Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Lindsay J. Edwards Date

T cell recognition of weak ligands activates negative regulatory pathways

By

Lindsay J. Edwards
Doctor of Philosophy

Graduate Division of Biological and Biomedical Science
Immunology and Molecular Pathogenesis

_____
Brian D. Evavold
Advisor

_____
John D. Altman
Committee Member

_____
Jeremy M. Boss
Committee Member

_____
Mandy L. Ford
Committee Member

_____
Aron E. Lukacher
Committee Member

T cell recognition of weak ligands activates negative regulatory pathways

By

Lindsay J. Edwards
B.S., Emory University, 2005

Advisor: Brian D. Evavold, Ph.D.

An abstract of
A dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies
of Emory University
in partial fulfillment of the requirements for the degree of Doctor of Philosophy in
Graduate Division of Biological and Biomedical Science
Immunology and Molecular Pathogenesis
2011

Abstract

T cell recognition of weak ligands activates negative regulatory pathways

By Lindsay J. Edwards

The outcomes of T cell interaction with antigen are determined by a complex integration of positive and negative signaling events downstream of the T cell receptor. The introduction of amino acid substitutions into the antigenic peptide provide an opportunity to alter T cell activation by modulating the affinity of the T cell receptor for the peptide: MHC complex or by altering the stability of the peptide: MHC interaction itself. Both of these strategies can generate ligands that drive atypical T cell activation by stimulating negative regulatory pathways, which we demonstrate with an antagonist in a model antigen system, an MHC variant peptide in a type 1 diabetes system and a myelin antigen in an in vivo model of multiple sclerosis. The effects of these weak ligands are evident as both antagonism and MHC variant peptide treatment induce a stable hypoproliferative phenotype. These two stimulations differ somewhat in their outcomes as antagonism drives a response in which IL-2 is highly produced, whereas the MHC variant peptide stimulates a classic anergic phenotype is which the T cells fail to produce IL-2 in response to an agonist ligand. Both antagonism and this form of anergy have been shown to rely on the tyrosine phosphatase SHP-1. As self antigens are generally thought to be relatively weak ligands, we sought to determine the effects of SHP-1 deficiency on the autoimmune disease experimental autoimmune encephalomyelitis (EAE). Despite data that SHP-1 is a negative regulator of T cell activation, we found that deletion in T cells decreased both the incidence and severity of EAE. This effect appears to depend upon the presence of CD4+Foxp3+ T cells, as depletion of these cells resulted in disease severity similar to that of control groups. These results suggest that the function of SHP-1 in T cell biology is more complex than simple negative regulation of activation, and that the consequences of SHP-1 activation may vary between subsets of T cells. Collectively, our data demonstrate that T cell stimulation by both weak ligands and self antigens can drive the activation of negative regulatory pathways, thus leading to a continuum of functional responses.

T cell recognition of weak ligands activates negative regulatory pathways

By

Lindsay J. Edwards
B.S., Emory University, 2005

Advisor: Brian D. Evavold, Ph.D.

A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in
Graduate Division of Biological and Biomedical Science
Immunology and Molecular Pathogenesis
2010

Table of Contents

Chapter 1

Introduction

T cells were once thought to be highly specific for a single antigen. Over time, as our knowledge of the intricacies of T cell activation has increased, we have come to realize that T cell activation is not a digital phenomenon. That is to say, a T cell is not either activated or inactivated at any given time. On the contrary, even at rest, a T cell is a complex mix of positive and negative signaling events. These same signaling events are activated or inactivated to varying degrees upon a T cell's encounter with antigen, and the balance between positive and negative signaling events determines the outcome of the interaction.

Stimulation with an agonist peptide rapidly activates both positive and negative signaling events. However, due to the nature of the interaction, the positive signaling events that are initiated are sufficient to either inhibit the negative signals or overbalance them to such an extent that the result is full activation of the T cell, as evidenced by proliferation and the development of effector functions such as cytokine production. As our lab and others have demonstrated, T cells can recognize a broad spectrum of ligands, encompassing stimuli that result in activation of only a subset of effector functions (partial agonists) or even inhibition of the T cell's ability to respond to agonist stimulation (antagonists) (1-3).

T cell recognition of both strong and weak ligands is an important aspect of T cell biology. Variants of agonist ligands occur frequently during viral infection, providing

impetus for the continued study of the biology of T cell responses to a broad spectrum of pMHC ligands. Studies of signaling in response to weak ligands has demonstrated that T cell recognition of ligands that vary in potency is quantitatively and qualitatively different, and not attributable to simple dose effects (4, 5) our unpublished data). Furthermore, the kinetic parameters of a given interaction between the trimolecular TCR:peptide:MHC complex correlate with the stimulatory capacity of the ligand (6, 7). The ability of T cells to respond to ligands that vary in potency affects the response to pathogens that are able to mutate their T cell epitopes, and may also be harnessed for therapeutic use in several different ways.

Binding kinetics of antigen recognition

The affinity of the interactions between TCR and peptide:MHC as well as between the antigenic peptide and MHC have long been recognized as playing a crucial role in the outcome of T cell stimulation. Initial measures of the kinetic parameters governing the TCR:peptide:MHC interaction primarily utilized surface plasmon resonance. This technique measures binding interactions with one purified protein immobilized on a solid surface and the interacting partner in the fluid phase. This setup allows for the molecules to interact in 3 dimensions (3D), and provides data allowing for the extrapolation of kinetic parameters such as on rate, off rate, half life and affinity. The measures of TCR:peptide:MHC interaction derived in 3D correlate to some extent with ligand potency, although discrepancies were commonly observed for weaker ligands (7-9).

In a typical cell-cell interaction as would occur in vivo, membrane bound receptor:ligand pairs interact in only 2 dimensions (2D), which is to say each of the proteins is anchored in the cell membrane. This type of interaction is significantly more constrained than the 3D interactions that can be observed by surface plasmon resonance. To measure binding interactions in a more physiologically relevant manner, several novel techniques have been developed in recent years that are capable of assessing 2D binding events. One of these methods is based on single molecule Förster resonance energy transfer (FRET), while the other is a mechanically based assay that visualizes binding via membrane stretching (6, 10). The single molecule FRET approach described by Davis and colleagues utilizes pMHC anchored to a planar lipid bilayer and intact T cells. The peptide is labeled with a fluorophore, and the T cell receptor is tagged by a fluorophore-labeled antibody fragment. By bringing these two labels into contact via TCR interaction with pMHC, one fluorophore served as a donor and the second as an acceptor to provide a FRET readout that allowed for calculation of various binding parameters (Fig. 1.1A).

The mechanical micropipette adhesion frequency assay utilizes an intact T cell and measures its binding to a red blood cell (RBC) that serves as a surrogate antigen presenting cell (7, 11-13). In this assay, the RBC coated with pMHC monomers is used as a biosensor to detect binding events. To determine adhesion frequency, a T cell is aspirated onto one micropipette and a pMHC coated RBC is aspirated onto a second micropipette. The two cells are brought into contact for a defined period of time, and the T cell is retracted to terminate the contact. If binding occurs, the soft RBC membrane elongates (Fig. 1.1B). By repeating this contact multiple times for each cell pair and for a

number of different cells pairs and interaction times, the kinetic parameters of the binding interaction can be determined, including on rate, off rate, half life of interaction as well as affinity. We have demonstrated that a number of these kinetic parameters strongly correlate with biological function (i.e., ligand potency), suggesting that 2D measurements are a more faithful representation of receptor ligand interactions as they occur physiologically than the 3D measurements that have been used in the past (7).

One major advantage of the 2D approaches is that it is not necessary to express and purify the individual proteins of interest. This aspect makes the assessment of polyclonal populations difficult and time consuming, if not impossible in three dimensional analysis. However, the micropipette assay is readily adaptable to polyclonal T cells, as we have recently published (12). The utility of the micropipette assay in determining the binding characteristics of polyclonal T cell populations may allow for more extensive profiling of autoreactive populations and their responsiveness to various therapeutic interventions as well characterization of responses following vaccination or during chronic infection.

Both the FRET and micropipette 2D approaches demonstrated that in both CD4 (6) and CD8 (7) T cells binding kinetics varied somewhat from what had been previously described using the 3D system, even when the same pMHC and TCRs were utilized. 2D analysis of TCR interaction with cognate pMHC resulted in rapid association and rapid dissociation and accordingly short half lives of interactions. This is in stark contrast to the kinetics observed in the 3D system, which displayed rapid on rates, but slow dissociation rates and thus longer interaction times for agonist ligands (7-9). The 2D interaction kinetics suggest a model in which T cells rapidly sample antigen, with strong

stimuli resulting in short dwell time and thus perhaps engaging a greater number of TCRs. In contrast, weaker ligands bound with slower off rates and longer half lives, suggesting that interaction with a weak ligand may ultimately limit the number of T cell receptors that encounter antigen. These differences in binding kinetics likely affect signaling downstream of the TCR as well, providing one means of translating the interaction with antigen into effector outcomes.

Escape mutants: naturally occurring variant peptides

Pathogens employ a number of different mechanisms to evade the immune system. Among the most common of these approaches is mutation of T cell epitopes. The stochastic nature of the mutations generated by pathogens can result in a broad spectrum of ligand stimulatory capacity, mimicking all of the classes of altered peptide ligands (weak and partial agonists, antagonists) initially described using synthetic peptides and model systems. Of course, the mutation itself may be detrimental to the fitness of the pathogen. However, the impact of such a mutation on the T cell response can have an equally dramatic effect on the maintenance or clearance of the infection.

Mutated T cell epitopes have been most widely studied in viruses, although escape epitopes have also been identified in bacteria and parasites (14-18). Viruses, particularly those with an RNA genome, are more likely than bacteria or parasites to develop mutations in T cell epitopes due to the error prone nature of their genomic replication (19). Mutations leading to the generation of an antagonist epitope have been described for several viruses and parasites (15, 16, 20-24). Antagonists are defined as altered peptide ligands that induce little to no T cell activation when given alone, but are

able to inhibit the T cell's ability to respond to agonist stimulation when both agonist and antagonist are present (2). The phenomenon of antagonism was initially described *in vitro*; however, the generation of an escape mutant during an ongoing viral infection could also present a situation in which a single T cell encounters the agonist ligand and the mutant. Inhibition of the T cell response due to the generation of antagonist ligands has been described in human immunodeficiency virus (16, 25, 26), as well as hepatitis B virus, (15), human T lymphotropic virus (20), *Plasmodium falciparum* (24), lymphocytic choriomeningitis virus (22, 27) and hepatitis C virus (21, 23, 28).

A related phenomenon that is distinct from classical antagonism but likewise mediated by variant peptides has been termed "immune interference". This means of immune evasion was initially described for a variant epitope from *Plasmodium falciparum* (29). Peptides that mediate this interference effectively block initial T cell priming events, thereby altering the effective repertoire in the infected individual. Another means by which escape mutants can alter the T cell repertoire is by selectively expanding cross reactive T cell populations (30). Similarly, a subsequent study utilizing a model antigen and an antagonist variant demonstrated that the presence of the antagonist ligand at the time of initial priming resulted in a failure of the T cells to develop normal effector functions, and the cells were ultimately eliminated via apoptosis (31). These studies emphasize that the presence of variant ligands at the time of infection (i.e., concurrent infection with multiple strains or subspecies) can be just as devastating to the immune response as the mutation of T cell epitopes during ongoing infection, and this situation likely plays an important role in the subsequent development of T cell memory.

The outcomes of T cell encounter with an epitope mutant vary dramatically depending on the T cell activation state, context of antigen encounter and nature of the antigenic ligand (i.e., affinity of the epitope for MHC and TCR). One frequently observed result of T cell encounter with a variant ligand is a shift in effector phenotype. For example, after encounter with a variant epitope from *Plasmodium falciparum*, CD4+ T cells were shown to shift from IFN-γ production to IL-10 secretion (29). In some systems, escape mutants have been demonstrated to induce anergy in the responding T cells (32), apoptosis (29, 33) or a failure of T cell activation (27).

These responses to mutant epitopes are driven by active negative signaling events or significant alterations in the kinetics of normally activating pathways. For example, we have demonstrated that in vitro stimulation of T cells with a viral escape mutant epitope from lymphocytic choriomeningitis virus resulted in abrogated positive signaling events (Erk1/2 phosphorylation) and augmented negative signaling events (SHP-1 phosphatase activity) (34). The induction of altered signaling pathways by variant ligands is a feature of my studies and will be discussed in greater detail in subsequent sections.

It is becoming increasingly clear that weak ligands play a significant role in shaping the immune response. As identification of antigenic epitopes is ongoing, it is impossible to know just how many variant peptides may exist in nature. However, continually increasing our understanding of the impact that variants have on the T cell response may allow us to harness this aspect of the immune response to develop more effective vaccine strategies, as well as treat infections, particularly chronic infections that are often resistant to common therapies. Furthermore understanding the aspects of T cell

binding to pMHC that determine the outcome of the interaction as well as the downstream consequences of these interactions will be crucial to overcoming the effects of epitope escape mutations during infection.

Signaling in response to weak ligands: quantitatively and qualitatively unique

The simplest explanation for the differences in T cell activation observed with weak ligands is that there is a dose effect. If this were the case, then a low dose of a highly stimulatory peptide should induce signaling events similar to a high dose of a weak ligand. However, this is not the case. Studies assessing signaling events downstream of T cell stimulation with weak ligands have repeatedly demonstrated that unique signaling patterns result from weak stimuli.

In the seminal study assessing signaling in response to altered peptide ligands, Allen and colleagues demonstrated that stimulation of T cell clones with an anergy inducing variant peptide resulted in a unique pattern of zeta chain phosphorylation (4). Similarly, in a different system, altered zeta chain phosphorylation was demonstrated upon stimulation with variant peptides representing a partial agonist and an antagonist (5). This unique pattern of zeta chain phosphorylation resulted in either a failure of ZAP-70 to associate with the T cell receptor complex or a lack of phosphorylation and activation of this kinase (4, 5). Importantly, stimulating cells with even a 100 to 1000-fold lower doses of wild type peptide did not mimic this effect (4, 5), emphasizing that signaling in response to weak ligands is not explained by a simple dose effect but may in fact represent an alternative pathway of cellular activation.

The altered phosphorylation patterns observed in these early studies of T cell responses to weak ligands could potentially be explained by several different mechanisms. The simplest explanation for decreased phosphorylation events is a decrease in kinase activity. Alternatively, the signal delivered by a weak ligand could activate one or more phosphatases, which would rapidly remove the activating phosphorylation events. Given the complex nature of the signaling events downstream of the TCR, it is likely that both of these mechanisms are playing a role. As yet, no comprehensive studies have assessed both the positive and negative signaling events that occur upon stimulation of a T cell with a weak ligand.

In addition to the altered phosphorylation pattern of zeta and the lack of ZAP-70 activity observed on stimulation with weak ligands, a number of other positive signaling mediators are also absent. For example, MAPK activation is transient on stimulation of a CD4+ T cell with a partial agonist (35). Similarly, in CD8+ T cells, stimulation with a weak agonist resulted in no detectable calcium flux or phosphorylation of Erk1/2, p38 or Jnk. However, cells stimulated with the weak ligand for 24h accumulated phosphorylated c-Jun to the same level as agonist-stimulated. This result suggests that even undetectable levels of early positive signaling events may somehow accumulate in distal mediators of T cell activation, thus driving the limited positive responses to weak or partial agonist ligands (9), our unpublished data).

A number of unique signaling events have been described in the setting of antagonism. Analysis of cells stimulated in this unique manner where both agonist and antagonist ligands are present suggest that the phenotype of antagonism is mediated by an active negative signal. Activation of Vav, SLP-76, LAT and Erk1/2 were not observed

by traditional biochemical methods on stimulation under antagonist conditions, although some minor phosphorylation events were observed at the T cell:APC interface using confocal microscopy (36). Calcium flux, however, is not required for the induction of antagonism (37), and this reduced or absent calcium flux may play a role in the blockade of proliferation and cytokine production (38). Additionally, antagonism resulted in altered LFA-1/ICAM-1 interactions (38) and failure to form mature T cell: APC conjugates (39). Other studies observed phosphorylation of Vav and SLP-76 upon antagonism, as well as stable T cell: APC conjugate formation (40-43), suggesting that some aspects of antagonism may be dependent upon the specific system or conditions.

Furthermore, we have demonstrated that extended culture of transgenic T cells under antagonist conditions can permanently alter the phenotype of the cells, in a manner similar to what has been described for anergy (44). Unlike the anergic phenotype, cells that have been antagonized and subsequently restimulated with agonist are capable of producing IL-2. In fact, these antagonized cells secrete more IL-2 than cells cultured on agonist, but are unable to proliferate, despite expressing the high affinity IL-2 receptor at similar levels as agonist cultured cells. Similar to anergy induced by APLs, the non-proliferative phenotype cannot be rescued by the provision of exogenous IL-2. This unique phenotype emphasizes once more that the encounter of T cells with variant ligands can have a profound effect on both the early signaling events as well as the resulting effector functions of the cells.

Antagonism of T cells with altered peptide ligands has also been associated with the induction of negative signaling mediators, specifically activation of the Src homology 2 domain containing protein tyrosine phosphatase (SHP-1) (45, 46). SHP-1 is a broadly

expressed phosphatase that is generally thought to function as a negative regulator of cellular activation. In hematopoeitic cells, SHP-1 has been shown to associate with a number of signaling intermediates including Lck, ZAP-70, various Jaks and Stats, the p85 subunit of PI3K, CDK2 and the IL-2 receptor complex (47-56). SHP-1 is thought to be activated by phosphorylation of its C-terminal tail by kinases including Lck, making it perhaps the most TCR proximal negative regulator (48, 57, 58). In addition to its role in T cell antagonism, we have also demonstrated that SHP-1 activity is required for anergy induction by an MHC variant peptide (59), and is upregulated in response to a viral escape mutant epitope (34). These data suggest that the negative signals driven by T cell interaction with weak ligands may be largely regulated by SHP-1.

Recently, overexpression of microRNA 181a has been demonstrated to abolish the inhibitory effect of an antagonist peptide, but was insufficient to convert the response to the level of the agonist (60). This microRNA was shown to decrease expression of a number of inhibitory mediators in T cells, including several phosphatases. However, SHP-1 expression was not affected. These data suggest a model in which the negative signals delivered by an antagonist peptide engage multiple pathways, perhaps with SHP-1 as the most TCR proximal regulator and other phosphatases controlling downstream signaling events, although the details of these pathways remain to be clearly determined.

Role of receptor number in recognition of weak ligands

Several studies have utilized T cells expressing two distinct TCRs in an attempt to determine whether T cell antagonism is a passive process mediated by competition for ligand binding or an active process mediated by the activation of a negative signal. By

using T cells expressing two receptors of different antigen specificity and/or MHC restriction, it is possible to deliver an agonist stimulus via one TCR and antagonist stimulus via the second TCR. Studies of this nature, while well conceived, gave mixed results. The initial studies of this setup failed to observe "cross-antagonism" in CD8+ dual TCR transgenic cells (61, 62). That is to say, when the dual receptor T cells were stimulated with an agonist for one receptor and an antagonist for the second receptor, no inhibition of cytolysis was observed. Subsequent studies by our lab and others, however, demonstrated potent cross-antagonism of proliferation and cytokine production in CD4+ dual receptor T cells (45, 63). It has been suggested that this apparent disconnect between CD4+ and CD8+ T cell antagonism is due at least in part to the assays utilized to measure antagonism. Cross-antagonism was consistently observed when the readout was proliferation or cytokine production, but not when the readout was cytolysis (45, 61-63). These data suggest that the regulatory mechanisms governing various effector outcomes differ, rendering proliferation more susceptible to inhibition by antagonist ligands than cytolysis. These differences could be driven by different signaling requirements or differences in kinetics of both input and output signals, as proliferation is necessarily a longer process than degranulation-mediated cytolysis.

One caveat to the dual TCR system is that these T cells tend to preferentially express one TCR over the other. That is, one of the TCRs typically represents 70% or more of the TCR on the cell surface. Although this issue is never directly addressed in the early studies assessing antagonism in dual TCR cells, it is evident from assessing the fluorescence intensity of the staining for the TCR that one beta chain is more highly expressed. Considering this, we sought to determine what role TCR level may play in

susceptibility to antagonism. By using antibodies to block specific TCRs, we demonstrated that antagonists as well as weak ligands require a significantly greater number of TCRs on the cell surface to exert their effects, as compared with agonist (64-66). Full proliferation could be stimulated with agonist peptide with as many as 95% of the TCRs blocked (65-67). This is in good accord with other studies demonstrating activation in response to a very few pMHC ligands, which is essentially the reciprocal experiment (68-70). However, blockade of only 50% of the available TCR was sufficient to dampen responses to weak agonists, as well as the ability of an antagonist to inhibit the T cell response to agonist (65).

These studies assessed TCR transgenic cells (AND, P14 and OTI) that have a very high affinity for their respective ligands. It has been postulated that autoreactive T cells are of low affinity for antigen due to the both central and peripheral tolerance mechanisms (71-73). Given this idea, we sought to determine if blocking the TCR of a self-reactive T cell would result in the inability of those T cells to respond to their "agonist" ligand. In accordance with the idea that autoreactive cells respond to their agonist ligand as if it were a weak agonist due to the low affinity of the TCR, we demonstrated that a 5% decrease in the number of TCR on the surface increased the EC50 by 3 fold, and a 75% reduction in TCR shifted the EC50 by more than 350 fold (66).

Together, these data are similar to the pharmacological idea of a spare receptor theory. The spare receptor theory postulates that a given ligand (i.e., pMHC in the case of T cell activation) can exert maximum biological effect while only engaging a small number of the available receptors (Fig. 1.2). This, of course, raises the issue of the

purpose of having such an excess of receptors. In the case of T cells, it seems likely that the importance of this receptor reserve is two-fold. First, a high number of receptors may allow for the recognition of self peptides and/or MHC that is required for survival signals as well as homeostatic proliferation (67, 74, 75). Secondly, excess receptors allow for the recognition of a broad spectrum of ligands (64-66). This is a key aspect of T cell protection from pathogens, as the number of T cell specificities in the body is limited. A system employing spare receptors allows for each T cell to potentially recognize a greater number of ligands through cross reactivity. This cross reactivity likely increases the risk of autoimmune disease, in addition to increasing the chance that the T cell repertoire will be able to respond to a pathogenic challenge. Similar to this, a recent study described a system in which infection with a virus was sufficient to induce autoimmune disease (76). In this system, mice transgenic for a TCR recognizing a myelin basic protein epitope were activated via a second, endogenous receptor specific for a viral epitope. This activation was sufficient to allow the cells to mediate tissue damage. The delicate balance between autoimmunity and immunity to pathogens lies at the heart of the T cell response. Peripheral tolerance mechanisms are typically able to keep autoimmunity at bay, allowing for the maximum available repertoire to respond to foreign antigens, although escape from tolerance can and does occur.

Models of T cell activation

Our understanding of how exactly T cells achieve activation is constantly evolving. A number of models have been put forth to explain the outcomes of a T cell interacting with a peptide: MHC ligand. These models must account for a number of parameters,

perhaps most importantly the ability of T cells to recognize a broad spectrum of ligands. Altan-Bonnet and colleagues have defined a “golden triangle” of key characteristics that must be incorporated into a useful model of T cell activation (77). These three critical components are speed, specificity and sensitivity of response.

To mount an effective T cell response to an ongoing infection, T cells must be rapidly activated upon encounter with specific antigen. In fact, phosphorylation events have been detected as rapidly at 4 sec following TCR binding to peptide: MHC (78). The ability of T cells to rapidly detect a specific peptide on an antigen presenting cells is a crucial aspect of effective T cell-mediated immunity.

T cells are both highly specific yet paradoxically degenerate in their recognition of antigen. To achieve full activation, a T cell recognizes a short peptide in the context of a specific MHC molecule. However, not all alterations of the antigenic peptide are sufficient to abrogate the T cell response (79). In fact, such amino acid substitutions are frequently remarkably well tolerated at both T cell receptor contact residues and MHC anchor positions. A large body of work has demonstrated that variant peptides containing both single and multiple amino acid substitutions can result in a variety of T cell responses, as detailed above (79). Collectively, these data indicate that the specificity of T cell recognition is a complex factor that must be carefully considered to accurately model T cell activation.

In addition to the specific nature of T cell recognition of antigen, full responses are possible, even to vanishingly small amounts antigen. In fact, effector functions can be triggered by as few as 1-400 pMHC ligands (68, 69). This is a critical component of the adaptive immune response, as it is necessary during infection for T cells to find minute

quantities of pathogen-derived antigens among a sea of self-derived peptides. The importance of sensitivity necessitates a means to amplify an initially minuscule signal. To this end, signaling cascades downstream of the TCR serve to multiply the initial signal delivered by interaction with a stimulatory peptide: MHC ligand.

To explain the ability of a T cell to translate small differences in the kinetics of interaction with a peptide: MHC ligand into robust differences in functional outcome, a series of positive and negative signaling feedback loops have been proposed. Data from Germain and colleagues suggested a model in which complete activation results in a positive feedback loop driven by Erk1/2 activity. In contrast, stimulation of T cells with weak or antagonist ligands results in a signaling program dominated by activity of the tyrosine phosphatase SHP-1. It is postulated that the balance between these positive and negative feedback loops determine the outcome of a T cell stimulation event.

The models of T cell activation discussed in this section seek to incorporate our knowledge of both the functional behavior of T cells as well as various biochemical and biophysical aspects of the interaction between a T cell receptor and its peptide: MHC ligand as well as the specific consequences of these interactions.

Kinetic proofreading Model

The kinetic proofreading model was first proposed as a means to explain T cell behavior by McKeithan in 1995 (80). The primary feature of the kinetic proofreading model is that a delay between the TCR's encounter with antigen and commitment to effector functions allows for a series of reversible steps during which ligand discrimination can occur. This model emphasizes the length of time that a TCR and

pMHC remain in contact, as early signaling events are readily reversed. Thus achieving full activation of all downstream components requires a certain amount of contact time, sometimes termed "dwell time". A number of studies have provided data in support of the kinetic proofreading model (81-84), although not all available data are consistent with these parameters (8, 85-87). Of interest, even data that don't directly support the kinetic proofreading model support the idea of dwell time as being a key component governing the outcome of TCR: pMHC interaction. Importantly, the kinetic proofreading model predicts that a low concentration of a high affinity ligand will not result in signaling events identical to a high concentration of a weaker ligand.

Several modifications of the kinetic proofreading model have been proposed over the last two decades. Among these, several groups proposed a version of the kinetic proofreading model in which the initial encounter with antigen induces both positive and negative signaling events (88, 89). If the interaction persists, the positive signal is propagated and activation is initiated. However, if the dwell time is insufficient to drive positive signaling events to completion, then the negative feedback loop that was initially activated dominates the response, resulting in no or incomplete activation of the T cell.

A further modification of the kinetic proofreading model that has been termed "temporal summation" proposes that T cells are capable of accumulating a series of minor stimulations to result in complete activation, despite early positive signaling events being undetectable (9, 90). The proposed mechanism of this summation model is that incomplete activating events that are sufficiently stimulatory result in a rise in activated molecules downstream. Although these activated downstream molecules are insufficient to result in full T cell activation, subsequent encounter with antigen may be sufficient to

activate a second, third or more waves of these so called "counter" molecules. Assuming the activation of the counter molecules does not reach baseline, subsequent waves of activation may be sufficient to ultimately reach the threshold required for the generation of T cell effector function (9, 90, 91). Thus, rather than a smooth and continuous increase in positive signaling events, oscillatory activation of downstream mediators may be sufficient to mediate activation under some circumstances.

