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Investigating the role of TCR signal strength in the CD8+ T Cell Response to a viral escape mutant

By Shayla K. Shorter
Doctor of Philosophy

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Investigating the role of TCR signal strength in the CD8+ T cell response to a viral escape mutant

By

Shayla K. Shorter

B.S., University of Maryland Baltimore County, 2008

Advisor: Brian D. Evavold, Ph.D.

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A dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies
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Abstract

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By Shayla K. Shorter

The ability to interpret signal strength and initiate a suitable response is an important feature of T cells. Distinct perception of antigen potency is critical for instructing T cell fate and thus the overall effector response to pathogens. This dissertation work investigates the mechanisms behind T cell responsiveness to antigen and how sensitivity of T cell perception of antigen impacts T cell outcome.

The first part of this work is focused on investigating the T cell response to an LCMV viral escape mutant epitope with diminished affinity for MHC but unchanged affinity for TCR. Rather than immunological ignorance, T cells can engage the mutant epitope and initiate a proliferative response that is prematurely curtailed. This response is characterized by deficient IL-2 and IFNγ production, increased apoptosis and an abrogated glycolytic response. Despite high TCR affinity for the variant epitope, levels of interferon regulatory factor-4 (IRF4) are not sustained in response to the variant indicating differences in perceived TCR signal strength. This study revealed a unique mechanism by which pathogens can escape immune surveillance by diminishing TCR signal strength and abrogating the antiviral response.

The second part of this work is focused on elucidating the role of the tyrosine phosphatase, Src homology region 2 domain-containing phosphatase-1 (SHP-1) in the anti-viral response to LCMV. As SHP-1 plays a central role in negatively regulating TCR activation, we hypothesized that removal of SHP-1 would enhance T cell responsiveness to the low potency LCMV 35A epitope. However, we found that SHP-1 deficiency did not lead to a significant increase in the tetramer specific response to the viral variant nor any other dominant epitopes in the LCMV D^b restricted response. We did not observe any change in the IFNγ or granzyme B effector response in our SHP-1 deficient model. Our results demonstrated that removal of a single negative regulator was insufficient to restore optimal T cell responsiveness to the viral variant 35A.

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"And we know that God causes all things to work together for good to those who love God, to those who are called according to His purpose." –Romans 8:28

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Note: Figure 4.2 was done by E.M. Kolawole

Note: Micropipette adhesion frequency assays in Figure 2.1 were performed by R. Andargachew

Introduction

T cells play a key role in mediating protective immunity in vertebrate organisms. These cells possess the unique ability to target specific antigenic motifs and initiate a variety of mechanisms to eradicate infectious agents. T cell receptor (TCR) engagement with antigen leads to a series of intracellular signaling cascades that instruct T cell proliferation, metabolic reprogramming, cytokine production and release of cytotoxic mediators. In this way, T cells play a critical role in recognizing and eliminating foreign antigens from the host.

T cell development generates a highly diverse T cell repertoire

The ability to recognize a diverse range of antigens stems from the unique development of the TCR in the thymus. Through a process of stochastic VDJ gene arrangement, α and β chain gene segments arrange to encode for a wide array of unique T cell receptors. It is estimated that this process can generate up to 10^{15} unique receptors on T cell progenitors[1]. The association of the αβ heterodimer creates a binding groove on the antigen binding surface of the receptor known as the complementary determining region (CDR). The unique CDR3 region creates the unique antigen specificity inherent to T cell clones.

With this immense diversity comes the need to prevent the generation of mature T cells with inappropriate reactivity to "self" antigens. To prevent this, thymocytes are

'educated' through a process known as thymic selection. In this process, thymocytes are tested for reactivity by engaging with a range of peptides presented on major histocompatibility molecules (MHC) on specialized antigen presenting cells which include B cells and various dendritic cells subsets. During positive selection, thymocytes are tested for the ability to bind to MHC on cortical dendritic cells. This checkpoint is essential in selecting progenitors that will be effective in recognizing antigen presented in context of MHC. Thymocytes also go through a negative selection process during which these immature T cells are tested for the ability to appropriately react to self peptides. Inherently self reactive T cells are either deleted in this selection round or converted into T regulatory cells (Tregs) that can regulate the function of other T cells to prevent pathology. During these selection processes, thymocytes gain the ability to co-express coreceptors CD4 and CD8 before becoming mature single positive CD4 or CD8 T cells.

T cell binding kinetics are important for T cell activation

T cell activation is a sensitive and intricately regulated process through the engagement of several pathways. The critical determinant of this process is engagement of TCR with peptide:MHC. This initial interaction is highly dependent on optimal binding of the peptide in the MHC binding groove as well as to optimal binding of the peptide:MHC complex to TCR. Co-receptors can play a role in stabilizing this interaction as the recruitment of Lck kinase brings the coreceptor into proximity for interaction with the TCR-peptide:MHC complex. While the affinity of

CD4 T cells isn't highly dependant on the CD4 co-receptor, CD8+ has been shown to significantly enhance fidelity of binding particularly with antigens of lower affinity[2].

The main parameters describing peptide: MHC interaction with TCR are k_{on} (association) and k_{off} (dissociation) and can be used to derive both affinity (probability of a binding event) and peptide half-life. One of the central tenants of both the kinetic proofreading model and serial triggering model of T cell activation is the important of time and peptide dissociation as a predictor of T cell activity. In the kinetic proofreading model, T cell activation requires peptide engagement time to reach a certain threshold in order for proximal TCR signaling events to accumulate and drive activation[3]. By this model, the strongest agonist would have longer dissociation times. The serial triggering model also purports the idea that TCRs require a specific amount of "dwell time" with peptide:MHC but it is intermediate duration as much longer or much shorter times prevented optimal T cell activation. The serial triggering model proposes that serial engagement of multiple TCRs by peptide:MHC occurs to achieve sustained signaling[4, 5]. There is still quite a bit of controversy over which model best encapsulates how T cell activation occurs as data has been presented to support and/or refute either position. Recently, a modified version of the kinetic proofreading model (induced kinetic proofreading model) was proposed which described TCR:peptide:MHC engagement causing TCR clusters to form thus increasing the likelihood of rebinding to new TCRs[6]. This proposed model seems to incorporate aspects of both the kinetic proofreading model as well as the serial triggering model to present an alternative for how antigen discrimination by T cells occurs.

Historically, receptor binding parameters have been measured using the surface plasmon resonance (SPR) technology[7]. In this assay, peptide:MHC molecules are immobilized to a surface and fluid with purified cognate receptors are allowed to flow over the surface. This method allowed for derivation of affinity, on and off rates as well as half lives. However, the three dimensional (3D) nature with which TCR receptors could engage with peptide:MHC is not an accurate example of how these molecules interact biologically. The use of two dimensional (2D) binding assays have provided a more physiologically relevant system as TCR and peptide:MHC are both adhered to a membrane that still allows for receptor diffusion and movement. Both fluorescence resonance energy transfer (FRET)[8] and the micropipette adhesion frequency assay allow for measurement of these parameters in a 2D setting[2, 9-11].. Two dimensional measurements of these parameters have correlated more closely with predicting T cell function than 3-dimensional (3D) measurements[2, 9]. 2D on-rates have been shown to have much wider range than predicted by 3D measurements. While the trend of off-rates is similar between 2D and 3D measurements, 2D off rates are much faster than those predicted by 3D measurements. So in general, 2D kinetics would predict peptide potency corresponds with shorter off rates and higher affinity.

The significance of TCR peptide:MHC binding kinetics can be seen in the profound effects modifications can have on T cell fate. By mutating critical residues that affect binding to either TCR or MHC allowed for better understanding of how these changes affected T cell fate [12, 13]. Altered peptide ligands (APL) are antigenic peptides that

have been mutated at TCR contact residues to alter the binding of that peptide to the cognate TCR. Studies found APLs could induce a range of biological effects[13-18]. These peptides induce differential responses (or lack thereof) that cover the full range from no response (antagonist) to agonist (full effector functions). Mechanistically, these modified antigens were found to induce differential patterns of signaling which translated into different overall T cell responses. For instance, the pattern of CD3 ζ chain phosphorylation can be modified by stimulation with different APLs. Peptide affinity was found to confer specific capabilities as in one scenario low affinity APLs were found to cause cytokine production but could not induce proliferation like the parent peptide[12, 19].

MHC variant peptides (MVP) on the other hand, are peptides that have been modified to alter binding to MHC. Like APLs, changes to destabilize or enhance binding to MHC can impact cell fate. For example, we have induced an anergic state in CD4+ T cells mediating disease in experimental autoimmune encephalomyelitis (EAE) and Type I diabetes by modifying immunogenic myelin and diabetogenic peptides, respectively [20, 21]. The unique property of MVPs is the ability to target more than a single clone. While changes to TCR contact sites may affect responsiveness to a specific clonal TCR, changes that affect MHC binding can impact a much wider population of T cells restricted by a certain MHC. For the purposes of this dissertation work, this distinction is important to highlight. The context in which this MVP is used will determine whether this broad effect is a positive or negative aspect. For instance, for the purpose of anergizing auto reactive T cells, this an

attractive feature to target a wider population. However, in the context of viral infection for example, this breadth of T cell incapacitation could be detrimental.

The study of APLs and MVPs has led to novel therapeutic interventions that utilize the ability to target antigen specific T cells with an APL to induce a specific response[21, 22]. Such interventions have been demonstrated in cancer and autoimmune settings where effector T cells are induced to either restore tumor specific responses or abrogate auto-reactive T cells[23]. In tumor and pathogen specific responses, T cell receptors have been designed to possess supra-optimal affinity for the target antigen and has shown success in both preclinical and clinical settings[24, 25]. Further studies found however that these T cells were more likely to be non-specifically activated causing treatment toxicity or in some cases, lose the ability to respond to the antigen altogether[24, 26, 27]. Though the clinical outcomes of these interventions have had mixed success, the idea of modifying T cell antigens to impact T cell function has led to a better understanding of T cell biology and overall immunity.

T cell activation is accompanied by changes in signaling and cellular metabolism

Signal transduction that follows pMHC triggering is an intricate network of biomolecular events with several levels of regulation. Kinases and phosphatases modify signaling components thus promoting or arresting signal transduction. This regulatory feedback mechanism is vital in preventing inappropriate and potentially detrimental activation of T cells that have the capacity to mediate immunopathology.

Immediately after antigen specific peptide:MHC engagement, several molecules proximal to the TCR are activated. CD3 ζ chains associated with the cytoplasmic tail of the T cell receptor are phosphorylated by the coreceptor associated Lck. ZAP70 is then rapidly recruited to this area and phosphorylates key residues on LAT which serves as a docking station for the many adaptor signaling molecules that feed into several pathways including MAPK, NF-κB Ca2+-calcineurin signaling networks. Calcium flux, initiated by the release of intracellular Ca2+ storage, helps to support the translocation of the transcription factors Erk, NFAT and NF-κB to the nucleus. Propagation of these signals initiates the structural reorganization, gene transcription and protein expression that lead to proliferation, cytokine production and effector function.

Given the potency of T cell responses and potential risk of immunopathology, the TCR regulates the extent of the responsiveness based on the signal from peptide:MHC engagement. One of the prominent ways to do this is through the use of negative regulators. For instance, Src homology 1 phosphatase (SHP-1) is a tyrosine phosphatase associated with receptor signaling pathways in various hematopoietic cells. SHP-1 was identified as a key negative regulator of proximal TCR signaling by targeting several early mediators (zap70, Lck, etc) resulting in cessation of further downstream signaling. Early characterization of this mechanism proposed a dual feedback system in which Erk (initiated after TCR ligation with potent agonist) and SHP-1 (initiated with weak stimulus) compete for binding on phosphorylated Lck. In

this manner, T cell signaling can be modulated. Recent reports have identified a new mechanism for SHP-1's role in abating TCR signaling[28, 29]. In this proposed model, SHP-1 is constitutively bound to THEMIS via adaptor protein GRB2 in a resting T cell. Upon activation, co receptor bound Lck phosphorylates the CD3 ζ chains, recruiting Zap70 to the phosphorylated sites. Zap70 phosphorylates tyrosine residues on LAT allowing for recruitment of the THEMIS-GRB2-SHP-1 complex.

Another key aspect of T cell activation is cellular metabolism. Like other cells, T cells require metabolic fuel in order to support the numerous activities needed to initiate and maintain T cell functions. Naive T cells primarily use oxidative phosphorylation as well as fatty acid oxidation to support basal level functionality. Upon antigen specific activation, T cells rapidly upregulate both glucose and amino acid transporters, to acquire energy rich substrates for glycolysis and glutaminolysis[30, 31]. This 'glycolytic switch' to aerobic glycolysis is a characteristic feature of proliferating cells. This switch to aerobic glycolysis is paradoxical as it occurs in an oxygen replete setting and yields a net of only 2 ATP molecules per glucose molecule which is significantly less than other methods of generating ATP[32]. However, aerobic glycolysis is rich in the production of several other biological precursors that are critical to the sustaining of other pathways important in the synthesis of nucleic acids, protein, and membrane synthesis.

Recent inquiries have highlighted the importance of metabolism in directing T cell fate. For example, CD4+ T -helper subsets were found to have distinct metabolic profiles that both influence and are influenced by local cytokine milieu as well as the

transcriptional profiles induced by TCR binding[33-35]. For instance, CD4 helper subsets primarily rely on glycolysis while T-regulatory cells also rely on lipid biosynthesis and oxidative phosphorylation during immune activation. Memory CD8+ T cells in contrast to the highly glycolytic precursors, are heavily reliant on fatty acid oxidation as a means of generating energy[36]. Mechanistically, memory T cells use FAO to make use of the stored mitochondrial energy. This 'spare respiratory capacity' is a unique feature of memory T cells and explains how memory T cells are able to rapidly mount a response upon re-encounter with antigen[37, 38].

