd2apFF and Cd4-cre+ Cd2apFF mice 60 days after LCMV-c13 infection. Reduction in focus forming units (FFU) in the presence of 1:10 dilution of plasma compared to untreated control (%Neutralization) is shown with mean and standard deviation. Cd2apFF: n=7, Cd4-cre+ Cd2apFF: n=9.
Fig 2.6. Analysis of GC Response in CD2AP deficient mice following LCMV-c13 infection

(A) Analysis of viral plaque forming units (PFU) in Cd2ap<sup>−/−</sup> and Cd4-cre<sup>+</sup> Cd2ap<sup>−/−</sup> mice at day 45 of LCMV-c13 infection. (B) Expression of PD-1 and CXCR5 in CD4 T cells at day 22 of LCMV-c13 infection in Cd2ap<sup>−/−</sup> and Cd4-cre<sup>+</sup> Cd2ap<sup>−/−</sup> mice (C-D) Absolute numbers of (C) CD4 T cells, and T<sub>FH</sub> cells (CXCR5<sup>+</sup>PD-1<sup>+</sup>) and (D) B cells at day 22 of LCMV-c13 infection in Cd2ap<sup>−/−</sup> and Cd4-cre<sup>+</sup> Cd2ap<sup>−/−</sup> mice. (E, F) Frequencies of Foxp3<sup>+</sup> T<sub>FH</sub> cells 22 days following LCMV-c13 infection of Cd2ap<sup>−/−</sup> and Cd4-cre<sup>+</sup> Cd2ap<sup>−/−</sup> mice. (G) Anti-LCMV IgG1 antibody titers of plasma from Cd2ap<sup>−/−</sup> and Cd4-cre<sup>+</sup> Cd2ap<sup>−/−</sup> mice 60 days after LCMV-c13 infection. (H) Frequencies of Ki67<sup>+</sup> T<sub>FH</sub> cells 22 days following LCMV-c13 infection of Cd2ap<sup>−/−</sup> and Cd4-cre<sup>+</sup> Cd2ap<sup>−/−</sup> mice. (I) Expression of ICOS and OX40 in Naive CD4 (PD-1<sup>−</sup>CD44<sup>lo</sup>) CD4 T cells and CXCR5<sup>+</sup>PD-1<sup>+</sup> T<sub>FH</sub> cells 22 days following LCMV-Armstrong infection in Cd2ap<sup>−/−</sup> and Cd4-cre<sup>+</sup> Cd2ap<sup>−/−</sup> mice. Data are representative of 2 independent experiments with n=3-6 mice per genotype.
Fig 2.7. Analysis of GC output following LCMV-c13 infection (A-B) Expression of B220, IgM, IgD, GL7, AA4.1, CD138, and CD24 of splenocytes (A) and bone marrow cells (B) of Cd2ap^{F/F} and Cd4-cre^{+} Cd2ap^{F/F} 60 days after LCMV-c13 infection. (C-D) Absolute numbers of (C) memory B cells in the spleen of (D) plasma cells in the bone marrow of Cd2ap^{F/F} and Cd4-cre^{+} Cd2ap^{F/F} 60 days after LCMV-c13 infection. Data are representative of 2 independent experiments shown as means and standard deviation.
Fig. 2.8. CD8 T cell response in CD2AP deficient mice is comparable to control mice following LCMV-c13 infection. (A) Expression of CD8, CD4, CD44 and PD-1 and binding of H-2D\(^b\) (gp33-41) in Cd2ap\(^{FF}\) and Cd4-cre\(^+\) Cd2ap\(^{FF}\) mice 22 days after LCMV-c13 infection. (B) Absolute quantification of (A) (C) Splenocytes from Cd2ap\(^{FF}\) and Cd4-cre\(^+\) Cd2ap\(^{FF}\) mice 22 days after LCMV-c13 infection were stimulated with gp33-41 peptide and the expression of IFN-\(\gamma\) and TNF-\(\alpha\) was analyzed. Data are representative of 2 independent experiments with n=4-6 mice per genotype.
Fig. 2.9 Cd2ap-deficiency enhances T_{FH} differentiation in a cell-intrinsic manner. (A) Schematic of experimental strategy for the generation of mixed BM chimeras and subsequent analysis of anti-LCMV responses. (B) Contribution of CD45.2 {Cd2ap}^{F/F} or {Cd4-cre} {Cd2ap}^{F/F} cells to B cells, activated CD4 T cells and T_{FH} cells in the mixed BM chimeras 22 days after LCMV-c13 infection. Representative Data from 2 independent experiments (n=4 of each genotype) are shown with means and standard deviations. Statistical difference was tested by Student's t-test. (C) A volcano plot showing differentially expressed genes between Cd2ap-deficient (KO, n=2) and -sufficient (WT, n=2) T_{FH} cells harvested from mixed BM chimeras 22 days after infection. Genes that were differentially expressed by >2-fold are shown.
Fig 2.10. *Cd2ap* deficiency causes sustained TCR signaling specifically in T<sub>H1</sub> cells *in vitro*. (A) Schematic of experimental strategy for culture of CD4 T cells. (B) Concentration of IFN-γ or IL-4 in supernatant measured by ELISA 24 hours after re-stimulation of polarized *Cd2ap<sup>F/F</sup>* and *Cd4-cre<sup>+</sup> Cd2ap<sup>F/F</sup>* T<sub>H1</sub> cells and T<sub>H2</sub> cells with plate-bound anti-CD3/anti-CD28 antibodies. (C) Immunoblotting showing phosphorylation of MEK1/2 in polarized *Cd2ap<sup>F/F</sup>* and *Cd4-cre<sup>+</sup> Cd2ap<sup>F/F</sup>* T<sub>H1</sub> or T<sub>H2</sub> cells following re-stimulation with plate-bound anti-CD3/anti-CD28 for the indicated times at 37°C. Anti-CD2AP, -CIN85 and ERK were used as control. Data are representative of three experiments. (D) Downregulation of surface TCR of *Cd2ap<sup>F/F</sup>* and *Cd4-cre<sup>+</sup> Cd2ap<sup>F/F</sup>* T cells that were polarized under indicated conditions. Cells were stimulated with plate-bound anti-CD3 and surface TCR levels were quantitated at the indicated time points. Data are representative of three experiments. (E, F) Intracellular staining for IFN-γ and TNF-α of polarized *Cd2ap<sup>F/F</sup>* and *Cd4-cre<sup>+</sup> Cd2ap<sup>F/F</sup>* T<sub>H1</sub> cells after re-stimulation with PMA and Ionomycin or plate-bound anti-CD3 and anti-CD28 for 4 or 24 hours in the presence of Brefeldin A for the last 2 hours before harvest. Representative plots (E) and statistical analyses with means and standard deviations (F) from three experiments are shown.
Fig 2.11 Intact CD8 and CD4 T cell response to LCMV Armstrong in T cell-specific Cin85 deficient mice. (A) Expression of IFN-γ, TNF-α, and IL-21 in splenocytes from day 8 LCMV-Armstrong following stimulation with gp61-80 peptide in Cin85 F/F and CD4-cre + Cin85 F/F animals. (B) Expression of CD4, CD8, and CD44, and binding of H-2D b (gp33-41) and H-2D b (np396-404) tetramers of splenocytes from Cin85 F/F and Cd4-cre + Cin85 F/F mice eight days after LCMV-Armstrong infection. (C) Expression of CD19, B220, GL7, and Fas eight days after LCMV-Armstrong infection in Cin85 F/F and Cd4-cre + Cin85 F/F mice. (D, E) Absolute quantification of cell numbers from Cin85 F/F and Cd4-cre + Cin85 F/F mice. Data are representative of 2 independent experiments of n=4-6 mice each shown as means and standard deviation.
Fig 2.12. Delayed clearance of LCMV-c13 in Vav1-icre Cin85^{F/F} mice. (A) Plasma viral abundance of LCMV determined by gp transcripts in Cin85^{F/F} and Vav1-icre^+ Cin85^{F/F}. (B-D) Frequencies and absolute numbers of (B) H-2D^b(gp33-41)-specific CD8^+ cells (C) I-A^b(gp66-77)-specific CD4^+ T cells and CXCR5^+PD-1^+CD4^+ T cells, and (D) Fas^+GL7^+ B cells in the spleen day 30 post-infection in LCMV-c13 infection in Cin85^{F/F} and Vav1-icre^+ Cin85^{F/F} mice. Data combined from three independent experiments of n=2-3 mice each.
Fig 2.13 *Cin85*-Deficiency results in defective clearance of LCMV-c13 and is correlated with decreased neutralizing antibody titer. (A, B) Analysis of LCMV abundance in plasma in *Cin85*F/F and *Cd4*-cre+ *Cin85*F/F mice as determined by LCMV gp transcript levels (A) or focus forming assay (B) at day 80. Horizontal lines indicate median. The limit of detection is shown by dashed lines. Statistical significance was tested by Mann Whitney U-test. (C) Frequencies of Fas⁺ GL7⁺ B220⁺ GC B cells at day 35 after LCMV-c13 infection. (D) anti-LCMV IgG antibody titers of plasma from *Cin85*F/F and *Cd4*-cre+ *Cin85*F/F mice 30 days after LCMV-c13 infection. (E) Neutralizing activity of plasma from *Cd2ap*F/F and *Cd4*-cre+ *Cd2ap*F/F mice 80 days after LCMV-c13 infection. Reduction in focus forming units (FFU) in the presence of 1:5 dilution of plasma compared to untreated control (% Neutralization) is shown with mean and standard deviation. Student t-test. Data combined from 3 independent experiments are shown with n=3-4 mice per genotype.
Chapter 3:

Modulation of CD8 T cell Function by PD-1 and Type I Interferon in Chronic LCMV Infection

The contents of this chapter are currently under preparation for submission to a peer-reviewed journal.
3.1 ABSTRACT

In response to persistent exposure to antigen, CD8 T cell responses are attenuated in part due to upregulation of the inhibitory receptor PD-1. PD-1 mediated inhibitor signaling on T cell responses has been associated with prolonged viral persistence or tumor progression, and blockade of PD-1 signaling during antigen persistence can lead to durable responses. However, it remains elusive whether PD-1-mediated signaling directly promotes viral persistence via suppression of immune responses or if it functions as a mechanism by which the host facilitates durable immunity by preventing tissue damage. Here we show that PD-1-mediated suppression is required for durable CD8 T cell responses against chronic viral infection. Treating mice infected by a chronic strain of LCMV with PD-1 blockade at the peak of viremia does not cause fatal immunopathology, but eventually resulted in disruption of lymphoid architecture and a reduction in the CD8 T cell response. Furthermore, transient blockade of type-I interferon completely prevented death of LCMV-infected mice treated with PD-1 blockade at the time of infection by attenuating CD8 effector function and, intriguingly a combination of the transient blocking IFNAR signaling and delayed PD-1 blockade, which alone would compromise antiviral immunity, accelerated viral clearance without causing immunopathology. These results indicate that harnessing CD8 T cells at early stages of chronic viral infection in paradoxically benefits long-term immunity and eventually results in enhanced control of viral infection.
3.2 INTRODUCTION

The host immune system responds to invading pathogens through a variety of effector mechanisms that not only control infection but also have the potential to cause host damage and the manifestations of disease \(^1\). The adaptive immune response is elicited following engagement of antigen receptors on B and T cells along with co-stimulatory signals and cytokines which activate a dynamic gene expression program necessary for clonal expansion and effector differentiation of antigen-specific naive T and B cells \(^2,3\). While robust adaptive responses often achieve clearance of pathogen, prolonged persistence of infections in the host in turn results in deregulated CD8 T cell responses known as T cell “exhaustion”, which is characterized by increased expression of PD-1 and other inhibitory receptors \(^4,5\) and decreased *ex vivo* cytokine production. A similar phenomenon is observed within the tumor microenvironment; tumor infiltrating lymphocytes (TIL) express inhibitor receptors in the presence of persistent antigen. These situations highlight a fundamental mechanism of T cell suppression involving extracellular modulation of intracellular signaling pathways.