Serial engagement

The serial engagement model proposes that a single peptide: MHC complex interacts with a TCR for a period sufficient to activate downstream signaling events. Following the dissociation of the initial pMHC: TCR complex, the same pMHC subsequently engages additional TCRs, ultimately resulting in an accumulated signal from a single or very few pMHC complexes interacting with many TCRs. One of the major strengths of this model is its ability to explain how a very small number of specific peptide: MHC complexes is able to fully activate a T cell. Studies have indicated that a very low density pMHC could drive the internalization of as many as 100-200 TCR (92, 93). However, the extent to which this internalization is the result of actual encounter with antigen is not certain (94).

Optimal dwell time

The kinetic proofreading model and the serial engagement model represent two important aspects of T cell activation. The kinetic proofreading model focuses on the behavior of a single receptor: ligand pair and the signaling events that occur downstream

of that interaction. On the other hand, the serial engagement model predicts that on the level of the entire cell, a certain half life or affinity of interaction would be ideal to interact with the greatest number of TCRs but still interact with each TCR long enough to sufficiently drive positive signaling events. This combination of long enough interaction to drive activation and short enough interaction to allow encounter with many receptors is known as the optimal dwell time (92, 95-97). The responses elicited by various dwell times are in the shape of a bell curve, such that either too short or too long a dwell time is not adequate to drive T cell activation.

Summary

The ability of T cells to recognize and respond to a broad spectrum of ligands is a critical aspect of T cell biology. Variant ligands are frequently generated by pathogens as a means to escape the T cell response, and therefore present as a unique target to boost immune responses against chronic pathogens. The use of variant ligands has provided novel insights into signaling pathways downstream of the TCR that may be applicable to the regulation of T cell responses. Related studies have demonstrated that the number of T cell receptors expressed on the surface also plays a crucial role in the T cell response to antigen, particularly self antigens and weak ligands. These aspects of T cell function continue to inform computational and theoretical models of T cell activation.

Furthermore, the insights into T cell biology that have been generated by exploration of variant peptides have shown promising results in the treatment of mouse models of several autoimmune diseases. Hopefully these insights will be parlayed into novel therapeutics for the treatment of autoimmunity and chronic infections, two classes

of disease that are notoriously difficult to effectively treat.

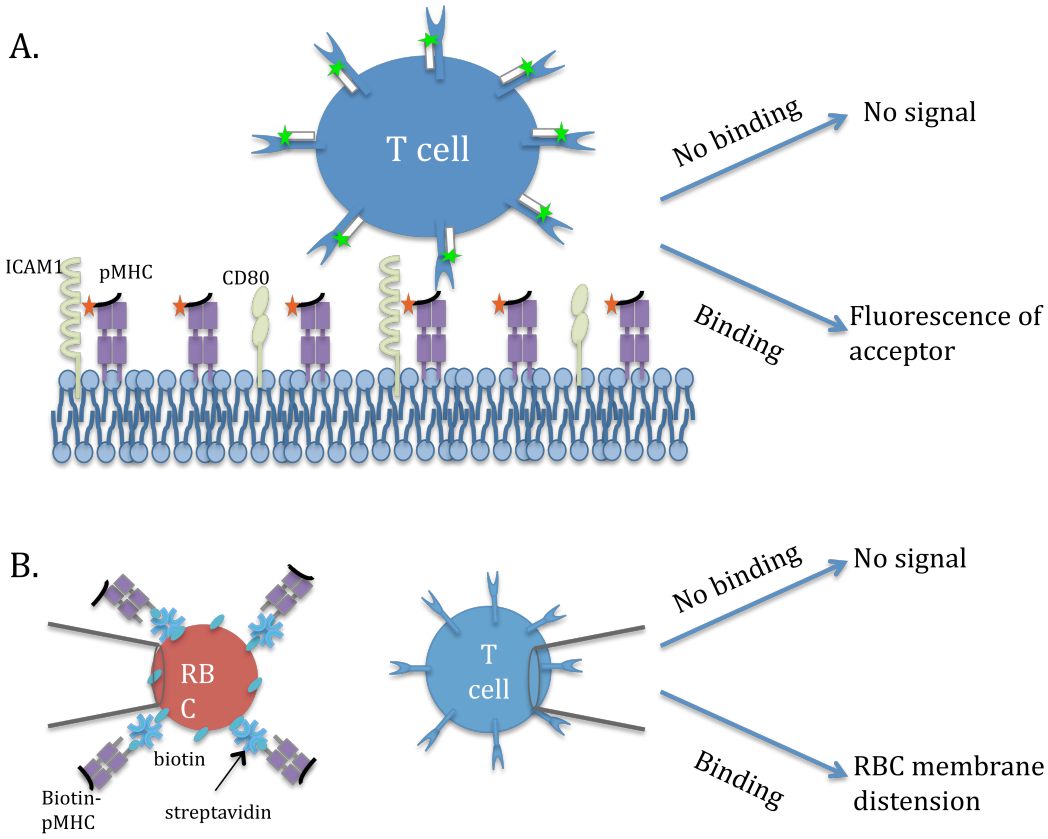

Figure 1.1 Two dimensional analysis of T cell: pMHC interactions

A. FRET-based two dimensional binding assay. In this assay, T cells labeled with a fluorescently anti-TCR antibody were allowed to act with a planar membrane setup expressing MHC with fluorescently labeled peptide, ICAM1 and CD80. If no binding occurred, no fluorescent signal was observed from the acceptor fluorophore. If binding occurred, the two fluorescent molecules were juxtaposed closely enough to allow for detection of the FRET signal. **B.** Micropipette-based adhesion frequency assay. In this assay, red blood cells are coated with biotin, followed by streptavidin, followed by biotinylated peptide:MHC monomers. The pMHC-coated red blood cell is aspirated onto one micropipette while a T cell is aspirated on a second micropipette. The cells are brought into contact and binding is observed upon retraction of the T cell by distension of the soft red blood cell membrane.

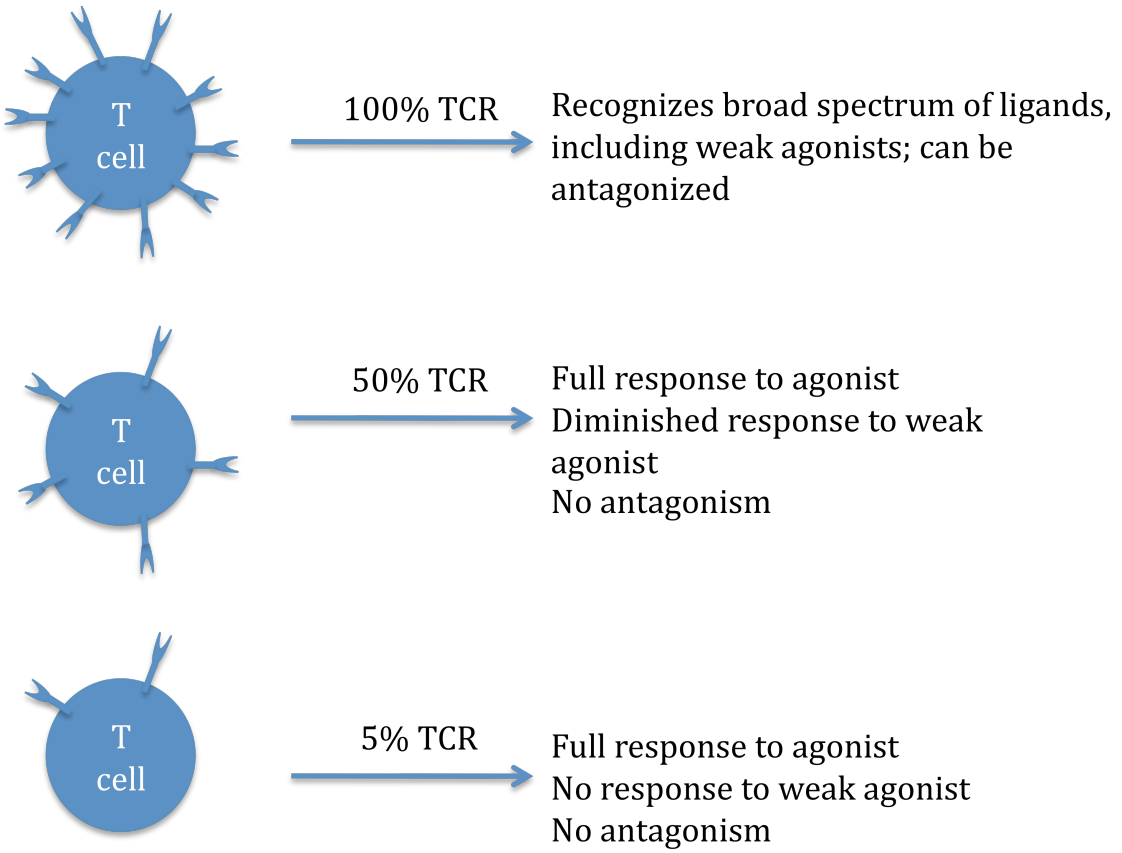

Figure 1.2 Spare receptor behavior of TCR

At physiological levels of TCR expression, T cells are able to recognize a broad spectrum of ligands. When T cells express approximately 50% of normal TCR numbers, responses to weak ligands are diminished and antagonism is abolished, but responses to agonist stimulation is unaffected. At 5% of normal TCR numbers, responses to low potency ligands are not observed; however, responses to agonist stimulation are unaffected.

Chapter 2

A unique unresponsive CD4+ T cell phenotype post TCR antagonism

Published in *Cellular Immunology* 2010; 261(1):64-8

Abstract

The functional outcomes of the T cell's interaction with the peptide:MHC complex can be dramatically altered by the introduction of a single amino acid substitution. Previous studies have described the varied effects of these altered peptide ligands (APL) on T cell responses. These outcomes of T cell interaction with an APL include the induction of clonal unresponsiveness (anergy) and inhibition of T cell responses (antagonism). The phenotype of peptide-induced anergy, i.e. low proliferation and low IL-2 production, has been extensively described, and a number of groups have demonstrated antagonism. However, the response of T cells to an agonist ligand after encountering an antagonistic stimulus has not been previously characterized. Here, we show that T cells post-antagonism fail to proliferate but produce large quantities of IL-2 upon stimulation with their wild type ligand. This unique phenotype is not due to differences in IL-2 receptor expression or rates of apoptosis, and cannot be overcome by the addition of recombinant IL-2. The response of CD4 T cells to agonist stimulation after encountering an antagonist is a novel phenotype, and is distinct from previously described forms of anergy.

Introduction

A T cell response is elicited following interaction of the T cell receptor (TCR) with its cognate ligand. The outcome of this interaction can be affected by introducing changes in the immunogenic peptide sequence (2, 98). Peptides in which amino acid substitutions have been made at a TCR contact residue are known as altered peptide ligands (APL)(98). APL have an affinity for MHC similar to that of the wild type peptide and are classified based on the potency of the T cell response they elicit. Agonist peptides stimulate T cells to levels similar to that of the wild type peptide, while weak agonists stimulate suboptimal proliferative and cytokine responses. Partial agonists stimulate only a subset of effector functions (98). This class of APL has been used to induce anergy, a state of unresponsiveness defined by a lack of proliferation and IL-2 production in response to an immunogenic stimulus (98). An anergic phenotype can also be induced by lack of costimulation (99-101), a low dose agonist ligand (102), or unstable peptides containing substitutions at their MHC anchor residues (103, 104).

In addition to partial agonists, which have been shown to induce T cell anergy, another category of APL that dampens the T cell response is antagonists. Antagonist ligands have been defined in vitro by their inability to stimulate T cell responses when presented alone and their ability to inhibit the T cell response to wild type peptide when both the antagonist and agonist are present (2, 105, 106). Epitopes behaving as antagonists have been identified in a number of infections, including HIV (26, 107), Hepatitis B and C (21, 108) and malaria (18). Additionally, antagonist ligands may play a role in maintaining peripheral tolerance (109).

Although the phenomenon of antagonism has been extensively described in a

number of systems, the exact mechanism by which antagonism occurs remains unclear. Studies utilizing dual-receptor expressing T cells suggest that the mechanism is one of active inhibition, rather than a passive effect of competition (45, 62, 63, 65). Further studies have indicated that antagonism impacts the signaling cascades normally activated upon T cell stimulation, activating a negative feedback loop involving the phosphatase SHP-1(48, 110, 111).

The response of a T cell under antagonist conditions is similar to the response of anergic cells to wild type stimulation. In both these conditions, T cells fail to proliferate and secrete little or no IL-2. To define the fate of these cells following rechallenge with agonist ligand has not been considered. We examine the phenotype of previously antagonized T cells in response to wild type stimulation. We have found that the phenotypes of anergy and antagonism differ dramatically when cells are rechallenged with wild type ligand post-antagonism. Both groups fail to proliferate upon stimulation with wild type peptide, but we demonstrate that previously antagonized cells make significant amounts of cytokines including IL-2. This distinct difference in cytokine production is a stark and important contrast between the phenotypes of antagonism and anergy. Thus, we describe a unique response pattern of T cells, a phenotype distinct from previously described forms of anergy.

Materials and Methods

Mice

3.L2 TCR transgenic mice were used at 6-12 weeks of age. The 3.L2 TCR is specific for Hb64-76 presented by I-E^k (112). Mice were bred and housed in the Emory University Department of Animal Resources facility according to federal guidelines.

Peptides

The wild type peptide, Hb64-76 (GKKVITAFNEGLK) and the antagonist 72I (GKKVITAFIEGLK) were purchased from Invitrogen (Carlsbad, CA). 72I is a single amino acid substitution, Asn (N)→Ile (I) at position 72.

Antagonism

Antagonism was performed as previously described by Sette and colleagues (2). Briefly, for antagonist conditions, cells were pre-pulsed with 1μM Hb64-76 for 2h at 37°C, washed twice in HBSS, then cultured with 10μM 72I or the indicated concentration of peptide for proliferation assays.

Cells and Reagents

3.L2 spleen cells ($2x10^6$/well) were cultured ex vivo with 1μM Hb64-76, pre-pulse only (1μM Hb64-76 for 2h, at 37°C) or under antagonist conditions (1μM Hb64-76 pre-pulse, 10μM 72I continuously) with 10 pg/mL IL-2 for 7 days in 24-well plates. Live cells were purified over a Ficoll gradient (Mediatech) and were restimulated with irradiated syngeneic splenocytes (2000 rad) and the indicated concentrations of peptide. Culture

media consisted of RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, .01M Hepes buffer, 100μg/mL gentamicin (Mediatech, Herndon, VA) and 5x10-5 M 2-mercaptoethanol (Sigma, St. Louis, MO).

Proliferation Assay

Naïve 3.L2 splenocytes ($3x10^5$/well) or cultured T cells ($5x10^4$/well) and irradiated syngeneic splenocytes (2000 rad, $5x10^5$/well) were cultured in 96-well plates with the indicated concentration of peptide at 37°C. Where indicated, recombinant IL-2 (BD Pharmingen, San Diego, CA) was added at 3.5ng/well. After 48h in culture, 0.4μCi/well of [^{3}H] thymidine was added. After an additional 18h, cells were harvested on a FilterMate harvester (PerkinElmer Life and Analytical Sciences, Wellesly, MA) and analyzed on a Matrix 96 Direct Beta Counter (PerkinElmer).

Cytokine ELISA

After one week in culture under the indicated conditions, 3.L2 T cells (1x106/well) were stimulated with irradiated syngeneic splenocytes (2000 rad, 5x106/well) and the indicated concentration of peptide for 24h (IL-2) or 48h (IFN-γ). Supernatants were incubated in triplicate on microtiter plates that had been coated with 50μl of purified anti-IL-2 (5 μg/mL, clone JES6-1A12, BD Pharmingen) or anti-IFN-γ (5 μg/mL, clone R4-6A2, BD Pharmingen) overnight at 4°C. Recombinant IL-2 or IFN-γ (BD Pharmingen) was used as a standard. Captured cytokines were detected using biotinylated anti-IL-2 (JES6-5H4, BD Pharmingen; 100 μg/mL, 100 μl/well) or biotinylated anti-IFN-γ (clone XMG1.2, BD Pharmingen; 100 μg/mL, 100 μl/well) followed by alkaline phosphatase-conjugated

avidin (Sigma, St. Louis, MO) and p-nitrophenylphosphate (pNPP) substrate (BioRad, Hercules, CA). Colorimetric change was measured at 405 nm on a Microplate Autoreader (Biotek Instruments, Winooski, VT).

Flow Cytometry

3.L2 T cells were stained with anti-CD25 (IL-2Rα)-FITC (clone PC61), anti-Vβ8.3-PE (clone 1B3.3) and anti-CD4-APC (clone RM4-5; all BD Pharmingen) at 24h and 48h after restimulation. Alternatively, cells were stained with PE conjugated CD122 (clone TM-b1) or CD132 (clone 4G3). For intracellular cytokine staining, cells were stimulated for 6h, then fixed and permeabilized with Caltag Fix and Perm cell permeabilization kit per the manufacturers instructions (Caltag, San Diego, CA). Permeabilized cells were stained with anti-CD4-PerCP and anti-IL-2-APC (clone JES6-5H4) or anti-IL-4-APC (clone 11B11; all BD Pharmingen). Annexin V and 7-AAD staining was performed according to manufacturer's instructions (BD Pharmingen). Flow cytometry was performed on a BD FACSCalibur (Franklin Lakes, NJ) and data were analyzed using FlowJo Software (Tree Star, San Carlos, CA). Data are gated on Vβ8.3+ or CD4+ cells as indicated.

Statistical analysis

Data were analyzed using GraphPad Prism. Statistical significance was determined by Student's *t* test or ANOVA, as indicated in the figure legends.

Results

Characterization of Antagonist Peptide

The response of 3.L2 T cells to both Hb64-76 and the APL 72I has been previously described (46, 112). As expected, the antagonist peptide alone induced minimal proliferation of 3.L2 T cells, whereas stimulation with the wild type peptide resulted in a dose dependent proliferative response (Fig. 2.1A, $p=0.0184$). Additionally, cells pre-pulsed with the wild type peptide were effectively antagonized (>95%) by addition of 72I (Fig. 2.1B). Thus the APL 72I acts as an antagonist, as it fails to stimulate proliferation when presented alone, and inhibits the T cell response to wild type peptide in a dose-dependent manner when both wild type and 72I are presented.

Phenotype Post-antagonism

Although T cell antagonism has been extensively described (62, 63, 106, 113-115), the response of these antagonized cells to subsequent stimulation with wild type ligand has not yet been described. Previous studies have shown that under antagonist conditions, T cells exhibit a dose dependent decrease in proliferation to wild type stimulus (45, 62, 63, 106, 113-115), altered cytokine production (98, 116), and altered signaling (26, 45, 106). To assess the effects of T cell antagonism on subsequent T cell function, we have characterized the proliferative and cytokine responses of previously antagonized T cells to rechallenge with wild type ligand.

After 7d in culture with either wild type peptide, pre-pulse alone or under antagonist conditions, cells were restimulated with wild type peptide. Cells cultured on wild type peptide show characteristic, dose-dependent proliferation upon restimulation

with wild type peptide. However, cells cultured on the antagonist condition show significantly blunted proliferation relative to wild type cultured cells (Fig. 2.2A, $p<0.05$). Antagonized cells secrete near normal or slightly elevated levels of the effector cytokine IFN-γ after 48h of wild type stimulation (Fig. 2.2B, $p=0.3878$).

The failure of pre-pulse only and antagonized cells to proliferate in response to wild type stimulation suggests a lack of IL-2, as is the case in classical anergy. As expected, cells cultured on pre-pulse alone produce little IL-2 after 24h of stimulation (Fig. 2.3, prepulse vs. antagonist, $p<0.001$). The lack of IL-2 production in the pre-pulse only condition coupled with the hypoproliferative response defines these cells as anergic. This is consistent with previous data indicating that anergy can be induced by a low dose of agonist ligand (102). Antagonized cells, however, secrete significantly greater amounts of IL-2 relative to wild type cultured cells after 24h of restimulation with wild type peptide (Fig. 2.3, Hb64-75 vs. antagonist $p<0.01$). This increased production of IL-2 contrasts sharply with the lack of proliferation exhibited by the antagonized T cells. This phenotype is clearly distinct from previously described forms of anergy, in which IL-2 production in blunted or entirely absent.

IL-2 and Antagonism

The differences in IL-2 production in these groups do not appear to be due to cell death, as no significant differences in Annexin V or 7-AAD staining were observed at 48h. (Fig. 2.4A). Additionally, these cells have not been skewed to a Th2 phenotype, as they are not producing IL-4 as measured by cytokine capture ELISA or intracellular cytokine staining (data not shown). The disconnect between IL-2 production and

proliferation that is observed in antagonized cells upon restimulation with wild type peptide suggests a disruption of the IL-2 signaling pathway. To address the mechanism of this unique phenotype, IL-2R levels were determined by flow cytometry. These studies indicated that expression of CD25, the high affinity IL-2R, is similar in both antagonized and wild type cultured cells across a range of doses at both 24h and 48h after restimulation (Fig. 2.4B, $p=0.4112$). Expression of CD122 and CD132 is also similar (data not shown), which implies that the disruption in the IL-2 pathway is not at the level of receptor expression.

Additionally, the anergic phenotype of cells cultured on pre-pulse alone can be overcome by the addition of exogenous IL-2 (Fig. 2.5; Hb64-76 vs. prepulse $p>0.05$), as has been described in other cases of low dose anergy (102). However, proliferation of antagonized cells cannot be rescued in this way (Fig. 2.5, Hb64-76 vs. antagonist $p<0.05$). These data further support the idea that a blockage in the IL-2 signaling pathway is responsible for the phenotype of hypoproliferation yet enhanced IL-2 production we have observed post-antagonism.

Discussion

In this study we have found that after stimulation under antagonist conditions, T cells respond to wild type antigen by producing high levels of IL-2 while also exhibiting a marked lack of proliferation. This lack of proliferation is similar in both anergized and antagonized cells, although these two groups of cells exhibit significant differences in their ability to produce and respond to IL-2. These findings indicate that antagonism is a distinctly different phenomenon than known forms of anergy.

Previous studies have described anergy induced by lack of costimulation (99-101, 117), low dose of agonist peptide (102), APL (98) and peptides containing substitutions at their MHC anchor residues (103, 104). Lack of proliferation and IL-2 production are key characteristics of all of the forms of anergy. Our data show that antagonized cells exhibit a phenotype of hypoproliferation and normal IFN-γ production that is similar to anergic cells. In most forms of anergy, including that induced by low doses of wild type peptide, proliferation can be rescued by the addition of exogenous IL-2 (98, 99). However, the lack of proliferation in antagonized cells is not reversible by the addition of exogenous IL-2. The failure of IL-2 to rescue the hypoproliferative phenotype post-antagonism is similar to APL induced anergy, suggesting that these phenotypes may share some mechanistic features. In most forms of anergy, for example the pre-pulse only condition, cells are unable to produce IL-2, but they remain able to respond to it (102). In contrast, antagonized cells secrete large quantities of IL-2, but have been rendered unresponsive to this growth factor. Furthermore, our data demonstrate that the presence of an antagonist is sufficient to at least partially overcome low dose induced anergy, suggesting that this phenotype is mechanistically distinct from low dose induced

anergy.

The failure of antagonized cells to respond to IL-2 from both endogenous and exogenous sources is not due to differences in apoptosis or decreased expression of IL-2R, as we have found that all three chains of the high affinity IL-2R are expressed at similar levels in both antagonized and wild type cultured cells. These data suggest an intracellular disruption of the IL-2 signaling pathway as the mechanism mediating the phenotype post-antagonism.

Several pathogens have been shown to generate escape epitopes that function as T cell antagonists, including HIV (26, 107), Hepatitis B and C (21, 108) and malaria (18). In the context of one of these infections, the phenotype of T cells that encounter their cognate ligand after interacting with an antagonist mutant epitope could dramatically impact the course of the infection. The data presented herein would suggest that these T cells would produce large quantities of IL-2, perhaps expanding or activating regulatory T cells (118, 119), and thereby potentiating the efficacy of the pathogen's escape strategy. Alternatively, this cytokine production could result in non-specific bystander activation, which could also result in subsequent failure to clear the infection and/or immunopathology. The phenotype that we have observed post-antagonism underscores the role of T cell epitope mutation in the escape of chronic infections from control by the immune system.

T cell unresponsiveness can result from many different pathways, as evidenced by the diverse mechanisms of anergy that have been previously described. In T cells that are anergic, the proliferative and IL-2 responses are significantly reduced. Similarly, T cell antagonism also results in a refractory state in which proliferation is blocked. Unlike

classical anergy, however, antagonized cells retain their ability to secrete the growth factor IL-2, despite their inability to respond to it. This inability to respond is similar to APL induced anergy, as well as MHC variant peptide induced anergy, which has been shown to be mediated by the negative regulatory phosphatase SHP-1 (59). SHP-1 has also been shown to play a role in the early stages of antagonism (46, 48). Furthermore, at least one report has demonstrated association of SHP-1 with the IL-2 receptor complex (56). Taken together, these data suggest that this phosphatase may play an important role in regulating the phenotype that we have described. In this way, the phenotype post-antagonism may be mechanistically similar to anergy induced by altered peptide ligands, as neither of these states can be overcome by IL-2.

The data presented herein demonstrate that the response of T cells post-antagonism is distinct from previously described forms of anergy, as antagonized cells respond to wild type stimulation by producing large quantities of IL-2, whereas anergized cells produce very little or no IL-2. The increased production of IL-2 by antagonized T cells in response to wild type stimulus contrasts sharply with the failure of these cells to proliferate. This cytokine hyperproduction coupled with the inability to proliferate represents a unique phenotype in T cell biology.

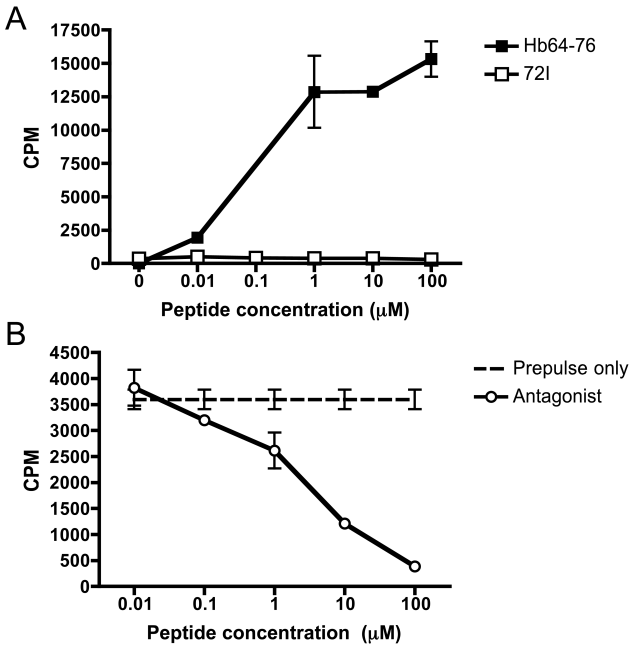

Figure 2.1 72I acts as a TCR antagonist.

A. Splenocytes were cultured with indicated concentrations of wild type peptide (Hb64-76) or antagonist peptide (72I) for 48h. Cells were cultured an additional 18h with [^{3}H] thymidine and proliferation was measured by [^{3}H] thymidine incorporation. p=0.0184 by Student's *t* test. B. Splenocytes were cultured on the indicated doses of antagonist peptide (72I) following a 2h pre-pulse with 1μM wild type peptide. The dashed line indicates proliferation caused by the pre-pulse alone. Proliferation was assayed as in part A. Assays were performed in duplicate and data are representative of 5 independent experiments.

