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CD4+ T cell affinity in autoimmunity and viral infection

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CD4+ T cell affinity in autoimmunity and viral infection

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Abstract

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By Joseph J. Sabatino, Jr.

Cellular immunity is mediated by engagement of the T cell receptor (TCR) with specific peptide:MHC (pMHC) antigen leading to the induction of T cell effector functions. The affinity of the TCR for antigen is believed to be a critical determinant for antigen-specificity. The existing models of clonal selection and affinity maturation posit that the highest affinity T cells dominate in a polyclonal T cell response. However, the minimal affinity required for T cell activation is unknown, therefore it is unclear to what extent low affinity T cells contribute to polyclonal T cell responses. Moreover, although it is postulated that T cells specific for self- and foreign-antigens differ in their respective affinities due to tolerance, it is unclear whether this is in fact the case. The answers to these vital questions have been hindered by the lack of sensitive techniques for the measure of individual T cells that comprise a polyclonal antigen-specific T cell population. Using a highly sensitive two-dimensional (2D) binding assay, the affinities of CD4+ T cells specific for central nervous system (CNS)-derived antigen and a viral-antigen were compared. These studies revealed that the autoreactive CD4+ T cells were overall of lower affinity than those specific for viral-antigen, underlying their differences in detection by pMHC II tetramers. Remarkably, the majority of CD4+ T cells in CNS autoimmune disease were cross-reactive for myelin- and neuronal-antigens, yet were primarily of low affinity. However, an extensive degree of T cell affinity diversity was observed in all polyclonal CD4+ T cell responses, suggesting that a range of low to high antigen-specific T cells is a hallmark of cellular immunity. These findings indicate that polyclonal CD4+ T cell responses are larger than previously believed and challenge the existing paradigms of T cell affinity in immunity. T cell affinity may therefore play a crucial role in the initiation, duration, and modulation of polyclonal T cell responses, which may critically alter the outcome and treatment of autoimmunity and infection.

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Chapter 1: Introduction

T cell antigen recognition is highly specific, but degenerate. T cells comprise the cell-mediated arm of adaptive immunity. Each T cell bears a clonotypic T cell receptor (TCR) that engages with a specific antigen. T cell antigens are composed of a specific peptide, which can be either self- or foreign-derived, that is presented by self-major histocompatibility complex proteins (MHC) on the surface of antigen presenting cells (APCs). Peptides bind to MHC molecules using certain anchor amino acids that provides sufficient stability and the proper orientation for TCR recognition [1] (Figure 1.1). Other amino acids are involved in TCR contact, with typically 3-4 amino acids required for TCR:pMHC recognition. One residue, termed the primary TCR contact, is almost always the most critical for a given antigen determinant [2, 3]. The other secondary contact residues are more permissive to changes.

Recognition of peptide:MHC (pMHC) antigen by a specific T cell elicits various effector functions from the T cell, including proliferation, cytokine release, and cytolysis. The exquisite specificity that T cells possess for antigen was demonstrated by showing that alteration of a single amino acid of a given epitope drastically alters T cell responsiveness [4-6]. Despite their fine specificity, these landmark studies also revealed that T cells exhibit a remarkable degree of reactivity to a number of different antigenic ligands, including both homologous [7-9] and non-homologous antigens [10-12]. T cell cross-reactivity, or degeneracy, is a reflection of the fact that T cell activation is not simply a binary event, but rather a spectrum of outcomes that require varying degrees of antigenic ligand strength through the TCR. Indeed, TCR ligation by pMHC can lead to a range of outcomes, including full activation, partial activation (e.g. cytokine secretion in

the absence of proliferation), anergy (persistent unresponsiveness to antigen stimulation), antagonism (inhibition of activation when agonist is simultaneously present), or no activation (i.e. null response) [3, 13].

The term altered peptide ligand (APL) was coined to describe antigen peptide analogues where the TCR contact residues have been changed [3] (Figure 1.1). APLs preserve the interaction with the MHC molecule, but prevent T cell proliferation while still being able to stimulate some effector functions, such as cytokine release or cytolysis [5, 6, 14, 15]. Agonists (i.e. wild-type antigen) represent a perfect fit between the pMHC ligand and the TCR, which allows for the activation of all downstream signaling pathways. Alteration of the primary contact residue typically results in a null response [2]. Partial agonists are generated by a conservative substitution of a secondary contact residue, which results in incomplete signal transduction [3, 13]. This can result in partial activation, such as cytokine production in the absence of proliferation. In some cases, partial agonists may act as antagonists, depending on the dose of antigen. Antagonists are peptides that are alone non-stimulatory, but completely block responsiveness of T cells when simultaneously presented with agonist [5]. Antagonists are generated by non-conservative substitution of a secondary contact residue resulting in unique signal transduction [3]. In certain cases, peptide analogs with greater stimulatory capacity than the wild-type antigen can be achieved. These are termed heteroclitic antigens [16] or superagonists and typically exist only for lower affinity T cells. Functional responsiveness, which is defined as the amount of antigen to achieve half-maximal activation [17], is therefore directly related to the strength of antigenic ligand stimulation. Thus, superagonists can shift the antigen dose curve so that T cells are fully activated at

doses lower than agonist, and can induce antigen-induced cell death (AICD) at concentrations that are agonistic for weak ligands.

In contrast to TCR contacts, a number of different MHC anchor residues (termed motifs) are capable of binding a given MHC allele. Despite the promiscuity of peptide:MHC binding, certain anchor motifs predominate among the various peptides bound to a particular MHC allele [18-20]. Thus, polymorphisms within the peptide binding groove enable MHC allelic variants to present different pools of antigens. Like TCR contacts, alteration of MHC anchor residues leads to various outcomes of T cell activation [21-24]. Although many groups use the term "APL" to refer to all peptide modifications, changes of MHC contacts are a distinct subset of analogs and are therefore referred to as MHC variant peptides or MVPs (Figure 1.1). These nomenclature differences are not trivial, however, as APLs and MVPs can lead to vastly different outcomes on polyclonal T cell responses. MVPs selectively alter the affinity of peptide:MHC binding with minimal effect on TCR:pMHC binding [25]. However, this changes the pMHC half-life, which alters the threshold for activation through the TCR [26]. As a result, antigenic ligand strength can be increased by increasing the affinity between peptide and MHC [16], while decreasing peptide:MHC affinity decreases stimulation strength [27].

T cell receptor affinity for antigen. The degree of T cell activation is in fact a reflection of the T cell receptor binding dynamics to pMHC antigen. As in all receptor-ligand interactions, these dynamics are time-dependent and are quantified according to three different parameters: on-rate, off-rate and affinity. The probability of TCR and pMHC forming a complex increases over time until equilibrium (or steady-state binding)

is reached. The on-rate (k_{on}) is a reflection of the amount of time required for TCR and pMHC to form a complex and is proportional to the initial slope of the curve. The off-rate (k_{off}) is inversely proportional to the time required to half steady-state binding. Affinity (K_a or its inverse, K_d) is perhaps the most commonly quantified parameter and is a measure of equilibrium.

Intrinsic TCR affinity is a thermodynamic parameter based on equilibrium binding (where $K_a = k_{on}/k_{off}$ and $K_d = k_{off}/k_{on}$) of the TCR and peptide:MHC (pMHC) [17] and can be measured by soluble or three-dimensional (3D) binding analysis, such as surface plasmon resonance (SPR). The earliest 3D studies revealed that the TCR has a strikingly low affinity for pMHC due to its relatively fast off-rate (Table 1.1) [28-33]. As a comparison to other receptor:ligand interactions, biotin:streptavidin binds with an extremely high affinity due to its very low k_{off} [34-36], while antibody:antigen interactions are somewhat lower affinity, but still overall very high (Table 1.1) [37]. In contrast, selectin:ligand [38] and LFA-1:ICAM-1 [39] interactions have more comparable 3D kinetics to that of TCR:pMHC (Table 1.1), a striking finding given the remarkable antigen specificity of the TCR. Most 3D studies have found no correlation between TCR affinity for pMHC and response potency [32, 33], but more potent antigen ligands correlated with longer TCR:pMHC half-lives (i.e. slower off-rates) [40-42]. Although large-scale 3D measurements of TCR:pMHC kinetics have indicated a strong correlation between on-rates and off-rates and ligand potency, the range of 3D off-rates was rather narrow [43]. Nonetheless, 3D TCR affinity is likely related to antigen potency as it has been found that increasing TCR affinity for pMHC results in increasing T cell responsiveness [44].

While pMHC tetramers are used for the identification of antigen-specific T cells [45], their binding is dependent on TCR avidity, a multivalent interaction that is likewise affected by TCR:pMHC kinetics [46-48]. Indeed, tetramers can be used to measure TCR kinetics. Scratchard analyses allow the measurement of TCR affinity (measured in K_d), while decay analyses can be used to measure off-rate. By comparing pMHC monomers, dimers, and tetramers, it was determined that each successive increase in antigen valency resulted in much stable complexes and slower dissociation rates [49]. However, a caveat of tetramer dissociation studies is that a competitive blocking agent (usually an anti-MHC antibody) must be used to prevent re-binding of the tetramer. A detailed methods analysis revealed that the dissociation kinetics should be unaffected by the blocking agent, a criterion that can only be met by using an anti-MHC Fab fragment [46]. It has been clearly demonstrated that the intensity of pMHC tetramer staining is directly correlated to TCR levels [50-52] and the affinity of the TCR for pMHC multimer [48-50, 53-63]. Thus, T cells with a high affinity for the tetramer will exhibit a higher MFI than those with a lower affinity. However, the affinity requirements for stable tetramer binding by a T cell are unclear, although it has been shown that CD8+ T cells require a $K_d \leq 40$ μM in order to be detectable by pMHC I tetramers [48, 64]. This therefore suggests that lower affinity T cells may be undetectable by tetramers [48, 65]. As such, tetramers would be unable to measure the affinity of T cells with insufficient TCR affinity.

In contrast to 3D analyses, which measure receptor-ligand binding in solution, two-dimensional (2D) techniques measure the TCR:pMHC interactions in the cell membrane-anchored context [66]. 2D binding can be measured in a number of different

ways, including fluorescence-based (e.g. Förster resonance energy transfer (FRET)) and mechanical-based (e.g. micropipette adhesion frequency) assays. Strikingly, 2D TCR:pMHC kinetics differ markedly from 3D kinetics. Whereas the 3D kinetics are low and slow, 2D TCR:pMHC measurements revealed much higher affinities, on-rates, and off-rates [67, 68]. Moreover, using the OT-I CD8+ TCR transgenic and a range of APLs varying in potency, it was shown that all three parameters of 2D binding were directly related to T cell functional outcome [67]. Thus, agonist pMHC have a higher 2D affinity, on-rate, and off-rate than weaker ligands, in contrast to 3D off-rate being inversely related to T cell responsiveness.

The differences between 2D and 3D TCR:pMHC dynamics are striking, yet the cause of the differences is not entirely clear. It is possible that TCR clustering on the T cell membrane, which has been suggested to occur via cholesterol and/or actin cytoskeleton interactions [69-71], impacts binding of the TCR to pMHC antigen. Evidence for this is suggested by the findings that depletion of cholesterol from the T cell membrane or actin depolymerization result in a decrease in 2D binding [67, 68]. Thus, it is possible that clustering of the TCR may enable cooperative binding, in which binding of one TCR in a cluster increases the likelihood of binding (and thus, increases the apparent affinity) of the other members of the TCR cluster. Regardless of whether 2D TCR:pMHC measurements are due to cooperativity, 2D assays provide a direct measure of the natural cellular environment of TCR:pMHC interactions and are thus more physiologically relevant.

The most indirect measure of T cell affinity is functional avidity or responsiveness, which, as previously described, is based on the sensitivity of a T cell

population to antigen. T cell receptor affinity is directly correlated with functional responsiveness and the degree of tetramer binding [53, 72]. Recently, it was shown that 2D TCR:pMHC binding kinetics were directly correlated with functional responsiveness [67]. However, functional avidity is also influenced by other protein interactions independent of TCR:pMHC interactions. For instance, a 50-fold increase was found in the functional avidity in the early stage of viral infection without the selection of higher avidity TCR clonotypes [73]. The increased functional avidity occurred in monoclonal T cells, suggesting TCR signaling-mediated changes in responsiveness, also known as tuning. Another study showed that the increase in TCR avidity following T cell activation was due to TCR reorganization and changes in the cholesterol content of the T cell membrane [74]. In addition, T cells can upregulate their expression of adhesion molecules, to result in increased T cell adhesion to APCs and thereby alter their functional responsiveness [75, 76]. Thus, although functional avidity is directly correlated with TCR affinity, it is not simply a direct reflection of TCR binding dynamics because of the effect of the molecular interactions downstream of TCR signaling.

Differences between the co-receptors of CD4+ and CD8+ T cells. In addition to the interaction of the TCR to the pMHC complex, the CD8 and CD4 co-receptors on the T cell surface dock with MHC I and MHC II, respectively. The co-receptors are vital as they interact with Lck on their cytoplasmic domains, which allows induction of T cell signaling following TCR:pMHC ligation [77-80]. Despite these similar roles, the two co-receptors vary significantly in their affinities for their respective MHC partners. On the one hand, the CD8 co-receptor has a low, but measureable 3D affinity for MHC I (Table 1.1) [33, 81, 82]. This has been validated by 2D affinity analysis where CD8 can bind

certain MHC I alleles with affinities on par with low affinity TCR:pMHC I interactions [83]. In contrast, the CD4 co-receptor has an undetectable 3D affinity for MHC II (Table 1.1) [84-86], despite the fact that it is known to bind MHC II [87-89]. For example, the comparative difficulty of detecting antigen-specific T cells by pMHC II versus pMHC I tetramers has been attributed to the differences in CD4 and CD8 binding [65]. Indeed, it has been demonstrated that CD8 binding can compensate for low affinity TCRs to facilitate CD8+ T cell responsiveness and pMHC I tetramer binding [44, 64, 73, 79, 90-92], whereas numerous studies have suggested that CD4 plays no role in pMHC II tetramer binding [48-50, 54, 93].

Diversity of the polyclonal T cell repertoire. Thus far, the dynamics of T cell:antigen interactions have been discussed. In order for a T cell response to be mounted, however, T cells specific for a multitude of potential antigens must be generated. The total size the of the mature αβ TCR-expressing T cell repertoire in the periphery of adult mice is estimated to be 8.5×10^7 cells [94] and the human T cell repertoire is estimated to be up to 10 times that amount [95]. Given that approximately 2 million different TCR clonotypes have been sequenced in adult mice [96], approximately 30 copies of each naïve T cell clone is estimated to exist on average [94]. Tetramer-based methods have estimated that the average naïve T cell frequency varies from 1 in 10^5-10^6 of the total T cell population [94, 97]. T cell repertoire diversity has been demonstrated in a variety of polyclonal immune responses [54, 61, 98], but it can vary greatly from one response to another. Anywhere from 5 to 500 different T cell clones have been estimated to participate in a polyclonal immune response upon encounter with a given antigen [61, 99-101]. Polyclonal T cell responses specific for both pathogen- and self-antigens

demonstrate a remarkable degree of diversity for their respective antigens. For instance, numerous TCR clonotypes have been found in a variety of viral infections, including CMV [91, 102], influenza [103], and EBV [91]. In addition, T cell diversity has been demonstrated in polyclonal myelin-specific T cell responses [104-106].

The models of clonal selection and affinity maturation contend that only those clones with TCR specificities for the inciting antigen are stimulated to expand and become the dominant population of effector cells during infection [102, 107]. The specificity of the TCR for its cognate antigen is in fact a reflection of its affinity for pMHC antigen and thus, only those T cell clones with a sufficient affinity for antigen are activated. Despite this affinity threshold, T cells possessing variable affinities for antigen have been suggested to comprise polyclonal T cell responses. As expected, high affinity clonotypes are present at high frequencies during immune responses [53, 54, 56, 61, 91, 102, 108-111]. Moreover, the T cell repertoire is believed to narrow upon secondary challenge, as the re-stimulated T cells have been reported to have higher affinities [54, 56, 61, 98, 107, 112]. However, even T cells with low functional avidity have been shown to expand and elicit effector functions [110, 111]. It has been suggested that increased functional TCR diversity provides a competitive advantage that increases immune protection [108] by preventing viral escape variants from emerging during infection [113-115] and contributing to heterologous immunity [116-118].

As described previously, the increased affinity in T cell responses is not only due to the competitive advantage of high affinity T cells, but also to the increased TCR binding affinity of a given activated T cell clone [73-76]. The resulting expanded antigen-specific T cell repertoire is therefore composed of a selected set of T cell

clonotypes, where the highest frequency of responding T cells are those with the greatest functional responsiveness. As a result, functional responsiveness is an average affinity of the most dominant clones, and therefore does not reflect the range of T cell affinities comprising a polyclonal response.

Self-reactive T cells can mediate autoimmune disease, such as multiple sclerosis. Numerous autoimmune diseases have been demonstrated to be induced by T cells specific for self-antigens, including type I diabetes, rheumatoid arthritis, and multiple sclerosis (MS). MS is a chronic neurological disease resulting from an inflammatory process directed against the central nervous system (CNS). The disease process is mediated primarily by autoreactive CD4+ T cells that target oligodendrocytes, the myelin-producing cells of the CNS [119]. The ensuing widespread demyelination and mulitfocal plaques result in the characteristic symptoms of MS, including vision loss, sensory disturbances, limb weakness, and gait instability. Several well-characterized animal models, including experimental autoimmune encephalomyelitis (EAE), have been used to further our understanding of the intricacies of MS [120, 121].

