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Understanding the Effects of Low Potency TCR Ligands

By

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Understanding the Effects of Low Potency TCR Ligands

By

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B.S., Georgia Institute of Technology, 2003

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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

2012

Abstract

Understanding the Effects of Low Potency TCR Ligands

By Kristen M Rosenthal

Antigen recognition and subsequent T cell activation is essential for productive cellular immunity, and depends on a productive encounter between T cell receptors (TCR) and antigens presented on major histocompatibility complexes (pMHC). Understanding how the quality and quantity of antigen can affect T cell activation, effector function, and survival is a necessary part of understanding T cell immunity and function. Moreover, it is hypothesized that T cells specific for self- or foreign-antigens differ in their affinities, although emerging evidence indicates that there is a range of affinities in any T cell response, with a larger subset of low affinity T cells comprising an injurious response to self-peptides. Here we utilize prototypical self-reactive (myelin) and viral-specific (LCMV) T cells from T cell receptor (TCR) transgenic mice (2D2 and SMARTA, respectively) to explore how differences in the quality and quantity of antigen can affect the T cell response. Despite their dramatically lower 2D affinity for their cognate ligand (>10,000 fold less than SMARTA T cells), 2D2 T cells respond with complete, albeit delayed, activation as assayed by either proliferation or cytokine production, and delayed or absent TCR signaling intermediates. Strikingly, the timing of T cells activation does not change with decreasing doses of antigen as it does with decreasing affinity, and only the magnitude of the response is affected. Finally, sustained access to antigen can offset low affinity for a ligand and allow for an accumulation of signaling intermediates to restore activation. These finding suggests that the affinity of TCR ligation directs the outcome of response in individual T cell clones, while the dose of antigen may influence the affinity profile during an immune response by tuning the scope of the responding T cell populations. Low affinity T cells may be able to escape tolerance mechanisms and enable autoimmune disease through a smoldering response to the ever-present self-antigens that propagate disease.

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Note: All figures and tables were generated by the author.

Chapter 1: Introduction

Antigen Recognition by T cells

T cells are crucial for the function of the adaptive immune response and essential in controlling infection. In order to maintain a close watch for invaders, T cells constantly travel through the circulatory and lymphatic system, interacting with host antigen presenting cells (APCs) to detect the presence of foreign antigen. In the lymphoid tissues, T cells will become activated following recognition of a foreign antigen presented by an MHC molecule on the surface of an APC [1]. Each T cell clone has a unique heterodimeric receptor on its surface that recognizes an antigen presented in the context of an MHC molecule. These T cell receptors (TCR) recognize a range of peptides when presented by major histocompatibility complexes (MHC) on the surface of APCs. The molecular subunits of MHC proteins form a peptide-binding groove and allow certain amino acids of a given peptide to bind with the MHC while leaving other amino acids of the peptide available to come in contact with the TCR on the surface of a T cell, usually with 3 to 4 amino acids required for TCR recognition. T cell activation is dependent on recognition of both the peptide and the MHC molecule by the TCR and this interaction is important in mediating the functions associated with these cells.

Through degenerate recognition of antigens, T cells can recognize an assortment of epitopes, including multiple sites within one peptide or completely distinct, non-related peptides [2]. Therefore, every T cell must be able to differentiate between a high and a low potency ligand in order to respond appropriately after TCR ligation [3-5]. The array of peptides for a specific TCR can include agonists, partial or weak agonists, and antagonists [6]

and the TCR can integrate the strength of the ligand binding to inform the appropriate response, ranging from full activation to anergy to antagonism [7, 8].

During an infection, ideal T cell activation through TCR ligation with the appropriate pMHC complex results in full activation with various effector functions associated with viral or bacterial clearance including proliferation, production of cytokines, and cytolysis. However, many parameters affect the T cell's response to TCR ligation by pMHC. The affinity of the TCR for the peptide:MHC complex as well as the dissociation rate of the peptide on the MHC molecule help determine the outcome of T cell activation [9-12]. Therefore, the stability of the peptide:MHC complex (pMHC) is crucial for generation of an immune response and depends on a combination of the affinity of the peptide to the MHC and the half-life of the molecules [13-15]. In addition, the density of both the TCR on the surface of the T cell and the density of pMHC on the APC can affect the T cell response [12, 16].

T Cell Receptor Affinity and Binding Parameters

The affinity of the TCR:pMHC complex is an important aspect of the parameters affecting the T cell response and there have been many methods used to explore this issue. T cell functional avidity, or the amount of antigen needed for half-maximal response, has been used as a surrogate measure of TCR affinity [17, 18]. The development of pMHC tetramers allowed the use of flow cytometry to gauge of TCR affinity via the extent of pMHC tetramer staining [1]. For more exact measurements of TCR binding parameters – the on-rate, off-rate, half-life, and affinity – other assays have been developed and include surface plasmon

resonance, use of single molecule Förster resonance energy transfer (FRET), and mechanical micropipette adhesion assays.

Initially, the affinity measurements were done using surface plasmon resonance, which utilizes purified proteins, one binding partner immobilized on a solid surface and the other in fluid phase. These measures of TCR:peptide:MHC interactions, derived in the three-dimensional (3D) fluid phase, have shown correlation between the T cell response and the TCR:pMHC off rate [9, 19-22]. However, other studies have shown correlations between the dissociation constant (Kd), but not off-rate, with the T cell response [23-27]. Overall, there is some correlation with ligand potency, although discrepancies were commonly observed for antagonist ligands [28-30] [21, 31-33].

The advent of two-dimensional (2D) assays to measure binding events allowed for a more constrained and physiologically relevant model of TCR:pMHC binding. One method, based on single molecule FRET, 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. When these two fluorophores are in close proximity through TCR interaction with pMHC, one fluorophore served as a donor and the second as an acceptor to provide a FRET readout that can be used in calculating various binding parameters [34].

The mechanical micropipette adhesion frequency assay utilizes an intact T cell and measures its binding to a red blood cell (RBC) coated with pMHC tetramers, which serves as a surrogate antigen-presenting cell [35-37]. T cell is aspirated onto one micropipette and a surrogate APC 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. 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. 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 [28].

The described 2D approaches demonstrate that 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 [28, 38]. The 2D analysis of TCR interaction with cognate pMHC resulted in rapid association and rapid dissociation and accordingly short half-lives of interactions - contrasting with the rapid on-rates, but slow dissociation rates and thus longer interaction times observed in the 3D system, [28-30]. The findings from these various affinity parameter measurements have influenced development of or altered various models of TCR:pMHC engagement models of T cell activation.

TCR Fidelity and Strength of Signal

The kinetic proofreading model, described by McKeithan et al, defines T cell activation based on the duration of the TCR:pMHC interactions [39]. According to this theory, T cell activation is a complex series of biochemical steps that need to overcome a rate limiting step, or threshold, in order to progress. When the TCR binds its cognate pMHC, a series of biochemical events is triggered. Complexes that are able to remain bound to the TCR for a longer period of time are able to fully activate the signaling cascade to overcome the

threshold signaling event needed for complete cellular activation; however, with lower affinity interactions, which dissociate rapidly, downstream signals are attenuated and signaling is incomplete.

The serial engagement model, first proposed by Lanzavecchia et al, addresses the ability of T cells to find their cognate ligand in the midst of a myriad of self-peptide:MHC complexes [40-42]. It was shown that in order for a T cell to be activated, 8000 TCR must be engaged or 1500 in the presence of co-stimulation; however, only 100 pMHC complexes are necessary for activation. This dichotomy led to the proposal that each pMHC complex can serially engage multiple TCR; therefore, T cells can become activated even with a low density of foreign antigen. Further, this model suggests that there is an optimal "dwell-time" in which the TCR should interact with its ligand [9]. If the interaction is too short, activation will not occur; however, if the interaction is too long, too few TCR will be engaged.

The confinement time model [43] incorporates data from TCR affinity measurements and postulates that rapid TCR rebinding to the same pMHC after chemical dissociation increases the effective half-life or ''confinement time'' of a TCR:pMHC interaction and results in T cell activation. Further, weaker ligands may have 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 [44]. These differences in binding kinetics may affect TCR signaling pathways and may provide one means of converting the interaction with antigen into the appropriate effector functions of the T cell.

The Importance of Time in the T cell:APC Interaction

It is apparent that there are multiple parameters that may affect how a T cell is activated once the TCR comes in contact with an appropriate pMHC; however, the exact details of how these factors directly relate to T cell responsiveness. The affinity of the TCR for its cognate pMHC plays a role and the subsequent formation of an immunological synapse allows signal integration to occur through TCR engagement with peptide:MHC complexes, leading to T cell activation [45, 46]. It has also been shown that the amount of time that a T cell interacts with DCs can influence the outcome of the effector response [47, 48] [49].

Current data suggests that T cells need prolonged encounters with an APC to lead to a response and that multiple encounters with APCs may facilitate a full response to antigen [48]. Studies using live cell imaging have shown that the priming of naïve T cells takes place in distinct phases characterized first by short interactions with dendritic cells (DCs) [47] [50], leading to more stable conjugates, and ultimately, resulting in renewed motility and cytokine secretion from the T cells [47]. Further, successive T cell encounters with DCs following the initial priming event correlate with the induction of effector function [49]. The MHC contacts may dictate the initial association and guide TCR:pMHC interactions in a way that is mainly independent of the peptide followed by more stabilized contacts, which convey specificity and influence T-cell activation by modulating the duration of binding. This two-step process for TCR recognition may facilitate the efficient scanning of diverse peptide:MHC complexes on the surface of cells and also makes TCRs inherently cross-reactive towards different peptides bound by the same MHC [51].

T cells require sustained periods of stimulation to differentiate into effector and memory T cells [52-54]; however, CD8 T cells require only transient stimulation with antigen to initiate proliferation [55-57], while CD4 T cells appear to require a longer encounter with

antigen for division to occur [52]. In vivo studies suggest that 36–60 h of antigen availability is sufficient for T cells to undergo the programming necessary for the acquisition of effector function and differentiation to memory cells [57-60]. Some data also suggests that more prolonged antigen exposure may be required for optimal expansion and effector differentiation [61-63]. In contrast, other data indicate that antigenic stimulation beyond 2 d results in a diminished population of effector CD4 T cells [64, 65]. Others have also shown that limiting access to antigen during the priming of CD4 T cells allows for maximal expansion but can result in defective effector differentiation and memory development [66]. Taken together, these studies indicate that T cell programming occurs very early during the T cell response and that antigen, even beyond the initial APC:T cell interaction, may continue to influence the process. Nevertheless, there remains ambiguity in terms of the durational requirements of antigen recognition by T cells for mounting an effective immune response.

The ambiguity around the duration of the TCR:pMHC interaction is compounded when analyzing differences between peptides that induce differential responses in a particular T cell population. There are many similarities in the early responses of T cells regardless of the activation status, despite the fact that the functional outcomes after antigen recognition are vastly different. Even under conditions of tolerance, T cells can form stable interactions with DCs and may receive early signals through the TCR, inducing activation marker expression, initiating proliferation, and promoting intracellular signaling; but, the discrimination of tolerance or activation is determined mainly by the duration and magnitude of the T cell:APC interaction. [67]. Biophysical analysis of these interactions has indicated that the duration of TCR engagement, and therefore the period of TCR signaling, is a prominent factor [48]. Prolonged periods of TCR engagement are required to achieve full effector potential and

proliferation, while shorter periods of engagement induce a state of partial activation, typified by CD69 expression [68]. It has also been suggested that TCR:MHC engagement sets up competing positive and negative feedback loops and, depending on which prevails, results in either digital amplification of the signal and activation of the T cell or abortion of the signal and a return to cellular quiescence [69]. Based on this data, the outcome of various feedback loops may rely on the duration of the TCR:pMHC contact and relate to the affinity of the pMHC for a given TCR.

T cell signaling

As mentioned in the previous section, engagement of the TCR can initiate a complex chain of biochemical events leading to the activation of the T cell. In a successful interaction, this complex cascade of events involving both kinases and phosphatases culminates in the activation of transcription factors that turn on genes which regulate cell division and cytokine production.

The TCR associates with CD3, composed of a γ, δ, and two ε chains, at the cell surface. The TCR and CD3 non-covalently associate with two TCR ζ chains, each of which contain three immunoreceptor tyrosine-based activation motifs (ITAMs) [5]. On engagement of the TCR with peptide:MHC, these ITAMs are brought into proximity of members of the src family kinases Lck and Fyn, which are associated with the CD4 or CD8 co-receptor at the surface of the T cell [70, 71]. Lck and Fyn can then phosphorylate specific tyrosine residues contained in the ITAMs to provide docking sites for other signaling molecules containing SH2 domains, which are necessary for binding the phosphorylated ITAMs. Also upon TCR

ligation, CD45, the phosphatase responsible for keeping Lck and Fyn inactive, is sequestered away from the CD3 complex and allows for activation of Lck and Fyn [72, 73].

Lck is an important player in TCR signal transduction and phosphorylation of ITAM residues in the CD3 and TCRζ chains leads to recruitment and activation of Zap70 and consequent propagation of downstream signals [74, 75]. Lck has been shown to control the activation threshold of naive T cells [76] and is an important part of signaling feedback loops through positive regulation (phosphorylation of Lck) by ERK, and negative regulation (dephosphorylation) by the phosphatase SHP-1 [77]. Fyn is also expressed at high levels in T cells; however, its function is less well understood and the absence of Fyn has only minor consequences [78]. Fyn has recently been implicated in the generation and maintenance of anergy [79] and influences the TCR:pMHC contact time required for commitment to full activation, correlating with changes in phosphorylation of the regulatory tyrosine of Lck which might alter its activity [80].

ZAP-70, a member of the Syk family protein tyrosine kinase, is another major player in the signaling pathways that result from TCR ligation [72, 81, 82]. ZAP-70 binds to the phosphorylated ITAMs and is then phosphorylated by Lck, which leads to the recruitment of vav, a small GTPase. Vav, along with WASP, leads to cytoskeletal reorganization, polarization, and capping [83]. Vav is also responsible for induction of the MAPK cascade, through activation of rac, which leads to activation of c-jun that, along with c-fos, forms the transcription factor AP-1 responsible for the transcription of IL-2 [5, 84].

ZAP-70 also phosphorylates LAT (linker of activated T cells), which is an essential scaffolding protein and adapter molecule that recruits many other signaling molecules,

including PLCγ, Grb2, and GADS [85-88]. PLCγ is responsible for cleaving the phospholipid PIP_2 into IP_3 and DAG, two important signaling molecules. IP_3 induces release of Ca2+, leading to activation of calcineurin that dephosphorylates NFAT, allowing trafficking to the nucleus to participate in activation of genes involved in cell division and cytokine production [5, 84]. DAG, on the other hand, activates protein kinase C (PKC), which then phosphorylates IkB to allow translocation of NFkB to the nucleus for transcription of pro-inflammatory cytokines [89]. Grb2 is an adaptor protein that activates ras, leading to the induction of the MAPK cascade through raf [90].

Although all of these molecules result in the functional activation of T cells, there are also many molecules involved in negatively regulating T cell signaling. The adaptor protein c-Cbl can inhibit signaling initiated by ZAP-70 to attenuate T cell signaling and activation [5, 84]. C-terminal Src kinase (Csk) can phosphorylate the C-terminal tyrosine of Src-family tyrosine kinases to inactivate them [91, 92]. Numerous phosphatases have also been implicated in inhibiting T cell activation, including SHP-1, SHP-2, PEP, and PTP-PEST [93] and may play an important part in negative feedback loops.

Src homology 2 domain containing protein tyrosine phosphatase (SHP-1) is involved in the negative regulation of responses in hematopoietic cells. The importance of SHP-1 for the maintenance of a functional immune system is highlighted by the naturally occurring SHP-1 mutations expressed by the motheaten strains of mice. Mice homozygous for the motheaten mutation, which have little or no SHP-1 function, die between 3-8 weeks of age due to abnormal B cell development, decreased NK cell activity, and hyper-responsive thymocytes and peripheral T cells [94-98]. SHP-1 is found to interact with many signaling pathways

including cytokine receptors, inhibitory receptors, Fc receptors, antigen receptors, and receptors with death domains [99, 100].

While the signaling pathways discussed above focused on signaling resulting from fully activated T cells, many groups are also interested in characterizing the molecular profile in cells that are sub-maximally stimulated. With multiple types of sub-par stimulation, cells can be rendered anergic, or refractory to future stimulation. According to most models of T cell activation, low affinity peptides fail to induce a full activation of signals downstream of the TCR due to a quick dissociation of the TCR:peptide:MHC complex [39]. However, more recent reports have suggested that T cell ligands not only trigger activating signals but also trigger a negative feedback loop involving the tyrosine phosphatase SHP-1 [77]. In this model, T cell stimulation results in the activation of Lck, which phosphorylates ZAP-70 as well as phosphorylating SHP-1, inducing a negative feedback loop. Phosphorylation of SHP-1 results in its activation, allowing for dephosphorylation of Lck substrates that then leads to dampening of the response [101]. This competition between the activating kinases, discussed previously, and the negative phosphatase SHP-1 is a direct result of the strength of the ligand, where kinase activity wins out to agonist peptides while phosphatase activity is enhanced by low affinity peptides [77].

Recent work in the lab suggests that that SHP-1 may have a role in controlling an autoimmune response to the self-peptide MOG [102] and that in CD8+ T cells the peak of SHP-1 activity occurs at 1 min in response to high affinity antigen SHP-1 but low affinity stimulation causes a delayed, but sustained peak of SHP-1 activity [103]. Others have shown a delay in certain signaling pathways after low affinity TCR stimulation and suggest that early activation events are induced by high- but not low-affinity ligands and some low-

affinity TCR ligands are less efficient at inducing proximal activation events than distal T cell responses [30]. These data are in opposition to predictions from previous theories regarding the kinetic proofreading or antigen discrimination models and suggest a model for T cell activation in which early signals of activation cannot be detected but still allow for a slow accumulation of later T cell signaling intermediates.

Self v Foreign T Cell Affinity

During positive selection of immature thymocytes, all T cells must have at least a minimal responsiveness to self-antigen in order to remain viable. However, during negative selection developing thymocytes with a high affinity for self-antigen are deleted in order to prevent activation against self-antigens in the periphery. Unfortunately, low affinity interactions with self-antigen in the thymus allow the escape of self-reactive CD4+ T cells into the periphery [104-109], which can result in the development of T cell-mediated autoimmune disease as demonstrated by transgenic mice expressing neo-self antigens. These experiments resulted in the deletion or tolerization of high affinity neo-self antigen-specific T cells, but allowed the preservation of lower affinity clonotypes [110-114].

Variable results regarding the issues around central tolerance have been demonstrated using various myelin antigens in murine models. One demonstration of central tolerance utilized the myelin antigens PLP. In mice and in humans, the protein DM20, a PLP splice variant lacking amino acids 116-150 is found in much higher levels in the thymus than the full-length native form of PLP [115-118], [119-121]. Consequently, the naïve $PLP_{139-151}$-specific $CD4^+$ T cell repertoire is extremely large in SJL mice with the MHC allele $H\text{-}2^s$ (estimated at 1 in 20,000) [115]. These findings are correlated with the increased susceptibility of SJL mice to EAE induction by $PLP_{139-151}$, as well as its immunodominance

over the $PLP_{178-191}$ epitope. The lack of central tolerance to this PLP epitope underlies the high antigen-specific precursor frequency and predisposes to CNS autoimmunity.