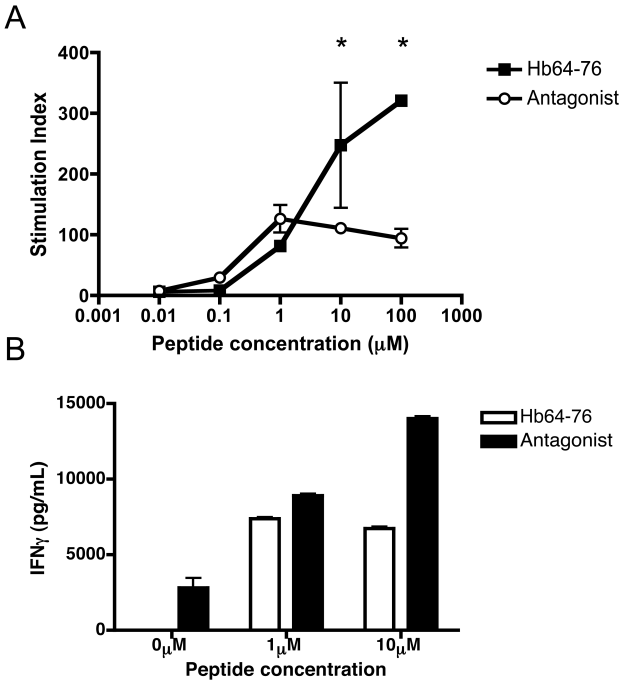

Figure 2.2 Antagonism causes blunted proliferation but normal IFN-γ production in response to wild type peptide.

A. After 7d in culture with either wild type peptide (1μM) or under antagonist conditions (1μM wild type prepulse plus continuous culture with 10μM antagonist peptide), and proliferation was assayed. Cells were stimulated with irradiated syngeneic splenocytes as antigen presenting cells and the indicated concentrations of wild type peptide (Hb64-76) for 48h. Cells were cultured an additional 18h with [^{3}H] thymidine and proliferation was measured by [^{3}H] thymidine incorporation. * indicates $p<0.05$ by Student's *t* test. Assays were performed in duplicate. Data are representative of 4 independent experiments. B. Cells were cultured and restimulated as described in A. IFN-γ production was assayed by cytokine capture ELISA 48h after restimulation. Differences are not statistically significant by Student's *t* test ($p=0.3878$). Assays were performed in triplicate and data are representative of 3 independent experiments.

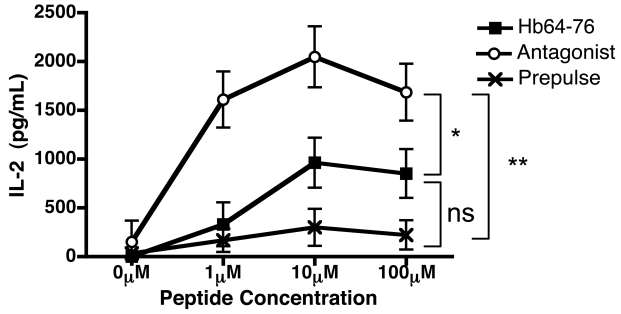

Figure 2.3 Antagonized cells exhibit altered IL-2 production.

Cells were cultured 7d with either wild type peptide (1μM) or under antagonist conditions (1μM wild type prepulse plus continuous culture with 10μM antagonist peptide) and then restimulated for 24h with irradiated syngeneic splenocytes and the indicated doses of wild type peptide. IL-2 production was assayed by cytokine capture ELISA. Data were analyzed by ANOVA with Bonferroni's Multiple Comparison Test. * $p<0.01$, ** $p<0.001$, ns=not significant. Assays were performed in triplicate and data are representative of 3 independent experiments.

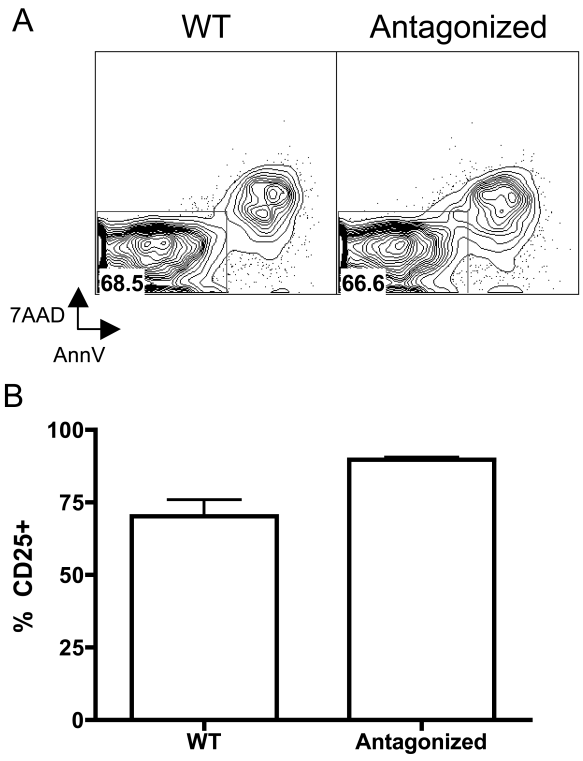

Figure 2.4 Antagonism does not induce apoptosis or alter IL-2R expression.

A. After 7d in culture on either wild type peptide (1μM) or under antagonist conditions (1μM wild type prepulse plus continuous culture with 10μM antagonist peptide), cells were restimulated with irradiated syngeneic splenocytes and 10μM wild type peptide. At 48h, cells were stained for Annexin V and 7-AAD. B. After 7d in culture on either wild type peptide (1μM) or under antagonist conditions (1μM wild type pre-pulse plus continuous culture with 10μM antagonist peptide), cells were restimulated with irradiated syngeneic splenocytes and 10μM wild type peptide. Cells were stained with anti-CD25-FITC at 24h after restimulation and analyzed by flow cytometry. All data were gated on Vβ8.3+ cells. Differences are not significant by Student's *t* test (p=0.4112). Data are representative of 3 independent experiments.

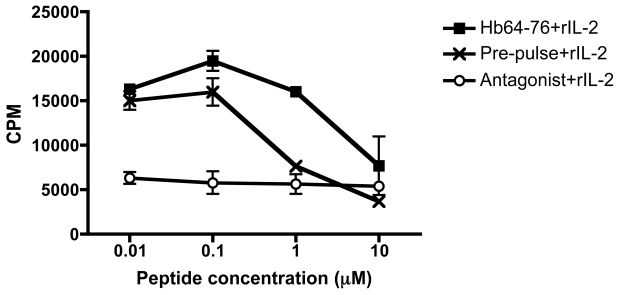

Figure 2.5 Exogenous IL-2 restores proliferation of pre-pulsed but not antagonized cells.

Cells were cultured for 7d on either wild type peptide (1μM), under antagonist conditions (1μM wild type prepulse plus continuous culture with 10μM antagonist peptide) or pre-pulsed with 1μM wild type and then washed. Cells were restimulated with irradiated syngeneic splenocytes and indicated amounts of wild type peptide with or without the addition of recombinant IL-2 for 48h. Cells were cultured an additional 18h with [^{3}H] thymidine and proliferation was measured by [^{3}H] thymidine incorporation. Differences between Hb64-76 and prepulse only were not significant. Differences between Hb64-76 and antagonist $p<0.05$. Data were analyzed by ANOVA with Dunnett's Multiple Comparison Test. Assays were performed in duplicate and data are representative of 3 independent experiments.

Chapter 3

Induction of unresponsiveness in diabetogenic T cells by MHC variant peptides.

Abstract

Autoreactive T cells are responsible for causing a number of autoimmune diseases, including type 1 diabetes. In an effort to ameliorate CD4+ T cell mediated autoimmune disease, we have developed a strategy to induce unresponsiveness in autoreactive T cells by destabilizing the peptide:MHC ligand recognized by the T cell receptor. By introducing amino acid substitutions into the immunogenic peptide at residues that determine binding to the MHC, we reduce the half life of the peptide:MHC complex, thereby resulting in abortive T cell activation. We have utilized this strategy to induce anergy in both monoclonal and polyclonal diabetogenic T cells. By treating activated T cells with an MHC variant peptide, the cells are rendered unresponsive to the wild type ligand, as measured by both proliferation and IL-2 production. Stimulation of T cells with MHC variant peptides initiates a signaling program dominated by negative signals, with only weak and/or delayed activating signals. These negative signaling events result in an anergic phenotype in which the T cells are not competent to signal through the IL-2 receptor, as evidenced by a stark lack of phospho-Stat5 upregulation despite high expression of the IL-2 receptor. This unique negative signaling cascade provides a chance to shut down the anti-self response, thereby providing opportunities for therapeutic intervention in T cell mediated autoimmune diseases.

Introduction

T cell mediated autoimmune diseases, including type 1 diabetes mellitus, are the result of inappropriate T cell activation in response to self antigens. Thymic education is incapable of completely eliminating autoreactive T cells, thus necessitating peripheral tolerance mechanisms, including induction of an unresponsive state termed anergy (120). T cell anergy was first defined as a hyporesponsive state in which T cells fail to proliferate or produce IL-2 in response to normally agonistic stimuli (121). In the years following this initial description, use of the term anergy expanded to include essentially any tolerant T cell state in which the cells survive for a prolonged period but remain functionally unresponsive (120).

An anergic phenotype can be induced in CD4+ T cells in a number of different ways. Among these are provision of a strong signal through the TCR in the absence of sufficient costimulation, by means of chemically fixed antigen presenting cells, antibody cross-linking or antibody blockade, low or high doses of antigen, altered peptide ligands and MHC variant peptides (3, 66, 99-104). Anergy induced by each of these methods results in a stable phenotypic shift resulting in cells that survive, but fail to divide or produce IL-2 in response to a normally strong stimulus. However, the anergic phenotype can frequently be overcome in common forms of anergy by the provision of IL-2, although this is not the case in anergy induced by MHC variant peptides (122, 123). Interestingly, antagonism with an altered peptide ligand results in a unique semi-anergic state in which antagonized T cells respond to agonist stimulation with abundant IL-2 production but limited proliferation (44). The variety of ways to induce anergy and the

subtle differences in the phenotypes generated by each model imply that the molecular mechanisms may vary.

Although the different means of inducing anergy result in a similar hyporesponsive phenotype, it is thought that the mechanisms regulating the induction and maintenance of this phenotype vary to an extent. For example, cells anergized by provision of TCR signal alone exhibit a blockade in the activation of the kinases Erk and Jnk and the GTP-ase Ras following CD3/CD28 cross-linking (124, 125). However, anergy induction by altered peptide ligands results in altered zeta chain phosphorylation (4), and a subsequent failure to recruit ZAP-70 to the TCR (4, 5). The mechanisms controlling the induction of anergy by MHC variant peptides are unknown.

Here we demonstrate that an MHC variant peptide can be utilized to induce anergy in a monoclonal diabetogenic T cell population. Stimulation with the variant peptide results in undetectable early phosphorylation events, with a concomitant increase total tyrosine phosphatase activity. Specifically, activity of the phosphatase SHP-1 is increased following stimulation with an anergy-inducing peptide. Unlike classical anergy, MHC variant peptide-induced anergy is not overcome by the addition of exogenous IL-2, and we demonstrate a lack of Stat5 phosphorylation following IL-2 stimulation of anergic cells. Thus we have characterized several novel aspects of the molecular mechanisms regulating CD4+ T cell response to MHC variant peptides.

Methods

Mice

BDC2.5 TCR transgenic (126) breeders were a kind gift from Mark Rigby. NOD mice were purchased from either Jackson Laboratory (Bar Harbor, Maine) or Taconic Farms (Germantown, New York). All mice were housed by the Emory University Division of Animal Resources. Breeding and experiments were carried out in accordance with protocols approved by the Emory University Institutional Animal Care and Use Committee and other applicable regulations.

Peptides and reagents

Peptides were synthesized in house using FMOC chemistry on a Peptide Technologies Incorporated Prelude synthesizer. Because the specific epitope recognized by BDC2.5 T cells was previously unknown, synthetic peptides were screened to identify sequences that stimulate the T cells. These epitope mimics have been termed mimotopes. The mimotope sequence utilized in these studies was AHHPIWARMDA (127). The sequence of the YPDV variant was AHYPIPADMDV.

Cell culture

BDC2.5 spleen cells ($2x10^6$/well) were cultured *ex vivo* with 1μM mimotope peptide for 2 wks in 24-well plates. Live cells were purified over a Ficoll gradient and

restimulated for 2wks with irradiated syngeneic splenocytes (3000 rad) and either 1μM mimotope peptide or 10μM variant peptide.

For proliferation assays, naïve or previously activated T cells and irradiated syngeneic splenocytes (3000 rad) were cultured in 96-well plates with the indicated concentration of peptide at 37°C. After 48h in culture, 0.4μCi/well of [^{3}H] thymidine was added. After an additional 18h, cells were harvested on a FilterMate harvester (Packard Instrument) and [^{3}H] thymidine incorporation was assessed on a 1450 LSC Microbeta TriLux counter (PerkinElmer). Where indicated, recombinant mouse IL-2 was added to a final concentration of 3.5ng/well.

Culture media consisted of RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, 0.01M Hepes buffer, 100μg/mL gentamicin (Mediatech, Herndon, VA) and $5x10^{-5}$ M 2-mercaptoethanol (Sigma, St. Louis, MO).

Cytokine ELISA

After induction of anergy, T cells were stimulated with irradiated syngeneic splenocytes (3000 rad) and 10μM mimotope peptide for 24h. Supernatants were incubated in triplicate on microtiter plates coated with purified anti-IL-2 (5 μg/ml clone JES6-1A12; BD Pharmingen). Recombinant IL-2 was used as a standard. Captured cytokines were detected using biotinylated anti-IL-2 (100 μg/ml JES6-5H4, 100 μl per well; BD Pharmingen) followed by alkaline phosphatase-conjugated avidin and p-nitrophenylphosphate (pNPP) substrate (Sigma). Colorimetric change was measured at dual wavelengths of 405 and 630 nm on a Microplate Autoreader (Biotek Synergy HT)

Western blots

At various times after stimulation, cells were lysed with a buffer containing 20mM Tris-HCl, 150mM NaCl, 1mM EDTA, 0.5% Igepal, protease inhibitor cocktail (Calbiochem) and sodium orthovanadate (1mM). Proteins were resolved on a 4-15% SDS-PAGE gel (BioRad) and transferred onto nitrocellulose. Blots were probed with antibodies to pErk1/2 (Cell Signaling Technologies) or β-actin (Sigma).

Flow cytometry

Cells were stimulated with 100μg recombinant mouse IL-2 for the indicated periods of time. $3x10^5$ to $5x10^5$ cells were fixed in a final concentration of 1.5% formaldehyde (Polysciences) for 30min-18h. Cells were then permeabilized in 100% ice cold methanol for 10 minutes. Cells were stained for 30 min. on ice with antibodies to CD4 (RM4-5, BD Biosciences), CD25 (clone PC61, BD Biosciences), p-p44/42 (D13.14.4E, Cell Signaling) and/or pStat5 (Y694, BD Biosciences). Staining buffer consisted of phosphate buffered saline containing 0.1% BSA and 0.05% sodium azide. Data was collected on a BD FACSCalibur and analyzed using FlowJo software (TreeStar).

pNPP assay

For whole cell lysate phosphatase activity, cell lysates were prepared as described for western blot, with lysis buffer not containing sodium orthovanadate. pNPP substrate solution was added to lysates, incubated overnight at 37°C and colorimetric change was

read at dual wavelengths of 405 and 630 nm on a Microplate Autoreader (Biotek Synergy HT).

SHP-1 activity assay

For specific SHP-1 activity, SHP-1 was immunoprecipitated from whole cell lysates prepared without the addition of sodium orthovanadate. Immunoprecipitation was performed with 2μg of anti-SHP1 antibody (Santa Cruz) and a Pierce Protein A/G plate based immunoprecipitation kit. The immunoprecipitated protein was reduced using pNPP Tyr Assay Buffer (Millipore) and incubated for 1h at 37°C with a pTyr containing substrate peptide (AEEEIpYGEFEA). Malachite green was added to assess the amount of free phosphate in the solution, and colorimetric change was assessed at 620nm on a BioTek microplate reader.

Statistical analysis

Data were analyzed using GraphPad Prism. Statistical significance was determined by Student's *t* test or ANOVA, as indicated in the figure legends.

Results

Peptide design

Peptide substitutions were designed based on existing studies of peptide binding to I-A^{g7} class II MHC molecules (128, 129). The parent peptide sequence and the variant utilized in this study are aligned in Figure 3.1. The binding groove of I-Ag7 exhibits a preference for medium, hydrophobic residues at p4, large, hydrophobic residues at p6 and an aromatic, hydrophobic or positively charged amino acid at p9 (128, 130). The YPDV variant contains the following substitutions: H→Y, W→P, R→D and A→V.

BDC2.5 T cells do not proliferate in response to variant peptide

To determine the immunogenicity of our variant peptide, we performed a proliferation assay. BDC2.5 splenocytes we activated for 14 days on 1μM mimotope peptide. Activated T cells were then re-challenged with either the parent mimotope or the variant peptide. The BDC2.5 T cells exhibited typical dose dependent proliferation to the wild type mimotope (Fig. 3.2). However, these cells had no proliferation above background at any of the doses of variant peptide tested, indicating that the YPDV variant is dramatically less stimulatory than the parent peptide.

Variant stimulation induces no detectable Erk1/2 phosphorylation

To characterize the mechanism by which MHC variant peptides induce anergy, we examined positive signaling events following initial encounter of activated T cells

with the variant peptide. We assessed phosphorylation of the MAP kinase Erk1/2 following stimulation of activated BDC2.5 T cells with either the mimotope or variant peptide. Following stimulation with the agonist peptide, we observed robust phosphorylation of Erk1/2 as rapidly as 1 minute, which waned to lower, but still detectable levels by 60 minutes of stimulation (Fig. 3.3A). However, upon stimulation with the variant peptide YDPV, we observed no detectable Erk1/2 phosphorylation at any time point (Fig. 3.3B) by western blot. To assess Erk1/2 phosphorylation on a more quantitative basis, we utilized phospho-flow cytometry. Similar to the data obtained by western blot, mimotope stimulation resulted in rapid Erk1/2 phosphorylation peaking at 30min before waning to background levels (Fig. 3.3C). No Erk1/2 phosphorylation above background was detectable in variant stimulated cells, even at later time points.

Variant stimulated cells exhibit increased total tyrosine phosphatase and specific SHP-1 phosphatase activity

The lack of a positive signal in the form of Erk1/2 phosphorylation following stimulation with the variant peptide may be the result of a lack of upstream positive signals sufficient to result in detectable Erk1/2 phosphorylation or an increase in negative signaling events such that Erk1/2 phosphorylation is reversed rapidly enough to be undetectable. Furthermore, a combination of these two situations may be occurring, as they are not mutually exclusive. Previous studies utilizing CD8+ T cells have demonstrated that positive signals delivered by a weak agonist are insufficient to result in detectable Erk1/2 phosphorylation (9). However, analysis of the activation of Jun/Jnk, signaling mediators downstream of Erk1/2, revealed that a "trickle through" of positive

signals was occurring such that although phosphorylation of Erk1/2 was not observed, phosphorylation of Jun/Jnk accumulated over time. We have observed a similar phenomenon in autoreactive CD4+ T cells in which T cells with a low affinity for their ligand have no detectable Erk1/2 phosphorylation, but accumulate phospho-Jnk/Jun over time and exhibit a delayed but still maximal upregulation of activation markers, cytokine production and proliferation (unpublished data). Likewise, we have also previously demonstrated that encounter of CD8+ T cells with a viral escape mutant antigen induces minimal Erk1/2 phosphorylation, but rather induces a negative signaling program dominated by the tyrosine phosphatase SHP-1 (34).

In T cell signaling, with a few notable exceptions (e.g., CD45), kinases drive the positive signaling events while phosphatases are responsible for negative regulation. To determine if treatment with our variant peptide upregulated a generally negative signaling profile, we assessed bulk T cell lysates for their phosphatase activity. To do this, activated BDC2.5 T cells were stimulated with antigen presenting cells and either mimotope or variant peptide. At various times following stimulation, cells were harvested and lysed, and phosphatase activity was assessed by incubating the lysates with p-nitrophenylphosphate (pNPP) as a substrate. Mimotope stimulation resulted in a low level of tyrosine phosphatase activity (Fig. 3.4A). Variant stimulated cells, however, exhibited a significantly different pattern of phosphatase activity. Activated BDC2.5 T cells restimulated with variant peptide exhibited an early peak of phosphatase activity that was approximately three times greater in magnitude than that produced by wild type stimulated cells. Additionally, phosphatase activity was sustained in variant stimulated cells throughout the 30 minute time course assayed (Fig. 3.4A).

To clarify the specific negative signaling events occurring in this system, we next assayed the activity of the phosphatase SHP-1. In this case, cells were stimulated as for the total phosphatase assay and lysed. SHP-1 was immunoprecipitated from the cell lysates, and its activity was assessed by incubating the immunoprecipitated protein with a phospho-tyrosine containing target peptide. Phosphate release was measured by malachite green. Similar to the bulk phosphatase activity, SHP-1 activity in mimotope stimulated cells peaked and then waned over time (Fig. 3.4B). Variant stimulated cells peaked at the same time point as mimotope treated cells, but maintained high levels of phosphatase activity for the duration of the assay (Fig. 3.4B).

Variant peptide treatment induces anergy

Anergy has been classically defined as an unresponsive state in which T cells do not proliferate or secrete IL-2 in upon challenge with agonist peptide. To induce anergy with our variant peptide, we first activate cells for two weeks with 1μM of mimotope (wild type) peptide. Live cells are then restimulated with additional antigen presenting cells and either wild type or variant peptide. Cells that were passed for two cycles on mimotope peptide exhibit typical dose dependent proliferation to mimotope peptide (Fig. 3.5A). However, cells cultured on the variant peptide and then rechallenged with mimotope show no proliferation above background (Fig. 3.5A).

Similar to the phenotype observed for proliferation, cell passed on wild type peptide secrete significant amounts of IL-2, while those cells cultured on variant peptide secrete very limited amounts of IL-2 (Fig. 3.5B). These data indicate that the YPDV variant peptide is similar to the MHC variant peptides we have previously described for

myelin antigens in experimental autoimmune encephalomyelitis (103, 131), i.e. it is minimally stimulatory and stimulation with variant is sufficient to induce an anergic phenotype characterized by minimal proliferation and IL-2 production in response to stimulation with the agonist ligand.

Anergic T cells are unresponsive to IL-2 stimulation

A key characteristic of MHC variant peptide induced anergy is the inability of IL-2 to overcome the unresponsive phenotype. In other forms of anergy, the provision of exogenous IL-2 is sufficient to restore the cells' ability to proliferate (122). The resistance of T cells anergized by MHC variant peptides to rescue by IL-2 is a critical aspect of their potential therapeutic utility, as MVP-anergized cells would not be reactivated during a subsequent immune response (123). The mechanism of this resistance to IL-2 stimulation has not been previously examined.

To verify that the anergized BDC2.5 T cells were resistant to IL-2 induced proliferation, we stimulated anergic cells with agonist ligand in the presence of 3.5ng/well recombinant IL-2. Mimotope cultured cells cultured without the addition of IL-2 exhibit a typical proliferation profile, while variant cultured cells have little proliferation above background (Fig. 3.6A). Addition of IL-2 to mimotope cultured cells increased background proliferation in cells not stimulated with peptide. However, variant cultured cells challenged with agonist peptide plus IL-2 did not exhibit proliferation above background, consistent with the inability of cells anergized by MHC variant peptides to be rescued by the addition of IL-2 (Fig. 3.6B).

To characterize the mechanisms mediating the IL-2 resistant phenotype of the anergized BDC2.5 cells, we first assessed their expression of the high affinity IL-2 receptor by staining for CD25. Both mimotope cultured and YPDV anergized cells expressed high levels of CD25 (Fig. 3.7A). Interestingly, the anergic cells maintained levels of CD25 that were slightly elevated relative to the mimotope cultured control cells. Thus, the lack of receptor expression does not explain the inability of the anergized cells to be rescued by the addition of exogenous IL-2.

Ligation of the IL-2 receptor drives both proliferative and survival signals (132). The proliferative program is mediated by signaling through Jak1 and Jak3, which phosphorylate Stat5. To determine if this aspect of signaling via the IL-2 receptor was disrupted in MHC variant peptide anergized cells, we stimulated the cells with IL-2 and assessed Stat5 phosphorylation by flow cytometry. Stimulation of mimotope cultured cells with IL-2 resulted in robust phosphorylation of Stat5 by 30 minutes (Fig. 3.7B and C). However, anergized cells displayed minimal Stat5 phosphorylation (Fig. 3.7B and C), suggesting that some blockade in this pathway is responsible for the inability of MVP-anergized cells to respond to IL-2 stimulation.

Discussion

The occurrence of autoimmune disease is a stark reminder that central tolerance is an imperfect means of protection from autoreactive T cells. In fact, T cells specific for self antigens can be identified even in healthy individuals (133). Due to the incomplete nature of thymic selection, peripheral tolerance mechanisms have evolved to prevent the activation of autoaggressive T cells. Perhaps the best studied of these peripheral tolerance mechanisms is anergy, a state of hyporesponsiveness characterized by limited proliferation and cytokine production. Previous studies of various ways of inducing T cell tolerance have revealed that the anergic phenotype can be generated by modifying a variety of the parameters that control T cell activation and development of effector function.

We have previously demonstrated that decreasing the stability of an antigenic peptide's interaction with the MHC is an effective strategy for inducing anergy in both monoclonal and polyclonal populations of T cells (103, 104, 123). We have expanded these findings in this study to characterize an MHC variant peptide capable of inducing anergy in a diabetogenic T cell population. In addition, we have demonstrated that MHC variant peptide-induced anergy cannot be overcome by the addition of exogenous IL-2. This failure to respond to IL-2 stimulation is not due to differences in expression of the high affinity IL-2 receptor, as expression levels are similar in agonist treated and anergized populations. However, following stimulation with IL-2, anergized cells failed to upregulate phosphorylation of Stat5, a key mediator of the IL-2 signaling pathway driving proliferation. This failure to display detectable levels of Stat5 phosphorylation

following IL-2 stimulation may be a result of either a failure to activate another kinase upstream of Stat5, increased activity of a phosphatase responsible for Stat5 dephosphorylation or a combination of these two. Given the role of signaling through Stat5 in helping drive T cell proliferation, it is possible this blockade in IL-2 signaling represents a key mechanism for limiting proliferation of anergic cells.

In addition to its role in maintaining tolerance, anergy can also be a consequence of various pathological states including infection and cancer (134, 135). Better understanding the mechanisms regulating the induction and maintenance of the anergic state may allow for therapeutic interventions to induce anergy as a means to treat autoimmune diseases or prevent graft rejection. Furthermore, these mechanistic insights may also be utilized as a means to reverse anergy, allowing for the revitalization of a functional T cell response.

MHC variant peptide anergy represents a unique approach to the induction unresponsiveness in T cells. Inducing anergy by destabilizing the interaction between the peptide and MHC results in a stable hypoproliferative phenotype that cannot be rescued by the addition of exogenous IL-2. This key feature would be of critical importance if used therapeutically, as bystander activation during an ongoing immune response would be less likely to result in expansion of the anergized population. Similarly, viral escape mutants or tumor antigens that have decreased affinity of peptide: MHC interaction anergize reactive T cells (34, 134, 135). Because T cells anergized by MHC variant peptides are resistant to stimulation via the IL-2 receptor, knowledge of the signaling events downstream of IL-2 stimulation of anergized cells can inform efforts to reinvigorate the T cell response.