EAE is induced by immunization of disease susceptible animals with myelin antigens in adjuvant [120, 122]. A number of different myelin antigens are used for different strains of animals. Mice are by far the most commonly used model, due to their rapid maturity, existence of a wide array of gene knockouts and transgenic knockins, and cost effectiveness. Three myelin antigens, proteolipid protein (PLP), myelin basic protein (MBP), and myelin oligodendrocyte glycoprotein (MOG) are the most commonly used in EAE (see Table 1.2) and reflect the most prevalent myelin-specific T cell responses in MS [119]. 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 an ordinal scale of 1) tail paralysis; 2) hind limb ataxia; 3) single hind limb paralysis; 4) complete hind limb paralysis and inability to right; and 5) moribund. A typical disease course for MOG_{35-55}-induced EAE is shown in Figure 1.2. 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 (also called adoptive transfer). Active immunization is carried out by subcutaneous injection of myelin peptide emulsified in complete Freund's adjuvant (CFA) containing heat-inactivated *M. tuberculosis*. In addition, intraperitoneal injection of pertussis toxin is usually given as an added adjuvant. Priming of myelin-reactive CD4+ T cells in the periphery leads to their trafficking across the blood-brain barrier (BBB) where they elicit a pro-inflammatory response to APCs presenting myelin antigen. Passive transfer involves stimulation and expansion of myelin-reactive CD4+ T cells *in vitro*, which are then injected i.v. into naïve syngeneic hosts. This therefore bypasses the peripheral activation step in EAE and leads to more rapid T cell infiltration of the CNS. Classic EAE, as it is sometimes known, involves extensive myelin-reactive T cell infiltration of the meninges and the spinal cord, with little involvement of the brain parenchyma [123]. *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 [124, 125].

In addition to damage to the myelin sheath, extensive axonal and neuronal injury have been reported in MS and EAE. One such target are 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 [126] and NF-L can induce EAE in a subset of mice [127]. In addition, autoantibodies to the neurofilament medium chain (NF-M) have also been detected in MS patients [128]. While immunization with NF-M does not induce active EAE in mice, it was recently reported that MOG_{35-55}-reactive CD4+ T cells are also cross-reactive to NF-M_{15-35} antigen in EAE [129].

Pro-inflammatory cytokines are a hallmark of MS and EAE. In both MS and EAE, IFNγ production by autoreactive CD4+ T cells has been implicated as a key mediator in disease pathogenesis. This pro-inflammatory role has been substantiated by numerous data including, exacerbations of MS symptoms following IFNγ treatment [130, 131], IFNγ production by CD4+ T cells (MS and EAE) re-stimulated with myelin antigens [132], abundant IFNγ-producing CD4+ T cells in peripheral lymphoid organs and CNS in MS and EAE [119, 133-136], correlation of peak IFNγ levels with peak of disease symptoms in MS [137] and EAE [132, 134, 136, 138, 139], and the induction of EAE by adoptive transfer of Th1, but not Th2 myelin-reactive T cells [140, 141].

While IFNγ clearly has strong pro-inflammatory effects on the immune system, this cytokine also has similarly potent down-regulatory effects. Loss of IFNγ has been shown to increase CD4+ T cell proliferation [142-144] as well as inhibit CD4+ T cell apoptosis [145, 146]. Although Th1 cells downregulate the IFNγR2 subunit [147, 148], IFNγ can mediate its effects indirectly by inhibiting T cell proliferation via the induction

of nitric oxide (NO) and indoleamine 2,3-dioxygenase (IDO) by APCs [149-151]. It has also been demonstrated that IFNγ can convert effector CD4+ T cells into regulatory Foxp3+ CD4+ T cells (Tregs) [152]. Such findings indicate that although IFNγ causes inflammation in the initiation of the immune response, it subsequently self-limits Th1-mediated inflammation during the effector phase of the response.

Consistent with these regulatory effects, IFNγ has been shown to have a strong anti-inflammatory role in EAE. For instance, treatment with anti-IFNγ mAb enhances EAE severity [153-157]. Likewise, studies have shown that IFNγ -/- and IFNγR -/- mice remain susceptible to EAE induction [145, 152, 158-160]. Interestingly, IFNγ neutralization decreases EAE incidence in IFNγR -/- mice, suggesting the possibility of an alternate IFNγ receptor [161]. Moreover, deficiency of IFNγ or its receptor has been shown to convert EAE-resistant mice to a susceptible phenotype [144, 149, 162]. These effects appear to be IFNγ signaling dependent as STAT1 -/- mice develop more severe EAE, whereas T-bet -/- mice are resistant to EAE [163, 164]. These results indicate that Th1 development, but not IFNγ signaling, is required for EAE induction and suggests an anti-inflammatory effect of IFNγ in this disease process.

The ubiquitous distribution of the IFNγR no doubt underlies the complexity of this highly pleiotropic cytokine. As a result, IFNγ exerts its effects on cells outside the immune system and the central nervous system (CNS) is a very important target of IFNγ in EAE. Like the immune system, IFNγ exerts both pro- and anti-inflammatory effects in the CNS. The pro-inflammatory effects of IFNγ are well-known and include increased blood-brain barrier permeability, chemokine induction, and expression of MHC I and II by glial cells [144, 165]. Interestingly, IFNγ has also been shown to protect mature

oligodendrocytes from immune-mediated damage [166, 167]. Given these pleiotropic effects, loss of IFNγ or IFNγR may facilitate the induction of EAE through not only the increased responsiveness of autoreactive CD4+ T cells, but also increased oligodendrocyte apoptosis with subsequent release of myelin antigens.

That IFNγ was not required for the induction of EAE pointed to the role of another pro-inflammatory cytokine in disease pathogenesis. The missing puzzle piece was found several years ago with the identification of IL-17-producing CD4+ T cells (termed Th17) as a key encephalitogenic mediator of EAE pathogenesis [168-171]. There appears to be a similar role for Th17 cells in MS [172-175]. For example, higher IL-17 production over IFNγ is required for CD4+ T cell infiltration into the brain parenchyma beyond the spinal cord [123].

Not only do IL-17 and IFNγ differ in their roles in EAE, they also mutually inhibit the production of the other [103, 169]. Thus, the increased severity of EAE in IFNγ-deficient mice can be explained by an increase in myelin-reactive Th17 cells. As a result, abrogation of IL-17 [170, 176] or deficits in the Th17 induction pathway ameliorate EAE substantially [177, 178]. Despite the overwhelming data demonstrating a critical role for IL-17 in EAE pathogenesis, the story may be more complicated than initially appreciated as at least one group has demonstrated that IL-17 is dispensible for EAE induction [179]. Instead, it is likely that both myelin-reactive Th1 and Th17 cells are required for EAE pathogenesis. Indeed, several recent studies have demonstrated that EAE pathogenesis is initiated by myelin-reactive Th1 cells, which are subsequently followed by pro-inflammatory myelin-reactive Th17 cells [180, 181].

CD4+ T cells in lymphocytic choriomeningitis virus infection. The critical role of T cells in fighting viral infections has been extensively demonstrated in the lymphocytic choriomeningitis virus (LCMV) model. LCMV is a non-cytopathic RNA virus that gains entry to cell 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 [182]. The resulting mutations lead to an increased predilection of clone 13 for infecting macrophages and higher virus yields, which consequently leads to chronic infection [183].

CD8+ cytotoxic lymphocytes (CTLs) alone can clear LCMV [184], but CD4+ T cells alone cannot [184, 185]. Nonetheless, CD4+ T cells play vital roles, particularly in the prevention of chronic infection [184, 186-190] and for LCMV-specific CTL memory in both acute [191-193] and chronic infections [190, 194]. LCMV-specific CD4+ T cells also aid in the clearance of virus by induction of neutralizing antibodies [194] and by mediating their own cytotoxic effects [195-197]. 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 the Armstrong and clone 13 strains of LCMV [198-201]. The nucleoprotein $(NP)_{309-328}$ epitope is a minimal secondary epitope in the Armstrong, but not the clone 13 model [198-201].

Central tolerance of myelin-specific T cells. Positive selection of immature thymocytes requires that all T cells must have at least a minimal responsiveness to self-antigen. However, developing thymocytes with a high affinity for self-antigen are deleted in order

to prevent autoimmunity. Nonetheless, low affinity interactions with self-antigen in the thymus allow the escape of self-reactive CD4+ T cells into the periphery [202-207], which allows the development of the wide array of T cell-mediated autoimmune diseases. This is demonstrated by transgenic mice expressing neo-self antigens, which results in the deletion or tolerization of high affinity neo-self antigen-specific T cells, but allows the preservation of lower affinity clonotypes [208-212].

The thymic expression of the three primary myelin antigens, PLP, MBP, and MOG, provides an excellent example of the imperfection of central tolerance in preventing autoimmunity. The expression of PLP has been definitively demonstrated in the thymus of mice [213-215] and humans [213]. Numerous studies have also demonstrated the thymic expression of Golli-MBP (expressing the N-terminal half of MBP) in mice [214, 216-218] and humans [213, 219, 220]. To date, MOG protein has not been detected in the thymus, however, it appears that at least MOG mRNA is expressed in the thymus of mice [215, 221] and humans [220, 222, 223]. The thymic expression of other myelin antigens has also been reported, including S100β [215, 222] and αβ crystalline [222]. NF-M mRNA and protein also been reported at low levels in human and murine thymic epithelial cells [224].

While the thymic expression of myelin antigens suggests the likelihood of central tolerance to these antigens, direct proof is variable. Perhaps the best demonstration of central tolerance to myelin antigens is in the case of PLP. Strikingly, a PLP splice variant lacking amino acids 116-150 and known as DM20, is found in much higher levels in the thymus than the full-length native form in both mice [215, 221, 225, 226] and humans [213, 220, 222]. Consequently, the naïve $PLP_{139-151}$-specific CD4+ T cell repertoire is

extremely large in H-2^s mice (estimated at 1 in 20,000) [225]. Furthermore, these findings are correlated with the high susceptibility of SJL mice to EAE induction by $PLP_{139-151}$, as well as its immundominance over the $PLP_{178-191}$ epitope. Thus, lack of central tolerance to this PLP epitope underlies the high antigen-specific precursor frequency and predisposes to CNS autoimmunity.

The degree of MBP-specific T cell deletion appears to be directly related to the amount of MBP expression in the thymus. For instance, higher levels of MBP were found in the thymus of mice resistant to EAE [227]. Studies in MBP -/- mice demonstrated that the loss of MBP allows the development of a high functional avidity MBP-specific CD4+ T cell repertoire [228]. 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 [229]. The degree of tolerance to MBP is directly related to the affinity of MBP antigen to MHC II [230], thus, low affinity MBP:MHC II interactions allowed the escape of low affinity MBP-specific CD4+ T cells [231, 232]. 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 [233].

Several studies have shown that MOG_{35-55} CD4+ T cell responses are more potent and more encephalitogenic when derived from MOG -/- mice on the 129 or B6 backgrounds [234]. However, 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 [106, 221]. Regardless, it appears that tolerance to MOG is at least partially incomplete, as exogenous delivery or expression of MOG_{35-55} in the periphery inhibits CD4+ T cell responsiveness to MOG_{35-55} and protects against EAE [106, 235]. Moreover, 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 [236, 237]. Moreover, no defect in transgenic T cell development was observed when 2D2 mice were crossed to a MOG -/- background [129]. 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.

Affinity differences between foreign- and self-specific CD4+ T cells. The clonal selection and avidity maturation models described previously are primarily based on studies of foreign antigen-specific T cell responses. It is unknown whether this selection of dominant T cell clonotypes also exists for T cells responding to self-antigens, because of the TCR affinity-based mechanisms of central and peripheral tolerance. Negative selection against self in the thymus 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. This postulate has been largely corroborated by a number of different affinity studies. For instance, 3D TCR measurements have suggested that myelin-specific TCRs [58, 238, 239] have lower affinities than viral-specific TCRs [239, 240]. 3D affinity 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 [58]. Interestingly, mice transgenic for the 172.10 clone develop spontaneous EAE at a much higher rate than those bearing the 1934.4 transgene [230,

241-243], suggesting that increased affinity for myelin antigen is associated with increased encephalitogenicity. The fidelity of pMHC II tetramer staining also 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 [244-247], HSV-2 [248], *B. burdgorferi* [249], and *S. typhimurium* [97]. In contrast, many studies have reported poor pMHC II tetramer detection of autoreactive CD4+ T cells in various disease models, including rheumatoid arthritis [62, 250], relapsing polychondritis [251], type 1 diabetes [59, 252], and EAE [253-258].

Most 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 [59]. Other studies in CD8+ T cells have demonstrated lower functional avidities of self-reactive CD8+ T cells versus those specific for pathogen [204, 259, 260]. Many studies have demonstrated the presence of both high and low functional avidity (defined as EC_{50} < 1μM and > 1 μM, respectively) populations in both EAE [57, 171, 261] and MS [105, 262, 263]. 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 [136].

A variation of functional responsiveness methods used to measure T cell avidity in EAE has been based on altering myelin antigen potency. For instance, it has been shown that increasing the expression of MBP_{Ac1-9}:IA^u or the affinity of MBP_{Ac1-9} for IA^u

induces the deletion or hyporesponsiveness of MBP-reactive CD4+ T cells [230, 243, 264] and protects against EAE [265]. A recent study using a TCR retrogenic system with five different MOG-specific TCRs differing in functional avidity (EC_{50} <1 μM to > 10 μM) indicated that the degree of bone marrow engraftment (i.e. T cell frequency) was the primary determinant on encephalitogenicity regardless of functional avidity [261]. A major drawback of this study, however, was that the degree of engraftment was different among the five different clones, thereby making it difficult to determine the role of TCR affinity in EAE. 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.

It is well known that the strength of the antigenic signal through the TCR can affect the phenotype of CD4+ T cells. For instance, it has been shown that antigen dose can profoundly skew CD4+ T cells toward Th1 or Th2 phenotypes [266, 267]. In addition, the strength of signal can affect expression of several surface proteins. It is therefore likely that the affinity of a given T cell for antigen is translated into the signaling cascade downstream of TCR engagement that results in CD4+ T cell effector functions. It also been suggested that affinity for self-antigen can alter Treg development in the thymus [268-270]. This suggests that altering the T helper phenotype may be associated with a certain range of affinities for antigen. It has been shown in numerous studies that stimulation of naïve CD4+ T cells with intermediate doses of soluble peptide antigen (0.05-5.0 μM) leads to the selective induction of Th1 cells [266, 267]. Moreover, numerous studies have shown that increasing the affinity of peptide antigen for MHC

results in Th1 polarization [271-273]. Recently, it was shown that higher functional avidity myelin-reactive CD4+ T cells are preferentially skewed toward a Th17 over a Th1 phenotype [123]. Thus, the affinity composition of self-reactive CD4+ T cells probably influences the cytokine profile of the population, which may significantly impact autoimmune disease pathogenesis.

Myelin-reactive T cell cross-reactivity. As previously described, T cells exhibit a remarkable degree of cross-reactivity to different antigens. It has been suggested that a given TCR clonotype can recognize 1 in 30,000 [274] to 1 in 100,000 [275] unrelated pMHC antigens. Autoreactive T cells, in particular, have been shown to be cross-reactive to a number of different antigens. For instance, APLs of MBP [25, 276] and PLP [225, 277] can activate myelin-specific CD4+ T cells. Cross-reactive pathogen-antigens have been shown in numerous cases to either predispose to EAE induction [278-281] or to induce overt EAE [282-284]. Moreover, human myelin-specific CD4+ T cells isolated from MS patients have been shown to be cross-reactive to a number of microbial antigens [285-292]. Such self-reactive T cell cross-reactivity to foreign-antigens, known as molecular mimicry, has been suggested to be a potential mechanism for breaking T cell tolerance.

Interestingly, autoreactive CD4+ T cells have been found to be cross-reactive to other self-antigens in both EAE [129] and type 1 diabetes [293]. Several studies have demonstrated that MOG -/- myelin or CNS homogenates can activate MOG_{35-55} CD4+ T cells and induce EAE [221, 234, 294], suggesting a role for cross-reactivity to another CNS autoantigen. Moreover, class-switching of anti-MOG antibodies was observed in MOG_{35-55} TCR (2D2) and IgH double transgenic mice crossed to a MOG -/- background

[295]. The cross-reactivity of MOG_{35-55} CD4+ T cells to another CNS self-antigen was recently definitively shown to be against the NF-M_{15-35} epitope [129].

