TIM-3 CO-STIMULATION PROMOTES SHORT-LIVED EFFECTOR T CELLS, RESTRICTS MEMORY PRECURSORS, AND IS DISPENSABLE FOR T CELL EXHAUSTION

by

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ABSTRACT

Tim-3 is highly expressed on a subset of T cells during T cell exhaustion, in settings of chronic viral infection and in tumors. Using LCMV Clone 13, a model for chronic infection, we have found that Tim-3 is neither necessary nor sufficient for the development of T cell exhaustion. Nonetheless, expression of Tim-3 was sufficient to drive resistance to PD-L1 blockade therapy during chronic infection. Strikingly, expression of Tim-3 promoted the development of short-term effector T cells, at the expense of memory precursor development, following acute infection with LCMV-Armstrong. These effects were accompanied by increased Akt/mTOR signaling in T cells with endogenous or ectopically expressed Tim-3. Conversely, Akt/mTOR signaling was reduced in effector T cells from Tim-3 deficient mice. Thus, Tim-3, while essential for optimal effector T cell responses, but may also contribute to T cell exhaustion is restricting the development of long-lived memory T cells. Taken together, our results suggest that Tim-3 is more similar to co-stimulatory receptors that are upregulated following T cell activation, rather than dominant inhibitory proteins such as PD-1. These findings have significant implications for the development of anti-Tim-3 antibodies as immunotherapy agents for the treatment of cancer, infections, and other matters of public health.
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PREFACE

I would like to thank my mentor, Dr. Lawrence Kane for guiding me through this process and giving me the freedom to explore science and discover at my own pace. I owe gratitude to those members of the Kane lab both past and present, specifically Kristia Hamilton, Binh Phong, and Andrea Workman who have been essential to my growth as a person and a scientist. My committee members have supported my intellectual growth and given invaluable advice throughout the life of this project. I am forever grateful to my family, especially my wife, Ellie Avery, for loving and supporting me in my pursuit of this degree and a career in academics.
1.0 INTRODUCTION

1.1 T CELL BIOLOGY

1.1.1 T cell development and the T cell receptor (TCR)

T cells originate from a hematopoietic stem cell in the bone marrow that evolves into an early lymphocyte progenitor. From there, cells with this lineage travel to the thymus via the blood and will become an early T cell lineage precursor. In the thymus, the T cell receptor (TCR) α and β gene segments rearrange to produce a TCR to be expressed on the surface of the cell. Using the TCR, both positive and negative selection of the T cell occurs. This ensures that mature T cells can recognize antigen displayed by the MHC complex on antigen presenting cells (APC) in the periphery.

The TCR is a defining element of T cells. Most T cells express an alpha (α) and beta (β) chain for the TCR, with each chain having a cell-proximal constant region and a cell-distal variable region. The very tip of the variable region on each chain has a hypervariable region that is the specific protein binding area for antigen and is unique to each naïve T cell. Other cell types with a TCR include gamma/delta (γ/δ) T cells (which express TCR γ and δ, rather than α and β), natural killer T cells (with a TCR that recognizes glycolipids in the CD1d presentation), and regulatory T (Treg) cells (a subset of T cells that contain αβ TCR but respond in a suppressive
rather than activating manner). Each of these cell types goes down a varied differentiation path based on the strength of TCR signaling through Akt during positive and negative selection\(^1\).

### 1.1.2 T cell activation and signaling

In addition to TCR\(\alpha\) and TCR\(\beta\) chains, the TCR is associated with the invariant chains of the CD3 complex, CD3\(\varepsilon\gamma\delta\) and CD3\(\zeta\). It is generally accepted that the TCR:CD3 complex consists of four dimers: TCR\(\alpha\beta\) (or TCR\(\gamma\delta\)), CD3\(\varepsilon\gamma\), CD3\(\varepsilon\delta\), and a CD3\(\zeta\) heterodimer. While the TCR\(\alpha\beta\) is responsible for recognizing antigen as presented by MHC molecules on APCs, the CD3 complex, in the presence of costimulatory molecules, is responsible for signaling through the TCR upon this recognition, which results in an activation signaling cascade. The CD3 chains contain residues and motifs within their cytoplasmic tails that are necessary for transport and activation. The immunoreceptor tyrosine-based activation motifs (ITAMs) are well-studied due to their role in T cell activation. Each CD3\(\varepsilon\), CD3\(\gamma\), and CD3\(\delta\) chain contains one ITAM, while CD3\(\zeta\) contains three. These motifs contain tyrosine residues that become phosphorylated upon antigen receptor and co-receptor (CD4 or CD8) ligation and provide docking sites for SH2 domain-containing proteins important for downstream signaling.

T cell activation is initiated when TCR\(\alpha\beta\) recognizes peptide:MHC complexes. Clustering of these complexes brings in the coreceptors CD8 and CD4 that bind to MHC class I or II molecules, respectively. These coreceptors are associated with tyrosine kinases such as Lck and Fyn that will phosphorylate the ITAMs. The increased phosphorylation of the ITAM residues allows docking zeta associated protein-70 (Zap-70), a protein kinase which contains two tandem SH2 domains that bind the phosphorylated ITAMs and whose kinase activity is further
potentiated through phosphorylation by Lck and Fyn. This initial activation recruits scaffolding proteins and other kinases to activate phospholipase C-gamma (PLCγ), an enzyme that can cleave PIP2 into the second messengers DAG and IP3. IP3 will then trigger calcium release from first intracellular stores followed by extracellular Ca\(^{2+}\) influx through activation of the CRAC channel for activation of the NFAT transcription factor. DAG will go on to recruit additional kinases and scaffolding proteins for eventual activation of NFκB and AP-1 transcription factors. Once activated, transcription factors can translocate into the nucleus where they regulate gene transcription important for the function of the T cell\(^1\). Ligation of the TCR is commonly known as “signal one”- of the three main signals T cells receive that dictate their function.

Signal two helps ‘license’ the T cell to survive this activation. This signal comes primarily through the co-stimulatory molecule CD28 that is in close proximity to the TCR on the cell surface. The ligands for CD28 include CD80 and CD86 on the surface of APCs. The CD28 cytoplasmic tail can also become phosphorylated by Lck, a kinase that links into the activation of PLCγ and the Akt pathway downstream. Akt will not only promote cell survival with its signals but will also inhibit cell death pathways.

Signal three provides more information about the specific type of infection and dictates the differentiation pathway of the activated T cell. Signal three usually comes in the form of cytokine receptor signaling. If cytokines such as IFNγ and IL-12 are present during activation, a T helper type 1 (Th1) response will ensue for CD4\(^+\) cells. Conversely, T helper type 2 (Th2) responses dominate when IL-4 is present. These cytokines bind their specific receptor dictating a signal that will activate additional transcription factors to modify a particular response.
### 1.1.3 Functional T cell responses

The outcome of TCR ligation is to develop a functional response in the form of cytokine production, proliferation, and cytotoxicity (specific for CD8\(^+\) T cells). Both CD4\(^+\) and CD8\(^+\) T cells are capable of secreting the pro-inflammatory cytokines IFN\(\gamma\) and TNF\(\alpha\). These cytokines are primarily driven through activation of NF\(\kappa\)B and function to recruit additional cells to the site of infection. Another cytokine, IL-2, is triggered primarily through the CD28 costimulation pathway. IL-2 is highly transcribed when three major transcription factors (NFAT, AP-1, and NF\(\kappa\)B) are activated and is essential for T cell survival and proliferation.

Proliferation and differentiation are essential properties of T cells, which must clonally expand and adjust their function to actively fight the specific pathogen present. Proliferation requires an increase in cell size and activation of cell cycle genes to begin rapid mitosis. Each of the daughter cells are created for an antigen-specific army. Once a T cell is activated, proliferates, and differentiates into an effector T cell, it no longer is native and does not require costimulation by CD28 to survive. The differentiation process will then direct CD4\(^+\) T cells into a specific helper subset dictated by the cytokines present. It is important to note that any one infection can produce multiple helper subtypes *in vivo*. In addition, each helper subset has a specific set of cytokines that are secreted for a specialized, adaptive immune response. In contrast to CD4\(^+\) T cells, effector CD8\(^+\) T cells are less plastic and once activated, generally differentiate into cytotoxic or memory T cells.

A primary adaptive function of CD8\(^+\) T cells is cytotoxicity through the production of cytotoxins such as perforin and granzymes. Cytotoxins, however, do not bind a receptor. Instead, these effector molecules kill their target cells by permeating their lipid bilayer. Because of this,
their production is tightly regulated. Specific antigen recognition by cytotoxic CD8+ T cells will result in a tight T cell:target cell conjugate followed by the release of cytotoxic granules that are polarized to be released directly to the target cell leading to its apoptosis. Together, these functional responses of T cells recruit other cells to the site of infection and target clearance of infected cells.

Quickly following T cell expansion and effective pathogen clearance, T cell contraction occurs. The antigen-specific T cells that remain are long-lived memory T cells. While they appear previously activated by expressing proteins such as CD44, they also express anti-apoptotic factors like Bcl-2 and cytokine receptors for homeostatic maintenance such as IL-7Rα. Memory T cells are quickly reactivated upon exposure to antigen and produce cytokines such as IFNγ and TNFα. Within the memory T cell population there are subsets with heterogeneous populations. Two main subsets of memory T cells are the effector memory (TEM) and central memory (TCM) that are distinguished both phenotypically and functionally. Central memory T cells express CD62L and CCR7, lymph node homing molecules and mainly produce IL-2 and Th2 cytokines. Effector memory T cells on the other hand, display potent cytotoxicity and Th1 cytokines when stimulated ex vivo. The mechanisms for determining T cell fate as a TEM or TCM are currently being explored. One possibility is that a TEM is capable of later becoming a TCM, a mechanism described by Ahmed and colleagues using LCMV and L. monocytogenes as a model. Thus, TCR signaling leads to proper effector function and differentiation of some T cells to the memory compartment.
1.2 T CELL EXHAUSTION

1.2.1 In the setting of chronic viral infection

While antigen-specific activation usually leads to efficient T cell activation, pathogen clearance, and subsequent memory, this process can become dysfunctional under some conditions. The phenomenon of “T cell exhaustion” was first described as a mechanism for viral immune evasion during chronic murine lymphocytic choriomeningitis virus (LCMV) infection\(^4\)\(^-\)\(^5\). It was first noted that some antigen-specific cells were deleted while others were maintained, albeit with reduced cytokine production and proliferative potential. After further study, it appears that T cell exhaustion is a mechanism of tolerance to prevent fatal immune pathology during chronic infection. A key component to this phenotype is the chronic exposure to antigen. While exhaustion is described in both CD4\(^+\) and CD8\(^+\) effector T cells, the primary focus of this work is in cytotoxic CD8\(^+\) T lymphocytes. Early work defining exhaustion distinguished the reduced effector phenotype from anergy, a non-responsive state of T cells. Based in large part on studies using LCMV as a model, functional T cell exhaustion is now usually characterized as a gradual loss of cytokine production, proliferative capability, and sometimes eventual apoptosis\(^6\).

The cytokines notably reduced at the protein level during CD8\(^+\) T cell exhaustion are IFN\(\gamma\), TNF\(\alpha\), and IL-2. However, the transcripts encoding these proteins appear to be upregulated\(^7\). Additionally, production of granzyme B (GnzB) does not appear to be affected by chronic T cell stimulation. As the definition of exhaustion continues to be refined, it is often termed ‘dysfunction’ rather than exhaustion as there is an increase in some functional capabilities, such as production of the anti-inflammatory cytokine IL-10\(^8\). In fact, production of IL-10 is critical to maintenance of chronic LCMV infection\(^9\).
Importantly, proliferative potential is also reduced in antigen-specific T cells during chronic viral infection. After initial expansion of antigen-specific cells, if antigen persists, further expansion is limited. Exhausted T cells notably reduce expression of IL-7 and IL-15 receptors for survival and proliferation\textsuperscript{10,11}. However, studies that isolate exhausted cells from a previously infected host and transfer them to a naive host show that when antigen is no longer present they maintain the ability to homeostatically proliferate\textsuperscript{12}. This confirms the need for persistent antigen exposure in exhausted T cell phenotypes\textsuperscript{13}.

Transcription factor expression is another characteristic that can differentiate effector T cells from exhausted CD8\textsuperscript{+} T cells. While Tbet is high in functional and activated CD8\textsuperscript{+} T cells, there is a reduction of Tbet in exhausted cells. This correlates with an increase in a related T-box transcription factor, Eomesodermin (Eomes) in exhausted cells. Tbet, and to a lesser extent Eomes, increases transcription of pro-inflammatory cytokines such as IFN\textgamma\textsuperscript{14}. In the absence of Tbet and Eomes, a tumor microenvironment produces more IL-17 than IFN\textgamma, resulting in a reduced CD8\textsuperscript{+} T cell infiltrate\textsuperscript{15}. More recently, expression of TCF1, a transcription factor that is normally lost in effector CD8\textsuperscript{+} T cells, was shown to be expressed in a population of cells that has central memory characteristics with self-renewal and anti-viral capabilities\textsuperscript{16,17}.

Along the path toward exhaustion, accompanying the loss of function is a corresponding increased expression of so-called checkpoint molecules. Proteins such as PD-1, LAG-3, CTLA-4, TIGIT, 2B4, and Tim-3 are the most commonly observed markers of dysfunctional CD8\textsuperscript{+} T cells in chronic viral infection. The level of surface expression is correlated to the extent of T cell exhaustion, with the most exhausted cells expressing multiple markers\textsuperscript{18}. Many of these cell-surface markers are also known as inhibitory receptors, although only some have defined mechanisms by which they reduce T cell function. While we often use expression of these
markers to define an exhausted population, they are also expressed on other cells types and some during effector T cell differentiation in acute viral infection. The checkpoint molecules are an example of phenotypic exhaustion, but the importance lies in the ability of the cells to respond to antigen.

A key aspect of exhaustion during chronic viral infection is the accompanying lack of memory T cell precursors. Memory T cells are those that respond most rapidly to restimulation with their cognate antigen. As antigen is constantly present and driving reactivation of memory cells during chronic infection, the pools of quiescent cells are depleted\(^9\). The long-lived memory T cells express IL-7R (CD127), CD122, Bcl-2, and CD27 and notably lack KLRG1\(^20\). However, overexpression of IL-7R does not rescue terminally differentiated T cells\(^21\). There are other factors that can dictate the fate decision to memory precursor (MPEC) or short-lived effector cells (SLEC) such as strength of TCR signal, inflammatory environment, and CD4 T cell help (reviewed in \(^20\)).

### 1.2.2 T cell exhaustion in the setting of solid tumors

Evolution of T cell exhaustion appears to be adaptive, so that the immune system will not cause fatal pathology when fighting some pathogens. However, it is not just infectious agents that can drive T cell exhaustion. Cancers employ numerous mechanisms to evade the immune system including triggering effector T cell exhaustion. The study of cancer has also provided many key insights into the mechanisms that cause and maintain T cell dysfunction. As opposed to tolerance or anergy, T cell exhaustion is a unique T cell fate in cancers and is highest within the tumor infiltrating lymphocytes (TILs) while virtually undetectable in the periphery of the same patients, indicating the role of antigen exposure in driving this phenomenon\(^22\). In addition, exhausted T
cells in cancer appear to be much more heterogeneous than those in viral infection. This could be due to the additional factors of suppression in the tumor microenvironment (TME) affecting T cell function.

First, tumor cells can produce metabolites such as indoleamine 2,3-dioxygenase and lactic acid that inhibit proliferation and aerobic glycolysis, respectively\textsuperscript{23, 24}. This competition for glucose resources and metabolic needs in the TME suppresses T cell function and promotes cancer progression\textsuperscript{25}. Second, regulatory T cells (T\textsubscript{regs}), a subset of CD4\textsuperscript{+} T cells that, through both cell intrinsic and extrinsic mechanisms, downregulate the function of effector T cells are present in the TME. T\textsubscript{regs} are not only present in higher numbers in the TME but are functionally more suppressive. Finally, tumor associated macrophages and other cells of the myeloid lineage are present and secrete inhibitory cytokines that reduce T cell function. The overall milieu of the TME is hypoxic, triggering changes in metabolism and more reactive oxygen species, further affecting T cell activation (reviewed in \textsuperscript{26}).

Evidence of T cell exhaustion is described in nearly all human malignancies. Melanoma was among the first tumors in which it was shown that antigen-specific TILs had the phenotypic markers of exhaustion\textsuperscript{27}. Because of the rapid advancements in understanding immune dysfunction in the TME, melanoma was also the first cancer shown to respond well to checkpoint blockade immunotherapy and be FDA-approved\textsuperscript{28}.

\section*{1.2.3 Checkpoint receptors}

\subsection*{1.2.3.1 Programmed cell death protein-1 (PD-1)}

Programmed cell death-1 (PD-1; CD276) was first discovered in 1992 in a study investigating genes involved in programmed cell death and is the most characterized checkpoint receptor in T
cell exhaustion\textsuperscript{29}. PD-1 is a cell surface marker that contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM) in its cytoplasmic tail that allows it to recruit SHP-2 phosphatase reducing T cell activation in a variety of disease settings\textsuperscript{30}. The known ligands of PD-1 are PD-L1 and PD-L2 which, when engaged, induce the inhibitory function of PD-1 on T cells. Blockade of this interaction effectively reinvigorates some of the effector function of the T cells\textsuperscript{31}. This is the pathway targeted in immunotherapy drugs such as nivolumab and pembrolizumab\textsuperscript{32}. However, genetic loss of PD-1/PD-L1 axis results in overwhelming autoimmunity and susceptibility to chronic viral infection due to immune pathology\textsuperscript{30, 31}.

