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ROLE OF TIM3 IN MEDIATING T CELL EXHAUSTION DURING CHRONIC *MYCOBACTERIUM TUBERCULOSIS* INFECTION

A Masters Thesis Presented

By

MIYE KATHLEEN JACQUES

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

JULY 7th 2017

IMMUNOLOGY AND MICROBIOLOGY

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IMMUNOLOGY AND MICROBIOLOGY PROGRAM

July 7, 2017

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Abstract

Mycobacterium tuberculosis infection is one of the leading causes of mortality worldwide. One third of the population is estimated to be infected, however only 5-10% of those individuals can transmit the disease. While T cell immunity initially limits mycobacterium growth, it is unclear why T cell immunity fails to sterilize the infection and prevent subsequent recrudescence. One hypothesis is T cell exhaustion is mediating the failure of T cell immunity late during infection. Here we show the development of T cell exhaustion during chronic infection, and that the inhibitory receptor T cell-immunoglobulin and mucin domain containing 3 (TIM3) mediates the development of T cell exhaustion. TIM3 accumulates on the surface of T cells throughout the course of infection and there is a subsequent decrease in effector cytokine production, such as IL-2, TNFα, and IFNγ. Furthermore, antibody blockade of TIM3 restores T cell function and improves bacterial control. Our results show that TIM3 is mediating T cell exhaustion during chronic TB infection and leading to suboptimal bacterial control.

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Jayaraman P, Jacques MK, Zhu C, Steblenko KM, Stowell BL, Madi A, Anderson AC, Kuchroo VK, and Behar SM. (2016) TIM3 Mediates T Cell Exhaustion during Mycobacterium tuberculosis Infection. PLoS Pathog 12(3): e1005490. http://doi:10.1371/journal.ppat.1005490

List of Abbreviations

Ag86B – Antigen 85B, *Mtb* immunodominant target of $CD4^+$ T cells

BSL-3 – Biosafety level 3

CFU – Colony forming units

CTLA-4 – Cytotoxic T-Lymphocyte Associated Protein 4

ESAT6 – Early Secreted antigenic target 6Kd, an immunodominant target of $CD4^+$ and $CD8^+$ T cells

HIV – Human Immunodeficiency virus

IFNγ – Interferon Gamma

IL-2 – Interleukin 2

Lag3 – Lymphocyte-activation gene 3

Mtb – Mycobacterium tuberculosis

PD1 – Programmed Cell Death 1

TB – Tuberculosis

TB10.4 – (EsxH) An immunodominant *Mtb* antigen which human and mice mount a $CD4^+$ and CD8+ T cell response to

TCR – T cell receptor

Th1 – T helper 1 subset of $CD4^+$ T cells

TIM3 – T cell Immunoglobulin and Mucin containing domain 3

TNFα – Tumor Necrosis Factor alpha

Tregs – T regulatory Cells

WT – Wild type, referring to a genetically unaltered mouse strain

Chapter 1: Introduction

Tuberculosis infection

Mycobacterium tuberculosis (Mtb) infection, clinically referred to as Tuberculosis (TB), is currently one of the leading causes of mortality globally[1]. Most individuals infected with *Mtb* contain the infection and present no clinical symptoms; this is termed latent TB[2]. However, the immune system of individuals with latent TB are unable to completely clear the bacterium and approximately 5-10% of latently infected people will go on to form active disease and transmit the bacterium[3, 4]. In addition, individuals in endemic countries have an increased risk of developing active disease following repeat exposures[5].

Mtb transmission occurs when an individual breathes in aerosolized droplets containing the bacterium. These droplets are deposited in the lungs, where the bacteria infect primarily alveolar macrophages. Following the initial infection innate immune cells, such as neutrophils, are recruited to the site of infection[6-8]. The adaptive immune response is delayed with recruitment of T cells to the lung following priming in the lung draining lymph node[9, 10]. Once T cells are primed and recruited back to the lung, they not only secrete cytokines to activate macrophages, but also form the outer shell of granulomas to contain the infection [11, 12]. With the help of T cells, macrophages in the granuloma induce bacterial killing through oxidative damage, induction of apoptosis, autophagy and efferocytosis[13-16]. Cytokines, such as Interferon gamma (IFNγ) produced by Th1 $CD4^+$ and $CD8^+$ T cells activate macrophages to clear mycobacteria, but this is still insufficient for sterilization.

T cells during TB

T cells are critical immune cells in the response to *Mycobacterium tuberculosis*. When mice are depleted of $CD4^+$, $CD8^+$, or both $CD4^+$ and $CD8^+$ T cells they are more susceptible to TB than mice that did not have T cells depleted[17]. Additional evidence from humans studies have also shown that $CD4^+$ T cells are necessary for protective immunity to TB. HIV infection results in the depletion of $CD4^+$ T cells in infected human patients[18, 19]. TB is one of the leading causes of mortality for individuals with HIV[1]. The depletion of $CD4^+$ T cells following HIV infection and the increase in TB susceptibility in co-infected patients, further supports the importance of T cells in immunity to TB[20].

The majority of the $CD4^+$ T cell response during TB is of the T helper 1 (Th1) subtype, in which most of the $CD4^+$ T cells are producing IFNγ[21-23]. Loss of IFNγ results in an increased susceptibility to TB infection[24, 25]. The transcription factor T-bet regulates the expression of IFNγ[26, 27]. Loss of T-bet during *Mtb* infection decreases IFNγ production and subsequently increases susceptibility to disease[28]. In addition to IFNγ, tumor necrosis factor alpha (TNFα) is required for optimal immunity against TB. Granuloma formation and containment of the bacteria is dependent on TNFα[29]. Furthermore, patients with latent TB that are given TNF inhibitory drugs for treatment of inflammatory diseases, such as crohn's and rheumatoid arthritis, converted to active disease following treatment[30].

Considering the importance of cytokines in modulating containment compared to the spread of the disease, the expression of effector cytokines needs to be carefully coordinated. T cells regulate cytokine expression through the expression of inhibitory receptors, such as PD1 and TIM3. PD1 and TIM3 are cell surface receptors that following binding with their respective

ligands, transmit a negative signal within the T cell to down regulating cytokine production and inhibit proliferation[31].

PD1 is one of the best characterized inhibitory receptor. It dampens the signals from the TCR by recruiting phosphatase SHP-2 to the activated PD1[32, 33]. This recruitment leads to the dephosphorylation of activation molecules downstream of TCR and CD28 signaling, such as Syk and phosphhatidylinosital-3-OH kinase. PD1 is a target for therapeutic intervention for late stage cancer with two monoclonal antibodies against PD1 gaining FDA approval. These antibodies block PD1 from binding to its ligands, PD-L1 or PD-L2, and stop the signaling downstream of PD1 therefore, preventing the loss of IFNγ, TNFα, and IL-2 production, and allowing for a continued effector immune response[34-36].

In addition to PD1, another inhibitory receptor that regulates T cell function is TIM3. TIM3 was originally described as a marker to distinguish Th1 $CD4^+$ T cells from other $CD4^+$ helper subtypes[37]. TIM3 is not exclusively expressed on CD4+ T cells and other cell types such as $CD8^+$ T cells, macrophages, dendritic cells, and NK cells upregulate TIM3[38-40]. TIM3 inhibits T cell proliferation and causes apoptosis after binding to its ligands, Galectin-9 or CEACAM-1[37, 41, 42]. Furthermore, TIM3 is associated with a reduction in IFNγ, TNFα, and IL-2 production during chronic viral infection[43, 44]. As PD1 and TIM3 are both associated in the reduction and regulation of cytokine production, these two inhibitory receptors are considered mediators of T cell exhaustion[31].

T cell exhaustion

T cell exhaustion was first described during chronic viral infection and was characterized as $CD8^+$ T cells that had a decrease in cytokine production, increase in inhibitory receptor

expression, and decrease in proliferation [45, 46]. An exhausted T cell develops during chronic infection where antigen is not completely cleared. In the mouse LCMV model of infection, mice that where infected with the Armstrong strain of LCMV (acute infection), developed functional $CD8^+$ T cells producing IFNγ, TNFα, and IL-2. Whereas, mice infected with the Clone 13 strain of LCMV (chronic infection) had dysfunctional CD8+ T cells with reduced IFNγ, TNFα, and IL-2 production[45]. IL-2 is one of the first cytokines lost and is associated with early stages of exhaustion, followed by TNFα during the intermediate stage, and finally at late stages a loss of IFNγ and cell death[47].