Comparison of peripheral tolerance to regulate autoreactive and pathogen-specific CD4+ T cells. Peripheral tolerance is required to prevent the activation of self-reactive T cells that escape deletion in the thymus. There are at least four different mechanisms that inhibit activated self-reactive CD4+ T cells in the periphery: anergy/exhaustion, phenotype skewing, deletion by apoptosis, and interactions with FoxP3+ regulatory CD4+ T cells (Tregs) [205, 296]. Anergy or unresponsiveness occurs during T cell activation in the absence of B7:CD28 co-stimulation or the upregulation of inhibitory co-stimulatory molecules, such as CTLA-4 and PD-1. Numerous studies have demonstrated that co-stimulation-dependent tolerance mechanisms are defective in MS [297-300] and EAE [301-311]. In addition to regulating persistently stimulated viral-specific T cells, PD-1 also negatively regulates self-specific T cells exposed to constant self-antigen in order to prevent autoimmune disease [312, 313]. Phenotype skewing from a pro-inflammatory Th1 phenotype to an anti-inflammatory Th2 phenotype is associated with recovery in EAE [314]. Recovery from EAE has been shown to be partly dependent on contraction of the encephalitogenic CD4+ T cell population via Fas-dependent mechanisms [315-317]. Lastly, Tregs, which are defined by the FoxP3+ lineage of CD4+ T cells [318], are a crucial aspect of peripheral tolerance by protecting against autoimmune disease [319-321].

In the case of infection, the goal of the adaptive immune response is the selective elimination of the pathogen and all pathogen-infected cells. Clearance of the pathogen (and thus removal of the antigen) leads to down-modulation and contraction of pathogen-

specific T cells and conversion to a resting, memory phenotype. In the case of a persistent, chronic infection, however, activated T cells are either rendered anergic, exhausted, or are deleted by various mechanisms. CD4+ T cell exhaustion in LCMV chronic infection has clearly demonstrated [47, 322, 323]. Functional unresponsiveness of CD4+ T cells occurs in a variety of human chronic infections (e.g. HCV, HIV), by the upregulation of inhibitory molecules like CTLA-4 and PD-1 on persistently activated virus-specific CD4+ T cells [324-328]. Deletion of peripheral T cells by AICD (e.g. Fas-dependent) has also been shown to occur in pathogen-specific CD4+ T cells during chronic viral infection [324, 329]. While downregulation of the immune response is obviously not beneficial for clearance of the pathogen, this is necessary in order to avoid immunopathology and autoimmune disease [330, 331].

Limitations of current treatments for MS. Immunomodulation of T cell responses is the goal of many drugs in use or in testing for MS. Some of these current therapies include administration of Glatiramer acetate (Copaxone), IFNβ (Avonex, Betaseron, Rebif), Vitamin D, steroids, mitoxanthrone (Novantrone), anti-α4 integrin (Tysabri), and FTY720 [332-337]. Interestingly, IFNβ treatment is dependent on IFNγ signaling and only appears to provide therapeutic benefit in Th1, but not Th17 dominated responses in EAE and MS [338]. While these treatments can diminish the frequency and severity of relapses, the mechanisms behind their actions remain relatively undefined with wide diversity of disease outcomes in patients. In general, these therapies act broadly and are not limited to the T cells specific for CNS antigens. Thus, an antigen-targeted therapy for treatment of MS would have obvious advantages related to the selective control of the T cells.

Manipulation of antigenic ligands to selectively target myelin-reactive CD4+ T cells in MS and EAE. Selective tolerization of myelin-reactive CD4+ T cells by antigen-specific therapies represents the Holy Grail of treatment for MS. Despite some successes in EAE, this quest remains elusive, as various antigen-specific strategies targeting myelin-specific T cells have failed to be clinically effective in human trials. APL-based strategies were first implemented shortly after the discovery that APLs could inhibit T cell function. As shown in Table 1.3, numerous studies have used myelin APLs to prevent or dowmmodulate EAE in various models. However, APL-based strategies have incurred a number of problems. Although myelin APLs could inhibit certain myelin-reactive clonotypes, they failed to block some myelin T cells and often activated others. Thus, myelin APLs were often non-protective (i.e. unable to treat EAE induced by wild-type myelin antigen) or were themselves encephalitogenic (i.e. capable of inducing EAE). These failures in fact reflect the diversity of the autoreactive T cell repertoire, which cannot be overlooked for the successful treatment of autoimmunity. Moreover, several APLs induced switching of myelin-reactive CD4+ T cells from a Th1 to a Th2 phenotype [277, 339]. In some situations this led to hypersensitivity reactions or anaphylaxis in animals with EAE [340, 341]. The drawbacks of APL therapies were alarmingly demonstrated in two phase II clinical trials for MS patients, which had to be halted midway due to worsening of neurological symptoms [342] or hypersensitivity reactions [343].

A converse strategy for targeting polyclonal myelin-reactive CD4+ T cells is instead to generate myelin antigen MVPs. As previously described, MVPs can increase or decrease ligand strength by altering peptide:MHC stability. Increasing the affinity of

myelin antigen for MHC can generate a superagonist, which can lead to the tolerization of myelin-reactive CD4+ T cells by AICD. For instance, MVPs in the MBP_{Ac1-11}:IA^u model increase the peptide affinity for IA^u and protect against EAE [16, 265, 344], whereas MBP_{72-85} MVPs protect against EAE in Lewis rats [345] (Table 1.4). A $PLP_{139-151}$ MVP superagonist also induces AICD of myelin-reactive CD4+ T cells in SJL mice during EAE [279]. MBP_{Ac1-11} MVPs can also be therapeutic in EAE by inducing apoptosis, upregulating the signaling inhibitor CD5, or cytokine deviation [243, 265, 346]. On the other hand, an MBP_{Ac1-11} subagonist was used to generate high avidity myelin-reactive CD4+ T cells, which were poorly encephalitogenic [347]. This may have been due to tolerance by AICD from higher concentrations of endogenous myelin antigens *in vivo*.

Alternatively, myelin MVPs can be generated which destabilize the peptide:MHC interaction. This was demonstrated using two different weakly binding MVPs for MOG_{35-55} (termed 45D) and $PLP_{139-151}$ (termed 145D). These MVPs anergized polyclonal myelin-reactive CD4+ T cells and ameliorated ongoing EAE in B6 [348, 349] and SJL mice [350], respectively. Moreover, these MVPs were very safe as neither caused deviation towards a Th2 phenotype or induced anaphylaxis even after repeated treatments [348, 350].

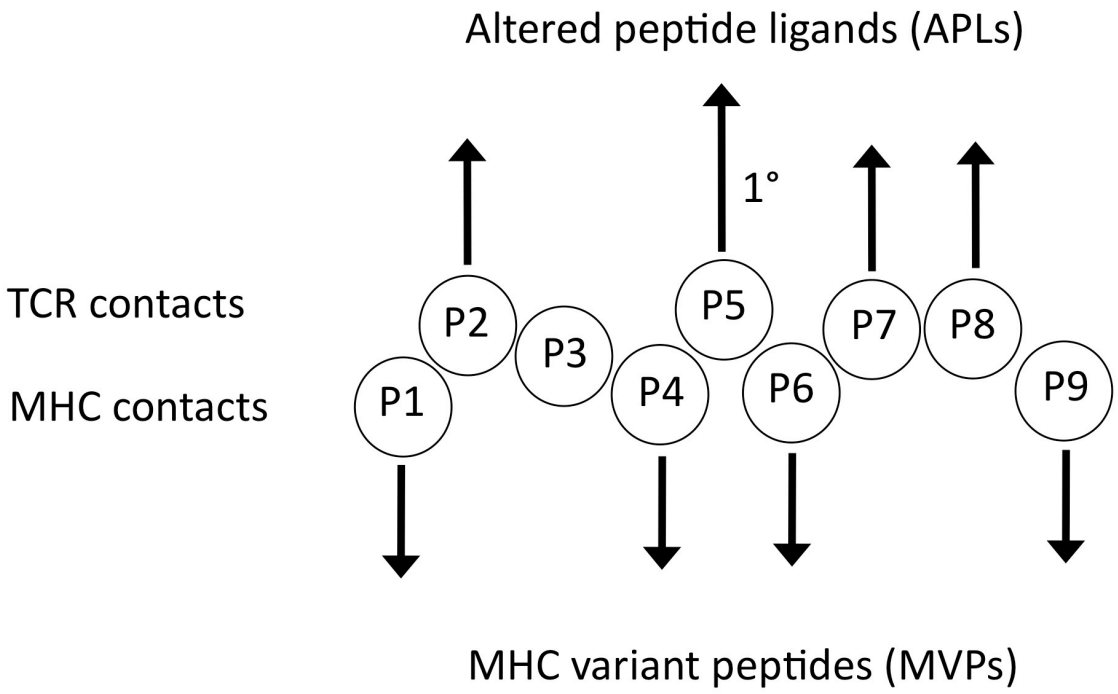

Figure 1.1. Alteration of TCR or MHC contact residues distinguishes altered peptide ligands and MHC variant peptides. Displayed are hypothetical TCR and MHC contact residues, which are based on the common motifs for IA^b. For instance, P5 is often the primary TCR contact. Alteration of any of the TCR contact residues generates altered peptide ligands or APLs. MHC contacts are typically formed by P1, P4, P6, and P9. Substitution of any of these residues generates MHC variant peptides or MVPs.

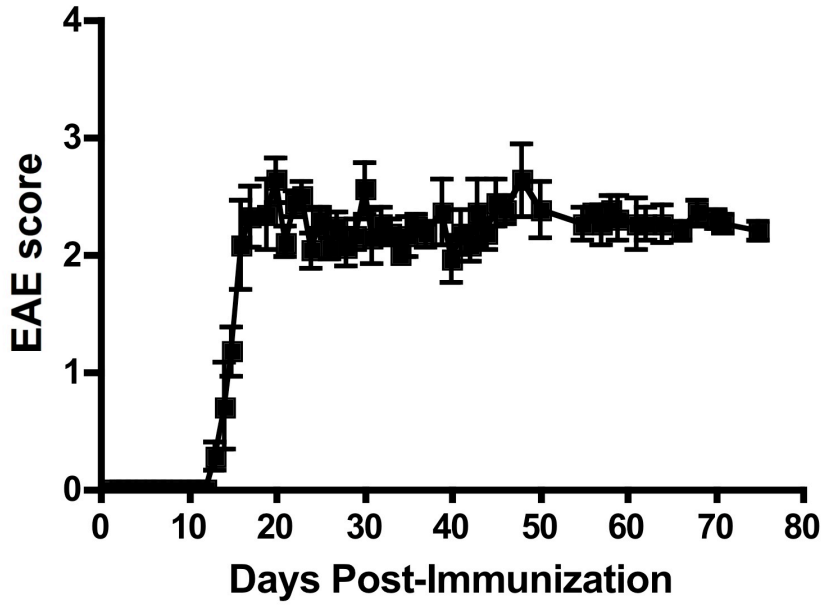

Figure 1.2. MOG_{35-55}-induced EAE disease course. Shown is a typical EAE score severity profile following immunization of B6 mice immunized with MOG_{35-55}.

Receptor	Ligand	K_d (μM)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})
Biotin	Streptavidin	10^{-9}	10^6 - 10^7	10^{-6}
Antibody	Antigen	10^{-3}	10^5-10^6	10^{-2}-10^{-4}
TCR	pMHC	1-10	10^3-10^5	0.01-0.2
Selectin	Ligand	0.3-100	10^5-10^6	1.4-10
LFA-1	ICAM-1	0.13	10^5	0.03
CD8	MHC I	50-200	$>10^5$	>18
CD4	MHC II	>200		

Table 1.1. Comparison of TCR:pMHC kinetics to other receptor-ligand interactions. Shown are the 3D kinetic parameters (K_d, k_{on}, and k_{off}) for the indicated receptor:ligand interactions.

Antigen	Mouse Strain	Haplotype
MOG_{35-55}	C57BL/6	IA^b
$PLP_{139-151}$	SJL/J	IA^s
MBP_{Ac1-11}	B10.PL/J	IA^u

Table 1.2. Summary of commonly used EAE models. Shown are the three most common myelin antigens, mouse strains and their haplotypes that are used to induce EAE.

EAE Model	APL	MHC II	Outcome	Reference
MBP_{87-99}	91A, 95A, 96A	$RT.D^{l}$	91A protective; 95A and 96A not protective	[351]
	96A[a]	IA^{s}	Protective against certain clones	[339]
	91A[b], 97A[c]	IA^{s}	Protective, non-encephalitogenic	[352]
$PLP_{139-151}$	Y144, L144	IA^{s}	Partially protective	[353]
	L144/R147	IA^{s}	Protective	[354]
	Q144	IA^{s}	Protective	[277]
MBP_{Ac1-11}	6G, 6T, 6V	IA^{uxs}	Encephalitogenic	[355]
	3D, 3I, 3K	IA^{u}	Protective	[356]
	3F, 3H, 3M, 3Y	IA^{u} or IA^{uxs}	Non-encephalitogenic	[25]
MOG_{35-55}	41A, 44A	IA^{b}	Protective	[357]
	47A	IA^{b}	Not protective and encephalitogenic	[348]

Table 1.3. Summary of altered peptide ligand therapies in EAE. Listed are the different types of myelin antigen-based APLs that have been used in treating EAE. The name of the APL, the MHC II restriction, and outcome of the studies are given. Encephalitogenicity refers to the ability of the APL to induce EAE, whereas protection refers to ability to treat EAE induced by wild-type myelin antigen.

[a]96A, [b]91A, and [c]97A are classified by their authors as APLs, but whether these are TCR contacts or MHC contacts is not clear. 96A is a partial agonist for certain clones of MBP_{87-99}-reactive CD4+ T cells. 91A appears to function as an antagonist, while 97A appears to function as a superagonist.

EAE Model	MVP	MHC II	Outcome	Reference
MBP_{Ac1-11}	4A	IA^{u} or IA^{uxs}	Protective, non-encephalitogenic	[16, 265, 344]
	4Y	IA^{u} or IA^{uxs}	Protective, non-encephalitogenic	[57, 358]
MBP_{72-85}	1028	$RT1.B^{L}$	Protective	[345]
MOG_{35-55}	45D	IA^{b}	Protective, non-encephalitogenic	[348, 349]
$PLP_{139-151}$	145D	IA^{s}	Protective	[350]

Table 1.4. Summary of MHC variant peptide therapies in EAE. Shown are the various studies using myelin antigen-based MVPs to treat EAE using the same parameters as in Table 1. Encephalitogenicity refers to the ability of the MVP to induce EAE, whereas protection refers to ability to treat EAE induced by wild-type myelin antigen.

Chapter 2. Loss of IFNγ enables the expansion of autoreactive CD4+ T cells to induce EAE by a non-encephalitogenic myelin variant antigen

Abstract

MHC variant peptides are analogs of immunogenic peptides involving alterations of the MHC binding residues, thereby altering the affinity of the peptide for the MHC molecule. Recently, our lab demonstrated that immunization of wild-type B6 mice with 45D, a low affinity MHC variant peptide of MOG_{35-55}, results in significantly attenuated EAE, yet IFNγ production is comparable to MOG_{35-55}-immunized mice. In light of these findings, we asked whether IFNγ was required for the reduced encephalitogenicity of the weak ligand 45D in EAE. Here we report that immunization of mice deficient in IFNγ or its receptor with 45D exhibit significant EAE signs compared to 45D-immunized wild-type B6 mice. Moreover, 45D-immunized IFNγ -/- and IFNγR -/- mice demonstrate MOG-tetramer positive CD4+ T cells within the CNS and display substantial numbers of MOG-specific CD4+ T cells in the periphery. In contrast, wild-type mice immunized with 45D exhibit reduced numbers of MOG-specific CD4+ T cells in the periphery and lack MOG-tetramer positive CD4+ T cells in the CNS. Importantly, the increased encephalitogenicity of 45D in mice lacking IFNγ or IFNγR was not due to deviation towards an enhanced IL-17-secreting phenotype. These findings demonstrate that IFNγ significantly attenuates the encephalitogenicity of 45D and are the first to highlight the importance of IFNγ signaling in setting the threshold level of responsiveness of autoreactive CD4+ T cells to weak ligands.

Introduction

Experimental autoimmune encephalomyelitis (EAE) is an experimental model of multiple sclerosis (MS) mediated primarily by autoreactive CD4+ T cells against myelin-producing oligodendrocytes in the central nervous system (CNS). Over the years, various approaches have been used to develop antigen-specific therapies for autoimmune diseases. The ultimate goal of such efforts is the induction of clonal anergy, thereby leaving non-autoreactive T cell clones unaffected. Such strategies include the use of peptides containing substitutions at the self-antigen TCR contact residues, termed altered peptide ligands (APLs). APLs vary in their potency for T cell activation and are categorized as full agonists, weak agonists, partial agonists, or antagonists [3]. APLs are capable of inducing T cell anergy [21, 359] as well as T cell antagonism [5]. However, the clinical use of this approach has been hampered by several issues, including the inability to induce anergy in a polyclonal T cell population [348], generation of APL-reactive T cell clones [342, 348, 360], and immune deviation to a Th2 phenotype and hypersensitivity reactions [277, 343, 360].

An alternate strategy involves the alteration of the self-peptide MHC anchor residues, which we refer to as MHC variant peptides. These variant peptides have altered affinity for the MHC molecule with little to no effect on the TCR contact regions, thereby allowing the selective targeting of all antigen-reactive T cells in a polyclonal population (i.e. complete anergy of all antigen-reactive cells). Previously, we demonstrated the ability of myelin-associated MHC variant peptides to induce anergy in myelin-reactive CD4+ T cells, which results in reduced encephalitogenicity in several EAE models [348, 350]. In the B6 MOG_{35-55} model, substitution of the MHC anchor residue at position 45

from serine to aspartic acid (termed 45D) results in a 200-fold reduction in the affinity for I-A^b [348] without altering TCR recognition [361]. The reduced half-life of 45D for the MHC molecule inhibits the proliferation and IL-2 secretion of MOG_{35-55}-reactive CD4 T cells without altering the Th1 phenotype [348]. Moreover, immunization of B6 mice with 45D does not result in the development of EAE [348], yet CD4+ T cells from these mice secrete significant amounts of IFNγ [349].