Importance of Overall Signal Strength

T cells are constantly being engaged with self-peptide MHC in the periphery yet they remain naïve. These tonic signals are important for maintaining a responsive population of naïve T cells. This system points to the idea that T cell activation isn't simply based on ability to bind antigen but rather to integrate a variety of external signals into one that reaches a certain activation threshold. It is thus the 'strength of signal' perceived by the TCR that instructs the appropriate pathways required for downstream T cell functions.

Many of the activation pathways described above are influenced by specifically by signal strength. For instance, T cell engagement with APC occurs in three distinct stages during which each stage sequentially confers effector phenotype traits. While strong agonists that deliver a strong signal proceed through the entire three step sequence, weaker agonists end the sequence prematurely which leads to a difference

in TCR signal propagation[39, 40]. Both the spatial organization of the proximal signalsome complex and regulation of signaling are correlated with the potency of the ligand bound to the TCR. TCR signal strength is even sufficiently sensitive to distinguish between peptide potency versus peptide density both of which elicit unique signaling signatures despite similarities in proliferation[41]. The ability of T cells to integrate extracellular signals into a highly sensitive and specific phenotypic program is quite impressive. This dissertation work is extension of the current data that attempts to explain how T cells integrate and transduce these signals into cell fate instructions. Understanding the intricacies of T cell responsiveness allow for the development of more targeted therapies to enhance T cell mediated immunity.

Chapter 2: Viral escape mutant epitope maintains TCR affinity for antigen yet curtails CD8 T cell responses

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Abstract

T cells have the remarkable ability to recognize antigen with great specificity and in turn mount an appropriate and robust immune response. Critical to this process is the initial T cell antigen recognition and subsequent signal transduction events. This antigen recognition can be modulated at the site of TCR interaction with peptide:major histocompatibility (pMHC) or peptide interaction with the MHC molecule. Both events could have a range of effects on T cell fate. Though responses to antigens that bind sub-optimally to TCR, known as altered peptide ligands (APL), have been studied extensively, the impact of disrupting antigen binding to MHC has been highlighted to a lesser extent and is usually considered to result in complete loss of epitope recognition. Here we present a model of viral evasion from CD8 T cell immuno-surveillance by a lymphocytic choriomeningitis virus (LCMV) escape mutant with an epitope for which TCR affinity for pMHC remains high but where the antigenic peptide binds sub optimally to MHC. Despite high TCR affinity for variant epitope, levels of interferon regulatory factor-4 (IRF4) are not sustained in response to the variant indicating differences in perceived TCR signal strength. The CD8+ T cell response to the variant epitope is characterized by early proliferation and up-

regulation of activation markers. Interestingly, this response is not maintained and is characterized by a lack in IL-2 and IFNγ production, increased apoptosis and an abrogated glycolytic response. We show that disrupting the stability of peptide in MHC can effectively disrupt TCR signal strength despite unchanged affinity for TCR and can significantly impact the CD8+ T cell response to a viral escape mutant.

Introduction

Although there are many contributing arms of an effective immune response, T cells are one of the most significant players. T cell receptors display an impressive breadth of specificity for a wide variety of antigens owed largely to the process of T cell development in the thymus where VDJ rearrangement can generate a diverse repertoire [42]. Engagement of TCR with cognate peptide: MHC initiates downstream signaling cascades leading to up-regulation of activation markers, cytokine production and proliferation [43, 44]. While full T cell activation is the result of a combination of signals derived from co-stimulation and cytokine signals, the initial TCR recognition of antigen is a critical aspect of this process and a determinate of T cell fate [45, 46]. Thus, modifications that affect the ability of TCR to bind peptide:MHC as in the case of altered peptide ligands (APL), can dramatically impact an ensuing T cell response. The effect of APL on T cell function have been characterized using peptide variants with mutated TCR contact residues [12, 13]. These studies demonstrated that TCR affinity for APL directly correlated with T cell function, with high affinity peptides defined as agonists that induced maximal T cell activation. Mutations that result in suboptimal binding to TCR have been shown to limit downstream signaling and gene expression involved in activation, proliferation and development of effector function. The result is a variety of outcomes including partial activation, T cell antagonism or anergy, where T cell activation is blocked by the initiation of negative signaling cascades [16]. These studies have led to a variety of therapeutics for anti-tumor and autoimmune responses with mixed success [47, 48]. Most include vaccination with altered peptide ligand epitopes and/or engineering T

cells to express receptors with supraoptimal affinity for peptide:MHC in effort to enhance or abrogate T cell responses [23, 49, 50].

The study of the affinity parameters that govern a productive recognition event is of great interest as these fundamental mechanisms inform our understanding of immune responses ranging from autoimmunity to viral infection. Though responses to antigens that have differential binding affinities to TCR have been studied extensively, understanding the impact of disrupting binding to MHC has been highlighted to a lesser extent. Like their APL counterparts, recognition of MHC variant peptides (MVP) can alter T cell phenotype, but in this case the TCR affinity for antigenic pMHC is likely maintained with the parameter of pMHC stability driving the sub-optimal triggering. For instance, we have previously shown an MHC variant peptide of myelin oligodendrocyte (MOG) can anergize polyclonal encephalitogenic T cells by triggering negative signaling mediated by Src homology tyrosine phosphatase (SHP-1)[20]. This suggests that unlike APLs that have effects on clonal TCRs and in MS patients caused exacerbation of clinical symptoms [22]. MVPs could be more effective in therapeutically abrogating polyclonal T cell mediated autoimmune disease. The advantage of manipulating pMHC stability to alter T cell activation state allows for a broader impact on polyclonal T cell responses rather than a single clone as would be in the case of APL recognition. While MVP-induced unresponsiveness in T cells may be beneficial in the context of autoimmunity, we hypothesized that a similar mechanism could be exploited by pathogens to subvert the T cell response.

In our study, we describe a strategy by which recognition of a lymphocytic choriomeningitis (LCMV) viral variant epitope with diminished affinity for MHC leads to a subpar outcome despite having similar TCR affinity as the wildtype epitope. This previously identified viral variant (henceforth referred to as 35A) contains a single amino acid mutation in position 35, changing a valine to alanine and resulting in a considerably lower affinity for H-2D^b [51]. We have also previously shown that the 35A epitope is not ignored but that this viral variant recognition results in modification of the SHP-1 tyrosine phosphatase which plays a role in mediating negative regulation of TCR signaling [52]. Here, we extend our findings to show that CD8+ T cells from the P14 TCR transgenic mice specific for the original GP33 epitope can recognize the mutated epitope with the same two dimensional (2D) affinity. Although TCR affinity tends to correlate with functional outcome for APL[9, 53], here recognition of the viral variant leads to diminished interferon regulatory factor 4 (IRF4) expression indicating that T cells interpret variant 35A as weak stimulus. Importantly, the 35A:D^b epitope rapidly decays displaying a half-life of 3 hours whereas the native GP33 peptide has a half-life of >5 hours. This high affinity TCR recognition of a less stable complex results in an early proliferative response and up-regulation of activation markers. This initial response to the variant is not sustained and instead culminates in abortive proliferation and death of responding effectors. The blunted response is characterized by diminished production of IL-2 and IFNγ as well as a decreased glycolytic response. We show a model in which disruption of peptide stability on the MHC leads to abrogation of the CD8+ T cell response to an LCMV viral escape mutant.

Results

Recognition of 35A epitope results in decreased TCR signal strength despite unchanged TCR affinity

Common methods of measuring affinity or avidity, by surface plasmon resonance (SPR) or tetramer fall-off assays, respectively have yielded much information on the kinetics and nature of TCR binding to peptide:MHC (pMHC). While affinity is generally accepted as a reliable predictor of functional outcome, there are cases that do not fit this predictive model. Measuring two-dimensional (2D) affinity using the micropipette adhesion frequency assay has been shown to more closely correlate with potency of T cells responses for both CD8 and CD4 T cells [9, 54-56]. Using this system, we measured the 2D affinity of P14 TCR transgenic CD8+ T cells for wildtype GP33 and mutant 35A epitope and found no significant difference (Fig 1A). This 2D affinity is also consistent with reports of identical avidity between the GP33 and 35A epitopes using pMHC tetramers [57]. As affinity is the outcome of the on rate and off rate of binding, it does not inform on the stability or half-life of the antigenic complex. Functional outcome is also dependent on the interaction between antigen and MHC, so we used the RMA-S MHC stability assay to determine the stability and half-life of the peptides on H-2D^b. Both wildtype GP33 peptide and 35A peptide were able to stabilize the expression of H-2D^b on the surface of the RMA-S cells across a range of doses (Fig 1B). However, mutant 35A epitope was highly unstable, as even at the highest dose that gave equal loading on MHC (10μm), the mutant 35A rapidly decayed with a half-life of ~3 hours while the wildtype GP33 epitope had a half-life of >5 hours (Fig 1C).

Given these binding parameters, we wanted to investigate how T cells would interpret an antigen that maintains high TCR affinity and tetramer avidity but fails to stably bind MHC with long half-life as comparable to wildtype. Using D^b restricted P14 TCR transgenic CD8+ T cells, we measured IRF4 levels in response to stimulation with both the wildtype GP33 epitope and the 35A epitope. IRF4 is a transcription factor involved in regulating various aspects of lymphocyte differentiation and expansion and has also been shown to correlate with TCR affinity for antigen [58-60]. Interestingly, while P14 T cells responding to 35A virus *in vivo* upregulated IRF4 expression at day 4 post infection, this expression was significantly less than in the response to Clone 13 infection (Fig 1D and 1E). This pattern of expression is also demonstrated *in vitro* as 35A stimulated cells expressed less IRF4 on day 3 (Fig 1F and 1G). It should be noted however that within 24 hours of stimulation *in vitro*, IRF4 expression in both GP33 and 35A primed cells is comparable (Fig 1G). This suggests that T effectors can initially be triggered by 35A but the MHC instability causes IRF4 expression to be prematurely downregulated. This pattern can be observed over a range of peptide doses (Fig 1H), with 35A peptide stimulated cells expressing significantly less IRF4 at equivalent doses of peptide. While IRF4 has been described as a metric for TCR affinity for antigen, we found that despite similar affinity for antigen, 35A recognition resulted in decreased maintenance of IRF4 up-regulation. In this case, IRF4 expression does not exclusively readout TCR affinity for antigen, but the overall signal potency perceived by the TCR which is determined by both TCR affinity and peptide stability on MHC.

Recognition of mutant epitope results in activation and abortive proliferation

We sought to examine whether the TCR signal derived from recognition of 35A was of sufficient strength to sustain T cell activation and expansion *in vivo*. We adoptively transferred 10^5 P14 transgenic T cells into congenic hosts and infected mice with a chronic LCMV strain, Clone 13, which contains the wild type GP33 epitope or the Clone 13 based variant virus 35A [61] (Fig 2A). Since we observed high mortality in animals infected with the traditional 2 x 10^6 PFU dose of Clone 13,we reduced the dose to 2 x10^3 PFU for each virus. By day 4 post infection, P14 T cells expanded in response to both viruses, albeit to a lesser extent to the variant virus (Fig 2A). As early as day 4 post infection, donor cells responding to Clone 13 not only rapidly proliferated but also up-regulated markers of activation including CD44, KLRG-1, CD25 and PD1 (Fig 2B). Despite its lag in cell expansion, donor cells responding to the variant virus also up-regulated these activation markers although to a lesser degree in the case of PD-1 and CD25. By day 8 post-infection, cells responding to both viruses had similar levels of CD44 and KLRG-1 while maintaining disparate expression of CD25 and PD-1. Even with evidence of activation, the response to the 35A variant virus resulted in significantly less accumulation of donor CD8+ T cells as compared to infection with the Clone 13 (Fig 2A). While many viral escape mutants are thought to destroy epitope recognition by mutating targeted residues, this data shows that T cells recognize 35A epitope but are sub-optimally activated leading to an inability to maintain the initiated response. The extent of this activation was further demonstrated by *in vitro* peptide stimulation. By day 3 post stimulation, P14 T cells

underwent 2-3 divisions to both the GP33 and 35A peptide (Fig 2C). As observed in the *in vivo* model, cells primed with wildtype GP33 peptide expand and rapidly lose CFSE fluorescence while proliferation of variant-primed cells fail to accrue a fully divided population after the initial 2-3 rounds of division. By day 6, there is a dramatic loss in cells responding to the 35A mutant peptide (Fig 2D). Despite this abrogated proliferation, phenotypic analysis shows that 35A variant primed cells up-regulate markers of activation in response to 35A stimulation (Fig 2E). Comparable levels of CD69, CD44, CD25, CD122 and Ki67 are expressed in T cells responding to both GP33 and 35A at 24 hours post stimulation. By day 3, 35A stimulated cells have a slightly less activated phenotype than its wildtype counterpart. By day 6, the disparity between the wild-type response and the variant response is more evident as variant primed cells are unable to sustain their activation response to antigen from day 3 to day 6.

35A Stimulation results in reduced viability

To investigate whether the stagnation of cell turnover in response to the 35A epitope was specifically due to death of the responding cells, we assayed for evidence of apoptosis. At day 3, cells stimulated with GP33 and 35A *in vitro* displayed comparable levels of viability as shown by staining with Annexin V and 7AAD (Fig 3A and 3B). However by day 6, less than 40% of the cells responding to the variant were viable (in comparison to approximately 80% viability in response to GP33). Stimulation with 35A is insufficient to maintain pathways that would support

viability as we also detected lower levels of the pro survival molecule Bcl-2 at day 3 post 35A stimulation (Fig 3B). This supports the conclusion that suboptimal recognition of the viral variant epitope aborted activation by favoring apoptosis over survival.