T cell exhaustion was initially discovered and characterized for $CD8^+$ T cells, and while $CD4^+$ T cells also have an exhausted phenotype, there are some differences from $CD8^+$ T cell exhaustion. Similar to that described for $CD8^+$ T cells, $CD4^+$ T cell exhaustion is characterized by an upregulation of inhibitory receptors and a decrease in the ability to produce the Th1 cytokines IFNγ and TNFα[48, 49]. In addition to the lack of Th1 cytokine production, there is an increase in IL-21 production within $CD4^+$ T cells during chronic viral infection[49-52]. There are also differences in inhibitory receptor expression on exhausted $CD4^+$ and $CD8^+$ T cells during chronic viral infection. Exhausted $CD8^+$ T cells express more inhibitory receptors such as TIM3, 2B4 and Lag3 during chronic viral infection. Whereas, $CD4^+$ T cells express more CTLA-4. The inhibitory receptor PD1 is highly expressed on both $CD4^+$ and $CD8^+$ T cells during chronic infection[48, 49].

T cell Exhaustion, PD1 and TIM3 during TB

The role of T cell exhaustion during TB infection has not been as well characterized as it has been for chronic viral infections or cancer. Work from Day et al, showed that human peripheral blood from patients with latent TB had increased in polyfuctional, $IFN\gamma^+TNF\alpha^+IL\text{-}2^+$,

$CD4^+$ T cells than patients with active TB[53]. Following treatment with antibiotics, individuals with active TB had an increase in polyfunctional T cells in the peripheral blood. The authors concluded that an increase in antigen load during active TB decreased percentage of polyfunctional T cells. Recent work from Moguche et al, demonstrates that high antigen load throughout the course of infection decreases T cell function and results in terminally differentiated T cells in the mouse model as well as humans[54]. The work by these two groups are the only studies examining T cell exhaustion during TB. Leaving many questions unanswered, such as what are drivers of T cell exhaustion during TB?

During an *Mtb* infection, the exhaustion associated inhibitory receptor PD1 is necessary for providing protective immunity. PD1-/- mice are highly susceptible to *Mtb* infection, due to a hyper-inflammatory lung environment[55]. Regulation of IFNγ is dependent on PD1 expression[56]. Therefore, PD1 maintains immune-homeostatic balance in preventing a hyper-inflammatory response during infection[55, 56]. PD1 binding to its ligand PD-L1/PD-L2 can negatively influence dendritic cell activation[57]. PD-L1/PD-L2 signaling within dendritic cells reduces the expression of maturation markers and increases the expression of the inhibitory cytokine IL-10[58]. Although PD1 has an essential immunoregualtory role, the specific mechanisms that alter T cell function in the context of TB remain unclear.

Unlike PD1, the T cell inhibitory receptor TIM3 has beneficial effects on macrophages during TB by regulating the proliferation of macrophages and dendritic cells[39]. During an *in vitro Mtb* infection, TIM3 activates infected macrophages to kill bacteria[59, 60]. Following binding of TIM3 to its ligand Galectin-9, which is expressed on the surface of macrophages, induces the expression of IL-1β within the macrophage and subsequently reduces bacterial

burden[59, 60]. While these studies indicate a positive role for TIM3 in activating macrophages, the effect of TIM3 signaling within T cells during the course of TB infection remains unclear.

Summary

T cell exhaustion drives failure of T cell immunity in chronic viral infection and cancer. Inhibitory receptors, such as PD1 and TIM3 mediate this exhausted phenotype during chronic infections. Immunity during TB is mediated by protective T cell responses. With 5-10% of individuals with latent TB reactivating to active pulmonary disease, we wanted to further understand the cause of this reactivation. We hypothesize that T cell exhaustion drives a failure in T cell immunity and that the inhibitory receptor TIM3 mediates this exhaustive phenotype. In this thesis, we show that the presence of TIM3 negatively affects the immune response and that blocking TIM3 reinvigorates T cells. Altogether, the work presented here will help in understanding the progression of disease that can pave the way for better vaccine and therapeutic development, as well as public health measures.

Chapter 2: TIM3 Mediates T cell exhaustion during *Mycobacterium tuberculosis* Infection

Preface

This chapter was adapted from the published manuscript:

Jayaraman P, Jacques MK, Zhu C, Steblenko KM, Stowell BL, Madi A, Anderson AC, Kuchroo VK, and Behar SM. (2016) TIM3 Mediates T Cell Exhaustion during Mycobacterium tuberculosis Infection. PLoS Pathog 12(3): e1005490. http://doi:10.1371/journal.ppat.1005490

The exact figures and the organization are different than the published manuscript. All experiments were designed and manuscript was prepared by MKJ, PJ and SMB. Below are authors contributions to all figures:

Figure 2.1: MKJ generated and analyzed all data presented

Figure 2.2: PJ generated and analyzed data presented in part A-B; MKJ generated and analyzed data presented in C-G

Figure 2.3: PJ generated and analyzed all data presented; PJ, CZ, and AM analyzed data presented in parts A-B

Figure 2.4: PJ generated and analyzed data presented in A-B; MKJ and PJ jointly generated and analyzed data presented in parts C-D; MKJ generated and analyzed data presented in part E

Figure 2.5: MKJ generated and analyzed data presented in A-B; MKJ and PJ jointly generated and analyzed data in D-G

Introduction

In countries with endemic tuberculosis there is an increased risk of developing clinically active disease following multiple exposures[5]. This chronic stimulation by antigen could reduce T cell immunity by inducing T cell exhaustion. T cell exhaustion was first described during chronic viral infection and is caused by constant antigen stimulation of T cells[46, 61]. T cell exhaustion is characterized by a step-wise progression of the loss of proliferation, cytokine production, and increase in inhibitory receptors. Cell surface hallmarks of an exhausted T cell include the expression of the inhibitory receptors PD1, TIM3, Lag3, and 2B4[31]. Antibody blockade of the inhibitory receptor TIM3 reverses the exhausted phenotype during chronic viral infection[40]. We hypothesize that T cell exhaustion is mediating the failure of T cell immunity during active TB disease. Here, we demonstrate in the mouse model of infection that T cells increase expression of PD1 and TIM3 throughout the course of infection and have decreased cytokine production. The inhibitory molecule TIM3 is helping to mediate this exhausted phenotype, as treatment of mice with anti-TIM3 reinvigorates T cells and reduces bacterial burden.

Results

Cytokine expression in CD4$^+$ T cells is diminished following chronic *Mtb* infection

One of the defining features of T cell exhaustion the decrease in cytokine production[31]. We were interested in understanding the changes in cytokine expression within the antigen specific and the broadly recruited T cells during the course of infection in the mouse model. To measure antigen specific responses, we looked at the response in CD4$^+$ T cells that recognized the immunodominant *Mtb* antigen ESAT6$_{1\text{-}20}$[62, 63]. At different time points throughout disease

progression, cytokine production was measured after a short re-stimulation with the $ESAT6_{1-20}$ peptide and the background from an unstimulated control was subtracted out. The cytokine production was then normalized to tetramer staining. During infection, IL-2 production by ESAT6 specific $CD4^+$ T cells was predominate at day 20, but by 4 and 17 weeks the amount of IL-2 was nearly undetectable. This loss in IL-2 production resulted in a reduction of triple producing ($IL\text{-}2^+IFN\gamma^+TNF\alpha^+$) cells. When measuring the broad response of T cells in the lungs following stimulation with anti-CD3/CD28, there was an overall reduction in IL-2 and TNFα throughout the course of infection. Along with an overall decrease in TNFα production, there was a decrease in T cells capable of producing both IFNγ and TNFα; this is characteristic of T cell exhaustion. While there was an overall decrease in $CD4^+$ T cells ability to produce IL-2 and TNFα, this was not due to a lack of $CD4^+$ T cells specific to *Mtb*. The frequency of tetramer positive T cells to the *Mtb* antigen ESAT6 was unchanging from week 2, 4, and 17. This change in cytokine was only observed within the $CD4^+$ T cell compartment and not within $CD8^+$ T cells. One possible explanation for the difference in cytokine responses between $CD4^+$ and $CD8^+$ T cells is the timing of response during infection. From survival data, we know that CD4+ T cells are critical early during the response to TB while $CD8^+$ T cells have an important role later during infection[17]. Therefore, a change in cytokine production amongst the CD8+ T cells may be observed at times later that 17 weeks post infection in the C57BL/6J mouse model.