The maintenance of IFNγ by anergized MOG-reactive CD4+ T cells is highly interesting given the numerous studies demonstrating a protective role for IFNγ in EAE [144, 145, 149, 152, 158-160, 162, 362]. In light of these findings, we reasoned that IFNγ may be required to prevent EAE induction by the tolerizing variant peptide 45D. Here we report that, in contrast to wild-type B6 mice, which have been shown to be extremely resistant to EAE induction by 45D, IFNγ -/- and IFNγR -/- mice were highly susceptible to the induction of EAE by 45D. Consistent with these findings, MOG tetramer-positive CD4+ T cells were found within the CNS of 45D-immunized IFNγ -/- and IFNγR -/- mice, but were not found in the CNS of 45D-immunized wild-type mice. Moreover, significantly higher absolute numbers of MOG-specific CD4+ T cells were found in the draining lymph nodes of IFNγ -/- and IFNγR-/- mice footpad-primed with 45D compared to wild-type mice. Lastly, immunization with 45D in an IFNγ-deficient environment did not cause skewing to an enhanced Th17 phenotype, demonstrating that the increased encephalitogenicity of 45D in IFNγ -/- and IFNγR -/- mice was mediated by the increased responsiveness of autoreactive CD4+ T cells. Thus, this illustrates the importance of IFNγ in negatively regulating the response of autoreactive CD4+ T cells to

weak ligands for myelin antigens and suggests a crucial role for IFNγ in the induction of peripheral tolerance.

Materials and Methods

Mice

Female wild-type, IFNγ -/- and IFNγR -/- C57BL/6 mice (H-2^b) were purchased from Jackson laboratory. Mice were housed in an Emory University Department of Animal Resources facility and used in accordance with an IACUC-approved protocol. Mice were used for experiments at 6-8 weeks of age.

Peptides

MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK) and 45D (MEVGWYRSPF**D**RVVHLYRNGK) were purchased from Biosynthesis Incorp. (Lewisville, TX) and the Emory University Microchemical Core Facility (Atlanta, GA). Peptides were analyzed by mass spectrometry and HPLC.

EAE induction

EAE was induced by s.c. immunization in the hind flanks on days 0 and 7 using 100 μg of MOG_{35-55} or 45D emulsified in CFA containing 2.5 mg/ml heat-inactivated *M. tuberculosis* (Difco, Detroit, MI). Mice also received 250 ng of pertussis toxin (List Biological, Campbell, CA) i.p. on days 0 and 2. Disease severity was assessed according to the following scale: 0, no disease; 1, flaccid tail; 2, hind limb weakness; 3, hind limb paralysis; 4, forelimb weakness; 5, moribund.

Cells and reagents

Culture medium consisted of RPMI medium 1640 (Mediatech, Herndon, VA) supplemented with 10% FBS (Hyclone, Logan, UT), 2 mM L-glutamine (Mediatech), 0.01 M HEPES buffer (Mediatech), 100 μg/ml gentamicin (Mediatech), and 2 x 10^{-5} M β-mercaptoethanol (Sigma-Aldrich). Flow cytometry was performed on a BD

FACSCalibur, and data were processed using FlowJo software (Tree Star, San Carlos, CA).

Proliferation assay

Ex vivo splenocytes from MOG_{35-55} or 45D-immunized mice (5 x 10^5 per well) were incubated in a 96-well plate with the indicated concentration of peptide. After 48 h in culture, cells were labeled with 0.4 μCi/well of [^{3}H]thymidine. After 18-24 hours, the plates were harvested on a FilterMate harvester (Packard, Meridien, CT) and analyzed on a 1450 LSC Microbeta TriLux counter (Perkin Elmer).

CNS mononuclear cell isolation

Mice were euthanized with CO_2 and perfused through the left ventricle with PBS. The brain and spinal cord were removed from each animal and homogenized on a 0.2 μm filter. Mononuclear cells were isolated over Percoll (Sigma-Aldrich, St. Louis, MO), washed and were stained with anti-CD45.2 FITC and anti-CD11b PerCP (BD-Bioscience).

Generation of MHC II tetramers

Constructs encoding the extra-cellular domains of murine I-A^b a and b chains were cloned into a bicistronic expression cassette. The β chain N-terminus contains a tethered MOG_{38-49} peptide and flexible linker; the β chain C-terminus contains the acidic strand of the leucine zipper [363] followed by a "ribosome skippage sequence" (RSS) from the insect virus *Thosea asigna* (TaV). The RSS directs chain stop and restart during translation, thereby separating the I-A β and α chains [364]. The α chain contains C-terminal modifications comprising the basic leucine zipper strand, the biotinylation signal peptide LNDIFEAQKIEWHE [365] and a 6X histidine tag. Protein expression and

tetramers were generated by the NIAID Tetramer Core facility (the detailed method for Class II tetramer production will be described elsewhere). The bicistronic expression construct was cloned into the SIN lentiviral vector pCMJJ (a gift from Joshy Jacob). Lentiviral particles were generated and used to transduce HEK 293T cells. Transduced cells were cultured in standard DMEM supplemented with 10% FCS, L-glutamine and Pennstrep™. Cultures were expanded on a Bellocell bioreactor (Bellco) and recombinant monomers purified by Ni++ column chromatography followed by affinity-purification on sepharose conjugated mAb 2H11 against the leucine zipper. Purified monomers were biotinylated and tetramerized to streptavidin conjugated to phycoerythrin.

Tetramer staining

Cells were incubated with either MOG_{38-49} or $OVA_{323-339}$ $I\text{-}A^b$ tetramer at a concentration of 4 μg/ml for 20 hours at 37 ºC. The cells were washed with buffer containing 1X PBS, 0.1% BSA, and 0.05% sodium azide. Cells were 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.

Determination of absolute numbers of tetramer positive CD4+ T cells

Wild-type, IFNγ -/- and IFNγR -/- mice were primed in the hind footpads and tail base using 100 μg of MOG_{35-55} or 45D emulsified in 1.25 mg/ml CFA. The inguinal and popliteal lymph nodes (LNs) were isolated 12-14 days post-priming, pooled together for each mouse and stained with MOG:$I\text{-}A^b$ or OVA:IA^b tetramers (see above). The percentage of live tetramer-positive cells was multiplied by the absolute number of LN cells determined by flow cytometric analysis using TruCOUNT tubes (BD Pharmingen). The following formula was used to calculate the absolute number of LN cells per mouse:

((number of cells collected/number of beads collected) x (number of beads per tube/test volume)).

Intracellular Cytokine Staining

Cells from the spleen and CNS of immunized mice were stimulated for 5 hours with 20 nM phorbol 12-myristate 13-acetate (Fisher Biotech, Fair Lawn, NJ) and 1 nM ionomycin at 37 ºC. After the first 30 minutes of stimulation, 10 μg/mL Brefeldin A was added. Cells were first stained extracellularly with anti-CD4 Ab (RM4-5, CD4 PerCP) and were then fixed and permeabilized using the Caltag Laboratories Fix & Perm Cell Permeabilization kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Cells were stained for intracellular cytokine using anti-IFNγ-FITC (DB-1) (BD Pharmingen), anti-IL-17-APC (eBio f7B7) (eBioscience), and anti-IL-4-PE (11B11) (BD Pharmingen) for 30 minutes on ice.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 4 (Software for Science, San Diego, CA). Mean clinical scores were compared using a one-tailed Wilcoxon matched pairs test and disease incidence was analyzed using a one-tailed paired t-test. Absolute numbers of MOG tetramer positive CD4+ T cells were compared using a one-tailed t-test.

Results

IFNγ -/- and IFNγR -/- mice are highly susceptible to EAE induction by 45D

To determine the role of IFNγ in regulating the encephalitogenicity of 45D, wild-type, IFNγ -/- and IFNγR -/- B6 mice were immunized with MOG_{35-55} or 45D and were monitored for EAE signs. Confirming our previous findings [348, 349], wild-type mice immunized with MOG_{35-55} developed significant EAE signs (average maximum score of 3.2 ± 0.3), in contrast to wild-type mice immunized with 45D, which displayed little to no signs of clinical EAE (average maximum score of 0.6 ± 0.3) (Figure 2.1 and Table 2.1). Moreover, IFNγ -/- and IFNγR -/- mice immunized with MOG_{35-55} developed rapid and severe EAE (average maximum score of 3.5 ± 0.2 and 4.3 ± 0.2, respectively), consistent with previous reports [149, 152]. Surprisingly, 45D-immunized IFNγ -/- and IFNγR -/- mice developed delayed, but considerable EAE signs (average maximum EAE score of 1.8 ± 0.6 and 1.5 ± 0.4, respectively) with incidences of 60% and 67%, respectively (Figure 2.1 and Table 2.1). The disease courses of IFNγ -/- and IFNγR -/- 45D-immunized mice were significantly more severe than wild-type 45D-immunized mice in terms of both EAE score ($p = 0.0017$ and $p= 0.0013$, respectively) and incidence ($p = 0.0052$ and $p = 0.0121$, respectively). Thus, the loss of IFNγ enables the induction of EAE by the weak myelin antigen 45D.

IFNγ -/- and IFNγR -/- 45D-immunized mice have substantial inflammatory infiltration in the CNS

In order to verify that the development of disease signs in IFNγ -/- and IFNγR -/- mice immunized with 45D was due to inflammation within the CNS, CNS mononuclear cell infiltration was assessed by flow cytometry. All MOG-immunized mice

demonstrated significant numbers of lymphocytes ($CD45.2^+$, $CD11b^{lo/-}$) in the CNS 20 days post-immunization, whereas these cell populations were minimally present in wild-type mice immunized with 45D (Figure 2.2). Consistent with the delayed, but significant onset of disease signs in 45D-immunized IFNγ -/- and IFNγR -/- mice, few lymphocytes were found in the CNS of these mice at day 15 (data not shown), but high percentages were present by day 20. Similar percentages (4.8-8.8%) of infiltrating macrophages ($CD45.2^+$, $CD11b^{hi}$) were found in all groups of mice (Figure 2.2). Thus, these results indicate that the development of EAE signs in 45D-immunized IFNγ -/- and IFNγR -/- mice correlated with substantial amounts of infiltrating lymphocytes in the CNS.

MOG-specific CD4+ T cells are found in the CNS of 45D-immunized IFNγ -/- and IFNγR -/- mice

The observation that 45D-immunized IFNγ -/- and IFNγR -/- mice develop significant EAE signs implies that primed CD4+ T cells from these mice are responsive to endogenous myelin components within the CNS. To test this, our lab has developed an I-A^b tetramer specific for MOG_{38-49}, which spans the dominant T helper epitope of MOG_{40-48} [366]. The DNA sequence for MOG_{38-49} was inserted into a construct for I-A^b, such that the resulting protein contains the MOG epitope tethered to the I-A^b molecule. This MOG tetramer is highly specific as it stains MOG-reactive CD4+ T cells, which show minimal staining with a control I-A^b tetramer specific for $OVA_{323-339}$. Conversely, the OVA tetramer stains OVA-reactive, but not MOG-specific CD4+ T cells (data not shown).

Class II tetramer staining revealed the presence of MOG-specific CD4+ T cells within the CNS (2-4%) of MOG_{35-55}-immunized wild-type, IFNγ -/- and IFNγR -/- mice

beginning at the onset of disease signs (data not shown). By day 20, MOG tetramer-positive CD4+ T cells of MOG-immunized wild-type, IFNγ -/- and IFNγR -/- mice reached levels of 7.4%, 10.1%, and 9.6%, respectively (Figure 2.3) and remained detectable in substantial amounts through day 30 (data not shown). MOG tetramer-positive CD4+ T cells were not detected at any time point in the CNS of wild-type mice immunized with 45D. In contrast, increased frequencies of MOG-specific CD4+ T cells were identified in IFNγ -/- and IFNγR -/- mice immunized with 45D beginning at day 20 (2.6% and 3.4%, respectively) (Figure 2.3) and persisted through day 30 (data not shown). Notably, the percentages of MOG-specific CD4+ T cells (7.4%) at the peak of disease in wild-type B6 mice were very similar to those detected with a recently reported MOG_{35-55} I-A^b tetramer [256], indicating that the core epitope used for the tetramer is sufficient for tracking MOG-reactive CD4+ T cells. These findings convincingly demonstrate that the induction of EAE by 45D in IFNγ -/- and IFNγR -/- mice is mediated by MOG-reactive CD4+ T cells in the CNS.

T cells from 45D-immunized IFNγ -/- and IFNγR -/- mice proliferate robustly in response to MOG and 45D

Given the marked increase in encephalitogenicity of 45D in IFNγ -/- and IFNγR -/- mice, the proliferative responses of T cells from wild-type, IFNγ -/- and IFNγR -/- mice immunized with either MOG_{35-55} or 45D were compared. As expected, wild-type MOG T cells proliferated in response to MOG_{35-55} re-stimulation, whereas wild-type 45D T cells did not (Figure 2.4). IFNγ -/- and IFNγR -/- MOG T cells showed very robust proliferation in response to MOG_{35-55} re-stimulation compared to wild-type MOG T cells. Strikingly, both IFNγ -/- and IFNγR -/- 45D T cells also proliferated strongly in response

to MOG_{35-55} re-stimulation (Figure 2.4), which was much greater than wild-type 45D T cells. Consistent with the increased proliferative capacity to MOG_{35-55}, IFNγ -/- and IFNγR -/- 45D T cells proliferated significantly in response to 45D (Figure 2.4). Interestingly, splenocytes from 45D-immunized IFNγ -/- and IFNγR -/- mice proliferated in response to concentrations of 45D as low as 10^{-4} to 10^{-3} μM. These results therefore indicate that loss of IFNγ increases the responsiveness of MOG-reactive CD4+ T cells from 45D-immunized mice.

Loss of functional IFNγ increases the number of MOG-specific CD4+ T cells that expand to 45D in the periphery

The increased responsiveness of splenocytes from 45D-immunized IFNγ -/- and IFNγR -/- mice to MOG_{35-55} suggests that the loss of IFNγ may lower the activation threshold of MOG-reactive CD4+ T cells in response to the weak ligand 45D. As a result, we would predict IFNγ deficiency would allow the generation of increased numbers of MOG-reactive CD4+ T cells in response to 45D in the periphery. To test this, wild-type, IFNγ -/- and IFNγR -/- mice were immunized in the hind footpads with MOG_{35-55} or 45D and the absolute number of MOG:I-A^b positive CD4+ T cells from the draining lymph nodes were quantified on days 12-14. There were greater numbers of MOG-specific CD4+ T cells in the LNs of MOG-primed IFNγ -/- and IFNγR -/- mice (31355 ±11622 and 41533 ± 8722, respectively) compared to wild-type mice (14716 ± 5548) (Figure 2.5). As predicted, significantly fewer MOG-specific CD4+ T cells were isolated from the LNs of 45D-primed wild-type mice (3263 ± 991) compared to 45D-primed IFNγ -/- (6645 ± 1145) and IFNγR -/- mice (7276 ± 1119) ($p = 0.0379$ and $p = 0.0221$, respectively). These results therefore indicate that loss of functional IFNγ allows

the increased generation of MOG-reactive CD4+ T cells in response to the normally tolerizing weak ligand 45D.

The increased encephalitogenicity of 45D in IFNγ -/- and IFNγR -/- mice is not due to increased pro-inflammatory IL-17 production

In contrast to a prototypic Th1-mediated disease process, a number of recent reports have implicated myelin-reactive IL-17-producing CD4+ T cells (Th17 cells) as the key encephalitogenic T cell population in EAE [168-170]. IFNγ has also been shown to inhibit the development of Th17 cells [103, 169], therefore we investigated whether the increased encephalitogenicity of 45D in the absence of IFNγ might be due to an enhanced Th17 phenotype. Splenocytes and CNS mononuclear cells were isolated at various time-points post-immunization and re-stimulated with PMA and ionomycin to compare the production of IFNγ, IL-17, and IL-4 by CD4+ T cells from each group of mice.

Markedly increased frequencies of CD4+ T cells producing IFNγ (27.8-28.2%, includes IFNγ+/IL-17- and IFNγ+/IL-17+) and IL-17 (20.2-26.3%, includes both IL-17+/IFNγ- and IL-17+/IFNγ+) were detected at day 20 within the CNS of all groups of mice, except from wild-type 45D-immunized mice (Figure 2.6). No IL-4 production was detected in any of the mice analyzed (data not shown). Similar frequencies of IL-17-producing CD4+ T cells were detected in the spleen (data not shown) and CNS of 45D-immunized IFNγ -/- (20.7%) and IFNγR -/- (22.8%) mice compared to MOG-immunized mice (20.2-26.3%) (Fig 2.6). Thus, while CD4+ T cells from 45D-immunized IFNγ -/- and IFNγR -/- mice produced substantial amounts of IL-17, there was no significant increase over the other groups that developed EAE. This indicates that the ability of 45D

to induce EAE in IFNγ -/- and IFNγR -/- mice is not due to an enhanced IL-17-secreting profile of encephalitogenic CD4+ T cells.