Response to variant characterized by low CD25 expression and deficient IL-2 and IFNγ production

T cells have several requirements for optimal activation, expansion, acquisition of effector function and survival and many are essential in the early stages of T cell activation. In particular, IL-2 is an important cytokine needed for both paracrine and autocrine support of cellular proliferation and survival [62-64]. In addition to decreased survival and proliferation, we noted that cells responding to 35A *in vitro* express lower levels of the high affinity IL-2 receptor, CD25 (Fig 4A). While an equal percentage of wildtype or 35A-stimulated cells had undergone at least 2-3 divisions at day 3, cells responding to 35A in each cycle demonstrated consistently lower CD25 expression in each division cycle (Fig 4B and 4C). Given that CD25 expression is regulated in part by IL-2 levels in an immune microenvironment [65], we next investigated IL-2 production by P14 CD8+ T cells following stimulation with either GP33 or 35A peptide. When we assayed IL-2 production after 24 hours of stimulation, GP33-stimulated effectors produced high levels of IL-2, up to 15ng/mL (Fig 4D). In contrast, we found a severe deficit in the ability of 35A stimulated cells to produce IL-2, as the cytokine was nearly undetectable in the supernatant of cultures stimulated with a range of doses of the variant peptide (Fig 4D). This defect in IL-2 secretion can be seen out to day 3 post peptide stimulation demonstrating that

cytokine production is absent and not delayed. We also noted that *in vitro* 35A primed cells were unable to produce the effector cytokine IFNγ at levels comparable to GP33 (Fig 4E), further supporting the idea that mutant epitope recognition initiates an early proliferative program but later results in incomplete acquisition of effector function and phenotype. We also assessed total IFNγ CD8+ T cells directly *ex vivo*. As there were limited numbers of 35A expanded cells to assay cytokine with a complete peptide dose response curve, we chose to stimulate the T cells using phorbal myristate acetate (PMA) and ionomycin. While low cell recovery in 35A infected animals prevented us from adequately assessing the IFNγ response at day 8, we found significantly fewer IFNγ + P14 T cells in 35A infected mice at day 4 post infection (Fig 4F and 4G). This data corroborates our findings in our *in vitro* model that suboptimal recognition of an MHC variant detrimentally impacts the ability of responding T cells to produce cytokine.

35A stimulated cells fail to meet metabolic demands for activation and proliferation

Lymphocytes require efficient means of generating energy to support the enormous demands of proliferation, effector function and growth [66]. Naïve T cells rely on oxidative phosphorylation to support basal cell functioning. Upon activation however, T cells undergo a glycolytic switch characterized by an increased reliance on glycolysis to generate the ATP and other biosynthetic precursors needed for cellular function and growth [66, 67]. In addition to being important for T cell proliferation

and survival, recent work demonstrated this glycolytic switch is critical for the production of IL-2 and IFNγ [68]. As proliferation and cytokine production were compromised in variant stimulated cells, we hypothesized that an altered metabolic state may be a contributing factor. To determine whether cells responding to variant 35A had defects in metabolism we utilized an extracellular flux analyzer to measure glycolysis in cells stimulated with peptide following sequential exposure to glucose, oligomycin and 2-deoxyglucose (2-DG) (Fig 5A and 5B). We consistently found variant primed cells had a lower rate of glycolysis, which was measured by the lactate-induced change in media acidity after the addition of glucose. The addition of the ATP synthase inhibitor, oligomycin, halts oxidative phosphorylation and forces the cell to perform at peak glycolytic capacity which is demonstrated by an increase in the extracellular acidification rate (ECAR) and calculated by subtracting basal ECAR measurements. We observed a significant decrease in glycolysis (Fig 5C) and a decrease in glycolytic capacity (Fig 5D) in CD8+ T cells stimulated with the variant epitope. These parameters of metabolic function are measures of cellular capacity to sustain continued expansion and acquisition of effector function via glycolytic metabolism. As limited T cell numbers prevented us from using the Seahorse based metabolic extracellular assay on *ex vivo* cells, we tested glycolytic function by using the 2-NBDG uptake assay[69]. This assay utilizes a fluorescent glucose analog 2-NBDG which is modified such that it can be taken up by glucose transporters but cannot be fully metabolized. P14 T cells from 35A variant infected mice demonstrated a defect in uptake of 2-NBDG (Fig 5E). In addition, we also observed both decreased size and granularity in 35A-stimulated cells (Fig 5F), which has been shown to correlate with lower rates of glycolysis and reflects a more quiescent metabolic

state[70]. Despite the identical 2D affinity and initial TCR engagement, lower pMHC stability affects the ability of variant stimulated cells to undergo glycolysis and thus meet the bioenergetic demands required for sustained proliferation and effector function.

Discussion

Viral immune evasion from CD8+ T cell responses can hamper effective viral clearance and development of sterilizing immunity. Thus, understanding the mechanisms underlying viral escape can inform the development of effective interventions. The focus of our model is a variant virus containing a single amino acid mutation in the D^b/GP33 epitope of lymphocytic choriomeningitis virus (LCMV). Infection with the variant 35A virus fails to induce a detectable D^b/GP33 response and previous studies concluded effector CD8+ T cells were unable to recognize the epitope due its diminished affinity for MHC [51]. However, we demonstrate that P14 CD8+ T cells can initiate several rounds of early division in response to 35A before undergoing a dramatic contraction. In our characterization, we found that variant 35A primed cells exhibited several deficiencies that contributed to the inability of CD8+ T cells to maintain a proliferative response. We observed a severe lack in IL-2 and IFNγ production along with a diminished capacity to undergo glycolytic metabolism. This is an intriguing phenotype as despite having similar affinity for TCR as the wildtype GP33 epitope, T cells have lower and altered IRF4 expression kinetics in response to the variant epitope revealing that 35A is interpreted as a suboptimal

TCR stimulus. Our observations define a viral escape method driven by suboptimal triggering of TCR rather complete evasion of the CD8 + T cell response

Optimal T cell recognition of cognate antigen is essential for achieving full activation of a T cell. Based on current models of T cell activation, peptide:MHC must be of sufficient antigenic quality and require sufficient interaction time to induce downstream signals for initiation of proliferation and cytokine production[44, 71-73]. Though CD8+ T cells need a remarkably short period of engagement with peptide:MHC to distinguish between ligands of similar TCR binding kinetics (< 3 min) and less than 2 hours to initiate a proliferative response [74, 75], T cells require extended time (~40 hours), in conjunction with co-stimulation and cytokine to acquire the properties of an effector cell [76, 77]. For instance, T cell-DC interactions occur in phases that are associated with the acquisition of distinct effector phenotypic qualities [39]. The stability of the peptide:MHC interaction affects the kinetics of the transition through these phases as unstable peptide:MHC complexes prevented long-lasting T cell-DC interactions and optimal T cell responsiveness. Models of T cell activation also rely on TCR affinity as a predictive metric of functional outcome. While TCR affinity for 35A is not different from the native GP33 epitope, 35A has a significantly shorter half-life on H-2D^{b} (Fig 1A). The decreased expression of IRF4 on 35A stimulated cells demonstrates that peptide stability on MHC causes TCR perception of antigen quality to be lower than would be predicted by TCR affinity (Fig 1D-H). Interestingly, the identical levels of IRF4 at 24 hours in the *in vitro* response suggests 35A is sufficient to initially produce a quality response but the subsequent decrease in IRF4 seen at day 3 indicates the substandard nature of the ligand.

Therefore, IRF4 is not simply a direct readout for TCR affinity for antigen, but it can also readout overall quality and strength of the TCR interaction with pMHC ligand, which in this case is greatly affected by the shorter half-life of 35A. While both wildtype GP33 and variant 35A can initiate 2-3 early rounds of preprogrammed cell division by day 3 post stimulation (Fig 2C), the proliferation of 35A-primed cells rapidly diminishes thereafter resulting in less cell accumulation at later time points (Fig 2A and 2D), day 8 *in vivo*, day 6 *in vitro* respectively. Our previous characterization of the intracellular signaling response to the variant epitope demonstrated deficient downstream intracellular signaling as evidenced by low pErk, low Egr1 expression and sustained SHP-1 activity which blocks further positive signaling downstream of the TCR [52]. Indeed, recent work has shown that T cells need as little as 3 minutes to interpret differences between closely related peptides and initiate an appropriate response based on signaling cascades engaged [78]. Therefore while some downstream propagation of TCR signaling occurs, variant peptide stimulation provides insufficient signal to support continued clonal expansion.

While IL-2 has been found to be expendable for the earliest rounds of division, maintenance of the proliferative response is IL-2 dependent [64]. We observed a severe deficit in the ability of 35A primed T cells to produce IL-2 (Fig 4D), as well as decreased expression of the high affinity IL-2Rα chain CD25 on cells responding to the variant (Fig 4A). In addition, the CD25 expression inversely correlated with number of division cycles (*in vitro*), resulting in fewer variant primed cells entering later division cycles (Fig 4C). Our data shows that while 35A recognition meets the

minimum threshold of activation to engage this “auto-proliferative” response, the response lacks sufficient signals to maintain an activated phenotype. Diminished production of IFNγ both in vitro (Fig 4E) and *in vivo* (Fig 4F and 4G) further highlights that variant cells are unable to completely acquire effector function.

The consequence of this low quality TCR stimulation by 35A is a significant increase in apoptosis, as evidenced by co-staining with Annexin V and 7AAD and diminished expression of the anti apoptotic protein Bcl-2 in 35A stimulated cells (Fig 3). Low Bcl-2 levels are indicative of skewing towards apoptotic signaling as T cells balance both Bcl-2 mediated pro-survival and Bax/Bim mediated pro-apoptotic death signals [79, 80]. Several studies have highlighted the ability of T cells to mount a response to low affinity antigens with the outcome being a contracted or restricted response mediated by decreased division cycles of responding cells and/or increased apoptosis [81-83]. However, in those studies, the variant had mutations at TCR contact residues that directly modulated TCR affinity. Here, we demonstrate that an epitope with reduced affinity for MHC rather than TCR can also negatively modulate the T cell response by inducing division and subsequent death of dividing cells. The study of MHC variants is particularly impactful as this type of mutational escape has the potential to effect a wider breadth of CTL responses, both on an individual and population level, than if the mutation affected a single TCR clone as APLs do. Additionally, it has been observed that the majority of escape mutations identified in HIV infections affect binding to HLA [84, 85]. Thus our model is particularly relevant for studying differences in how disruption of MHC binding to viral antigens differ from that of

TCR binding to MHC:peptide and how that may affect development of therapeutic and prophylactic interventions.

Recent work has highlighted the importance of cellular metabolism in modulating T cell fate decisions[66, 67, 70, 86]. The transition from a naïve to an activated CD8+ T cell is associated with metabolic reprogramming to a glycolytic phenotype. Additionally, several studies have connected the ability of T cells to produce cytokines with its mode of generating metabolic fuel [68, 87]. Although switching from mainly oxidative phosphorylation to aerobic glycolysis is important for sustaining proliferation and survival, it is most critically required for cytokine production. Since we observed a deficit in both IL-2 and IFNγ production in our 35A stimulated cells, we investigated whether metabolic differences could further explain the defective CD8+ T cell response to variant 35A at later time points. Our results indicated that variant-primed cells were lacking in their ability to undergo glycolysis and also were inefficient in glucose uptake (Fig 5). This may be due to less glucose receptor expression and/or inability to efficiently metabolize glucose into the lactate end product. As aerobic glycolysis is important for generation of ATP as well as secondary byproducts used for synthesis of lipids, nucleotides and other proteins, the various metabolic defects in variant primed cells provide an explanation for why they fail to thrive later in the response. It should also be noted that IRF4 expression has been found to regulate transcription of enzymes involved in metabolic function like glucose transport receptors and HIF-1, accordingly [60]. Our observation showed IRF4 correlated with both peptide dose and peptide potency, with the variant response found to have the lowest IRF4 expression (Fig 1). Based on these observations, it is

plausible that lower IRF4 expression in variant primed cells may contribute to the inability of the cells to efficiently produce the glycolysis-derived energy needed to fuel proliferation and survival.

Targeting the metabolic state of immune cells for therapeutic purposes has shown great promise in models of viral infection, cancer, and autoimmunity [88, 89] Our results found that T cells responding to the variant 35A have decreased glycolysis and glycolytic capacity as compared to the response to the wildtype epitope. We might then hypothesize that therapy to enhance glycolysis may allow for increased survival of T cells confronted with MHC variants. It should be noted that other studies have found that blocking glycolysis with 2-deoxyglucose (2-DG) augments the formation of memory CD8+ T cell responses while enhancing glycolysis led to an increase of short term CD8+ effectors during primary response [70]. It has also been shown that glycolytic flux is essential for the acquisition of IFNγ producing abilities, particularly by memory CD8+ T cells undergoing secondary re-challenge [68, 90]. Thus, therapies to modulate T cell metabolic programs require careful consideration of the target cell population.

In conclusion, this study demonstrates that immunological ignorance is not simply a passive form of viral evasion, but rather an active process by which viral variants disrupt optimal T cell recognition leading to the deletion of activated effectors through modulation of critical pathways of T cell survival and activation. Recovery of the response to viral variant may require a multi-targeted approach involving both

increasing cellular perception of signal strength and modulation of the bioenergetic program.