The PD-1 pathway was originally found to be involved in T cell apoptosis and cell death in a variety of *in vitro* experiments \(^6\), however, later, it was found to play a role in regulation of T cell priming *in vivo* \(^7,8\). Early studies revealed PD-1 to act in an inhibitory fashion; its loss promoted T cell priming and activation. These studies were consistent with genetic loss of PD-1 or its ligands PD-L1/L2 which results in varying degrees of autoimmunity depending on the strain of mice \(^9,10\). Thus, PD-1 became a canonical inhibitory molecule which acted to suppress T cell responses during T cell activation and also to a certain extent in peripheral tissues in maintaining immune tolerance. These effects are thought to be mediated through the ITIM motif
of PD-1 which serves to recruit SHP-2 phosphatase to inhibit signaling via the CD28-B7 pathway and/or direct inhibition of TCR signaling.

A significant body of evidence has implicated the PD-1 pathway in tumor control. Overexpression of PD-L1 in certain tumor lines was shown to promote tumor growth in a T cell dependent manner, and genetically deficient mice have reduced tumor burden in some models\textsuperscript{11,12}. The role of PD-L1 expression on host or tumor depend on a variety of factors, specific to each tumor type, and highlight the multifaceted role PD-1 plays in the antitumor response\textsuperscript{13-15}. These studies led to the development of checkpoint blockade inhibitors in the clinic, which have resulted in significant and durable effects in a subset of patients with metastatic cancer. While the effects of checkpoint blockade are clear, a mechanistic understanding of how the immune response is altered remains not as well understood. Specifically, the relevance to T cell priming and “reinvigoration” of exhausted T cells is not known.

Chronic viral infections result in prototypic T cell “exhaustion” whose molecular and cellular basis has been a subject of intense investigation. It is generally thought development of high-affinity neutralizing antibodies function in conjunction with CD8 T cell responses are required for significant viral control. Infection of mice with rapidly-replicating LCMV strains results in CD8 T cell exhaustion and a long-term viremia that can be eventually cleared depending on dose and strain of both mouse and virus\textsuperscript{16-18}. A mechanistic link to T cell exhaustion and viral persistence is exemplified by the inhibition PD-1:PD-L1 interactions which leads to so-called “re-invigoration” of CD8 T cells and enhanced control of viremia in certain settings\textsuperscript{19-21}. Alternatively, acquisition of the exhausted state may be an adaptation strategy taken by the host, which eventually allows for durable control of persistent antigen without excess tissue damage associated with antiviral responses\textsuperscript{5}. Thus, the ultimate consequences of
PD-1-mediated suppression of T cell responses are subject to modulation by host factors which remain incompletely defined.

In response to infection by LCMV clone 13, a variant stain of LCMV-Armstrong causing chronic infection, CD8 T cells upregulate PD-1 expression by day 5 after infection and continue to express PD-1 until virus is spontaneously cleared in C57BL/6 mice around days 80-100 after infection. However, the role of PD-1 during the establishment of exhaustion remains incompletely understood in part due to fatal immunopathology caused by blocking PD-1 interaction with its ligand PD-L1 during LCMV infection. It should be noted that death due to rapidly-replicating LCMV can also be caused by mechanisms other than blockade of PD-1 as evidence by death of certain strains of mice, and with treatment with exogenous IL-21. Nevertheless, this lethal phenotype has been attributed to a consequence of high viral load as it does not occur in LCMV-Armstrong infected mice. However, death does not occur following PD-L1 blockade during chronic phase of the infection (>23) or in the context of CD4 T cell depletion (>D45) in which viral load is extremely high indicating PD-L1 blockade is not sufficient to cause death in all contexts.

In this work, we sought to clarify the function of PD-1 throughout the course of LCMV-c13 infection in mice. We establish that the function of PD-1 during the late acute phase (D8-22) is required for durable control of infection, in contrast to its function in the chronic phase (>23) where it promotes viral persistence. Importantly, we find that although PD-1 protects the animal from death immediately following infection, this is not clearly the case following acute inoculation, potentially reflecting a specific phenomenon during the T cell priming phase. We found evidence of diminished adaptive immune responses correlated with tissue damage induced by PD-L1 blockade. This damage was reversed by blockade of Type I Interferon, and
combination blockade with anti-PD-L1 accelerated viral clearance. These results suggest the functional outcome of PD-L1 signaling can be modulated by other host factors, including Type I Interferon.
3.3 RESULTS

**PD-L1 blockade at peak of viremia results in enhanced CD8 T cell response but not significant lethality**

To understand the difference in phenotype observed between blockade of the PD-1 pathway late in LCMV-c13 infection compared to infection of PD-1 or PD-L1 deficient mice, we first tested whether this effect could be trivially explained by incompleteness of PD-1 blockade. A previous report using antibody blockade at the beginning of infection reported significant core body temperature loss, however, did not measure lethality. Using two different antibody treatment strategies: 200ug everyday for 5 days or 200ug every other day for 8 days (data not shown) we observed complete lethality of treated mice 10 days after infection (Fig. 1A). Thus, antibody mediated blockade is sufficient to induce lethality following LCMV-c13 infection recapitulating previously reported genetic knockout experiments.

While blockade of the PD-1 pathway “reinvigorates” exhausted T cells, it has also been implicated in early phases of T cell priming. Another possibility is that loss of PD-1 signaling prevents exhaustion, resulting in a more “pathogenic” phenotype. However, the prevention of exhaustion is likely not the case as competitive transfer experiments of WT and PD-1 deficient T cells into the same environment, paradoxically results in enhanced exhaustion. We thus, analyzed the PBMC of mice at Day 5 of infection, before the mice exhibit signs of disease, and found that anti-PD-L1 treated mice had an increased frequency of both CD8 T cells and LCMV-specific CD8 T cell compared to control mice (Fig. 1, B and C). This result indicates that PD-1 deficiency at the beginning of infection results in enhanced priming and accumulation of antigen-specific cells which could be sufficient to explain lethality.
As previously mentioned, blockade of the PD-1 pathway following infection with acute strains of LCMV such as WE and Armstrong does not result in lethality dependent on CD8 T cells. This difference is likely due to the vastly different viral loads attained with greater than $10^5$ PFU/mL of serum with LCMV-c13 and $<10^2$ PFU/mL with the acute strains. Thus, a commonly held view is that a high viral load in the context of PD-1 deficiency is sufficient to induce lethal disease$^{19,23}$. We tested this hypothesis directly by blocking PD-L1 (200ug every third day) beginning on Day 8, at the peak of viremia. Treatment beginning at Day 8 did not result in significant mortality, with $>80\%$ of mice surviving the course of treatment (Fig. 2A). Thus, blockade of the PD-1 pathway at this time point is not sufficient to induce lethality after Day 8, similar to results obtained with blockade beginning at Day 23$^{19}$.

It is possible that micro-environmental factors prevent PD-L1 induced lethality or decrease efficacy of blockade which could explain our results. We thus wished to test whether the CD8 T cells could still mediate significant lethality. To determine if this was the case, we transferred CD8 Effector T cells from mice infected with the acute LCMV-Armstrong strain into Day 8 LCMV-c13 infected mice, and treated with anti-PD-L1. We found that anti-PD-L1 treatment along with transferred CD8 T cells induced lethality in 2-5 days. We conclude that efficacy of blockade is not impeded by microenvironmental factors affecting antibody administration but rather alteration of host CD8 T cell response in a cell-intrinsic or extrinsic-manner (Fig. 2B).

We were next interested in determining how PD-L1 blockade at Day 8 affected the adaptive immune response. Analysis of PBMC at Day 14 of infection revealed significant expansion of bulk and antigen-specific T cells in PD-L1 treated mice (Fig. 3, A and B). LCMV-specific T cells from treated mice also exhibited higher expression of PD-1 indicating additional
TCR-dependent activation (Fig. 3B). Consistent with an increased CD8 T cell response we found that treated mice did not begin to re-gain weight following after the initiation of blockade at Day 8 (Fig. 3C), suggesting that immunopathological disease was enhanced. Correspondingly, we found increased ALT activity in the serum of treated mice, a marker of liver damage mediated by CD8 T cells (Fig. 3D). When we measured viral load in the serum at Day 14, we found them to be comparable (Fig. 3E). Thus PD-L1 blockade beginning at Day 8 results in a non-fatal immunopathology, with no significant alteration of viral load.

We were then interested in determining the resultant effects following two weeks of blockade initiated on Day 8. In contrast to the expanded antigen-specific cells at Day 14, we did not observe significant differences in the PBMC at Day 22 (Fig. 4, A and B). However, gp33-specific cells did express higher amounts of PD-1 and LAG-3 consistent with enhanced activation (Fig. 4, A and B), also observed on Day 14. The high levels of PD-1 suggested T cells were still in an environment with persistent antigen; we found no statistical difference in viral load compared to control (Fig. 4C). These results indicate release from PD-1 promotes a temporary T cell expansion that quickly contracts back to control levels and importantly does not significantly impact viral load.

To more closely assess the effect of anti-PD-L1 blockade initiated on Day 8 we analyzed the spleens of mice immediately at the end of treatment at Day 22. Grossly, we observed a significant reduction in spleen size in anti-PD-L1 treated mice (Fig. 5A). This size difference was also reflected in total splenocyte number which was reduced by 2 fold compared to control (Fig. 5B). Given that CD8 T cells mediate significant damage to lymphoid tissue early in LCMV-c13 infection, these results indicate PD-L1 blockade potentiates this effect. Consistent with similar frequencies of CD8 and gp33- and gp276- specific CD8 T cells in the blood day 22,
we found similar results in the spleen at this timepoint (Fig 5, C and D). However, given the reduction in splenocyte number, absolute numbers of CD8 T cells were reduced (Fig. 5, C and D). Previous reports have suggested loss of PD-1 signaling to promote loss of a T cell progenitor pool in a cell-intrinsic manner\textsuperscript{26-29}. To assess if this was the case following treatment, we analyzed the TCF1+ progenitor gp33-specific pool using flow cytometry and did not observe any differences in frequency between control and treated mice (Fig. 5C). When we measured PD-1 expression on the progenitor-like TCF-1+ and terminal Tim-3+ subset we found higher expression in anti-PD-L1 treated mice consistent with increased activation on both subsets (Fig. 5E). These results suggest that in the context of blockade of PD-L1 the CD8 T cell response is able to maintain a progenitor-like pool of cells and could be having an impact on both populations.

Our results suggested that following treatment CD8 T cells were activated, as determined by increased PD-1 expression, but could not accumulate in a durable manner. In support of continued activation that was ongoing at Day 22, we found increased frequency of gp33-specific Ki-67+ cells in anti-PD-L1 treated mice compared to control (Fig. 6A). In addition, following \textit{ex vivo} stimulation with gp276-284 peptide we found an increased frequency of IFN\textgamma+ and IFN\textgamma+ TNF\alpha+ cells (Fig. 6B). These results suggest the CD8 T cell response is enhanced following blockade, however, its functional impact is unclear.

Given the role of PD-1 in preventing tissue damage, and our results we hypothesized unleashed CD8 T cell responses would negatively impact other arms of the immune response. We thus analyzed the germinal center response at Day 22 and found significantly reduced Fas+GL7+ GC B cells in anti-PD-L1 treated mice (Fig. 7A). In addition, immunofluorescence
revealed reduced follicle size and irregular borders between follicle and red pulp in treated mice (Fig. 7B). We suspect CD8 T cell mediated cytokine secretion of direct killing could lead to altered stromal compartment and indirectly or directly affect the B cell response.