1.2.3.2 Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4)

CTLA-4 is a close homologue of CD28 that can bind to B7-1/B7-2 ligands. Unlike CD28, CTLA-4 is only expressed on T cells after activation. Because some antibodies against CTLA-4 enhances T cell activation, it was first thought of as a co-stimulatory molecule\textsuperscript{33}; however, further work indicated that activation of CTLA-4 signaling by cross-linking the receptor resulted in inhibition of T cell activation by competing with CD28\textsuperscript{34}. Genetic loss of CTLA-4 results in a fatal lymphoproliferative disease\textsuperscript{35}. The first immunotherapy for melanoma patients was an antibody against CTLA-4 (ipilimumab), which was FDA-approved in 2010\textsuperscript{28}.

1.2.3.3 Lymphocyte-activation gene-3 (LAG-3)

Also known as CD223, LAG-3 was discovered on activated T cells and found to be structurally similar to CD4\textsuperscript{36}. Its intracellular domain is unique and the signaling ability has yet to be defined. However, LAG-3 is able to bind to MHC II with greater affinity than CD4\textsuperscript{37}. As for CD8\textsuperscript{+} T cells, blockade of LAG-3 along with PD-1 resulted in synergistic restoration of function\textsuperscript{38}. LAG-
3 deficient mice exhibit increased numbers of T cells, macrophages, granulocytes, and DCs when aged to 16 weeks and was measured out to 52 weeks of age\textsuperscript{39}. LAG-3 also plays an essential role in the prevention of autoimmunity (i.e. Type-I diabetes)\textsuperscript{40}. There are several clinical trials currently using LAG-3 blocking monoclonal antibodies (mAbs) alone or in combination with other FDA-approved immunotherapies for cancers ranging from hematopoetic, gastric, lung cancer, and glioblastoma\textsuperscript{41}.

1.2.3.4 T cell immunoreceptor with Ig and ITIM domains (TIGIT)
A more recent addition to the checkpoint receptor category is T cell immunoreceptor with Ig and ITIM domains (TIGIT), which contains an ITIM domain in its cytoplasmic tail and binds to its ligand polio virus receptor (PVR). PVR is highly expressed in the adrenal glands, lungs, and placenta, but is also found in multiple other tissues. It is thought to reduce T cell function by inducing DCs to make the anti-inflammatory cytokine IL-10\textsuperscript{42}. The expression of TIGIT on T cells is correlated with disease progression in HIV\textsuperscript{43}. Blockade of TIGIT alone or in conjunction with PD-1 enhanced melanoma-specific CD8\textsuperscript{+} T cell function\textsuperscript{44}. Phase I clinical trials have already begun to evaluate the potential of TIGIT as a target in immunotherapy, either alone or in conjunction with anti-PD-1.

1.2.3.5 2B4
Also known as CD244, 2B4 is known primarily for its expression on natural killer cells, where its ligation increases killing\textsuperscript{45}. In addition to NK cells, 2B4 is also expressed on CD8\textsuperscript{+} T cells in different settings of chronic antigen stimulation. 2B4 belongs to the signaling lymphocyte activation molecule (SLAM) family of proteins that exhibit between two and four extracellular Ig domains. 2B4 contains a tyrosine rich cytoplasmic tail with four ITSM motifs and CD48 is the
only known ligand\textsuperscript{46}. The ITSM motifs can recruit phosphatases or kinases, causing 2B4 to have conflicting effects on T cell function. Current work uses high throughput approaches to understand what causes the switch in signaling for 2B4\textsuperscript{47}.

### 1.3 T CELL (TRANSMEMBRANE) IMMUNOGLOBULIN AND MUCIN DOMAIN CONTAINING PROTEIN – 3 (TIM-3)

#### 1.3.1 TIM-family proteins

The “Tim” family proteins got their name because the genes contain coding regions for T cell membrane glycoproteins, an immunoglobulin variable domain (IgV) and a mucin domain. They were first identified in a region of genes for asthma susceptibility named \textit{T cell and airway phenotype regulator (Tapr)}, found on mouse chromosome 11 and human chromosome 5q\textsuperscript{48}. The murine region contains eight genes, four non-coding genes and four coding for Tim proteins 1-4. The three human genes all produce functional proteins (Tim-1, Tim-3, Tim-4). Tim-1 and Tim-2 in the mouse are homologous to human Tim-1, while Tim-3 and Tim-4 in the mouse are homologous to human Tim-3 and Tim-4, respectively. Overall, there is a 63% amino acid sequence match between mouse and human Tim-3 (HAVCR2). At the time of discovery of the \textit{Tapr} region, alignment analysis matched with described proteins, kidney injury molecule-1 (Kim-1) and Hepatitis A virus cellular receptor-1 (HAVCR1), corresponding to Tim-1.
1.3.2 Discovery of Tim-3

Tim-3 was first described in a study where rats were immunized with T helper type-1 (Th1) cells, followed by screening for antibodies that would bind Th1, but not Th2, T cells\(^49\). After isolation and cloning of the antigen, the gene was found to have homology to the already described kidney injury molecule-1 (Kim-1, \(HAVcr-1\)). In the initial studies, Tim-3 was defined as a negative regulator, as antibodies to Tim-3 or Tim-3-Ig fusion proteins, administered to mice with experimental autoimmune encephalomyelitis (EAE) exacerbated the disease\(^49\). Further, when T cells were stimulated in Th1-polarizing conditions, Tim-3 was present on the surface during stimulation but decreased during resting periods. Only after multiple rounds of stimulation \textit{in vitro} was Tim-3 stably expressed on the surface of Th1 T cells\(^50\). Thus, this study suggested that chronic activation was required to achieve stable expression of Tim-3.

1.3.3 Tim-3 protein structure and signaling

Tim-3 is a type I transmembrane protein with an extracellular C-terminus and an intracellular N-terminus. The extracellular portion contains an IgV domain and a highly glycosylated mucin-like region. The IgV domain consists of two distinct anti-parallel beta-sheets connected by loops. The two sheets face one another, creating a pocket where ligand binding occurs on the outside, inside, or on top of the protein structure\(^51, 52\). The cysteine residues in the IgV domain are conserved through each of the Tim family proteins and form the di-sulfide bonds at the top of the domain\(^48\). These bonds also flank a hydrophobic loop that can penetrate opposing cell membranes.

Tim-3 has no defined motifs that are traditionally activating or inhibitory, however there are five tyrosine residues in its cytoplasmic tail that have the potential to become phosphorylated.
and allow for binding of proteins\textsuperscript{53, 54, 55}. Work by the Kane lab and others have shown that these residues can indeed become phosphorylated upon T cell activation\textsuperscript{54, 55}. The p85 domain of PI3K is able to bind to the tail of Tim-3 in a phosphorylation-dependent manner\textsuperscript{54}. Many proteins have been described to bind to the cytoplasmic tail of Tim-3 independent of tyrosine phosphorylation. The Src family kinase Fyn can constitutively bind the tail of Tim-3\textsuperscript{54}. In addition, the Tec family kinase, Itk has been shown to bind the cytoplasmic tail of Tim-3 upon ligation by galectin-9\textsuperscript{55}. Further, the protein chaperone, human leukocyte antigen B (HLA-B)-associated transcript 3 (Bat3) was described to bind the cytoplasmic tail of Tim-3 independent of phosphorylation\textsuperscript{56}. Interestingly, binding of Bat3 appear to rescue the loss of IFN\textgreek{g} production seen during galectin-9 ligation via its association with Lck. Strikingly, genetic loss of Bat3 results in spontaneous expression of Tim-3, suggesting it can not only associate, but also regulate Tim-3 expression\textsuperscript{56}.

The binding of the Lck, Fyn, or Itk kinases to Tim-3 indicates an activation signal is being sent downstream, and indeed that is what is seen. An enhancement in activation of PLC\textgreek{g}1, Akt, and S6 signaling molecules is observed when Tim-3 is present on the surface of a T cell leading to enhanced activation of transcription factors NF\textgreek{b} and NFAT/AP-1. This increased signaling leads to enhanced T cell function, as T cells expressing Tim-3 on the cell surface produced more IL-2 and IFN\textgreek{g} than those without Tim-3\textsuperscript{54}.

While the studies on the intrinsic effects of Tim-3 on TCR signaling and T cell activation are thus far limited, there are some patient data linking Tim-3 expression with T cell activation. Tim-3\textsuperscript{+} \textit{M. tuberculosis}-specific T cells have increased phosphorylation of signaling molecules such as p38, Stat3, Stat5, and Erk1/2 at baseline, and after peptide stimulation, compared to Tim-3\textsuperscript{−} T cells from the same patients\textsuperscript{57}. Although Tim-3\textsuperscript{+} T cells do not reactivate well from patients with HIV, they do have a higher baseline phosphorylation of p38, Stat5, and Erk\textsuperscript{58}. Additionally,
data from Tim-3⁺ acute myeloid leukemia cells shows an enhancement in the PI3K/mTOR pathway over Tim-3⁻ counterparts⁵⁹.

Known regulators of Tim-3 expression include the Th1 master transcription factor, Tbet, as well as nuclear factor, IL-3 regulated (NFIL3)⁶⁰, ⁶¹. Tbet is a t-box transcription factor that is rapidly activated during TCR stimulation in the presence of Th1 differentiating cytokines. While Tbet can drive initial expression of Tim-3 during T cell exhaustion, Tbet expression eventually decreases, leaving questions for how high Tim-3 expression is further maintained. When cytokine expression was screened against increased Tim-3 transcription, the anti-inflammatory cytokine IL-27 was identified as well as IL-10, a cytokine secreted by exhausted T cells that can promote tolerance. Notably, IL-27R⁻/⁻ mice do not have Tim-3⁺PD-1⁺ TILs and are more resistant to tumor growth⁶¹.

1.3.4 Tim-3 Ligands

Thus far, all known ligands for Tim-3 interact with the IgV domain at the N-terminus. The exact epitopes for ligands have not been determined but can be predicted using software and in silico analysis. The first described ligand for Tim-3 is galectin-9, identified using Tim-3-Ig fusion proteins⁶². In this work, galectin-9 was shown to cause cell apoptosis and necrosis. Exogenous galectin-9 ameliorated EAE, while knock-down of galectin-9 exacerbated disease. Galectin-9 is an S-type lectin that binds glycoproteins and is highly expressed on a variety of cell types, including naïve T cells⁶². Work in a variety of different cell types indicates that the amount of galectin-9 present can dictate the effect⁶³. In subsequent studies, it was shown that galectin-9 can bind T cells in the absence of Tim-3⁶⁴, ⁶⁵. This promiscuity is a characteristic of other Tim-3 ligands as well.
Phosphatidylserine (PS) is a constitutive component of the cell membrane but is only exposed on the cell surface upon initiation of apoptosis. Tim-3 on the surface of phagocytes (particularly CD8+ DCs) binds PS and initiates phagocytosis of apoptotic cells. Through this mechanism, Tim-3/PS interactions are thought to play a role in peripheral tolerance.

While galectin-9 and PS are associated with apoptosis, a non-inflammatory mechanism, another described Tim-3 ligand, HMGB1, has been studied for its role in pro-inflammatory settings. High mobility group protein-1 (HMGB1) normally resides in the nucleus, where it can regulate transcription as a DNA-binding protein. However, during inflammation, HMGB1 can be found in the cytoplasm and the extracellular space. In this setting, HMGB1 triggers toll-like receptors as an “alarmin.” Research in the effectiveness of DC vaccines in tumors found that Tim-3 was highly expressed on tumor-associated DCs and suppresses the DC’s response to some TLR stimulation. This mechanism was further supported by blockade or deletion of Tim-3 resulting in enhanced efficacy of DC vaccines in a murine tumor model. This effect of Tim-3 is dependent on binding to HMGB1 and not any other described Tim-3 ligand.

A more recently described ligand for Tim-3 is carcinoembryonic antigen-related cell-adhesion molecule-1 (CEACAM-1), a protein previously shown to negatively regulate T cell function by recruiting SHP phosphatases to reduce T cell signal strength. CEACAM-1 is also expressed on many other cell types and is associated with angiogenesis and apoptosis during tumor development. CEACAM-1 is similar to Tim-3 in protein structure, with a heavily glycosylated IgV-like domain on the extracellular portion. This glycosylation can promote dimer formation of CEACAM-1. It has been shown that Tim-3 and CEACAM-1 can be co-expressed on the surface of T cells and these two proteins interact in cis or trans. Evidence for this regulation include studies using Tim-3/CEACAM-1 co-blockade by antibodies, resulting in
enhanced T cell responses to murine colorectal cancer models. However, the most striking finding is that in genetic deletion of CEACAM-1, Tim-3 expression was completely abrogated\textsuperscript{74}.

A recent systematic study of overlapping epitopes for Tim-3 antibodies and ligands found that three current antibodies to Tim-3 have distinct epitopes and block only the interaction between Tim-3 and CEACAM-1 or PS, but not interactions with galectin-9\textsuperscript{75}. By performing hydrogen-deuterium exchange followed by mass spectrometry, more specific ligands binding sites were mapped\textsuperscript{75}. Based on the crystal structure of Tim-3, there is evidence that additional ligands exist. As discussed above, none of the known ligands are specific for Tim-3 and can bind other molecules. Very little signaling data exist to indicate whether these ligands act agonistically or antagonistically to the “true” signaling function of Tim-3, and whether different ligands have divergent functions in this regard.

\textbf{1.3.5 Tim-3 in autoimmunity}

As described above, Tim-3 was discovered in a screen for Th1-specific molecules. The specific disease setting Tim-3 was described in was a mouse model of multiple sclerosis known as experimental autoimmune encephalitis (EAE)\textsuperscript{49}. Tim-3 is expressed on the antigen-specific T cells infiltrating the central nervous system. When Tim-3 Ig-fusion proteins were administered to mice with EAE, exacerbation of the disease was observed. Additionally, when Bat3, a cytosolic binding partner of Tim-3, was overexpressed, there was enhanced Th1 cell function\textsuperscript{56}. Extending these studies to multiple sclerosis in humans led to the finding that antigen-specific T cells of the cerebral spinal fluid showed reduced Tim-3 expression compared to healthy controls\textsuperscript{76}. Interestingly, patients that were treated with glatiramer acetate or IFN\textbeta saw a rebound in Tim-3 expression on peripheral blood T cells\textsuperscript{77}. Tim-3 is described as a negative regulator in T cells
during MS, but it is not present on the T cells unless the patients are treated with immunomodulators. This paradoxical expression of Tim-3 has led to multiple interpretations of this work over the years. Since its discovery, Tim-3 has also been described in other settings of autoimmunity, as described below.

In addition to Th1 cells, Tim-3 is also expressed on Th17 cells, a known player in the pathogenesis of many autoimmune disorders. The effects of targeting Tim-3 in EAE sparked investigation into the autoimmune disorder systemic lupus erythematosus (SLE). Tim-3 expression was apparent on the peripheral T cells from SLE patients, and galectin-9 had been shown to ameliorate disease in a lupus disease model. However, when more closely dissected, galectin-9 was found to be therapeutic, through apoptosis of plasma cells, independent of Tim-3. Similar to SLE, T cells from synovial fluid of rheumatoid arthritis (RA) patients also express Tim-3, but to a smaller degree than healthy controls. This caused Tim-3 expression to be inversely correlated with disease severity. It is important to note that Tim-3 is also expressed on other cell types, such as innate immune cells, that are important for the pathology of autoimmunity.

When investigating Tim-3 and its relation to the ligand CEACAM-1, Huang et al. noticed accelerated morbidity and mortality in Tim3 transgenic mice subjected to the DSS colitis model. However, work done previously showed that when Tim-3 was overexpressed, DSS colitis was attenuated by decreasing the inflammatory macrophage response. Using DSS-treated mice as a control, Tim-3 expression was found to be lower in patients with ulcerative colitis than in healthy controls.

Much of the present data on Tim-3 in autoimmunity is correlative. It also assumes that Ig-fusion proteins, or antibodies work in an antagonistic way, while ligands such as galectin-9 act
on Tim-3 in an agonistic way. Due to the paucity of information on exactly how Tim-3 signals in any particular setting, it is presently unclear how the treatments are affecting a particular cell type.

1.3.6 Tim-3 in viral infection

Robust Tim-3 expression on T cells is also observed in human chronic viral infections, where T cell exhaustion is evident. The most well-documented examples include HIV and hepatitis-infected patients. HIV-infected individuals express Tim-3 on antigen-specific T cells and expression correlates with disease progression, viral load, and inversely with HAART. Studies specifically focused on CD8+ T cells in HIV patients show that Tim-3 expression is correlated with a reduced ability to degranulate, yet Tim-3+CD8+ T cells also contain more perforin. In vitro stimulation of these cells in the presence of a Tim-3 antibody can partially rescue the exhausted phenotype. However, ligation of Tim-3 by galectin-9 indicated a reduced susceptibility of T cells to HIV infection by reducing virus co-receptor expression. A better analysis of the kinetics of Tim-3 expression was obtained when the rhesus macaque SIV model was used to show Tim-3 expression in the acute and chronic phase of the infection. Tim-3+CD8+ cells were also the most dysfunctional T cells in this system.

Similarly, with hepatitis infection, Tim-3 is thought to play a negative role on the T cells. Tim-3 is expressed on T cells from patients with hepatitis A, B, or C infections. T cells from patients with HBV or HCV exhibited increased effector function when treated ex vivo with a Tim-3 Fc-fusion protein. This finding was corroborated by knock down of Tim-3 in a mouse model of HBV. With the defined link between chronic HBV and hepatocellular
carcinoma, Tim-3 is a prognostic marker for poor survival in HCC patients, with specific polymorphisms more prevalent in those with advanced disease\textsuperscript{95, 96}.