Chapter 2 Figures

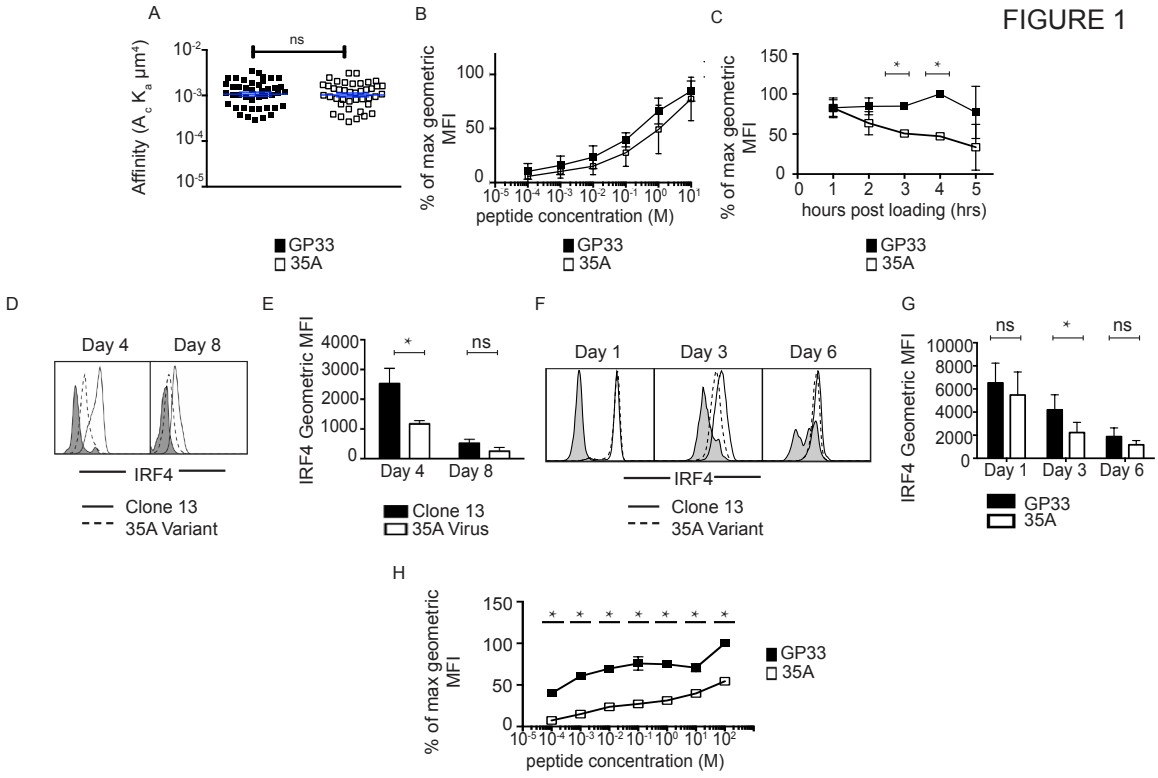

Fig 1. Variant peptide 35A maintains the same affinity for TCR as native GP33 epitope despite MHC instability and decreased IRF4 expression

(A) Affinity of GP33 and 35A epitope using the two-dimensional (2D) micropipette adhesion system. Average of 5 independent experiments. Total of 43 pairs tested for GP33 and 41 pairs for 35A. p=0.775 n.s.(two tailed unpaired T test) (B) Stability of peptide on MHC was measured using an RMAS assay by loading different concentrations of peptide on empty class I and assaying surface expression of H-2D^b after 30 minutes post loading. Average of two independent experiments. (C) 10μm of each peptide is loaded onto RMAS cells and the gMFI of H-2D^b is measured over time

to assess peptide stability and half life. Average of 3 independent experiments. *p<0.05 (Two way ANOVA Holm-Sidak)

(D) and (E) 10^5 P14 CD8+ T cells were adoptively transferred into congenic hosts and infected with 2 x10^3 PFU Clone 13 or 35A variant. At 4 and 8 days post infection, spleens were harvested analyzed by flow cytometry for IRF4 expression on donor cells. Average of 3-4 independent experiments with 3-4 mice pooled per group. *p<0.05 (Paired t test)(F) and (G) P14 CD8 T cells were stimulated *in vitro* with 10μm wildtype GP33 peptide or variant 35A peptide and harvested at indicated time points and analyzed by flow cytometry for IRF4 expression. Average of 3-4 independent experiments. *p<0.05 (Paired t test) (H) P14 T cells were stimulated with GP33 or 35A peptide over a range of doses and analyzed for IRF4 expression at day 3. Data shown is the average of 2 independent experiments. *p<0.05 (Multiple t tests) Gray shaded histograms refer to cells isolated from uninfected control hosts or unstimulated cells. Error bars on all plots indicate ± SEM

FIGURE 2

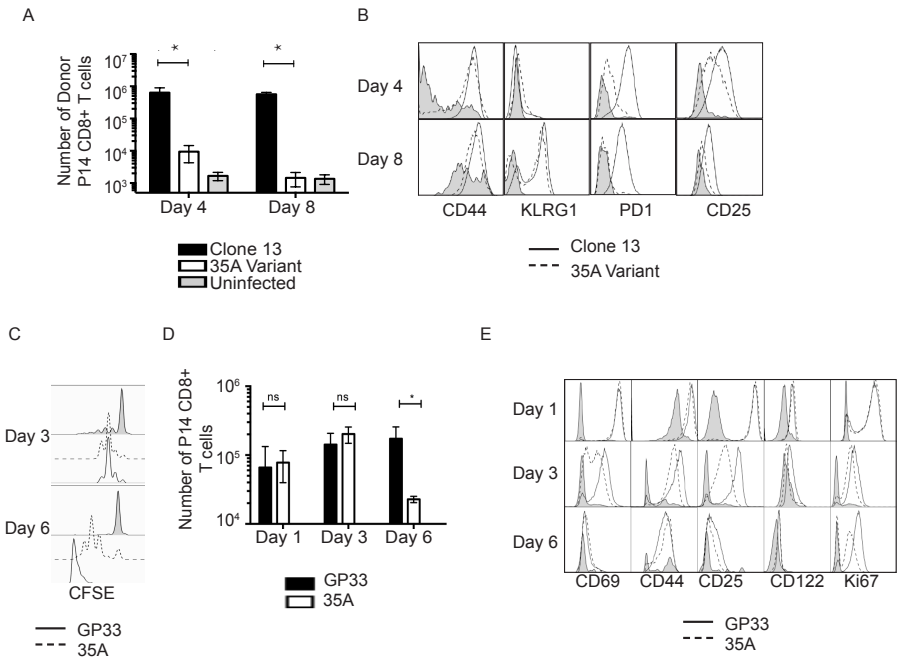

Fig 2. Recognition of 35A induces activation and proliferation of P14 CD8+ T cells without accumulation.

(A) 10^5 P14 Thy1.1 CD8+ T cells were adoptively transferred into C57BL/6 hosts and infected one day later with 2 x10^3 PFU Clone 13 or 35A variant virus i.v. At 4 and 8 days post infection, spleens were harvested and analyzed for recovery of donor CD8+ T cells (A) and phenotypic analysis (B). Data from 3-5 experiments shown. n=2-3 mice per group. *$p<0.05$ (2 way ANOVA multiple comparisons). Error bars indicate ± SEM. (C) P14 T cells were stimulated *in vitro* with 10μm GP33 or 35A peptide and harvested at day 3 or 6 to evaluate division by CFSE (C) total CD8+ T cell expansion (D) and phenotypic analysis (E). Representative data is shown in (C and E) or average of 3 experiments shown in (D) with samples run in duplicate for total cell number counts. *$p<0.05$ (2 way ANOVA multiple comparisons). Error bars indicate ± SEM. Gray shaded histograms refer to cells isolated from uninfected control hosts or unstimulated cells.

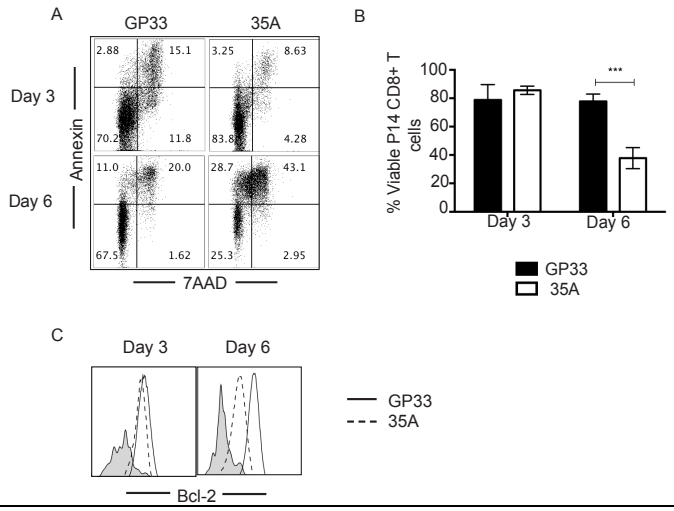

FIGURE 3

Fig 3. 35A Stimulation results in reduced viability in responding cells.

P14 CD8+T cells were stimulated with 10μm of GP33 or 35A peptide *in vitro* and evaluated for viability at day 3 and 6 post stimulation by staining with Annexin and 7AAD (A) or using Zombie Yellow cell viability dye (B). Representative flow plot shown in (A). Total of 4 experiments. ***p=0.002 (Multiple T tests). Error bars indicate ± SEM. Cells were also assayed for the detection of pro-survival Bcl-2 molecule (C) Representative flow plot shown. Total of 2 experiments. Gray shaded histograms refer to unstimulated cells.

FIGURE 4

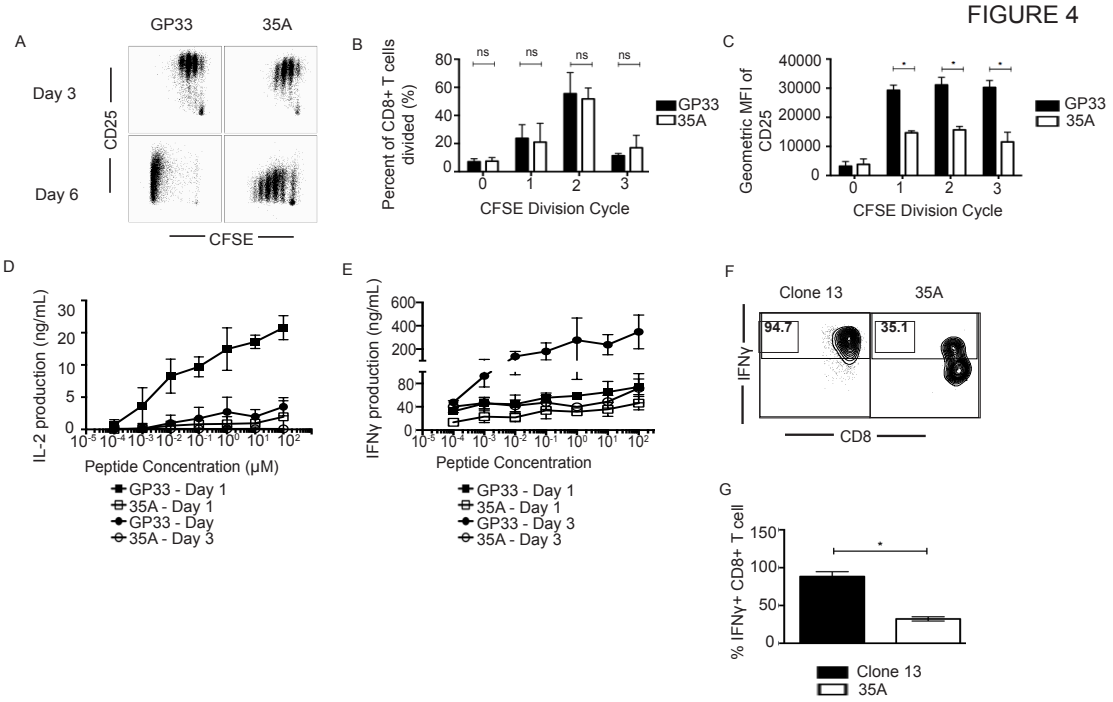

Fig 4. CD25 expression, IL-2, and IFNγ are decreased after 35A stimulation despite equivalent early rounds of division

(A) CD25 expression was assayed on P14 T cells after 3 and 6 days of *in vitro* stimulation with 10μm peptide and correlated with division cycles indicated by CFSE. (B) and (C) Graphical representation of Fig 3A. Average of 2 experiments. *$p < 0.05$ (Two way ANOVA-Holm Sidak). (D) IL-2 production after 24 and 72 hours of *in vitro* stimulation with peptide measured with ELISA. Average of 3-4 experiments with samples run in duplicate for each time point. (E) IFN**γ** production after 24 and 72 hours of in vitro stimulation with peptide measured via ELISA. Average of 2 experiments with samples run in duplicate for each time point. (F) Representative flow plot of *in vivo* IFN**γ** production at day 4 post infection. (G) Graphical representation of *in vivo* IFN**γ.** Average of 2 experiments with 3 mice pooled for each sample. *p=0.0153 (Unpaired t test). Error bars indicate ± SEM.