To test if the reduction in CD8 T cell response was cell-intrinsic we performed a transfer of PD-1+ cells from control and anti-PD-L1 treated mice, into congenic recipients, followed by LCMV-c13 infection (Fig 8A). Importantly, we did not observe any differences in the expansion of transferred cells assessed by frequency of CD45.2 cells in the PBMC on Day 8 after infection (Fig. 8B).

We were next interested in determining the mechanism underlying the detrimental consequences of PD-1 blockade. Kamphorst et al., found that the reduction in viral load and CD8 T cell expansion following PD-1 blockade was completely dependent on CD28-B7 mediated signaling in the context of CD4 depletion. However, it cannot be ruled out that PD-1 also directly inhibits TCR dependent signaling and thus co-stimulation independent cytokine secretion or perforin mediated killing. To test these ideas, we treated mice at Day 8 with anti-PD-L1 along with anti-B7 to assess the CD28 dependency of PD-L1 blockade. We analyzed the spleens of mice after two doses of antibody injection on Day 14. Similar to analysis at Day 22, the spleens from anti-PD-L1 treated mice at Day 14 were significantly smaller, also reflected in the total splenocyte number (Fig. 9A). However, the absolute number gp33-specific cells were increased consistent with the increased frequency in the PBMC at this time point (Fig. 9, B and C). Importantly, co-blockade of PD-L1 and B7 also resulted in significantly lower number of splenocytes suggesting anti-PD-L1 blockade mediates a detrimental effect on total spleen number independent of B7 signaling (Fig. 9, B and C). B7 was efficacious as the number of gp33-specific CD8 T cells was similar to control animals, demonstrating inhibition of CD8
The decreased splenocyte number suggested that blockade of PD-1 inhibits not only CD28 but also another pathway in vivo. Given that PD-1 expression is directly induced by the TCR, we suspect TCR-dependent signaling is also directly inhibited as we noted increased PD-1 expression on gp33-specific T cells following treatment both anti-PD-L1 single treatment and anti-PD-L1/anti-B7 combined treatment (Fig. 9D). We thus suspect enhanced cytokine secretion, IFNγ or TNFα, or direct perforin-mediated cytotoxicity could be contributing to loss of spleen size due to effects on stromal cells.

The detrimental effect of anti-PD-L1 on the adaptive immune response suggested to us that there could be a loss of viral control after the cessation of blockade. Indeed, we observed at later time points beginning on Day 37 significantly higher viral loads in the serum in anti-PD-L1 treated mice (Fig 10A). By Day 80 when most control mice had resolved viremia, a majority of anti-PD-L1 mice still exhibited high viremia (Fig. 10A). As a control, we treated mice with anti-PD-L1 from Day 23-37 and as expected observed accelerated control of virus in the serum (Fig 10A). Our results indicate that the functional outcome of PD-L1 blockade has divergent effects depending on the timing in infection.

To help understand how blockade from Day 8-22 could have a substantial lasting impact on viral control following cessation of treatment we analyzed the PBMC of mice at Day 37. We detected a significant reduction in total CD8 T cell frequency and gp276-tetramer binding T cells in the blood in anti-PD-L1 treated mice at this time-point (Fig. 10, B and C). Importantly, we observed an increase in gp276 cells compared to control in mice treated with anti-PD-L1 initiated on Day 23 (Fig. 10, B and C). In addition, when we analyzed the PBMC of mice at
Day 80 of infection, we found continued loss of CD8 T cells in the blood in viremic mice that received anti-PD-L1 treatment from Day 8-22 (Fig. 10D).

To determine lasting consequences of PD-L1 blockade from Day 8-22 we performed flow Cytometry of splenocytes at Day 37. We found there to be decreased total splenocytes, but a preserved frequency of CD8 T cells and gp33-specific T cells (Fig 11, A and B). However, we did find an increased frequency of Tim-3+ gp33-specific cells (Fig 11C). Tim-3+ cells have been termed “terminally exhausted”, which could reflect an intrinsic dysregulation of cells, or also an extrinsic alteration which promotes their accumulation following blockade. Nevertheless, the data indicate a detrimental effect on the CD8 T cell response well-after cessation of blockade. Importantly, the frequency of TCF1+ stem-cell like cells at this time point was similar, suggesting this pool is protected from depletion in the context of PD-L1 blockade. Previously, it was shown genetically PD-1 deficient T cells were depleted of the Tbet$^\text{hi}$ population, however, under our treatment regimen we found a nonsignificant trend toward a decrease in the Tbet$^\text{hi}$ population (Fig. 11C). As a whole, our data point toward CD8 T cell mediated disruption of lymphoid organs, that in a cell-extrinsic manner diminishes the adaptive immune response resulting in a long-term viremia.

We next wondered whether we could potentially separate the protective versus pathological effects of the CD8 T cell response mediated by blockade of the PD-1 pathway. We hypothesized modulators of the cytokine microenvironment could either influence T cell differentiation or the effect T cells have on tissue. Type I Interferon has a well-established role in the induction of genes that directly inhibit viral activity, however, it also promotes immunopathology in certain settings$^{18}$. anti-IFNAR at the beginning of infection, paradoxically promotes accelerated viral clearance$^{30,31}$, however the mechanism remains unclear as Type I
interferon induces known immunosuppressive genes such as IL-10 and PD-L1. In addition, Type I interferon has been shown to facilitate effector differentiation of T cells in an intrinsic-manner, promoting both cytokine secretion and cytotoxicity.

We thus first wished to more closely examine the response of Ifnar\(^{-/-}\) mice to LCMV-c13 infection. At Day 8 following LCMV-c13 infection IFNAR deficient mice exhibited increased spleen size, and increased splenocyte number compared to control (Fig. 12A). However, we found decreased frequency of CD8 T cells and gp33-specific T cells in the spleen consistent with an impaired T cell response (Fig 12, B and C). As previously reported, we found an increase in frequency of TCF1+ cells and decrease in Tim-3+ cells within gp33 specific cells (Fig 12, B and C). The combined effect however, indicated, that the TCF1+ gp33-pool of cells was similar in absolute number (Fig 12D) between WT and IFNAR deficient animals, with a severe reduction in Tim-3+ cells which express perforin and granzyme and likely contribute to immunopathological damage. When we measured PD-L1 expression on APC populations we found there to be an approximately 2 fold decrease in gMFI within CD11c+MHC-II+CD11b+ “dendritic cells” (Fig 13, A and B). However, this reduction was relatively modest in comparison to total amount of PD-L1 present on the splenocytes.

To determine if immunopathological damage and/or lethality caused by PD-L1 blockade required Type I Interferon, we treated mice with an anti-IFNAR (MAR1) antibody following infection. We administered 1mg of anti-IFNAR 1 day before LCMV-c13 infection and treated with mice with anti-PD-L1 every other day for 8 days. We observed an almost complete rescue of lethality in anti-IFNAR/anti-PD-L1 mice compared to anti-PD-L1 which demonstrate 100%
lethality by Day 10 (**Fig. 14A**). The role of Type I Interferon in LCMV-c13 includes both induction of PD-L1 and IL-10, we tested whether blockade of IL-10 could also rescue anti-PD-L1 induced lethality. When we treated mice with anti-IL-10R and anti-PD-L1, the mice exhibited complete lethality similar to anti-PD-L1 alone (**Fig 14B**). Thus, the function of Type I IFN in inducing lethality appears to be independent of IL-10 induction in this setting.

Similar to results obtained in the spleen at Day 8 in IFNAR deficient mice, we found a decreased frequency of CD8 T cells and gp33-specific cells in anti-IFNAR treated mice (**Fig 15, A and B**). While blockade of IFNAR reduced PD-L1 expression on DCs and macrophages, we found anti-IFNAR/anti-PD-L1 double blockade to still expand gp33-specific cells suggesting significant PD-L1 mediated signaling was still ongoing (**Fig 15, A and B**). The expression of PD-1 was significantly higher in anti-IFNAR and with combined treatment, potentially indicating higher viral load or an intrinsic effect on PD-1 expression. Consequently, we measured viral burden at Day 8 of the infection anti-IFNAR treated mice had ~5 fold higher viral load compared to control (**Fig 15C**) likely reflecting lack of antiviral gene induction, which could explain higher PD-1 expression. Notably, increased viral load was also observed in mice with combined anti-IFNAR/anti-PD-L1 treatment on Day 8, suggesting enhanced CD8 T cell responses are unable to effectively control virus in this setting (**Fig 15C**).

We were next interested in determining the effect of combined treatment on long-term viral control. As expected, we found enhanced viral clearance at Day 22 of infection in anti-IFNAR treated mice compared to control (**Fig 15C**). However, the combined treatment also exhibited enhanced clearance compared to control at Days 22 and 37, but was not significantly different than anti-IFNAR alone.
Given the rescue of lethality with anti-IFNAR blockade, we next tested with anti-IFNAR blockade could rescue the detrimental effect of PD-L1 blockade initiated on Day 8. Indeed, we found blockade of IFNAR at the beginning of infection and anti-PD-L1 treatment on Days 8-22 resulted in lower viral loads compared to mice treated with anti-IFNAR alone (Fig 16A). Consistent with enhanced viral clearance, the splenic reduction caused by anti-PD-L1 treatment alone was reversed with combined anti-IFNAR (Day -1) and anti-PD-L1 Day8-22 (Fig 16, B and C). We suspect the effect of IFNAR on the host could be multi-factorial; diminished CD8-intrinsic signaling results in a diminished CD8 response, and non-hematopoietic tissues are relatively protected from effector arms the immune response with potential to cause damage ¹⁸.
3.4 DISCUSSION

Herein we demonstrate a critical function of PD-1 mediated restriction of CD8 T cells required for induction of protective immunity. At early time points, Enhanced activation of CD8 T cells could be elicited with blockade of PD-L1 however, however, viral load was not altered and instead damage to the host was observed. Blockade of Type I Interferon both abrogated lethality and promoted viral clearance in the context of PD-1 blockade. Our results suggest manipulation of the host microenvironment can ameliorate detrimental consequences of checkpoint blockade while preserving the beneficial aspects; a current issue with cancer immunotherapy.

Enhanced clearance is observed anti-PD-L1 administered over the course of 2 weeks beginning on Day 23 of infection. Moreover, a similar effect is observed in a well-established a model of persistent infection throughout the life of the animal by depletion of CD4 T cells at the beginning of infection. Enhanced viral control is associated with increased antigen-specific CD8 T cell numbers and ex vivo cytokine production (likely reflecting increase LCMV-specific T cells). What explains the difference in outcome following treatment with blockade beginning at Day 8? These scenarios are likely different from early time-points (Day0-15) after the infection as the cytokine and co-stimulation in the microenvironment are substantially lower\(^\text{32}\). Thus, while PD-1 acts as a dominant negative regulator of T cell function, this serves to dampen activation signals from the microenvironment which promote T cell pathogenicity. However, when cytokine and co-stimulation signals wane, release of PD-1 signaling improves viral control without causing significant tissue damage.

We have now defined three phases of the infection in which PD-1 can be considered to have different functions. At the beginning of the infection PD-1 prevents death due to circulatory
failure caused by damage to the lung. From Day 8-22, PD-1 prevents damage to lymphoid organs, and thus the adaptive immune response which ultimately is required for viral control. Following this period, PD-1 acts in a negative fashion, with its blockade promoting viral clearance.