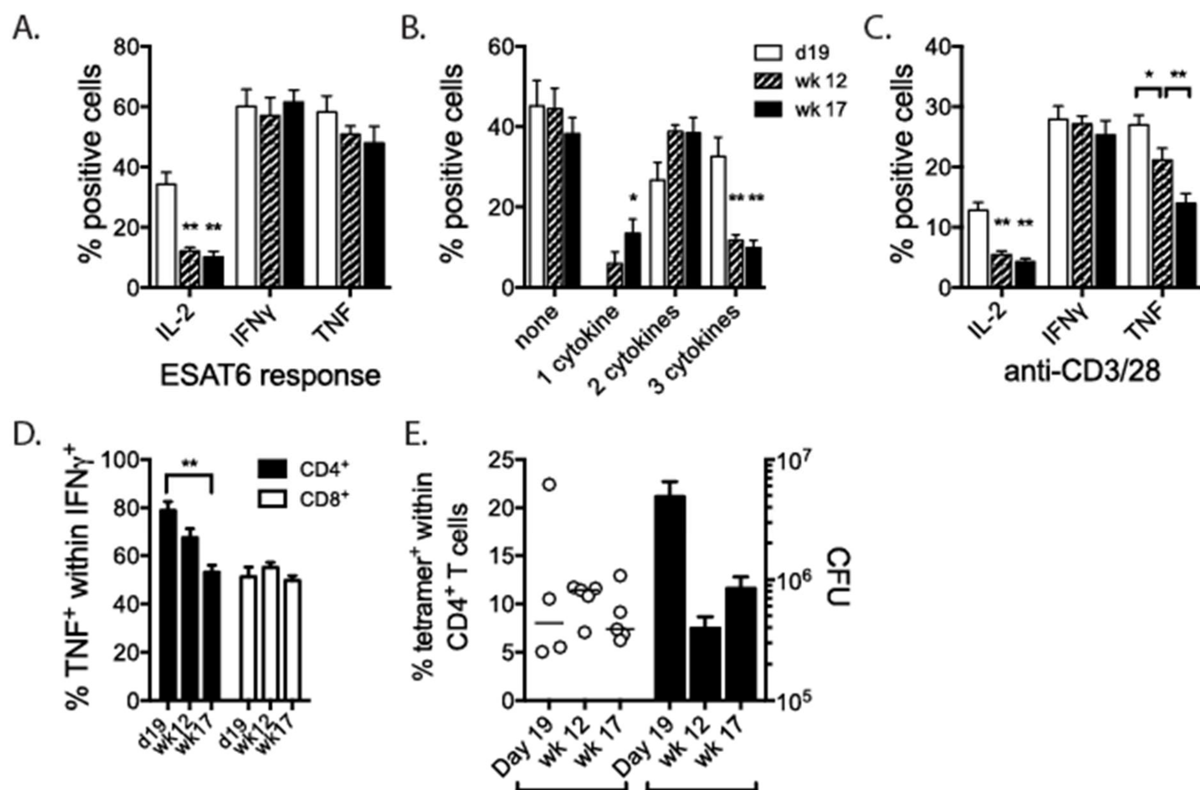

Fig 2.1. Cytokine expression in antigen-specific CD4⁺ T cells is diminished following chronic Mtb infection. (A) The fraction of ESAT-specific CD4⁺ T cells that make IL-2, IFNγ, and TNF on d19 (unfilled), w12 (striped), or w17 (filled) post infection. (B) The fraction of the number of cytokines being produced by ESAT6-specefic CD4⁺ T cells. (C) The fraction of CD4⁺ T cells producing IL-2, IFNγ, and TNF on d19 (unfilled), w12 (striped), or w17 (filled) post infection. (D) The percentage of IFNγ-producing CD4⁺ and CD8⁺ T cells that also make TNF over the course of infection. (E) The fraction of ESAT6-specific CD4⁺ T cells and bacterial burden in the lungs as d19, w12, and w17 post infection. All data is representative of three independent experiments with at least five mice per time point. *p<0.05, **p<0.01, ***p<0.001, one-way anova compared. Bars represent mean ± SEM. The "background" cytokine production, defined as cytokine production that occurs in the absence of specific stimulation was subtracted for each sample before calculations or normalizations were performed.

T cells express multiple inhibitory receptors throughout the course of *Mtb* infection

T cell exhaustion is not only mediated by a change in cytokine expression, but also an increase in inhibitory receptors[31]. To evaluate if the loss of T cell cytokine production is correlated with an increase in inhibitory receptors, we measured the expression of five inhibitory receptors, PD1, TIM3, Lag3, 2B4, CTLA-4, over the course of infection in C57BL/6J mice (Figure 2.2A). The receptors PD1, TIM3, and Lag3 were highly expressed on both $CD4^+$ and $CD8^+$ T cells, with the expression increasing late (44wpi) during infection. 2B4 and CTLA-4

were expressed by a small percentage of $CD4^+$ T cells and even fewer $CD8^+$ T cells expressed these two inhibitory receptors (Figure 2.2A).

T cells in the late stages of exhaustion express multiple inhibitory receptors[31]. At points during infection more than 30% of $CD4^+$ T cells expressed three or more inhibitory receptors and about 20% expressed five or more (Figure2.2B). In contrast, less than 10% of $CD8^+$ T cells expressed three or more inhibitory receptors. Supporting the findings from Crawford et al, that inhibitory receptors are regulated differently in $CD4^+$ and $CD8^+$ T cells[49].

Next, we wanted to focus on the two highly expressed receptors on both $CD4^+$ and $CD8^+$ T cells, PD1 and TIM3. We measured the expression levels of TIM3 and PD1 throughout the course of disease progression. In the first 12 week of infection, $CD4^+$ T cells express high levels of PD1, whereas $CD8^+$ T cells express high levels of TIM3 (Figure 2.2C, D). Both inhibitory receptors are upregulated following *Mtb* infection, as age matched uninfected mice did not have an increase in inhibitory receptor expression (Figure 2.2C, E).

At 43 weeks post infection, there was a significant increase in the percentage of $CD4^+$ and $CD8^+$ T cells expressing TIM3 and PD1 within the lung parenchyma compared to the vasculature space (Figure 2.2F, G). This finding is consistent with the results from Sakai et al, that there is an enrichment of PD1+ $CD4^+$ T cells in the lung parenchyma[64]. In addition to an increase in PD1 within the parenchymal space there is also an increase in TIM3 on both $CD4^+$ and $CD8^+$ T cells. TIM3 was mainly co-expressed with PD1 on $CD4^+$ T cells within the parenchyma, whereas the $CD8^+$ T cells had distinct populations of PD1 and TIM3 single expressing cells and PD1 and TIM3 co-expressing cells. Thus, our data indicates that both $CD4^+$ and $CD8^+$ T cells express multiple inhibitory receptors and have distinct populations of TIM3 and PD1 expression.