In order to model acute and chronic viral infection in mice, LCMV is often utilized. This is a well-described model where Tim-3 expression on T cells is comparable to what is observed in humans with chronic viral infection. An elegant kinetic analysis in LCMV-Armstrong and LCMV-Clone 13 revealed transient expression of Tim-3 during acute infection, and sustained expression during chronic infection\textsuperscript{8}. Additionally, in chronic LCMV infection, treatment with Tim-3 Fc-fusion protein alone did not reinvigorate exhausted T cells. However, when combined with PD-L1 blockade there was a synergistic effect\textsuperscript{8}.

In a mouse model of herpes simplex virus (HSV-1) infection, latently infected trigeminal ganglia have Tim-3\textsuperscript{+}CD8\textsuperscript{+} T cells with abundant galectin-9 in the area. The authors indicate that this interaction may affect reactivation of HSV-1 as galectin-9 knock-out mice had delayed viral reactivation\textsuperscript{97}. Exhaustion has not been clearly defined in HSV infection, which rather appears to associate with a unique phenotype, compared to that seen in a chronic viral infection with hepatitis or HIV.

\subsection*{1.3.7 Tim-3 in tumors}

As polymorphisms of Tim-3 are implicated in viral infections, they have also been linked to increased risk of non-small cell lung carcinoma (NSCLC) and pancreatic cancer in some populations\textsuperscript{98, 99}. Along with PD-1, Tim-3 is a marker of the most dysfunctional TILs during cancer\textsuperscript{27}. Tim-3 expression has been observed in almost every human cancer investigated, with the most-studied being melanoma, NSCLC, and renal cell carcinoma\textsuperscript{27, 100, 101}. Tim-3 is also described in head and neck, gastric, and prostate cancers as well as lymphoma\textsuperscript{102, 103, 104, 105, 106}. 
Interestingly, Tim-3 has not been observed to be expressed on the peripheral T cells of patients with cancer, suggesting antigen and/or the TME is necessary to maintain expression.

As with other checkpoint receptors, the therapeutic potential of mAbs to Tim-3 has been investigated. In the CT26 mouse tumor model, Tim-3 mAb functioned synergistically with anti-PD-1 treatment to increase effector function of exhausted TILs\textsuperscript{107}. When melanoma-specific exhausted T cells were treated \textit{ex vivo} with Tim-3 mAb alone, some effector function was recovered, although Tim-3 mAb was most effective in combination with PD-1 blockade\textsuperscript{27}. This was particularly notable as PD-1 blockade on its own is not fully effective\textsuperscript{108}. Due to substantial pre-clinical work, there are currently multiple clinical trials to investigate the use of Tim-3 blockade in conjunction with FDA-approved PD-1 and/or CTLA-4 blockade.

Tim-3 has also been shown to mark a more suppressive subset of regulatory T cell (T\textsubscript{reg})\textsuperscript{109, 110}. While a small population of T\textsubscript{reg}s in naïve mice express Tim-3, this population increases during infection and is the majority of T\textsubscript{reg}s in the tumor microenvironment\textsuperscript{105, 110}. Additionally, this correlates with Tim-3\textsuperscript{+} T\textsubscript{reg}s indicating poor prognosis in multiple types of cancers\textsuperscript{105}.

\textbf{1.3.8 Tim-3 in bacterial infections}

Some of the first evidence that Tim-3 may not solely function as a negative regulator came from studies in tuberculosis (TB). In the mouse model of \textit{mycobacterium tuberculosis} (mTb) infection, Tim-3 was highly expressed on lung-infiltrating T cells. When treated with galectin-9 or a Tim-3 Ig-fusion protein, bacterial burden was reduced through enhanced macrophage activation\textsuperscript{111}. Similarly, the same group observed similar results with human macrophages from TB-infected patients\textsuperscript{112}. Evidence indicates that it may not only be Tim-3\textsuperscript{+} macrophages with anti-TB
properties. Tim-3 is expressed on more peripheral T cells from TB infected patients, compared to healthy controls, and those T cells exhibit an effector memory phenotype. Furthermore, Tim-3$^+$ T cells have more cytokine production compared to their Tim-3$^-$ counterparts, which can be enhanced by Tim-3 ligation with mAbs$^{57}$. TB represents a chronic bacterial infection, but Tim-3 is also expressed in acute bacterial infections.

Listeria monocytogenes is an acute bacterial infection that commonly affects humans. In a murine model of L. monocytogenes, Tim-3 was associated with activation of the T cells. In Tim-3 deficient mice, fewer antigen-specific T cells were present during acute infection and even fewer were reactivated upon restimulation$^{113}$. This phenotype was still present when Tim-3 deficient T cells were transferred to a naïve host followed by infection. Investigation of Tim-3 in infectious and non-infectious disease settings has revealed the complexity of roles Tim-3 can play, both cell-type and disease dependent.

1.4 CANCER IMMUNOTHERAPY

Immunotherapy is a generic term for any treatment that stimulates the immune response. Specifically for cancer, the goal is to induce an immune response to fight (and potentially clear) the malignancy. The idea of immunotherapy is credited to William B. Coley, now known as the “father of immunotherapy”. In the late 19th century this surgeon, who was plagued by the death of his cancer patients, noticed something peculiar. He saw that more than 40 of his patients that had a streptococcal skin infection spontaneously went into remission from their sarcoma. In a time where little regulation was in place for clinical trials, Coley began experimenting with intentionally infecting his cancer patients. After various trials, and some deaths, he achieved
more than 50% response rate in multiple cancers using a mixture of heat killed *Streptococcus pyogenes* and *Bacillus prodigious*\textsuperscript{114}.

Unfortunately, the idea of immune activation as a therapy was largely dismissed, due to the advent of chemotherapy and radiation, until the theory of cancer immunosurveillance was proposed in 1957 by Burnet and Thomas. This theory stated that the immune system has the capability to prevent the majority of tumors by surveying and killing abnormal somatic growth by recognizing tumor neoantigens\textsuperscript{115}. Neoantigens are peptides produced by cancer cells either by somatic mutation or introduction of oncolytic virus genes that are not normally expressed by the human genome. This novelty makes them promising targets for cancer, as we can discriminate cells making neoantigens from otherwise healthy tissue.

Again, due to the lack of technology to support these claims, immune therapies for cancer would again be suspended from development until the discovery of IL-2. In 1976, the discovery of the soluble T cell growth factor, IL-2, revolutionized immunology\textsuperscript{116}. Scientists could now culture and study T lymphocytes in a way they could not previously. Creation of recombinant IL-2 allowed it to be used as an immunotherapy in patients, and by stimulating T cell growth, 1984 brought patients who responded to complete remission\textsuperscript{117}. Significant toxicities were found associated with IL-2 treatment, that were reversed with cessation of treatment\textsuperscript{118, 119}. Even then, IL-2 as a biologic and immunotherapeutic agent was not FDA-approved for cancer until the 1990’s.

Tumor necrosis factor-alpha (TNF\(\alpha\)) was first discovered as one the soluble factors in “Coley’s toxins” that had anti-tumor effects\textsuperscript{120}. However, early attempts to use it in cancer therapies found it to be exceedingly inflammatory and caused more morbidity than treatment\textsuperscript{121}. In 1992, Lienard and colleagues pioneered TNF\(\alpha\) in isolated limb perfusion for treatment of
melanoma and sarcoma\textsuperscript{122}. This has proved to be an effective treatment with significant effects on overall response rate.

IL-2 and TNF\textsubscript{α} therapy are in a category with other cytokines including interferons known as adjuvants, that non-specifically activate the immune system. Other adjuvant therapies include Bacille Calmette-Guerin and Imiquimod, used to treat bladder and skin cancer, respectively. In the last 30 years, immunotherapy has proved exciting, sometimes successful, and also extremely complicated. More importantly, translational treatments have provided hope to patients. The majority of popular immunotherapies focus on targeting the adaptive immune system. Some of the most researched areas where there are now FDA-approved drugs include immunotherapy using monoclonal antibodies, checkpoint inhibitors, and cancer vaccines.

\subsection*{1.4.1 Monoclonal antibodies and checkpoint inhibitors}

Antibodies are a hallmark of the adaptive immune response. When cancer antigens were discovered, the idea of producing antibodies to them followed quickly. After the work of Milstein and Kohler, scientists could now manufacture mAbs in the lab using hybridomas\textsuperscript{123}. Large scale production of antigen-specific antibodies allowed physicians to treat patients, leading to Rituximab, the first FDA approved mAb to treat non-Hodgkin’s lymphoma in 1997\textsuperscript{124}. The potential mechanisms by which these antibodies work include antibody-dependent cellular cytotoxicity (ADCC), opsonization, and subsequently increased cross-presentation of antigen to cytotoxic T cells\textsuperscript{125}.

While the drug type (mAb) may be similar, immunotherapeutic antibodies have various mechanisms of action depending on what they target. In some cases, the antibodies block an oncogenic pathway, such as with Cetuximab. This mAb is specific for EGFR and prevents
phosphorylation of the receptor and downstream signaling, which normally keep the cancer cell alive to promote anti-tumor activity in the site\textsuperscript{126}. Another revolutionary mAb was Pertuzumab, a receptor dimerization inhibitor that also blocks an oncogenic pathway. It is specific for HER2, a protein expressed on several types of cancer cells\textsuperscript{127}. Some mAbs work by blocking the angiogenesis pathway, such as with VEGF/VEGFR inhibitors. Bevacizumab and Ramucirumab block ligand interactions or cause a conformational change in VEGF, respectively. Both reduce tumor vasculature and prevent new angiogenesis\textsuperscript{128}.

Instead of preventing oncogenic pathways, other mAbs can enhance the immune response by inhibiting negative regulators of anti-tumor immunity. These include mAbs targeting so-called “checkpoint” molecules and are currently the basis of extensive research. The first checkpoint inhibitor to be FDA-approved was Ipilimumab, specific for CTLA-4, a protein homologous to CD28 and which competes for binding to CD80/CD86, preventing costimulation and dampening the T cell response\textsuperscript{34, 129}. This drug’s success in metastatic melanoma led to a huge growth in research into checkpoint blockade therapy, as CTLA-4 blockade, while useful, does not work in all patients.

Arguably the most exciting checkpoint blockade therapy to date involves blocking the PD-1/PD-L1 axis. PD-1 is a well-documented inhibitor of T cell responses and drugs such as nivolumab and pembrolizumab prevent PD-1 from interacting with its ligands PD-L1/PD-L2\textsuperscript{130}. While first approved for combination therapy against metastatic melanoma, it has also been effective in non-small cell lung carcinoma, classical Hodgkin’s lymphoma, and head and neck squamous cell carcinomas. While PD-1 mAbs can bind T cells, the cancer cells themselves express high levels of its ligand PD-L1, leading to effectiveness of PD-L1 antibodies, including atezolizumab in urothelial carcinoma\textsuperscript{131, 132}. 
While the above antibodies are ‘naked’ mAbs, others are still working on development of radiolabeled and drug-conjugated mAbs, which use the antibody to guide a drug to its specific target, avoiding some of the caustic side-effects of systemic chemotherapy drugs. Ado-trastuzumab emtansine specifically delivers a microtubule-inhibiting drug to HER2 positive cells, reducing some of the toxicity seen with the drug alone. Another use of these labeled antibodies is simply to track and follow the cancerous cells to best image and target them for destruction.

Overall, monoclonal antibodies offer a large variety of treatments to many cancers, particularly with combination therapies. Having available drugs with different mechanisms of action provides an advantage when dealing with a constantly adapting and mutating disease such as cancer. As the cancer develops resistance to one therapy, we have multiple options to target another pathway or receptor that could offer some response. Immunotherapy offers less toxicity than traditional chemotherapy, however it still has potential side effects. Most of those side effects are due to the activation of an otherwise quiescent immune system, resulting in autoimmune morbidities such as inflammatory bowel disease and non-specific inflammation. As research continues to investigate other checkpoint molecules, there will be more opportunity for further drug development against other targets. Yet it is important that we first understand how these proteins work before we can efficiently target them and reduce chances of off-target or toxic effects.

1.4.2 Cancer vaccines

Vaccinations are often effective for prevention of infectious diseases, and the discovery of oncogenic viruses led to the idea of cancer vaccines. One of the first oncogenic viruses
discovered was Epstein-Barr virus (EBV). While most commonly known for causing mononucleosis, EBV also contributes to the development of Burkitt’s lymphoma in developing countries\textsuperscript{135}. An EBV vaccine has only made it as far as Phase II trials with little efficacy, proving difficult to develop\textsuperscript{136}. In 1983, zur Hausen and colleagues made the link between women with cervical cancer and human papilloma virus (HPV) infection\textsuperscript{137}. It would not be until 1999 when worldwide studies showed that \textasciitilde 99\% of cervical cancers had some HPV strain infection\textsuperscript{138}. Although vaccinations for bovine papilloma viruses were available in the 1950’s, an HPV vaccine was not FDA-approved until 2006\textsuperscript{139}. As a relatively new vaccine, its durability is not really known at this time. If HPV vaccines follow the effectiveness of hepatitis vaccinations reducing the risk of developing hepatocellular carcinomas, it will be promising\textsuperscript{140}.

In addition to the possibility of vaccinating healthy patients, cancer vaccines can boost the immune system of those already with disease. This possibility came as a consequence of understanding tumor antigens. The only (mildly) successful cancer vaccine to date is sipuleucel-T, for castration-resistant prostate cancer\textsuperscript{141}. It extends life by just a few months on average, leaving much room for improvement. Vaccine developers are becoming more creative now and using more individualized approaches. One vaccine strategy is against self-neoantigens that showed strong efficacy in a small group of patients\textsuperscript{142, 143}. Samples of tumors were subjected to whole exome and RNA sequencing to compare possible neoantigens. Based on this, one group chose to use a peptide-based vaccine, while another used an RNA-based vaccine, both of which had similar efficacy in their small study groups of melanoma patients\textsuperscript{142, 143}.

Cancer vaccines are not a new concept, but rather an elusive one. If an infectious agent is determined to be related to a cancer, preventative vaccines appear successful. However, once malignancy has set in, activating the immune response with a vaccine is much more difficult.
1.5 LYMPHOCYTIC CHORIOMENINGITIS VIRUS (LCMV)

LCMV is an enveloped Baltimore class V (-) ssRNA virus of the family *Arenaviridae*. It was first isolated in 1933 during investigation of St. Louis encephalitis. Its name is suggestive of the symptoms displayed by patients with this virus: inflammation and cellular infiltrate in the meninges. While human infection is exceedingly rare, primarily seen in immunocompromised patients, the prevalence in wild rodents is about 5%. Because LCMV is infectious to mice, it serves as a model with which we can study immune responses to viral infection.

LCMV is credited with aiding in discovery of numerous fundamental principles in immunology. For example, Zinkernagel and Doherty earned a Nobel Prize in 1966 for their work using LCMV to define MHC restriction and determine its necessity in T cell recognition and activation. Early on, it became clear that the ability of a rodent to clear the infection was dependent on the mouse strain, the viral strain, as well as the mode of transmission. While there are many described strains of the virus, the most commonly used are LCMV-Armstrong (Arm) and LCMV-Clone 13 (Cl13). The former refers to the original strain discovered by Charles Armstrong in 1934, while the latter was isolated by Rafi Ahmed in 1984.

LCMV-Arm causes a robust immune response in C57Bl/6 mice that is cleared after a week of infection, leaving both memory B and T cells. However, if there is vertical transmission from mother to fetus or intracranial infection the virus can become persistent. In addition, research into the necessity of CD4+ T cell help revealed that CD4 depletion resulted in a chronic infection of LCMV-Arm. LCMV-Arm is a more neurotropic virus, while LCMV-Cl13 tracks to other organs such as the spleen and liver. Sequence analysis shows only two amino acid differences between Armstrong and Clone 13 strains. There is a lysine to glutamine (K→Q) mutation in the polymerase protein and phenylalanine to leucine (F→L) substitution in the
glycoprotein$^{148}$. These small changes affect the viral tropism and the way the antigen is processed and presented to T cells. LCMV-Cl13 creates a chronic infection in rodents and can be fatal when CD4$^+$ T cells are depleted$^{149}$. This chronic LCMV models some features of the human immune responses to viruses such as HIV and Hepatitis C.

The immunodominant epitopes of LCMV are the peptides that result in the greatest clonal expansion of T cells specific for those sequences. Glycoprotein amino acids 33-41 (GP33) is the dominant epitope, inducing the most antigen-specific T cells$^{150}$. Other common epitopes include nucleoprotein (NP396) and GP276. Knowing the dominant epitopes not only provides us with the information on how to stimulate antigen-specific cells, but also to recognize them. LCMV was critical in the advent of tetramer technology to identify antigen-specific T cells without activation. Tetramers contain a central streptavidin molecule usually labeled with a fluorophore that binds four biotinylated MHC molecules that are specific to its cognate TCR. Having four MHC molecules increases the avidity of the complex, creating a more stable conjugation that can be detected using flow cytometry.