Discussion

45D is an MHC variant peptide of MOG_{35-55} that has an approximately 200-fold reduction in the affinity for I-A^b and is weakly encephalitogenic in wild-type B6 mice [348]. In addition, treatment of MOG-reactive T cells with 45D *in vitro* results in clonal anergy as measured by decreased proliferation and IL-2 production [348], suggesting that the decreased affinity of 45D for the MHC molecule leads to negative signaling and a permanent change in the phenotype of MOG-reactive CD4+ T cells. Interestingly, the anergized CD4+ T cells retain normal IFNγ levels upon re-stimulation with MOG or 45D [348, 349], indicating the retention of certain cytokine effector functions. The importance of the preservation of IFNγ production by 45D is highlighted by numerous studies revealing a protective, anti-inflammatory role of IFNγ in EAE [144, 145, 149, 152, 158-160, 162, 362]. Moreover, it has been reported that the poor encephalitogenicity of a high affinity myelin-associated MHC variant peptide can be reversed by anti-IFNγ Ab [367]. Given the protective role of IFNγ in EAE and the ability of 45D to maintain IFNγ production by MOG-reactive CD4+ T cells, we hypothesized that IFNγ was required to inhibit the responsiveness of myelin-reactive CD4+ T cells to weak MHC variant peptides and thereby prevented the induction of EAE.

Here we demonstrate that loss of either IFNγ or IFNγR results in a significant increase in the susceptibility of B6 mice to EAE induction by 45D (Figure 2.1 and Table 2.1), confirming our hypothesis that IFNγ reduces the encephalitogenicity of this weak ligand. That 45D induces EAE in the absence of functional IFNγ suggests that this weak ligand activates myelin-reactive CD4+ T cells that are responsive to endogenous MOG_{35-55} within the CNS. Indeed, the increased encephalitogenicity of 45D in IFNγ -/- and

IFNγR -/- mice is reflected by the detection of significant percentages of MOG_{38-49} tetramer-positive CD4+ T cells in the CNS of these mice (Figure 2.3). Importantly, we have ruled out deviation towards an enhanced Th17 phenotype as a mechanism for the increased encephalitogenicity of 45D in the absence of IFNγ since similar frequencies of IL-17-producing CD4+ T cells were found in the spleen and the CNS of all mice that developed EAE, regardless of the presence of IFNγ signaling or the peptide used for immunization (Figure 2.6).

The detection of MOG-specific CD4+ T cells in the CNS of IFNγ -/- and IFNγR -/- mice immunized with 45D was mirrored by the demonstration of MOG-reactive CD4+ T cells in the periphery of these mice (Figure 2.4 and 2.5). Due to the time frame of the lymph node priming experiments, primed CD4+ T cells have not entered the CNS to mediate the release of endogenous CNS antigens. Thus, the increased frequency of MOG-specific CD4+ T cells in 45D-primed IFNγ -/- and IFNγR -/- mice is likely due solely to increased expansion in response to local Ag. Nonetheless, differences in naïve precursors cannot be excluded. The increased numbers (approximately 2-fold) of MOG-specific CD4+ T cells in the periphery of IFNγ -/- and IFNγR -/- mice compared to wild-type mice primed with 45D suggests that loss of IFNγ allows the generation of numerous MOG-reactive CD4+ T cells that can subsequently traffic to the CNS to elicit their effector functions. The number of MOG-specific CD4+ T cells in the 45D-primed IFNγ-deficient mice, however, is much lower than those found in MOG-primed mice. While this number of myelin-reactive CD4+ T cells is lower than that induced with MOG_{35-55}, this level of response is still sufficient to induce EAE, albeit at a slower rate.

There are several potential mechanisms we are actively pursuing to explain the increased expansion of MOG CD4+ T cells in response to a non-encephalitogenic peptide in the absence of IFNγ. IFNγ could limit CD4+ T cells responses via the induction of nitric oxide [146, 362, 368] or the tryptophan-catabolizing enzyme indoleamine-2,3-dioxygenase (IDO) on relevant APCs [151]. It is also possible that loss of IFNγ signaling protects against IFNγ-induced apoptosis [145, 146, 369], thereby allowing greater CD4+ T cell expansion. While it is intriguing that the increased EAE severity in IFNγ -/- mice was correlated with decreased CD4+ CD25+ FoxP3+ regulatory T cells [152], our preliminary studies thus far have not revealed decreased frequencies of FoxP3+ CD4+ T cells in 45D-immunized IFNγ -/- and IFNγR -/- mice, however, this will require further investigation.

We purport that functional IFNγ deficiency increases the encephalitogenicity of 45D by permitting the expansion of MOG-reactive CD4+ T cells in response to priming with the weak ligand in the periphery. Nonetheless, it is conceivable that IFNγ may regulate the encephalitogenicity of 45D by effects in the CNS in addition to effects on CD4+ T cell proliferation. Despite well-known pro-inflammatory effects of IFNγ in the CNS [144], [165], IFNγ also has protective effects on mature oligodendrocytes in the CNS [166, 167]. Consequently, it is possible that the loss of IFNγ or IFNγR may increase oligodendrocyte apoptosis leading to the enhanced release of myelin antigens, thereby allowing the enhanced activation and/or CNS recruitment of CD4+ T cells primed with 45D.

Here we demonstrate that loss of IFNγ or IFNγR endows 45D, a myelin variant peptide of MOG_{35-55} that is normally poorly encephalitogenic, with the ability to induce

EAE. We have gone on to show that the increased EAE susceptibility of IFNγ -/- and IFNγR -/- mice following immunization with 45D is due to the expansion of MOG-reactive CD4+ T cells in the periphery. Although numerous studies have suggested a protective role for IFNγ in EAE, we have provided the first evidence suggesting that loss of functional IFNγ increases the responsiveness of autoreactive CD4+ T cells to weak ligands, thereby enabling the induction of autoimmune disease in response to normally tolerizing peptides. Moreover, our findings suggest a fundamental role of IFNγ in setting the activation threshold of autoreactive CD4+ T cells thereby affecting the tolerizing abilities of weak ligands. Consequently, this suggests that loss of IFNγ converts a negative, anergizing signal into a positive proliferation-inducing signal in myelin-reactive CD4+ T cells responding to weak ligands. Finally, given that the absence of IFNγ significantly increases the encephalitogenicity of 45D, these findings imply that IFNγ is required for the induction of anergy by low affinity MHC variant peptides.

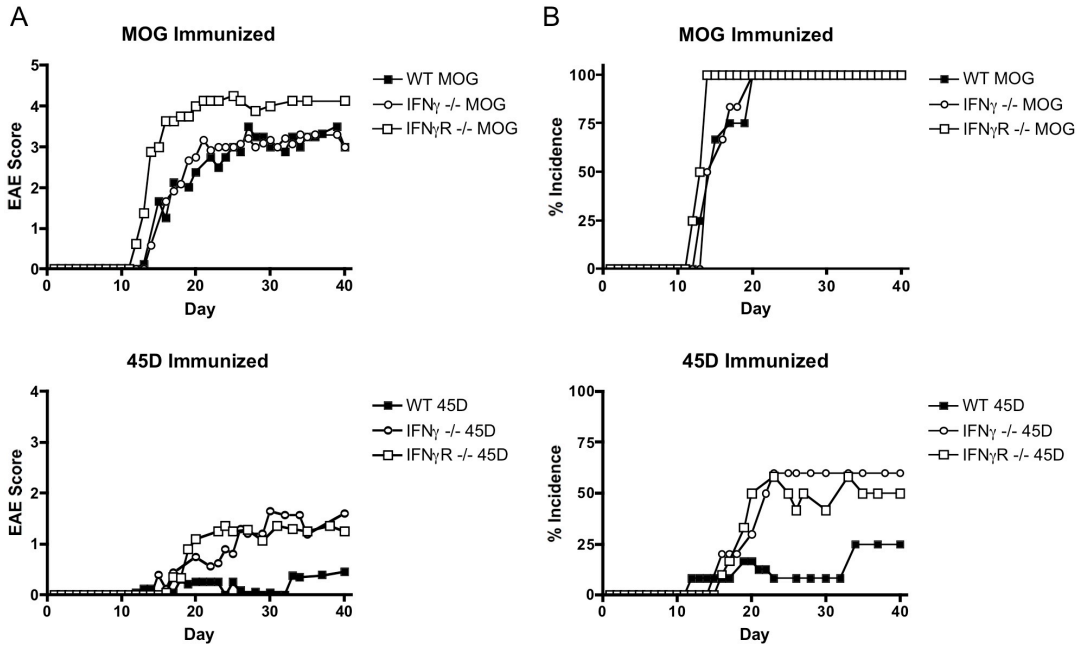

Figure 2.1. IFNγ -/- and IFNγR -/- mice develop significant EAE following 45D immunization. Wild-type B6, IFNγ -/- or IFNγR -/- mice were immunized with 100 μg MOG_{35-55} or 45D on days 0 and 7. Mice also received 250 ng Pertussis toxin on days 0 and 2. Mice were monitored for disease signs (A) and incidence (B) as described in *Materials and Methods*. Compared to wild-type mice immunized with 45D, 45D-immunized IFNγ -/- and IFNγR -/- mice developed EAE with significantly greater severity ($p = 0.0017$ and $p = 0.0013$, respectively) and incidence ($p = 0.0052$ and $p = 0.0121$, respectively). Data for each group is the average of 2-3 independent experiments.

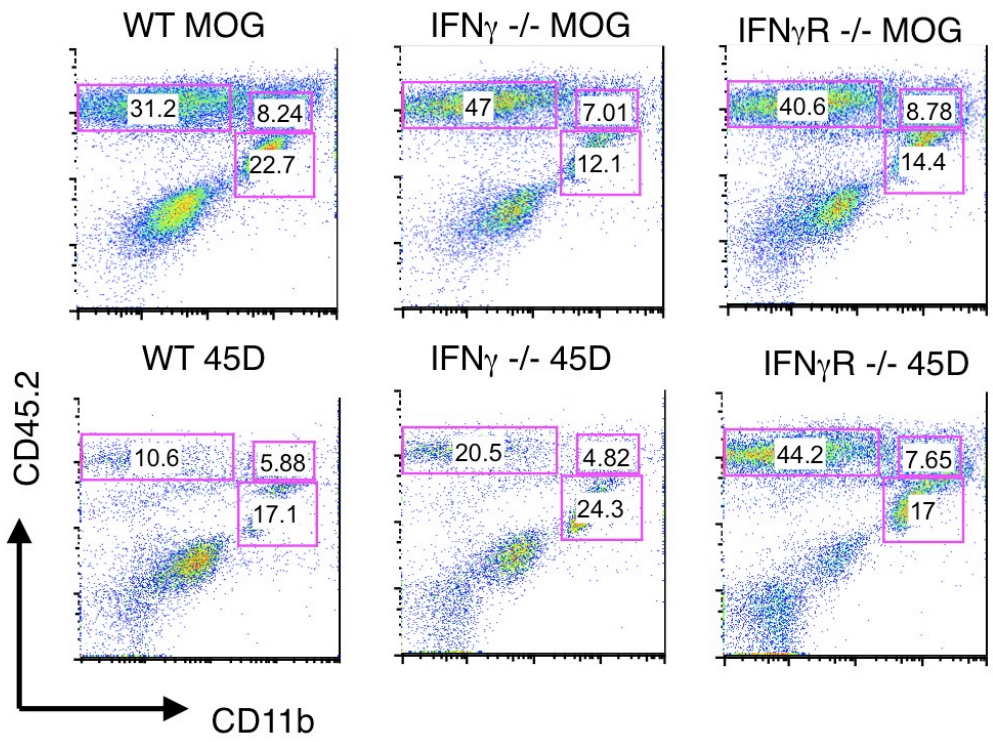

Figure 2.2. 45D induces significant lymphocyte infiltration in the CNS of IFNγ -/- and IFNγR -/- mice. CNS tissue was isolated 20 days post-immunization and stained with CD45.2 and CD11b to assess percentages of infiltrating lymphocytes (CD45.2+, $CD11b^{lo/-}$), macrophages (CD45.2+, $CD11b^{hi}$), and resident microglia ($CD45.2^{int}$, $CD11b^{hi}$). Results shown are representative of two independent experiments with each group containing CNS tissue pooled from 2 mice.

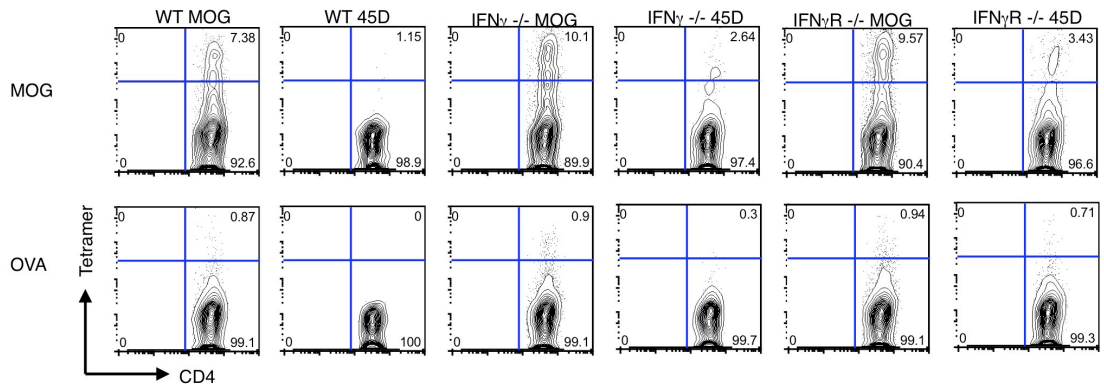

Figure 2.3. MOG tetramer-positive CD4+ T cells are found in the CNS of 45D-immunized IFNγ -/- and IFNγR -/- mice. Mononuclear cells were isolated from the CNS of mice 20 days post-immunization as described in *Materials and Methods*. CD4+ T cells were stained with a MOG_{38-49} I-A^b tetramer or an $OVA_{323-339}$ I-A^b control tetramer. Tetramer-positive cells are gated on CD4 positive, 7-AAD negative cells. Results shown are representative of 2 independent experiments with CNS tissue pooled from 2 mice in each group.

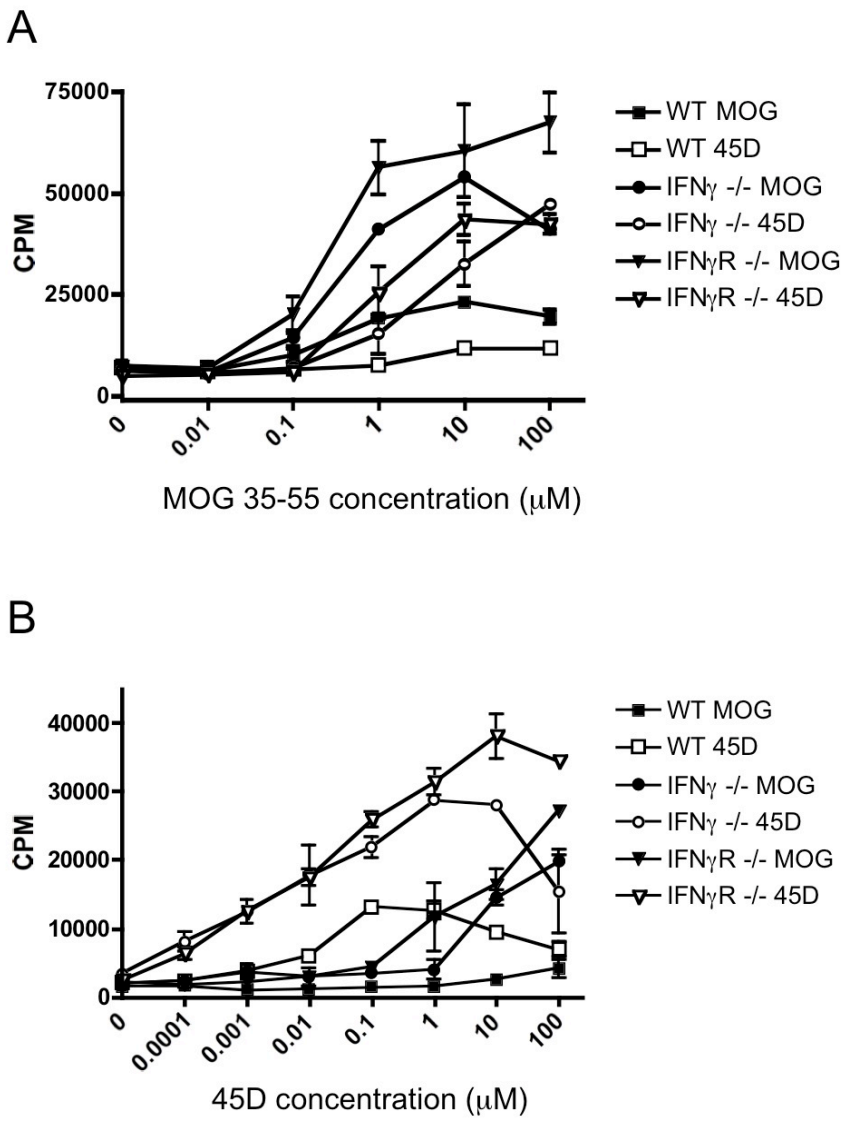

Figure 2.4. Splenocytes from 45D-immunized IFNγ -/- and IFNγR -/- mice proliferate significantly in response to MOG_{35-55}. Fifteen days post-immunization, 5 x 10^5 splenocytes (*ex vivo*) were cultured for 48 hours with the indicated concentrations of MOG_{35-55} (A) or 45D (B). Proliferation was assessed by [^{3}H]-thymidine incorporation over the next 18-24 hours. Data shown is representative of 3 independent experiments.