			P1			P4		P6			P9
Parent	A	H	**H**	P	I	**W**	A	**R**	M	D	**A**
Variant			**Y**			**P**		**D**			**V**

Figure 3.1 Alignment of parent and variant peptide sequences.

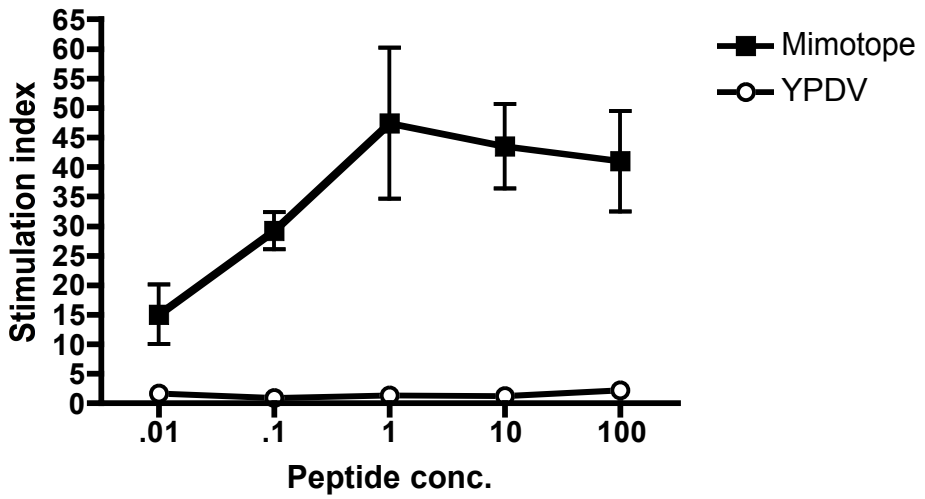

Figure 3.2 BDC2.5 T cells do not proliferate in response to variant peptide.

A. Sequence of the mimotope and variant peptides. Red indicates predicted MHC anchor residues. **B.** BDC2.5 splenocytes were stimulated with a range of concentrations of both wild type and variant peptide. Proliferation was assessed by [^{3}H] thymidine incorporation as described in the methods. Data represent the average of two experiments, each performed in duplicate.

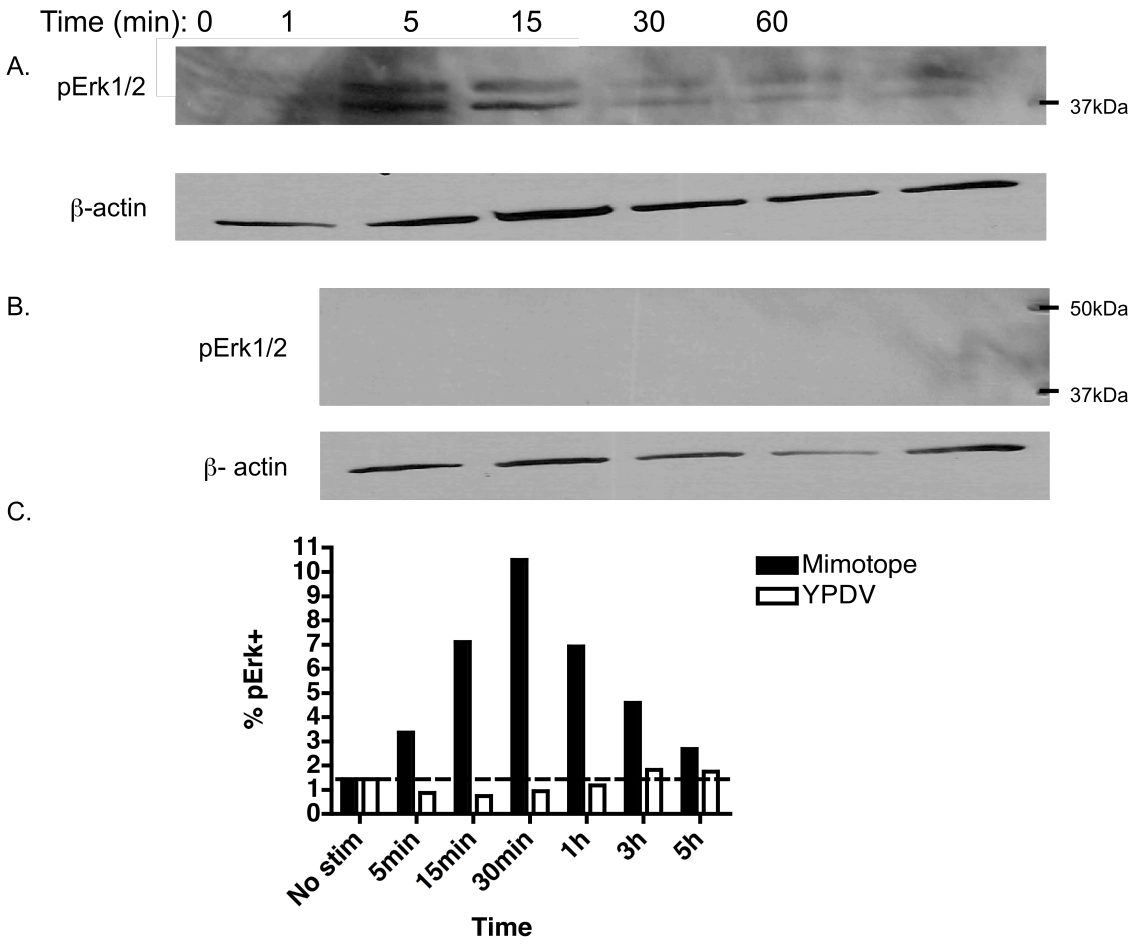

Figure 3.3 Variant stimulation induces no detectable Erk1/2 phosphorylation. BDC2.5 splenocytes were stimulated with mimotope peptide for 14d. Live cells were stimulated with syngeneic splenocytes and either 1μM mimotope (**A**) or 10μM variant (YPDV, **B**) for the indicated times. **C.** BDC2.5 T cells were stimulated as in parts A and B. Cells were fixed and permeabilized and Erk1/2 phosphorylation was determined by flow cytometry. Y-axis represents percent of CD4+ T cells that are pErk1/2+

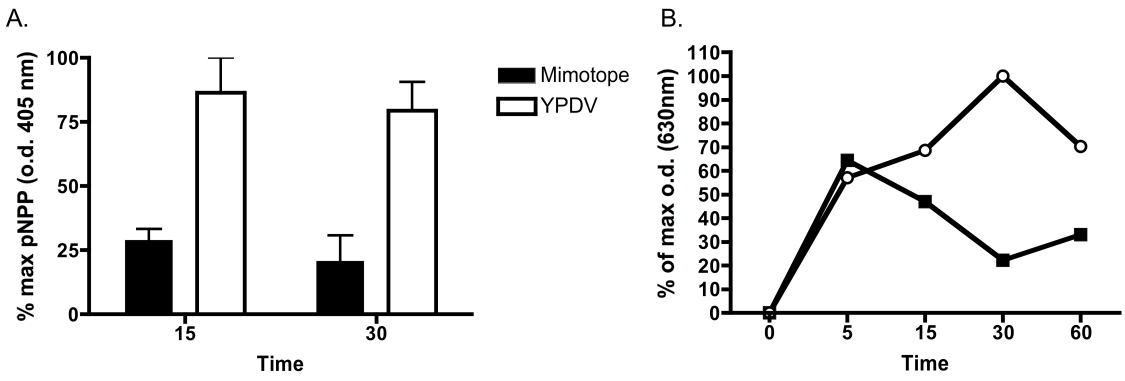

Figure 3.4 Variant stimulated cells exhibit increased total tyrosine phosphatase and specific SHP-1 phosphatase activity.

A. BDC2.5 T cells were stimulated as in Figure 3. pNPP solution was added to whole lysates and allowed to develop overnight at 37°C. Colorimetric change was assessed at 405nm. **B.** Cells were stimulated as in Figure 3. After cell lysis, SHP-1 was immunoprecipitated and incubated with a pTyr containing substrate peptide. Malachite green was added and amount of free phosphate was determined by colorimetric change at 620nm.

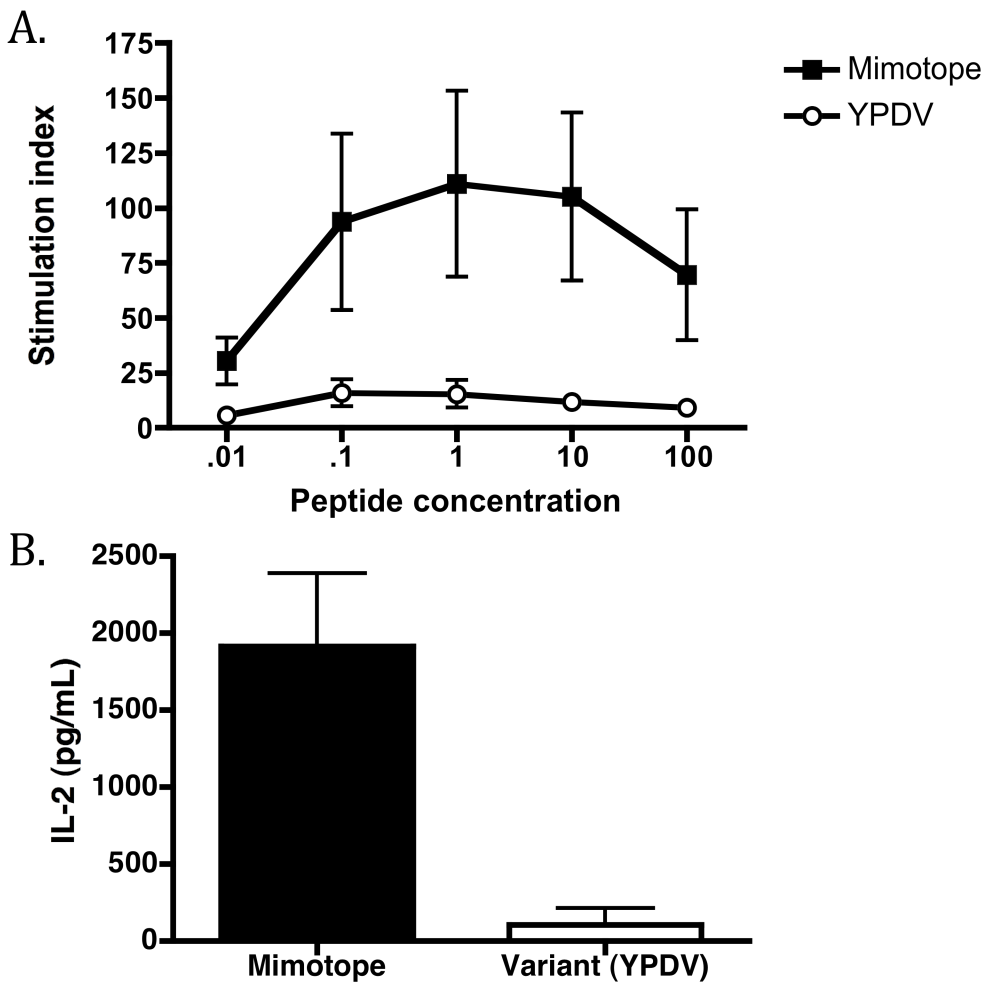

Figure 3.5 Variant peptide treatment induces anergy.

A. BDC2.5 cells treated with either wild type mimotope or variant (YPDV) were restimulated with the indicated concentrations of wild type peptide. Proliferation was assessed by [^{3}H] thymidine incorporation. Data represents average of four independent experiments performed in duplicate. **B.** Wild type or variant treated BDC2.5 T cells were restimulated with 10μM wild type peptide. Supernatants were harvested after 24h and IL-2 production was determined by ELISA. Data is representative of at least 3 independent experiments.

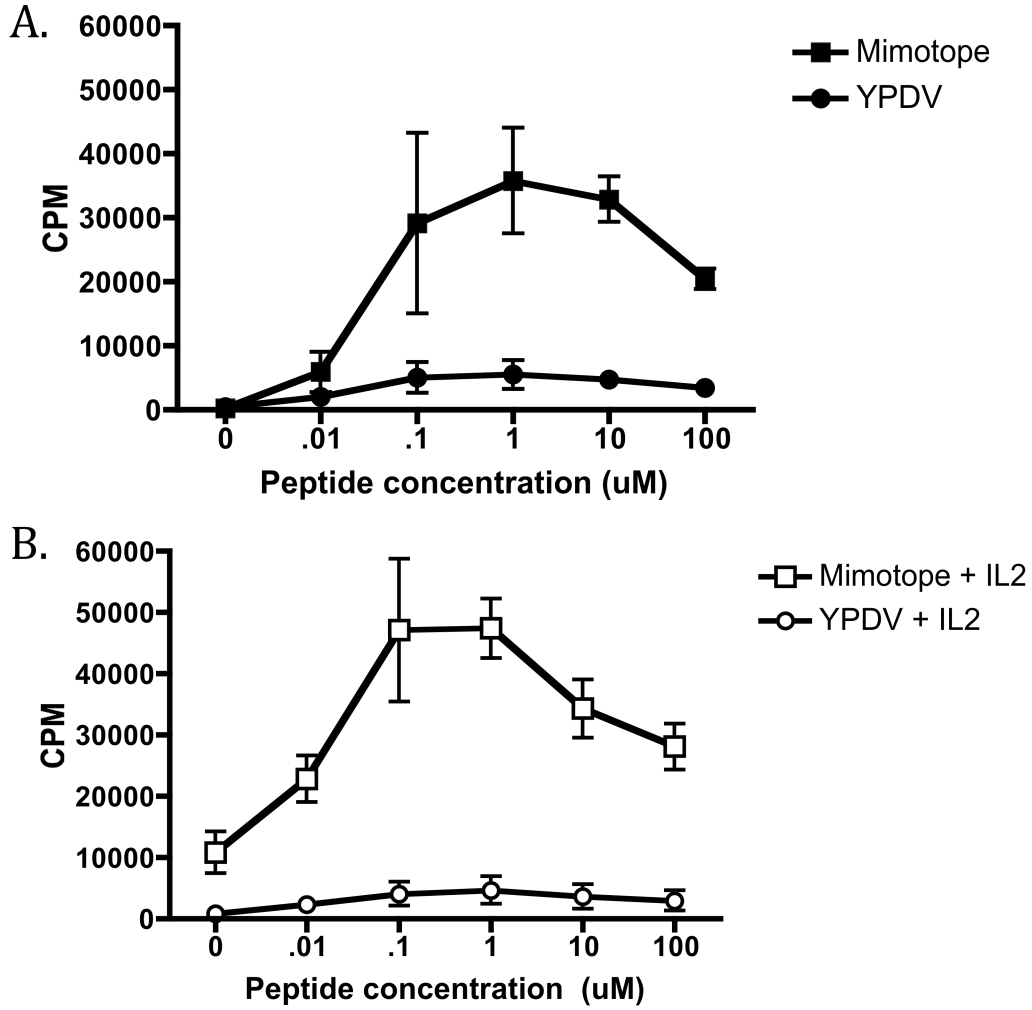

Figure 3.6 Addition of exogenous IL-2 does not rescue proliferation of anergized T cells.

BDC2.5 T cells were stimulated for 14d with 1μM mimotope peptide. Live cells were restimulated for an additional 14d with either 1μM mimotope of 10μM variant peptide plus irradiated antigen presenting cells. Live cells were restimulated with irradiated antigen presenting cells and indicated doses of mimotope without the addition of IL-2 (**A**) or with IL-2 (**B**).

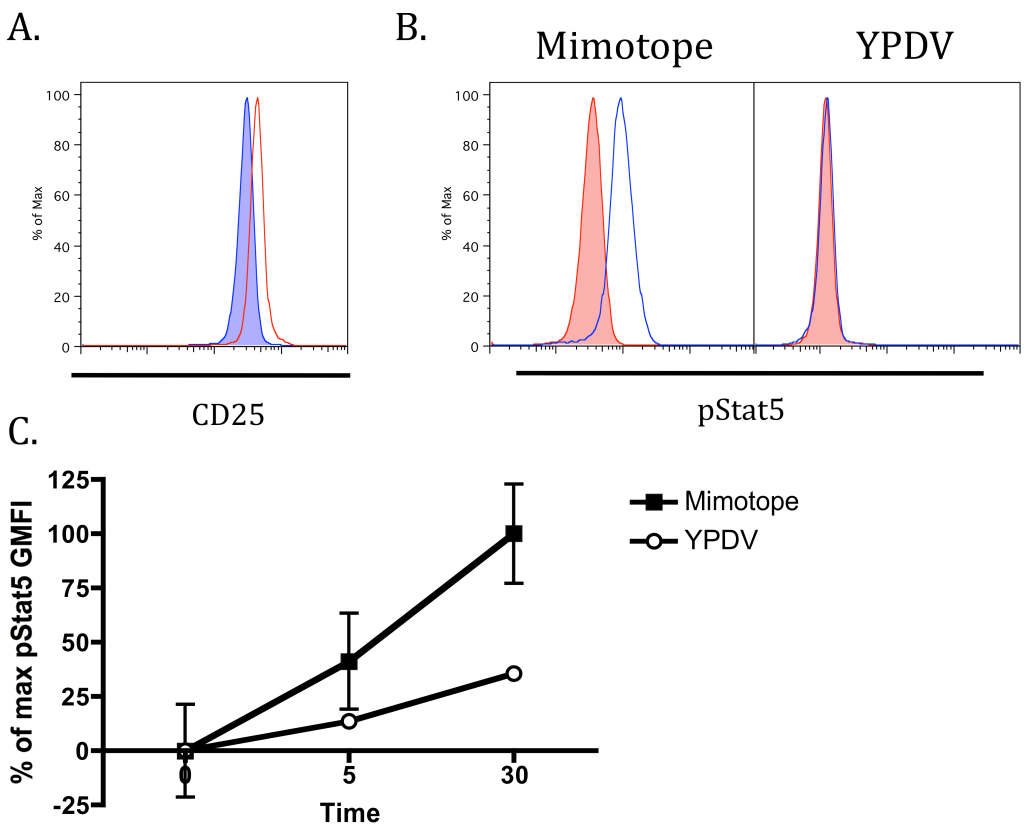

Figure 3.7 MHC variant peptide anergized cells are resistant to signaling through the IL-2 receptor.

A. Expression of CD25 is slightly elevated in variant anergized cells (red line, open histogram) as compared to mimotope-cultured cells (blue, filled histogram). B. 30 minutes following stimulation with 100ng/ml recombinant IL-2 mimotope cultured cells exhibit significant Stat5 phosphorylation (blue, open histogram) compared with unstimulated cells (red, filled histogram). Anergized cells stimulated under the same conditions exhibit no Stat5 phosphorylation above background (blue, open histogram: IL-2 stimulated; red, filled histogram: no stimulation). C. Average of percent maximum pStat5 geometric mean fluorescence intensity. Data represents average of two independent experiments.

Chapter 4

T cell specific deletion of the phosphatase SHP-1 decreases incidence and severity of experimental autoimmune encephalomyelitis

Abstract

T cell activation is regulated by a dynamic balance between kinase and phosphatase activity. The tyrosine phosphatase SHP-1 is a critical negative regulator of the immune system, and a proximal regulator of T cell receptor signaling. Here, we have utilized two novel models to study the deletion of SHP-1 specifically in mature T cells. We have bred mice bearing a floxed SHP-1 allele that deletes the majority of the gene with mice bearing an inducible Cre (Mx1 promoter). We also utilized a dominant negative allele of SHP1 whose expression requires Cre-driven recombination. By transferring mature T cells from these mice into T cell deficient hosts and subsequently inducing Cre expression we have isolated the effect of loss of SHP-1 activity to T cells. We have determined that selective loss of this phosphatase in mature T cells dramatically attenuates experimental autoimmune encephalomyelitis (EAE). Both severity and incidence were decreased significantly in the conditional knockout and dominant negative mice relative to controls (mean max score 1.0, 1.7, and 2.7, respectively and mean incidence 29, 37 and 79%). These data differ from results in mice heterozygous for the motheaten allele, a naturally occurring SHP-1 mutant, which exhibit EAE severity that is similar to or slightly worse than controls. Depletion of CD25+ cells prior to adoptive transfer abrogated protection from disease in conditional knockout animals.

Collectively, this suggests that lack of SHP-1 activity in T cells may protect from disease, while deletion in other cell types may exacerbate susceptibility or symptoms.

Introduction

T cell activation is regulated by an intricate balance between activating and inhibitory signals. Engagement of the TCR with an agonist ligand stimulates the activation of a series of kinases, ultimately resulting in gene transcription, proliferation, cytokine production and the development of other effector functions (136). Phosphatase activity is also stimulated upon activation, which ultimately allows the cell to return to a resting state. In the case of a low affinity interaction, however, the balance between kinase and phosphatase activity is thought to shift such that phosphatase activity is favored (48). Thus a strong ligand stimulates a response in which there is a positive feedback loop supporting Lck and downstream Erk activation. The opposite is true in the case of a low affinity ligand—a negative feedback loop mediated by the tyrosine phosphatase SHP-1 activity dominates, resulting in dephosphorylation of Lck and other Src family members, causing a dampening of the signal delivered.

Src homology 2 domain containing protein tyrosine phosphatase (SHP-1) is a tyrosine phosphatase that is a critical regulator of immune cell signaling (137). The importance of SHP-1 is highlighted by a naturally occurring mutant known as the motheaten mouse. These animals display severe autoimmunity initially characterized by neutrophilic skin lesions and usually die by 3-8 weeks of age of a macrophage driven pneumonitis that is independent of the presence of T and B cells (138-142). SHP-1 has been shown to dephosphorylate a number of targets downstream of the TCR including Lck, Zap-70, PI3K and LAT (49, 50, 53, 143, 144), ultimately serving to regulate the

threshold of signaling required for T cell activation (145, 146) and discriminate between ligands of varying potencies (48).

That SHP-1 plays a pivotal role in regulating T cell signaling events is clear; the impact of the loss of SHP-1 on T cell function is less clear. Early descriptions of the homozygous motheaten mice indicated that both B and T cells were hyporesponsive to a variety of stimuli including mitogens (140, 141, 147). Subsequent studies have demonstrated that even partial loss of SHP-1 can have a dramatic effect on the polarization of T cells to a Th1 or Th2 phenotype (148-150) as well as the generation and function of regulatory T cells (151, 152). Studies of several autoimmune disease animal models including EAE and experimental autoimmune myasthenia gravis have revealed that disease severity is typically unaltered (59, 153) to slightly more severe (154) in mice heterozygous for the motheaten mutation than in their wild type counterparts, although increased sensitivity to antigen was noted in all three studies.

In this study, we demonstrate that selective deletion of the putative negative regulatory tyrosine phosphatase SHP-1 in T cells reduces both the incidence and severity of experimental autoimmune encephalomyelitis, a disease driven primarily by CD4+ T cells. Despite the dramatic differences in disease severity in mice with T cells lacking SHP-1 compared to SHP-1 sufficient controls, total, lymphocytic and macrophage infiltrates into the CNS were similar in frequency and absolute numbers between groups, and no differences were observed in microglia number or frequency. Similarly, no differences were observed in infiltration of CD4+Foxp3+ T cells into the CNS, and frequencies and absolute numbers of these cells were similar between groups in the periphery. However, depletion of the Foxp3+ population prior to adoptive transfer

resulted in more severe disease in animals receiving SHP-1 deficient T cells, supporting the idea that SHP-1 deficient CD4+Foxp3+ T cells may have increased regulatory ability relative to wild type CD4+Foxp3+ T cells. Overall, these data suggest that classification of SHP-1 strictly as a negative regulator is a oversimplification and emphasizes the complexities of the T cell response at both the level of the signaling network as well as the panoply of functional outcomes and interplay between different subsets of cells.

Materials and methods

Mice

The SHP-1dominant negative transgene (D4) is expressed under the human elongation factor-1a promoter, which is expressed very well in all murine cell types including the T cell lineage. Between the promoter and the transgene, a transcriptional/ translational STOP cassette was inserted with flanking *loxP* sites. The expression of the dominant negative protein is blocked until Cre expression induces removal of the STOP cassette. D4 mice were a kind gift of Ulrike Lorenz (University of Virginia, Charlottesville, VA). Floxed SHP-1 allele and Mx1-Cre mice were purchased from Jackson labs (Bar Harbor, ME). Both D4 and Floxed SHP-1 mice were interbred with Mx1-Cre to generate Cre+D4+ or Cre+Flox(homozygous) offspring. Cα-/- mice were purchased from Jackson Labs and subsequently bred in our colony. All animals were utilized in accordance with a protocol approved by the Emory University Institutional Animal Care and Use Committee and housed by the Emory University Department of Animal Resources.

Western blots

At various times after stimulation, cells were lysed with a buffer containing 20mM Tris-HCl, 150mM NaCl, 1mM EDTA, 0.5% Igepal, protease inhibitor cocktail (Calbiochem) and sodium orthovanadate (1mM). Proteins were resolved on a 4-15% SDS-PAGE gel (BioRad) and transferred onto nitrocellulose. Blots were probed with antibodies to SHP-1 (Santa Cruz) or β-actin (Sigma).

SHP-1 activity assay

For specific SHP-1 activity, SHP-1 was immunoprecipitated from whole cell lysates prepared without the addition of sodium orthovanadate. Immunoprecipitation was performed with 2μg of anti-SHP1 antibody (Santa Cruz) and a Pierce Protein A/G plate based immunoprecipitation kit. The immunoprecipitated protein was reduced using pNPP Tyr Assay Buffer (Millipore) and incubated for 1h at 37°C with a pTyr containing substrate peptide (AEEEIpYGEFEA). Malachite green was added to assess the amount of free phosphate in the solution, and colorimetric change was assessed at 620nm on a BioTek microplate reader.

Cell purification and adoptive transfer

T cells were purified from spleens by negative selection with MACS magnetic separation using either a Pan T cell enrichment kit or a CD4 T cell enrichment kit. Transferred cell populations were 90-98% CD3ε+ or CD4+ respectively. Approximately $3x10^6$ cells were delivered retro-orbitally to gender matched Cα-/- recipients. Twenty-four hours after adoptive transfer, recipient mice were treated with 250μg Poly I:C (Sigma) intraperitoneally. Poly I:C treatment was repeated every 3 days for a total of 3 treatments. For experiments in which CD25+ cells were depleted, biotin-anti-CD25 antibody (clone PC61, ebioscience) was added to the MACS biotin antibody cocktail at a dilution of 1:1000. Otherwise, the purification was carried out according to the manufacturer's instructions. Transferred populations were less than 1% CD25+ and 3% or less Foxp3+.

EAE

EAE was induced 7-10 days following the final Poly I:C injection. Disease was induced by injection of 150µg of MOG35-55 peptide emulsified in complete Freund's adjuvant supplemented with heat killed *Mycobacterium tuberculosis* to a final concentration of 2.5mg/ml. Mice received injections of MOG/CFA on days 0 and 7. Additionally, mice received 250ng pertussis toxin intraperitoneally on days 0 and 2. EAE was assessed on the following scale: 0-asymptomatic, 1-flaccid tail, 2-hind limb weakness, 3-partial hind limb paralysis, 4-complete hind limb paralysis plus forelimb weakness or an inability to right herself, 5-moribund.

Isolation of CNS infiltrating cells

To determine leukocytic infiltration into the CNS, mice were sacrificed at the indicated time points by carbon dioxide inhalation. Mice were perfused with PBS through the left ventricle and brains and spinal cords were collected. CNS tissue was homogenized and the cellular infiltrate was isolated via percoll gradient as previously described (155). Isolated cells were subsequently stained for flow cytometry or stimulated to assess cytokine production.