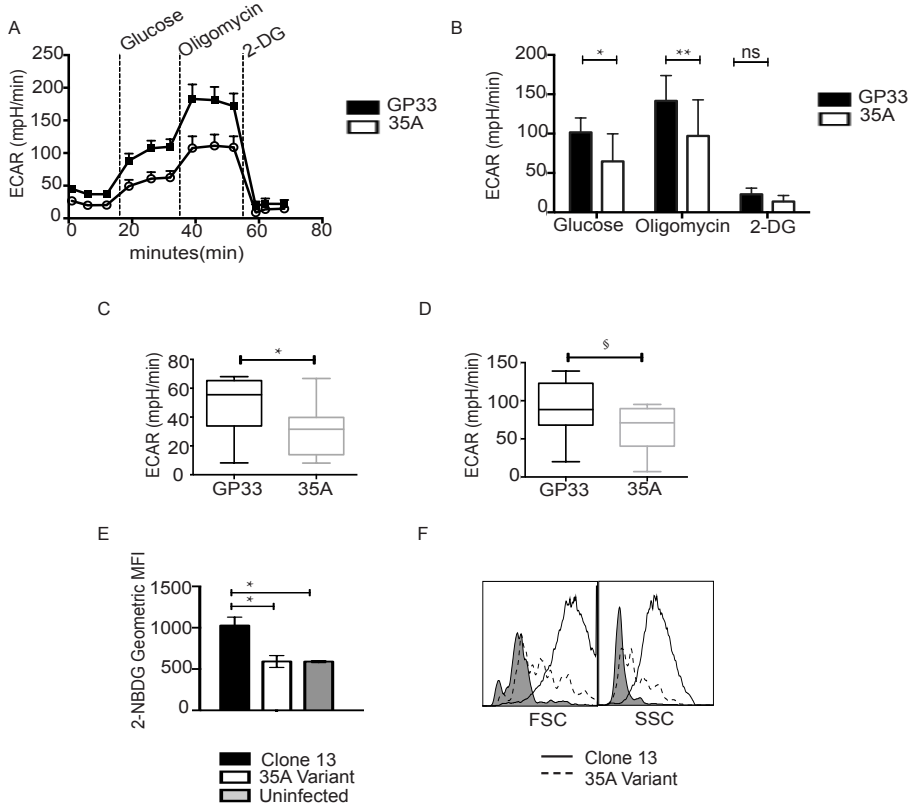

FIGURE 5

Fig 5. Variant 35A stimulated cells demonstrate a decrease in metabolic fitness

P14 CD8+ T cells were assayed for glycolytic function using the Seahorse extracellular flux assay. (A) Glycolysis stress test assay was performed by sequentially adding the indicated reagents to purified CD8+ cells primed *in vitro* with either GP33 or 35A at day 3. Representative data shown (B) Graphical representation of stress test assay. * $p < 0.05$, **$p < 0.01$ (Two way ANOVA-Holm Sidak test). Data shown is the average of 8 experiments with samples run in quadruplicate. Glycolysis (C) and glycolytic capacity (D) were calculated from stress test ECAR values. Glycolysis was calculated by measuring the change in ECAR after addition of glucose and subtracting the basal ECAR levels. Glycolytic capacity is defined as difference between the

maximum ECAR achieved after addition of oligomycin and basal ECAR. *p< 0.05, §p=0.07 (Wilcoxon matched pairs signed rank test) Data shown is the average of 8 experiments with samples run in quadruplicate. (E) Glucose uptake was measured *ex vivo* from infected mice through the detection of fluorescent 2-NBDG by flow cytometry. Two independent experiments with 3 pooled mice in each experiment. Multiple t tests *p < 0.05 (F) *Ex vivo* cells were also analyzed for cell granularity (SSC) and size (FSC) using flow cytometry. Representative data shown. Error bars indicate ± SEM. Gray shaded histograms refer to cells isolated from uninfected control hosts or unstimulated cells.

Materials and Methods

Mice

P14 CD45.1 or P14 Thy 1.1 TCR transgenic mice[91] were housed and bred in the Emory University Department of Animal Resources facility and used in accordance with the Institutional Animal Care and Use Committee–approved protocols. C57BL6 mice used as hosts for adoptive transfers were purchased from National Cancer Institute (NCI) or The Jackson Laboratory. Mice were used between 8-15 weeks of age.

Ethics Statement: This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Emory University Institutional Animal Care and Use Committee (IACUC) Approval number: DAR-

2002722-051317GN. All intravenous injections and euthanasia techniques (CO_2 exposure) were done according to the approved IACUC protocols and all efforts were made to minimize undue suffering. Mice were monitored daily for signs of adverse health effects (reduced grooming, excessive weight loss, lethargy)

Peptides

Peptides were synthesized at Emory University on a Peptide Technologies Incorporated Prelude synthesizer. The GP33-41 sequence (KAVYNFATM) and the 35A variant sequence (KAAYNFATM) were used at a concentration of 10μm unless otherwise indicated.

Virus

Lymphocytic choriomeningitis virus (LCMV) Clone 13 and variant 35A were kind gifts from Dr. Rafi Ahmed (Emory University) and were made as described in [92, 93]

Adoptive Transfers

CD8+ T cells were purified from P14 TCR transgenic animals using the EasySep CD8+ T cell Enrichment Kit (StemCell) and transferred intravenously to congenic C57Bl/6 hosts. Mice were intravenously infected with $2x10^3$ PFU of either virus 24 hours after adoptive transfer. Spleens were harvested at indicated time points for analysis of donor cell expansion and phenotype.

Cell Culture

P14 splenocytes were stimulated for one hour with peptide and then washed and re-plated in 96 well plates. For analysis of CFSE division, cells were labeled with 2.5mM CFSE for 3 minutes prior to peptide stimulation. Cells were subsequently incubated with fetal bovine serum (FBS) then washed with media. Cells were then cultured in 37°C incubator for indicated time periods after which cells were harvested for phenotypic analysis.

Enzyme-linked immunosorbent assay (ELISA)

To measure IL-2 and IFNγ, supernatants from cell cultures were added to plates previously coated with purified anti-IL 2 or anti- IFNγ antibody. After overnight incubation, biotinylated anti-IL2 or anti- IFNγ antibody (Ebioscience) were added followed by alkaline phosphatase-conjugated avidin and p-nitrophenylphosphate substrate (Sigma). Cytokine concentrations were determined based on standard curve derived from recombinant stocks of IL-2 and IFNγ.

Flow cytometry

Cells were stained on ice with antibodies to surface markers (CD25 (PC61), CD44 (IM7), KLRG1 (2F1), PD1(29F.1A12), CD69 (H1.2F3), CD122 (TM-b1), H-2D^{b} (28-14-8) THY1.1 (HIS51) for 30 minutes on ice. For detection of intracellular markers (Bcl-2 (3F11), IRF4 (3E4), Ki67 (B56), IFNγ (XMG1.2)), cells were fixed and permeabilized using the Transcription Factor Staining Set (Ebioscience). Annexin V, 7AAD and Zombie Yellow Viability Kit (Biolegend) were utilized according to manufacturers instructions. All antibodies were purchased from BD or Ebioscience.

Metabolic Assays

For the 2-NBDG uptake assay, cells were re-suspended in glucose free media and allowed to incubate with 10μm of 2-NBDG for 20 minutes. Cells were washed with glucose free media and analyzed by flow cytometry. The glycolysis stress test was performed by purifying CD8+ T cells using the EasySep CD8+ T cell Enrichment (StemCell) and adhering them to a 96 well plate with CellTak tissue adhesive (Corning). Cells were incubated in a CO_2 free incubator for a minimum of 30 minutes. Samples were then run on the Seahorse XF extracellular analyzer 96^e where glucose, oligomycin and 2-deoxyglucose (2-DG) were sequentially added to cells. Changes in glycolytic activity were measured by changes in extracellular acidification rate (ECAR). Glycolysis was calculated by measuring the change in ECAR after addition of glucose and subtracting the basal ECAR levels. Glycolytic capacity is defined as the difference between maximum ECAR achieved after addition of oligomycin and basal ECAR levels.

Two-dimensional (2D) micropipette adhesion frequency assay

Human RBCs (obtained from healthy volunteers) were biotinylated using Biotin-X-NHS (EMD4 Biosciences) and subsequently labeled with streptavidin (Thermo Scientific) and biotinylated peptide:MHC-I monomers. P14 T cells were purified from spleens using the EasySep CD8+ T cell Enrichment Kit (Stemcell). Single cells were aspirated onto opposing glass micropipettes in a cell chamber mounted on the stage of an inverted light microscope. An electronically controlled piezoelectric actuator controlled repeated T cell contact with a stationary pMHC-coated RBC for two

seconds. Binding events were visualized as an elongation of the RBC membrane upon retraction of the T cell. Cells were brought into contact 50 times in order to calculate an adhesion frequency (P_a). Quantification of pMHC ligand (m_l) and TCR-α receptor (m_r) densities were determined by flow cytometry using BD QuantiBRITE PE Beads for standardization (BD Biosciences). Surface densities as well as adhesion frequency (Pa) were then used to calculate two-dimensional affinity using the following equation: $A_c K_a = -\ln[1 - P_a(\infty)/m_r m_l$. Geometric mean affinities are reported ± SEM. This assay is also described in detail in [9] and[94]

RMA/S MHC Class I Stability Assay

RMA/S cells were incubated at room temperature overnight to allow for surface expression of empty H-2D^b. Cells were plated at 2x10^5 cells per well in a flat bottom 96 well plate and loaded with peptides of interest. Cells were placed at 37°C to induce downregulation of unoccupied MHC molecules and harvested at indicated time points to assay for expression of H-2D^b by flow cytometry. Half life was calculated by using data from Figure 1C and is expressed in the following equation: $t_{1/2} = (t * \ln(2))/(\ln(N_0/N_f))$, where $t_{1/2=}$ Half life, t=time elapsed, N_0=initial H-2D^b expression after loading, N_f=final H-2D^b expression at final time point assayed

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6 software (Software for Science). Significance was calculated using two-way ANOVA, unpaired t test, and Wilcoxon matched pairs signed rank tests.

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Conflict of Interest

The authors declare no commercial or financial conflict of interest.

Chapter 3: SHP-1 deficiency does not restore the CD8+ T cell response to LCMV viral variant 35A

Abstract

Viruses employ various mechanisms to evade recognition by host immune effectors and establish persistence in the host. We have previously shown that CD8+ T cells responding to a viral variant of lymphocytic choriomeningitis virus (LCMV) have high SHP-1 activity. Src homology region 2 domain-containing phosphatase-1 (SHP-1) is a non-receptor phosphatase found in hematopoietic cells and is thought to play a primarily negative regulatory role in many signaling pathways. SHP-1 exerts important negative regulation on proximal TCR signaling by blocking early T cell activation. Because of its role in dampening TCR signaling, we hypothesized that removal of SHP-1 in T cells would restore the response to the viral variant. Using CD4 Cre Fl SHP-1 mice (SHP-1 KO), we evaluated the response of CD8+ T cells to the viral escape mutant in the absence of SHP-1. Loss of SHP-1 did not significantly increase expansion of the polyclonal SHP-1 KO or P14 TCR transgenic cells in infection with the 35A virus. We also failed to see any significant difference in the antigen specific expansion and effector function (IFNγ or granzyme B) to the other dominant D^b restricted epitopes, GP33, GP276, and NP396, in the SHP-1 deficient models. These observations suggest that removal of a negative regulator may not have the expected therapeutic benefit of an enhanced T cell response.

Introduction

As T cell receptors lack inherent activity, kinases and phosphatases play an integral role in transducing signals from the TCR to downstream pathways. The addition and removal of phosphates from key residues allow for termination or activation of particular signaling cascades. For instance, after peptide:MHC engagement, the co-receptor brings Lck in proximity to the CD3 zeta chains which contain immunoreceptor tyrosine-based activation motif (ITAM). Phosphorylation of ITAMs initiates recruitment of other signal molecules that also have ITAM or ITIM motifs. These residues also often serve as docking sites that recruit other molecules to the site. Src homology region 2 domain-containing phosphatase-1 (SHP-1) is a non-receptor phosphatase that is involved in negatively regulating a variety of cellular signaling pathways. With respect to T cells, it is a phosphatase that plays a significant role in the early signaling steps of TCR activation. Its activity of dephosphorylating Lck negatively regulates or terminates TCR signaling by aborting further downstream signaling from the receptor.

While negative regulation of the TCR is a needed control mechanism, inappropriate inhibition of T cell activation can be detrimental to the development of a productive immune response. SHP-1 activity has been found to play a role in both antagonism and anergy of T cells. While this mechanism can be manipulated for therapeutic purposes as in the case of models of Multiple sclerosis or Type I diabetes, the handicapping the T cells via a viral variant has been observed and could prove detrimental. Thus, efforts to interrupt phosphatase activity could prove to have

therapeutic benefit.

Our lab has previously demonstrated that CD8+ T cells responding to the 35A lymphocytic choriomeningitis virus (LCMV) viral escape mutant triggers sustained activation of SHP-1. In an effort to restore responsiveness to this viral escape mutant, we hypothesized that T cell specific SHP-1 deficiency would enhance the response to this viral variant. Utilizing a mouse model in which a floxed SHP-1 construct is under the control of a CD4 promoter, our CD4 Cre Fl SHP-1 mice lose SHP-1 in all T cells after the expression of CD4 in the thymus. We found that loss of SHP-1 did not enhance the response to the 35A viral variant. Further analysis revealed that antigen specific expansion and effector function to the other dominant D^b restricted epitopes, GP33, GP276, and NP396, were also not perturbed by SHP-1 deficiency. These observations highlight the potentially complex role that negative regulators may play in controlling T cell responses and supports the idea that targeting of a single biomarker might not overcome negative regulation.