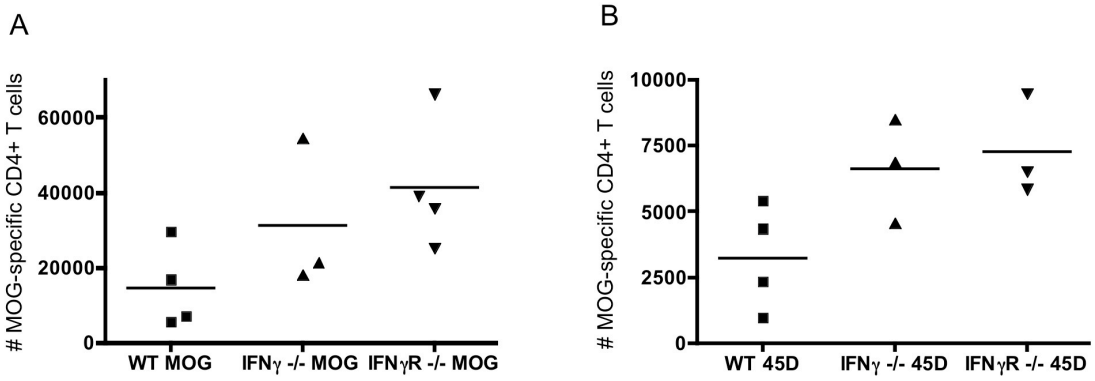

Figure 2.5. Loss of IFNγ increases absolute numbers of MOG:I-A^b tetramer positive CD4+ T cells in the periphery. Wild-type, IFNγ -/- and IFNγR -/- mice were primed in the footpad and tail base with MOG_{35-55} or 45D and draining LNs (popliteal and inguinal) were harvested 12-14 days later. The percentage of MOG tetramer-positive CD4+ T cells was multiplied by the absolute number of cells from the LNs of each mouse as described in *Materials and Methods*. The absolute number of MOG-specific CD4+ T cells from IFNγ -/- 45D and IFNγR -/- 45D mice was significantly higher compared to wild-type 45D mice ($p = 0.0379$ and $p = 0.0221$, respectively).

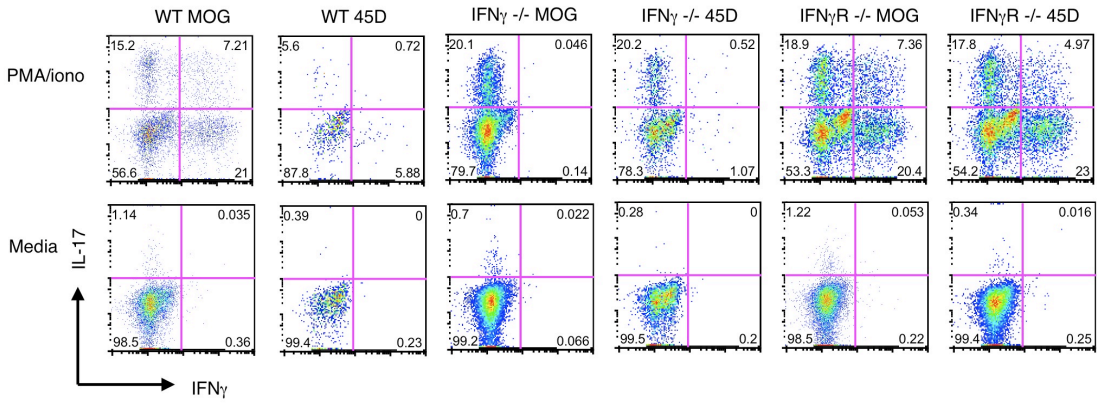

Figure 2.6. CD4+ T cells from 45D-immunized IFNγ -/- and IFNγR -/- mice do not demonstrate an enhanced IL-17-secreting phenotype. Splenocytes and CNS mononuclear cells were isolated at 20 days post-immunization and stimulated for 5 hours with PMA/ionomycin or media alone in the presence of Brefeldin A. Displayed flow plots are gated on CD4 positive cells.

Group	Disease Incidence[a]	Avg Max Severity[b] (± SEM)	Avg Day of Onset (± SEM)
WT MOG	5/5	3.20 ± 0.26	16.4 ± 1.0
WT 45D	3/12	0.58 ± 0.31	21.7 ± 6.3
IFNγ -/- MOG	6/6	3.50 ± 0.18	15.8 ± 1.1
IFNγ -/- 45D	6/10	1.80 ± 0.56	19.5 ± 1.3
IFNγR -/- MOG	4/4	4.25 ± 0.25	13.3 ± 0.5
IFNγR -/- 45D	8/12	1.54 ± 0.41	19.1 ± 0.9

Table 2.1. EAE incidence, average severity and day of onset in wild-type, IFNγ -/- and IFNγR -/- mice immunized with MOG or 45D.

[a]$p = 0.0846$ between WT 45D and IFNγ -/- 45D and $p = 0.0417$ between WT 45D and IFNγR -/- 45D.

[b]$p = 0.0536$ between WT 45D and IFNγ -/- 45D and $p = 0.0573$ between WT 45D and IFNγR -/- 45D.

Chapter 3. High prevalence of low affinity peptide:MHC II tetramer-negative effectors during polyclonal CD4+ T cell responses

Abstract

T cell affinity for antigen initiates adaptive immunity. However, the contribution of low affinity cells to a response is unknown as it has not been possible to assess the entire affinity range of a polyclonal T cell repertoire. Here, we used a highly sensitive two-dimensional (2D) binding assay to identify low affinity cells in polyclonal autoreactive and pathogen-reactive CD4+ T cells specific for myelin oligodendrocyte glycoprotein (MOG) and lymphocytic choriomeningitis virus (LCMV) antigens, respectively. Low affinity CD4+ T cells, below detection with peptide:MHC class II tetramers, were more frequent than high affinity responders and contributed significant effector cytokines at the peak of both primary antigen-specific responses. We further demonstrated that MOG- and LCMV-specific CD4+ T cells similarly possessed more than 100-fold wide ranges in their affinities, only differing in the frequencies of low and high affinity cells. Thus, low as well as high affinity CD4+ T cells are critical effectors in autoimmune and pathogen-specific responses.

Introduction

Determining the affinities of T cells in polyclonal responses is essential for understanding the outcome of cell-mediated immunity directed towards both self- and foreign-antigens. The prevailing models of clonal selection and avidity maturation suggest that cells bearing the highest affinity T cell receptors (TCRs) for antigen are selectively expanded [54, 61, 91], but the range in affinities of the T cells and the frequency of low affinity responders is unknown. The potential importance of low affinity T cells in immunity is supported by the findings that monoclonal CD8+ T cells can initially proliferate to low affinity antigens [111]. In addition to foreign-antigens, T cells specific for self-peptides that drive autoimmune disease could comprise another subset of low affinity cells as a result of tolerance mechanisms [209, 212, 230]. Thus, low affinity T cells may potentially contribute to responses to both foreign- and self-antigens.

Insight into TCR affinity for antigen has been provided by three-dimensional (3D) and 2D technologies, such as surface plasmon resonance, Förster resonance energy transfer (FRET), or micropipette-based assays [41, 67, 68, 370]. However, studies have to date only considered monoclonal TCRs and cannot reveal T cell frequency and breadth of affinities comprising an antigen-specific polyclonal population. Peptide:MHC (pMHC) tetramers based on an enhanced TCR avidity via multivalent interactions provide the most valuable technique for detecting the frequency of polyclonal antigen-specific T cells [45, 97], but their engineered avidity may well limit their sensitivity to detect low affinity T cells.

Recently, we used a 2D-based affinity analysis, which measures TCR:pMHC binding in the cell membrane-anchored context [67, 68], to define a 1000-fold range in affinities corresponding to response levels between a monoclonal CD8+ T cell and a panel of altered peptide ligands [67]. Here, we harnessed the sensitivity of the 2D binding assay to define the antigen-specific frequencies and affinities of two polyclonal IA^b-restricted CD4+ T cell populations specific for a self-antigen, myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$, which induces experimental autoimmune encephalomyelitis (EAE) [366], and a foreign-antigen, glycoprotein $(GP)_{61-80}$, the dominant T helper epitope for LCMV [198, 200]. The 2D analysis revealed significantly larger frequencies of antigen-reactive CD4+ T cells for both antigens compared to pMHC II tetramers. Polyclonal T cell affinities were diverse with a fraction identified as high affinity and tetramer-positive and many as low affinity and tetramer-negative. Despite the affinity variability, the entire affinity span of antigen-specific T cells contributed to the effector cytokine response.

Results and Discussion

pMHC II tetramers underestimate the frequencies of polyclonal CD4+ T cells

Polyclonal antigen-reactive CD4+ T cell frequencies are traditionally measured by pMHC II tetramers or functional studies. To compare the MOG_{35-55} and GP_{61-80} CD4+ T cell responses, we generated polyclonal CD4+ T cells (see Methods) and investigated the response with MOG_{38-49}:IA^b and GP_{66-77}:IA^b tetramers encompassing the respective core CD4+ T cell epitopes [240, 257, 366] (Figure 3.1A). After one week of stimulation *in vitro*, three times as many GP_{61-80} CD4+ T cells were identified by tetramer than MOG_{35-55} CD4+ T cells (32.4 ± 7.7% vs. 9.6 ± 2.8%) (Figure 3.1B). Of interest, the tetramer-positive frequency of MOG_{35-55} CD4+ T cells remained static (11.2 ± 2.4%) on repeated rounds of *in vitro* re-stimulation with antigen, whereas the tetramer-positive GP_{61-80} CD4+ T cells expanded to 59.2 ± 7.0% of the culture (Figure 3.1B). The differences between GP_{61-80} and MOG_{35-55} CD4+ T cells mirrored other studies in which CD4+ T cells specific for foreign-antigens [97, 200] were more prevalent and easier to detect by tetramer than those specific for self-antigens [59, 62, 256]. The tetramer data seemed strikingly low for the self-reactive CD4+ T cells and raised the possibility that the pMHC II tetramers were not detecting all antigen-specific CD4+ T cells.

As an alternative measure of determining the frequency of antigen-specific CD4+ T cells, we employed the micropipette adhesion frequency assay, which measures the 2D interactions of receptor-ligand interactions [67, 83, 371]. In this assay, a single T cell was brought in and out of contact with a red blood cell (RBC) coated with pMHC II to yield an adhesion frequency (the percentage of adhesions out of the total number of contacts, described in Methods). Following stimulation with antigen for one week *in*

vitro, polyclonal MOG_{35-55} and GP_{61-80} CD4+ T cells bound only with their respective antigens, but not RBCs alone or irrelevant antigen (Figure 3.1C-D). 2D analysis revealed a range in adhesion frequencies and antigen-specific CD4+ T cell frequencies were determined by the percentage of T cell binding above irrelevant antigen background (> 0.1, see $hCLIP_{103-117}$:IA^b in Figure 3.1C-D). Surprisingly, the majority of the MOG_{35-55} CD4+ T cells were antigen-specific (69.2 ± 6.0%), 8-fold higher than the tetramer-based frequencies measured in parallel (8.7 ± 1.6%) (Figure 3.1E). Moreover, the micropipette assay identified a similar wide range in adhesion frequencies and a 2.5-fold increase over tetramer-positive GP_{61-80} CD4+ T cells (76.9 ± 3.4% vs. 30.9 ± 8.8%) (Figure 3.1E). Thus, 2D binding demonstrated that most CD4+ T cells in short-term cultures were antigen-specific in contrast to the differences in frequency identified using pMHC II tetramer (Figure 3.1B).

Peptide:MHC II tetramer-negative CD4+ T cells are low affinity, but elicit robust effector responses

The degree of tetramer binding is critically dependent on TCR avidity [50, 54], a parameter determined by intrinsic TCR affinity and TCR levels. However, MOG_{35-55} CD4+ T cells and GP_{61-80} CD4+ T cells expressed similar levels of surface TCRs (Figure 3.2). To determine whether TCR affinity caused the deficiency in tetramer detection of polyclonal CD4+ T cells, we used the micropipette-based assay to define 2D affinities. The effective 2D affinity (in μm^4) of the T cell was derived from the adhesion frequency at equilibrium and expressed as a product of the two cells' contact areas and 2D affinity. The 2D affinities were derived at 5s since binding of both T cell populations reached equilibrium within seconds of contact (Figure 3.3). The adhesion frequency was

dependent on the molecular densities of TCR and pMHC, and can potentially detect a dynamic range of affinities spanning six orders of magnitude (10^{-2} to 10^{-8} μm^4) [67] with lower affinity T cells detected by increasing the pMHC densities.

For comparable adhesion frequencies, GP_{61-80} CD4+ T cells (Figure 3.1D) required lower levels of antigen than MOG_{35-55} CD4+ T cells (Figure 3.1C), indicative of overall higher affinity. Indeed, the population-averaged effective 2D affinity of GP_{61-80} CD4+ T cells was 26-fold higher than MOG_{35-55} CD4+ T cells ($4.21 \pm 1.48 \times 10^{-4}$ vs. $1.63 \pm 0.48 \times 10^{-5}$ μm^4, Figure 3.4A). T cell functional avidity (inverse of antigen concentration for half-maximal response, $1/EC_{50}$), which is often used as a surrogate for TCR affinity [57, 101, 109], paralleled the effective 2D affinities as GP_{61-80} CD4+ T cells had a 61-fold greater functional avidity than MOG_{35-55} CD4+ T cells (Figure 3.4B).

Peptide:MHC tetramers provide a useful tool for separating the polyclonal MOG_{35-55} and GP_{61-80}-specific CD4+ T cells in two distinct populations based on reactivity to the tetramer (Figure 3.5). Essentially all tetramer-positive CD4+ T cells (>96%) were detected by 2D binding showing high adhesion frequencies at low antigen densities (Figure 3.4C-E). Importantly, the majority of tetramer-negative CD4+ T cells ($68.6 \pm 6.2\%$ of MOG_{35-55} and $75.0 \pm 0.0\%$ of GP_{61-80}) also bound to antigen in the micropipette assay (Figure 3.4C-E). Nevertheless, tetramer-negative MOG_{35-55} and GP_{61-80} CD4+ T cells had lower effective 2D affinities ($6.67 \pm 3.14 \times 10^{-6}$ and $8.36 \pm 2.49 \times 10^{-6}$ μm^4, respectively) than their tetramer-positive counterparts ($1.48 \pm 0.27 \times 10^{-4}$ and $3.91 \pm 0.83 \times 10^{-4}$ μm^4, respectively) (Figure 3.4F). Regardless of myelin- or viral-antigen specificity, all CD4+ T cells required a 2D affinity exceeding 1.52×10^{-5} μm^4 in order to bind tetramer (Figure 3.4F). Thus, pMHC II tetramers underestimated the frequency of

MOG_{35-55} and GP_{61-80} CD4+ T cells as their reactivity was only accurate to the extent that T cell responses were dominated by high affinity T cells. The presence of high affinity and low affinity (henceforth broadly distinguished by the 2D affinity cut-off for tetramer binding) MOG_{35-55} and GP_{61-80} CD4+ T cells indicated that both populations were comprised of T cell clonotypes possessing a large (>100-fold), mostly overlapping span of affinities (7.92×10^{-7} to 2.01×10^{-4} for MOG_{35-55} and 5.37×10^{-6} to 5.53×10^{-4} for GP_{61-80}) (Figure 3.4F). These ranges of 2D affinities correspond to those recently reported for full to weak agonists in the OT-I CD8+ T cell model [67].

Despite their lower affinity, the tetramer-negative CD4+ T cells elicited robust effector functions as similar percentages of tetramer-positive and tetramer-negative MOG_{35-55} (54.6 ± 7.4% and 58.7 ± 19.1%, respectively) and GP_{61-80} (81.4 ± 4.8% and 65.6 ± 5.7%, respectively) CD4+ T cells produced TNFα and/or IFNγ following antigen stimulation (Figure 3.4G). Considering that tetramer-negative cells were not exclusively antigen-reactive by 2D binding analysis, cytokine was produced at a higher frequency than the tetramer-positive populations.

Low affinity tetramer-negative CD4+ T cells in the CNS dominate during peak EAE

An added strength of the micropipette assay is that its increased sensitivity to antigen allows assessment of *ex vivo* T cell responses. CD4+ T cells isolated from the CNS during peak EAE bound specifically to MOG_{38-49}:IA^b, but not irrelevant antigen by both tetramer (Figure 3.6A) and 2D analysis (Figure 3.6B). The average frequency of tetramer-positive MOG_{35-55} CD4+ T cells was 8.2 ± 1.3% (Figure 3.6C), similar to previous findings [256, 257]. In parallel with the *in vitro* data, 8-fold more CNS-infiltrating MOG_{35-55} CD4+ T cells were detected by 2D binding (63.6 ± 6.9%) (Figure

3.6C). Moreover, the population-averaged effective 2D affinity of MOG_{35-55}-specific CD4+ T cells from the CNS was $7.95 \pm 2.77 \times 10^{-6}$ μm^4, which was comparable (2.1-fold lower) to the *in vitro* analysis (Figure 3.4A). This affinity level explains the low degree of tetramer staining as it was below the threshold for tetramer binding (Figure 3.4F). Thus, the majority of CD4+ T cells penetrating the CNS at the peak of EAE were in fact MOG_{35-55}-specific.