Another myelin antigen, MBP, highlights that the degree of T cell deletion appears to be directly related to the amount of antigen expression in the thymus. Higher levels of MBP were found in the thymus of mice resistant to EAE [122]; however, studies in $MBP^{-/-}$ mice demonstrated that the loss of MBP allows the development of a high functional avidity MBP-specific $CD4^+$ T cell repertoire [123]. Moreover, the degree of MBP-specific TCR transgenic thymocyte deletion was shown to be dependent on the amount of MBP gene expression, which directly impacted the size and functional avidity of the responding repertoire [124]. Moreover, the degree of tolerance to MBP is directly related to the affinity of MBP antigen to MHC II [125], thus, low affinity MBP:MHC II interactions allowed the escape of low affinity MBP-specific CD4+ T cells [126, 127]. However, tolerance to MBP appears to be more complex than mere self-antigen expression in thymus. In fact, it seems that central as well as peripheral tolerance to MBP is due to presentation of exogenous MBP antigen, leading to progressive elimination of MBP-specific $CD4^+$ T cells with age [124].

Finally, the myelin-specific antigen MOG shows how complicated the issues around central tolerance may be, as MOG protein has not been detected in the thymus although, at the very least, MOG mRNA is expressed in the thymus of mice [117, 118] and humans [120, 121, 128]. Several studies have shown that MOG_{35-55} $CD4^+$ T cell responses are more potent and encephalitogenic when derived from $MOG^{-/-}$ mice on the 129 or B6 backgrounds [129]. While other studies have indicated that MOG_{35-55} $CD4^+$ T cells generated in $MOG^{-/-}$ mice showed no difference in MOG_{35-55} responsiveness or in the TCR repertoire [118, 130]. Regardless of this discrepancy, some data suggests that tolerance to MOG is partially

incomplete, as exogenous delivery or expression of MOG_{35-55} in the periphery inhibits $CD4^+$ T cell responsiveness to MOG_{35-55} and can protect against EAE [130, 131]. Additionally, two different MOG_{35-55}-specific TCR transgenic mice (termed 2D2 and 13A) have been shown to develop mature MOG_{35-55} $CD4^+$ T cells [132, 133] and no defect in transgenic T cell development was observed when 2D2 mice were crossed to a $MOG^{-/-}$ background [134]. Based on these observations, it is possible that high affinity MOG_{35-55} $CD4^+$ T cells are purged from the mature T cell repertoire, but a substantial portion of $CD4^+$ T cells with low affinity for MOG_{35-55} likely survive thymic deletion. These data implies that autoreactive T cells must possess an overall lower affinity for self-antigens compared to that of pathogen-specific T cells for their antigens and this has been corroborated by a number of different studies.

For instance, in experiments examining the 3D affinity of various TCR have suggested that myelin-specific TCRs [135-137] have lower affinities than viral-specific TCRs [137, 138]. Comparisons of the TCRs from two different MBP_{Ac-11} (IA^u-restricted) transgenic mice indicated that the 172.10 clone had a much higher affinity than that of its 1934.4 counterpart [135] and mice transgenic for the 172.10 clone develop spontaneous EAE at a much higher rate than those bearing the 1934.4 transgene [125, 139-141]. This suggests that an increased affinity for myelin antigen may be associated with increased encephalitogenicity.

As mentioned previously, the development of tetramers has led to the ability to gauge TCR affinity via the extent of pMHC tetramer staining, which can varies dramatically across different $CD4^+$ T cell specificities. On the one hand, pMHC II tetramers have been found to identify most pathogen-specific $CD4^+$ T cells, including those specific for influenza [142-

145], HSV-2 [146], *B. burdgorferi* [147], and *S. typhimurium* [148]. In contrast, many studies have reported poor pMHC II tetramer detection of autoreactive CD4+ T cells in various disease models, including rheumatoid arthritis [149, 150], relapsing polychondritis [151], type 1 diabetes [152, 153], and EAE [154-159].

Additionally, many studies have compared the affinities of self- and foreign-antigen specific T cells by functional responsiveness. For instance, one study has shown that autoreactive $CD4^+$ T cell hybridomas displayed a wider range of affinities for self-antigen compared to pathogen-specific hybridomas, yet the autoreactive hybridomas possessed an overall lower functional avidity [153]. Other studies in $CD8^+$ T cells have demonstrated lower functional avidities of self-reactive $CD8^+$ T cells versus those specific for pathogen [106, 160, 161]. Many studies have demonstrated the presence of both high and low functional avidity (defined as $EC_{50} < 1\mu M$ and $> 1\ \mu M$, respectively) populations in both murine models of autoimmunity [17, 18, 162] and in human disease [163-165]. Interestingly, the functional avidity of myelin-reactive CD4+ T cells was not found to differ in the periphery (blood and spleen) and the CNS, nor did the functional avidity change over time [166]. Overall, these studies demonstrate that not only is deletion of self-reactive T cells incomplete, that but self-reactive T cells appear to demonstrate an altered pattern of avidity maturation in which low affinity clonotypes dominate the polyclonal response compared to foreign specific T cells.

CD4 T Cells and Infection

T cells are necessary for the initiation and maintenance of effective immune responses to rid the body of foreign pathogens. The crucial role of T cells in fighting viral

infections has been demonstrated in the lymphocytic choriomeningitis virus (LCMV) model. LCMV is a non-cytopathic RNA virus that gains entry to cells via binding of the viral glycoprotein (GP) to α-dystroglycan on the cell surface. Two of the most common LCMV strains, known as Armstrong and clone 13, are used to study acute and chronic viral infections, respectively. Interestingly, the virus strains differ in only 2 amino acids, one in the polymerase gene, and one in the glycoprotein gene [167]. The resulting mutations lead to an increased predilection of clone 13 for infecting macrophages and higher virus yields, which consequently leads to chronic infection [168].

$CD8^+$ cytotoxic lymphocytes (CTLs) can clear LCMV alone [169], but $CD4^+$ T cells cannot [169, 170]. Regardless, $CD4^+$ T cells do play a vital role in controlling and ultimately ridding the body of infection. $CD4^+$ T cells are important in the prevention of chronic infection [169, 171-175] and for LCMV-specific CTL memory in both acute [176-178] and chronic infections [175, 179]. LCMV-specific $CD4^+$ T cells can also aid in the clearance of virus by induction of neutralizing antibodies [179] and by mediating their own cytotoxic effects [180-182]. Although a number of $CD4^+$ epitopes have been identified, the glycoprotein $(GP)_{61-80}$ epitope is by far the most immunodominant epitope in B6 mice infected with either the Armstrong and clone 13 strains of LCMV [183-186]. The nucleoprotein $NP_{309-328}$ epitope is a minimal secondary epitope in the Armstrong, but not the clone 13 model [183-186].

CD4 T Cells and Autoimmunity

Although T cells are necessary for the initiation and maintenance of effective immune responses to exterminate foreign pathogens, they have also been implicated in initiating and

maintaining autoimmunity. A variety of autoimmune diseases have been demonstrated to be mediated by T cells specific for self-antigens, including type I diabetes, rheumatoid arthritis, and multiple sclerosis. Multiple sclerosis is a chronic neurological disease resulting from an inflammatory process directed against the central nervous system (CNS). The disease process is thought to be mediated primarily by autoreactive $CD4^+$ T cells that target oligodendrocytes, the myelin-producing cells of the CNS [187]. The resulting demyelination and multifocal lesions result in the symptoms of MS, which can include vision loss, sensory disturbances, limb weakness, and gait instability.

Several autoimmune disorders have been studied using animal models of disease, including the murine model experimental autoimmune encephalomyelitis, or EAE, which mimics the human disease multiple sclerosis [188-191]. EAE is initiated in disease susceptible animals with various myelin antigens. Three myelin antigens, proteolipid protein (PLP) [192], myelin oligodenrocyte glycoprotein (MOG) [193], or myelin basic protein (MBP) [194] have previously been mentioned and are the most commonly used in EAE. Disease susceptibility is primarily determined by the MHC II allele, highlighting the primary role of $CD4^+$ T cells in the disease process. EAE presents as an ascending paralysis, which is typically scored using a numerical scale of 1) tail paralysis or hind limb ataxia; 2) tail paralysis and hind limb ataxia; 3) single hind limb paralysis; 4) complete hind limb paralysis or inability to right; and 5) moribund. Each model has different phenotypes (e.g. acute, chronic, relapsing-remitting), which are useful for studying the heterogeneous types of MS.

EAE can be induced in two different ways: active immunization or passive transfer of the disease. With active immunization, the disease is induced in susceptible strains of mice upon immunization with a myelin peptide emulsified in complete Freund's adjuvant (CFA), which

contains heat-inactivated *M. tuberculosis*, and injected into the hind flank of the mouse. In addition to peptide, pertussis toxin is also administered to the animals. Pertussis toxin affects G-protein coupled receptors on T cells, which alters their migration and trafficking patterns, as well as possibly permeabilizing the blood-brain barrier for easier entry into the central nervous system, or CNS. Passive transfer involves stimulation and expansion of myelin-reactive $CD4^+$ T cells *in vitro*, followed by adoptive transfer of these cells (injected intravenously) into naïve syngeneic hosts. With adoptive transfer, the peripheral activation of $CD4^+$ T cells is unnecessary EAE and the previously activated, myelin-specific cells can traffic directly to the CNS. Classic EAE involves extensive myelin-reactive T cell infiltration of the meninges and the spinal cord, with little involvement of the brain parenchyma [195]. *De novo* CNS myelin antigen processing is required for EAE induction, as defects in MHC II antigen processing prevents the induction of active EAE, but myelin-reactive $CD4^+$ T cells generated in this context can passively transfer EAE in wild-type hosts [196, 197].

Once antigen-specific T cells infiltrate the CNS, the inflammatory reaction can also be characterized by the activation of resident microglia and an accumulation of macrophages from the periphery [198]. Together with the $CD4^+$ T cells, these cells secrete inflammatory mediators and induce tissue damage in the CNS [199]. The resulting inflammatory lesions and destruction of oligodendrocyte populations, which produce the myelin insulating nerves, triggers many disease symptoms, culminating in paralysis due to the disruption in the electrical impulses sent down the axons [198, 200-202].

In addition to damage to the myelin sheath, extensive axonal and neuronal injury has been reported in MS and EAE [203]. Targets that have been implicated in this pathway

include peptides derived from neurofilaments, which are cytoskeletal proteins found within the peripheral nervous system (PNS) and CNS. For instance, T cells specific for the light chain form of neurofilaments (termed NF-L) have been reported in MS patients [204] and NF-L can induce EAE in a subset of mice [205]. Additionally, autoantibodies to the neurofilament medium chain (NF-M) have also been detected in MS patients [206] and, while immunization with NF-M does not induce active EAE in mice, it was recently reported that a subset of MOG_{35-55}-reactive $CD4^+$ T cells can cross-react with NF-M_{15-35} antigen during EAE [134].

Chapter 2: Low 2-Dimensional CD4 T Cell Receptor Affinity for Myelin Sets in Motion Delayed Response Kinetics

Published in *PLoS One:* Rosenthal KM , Edwards LJ , Sabatino JJ Jr, Hood JD , Wasserman HA , Zhu , Evavold BD. (2012) PLoS ONE 7(3): e32562. doi:10.1371/journal.pone.0032562

Abstract

T cells recognizing self-peptides that mediate autoimmune disease and those that are responsible for efficacious immunity against pathogens may differ in affinity for antigen due to central and peripheral tolerance mechanisms. Here we utilize prototypical self-reactive (myelin) and viral-specific (LCMV) T cells from T cell receptor (TCR) transgenic mice (2D2 and SMARTA, respectively) to explore affinity differences. The T cells responsive to virus possessed >10,000 fold higher 2D affinity as compared to the self-reactive T cells. Despite their dramatically lower affinity for their cognate ligand, 2D2 T cells respond with complete, albeit delayed, activation (proliferation and cytokine production). SMARTA activation occurs rapidly, achieving peak phosphorylation of p38 (1 minute), Erk (30 minutes), and Jun (3 hours) as well as CD69 and CD25 upregulation (3 and 6 hours, respectively), with a corresponding early initiation of proliferation. 2D2 stimulation with MOG results in altered signaling – no phospho-Erk or phospho-p38 accumulation, significantly delayed activation kinetics of Jun (12 hours), and delayed but sustained SHP-1 activity – as well as delayed CD69 and CD25 expression (12-24 hours), and slow initiation of proliferation. This delay was not intrinsic to the 2D2 T cells, as a more potent antigen with >100-fold increased 2D affinity restored rapid response kinetics in line with those identified for the viral antigen. Taken together, these data demonstrate that time can offset low TCR affinity to attain full

activation and suggest a mechanism by which low affinity T cells participate in autoimmune disease.

Introduction

Every T cell must be able to differentiate between high and low potency ligands to generate the appropriate response after T cell receptor (TCR) binding [3, 4, 207], as antigens vary in their capacity to stimulate a given T cell. The library of peptides for a specific TCR includes agonists, partial or weak agonists, and antagonists [6]. In the case of CD8+ OT-I T cells, these ligands can span a >1,000 fold range in effective 2D affinity [28]. The TCR can thus integrate the strength of ligand binding and impart the appropriate response, ranging from full activation to anergy to antagonism [7, 8].

Generally, self-reactive T cells mediating autoimmune disease are thought to be lower affinity than pathogen-specific T cells due to various tolerance mechanisms [208, 209]. Recently, we utilized a micropipette-based assay (where both the TCR and pMHC are membrane-bound) to assess the effective 2D affinity of T cells during various immune responses [36]. This report identified participation of lower affinity T cells in both pathogenic and autoimmune responses, although stimulation with a myelin-specific self-peptide important in murine EAE induction (MOG_{35-55}) results in a greater frequency of tetramer negative low affinity T cells as compared to a pathogen-derived epitope in murine LCMV (gp_{61-80}) [36].

When shaping an immune response, both the TCR affinity and duration of antigen encounter play roles in directing the outcome of T cell activation. During an acute infection, the presence of foreign antigen is transient and allows for robust T cell expansion followed

by contraction to the memory state as pathogen is cleared [210]. During chronic infections, pathogenic antigens can be present for an extended time, which can lead to deletion or exhaustion of the T cells [211-213]. Self-peptide antigens are constantly produced and presented [214, 215], yet intriguingly, T cells that propagate autoimmune disease can seemingly avoid exhaustion or regulation.

As a first step to understand the apparent differences in T cell activation, we utilized LCMV specific (SMARTA) [170, 183] and myelin-reactive (2D2) [132] $CD4^+$ TCR transgenic mouse models and identified a >10,000 fold lower effective 2D affinity in 2D2 T cells that resulted in a substantial decrease in functional sensitivity to myelin and a complete absence of peptide:MHC class II tetramer reactivity. In spite of this dramatically decreased affinity for cognate ligand, 2D2 T cells successfully proliferated and produced cytokines, although with a temporal delay in the T cell response that manifested as an absence of detectable phosphorylated Erk and p38 and significantly delayed activation kinetics of SHP-1, Jun, CD69, and CD25. In contrast, activation occurred rapidly in the pathogen specific T cells. The delay in response to myelin was not intrinsic to the self-reactive T cells, as the 2D2 response to a more potent antigen with a >100-fold increase in effective 2D affinity gave rapid response kinetics. Moreover, the low-affinity MOG peptide must be displayed for a protracted time to initiate a robust response. Our data demonstrate that extended time of antigen presentation can compensate for lower TCR affinity for self and allow for accumulation of signals and eventual full activation of $CD4^+$ T cells.

Results

The relative 2D affinity of CD4$^+$ T cells. Using a micropipette-based binding assay the effective two-dimensional (2D) affinity, which measures receptor:ligand binding in the context of a cell membrane, can define differences in affinity of TCR:pMHC with greater resolution than other measurements [28, 36]. In this assay, a single T cell is brought in and out of contact with a red blood cell (RBC) coated with pMHC class II monomers to yield an adhesion probability (the percentage of adhesions out of the total number of contacts). The adhesion probability allows for derivation of the effective 2D affinity of the TCR:pMHC [28, 34, 102]. SMARTA TCR interacts with gp_{66-77}:I-A^b with high affinity (Figure 2.1a). In contrast, adhesion of 2D2 CD4$^+$ T cells to RBCs coated with >2000 molecules/um^2 MOG_{38-49}:I-A^b was still negligible, indicating a $<10^{-8}$ μm^4 affinity (Figure 2.1a). It has recently been shown that, in addition to MOG, 2D2 T cells can respond better to a CNS self-epitope from the neurofilament medium chain (NFM) [134]. NFM_{15-35} contains 6 out of 9 identical amino acid residues to the core epitope of MOG_{35-55} and conserves all of the recognized TCR contact residues [134]. In contrast to MOG_{35-55}, the adhesion between 2D2 TCR and NFM_{18-30}:I-A^b is measurable in the micropipette assay and results in an affinity approximately 80-fold lower than that of SMARTA T cells ($9.22x10^{-6}$ μm^4 compared to $7.32x10^{-4}$ μm^4) (Figure 2.1a).

Another gauge of TCR affinity uses the extent of peptide:MHC II tetramer staining detected by flow cytometry [216-218]. Previously, pMHC II tetramers were shown to detect MOG_{35-55} CD4$^+$ T cells in EAE [36, 157, 158], but on detailed analysis, MOG:I-A^b tetramer failed to identify most of the MOG-specific T cells [36]. In accordance with the undetectable 2D affinity of MOG:I-A^b interaction with 2D2 TCR, there was a lack of MOG:I-A^b tetramer

staining in 2D2 T cells, whereas all of the SMARTA T cells were positive for tetramer staining (Figure 2.1b).

Peptides, particularly longer peptides, may bind to MHC class II molecules in multiple 9-mer epitopes, or registers [219-223]. To define the 2D2 T cells' response to the core epitope engineered into the MOG_{38-49}:I-A^b monomer, we assessed the proliferative capacity of 2D2 T cells to a panel of overlapping, truncated peptides from the MOG_{35-55} sequence (Table I). This assay revealed that the core epitope for 2D2 T cells is MOG_{39-48}, which is the basis of the MOG:I-A^b construct (Table I), and demonstrated that the absence of MOG:I-A^b reactivity in tetramers or by 2D micropipette analysis was not due to the register of the antigenic epitope.