Results

Recognition of viral variant is characterized by sustained SHP-1 activity

Previous work in our lab identified increased SHP-1 activity when P14 TCR transgenic CD8+ T cells are stimulated with 35A peptide in vitro. While SHP-1 activity is a standard response initiated post TCR engagement, SHP-1 activity is rapidly down-regulated in T cell responses to agonist peptides like GP33. In contrast, 35A stimulation results in a sustained SHP-1 response over several minutes which I

confirmed in Figure 1. Further characterization revealed that in response to the wildtype GP33 epitope, the catalytic cysteine of SHP-1 required for de-phosphorylating substrates is rapidly inactivated by oxidation. This step prevents SHP-1 from blocking TCR signal transduction. In 35A-stimulated cells however, SHP-1 remained in a reduced state allowing for active SHP-1 function and lack of full TCR signal propagation.

SHP-1 deficiency does not restore the response to the 35A viral variant

Since 35A recognition was characterized by sustained SHP-1 activity, we tested whether SHP-1 deficiency could enhance T cell responsiveness to the 35A antigen. We decided to test the effect of SHP-1 deficiency in a monoclonal response using P14 TCR transgenic mice that were bred to CD4 Cre FLSHP-1 mice, hereafter referred to as P14 FLSHP-1. We adoptively transferred CD8+ T cells from P14 FLSHP-1 mice into Thy1.1 recipients and infected with either Clone 13 (contains the D^b restricted gp33-43 epitope) or 35A viral mutant (contains the valine to alanine mutation in position 35 in the D^b/gp33-43 epitope). We saw no difference in expansion in response 35A infection (Figure 3). We also failed to observe a difference in the response to the wildtype GP33 epitope.

To test whether SHP-1 deficiency could restore the response to the variant 35A epitope in a polyclonal setting, we infected B6 or SHP-1 knockout mice with LCMV Armstrong or 35A viral mutant. Interestingly, we found no difference in the D^b/gp33 tetramer response to the 35A viral escape mutant. As in our P14 experiments, we

again found no difference in the tetramer response to the original gp33 epitope (Figure 2).

SHP-1 deficiency does not enhance the responsiveness to dominant LCMV CD8+ epitopes at day 8 post infection

To better understand how loss of SHP-1 affects the response to other LCMV epitopes, we infected mice with B6 or FLSHP-1 mice with Clone 13 or 35A virus. We found no difference in the number of tetramer positive cells for the D^b restricted GP33, GP276 or NP396 epitopes (Figure 4). We also assayed whether the absence of SHP-1 impacted effector function. IFNγ is known to be a potent cytokine in antiviral responses and cytotoxic CD8+ utilize granzyme to target infected cells. We found no significant difference in the numbers of IFNγ producing cells or granzyme B positive cells in either the SHP-1 knockouts or B6 mice (Figure 5).

Naïve SHP-1 deficient mice have "virtual memory" CD44hi CD8+ T cells

While our B6 and SHP-1 knockout mice were phenotypically similar in their responses to LCMV infection, we noted that naïve mice had a higher frequency of CD44hi CD8+ T cells (Figure 6). We observed this population of CD44hi CD8+ T cells both in naïve polyclonal CD4 Cre FLSHP-1 mice as well as our transgenic P14 CD4 Cre FLSHP-1 mice. These cells have been previously described as 'innate CD8+ memory' cells and are thought to acquire phenotypic memory markers and expand due to excess IL-4 in the periphery[95]. Canonical memory CD8+ T cells are CD44hi

CD62L lo and rapidly expand after rechallenge, reaching their peak at day 5 post infection[75]. To assay whether the CD44hi cells we observed in the naïve polyclonal CD4Cre FLSHP-1 mice reached peak expansion at an earlier timepoint, we infected B6 and SHP-1 knockout mice with LCMV Armstrong and enumerated tetramer specific cells at day 5. Even at day 5 post infection, we observed no significant difference in the number of D^b/gp33 specific cells detected (Figure 7). While the SHP-1 knockouts have cells that are phenotypically similar to true memory cells, there is no increased expansion of these cells detected at the conventional peak of infection for a secondary rechallenge response.

Discussion

Regulation of TCR activation is a necessary and important part of maintaining efficient immune responses. However, in cases where inappropriate regulation hampers productive immunity, there is a need to overcome these regulatory mechanisms. SHP-1 has been implicated in the regulatory mechanism behind several T cell phenotypes in which an aspect of T cell function is dampened including anergy, antagonism, and exhaustive phenotypes[20, 96, 97].

We have previously investigated the CD8 T cell response to the LCMV viral variant 35A and observed increased SHP-1 activity as a characteristic of the response[52]. We hypothesized that removal of this negative regulator of TCR proximal signaling would enhance responsiveness to the viral variant. However, we were unable to detect a measurable Db/gp33 specific response to the mutant 35A epitope in either a polyclonal or monoclonal SHP-1 knockout models (Figure 2 and Figure 3). In

addition, we did not observe any change in the tetramer response to the two other dominant LCMV epitopes, GP276 and NP396 (Figure 4). We also tested whether SHP-1 deficiency impacted the effector function. We found no change in the numbers of IFNγ or Granzyme B positive cells (Figure 5). The observation of CD44hi CD8+ 'innate CD8+ memory' T cells in our naïve CD4Cre FLSHP-1 mice led us to hypothesize that these cells could be responding with earlier kinetics like conventional memory T cells. We infected mice with LCMV and assayed the tetramer specific Db/gp33 response and found no significant difference in the number of tetramer specific cells expanded at day 5 post infection.

These data suggest that removal of SHP-1 in this model does not directly impact the ability of CD8+ T cells to respond to LCMV antigens as measured by tetramer, IFNγ and Granzyme B. This was an unusual finding as many negative regulators of TCR have been targeted with the intent of increasing T cell responsiveness. For instance, programmed death receptor 1 (PD-1) is an inhibitory receptor expressed on T cells after activation and functions in negatively regulating TCR signaling. While transient expression of this marker is normal, sustained expression of this receptor on T cells leads to a restriction of effector functionality like cytokine production. As the PD-1 receptor lacks inherent signaling ability, SHP-1 and SHp-2 provide the inhibitory function that PD-1 expression denotes[98]. Current therapies to overcome this "chronic exhaustion" include PD1 blockade treatment which has been shown to overcome this negative regulation of TCR functionality and restore cytotoxic abilities of CD8+ T cells in both anti-tumor and anti pathogen responses. Other common negative regulators that have been targeted for treatment of disease include CTLA-4

which has been targeted for the alleviation of allograft responses in transplant as well as autoimmunity in both animal and clinical models[99].

SHP-1 itself has also been highlighted as a potential therapeutic target in the treatment of murine models of viral infection[100] as well as anti-tumor responses[101]. In these studies, investigators observed SHP-1 deficiency caused increase expansion of T cells mediated by a decrease in the TCR activation threshold. This response allowed for more sensitive response to low potency or low dose antigen. They also observed a significant increase in short lived effectors (SLEC) at the peak of CD8+ T cell expansion. A similar response was observed in the tumor model; namely an increase in SLEC in response to models of leukemia and a solid liver tumor. Similar to our observations, the investigators did not detect any significant difference cytokine production or degranulation ability in the absence of SHP-1. In the above two studies, the Cre recombinase construct was expressed under the control of the distal Lck promoter[102] rather than the CD4 promoter that we utilized. The distal Lck promoter causes Cre to be expressed later than that of CD4 driven Cre constructs. This could differentially affect the highly regulated thymocyte development process thus yielding T cells with different levels of responsiveness.

To fully elucidate the effect of SHP-1 on the antiviral response, several other areas need to be studied. First, the effect of SHP-1 deficient CD4 T cells needs to be explored and particular their impact of CD8 T cells in a primary response. Our CD4 Cre model results in loss of SHP-1 in any CD4 expressing cell type including CD4,

CD8 and NKT cells. Isolating the effect of a single population will help us to understand the contributions (or lack thereof) from each group lacking SHP-1. This study would also benefit from a closer and more direct assay of effector cytotoxic function in the knockout mice. Though we (and others) did not observe any change in cytokine production it would be worth performing killing assays and measuring viral titers to assess functionality of the effectors.

Another consideration in this model is the dual loss of SHP-1 in both CD8 and CD4 T cells since we are using a Cre mouse that is under the control of the CD4 promoter. For these studies, we focused primarily on the CD8 T cell response as it is the dominant population that is critical for the LCMV anti-viral response. CD4 T cells also participate in the response but are not critical for the primary response. The importance of CD4 T cell help is evident as loss of CD4+ leads to deficient CD8+ T cell memory response and persistent infection in the case of CL13. This model would benefit from further exploration into how SHP-1 deficiency affects CD4 T cells in the LCMV response as well as how they affect the generation of CD8 T cell memory. If there is a gross defect in the CD4+ compartment, it would most likely be seen upon rechallenge of memory population.

We failed to observe any enhanced responsiveness to the 35A viral variant in the absence of SHP-1 thus far. This reveals an interesting observation about the potential consequences of removing a negative regulator. More work is needed to better elucidate the role of SHP-1 in the anti-viral response.

Chapter 3 Figures

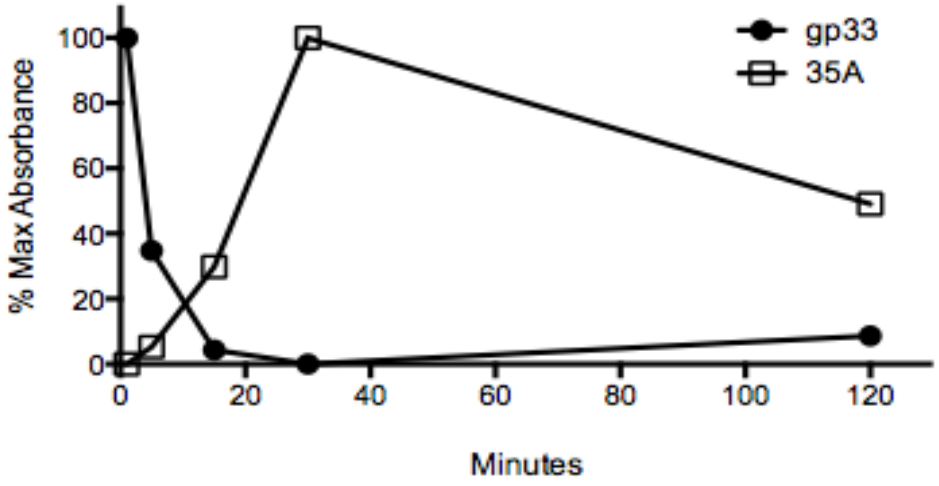

Figure 1. 35A Recognition induces sustained SHP-1 Activity.

Naïve P14 CD8+ T cells were stimulated with peptide for the indicated time period. SHP-1 was immunoprecipitated from cell lysates and used to act on tyrosine phosphorylated substrate. Malachite green was added for colorimetric detection of free phosphate which serves as metric of phosphatase activity.

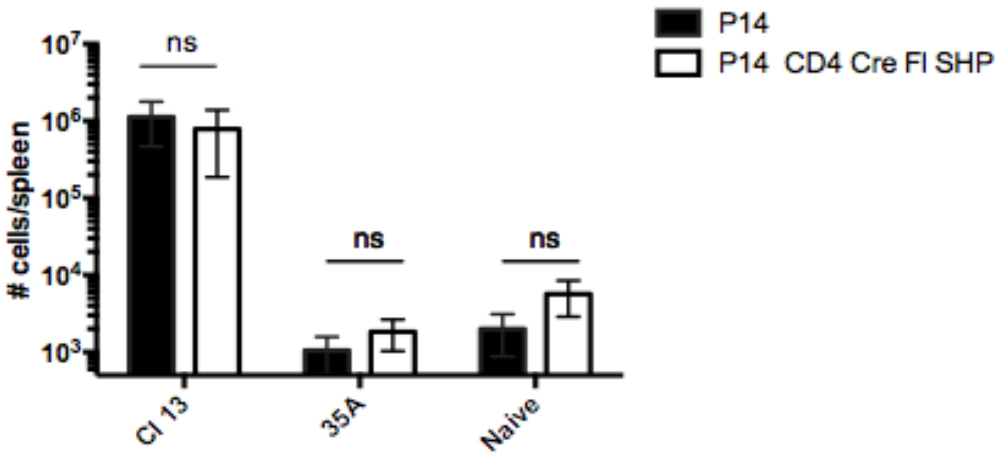

Figure 2. SHP-1 deficiency does not enhance the monoclonal P14 response to viral variant 35A

P14 or P14 FLSHP-1 CD8+ T cells were purified and transferred into Thy1.1 congenic recipients. Hosts were subsequently infected with either Clone 13 or 35A virus. Spleens were harvested at day 8 for flow cytometry analysis of donor cells. Average of 2 experiments. (Multiple t test)

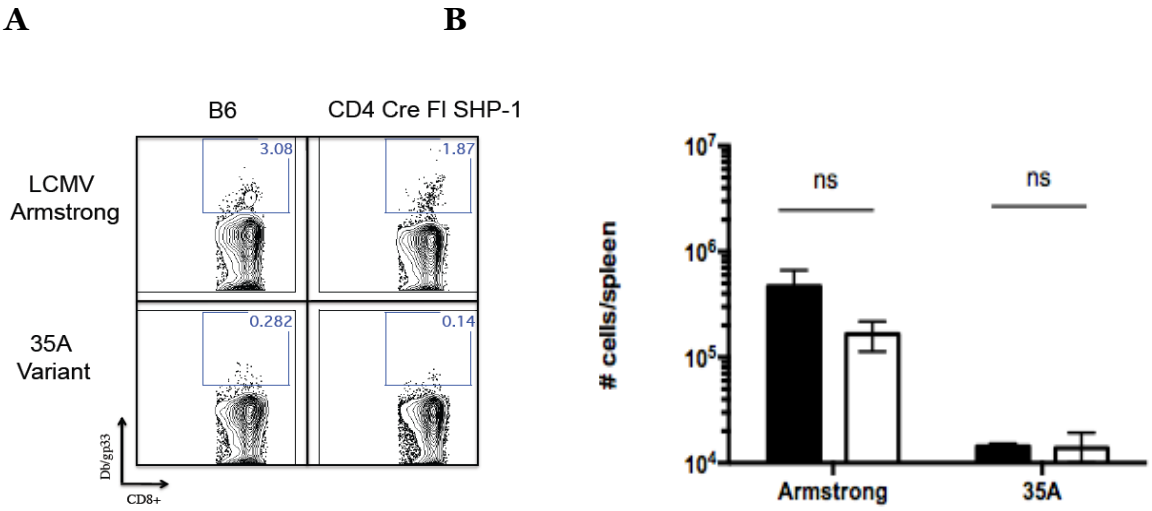

Figure 3. SHP-1 deficiency does not enhance the polyclonal response to viral variant 35A.