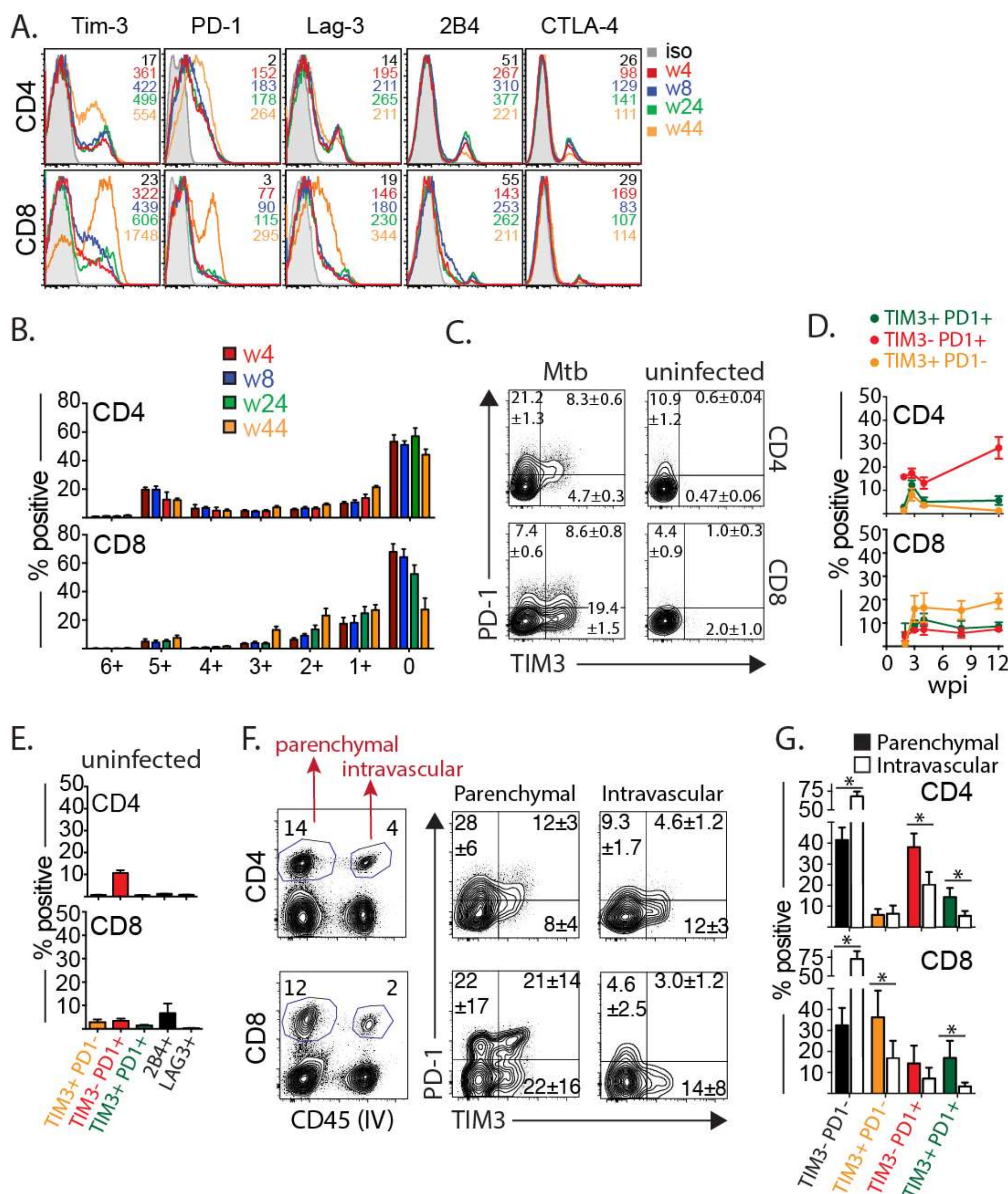

Fig 2.2. T cells express multiple inhibitory receptors throughout course of *Mtb* infection. (A) Expression of inhibitory receptors (TIM3, PD1, Lag-3, 2B4, and CTLA-4) by $CD4^+$ and $CD8^+$ T cells at week 4, 8, 24, or 44 post-Mtb infection. (B) Individual populations of $CD4^+$ and $CD8^+$ T cells grouped according to total number of inhibitory receptors expressed. Data was generated by boolean gating analysis of the simultaneous expression of multiple inhibitory receptors (TIM3, PD1, Lag-3, 2B4, CTLA-4 and CD160) on $CD4^+$ and $CD8^+$ T cells from lungs of Mtb infected mice at weeks 4, 8, 24 and 44 after infection. (C) Expression of PD1 and TIM3 on gated pulmonary CD4 and CD8 T cells in Mtb infected (12wpi) mice and age-matched uninfected mice. Representative FACS plots shown for one out of 5-6 mice per group (D) Frequency of $CD4^+$ or $CD8^+$ T cells that express TIM3 or PD1 at different time points after Mtb infection. (E) Frequency of $CD4^+$ or $CD8^+$ T cells that express TIM3, PD1, 2B4, or LAG-3 in uninfected mice (F) Left panel, representative FACS plots of $CD4^+$ and $CD8^+$ T cells at 43 wpi that stained for CD45 antibody injected intravenously. Right panel, frequency of TIM3 and PD1 within CD45+ (intravascular) or CD45- (parenchymal) $CD4^+$ and $CD8^+$ T cells. (G) Frequency of $CD4^+$ or $CD8^+$ T cells that express TIM3 or PD1 in the parenchymal or intravascular space after Mtb infection. Data is representative of 4 (A, B) or 3 (C-E, F, G) independent experiments, each involving 5-8 mice per time point and per group. *$p<0.05$, by one-way anova compared. Bars represent mean ± SEM.

Two distinct subsets of TIM3 expressing T cells exhibit divergent molecular signatures

The decrease in cytokine expression and increase in inhibitory receptor expression of T cells in the parenchyma at post 40 weeks infection suggests that the T cells are exhausted. To determine if TIM3+PD1+ T cells were molecularly distinct from TIM3+PD1- or TIM3-PD1+ T cells, Pushpa Jayaraman, PhD, sorted four distinct populations (TIM3+PD1+, TIM3-PD1+, TIM3+PD1-, TIM3-PD1-) from the lungs of infected mice. The transcriptional profile of each population was analyzed by Nanostring without re-stimulation of the T cell populations[65, 66]. There was a distinct transcriptional profile for each population. TIM3+PD1+ $CD4^+$ and $CD8^+$ T cells had a more exhausted transcriptional profile, with increases in exhaustion related receptors Lag3, TIGIT, and CTLA-4, as well as the exhaustion associated transcription factor Blimp1 (prdm1) (Figure 2.3A, B)[47, 48]. In contrast, TIM3+PD1- T cells expressed a molecular signature of effector T cell, within both $CD4^+$ and $CD8^+$ TIM3+PD1- T cells having increased transcripts of IFNγ and TNFα. The transcriptional data was confirmed at the protein level with increases in IFNγ and TNFα in TIM3+PD1- T cells following intracellular cytokine flow cytometry staining. Additionally, TIM3+PD1+ T cells had a decrease in IFNγ and TNFα expression, but had an increase in the inhibitory cytokine IL-10 (Figure 2.3C, D). Thus, transcriptional and protein levels of effector cytokines and additional inhibitory receptors (Figure 2.3), strongly indicate that TIM3+PD1- T cells are functionally distinct from TIM3+PD1+ T cells, and that TIM3+PD1+ T cells are becoming exhausted during chronic *Mtb* infection.