Importantly, it appears the blockade of PD-1 is not required for viral clearance. Immunocompetent mice can clear the virus without administration of blockade suggesting the host engages other mechanisms to sustain a durable immune response. In the context of CD4 depletion, the reduction of viral load is transient, and rebounds to similar levels following blockade. Given that PD-1 expression is very-well correlated to presence of antigen, it is unclear how T cells would modulate their levels in an ongoing immune response to achieve a physiologic effect. In addition, PD-L1 is constitutively expressed, and while Type I and II Interferons can increase expression in certain cell types, it appears the functional relevance is unclear. Thus one way to conceptualize the PD-1 pathway is strictly that of a tolerance enforcer. The danger of immunopathological damage is a stronger evolutionary pressure then enhanced clearance of virus on the order of weeks. Given that chronic viral infections also require significant help from the B cell response in the form of high-affinity neutralizing antibodies, enhancing CD8 T cell responses as a single modality is likely not to have significant therapeutic benefit, unless the clearance process is already initiated.

Our results have potential implications for checkpoint blockade therapy in cancer. Immune related adverse events (IRAEs) to checkpoint blockade is a significant problem to successful therapy. It is possible blockade of Type I Interferon, or other immune signaling pathways could selectively inhibit these adverse events, while preserving therapeutic benefit. However, mouse orthotopic tumor models, often require Type I Interferon for a robust immune
response to the tumor. The function of Type I Interferon has typically been associated with enhanced CD8 T cell priming\textsuperscript{33,34}, which is of unclear relevance to checkpoint blockade therapy in human tumors. When viewed from a different angle, targeted cytokine therapy into the tumor microenvironment could boost responses to checkpoint blockade. Overall, our results indicate that while the PD-1 pathway acts a strong inhibitor of T cell function, its pliability is subject to other host factors which can ultimately effect the physiologic response.
3.5 MATERIALS AND METHODS

Mice and Infection

Male C57BL/6N and B6-CD45.1 mice were purchased from Charles River Laboratory. Ifnar−/− mice were obtained from Jackson Laboratories. To generate BM chimeras, B6-CD45.1 mice were lethally irradiated (10.5 Gy) and reconstituted with donor BM cells for at least 8 weeks before experiments. All mice were housed in a specific pathogen-free facility at Washington University in St. Louis, and were analyzed at 8 to 10 weeks of age, unless stated otherwise. All experiments were performed according to a protocol approved by Washington University’s Animal Studies Committee.

Mice were infected with 2 × 10⁵ plaque-forming units (PFU) of LCMV-Armstrong strain via the intraperitoneal route or 2 × 10⁶ (PFU) of LCMV-c13 by intravenous injection. For the quantification of plasma viral load, RNA was extracted from 10 μL of plasma using Trizol (Life Technologies). Before RNA extraction a spike-in of RNA extracted from 293T cells expressing gfp mRNA was added to the plasma samples. The amounts of the LCMV gp transcript relative to that of “spiked-in” gfp RNA were determined by real-time qRT-PCR as previously described (Chou et al., 2014; McCausland and Crotty, 2008)

Adoptive Transfer

CD8 T cells were positive selected using Flow Comp Dynabeads (Thermo Fischer) using the manufacturer’s suggested protocol. The indicated number of cells were transferred intravenously into recipient mice.

Treatments
a-PD-L1 (BioXcell, 29F.1.G2) was used at 200ug per dose. Anti-IFNAR (MAR1, Leinco) was used at 1mg per dose given at the indicated time. Anti-IL-10R (BioxCell, 10B.A2) was used at 500ug per dose at the indicated days.

Histology

Organs were fixed in 4% PFA overnight, then dehydrated in 70% ethanol. Samples were subject to H&E staining using standard methods performed by the Developmental Biology Histology Core at Washington University in St. Louis.

ALT Assay

ALT measurement was made using Sigma ALT Serum Kit using manufacturer’s recommended instructions.

Cell Preparation and Flow Cytometry

Single-cell suspensions were prepared by manual disruption of spleens with frosted glass slides followed by removal of debris through 80 micron nylone mesh. Absolute cell counts were determined using Vi-Cell (Becton-Dickson). The following flurochrome-conjugated antibodies were purchased from Biolegend, BD, or Thermo Fisher. Anti-Rabbit IgG-A488 (Thermo Fischer). Intranuclear staining was performed using Foxp3 Fixation/Permeabilization Kit (Thermo Fischer) according to manufacturer’s protocol.

Intracellular Cytokine Staining

Splenocytes were stimulated with 1ug/mL of gp33 or gp276 peptide for 2 hours before the addition of Brefeldin A (Biolegend). 5 hours post-stimulation cells were subject to surface
antibody staining, followed by LIVE/DEAD Aqua staining (Thermofisher) for 30 minutes at 4°C before being fixed with 4% PFA for 10 minutes at RT. Cells were washed twice with 0.03% saponin in 2% FBS/PBS before being stained with the indicated antibodies in 0.3% Saponin in 2% FBS/PBS for 20 min at 4°C.
3.6 ACKNOWLEDGEMENTS

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3.7 AUTHOR CONTRIBUTIONS

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Writing ± review & editing: Saravanan Raju, Takeshi Egawa.
3.8 REFERENCES


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Figure 3.1. α-PD-L1 treatment beginning on Day 0 of LCMV-c13 infection results in lethality. (A) (Top) Schematic of treatment regiment (Bottom) Survival curve of mice following LCMV-c13 infection (B,C) Flow cytometry analysis (B) and quantification (C) showing expression of indicated molecules from PBMC on Day 5 from mice in A. Each symbol represents an individual mouse; small horizontal lines indicate the mean (± s.d.). Data are representative of 4 independent experiments.
Figure 3.2. α-PD-L1 treatment beginning on Day 8 does not result in significant lethality.  
(A) (Top) Schematic of treatment regimen (Bottom) Survival curve of mice following LCMV-c13 infection, data is partially reproduced from Figure 3.1 (B) (Top) Schematic of transfer and treatment regimen for recipient mice (Bottom) Survival curve of mice following adoptive transfer of effector CD8+ T cells. Data are combined from at least two independent experiments with n>5 mice per group.
Figure 3.3. α-PD-L1 treatment beginning on Day 8 results in enhanced CD8 T cell responses and disease but not viral control on Day 14. (A,B) FACS plots (A) and quantification (B) showing expression of indicated molecules or tetramer binding from PBMC on Day 14 after LCMV-c13 infection. (C) Weights of mice following LCMV-c13 infection. (D) ALT activity in serum on Day 14 of LCMV-c13 infection (D) Plasma viral load as measured by LCMV gp transcript in plasma of mice on Day 14 of LCMV-c13 infection. Each symbol represents an individual mouse; small horizontal lines indicate the mean (± s.d.).
Figure 3.4. α-PD-L1 treatment from Day 8-22 results in unaltered viral load and CD8 Frequencies in PBMC. (A,B) FACS plots (A) and quantification (B) showing expression of indicated molecules or tetramer binding from PBMC on Day 22 after LCMV-c13 infection. (D) Plasma viral load as measured by LCMV gp transcript in plasma of mice on Day 22 of LCMV-c13 infection. Each symbol represents an individual mouse; small horizontal lines indicate the mean (± s.d.).
Figure 3.5 Diminished spleen size and absolute CD8 T cell response following PD-1 blockade from Day 8-22. (A) Gross Images of spleens from control and anti-PD-L1 treated mice 22 days following LCMV-c13 infection. (B) Quantification of absolute number of splenocytes from control and anti-PD-L1 treated mice 22 days following LCMV-c13 infection. (C-E) (C,E) Flow Cytometry of splenocytes from control and anti-PD-L1 treated mice at Day 22 of infection. (D) Quantification of frequencies and Absolute numbers of the indicated cell populations. Each symbol represents an individual mouse; small horizontal lines indicate the mean (± s.d.).
Fig. 3.6. α-PD-L1 treatment from Day 8-22 results in enhanced CD8 T cell functions at the end of treatment. (A) Analysis of Ki-67 expression in gp33-specific CD8 T cells on Day 22. (B) Analysis of cytokine production following stimulation of splenocytes with gp276-284 peptide. Each symbol represents an individual mouse; small horizontal lines indicate the mean (± s.d.).
Figure 3.7. α-PD-L1 treatment from Day 8-22 results in a diminished GC response, altered splenic architecture. (A, B) Analysis of surface protein expression in B220+ cells. (B) H&E staining of spleen and lung sections from Day 22 of LCMV-c13 infection. Each symbol represents an individual mouse; small horizontal lines indicate the mean (± s.d.).
Figure 3.8. α-PD-L1 treatment from Day 8-22 does not significantly impact intrinsic CD8 proliferative capacity. (A) Schematic of experiment in (B). (B) Analysis of CD45.2 frequency on Day 8 of PBMC of congenic CD45.1 mice following LCMV-c13 infection. Each symbol represents an individual mouse; small horizontal lines indicate the mean (± s.d.).
Figure 3.9 α-PD-L1 induced splenic reduction is B7 independent, but increased PD-1 expression on CD8 T cells is not. (A-C) Absolute number of splenocytes (A), CD8 T cells (B) and gp33-specific T cells (C) on Day 14 of LCMV-c13 infection following treatment initiated on Day 8. (D) Expression of PD-1 on gp33-specific CD8 T cells. Each symbol represents an individual mouse; small horizontal lines indicate the mean (± s.d.).
Figure 3.10. Long-term defective viral control and diminished CD8 T cell responses following α-PD-L1 treatment Days 8-22. (A) Viral load assessed by LCMV gp transcript in serum of mice following LCMV-c13 infection. (B, C). Flow cytometry analysis of PBMC on Day 37 of LCMV-c13 infection. (D) Flow cytometry analysis of PBMC on Day 80 of LCMV-c13 infection. Each symbol represents an individual mouse; small horizontal lines indicate the mean (± s.d.).
Figure 3.11. Accumulation of Tim-3+ cells on Day 37 following PD-1 blockade from days 8-22. (A-C) (A) FACS plots measuring expression of indicated molecules or binding of tetramer. (B) (Left) Absolute number of splenocytes (Right) Quantification of (A). (C) Quantification of (A). Each symbol represents an individual mouse; small horizontal lines indicate the mean (± s.d.).
Figure 3.12 IFNAR deficient mice display increased spleen size, but diminished CD8 T cell responses on Day 8 of LCMV-c13 infection. (A) Absolute splenocyte number. (B) Flow Cytometry analysis of expression of indicated molecules or tetramer binding (C, D) Quantification of frequency (C) and absolute number (D) of B. Each symbol represents an individual mouse; small horizontal lines indicate the mean (± s.d.).
Figure 3.13. PD-L1 expression is slightly reduced on a subset of Dendritic Cells in IFNAR deficient animals on Day 8 of LCMV-c13 Infection. (A) Flow Cytometry analysis of expression of indicated molecules (B) Expression of PD-L1 on indicated subsets of cells.
Figure 3.14. IFNAR but IL-10 signaling is required for α-PD-L1 induced lethality following LCMV-c13 infection. (A,B) Survival curves of mice following LCMV-c13 infection. Data are pooled from at least two independent experiments with (n>6 mice per cohort in total).
Figure 3.15. Diminished CD8 T cell response caused by IFNAR deficiency is rescued by α-PD-L1 blockade. (A, B) Flow Cytometry analysis of expression of indicated molecules or tetramer binding. (C) Viral load assessed by LCMV gp transcript in serum of mice following LCMV-c13 infection. Each symbol represents an individual mouse; small horizontal lines indicate the mean (± s.d.).
Figure 3.16. α-IFNAR treatment reverses detrimental effects of α-PD-L1 blockade at Day 8 and enhanced viral clearance. (A) Viral load assessed by LCMV *gp* transcript in serum of mice following LCMV-c13 infection. (B,C) Gross Spleen Size (B), and absolute quantification of splenocytes (C) on Day 22 of LCMV-c13 infection. Each symbol represents an individual mouse; small horizontal lines indicate the mean (± s.d.).
Chapter 4:

Analysis and Function of T cell subset dynamics in viral infection
4.1 ABSTRACT

CD8 T cell differentiation is characterized by significant phenotypic and functional heterogeneity that is likely important for acute pathogen control. However, in chronic viral infection, the nature of phenotypic heterogeneity within “exhausted” T cells is much less clear. Herein, we perform an in-depth analysis of the CD8 T cell response in chronic viral infection and define the behavior of exhausted T cell subsets \textit{in vivo}. Unexpectedly, in non-competitive settings we observe a long-term persistence of “terminally exhausted” Blimp-1$^{+}$Tim-3$^{+}$ cells. Moreover, we find that TCF-1$^{+}$ CD8 T cells to exhibit low intrinsic turnover in the context of persisting antigen, with “terminally exhausted” Blimp-1$^{+}$Tim-3$^{+}$ cells exhibiting significant entry into cell-cycle. These results led us to investigate the diversity of CD8 T cells within the TCF1$^{−}$ exhausted cells, leading us to define a novel subset of CX3CR1 expressing cells which are exclusively found in the TCF1$^{−}$ compartment. Without CD4 T cell help these CX3CR1$^{+}$ cells are selectively depleted and correlated with the accumulation of Tim-3$^{hi}$ cells. We further show that IL-21 deficient animals are unable to maintain significant numbers of CX3CR1 expressing CD8 T cells and are unable to clear chronic viral infection. These results revise our current understanding of T cell exhaustion, and implicate a dynamic regulation of CD8 T cell turnover in control of chronic viral infection.
4.1 INTRODUCTION

CD8 T cells provide pathogen control to the host that is mediated through cytokine secretion and direct cytotoxicity. \textsuperscript{1-3} Signaling through the T cell antigen receptor (TCR) in naïve antigen-specific T cells initiates a program of population expansion, and effector differentiation. \textsuperscript{4} Following pathogen clearance higher frequencies of antigen-specific CD8 T cells remain as so-called memory CD8 T cells. This process results in phenotypic heterogeneity of antigen-experienced CD8 T cells. \textsuperscript{4} Many studies have defined the function of these subsets as defined by various cell surface molecules. \textsuperscript{5} However, the relative contribution of CD8 T cell subsets to both pathogen control in the acute and memory phase remains unclear. Importantly, the idea that unique subsets divide labor in the immune response has been more recently applied to “exhausted” T cells present in chronic infection. \textsuperscript{6-8} However, whether the same principles can be directly applied is not abundantly clear.

A general paradigm of T cell effector differentiation has emerged from half a century of study using acute LCMV-Armstrong as a model infection. \textsuperscript{9} Antigen-specific CD8 T cell remain at increased frequencies compared to naïve mice following resolution of infection and have been termed “memory” T cells strictly based on a timepoint >30 days after the acute infection. \textsuperscript{10} However, there is a substantial loss of antigen-specific cells compared to the peak of the response; this phenomenon suggested a subset of cells present at the peak of the response were “endowed” with memory potential. \textsuperscript{11} Consequently, Ahmed and colleagues identified IL-7R expression at the peak of the response to correlate best with long-lived potential following transfer into recipient mice. \textsuperscript{12} This result forms the basis for discriminating between subsets of T cells in the effector phase; IL7Ra (CD127)+KLRG1– cells are Memory precursor effector ce
(MPEC) and IL7R-KLRG1+ are short lived effector cells (SLEC) \textsuperscript{13}. While these cells are essentially defined by phenotyping markers, and transcription factor expression, their ultimate role in the both the acute and memory phase response in the endogenous response has not been clear outside of transfer experiments.

During the memory phase antigen-specific cells also exhibit significant heterogeneity. Lanzavecchia and colleagues established the use of CD62L to distinguish between T Effector Memory cells (TEM) and T Central Memory cells (TCM) \textsuperscript{14}. TEM cells are CD62L– cells that exist in peripheral tissues as well as lymphoid organs, while TCM cells are predominant in lymphoid organs as their name suggests. Functionally, the most salient difference is that TCM cells are able to undergo significantly higher expansion in re-challenge experiments. TEM cells are thought to be more “effector-like”, they readily produce cytokines and granzyme, however the significance of this depends on type of infection introduced.

More recently, several groups have sought to more deeply understand memory T cell heterogeneity, which has led to another conceptual shift in our understanding. Specifically, the use of CD43 and CD27 has divided the memory subset into different type of populations than simply CD62L \textsuperscript{15,16}. In addition, the same markers used in the effector phase to identify MPEC and SLEC, also have use memory phase. CD127+KLRG1– cells have enhanced proliferative capacity following transfer compared to KLRG1+ cells, and tend to persist better \textsuperscript{17,18}. In summary, there appears to be more than anticipated heterogeneity in cells in the memory phase, although a general concept can be derived: cells with higher proliferative capacity and persistence generally expression IL7Ra but cannot exert immediate effector activity, and cells with immediate effector activity tend to proliferate less.
An idea that originally did not receive significant attention was the potential for “plasticity” or alteration of phenotype between subsets of cells in both late effector phase, and during the memory phase. Using KLRG1 and CD127 the frequency of cells with each phenotype inverts following infection; KLRG1+ cells are most prevalent early (>60-80%) and decline to (<10%) while CD127+ cells slowly increase their frequency concurrently. This phenomenon has typically been interpreted as a “selective” loss of KLRG1 (short-live effectors), and a preferential survival of CD127 cells. However, it has not been definitively shown, that there is not interconversion between these phenotypes in vivo which could alter our interpretation of the nature of these cells. Two recent studies have given credence to plasticity; the Goldrath group has found the transcription factor Id2 expressed in KRLG1+ cells in the memory phase to “reinforce” effector differentiation, as deletion of ID2 led to “dedifferentiation” to CD127+ cells. In addition, an elegant use of KLRG1-Cre with fate-mapping led Flavell and co-workers to discover CD127+ cells in all phases of effector and memory response to be labelled suggesting a time-period of KLRG1 expression. Notably, these traced cells were similar to non-traced CD127+ cells. An important caveat here is that nearly all cells express KLRG1 to intermediate levels very early in the infection, and uniformly downregulate CD127, thus whether KLRG1 conversion occurs later in the response is unclear.

While the heterogeneity in CD8 T cell response to acute infection has been studied in relatively great detail, we are now starting to understand the heterogeneity in response to chronic viral infection. The generation of “exhausted” T cells that express PD-1 during antigen persistence was originally thought to be a monolithic phenenomenon. The acquisition of the exhausted phenotype correlates well with and is thought to promote viral persistence, although, whether this is causative is unclear. These, antigen-specific T cells gradually lose ability to
secrete cytokine and mediate cytolysis while also losing ability to proliferation and becoming susceptible to apoptosis \(^\text{20}\). However, the original idea that cells have lost all function and are unable to proliferate were mostly demonstrated \textit{ex vivo} or in transfer settings. A seminal study by the Zehn group demonstrate equivalent, if not enhanced per-cell protection, and proliferative capacity by exhausted T cells compared to memory T cells following transfer into T cell deficient recipients\(^\text{21}\). In addition, an oft forgotten finding is that mice infected with virulent LCMV-c13 can control viremia in 60-80 days in a B and T cell dependent manner. Thus, within the exhausted population of CD8 T cells exists the capacity to control viral replication and host burden.

The first indication there could be different functional subsets in chronic LCMV infection arose from a study by Wherry and colleagues who found the amount of PD-1 expression on antigen-specific T cells could predict expansion following transfer into recipient mice \{Blackburn:dj\}. These results supported the idea of a “terminally exhausted” population and a more protected subset of cells which could still be “reinvigorated”. This study was extended with the usage of the T-box transcription factors T-bet and Eomes to distinguish between a “progenitor-like” and “terminal” subset \(^\text{22}\). Tbet\(^{\text{hi}}\) cells had lower PD-1 expression compared to Eomes\(^{\text{hi}}\) cells. Based on the use of adoptive transfer into naïve an infection matched mice the authors developed a model whereby Tbet\(^{\text{hi}}\) cells acted as progeny to Eomes\(^{\text{hi}}\) cells with both Tbet and Eomes expression required for viral control in genetic deletion experiments. However, it should be noted that CD8 T cell intrinsic expression of these factors was not clearly shown to be required for viral clearance.

In addition to the use of T-bet and Eomes expression in understanding T cell dynamics during chronic viral infection, several groups have found a TCF-1\(^{+}\)CXCR5\(^{+}\) subset of cells that
exhibits stem-cell like features, in some ways similar to the T-bethi cells\textsuperscript{6-8}. However, the TCF-1\textsuperscript{+} cells have been shown to express high levels of Eomes rather than T-bet suggesting a potential hierarchy of heterogeneity more complex than expected\textsuperscript{8}. The discrepancy between these two ideas and a resolution of them is currently lacking.

In this section, we first provide a more thorough analysis of the CD8 T cell response to LCMV-c13 infection making comparisons at different time points of infection and in the well-studied context of CD4 depletion. We shed light on the dynamics between these subsets using a combination of \textit{in vivo} BrdU labeling, and genetic lineage tracing. Conceptually we find a functional T cell response to likely be driven by both cytokine and antigen-driven dynamics without concern for “loss-of-function” on a per-cell basis.
4.3 RESULTS

Memory CD8 T cell Subsets and Heterogeneity in Homeostatic Turnover

We first were interested in a formal analysis using both transcription factor and cell-surface marker expression of memory T cell subsets and their dynamics following acute LCMV-Armstrong infection. To this end, we infected Prdm1-YFP reporter mice with LCMV-Armstrong, and from Day 40-44 administered BrdU in the drinking water (Fig 1A). Analysis of splenocytes on Day 44 revealed increased frequency of gp33-tetramer binding CD8 T cells that did not express PD-1 indicating a resolved infection (Fig 1B). These cells were uniformly CD44hi, however, we noted three unique populations that could be defined using CD62L and CD127. Thus, as others have observed there is heterogeneity within the TEM compartment (CD62L−) cells (Fig 1B). This compartment can be subdivided by CD127+ and CD127− expression; CD127− are almost exclusively KLRG1+ and YFP+. CD127+ is typically correlated with long-lived potential so the usage of CD62L+ (TCM) could be functionally irrelevant. We noted a very close correspondence between TCF1 expression and CD127 expression. TCF1 is a HMG-box transcription factor that promotes memory T cell formation; and is correlated with cells that have high proliferative capacity. TCF-1 has been shown to repress Prdm1, however, we observed YFP+TCF-1+ cells these cells could also be defined by CD127+KLRG1+ (Fig 1B). Thus, a relatively stable intermediate state exists in which both TCF and Prdm1 are expressed potentially lending to characteristics of cells expressing each factor by itself.

When we analyzed the frequency of BrdU+ within each memory T cell subset, we found TCF1+YFP− cells to have the highest BrdU incorporation (~5%) during this time period,
consistent with homeostatic turnover of this population (Fig 1C). TCF1$^+$YFP$^+$ cells had approximately half as much BrdU incorporation, while YFP$^+$TCF1$^-$ (KLRG1$^+$CD127$^-$) cells had <0.5% BrdU incorporation (Fig 1C). These results suggest that the KLRG1$^+$CD127$^-$ population is relatively stable at this timepoint, with likely little contribution from other subsets, and low overall turnover. Sarkar et al. reported KLRG1$^+$ during the effector phase to have high BrdU incorporation, highlighting, a contrast between cell-surface phenotype and proliferation at different timepoints. Thus, as others have implied, the TCF1$^+$CD127$^+$ likely persists due to increased homeostatic turnover. Importantly, whether KLRG1$^+$CD127$^-$ cells are intrinsically short-lived is unclear. As they do not express CD127, they cannot respond to IL-7 to proliferate, and declining amounts of antigen following infection could reflect their loss. Conversely, in the presence of antigen, these cells could potentially exhibit increased half-life.