B6 or CD4-Cre FL-SHP-1 animals were infected with LCMV Armstrong or viral variant 35A. A) Spleens were harvested at day 8 and stained for tetramer. B) Total numbers of tetramer positive cells were enumerated. Average of 2 experiments. (Multiple t test)

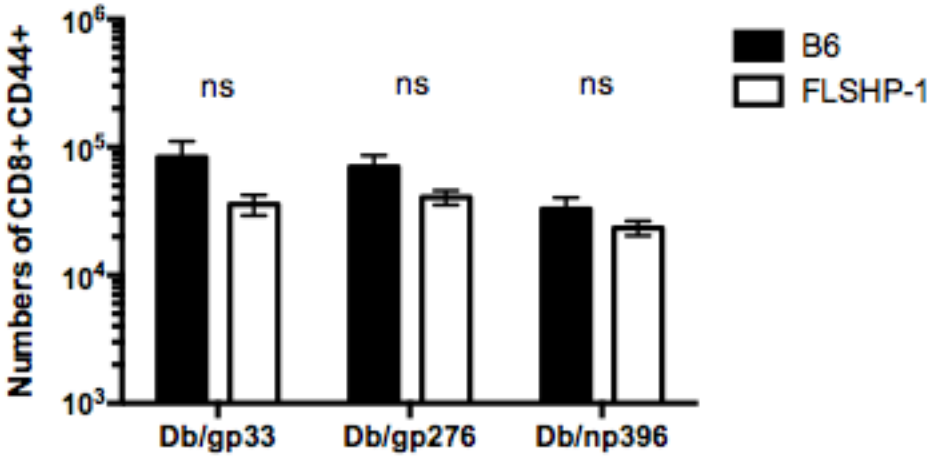

Figure 4. SHP-1 deficiency does not change the number of cells expanded at day 8 Clone 13 post infection.

B6 or CD4-Cre FL-SHP-1 animals were infected with Clone 13 or viral variant 35A. Spleens were harvested at day 8 and total numbers of tetramer positive cells were enumerated. Average of 4 experiments. (Multiple t test)

A **B**

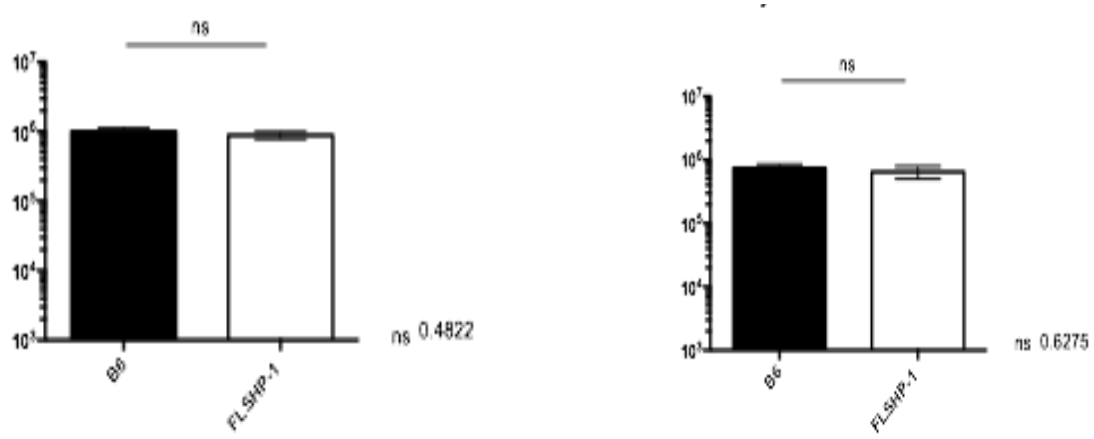

Figure 5. SHP-1 deficiency does not significantly change numbers of IFNγ + or Granzyme B+ cells

B6 or CD4-Cre FL-SHP-1 animals were infected with LCMV Armstrong or viral variant 35A. Spleens were harvested at day 8 and stimulated for 5 hours with peptide and BFA in an intracellular cytokine assay. A) Numbers of IFNγ positive cells B) Numbers of Granzyme B positive cells. Average of 2 experiments. (Student t test)

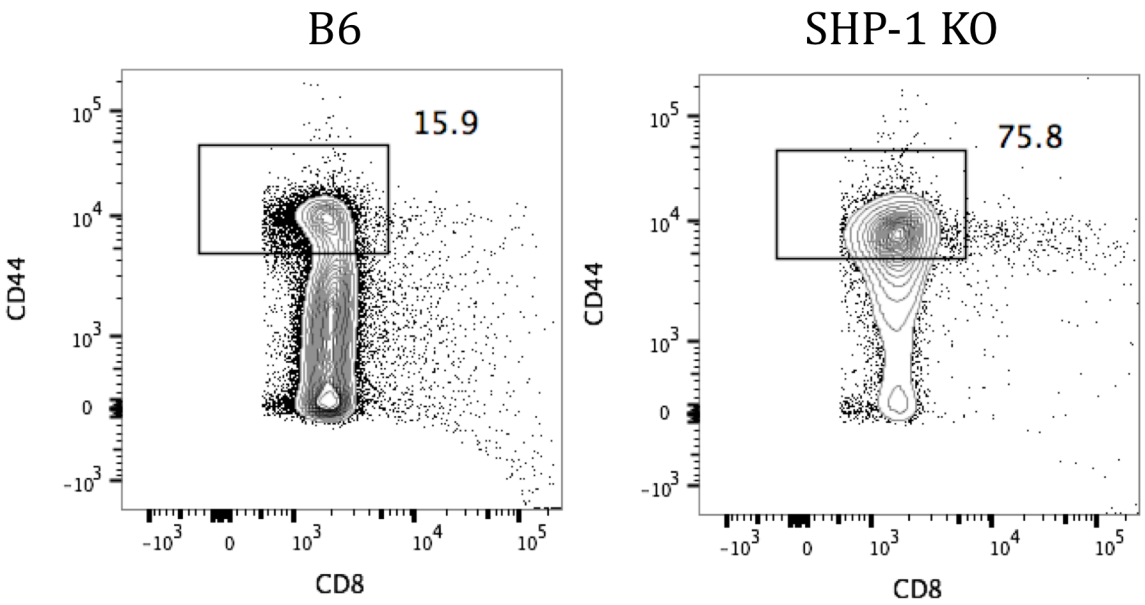

Figure 6. Naïve CD4 Cre FLSHP-1 mice have higher frequency of CD44hi CD8+ T cells.

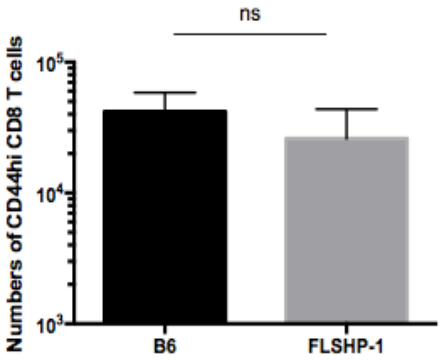

Figure 7. SHP-1 knockouts display no significant difference in numbers of antigen specific cells expanded at day 5 post Armstrong infection.

Materials and Methods

Mice

CD4 Cre-FLSHP-1 , Mx Cre FLSHP-1, and P14 Thy1.1 mice, were housed and bred in the Emory University Department of Animal Resources facility and used in accordance with the Institutional Animal Care and Use Committee–approved protocols. C57BL6 mice used as hosts for adoptive transfers or controls were purchased from the National Cancer Institute (NCI). Mice were used between 8-15 weeks of age.

Ethics Statement: This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Emory University Institutional Animal Care and Use Committee (IACUC) Approval number: DAR-2002722-051317GN. All intravenous injections and euthanasia techniques (CO_2 exposure) were done according to the approved IACUC protocols and all efforts were made to minimize undue suffering. Mice were monitored daily for signs of adverse health effects (reduced grooming, excessive weight loss, lethargy)

Peptides

Peptides were synthesized at Emory University on a Peptide Technologies Incorporated Prelude synthesizer. The GP33-41 (KAVYNFATM), GP276-286 (SGVENPGGYCL) and NP396-404 (FQPQNGQFI) peptides were used at a concentration of 10μm unless otherwise indicated.

Virus

Lymphocytic choriomeningitis virus (LCMV) Clone 13 and variant 35A were kind gifts from Dr. Rafi Ahmed (Emory University) and were made as described in [92, 93]. CD4 Cre FLSHP-1 mice and controls were directly infected with $2x10^6$ pfu of either Clone 13 or 35A.

Adoptive Transfers

CD8+ T cells were purified from P14 Thy1.1 TCR transgenic animals using the EasySep CD8+ T cell Enrichment Kit (StemCell) and transferred intravenously to congenic C57Bl/6 hosts. Mice were intravenously infected with $2x10^3$ PFU of either virus 24 hours after adoptive transfer. Spleens were harvested at indicated time points for analysis of donor cell expansion and phenotype.

Tetramers

Monomers were kind gifts from Dr. Rafi Ahmed (Emory University) and were used to make Db/GP33, Db/276, and Db/NP396 tetramers as prescribed by the NIH Tetramer Core Facility. One to two million cells were stained in 100ul volume at 1:100 ratio.

Intracellular Staining Assay (ICS)

One million cells are stimulated with peptide or PMA and ionomycin for 30 minutes before BFA is added. Cells are incubated for an additional 4.5 hours before staining for surface markers on ice. Cells are then fixed and permeabilized for intracellular

staining using the FoxP3 Transcription Factor Staining Kit (Ebioscience or Tonbo Sciences)

Phosphatase Activity Assay

Db-transfected fibroblasts were prepulsed with 10uM GP33-41 or 35A uM peptides for one hour. Purified naive P14 CD8 T cells were added to the fibroblasts and spun in centrifige at 600 rpm for 1 min to begin timecourse. Cells were removed from mM Na2EDTA, 0.5% Igepal, 1 mM Na3VO4, and 1/100 protease inhibitor cocktail I (Calbiochem)) and spun at 14,000 rpm for 5 min. SHP-1 was immunoprecipitated by coating 96 well plates from the Protein A/G Plate Immuno-precipitation Kit (Pierce) with 0.2ug of C19 anti-SHP-1 antibody (Santa Cruz Biotechnology) overnight. Plates were then coated with cell lysates for two hours at room temperature. Plates were then washed with 200ul per well IP buffer. PnPP Tyr Assay Buffer (Millipore) and 5 ul/well pTyr substrate peptide were added and incubated for 1 hour at 37C.
After transferring to a clean 96 well plate, 100ul of Malachite green solution (Millipore) was added to each well. Free phosphate levels were detected by the colormetric change and compared to a standard curve for quantification.

Chapter Four: Discussion and Conclusion

T cells maintain an intricate balance of maintaining specificity to recognize cognate antigen but also with sufficient TCR degeneracy to cover a wide range potential non-self entities. In addition, several regulatory mechanisms are in place to regulate the nature of the response such that the response is appropriate for the antigenic target.

This dissertation explored the question of how T cell sensitivity to antigen is impacted by changes in peptide affinity for MHC and in the removal of a negative regulator (SHP-1). It is through an understanding of how TCR binding to antigen is translated into a biological response that we can get a better idea of how pathogens subvert the immune response and how we can utilize this knowledge to improve T cell based therapies.

In Chapter 2, we presented a study that described a mechanism by which an LCMV viral variant evades CD8+ T cell immune surveillance by sub-optimally stimulating responding effectors. While 2D TCR affinity for the wildtype GP33 and the mutant 35A epitope are not significantly different, the variant has a significantly shorter half-life on H-2D^{b}. The unstable nature of the peptide on MHC classifies the peptide as an MHC variant peptide (MVP). One of our key findings is that diminished IRF4 expression as a characteristic of 35A stimulated cells despite unchanged TCR affinity. While the transcription factor IRF4 plays a role in the differentiation and function of many different cell types[103-109], IRF4 has most recently been identified as a read-

out of TCR affinity[60, 110]. This discovery was made when investigators stimulated OT-1 transgenic T cells (specific for the SINFEKEL epitope of ovalbumin) with altered peptide ligands of varying affinities and demonstrated that IRF4 expression was both affinity dependent and peptide concentration dependant with higher affinity/concentration of antigen leading to higher IRF4 expression.