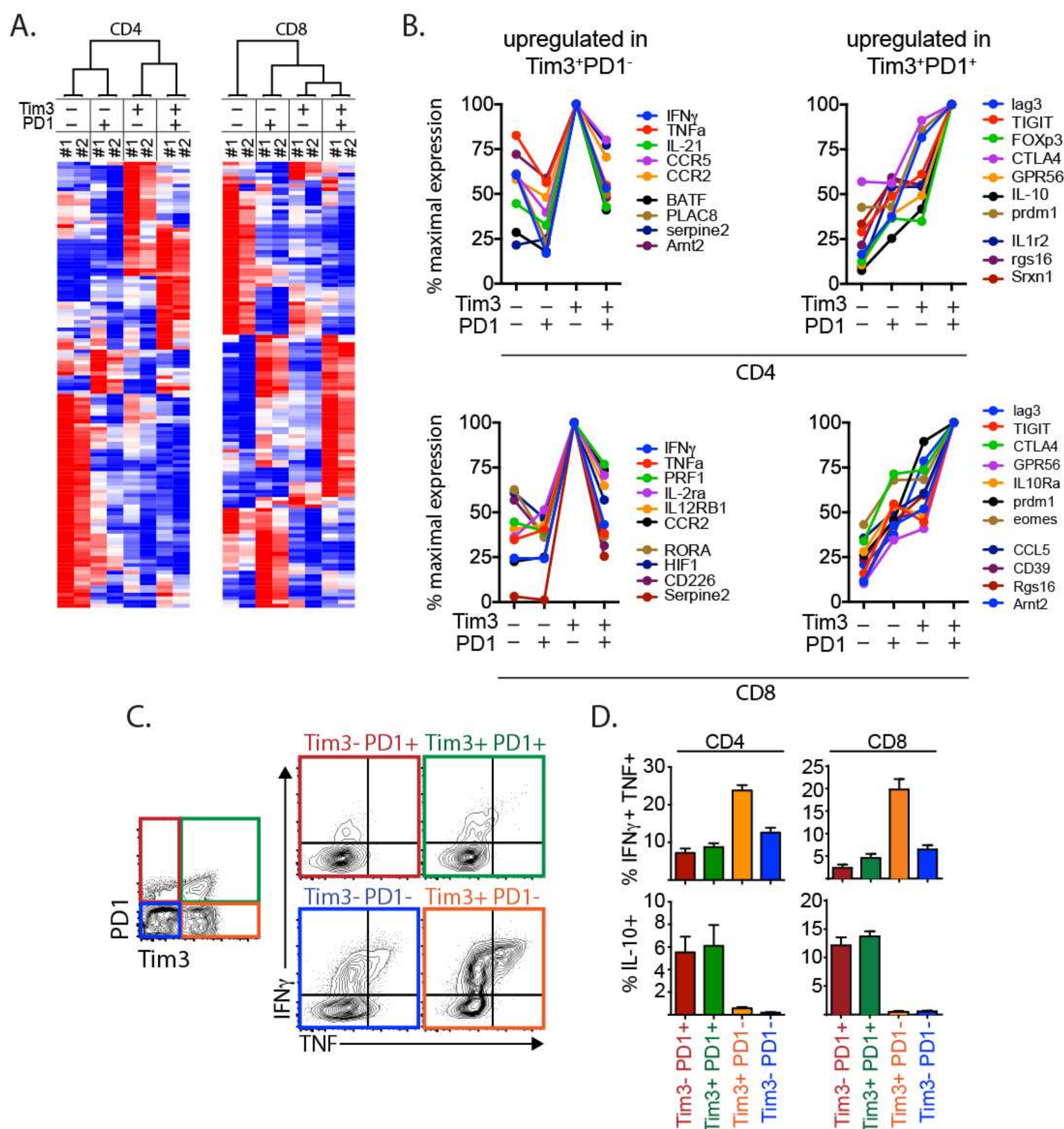

Fig 2.3. Two distinct subsets of TIM3 expressing T cells exhibit divergent molecular signatures. (A) $CD4^+$ and $CD8^+$ T cell populations sorted based on their TIM3 and PD1 expression was subjected to nanostring code set and gene expression analyses. Heat map of differentially expressed genes by $TIM3^-PD1^-$, $TIM3^-PD1^+$, $TIM3^+PD1^-$ and $TIM3^+PD1^+$ $CD4^+$ or $CD8^+$ populations are shown. Blue indicates low relative expression and red, high relative expression. #1 and #2 indicate data from two independent experiments. (B) Fold expression of genes were normalized with respect to highest value among the four TIM3/PD1-expressing populations. Value of 100 indicates preferential expression of a gene set to a particular TIM3/PD1 expressing population and allows assessing population-specific gene patterns. Data is representative of 2 independent experiments. (C) $CD4^+$ or $CD8^+$ T cells from the lungs of mice 45 weeks after Mtb infection were stimulated with anti-CD3/CD28 mAbs in vitro and their expression of TIM3 and PD1, and production of IFNγ and TNF analyzed by flow cytometry. Representative gating showing the cytokine production by each of the TIM3/PD1-expressing T cell populations. (D) Frequency of TIM3/PD1-expressing $CD4^+$ or $CD8^+$ T cells that produce $IFN\gamma^+TNF^+$ or IL-10. Data in C and D is representative of 3 independent experiments with 5-8 mice per time point per experiment. Bars represent mean ± SEM.

TIM3 impairs clearance of Mtb

To understand how TIM3 affects host resistance to TB, we compared TIM3-/- and wild type (WT) mice following low dose aerosol infection. We found that TIM3-/- mice were more resistant TB disease. At four weeks post infection, TIM3-/- mice had a lower bacterial burden in both the lung and the spleen than WT mice (Figure 2.4A). Additionally, WT mice succumbed to infection earlier than TIM3-/- mice with a median survival of 257 versus 307 days (Figure 2.4B).

Blockade of inhibitory receptor signaling using monoclonal antibodies can restore T cell function, termed as reinvigoration [34, 40]. If TIM3 is mediating T cell exhaustion and that is impairing T cell mediated clearance of *Mtb*, we sought to determine if antibody blockade of TIM3 would improve bacterial clearance. Using the C57BL/6J mouse strain, we treated mice with anti-TIM3 antibody (clone 5D12) or an isotype control (mIgG1) starting at 12wpi. The treatment start time was determined following the kinetics of TIM3 expression, with 12 weeks correlating with an increase in TIM3 expression and decrease in cytokine production (Figures 2.1, 2.2). Antibody was given every three days for two weeks after which the mice were sacrificed (Figure 2.4C). Following treatment mice that received anti-TIM3 had a significant decrease in bacterial burden compared to isotype control or no treatment (Figure 2.4D).

TIM3 is not only expressed on T cells, but also on macrophages and dendritic cells. Blockade of TIM3 with the anti-TIM3 antibody (clone 5D12) can activate macrophages[37]. To confirm that the decrease in CFU from TIM3 antibody blockade was mediated by T cells and not through activation of macrophages, TCRα-/- mice were treated with anti-TIM3 every 3 days for 2 weeks. Following treatment, there was no difference in bacterial burden between anti-TIM3

treated and isotype control mice. Thus, the reduction in bacterial burden following antibody treatment was mediated by T cells and not through the activation of macrophages.

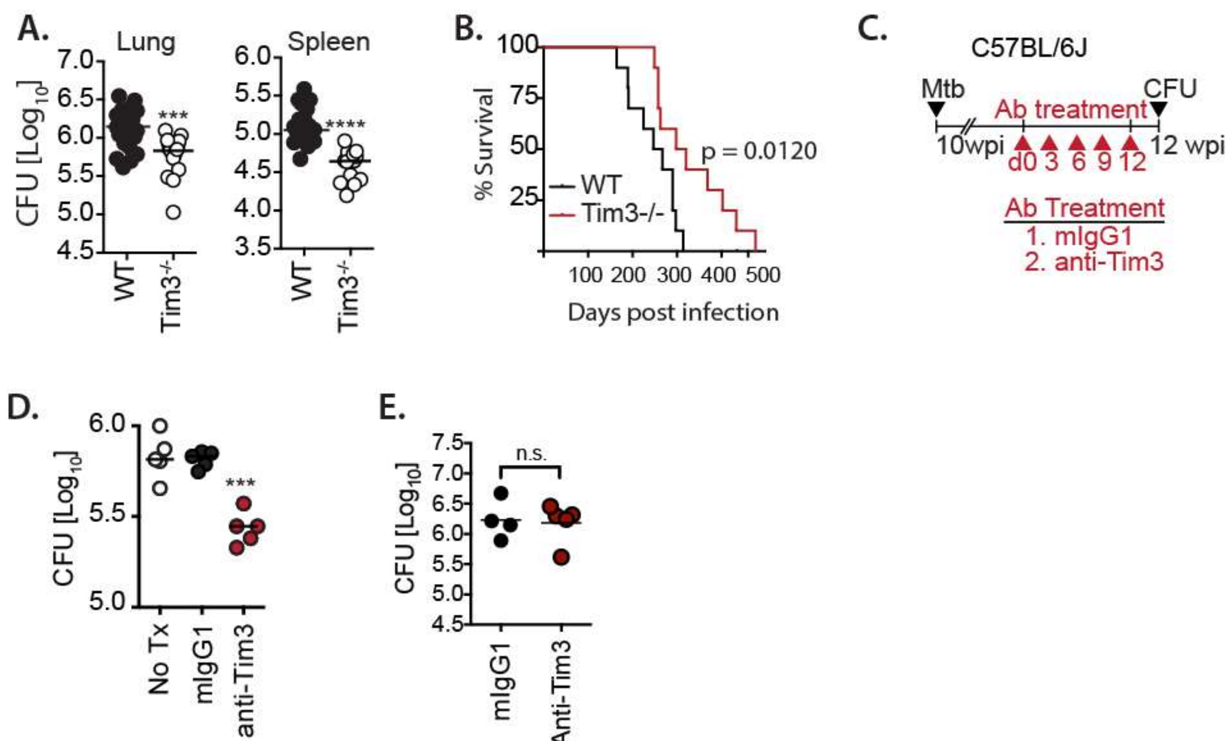

Fig 2.4. TIM3 impairs clearance of Mtb. (A) Bacterial load in the lungs and spleens of WT BALB/c and TIM3$^{-/-}$ mice at wk4 post-infection. (B) Percent survival in Mtb infected WT and TIM3$^{-/-}$ mice. (C) Therapeutic protocol for TIM3 blockade in C57BL/6J mice. Beginning at week 10 post-infection, chronically infected mice were treated every third day for two weeks with isotype-matched control antibody or anti-TIM3 mAb. (D) Bacterial load in the lungs of C57BL/6J mice treated with isotype-matched control antibody or anti-TIM3 mAb. (E) Bacterial load in the lungs of TCRα$^{-/-}$ mice treated with murine IgG1 control antibody (mIgG1) or anti-TIM3 mAb. No Tx, No treatment. Data is representative of 5 (A), 1 (B), 2 (D), independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 for: (A) student's t-test; (B) Log-rank (Mantel-Cox) test; (D) one-way Anova with Dunnett's post-test. Bars represent mean ± SEM.