**Identification of heterogeneity within TCF1$^-$ subset in chronic viral infection**

Our analysis of memory T cell heterogeneity prompted us to investigate the heterogeneity within exhausted T cells during LCMV-c13 infection. Specifically, we sought to more accurately define antigen-specific CD8 T cells by both transcription factor and surface marker expression. Genetic deficiency experiments have suggested there to be a potential hierarchy of progenitor subsets to maintain the CD8 T cell response which include cells expression T-bet and TCF1. In addition the use of the inhibitor receptor Tim-3 expressed on Blimp-1$^+$ cells has identified “terminally exhausted” cells. The relationship between these markers, and relationship to PD-1 expression are not well-defined.

To closely examine the heterogeneity in CD8 T cell response, we infected mice with LCMV-c13 to establish chronic infection, and analyzed the spleens of mice on Day 30 when
viral load is high in the serum. Within gp33-specific CD8 T cells, we confirmed the presence of reciprocal T-bet<sup>hi</sup> and Eomes<sup>hi</sup> subsets (Fig 2A). In addition, we validated that T-bet<sup>hi</sup> cells expressed lower amounts of PD-1 compared to Eomes<sup>hi</sup> cells. When we analyzed the expression of TCF-1 and Tim-3<sup>+</sup> we found 3 distinct populations of cells. The TCF-1<sup>+</sup>Tim-3<sup>−</sup> and TCF1<sup>−</sup>Tim-3<sup>+</sup> populations have been previously described, however, we found a substantial fraction of cells that were TCF-1<sup>−</sup>Tim-3<sup>−</sup> (DN) (Fig 2B). Unexpectedly, we found DN cells to exhibit the lowest expression of PD-1 among the three subsets as defined by TCF-1 and Tim-3. Although, we could confirm lower PD-1 expression in TCF-1<sup>+</sup> cells compared to Tim-3<sup>+</sup> cells (Fig 2B). When we examined the expression of T-bet and Eomes within each of these populations we noted that the DN fraction has the highest fraction of T-bet<sup>hi</sup> cells while the TCF-1<sup>+</sup> population had the lowest (Fig 2C). Using these markers, we found T-bet<sup>hi</sup>TCF-1<sup>−</sup>Tim-3<sup>−</sup> to have the lowest PD-1 expression indicating that a linear expression of PD-1 initiating with the stem-cell like TCF-1<sup>+</sup> population may not be the case (Fig 2D). These results demonstrate previously unappreciated heterogeneity within “exhausted” T cells and beg the question of how these subsets are regulated and contribute to viral clearance.

**“Terminally Exhausted” Tim-3<sup>+</sup> cells are not-intrinsically short-lived**

Our analysis confirmed that TCF1<sup>−</sup>Tim-3<sup>+</sup> cells express the highest levels of PD-1 which has been associated with a terminally exhausted state incapable of proliferation. For approximately 10-15 years following the description of “exhausted” T cells their presence in the host was not thought to require significant factors to maintain their presence other than antigen. However, now it is currently thought that exhausted cells require continuous replenishment from a progenitor pool<sup>7,8,22</sup>. This concept implies Tim-3<sup>+</sup> cells are inherently prone to cell death, and thus antigen-specific cells would decay without input. Importantly, it remains unclear whether
cells lacking expression of T-bet and/or TCF-1 are intrinsically short-lived. These models rely upon the use of transfer studies, while illuminating, are subject to caveats of competition with endogenous T cells which could confound interpretation of the situation in an endogenous mouse when performing genetic experiments.

We thus sought to directly test the ability of Tim-3 cells to persist in a host. To this end, we first infected mice with LCMV-c13 to establish a persistent infection for 4 weeks. We then sorted CD8 T cells expressing PD-1\textsuperscript{+}Tim3\textsuperscript{+} of PD-1\textsuperscript{+}Tim-3\textsuperscript{–} (including TCF-1\textsuperscript{+} progenitor cells) and transferred \~10\textsuperscript{5} cells into T cell deficient, TCR\textgreek{b}\textgreek{d}\textsuperscript{–}/\textsuperscript{–} recipients (Fig 3A). The benefit of this model is that these mice do not have endogenous T cells which could potentially compete for LCMV antigen with transferred cells, allowing us to determine whether Tim-3 cells can persist. One day after transfer, we infected mice with LCMV-Armstrong, which established a persistent infection, in these immune-incompetent hosts (Fig 3A). As expected, we observed a greater expansion of Tim-3\textsuperscript{–} cells compared to Tim-3\textsuperscript{+} cells by analysis of the PBMC at Day 8 following infection (Fig 3, B and C). However, Tim-3\textsuperscript{+} cell were present in appreciable frequencies at this timepoint (Fig 3, B and C). When we then analyzed recipient mice approximately 3 weeks post-transfer, we found similar total frequencies of transferred Tim-3\textsuperscript{+} cells in the recipients (Fig 3, B and C). A caveat of these studies, is that Tim-3\textsuperscript{+} could be undergoing homeostatic proliferation in the lymphopenic environment, rather than direct antigen-induced survival, nevertheless, they establish Tim-3\textsuperscript{+} cells can persist. These data suggest that the assumption that Tim-3\textsuperscript{+} cells are short-lived, and require replenishment from a progenitor pool might need to be revised.

TCF1– are in active cell cycle compared to TCF-1\textsuperscript{+} cells
Our experiments indicate that Tim-3\(^+\) cells could be more resilient than currently thought. It is possible Tim-3\(^+\) cells possess the ability to proliferate in addition to TCF-1\(^+\) cells. Current models suggest TCF1\(^+\) progenitor cells produce TCF1\(^-\)Blimp-1\(^+\) cells to “sustain” the CD8 T cell response. This model is derived from a series of studies which utilized either germline TCF7 or conditional TCF7 knockouts using CD4-Cre\(^7,8\). In the setting of TCF7 deficiency the initial expansion of CD8 T cells was unaltered with the generation of Tim-3\(^+\) cells, however, this response is significantly reduced later in the infection (>30 days). Importantly, Shwartzberg and colleagues showed that the antigen-specific CD4 T cell response is severely reduced in CD4-Cre \(Tcfl^{7/F}\) mice potentially indicating a cell-extrinsic effect of TCF7 loss. In support of a cell-intrinsic role of TCF7 in CD8 T cell maintenance, several groups have performed transfer experiments or mixed bone marrow chimeras with WT and TCF7 deficient cells which demonstrates a competitive disadvantage of these cells. However, the relevance of these transfer experiments to the endogenous T cell response is not extremely clear, as competition with endogenous cells is not a factor.

While the TCF1\(^+\) progenitor cells have been unequivocally shown to produce Tim-3 progeny and also endowed with the ability to self-renew, the rate of this process is unclear. Their stem-cell like features include expression of \(Kit, Sox4,\) and \(Lef\), but their proliferative rate remains unclear\(^6\). It is possible that TCF1\(^+\) cells are cycling at high rate to replace Tim-3 cells or act more similar to LT-HSCs which exhibit quiescence and replenish mature cells more slowly\(^{24,25}\). In addition, it has not been determined whether Tim-3\(^+\) cells also possess the capacity to proliferate which would require a revision to the nature of a progenitor-progeny relationship.
To assess the proliferative dynamics of the TCF1+ and TCF− subsets we analyzed the frequency of Ki-67+ cells in each subset in the chronic phase of the infection (~4 weeks following infection). Consistent with TCF1+ were ~5-10% Ki-67+ on Day 22 which was also observed for DN cells (Fig 4A). However, Tim-3+ cells were ~20-30% Ki-67+ suggesting a significantly higher entry into cell cycle (Fig 4A). These results are consistent with the TCF1 being a quiescent population, unexpectedly, the Tim-3 cells as an effector cell that maintains the ability to proliferate in vivo. As Ki-67 is a marker of non-G0 cells, we wished to confirm directly if Tim-3+ cells were actively proliferating by assessing BrdU incorporation. We found the frequency of BrdU+ cells following 24 hour of 1mg BrdU pulse was similar to Ki67 frequency with 20% of Tim-3+ cells and 5% of TCF1+ and DN cells being labelled with BrdU (Fig 4B).

However, we were concerned that Ki-67+ and BrdU labelled Tim-3+ cells could have recently differentiated from the TCF-1+ population over the course of 24 hours and changed their phenotype; and thus, are not bona fide Tim-3+ cells but simply recent emigrants from the TCF-1+ pool of cells. We thus performed a 2 hour BrdU pulse to assess cell-cycle progression on a time-scale where cellular phenotype is relatively stable. Our analysis revealed a similar but decreased trend with 6-8% of Tim-3+ cells labelled with BrdU with only 2% of TCF-1+ cells labelled (Fig 4C). These data indicate that Tim-3+ cells actively cycle in vivo and that the TCF-1+ and DN cells are relatively quiescent and draw into question the importance of the role of the TCF1+ population in sustaining the overall response.

**CD4 T cell deficiency results in altered CD8 T cell subset distribution**

CD4 T cells have an established role in several viral and tumor contexts. For example, when CD4 T cells are depleted at the initiation of infection with LCMV-c13 a permanent viremia
is established and CD8 T cells have diminished ex vivo cytokine production, and are so-called “helpless” T cells. Given the importance of CD4 T cells in directing immune responses we wished to perform a direct comparison between CD8 T cell responses in mice sufficient and deficient CD4 T cell responses.

To this end, we examined the CD8 T cell response in chronic phase of the infection (Day 15-30) when viral loads are similar between both groups. Importantly, at Day 30 we did not find a significant reduction in the absolute number of CD8 T cells or gp33-specific T cells (Fig 5, A, B, E). However, when we analyzed the expression of TCF1 and Tim-3 within gp33-specific cells we observed an altered distribution of cells in CD4 Depleted mice. Specifically, we observed an increase in the frequency of TCF-1+ cells in CD4 depleted mice, and a reduction in the frequency of DN cells (Fig 5, B and D). The loss of DN cells suggested to us that expression of T-bet would be lost in CD4 depleted mice as the DN subset of cells is substantially enriched for T-bet expressing cells. Indeed, when we analyzed the expression of T-bet and Eomes we found an almost complete loss of T-bet expressing cells in mice depleted of CD4 T cells (Fig. 5C). Thus, CD4 T cells play a role in the induction of T-bet expression and maintenance of the DN subset in CD8 T cells during the chronic phase of LCMV-c13 infection.

The relative expansion of the TCF-1+ population suggests that CD4 help may be required to mobilize this population into TCF-1- effector cells and without their presence results in a “build-up” of this population. Importantly, we found an accumulation of Tim-3hi cells in CD4 depleted mice suggesting their turnover to be reduced (Fig 5, B and D). Thus, in CD4 depletion following initial inflammation following the infection, there is an accumulation of Tim-3+ cells and TCF1+ cells with a selective loss of DN cells.
**CX3CR1 marks a subset of exhausted DN cells that express T-bet**

Our results from CD4 depletion indicated that a previously uncharacterized population of antigen-specific cells (double negative, TCF-1−Tim-3−) was severely reduced compared to control mice. We wished to further characterize this population to determine its function and relevance to CD8 T cell turnover during chronic viral infection. To assist in our studies, we wished to find a cell-surface marker which we could use as a tool to investigate the nature of these cells. An important consideration would be to find a marker that would only be expressed in TCF1− (Blimp-1+) population of cells. Given that DN cells were enriched for T-bet expressing cells, we analyzed the expression of genes that are T-bet dependent in effector CD8 T cells from acute viral infection.