Flow cytometry, Foxp3 and tetramer staining

For surface staining, buffer consisted of PBS containing 0.1% BSA and 0.05% sodium azide. Cells were stained for 30 minutes on ice with the following antibodies: CD25, CD4, CD8, CD45.2, CD11b.

For Foxp3 staining, cells were fixed and permeabilized with the eBioscience Foxp3 fixation and permeabilization kit per manufacturer's instructions. For tetramer staining, cells were incubated overnight in complete medium with 4μg/ml MOG 35-55: I-A^b or human CLIP: I-A^b as a control. Cells were subsequently surface stained as described above. Data were collected on a BD FACSCalibur and analyzed with FlowJo software (Treestar).

Culture media consisted of RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, 0.01M Hepes buffer, 100μg/mL gentamicin (Mediatech, Herndon, VA) and $5x10^{-5}$ M 2-mercaptoethanol (Sigma, St. Louis, MO).

Statistical analysis

Data were analyzed using GraphPad Prism. Statistical significance was determined by Student's *t* test or ANOVA, as indicated in the figure legends.

Results

Deletion of SHP1

To verify loss of SHP-1 in the conditional deletion mutants utilized in these studies, we analyzed SHP-1 expression in cells from poly I:C treated Mx1-Cre+ fl/fl mice. Cells were lysed and proteins separated by SDS-PAGE. Following transfer to nitrocellulose membranes, blots were probed using an antibody recognizing the C-terminal end of SHP-1. Samples from conditional knockout animals expressed minimal SHP-1 protein relative to controls (Fig. 4.1A).

In the case of the dominant negative SHP-1 animals (Mx1-Cre+ D4), the two wild type copies of the SHP-1 gene are still present. Therefore, we assessed loss of SHP-1 function by phosphatase assay. To determine SHP-1 specific activity, lysates from T cells stimulated by anti-CD3 were immunoprecipitated using an antibody specific for SHP-1. Following immunoprecipitation, the purified SHP-1 was provided a target peptide containing a phosphorylated tyrosine residue. After incubation of the immunoprecipitated phosphatase with the target peptide, free phosphate was assessed by malachite green. As shown in Figure 4.1B, T cells from control animals upregulate specific SHP-1 activity following TCR cross-linking, with a peak at 5 minutes. However, mice expressing the dominant negative form of SHP-1 display little specific phosphatase activity above background, and exhibit altered kinetics of phosphatase activity.

Reduced severity and incidence of EAE

To isolate the T cell intrinsic effects of SHP-1 deletion, we adoptively transferred naïve T cells purified from the spleens of animals of the genotype Mx1-Cre+ fl/fl or Cre- littermate controls. Following purification, cells were stained, purity was assessed by flow cytometry, and transferred T cells were 90-98% CD3ε+. Purified T cells were injected into T cell deficient Cα-/- hosts. Beginning 24h after adoptive transfer, mice received injections of 250μg poly I:C i.p. every third day for a total of three injections. At least 10 days following the final poly I:C injection, EAE was induced by delivering 150μg MOG35-55 emulsified in CFA on the day of induction and 7 days later. Pertussis toxin was delivered i.p. at a dose of 250ng on the day of induction and two days later. Mice were assessed for disease symptoms beginning on day 10 following disease induction.

As shown in Figure 4.2, mice receiving cells that were subsequently SHP-1 deleted displayed significantly less severe disease scores, with knockouts having an average maximal score of 1.7 +/- 0.3 and controls having an average maximal score of 2.5 +/- 0.2 (Table 4.1). This difference remains statistically significant even when mice that never developed symptoms were excluded from the analysis (data not shown). In addition to decreased disease severity, mice with T cells deficient in SHP-1 also exhibited decreased incidence of disease compared to SHP-1 sufficient controls (Fig. 4.2, middle column). Despite the differences in disease severity and incidence, the deletion of SHP-1 did not affect the average day of onset in mice that became symptomatic (15.7 +/- 0.7 and 14.9 +/- 0.5 for SHP-1 deficient and control groups respectively, Fig. 4.2 right column and Table 4.1).

In an effort to recapitulate these results with the more widely studied motheaten mutation as well as a dominant negative mutant, we performed a similar adoptive transfer experiment using cells purified from motheaten homozygous animals and mice carrying a dominant negative mutant SHP-1. Cells were purified and adoptively transferred as described above, and EAE was induced 24h following adoptive transfer for recipients of motheaten cells. In dominant negative recipients, poly I:C treatment was carried as described above to induce Cre expression. As with the conditional knockouts, mice receiving SHP-1 dominant negative or motheaten T cells exhibited less severe disease as well as decreased incidence relative to controls (Fig. 4.2B and C). Also similar to the conditional knockouts, dominant negative SHP-1 recipients and controls had similar day of onset (16.3 vs. 14.2, Fig. 4.2B, right column), as did motheaten and control recipients (12.3 vs. 12.2, Fig. 4.2C, right column).

Similar mononuclear infiltrates in SHP1 KO

To determine the mechanisms regulating decreased disease severity and incidence in animals with SHP-1 deficient T cells, we assessed mononuclear infiltrates into the brain and spinal cord. Infiltrates were isolated from CNS tissue by a percoll gradient, enumerated by counting in the presence of trypan blue, stained and phenotyped by flow cytometry. The total mononuclear infiltrate was similar in SHP-1 sufficient and deficient groups, with an average of $3.6x10^6$ and $3.2x10^6$ cells, respectively (Fig. 4.3A). Frequencies and absolute numbers of infiltrating macrophages were similar between groups, with an average of 3.3% and $13x10^4$ +/- $4.4x10^4$ in controls and 3.1% and $6x10^4$

+/- $1.6x10^4$ in knockouts (Fig. 4.3B). Similarly, no differences were observed in the frequency or absolute numbers of resident microglia (Fig. 4.3C).

T cell infiltration of the CNS

As T cells are crucial mediators of EAE and the population manipulated in these studies, we assessed lymphocytic infiltration into the CNS. As shown in Figure 4.4A, the frequencies and absolute numbers of lymphocytes in the CNS as determined by CD45+CD11b- phenotype were similar in controls (11.3%, $5.7x10^5$ +/- $2.7x10^5$) and knockouts (10.1%, $2.4x10^5$ +/- $0.78x10^5$). Further, no statistically significant differences were observed in the frequency (21.9% +/- 8.0 vs. 10.3% +/- 4.2) or total number ($2.1x10^5$ +/- $.6x10^5$ vs. $1.3x10^5$ +/- $0.4X10^5$) of CD4+ T cells in controls as compared to mice with SHP-1 deficient T cells (Fig. 4.4B). Similarly, no differences were observed in CD8+ T cell infiltrates into the CNS (Fig. 4.4C).

Infiltration of Foxp3+ and tetramer binding T cells

SHP-1 deficiency has previously been associated with increased regulatory capacity of Foxp3 expressing CD4+ T cells (152). Similar to total CD4+ T cells, neither the frequencies nor the absolute number of CD4+Foxp3+ cells infiltrating the CNS was not significantly different in controls and mice with SHP-1 deficient T cells (Fig. 4.5A and B). Similarly, the frequency of MOG35-55:I-A^b tetramer positive cells was not significantly different between groups (Fig. 4.5C).

Depletion of CD25+ cells exacerbates EAE in SHP-1 deficient mice

To determine if the SHP-1 deficient CD4+Foxp3+ population was functionally more suppressive effect in vivo, we depleted the CD25+ population of T cells prior to transfer. This approach was an efficient means of depleting Foxp3 expressing cells from naive spleens, as shown in (Fig. 4.6). CD25 depleted populations were less than 1% CD25+ and 3% or less expressed Foxp3. CD25 replete or CD25 depleted populations were adoptively transferred, Cre expression was induced and disease was initiated as described above. As before, animals in the control group manifested a higher incidence and more disease than animals receiving T cells deficient in SHP-1. The disease incidence and severity were not significantly different between control CD25 depleted and non-depleted groups (Fig. 4.7A). However, the depletion of CD25+ cells prior to adoptive transfer resulted in increased disease severity in recipients of SHP-1 knockout T cells, suggesting this population may play a role in the suppression of disease in the absence of SHP-1 (Fig. 4.7B). No significant differences in the day of onset were observed between any of the groups (Fig. 4.7C).

Interestingly, in the control CD25 depleted group, there appears to have been a recrudescence of the Foxp3+ population in both the spleen and the CNS (Fig. 4.8). It remains to be determined if these Foxp3+ cells represent an expansion of the small contaminating population of Foxp3 expressing cells initially transferred or if this is a newly generated population that has been induced to express Foxp3. However, minimal generation of a Foxp3 expressing population was observed in mice receiving CD25 depleted SHP-1 deficient cells. The maximum frequency of CD4+Foxp3+ cells in the CD25 depleted SHP-1 deficient animals was approximately 3% compared to an average of 14% CD4+Foxp3+ cells in the CNS of the control CD25 depleted group.

Discussion

Previous studies of the impact of loss of SHP-1 on EAE have utilized motheaten mice, which carry a naturally occurring point mutation in the SHP-1 gene (138). Studies of mice homozygous for the motheaten mutation have been limited as the mice die by 3 to 8 weeks of age, depending on the allele. Previously, EAE has been assessed in mice heterozygous for the motheaten mutation (me/+) (59, 154). In these studies, EAE was more severe in me/+ mice than in controls, and me/+ T cells were more sensitive to antigen. However, there are several important caveats to using motheaten mice. First, T cells in motheaten heterozygous animals go through thymic development with reduced levels of SHP-1. As T cell signaling plays an important role in the outcome of thymic selection, it is possible that the deletion of SHP-1 may affect the T cell repertoire in these animals. Secondly, as early as one day after birth motheaten mice begin to develop neutrophil mediated skin lesions, with macrophage mediated pneumonitis developing as early as day 3 (138). There is also a concomitant overabundance of neutrophils and macrophages in the circulation as well as increased serum antibody titers (138, 147). In addition to SHP-1 being deficient in all hematopoietic cells in the motheaten mice, the inflammatory environment in these animals could also affect subsequent T cell behavior. Indeed, T cells harvested from motheaten homozygous mice express several markers of activation immediately *ex vivo* (data not shown). A further complication in utilizing motheaten mice for studies of demyelinating disease is the fact that SHP-1 is expressed in oligodendrocytes, the cells that form the myelin sheath (156). It has been noted that motheaten mice inherently have a myelination defect characterized by dysmyelination

and decreased myelin basic protein expression (157). Thus it is likely that these alterations in the myelin sheath may affect the susceptibility of motheaten mice to EAE independent of a T cell intrinsic effect.

To circumvent the problems inherent in the motheaten system, we have utilized an adoptive transfer approach in which motheaten or conditional SHP-1 knockout T cells are transferred into T cell deficient hosts. With this approach, we have demonstrated decreased incidence and severity of disease in animals receiving SHP-1 deficient T cells. This result stands in contrast to the proposed role of SHP-1 as a strictly negative regulator of T cell activation. Despite the decreased severity of disease, both lymphocytic and myeloid infiltration into the CNS was similar in SHP-1 deficient and control animals. Given recent data suggesting that SHP-1 may control the regulatory capacity of CD4+Foxp3+ T cells (152), we sought to deplete this population prior to adoptive transfer. Depletion of this population resulted in increased incidence and severity of disease relative to mice receiving the intact CD4+ T cell population. These data support the notion that SHP-1 is an important component controlling the regulatory capacity of CD4+Foxp3+ T cells, but calls into question its role in regulating the activation of effector T cell populations. Recent studies of the CD8+ anti-viral T cell response utilizing a similar adoptive transfer strategy observed only minor differences between SHP-1 deficient T cells and controls (158), supporting the idea that SHP-1 deficiency may play a more subtle role in the control of T cell activation than previously appreciated.

We have previously demonstrated that SHP-1 activity is rapidly upregulated upon T cell stimulation with weak ligands (34) and that this phosphatase is required for the

induction of anergy by MHC variant peptides (59), together suggesting a critical role for SHP-1 in the control of T cell responses to ligands that are not highly stimulatory. Although self peptides that generate autoimmune disease are generally thought of as agonists, recent data has suggested that a large component of the polyclonal response in MOG 35-55 induced EAE is, in fact, lower affinity (12). Given the low affinity of many of the MOG-specific T cells, it is possible that signaling downstream of the TCR in these cells behaves more similarly to that observed following stimulation with weak ligands in other systems, suggesting that kinetics may be altered and that SHP-1 may play a different role depending on the affinity of each individual cell.

It has been clearly demonstrated that SHP-1 plays a critical role in setting the TCR signaling threshold and regulating thymic development (143, 145, 146). In our adoptive transfer inducible deletion model, SHP-1 function is intact through thymic development and is only deleted in mature T cells. Thus, the input population for both SHP-1 knockout and control groups should be similar in their affinity profile. However, it is possible that following deletion of SHP-1 the population dynamics are altered such that a higher or lower affinity population dominates in knockout animals, precluding the development of overt disease despite significant infiltration of T cells into the CNS. The specific impacts of high versus low affinity T cells during autoimmune disease is an intense area of study, and the effects of signaling differences on these populations is unknown.

In addition to its role as a negative regulator of effector T cell signaling, SHP-1 has recently be shown to play an important role in the control of regulatory T cell function (152). This study observed very few alterations between Tregs from wild type

controls and moth-eaten homozygous mice by microarray, although differences in several surface markers were noted (primarily adhesion molecules). These data suggest that the alterations in regulatory T cell function in mice lacking SHP-1 are not developmental or even transcriptional in nature, but rather function by affecting cytoplasmic signaling events. The exact mechanisms of the increased regulatory capacity of Tregs lacking SHP-1 are not entirely clear, although the SHP-1 deficient cells are phenotypically more activated and, due to increased expression of adhesion molecules, more readily form conjugates, thus allowing them to more readily interact with and potentially affect antigen presenting cells.

Regulatory T cells exert a number of effects that can alter the course of an immune response. In our adoptive transfer model, it is unclear what mechanisms the SHP-1 deficient cells may be employing to alter the course of disease. Our data demonstrate that CD4+Foxp3+ T cells are readily found in the CNS of control animals with severe disease as well as asymptomatic animals with SHP-1 deficient T cells. The role of regulatory T cells in controlling neuroinflammation is not fully understood, but current studies indicate that these cells may have a neuroprotective function due to their production of neurotrophic factors including brain derived neurotrophic factor (BDNF) and glial cell derived neurotrophic factor (159) and suppression of microglial activation (160). Studies of multiple sclerosis patients have observed decreased BDNF secretion (161), and increased BDNF expression was observed following treatment with the glatiramer acetate in patients that responded to treatment (162, 163). Production of BDNF by immune cells in the CNS is thought to limit neuroinflammation by protecting neurons from undergoing cell death (164), promoting remyelination (165) and limiting

class II MHC upregulation in microglia (166). The role of SHP-1 in controlling BDNF production by regulatory or effector T cells is unknown, but provides an interesting avenue for potential therapeutic intervention as a pharmacological inhibitor of SHP-1 is available (167).

The paradoxical results observed in this study, namely that removing a negative regulator protected from disease rather than exacerbating it, emphasizes the complex nature of the signaling cascades required for T cell activation, as well as the importance of the interplay between different subsets of cells, even within the T cell population.

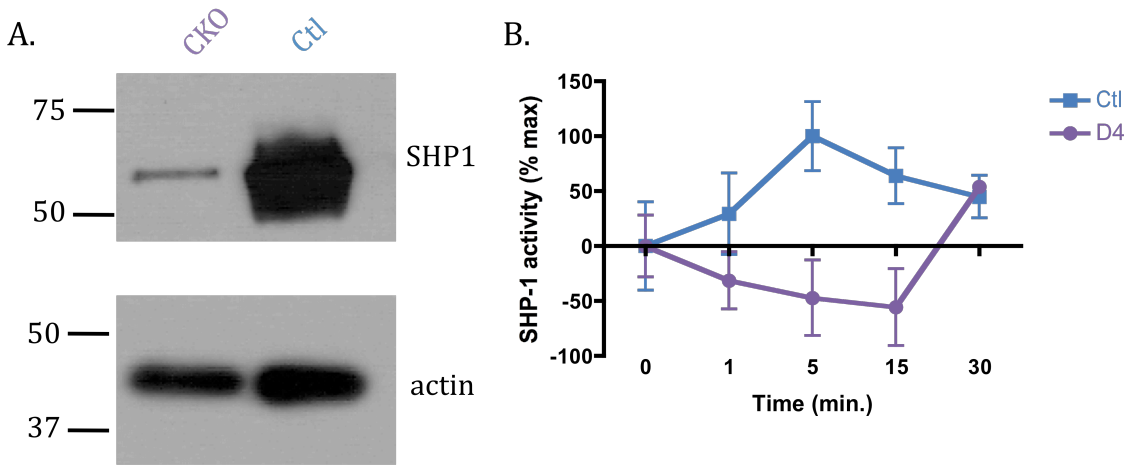

Figure 4.1 SHP-1 expression and activity are decreased in conditional deletion animals.

A. Floxed SHP-1 T cells have dramatically reduced expression of SHP-1 protein. **B.** Dominant negative expressing T cells have limited SHP-1 phosphatase activity.

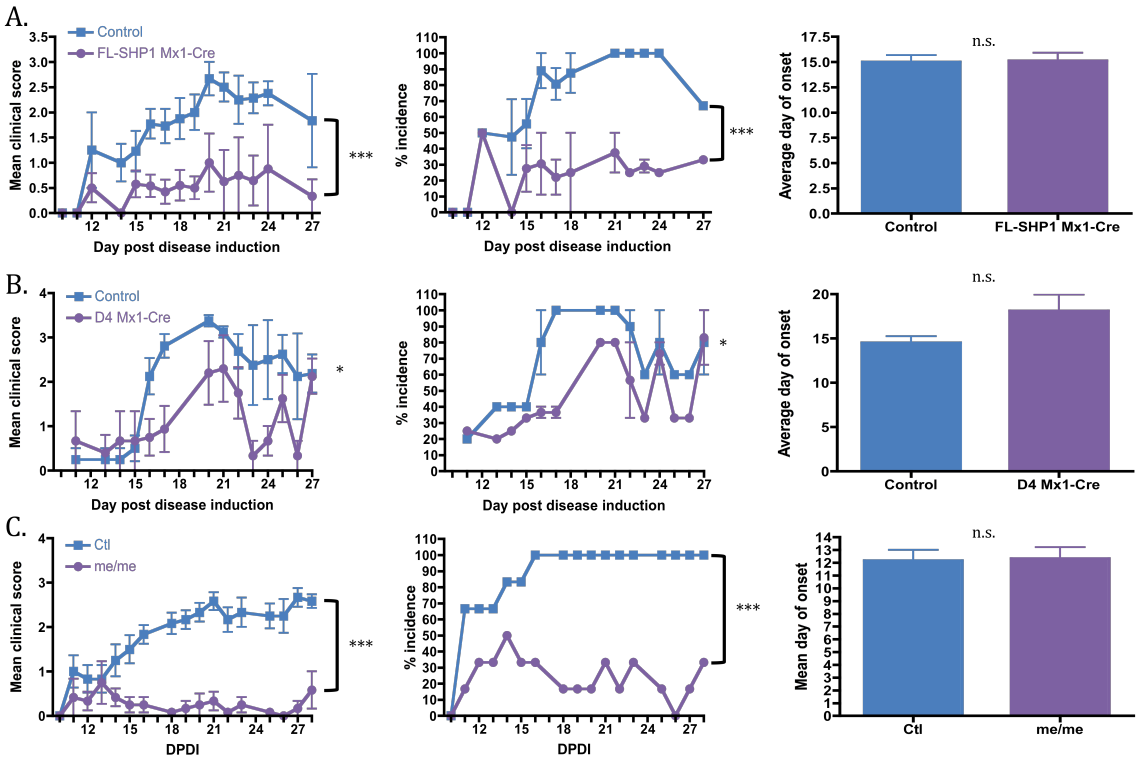

Figure 4.2 Mice with SHP-1 deficient T cells exhibit decreased incidence and severity of EAE.

Clinical scores following EAE induction (left column), disease incidence (center) and average day of disease onset (right) in mice with T cells from Floxed SHP-1 Mx1-Cre (**A**), SHP-1 dominant negative (**B**) or motheaten donors (**C**). Day of onset data include only symptomatic mice. Ctl indicates mice that received T cells from wild type donors. *** $p<0.0004$; *$p<0.04$; n.s.=not significant Data were analyzed by Mann-Whitney test. Data are averages of:

Floxed: 3 independent experiments, Ctl n=13, CKO n=11

Dominant negative: 2 independent experiments, Ctl n=9, CKO n=8

Motheaten: Representative of 2 independent experiments, Ctl n=6, me n=6 (for data shown)

	Mean high score	Mean day of onset	Mean day of peak
Control n=16	2.532 +/- 0.155	14.94 +/- 0.487	18.19 +/- 0.813
FL-SHP1 Mx1-Cre n=21	1.738 +/- 0.294	15.69 +/- 0.657	17.94 +/- 0.820

Table 4.1 Summary of EAE data for FL-SHP1 Mx1-Cre

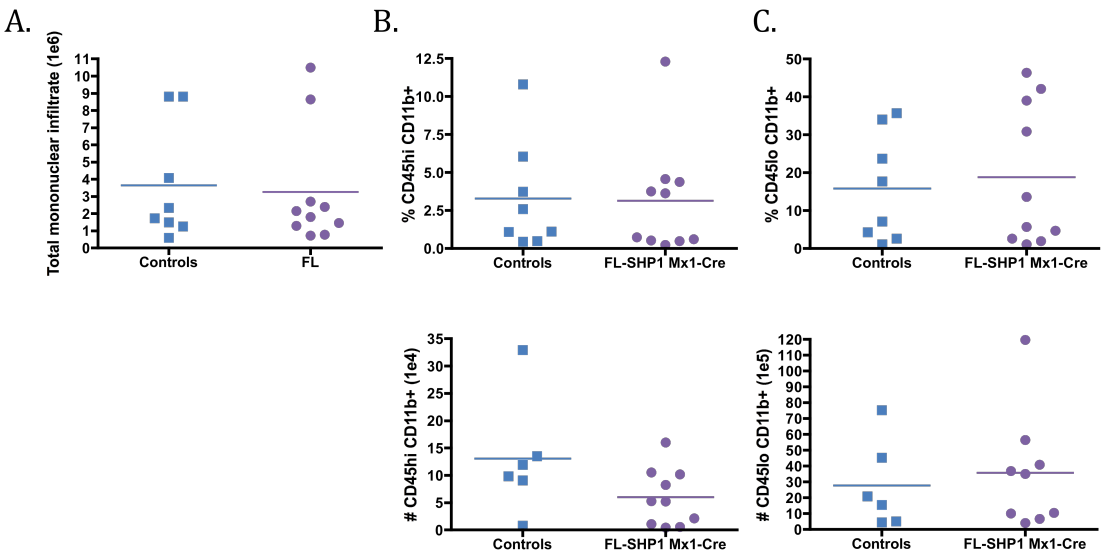

Figure 4.3 Macrophages and microglia in the CNS at peak of EAE are not altered by T cell-specific SHP-1 deletion.

A. Total mononuclear infiltrate in the brain and spinal cord following isolation by percoll gradient. Frequency (top) and absolute numbers (bottom) of CNS-infiltrating macrophages (**B**), CNS-resident microglia (**C**) cells at peak of disease (day 21-28). CKO indicates FL-SHP1 Mx1-Cre.

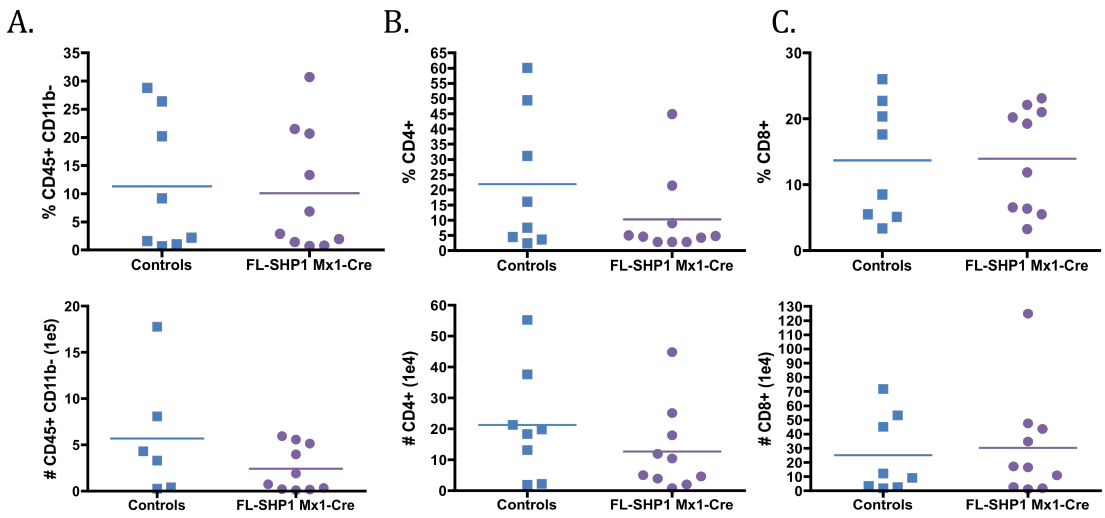

Figure 4.4 Lymphocytic infiltrates in the CNS at peak of disease are not altered by T cell-specific SHP-1 deletion.

Frequency (top) and absolute numbers (bottom) of CNS-infiltrating infiltrating lymphocytes (**A**), CD4+ T cells (**B**), and CD8+ T cells (**C**). CKO indicates FL-SHP1 Mx1-Cre.

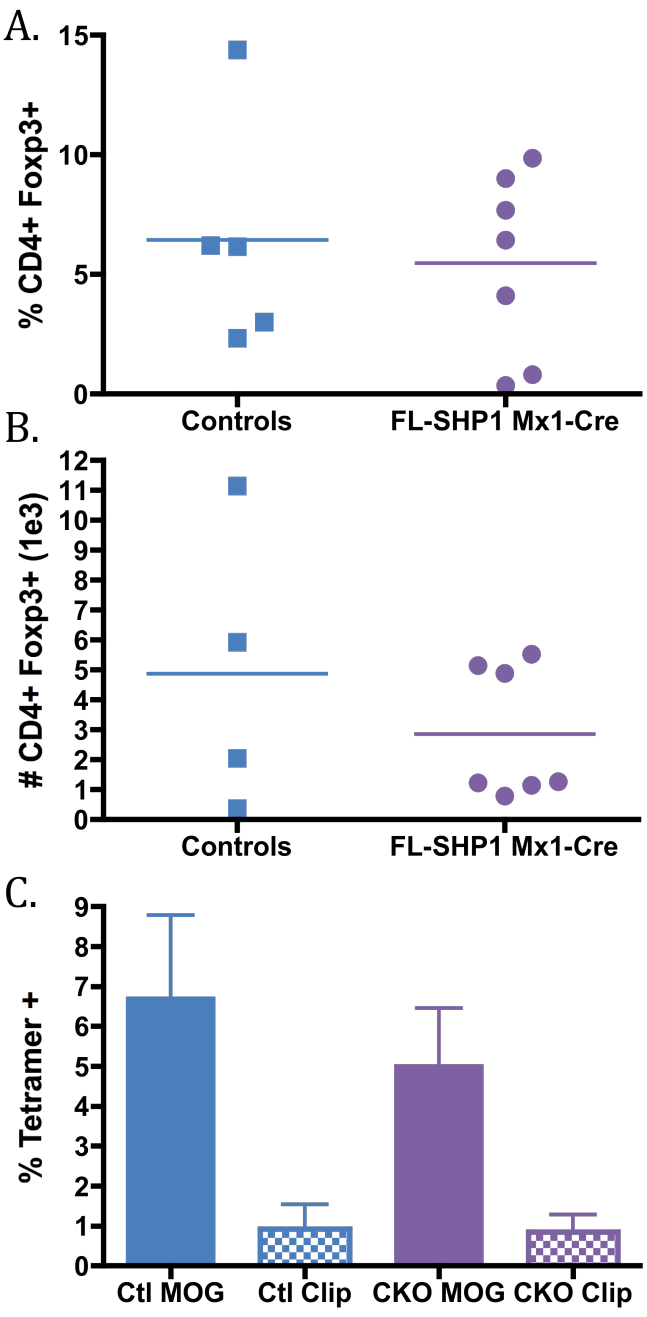

Figure 4.5 CNS infiltrating Foxp3+ and tetramer positive T cells are not altered by T cell-specific SHP-1 deletion.