TIM3 Blockade improves T cell function and disease outcome in susceptible mouse strains

C57BL/6J mice are relatively resistant to tuberculosis and can contain the infection for upwards of a year. Although we cannot currently access to what degree T cell exhaustion mediates the C57BL/6J mouse's demise, we predict that the T cell exhaustion develops in part to chronic antigen stimulation. We hypothesize that T cell exhaustion develops earlier in susceptible mouse strain, such as the C3HeB/FeJ, in which these mice maintain a higher bacterial

burden and succumb to infection earlier compared to C57BL/6J[67-69]. There was an increase in TIM3 and PD1 expression on $CD4^+$ and $CD8^+$ T cells throughout the course of infection in the C3HeB/FeJ mice, with progressive increases from week 4 to week 12 (Figure 2.2A, B). Additionally, there was an increase in the percentage of TIM3+PD1+ $CD8^+$ T cells and less TIM3+PD1- $CD8^+$ T cells in the C3HeB/FeJ mice compared to C57BL/6J mice (Figure 2.2D, 2.5B), which is consistent with the findings that chronically elevated antigen levels promote T cell exhaustion[53, 54, 70].

We next determined if TIM3 blockade would alter host resistance in the susceptible C3HeB/FeJ mouse. Four weeks post infection, mice were treated with either anti-TIM3 (clone 5D12) or an isotype control (mIgG1). The antibody was administered every three days for two weeks. Following antibody treatment the mice were sacrificed and T cell responses were measured (Figure 2.5C). Following antibody treatment, mice treated with the anti-TIM3 antibody had a significant increase in cytokine production in both the CD4+ and CD8+ T cell compartments following re-stimulation with either a MHC-Class I restricted ($CFP10_{32\text{-}39}$) or MHC-Class II restricted ($ESAT6_{53\text{-}71}$) peptide epitopes, or anti-CD3/CD28. There was a TB specific increase (peptide stimulation) and overall global increase (anti-CD3/CD28 stimulation) in all three exhaustion associated cytokines, IL-2, TNF, and IFNγ, following treatment with anti-TIM3 treatment(Figure 2.5G). The increase in cytokine production was also correlated with a significant reduction in bacterial burden in both the lung and spleen (Figure 2.5D, E, F). Overall, our results suggest that TIM3 is helping to mediate an exhausted phenotype, and by blocking TIM3 T cells are reinvigorated. Furthermore, our data shows that T cell exhaustion is mediating impairment of T cell immunity during *Mtb* infection, but also therapeutic targeting of inhibitory T cell signals can reverse exhaustion and improve bacterial control.

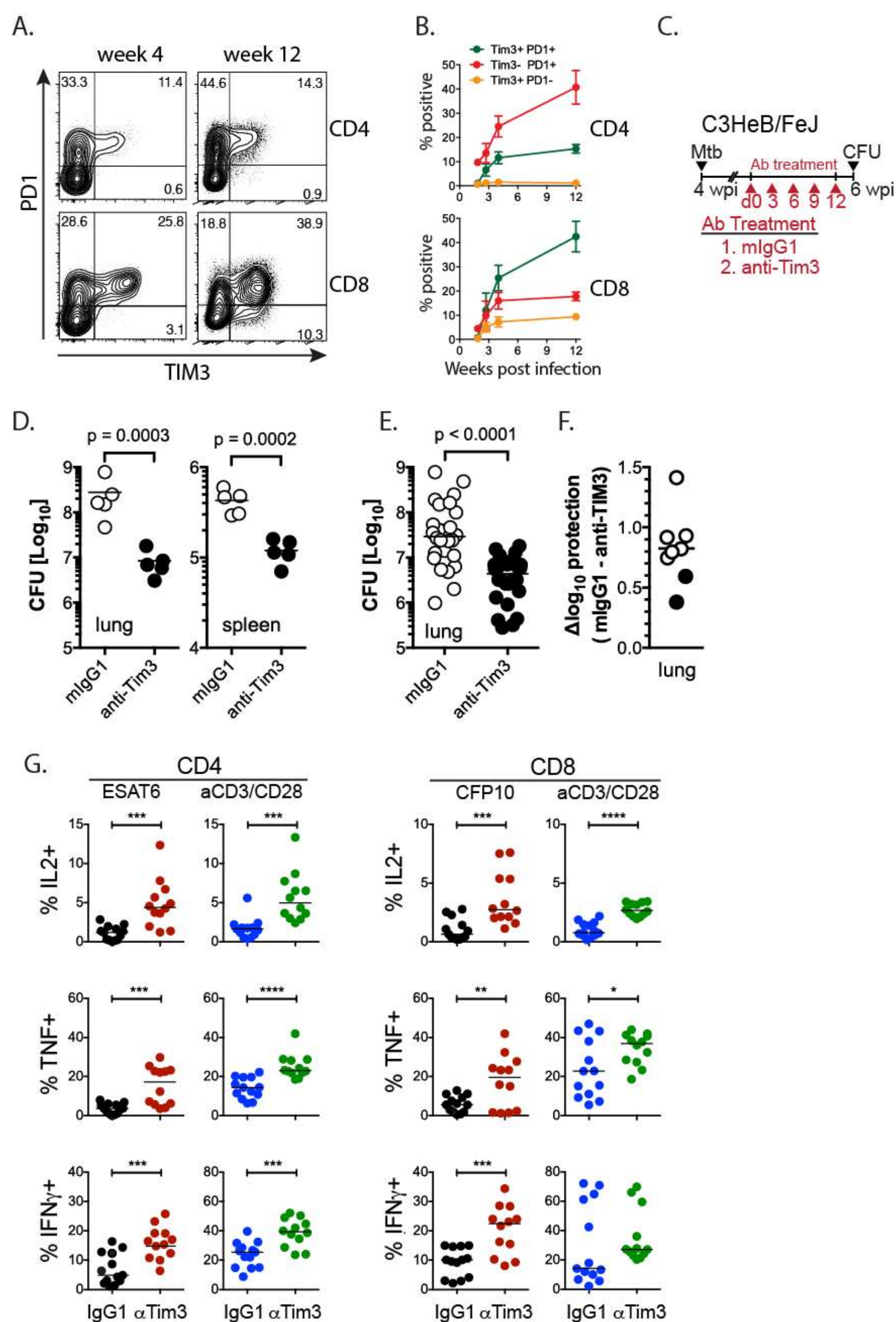

Fig 2.5. TIM3 blockade improves T cell function and disease outcome. (A) Representative contour plots for PD1 and TIM3 expression on pulmonary $CD4^+$ and $CD8^+$ T cells in susceptible C3HeB/FeJ mice, 4 and 12 weeks after Mtb infection. (B) Frequency of $CD4^+$ or $CD8^+$ T cells that are positive or negative for TIM3 and PD1 expression at different times post Mtb infection is plotted. Each point represents the mean ± SEM of 5 mice per strain per time point, and is representative of 2-3 independent experiments. (C) Protocol for TIM3 blockade in C3HeB/FeJ mice. (D) Data from a representative experiment shows the bacterial loads in lung and spleen. (E) Cumulative results from all blocking experiments performed in C3HeB/FeJ mice representing 26 mice/group from six independent experiments. Each point represents lung CFU from an individual mouse. $p<0.0001$ by unpaired t-test after $\log_{10}$ transformation. (F) The $\Delta\log_{10}$ protection [control CFU – treatment CFU] from eight independent experiments. Black circles, C57BL/6 experiments; white circles, C3HeB/FeJ experiments. (G) Production of IFNγ, TNF and IL-2 by $CD4^+$ and $CD8^+$ T cells from the lungs of infected C3HeB/FeJ mice that had been treated as described above. T cells were stimulated in vitro with $ESAT6_{53-71}$ or $CFP10_{32-39}$ peptides (recognized by $CD4^+$ or $CD8^+$ T cells, respectively) or anti-CD3/28 mAbs. Data is from 12-13 mice from three independent experiments tested by unpaired t-test: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, <0.0001. Bars represent median.