In addition to viral clearance, the T cell response is critically different in acute versus chronic LCMV infection. LCMV-Cl13 causes antigen-specific T cells to exhibit the exhaustion phenotype discussed earlier, a phenotype not present in mice infected with LCMV-Arm$^{147}$. In contrast, LCMV-Arm induces functional memory T cells that reactivate quickly upon peptide re-stimulation with APC. Mice exposed to LCMV-Arm that have functional memory are protected from challenge with LCMV-Arm, LCMV-Cl13, and any other heterologous infection that utilizes an LCMV-immunodominant epitope as its main antigen$^{151}$. LCMV has been, and continues to be, an infection model for dissecting anti-viral immune responses.
1.6 TIM-3 INDUCTION AND KNOCKOUT MOUSE MODELS

1.6.1 Flox-Stop-Flox Tim-3 (FSF-Tim3) and Cre mice

Tim-3 protein not present on naïve T cells and requires chronic antigen stimulation to maintain stable expression\(^5\). In the process of inducing Tim-3 expression, many other checkpoint receptors are also expressed\(^3\). Therefore, to study the intrinsic effects of Tim-3, apart from other receptors, we generated an inducible Tim-3 mouse model. A cDNA was generated previously\(^5\) that contains a flag-tagged murine Tim-3 sequence. This construct was then preceded by a floxed-stop cassette and targeted to the Rosa26 locus in C57Bl/6 embryonic stem (ES) cells, by scientists at genOway (France). Targeted C57Bl/6 ES cells were injected, and targeted mice were derived at University of California Davis through the MMRC. FSF-Tim3 mice have no obvious baseline phenotype and endogenous Tim-3 expression is intact in a normal manner.

Utilizing various Cre systems, I confirmed that the stop cassette is efficiently removed, driving high (and irreversible) Tim-3 expression. Tat-Cre is a protein transduction system that targets Cre directly to the nucleus of cells using the HIV protein trans-activator of transcription (Tat) as a delivery system\(^1\). Tat is encoded in the HIV genome, contains a cell-penetrating peptide, and a nuclear localization signal to effectively deliver the Cre\(^1\). For in vivo recombination, Cre is expressed under a cell type specific promoter and when present excises the stop cassette in the same irreversible manner. For the purposes of our experiments, E8i-Cre and CD4-Cre were used. E8i-Cre is only expressed in mature CD8\(^+\) T cells as the cells exit the thymus\(^1\). CD4-Cre expresses Cre starting at the double-positive stage of T cell development in the thymus, therefore Tim-3 is expressed on all CD4\(^+\) and CD8\(^+\) αβ T cells, including T\(_{\text{reg}}\)\(^1\).
Figure 1: Flox-Stop-Flox Tim-3 mouse model.

The Tim-3 cassette preceded by the floxed stop codon was knocked into the \textit{Rosa26} locus of C57BL/6 ES cells. Under Cre-mediated recombination the stop codon is removed driving transcription of a flag-tagged murine Tim-3.
Figure 2: Confirmation of FSF-Tim3 mouse model and normal T cell development in FSF-Tim3/CD4Cre mice.

(A) Cell numbers counted in lymphoid compartments of FSF-Tim3/CD4-Cre or CD4-Cre alone (n=2 mice, mean ± SD). (B) CD4+ and CD8+ T cells in the indicated compartments from CD4-Cre and FSF-Tim3/CD4-Cre mice. Shown is gating on CD3+ viable cells (Left) and Tim-3 expression in CD3+CD4+ or CD3+CD4- cells in the indicated compartment (Right). Data are representative of two mice per group in two independent experiments.156
Figure 3: Normal T cell development in FSF-Tim3/E8iCre mice.

(A) Cell numbers from selected lymphoid organs of 6-wk old mice of the indicated genotypes. (B) Representative flow diagrams of CD4 and CD8 expression (Left) and the flag expression in those populations. Cells quantitated and analyzed as in Fig. 2.156
1.6.2 Tim-3 KO mice

To determine the necessity of Tim-3 in immune responses, Tim-3 KO mice were generated by a collaborating lab\textsuperscript{113}. Briefly, a construct targeting the Tim-3 locus (Havcr2 gene) was injected into mouse strain 129 ES cells and selected for using antibiotic resistance. Surviving stem cells were injected into blasts from C57Bl/6 mice into pseudo-pregnant mice and chimeras were derived. Chimeras were then back-crossed to C57Bl/6 mice and have been used at this point at greater than 15 generations. These mice were phenotyped and described by the Colgan lab at University of Iowa\textsuperscript{113}. However, because stem cells from 129 background mice were targeted, and the Tim family genes are closely linked, there was some controversy over the conclusions of the work\textsuperscript{157}. This controversy was settled by additional experiments done by the Colgan lab to ensure that lack of Tim-3 was indeed the cause of the described phenotype\textsuperscript{158}. Tim-3 KO mice were subject to our experimental system for the work here.
2.0 STATEMENT OF THE PROBLEM

T cell exhaustion is a phenomenon seen in multiple settings where chronic T cell stimulation occurs. This includes, but is not limited to, chronic viral infection, solid and leukemic malignancies, as well as autoimmune settings. This is an adaptive response to prevent overwhelming immune pathology, killing the affected person. In cancer, the field of immunotherapy aims to activate or reinvigorate the patient’s immune system to battle the malignancy. The concept of immunotherapy can also be applied to chronic viral infections and autoimmunity. There are multiple types of immunotherapy based on the mechanism targeted. One such target is checkpoint receptors or cell-surface proteins associated with the most dysfunctional antigen-specific T cells. Blockade of these receptors using monoclonal antibodies can result in enhanced effector T cell function. Antibodies against PD-1/PD-L1 interaction as well as CTLA-4 are FDA-approved drugs for treatment of certain cancers. However, they do not work for all patients, and their exact mechanism of action is largely unknown. It is necessary to explore other checkpoint receptors for possible combination therapies to enlarge the responsive population.

One such checkpoint receptor is Tim-3. The majority of published work on Tim-3 in T cells associates it with a negative effector cell phenotype, Tim-3 is expressed on the most dysfunctional T cells in cancer, chronic viral infection, and autoimmunity. In addition, antibodies and fusion proteins specific for Tim-3 appear to increase T cell effector function in vivo and in
*vitro* and can work synergistically with PD-1 blockade. However, recent data from multiple labs has implicated a potential positive role for Tim-3 on the surface of T cells. Two complicating factors to studying the intrinsic effects of Tim-3 include the need for persistent antigen to induce Tim-3 expression, and the subsequent upregulation of additional checkpoint. In order to study the intrinsic effects of Tim-3, we developed a novel Flox-Stop-Flox Tim-3 mouse model (FSF-Tim3) that induces Tim-3 expression using Cre-mediated recombination, without the need for antigen exposure. We used this mouse in conjunction with Tim-3 KO mice to study the necessity and sufficiency for Tim-3 in acute and chronic LCMV infection.

We hypothesized that if Tim-3 negatively regulates T cell function, induction of Tim-3 will drive T cell dysfunction and cause an acute viral infection to become chronic. Additionally, the loss of Tim-3 would result in enhanced effector T cell function during chronic LCMV infection. We addressed these hypotheses using murine LCMV infection (both acute and chronic strains). The data we have generated here will impact the field of immunotherapy and further the understanding of Tim-3 function on T cells.
3.0 TIM-3 IS ASSOCIATED WITH T CELL ACTIVATION IN VITRO

3.1 INTRODUCTION

Until recently, expression of Tim-3 has primarily been associated with reduced T cell activation. However, study of the intrinsic effects of Tim-3, using ectopic expression and CD3/CD28 stimulation, found enhanced T cell effector function. This corresponded with increased transcription factor activity and enhanced phosphorylation of some T cell signaling molecules such as ERK and S6. This co-stimulating effect of Tim-3 was dependent on the phosphorylation of two tyrosine residues in the cytoplasmic tail of Tim-3, Y256 and T263, which constitutively bind Fyn kinase and p85 in a phosphorylation-dependent manner. In addition, expression of Tim-3 correlated with enhanced effector T cell function in tuberculosis and murine *L. monocytogenes* infections. To directly determine if endogenous Tim-3 expression was associated with T cell activation through TCR engagement, we utilized the Nur77GFP reporter mice. Nur77 is an orphan nuclear receptor that is upregulated upon antigen receptor ligation, but is not affected by inflammatory stimuli. Nur77GFP mice were generated independently by two different labs in order to investigate the effect of TCR signal strength on T and B cell differentiation. We generated phenotypically exhausted T cells *in vitro* using repeated anti-TCR antibody stimulation resulting in stable, endogenous Tim-3 expression. Using flow cytometry, we found that higher Tim-3 expression is associated with higher expression of the Nur77-driven GFP.
reporter. This suggested the presence of enhanced TCR signaling when Tim-3 is expressed on the surface.

3.2 METHODS

3.2.1 Antibodies, mice, and reagents

Mice were bred in-house under SPF conditions and used at 6-8 weeks of age. The Nur77GFP mice used were C57BL/6-Tg(Nr4a1-EGFP/cre)820Khog/J originally purchased from Jackson Laboratory (Bar Harbor, ME) and then bred in-house. Antibodies used: Tonbo: GhostDye, αCD28 (purified & biotinylated) clone 37.51, αCD3 (purified & biotinylated) clone 2C11, αCD4 (GK1.5), αCD8 (53-6.7). eBioscience: αCD69 (H1.2F3). Biolegend: αPD-1 (RMP1-30), R&D Systems: αTim-3 (215008). Millipore: α-phosphor-tyrosine (4G10) and streptavidin. Cell signaling: αpS6 Ser235/236, (D57.2.2E). Sigma-Aldrich: α-β-actin (AC-15). Invitrogen: αpAkt Ser473, (14-6).

3.2.2 In vitro T cell stimulation and Tat-Cre protein transduction

For repeat stimulations to generate phenotypically exhausted T cells, CD25-depleted splenocytes (depletion using CD25 microbead kit, Miltenyi Biotec) were stimulated for three days with plate bound αCD3 and soluble αCD28 followed by a seven-day rest with IL-2. This process was repeated for a second stimulation and rest, with the final analysis performed after a tertiary stimulation for 24 hours. For protein transduction, whole splenocytes were treated in vitro
overnight with 1.5μM Tat-Cre (Excellgen). For short stimulations (up to four hours), whole lymph nodes were processed and stimulated with biotinylated αCD3/CD28, plus streptavidin to cross-link (at 1:1:5 ratio) in serum-free RPMI (Corning). After stimulation, cells were processed for western blot or flow cytometric analyses.

### 3.2.3 Flow cytometry and western blotting

For flow cytometry, fluorescently-conjugated antibodies against cell surface markers were incubated with cells, followed by 1.5% PFA fixation and methanol permeabilization for intracellular phospho-flow staining. Cells were analyzed on a BD LSR II using FACS DIVA software. Post-run analysis was performed using FlowJo software. For western blotting, cells were lysed in RIPA buffer in the presence of protease and phosphatase inhibitors. Clarified lysates were then run on SDS/PAGE reducing gels and blotted onto PVDF membrane, blocked with BSA and probed for the indicated proteins. Blots were imaged using a Protein Simple FluorChem M and Alpha View software.

### 3.2.4 Statistical analysis

Two to five biological replicates were used for all experiments. Statistical analyses were performed using GraphPad Prism software. Paired and unpaired Student’s t test and one-way ANOVA were used for data analysis and determination of p-values as appropriate and indicated in figure legends.
3.3 RESULTS

3.3.1 Endogenous Tim-3 expression is correlated with enhanced TCR signaling and T cell activation

In order for Tim-3 to be stably expressed by T cells, multiple rounds of TCR stimulation are required\textsuperscript{50}. Because of the need for IL-2 expansion, CD25\textsuperscript+ cells needed to be depleted to prevent selective expansion of T\textsubscript{regs}. Efficiency of CD25 depletion was >99% (Fig. 4). With successive stimulations there was an increase in the frequency of Tim-3 expressing cells after each successive round of T cell stimulation (Fig. 5). To confirm that these cells were indeed activated, we corresponded the Tim-3 expression with PD-1, and those cells expressing the highest levels of Tim-3 also express PD-1 (Fig. 5A-C). Using flow cytometry, we found that this increase in Tim-3 expression was associated with an increase in GFP when under the control of Nur77. Following the primary stimulation, only about 10% of the CD8\textsuperscript+ T cells expressed low levels of Tim-3; however all Tim-3\textsuperscript+ cells were also Nur77\textsuperscript{GFP+} (Fig. 5A). This same trend was observed following the secondary stimulation, after which there were both Tim-3\textsuperscript{Lo} and Tim-3\textsuperscript{HI} cells with correlating levels of Nur77\textsuperscript{GFP} (Fig. 5B). After the tertiary stimulation, ‘resting’ cells had various levels of Tim-3 expression, while the stimulated cells all became Tim-3\textsuperscript{HI} by this time (Fig. 5C). Resting T cells with the highest Tim-3 expression following the second stimulation also had the highest baseline Nur77\textsuperscript{GFP} activation. Because Nur77 appears to integrate overall T cell activation through the TCR\textsuperscript{160}, we can conclude that the level of Tim-3 expression correlates with signal strength in the T cells at rest and during stimulation.
Figure 4: Confirming CD25$^+$ depletion for in vitro stimulations.

Lymph nodes were harvested from Nur77$^{GFP}$ mice and processed into single cell suspensions. The cells were subjected to magnetic depletion of CD25$^+$ cells then stained for flow cytometry. CD4 and CD25 was measured in cells stained before and after depletion and gated on TCR$^+$ live cells.
Figure 5: Endogenous Tim-3 expression is associated with enhanced T cell activation.

Purified CD3^+CD25^- T cells were isolated from Nur77^{GFP} transgenic mice and stimulated in vitro. (A) After three days, cells were stained with fluorescently conjugated antibodies to endogenous PD-1 and Tim-3 in the CD8^+ live population and analyzed for Nur77^{GFP} expression. (B) Remaining cells were rested for seven days in IL-2 (100IU/ml) then restimulated for three days for the same analysis. (C) Remaining cells were rested for seven days in IL-2 and restimulated (tertiary stim) for 24 hours with the same analysis of Tim-3, PD-1 and Nur77^{GFP}. Data are representative of five biological replicates from five independent experiments.156
3.3.2 Enforced Tim-3 expression results in enhanced mTOR signaling

Since endogenous Tim-3 expression was associated with more T cell activation (Fig. 5), we aimed to determine if overexpression of Tim-3 could increase the activation of αCD3/CD28-stimulated T cells. Using the FSF-Tim3 mouse model (described in chapter 1.5, Fig. 1, 2), I bred these mice to CD4-Cre mice, effectively inducing Tim-3 expression on all αβ⁺ T cells without the need for chronic antigen exposure (Fig. 2). I isolated bulk CD3⁺ T cells (using negative selection) from lymph nodes of CD4-Cre or FSF-Tim3/CD4-Cre mice, stimulated the cells with αCD3/CD28 for short periods of time, and observed enhanced total phosphotyrosine (Fig. 5A). At baseline, Tim-3 induced cells had slightly more phosphor-tyrosine (similar to what we saw with Nur77GFP). However, they continued to enhance phosphorylation upon stimulation over that of Cre-only T cells. When narrowing down to specific downstream signaling pathways, there is evidence in the literature that Tim-3 may play a role in signaling to Akt and mTOR⁵⁴, ⁵⁹. Therefore, we investigated phosphorylation of Akt at serine 473, an mTORC2 target protein, and phosphorylation of ribosomal protein S6 at serines 235/236, an mTORC1 target protein. We found that both proteins had increased phosphorylation upon αCD3/CD28 activation of T cells with enforced Tim-3 expression (Fig. 6B,C). These data indicate that the presence of Tim-3 places the T cell in a ‘poised’ position to activate as well as further enhances T cell activation after stimulation through the TCR.
Naïve CD3⁺ T cells were isolated from lymph nodes of CD4Cre or FSF-Tim3/CD4-Cre mice and stimulated \textit{in vitro} with αCD3/CD28 for the indicated times. Cells were then lysed and analyzed by western blot for (A) total phosphotyrosine (B) pAkt (Ser473), Tim-3 or (C) pS6 (Ser235/236) and β-actin loading control. Data are representative of three independent experiments.¹⁵⁶
3.3.3 Enhanced activation of TCR signaling by Tim-3 is T cell-intrinsic

Our initial analyses were performed with FSF-Tim3 mice bred to CD4-Cre, which leads to expression of Tim-3 on all αβ+ T cells, including Tregs. In order to determine whether the stimulatory effect of Tim-3 on T cell activation was cell-intrinsic, we bred the FSF-Tim3 mice to E8i-Cre. This induces Tim-3 expression only on mature CD8+ T cells as they exit the thymus (Fig. 3). When T cells were stimulated with αCD3/CD28 for four hours, phosphorylation of S6 was observed (Fig. 6C) Tim-3-induced CD8+ T cells had increased pS6 (Fig. 7A) as well as enhanced expression of the early activation marker, CD69 (Fig. 7B), over that of Cre-only CD8+ T cells or CD4+ T cells within the same mouse. Although there were no gross effects on T cell development when Tim-3 was induced (Fig. 3), to rule out any unobserved issues, we used splenocytes from FSF-Tim3 mice (and no Cre Tg) and treated with Tat-Cre fusion protein162. Treating FSF-Tim3 T cells with Tat-Cre in vitro resulted in effective nuclear delivery of Cre protein. Cre delivery caused a portion of CD8+ live T cells to induce Tim-3 expression (Fig. 7C). When gating on those with higher Tim-3 expression the level of pS6 and CD69 was increased (Fig. 7C). These data demonstrate that the enhanced T cell activation in the presence of Tim-3 is T cell-intrinsic.
Figure 7: Enhanced TCR signaling by Tim-3 is cell-intrinsic.