Frequency (**A**) and absolute numbers (**B**) of CNS-infiltrating CD4+Foxp3+ cells at peak of disease (day 21-28). Frequency of MOG/I-A^b tetramer staining cells (**C**) in the CNS at peak of disease. CKO indicates FL-SHP1 Mx1-Cre.

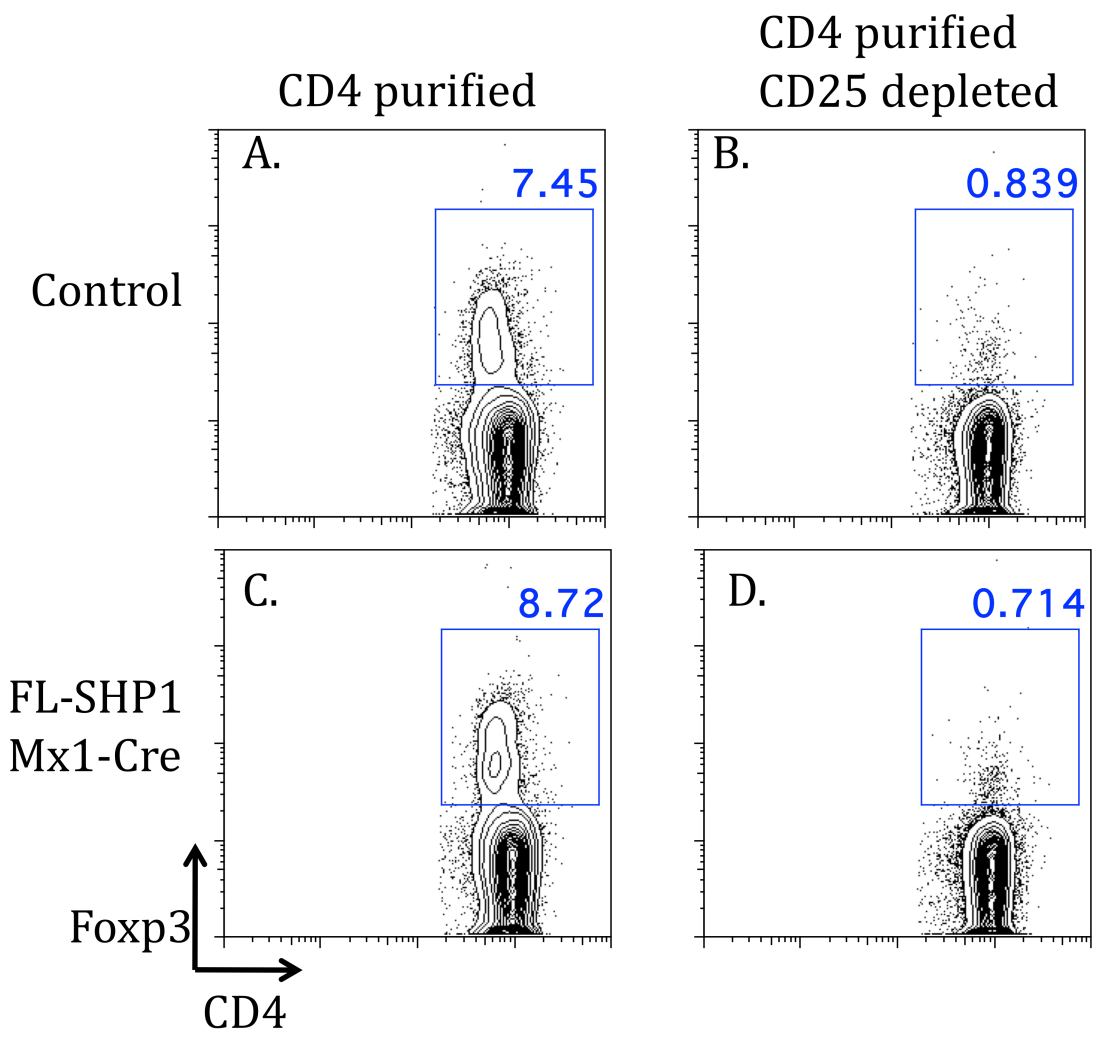

Figure 4.6 Depletion of CD25+ cells effectively depletes the Foxp3+ population.

Cells from control (**A** and **B**) or FL-SHP1 Mx1-Cre (**C** and **D**) spleens purified for CD4+ T cells (**A** and **C**) or also depleted of CD25+ cells (**B** and **D**).

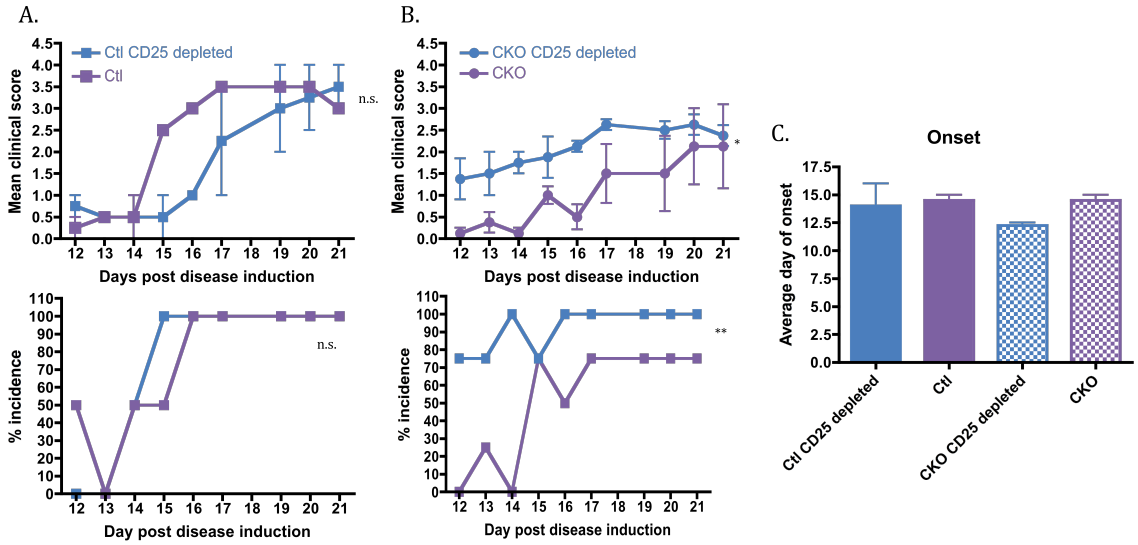

Figure 4.7 Depletion of CD25+ cells exacerbates EAE in animals with SHP-1 deficient T cells.

Clinical scores following EAE induction (top) and disease incidence (bottom) in mice with control or control CD25 depleted T cells (**A**) or total or CD25 depleted T cells from Floxed SHP-1 Mx1-Cre (**B**). **C.** Average day of onset. Day of onset data include only symptomatic mice. There are no significant differences between any groups in the average day of onset. ** $p<0.002$; *$p<0.01$; n.s.=not significant

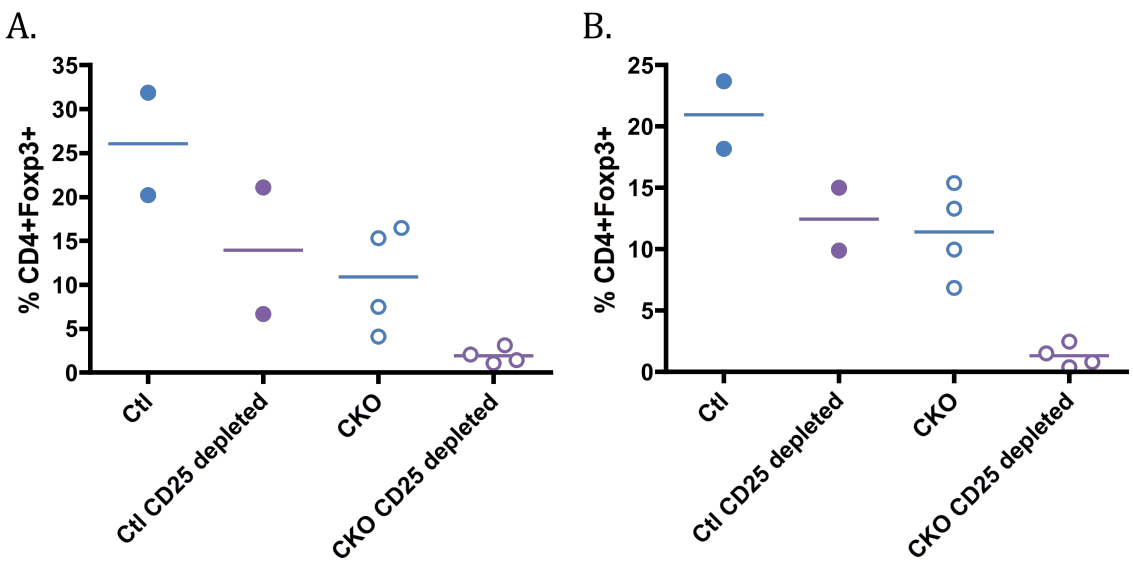

Figure 4.8 The frequency of CD4+Foxp3+ cell in mice receiving CD25 depleted T cells increases under control but not SHP-1 deficient conditions.

Frequency of CD4+Foxp3+ T cells in the CNS (**A**) and spleen (**B**) at peak of disease.

Chapter 5

Discussion

T cell activation is a critical aspect of the development of effective immunity to a variety of pathogens, as well as a key component governing the development of T cell mediated autoimmune diseases. There are a number of parameters that control the outcome of a T cell's interaction with an antigen presenting cell. These include: 1) The affinity of the T cell receptor for the peptide: MHC complex (Fig. 5.1); 2) The affinity of the peptide: MHC interaction (Fig. 5.1); 3) Signaling events within the T cell downstream of the TCR, various costimulatory pathways and cytokine receptors (Fig. 5.2). By altering any of these events, we can modulate the outcome of the T cell: APC interaction to improve or impair the resulting T cell function.

The affinity of the T cell receptor for the peptide: MHC complex is one of the earliest parameters governing T cell activation. Initial studies of TCR: pMHC affinity utilized surface plasmon resonance, a technique in which one protein is immobilized on a solid support and the interacting protein is provided in the fluid phase. This approach provides information regarding a number of kinetic parameters (on rate, off rate, half life), however it fails to take into consideration the complexities imparted by the fact that both of these complexes are normally anchored in the plasma membrane. The kinetic measurements obtained by this three dimensional approach correlate to some extent with the functional outcomes of T cell interactions with the ligands tested, although rarely was a clear correlation between kinetic measurements and T cell function for antagonist ligands observed (7-9).

To study the kinetics of TCR: pMHC in a more relevant membrane anchored system, we utilized the micropipette adhesion frequency assay. In this assay, pMHC is anchored to the membrane of a red blood cell through a biotin-streptavidin interaction, and intact T cells are brought into contact for a defined period of time (10). In this assay, adhesion is measured by elongation of the RBC membrane. By measuring many cell pairs, various concentrations of antigens and multiple contact times, we can generate kinetic data including on and off rates, half life of interaction and affinity of the interaction. This two dimensional approach to studying TCR: pMHC affinity has provided a wealth of novel information about the biophysical parameters that control T cell activation and is a more faithful representation of the natural interaction of a T cell with an antigen presenting cell.

Three dimensional analysis of TCR: pMHC interactions showed the best degree of correlation between functional response and off rates, which were slow for agonist ligands and more rapid for progressively weaker ligands (168 2001, 169). In contrast, 2D off rates were most rapid for agonist ligands and progressively slower for weaker ligands (6, 7). These 2D kinetic parameters span a much larger dynamic range than the previously utilized 3D approach, and functional T cell responses correlated well the effective 2D affinity, on rate and off rate for a range of peptides from full agonist to antagonist (7). The rapid off rate observed in the 2D system for agonist ligands provides support for the serial engagement model of T cell activation, suggesting that T cells rapidly sample antigen, interacting with stimulatory peptides for only a short time before disengaging. This rapid off rate would allow many TCRs to engage a few agonist ligands, thus driving complete T cell activation. Additionally, equilibrium dissociation

constants derived from 3D measurements suggested that the TCR: pMHC interaction is relatively low affinity. However, 2D measurements suggest that the affinity of the TCR: pMHC interaction is of similar affinity to the LFA-1/ICAM-1 interaction, a binding pair traditionally considered to be high affinity (7). Together these data emphasize the importance of context in regulating the biophysical parameters of interactions between membrane bound proteins. Furthermore, these differences between 2D and 3D measurements can serve to inform our understanding of how exactly T cell receptors "see" peptide: MHC complexes, and how changes in the interactions can alter downstream events.

Alterations in the interaction between TCR and pMHC can be introduced experimentally and therapeutically by introducing amino acid substitutions at the residues of the antigenic peptide that serve as contracts for the TCR (Fig. 5.1). Peptides containing amino acid substitutions at the TCR contact residues have been termed altered peptide ligands (1-3) and can result in antigens with a range of stimulatory capacities, including agonists, weak agonists, partial agonists and antagonists. Altered peptide ligands have been identified as an immune escape strategy in a number of pathogens (14-18).

A number of the epitope escape mutations that have been identified behave as antagonists (15, 16, 20-24). Our data in a model antigen system suggest that stimulation of T cells under antagonist conditions can significantly alter subsequent responses to agonist ligands. Rather than inducing a typical anergic phenotype in which the T cells do not proliferate or produce IL-2 in response to agonist stimulation, antagonism drives a phenotype in which the T cells are hypoproliferative but produce copious amounts of IL-

2 following agonist stimulation (Chapter 2, (44). This elevated IL-2 production could have a number of potential impacts during an ongoing infection. Increased IL-2 could drive bystander activation of other T cells or alter the frequency or function of regulatory T cells. These possibilities must be carefully considered when altered peptide ligands are used therapeutically.

Variant peptide therapies have been described in animal models of multiple sclerosis (170-172), myasthenia gravis (173, 174), allergy (175, 176), type 1 diabetes (177, 178), arthritis (179-181), and psoriasis (182). These efforts have generally been very successful in animal models of autoimmune disease, but have often been less successful in clinical trials (183-185).

Initial studies of peptide therapies in experimental autoimmune encephalomyelitis (EAE), a rodent model of multiple sclerosis, utilized altered peptide ligands with one or more amino acid substitutions in an attempt to ameliorate disease. This approach, which frequently resulted in immune deviation from a Th1 to a Th2 phenotype, was effective in EAE induced by several different antigenic epitopes, including proteolipid protein (171) and myelin basic protein (170, 172). An altered version of the myelin basic protein epitope was subsequently utilized in clinical trials as a means to treat multiple sclerosis (183, 184). The phase I trial was ultimately too small and too short lived to assess efficacy, and was halted due to adverse reactions, including exacerbation of disease and some hypersensitivity responses (183). A subsequent phase II trial demonstrated no significant differences in frequency or number of relapses, although a decrease in volume and number of lesions following treatment and a shift to a Th2 phenotype in responding T cells was observed (184, 186). The Th2 phenotype of cells from these patients was

maintained long term (2-4.5 years) (187). The phase II trial was also halted due to adverse reactions, particularly hypersensitivity responses in approximately 9% of patients. This adverse reaction most frequently manifested as itching or rash, and seemed to correlate with the number of injections the patient had received (184). Interestingly, injection of some unaltered self peptides or altered peptide ligands resulted in hypersensitivity in the form of anaphylaxis in several mouse models of autoimmunity (type 1 diabetes and EAE) (104, 188-190), suggesting that hypersensitivity may be a typical rather than an atypical response to peptide therapies (wild type or altered) during ongoing autoimmune disease. Although subsequent studies have successfully engineered peptide therapies that did not cause anaphylaxis in mice (104, 191), clinical trials utilizing altered peptide ligands to treat multiple sclerosis have not been revived.

An additional problem with therapeutic use of altered peptide ligands, which typically contain amino acid substitutions at the TCR contact residues, is the outgrowth of T cell clones specific for the APL itself (183). This type of response could at best impede therapeutic efficacy and at worst exacerbate ongoing autoimmune disease. To circumvent this problem, we have utilized an approach in which amino acid substitutions are made at MHC anchor residues rather than TCR contacts (Fig. 5.1). In this way, the peptide:MHC interaction can be destabilized, resulting in the decreased ability of the T cells to be activated. In fact, we have demonstrated that in addition to inducing anergy in autoreactive T cells, MHC variant peptides can prevent the induction of EAE when used in a vaccination strategy as well as decrease disease severity when given in soluble form during ongoing EAE (103, 104). Outgrowth of variant specific populations has not been observed, likely because the face of the peptide recognized by the T cells is not

dramatically altered by the substitutions that have been introduced. Furthermore, no evidence of anaphylaxis was observed upon treatment with MHC variant peptides, even though altered peptide ligands in the same system did cause rapid death due to an anaphylactic reaction (104). Perhaps these and other improvements on peptide therapies will lead to new clinical trials, with the hope of treating diseases for which current therapies are of limited efficacy.

To expand the use of MHC variant peptides to other autoimmune diseases, we sought to identify a peptide that would effectively anergize a diabetogenic T cell. We successfully anergized BDC2.5 T cells with an MHC variant peptide containing amino acid substitutions at all four MHC anchor residues (Chapter 3). To further characterize the initial signaling events regulating the induction of anergy by treatment with an MHC variant peptide, we assessed both positive and negative signaling events downstream of the TCR. These studies revealed no detectable Erk1/2 phosphorylation following stimulation with the variant peptide by western blot or phospho-flow cytometry. However, total tyrosine phosphatase activity as well as specific SHP-1 phosphatase activity was transient in agonist stimulated cells but sustained in variant treated cells. Together, these data suggest that MHC variant peptide anergy induces a program dominated by negative signaling events in which positive signaling events such as Erk1/2 phosphorylation are either too transient or too minor to detect by the methods utilized (Summarized in Fig. 5.2). The sustained activation of the tyrosine phosphatase SHP-1 upon treatment with an MHC variant peptide is similar to the negative feedback loop that is activated during T cell antagonism (45, 46). How this increased phosphatase activity at early times following T cell activation affects subsequent signaling events and overall T

cell responses remains to be determined.

Following treatment with the MHC variant peptide, the BDC2.5 T cells exhibited an anergic phenotype characterized by limited proliferation and minimal IL-2 production. Our previous data as well as the data generated in the BDC2.5 system demonstrate that MHC variant peptide induced anergy cannot be rescued by the provision of exogenous IL-2 (123)(Fig. 3.6). To determine the mechanism regulating the unresponsiveness of the cells to IL-2, we sought to assess signaling downstream of the IL-2 receptor. Despite the slightly higher levels of IL-2 receptor expressed by anergized cells, the cells exhibit no upregulation of phospho-Stat5 following stimulation with recombinant IL-2. These data suggest that the MHC variant peptide anergized cells are refractory to signaling through the IL-2 receptor. Our data demonstrating increased total tyrosine phosphatase activity as well as increased SHP-1 activity in response to variant stimulation as well as prior evidence that SHP-1 may associate with the IL-2 receptor (56) suggest a mechanism by which increased phosphatase activity blocks inhibits phosphorylation events required for activation downstream of the IL-2 receptor. Although this mechanism has not yet been directly demonstrated, it is possible this means of shutting down T cell activation could be exploited therapeutically as a means to inhibit unwanted responses.

The signaling events following stimulation with both altered peptide ligands and MHC variant peptides emphasize that T cell activation is a complex process that depends upon the balance between positive and negative signaling events. Our knowledge of the role that negative regulators including the tyrosine phosphatase SHP-1 play in the T cell response to weak ligands is limited, although the current evidence demonstrates that SHP-1 is required for the induction of anergy by MHC variant peptides (59).

Furthermore, how this negative regulator controls the T cell response to self antigens in vivo models has only been studied in mice partially deficient in SHP-1 in all cell types (59, 153, 154). To isolate the T cell intrinsic effects of SHP-1 on the autoimmune response we have utilized an adoptive transfer approach with three types of SHP-1 mutants. We have utilized two inducible deletion systems as well as cells from the commonly used motheaten mouse, a naturally occurring SHP-1 mutant. All of these approaches have revealed that T cell specific deletion of SHP-1 results in abrogation of EAE, as mice with SHP-1 deficient T cells exhibit reduced incidence and severity of disease.

Despite the drastic differences in disease severity between control and SHP-1 deficient animals, infiltration into the CNS by macrophages and lymphocytes was similar between groups, and no significant differences were observed in frequency or absolute numbers of CNS infiltrating CD4+, CD8+ or CD4+Foxp3+ T cells. Given recent data that SHP-1 deficiency increases the regulatory capacity of CD4+Foxp3+ T cells (152), we sought to determine if this population played a role in the suppression of EAE in mice with SHP-1 deficient T cells. Depletion of the CD25+ population prior to adoptive transfer successfully depleted the majority of Foxp3+ T cells, allowing us to assess the role of this population in our adoptive transfer model. Indeed, disease severity in mice receiving SHP-1 deficient CD25 depleted T cells was significantly more severe than mice receiving SHP-1 deficient T cells that were not CD25 depleted. No significant difference was observed between CD25 depleted and non-depleted groups that received wild type control T cells. These data suggest that the CD4+Foxp3+ population is playing a role in the suppression of disease when the T cell population is SHP-1 deficient.

The details of this mechanism, however, have not yet been determined. Increased suppressive capacity of polyclonal SHP-1 deficient regulatory T cells has not been demonstrated in this system. Furthermore, the susceptibility of SHP-1 deficient effector T cells to suppression by the regulatory T cell population is unknown. It is possible that SHP-1 deficiency makes effector T cells more prone to regulation by even wild type regulatory T cells, thus resulting in an additive effect when both the effector and regulatory population are SHP-1 deficient. Future studies will attempt to address these issues both *in vitro* and *in vivo*. Due to the complexities of the *in vivo* system, it is possible that the contribution of the CD4+Foxp3+ population is not the only factor dampening disease in mice with SHP-1 deficient T cells.

T cells can secrete a variety of cytokines, including neurotrophic factors that could specifically affect neuroinflammation. Among these, brain derived neurotrophic factor (BDNF) has been shown to play an important role in regulating microglial activation in the CNS as well as protecting neurons from apoptosis and promoting remyelination (160, 164-166). Interestingly, BDNF secretion is decreased in cells from multiple sclerosis patients (161), and increased in patients that responded well to treatment with glatiramer acetate, but not in non-responders (162, 163). It has been demonstrated that regulatory T cells can produce both BDNF and the closely related and also neuroprotective glial cell derived neurotrophic factor (159). However, the role of SHP-1 in regulating T cell production of neurotrophic factors is unknown. If future experiments demonstrate a role for SHP-1 in controlling this pathway, a pharmacologic inhibitor of SHP-1 may be successful at regulating neuroinflammation.

Encephalitogenic T cells may be either Th1 or Th17 phenotype, and shifts in the

ratio of cells producing these cytokines could alter the course of disease. Although we have not observed differences in IFNγ or IL-17 production (data not shown), recent evidence suggests that production of GM-CSF may play a role in the encephalitogenicity of T cells in the EAE model (192). T cell production of GM-CSF is driven by RORγt, but as with the neurotrophic factors, the role of SHP-1 in regulating this pathway is unknown.

T cell activation and function are very complex and context dependent. Our studies of the kinetic parameters of TCR: pMHC interaction demonstrate that proteins can interact very differently in three dimensions as opposed to the two dimensional context of the plasma membrane (7). Our observations that 2D interactions between TCR and agonist pMHC have rapid on and off rates allow for a revision to the serial engagement model of T cell activation in that T cells rapidly engage and disengage a stimulatory ligand thus allowing a few of those ligands to engage many T cell receptors driving full activation. By the same token, weaker ligands interacting for a longer period of time may be able to perform a summation of weak signals to achieve full or partial activation. Alternatively, longer interactions may allow for the accumulation of negative feedback mediators thus abrogating or preventing T cell activation.

The optimal dwell time model predicts that if a particular ligand interacts with the TCR for too long or too short a period of time, activation will be limited. Thus, if a T cell interacts with a weak ligand for an extended period of time, an insufficient number of receptors may be engaged to support activation. Further supporting this notion, we have demonstrated in multiple systems that weaker ligands require engagement of greater numbers of T cell receptors than do strong ligands (64, 65 2008). That a strong ligand requires fewer TCRs to achieve activation may also play a role in the rapidity of the T

cell response to antigens that are recognized strongly, a characteristic that is important in quickly clearing pathogens. The longer interaction time and increased TCR number required for activation by weaker ligands may serve as a check point to delay or prevent unnecessary responses, e.g. if the pathogen has already been cleared, or decrease the likelihood that autoreactive cells will be activated.

Once a T cell has encountered antigen, it is evident that both positive and negative signaling events occur, likely simultaneously. These opposing events provide a means by which kinetic proofreading can be undertaken. Strong and/or repeated interactions with a pMHC ligand is thought to drive a primarily positive signaling program, activating a positive feedback loop and limiting the impact of the negative signaling events. However, weaker ligands with different interaction kinetics are capable of driving a signaling program dominated by negative regulators. It is the balance between these positive and negative signaling events that determines the outcome of a given T cell stimulation event (Fig. 5.2).

The combination of the kinetic proofreading, serial engagement and optimal dwell time models and the data described herein allow us to develop a more comprehensive model of T cell activation. Our studies of antagonism and anergy indicate that T cells are capable of persisting despite very limited or undetectable positive signaling events, data that provide novel opportunities to study the factors and signaling events necessary for T cell survival. The increased activity of tyrosine phosphatases and specifically SHP-1 in T cells treated with an MHC variant peptide clearly demonstrate that negative regulators play an important role in determining the outcome of T cell interaction with a weak ligand. Furthermore, these results extend our knowledge regarding how anergy is

induced and maintained, information that may be exploited as a means to either overcome anergy in conditions such as chronic infection or cancer or induce anergy as a means to treat autoimmune disease or prevent graft rejection.

Given the importance of the phosphatase SHP-1 in regulating signaling events downstream of both the TCR and a number of cytokine receptors, we sought to better understand the role that this phosphatase plays in the functional response of autoreactive T cells. We predicted that deletion of a negative regulator would amplify the T cell response, thus exacerbating disease. However, this approach revealed that T cell specific deletion of SHP-1 abrogated both disease severity and incidence, without altering the average day of onset. The current data suggest that the effects of SHP-1 deletion in effector and regulatory T cells may differ, emphasizing once more the complexity of the T cell response. Currently, data are scarce regarding the kinetic parameters and antigen specificity of regulatory T cell populations, and studies of these factors as well as the signaling events regulating their function are ongoing. It is possible that the signaling events downstream of interaction with pMHC are different in effector and regulatory T cells, or that the same signaling events can mediate different functional responses depending on the context.