Many studies have shown there are specific TCR signaling signatures that are dictated in a TCR affinity manner. For instance, the amount of CD3 zeta chain phosphorylation has been shown to correlate with the affinity of the ligand binding the TCR[111, 112]. Other proximal signaling pathways are also directly impacted by TCR affinity, including Lck phosphorylation, calcium flux and extent of ERK recruitment. But perhaps what is most notable is understanding the ability of T cells to discern between ligands of similar affinity and respond differentially based on binding parameters and other external signals[113]. The T cell interprets antigenic potency and translates that into the downstream signaling events known as signal strength. In fact, T cells induce distinct signaling patterns in response to low quantity of antigen (less antigen/less TCR occupancy) versus low quality (altered binding parameters, etc)[41]. Our model is the first to demonstrate that IRF4 is not simply a readout of TCR affinity but of overall signal strength as in this case 35A is an MHC variant peptide with unchanged affinity for TCR.

In addition to being a metric of TCR signal strength, IRF4 plays direct and indirect roles in the maintenance of other cellular systems. IRF4 deficient mice have been shown to have defects in maintaining proliferative expansion, IL-2 sensitivity as well

as sustaining a glycolytic response. While IRF4 knockout T cells were able to initially divide, this proliferation could not be maintained past 3 days[59]. The effect of protracted proliferation is due in part to IRF4's role in repressing cyclin dependant kinase (CDK) inhibitors which negatively regulate cell cycling. This phenotype is further diminished by increased apoptosis caused by an increase in proapoptotic molecule BIM, which would normally be repressed by IRF-4 [59]. IRF4 has also been found to directly regulate the transcription of genes involved in glycolytic metabolism including glucose transport receptors and HIF-1a[60, 110]. This parallels our observation of decreased glucose uptake and glycolysis in variant 35A stimulated cells which highlights another way IRF4 translates the 'weak' signal to a suboptimal metabolic profile. While the previous studies would only link antigen of decreased affinity to this abrogated T cell activation phenotype, we now add MHC variant peptide stimulation as an additional method of delivering a reduced strength of signal. While stimulation with variant 35A initially upregulates IRF4 at comparable levels to GP33 stimulated cells at 24 hours post stimulation, the lack of sustained expression leads to a similar phenotype seen in IRF4 deficient cells.

It would be of interest to assess IRF4 expression and the resulting T cell phenotype in response to other viral escape epitopes, particularly those that disrupt binding to the TCR. Thus far, the phenotype (low IL-2, diminished metabolic response, curtailed proliferation) we have characterized for the 35A MVP is similar to that of APLs in other systems[82, 83], While the studies with APL variants of OT-1 would suggest that the phenotype of other APLS would be the same, it would be necessary to properly test this assumption with the appropriate experiments. We would

hypothesize that whether the resulting response to MVPs are similar to APLS, the potential impact an MVP can have a on a T cell population is larger than its counterpart. In the context of an anti-pathogen response, MVP epitopes that hamper T cell function would affect an entire population of effectors that are restricted by that MHC while APL mutations would primarily just affect a single clonotype. It has been reported that most HIV viral escape occurs with mutated epitopes that alter binding to HLA rather than the TCR[84]. It is not clear if this finding is applicable to other viruses, but given the gravity of such public health issues as the HIV pandemic, it is important to consider even the smallest nuances in order to design more effective therapies.

To date, immune therapies to bolster immunity have been used in a variety of settings. With their ability to directly modify T cell function, APL based therapies have been found to be beneficial in both animal and human models of autoimmune disease and cancer[47]. In some cases, these therapies have led to unintended negative consequences as in the clinical trial for MS where APL treatment led to exacerbation of symptoms[22]. We have previously shown efficacy using MVPs to anergize autoreactive T cells in models of both MS and Type I diabetes. In the case of pathogen infection, this study highlights potential therapeutic targets for treatment of viral infection that might be populated with viral quasi-species with epitopes that bind weakly to MHC. Targeting deficient IL-2 signals has been used in both antiviral and antitumor treatments often in combination with other immunomodulatory agents like PD-L1 blockade[114-116]. With the recent interest in metabolic underpinnings of immunity, several studies have highlighted the utility of agents to

impact glycolysis, mitochondrial function, and fatty acid oxidation to inhibit or promote different T cell populations[88]. Taken together, there are a multitude of ways to approach restoring the response to a viral variant.

One such method was the subject of our study in Chapter 3 which was an extension of previously published finding we observed in the CD8+ T cell response to viral variant 35A[52]. Upon stimulation with 35A, T cells exhibited sustained SHP-1 activity while the response to the wildtype GP33 epitope had a much earlier downregulation of SHP-1 activity. We hypothesized that SHP-1 was restricting an adequate response to the 35A epitope and removal of the phosphatase would revive effector responsiveness. Utilizing both monoclonal and polyclonal models of T cell specific SHP-1 deficiency, we interrogated the CTL response to 35A. We found no increase in tetramer specific responses to the variant epitope. We also found no difference in the response to the main Db/restricted LCMV epitopes (Db/gp33, Db/gp276 and Db/np396). We also failed to observe any difference in the number IFNγ or Granzyme B producing effectors in the anti-viral response.

We did observe increased frequency of CD44hi CD8+ T cells in the periphery in naïve CD4Cre FLSHP-1 mice. These cells have been described as “innate memory” CD8+ T cells[117, 118]. These cells phenotypically resemble conventional memory CD8+ T cells (CD44hi, CD62L+, CD122+) that form after resolution of an antigen driven effector response. These innate memory cells can be driven by either lymphopenic or high IL-4 conditions. Another group reported that knocking out SHP-1 facilitated an increase in IL-4 which led to the increased population of these innate memory T

cells[95]. These cells were reported to be hyperresponsive to antigen. To address the possibility that the SHP-1 knockout peak CD8+ T cell response was higher/peaked earlier, we infected mice and looked for tetramer specific responses at day 5 instead of day 8. We found no difference in the number of cells responding to the Db/gp33 epitope between wildtype B6 animals and SHP-1 knockout animals. Studies to probe effector function directly (IFNγ, TNFα, Granzyme B) would ascertain whether the SHP-1 knockouts have a greater effector response despite having the same number of tetramer specific cells.

Others have reported T cell hyper-responsiveness in other models of SHP-1 deficiency. These models have been mostly based on the motheaten mouse me/me, that contains a natural mutation yielding non-functional SHP-1 phosphatase[119, 120]. Because SHP-1 is absent from all hematopoietic cells, these animals have massive immune hyperactivity overall. It is plausible that the T cell assays done using motheaten T cells came from a systemic inflammatory microenvironment thus affecting how those T cells respond in subsequent assays. An increase in T cell expansion has been reported in other models of SHP-1 deficiency where the FLSHP-1 construct was expressed under the control of a distal Lck promoter. While both dLck Cre and CD4 Cre would result in the loss of SHP-1 during thymocyte development, our CD4 Cre would induce SHP-1 deletion at the double negative (DN) stage while dLck would introduce this deletion at the later single positive (SP).

Conversely, another group found T cells deficient in the protein tyrosine phosphatase PTP-PEST are hypo responsive to antigen stimulation and cause less severe disease in

EAE[121]. We also have preliminary data using a conditional SHP-1 knockout in which Cre expression is controlled by type I interferon inducible Mx promoter. We transferred untouched cells from Mx Cre FLSHP-1 mice or B6 mice into TCR α knockout mice. Poly IC was administered to induce Type I IFN response. After one week, mice were infected with LCMV Armstrong. As with the CD4 Cre FLSHP-1 mice, we did not observe any change in expansion of tetramer specific cells nor did observe any change in the proportion of KLRG1+ short-lived effector cells (SLEC) (Figure 1). This data also suggests that the phenotype we are observing is not simply a by product of a single Cre regulated SHP-1 deficiency but instead may highlight a conserved mechanism in need of additional study.

These data demonstrate the complexity of T cell response to antigen engagement. This dissertation shows how TCR perception of antigen is a product of multiple physical and biological factors and can be modified by changing antigen stability on MHC (Figure 2). Our data also shows more study of how modifying TCR negative regulators impact T cell responses in different contexts. This work highlights how potential aspects of T cell recognition and response can be modulated for therapeutic purposes.

Figure 1.

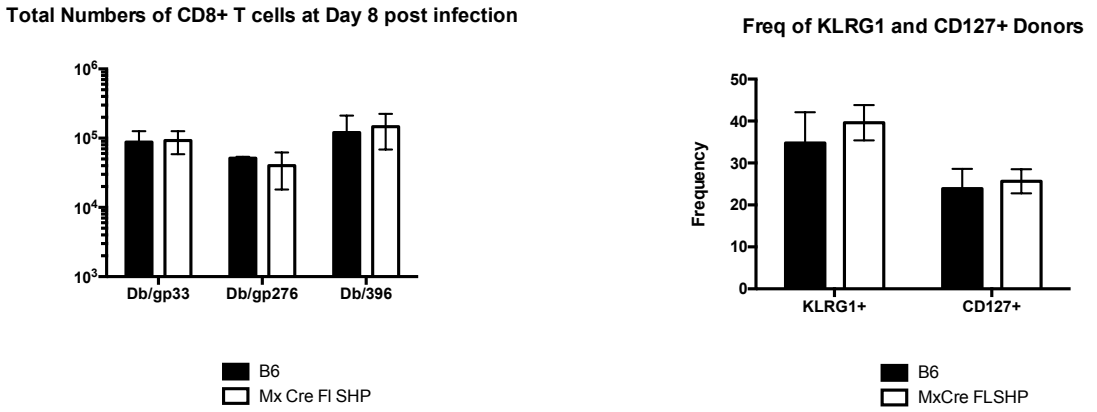

Figure 1. Conditional SHP-1 deficiency does not affect T cell expansion or composition

CD8+ T cells were purified from MxCre FLSHP-1 or B6 mice and adoptively transferred in Tcr α knockout mice. Mice were treated with three doses of 250ug Poly IC every two days followed by one 'rest' week. Mice were then infected with 2 x 10^5 pfu LCMV Armstrong and spleens were harvested at day 8 post infection to assay response. A) Total numbers of CD8+ CD44hi tetramer positive cells B) Percentage of KLRG1+ (SLEC) or CD127+ (MPEC) cells.

Figure 2.

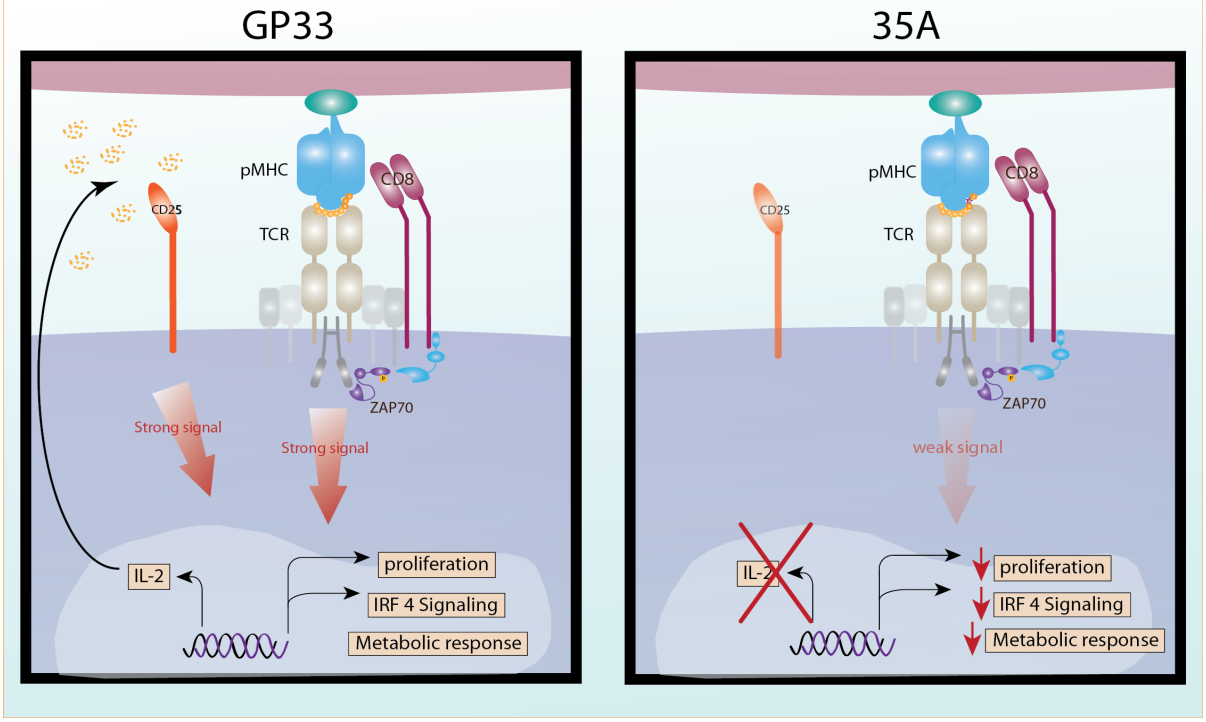

Figure 2: 35A diminishes signal strength by disrupting MHC binding (Model)

The wildtype GP33 epitope stably binds to both TCR and MHC. This engagement induces downstream signaling that leads to optimal proliferation, cytokine production, and metabolic response. The mutant 35A epitope maintains affinity for TCR but has diminished half life on MHC. Though TCR affinity for 35A is unchanged, the MHC instability diminishes the quality of the response perceived by the T cell and proximal signaling events are abrogated by high SHP-1 activity. Failure to maintain IRF-4 despite high initial expression demonstrates the partial stimulatory ability of 35A. The ensuing response to the variant epitope is

characterized by early proliferation and up-regulation of activation markers. Interestingly, this response is not maintained and is characterized by a lack in IL-2, increased apoptosis and an abrogated metabolic response. We show that disrupting the stability of peptide in MHC can effectively decrease TCR signal strength despite unchanged affinity for TCR and can significantly impact the CD8+ T cell response to a viral escape mutant.

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