In order to define the effector function of low affinity MOG_{35-55} CD4+ T cells in the CNS, we isolated CD4+ T cells from the CNS at the peak of EAE and assessed their ability to produce IFNγ and TNFα (Figure 3.6D, representative experiment). Approximately 2.4-fold more CD4+ T cells produced cytokine in response to MOG_{35-55} peptide stimulation than were detected by tetramer ($19.0 \pm 3.6\%$ vs $8.0 \pm 0.7\%$, in parallel experiments) (Figure 3.6E). Since not all antigen-specific CD4+ T cells produced cytokine (Figure 3.4G), this indicated that the majority of pro-inflammatory cytokine-secreting MOG_{35-55}-specific CD4+ T cells were of low affinity. Though our studies did not directly address the effect of thymocyte negative selection or peripheral events in shaping T cell affinity, the mere presence of high affinity MOG_{35-55} CD4+ T cells suggested that their clonal deletion was absent or incomplete. Moreover, the stable existence of high affinity self-reactive T cells refines our ideas on clonal selection and avidity maturation [54, 61, 91], since they did not dominate over their low affinity counterparts even on extended culture *in vitro* (Figure 3.1B).

High frequency of LCMV-specific tetramer-negative CD4+ T cells at the peak effector phase

The acute GP_{61-80} LCMV-specific CD4+ T cell response was analyzed at its peak, 8 days post-infection [101, 200]. CD4+ T cells from the spleen bound specifically to GP_{66-77}:IA^b by tetramer (Figure 3.7A) and 2D analysis (Figure 3.7B). Interestingly, while 8.9 ± 0.4% of CD4+ T cells were detected by pMHC II tetramer, 33.7 ± 4.8% were in fact GP_{61-80}-specific by 2D adhesion analysis (Figure 3.7C). The average effective 2D affinities ($1.65 \pm 0.79 \times 10^{-4}$) of the GP_{61-80} CD4+ T cells in LCMV infection were again 2.6-fold less than *in vitro* GP_{61-80} CD4+ T cells (Figure 3.4A). Although viral-specific CD4+ T cells displayed an overall higher 2D affinity than myelin-specific CD4+ T cells, pMHC II tetramer nonetheless underestimated their frequency by 4-fold. Like MOG_{35-55} CD4+ T cells, the presence of low affinity GP_{61-80} CD4+ T cells demonstrated a lack of exclusive dominance by higher affinity T cell clones during a primary pathogen-specific response or on successive selective cycles with antigen *in vitro*. Thus, it is appears that breadth in affinity is maintained within a CD4+ polyclonal population, at least during the peak effector response.

To determine the contribution of low affinity T cells to overall effector function, splenocytes 8 days post-infection were sorted into GP_{66-77}:IA^b tetramer-positive and – negative CD4+ T cells and assessed for cytokine production (TNFα and IFNγ) in response to GP_{61-80} stimulation (representative experiment, Figure 3.7D). We normalized the percentage of cytokine producing tetramer-positive and tetramer-negative cells to the total number of CD4+ T cells (see Methods) since at least 75% of the tetramer-negative CD4+ T cells in the spleen were not antigen-specific by 2D analysis (Figure 3.7C). The cytokine-adjusted frequencies of tetramer-positive (4.9 ± 0.7%) and tetramer-negative (3.7 ± 1.0%) CD4+ T cells were in fact essentially the same (Figure 3.7E). Our

demonstration that tetramer-negative LCMV-specific CD4+ T cells were major contributors to the effector cytokine response indicates that the approximately equal pMHC II tetramer and cytokine responses in LCMV infection [109, 200] is fortuitous as opposed to being an indication that they are the same high affinity responding T cell population.

In conclusion, we applied a micropipette-based 2D binding technology to measure the affinities and frequencies of polyclonal CD4+ T cells by taking advantage of its capability over other measures to resolve low affinity monoclonal TCR:pMHC interactions [67]. 2D analysis resolved the affinity of polyclonal T cell responses and revealed that low affinity CD4+ T cells participated during autoimmunity and viral infection, although they were accompanied by high affinity counterparts in both cases. Thus, 2D analysis revealed significantly larger numbers of antigen-specific CD4+ T cells than can be quantified using pMHC II tetramers. Our data imply that heterogeneity in TCR affinity is important since both self- and foreign-antigens encompassed more than 100-fold wide affinity ranges. These findings may raise issues over the accuracy that a monoclonal T cell population possessing a single affinity completely reflects immune processes comprised of diverse polyclonal populations of cells with a range of affinities. Characterization of low affinity T cell frequency therefore allows for increased understanding of the polyclonal T cell response, and ultimately enables defining how the affinity range impacts cell-mediated immunity.

Materials and Methods

Mice

C57BL/6 mice were purchased from the National Cancer Institute and housed in an Emory University Department of Animal Resources facility and used in accordance with an IACUC-approved protocol.

Peptides

MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK) and GP_{61-80} (GLNGPDIYKGVYQFKSVEFD) peptides were synthesized on a Prelude peptide synthesizer (Protein Technologies, Inc.).

Cells and reagents

All cells were cultured in RPMI 1640, supplemented with 10% FBS, 2mM L-glutamine, 0.01 M HEPES, 100 μg/ml gentamicin (all Mediatech), and 20 μM 2-ME (Sigma-Aldrich). Polyclonal T cell lines were generated by footpad priming 2-3 month old male mice with 100 μg of the indicated peptide emulsified in CFA containing 1 mg/ml heat-killed *M. tuberculosis*. Draining lymph nodes were harvested 10-14 days later and stimulated for one week with 1 μM of the priming peptide and IL-2. Cells were CD4 purified by positive selection microbeads (Miltenyi Biotec). CD4+ T cells were re-stimulated weekly with irradiated syngeneic splenocytes (3000 rad) and 1 μM peptide and IL-2. Proliferation was measured by ^{3}H-thymidine incorporation as previously described [257].

Tetramer and cell surface staining

MOG_{38-49}:IA^b (generated from a previously described construct) [257], GP_{66-77}:IA^b, $hCLIP_{103-117}$:IA^b monomers and tetramers were provided by the NIAID Tetramer Core

Facility at Emory University (Atlanta, GA). CD4+ T cells were incubated with 4 μg/ml MOG_{38-49}:IA^b (8-20 h), GP_{66-77}:IA^b tetramers (3-4 h), or $hCLIP_{103-117}$:IA^b in complete RPMI at 37 °C. The following antibodies and stains were used for analysis: CD4 (RM4.5), CD8α (53-6.7), B220 (RA3-6B2), CD11b (M1/70), 7-AAD (all BD Biosciences), TCRβ (H57-597; eBioscience), MHC II (M5/114.15.2; BioLegend). All flow cytometric analysis was performed on a FACSCalibur (BD) and data were analyzed using FlowJo (Tree Star). Cell sorting on a FACSAria II was performed by the Emory University Flow Cytometry Core.

Intracellular cytokine detection

CD4+ T cells were stimulated with IA^b-expressing fibroblasts (clone FT7.1C6) [372] and 0 or 10 μM of peptide for 5 h at 37 °C in the presence of 10 μg/ml brefeldin A. T cells were stained with CD4, fixed/permeabilized using the Caltag Laboratories Fix and Perm Cell Permeabilization kit (Invitrogen), and stained for intracellular IFNγ (XMG1.2) and TNFα (MP6-XT22) (both BD Biosciences).

Ex vivo CD4+ T cell analysis in EAE and LCMV

EAE was induced in 6-8 week old female as previously described [257, 348]. For LCMV infection, 2-3 month old male mice were injected i.p. with 2×10^5 pfu LCMV Armstrong [373], kindly provided by Dr. Rafi Ahmed (Emory University). At the time of analysis, all mice were euthanized and perfused with saline. At 21 days post-immunization for EAE, brains and spinal cords were harvested and pooled together from 8-10 mice per experiment. Infiltrating mononuclear cells from the CNS were isolated over Percoll (Sigma-Aldrich). Eight days post-LCMV infection, spleens were harvested and pooled together from 2 mice per experiment. All cells were CD4-purified as above

to 90-95% purity. Cells were stained with tetramer as before, washed and stained with CD4 FITC and a dump channel cocktail of CD8α, B220, CD11b (all PerCP) and 7-AAD. For normalizing the percentage of cytokine-producing tetramer-positive and tetramer-negative cells to the overall percentage of CD4+ T cells in the spleen in LCMV infection, the average percentage of CD4+ T cells in each tetramer fraction were multiplied by the average percentage of cytokine-producing cells in the respective tetramer populations.

Cell preparation for micropipette adhesion frequency assay

Human RBCs were isolated in accordance with the IRB at the Georgia Institute of Technology and prepared as previously described [67, 83]. RBCs coated with various concentrations of Biotin-X-NHS (Calbiochem) were coated with 0.5 mg/ml streptavidin (Pierce), followed by 1-2 μg of pMHC II monomer. The pMHC-coated RBCs were stained with anti-MHC II FITC Ab and T cells were stained with anti-TCR FITC Ab. The site densities of IA^b monomers per RBC and TCRs per T cell were derived using FITC MESF beads (Bangs Labs) as previously described [67, 83] and normalized for the F/P ratios of the antibodies.

2D TCR affinity analysis

The details of the micropipette adhesion frequency assay are described in detail elsewhere [67, 83, 371]. Briefly, a pMHC-coated RBC and T cell were placed on apposing micropipettes and brought into contact by micromanipulation for a controlled contact area (A_c) and time (t). The T cell was retracted at the end of the contact period and the presence of adhesion (indicating TCR:pMHC ligation) was observed microscopically by elongation of the RBC membrane. This contact-retraction cycle was carried out 50 times per T cell-RBC pair to calculate an adhesion frequency (P_a). The

contact area was kept constant for all experiments so it would not affect the affinity comparison. For each experiment, an average P_a was calculated based only on T cells that bound specifically to antigen. The population-averaged 2D affinity (A_cK_a) using the average P_a at equilibrium (where $t \rightarrow \infty$) was calculated using the following equation:

$$A_cK_a = ln[1-P_a(\infty)]/(m_rm_l),$$

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

Statistical analysis

Statistical analyses were performed using GraphPad Prism 4. Two-way student's t-tests were used for all statistical comparisons.

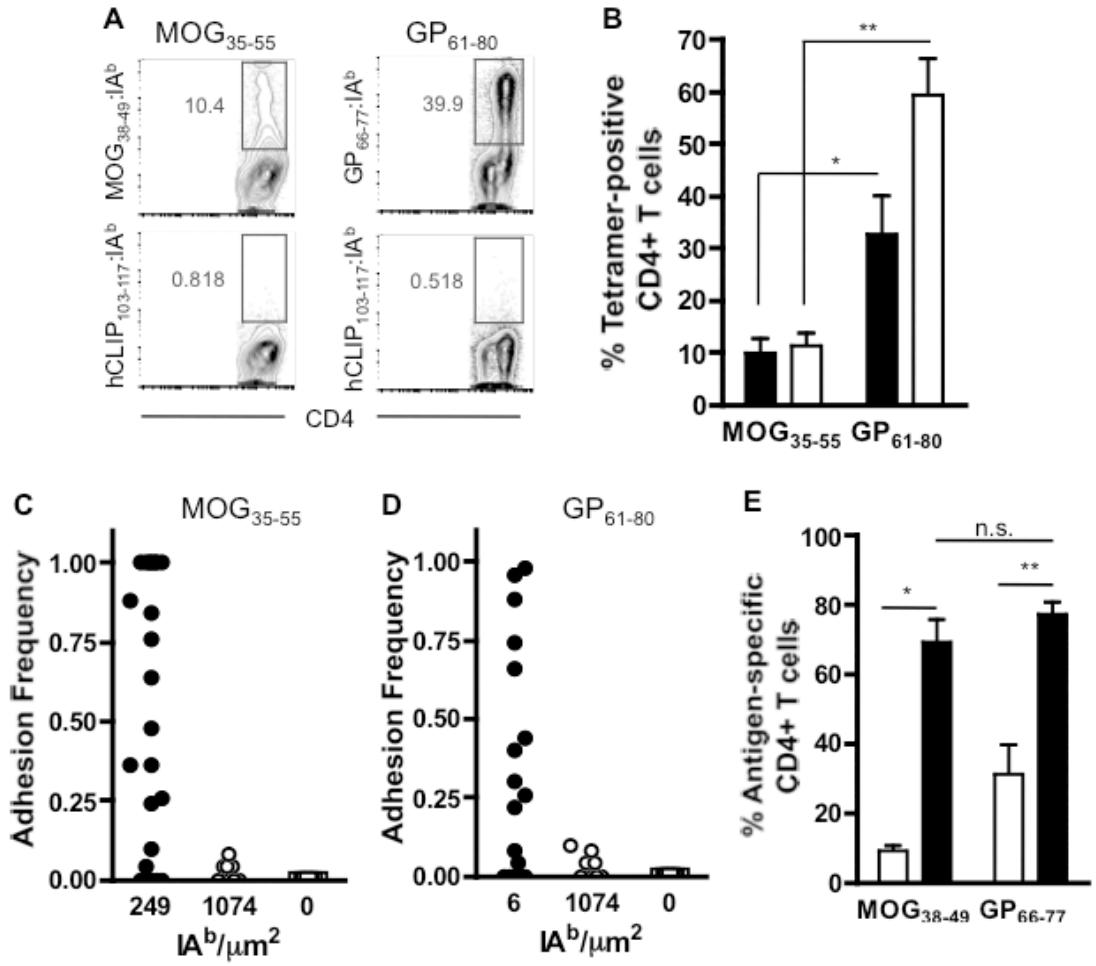

Figure 3.1. Tetramer versus 2D detection of polyclonal MOG_{35-55} and GP_{61-80} CD4+ T cells. Polyclonal MOG_{35-55} and GP_{61-80} CD4+ T cells (after one week in culture) were stained with the indicated tetramers in representative experiments (A). MOG_{35-55} and GP_{61-80} CD4+ T cells were re-stimulated with antigen for 1 week (single stim, ■) or for 4 consecutive weeks (repeated stim, □) and the mean percentages ± SEM of tetramer-positive CD4+ T cells were measured in at least four independent experiments per group (*p=0.03; **p=0.0001) (B). Polyclonal MOG_{35-55} CD4+ T cells were tested for adhesion to the indicated concentrations ($IA^b/\mu m^2$) of MOG_{38-49}:IA^b (●), $hCLIP_{103-117}$:IA^b (○), and no IA^b (□) in a representative experiment (C). Representative adhesion frequencies of

polyclonal GP_{61-80} CD4+ T cells binding to GP_{66-77}:IA^b (●), $hCLIP_{103-117}$:IA^b (O), and no IA^b (□) (D). The average frequency ± SEM of antigen-specific binding by tetramer (□) and 2D analysis (■) was measured in parallel for polyclonal CD4+ T cells after one week of passage *in vitro* (E) (*p=0.0002; **p=0.0003; n.s = not significant). Data were based on four (MOG_{35-55}) and three (GP_{61-80}) independent experiments.

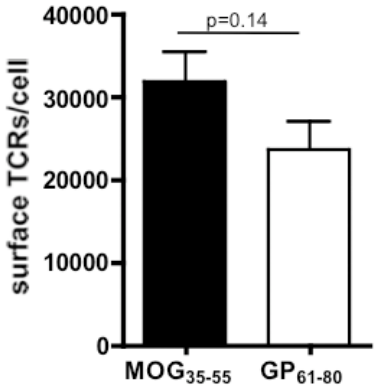

Figure 3.2. TCR levels of MOG_{35-55} and GP_{61-80} CD4+ T cells. Polyclonal MOG_{35-55} and GP_{61-80} CD4+ T cells were re-stimulated for one week *in vitro* with their respective antigens and surface TCR levels were quantified using TCRβ FITC antibody and FITC calibration beads (see Methods). Results are expressed as mean ± SEM and are based on ≥ 5 independent experiments.

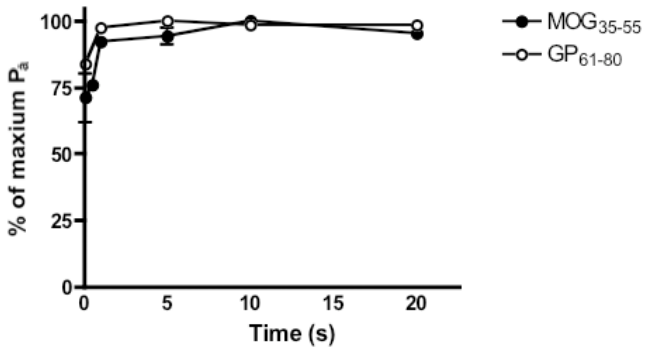

Figure 3.3. Kinetics of maximal adhesion frequency (P_a) of polyclonal CD4+ T cells. Polyclonal MOG_{35-55} (●) and GP_{61-80} (O) CD4+ T cells were brought into contact with antigen-coated RBCs for the indicated contact times and their measured adhesion frequencies were expressed as a percent of the maximum.