Functional avidity of $CD4^+$ T cells. T cell functional avidity, defined by the amount of antigen needed for half-maximal response, is often used as a surrogate of TCR affinity for expressing the potency of an antigen [17, 18, 224, 225]. SMARTA splenocytes showed some proliferation at the lowest dose of gp61 tested (30 pM) while 2D2 splenocytes did not respond until much higher doses of MOG were reached (0.3-1 μM) (Figure 2.2a). Indeed, the EC_{50} of 2D2 T cells for MOG was more than 3,000 times higher than that of SMARTA T cells (3.3 μM compared to 0.001 μM) (Figure 2.2b). Although MOG:2D2 binding is too weak to be detected by either flow cytometry or 2D micropipette analysis, stimulation with this self-peptide still resulted in activation of 2D2 T cells. 2D2 cells responded to lower doses of NFM (starting at 1 nM) and by EC_{50} required approximately 10-fold more peptide than that of SMARTA cells (~0.01 vs 0.001 μM) and 100-fold less peptide than that of 2D2 cells stimulated with MOG (~3 μM) (Figure 2.2a and c). Additionally, antigen-dependent cytokine production, assessed by IL-2 production, paralleled the proliferative capacity of the cells

(Figure 2.2d). A similar trend was observed with IFN-γ –in that 2D2:MOG stimulation resulted in less cytokine production than either SMARTA:gp61 or 2D2:NFM across the range of antigen concentrations (Figure 2.2e). Some have previously reported that low affinity T cell stimulation can result in cytokine skewing to a Th2 response [226]; however further cytokine analysis revealed 2D2:MOG produced lower amounts of IL-2 and IFN-γ than either SMARTA:gp61 or 2D2:NFM ($P = 0.0018$ and $P = 0.005$, respectively) with only minimal levels of IL-4 produced regardless of the antigen (Figure 2.2f). Taken together, this suggests that although 2D2:MOG results in less cytokine production, there is no evidence of phenotypic skewing.

Thus, 2D2 T cells have a relatively high functional avidity for NFM, with proliferation and IL-2 production closer to the corresponding functional avidity of SMARTA cells than to 2D2 cells for MOG (Figure 2.2). The hierarchy of the ligands shows that SMARTA:gp61, a TCR:foreign antigen interaction, is the most potent, followed by 2D2:NFM and, finally, 2D2:MOG. Overall, the effective 2D affinities of these $CD4^+$ T cell clones for their ligand correlates to some extent with their functional avidity (Figure 2.2c).

Altered signaling events in low affinity $CD4^+$ T cells. Erk plays a key role in positive T cell signaling events [69, 227], although it has been noted that in human cells Erk signaling can contribute to limitation of naïve T cell activation [228]. To explore the impact that TCR affinity has on T cell activation, phosphorylation of Erk in the MAP kinase pathway was analyzed. Phosphorylation events were visualized by flow cytometry to allow for detection and isolation of $CD4^+$ T cells from other cell types [229]. Upon activation with gp61, SMARTA $CD4^+$ T cells showed rapid phosphorylation of Erk1/2 by 5 min, peaking at 30 min, and remaining phosphorylated through 6 h (Figure 2.3a and c). Conversely, stimulation

of 2D2 $CD4^+$ T cells with MOG showed no appreciable accumulation of pErk at any time, from 5 min through 24 hours (Figure 2.3a and c). Activation of 2D2 $CD4^+$ T cells with the higher 2D affinity NFM ligand resulted in up-regulation of pErk by 5 to 15 min, peaking at 60 min before slowly declining by 24 h (Figure 2.3a and c). The NFM response confirms that the 2D2 cells are not intrinsically deficient in their signaling capacity. This data highlights that, despite proliferative and cytokine responses (Figure 2.2), there is a dramatic difference in the initial signaling program of high (SMARTA and NFM) versus low (MOG) affinity agonists.

Although there is no notable accumulation of phosphorylated Erk upon 2D2 TCR ligation with MOG, T cell stimulation with low affinity ligands can allow highly reversible events, such as phosphorylation of Erk, to revert quickly back to the basal state, thus precluding detection by biochemical methods [30]. To determine if Erk phosphorylation plays a role in 2D2 T cell activation with MOG antigen, we assessed proliferation in the presence of the MEK1/2 inhibitor, U0126. At 10 μM, this inhibitor specifically targets MEK1/2 to inhibit the activation of Erk1/2 without acting on p38 MAPK, JNK, protein kinase C, or other pathways [230]. Treating 2D2 T cells with 10 μM U0126 prior to MOG stimulation resulted in a marked decrease in the amount of 2D2 proliferation (Figure 2.3e). Despite the lack of detectable pErk in 2D2:MOG cells, these results demonstrate that this pathway is nonetheless utilized for activation.

Another mediator of T cell activation, c-Jun, is an important target of Erk and the MAPK intermediate, JNK [231-233]. To further explore the signaling profile during low potency peptide interactions in self-reactive T cells, we assessed the kinetics of c-Jun phosphorylation [30, 234]. With either SMARTA:gp61 or 2D2 triggered by NFM, c-Jun is

phosphorylated within 1 hour, while phosphorylated c-Jun (p-c-Jun) is not detected in 2D2 $CD4^+$ T cells stimulated with MOG until 3-6 hours later (Figure 2.3b and d). Interestingly, the magnitude of the response also correlates with the effective 2D affinity of the ligands, as more cells express both pErk and p-c-Jun in SMARTA:gp61, followed by 2D2:NFM and finally 2D2:MOG.

To further define the delayed signal transduction in T cells with a low affinity for antigen, we assessed the kinetics of p38 MAPK phosphorylation, which can influence differentiation of Th1 cells, IFN-γ production and possibly proliferation [90, 235]. Analogous to the phosphorylation kinetics of Erk, phosphorylation of p38 MAPK was similar in SMARTA:gp61 and 2D2:NFM but absent in 2D2:MOG (Figure 2.4a and b). The percent of maximal phosphorylation of either Erk or p38 is shown to compare the kinetics of the response for each positive signaling mediator. For both SMARTA:gp61 and 2D2:NFM, phosphorylation of p38 peaked by 5 minutes and declined to baseline by 30 to 60 minutes (Figure 4b) while phosphorylation of Erk peaked by 30 to 60 minutes before declining (Figure 4a and Figure 3a and c). However, with 2D2:MOG, there is no apparent accumulation of phosphorylated p38 throughout the time course (Figure 2.4b), similar to the lack of detectable pErk accumulation (Figure 2.3 and 2.4a).

Previously, we have shown that the negative regulator SHP-1 plays a role in controlling an autoimmune response to MOG [102], and in CD8+ T cells that the peak of SHP-1 activity occurs at 1 min in response to antigen [103]. Here, the kinetics of SHP-1 activity was analyzed following T cell activation. The phosphatase assay employed allows for determination of the amount of free phosphate released by immunoprecipitated SHP-1 from cell lysates using a SHP-1 specific phosphorylated substrate. Both SMARTA:gp61 and

2D2:NFM have up-regulated SHP-1 activity at 1 minute (Figure 2.4c). Strikingly, the peak of SHP-1 activity was delayed in 2D2 MOG stimulation (15 minutes) as compared to SMARTA:gp61 and 2D2:NFM (Figure 2.4c). In addition to the delay in peak activation, SHP-1 remains active in 2D2 MOG stimulation throughout the 30-minute time course. This data shows that multiple detectable signaling pathways are delayed or undetectable in 2D2:MOG activation, including both positive and negative feedback loops.

Expression of activation markers in low affinity CD4$^+$ T cells. This delay with signaling events could eventually translate to delays in downstream responses. We analyzed the expression of CD69, a T cell activation marker downstream of the Ras/Erk MAP kinase pathway [236] by flow cytometry and found that 2D2 T cells stimulated with MOG showed a delay in CD69 up-regulation (Figure 2.5a). Both SMARTA:gp61 and 2D2:NFM stimulation resulted in complete up-regulation of CD69 by 4 hours whereas stimulation with MOG did not completely up-regulate CD69 until 24 hours (Figure 2.5a). The magnitude of the CD69 response was comparable at high doses of peptide at 24 hours with each peptide, but 2D2:MOG needed 10- to 100- fold more antigen for maximal response (Figure 2.5c).

Similarly, the high affinity IL-2 receptor and marker of T cell activation, CD25, was completely up regulated by 24 hours for all peptides, yet MOG displayed a significant delay (Figure 2.5b and d). In SMARTA cells, activation with gp_{61-80} resulted in peak CD25 expression by 6 hours (Figure 2.5b). This was similar to the kinetics of CD25 expression in 2D2 cells stimulated with NFM; however, 2D2:MOG did not attain peak expression of CD25 until much later (24 hours) (Figure 2.5b). Analogous to the delay in MAP kinase signaling, up-regulation of activation markers was delayed in 2D2:MOG as compared to 2D2:NFM or SMARTA:gp61.

Delayed initiation of proliferation in low affinity $CD4^+$ T cells. To extend our understanding of how low affinity interactions may be time-dependent, the kinetics of cellular proliferation was analyzed by CFSE dilution assay. In this assay there was a delay in proliferation in 2D2 T cells with MOG as compared to either SMARTA:gp61 or 2D2:NFM (Figure 2.6a). At 48 hours, the majority of SMARTA:gp61 and 2D2:NFM $CD4^+$ T cells had entered cycle and undergone between one and two divisions; however, most of the $CD4^+$ T cells in MOG activation remained undivided (Figure 2.6a). By 72 hours, all of the cells that entered cycle underwent approximately the same number of divisions regardless of the initiating peptide (Figure 2.6a). By day 3, there was a noticeable increase over baseline in the cell numbers of both SMARTA:gp61 and 2D2:NFM but not in 2D2:MOG, probably due to the increased fraction of cells entering cycle (Figure 2.6b). Interestingly, the total number of live $CD4^+$ T cells after 7 days in culture was similar, in that SMARTA:gp61 ($3.7x10^6 \pm 4.9x10^5$ cells) and 2D2:NFM ($3.2x10^6 \pm 4.1x10^5$ cells) have a slightly greater, though not statistically significant, number of cells than 2D2:MOG ($2.\ 6x10^6 \pm 2.3x10^5$ cells) (Figure 2.6b). These data demonstrate that although the initiation of cellular proliferation is delayed in 2D2 T cells stimulated with MOG, the cells that do eventually enter the proliferative cycle retain the ability to divide and can eventually reach similar cell numbers of higher affinity TCRs.

Cell division in T cells has been shown to be programmed on presentation of antigen by APCs, requiring as little as a few hours to trigger a response [55, 56, 237]. Stimulation of 2D2 cells with NFM, but not MOG, allows for complete up-regulation of CD69 and CD25 by approximately 6 hours, whereas both peptides up-regulate activation markers by 24 hours (Figure 2.5a and b). To explore the importance of the time of antigen presentation as it relates

to the apparent delay in MOG:2D2, the extent of activation was examined at 24 hours after various periods of T cell to APC contact time. $CD4^+$ T cells were allowed 3, 6, 12 or 24 hours of contact time before analysis of CD69 and CD25 expression at 24 hrs. In 2D2:NFM, nearly half of the cells up-regulated CD69 and CD25 within 3 hours of APC contact time; however it took at least 12 hours for 2D2:MOG to up-regulate the activation markers to the same extent (Figure 2.6c). Within 6 to 12 hours of contact time, nearly all of NFM:2D2 $CD4^+$ T cells up regulated CD69 and CD25. At 24 hours of APC contact time, both NFM and MOG allowed near complete activation of the cells (Figure 2.6c). This data shows that 2D2 T cells stimulated with a low affinity peptide need an extended period of time in contact with pMHC to reach maximal activation in comparison to higher affinity interactions.

Discussion

It is generally assumed that auto-reactive T cells are of lower affinity than T cells specific for foreign antigen. Our lab has shown that in responses to a foreign antigen, gp61, or a self-peptide, MOG, the polyclonal T cell repertoire encompasses a similar wide breadth of affinities with the response to MOG including more low affinity T cell clones [36]. To further understand the relationship of affinity to T cell response, we examined viral specific and self-reactive TCR transgenic T cells (SMARTA and 2D2, respectively). We found that 2D2 T cells were of low affinity, compared to SMARTA T cells, and failed to be detected in the 2D micropipette assay or to react with a peptide-specific tetramer by flow cytometry. Surprisingly given their low affinity, we and many others have employed MOG-activated 2D2 T cells to induce EAE, indicating low TCR affinity for antigen does not preclude autoimmune disease [102, 132, 134, 238-240].

An alternative explanation for the lack of tetramer staining and 2D binding in 2D2 cells could relate to the findings that peptides presented by MHC class II, especially murine I-A^b, can bind the MHC in different registers through the use of different MHC anchor residues [219-222]. Epitope mapping in 2D2 T cells revealed that the core encompasses MOG_{39-48}, the epitope engineered in the MOG:I-A^b tetramer (Table I). Additionally, we have found that the MOG:I-A^b monomer identifies the majority (>70%) of the $CD4^+$ T cells in the CNS at the peak of EAE [36]. The lack of tetramer staining and undetectable 2D affinity is unlikely due to an alternative register recognized by 2D2 T cells, but instead caused by the considerably lower affinity of this TCR to MOG.

The low affinity of 2D2 T cells for MOG led to a qualitative difference in the kinetics of T cell activation (Figure 2.3, 2.4, 2.5, 2.6), apparent as a delay in signaling, up-regulation of activation markers, and subsequent proliferation. However, analysis at later time points show that the T cells can compensate for this apparent early defect (Figure 2.6). This indicates that, under appropriate conditions that include continued access to antigen (Figure 2.6), time can offset low affinity for a ligand and allow for a slow accumulation of signaling intermediates that eventually lead to a complete response. This idea of qualitative as opposed to quantitative differences in peptide antigens as it relates to T cell responses has also been reported elsewhere and suggests that following CD4 TCR ligation, signaling pathways can diverge to allow for various functional outcomes of demonstrable TCR:antigen interactions [30, 241, 242].

There has been much interest in determining the early signaling events in T cells after TCR ligation; however, the kinetics of the various signaling intermediaries involved and how timing affects the net outcome of signaling is still under investigation. The timing of signal propagation through both positive and negative mediators may play a key role in modulating T cell activation with low potency ligands. In this study we aimed to assess a few of the many potential differences in activation kinetics of a high affinity viral-specific T cell from SMARTA mice to a low affinity self-reactive T cell from 2D2 mice. Mathematical modeling of TCR signal transduction theorizes that positive and negative feedback loops activated during T cell interactions with antigen presenting cells allow for discrimination between a range of ligand affinities [69, 243]. In fact, one model proposes a balance between the positive signals driven by Erk activation and negative signals driven by SHP-1 to regulate T cell activation [69]. Here, we show that even with undetectable Erk or p38 phosphorylation

and delayed, but sustained SHP-1 activity, self-reactive T cells that encounter low affinity ligands can still undergo full activation. This suggests that these feedback mechanisms may be more complicated than initially described and that a smoldering positive signal may be able to drive T cell activation under the appropriate conditions.

Importantly, in addition to differences in positive signaling, we show that there is delayed, but sustained up-regulation of SHP-1 activity in 2D2:MOG interactions, but not in NFM (or SMARTA:gp61) stimulation (Figure 2.4). We, and others, have shown an important role for SHP-1 in regulating responses to low potency antigens [77, 102, 103, 244]. Specifically, we have shown that an LCMV mutant epitope allowing for viral escape induces delayed SHP-1 activation in CD8+ T cells [103]. In these experiments, the quick burst of SHP-1 activity in agonist stimulation is not unexpected, as this denotes the initial burst of signaling initiated by a potent pMHC complex. The quick recovery of SHP-1 to an inactive state during strong peptide interactions (SMARTA:gp61 and 2D2:NFM) supports the licensing of that cell to undergo activation and proliferation. The delayed but sustained activation of SHP-1 following MOG stimulation indicates an altered signaling profile in these cells and corresponds with the lower functional avidity of 2D2 T cells for MOG. We suggest that the interplay between delayed or negligible levels of positive signaling (c-Jun or Erk and p38, respectively) and the sustained negative activity of SHP-1 synergistically affects the outcome of T cell signaling in low affinity TCR interactions; however, given adequate time cells are able to overcome this delay in activation to divide and survive.

The absence of measured MOG reactivity using specific tetramer or the micropipette analysis may very well indicate the importance of time for these affinity measures. Delayed on-rates or very rapid off-rates could affect the ultimate outcome of T cell stimulation.

However, with the current assays it is difficult to distinguish these possibilities. Peptide:MHC tetramers work by increasing the avidity of the TCR:pMHC by allowing an improved chance of interaction between TCR and the multimeric structure [148, 245], which would be dependent on the binding kinetics [36]. Time is similarly important for the micropipette measurements as low affinity translates to a low binding probability [28, 36]. Longer antigen exposure time equates to a larger total number of TCR:pMHC encounters, hence a larger cumulative number of TCR bonds, which increases the binding probability [37]. Previously, we have shown that 2D2 T cells require high expression levels of TCR for response, supporting a requirement of an increased number of TCR bonds [102]. Thus, the persistent exposure of an autoimmune antigen may be able to compensate for its low affinity to yield a cumulative number of TCR bonds sufficient for T cell activation.

We have reported that the average TCR affinity for a polyclonal MOG-specific population is on the order of 10^{-5} μm^4 [36]. This implies that 2D2 cells represent the lowest affinity T cells in the spectrum of polyclonal TCR affinities during an autoimmune response. When compared to the CD8+ OT-I system, the 2D2 affinity for MOG is even below the level reported for the ovalbumin TCR antagonists [28]. This indicates that 2D2 T cells are of very low affinity and raises the issue of how T cells with such low affinity are relevant to T cell responses. Normally, during an acute infection the foreign antigen is transiently expressed for a limited amount of time [113, 212]. Self-antigens on the other hand are constantly available to be presented to T cells, potentially extending the length of time for triggering of the T cell [214, 215]. Taken together, this data implies that analysis of T cell activation over a short time frame may not allow for a full understanding of agonistic properties of antigens,

specifically in autoreactive T cells that are able to encounter low affinity peptides over extended periods of time.

Interestingly, the importance of time and the availability of antigen in the ability of T cells to reach the thresholds for signaling events highlights the fact that autoreactive T cells may be able to use time, through either sustained or short repeated engagements, to achieve a response [41, 42, 47]. Upon multiple instances of stimulation, high affinity T cells undergo exhaustion as one method to limit damage to the host in response to ineffective clearance of an infection [246, 247]. Potentially, the smoldering T cell response observed during autoimmune disease may result, at least in part, from the activation of very low affinity T cells that can escape tolerance mechanisms given sufficient time and access to self-antigens.

Materials and Methods

Transgenic Mice

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. MOG35-55 specific TCR transgenic 2D2 mice (Jackson Labs, C57BL/6-Tg(Tcra2D2,Tcrb2D2)1Kuch/J) and gp61-80 specific TCR transgenic SMARTA mice [17,18] were bred, housed and used with specific approval from the Institutional Animal Care and Use Committee-approved protocol of the Emory University Department of Animal Resources facility (IUCAC Number: DAR-2000870-061414). All mice were used for experiments at 6-8 weeks of age.

Peptides and Reagents

LCMV gp61-80 (GLNGPDIYKGVYQFKSVEFD) and mouse NFM15-35 (RRVTETRSSFSRVSGSPSSGF) and MOG35-55 (MEVGWYRSPFSRVVHLYRNGK) were synthesized in-house using F-moc chemistry on the Prelude peptide synthesizer (Protein Technologies). Culture medium consisted of RPMI 1640 medium (Mediatech) supplemented with 10% FBS (HyClone), 2 mM L-glutamine (Mediatech), 0.01 M HEPES buffer (Mediatech), 100 µg/ml gentamicin (Mediatech), and $2x10^{-5}$ M 2-ME (Sigma-Aldrich). Oxidation buffer consisted of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 0.5% Igepal, 1 mM Na_3VO_4, and 1/100 protease inhibitor cocktail I (Calbiochem) to inhibit degradation of cellular proteins following lysis of the cells.