Whole lymph nodes from E8iCre of FSF-Tim3/E8iCre mice were processed and stimulated *in vitro* for four hours, followed by flow cytometric analysis of (A) pS6 (Ser235/236) or (B) CD69 in the CD4+ and CD8+ populations. (C) Splenocytes from WT or FSF-Tim3 mice were treated with Tat-Cre overnight then stimulated the same as above. After gating on Tim-3 expression in the CD8+ live population, pS6 and CD69 were analyzed. Data are representative of three independent experiments with two biological replicates each. **p < 0.01, two-tailed unpaired Student’s *t* test.156
3.4 DISCUSSION

There is still a paucity of information regarding the mechanism by which Tim-3 can regulate T cell function. Although Tim-3 expression is often associated with reduced T cell effector function, several pieces of signaling data in the literature suggest Tim-3 may be co-activating based in some disease settings. By first taking a broad look at TCR activation in the presence of Tim-3, we saw that increased Tim-3 expression correlates with increased Nur77\text{GFP}. This indicates that, regardless of effector function, TCR signaling is activated to a higher degree in Tim-3$^+$ T cells. Inducing Tim-3 on naïve T cells also raised the basal activation state, as measured by total phosphotyrosine levels. However, these T cells were still able to further activate upon TCR stimulation, to a higher degree than those without Tim-3 expression. While the literature alludes to increased baseline activation in Tim-3$^+$ T cells in various disease states, these cells are typically unable to reactivate to the same level as their Tim-3$^-$ counterparts$^{58}$. By circumventing the need for chronic antigen exposure to express Tim-3, we show that if Tim-3 is present during primary activation T cells have increased mTOR pathway activity. This confirms previous work reported by the Kane lab with ectopic expression of Tim-3 in T cell lines$^{54}$. While we did not specifically measure levels of pErk, based on the increased total phosphotyrosine levels when Tim-3 is present, multiple pathways are likely activated. Furthermore, we observed increased CD69 expression following Tim-3 induction. CD69 is one of the earliest markers of T cell activation, and is upregulated through protein kinase C, and NFκB/AP-1 transcription factors$^{163}$. This also indicates that multiple pathways are activated by the presence of Tim-3. Work in the Kane lab suggests that this could be through the constitutive binding of Fyn kinase or the phosphorylation-dependent recruitment of PI3K to the cytoplasmic tail of Tim-3$^{54}$. 


Together, these data support the idea that Tim-3 can enhance intracellular signaling in T cells in a cell-intrinsic manner.
4.0 TIM-3 EXPRESSION IS ASSOCIATED WITH T CELL ACTIVATION IN LCMV-ARMSTRONG INFECTION

4.1 INTRODUCTION

While Tim-3 is most notably expressed on persistently stimulated T cells during chronic viral infections and in the tumor microenvironment, Tim-3 is also expressed during acute infections. In humans with acute West Nile virus infection, Tim-3 is associated with immune activation and an increased number of symptoms\textsuperscript{164}. Non-human primates with acute SIV infections express Tim-3 on activated T cells that exhibit polyfunctional cytokine responses\textsuperscript{89}. Other groups have used murine LCMV-Arm and LCMV-Cl13 infection to compare checkpoint receptor expression (including Tim-3) between acute and chronic infection. In addition to other activation markers, both Tim-3 and PD-1 are expressed on T cells during acute infection. The acute virus is typically cleared within eight days following infection, with a subsequent decrease in Tim-3 expression\textsuperscript{8}. In memory T cell formation during the contraction phase, upregulation of CD44 and loss of CD62L indicate the antigen-experienced population. Within that population, KLRG1 is associated with the most terminally differentiated T cells and is indicative of short-lived effector T cells (SLECs). CD127 (IL-7R) is a receptor for the homeostatic cytokine IL-7 and marks long-lived memory precursor effector cells (MPECs). We aimed to determine if any Tim-3 expression on T cells remained after virus is cleared following an acute infection and the phenotype of those
Tim-3 expressing cells. Additionally, the association between Tim-3 and effector phenotype upon \textit{in vivo} memory recall response was investigated.

\section{4.2 MATERIALS AND METHODS}

\subsection{4.2.1 Mice and infections}

C57Bl/6 mice were bred in-house under SPF conditions and used at 6-8 weeks of age in equal numbers of males and females. All animal procedures were conducted in accordance with NIH and University of Pittsburgh IACUC guidelines. LCMV-Arm was obtained from Rafi Ahmed, Emory University, and propagated as described previously \textsuperscript{147}. Mice were infected with $2 \times 10^5$ LCMV-Arm PFU i.p. and were analyzed at the indicated times. \textit{L. monocytogenes}-GP33 (LM-GP33) was obtained from Susan Kaech, Yale University, and propagated as described previously \textsuperscript{151}. Mice challenged with LM-GP33 received $2 \times 10^6$ CFU i.v. and were harvested at day 4 after challenge.

\subsection{4.2.2 Antibodies and reagents}

Antibodies used: \textit{Tonbo}: GhostDye, $\alpha$CD8 (53-6.7), $\alpha$CD44 (IM7), $\alpha$CD62L (MEL-14), $\alpha$CD127 (A7R34), $\alpha$KLRG1 (2F1). \textit{Biolegend}: $\alpha$PD-1 (RMP1-30), \textit{R&D Systems}: $\alpha$Tim-3 (215008). Tetramers were originally made from monomers, a gift from Rafi Ahmed, Emory University. Subsequently, monomers were obtained from the NIH tetramer core. Tetramer
analysis was performed with a pool of three tetramers specific to GP33, NP396, and GP276, except where indicated.

4.2.3 Statistical analysis

Biological replicates were used for all experiments. Statistical analyses were performed using GraphPad Prism software. Paired and unpaired Student’s *t* test and one-way ANOVA were used for data analysis and determination of p-values, as indicated.

4.3 RESULTS

4.3.1 Endogenous Tim-3 is expressed on highly phenotypically activated T cells

Tim-3 has typically been associated with other checkpoint receptors and seen in chronic viral infection or tumors. LCMV-Arm acute infection, however, can also produce Tim-3⁺CD8⁺ T cells during the primary response. We observed that at 30 days post-infection, long after the virus has been cleared, ~5% of CD8⁺ T cells still have measurable Tim-3 expression (Fig. 8A). These T cells also express high levels of CD44 and KLRG1, moderately high levels of CD127 (IL-7Rα), and lack CD62L expression (Fig. 8B). By this time point after infection, there also remain some antigen-specific cells (tetramer⁺). When comparing cells that were specific for GP33, GP276, or NP396 to those that were not, we found the tetramer⁺ population to have more Tim-3 expressing cells and higher levels of Tim-3 (%positive and MFI) (Fig. 8C). Thirty days after LCMV-Arm infection, C57Bl/6 mice were challenged with *L. monocytogenes* expressing the GP33 epitope
(LM-GP33) and splenocytes were analyzed four days post-challenge. Within the GP33 antigen-specific (tetramer+) population, the vast majority of cells expressed both Tim-3 and KLRG1, compared to the tetramer− population (Fig. 8D). These data suggest that Tim-3 marks activated antigen-specific T cells produced upon primary infection and denotes activated cells within the memory compartment.
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Figure 8: Tim-3 expression is associated with an effector memory phenotype.

C57Bl/6 mice were infected with LCMV-Arm. After 30 days, spleens were harvested for flow cytometric analysis (n=5 mice, mean ± SD). (A) Tim-3 expression was analyzed within the CD8\(^+\) population. (B) When gating on CD8\(^+\) live cells, we examined activation and differentiation markers in the Tim-3\(^+\) populations. (C) Tim-3 expression was evaluated in tetramer\(^-\) and tetramer\(^+\) (pooled: GP33, NP396, GP276) populations analyzed as percent positive, and overall mean fluorescence intensity (MFI) (n=5 mice, mean ± SD). (D) C57Bl/6 mice previously infected with LCMV-Arm (>d30 p.i.) were challenged with LM-GP33 and analyzed for Tim-3 and KLRG1 in the (GP33) Tet\(^+\) and Tet\(^-\) CD8\(^+\) populations four days post-challenge. Data are representative of three independent experiments. **p < 0.01, two-tailed Student’s t test.
4.4 DISCUSSION

To our knowledge, this is the first report of Tim-3 expression in a memory T cell population. Long after the LCMV-Arm virus was cleared, a small population of Tim-3-expressing CD8+ T cells remained, which appear to be SLECs. However, the relatively high CD127 expression leads us to believe that these cells could also be fairly long-lived in the absence of measurable antigen. This also fits with the published work of Mario Ostrowski indicating that common γ-chain cytokines are able to induce Tim-3 expression independent of TCR activation165. The tetramer+ CD8+ T cells that remain at this time point also contain a significant portion of Tim-3+ cells compared to the tetramer− CD8+ T cells, which is consistent with the antigen-experienced phenotype we see in the Tim-3+ T cells that remain. Upon challenge, it is possible that these are the memory T cells primed to respond quickest. The majority of antigen-specific T cells during an in vivo memory recall expressed both Tim-3 and the terminal differentiation marker, KLRG1. These data suggest that not only is Tim-3 a marker of both acute and chronically activated T cells, it is also expressed on a subset of memory T cells. This raises the possibility that Tim-3 is playing a role in memory T cell formation or stability and that the presence of Tim-3 may allow these T cells to reactivate quickly upon antigen re-exposure. During chronic infection, high Tim-3 expression and memory T cell depletion have both been noted, but never linked to one another. Thus, the next question we asked was whether Tim-3 was necessary for proper memory T cell formation.
5.0 TIM-3 IS REQUIRED FOR OPTIMAL ACUTE RESPONSE TO PRIMARY AND SECONDARY INFECTIONS

5.1 INTRODUCTION

Tim-3 is not commonly associated with acute viral infection, however there is transient expression of Tim-3 during primary activation of T cells. In fact, for acute *L. monocytogenes* bacterial infection, Tim-3 is necessary to mount optimal T cell responses. Once the infection is cleared, and the antigen is no longer present, expression of Tim-3 is dramatically lowered on most cells. It has yet to be reported if Tim-3 plays a role in memory T cell formation; however, Tim-3 expression is associated with T cells of the short-lived effector cell (SLEC) phenotype in tuberculosis, SIV, and after *in vitro* stimulation. Presumably, those Tim-3+ T cells that express Tim-3 are not maintained past the acute response. However, based on the data presented in Chapter 4, we know that a small percentage of CD8+ T cells do maintain low levels of Tim-3 expression after pathogen clearance. These T cells also appear to have an effector-memory phenotype. Using a Tim-3 global knockout mouse, we asked if Tim-3 was necessary for T cell responses to acute LCMV-Arm infection, memory T cell formation and recall responses.
5.2 MATERIALS AND METHODS

5.2.1 Mice and infections

C57Bl/6 and Tim-3 KO mice were bred in-house under SPF conditions and used at 6-8 weeks of age in equal numbers of males and females. All animal procedures were conducted in accordance with NIH and University of Pittsburgh IACUC guidelines. LCMV-Arm was obtained from Rafi Ahmed, Emory University, and propagated as described previously147. Mice were infected with 2x10^5 PFU LCMV-Arm i.p. and analyzed at day indicated. L. monocytogenes-GP33 (LM-GP33) was obtained from Susan Kaech, Yale University, and propagated as described previously151. Mice challenged with LM-GP33 received 2x10^6 CFU i.v. and were analyzed at day 4 post-challenge.

5.2.2 Antibodies and reagents

Antibodies used: Tonbo: GhostDye, αCD8 (53-6.7), αCD44 (IM7), αCD62L (MEL-14), αCD127 (A7R34), αKLRG1 (2F1), αIFNγ (XMG1.2), αIL-2 (JES6-5H4). R&D Systems: αTim-3 (215008). BD Biosciences: αTNFα (MP6-XT22), αCD107a (1D4B). Cell Signaling Technology: αpS6 Ser235/236, (D57.2.2E). Tetramers were made from monomers, initially a gift from Rafi Ahmed, Emory University. Subsequently, monomers were obtained from the NIH tetramer core. Tetramer analysis is pooled of three tetramers specific to GP33, NP396, and GP276 except where indicated.
5.2.3 Stimulation and flow cytometry

The phenotype of splenocytes was analyzed directly *ex vivo* by flow cytometry. For analysis of cytokine production, splenocytes were stimulated with 100 ng/ml of pooled LCMV-specific peptides (GP33, GP276, and NP396) or just GP33 after LM-GP33 infection. Stimulation was for five hours at 37°C in complete media in the presence of Golgi Plug (BD Biosciences). For flow cytometric analysis of cytokines, fluorescently-conjugated antibodies against cell surface markers were incubated with cells followed by fixation and permeabilization with BD cytofix/cytoperm solution and incubation with antibodies against intracellular cytokines on ice. For flow cytometric analysis of phosphor-S6 (pS6), fluorescently-conjugated antibodies against cell surface markers were incubated with cells followed by fixation with 1.5% PFA, permeabilization in ice-cold methanol, then incubation with antibodies against pS6 at room temperature.

5.2.4 Statistical analysis

Biological replicates were used for all experiments. Statistical analyses were performed using GraphPad Prism software. Paired and unpaired Student’s *t* test and one-way ANOVA were used for data analysis and determination of p-values as appropriate.
5.3 RESULTS

5.3.1 Tim-3 KO T cells retain most effector function during acute LCMV-Arm infection

Based on previous data that Tim-3 KO T cells do not have optimal responses to acute *L. monocytogenes* infection\(^\text{113}\), we investigated whether this was observed in LCMV-Arm infection. We infected C57Bl/6 or Tim-3 KO mice with LCMV-Arm and found that in splenocytes at day 8 p.i., there were no differences in the percentages of tetramer\(^+\)CD8\(^+\) or CD44\(^+\)CD8\(^+\) T cells. Additionally, when splenocytes were stimulated with LCMV peptides, cytokine production among antigen-specific T cells was unchanged (Fig. 9A). Within the antigen-experienced (CD8\(^+\)CD44\(^+\)CD62L\(^-\)) population, there were equivalent percentages of SLEC (KLRG1\(^+\)CD127\(^-\)) and MPEC (KLRG1\(^-\)CD127\(^+\)) populations (Fig. 9B). Thus, there appears to be no defect in the acute CD8\(^+\) T cell response of Tim-3 KO mice.
Figure 9: Phenotype of effector CD8+ cells phenotype is unchanged during LCMV-Arm infection

(A) Tetramer+ (pooled: GP33, NP396, GP276), CD44+, and IFNγ+TNFα+ T cells were analyzed within the CD8+ live cell population. Cytokines were detected after stimulation with pooled LCMV peptides in the presence of Golgi Plug (n=4-5 mice, mean ± SD).

(B) Effector/memory markers CD127 and KLRG1 were analyzed in the CD8+CD44+CD62L− population. Representative flow plots (left), and summary data (right) (n=3-5 mice, mean ± SD). Data are representative of two independent experiments.

C57Bl/6 or Tim-3 KO mice were infected with 2x10^5 PFU LCMV-Arm and spleens were harvested on day 8 post-infection. (A) Tetramer+ (pooled: GP33, NP396, GP276), CD44+, and IFNγ+TNFα+ T cells were analyzed within the CD8+ live cell population. Cytokines were detected after stimulation with pooled LCMV peptides in the presence of Golgi Plug (n=4-5 mice, mean ± SD). (B) Effector/memory markers CD127 and KLRG1 were analyzed in the CD8+CD44+CD62L− population. Representative flow plots (left), and summary data (right) (n=3-5 mice, mean ± SD). Data are representative of two independent experiments.
5.3.2 Tim-3 KO T cells are deficient in memory recall response \textit{in vitro}

To investigate whether Tim-3 is necessary for memory T cell formation and an \textit{in vitro} recall response, we infected C57Bl/6 or Tim-3 KO mice with LCMV-Arm and harvested at day 30 p.i.. Viral titer is undetectable in the spleen by day 16 post-infection (data not shown) and T cell contraction has also occurred by this point. Therefore, at 30 days, there should not be an active effector CD8$^+$ cell response$^{167, 168}$. We found that Tim-3 KO mice had significantly fewer CD44$^+$CD8$^+$ previously activated T cells and fewer antigen-specific (tetramer$^+$) T cells (Fig. 10A, B). When splenocytes were stimulated with LCMV peptides, significantly fewer Tim-3 KO CD8$^+$ T cells were able to produce cytokines in response to activation (Fig. 10C). Thus, Tim-3 KO CD8$^+$ T cells appear to be defective in memory T cell formation and recall response.
Figure 10: Tim-3 KO T cells have a poor recall response to LCMV-Arm.

C57Bl/6 or Tim-3 KO mice were infected with 2x10^5 PFU of LCMV-Arm and spleens were harvested at ≥30 d.p.i., processed, and analyzed for: (A) %CD44+ cells in the CD8+ live cell population. (B) %Tetramer+ (pooled: GP33, NP396, GP276) cells in the CD8+ live cell population. (C) %IFNγ+TNFα+ cells in CD8+ live population after LCMV peptide (pooled: GP33, NP396, GP276) stimulation in the presence of Golgi Plug. Representative flow cytometry plots (left) and summary data (right) (n=4-5 mice, mean ± SD). *p < 0.05, **p < 0.01, two-tailed Student’s t test.
5.3.3 Tim-3 KO CD8\(^+\) T cell memory pool contains a higher percentage of short-lived effector cells

Due to the reduced recall response in Tim-3 KO T cells, we aimed to determine the percentage of SLECs and MPECs present 30 days post-infection with LCMV-Arm. We gated on antigen-experienced (CD8\(^+\)CD44\(^+\)CD62L\(^-\)) T cells and saw that Tim-3 KO T cells had fewer SLECs (KLRG1\(^+\)CD127\(^-\)) and a correspondingly higher proportion of MPECs (KLRG1\(^-\)CD127\(^+\)) (Fig. 11A). This was surprising, considering there was no observable differences in these populations at day 8 post-infection (Fig. 9B).