As our knowledge of signaling events in T cells grow, additional modifications to the kinetic proofreading model may be necessary to explain the behavior of different subsets of T cells, cells at different stages of differentiation or activation, and in unusual contexts such as the CNS. Each of these specific contexts may also require modifications of our understanding of the importance of serial engagement and optimal dwell time. Understanding the basic mechanisms regulating T cell activation should provide a

number of novel approaches to developing therapies to treat a variety of conditions including autoimmune disease and chronic infections.

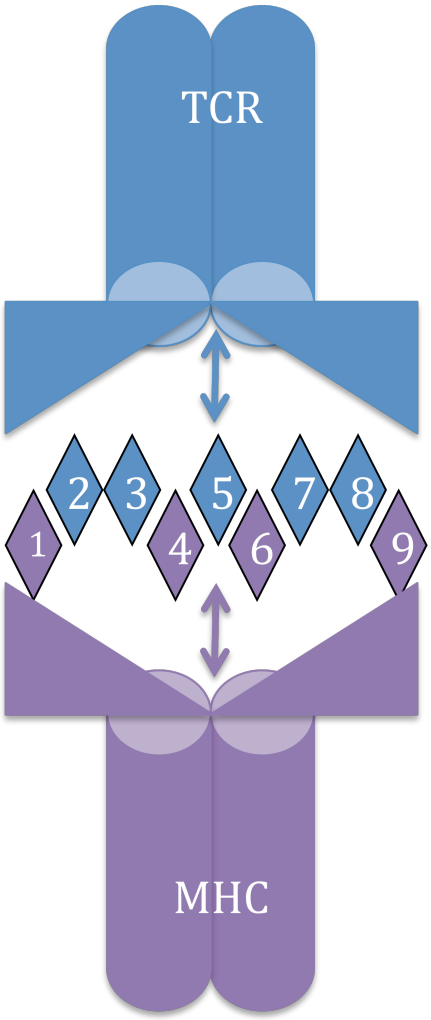

Figure 5.1 The stimulatory capacity of an antigenic peptide is determined by its interactions with both the MHC as well as the TCR.

Altering the amino acids that interact with either aspect can dramatically alter the ability of a T cell to recognize the ligand. Peptides containing substitutions at TCR contacts have been termed altered peptide ligands (APL), while peptides containing substitutions at MHC anchor residues are known as MHC variant peptides (MVP).

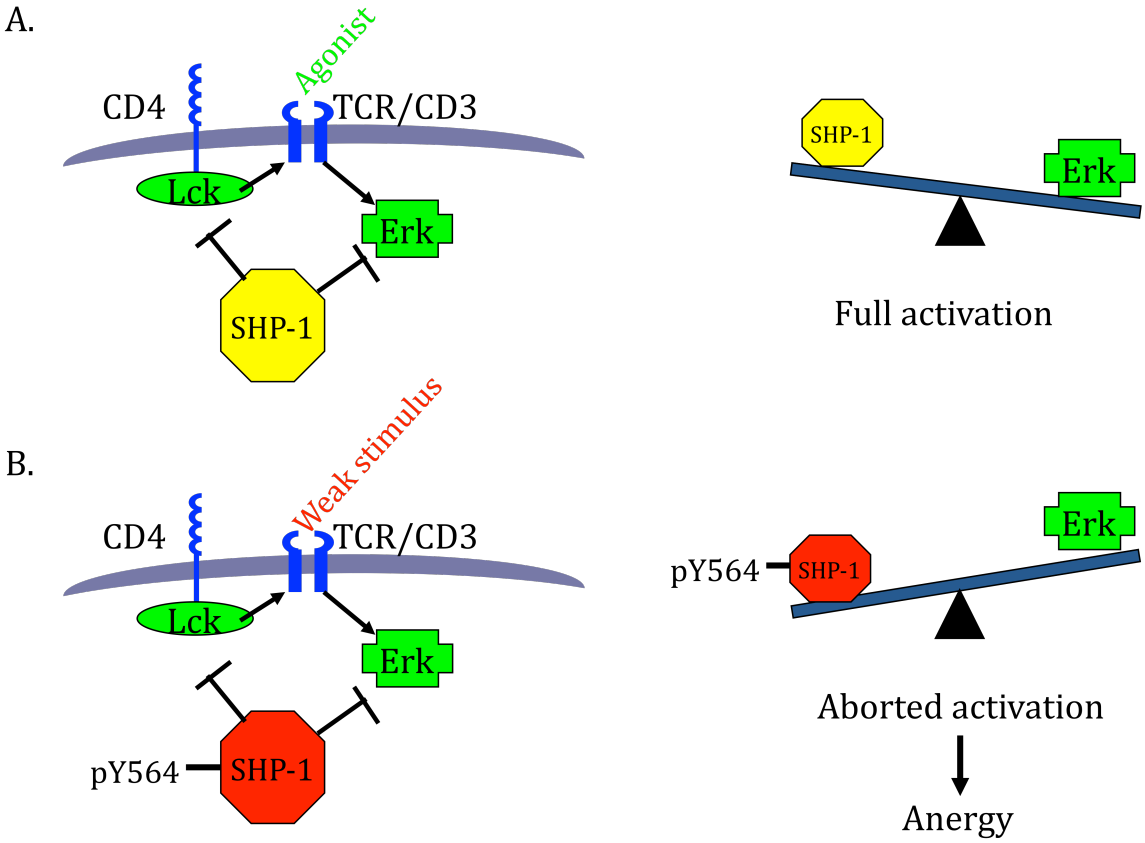

Figure 5.2 Model of T cell signaling events following stimulation with and strong or weak ligand

A. Following stimulation with an agonist ligand, positive signaling via Erk1/2 dominates the relatively minor negative signaling events through SHP-1. **B.** Stimulation with a weak ligand drives activation of SHP-1 more effectively, resulting in limited positive signaling events and abortive activation.

References

1. Evavold, B. D., and P. M. Allen. 1991. Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand. *Science* 252:1308-1310.
2. De Magistris, M. T., J. Alexander, M. Coggeshall, A. Altman, F. C. A. Gaeta, H. M. Grey, and A. Sette. 1992. Antigen analog-major histocompatibility complexes act as antagonists of the T cell receptor. *Cell* 68:625-634.
3. Sloan-Lancaster, J., B. D. Evavold, and P. M. Allen. 1993. Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells. *Nature* 363:156-159.
4. Sloan-Lancaster, J., A. S. Shaw, J. B. Rothbard, and P. M. Allen. 1994. Partial T cell signaling: altered phospho-zeta and lack of zap70 recruitment in APL-induced T cell anergy. *Cell* 79:913-922.
5. Madrenas, J., R. L. Wange, J. L. Wang, N. Isakov, L. E. Samelson, and R. N. Germain. 1995. Zeta phosphorylation without ZAP-70 activation induced by TCR antagonists or partial agonists. *Science* 267:515-518.
6. Huppa, J. B., M. Axmann, M. A. Mörtelmaier, B. F. Lillemeier, E. W. Newell, M. Brameshuber, L. O. Klein, G. J. Schütz, and M. M. Davis. 2010. TCR–peptide–MHC interactions in situ show accelerated kinetics and increased affinity. *Nature* 463:963-967.
7. Huang, J., V. I. Zarnitsyna, B. Liu, L. J. Edwards, N. Jiang, B. D. Evavold, and C. Zhu. 2010. The kinetics of two-dimensional TCR and pMHC interactions determine T-cell responsiveness. *Nature* 464:932-936.
8. Alam, S. M., P. J. Travers, J. L. Wung, W. Nasholds, S. Redpath, S. C. Jameson, and N. R. Gascoigne. 1996. T-cell-receptor affinity and thymocyte positive selection. *Nature* 381:616-620.
9. Rosette, C., G. Werlen, M. A. Daniels, P. O. Holman, S. M. Alam, P. J. Travers, N. R. Gascoigne, E. Palmer, and S. C. Jameson. 2001. The impact of duration versus extent of TCR occupancy on T cell activation: a revision of the kinetic proofreading model. *Immunity* 15:59-70.
10. Chesla, S. E., P. Selvaraj, and C. Zhu. 1998. Measuring two-dimensional receptor-ligand binding kinetics by micropipette. *Biophys J* 75:1553-1572.
11. Huang, J., L. J. Edwards, B. D. Evavold, and C. Zhu. 2007. Kinetics of MHC-CD8 interaction at the T cell membrane. *J Immunol* 179:7653-7662.
12. Sabatino, J. J., J. Huang, C. Zhu, and B. D. Evavold. 2011. High prevalence of low affinity peptide-MHC II tetramer-negative effectors during polyclonal CD4+ T cell responses. *Journal of Experimental Medicine* 208:81-90.
13. Jiang, N., J. Huang, L. J. Edwards, B. Liu, Y. Zhang, C. D. Beal, B. D. Evavold, and C. Zhu. 2011. Two-Stage Cooperative T Cell Receptor-Peptide Major Histocompatibility Complex-CD8 Trimolecular Interactions Amplify Antigen Discrimination. *Immunity* 34:13-23.
14. Bertoletti, A., A. Costanzo, F. V. Chisari, M. Levrero, M. Artini, A. Sette, A.

Penna, T. Giuberti, F. Fiaccadori, and C. Ferrari. 1994. Cytotoxic T lymphocyte response to a wild type hepatitis B virus epitope in patients chronically infected by variant viruses carrying substitutions within the epitope. *J Exp Med* 180:933-943.

15. Bertoletti, A., A. Sette, F. V. Chisari, A. Penna, M. Levrero, M. De Carli, F. Fiaccadori, and C. Ferrari. 1994. Natural variants of cytotoxic epitopes are T-cell receptor antagonists for antiviral cytotoxic T cells. *Nature* 369:407-410.
16. Klenerman, P., S. Rowland-Jones, S. McAdam, J. Edwards, S. Daenke, D. Lalloo, B. Koppe, W. Rosenberg, D. Boyd, A. Edwards, P. Giangrande, R. E. Phillips, and A. J. McMichael. 1994. Cytotoxic T-cell activity antagonized by naturally occurring HIV-1 Gag variants. *Nature* 369:403-410.
17. Johanns, T. M., J. M. Ertelt, J. C. Lai, J. H. Rowe, R. A. Avant, and S. S. Way. 2010. Naturally occurring altered peptide ligands control Salmonella-specific CD4+ T cell proliferation, IFN-gamma production, and protective potency. *J Immunol* 184:869-876.
18. Gilbert, S. C., M. Plebanski, S. Gupta, J. Morris, M. Cox, M. Aidoo, D. Kwiatkowski, B. M. Greenwood, H. C. Whittle, and A. V. Hill. 1998. Association of malaria parasite population structure, HLA, and immunological antagonism. *Science* 279:1173-1177.
19. Domingo, E., and J. J. Holland. 1997. RNA virus mutations and fitness for survival. *Annu Rev Microbiol* 51:151-178.
20. Kubota, R., S. S. Soldan, R. Martin, and S. Jacobson. 2000. An altered peptide ligand antagonizes antigen-specific T cells of patients with human T lymphotropic virus type I-associated neurological disease. *J Immunol* 164:5192-5198.
21. Frasca, L., P. Del Porto, L. Tuosto, B. Marinari, C. Scotta, M. Carbonari, A. Nicosia, and E. Piccolella. 1999. Hypervariable region 1 variants act as TCR antagonists for hepatitis C virus-specific CD4+ T cells. *J Immunol* 163:650-658.
22. Ciurea, A., L. Hunziker, M. M. Martinic, A. Oxenius, H. Hengartner, and R. M. Zinkernagel. 2001. CD4+ T-cell-epitope escape mutant virus selected in vivo. *Nat Med* 7:795-800.
23. Grakoui, A., N. H. Shoukry, D. J. Woollard, J. H. Han, H. L. Hanson, J. Ghrayeb, K. K. Murthy, C. M. Rice, and C. M. Walker. 2003. HCV persistence and immune evasion in the absence of memory T cell help. *Science* 302:659-662.
24. Lee, E. A., K. L. Flanagan, G. Minigo, W. H. Reece, R. Bailey, M. Pinder, A. V. Hill, and M. Plebanski. 2006. Dimorphic Plasmodium falciparum merozoite surface protein-1 epitopes turn off memory T cells and interfere with T cell priming. *Eur J Immunol* 36:1168-1178.
25. Sewell, A. K., G. C. Harcourt, P. J. Goulder, D. A. Price, and R. E. Phillips. 1997. Antagonism of cytotoxic T lymphocyte-mediated lysis by natural HIV-1 altered peptide ligands requires simultaneous presentation of agonist and antagonist peptides. *Eur J Immunol* 27:2323-2329.
26. Purbhoo, M. A., A. K. Sewell, P. Klenerman, P. J. Goulder, K. L. Hilyard, J. I. Bell, B. K. Jakobsen, and R. E. Phillips. 1998. Copresentation of natural HIV-1 agonist and antagonist ligands fails to induce the T cell receptor signaling cascade. *Proc Natl Acad Sci U S A* 95:4527-4532.
27. Puglielli, M. T., A. J. Zajac, R. G. van der Most, J. L. Dzuris, A. Sette, J. D.

Altman, and R. Ahmed. 2001. In vivo selection of a lymphocytic choriomeningitis virus variant that affects recognition of the GP33-43 epitope by H-2Db but not H-2Kb. *J Virol* 75:5099-5107.

28. Wang, S., R. Buchli, J. Schiller, J. Gao, R. S. VanGundy, W. H. Hildebrand, and D. D. Eckels. 2010. Natural epitope variants of the hepatitis C virus impair cytotoxic T lymphocyte activity. *World J Gastroenterol* 16:1953-1969.
29. Plebanski, M., E. A. Lee, C. M. Hannan, K. L. Flanagan, S. C. Gilbert, M. B. Gravenor, and A. V. Hill. 1999. Altered peptide ligands narrow the repertoire of cellular immune responses by interfering with T-cell priming. *Nat Med* 5:565-571.
30. Haanen, J. B., M. C. Wolkers, A. M. Kruisbeek, and T. N. Schumacher. 1999. Selective expansion of cross-reactive CD8(+) memory T cells by viral variants. *J Exp Med* 190:1319-1328.
31. Haribhai, D. 2004. Functional Reprogramming of the Primary Immune Response by T Cell Receptor Antagonism. *Journal of Experimental Medicine* 200:1371-1382.
32. Bouhdoud, L., P. Villain, A. Merzouki, M. Arella, and C. Couture. 2000. T-cell receptor-mediated anergy of a human immunodeficiency virus (HIV) gp120-specific CD4(+) cytotoxic T-cell clone, induced by a natural HIV type 1 variant peptide. *J Virol* 74:2121-2130.
33. Ream, R. M., J. Sun, and T. J. Braciale. 2010. Stimulation of naive CD8+ T cells by a variant viral epitope induces activation and enhanced apoptosis. *J Immunol* 184:2401-2409.
34. Schnell, F. J., N. Alberts-Grill, and B. D. Evavold. 2009. CD8+ T cell responses to a viral escape mutant epitope: active suppression via altered SHP-1 activity. *J Immunol* 182:1829-1835.
35. Montixi, C., C. Langlet, A. M. Bernard, J. Thimonier, C. Dubois, M. A. Wurbel, J. P. Chauvin, M. Pierres, and H. T. He. 1998. Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains. *Embo J* 17:5334-5348.
36. Smyth, L. A., L. Ardouin, O. Williams, T. Norton, V. Tybulewicz, and D. Kioussis. 2002. Inefficient clustering of tyrosine-phosphorylated proteins at the immunological synapse in response to an antagonist peptide. *Eur J Immunol* 32:3386-3394.
37. Wulfing, C., J. D. Rabinowitz, C. Beeson, M. D. Sjaastad, H. M. McConnell, and M. M. Davis. 1997. Kinetics and extent of T cell activation as measured with the calcium signal. *J Exp Med* 185:1815-1825.
38. Sumen, C. 2004. T cell receptor antagonism interferes with MHC clustering and integrin patterning during immunological synapse formation. *The Journal of Cell Biology* 166:579-590.
39. Ehrlich, L. I., P. J. Ebert, M. F. Krummel, A. Weiss, and M. M. Davis. 2002. Dynamics of p56lck translocation to the T cell immunological synapse following agonist and antagonist stimulation. *Immunity* 17:809-822.
40. Grey, H. M., J. Alexander, K. Snoke, A. Sette, and J. Ruppert. 1993. Antigen analogues as antagonists of the T cell receptor. *Clin Exp Rheumatol* 11 Suppl 8:S47-50.

41. Ruppert, J., J. Alexander, K. Snoke, M. Coggeshall, E. Herbert, D. McKenzie, H. M. Grey, and A. Sette. 1993. Effect of T-cell receptor antogonism on interaction between T cells and antigen-presenting cells and on T-cell signaling events. *Proceedings from the National Academy of Science* 90:2671-2675.
42. Huang, J., K. Sugie, D. M. La Face, A. Altman, and H. M. Grey. 2000. TCR antagonist peptides induce formation of APC-T cell conjugates and activate a Rac signaling pathway. *Eur J Immunol* 30:50-58.
43. Huang, J., D. Tilly, A. Altman, K. Sugie, and H. M. Grey. 2000. T-cell receptor antagonists induce Vav phosphorylation by selective activation of Fyn kinase. *Proc Natl Acad Sci U S A* 97:10923-10929.
44. Edwards, L. J., and B. D. Evavold. 2010. A unique unresponsive CD4+ T cell phenotype post TCR antagonism. *Cell Immunol* 261:64-68.
45. Dittel, B. N., I. Stefanova, R. N. Germain, and C. A. Janeway, Jr. 1999. Cross-antagonism of a T cell clone expressing two distinct T cell receptors. *Immunity* 11:289-298.
46. Kilgore, N. E., J. D. Carter, U. Lorenz, and B. D. Evavold. 2003. Cutting edge: dependence of TCR antagonism on Src homology 2 domain-containing protein tyrosine phosphatase activity. *J Immunol* 170:4891-4895.
47. Somani, A. K., J. S. Bignon, G. B. Mills, K. A. Siminovitch, and D. R. Branch. 1997. Src kinase activity is regulated by the SHP-1 protein-tyrosine phosphatase. *J Biol Chem* 272:21113-21119.
48. Stefanova, I., B. Hemmer, M. Vergelli, R. Martin, W. E. Biddison, and R. N. Germain. 2003. TCR ligand discrimination is enforced by competing ERK positive and SHP-1 negative feedback pathways. *Nat Immunol* 4:248-254.
49. Plas, D. R., R. Johnson, J. T. Pingel, R. J. Matthews, M. Dalton, G. Roy, A. C. Chan, and M. L. Thomas. 1996. Direct regulation of ZAP-70 by SHP-1 in T cell antigen receptor signaling. *Science* 272:1173-1176.
50. Brockdorff, J., S. Williams, C. Couture, and T. Mustelin. 1999. Dephosphorylation of ZAP-70 and inhibition of T cell activation by activated SHP1. *Eur J Immunol* 29:2539-2550.
51. Jiao, H., K. Berrada, W. Yang, M. Tabrizi, L. C. Platanias, and T. Yi. 1996. Direct association with and dephosphorylation of Jak2 kinase by the SH2-domain-containing protein tyrosine phosphatase SHP-1. *Mol Cell Biol* 16:6985-6992.
52. Klingmuller, U., U. Lorenz, L. C. Cantley, B. G. Neel, and H. F. Lodish. 1995. Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals. *Cell* 80:729-738.
53. Cuevas, B., Y. Lu, S. Watt, R. Kumar, J. Zhang, K. A. Siminovitch, and G. B. Mills. 1999. SHP-1 regulates Lck-induced phosphatidylinositol 3-kinase phosphorylation and activity. *J Biol Chem* 274:27583-27589.
54. Caron, D., P. E. Savard, C. J. Doillon, M. Olivier, E. Shink, J. G. Lussier, and R. L. Faure. 2008. Protein tyrosine phosphatase inhibition induces anti-tumor activity: evidence of Cdk2/p27 kip1 and Cdk2/SHP-1 complex formation in human ovarian cancer cells. *Cancer Lett* 262:265-275.
55. Simoneau, M., J. Boulanger, G. Coulombe, M. A. Renaud, C. Duchesne, and N. Rivard. 2008. Activation of Cdk2 stimulates proteasome-dependent truncation of tyrosine phosphatase SHP-1 in human proliferating intestinal epithelial cells. *J*

Biol Chem 283:25544-25556.
56. Migone, T. S., N. A. Cacalano, N. Taylor, T. Yi, T. A. Waldmann, and J. A. Johnston. 1998. Recruitment of SH2-containing protein tyrosine phosphatase SHP-1 to the interleukin 2 receptor; loss of SHP-1 expression in human T-lymphotropic virus type I-transformed T cells. *Proceedings of the National Academy of Sciences of the United States of America* 95:3845-3850.
57. Zhang, Z., K. Shen, W. Lu, and P. A. Cole. 2003. The role of C-terminal tyrosine phosphorylation in the regulation of SHP-1 explored via expressed protein ligation. *J Biol Chem* 278:4668-4674.
58. Liu, Y., M. J. Kruhlak, J. J. Hao, and S. Shaw. 2007. Rapid T cell receptor-mediated SHP-1 S591 phosphorylation regulates SHP-1 cellular localization and phosphatase activity. *J Leukoc Biol* 82:742-751.
59. Wasserman, H. A., C. D. Beal, Y. Zhang, N. Jiang, C. Zhu, and B. D. Evavold. 2008. MHC variant peptide-mediated anergy of encephalitogenic T cells requires SHP-1. *J Immunol* 181:6843-6849.
60. Li, Q. J., J. Chau, P. J. Ebert, G. Sylvester, H. Min, G. Liu, R. Braich, M. Manoharan, J. Soutschek, P. Skare, L. O. Klein, M. M. Davis, and C. Z. Chen. 2007. miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell* 129:147-161.
61. Daniels, M. A., S. L. Schober, K. A. Hogquist, and S. C. Jameson. 1999. Cutting Edge: A Test of the Dominant Negative Signal Model for TCR Antagonism. *J Immunol* 162:3761-3764.
62. Yang, W., and H. M. Grey. 2003. Study of the Mechanism of TCR Antagonism Using Dual-TCR-Expressing T Cells. *J Immunol* 170:4532-4538.
63. Robertson, J. M., and B. D. Evavold. 1999. Cutting edge: dueling TCRs: peptide antagonism of CD4+ T cells with dual antigen specificities. *J Immunol* 163:1750-1754.
64. McNeil, L. K., and B. D. Evavold. 2003. TCR reserve: a novel principle of CD4 T cell activation by weak ligands. *J Immunol* 170:1224-1230.
65. Jones, D. S., P. Reichardt, M. L. Ford, L. J. Edwards, and B. D. Evavold. 2008. TCR antagonism by peptide requires high TCR expression. *J Immunol* 181:1760-1766.
66. Wasserman, H. A., and B. D. Evavold. 2008. Induction of anergy by antibody blockade of TCR in myelin oligodendrocyte glycoprotein-specific cells. *J Immunol* 180:7259-7264.
67. Labrecque, N., L. S. Whitfield, R. Obst, C. Waltzinger, C. Benoist, and D. Mathis. 2001. How much TCR does a T cell need? *Immunity* 15:71-82.
68. Harding, C. V., and E. R. Unanue. 1990. Quantitation of antigen-presenting cell MHC class II/peptide complexes necessary for T-cell stimulation. *Nature* 346:574-576.
69. Demotz, S., H. M. Grey, and A. Sette. 1990. The minimal number of class II MHC-antigen complexes needed for T cell activation. *Science* 249:1028-1030.
70. Kimachi, K., M. Croft, and H. M. Grey. 1997. The minimal number of antigen-major histocompatibility complex class II complexes required for activation of naive and primed T cells. *Eur J Immunol* 27:3310-3317.
71. Liu, G. Y., P. J. Fairchild, R. M. Smith, J. R. Prowle, D. Kioussis, and D. C.

Wraith. 1995. Low avidity recognition of self-antigen by T cells permits escape from central tolerance. *Immunity* 3:407-415.

72. Bouneaud, C., P. Kourilsky, and P. Bousso. 2000. Impact of negative selection on the T cell repertoire reactive to a self-peptide: a large fraction of T cell clones escapes clonal deletion. *Immunity* 13:829-840.
73. Zehn, D., and M. J. Bevan. 2006. T cells with low avidity for a tissue-restricted antigen routinely evade central and peripheral tolerance and cause autoimmunity. *Immunity* 25:261-270.
74. Tanchot, C., F. A. Lemonnier, B. Perarnau, A. A. Freitas, and B. Rocha. 1997. Differential requirements for survival and proliferation of CD8 naive or memory T cells. *Science* 276:2057-2062.
75. Ernst, B., D. S. Lee, J. M. Chang, J. Sprent, and C. D. Surh. 1999. The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. *Immunity* 11:173-181.
76. Ji, Q., A. Perchellet, and J. M. Goverman. 2010. Viral infection triggers central nervous system autoimmunity via activation of CD8+ T cells expressing dual TCRs. *Nat Immunol* 11:628-634.
77. Feinerman, O., R. N. Germain, and G. Altan-Bonnet. 2008. Quantitative challenges in understanding ligand discrimination by alphabeta T cells. *Mol Immunol* 45:619-631.
78. Huse, M., L. O. Klein, A. T. Girvin, J. M. Faraj, Q. J. Li, M. S. Kuhns, and M. M. Davis. 2007. Spatial and temporal dynamics of T cell receptor signaling with a photoactivatable agonist. *Immunity* 27:76-88.
79. Evavold, B. D., J. Sloan-Lancaster, and P. M. Allen. 1993. Tickling the TCR: selective T-cell functions stimulated by altered peptide ligands. *Immunol Today* 14:602-609.
80. McKeithan, T. W. 1995. Kinetic proofreading in T-cell receptor signal transduction. *Proc Natl Acad Sci U S A* 92:5042-5046.
81. Matsui, K., J. J. Boniface, P. Steffner, P. A. Reay, and M. M. Davis. 1994. Kinetics of T-cell receptor binding to peptide/I-Ek complexes: correlation of the dissociation rate with T-cell responsiveness. *Proc Natl Acad Sci U S A* 91:12862-12866.
82. Lyons, D. S., S. A. Lieberman, J. Hampl, J. J. Boniface, Y. Chien, L. J. Berg, and M. M. Davis. 1996. A TCR binds to antagonist ligands with lower affinities and faster dissociation rates than to agonists. *Immunity* 5:53-61.
83. Kersh, G. J., E. N. Kersh, D. H. Fremont, and P. M. Allen. 1998. High- and low-potency ligands with similar affinities for the TCR: the importance of kinetics in TCR signaling. *Immunity* 9:817-826.
84. Ding, Y. H., B. M. Baker, D. N. Garboczi, W. E. Biddison, and D. C. Wiley. 1999. Four A6-TCR/peptide/HLA-A2 structures that generate very different T cell signals are nearly identical. *Immunity* 11:45-56.
85. Holler, P. D., and D. M. Kranz. 2003. Quantitative analysis of the contribution of TCR/pepMHC affinity and CD8 to T cell activation. *Immunity* 18:255-264.
86. Sykulev, Y., Y. Vugmeyster, A. Brunmark, H. L. Ploegh, and H. N. Eisen. 1998. Peptide antagonism and T cell receptor interactions with peptide-MHC complexes. *Immunity* 9:475-483.