2D TCR affinity analysis by micropipette adhesion frequency assay

Human red blood cells (RBCs) were isolated from healthy volunteers at the Georgia Institute of Technology in accordance with specific approval from the Georgia Institute of Technology Institutional Review Board (protocol number: H07343) and prepared as previously described [5]. In accordance with ethical guidelines, written and informed consent was obtained from all anonymous volunteers prior to blood collection. RBCs were coated with various concentrations of biotin-X-NHS (Calbiochem), followed by 0.5 mg/ml streptavidin (Pierce) and then 1-2 μg of pMHC II monomer. The MOG_{38-49}:I-A^b, NF-M_{18-30}:I-A^b, GP_{66-77}:I-A^b monomers were provided by the NIAID Tetramer Core Facility at Emory University. The pMHC-coated RBCs were stained with anti-MHC II FITC Ab (M5/114.15.2; BioLegend) and T cells were stained with anti-TCRβ FITC Ab (H57-597; eBioscience). The site densities of I-A^b monomers per RBC and TCRs per T cell were derived using anti-FITC MHC II, anti-TCR antibodies, and FITC MESF beads (Bangs Labs) and normalized for the F/P ratios of the antibodies.

The details of the micropipette adhesion frequency assay have been described [28, 34]. Briefly, the adhesion was measured following contact of a single T cell and pMHC-coated RBC on opposing micropipettes. At the end of the contact time, the T cell was retracted and the presence of adhesion (indicating TCR:pMHC ligation) was observed microscopically by elongation of the RBC membrane. The adhesion frequency (P_a) was calculated by performing the contact-retraction 50 times per T cell-RBC pair. A 5 second contact time was chosen in all experiments because the P_a had reached equilibrium and remained constant despite further increase in contact time. The effective 2D affinity (A_cK_a) was calculated using the average P_a according to the following equation:

$$A_c K_a = -\ln[1 - P_a(5s)]/(m_r m_l)$$

where m_r and m_l reflect the receptor (TCR) and ligand (pMHC) densities, respectively.

T cell Tetramer Staining

As performed previously, splenocytes from SMARTA or 2D2 mice were incubated for 7 days at 37°C with either gp61-80 or MOG35-55, respectively [36]. Live, previously activated cells were isolated using a Ficoll gradient, washed, and stained for tetramer analysis. Live cells were incubated with 4 μg/ml MOG_{38-49}:I-A^b (8-20 h) [158], GP_{66-77}:I-A^b tetramers (3-4 h), or $hCLIP_{103-117}$:I-A^b (NIAID Tetramer Core Facility at Emory University, Atlanta, GA) in complete RPMI at 37 °C. The cells were washed with buffer containing 1X PBS, 0.1% BSA, and 0.05% sodium azide. Cells were then stained with anti-CD4-APC (RM4.5) (BD-Bioscience) and 7-AAD for 30 minutes on ice. The percentage of tetramer-PE positive cells was determined in live (7-AAD negative) CD4-positive populations. All flow cytometric analysis was performed on a FACSCalibur (BD) and data were analyzed using FlowJo (Tree Star).

T cell Proliferation

For [^{3}H]-thymidine uptake, $6x10^5$ naive splenocytes from 2D2 or SMARTA mice were incubated in a 96-well plate with the indicated concentration of peptide. In some assays (as indicated), cells were pretreated for 30 min with the MEK inhibitor U0126 (Promega) at 10 μM. After 48 h in culture, cells were labeled with 0.4 μCi/well [^{3}H]-thymidine. After 18–24 h, the plates were harvested on a FilterMate harvester (Packard Instrument) and analyzed on a 1450 LSC Microbeta TriLux counter (PerkinElmer) [248].

For CFSE analysis, naïve splenocytes from either 2D2 or SMARTA mice were labeled with CFSE and $1.5x10^6$ cells were incubated in 24-well plates with 10 µM peptide for a given time period before being stained with CD4 APC and 7-AAD and analyzed on a FACSCalibur.

T cell IL-2 ELISA

Splenocytes ($1.5x10^6$) from 2D2 or SMARTA mice were incubated in a 24-well plate with the indicated concentration of peptide. After 24 h in culture, supernatants were removed and placed on microtiter plates coated with purified anti-IL-2 (5 µg/ml clone JES6-1A12; BD Pharmingen) overnight at 4°C. Recombinant IL-2 (BD Pharmingen) 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) and detected using alkaline phosphatase-conjugated avidin (Sigma-Aldrich) and *p*-nitrophenyl phosphate substrate (Bio-Rad). Colorimetric change was measured at dual wavelengths of 405 and 630 nm on a Microplate Autoreader (Biotek Synergy HT) [248].

Analysis of T cell signaling

For time courses that included short peptide stimulation (≤60 min), fibroblasts transfected with $I\text{-}A^b$ (clone FT7.1C6) [249] were plated out in 24-well plates and incubated until confluent (24 h), pre-pulsed with the indicated dose of antigen for 1–2 h and washed. Naive splenocytes were run over a Ficoll gradient and $3x10^6$ cells were added to each well of pre-pulsed fibroblasts. Cells were spun at 600 rpm for 1 min to begin peptide stimulation and allowed to incubate at 37°C for the indicated time points. For time courses with only long

peptide stimulation (≥60 min), naïve splenocytes ($3x10^6$) were stimulated with the indicated dose of antigen for the duration of the time course.

For analysis of protein phosphorylation, cells were taken off fibroblasts at the indicated time points and approximately 300,000 - 500,000 splenocytes were stained for intracellular signaling events. Cells were fixed for 10 min with methanol free formaldehyde at room temperature and permeabilized with 100% ice-cold methanol for 10 min on ice. Cells were then stained with antibodies to CD4 (RM4-5, BD Biosciences), p-p44/42 (D13.14.4E, Cell Signaling), p-c-Jun (KM-1, Santa Cruz Biotechnology), and/or phospho-p38 MAPK (3D7, Cell Signaling) for 30 min on ice, washed, and immediately analyzed by flow cytometry. Flow cytometry was performed on a BD FACSCalibur and data were processed using FlowJo software (Tree Star). FACS wash consisted of PBS, 0.05% sodium azide, and 0.1% BSA.

For analysis of SHP-1 activity, cells were taken off pre-pulsed fibroblasts at the indicated time points, lysed in oxidation buffer and spun at 14,000 rpm for 5 min. SHP-1 was immunoprecipitated with 2 µg of anti-SHP-1 Ab (C19, Santa Cruz biotechnology) overnight, collected with protein A beads for 1 h, and protein A beads were washed once with oxidation buffer and twice with wash buffer (25 mM HEPES (pH 7.2), 50 mM NaCl, and 2.5 mM EDTA). SHP-1 substrate peptide (AEEEIpYGEFEA) was added at a final concentration of 1 mM in Tyr assay buffer with 5 mM DTT (Upstate Biotechnology) and incubated with immunoprecipitated SHP-1 for 1 h at 37°C. Released phosphate was detected by addition of malachite green (Upstate Biotechnology) [101, 103, 250].

Analysis of Surface Markers

For continuous peptide stimulation, splenocytes ($3x10^6$) from 2D2 or SMARTA mice were stimulated for the indicated time points in 24-well plates, washed in FACS buffer and surface stained for CD4 (RM4-5, BD Bioscience), CD25 (PC61, BD Bioscience), and CD69 (H1.2F3, BD Bioscience) for 30 min on ice. Cells were then washed, stored at 4 °C and run on a flow cytometer within 24 hours.

For stimulation with various APC contact times, splenocytes ($3x10^6$) from 2D2 mice were stimulated for the indicated time points in a 24-well plate prior to $CD4^+$ MACS purification carried out as per manufacturer's instructions ($CD4^+$ T Cell Isolation Kit, MACS Miltenyi Biotec). The isolated $CD4^+$ T cells were then resuspended in R10, placed in a well in the 24-well plate and incubated for the remaining time in the 24 hour time course, to allow for further protein production and up-regulation after the limited stimulation time. At 24 hours, the remaining cells were also purified and all cells were stained on ice for 30 minutes with CD69 FITC, CD4 PE, 7AAD, CD25 APC and analyzed on a FACSCalibur flow cytometer.

Statistical Analysis

All data analysis was performed on GraphPad Prism (Software for Science).

Acknowledgements

We thank the members of the Evavold lab for many helpful discussions and critical reading of this manuscript. We also thank the NIH Tetramer Core Facility at Emory University for providing MHC monomers.

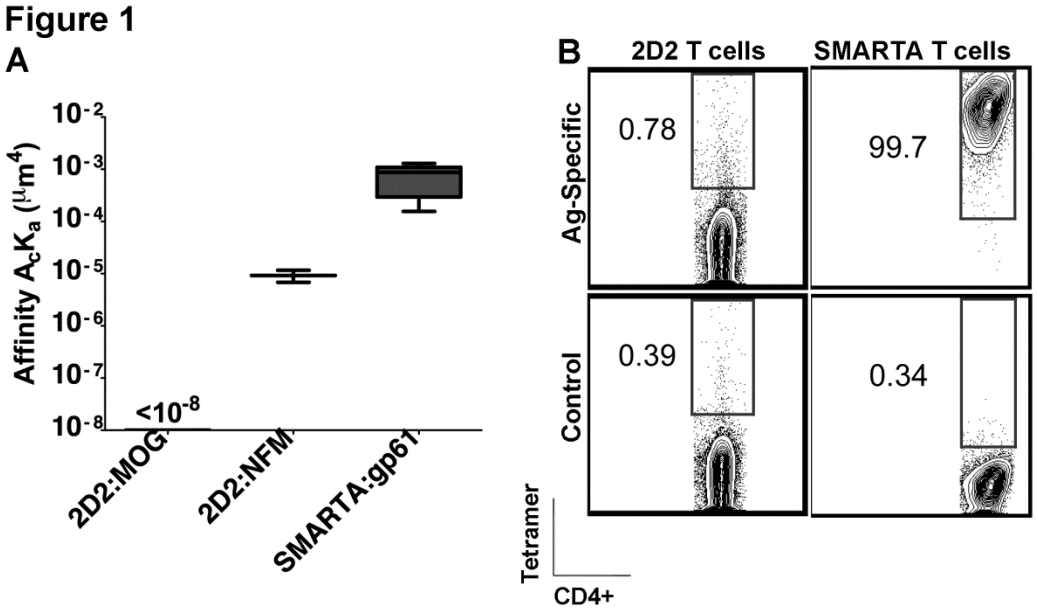
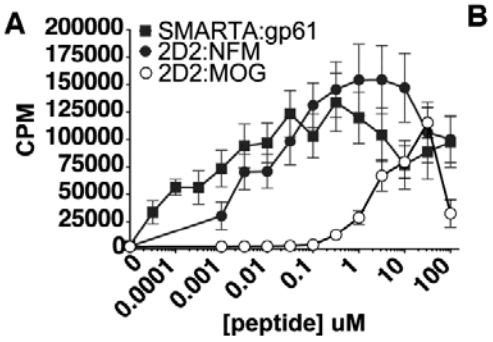
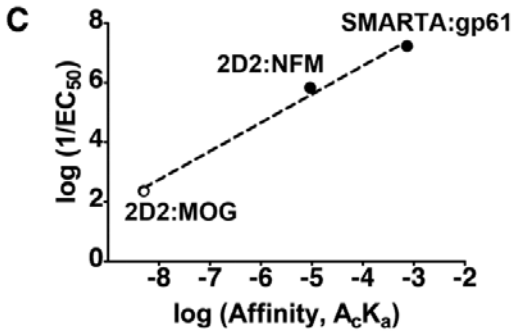


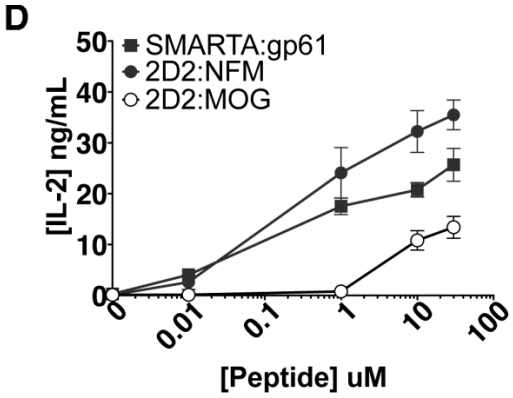
Figure 2.1 - The effective 2D affinity of 2D2 and SMARTA CD4+ T cells differs. (A) 2D2 or SMARTA T cells were stained with antigen-specific I-A^b tetramer (MOG_{38-49} or gp_{66-77}, respectively) or a negative control I-A^b tetramer and analyzed by flow cytometry, gated on $CD4^+$ cells. (B) Human RBCs were coated with the indicated pMHC complex and brought into contact with the corresponding T cell by micropipette numerous times. The resulting adhesion frequency was used to derive the effective 2D affinity (A_cK_a, in μm^4). All experiments were performed at least three times.

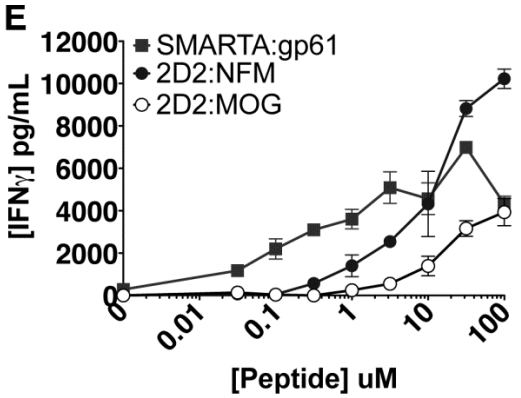
Figure 2



SMARTA:gp61	2D2:NFM	2D2:MOG
0.001	0.012	3.336







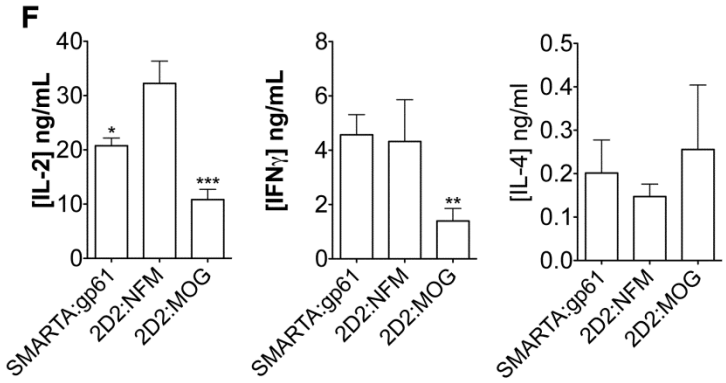


Figure 2.2- 2D2 splenocytes stimulated with MOG have a low functional avidity. (A) $6x10^5$ splenocytes from SMARTA or 2D2 mice were stimulated with various doses of the indicated antigen for 72 hrs. and ^{3}H-Thy was added during the last 18 hrs. to assess proliferation. (B) The concentration of peptide needed to reach the half-maximal response (EC_{50} values, μM) was derived from the above proliferation assay using GraphPad Prism. (C) The reciprocal EC_{50} was plotted against the effective 2D affinity. The open circle for 2D2:MOG denotes the uncertainty of 2D2:MOG affinity, as it was below 10^{-8} μm^4, the limit of detection for this assay. (D) $1.5x10^6$ splenocytes were stimulated for 24 hrs. and supernatants were harvested to determine the amount of IL-2 by ELISA. (E) $1.5x10^6$ splenocytes were stimulated for 24 hrs. and supernatants were harvested to determine the amount of IFN- γ by ELISA. (F) $1.5x10^6$ splenocytes were stimulated with 10 μM of the indicated antigen and supernatants were harvested (24 hrs. for IL-2 and IFN-γ or 48 hrs. for IL-4) to determine the amount of cytokine by ELISA (*P* value: * = 0.025, ** = 0.005, *** < 0.002). All experiments were performed at least three times.

Figure 3

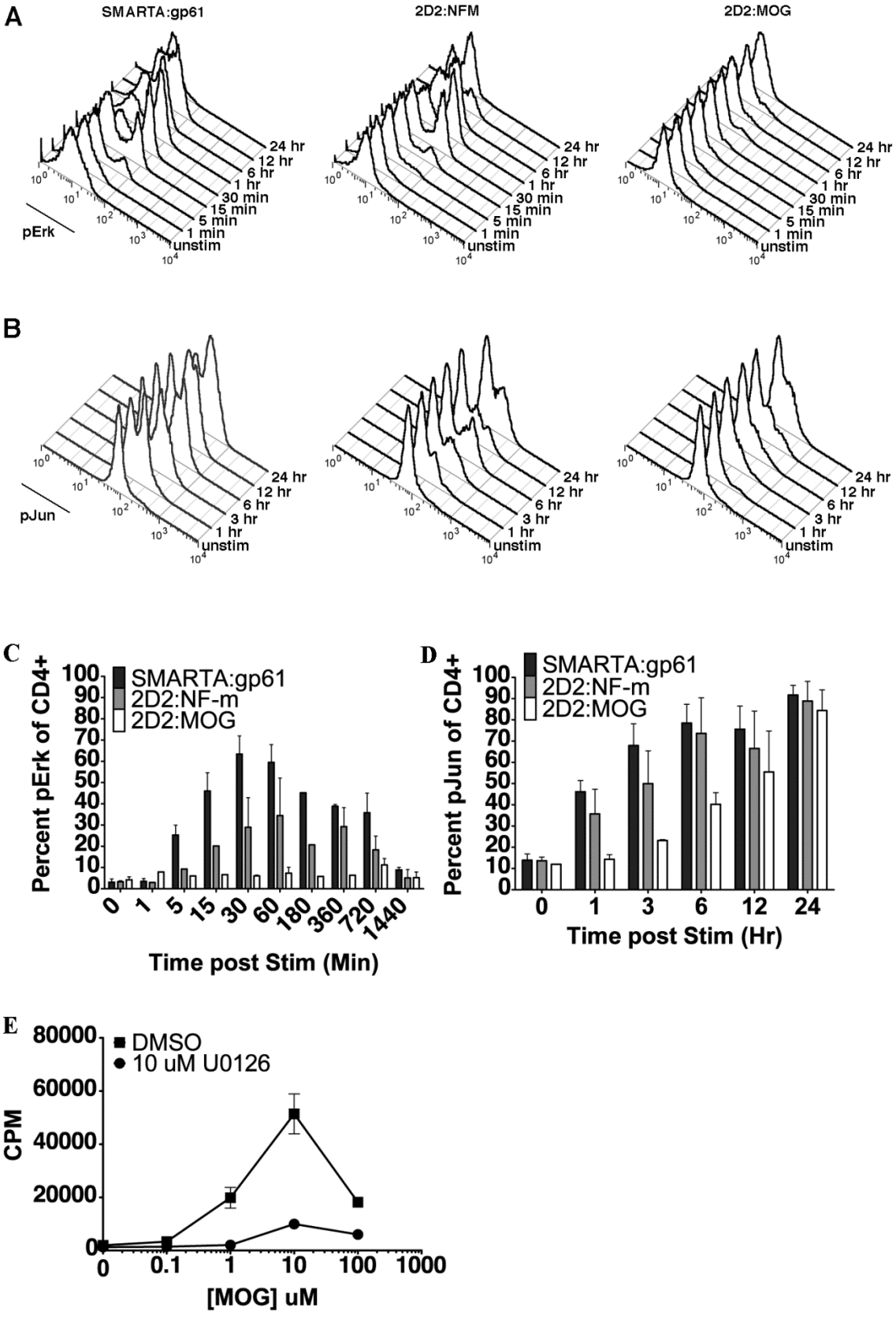

A
SMARTA:gp61
2D2:NFM
2D2:MOG
pErk
24 hr
12 hr
6 hr
1 hr
30 min
15 min
5 min
1 min
unstim
B
pJun
24 hr
12 hr
6 hr
3 hr
1 hr
unstim
C
Percent pErk of CD4+
SMARTA:gp61
2D2:NF-m
2D2:MOG
Time post Stim (Min)
D
Percent pJun of CD4+
SMARTA:gp61
2D2:NF-m
2D2:MOG
Time post Stim (Hr)
E
DMSO
10 uM U0126
CPM
[MOG] uM

Figure 2.3 - 2D2 cells stimulated with MOG have no detectible pErk and delayed phosphorylation of c-Jun. Splenocytes from SMARTA or 2D2 mice were stimulated with 10 μM of the indicated antigen and signaling events were assessed. (A) A representative plot of pErk expression assessed at various time points by flow cytometry, gated on $CD4^+$ cells. (B) A representative plot of p-c-Jun expression assessed at various time points by flow cytometry, gated on $CD4^+$ cells. Graphical representation of averaged p-Erk (C) and p-c-Jun (D) expression are from at least three independent experiments at various time points. (E) Cell proliferation was assessed after treatment with the Erk-specific MEK inhibitor U0126. All experiments were repeated at least three times.