Discussion

TIM3 is a key negative regulatory of T cell responses in chronic viral infections, cancer and autoimmune disease[40, 43, 71]. Here we show that TIM3 expression increase over the course of infection, and the increase in expression is also correlated with an increase in expression of the inhibitory receptor PD1. We did find that T cells co-expressing PD1 and TIM3 were functionally distinct from T cells expressing only TIM3, with TIM3+PD1+ T cells having a more exhausted profile. Further supporting the evidence that increased inhibitory receptor expression on an individual T cell decreases function[31]. In addition to the increase in inhibitory receptors, there was an overall decrease in effector cytokine, IL-2, TNFα, and IFNγ, production which is associated with T cell exhaustion[34, 46, 70].

The data presented here represents a correlation between a decrease in T cell function and an increase in bacterial burden. When addressing the overall question of why/how *Mtb* infection develops into disease, it is still unclear as to whether the T cell exhaustion is increasing bacterial burden or bacterial burden is causing T cell exhaustion. It could be that these two events are not mutually exclusive, as a larger bacterial burden can lead to an abundance of antigen that inhibits T cell function, and then once T cells lose function there is a further increase in bacterial burden. The C3Heb/FeJ mouse model is genetically more susceptible to disease compared to the C57BL/6J mouse[68, 69]. C3Heb/FeJ mice have a higher bacterial burden, almost one log, at the same time point compared to C57BL/6J mice[68]. In the C3Heb/FeJ mouse, it was observed that T cell exhaustion occurred earlier and that treatment of the mice with anti-TIM3 early during infection could reverse this exhausted phenotype. Further suggesting that the high bacterial burden and dysfunctional T cells are not mutually exclusive. The reinvigoration of T cells following antibody treatment strongly supports the finding that T cell exhaustion develops during

chronic *Mtb* infection. A further understanding of the mechanisms of T cell immunity failure can lead the way to the development of better therapeutics and preventive medicine for TB.

Methods

Ethics Statement. The animal studies were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School (Animal Welfare Assurance no. A3306-01), using the recommendations from the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the Office of Laboratory Animal Welfare.

Mice and Infections. Six- to eight-week old C57BL/6J, Balb/c, C3HeB/FeJ, or B6.129S2-Tcra^{tm1Mom}/J were purchased from Jackson laboratories; TIM3$^{-/-}$ mice (originally from Millennium Pharmaceuticals), were bred locally. All in vivo infections were performed using virulent Mtb (Erdman strain) by the aerosol route with ~200 CFU by an aerosol-generation device (Glas-Col). At different times post infection, mice were euthanized by carbon dioxide inhalation and lungs and spleens were aseptically removed. Organs were individually homogenized in 0.9% NaCl/0.02% Tween 80 with MiniBead Beater 8 (Biospec Products) and viable bacteria were enumerated by plating 10-fold serial dilutions of organ homogenates onto Remel 7H10 *Mtb* plates (R01610; Fisher Scientific) or Middlebrook 7H11 *Mtb* plates (W40; Hardy Diagnostics). Colonies were counted after 21 d.

In Vivo Blockade of the TIM3 pathway. All TIM3 blockade experiments were performed in chronically infected B6 (12 wks post-infection) and C3HeB/FeJ (4 wks post-infection), and TCRα-/- (1 day post infection). For TIM3 blockade, 500 µg of anti-TIM3 mAb (5D12; prepared in house) or moues IgG1 isotype control were injected intraperitoneally on d0 of treatment and 100 µg every

3d for 2 wk. The ability of anti–TIM3 mAb to block the TIM3 pathway was previously demonstrated [71]. TIM3 blockade in TCRα-/- mice were performed one day post infection with 500 μg of anti-TIM3 mAb (5D12) or mouse IgG1 isotype control were injected intraperitoneally on d0 of treatment and 100 μg every 3d for 2 wk. Lung CFU from anti-Tim3 and isotype treated mice was measured 21 days post infection.

MHC tetramers, Abs, and flow cytometry. Anti-CD3 (clone 145-2C11), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD19 (6D5), anti-CD62L (MEL-14), anti-CD44 (IM7), anti-CD45RB (C63-16A), anti-CD127 (A7R34), anti-TIM3 (2C12), anti-TIM3 (5D12; V. Kuchroo), anti-PD1 (29F.1A12), anti-Lag3 (C9B7W), anti-2B4 (M2B4CB6), anti-CD160 (7H1), anti-CTLA4 (UC10-4B9), anti-IFNγ (XMG1.2), anti-TNF (MP6-XT22), anti-IL2 (JES6-5H4), anti-IL-10 (JES6-16E3), anti-CD107a (1D4B), CD107b (M3/84), rat anti-mouse CD16/CD32 (Fc-Block) were purchased from biolegend. I-A^b ESAT-$6_{1–20}$, K^b TB10.3/$4_{4–11}$, and K^k CFP$10_{32–39}$ MHC tetramers were produced by the National Institute of Allergy and Infectious Diseases Tetramer Core Facility (Emory University, Atlanta, GA). For staining with I-A^b ESAT-$6_{1–20}$ tetramer, cells were incubated with tetramer at 1:200 dilution in complete media containing 10% FCS for 1 h at 37°C prior to staining with surface Abs. Cells were stained with MHC class I tetramers at 4°C for 30 min. For intracellular staining of cytokines IFNγ, TNF, IL-2 or IL-10, lung mononuclear cells from infected mice were cultured in complete media at 37°C with or without 10 μM peptide (to interrogate antigen-specific T cell responses, see Table 1) or 1 μg/ml anti-CD3/CD28 mAbs (to interrogate polyclonal T cell responses). Peptides used in this study are listed in Table 1. After 1 h, 50 μl brefeldin A (25 μg/ml; Sigma-Aldrich) was added and cells were cultured for an additional 4 h. After activation, the cells were washed and stained for extracellular and intracellular markers

according to manufacturer's instructions. Samples were acquired on an LSRII flow cytometer (Becton Dickinson, Franklin Lakes, NJ) or the MACSQuant Analyzer (Miltenyi Biotec, San Diego, CA) and analyzed with FlowJo software (Tree Star, Ashland, OR).

Table 2.1. *Mycobacterium tuberculosis* peptide epitopes used to stimulate T cells ex vivo for Intracellular cytokine staining assays

Mouse strain	MHC restriction	Epitope name	Epitope sequence
C57BL/6J	K^b	$TB10.4_{4-11}$	IMYNPAM
	$I\text{-}A^b$	$ESAT6_{1-15}$	MTEQQWNFAGIEAAA
C3HeB/FeJ	K^k	$CFP10_{32-39}$	VESTAGSL
	MHC II	$ESAT6_{53-71}$	GVQQKWDATATELNNALQN

Nanostring. To assess differences between specific populations, T cells were first purified from lungs of Mtb infected mice were MACS purified and stained for cell surface markers such as CD4, CD8, TIM3 or PD1 from different strains of mice: C57BL/6J or C3HeB/FeJ. Stained T cells were then sorted into distinct populations under BSL-3 conditions. RNA was isolated from sorted populations using Qiagen RNeasy kit according to manufacturer's instructions. The T cell exhaustion signature was constructed based on Affymetrix microarray dataset from D^b-restricted GP33-specific $CD8^+$ T cells on day 6, day 8, day 15, and day 30 following acute (Armstrong) or chronic (clone 13) LCMV infection[72]. The Marker Selection module of GeneE was used to rank genes by their signal-to-noise ratio [73]. A cutoff of 2 fold change (either up or down regulated), as well as, FDR<0.2 was applied to filter significantly differential expressed genes. Exhaustion

signature was defined as a combined list of all the differential expressed genes (DE) between chronic and acute between each of the 4 time points. The number of DE genes for each time point is: day 6, 62 genes; day 8, 139 genes; day 15, 547 genes; day 30, 176 genes with a total of 879 unique DE genes. From this list of 879 viral exhaustion signature, 29 genes were also included in the Nanostring set (Figure 2.3).