87. Baker, B. M., S. J. Gagnon, W. E. Biddison, and D. C. Wiley. 2000. Conversion of a T cell antagonist into an agonist by repairing a defect in the TCR/peptide/MHC interface: implications for TCR signaling. *Immunity* 13:475-484.
88. Rabinowitz, J. D., C. Beeson, C. Wulfing, K. Tate, P. M. Allen, M. M. Davis, and H. M. McConnell. 1996. Altered T cell receptor ligands trigger a subset of early T cell signals. *Immunity* 5:125-135.
89. Van Parijs, L., Y. Refaeli, J. D. Lord, B. H. Nelson, A. K. Abbas, and D. Baltimore. 1999. Uncoupling IL-2 signals that regulate T cell proliferation, survival, and Fas-mediated activation-induced cell death. *Immunity* 11:281-288.
90. Borovsky, Z., G. Mishan-Eisenberg, E. Yaniv, and J. Rachmilewitz. 2002. Serial triggering of T cell receptors results in incremental accumulation of signaling intermediates. *J Biol Chem* 277:21529-21536.
91. Faroudi, M., R. Zaru, P. Paulet, S. Muller, and S. Valitutti. 2003. Cutting edge: T lymphocyte activation by repeated immunological synapse formation and intermittent signaling. *J Immunol* 171:1128-1132.
92. Valitutti, S., S. Muller, M. Cella, E. Padovan, and A. Lanzavecchia. 1995. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature* 375:148-151.
93. Itoh, Y., B. Hemmer, R. Martin, and R. N. Germain. 1999. Serial TCR engagement and down-modulation by peptide:MHC molecule ligands: relationship to the quality of individual TCR signaling events. *J Immunol* 162:2073-2080.
94. San Jose, E., A. Borroto, F. Niedergang, A. Alcover, and B. Alarcon. 2000. Triggering the TCR complex causes the downregulation of nonengaged receptors by a signal transduction-dependent mechanism. *Immunity* 12:161-170.
95. Coombs, D., A. M. Kalergis, S. G. Nathenson, C. Wofsy, and B. Goldstein. 2002. Activated TCRs remain marked for internalization after dissociation from pMHC. *Nat Immunol* 3:926-931.
96. Sousa, J., and J. Carneiro. 2000. A mathematical analysis of TCR serial triggering and down-regulation. *Eur J Immunol* 30:3219-3227.
97. Kalergis, A. M., N. Boucheron, M. A. Doucey, E. Palmieri, E. C. Goyarts, Z. Vegh, I. F. Luescher, and S. G. Nathenson. 2001. Efficient T cell activation requires an optimal dwell-time of interaction between the TCR and the pMHC complex. *Nat Immunol* 2:229-234.
98. Sloan-Lancaster, J., and P. M. Allen. 1996. Altered peptide ligand-induced partial T cell activation: molecular mechanisms and role in T cell biology. *Annu Rev Immunol* 14:1-27.
99. Jenkins, M. K., and R. H. Schwartz. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J Exp Med* 165:302-319.
100. Jenkins, M. K., C. Chen, G. Jung, D. L. Mueller, and R. H. Schwartz. 1990. Inhibition of antigen-specific proliferation of type 1 murine T cell clones after stimulation with immobilized anti-CD3 monoclonal antibody. *Journal of Immunology* 144:16-22.
101. Quill, H., and R. H. Schwartz. 1987. Stimulation of normal inducer T cell clones

with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferation nonresponsiveness. *Journal of Immunology* 138:3704-3712.

102. Korb, L. C., S. Mirshahidi, K. Ramyar, A. A. S. Akha, and S. Sadegh-Nasseri. 1999. Induction of T Cell Anergy by Low Numbers of Agonist Ligands. *The Journal of Immunology* 163:6401-6409.
103. Ford, M. L., and B. D. Evavold. 2003. Regulation of polyclonal T cell responses by an MHC anchor-substituted variant of myelin oligodendrocyte glycoprotein 35-55. *J Immunol* 171:1247-1254.
104. Margot, C. D., M. L. Ford, and B. D. Evavold. 2005. Amelioration of established experimental autoimmune encephalomyelitis by an MHC anchor-substituted variant of proteolipid protein 139-151. *J Immunol* 174:3352-3358.
105. Jameson, S. C., and M. J. Bevan. 1995. T cell receptor antagonists and partial agonists. *Immunity* 2:1-11.
106. LaFace, D. M., C. Couture, K. Anderson, G. Shih, J. Alexander, A. Sette, T. Mustelin, A. Altman, and H. M. Grey. 1997. Differential T cell signaling induced by antagonist peptide-MHC complexes and the associated phenotypic responses. *Journal of Immunology* 158:2057-2064.
107. Norris, P. J., J. D. Stone, N. Anikeeva, J. W. Heitman, I. C. Wilson, D. F. Hirschkorn, M. J. Clark, H. F. Moffett, T. O. Cameron, Y. Sykulev, L. J. Stern, and B. D. Walker. 2006. Antagonism of HIV-specific CD4+ T cells by C-terminal truncation of a minimum epitope. *Mol Immunol* 43:1349-1357.
108. Burroughs, N. J., and D. A. Rand. 1998. Dynamics of T-cell antagonism: enhanced viral diversity and survival. *Proc Biol Sci* 265:529-535.
109. Haque, S. J., P. Harbor, M. Tabrizi, T. Yi, and B. R. Williams. 1998. Protein-tyrosine phosphatase Shp-1 is a negative regulator of IL-4- and IL-13-dependent signal transduction. *J Biol Chem* 273:33893-33896.
110. Wylie, D. C., J. Das, and A. K. Chakraborty. 2007. Sensitivity of T cells to antigen and antagonism emerges from differential regulation of the same molecular signaling module. *Proc Natl Acad Sci U S A* 104:5533-5538.
111. Lipniacki, T., B. Hat, J. R. Faeder, and W. S. Hlavacek. 2008. Stochastic effects and bistability in T cell receptor signaling. *J Theor Biol* 254:110-122.
112. Kersh, G. J., D. L. Donermeyer, K. E. Frederick, J. M. White, B. L. Hsu, and P. M. Allen. 1998. TCR transgenic mice in which usage of transgenic alpha- and beta-chains is highly dependent on the level of selecting ligand. *J Immunol* 161:585-593.
113. Alexander, J., J. Ruppert, K. Snoke, and A. Sette. 1994. TCR antagonism and T cell tolerance can be independently induced in a DR-restricted, hemagglutinin-specific T cell clone. *International Immunology* 6:363-367.
114. Bachmann, M. F., D. E. Speiser, A. Zakarian, and P. S. Ohashi. 1998. Inhibition of TCR triggering by a spectrum of altered peptide ligands suggests the mechanism for TCR antagonism. *Eur J Immunol* 28:3110-3119.
115. Gebe, J. A., S. A. Masewicz, S. A. Kochik, H. Reijonen, and G. T. Nepom. 2004. Inhibition of altered peptide ligand-mediated antagonism of human GAD65-responsive CD4+ T cells by non-antagonizable T cells. *Eur J Immunol* 34:3337-3345.

116. Takato-Kaji, R., M. Totsuka, W. Ise, M. Nishikawa, S. Hachimura, and S. Kaminogawa. 2005. T-cell receptor antagonist modifies cytokine secretion profile of naive CD4+ T cells and their differentiation into type-1 and type-2 helper T cells. *Immunol Lett* 96:39-45.
117. Jenkins, M. K., P. S. Taylor, S. D. Norton, and K. B. Urdahl. 1991. CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J Immunol* 147:2461-2466.
118. Furtado, G. C., M. A. Curotto de Lafaille, N. Kutchukhidze, and J. J. Lafaille. 2002. Interleukin 2 signaling is required for CD4(+) regulatory T cell function. *J Exp Med* 196:851-857.
119. Yagi, H., T. Nomura, K. Nakamura, S. Yamazaki, T. Kitawaki, S. Hori, M. Maeda, M. Onodera, T. Uchiyama, S. Fujii, and S. Sakaguchi. 2004. Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. *Int Immunol* 16:1643-1656.
120. Schwartz, R. H. 2003. T cell anergy. *Annu Rev Immunol* 21:305-334.
121. Lamb, J. R., B. J. Skidmore, N. Green, J. M. Chiller, and M. Feldmann. 1983. Induction of tolerance in influenza virus-immune T lymphocyte clones with synthetic peptides of influenza hemagglutinin. *J Exp Med* 157:1434-1447.
122. Beverly, B., S. M. Kang, M. J. Lenardo, and R. H. Schwartz. 1992. Reversal of in vitro T cell clonal anergy by IL-2 stimulation. *Int Immunol* 4:661-671.
123. Ryan, K. R., and B. D. Evavold. 1998. Persistence of peptide-induced CD4+ T cell anergy in vitro. *J Exp Med* 187:89-96.
124. Li, W., C. D. Whaley, A. Mondino, and D. L. Mueller. 1996. Blocked signal transduction to the ERK and JNK protein kinases in anergic CD4+ T cells [see comments]. *Science* 271:1272-1276.
125. Fields, P. E., T. F. Gajewski, and F. W. Fitch. 1996. Blocked Ras activation in anergic CD4+ T cells. *Science* 271:1276-1278.
126. Katz, J. D., B. Wang, K. Haskins, C. Benoist, and D. Mathis. 1993. Following a diabetogenic T cell from genesis through pathogenesis. *Cell* 74:1089-1100.
127. Mallet-Designe, V. I., T. Stratmann, D. Homann, F. Carbone, M. B. Oldstone, and L. Teyton. 2003. Detection of low-avidity CD4+ T cells using recombinant artificial APC: following the antiovalbumin immune response. *J Immunol* 170:123-131.
128. Harrison, L. C., M. C. Honeyman, S. Trembleau, S. Gregori, F. Gallazzi, P. Augstein, V. Brusic, J. Hammer, and L. Adorini. 1997. A peptide-binding motif for I-A(g7), the class II major histocompatibility complex (MHC) molecule of NOD and Biozzi AB/H mice. *J Exp Med* 185:1013-1021.
129. Levisetti, M. G., A. Suri, S. J. Petzold, and E. R. Unanue. 2007. The insulin-specific T cells of nonobese diabetic mice recognize a weak MHC-binding segment in more than one form. *J Immunol* 178:6051-6057.
130. Chang, K. Y., A. Suri, and E. R. Unanue. 2007. Predicting peptides bound to I-Ag7 class II histocompatibility molecules using a novel expectation-maximization alignment algorithm. *Proteomics* 7:367-377.
131. Kilgore, N. E., M. L. Ford, C. D. Margot, D. S. Jones, P. Reichardt, and B. D. Evavold. 2004. Defining the parameters necessary for T-cell recognition of ligands that vary in potency. *Immunol Res* 29:29-40.

132. Rochman, Y., R. Spolski, and W. J. Leonard. 2009. New insights into the regulation of T cells by gamma(c) family cytokines. *Nat Rev Immunol* 9:480-490.
133. Danke, N. A., D. M. Koelle, C. Yee, S. Beheray, and W. W. Kwok. 2004. Autoreactive T cells in healthy individuals. *J Immunol* 172:5967-5972.
134. Oxenius, A., R. M. Zinkernagel, and H. Hengartner. 1998. Comparison of activation versus induction of unresponsiveness of virus-specific CD4+ and CD8+ T cells upon acute versus persistent viral infection. *Immunity* 9:449-457.
135. Staveley-O'Carroll, K., E. Sotomayor, J. Montgomery, I. Borrello, L. Hwang, S. Fein, D. Pardoll, and H. Levitsky. 1998. Induction of antigen-specific T cell anergy: An early event in the course of tumor progression. *Proc Natl Acad Sci U S A* 95:1178-1183.
136. Chan, A. C., D. M. Desai, and A. Weiss. 1994. The role of protein tyrosine kinases and protein tyrosine phosphatases in T cell antigen receptor signal transduction. *Annu Rev Immunol* 12:555-592.
137. Poole, A. W., and M. L. Jones. 2005. A SHPing tale: perspectives on the regulation of SHP-1 and SHP-2 tyrosine phosphatases by the C-terminal tail. *Cell Signal* 17:1323-1332.
138. Green, M. C., and L. D. Shultz. 1975. Motheaten, an immunodeficient mutant of the mouse. I. Genetics and pathology. *J Hered* 66:250-258.
139. Sidman, C. L., L. D. Shultz, and E. R. Unanue. 1978. The mouse mutant "motheaten". I. Development of lymphocyte populations. *J Immunol* 121:2392-2398.
140. Sidman, C. L., L. D. Shultz, and E. R. Unanue. 1978. The mouse mutant "motheaten." II. Functional studies of the immune system. *J Immunol* 121:2399-2404.
141. Davidson, W. F., H. C. Morse, 3rd, S. O. Sharrow, and T. M. Chused. 1979. Phenotypic and functional effects of the motheaten gene on murine B and T lymphocytes. *J Immunol* 122:884-891.
142. Yu, C. C., H. W. Tsui, B. Y. Ngan, M. J. Shulman, G. E. Wu, and F. W. Tsui. 1996. B and T cells are not required for the viable motheaten phenotype. *J Exp Med* 183:371-380.
143. Lorenz, U., K. S. Ravichandran, S. J. Burakoff, and B. G. Neel. 1996. Lack of SHPTP1 results in src-family kinase hyperactivation and thymocyte hyperresponsiveness. *Proc Natl Acad Sci U S A* 93:9624-9629.
144. Kosugi, A., J. Sakakura, K. Yasuda, M. Ogata, and T. Hamaoka. 2001. Involvement of SHP-1 tyrosine phosphatase in TCR-mediated signaling pathways in lipid rafts. *Immunity* 14:669-680.
145. Carter, J. D., B. G. Neel, and U. Lorenz. 1999. The tyrosine phosphatase SHP-1 influences thymocyte selection by setting TCR signaling thresholds. *Int Immunol* 11:1999-2014.
146. Johnson, K. G., F. G. LeRoy, L. K. Borysiewicz, and R. J. Matthews. 1999. TCR signaling thresholds regulating T cell development and activation are dependent upon SHP-1. *J Immunol* 162:3802-3813.
147. Shultz, L. D., and M. C. Green. 1976. Motheaten, an immunodeficient mutant of the mouse. II. Depressed immune competence and elevated serum immunoglobulins. *J Immunol* 116:936-943.

148. Kamata, T., M. Yamashita, M. Kimura, K. Murata, M. Inami, C. Shimizu, K. Sugaya, C. R. Wang, M. Taniguchi, and T. Nakayama. 2003. src homology 2 domain-containing tyrosine phosphatase SHP-1 controls the development of allergic airway inflammation. *J Clin Invest* 111:109-119.
149. Yu, W. M., S. Wang, A. D. Keegan, M. S. Williams, and C. K. Qu. 2005. Abnormal Th1 cell differentiation and IFN-gamma production in T lymphocytes from motheaten viable mice mutant for Src homology 2 domain-containing protein tyrosine phosphatase-1. *J Immunol* 174:1013-1019.
150. Kubota, T., H. Taiyoh, A. Matsumura, Y. Murayama, D. Ichikawa, K. Okamoto, H. Fujiwara, H. Ikoma, M. Nakanishi, S. Kikuchi, C. Sakakura, T. Ochiai, Y. Kokuba, H. Taniguchi, T. Sonoyama, K. Matsumoto, T. Nakamura, and E. Otsuji. 2009. NK4, an HGF antagonist, prevents hematogenous pulmonary metastasis by inhibiting adhesion of CT26 cells to endothelial cells. *Clin Exp Metastasis* 26:447-456.
151. Carter, J. D., G. M. Calabrese, M. Naganuma, and U. Lorenz. 2005. Deficiency of the Src homology region 2 domain-containing phosphatase 1 (SHP-1) causes enrichment of CD4+CD25+ regulatory T cells. *Journal of Immunology* 174:6627-6638.
152. Iype, T., M. Sankarshanan, I. S. Mauldin, D. W. Mullins, and U. Lorenz. 2010. The protein tyrosine phosphatase SHP-1 modulates the suppressive activity of regulatory T cells. *J Immunol* 185:6115-6127.
153. Deng, C., B. Wu, H. Yang, R. Z. Hussain, A. E. Lovett-Racke, P. Christadoss, and M. K. Racke. 2003. Decreased expression of Src homology 2 domain-containing protein tyrosine phosphatase 1 reduces T cell activation threshold but not the severity of experimental autoimmune myasthenia gravis. *J Neuroimmunol* 138:76-82.
154. Deng, C., A. Minguela, R. Z. Hussain, A. E. Lovett-Racke, C. Radu, E. S. Ward, and M. K. Racke. 2002. Expression of the tyrosine phosphatase SRC homology 2 domain-containing protein tyrosine phosphatase 1 determines T cell activation threshold and severity of experimental autoimmune encephalomyelitis. *J Immunol* 168:4511-4518.
155. Ford, M. L., T. M. Onami, A. I. Sperling, R. Ahmed, and B. D. Evavold. 2003. CD43 modulates severity and onset of experimental autoimmune encephalomyelitis. *J Immunol* 171:6527-6533.
156. Wishcamper, C. A., J. D. Coffin, and D. I. Lurie. 2001. Lack of the protein tyrosine phosphatase SHP-1 results in decreased numbers of glia within the motheaten (me/me) mouse brain. *J Comp Neurol* 441:118-133.
157. Massa, P. T., C. Wu, and K. Fecenko-Tacka. 2004. Dysmyelination and reduced myelin basic protein gene expression by oligodendrocytes of SHP-1-deficient mice. *J Neurosci Res* 77:15-25.
158. Fowler, C. C., L. I. Pao, J. N. Blattman, and P. D. Greenberg. 2010. SHP-1 in T cells limits the production of CD8 effector cells without impacting the formation of long-lived central memory cells. *J Immunol* 185:3256-3267.
159. Liu, J., N. Gong, X. Huang, A. D. Reynolds, R. L. Mosley, and H. E. Gendelman. 2009. Neuromodulatory activities of CD4+CD25+ regulatory T cells in a murine model of HIV-1-associated neurodegeneration. *J Immunol* 182:3855-3865.

160. Reynolds, A. D., R. Banerjee, J. Liu, H. E. Gendelman, and R. L. Mosley. 2007. Neuroprotective activities of CD4+CD25+ regulatory T cells in an animal model of Parkinson's disease. *J Leukoc Biol* 82:1083-1094.
161. Azoulay, D., N. Urshansky, and A. Karni. 2008. Low and dysregulated BDNF secretion from immune cells of MS patients is related to reduced neuroprotection. *J Neuroimmunol* 195:186-193.
162. Aharoni, R., B. Kayhan, R. Eilam, M. Sela, and R. Arnon. 2003. Glatiramer acetate-specific T cells in the brain express T helper 2/3 cytokines and brain-derived neurotrophic factor in situ. *Proc Natl Acad Sci U S A* 100:14157-14162.
163. Blanco, Y., E. A. Moral, M. Costa, M. Gomez-Choco, J. F. Torres-Peraza, L. Alonso-Magdalena, J. Alberch, D. Jaraquemada, T. Arbizu, F. Graus, and A. Saiz. 2006. Effect of glatiramer acetate (Copaxone) on the immunophenotypic and cytokine profile and BDNF production in multiple sclerosis: a longitudinal study. *Neurosci Lett* 406:270-275.
164. Gravel, C., R. Gotz, A. Lorrain, and M. Sendtner. 1997. Adenoviral gene transfer of ciliary neurotrophic factor and brain-derived neurotrophic factor leads to long-term survival of axotomized motor neurons. *Nat Med* 3:765-770.
165. McTigue, D. M., P. J. Horner, B. T. Stokes, and F. H. Gage. 1998. Neurotrophin-3 and brain-derived neurotrophic factor induce oligodendrocyte proliferation and myelination of regenerating axons in the contused adult rat spinal cord. *J Neurosci* 18:5354-5365.
166. Neumann, H., T. Misgeld, K. Matsumuro, and H. Wekerle. 1998. Neurotrophins inhibit major histocompatibility class II inducibility of microglia: involvement of the p75 neurotrophin receptor. *Proc Natl Acad Sci U S A* 95:5779-5784.
167. Pathak, M. K., and T. Yi. 2001. Sodium stibogluconate is a potent inhibitor of protein tyrosine phosphatases and augments cytokine responses in hemopoietic cell lines. *J Immunol* 167:3391-3397.
168. Davis, M. M., J. J. Boniface, Z. Reich, D. Lyons, J. Hampl, B. Arden, and Y. Chien. 1998. Ligand recognition by alpha beta T cell receptors. *Annu Rev Immunol* 16:523-544.
169. Stone, J. D., A. S. Chervin, and D. M. Kranz. 2009. T-cell receptor binding affinities and kinetics: impact on T-cell activity and specificity. *Immunology* 126:165-176.
170. Karin, N., D. J. Mitchell, S. Brocke, N. Ling, and L. Steinman. 1994. Reversal of experimental autoimmune encephalomyelitis by a soluble peptide variant of a myelin basic protein epitope: T cell receptor antagonism and reduction of interferon gamma and tumor necrosis factor alpha production. *J Exp Med* 180:2227-2237.
171. Nicholson, L. B., J. M. Greer, R. A. Sobel, M. B. Lees, and V. K. Kuchroo. 1995. An altered peptide ligand mediates immune deviation and prevents autoimmune encephalomyelitis. *Immunity* 3:397-405.
172. Brocke, S., K. Gijbels, M. Allegretta, I. Ferber, C. Piercy, T. Blankenstein, R. Martin, U. Utz, N. Karin, D. Mitchell, T. Veromaa, A. Waisman, A. Gaur, P. Conlon, P. J. Fairchild, D. C. Wraith, A. O'Garra, C. G. Fathman, and L. Steinman. 1996. Treatment of experimental encephalomyelitis with a peptide analogue of myelin basic protein. *Nature* 379:343-346.

173. Kirshner, S. L., E. Zisman, M. Fridkin, M. Sela, and E. Mozes. 1996. Altered peptide ligands of a myasthenogenic epitope as modulators of specific T-cell responses. *Scand J Immunol* 44:512-521.
174. Faber-Elmann, A., M. Paas-Rozner, M. Sela, and E. Mozes. 1998. Altered peptide ligands act as partial agonists by inhibiting phospholipase C activity induced by myasthenogenic T cell epitopes. *Proc Natl Acad Sci U S A* 95:14320-14325.
175. Ikagawa, S., S. Matsushita, Y. Z. Chen, T. Ishikawa, and Y. Nishimura. 1996. Single amino acid substitutions on a Japanese cedar pollen allergen (Cry j 1)-derived peptide induced alterations in human T cell responses and T cell receptor antagonism. *J Allergy Clin Immunol* 97:53-64.
176. Kinnunen, T., K. Jutila, W. W. Kwok, M. Rytkonen-Nissinen, A. Immonen, S. Saarelainen, A. Narvanen, A. Taivainen, and T. Virtanen. 2007. Potential of an altered peptide ligand of lipocalin allergen Bos d 2 for peptide immunotherapy. *J Allergy Clin Immunol* 119:965-972.
177. Geluk, A., K. E. van Meijgaarden, B. O. Roep, and T. H. Ottenhoff. 1998. Altered peptide ligands of islet autoantigen Imogen 38 inhibit antigen specific T cell reactivity in human type-1 diabetes. *J Autoimmun* 11:353-361.
178. Kim, H. J., J. P. Antel, P. Duquette, D. G. Alleva, P. J. Conlon, and A. Bar-Or. 2002. Persistence of immune responses to altered and native myelin antigens in patients with multiple sclerosis treated with altered peptide ligand. *Clinical Immunology* 104:105-114.
179. Prakken, B. J., S. Roord, P. J. van Kooten, J. P. Wagenaar, W. van Eden, S. Albani, and M. H. Wauben. 2002. Inhibition of adjuvant-induced arthritis by interleukin-10-driven regulatory cells induced via nasal administration of a peptide analog of an arthritis-related heat-shock protein 60 T cell epitope. *Arthritis Rheum* 46:1937-1946.
180. Ohnishi, Y., A. Tsutsumi, I. Matsumoto, D. Goto, S. Ito, M. Kuwana, Y. Uemura, Y. Nishimura, and T. Sumida. 2006. Altered peptide ligands control type II collagen-reactive T cells from rheumatoid arthritis patients. *Mod Rheumatol* 16:226-228.
181. Boots, A. M., H. Hubers, M. Kouwijzer, L. den Hoed-van Zandbrink, B. M. Westrek-Esselink, C. van Doorn, R. Stenger, E. S. Bos, M. J. van Lierop, G. F. Verheijden, C. M. Timmers, and C. J. van Staveren. 2007. Identification of an altered peptide ligand based on the endogenously presented, rheumatoid arthritis-associated, human cartilage glycoprotein-39(263-275) epitope: an MHC anchor variant peptide for immune modulation. *Arthritis Res Ther* 9:R71.
182. Yu, Z., M. Maoui, Z. J. Zhao, Y. Li, and S. H. Shen. 2006. SHP-1 dephosphorylates 3BP2 and potentially downregulates 3BP2-mediated T cell antigen receptor signaling. *Febs J* 273:2195-2205.
183. Bielekova, B., B. Goodwin, N. Richert, I. Cortese, T. Kondo, G. Afshar, B. Gran, J. Eaton, J. Antel, J. A. Frank, H. F. McFarland, and R. Martin. 2000. Encephalitogenic potential of the myelin basic protein peptide (amino acids 83-99) in multiple sclerosis: results of a phase II clinical trial with an altered peptide ligand. *Nat Med* 6:1167-1175.
184. Kappos, L., G. Comi, H. Panitch, J. Oger, J. Antel, P. Conlon, and L. Steinman. 2000. Induction of a non-encephalitogenic type 2 T helper-cell autoimmune

response in multiple sclerosis after administration of an altered peptide ligand in a placebo-controlled, randomized phase II trial. The Altered Peptide Ligand in Relapsing MS Study Group. *Nat Med* 6:1176-1182.

185. Walter, M., A. Philotheou, F. Bonnici, A. G. Ziegler, and R. Jimenez. 2009. No effect of the altered peptide ligand NBI-6024 on beta-cell residual function and insulin needs in new-onset type 1 diabetes. *Diabetes Care* 32:2036-2040.
186. Crowe, P. D., Y. Qin, P. J. Conlon, and J. P. Antel. 2000. NBI-5788, an altered MBP83-99 peptide, induces a T-helper 2-like immune response in multiple sclerosis patients. *Ann Neurol* 48:758-765.
187. Kim, H. J., J. P. Antel, P. Duquette, D. G. Alleva, P. J. Conlon, and A. Bar-Or. 2002. Persistence of immune responses to altered and native myelin antigens in patients with multiple sclerosis treated with altered peptide ligand. *Clin Immunol* 104:105-114.
188. Pedotti, R., D. Mitchell, J. Wedemeyer, M. Karpuj, D. Chabas, E. M. Hattab, M. Tsai, S. J. Galli, and L. Steinman. 2001. An unexpected version of horror autotoxicus: anaphylactic shock to a self-peptide. *Nat Immunol* 2:216-222.
189. Pedotti, R., J. J. DeVoss, S. Youssef, D. Mitchell, J. Wedemeyer, R. Madanat, H. Garren, P. Fontoura, M. Tsai, S. J. Galli, R. A. Sobel, and L. Steinman. 2003. Multiple elements of the allergic arm of the immune response modulate autoimmune demyelination. *Proc Natl Acad Sci U S A* 100:1867-1872.
190. Liu, E., H. Moriyama, N. Abiru, D. Miao, L. Yu, R. M. Taylor, F. D. Finkelman, and G. S. Eisenbarth. 2002. Anti-peptide autoantibodies and fatal anaphylaxis in NOD mice in response to insulin self-peptides B:9-23 and B:13-23. *J Clin Invest* 110:1021-1027.
191. Leech, M. D., C. Y. Chung, A. Culshaw, and S. M. Anderton. 2007. Peptide-based immunotherapy of experimental autoimmune encephalomyelitis without anaphylaxis. *Eur J Immunol* 37:3576-3581.
192. Codarri, L., G. Gyulveszi, V. Tosevski, L. Hesske, A. Fontana, L. Magnenat, T. Suter, and B. Becher. 2011. RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nat Immunol* 12:560-567.