Figure 4

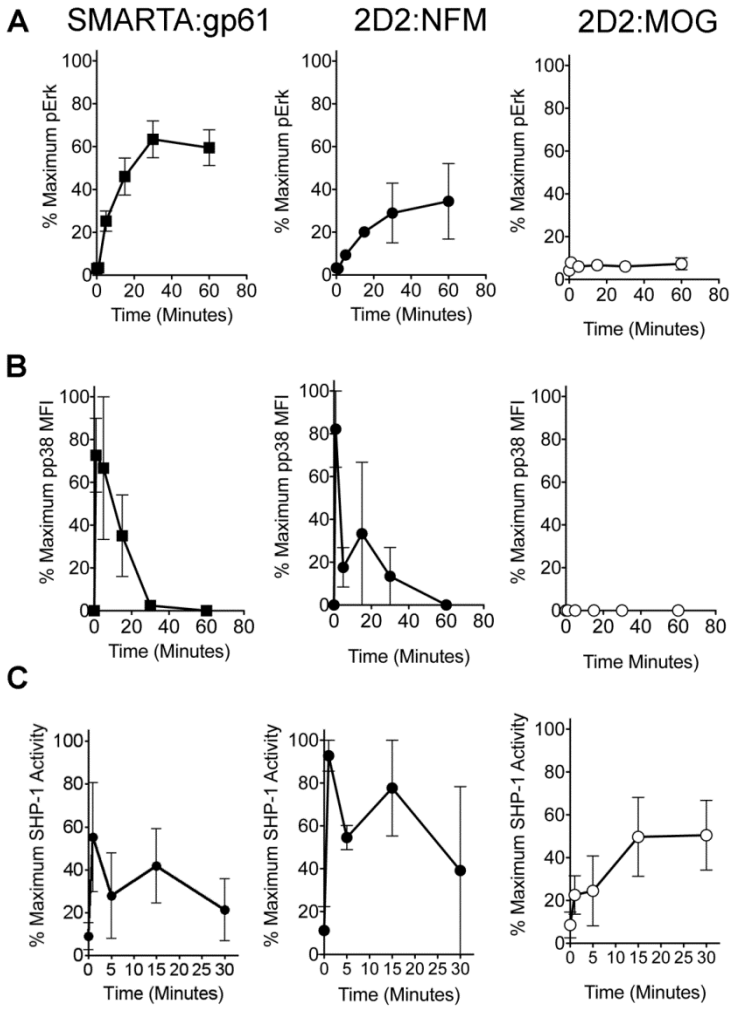


Figure 2.4 - 2D2 T cells stimulated with MOG have an altered signaling program. (A) Splenocytes from SMARTA or 2D2 mice were stimulated with 10 µM of the indicated antigen and the percent of maximal Erk phosphorylation was assessed at various time points by flow cytometry (gated on $CD4^+$ cells). (B) Splenocytes from SMARTA or 2D2 mice were stimulated with 10 µM of the indicated antigen and the percent of maximal p38 MAPK phosphorylation was assessed at various time points by flow cytometry (gated on $CD4^+$ cells). (C) Splenocytes from SMARTA or 2D2 mice were stimulated with 10 µM of the indicated antigen and SHP-1 phosphatase activity was assessed at various time points using a

colorimetric assay for free phosphate with a phosphorylated peptide substrate specific for SHP-1. The percent of maximal response was assessed to highlight the kinetics of the signaling response and was calculated using GraphPad Prism/ All experiments were repeated at least three times.

Figure 5

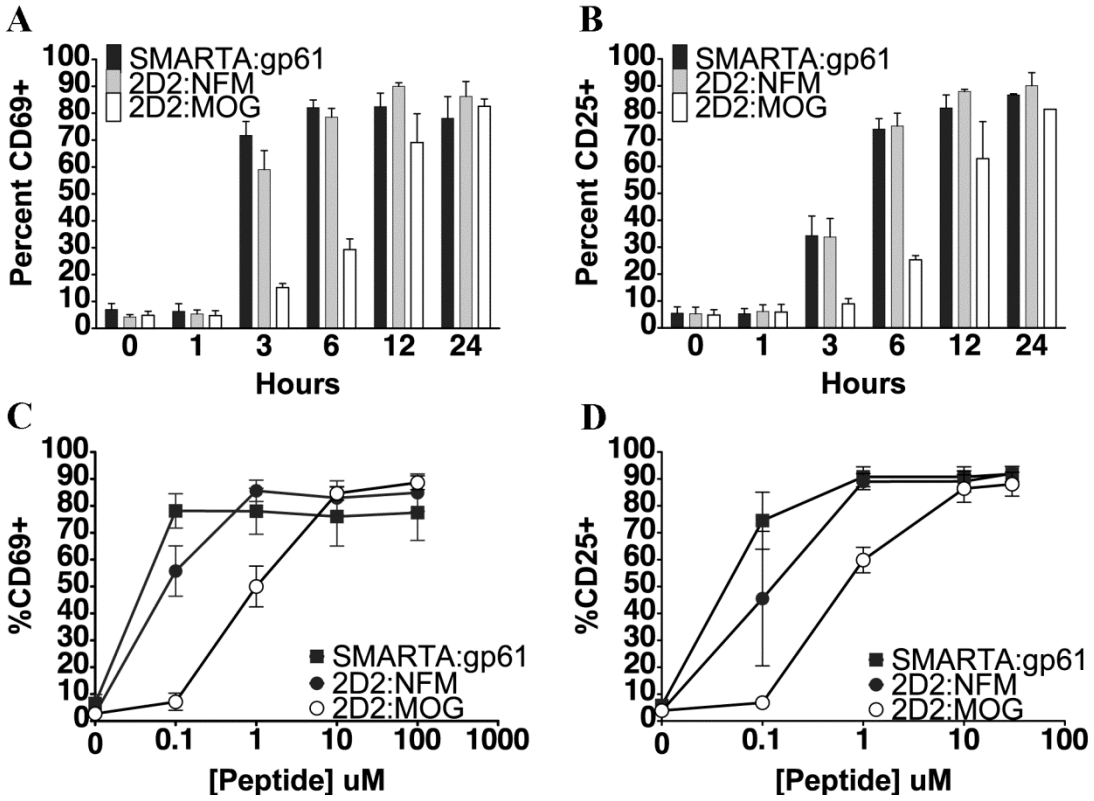


Figure 2.5 - 2D2 CD4+ T cell stimulation with MOG results in delayed expression of activation markers. SMARTA or 2D2 splenocytes were stimulated with 10 µM antigen for various time points and CD69 (A) and CD25 (B) expression on $CD4^+$ cells was analyzed by flow cytometry. SMARTA or 2D2 splenocytes were stimulated with various concentrations of antigen for 24 hrs. and CD69 (C) and CD25 (D) expression on $CD4^+$ cells was analyzed by flow cytometry. Experiments were performed at least three times.

Figure 6

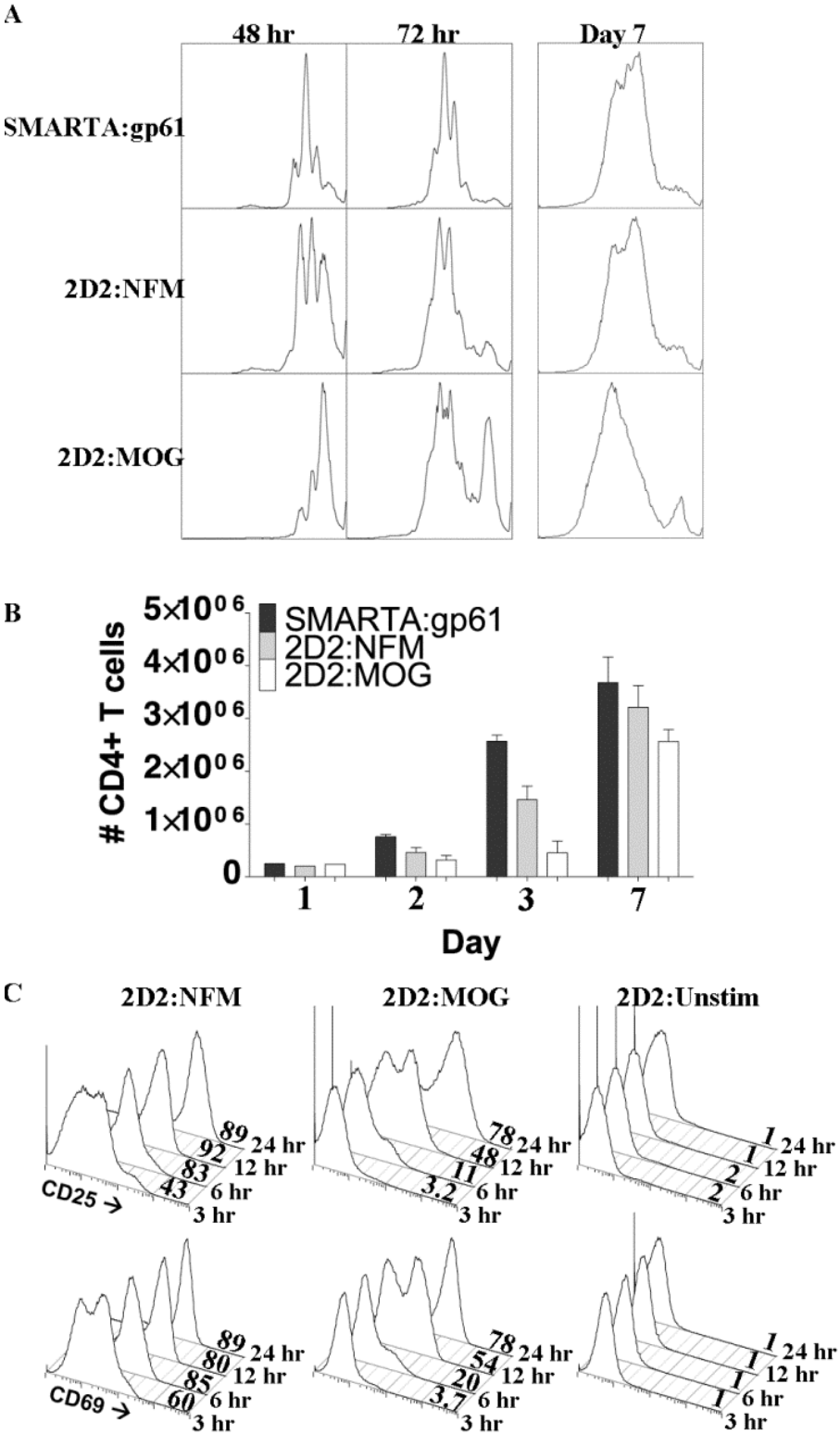

A
48 hr
72 hr
Day 7
SMARTA:gp61
2D2:NFM
2D2:MOG
B
CD4+ T cells
5x10^06
4x10^06
3x10^06
2x10^06
1x10^06
0
SMARTA:gp61
2D2:NFM
2D2:MOG
1
2
3
7
Day
C
2D2:NFM
2D2:MOG
2D2:Unstim
CD25 →
89
92
83
43
24 hr
12 hr
6 hr
3 hr
78
48
11
3.2
1
1
2
2
CD69 →
89
80
85
60
78
54
20
3.7
1
1
1
1

Figure 2.6 - MOG stimulation results in delayed initiation of proliferation but eventual accumulation of CD4+ T cells. Splenocytes from SMARTA or 2D2 mice were CFSE labeled and stimulated with 10 μM of the indicated antigen for various times. (A) Representative plots from at least three independent experiments show CFSE dilution of $CD4^+$ cells, assessed by flow cytometry, to detect proliferation at indicated times. (B) On various days, total $CD4^+$ T cell numbers were assessed using BD Trucount tubes (BD Biosciences) to gauge cellular accumulation after peptide stimulation (On Day 7, SMARTA:gp61 and 2D2:NFM, p=0.1; SMARTA:gp61 and 2D2:MOG p=0.5; 2D2:NFM and 2D2:MOG p=0.2). (C) After the indicated APC contact time, CD4+ T cells were MACS purified to remove the cells from the APCs. The percent of CD25+ and CD69+ CD4+ T cells was assessed by flow cytometry at 24 hours, as indicated on the x-axis. Experiments were performed at least three times; p values were generated using student's t-test on GraphPad Prism.

MOG Epitope	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	Proliferative Response
35-55	M	E	V	G	W	Y	R	S	P	F	S	R	V	V	H	L	Y	R	N	G	K	+++
37-50			V	G	W	Y	R	S	P	F	S	R	V	V	H	L						+++
37-46			V	G	W	Y	R	S	P	F	S	R										-
38-47				G	W	Y	R	S	P	F	S	R	V									+
39-48					W	Y	R	S	P	F	S	R	V	V								+++
40-49						Y	R	S	P	F	S	R	V	V	H							-
41-50							R	S	P	F	S	R	V	V	H	L						-
41-55							R	S	P	F	S	R	V	V	H	L	Y	R	N	G	K	-
42-50								S	P	F	S	R	V	V	H	L						-
44-54										F	S	R	V	V	H	L	Y	R	N	G		-

Table I - The 2D2 core MOG epitope is MOG39-48. Splenocytes were harvested and dose response curves were generated with the indicated peptides up to a maximal concentration of 100 μM to determine the proliferative capacity of 2D2 CD4+ T cells. The nested sets of peptides were generated from the known full length MOG35-55 epitope. Three pluses represent proliferation similar to the parent epitope, with each deduction of a plus representing a log shift in the dose required for maximal proliferation. A minus represents no proliferation above background.

Chapter 3: Dose and Affinity of Antigenic Ligands Guide Differential Effects on T cell Activation and Signaling

Abstract

T cells with low affinity for a specific antigen are thought to require higher concentrations of antigen for complete activation as compared to higher affinity T cell receptors. However, it has also been suggested that the decreased level of stimulation found with low affinity interactions is not necessarily what occurs with a low dose of antigen. Here, we examine low and high affinity T cell populations to explore the qualitative and quantitative differences in activation during various stimulation conditions. Activation kinetics — as measured by activation markers, proliferation, and signaling intermediates — of either a low (2D2:MOG) or high affinity (SMARTA:gp61) interaction does not change with varying doses of antigen, and only the magnitude of the response is affected. However, a low affinity TCR interaction does result in a slower onset of activation compared to a higher affinity antigen. Although the kinetics of signaling does not change with decreasing doses of antigen, the ability to utilize kinase inhibitors to dampen activation of T cells can depend on the dose of antigen, as inhibition of Erk or multiple kinases is more efficient at lower levels of stimulation. Interestingly, polyclonal populations of T cells are differentially affected by the multi-kinase inhibitor, imatinib mesylate, in that cells with low affinity for the stimulating antigen are more amenable to inhibition of proliferation than high affinity cells. This data suggests that there are qualitative and quantitative differences in the way low affinity peptides trigger TCR signaling, and that inhibition of signaling intermediates may differentially affect low and high affinity T cells during an immune response.

Introduction

T cell activation is essential in mounting a productive immune response, and depends on a productive encounter between T cell receptors (TCR) and antigens presented on major histocompatibility complexes (pMHC). Understanding how the TCR affinity for a given antigen and the dose of antigen can affect T cell activation, effector function, and survival has become an integral part of understanding T cell immunity and function. Recently, there have been many attempts to explain observations regarding T cell antigen potency, specificity, and efficacy of T cell activation [251-255]. The overriding theme of these investigations focuses on the importance of the influence of the potency of antigen and time of TCR:pMHC interactions in T cell activation.

We, and others, have shown that the pMHC affinity for TCR can influence the amplitude and kinetics of a T cell response, in that low affinity ligands cause a delay in T cell signaling, activation, and downstream functions such as proliferation [30, 255-258]. Further, T cells with low affinity for a ligand require higher doses of antigen for complete stimulation compared to high affinity TCR [25, 259]. The dose of ligand present during TCR stimulation can also affect the magnitude of the T cell response without changing the outcome [30, 241, 242]. However, other groups have shown that the potency of stimulation can affect the differentiation of $CD4^+$ T helper cells [226, 260]. Taken together, this data suggests that the affinity and the dose of a given TCR ligand can both contribute to the T cell response, but there are intrinsic differences in how these parameters can be used to yield a maximal response to a given ligand.

Previously we showed that even though the low affinity interaction with 2D2 T cells and the myelin-specific self-peptide important in murine EAE induction (MOG_{35-55}) does have a delayed initiation of activation, these cells are able to catch up to high affinity stimulation of SMARTA T cells with the pathogen-derived epitope in murine LCMV (gp_{61-80}) if given enough time [255]. Here, we look more closely at how the dose of either the high affinity viral antigen or the low affinity self-antigen can affect various outcomes of TCR stimulation. Similar to previous studies, a low affinity TCR interaction results in a slower activation kinetics compared to a higher affinity antigen. Importantly, we find that the kinetics do not change with varying the dose of either a high affinity or low affinity ligand, and only the magnitude of the response changes (eg, activation markers, proliferation, or signaling intermediates). However, the efficacy of kinase inhibitors to dampen T cell response can depend on either the antigen concentration or the affinity of the TCR, as inhibition of Erk or multiple kinases, with imatinib mesylate, is more efficient at lower levels of stimulation. These data suggest that there are qualitative and quantitative differences regarding low and high affinity ligands in T cell response, and suggest these inherent differences may be exploited with signaling intermediate inhibitors.