![Figure 11](image.jpg)

**Figure 11: Tim-3 KO T cells produce fewer short-lived effector CD8\(^+\) T cells.**

Spleens from mice infected with 2x10\(^5\) PFU LCMV-Arm were harvested at day 30 p.i. and processed for flow cytometry. (A) Representative flow cytometry plots for gating of KLRG1 and CD127 effector vs. memory populations in CD8\(^+\)CD44\(^+\)CD62L\(^-\) population (left) and summary data (right) (n=5-6 mice, mean ± SD). *p < 0.05, **p < 0.01, two-tailed Student’s t test.}156
5.3.4 Tim-3 KO memory T cells have a poor in vivo recall response

To investigate the in vivo memory recall response of Tim-3 KO T cells, we challenged C57Bl/6 or Tim-3 KO mice, previously infected with LCMV-Arm, with L. monocytogenes-GP33 (LM-GP33). This heterologous infection allows us to assess only the memory recall of T cells without secondary input from plasma cells or antibodies. Four days after challenge, we found that the percentage of CD8+ Tim-3 KO T cells that produced IFNγ, TNFα or IL-2 after GP33 peptide re-stimulation was significantly less than WT cells. Furthermore, fewer CD8+ T cells from Tim-3 KO mice expressed the degranulation marker CD107a or appeared activated as indicated by a decrease in the percentage of pS6+ cells.
Figure 12: Tim-3 KO CD8+ T cells have reduced *in vivo* recall response.

Mice previously infected with 2x10^5 PFU LCMV-Arm (≥ 30 d.p.i.) were challenged with 2x10^6 CFU LM-GP33 i.v.. Splenocytes were harvested and analyzed four days post challenge. (A) After GP33 peptide stimulation, %IFNγ^+TNFα^+ and %IL-2^- analyzed or (B) %CD107a^- and %pS6^- in CD8^- live population. Each point is a biological replicate. Representative of 3 independent experiments mean ± SD. *p < 0.05, **p < 0.01, two-tailed unpaired Student’s t test.
5.4 DISCUSSION

Interestingly, Tim-3 was not necessary for the acute T cell responses during the LCMV-Arm infection. This is in contrast to the report of Tim-3 deficient T cells having poor responses to *L. monocytogenes* infections\(^57, 113\). Although both are considered canonical Th1 activators, these data could suggest that Tim-3 has a different role in different types of infections and immune responses. A significant caveat remains that this is a global Tim-3 KO mouse, and Tim-3 is known to play important roles on a multitude of immune cells such as macrophages and dendritic cells that have a significant role in LCMV infection\(^148, 170\). It remains to be determined what specific role Tim-3 may have on these additional cells during an LCMV infection.

However, following the acute phase of the infection, we did find variation in the memory T cell compartment. Tim-3 KO mice had fewer tetramer\(^+\) and CD44\(^+\) CD8\(^+\) T cells, suggesting that fewer LCMV-specific memory T cells survived the contraction phase of the adaptive T cell response. This was confirmed by the significant reduction in CD8\(^+\) T cells able to produce cytokines upon *in vitro* peptide stimulation. Similar results were obtained by Colgan and colleagues when investigating the ability of Tim-3 to produce optimal Th1 responses in acute LCMV infection\(^171\). Within the antigen-experienced pool of Tim-3 KO CD8\(^+\) T cells, there was a smaller proportion of SLECs and subsequently more MPECs. This phenotype was maintained even 90 days post-infection with LCMV-Arm (data not shown). The disconnect between an efficient acute T cell response and poor memory recall suggests a role for Tim-3 in memory T cell formation or recall response. Based on the data from Chapters 4 and 5, we believe that Tim-3 is necessary to optimally reactivate long-lived memory T cells. We next asked if Tim-3 was necessary for the development of T cell exhaustion.
6.0 TIM-3 KO MICE EXHIBIT SEVERE T CELL EXHAUSTION AND ARE LESS RESPONSIVE TO PD-L1 IMMUNOTHERAPY

6.1 INTRODUCTION

T cell exhaustion is characterized by loss of effector cell function and the expression of multiple checkpoint molecules. Tim-3 is co-expressed with other checkpoint molecules on the most dysfunctional T cells in multiple settings of T cell exhaustion. Genetic deletion of the checkpoint molecule PD-1 does not prevent the development of exhaustion, but rather results in a more severe phenotype with reduced effector T cell function\textsuperscript{172}. We aimed to determine if genetic deletion of Tim-3 would prevent or ameliorate T cell exhaustion.

In addition, PD-L1 blockade partially rescues CD$^8^+$ T cell responses during chronic viral infection with LCMV-CI13 and promotes viral clearance\textsuperscript{31}. The addition of a Tim-3 Ig-fusion protein in addition to PD-L1 blockade had a synergistic effect for even more T cell recovery\textsuperscript{8}. This is consistent with reports of a compensatory increase in Tim-3 expression in patients receiving PD-1 blockade therapy \textsuperscript{173}. Therefore, we asked whether the Tim-3 deficient mouse model could enhance efficacy of PD-1 pathway blockade in a model of chronic LCMV infection.
6.2 MATERIALS AND METHODS

6.2.1 Mice and infections

C57Bl/6 and Tim-3 KO mice were bred in-house in SPF conditions and used at 6-8 weeks of age in equal numbers of males and females. All animal procedures were conducted in accordance with NIH and University of Pittsburgh IACUC guidelines. LCMV-C113 was obtained from Rafi Ahmed, Emory University, and propagated as described previously \(^{147}\). Mice were infected with 2x10^5 PFU LCMV-C113 i.v. at day 0.

6.2.2 Antibodies and reagents

Antibodies used: Tonbo: GhostDye, αCD8 (53-6.7), αIFNγ (XMG1.2). eBioscience: αEomes (Dan 11mag), αLAG-3 (C9B7W), αTIGIT (GIGD7). R&D Systems: αTim-3 (215008). BD Biosciences: αTNFα (MP6-XT22), αCD107a (1D4B). Biolegend: αPD-L (RMP1-30), αTbet (4B10). BioXcell: αPD-L1 (10F.9G2) and Isotype (LTF-2) purified and in vivo ready. Tetramers were initially made from monomers, a gift from Rafi Ahmed, Emory University. Subsequently, monomers were obtained from NIH tetramer core. Tetramer analysis is of three pooled tetramers specific to GP33, NP396, and GP276 except where indicated.

6.2.3 Stimulation and flow cytometry

The phenotype of splenocytes was analyzed directly ex vivo by flow cytometry. For analysis of cytokine production, splenocytes were stimulated with 100 ng/ml pooled peptides (GP33,
GP276, and NP396). Stimulation was for five hours at 37°C in complete media in the presence of Golgi Plug. For intracellular flow cytometry, fluorescently-conjugated antibodies against cell surface markers were incubated with cells followed by fixation and permeabilization with BD cytofix/cytoperm (for cytokines) or eBioscience Foxp3 staining kit (for transcription factors).

6.2.4 PDL1 blockade and measuring viral titer

Anti-PDL1 or isotype treatment began day 23 post-LCMV-Cl13 infection and continued every 3 days for 2 weeks. Mice received 200µg of antibody i.p.. Mice were randomized to treatment or control group and harvested 2 days after the last treatment. Viral titer was measured in the blood at time of necropsy. RNA was isolated from the blood using Trizol LS reagent followed by qPCR for the GP protein (FOR: 5’- CATTACCTGGACTTTGTCAGACTC -3’; REV 5’-GCAACTGCTGTGTTCCCGAAAC -3’) using SybrGreen reagent (Applied Biosystems). To calculate viral copies, the CT values are compared to a standard curve as described previously174.

6.2.5 Statistical analysis

Three to five biological replicates were used for all experiments. Statistical analyses were performed using GraphPad Prism software. Paired and unpaired Student’s t test and one-way ANOVA were used for data analysis and determination of p-values, as appropriate.
6.3 RESULTS

6.3.1 Tim-3 is not required for development of functional T cell exhaustion

The deficit of Tim-3 KO T cells in acute viral infection prompted us to determine whether T cells are able to become exhausted without Tim-3. Therefore, we infected C57Bl/6 and Tim-3 KO mice with LCMV-Ci13 to study chronic infection (≥30 d.p.i.). We found that Tim-3 KO mice consistently lost more weight during the acute timing of the infection (Day 8 post-infection) and took longer to recover from this weight loss (Fig. 13A). At 30 days post-infection, Tim-3 KO mice trended higher in viral titer, although no significant differences were observed (Fig. 13B). However, we found that Tim-3 KO mice had significantly fewer antigen-specific CD8+ T cells (Fig. 13C). A hallmark of T cell exhaustion is the presence of antigen-specific cells, with only a smaller percentage of them producing cytokine upon stimulation with pooled peptides. Typically, ability to produce IL-2 and TNFα is lost first followed by IFNγ, so by measuring TNFα+IFNγ+ upon peptide restimulation we can measure the polyfunctional CD8+ T cells that remain167. Loss of cytokine production was evident in the C57Bl/6 mice yet was more profound in the Tim-3 KO mice (Fig. 13D). Further, we observed a reduction in Tbet and a corresponding increase in Eomes in both C57Bl/6 and Tim-3 KO CD8+ and CD8+PD1+ T cells (Fig. 13E), which is typical throughout the development of T cell exhaustion.
Figure 13: Tim-3 KO mice have exacerbated T cell exhaustion.

Mice were infected with $2 \times 10^5$ PFU LCMV-Cl13 and (A) followed throughout the course infection for weight loss. Blood, spleen and lymph nodes were harvested ≥30 d.p.i. (B) Viral titer was measured in the blood using qPCR. Data are presented as mean ± SEM. (C) %Tetramer$^+$ (pooled: GP33, NP396, GP276) and (D) %IFN$^+$TNF$^+$ live CD8$^+$ was analyzed. Cytokines were measured after pooled peptide (GP33, NP396, GP276) restimulation in vitro. (E) MFI of Tbet and Eomes were measured in live CD8$^+$ and CD8$^+$PD1$^+$ populations. Representative plots are shown on the left, and summary data on the right. Each point indicates a biological replicate. Data are presented as mean ± SD. Data representative of three independent experiments in A-C, E. Data are pooled from three experiments in D. *p < 0.05, **p < 0.01, two-tailed unpaired Student’s t test.156
6.3.2 Tim-3 is not necessary for phenotypic expression of exhaustion checkpoint markers

After ≥30 days of LCMV-Cl13 infection, chronically infected Tim-3 KO T cells are more functionally exhausted (Fig. 13). However, in Tim-3 KO mice 30 days post-infection compared to C57Bl/6 mice expression of other checkpoint receptors within the antigen-specific T cells did not change (Fig. 14A-C).

![Figure 14: Global knockout of Tim-3 does not affect the expression of other checkpoint receptors.](image)

C57Bl/6 and Tim-3 KO mice were infected with LCMV-Cl13, spleens and lymph nodes were harvested at ≥ 30 d.p.i.. (A) %PD-1+ (B) %TIGIT+ and (C) %LAG3+ were assessed in live Tetramer CD8+ cells (n=4-5 mice per group, presented as mean ± SD).156

6.3.3 Tim-3 is necessary for therapeutic response to PDL1 blockade during chronic viral infection

To determine whether Tim-3 KO mice respond to PD-L1 blockade, we treated chronically infected C57Bl/6 and Tim-3 KO mice with αPD-L1 or isotype control *in vivo* every 3 days for 2 weeks. In contrast to Tim-3 sufficient animals, Tim-3 KO mice were unable to significantly expanded their antigen-specific T cell pool (Fig. 15A). Typically, in LCMV-cl13 infection, only 5-10% of the antigen specific cells will produce both TNFα and IFNγ167. Consistent with this, Tim-3 KO mice had significantly fewer T cells respond to peptide restimulation after PD-L1
blockade (Fig. 15B). While WT mice had a 2.9-fold increase in the IFNγ/TNFα producing CD8+ T cells, cytokine-producing CD8+ T cells from Tim-3 KO mice only increased by 1.2-fold. These data suggest that Tim-3 expression is required for effective PD-L1 blockade.

C57Bl/6 and Tim-3 KO mice were infected with 2x10^5 PFU LCMV-Cl13 and treated with αPDL1 or isotype every 3 days for 2 weeks beginning on day 23 p.i.. Spleens and lymph nodes were harvested 2 days after the final treatment, day 36 post-infection. (A) %Tetramer+ (pooled: GP33, NP396, GP276) and (B) following peptide (pooled: GP33, NP396, GP276) stimulation, %IFNγ+TNFα+ cells were assessed in the live CD8+ population (n=3-4 mice per group, mean ± SD). Representative of two independent experiments n=3-4 per group. *p < 0.05, **p < 0.01, two-tailed Student’s t test.156

Figure 15: Tim-3 is necessary for optimal response to PDL1 blockade.
6.4 DISCUSSION

Tim-3 KO mice have enhanced pathogenesis of LCMV-Cl13 indicated by enhanced weight loss and the increased viral titer. Typically, weight loss in mice with chronic LCMV has been associated with enhanced immune responses\textsuperscript{175}. However, examination of the CD8\textsuperscript{+} T cell responses we found fewer LCMV-specific CD8\textsuperscript{+} T cells in Tim-3 KO mice. Strikingly, the response to \textit{in vitro} peptide restimulation was nearly abolished. Consistent with this finding, less than 1\% of LCMV-peptide stimulated CD8\textsuperscript{+} T cells had the capacity to produce TNF\textalpha and IFN\gamma in Tim-3 KO mice. Unlike the PD-1 KO mice, loss of Tim-3 did not affect expression of other checkpoint molecules suggesting that no there were no additional compensatory changes in measured T cell inhibitory pathways present. These data suggest that in the absence of Tim-3, CD8\textsuperscript{+} T cell responses to infection, is decreased and fewer CD8\textsuperscript{+} are functional upon reactivation. Tim-3 deletion did not affect the expression of Tbet or Eomes transcription factors in exhausted CD8\textsuperscript{+} T cells, leaving the potential mechanism for this response unknown.

It is important to note that this is a global Tim-3 KO mouse and Tim-3 is known to play a role in phagocytic cells\textsuperscript{66}. In fact, LCMV-cl13 preferentially infects dendritic cells and can affect antigen presentation to CD8\textsuperscript{+} T cells\textsuperscript{176}. Therefore, it will be important to evaluate the T cell intrinsic effects of Tim-3 KO using adoptive T cell transfers or a conditional Tim-3 KO mouse. Additionally, understanding the cytotoxic potential of T cells lacking Tim-3 could be done through \textit{in vivo} cytotoxicity assays.

Tim-3 KO mice exhibit reduced therapeutic response to PD-L1 blockade indicating that Tim-3 is necessary for proper re-activation of CD8\textsuperscript{+} T cells. Additionally, supported by the data in chapter five, improper memory T cell subsets are created and therefore poor recall responses are observed. Memory T cell pools are known to be depleted during chronic viral infection\textsuperscript{19}. 73
Therefore, the loss of Tim-3 could be further exacerbating this phenotype. Interestingly, during PD-L1 blockade, an increase in Tim-3 expression is seen in patients\textsuperscript{173}. It is thought that this increase is a marker of activation of the T cells. Here we show that when Tim-3 is genetically deleted in mice, PD-L1 blockade is less effective. Whether this is due to defects in initial activation, or the requirement for Tim-3 in reactivation remains to be known. Overall, genetic deletion of Tim-3 results in more severe T cell exhaustion and that Tim-3 is necessary for optimal response to PD-L1 blockade.
7.0 TIM-3 OVEREXPRESSION DOES NOT DRIVE T CELL EXHAUSTION, AND PROMOTES RESISTANCE TO PD-L1 BLOCKADE

7.1 INTRODUCTION

The most dysfunctional subset of exhausted T cells express Tim-3 and multiple other checkpoint receptors. Although signaling through Tim-3 suggests a co-stimulatory role, it is plausible that Tim-3 inhibits T cell function in some settings. Currently, Tim-3 is stably expressed on T cells when there is persistent antigen exposure. Therefore, to investigate the intrinsic effects of Tim-3 in naïve mice, we developed the FSF-Tim3 mouse model to induce Tim-3 expression under Cre-mediated recombination. By crossing FSF-Tim3 mice with CD4-Cre or E8i-Cre, Tim-3 is efficiently induced on all αβ+ T cells, or CD8+ T cells, respectively. These murine models of Tim-3 induction have no obvious effects on T cell development (Fig. 2,3). We aimed to address whether Tim-3 alone is sufficient to promote T cell exhaustion and whether overexpression of Tim-3 would further exacerbate exhaustion during chronic LCMV infection.

Blocking the PD-1/PD-L1 axis is an FDA-approved therapy for multiple cancers and is effective in the restoration of T cell function in murine LCMV-Cl13 infection\textsuperscript{177, 178, 179}. Some patients treated with PD-1 blockade exhibit a compensatory increase in Tim-3 expression\textsuperscript{173}. We hypothesized that induced Tim-3 expression in FSF-Tim3/E8i-Cre mice infected with LCMV-Cl13 would result in resistance to the effects of PDL1 blockade.
7.2 MATERIALS AND METHODS

7.2.1 Mice and infections

C57Bl/6 and Tim-3 KO mice were bred in-house in SPF conditions and used at 6-8 weeks of age in equal numbers of males and females. All animal procedures were conducted in accordance with NIH and University of Pittsburgh IACUC guidelines. LCMV-Cl13 was obtained from Rafi Ahmed, Emory University, and propagated as described previously\(^{147}\). Mice were infected with 2x10^5 PFU LCMV-Cl13 i.v. at day 0.

7.2.2 Antibodies and reagents

Antibodies used: Tonbo: GhostDye, αCD8 (53-6.7), αIFNγ (XMG1.2). eBioscience: αLAG-3 (C9B7W), αTIGIT (GIGD7). R&D Systems: αTim-3 (215008). BD Biosciences: αTNFα (MP6-XT22). Biolegend: αPD-1 (RMP1-30). BioXcell: αPDL1 (10F.9G2) and Isotype (LTF-2) purified and in vivo ready. Tetramers were initially made from monomers, a gift from Rafi Ahmed, Emory University. Subsequently, monomers were obtained from the NIH tetramer core. Tetramer analysis is of three pooled tetramers specific to GP33, NP396, and GP276 except where indicated.