Specifically, we focused on both KLRG1 and CX3CR1, both well-characterized genes to be T-bet dependent. A previous study using the Cx3cr1gfp/+ reporter mouse reported ~40% of gp33-specific cells to express cx3cr1 at Day 40 after infection and claimed these cells were memory counterparts in chronic infection. We first examined expression of CX3CR1 and KLRG1 on PD-1 expression CD8 T cells 4 weeks following LCMV-c13 infection. We found approximately 20% of PD-1+ to express CX3CR1, and approximately 5% to express KLRG1. Notably, KLRG1+ cells were almost exclusively CX3CR1* (Fig 6A). In acute viral infection KLRG1 and CX3CR1 expression are very well correlated, however, in chronic viral infection KLRG1 expression is only restricted to a small subset of CX3CR1 expression (Fig 6A). As an additional validation of CX3CR1 expression on exhausted CD8 T cells we utilized Cx3cr1gfp/+ reporter mice. Two weeks following LCMV-c13 infection we detected GFP expression in PD-1+ cells from PBMC with a subset of these cells staining positive with CX3CR1 antibody (Fig 6B). Finally, we utilized the Prdm1-YFP reporter and found CX3CR1 expression was almost
exclusively found in YFP+ cells in both the Tim-3 positive and negative compartment (Fig 6C). This result contrasts with the idea that CX3CR1 marks “memory-like” cells in chronic viral infection as TCF-1+ cells did not express CX3CR1. Thus, similar to other cell-surface molecules such as Tim-3, and 2B4, CX3CR1 is almost exclusively expressed on TCF1lo cells, however its expression pattern varied.

Given the readily detectable CX3CR1 expression, we decided to focus on further analysis of CX3CR1 expression during chronic viral infection. We first performed a more in-depth analysis examining CX3CR1 expression and its relation to exhaustion-related transcription factors and cell surface molecules. When we analyzed mice on Day 22 following LCMV-c13 infection we found CX3CR1 expression to be heavily enriched in both DN (TCF-1−Tim3−) and Tim-3+ cells and relatively sparse within TCF-1+ cells, similar to results obtained with Prdm1-YFP reporter mice (Fig 7, A to C). We noted a direct correlation between CX3CR1 expression and T-bet expression as expected (Fig 7C). Using Tim-3 and CX3CR1 we were able to define a population of cells, TCF-1−Tim-3−CX3CR1+ that were almost exclusively T-bethi.

We next were interested in determining whether CX3CR1 cells were enriched for a population of cells that were quiescent or actively proliferating. On Day 21 of LCMV-c13 infection we treated mice intraperitoneally with 1 mg of BrdU twice over the course of 24 hours, and analyzed mice immediately after. When we analyzed gp276-specific cells on Day 22, we found CX3CR1+Tim-3+ cells to exhibit the highest amount of BrdU labelling ~35%. CX3CR1+Tim-3+ cells that are T-bethi had similar BrdU incorporation to CX3CR1−Tim-3+ cells (Fig 8, A and B). Interestingly, the CX3CR1− DN population had the lowest BrdU incorporation similar to the TCF-1+ memory-like population (Fig 8, A and B). We thus define, an extremely
proliferative population of cells which likely reflects proper mobilization and turnover of the CD8 T cell response facilitating viral clearance.

To assess the proliferative rate over several days during the chronic phase of LCMV-c13 infection we performed sequential BrdU pulse followed by an EdU pulse. We hypothesized cells incorporating BrdU would also incorporate EdU and a majority of cells would be dual-labelled. However, we found only a third of BrdU$^+$ cells were also labelled with EdU$^+$ suggesting intermittent periods of proliferation and quiescence within the PD-1$^+$ population (Fig 8C). In addition we found EdU$^+$BrdU$^-$ cells over this time period indicating quiescence and entry into cell-cycle over 2-3 days. When we used CX3CR1 or KLRG1 to determine if there was enrichment for a given labelled population we did not note any differences (Fig 8C). These results suggest relatively desynchronized proliferation within the exhausted T cell compartment, with each cell engaging cell-cycle periodically. It remains to be determined what signals regulate entry into cell-cycle or whether it is simply a passive process.

**IL-21 deficiency results in loss of CX3CR1$^+$ cells and defective viral control**

The high proliferative rate of CX3CR1 expressing cells suggested that this subset could be vital for eventual viral control. Given that this CX3CR1 is enriched in the DN population, we first asked whether CX3CR1 cells were diminished in CD4 depleted mice which could partially account for the lack of viral control. Indeed, when we analyzed CX3CR1 expression on Day 30 of infection we found a severe depletion of CX3CR1 expressing cells within the TCF1$^-$ pool of gp33-specific cells (Fig 9A). Thus, CD4 help maintains the presence of a highly proliferative CX3CR1$^+$ population whose deficiency could lead to defective viral control.
We were next interested in determining how CD4 T cells promote the generation of T-bet expressing CX3CR1 cells. IL-21 produced heavily by CD4 T cells is a critical factor required for viral clearance following LCMV-c13 infection. While IL-21R is expressed on many lymphoid cell types, several reports have reported a cell-intrinsic role for IL-21 signaling in “maintenance” of a CD8 T cell response. We thus hypothesize IL-21 signaling could promote the generation and/or maintenance of CXCR1 expressing exhausted T cell cells.

To this end we first infected WT and IL-21R deficient animals with LCMV-c13, and validated a complete lack of LCMV-c13 clearance from the serum of mice 80 days following initial infection (Fig 9B). When we analyzed mice 30 days after infection when viral titers are similar between WT and IL21R KO, we first noted increased splenocyte numbers in IL-21R KO mice (Fig 9C). This result was not previously reported, but was very robust in our hands. When we analyzed gp33-specific CD8 T cells we found there to be a reduced frequency in the spleen, but given larger spleen size, we did not find an absolute reduction of gp33-specific cells in the spleen (Fig 9, D and E). However, when we measured CX3CR1 expressing cells within the gp33-specific compartment, we found a similar reduction but with less severity in IL-21R deficient mice compared to CD4 depletion alone (Fig 9, D and E). Thus IL-21 produced by CD4 T cells likely promotes the maintenance of a CX3CR1+ population of proliferating antigen-specific CD8 T cells required for viral clearance.
4.4 DISCUSSION

Herein we provide a detailed cellular evaluation of the CD8 T cell response to chronic viral infection. Specifically, we closely analyze the proliferative dynamics directly \textit{ex vivo} using a combination of proliferative markers and BrdU incorporation. These results indicated previously underappreciated ongoing proliferation within the “exhausted” T cells. Moreover, we find the so-called “terminally exhausted” subset of Tim-3\(^+\) cells to exhibit the highest \textit{in vivo} proliferation. These data potentially indicate a need to revise our current conceptualization of exhausted T cells.

Much of the knowledge we have concerning the function of exhausted T cells has come from both \textit{ex vivo} stimulation and/or transfer studies. These data have revealed poor cytokine production and proliferation \textit{in vitro} compared to effector cells in acute infection. In addition, they have been thought to exhibit poor persistence in naïve mice relative to memory cells generated after acute infection. However, more recently, these concepts have needed significant revision. Zehn and colleagues were able to show proliferative capacity and protection provided to immune-deficient mice by exhausted T cells at a comparable level to memory T cells\(^{21}\). In addition significant numbers of exhausted cells persisted in naïve mice for over 50 days and were detectable after recall challenge\(^{21}\).

These data have strengthened the view that a subset of cells “maintains” protective capacity in chronic infection. TCF-1 expressing cells exhibit proliferative capacity and self-renewal upon transfer thus fulfilling this role\(^{6,8}\). However, a distinction must be made between proliferative capacity and \textit{in vivo} proliferation. Our results show that \textit{in vivo} proliferation in the context of persistent antigen well into the chronic phase of the infection is carried out mainly by
TCF1− cells, specifically CX3CR1+Tim-3+ cells. These data are reminiscent of results obtained from transfer studies examining contribution of HSCs to hematopoiesis. The generation of bone marrow chimeras involving transfer of HSCs into irradiated mice induces an “emergency” hematopoiesis scenario that strongly promotes HSC proliferation to fill the cellular niche. However, direct measurements of hematopoiesis in steady-state animals have revealed much lower contribution to mature subsets. It appears this process is carried out by downstream progenitor cells which exhibit high proliferative rates.

Our results support a stem-cell like role for TCF-1+ cells, however, where exactly on the hierarchy of cells this subset exists is unclear. We detected similarly low levels of turnover within TCF1−Tim3− CX3CR1+(T-bethi) and CX3CR1− compartments as previously published. While both loss of TCF1 and T-bet in the germline promote viral persistence the cell-intrinsic role for these factors in the CD8 T cell response remain to be determined as they are expressed in several other immune lineages. Importantly, the ability to maintain quiescence is correlated with an enhanced “recall” proliferation in transfer environments. We do not have a clear explanation as to the mechanistic basis underlying differences in transfer-induced proliferation following infection of a recipient compared to proliferation well-into persistent antigen. We suspect this could be do with mimicking conditions of T cell priming following infection that are more conducive for quiescent cells, such as naïve T cells.

An important question that remains is what extrinsic factors regulate CD8 T cell dynamics during persistent infection. We have identified CD4 help to be a critical factor in maintaining the CX3CR1+ subset of T-bet expressing cells, in part through production of IL-21. Interestingly, in both of these scenarios we do not observe a reduction in antigen-specific CD8 T cell responses in the spleen. Instead, we observe an altered distribution of T cell subsets which
we propose could be a functional readout of exhaustion. We hypothesize this to be the case as the
generation of Tim-3+ cells and decreased cytokine production is observed very early in the
response to LCMV-c13. However, generally CD8 T cells can still mediate clearance of virus.
Thus, under conditions of high viral load “exhausted” cells will generally be present, however,
their functional capacity in reducing viral load is likely determined by the extent to which
microenvironmental factors induce turnover.
4.5 MATERIALS AND METHODS

Please see Section 3.5 for a list of overlapping Materials and Methods.

*Mice*

*IL21r−/−, Prdm1-YFP, Cx3cr1gfpy/* were obtained from Jackson laboratories. TCRβδ−/− were bred in house.

*Cell Sorting and Transfer*

CD8 T cells were pre-enriched using Flow Comp Dynabeads (Thermo Fisher) followed by sorting on an Aria-III (BD). Sorted cells were transferred intravenously into recipient mice at the indicated cell numbers.

*Antibody Treatments*

α–CD4 (GK1.5, Leinco) was injected at a dose of 200µg intraperitoneally on Day −1 and +1 of LCM-c13 infection.

*BrdU and EdU Labeling and Detection*

BrdU (Sigma) or EdU (Cayman) was administered i.p. at a dose of 1 mg to mice which were sacrificed at the indicated time-points. Cells were processed using aAPC BrdU Flow Kit (BD Pharmingen) and a Click-iT Plus EdU Pacific Blue Flow Cytometry Kit (Invitrogen). BrdU was detected using an AF647-conjugated anti-BrdU(MoBU-1, Invitrogen) antibody for dual BrdU and EdU detection.
4.6 ACKNOWLEDGEMENTS

We thank Daniel J. Verbaro for insightful discussions, and Jim Chou for technical expertise.