Results

Kinetics of activation with varying doses of antigen. We have previously shown that stimulation of 2D2 $CD4^+$ T cells with the low affinity self-peptide, MOG_{35-55}, results in delayed kinetics of activation and proliferation [255]. To determine whether the kinetics of activation in 2D2:MOG was merely a reflection of a lowered level of stimulation, we compared the kinetics of CD69 expression, a marker of T cell activation, at various doses of either MOG in 2D2 T cells or the high affinity, pathogen-derived epitope gp61 in SMARTA T cells. Expression of CD69 had similar kinetics at each dose of antigen tested in either T cell clone and only the magnitude of the response changed with either MOG or gp61 (Figure 3.1a and b, respectively). Although the kinetics of CD69 expression with 2D2:MOG was delayed compared to SMARTA:gp61, there was no change in the kinetics of expression at each dose of peptide and only the number of cells that respond changed in a dose dependent manner.

In addition to the activation marker CD69, we also analyzed the kinetics of the proliferative potential of SMARTA $CD4^+$ T cells with progressively lower doses of the high affinity peptide, gp61 (Figure 3.2). The cellular proliferation was visualized using CFSE dilution and showed that the kinetics of division were similar, while only the number of cells that entered proliferative cycles were reduced with decreasing doses of antigen (Figure 3.2). On day two after stimulation, there are three clearly defined proliferative peaks with both 10 uM gp61 and 0.1 uM gp6, although there are less cells present in each cycle of proliferation with 0.1 uM gp61 and fewer cells have entered into cycle, but there are too few proliferating cells present at 0.001 uM gp61 to visualize any distinction (Figure 3.2). On day three, all doses of gp61 resulted in approximately five rounds of division, although there are fewer

cells present in each cycle of proliferation as the dose of gp61 decreases (Figure 3.2). By day seven of stimulation, the number of divisions is more difficult to distinguish but most cells that entered into the proliferative cycle have fully divided at each dose of antigen and fewer cells entered cycle with each decrease in peptide concentration (Figure 3.2). This data suggests that although the magnitude of activation is decreased with decreasing doses of a high affinity peptide, there is no change in the absolute kinetics of the response.

Kinetics of cellular signaling with varying doses of antigen. Differences in activation could translate to differences in the kinetics of T cell signaling, so we also examined whether dose of antigen can alter the kinetics of cellular signaling events. However, at all doses of gp61 tested the kinetics of Erk and c-Jun phosphorylation remain the same and only the magnitude of the response changes (Figure 3.3a and b). This trend can be seen in other transgenic T cells, including other CD4+ cells (ie, OT II) and CD8+ cells (ie, P14) (data not shown). The dose at which SMARTA cells have maximal proliferation (Figure 2.1) also allows for near maximal accumulation of phosphorylated Erk (1 μM gp61), whereas in 2D2:MOG there is no appreciable accumulation of pErk at maximal proliferation (10 μM MOG) (Figure 3.3c). At approximately one log above the EC_{50} there is no noticeable pErk present with either MOG:2D2 (10 μM) or gp61:SMARTA (0.01 μM) (Figure 3.3c). However, in 2D2:MOG stimulation the lack of pErk does not preclude p-c-Jun accumulation, unlike in gp61:SMARTA activation where there is no detectable p-c-Jun (Figure 3.3d). Importantly, at any dose of gp61 in which no phosphorylated Erk is detectable, there is a corresponding lack of p-c-Jun accumulation as well. Taken together, this data suggests that low dose of a high affinity peptide does not translate to a higher dose of a low affinity peptide and that there can be qualitative differences in the way low affinity peptides trigger TCR signaling.

Inhibition of Erk in differing doses of low affinity ligands. We have previously shown that although there is no detectible phosphorylation of Erk in 2D2 T cells stimulated with the low affinity MOG antigen, inhibition of this protein does affect the proliferative potential upon stimulation (Figure 2.3e) and [255]. Here, we utilized the Erk inhibitor, U0126, to determine the effects of the Erk inhibitor on cellular survival and proliferation. In 2D2 cells, stimulation with MOG with or without the inhibitor does not result in pErk or pc-Jun expression; however, the inhibitor can inhibit Erk phosphorylation and downstream signaling events, such as phosphorylation of c-Jun, in 2D2 cells stimulated with a higher affinity CNS self-epitope from the neurofilament medium chain, NFM (Figure 3.4a). Further, this inhibition of signaling does not result in an increase in cell death, as measured by 7-AAD staining (Figure 3.4b).

As shown in Figure 2.3, the addition of U0126 dramatically decreases the proliferative potential of 2D2 cells stimulated with MOG antigen, regardless of the lack of p-Erk expression in these cells (Figure 2.3e) and [255]. To further investigate the proliferative potential of 2D2 cells stimulated with MOG, we utilized CFSE dilution assays. At 1 μM stimulation in the presence of U0126, there is a dramatic decrease in the number of rounds of cellular division with most cells only undergoing between 3-5 divisions compared to complete dilution of CFSE with 1 μM MOG alone (Figure 3.4c). However, with either 100 or 10 μM of MOG, there is a less pronounced difference in the amount of CFSE dilution (Figure 3.4c). This data suggests that alteration of signaling events can lead to more profound changes at lower doses of antigen, and that a reduced magnitude of signaling may allow for greater inhibition of the signaling intermediates.

Effects of imatinib mesylate on proliferation. To further define the role of signaling inhibition in T cell proliferation, we utilized one of the earliest approved, and extensively studied targeted therapies in oncology to investigate the effects of kinase inhibition on various doses of T cell stimulation. Imatinib is an inhibitor of tyrosine kinases, including Abl, ARG, c-kit, and Lck [261]. Although imatinib was originally designed to treat chronic myelogenous leukemia (CML), others have shown that this inhibitor can decrease T cell proliferation and cytokine production [261, 262], with a dose dependent decrease in proliferation of T cells following anti-CD3 stimulation [261]. However, few groups have looked at how the strength of TCR stimulation affects the inhibitory nature of these targeted agents.

Here, we used serially decreasing doses of plate-bound anti-CD3 and soluble anti-CD28 to stimulate naïve C57B/6 splenocytes, treated with 10 μM STI-571 or vehicle only, to assess proliferation by ^{3}H-thymidine incorporation. Similar to inhibition with the U0126, STI-571 dramatically decreased T cell proliferation (Figure 3.5a). In addition to cellular proliferation, STI-571 reduced the amount of IFN-γ produced upon anti-CD3/anti-CD28 ligation (Figure 3.5b). Similar to the effects seen with the Erk inhibitor, STI-571 is more effective at inhibiting proliferation with lower doses of a stimulatory agent. In assessing the proliferative potential of naïve C57B/6 splenocytes using CFSE dilution, there is little effect on the number of divisions when the highest dose of anti-CD3/anti-CD28 is used (10 μg anti-CD3); however, with decreasing doses of stimulatory antibody, the inhibitory effect of STI-571 increases (Figure 3.5c). Again, this data suggest that inhibition of signaling events can lead to more profound changes as the strength of stimulation decreases.

Imatinib differentially affects low and high affinity T cells. Here, we have shown that inhibition of signaling intermediates may affect cells differently, depending on the strength of stimulation; however, it is also important to understand how affinity of the TCR:pMHC interaction may be affected by the inhibition of these molecules. Our lab has recently shown that MOG-induced EAE in C57B/6 mice results in a polyclonal T cell repertoire encompassing a wide breadth of affinities, with T cells that engage the pMHC with a range of affinities [36]. We utilized this system to understand how high and low affinity T cells are affected by treatment with the kinase inhibitor, imatinib.

As a whole, treatment with STI-571 successfully decreased the amount of MOG-induced proliferation, resulting in fewer cells that entered cycle and a reduction in the number of divisions, as assessed by CFSE dilution on day 3 after stimulation (Figure 3.6a). However, the high affinity MOG-reactive cells, as defined by $CD4^+$ T cells that can be identified by MOG:I-A^b tetramer staining [36], had no appreciative inhibition of proliferation (Figure 3.6b). Interestingly, treatment with STI-571 decreased the proliferative potential of low affinity, tetramer negative T cells, with fewer cells that entered cycle and those that did proliferate underwent fewer divisions after treatment with imatinib (Figure 3.6c). This data suggests that inhibition of key signaling intermediates may differentially affect high and low affinity $CD4^+$ T cells within a specific population.

Discussion

It is known that both the affinity and dose of antigen can influence the signaling and downstream effector responses of T cells [30, 252, 263]. The dose of initiating antigen is inversely proportional to the overall TCR affinity, in that low affinity TCR are thought to require higher doses of antigen for complete stimulation as compared to T cells expressing high affinity TCR for their cognate ligand [25, 259]. However, understanding the individual effects of quality and quantity of antigen can be complicated by the direct correlation of each parameter on T cell activation. Here, we show that the kinetics of T cell activation and corresponding activation of signaling intermediates are inherent to the affinity of the interaction and not to the dose of the initiating antigen, which affects the magnitude or the response (Figures 3.1-3.2). These data suggests that the affinity of TCR ligation directs the outcome of response in individual T cell clones, while the dose of antigen may influence the affinity profile during an immune response [36, 259] by tuning the scope of the responding T cell populations. Importantly, this data highlights that activation of a T cell with a highly efficacious peptide at a low dose does not equate to a low efficacious peptide at a high dose.

Recently, another group has published data suggesting that there may, in fact, be important differences in the contribution of pMHC potency and density in T cell activation. Gottschalk and colleagues show that although there is no apparent difference in low doses of high affinity ligands and high doses of low affinity in the initiation of proliferation [253] or Akt phosphorylation [258], there were distinct influences of pMHC potency on the in vivo IL-2 response [253]. Further, microarray data highlighted a subset of genes that were similarly expressed in in cells stimulated by either low-density or low-potency pMHC, while another subset of genes demonstrated discrimination between these two types of weak stimuli

[253]. While this data is interesting and certainly highlights the distinction of peptide quality versus quantity, a deeper look at the kinetics of both T cell signaling and initiation of proliferation suggests that there are intrinsic differences in the way a low dose of a high affinity ligand and a high dose of a low affinity ligand arrive at a similar response (Figures 3.1-3.3). Specifically, in looking at various time points in T cell activation – through signaling intermediates, activation markers, or proliferation – there is a variation in kinetics with differing affinities, but not with differing doses of ligands. The same end result in proliferation or activation markers at later time points highlights the ability of time of antigen encounter to compensate for a slow accumulation of signals in low affinity interactions, while maintaining the possibility of ligand discrimination by the TCR.

The potential for slow accumulation of signaling intermediates to produce a complete response results in a lack of detectible activation of other intermediates by biochemical methods, while this lack of detection (ie, phosphorylation of Erk) does not necessarily define their actions in cells. For example, inhibition of Erk does result in an attenuated response even though phosphorylation is not detectable in MOG:2D2 stimulation (Figure 3.3) [255]. This inhibition of proliferation is not caused by toxicity to the cells, as there is no increase in the amount of 7-AAD positive T cells (Figure 3.3). Interestingly, inhibition of Erk, or other signaling events with the kinase inhibitor STI-571, can lead to more profound changes in the ability for T cells to divide and proliferate at lower doses of stimulation (Figures 3.4 and 3.5). This suggests that although there is no change in the kinetics of response with decreasing doses of antigen, there is a potential for differential pMHC density to contribute to the robustness of response.

Many papers have shown that ligand discrimination depends on TCR proximal events, and differential recruitment and phosphorylation of signaling intermediates can occur during stimulation with weaker ligands [20, 30, 77, 242, 255]. We have seen here that kinase inhibition is more effective in reducing T cell activation at lower doses of stimulating antigen. Further, in a polyclonal T cell population specific for the self-peptide MOG_{35-55}, low affinity T cell clones are more sensitive to kinase inhibition than high affinity T cell clones at a given pMHC density in vitro. This suggests that for antigens of sub-par quality or quantity, there may be additional need for maximal signaling capabilities, beyond those needed for a higher potency ligand.

Here, we show that there is differential kinetics in activation – evaluated through signaling intermediates, activation markers, and rates of cell division – when examining the quality, but not the quantity of pMHC. The interplay of each parameter can direct the potency of the overall response to a ligand, with the TCR affinity directing the outcome in individual T cell clones, while the dose of antigen directs the response of the overall T cell population and robustness of the response. Further, this distinction of low affinity versus low doses of pMHC confirms a model of a temporal differentiation of ligand potency, while explaining the potential for a response to lower affinity ligands, given enough time to integrate the low level of signaling.

Materials and Methods

Transgenic Mice

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. MOG_{35-55} specific TCR transgenic 2D2 mice (Jackson Labs, C57BL/6-Tg(Tcra2D2,Tcrb2D2)1Kuch/J), gp_{61-80} specific TCR transgenic SMARTA mice [17,18] and C57B/6 mice were bred, housed and used with specific approval from the Institutional Animal Care and Use Committee-approved protocol of the Emory University Department of Animal Resources facility (IUCAC Number: DAR-2000870-061414). All mice were used for experiments at 6-8 weeks of age.

Peptides and Reagents

LCMV gp_{61-80} (GLNGPDIYKGVYQFKSVEFD), and mouse NFM_{15-35} (RRVTETRSSFSRVSGSPSSGF) and MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK) were synthesized in-house using F-moc chemistry on the Prelude peptide synthesizer (Protein Technologies). Culture medium consisted of RPMI 1640 medium (Mediatech) supplemented with 10% FBS (HyClone), 2 mM L-glutamine (Mediatech), 0.01 M HEPES buffer (Mediatech), 100 µg/ml gentamicin (Mediatech), and $2x10^{-5}$ M 2-ME (Sigma-Aldrich).

Analysis of Surface Markers

Splenocytes ($3x10^6$) from 2D2 or SMARTA mice were stimulated for the indicated time points in 24-well plates, washed in FACS buffer and surface stained for CD4 (RM4-5,

BD Bioscience) and CD69 (H1.2F3, BD Bioscience) for 30 min on ice. Cells were then washed, stored at 4 °C and run on a flow cytometer within 24 hours.

T cell Proliferation

For [^{3}H]-thymidine uptake, 6x10^5 naive splenocytes from 2D2 or SMARTA mice were incubated in a 96-well plate with the indicated concentration of peptide, or anti-CD3 and anti-CD28. In some assays (as indicated), cells were pretreated for 30 min with the carrier (DMSO), 10 μM of the MEK inhibitor U0126 (Promega), or imatinib mesylate (10 uM) prior to incubation with peptide. After 48 h in culture, cells were labeled with 0.4 μCi/well [^{3}H]-thymidine. After 18–24 h, the plates were harvested on a FilterMate harvester (Packard Instrument) and analyzed on a 1450 LSC Microbeta TriLux counter (PerkinElmer) [248].

For CFSE analysis, naïve splenocytes from either 2D2 or SMARTA mice were labeled with CFSE and 1.5x10^6 cells were incubated in 24-well plates with peptide or plate bound anti-CD3 and soluble anti-CD28 for a given time period before being stained with CD4 APC and 7-AAD and analyzed on a FACSCalibur. In some assays (as indicated), cells were pretreated for 30 min with the carrier (DMSO), 10 μM of the MEK inhibitor U0126 (Promega), or imatinib mesylate (10 uM) prior to incubation with peptide.

Analysis of T cell signaling

Fibroblasts transfected with I-A^b (clone FT7.1C6) [249] were plated out in 24-well plates and incubated until confluent (24 h), pre-pulsed with the indicated dose of antigen for 1–2 h and washed. Naive splenocytes were run over a Ficoll gradient and 3x10^6 cells were

added to each well of pre-pulsed fibroblasts. Cells were spun at 600 rpm for 1 min to begin peptide stimulation and allowed to incubate at 37°C for the indicated time points. For time courses with only long peptide stimulation (≥60 min), naïve splenocytes ($3x10^6$) were stimulated with the indicated dose of antigen for the duration of the time course.

For analysis of protein phosphorylation, cells were taken off fibroblasts at the indicated time points and approximately 300,000 - 500,000 splenocytes were stained for intracellular signaling events. Cells were fixed for 10 min with methanol free formaldehyde at room temperature and permeabilized with 100% ice-cold methanol for 10 min on ice. Cells were then stained with antibodies to CD4 (RM4-5, BD Biosciences), p-p44/42 (D13.14.4E, Cell Signaling), and/or p-c-Jun (KM-1, Santa Cruz Biotechnology) for 30 min on ice, washed, and immediately analyzed by flow cytometry. Flow cytometry was performed on a BD FACSCalibur and data were processed using FlowJo software (Tree Star). FACS wash consisted of PBS, 0.05% sodium azide, and 0.1% BSA.

Analysis of polyclonal MOG-specific T cells

C57B/6 mice were primed in the hind footpads and tail base using 100 μg of MOG_{35-55} emulsified in CFA. The inguinal and popliteal lymph nodes (LNs) were isolated 10-12 days post-priming and a single cell suspension was generated. Cells were then incubated for 7 days at 37°C with MOG_{35-55} and live cells were isolated after 7 days using a Ficoll gradient, washed, and stained for CFSE dilution assay and tetramer analysis.

Live cells were labeled with CFSE, pretreated for 30 min with the carrier (DMSO) or 10 uM STI-571 (imatinib mesylate) and $1.5x10^6$ cells were incubated in 24-well plates with peptide. After 3 days of stimulation, cells were incubated with 4 μg/ml MOG_{38-49}:$I\text{-}A^b$ [158]

or $hCLIP_{103-117}$:I-A^b (NIAID Tetramer Core Facility at Emory University, Atlanta, GA) in complete RPMI at 37 °C overnight. The cells were then washed with buffer containing 1X PBS, 0.1% BSA, and 0.05% sodium azide and stained with anti-CD4-APC (RM4.5) (BD-Bioscience) and 7-AAD for 30 minutes on ice for analysis by flow cytometry. All flow cytometric analysis was performed on a FACSCalibur (BD) and data were analyzed using FlowJo (Tree Star).

Statistical Analysis

All data analysis was performed on GraphPad Prism (Software for Science).

Acknowledgements

We thank Ruth Napier and Dan Kalman from the Kalman lab at Emory University for the generous gift of STI-571 (imatinib mesylate) and countless hours of discussion regarding the effects of imatinib on cellular signaling and disease processes. We also thank the NIH Tetramer Core Facility at Emory University for providing MHC monomers.