7.2.3 Stimulation and flow cytometry

The phenotype of splenocytes was analyzed directly ex vivo by flow cytometry. For analysis of cytokine production, splenocytes were stimulated with 100 ng/ml pooled peptides (GP33,
GP276, and NP396). For five hours at 37°C in complete RPMI media in the presence of Golgi Plug (BD Biosciences) cells were stimulated. For intracellular flow cytometry, fluorescently-conjugated antibodies against cell surface markers were incubated with cells followed by fixation and permeabilization using the BD cytofix/cytoperm kit.

7.2.4 PDL1 blockade and measuring viral titer

Anti-PDL1 or isotype treatment began day 23 post LCMV-Cl13 infection, and continued every 3 days for 2 weeks, at 200 µg i.p. Mice were randomized to treatment or control group and harvested 2 days after the last treatment. Viral titer was measured from tail bleeds at day 8 and 16 p.i. and in the blood at the time of necropsy. RNA was isolated from the blood using Trizol LS reagent, followed by qPCR for the GP protein using SybrGreen reagent (Applied Biosystems). To calculate viral copies, the CT values are compared to a standard curve as described previously174.

7.2.5 Statistical analysis

Four to six biological replicates were used for all experiments. Statistical analyses were performed using GraphPad Prism software. Paired and unpaired Student’s t test and one-way ANOVA were used for analysis and determination of p-values, as appropriate.
7.3 RESULTS

7.3.1 Tim-3 induction on T cells does not affect pathogenesis of LCMV in mice

It has been shown that mice infected with LCMV-C113 lose more weight with an intermediate dose (2x10^5 PFU) than with a high dose (2x10^6 PFU), due to increased immunopathology. Using weight loss as a measure of pathogenesis, we found that CD4-Cre and FSF-Tim3/CD4-Cre mice had a similar decrease in percent of their original body weight and both groups regained their weight with similar kinetics (Fig. 16A), although, they do not recover all of the weight lost by the date of sacrifice. We measured the viral titer in the peripheral blood from mice in each group on days 8, 16, and 30 (necropsy). Viral copies were determined by RNA isolation and qPCR, and viral copies were determined using a standard curve for the GP protein of LCMV. However, we found no significant difference in the viral titer in the blood of CD4-Cre and FSF-Tim3/CD4-Cre mice at either of the time points (Fig 16B). These data suggest that overexpression of Tim-3 at the time of, and throughout the course of infection does not affect the overall pathogenesis or viral titer of LCMV-C113 in mice.
Figure 16: Tim-3 overexpression in LCMV-CI13 infected mice does not affect weight loss or viral titer.

FSF-Tim3/CD4Cre or CD4-Cre only mice were infected with 2x10⁵ PFU LCMV-CI13 at day 0. (A) Mice were weighed every other day throughout the course of infection (mean ± SD). Representative of three independent experiments. (B) Tail bleeds were taken from each mouse at day 8 and 16 p.i. and harvested from abdominal aorta at day 30 p.i. RNA was isolated from blood and virus was measured using qPCR and compared to the GP standard curve to determine absolute viral copies (mean ± SEM). Data pooled from three independent experiments.¹⁵⁶
7.3.2 Tim-3 induction on T cells does not affect T cell exhaustion phenotype

To investigate the effect of Tim-3 induction on T cell exhaustion, we analyzed the T cell compartment from LCMV-Cl13 infected CD4-Cre and FSF-Tim3/CD4-Cre mice. There was no significant difference in %Tetramer^+CD8^+ T cells nor the %TNFα^+IFNγ^+ CD8^+ T cells upon pooled peptide stimulation between CD4-Cre and FSF-Tim3/CD4-Cre mice (Fig. 17A, B). Within the Tetramer^+CD8^+ population, we analyzed the expression of other checkpoint molecules and found similar percentages of PD-1, TIGIT, and LAG3 expressing cells in both mice (Fig. 17C). Thus, induction of Tim-3 on all αβ^+ T cells does not affect the T cell exhaustion phenotype.

![Figure 17: Tim-3 expression does not drive a T cell exhaustion phenotype.](image)

Fos-Tim3/CD4-Cre and CD4-Cre mice were infected with 2x10^5 PFU LCMV-Cl13, spleens and lymph nodes were harvested at day 30 p.i. and processed for (A) %Tetramer^+ (pooled: GP33, NP396, GP276) in the CD8^+ live population. (B) %IFNγ^+TNFα^+ after peptide (pooled: GP33, NP396, GP276) stimulation in the CD8^+ live population. (C) %PD1^+ (left), %TIGIT^+ (middle), %LAG3^+ (right) in the tetramer^+CD8^+ live population (n=5-6 mice in each group, mean ± SEM). Representative of three independent experiments.156
7.3.3 Inducing Tim-3 on T cells enhances resistance to PD-L1 blockade

PD-1 blockade in patients and in a mouse model of head and neck squamous cell carcinoma (HNSCC) exhibit compensatory upregulation of Tim-3 in the CD8+ T cell compartment. Given the extensive literature deeming Tim-3 a negative regulator in the tumor setting, we hypothesized that inducing Tim-3 expression on CD8+ T cells using FSF-Tim3/E8i-Cre mice would promote resistance to the effects of PD-1/PD-L1 blockade. We measured the viral copies in the blood upon necropsy and found a ~24-fold reduction in viral titer in PD-L1 treated E8i-Cre mice compared to isotype control (Fig. 18A). In striking contrast, FSF-Tim3/E8i-Cre mice exhibited only a ~5-fold reduction in viral titer with PD-L1 treatment, whereas ~20% was seen in the E8i-Cre. Although in the isotype groups the viral titer of FSF-Tim3/E8i-Cre mice appear higher, these results were not significant and when multiple experiments were combined there is significant overlap between groups (Fig. 16B). When splenic CD8+ T cells were restimulated with pooled peptides, a modest ~2-fold increase in TNFα+IFNγ+ producing T cells were seen in both PD-L1 treated groups, though slightly more in the E8i-Cre mice (Fig. 18B). It has been reported that effector CD8+ T cells with intermediate PD-1 expression expand in response to PD-1/PD-L1 blockade. Within the antigen-specific CD8+ T cells, E8i-Cre mice showed fewer %PD-1HI cells, and expansion of PD-1Int, while Tim-3 induced CD8+ T cells did not change (Fig. 18C). Together, these data suggest that Tim-3 overexpression on T cells cannot significantly impact the T cell exhaustion phenotype but does affect the therapeutic response to PD-L1 blockade.
Figure 18: Overexpression of Tim-3 on T cells results in resistance to PDL1 blockade.

FSF-Tim3/E8i-Cre and E8i-Cre mice were infected with 2x10^5 PFU LCMV-Cl13 and treated with αPDL1 or isotype every 3 days for 2 weeks beginning day 23 p.i. and spleens and lymph nodes harvested 2 days after the final treatment. (A) Viral copies in the blood measured at necropsy by qPCR (B) following peptide (pooled: GP33, NP396, GP276) stimulation, %IFNγ+TNFα+ were assessed in the CD8+ live population (n=3-4 mice per group, mean ± SD). (C) Representative flow cytometry analysis of %PD1HI in pooled Tetramer+CD8+ splenic T cells. Representative of two independent experiments. No statistically significant results were found, two-tailed Student’s t test.156
Inducing Tim-3 expression on all αβ+ T cells or CD8+ T cells alone did not precipitate an obvious phenotype or developmental effect in naïve mice (Fig. 1, 2). Typically, Tim-3 is not expressed on naïve effector T cells but is induced upon activation. With Tim-3 expression on T cells evident in both acute and chronic viral infections, we anticipated overexpression of Tim-3 might affect T cell function in an LCMV infection. Tim-3 overexpression was not able to drive any T cell dysfunction during acute LCMV infection (data not shown) and did not affect the pathogenesis or T cell function during chronic LCMV infection. We did not assess if Tim-3 affects bystander CD8+ T cell activation, though we do not expect differences because cytokine levels were not affected by Tim-3 overexpression. It is likely that Tim-3 does not supply strong activating or inhibitory signals to T cells, but rather fine-tunes differentiation signals. In an infection model that causes such robust immune activation such as LCMV, it is difficult for one molecule to perturb the entire system. Due to redundancy in the genome and protein function, there is likely compensatory effects of Tim-3 induction other than checkpoint receptors that we are not able to measure. Nonetheless, our work provides evidence that Tim-3 alone is not able to drive exhaustion and overexpression cannot exacerbate T cell exhaustion, neither functionally nor phenotypically.

Immune checkpoint blockade has made monumental progress in the treatment of advanced cancers providing some hope of durable responses. PD-1/PD-L1 blockade has made significant strides in cancer treatment, despite not fully understanding the mechanisms that are responsible for the therapeutic efficacy. Although the mechanisms for effectiveness are largely unknown, pre-clinical and patient ex vivo models indicate potential cross-talk between the PD-1 and Tim-3 signaling pathways. Contributing to this phenomenon, we found that
overexpression of Tim-3 in mice infected with chronic LCMV, promoted resistance to PD-L1 blockade. Tim-3 overexpression prevented reduction in viral titer as well as PD-1\textsuperscript{int}CD8\textsuperscript{+} population expansion, hallmarks of PD-1 blockade. This PD-1\textsuperscript{int} population can also be defined by expression of chemokine receptor, CXCR5, and maintained expression of transcription factor, TCF1\textsuperscript{16}. We did not detect any differences in these two markers when Tim-3 was overexpressed (data not shown). This may not be surprising, as in a comprehensive transcriptome analysis, chemokine receptors do not appear to be differentially expressed during acute or chronic LCMV infection\textsuperscript{182}. These data provide more evidence for cross-talk between Tim-3 and PD-1 signaling pathways. However, it remains to be known whether the proteins are working in concert or antagonistically to one another.
8.0 TIM-3 PROMOTES SHORT-LIVED EFFECTOR T CELL GENERATION AT THE EXPENSE OF LONG-LIVED MEMORY

8.1 INTRODUCTION

The evidence in previous chapters supports a conflicting notion that Tim-3 is primarily a co-stimulatory molecule. Several have shown Tim-3 expression is correlated with dysfunctional T cell responses (reviewed in 183). Additionally, antibodies preventing Tim-3/ligand interactions result in enhanced T cell function in settings of chronic stimulation. However, in Chapter 3, we noted that the CD8⁺Tim-3⁺ population persisted long after the LCMV-Arm was cleared. These cells are activated, antigen-experienced (CD44⁺CD62L⁻), and have high KLRG1 expression, a marker of short-lived effector T cells. Therefore, we aimed to investigate the effects of Tim-3 induction on the development of T cell memory after acute LCMV infection. We hypothesized that Tim-3 could be acting in a co-stimulatory role to promote differentiation of short-lived effector T cells. If Tim-3 is co-stimulating T cells through the mTOR/Akt signaling pathway during TCR stimulation, acute viral infection would result in memory T cell pool depletion via the promotion of differentiation into SLECs. Akt is an essential modulator of T cell fate to SLEC or MPEC184, 185. Therefore, if Tim-3 is enhancing Akt signaling, it could act as an activator for driving SLEC production. It is known that memory T cell populations are markedly reduced
during T cell exhaustion\textsuperscript{20}, therefore this could provide a mechanism where Tim-3 is contributing to exhaustion by being a co-activating molecule.

8.2 MATERIALS AND METHODS

8.2.1 Mice and infections

C57Bl/6, FSF-Tim3, and FSF-Tim3/Cre mice were bred in-house under SPF conditions and used at 6-8 weeks of age in equal numbers of males and females. All animal procedures were conducted in accordance with NIH and University of Pittsburgh IACUC guidelines. LCMV-Arm was obtained from Rafi Ahmed, Emory University, and propagated as described previously\textsuperscript{147}. Mice were infected with $2 \times 10^5$ PFU LCMV-Arm i.p. at day 0 and analyzed at day indicated.

8.2.2 Antibodies and reagents

Antibodies used: Tonbo: GhostDye, $\alpha$CD8 (53-6.7), $\alpha$CD44 (IM7), $\alpha$CD62L (MEL-14), $\alpha$CD127 (A7R34), $\alpha$KLRG1 (2F1), $\alpha$IFN$\gamma$ (XMG1.2). Biolegend: $\alpha$Tbet (4B10), R&D Systems: $\alpha$Tim-3 (215008), BD Bioscience: $\alpha$TNF$\alpha$ (MP6-XT22), $\alpha$Eomes (Dan 11mag), Cell Signaling Technology: $\alpha$pS6 Ser235/236, (D57.2.2E). Tetramers were originally made from monomers, a gift from Rafi Ahmed, Emory University. Subsequently, monomers were obtained from the NIH tetramer core. Tetramer analysis was performed with a pool of three tetramers specific to GP33, NP396, and GP276, except where indicated.
8.2.3 Stimulation, Rapamycin treatment, and flow cytometry

Rapamycin (LC Laboratories) was dissolved in ethanol and then further diluted in Tween-80/PEG-400 (10:80) and sterile-filtered. For in vivo rapamycin treatment, mice were injected every day with 2.5 µg of rapamycin i.p. starting one day before LCMV-Arm infection and extending until the day of necropsy. The phenotype of splenocytes was analyzed directly ex vivo by flow cytometry. For analysis of cytokine production, splenocytes were stimulated with 100 ng/ml of pooled peptides (GP33, GP276, and NP396). Stimulation was for five hours at 37°C in complete RPMI media in the presence of Golgi Plug. For intracellular flow cytometry, fluorescently-conjugated antibodies against cell surface markers were incubated with cells followed by fixation and permeabilization with BD cytofix/cytoperm kit (for cytokines) or eBioscience Foxp3 staining kit (for transcription factors).

8.2.4 Statistical analysis

Biological replicates were used for all experiments. Statistical analyses were performed using GraphPad Prism software. Paired and unpaired Student’s t test and one-way ANOVA were used for data analysis and determination of p-values, as indicated.
8.3 RESULTS

8.3.1 Induction of Tim-3 on T cells results in higher proportion of short-lived effector cells

Given the enhanced TCR signaling seen in Tim-3 induced mice, we hypothesized that Tim-3 alone might drive T cell exhaustion during an otherwise acute viral infection. We infected FSF-Tim3/CD4-Cre and CD4-Cre mice with LCMV-Arm and found no difference in the ability of the mice to clear the virus (data not shown). To evaluate the ability of Tim-3 overexpression in mice to differentiate memory T cells, we sacrificed mice 30 days post-infection with LCMV-Arm. When Tim-3 is overexpressed on all αβ⁺ T cells (using FSF-Tim3/CD4-Cre), we observed significantly more short-lived effector cells (SLECs) (KLRG1⁺CD127⁻) in the antigen-experienced (CD8⁺CD44⁺CD62L⁻) population (Fig. 19A). This was concurrent with a proportional reduction in the transitional KLRG1⁺CD127⁺ population (Fig. 19A). To determine if this was CD8⁺ T cell intrinsic, we repeated the same infection with FSF-Tim3/E8i-Cre mice inducing Tim-3 on only CD8⁺ T cells. Again, we found an increase in SLEC cells, with significantly fewer long-lived memory precursor T cells (MPECs) in the antigen-experienced population (Fig. 19B). The same significant populations were observed when gating on Tetramer⁺CD8⁺ T cells (data not shown). These data suggest that Tim-3 overexpression is driving terminal differentiation of CD8⁺ T cells to the effector phenotype.
Figure 19: Tim-3 promotes the formation of terminal effector CD8+ T cells.

FSF-Tim3/CD4-Cre or FSF-Tim3/E8i-Cre and the appropriate Cre control mice were infected with 2x10^5 PFU LCMV-Arm i.p. and splenocytes harvested ≥ day 30 p.i. and processed for flow cytometry. KLRG1^−CD127^− (SLEC), KLRG1^CD127^+ (transitional) and KLRG1^CD127^+ (MPEC) populations were evaluated in the antigen-experienced (CD44^−CD62L^−) CD8+ population of (A) FSF-Tim3/CD4-Cre and CD4-Cre mice or (B) FSF-Tim3/E8i-Cre and E8i-Cre mice. Representative flow plots on the left, with summary data on the right. Each point represents an individual mouse (mean ± SD). Data are representative of three independent experiments. *p < 0.05, ** p < 0.01, two-tailed unpaired Student’s t test.156
8.3.2 Tim-3 induced T cells exhibit enhanced activation signaling

To determine a possible mechanism for the enhanced SLEC phenotype seen in Tim-3 induced mice, we evaluated several intracellular signaling molecules. The Th1 transcription factor, and known regulator of Tim-3 expression, Tbet was expressed at similar levels in FSF-Tim3/CD4-Cre and CD4-Cre mice (Fig. 20A, left). However, using mean fluorescence intensity, Tim-3 induced mice had higher Tbet:Eomes ratio (Fig. 20A, right) due to a significantly reduced Eomes expression (Fig. 20A, left). Using LCMV peptide restimulation in vitro, there was no significant difference in IFNγ and TNFα production (Fig. 20B). However, there was a significant increase in phosphorylated S6 (pS6) expression upon peptide restimulation in the Tim-3 induced CD8+ T cells (Fig. 20C). These data provide evidence of enhanced mTOR activation signaling in memory T cells after LCMV-Arm infection when Tim-3 is overexpressed.
Figure 20: Enhanced activation signaling in Tim-3 induced SLECs.