Statistics. CFU data were $\log_{10}$ transformed before analysis. The Prism software program (GraphPad Software) was used to perform Student's t tests and one-way ANOVA and Bonferroni's multiple comparison post-test. A p-value of <0.05 was considered significant. The log-rank (Mantel-Cox) test was used for statistical analysis for survival experiments.

Chapter 3: Discussion

Tuberculosis is one of the leading causes of mortality worldwide[1]. While many people who are infected can contain the infection, about 10% of those infected will go on to develop active disease[1]. One key question in preventing the disease is: why do some individuals progress on to active disease? One possible explanation for this failure in T cell immunity is the development of T cell exhaustion[70]. Here we show that TIM3 negatively impacts T cell immunity and mediates T cell exhaustion during TB infection.

Expression of TIM3 has been associated with an increase in T cell exhaustion during chronic viral infections and cancer[40, 74, 75]. Our results confirm this finding and that TIM3 is leading to a failure in immunity during an *Mtb* infection. Interestingly, we observed distinct populations of $CD4^+$ and $CD8^+$ T cells that were either TIM3+PD1- or TIM3+PD1+. The varying expression of TIM3 and PD1 is observed in late stages of infection in the C57BL/6J mouse, and the kinetics of TIM3+PD1+ cell expression vary between $CD4^+$ and $CD8^+$ T cells. At 12 weeks post infection both $CD4^+$ and $CD8^+$ T cells have similar percentages of TIM3+PD1+ cells. By >40wpi there was a significant increase in TIM3+PD1+ CD8 T cells compared to 12wpi whereas, the percentage in TIM3+PD1+ $CD4^+$ T cells did not increase from 12 wpi. The variation in TIM3+PD1+ expression between $CD4^+$ and $CD8^+$ T cells can be due to the differences in the progression of $CD4^+$ and $CD8^+$ T cell exhaustion. In chronic LCMV models, exhausted $CD8^+$ T cells expression more TIM3 than exhausted $CD4^+$ T cells[49]. Which aligns with our findings in the TB model. Future work will evaluate the expression of TIM3 and PD1 on TB specific cells following tetramer staining, as the expression TIM3+PD1+ may vary among the antigen specific T cells.

As we have shown, TIM3+PD1- T cells have a less exhausted and more activated T cell phenotype following RNA profiling by nanostring analysis, and at the protein level by intracellular cytokine flow cytometry staining (Figure 2.3). In cancer and chronic viral infections, like HCV, HBV, and HIV, TIM3+ cells also co-express PD1, and each of these populations have different functional outcomes[40, 43, 74, 76, 77]. In cancer models, TIM3+PD1- T cells maintain a proliferative capacity, whereas TIM3+PD1+ T cells lose proliferative function[78]. This is consistent with our results that during chronic *Mtb* infection, different expression of TIM3 and PD1 alters T cell immunity. Late stage T cell exhaustion is also characterized as T cells expressing multiple inhibitory receptors[31]. Our data supports the possibility that within the polyclonal T cell populations TIM3+PD1- T cells are in the early stages of exhaustion having not lost the ability to produce many effector cytokines, whereas TIM3+PD1+ T cells are at the late stages of exhaustion. Due to low cell numbers following sorting, we were unable to sort the four different TIM3 and PD1 expressing populations following tetramer staining for antigen specific T cells. Future work will evaluate the changes in functionality of different TIM3 and PD1 expressing TB-antigen specific $CD4^+$ and $CD8^+$ T cells using adoptive transfer models with TCR transgenic T cells. This will allow for the collection of enough cells to do nanostring analysis[54, 79-81].

Our results from the genetic ablation of TIM3 contrast that of PD1 or PDL1. PD1-/- mice were highly susceptible to disease and most of the animals succumbed to infection because of an increased inflammatory response in the lung[55]. The over stimulatory effect is seen even following transfer of PD1-/- T cells into infected recipient mice[56]. Based on the different susceptibilities of PD1-/- vs. TIM3-/- to *Mtb* infection, it is clear that TIM3 and PD1 have distinct roles during infection, and PD1 has an important immunoregulatory role.

Blockade of inhibitory receptors with monoclonal antibodies have been shown to reinvigorate exhausted T cells. Here we show that treating both resistant (C57BL/6J) and susceptible (C3Heb/FeJ) mouse strains with α-TIM3 there is an overall increase in cytokine production and decrease in bacterial burden. It is unclear however if the antibodies are working directly on the effector T cells or on other cell types, such as antigen presenting cells or T regulatory cells (Tregs). It is known that α-TIM3 can activate macrophages and we evaluated the effect of α-TIM3 on antigen presenting cells ability to decrease bacterial burden by treating TCRα-/- mice with α-TIM3[71]. We observed no difference in bacterial burden, suggesting that α-TIM3 is impacting T cells ability to reduce bacterial burden (Figure 2.4E). Additionally, it is unclear whether or not α-TIM3 is effecting Tregs. In current clinical usage of antibodies against inhibitory receptors it is unclear of the effect the antibodies have on Tregs[82]. Further analysis of T regulatory cells, such as numbers and function, following α-TIM3 or isotype treatment is needed to fully understand the role Tregs have during the onset of T cell exhaustion.

To further understand the role of TIM3 during chronic *Mtb* infection, we can evaluate the effect that TIM3's binding partners have during infection. TIM3 has two binding partners, Galectin-9 and CEACAM-1[41, 42]. Both of these ligands have been reported to inhibit T cell function, but have separate binding locations on TIM3[41, 42, 83]. In our work presented here, we did not measure the amount Galectin-9 and CEACAM-1 throughout the course of infection. Previous work from our lab showed that in an *in vitro* model system, Galectin-9 present on infected macrophages can activate macrophages to induce IL-1β[59, 60]. The net effect on survival of TIM3-/- mice can be a combination of T cell exhaustion, but also a decrease in Galectin-9 expression. Future work will investigate the role of Galectin-9 and CEACAM-1 during *in vivo* infections, using genetic knockout mice and T cell adoptive transfer models.

T cell exhaustion is driven by chronic antigen stimulation. We observed that inhibitory receptor expression was increased on T cells in the susceptible mouse strain, C3HeB/FeJ[67, 68]. This was correlated with an increased bacterial burden, suggesting that antigen load is driving the exhausted phenotype. Our results support recent findings by Moguche et al, that antigen load and expression drives T cell exhaustion. They found that T cells specific for the immunodominant antigen ESAT6 became exhausted, whereas T cells specific for Ag85B did not become exhausted[54]. ESAT6 is expressed by the bacteria continuously throughout the course infection, whereas *Mtb* down-regulates the expression of Ag85B within the first 3 weeks of infection, further supporting the role of antigen load during the onset of exhaustion[84-86]. Using a similar adoptive transfer of TCR transgenic T cell model system, future studies can evaluate the role of TIM3 on mediating T cell exhaustion on antigen specific cells and the role antigen stimulation has on TIM3 expression.

Research on the impact of T cell exhaustion during infection has focused on chronic viral pathogens, which are characterized with high antigen loads and increased inhibitory receptors that negatively regulate T cell function. Unlike LCMV infection, in which acute or chronic infections can be established through viral strain, it is difficult to discern if T cell exhaustion is developing during TB. Here, we show that T cell exhaustion develops late during infection, with an increase in TIM3 and PD1 expressing cells, and particularly amongst $CD4^+$ T cells having a progressive but reversible loss of cytokine production. Further understanding of the differences in TIM3 expressing cells, both those that co-express PD1 or do not express PD1, can lead to a deeper understanding of T cell exhaustion during TB. Continued research into the failure of immunity during TB will lead to improved vaccine and host directed therapy development, as well as overall public health measures.

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