Figures

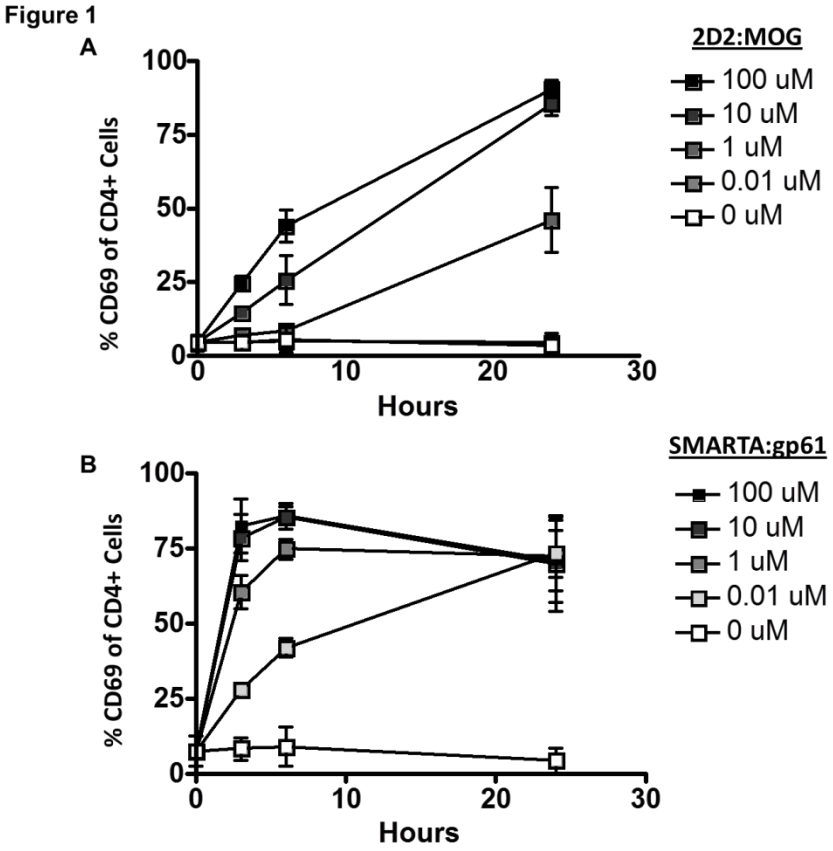


Figure 3.1 – Dose of antigen affects the magnitude, but not kinetics of activation marker expression. $1.5x10^6$ splenocytes were stimulated with the indicated concentrations of antigen for various time points and CD69 expression in 2D2 (A) or SMARTA (B) $CD4^+$ cells was analyzed by flow cytometry.

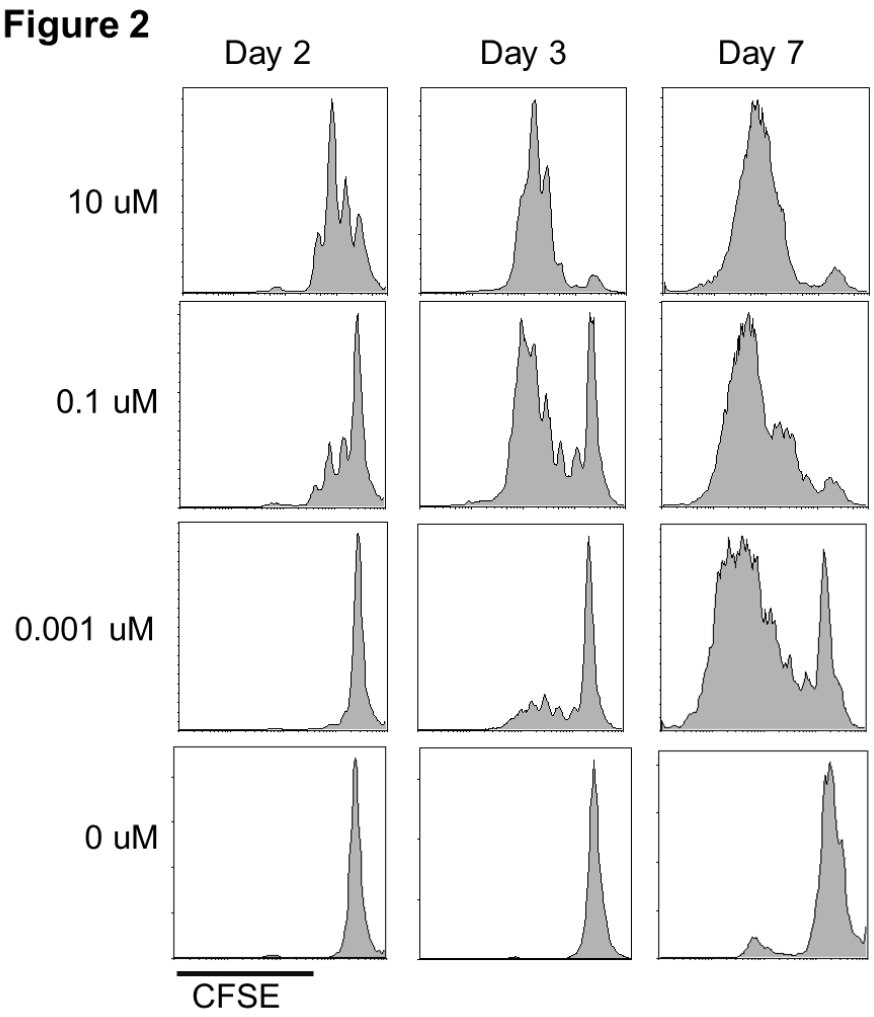


Figure 3.2 – Dose of antigen affects the magnitude, but not kinetics of T cell proliferation. Splenocytes from SMARTA were CFSE labeled and stimulated with the indicated concentration of antigen for various times. Representative plots show CFSE dilution of $CD4^+$ cells, assessed by flow cytometry, to detect proliferation on the indicated days.

Figure 3

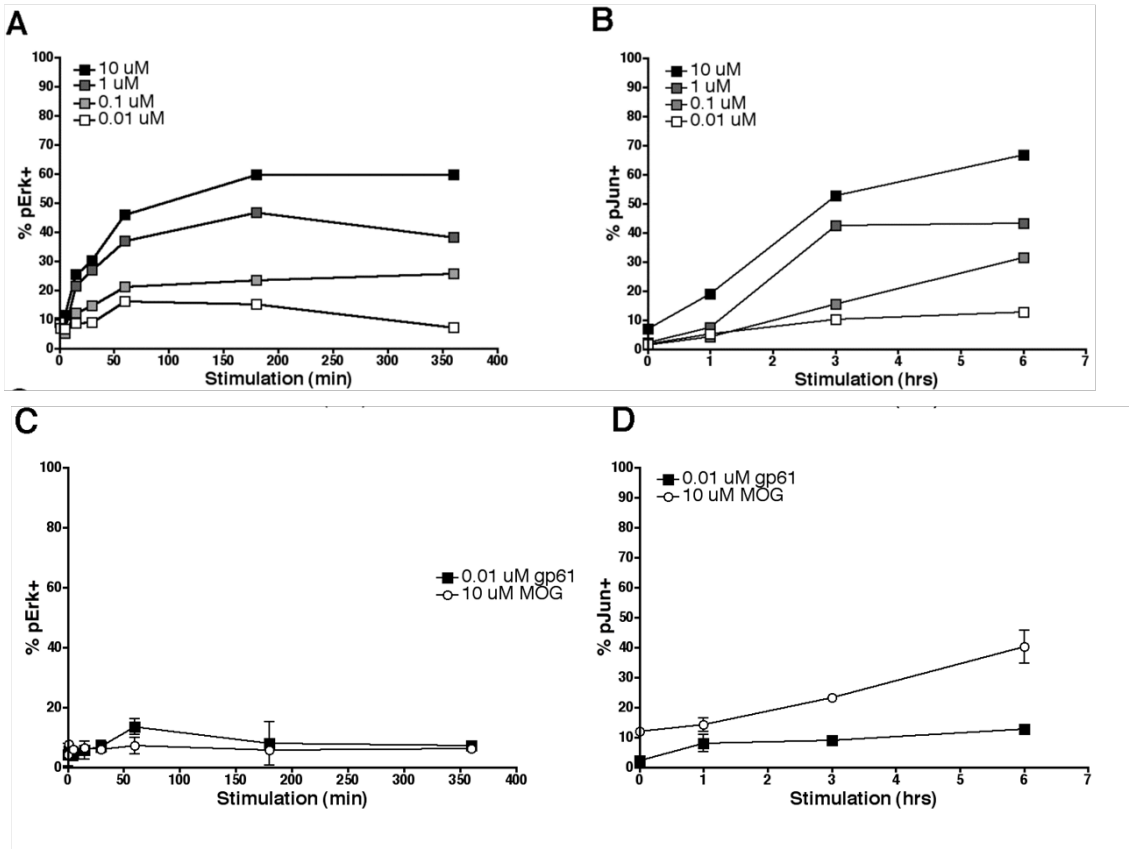


Figure 3.3 – Dose of antigen affects the magnitude, but not kinetics of T cell signaling.

Splenocytes from SMARTA or 2D2 mice were stimulated with the indicated concentration of cognate ligand and signaling events were assessed. Graphical representation of averaged p-Erk (A) or p-c-Jun (B) expression in SMARTA cells was assessed at various time points by flow cytometry, gated on $CD4^+$ cells. Graphical representation of averaged p-Erk (C) or p-c-Jun (D) expression in SMARTA cells compared to 2D2 cells as assessed by flow cytometry, gated on $CD4^+$ cells.

Figure 4

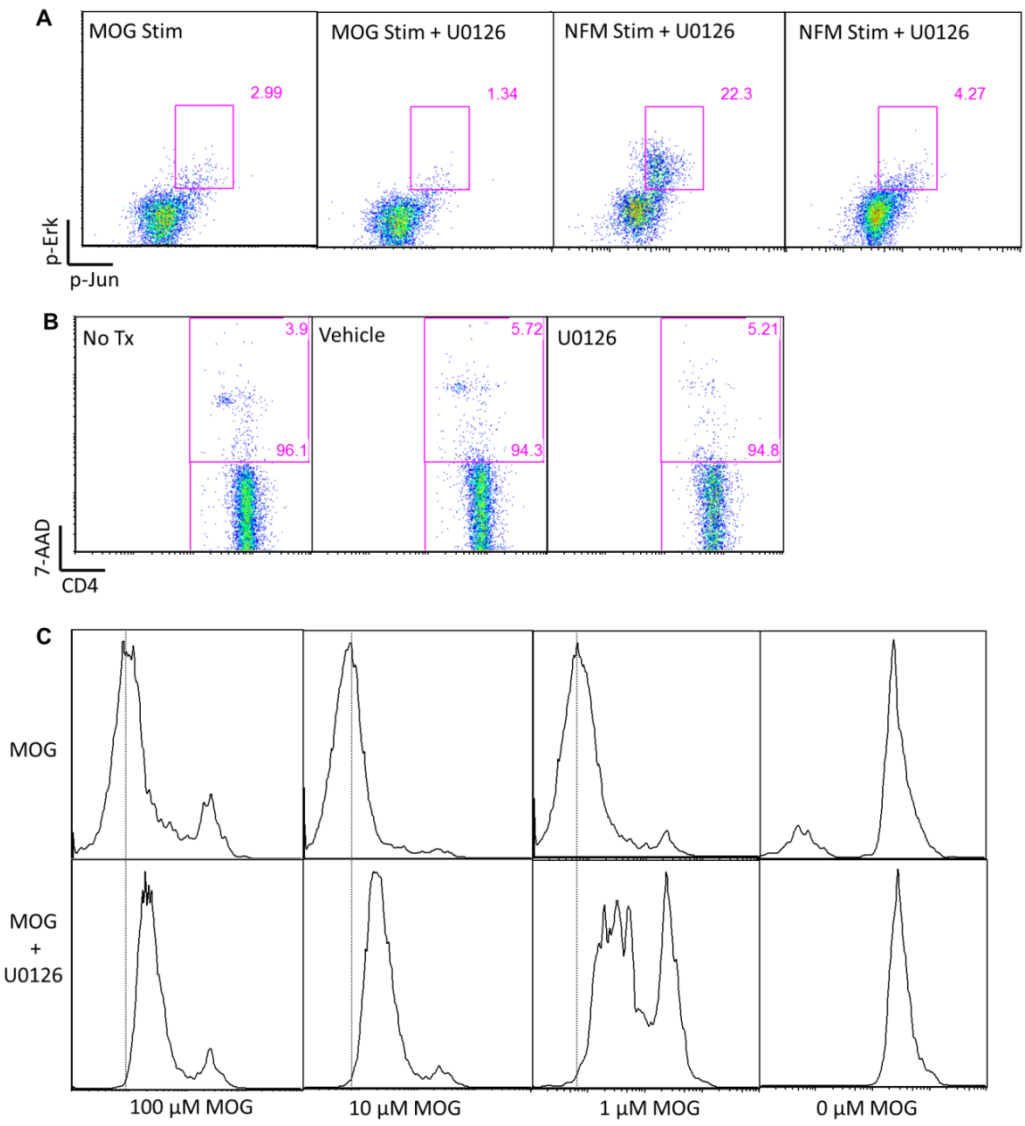


Figure 3.4 – Erk inhibition decreases proliferative ability in 2D2 T cells. Splenocytes from 2D2 mice were stimulated with the indicated concentration of antigen after treatment with Erk inhibitor. (A) Splenocytes were treated with vehicle control or U0126 and signaling events were assessed after 1 hour of peptide stimulation. (B) Splenocytes were treated with vehicle control or U0126 and 7-AAD staining was assessed after 1 hour of MOG stimulation.

(C) Splenocytes were treated with vehicle control or U0126 and cell division was assessed via CFSE dilution on day 5 after MOG stimulation.

Figure 5

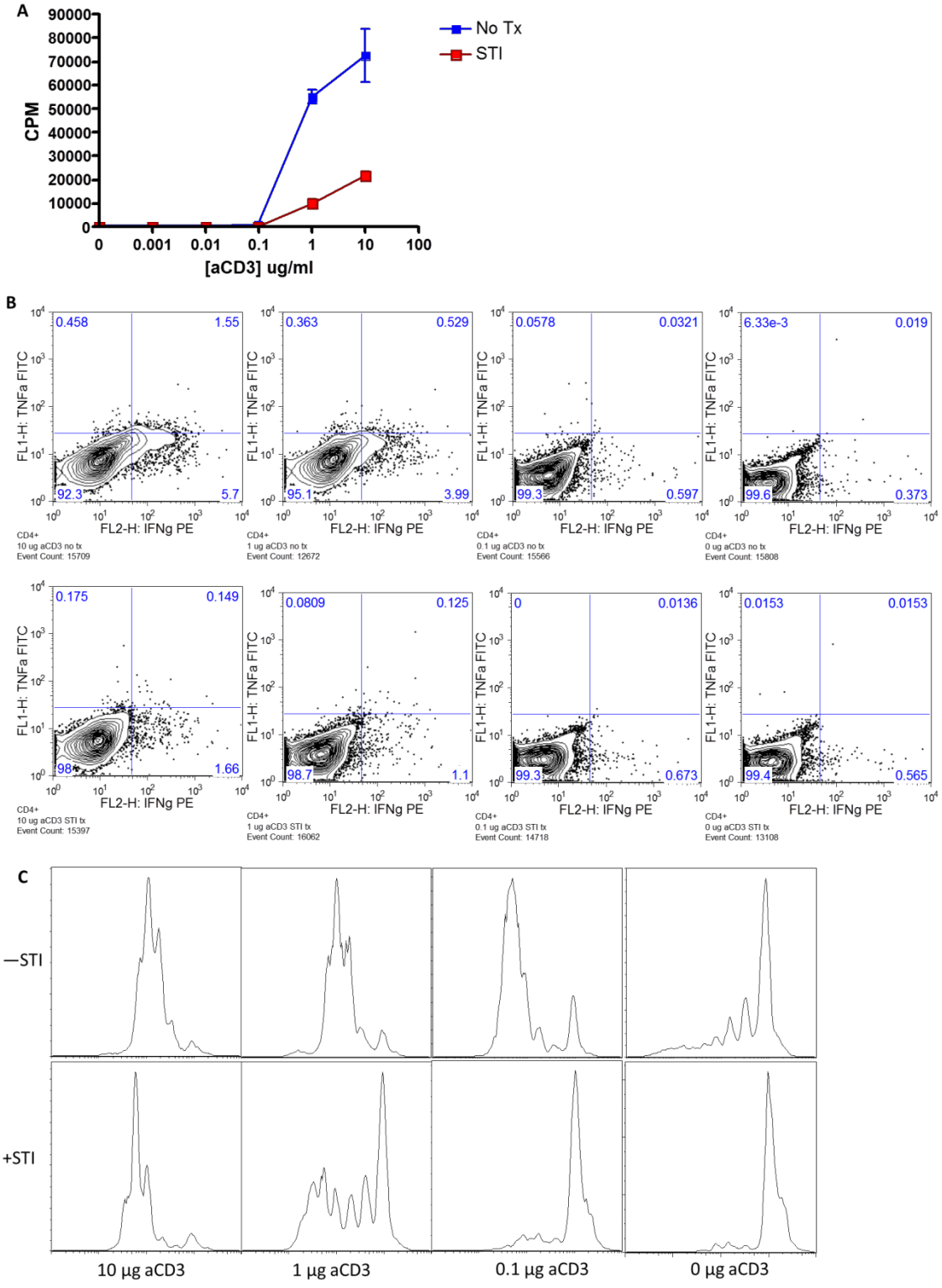

A
CPM
[aCD3] ug/ml
No Tx
STI
B
FL1-H: TNFa FITC
FL2-H: IFNg PE
0.458
1.55
92.3
5.7
CD4+
10 ug aCD3 no tx
Event Count: 15709
0.363
0.529
95.1
3.99
CD4+
1 ug aCD3 no tx
Event Count: 12672
0.0578
0.0321
99.3
0.597
CD4+
0.1 ug aCD3 no tx
Event Count: 15566
6.33e-3
0.019
99.6
0.373
CD4+
0 ug aCD3 no tx
Event Count: 15808
0.175
0.149
98
1.66
CD4+
10 ug aCD3 STI tx
Event Count: 15397
0.0809
0.125
98.7
1.1
CD4+
1 ug aCD3 STI tx
Event Count: 16062
0
0.0136
99.3
0.673
CD4+
0.1 ug aCD3 STI tx
Event Count: 14718
0.0153
0.0153
99.4
0.565
CD4+
0 ug aCD3 STI tx
Event Count: 13108
C
—STI
+STI
10 µg aCD3
1 µg aCD3
0.1 µg aCD3
0 µg aCD3

Figure 3.5 – Imatinib decreases activation of CD4+ cells stimulated with anti-CD3 and anti-CD28. C57B/6 splenocytes were treated with 10 μM STI-571(imatinib mesylate) or vehicle only for 30 minutes prior to stimulation with decreasing concentrations of anti-CD3 and anti-CD28. (A) Proliferation was assessed using ^{3}H-thymidine incorporation. (B) IFN-γ production was assessed using intracellular cytokine staining and visualized by flow cytometry. (C) Rate of cellular division was assessed on day 3 using CFSE dilution and visualized by flow cytometry.