FSF-Tim3/CD4-Cre and CD4-Cre mice were infected with 2x10^5 PFU LCMV-Arm i.p. splenocytes harvested ≥ day 30 p.i. and processed for flow cytometry. (A) The ratio of Tbet:Eomes (right) was plotted based on the mean fluorescence intensity (MFI) of Tbet and Eomes (left) in the CD8^-CD44^-CD62L^-KLRG1^-CD127^- population. (B and C) Splenocytes were stimulated with LCMV peptides (pooled: GP33, NP396, GP276) for five hours and then analyzed for IFNγ/TNFα (B) or pS6 (C). Each symbol represents an individual mouse (mean ± SD). Data are representative of three independent experiments. *p < 0.05, **p < 0.01, two-tailed unpaired Student’s t test.
8.3.3 Inhibition of mTOR rescues Tim-3 induced SLEC formation

To confirm that the increased SLEC population when Tim-3 is overexpressed in CD8+ T cells is due to enhanced mTOR signaling, we used the mTOR inhibitor rapamycin. Rapamycin is known to increase the MPEC frequency during LCMV-Arm infection\textsuperscript{187}. FSF-Tim3/E8i-Cre mice were treated with rapamycin or vehicle control throughout the course of LCMV-Arm infection and harvested 30 days post-infection. We found that rapamycin treatment effectively reduced the SLEC population and increased the MPEC population (Fig. 21A). The %Tetramer\textsuperscript{+}CD8\textsuperscript{+} population was doubled in Tim-3 induced mice treated with rapamycin compared to vehicle control (Fig. 21B). Because mTOR is a known regulator of Tbet\textsuperscript{188}, we saw significant reduction in Tbet expression in the KLRG1\textsuperscript{+}CD127\textsuperscript{+}CD8\textsuperscript{+} population of the Tim-3 induced, rapamycin-treated mice (Fig. 21C). Tim-3 induction on CD8\textsuperscript{+} T cells enhances the SLEC population that is rescued by rapamycin treatment. These data suggest a mechanism for Tim-3 promoting differentiation of T cells into short-lived effector T cells through the mTOR signaling pathway.
Figure 21: Rapamycin reduces SLECs in mice overexpressing Tim-3.

FSF-Tim3/E8i-Cre mice were infected with $2 \times 10^5$ PFU LCMV-Arm i.p. and treated with rapamycin or vehicle control i.p. daily starting day -1 pre-infection and continuing to day 30 p.i.. Splenocytes harvested at day 30 p.i. and processed for flow cytometry. (A) KLRG1$^+$CD127$^-$ (SLEC), KLRG1$^+$CD127$^+$ (transitional) and KLRG1$^-$CD127$^+$ (MPEC) populations were evaluated in the antigen-experienced (CD44$^+$CD62L$^-$) CD8$^+$ population. (B) %Tetramer$^+$ population (pooled: GP33, NP396, GP276) was evaluated in CD8$^+$ live population. (C) MFI of Tbet was determined in the CD8$^+$CD44$^+$CD62L$^-$KLRG1$^+$CD127$^+$ population. Each symbol represents an individual mouse (mean ± SD). Data are representative of two independent experiments. *p < 0.05, **p < 0.01, ****p < 0.0001, two-tailed unpaired Student’s t test.
8.4 DISCUSSION

Since the discovery of Tim-3 as a marker of Th1-specific cells, it has often been associated with defective T cell responses. However, recent work has also found a positive role for Tim-3 in T cell effector function\textsuperscript{112, 113, 171}. Work by our lab as well as others have defined Tim-3 as promoting effector T cell signaling\textsuperscript{54, 55}. In this chapter, we provide a novel mechanism by which Tim-3 can behave as a co-activating molecule and that paradoxically contributes to T cell exhaustion. Overexpression of Tim-3 during acute LCMV infection did not affect clearance of the virus or effector T cell function, however it did skew the percentages of memory T cells towards a SLEC phenotype T cells at the expense of MPECs. These SLECs in Tim-3 induced mice had enhanced activation of the mTOR pathway, which is known to drive effector T cell differentiation\textsuperscript{187}. Additionally, the ratio of the T-box transcription factors Tbet:Eomes was skewed higher in effector CD8\textsuperscript{+} T cells of Tim-3 induced mice by the reduction in Eomes expression. Both transcription factors are known for regulating T cell memory differentiation\textsuperscript{189}. Our data further supports this mechanism by treating Tim-3 induced mice with rapamycin, a known mTOR inhibitor, showing the rescue of the SLEC phenotype induced by Tim-3 overexpression. These data merge the possibility of Tim-3 as a co-activator, while still contributing to T cell exhaustion via mTOR activation and the depletion of memory T cell pools.
9.0 FINAL DISCUSSION AND PUBLIC HEALTH RELEVANCE

9.1 TIM-3 REGULATES EFFECTOR T CELL FUNCTION AND MEMORY T CELL FORMATION

The goal of this work was to dissect the intrinsic effects of Tim-3 and reconcile the conflicting literature of Tim-3 as a negative or positive regulatory molecule in T cell function. Tim-3 was initially discovered in a screen for Th1 specific molecules\textsuperscript{49} and using the mouse model of multiple sclerosis (EAE), Tim-3 Ig-fusion proteins caused exacerbated disease. Since then, Tim-3 is described in almost any disease state that activates the immune system. Specifically, Tim-3 is expressed on persistently stimulated T cells in chronic viral infection and cancer and is associated with the most dysfunctional T cells. The phenomena of T cell exhaustion is unique and heterogeneous with the key factors being progressive loss of effector T cell function and expression of checkpoint molecules\textsuperscript{6}. However, the specific mechanisms and populations in T cell exhaustion are still being defined with the help of a large data set and sequencing analysis\textsuperscript{182}. Two complicating factors to dissecting the intrinsic effects of Tim-3 are the need for persistent antigen exposure for Tim-3 expression\textsuperscript{13, 190} and the corresponding upregulation of other checkpoint molecules during the disease. Therefore, to circumvent these factors, we developed a genetic mouse model for induction of Tim-3 expression. Using Tim-3 induction and Tim-3 KO mice we studied the role for Tim-3 in acute and chronic LCMV infection.
By first investigating endogenous Tim-3 expression in association with TCR signaling, we used Nur77\textsuperscript{GFP} mice as a readout for TCR activation. We found that Tim-3 is associated with the most activated T cells. This information provided evidence that Tim-3 does not necessarily mean an inactivated T cell. Endogenous Tim-3 expression in the CD8\textsuperscript{+} T cells of mice previously exposed to LCMV-Arm also exhibited an activated memory phenotype. This phenotype was perplexing as the high expression of CD44 and KLRG1 on the cells indicated short-lived effector T cells, there was also a high expression CD127 (IL-7R\textalpha), a homeostatic cytokine receptor necessary for longer T cell survival. It is possible that CD127 expression could be participating in driving Tim-3 expression, independent of antigen exposure\textsuperscript{165}. Thus, the discovery of Tim-3 expression on long-lived memory T cells is novel and represents a subset with a SLEC phenotype that needs to be investigated further (Fig. 22A).

Signaling evidence that Tim-3 is co-activating in TCR stimulation exists in the literature with enhanced MAPK and mTOR pathway activation in infectious and cancerous settings\textsuperscript{54, 57, 58, 59}. Using the FSF-Tim3 mouse we induced Tim-3 expression on only T cells and found that overall phospho-tyrosine levels were higher, specifically pAkt and pS6 mTOR target proteins. This is a T cell intrinsic effect that we also saw in Tim-3 induced T cell memory recall from LCMV-Arm infected mice. This enhancement of the mTOR pathway by Tim-3 also affected Eomes transcription factor expression causing a higher Tbet:Eomes ratio, important in T cell memory differentiation\textsuperscript{189}. We did not directly assess the effects of Tim-3 on apoptosis, though it is noted that there were no differences in cell numbers or viability staining in Tim-3 deficient or overexpressing cells. Tim-3 is known to bind phosphatidylserine, expressed by apoptotic cells\textsuperscript{191}, and mAbs specific for Tim-3 can prevent this interaction\textsuperscript{75}. It would be important in the future to evaluate known markers for evaluating apoptosis such as Bcl2 and caspases. This could be a
potential mechanism for driving SLEC differentiation. Overall, the presence and induction of Tim-3 appears to enhance T cell activation during TCR stimulation.

Based on the signaling data, and the necessity for Tim-3 in acute and chronic viral infection, we expected heightened effector T cell function by inducing Tim-3 expression. However, cytokine production and activation/exhaustion markers during acute or chronic LCMV infection were not affected by Tim-3 induction. This could be explained by compensatory changes that we were unable to measure, requiring RNA sequencing in Tim-3 induced T cells to explore. Also, Tim-3 induction could be affecting other signaling pathways besides mTOR as suggested by previous work. Although this is controversial in the literature on the intrinsic effects of Tim-3 on NFAT and NFkB activation. Which leads us to believe that the role of Tim-3 on T cells can be context dependent. We did not specifically measure any changes in ligand expression during infection of Tim-3 deficient or overexpressing mice. The cytoplasmic tail of Tim-3 remains elusive and complicated as it can bind kinases, protein chaperones, and possibly much more. Future work with mice expressing truncated forms of Tim-3 will help dissect the requirements of Tim-3 signaling in the phenotypes described here.
Figure 22: Model for the T cell-intrinsic effect of Tim-3 on T cell activation, memory, and exhaustion.

(A) The endogenous expression kinetics of Tim-3, showing no expression on naïve T cells, transient expression in effector T cells, low level on a small percentage of memory T cells, and the highest expression on exhausted T cells. 

(B) When Tim-3 is enforced on T cells using Cre, there is a push toward more SLECs, associated with terminal differentiation. 

(C) In the setting of Tim-3 KO mice, fewer T cells are initially activated, with poor memory recall response in both memory and exhausted T cell settings.156
In mice genetically deficient for Tim-3, we produced data consistent with published literature that Tim-3 is necessary for optimal T cell responses\textsuperscript{57,113}. Without Tim-3, T cells from acute and chronically infected mice had poor cytokine recall response (Fig. 22C). This was particularly surprising in the LCMV-Cl13 infected mice that already had profound T cell exhaustion. Loss of Tim-3 exacerbated this phenotype, supporting the idea that in the absence of Tim-3 T cell exhaustion will still develop. Tim-3 KO mice were also less responsive to PD-L1 blockade. The reduced CD44\textsuperscript{+} population and reduced response to PD-L1 blockade, led us to postulate that without Tim-3 expression, fewer antigen-specific T cells are able to respond and differentiate in response to initial activation (Fig. 22C). This is also supported by fewer KLRG1\textsuperscript{+} SLECs in the memory population of LCMV-Arm infected mice in the absence of Tim-3. One possibility we did not explore was the effect of Tim-3 on altering the T cell repertoire during infection. We analyzed T cells specific for the top three immunodominant epitopes of LCMV. However, we know that during chronic infection this can be skewed\textsuperscript{167}. The reduced antigen-specific T cells in the Tim-3 KO mice could be due to increased specificity for epitopes we did not measure. This would be important to investigate in the future.

Both the \textit{in vivo} effect of Tim-3 deficiency and overexpression surprisingly, led to resistance to the effect of PD-L1 blockade during chronic LCMV infection. The Tim-3 KO situation would need to be confirmed as a T cell intrinsic effect, but we believe the resistance could be due to the requirement for compensatory Tim-3 upregulation during PD-L1 blockade\textsuperscript{173}. In the overexpression system, Tim-3 could be sequestering kinases away from the TCR to enhance signaling during blockade. We hypothesize that the mechanisms for resistance could be due to Tim-3/PD-1 cross-talk signaling. Nonetheless, this is an example of the juxtaposition of Tim-3 as a positive regulator yielding a negative T cell functional outcome.
Due to reduced SLEC population of Tim-3 KO mice after LCMV-Arm infection, we explored the memory T cell differentiation populations in Tim-3 induced mice. We found that overexpression of Tim-3 appears to drive SLEC differentiation at the expense of long-lived memory T cells. This finding allowed us to link the enhanced signaling of Tim-3+ T cells with the apparent reduction of T cell function. Tim-3 positively regulates the mTOR pathway in TCR stimulated cells pushing differentiation to SLECs. The more Tim-3 expression, the more depletion of long-lived memory T cell pools, a documented phenotype in T cell exhaustion. Together these data provide a mechanism in which Tim-3 is required for optimal T cell activation but contributes to exhaustion via differentiation of SLECs and depletion of long-lived memory T cells (Fig. 22B). Tim-3 is a potential immunotherapeutic target for disease settings with chronic antigen exposure.

9.2 TIM-3 AS A TARGET FOR IMMUNOTHERAPY

The data presented here, in conjunction with already published data, give strong evidence for a co-stimulatory role of Tim-3 on T cells. However, this co-stimulatory role leads to terminal differentiation of T cells to short-lived effector cells during acute LCMV infection. The progressive loss of memory T cells is a documented phenotype during chronic viral infections and in the tumor microenvironment. Therefore, antibodies specific for Tim-3 could be working by preventing the co-stimulatory function and driving the terminal effector differentiation. Current immunotherapies targeting Tim-3 are commonly used in conjunction with blockade of other checkpoint molecules. One successful checkpoint blockade therapy in cancer is targeting the PD-1/PD-L1 axis with monoclonal antibodies. Significant preclinical and translational work
has shown that PD-1 has a powerful inhibitory role on activated T cells. However, genetic absence of PD-1 does not prevent exhaustion. More importantly, PD-1 KO T cells have a strong initial response to antigen but poor recall response and are not maintained \textit{in vivo}\textsuperscript{172}. It is possible that PD-1 blockade therapies not only prevent the co-inhibitory function of PD-1, but also promotes terminal differentiation of cells down a path targeted for death. This provides a precedent for a role of checkpoint molecules in memory T cell formation.

Evidence suggests that Tim-3 targeting alone does not provide ample T cell reinvigoration\textsuperscript{8}. In a chronic T cell stimulation, if PD-1 is dampening TCR signals, and Tim-3 is providing a ‘differentiate’ signal, blockade of both these pathways could reanimate a T cell and keep it alive. The possibility of Tim-3/PD-1 cross-talk could also explain why co-blockade results in a synergistic effect.

Tim-3 is also expressed on T\textsubscript{regs} and a variety of other cells types that play a role in the tumor microenvironment. Therefore, delineating what Tim-3 does on the surface of these cells will be essential to fully understanding how the checkpoint blockade could work.
APPENDIX: ABBREVIATIONS USED

ADCC – Antibody dependent cell-mediated cytotoxicity
AP-1 – Activator protein - 1
APC – Antigen presenting cell
ARM - Armstrong
BAT – HLA-B-associated transcript
BCL – B cell lymphoma
CCR – Chemokine receptor
CD – Cluster differentiation
CDNA – complementary deoxyribonucleic acid
CEACAM – Carcinoembryonic antigen-related cell adhesion molecule
CL13 – Clone 13
CRAC – Calcium release activated channel
CTLA – Cytotoxic T-lymphocyte associated protein
DAG – Diacylglycerol
DC – Dendritic cell
DSS – Dextran sulfate sodium
EAE – experimental autoimmune encephalomyelitis
EBV – Epstein-Barr virus
EOMES - Eomesodermin
ERK – Extracellular signal regulated kinase
FDA – Food and Drug Administration
FSF – Flox-Stop-Flox
GnzB – Granzyme B
GP - Glycoprotein
HAART – Highly active antiretroviral therapy
HAVCR – Hepatitis A virus cellular receptor
HBV – Hepatitis B virus
HCC – Hepatocellular carcinoma
HCV – Hepatitis C virus
HIV – Human immunodeficiency virus
HLA – Human leukocyte antigen
HMGB1 – High mobility group box protein
HNSCC – Head and neck squamous cell carcinoma
HPV – Human papilloma virus
HSV – Herpes simplex virus
IFN - Interferon
IgV – Immunoglobulin variable domain
IL - Interleukin
IP3 – Inositol triphosphate
ITAM – Immunoreceptor tyrosine-based activation motif
ITIM – Immunoreceptor tyrosine-based inhibitory motif
ITK – Interleukin-2 inducible T cell kinase
ITSM – Immunoreceptor tyrosine-based switch motif
KIM – Kidney injury molecule
KLRG1 – Killer-cell lectin like receptor G
KO - Knockout
LAG – Lymphocyte-activation gene
LCMV – Lymphocytic choriomeningitis virus
LM – Listeria monocytogenes
mAb – Monoclonal antibody
MFI – Mean fluorescence intensity
MHC – major histocompatibility complex
MPEC – Memory precursor cells
mTORC – Mammalian target of Rapamycin complex
NFAT – Nuclear factor of activated T cells
NFIL3 – Nuclear factor, interleukin 3 regulated
NFkB – Nuclear factor kappa-light chain enhancer of activated B cells
NK – Natural Killer
NP - Nucleoprotein
NSCLC – Non-small cell lung carcinoma
PD – Programmed cell death
PI3K – Phosphoinositide-3-kinase
PLCγ – Phospholipase C gamma
PS - Phosphatidylserine
RA – Rheumatoid arthritis
SLAM – Signaling lymphocytic activation molecule
SLE – Systemic lupus erythematosus
SLEC – Short-lived effector cells
ssRNA – Single-stranded ribonucleic acid
STAT – Signal transducer and activator of transcription
TAPR – T cell and airway phenotype regulator
TB - Tuberculosis
TBET – T-box containing protein
TCF – T cell factor
T_{CM} – Central memory T cell
TCR – T cell receptor
T_{EM} – Effector memory T cell
TIGIT – T cell immunoreceptor with Ig and ITIM domains
TIL – Tumor infiltrating lymphocyte
TLR – Toll-like receptor
TME – tumor microenvironment
TNF – Tumor necrosis factor
Th – T helper cell
Tim – T cell immunoglobulin and mucin domain
Treg – Regulatory T cell
VEGF/VEGFR – Vascular endothelial growth factor / Receptor
Zap-70 – Zeta-chain-associated protein kinase 70
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