4.7 AUTHOR CONTRIBUTIONS

All work was performed by S.R. and T.E.
4.8 REFERENCES


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Figure 4.1. TCF1+ Blimp-1- subset of Memory T cells exhibit highest amount of homeostatic proliferation. (A) Schematic of BrdU labelling experiment of Prdm1-YFP mice infected with acute LCMV-Armstrong. (B) Flow Cytometry measuring expression of indicated proteins or binding of tetramer from mice in A. (C) BrdU incorporation in indicated memory populations measured by Flow Cytometry from mice in A.
Figure 4.2. Resolution of TCF-1, T-bet, and Eomes expression in Exhausted T cell subsets. (A-D) Flow cytometry measuring expressing of indicated proteins or tetramer binding in on Day 30 in splenocytes from mice infected with LCMV-c13. Data are representative of >20 mice over 5 independent experiments.
Figure 4.3. PD-1⁺Tim-3⁺ cells are able to persist in competition-free host with persistent antigen. (A) Schematic of experiment (B) Flow cytometry analysis measuring frequency of Cd8β expressing cells in PBMC in TCRβδ⁻/⁻ at indicated times following LCMV-Armstrong infection. (C) Quantification of B. Each symbol represents an individual mouse; small horizontal lines indicate the mean (± s.d.).
Figure 4.4. Increased Ki-67+ and BrdU labelling within Tim-3+ cells during chronic phase of LCMV-c13 infection. (A-C) Analysis of gp33-specific CD8 T cells 4 weeks after LCMV-c13 infection of WT mice examining expression of Ki-67 (A), BrdU incorporation following a 24 pulse (B), and a 2 hour pulse (C). Each symbol represents an individual mouse; small horizontal lines indicate the mean (± s.d.).
Figure 4.5. Lack of CD4 T cell help results in loss of TCF-1–Tim-3– (DN) and T-bet expression in antigen-specific CD8 T cells on Day 30. (A) Absolute number of splenocytes on Day 30 following LCMV-c13 infection of WT mice. (B, C) Flow cytometry measuring expression of indicated proteins or tetramer binding in splenocytes. (D, E) Frequency (D) and absolute number (E) from B. Each symbol represents an individual mouse; small horizontal lines indicate the mean (± s.d.).
Figure 4.6. CX3CR1 is expressed in exhausted PD-1\(^+\) cells almost exclusively within Blimp-1\(^+\) cells. (A-C) Flow cytometry measuring expression of indicated molecules in splenocytes following LCMV-c13 infection on Day 28 (A). (B) Analysis of CX3CR1 expression in PBMC in Cx3cr1\(^{gfp/+}\) mice infected with LCMV-c13 on Day 14. (C) Flow cytometry measuring expression of indicated molecules in splenocytes from Prdim1-YFP mice following LCMV-c13 infection on Day 28.
Figure 4.7. CX3CR1 expression is restricted to TCF1– cells and correlated with expression of T-bet in exhausted CD8 T cells. (A-D) Flow cytometry measuring expressing of indicated proteins or tetramer binding in on Day 22 in splenocytes from mice infected with LCMV-c13. Data are representative of >10 mice over 3 independent experiments.
Figure 4.8. CX3CR1+Tim-3+TCF-1- cells are most actively engaged in cell-cycle and exhibit desynchronized proliferation. WT mice were infected with LCMV-c13 and subject to a 24 hour BrdU pulse. (A,B) Flow cytometry measuring expressing of indicated proteins or tetramer binding in splenocytes on Day 22 (A) and assessment of BrdU incorporation in indicated subsets (B). (C) (Top) Schematic of BrdU and EdU sequential labeling 4 weeks following infection (Bottom) Assessment of BrdU and EdU incorporation by Flow Cytometry in indicated cell populations.
Figure 4.9. IL-21 deficiency results in loss of CX3CR1 expressing cells in chronic phase of LCMV-c13 infection and is correlated with loss of viral control. (A) Flow cytometry measuring expressing of indicated proteins or tetramer binding in splenocytes on Day 30 of LCMV-c13 infection. (B) Viral load on Day 80 of LCMV-c13 infection as measured by qPCR of LCMV gp transcript. (C-E) Analysis of Il21r−/− mice following infection with LCMV-c13 on Day 30 of infection. (C) Absolute number of splenocytes. (D-E) Flow cytometry measuring expressing of indicated proteins or tetramer binding in splenocytes on Day 30 of LCMV-c13 infection with frequencies and absolute numbers in E.
Chapter 5:

Discussion
In this work, we have sought to dissect signaling mechanisms within the adaptive immune system. In Chapter 2, we focus on understanding the role of the adaptor molecule CD2AP, and find that it regulates TCR signaling in CD4 T cells, and modulates T_{FH} differentiation during chronic viral infection. Loss of CD2AP enhances the germinal center response and generation of neutralizing antibodies which facilitate clearance of chronic viral infection. In Chapter 3 and 4, we turned our attention toward CD8 T cells and their role in both viral control and host damage. We describe a pivotal role for PD-1 in promoting immune homeostasis and generation of a durable immune response. We further define this function to be of significance in the context of Type I Interferon highlighting the need to consider microenvironmental factors in checkpoint blockade. Finally, we reassessed conventional views regarding the CD8 T cell response and took a close look at cell turnover. Our studies shed light on novel subset of “exhausted” T cells that correlates well with an enduring immune response.

The immune system is relatively unique as it exists in a state of relative quiescence until certain signals are received which rapidly mobilizes effector functions of tissue and cells. These signals are initiated by recognition relatively conserved molecular patterns among fungi, bacteria, and viruses by the innate immune system. However, the adaptive immune system, is able to recognize all potential molecular moieties that are unique to each pathogen – the central function of the TCR and BCR. Thus, a central question in adaptive immunity is mechanisms underlying and regulating T and B cell function in the integrated environment of the host. Ultimately, these signals are transduced into global transcriptional and epigenetic changes that may become “fixed” and define lineages. However, a substantial body of evidence indicates that the continued presence or absence of other stimulatory or inhibitory signals can have
significantly alter the ensuing response giving credence to continued plasticity of the immune response\textsuperscript{1-3}.

While many of the central signaling molecules that are involved in signal transduction in T and B cells are known, how this network is modulated in the context of an in vivo immune response is under-studied. Our work, have revealed how the signaling adaptor CD2AP can modulate T\textsubscript{FH} likely through modulation of TCR signals. It is unclear whether this is the sole function of CD2AP in CD4 T cells, or whether it also plays a role in modulating other signaling pathways in T\textsubscript{FH} differentiation which is then finally integrated with TCR signals. The concept that modulation and strength of TCR signal influences T\textsubscript{FH} development and maintenance has emerged over the past several years\textsuperscript{4,5,6}. However, whether intracellular signaling modules can influence the TCR independent of TCR affinity for antigen is not as clear. It will be interesting to determine the extent to which accessory molecules can be induced and/or regulated to ultimately control the functions of T cells. Importantly, we find a potentially opposite role for the related CMS-family member CIN85 in the response to LCMV-c13 infection. While we did not formally demonstrate T\textsubscript{FH} differences we did find evidence of diminished neutralizing antibodies in CIN85 deficient mice. Further analysis will be required to delineate the role of CIN85 in CD4 T cells. A previous report has implicated CIN85 in regulation TGF-\(\beta\) signaling via recycling of TGF-\(\beta\)RI to the cell surface; this could play a role in T cells in modulating T\textsubscript{FH} function\textsuperscript{7}.

Notably, we did not observe consistently enhanced T\textsubscript{FH} differentiation in all immunization contexts. We do not have a sufficient explanation for the underlying mechanism behind this phenomenon. However, a previous report, described similar behavior of DENND1b in specifically regulating T\textsubscript{H2} signaling while being expressed in other T helper subsets\textsuperscript{8}. A
provocative, but speculative, explanation could lie in the assembly of other molecules in a cell-type specific manner that ultimately directs the function of individual signaling proteins.

Both in vitro and most in vivo studies initiate TCR signal transduction in a strong single bolus either through crosslinking antibodies, bolus peptide, or acute infection. These types of experiments provide a certain kind of stimulation to the TCR that can be assayed by proliferation or direct ex vivo cytokine production. However, the nature of T cells in providing protection to the host likely occur under different scenarios of weak but continued stimulation occurring over a time-line of days to weeks to months. The function of T cells in the clearance of acute pathogens is also not abundantly clear with significant control exerted by T cells only well-described in chronic viral infections or cancer \(^9\) \(^10\) \(^11\) \(^12\). Thus, a potentially important and under-developed area of research is understanding the modulation of TCR signal transduction in a variety of contexts especially involving persistent stimulation.

Chronic viral infections and developed tumors provide persistent antigen stimulation to T cells. As a surrogate marker, PD-1 expression, can identify cells with ongoing TCR signaling \(^13\). PD-1 is expressed by both CD4\(^+\) and CD8\(^+\) T cells, and in these persistent antigen contexts, a commonly held view is that these cells are dysfunctional \(^14\). However, more recently, this idea has come under scrutiny with the following developments. CD4 T cells simply alter their cytokine profile to facilitate a more productive immune response; specifically IL-2 production is exchanged for IL-21 production \(^15\). CD8 T cells that are “exhausted” have been demonstrated to show excellent activity when transferred into permissive environments \(^16\). Thus, the notion that T cells become deregulated at an intrinsic level is unclear.
Our studies investigating the major negative regulator of T cell function, PD-1 in chronic LCMV infection, have revealed a pivotal role for PD-1 in promoting a durable adaptive immune response. At a mechanistic level, the previously described functions of PD-1 in preventing lethal immunopathology hold, but the ultimate consequences of tissue damage appear to depend on the context within the infection. These results also indicate that “more” is not necessarily better for control of virus. Blockade of PD-L1 at early time points of infection clearly result in exaggerated CD8 T cell responses, but have almost no impact on viral control, and instead simply promote damage. While it is hard to negative effects of damage on other arms of the immune system and an enhanced CD8 T cell response, the idea that CD8 T cells mediate a significant impact on viral clearance has not been well-established. The function of CD4+ T cells and B cells in production of neutralizing antibodies likely has a stronger role, with CD8 T cells acting to promote elimination of reservoirs of virus.

A common theme in the immune system is how the cytokine milieu ultimately directs immune responses. We have shown how Type I Interferon can modulate the ultimate effect of PD-1 blockade in the host. While the mechanistic basis for this effect is unclear, the implications are provocative. Notably, while we observe an effect on the CD8 T cell response in settings of IFNAR deficiency it is possible effects on other hematopoietic or non-hematopoietic cells contribute to the phenotype. Thus, one way in which to modulate the physiologic consequences of PD-1 blockade maybe to selectively target cytokine pathways in different cell types to either promote or diminish the effect of CD8 T cells on each tissue. While Type I Interferon has been proposed a “signal 3” for CD8 T cell activation, recent reports indicate it induces inhibitory ligands for NK cells to prevent killing \textit{in vivo}^{17}. Given the relative redundancy of co-stimulatory signals for Cd8 T cell function, it is possible to block IFNAR to promote a tolerogenic state, but
enhanced CD8 T cell function via co-stimulation with agonistic TNFR superfamily or B7 antibodies.

The concept that “exhausted” T cells lose proliferative potential and are susceptible to apoptosis intrinsically needs significant revision. The discovery of stem-cell like population within PD-1 expressing cells with robust proliferative capacity and self-renewal draw into question the use of the term “dysregulated”. The so-called “terminal” Blimp-1+ cells in chronic have decreased proliferative capacity in transfer settings, however, the relevance of this phenomenon in in vivo responses is unclear. These cells possess the higher BrdU incorporation and frequency of Ki-67+ cells than the stem-cell like population with proliferative capacity.

We propose an altered view of the T cell response to persistent antigen. Antigen abundance generally provides a sufficient survival signal to cells such that even Blimp-1+ cells remain. However, we rather focus on “dynamics” and continued proliferation as determining the extent of “exhaustion”. Specifically the loss of the cytokine and co-stimulatory milieu provided by other cells such CD4+ T cells and B cells alter the nature of the CD8 T cell response. This results in the presence of “old” cells that are not continually replaced to facilitate a durable immune response. A key question that remains is what do we mean by “old” cells — and whether these cells could be considered truly intrinsically dysfunctional. We suspect this could be the case, but another interpretation would be “old” cells upregulate numerous co-inhibitory receptors that effectively suppress effector signaling to levels that are not physiologically relevant for control of virus or tumor. Further work will need to be done to assess whether this model is a useful way to think of the T cell “exhaustion”. The generation of tools to effectively prevent any co-inhibitory receptor from signaling, while difficult, could be of immense help.
5.2 REFERENCES