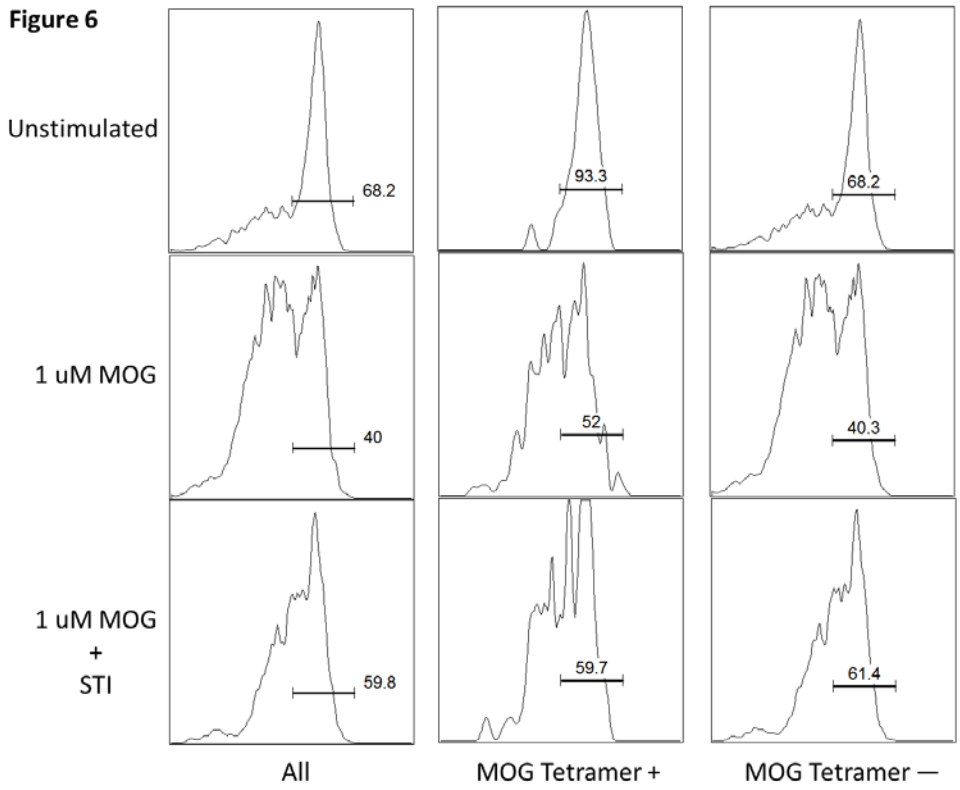


Figure 3.6 – Imatinib differentially affects high and low affinity T cell populations.

C57B/6 splenocytes specific for MOG_{35-55} were treated with 10 μM STI-571(imatinib mesylate) or vehicle only for 30 minutes prior to stimulation with 1 μM MOG. On day 3 after peptide stimulation, cells were stained with MOG:I-A^b tetramer to visualize high and low affinity T cell populations and CFSE dilution was assessed by flow cytometry.

Chapter 4: Discussion

T cell activation is critical to the development of effective cellular immunity against invading pathogens, as well as a key component instigating the development of T cell mediated autoimmune diseases. While there are a number of parameters that may influence the outcome of the interaction between a T cell and an activating antigen presenting cell, the parameters affecting the potency of the pMHC antigenicity has been called to the forefront of T cell biology. Understanding how the quality and quantity of the activating pMHC, as well as the downstream signaling events after TCR ligation, can be channeled by the responding T cell for maximal effector function may be key in increasing responses to chronic infections, infiltrating tumors or dampening responses to ubiquitous self-antigens during autoimmune disease.

Recently, many groups have attempted to further understand how the T cell receptor (TCR) can integrate slight variations in antigen potency for accurate ligand discrimination for an appropriate response. There is ample evidence that affinity of the TCR ligand can affect the kinetics of downstream signaling [30, 77, 242, 255, 264], and also that the dose of inciting antigen can affect the quality of the response [30, 253, 259, 263]. Here, utilize two transgenic $CD4^+$ T cells, 2D2 and SMARTA, to we compare the low affinity, myelin self-antigen MOG_{35-55} to the high affinity, LCMV epitope gp_{61-80} and understand how these types of ligands can affect T cell activation kinetics.

The 2D2:MOG system has been used extensively in the literature to induce murine experimental autoimmune encephalomyelitis (EAE) [102, 132, 134, 238-240]. Consistent with the literature, we show that although the 2D2 TCR is of extremely low affinity for MOG (>10,000 times less than that of SMARTA:gp61), this antigen is still effective in inducing T

cell proliferation and cytokine production (Chapter 2 and 3). In comparison to the high affinity SMARTA:gp61 interaction, however, the activation of 2D2 T cells is significantly delayed. The kinetics of SMARTA TCR signaling were rapid – achieving peak phosphorylation of p38 (1 minute), Erk (30 minutes), and Jun (3 hours) – and corresponded to fast CD69 and CD25 upregulation (3 and 6 hours, respectively). Comparatively, 2D2 stimulation with MOG resulted in altered signaling kinetics with no phospho-Erk or phospho-p38 accumulation, significantly delayed c-Jun phosphorylation (12 hours), and delayed but sustained SHP-1 activity, as well as delayed CD69 and CD25 expression (12-24 hours) (Chapter 2). Importantly, an internal control for 2D2 T cell activation (the higher affinity neurofilament peptide NFM_{18-30}) showed that this delay was not intrinsic to the 2D2 T cells, as the more potent antigen (with a >100-fold increase in effective 2D affinity) could induce rapid response kinetics, in line with those identified for SMARTA:gp61 stimulation (Chapter 2). Taken together, these data highlight the potential disparity in T cell activation by high affinity pathogenic antigens and lower affinity self-peptides that could potentiate autoimmune disease.

One important caveat to this and other data regarding T cell behavior and signaling profiles is that most of the work done to date has utilized transgenic models of T cell activation, where the data is difficult to extrapolate to other T cell clones or to polyclonal populations of cells. For example, 2D2 T cells are only one of many T cell clones that may influence EAE during a polyclonal response, as our lab has previously shown that there are numerous affinities present during a polyclonal response, including T cells that bind to MOG:I-A^b tetramer as well as low affinity cells that are not detectable by tetramer staining [36]. Further, the effective 2D affinity of 2D2:MOG interactions is much lower (a $<10^{-8}$ μm^4)

than that of a polyclonal MOG-specific population ($1.63 \pm 0.48 \times 10^{-5}$ μm^4) (Figure 2.1) and [36]. Therefore, expanding these findings to polyclonal populations of T cells, or utilizing human T cell populations may enable a more complete understanding of how the quality and quantity of antigen can affect an overall T cell response.

Self-reactive T cells mediating autoimmune disease are thought to be lower affinity than pathogen-specific T cells due to various tolerance mechanisms [208, 209]. However, recent work from our lab showed that immunization with the foreign antigen gp61 or the self-peptide MOG resulted in a polyclonal T cell repertoire with a wide breadth of affinities, although the response to MOG included more low affinity T cell clones [36]. The slower kinetics of low affinity activation suggests that higher affinity effector $CD4^+$ T cells may initiate or drive the initial response in early phases of viral infection or autoimmunity, where the amount of antigen available may be low. As viral replication increases or damage to internal organs during an autoimmune response releases more self-antigen, low affinity responders may be subsequently recruited over time as antigen dose increases (Figure 4.1). Importantly, we show that the dose of antigen directs the magnitude of the T cell response without affecting the kinetics of signaling, activation marker expression, or even proliferation of the responding T cells (Chapter 3). These data highlight that activation of a T cell with a highly efficacious peptide at a low dose does not equate to a low efficacious peptide at a high dose. Further, the affinity of pMHC guides the outcome and rate of response in individual T cell clones, while the dose of antigen may drive the affinity profile during an immune response by regulating the span of the responding T cell populations.

In cases where tolerance is broken and low affinity, autoreactive T cells are allowed to mediate an autoimmune response; one would posit that regulatory mechanisms may be

employed to dampen the unwanted response to self. However, the ongoing damage mediated by self-reactive $CD4^+$ T cells during autoimmune disease suggests that these mechanisms are not engaged or are ineffective in controlling low affinity T cells. It is possible that these mechanisms of regulation are suboptimal in autoreactive T cells. While peripheral tolerance may be capable of keeping low affinity autoreactive T cells somewhat controlled [265], several studies demonstrated that these low affinity, self-reactive T cells are less susceptible to central and peripheral tolerance [266, 267]. Others have suggested that failure of self-tolerance is a result of prolonged T cell–APC interactions, while transient interactions and signaling can lead to a tolerant phenotype [67]. Generally, low-potency pMHC results in a decreased frequency of long-lasting contacts with DCs [253], but we see that the time 2D2 T cells are allowed to interact with APCs loaded with MOG antigen plays an important role in allowing for an accumulation of signaling intermediates and full activation of the T cells (Figure 2.6). This begs the question, how do some self-reactive T cells create stable, long-lasting contacts with an APC to initiate activation?

Obviously, there are models of autoimmunity, like 2D2-intitated EAE, where the T cells are allowed enough time to engage appropriate APCs and initiate an autoimmune response. However, the original papers describing 2D2 TCR transgenic mice highlight the low incidence of spontaneous experimental autoimmune encephalomyelitis (EAE) (4%), with slightly higher incidence the alternative form of EAE, called optic neuritis (30%) in these mice [132, 268]. Interestingly, the density of MOG can differ among different areas of the CNS [132] and that the higher affinity neuronal antigen NF-M is found in the grey matter of the CNS as well as found in the peripheral nervous system [269, 270]. It is plausible that differing densities and potencies of self-antigens present during an autoimmune response

may impact the initiation and potentiation phases of autoimmune disease, and these differences may allow for a more permissive environment for low affinity T cells to engage APCs for an extended time.

In addition to the requirement for prolonged TCR:pMHC contact time in low affinity interactions, it has been noted that a T cell recall response required a substantially shorter duration of antigen availability compared with the respective primary response in those cells [63]. It would be expected that a similar delay in low affinity T cells signaling would accompany a recall response when compared to high affinity interaction; however, as memory T cells are more readily activated than naive T cells, this may alter the kinetics of the low affinity interaction. Previous studies support the idea that TCR-associated signal transduction components are modified in memory T cells as compared with naive T cells [271-273], and that continued antigenic stimulation had a negative impact on memory development [63]. Based on this limited data, it would be interesting to continue studying the impact of low or high affinity interactions on signaling components in recall responses and long-term memory T cells.

In addition to expanding the understanding of how antigen potency may affect signaling at different points in the life cycle of a T cell, there are multiple branches of T cell signaling that come in to play at different points during the signaling cascade that should be further explored. We show that there are differential effects of low affinity interactions on various signaling intermediates, in that phosphorylated Erk and p38 failed to accumulate while there was delayed activation of others (ie, c-Jun and the negative regulator SHP-1). While we focused on the canonical T cell signaling intermediates, Erk and p38, there are many other important mediators of T cell activation and inactivation that may be interesting to evaluate.

For instance, peripheral T cells from mice deficient in SHP-1 show increased IL-2 production as well as prolonged activation of Lck and Fyn kinases and overall increases intracellular tyrosine phosphorylation [274]. We have shown previously that SHP-1 plays a role in controlling an autoimmune response to MOG [102], so understanding the effects of delayed, but sustained SHP-1 on T cell signaling may be an important advance in controlling autoimmune disease.

Understanding how signaling intermediates interact and how altering the kinetics of their interactions affects the final outcome in regards to effector function is the next logical step in exploring the kinetics of T cell activation. Currently, there are many reports of spatial features necessary for signaling propagation; however, uncovering the fourth dimension of time may be more difficult with the myriad interactions that take place in one given interlude. Breaking apart the positive and negative signaling loops may allow for an initial evaluation of how time affects the overall signaling pathways. One may explore how the kinetics of the Erk/SHP-1 feedback loop are affected by the delayed, but sustained SHP-1 activity and how this may be enhanced for better control of EAE. However, other signaling intermediates that interact with negative mediators of T cell signaling, like Fyn, which preferentially interacts with negative regulators, such as the PAG/Csk [275] and SAP/SLAM [276] may also hold some clues as to how the kinetics of activation are just as important as the players involved in signaling.

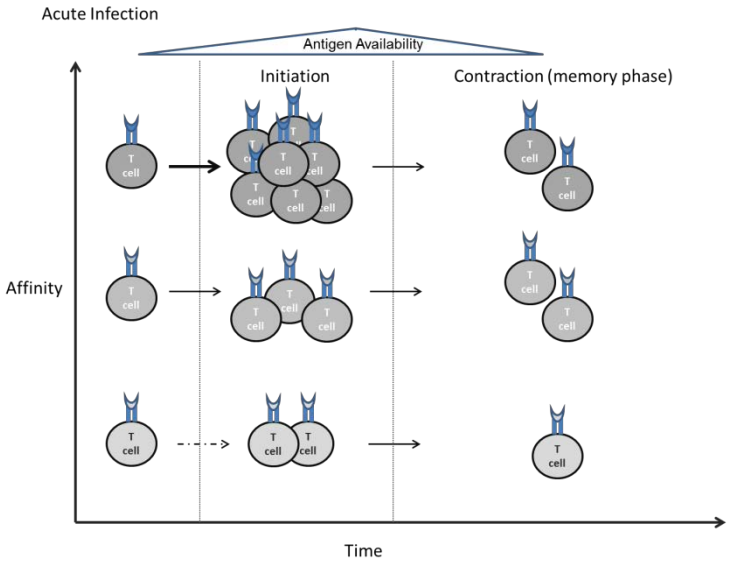
Some have suggested that the primary role of Fyn is to dampen T-cell responses, while Lck acts to amplify them; however, others show that Fyn can provide at least partial signals during stimulation, through partial phosphorylation of LAT to separate the activation of the ERK pathway from the inositol phosphate/protein kinase C pathways [76] or that Fyn plays a

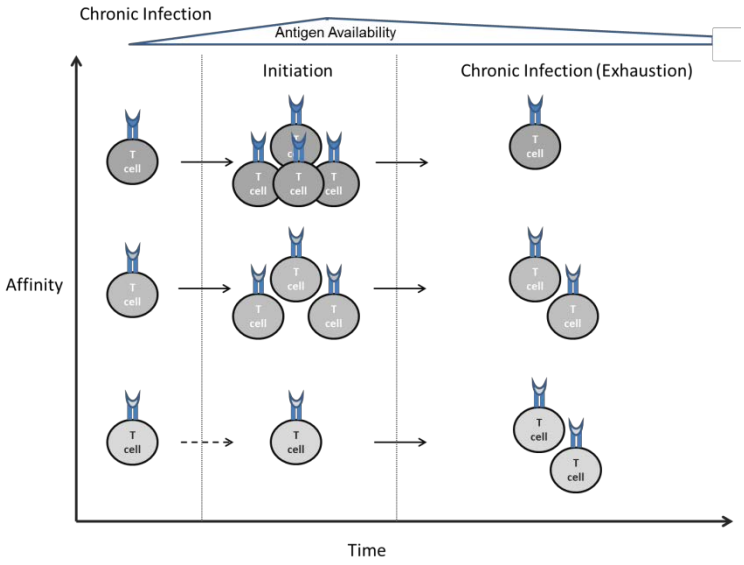
role during thymocyte development and is required for optimal responses to low affinity/avidity ligands [277]. It is tempting to posit that the kinetics of the inciting antigen may induce differential outcomes of Fyn activation, based on the contact time of the TCR:pMHC complex.

The slower kinetics of low affinity activation suggests that higher affinity effector $CD4^+$ T cells may initiate or drive the initial response in early phases of viral infection or autoimmunity, where the amount of antigen available may be low, and as antigen load increases during viral replication or damage to organs during an autoimmune response, low affinity responders may be subsequently recruited. Here, we show that the dose of antigen can direct the magnitude of the T cell response without affecting the kinetics (Chapter 3) and the affinity of pMHC guides rate of response in individual T cell clones. This suggests a differential regulation of the affinity profile during an immune response by via the quality and quantity of antigen, which controls the scope of the responding T cell populations during various stages of an immune response (Figure 4).

In line with a deeper understanding of how the kinetics of a T cell response can affect the outcome in low versus high affinity interactions, we have shown that kinase inhibition is more effective in reducing T cell activation at lower doses of stimulating antigen. Further, in a polyclonal T cell population specific for the self-peptide MOG_{35-55}, low affinity T cell clones are more sensitive to kinase inhibition than high affinity T cell clones at a given pMHC density in vitro. Harnessing this increased sensitivity of low affinity T cells to various therapies targeting signaling intermediates may allow for a targeted approach to controlling low affinity T cells during an autoimmune response. In fact, a recent study has shown IFN-beta therapy can induce compensatory changes in Ca^{2+} influx kinetics and lymphocyte K^+

channel function in MS patients, pushing the kinetics of response to a profile more similar to those of healthy individuals [278]. Exploring how the current MS immunotherapies may affect the kinetics of T cell signaling and resulting effector function could create a baseline knowledge of altered signaling profiles in FDA approved therapies and a rationale for further development of agents that may differentially target a slower, or smoldering T cell response over the more robust responses seen with high affinity T cells important for pathogen clearance and effective immunity against foreign invaders. Inhibition of low affinity T cell clones by targeted therapy may allow for an altered affinity profile that may be more amenable to intrinsic regulatory actions of the immune system and dampen or even halt the progression of disease and organ damage that is a hallmark of inflammatory autoimmune diseases.


Acute Infection
Antigen Availability
Initiation
Contraction (memory phase)
Affinity
Time


Chronic Infection
Antigen Availability
Initiation
Chronic Infection (Exhaustion)
Affinity
Time

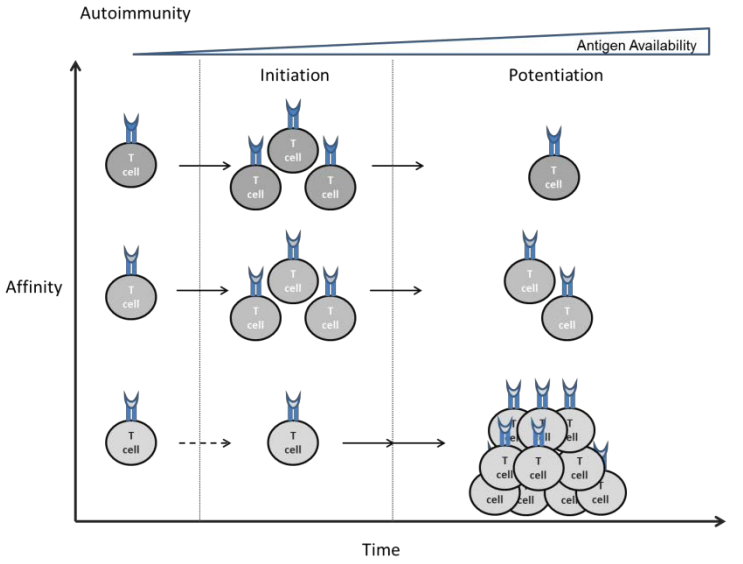

Autoimmunity
Antigen Availability
Initiation
Potentiation
Affinity
Time

Figure 4.1 – A model of polyclonal T cell population development over time. (A) High affinity effector $CD4^+$ T cells drive the response to an acute viral infection. (B) High affinity effector $CD4^+$ T cells initiate the initial response in early phases of viral infection, but a larger range of TCR affinities remain with extended antigen exposure, although these remaining T cells become exhausted. (C) High affinity effector $CD4^+$ T cells may drive the initial response to self-antigen, but low affinity responders are recruited over time as antigen dose increases during the autoimmune response.

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