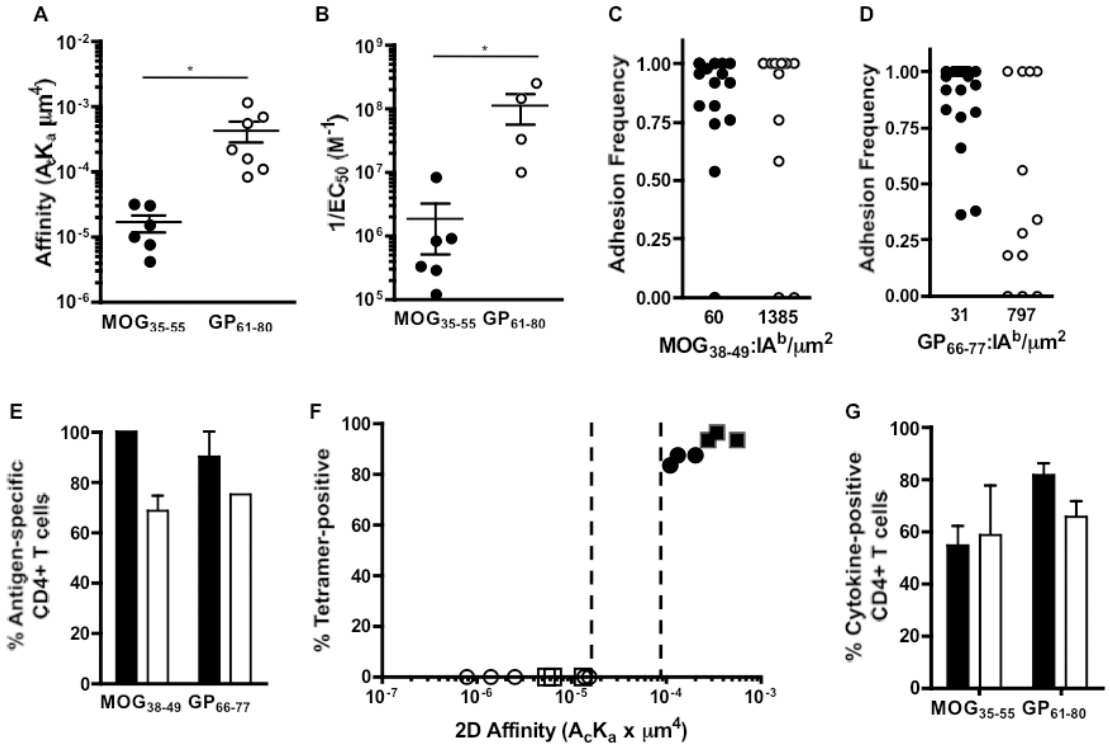

Figure 3.4. 2D affinity and antigen-specificity of tetramer-positive and tetramer-negative CD4+ T cells. The average affinities ± SEM of polyclonal MOG_{35-55} and GP_{61-80} CD4+ T cells were based on 62 MOG_{35-55} CD4+ T cells (six independent experiments) and 54 GP_{61-80} CD4+ T cells (three independent experiments encompassing 7 different antigen densities) (A) (*p=0.03). The mean ± SEM functional avidities (1/EC_{50}, based on proliferation) of polyclonal MOG_{35-55} (1.8 ± 1.3 x 10^6 M^{-1}) and GP_{61-80} (1.1 ± 0.6 x 10^8 M^{-1}) CD4+ T cells were based on six and four independent experiments, respectively (B) (*p=0.04). Representative adhesion frequencies of tetramer-positive (●) and tetramer-negative (O) sorted MOG_{35-55} (C) and GP_{61-80} (D) CD4+ T cells were carried out at the indicated antigen densities. The average percentage ± SEM of antigen-specific 2D binding of tetramer-positive (■) and tetramer-negative (□) MOG_{35-55} and GP_{61-80} CD4+ T cells was based on three (MOG_{35-55}) and two (GP_{61-80}) independent experiments (E). The average 2D affinities of tetramer-positive (black symbols) and tetramer-negative

(white symbols) MOG_{35-55} (circles) and GP_{61-80} (squares) CD4+ T cells were based on at least three independent experiments per group (F). The affinity threshold for tetramer binding is shown by the dotted lines, representing the lowest affinity for tetramer-positive CD4+ T cells ($> 1.1 \times 10^{-4}$ μm^4) and the highest affinity for tetramer-negative CD4+ T cells ($< 1.52 \times 10^{-5}$ μm^4). The average percentage ± SEM of cytokine-producing (IFNγ and TNFα) tetramer-positive (■) and tetramer-negative (□) MOG_{35-55} and GP_{61-80} CD4+ T cells was based on three and two independent experiments, respectively (G).

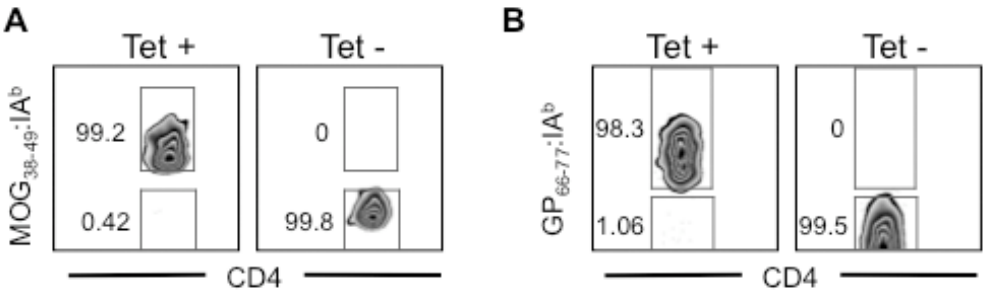

Figure 3.5. Sorting of CD4+ T cells by tetramer binding. Polyclonal MOG_{35-55} (A) and GP_{61-80} (B) CD4+ T cells were sorted into tetramer-positive and tetramer-negative populations using a FACSAria II cell sorter. The purity of representative post-sort populations is shown.

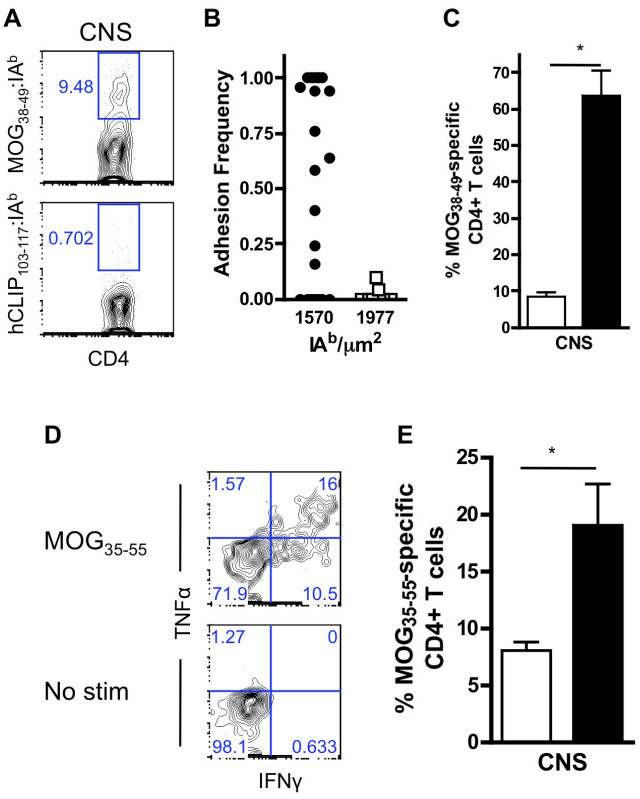

Figure 3.6. Dominance of pro-inflammatory low affinity myelin-reactive CD4+ T cells during EAE. Representative adhesion frequencies of CNS-infiltrating CD4+ T cells to the indicated densities of MOG_{38-49}:IA^b (●) and $hCLIP_{103-117}$:IA^b (□) (A). The average frequency ± SEM of MOG_{35-55}-specific binding by tetramer (□) and 2D analysis (■) was based on 3 experiments done in parallel, and CNS tissue was pooled together from 6-10 mice per experiment (B) (*p=0.004). Representative frequency of cytokine-producing (IFNγ and TNFα) CD4+ T cells isolated from the CNS during acute EAE following stimulation with MOG_{35-55} or no peptide (no stim) (C). The average percentage ± SEM of CNS-infiltrating MOG_{35-55} CD4+ T cells was compared in parallel by MOG_{38-49}:IA^b tetramer and the percent producing cytokine (IFNγ and TNFα) upon stimulation with MOG_{35-55} (no stim background subtracted) from two independent experiments (CNS tissue pooled from 12-24 mice) (D).

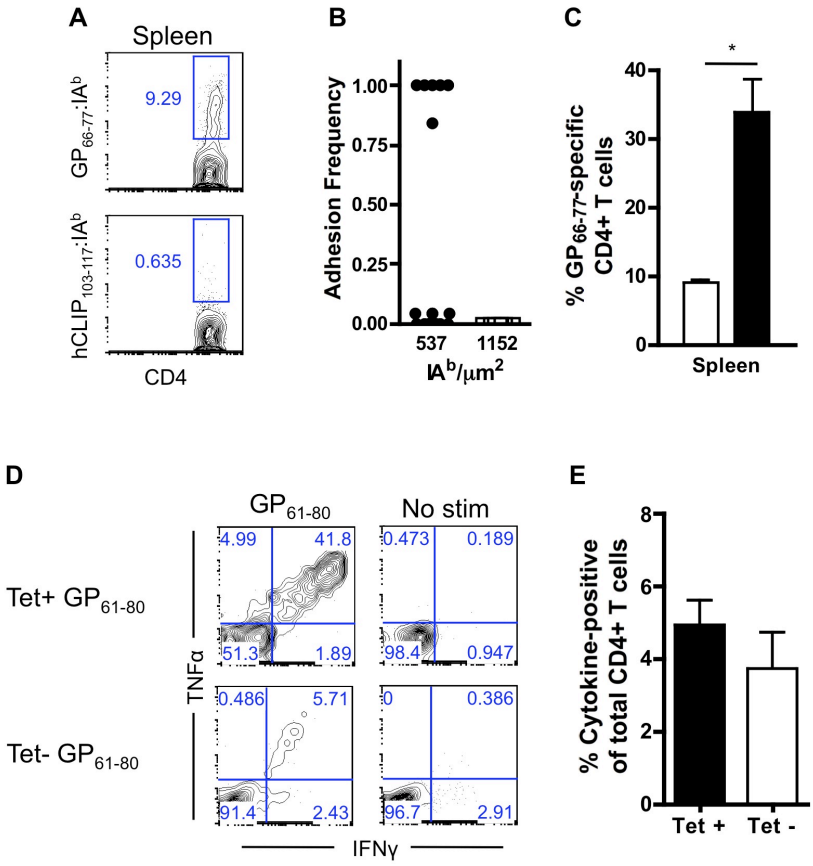

Figure 3.7. Low affinity viral-specific CD4+ T cells contribute significant effector responses during LCMV infection. Representative 2D binding (A) of CD4+ T cells from the spleen at day 8 LCMV to GP_{66-77}:IA^b (●) and $hCLIP_{103-117}$:IA^b (□). The average frequency ± SEM of GP_{61-80} CD4+ T cells in the spleen during LCMV infection by tetramer and 2D analysis (B) was done in parallel in three independent experiments (2 spleens pooled per experiment) (*p=0.007). Tetramer-positive and tetramer-negative sorted GP_{61-80} CD4+ splenocytes at day 8 post-infection were stimulated with 10 μM peptide or media alone (no stim) and assessed for cytokine production (IFNγ and TNFα) in a representative experiment (C). Average percentages ± SEM of cytokine producing tetramer-positive (■) and tetramer-negative (□) CD4+ T cells out of total CD4+ T cells in the spleen were based on 2 experiments (2 spleens pooled per experiment) (D).

Chapter 4: Low affinity cross-reactive myelin- and neuronal-antigen-specific CD4+ T cells predominate during CNS autoimmunity

Abstract

Cross-reactivity has been suggested to be a hallmark of self-reactive T cells, which may be a mechanism for breaking tolerance and the induction of autoimmunity. Recently, it was demonstrated that autoreactive CD4+ T cells specific for myelin-antigen were also highly cross-reactive to a neuronal-antigen in experimental autoimmune encephalomyelitis (EAE). In contrast to myelin-reactive TCR transgenic CD4+ T cells, which were highly responsive to the neuronal peptide, we found that the majority of CD4+ T cells generated to myelin-antigen were only weakly responsive to neuronal antigen. We demonstrate that CD4+ T cells specific for these self-antigens were of overall low affinity, which precluded their identification by pMHC II tetramers. Nonetheless, the majority of autoreactive CD4+ T cells infiltrating the CNS during acute EAE were specific for both self-antigens. These findings suggest that recognition of myelin- and neuronal-antigens in the CNS likely drives the expansion of cross-reactive CD4+ T cells, which are predominantly of low affinity.

Introduction

Despite exquisite specificity for cognate antigen, T cells exhibit a remarkable degree of cross-reactivity to alternate antigenic ligands [4, 5, 10]. Autoreactive T cells, in particular, have been highlighted for their cross-reactivity [25, 276, 278, 285, 286]. Cross-reactivity has typically been suggested as a means for increasing the repertoire of antigen-specific T cells as well as a mechanism of foreign-antigens to break tolerance to self. Recently, it was recently shown that autoreactive T cells could be responsive to two self-antigens as TCR transgenic CD4+ T cells specific for myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ were also responsive to another CNS self-antigen, neurofilament-M $(NF\text{-}M)_{15-35}$ [129], in the EAE mouse model for multiple sclerosis (MS).

Almost all studies demonstrating functional cross-reactivity have been based on responsiveness of monoclonal T cells. The frequency of cross-reactivity for a given TCR clonotype has previously been estimated [274, 275]. However, polyclonal T cell responses are comprised of T cell clonotypes bearing a variety of different TCRs. In light of this TCR heterogeneity, it remains unclear to what extent cross-reactivity occurs in a given polyclonal antigen-specific population. We recently demonstrated that polyclonal CD4+ T cells responses, including those specific for MOG_{35-55}, are comprised of a diverse array of TCR affinities (Chapter 3). Therefore it is also unknown how T cell affinity for one antigen impacts cross-reactivity to another antigen.

In this study, we examined the cross-reactivity of 2D2 TCR transgenic and polyclonal CD4+ T cells to the MOG and NF-M antigens. We found that polyclonal CD4+ T cells primed to MOG_{35-55} or $NF\text{-}M_{15-35}$ showed low functional cross-reactivity to

the alternate self-antigens. This differed starkly from 2D2 CD4+ T cells, which responded much more robustly to NF-M than MOG. Using a 2D-based assay of T cell affinity, we also showed that the majority of MOG and NF-M-specific CD4+ T cells were of low overall affinity. However, a high percentage of CNS-infiltrating CD4+ T cells during EAE were specific for both MOG and NF-M. Thus, our data demonstrate that the majority of autoreactive CD4+ T cells in EAE are cross-reactive to myelin- and neuronal-antigens, but of overall low affinity.

Results

Functional cross-reactivity differences between 2D2 TCR transgenic and polyclonal MOG- and NF-M-specific CD4+ T cells

The core sequences of MOG_{40-48} and NF-M_{20-28} share a remarkable degree of sequence homology, which underlies the previously reported cross-reactivity of MOG_{35-55}-specific 2D2 TCR transgenic CD4+ T cells to NF-M_{15-35} [129]. In agreement with these published observations, we have also demonstrated that naïve 2D2 CD4+ T cells proliferate much more strongly to NF-M_{15-35} than MOG_{35-55} (Figure 4.1A). Similar responsiveness was also detected to NF-M_{18-30} (data not shown), indicating the core NF-M epitope was located within that sequence, as previously suggested [129]. The superagonist responsiveness to NF-M is remarkable given that the 2D2 transgenic TCR was cloned out of a polyclonal CD4+ T cell population expanded only to MOG_{35-55} peptide [236]. Thus, it is possible that polyclonal MOG_{35-55} CD4+ T cells would be cross-reactive to NF-M_{15-35}, as has been previously reported [129]. However, we have observed a minimal amount of functional cross-reactivity of polyclonal MOG_{35-55} CD4+ T cells only to high concentrations (10-100 μM) of NF-M_{15-35} *in vitro* (Figure 4.1B). Moreover, we have not observed any functional cross-reactivity between polyclonal NF-M_{15-35} CD4+ T cells to MOG_{35-55} (Figure 4.1C).

Low degree of cross-reactive MOG and NF-M tetramer binding

Despite their responsiveness to MOG_{35-55}, 2D2 TCR transgenic CD4+ T cells are undetectable by MOG_{38-49}:IA^b tetramer (Figure 4.2A). In order to detect NF-M_{15-35} CD4+ T cells, we generated an NF-M_{18-30}:IA^b tetramer centered on the truncated sequence previously identified [129]. Similar to the MOG tetramer data, 2D2 T cells

were undetectable by the NF-M tetramer under any conditions tested (Figure 4.2A). We next compared the ability of MOG_{38-49}:IA^b and NF-M_{18-30}:IA^b tetramers to detect *in vitro* polyclonal MOG_{35-55} and NF-M_{15-35} CD4+ T cells, respectively (Figure 4.2B, representative experiment). In agreement with our previous results (Chapter 3), approximately 10% (9.4 ± 1.4%) of MOG_{35-55} CD4+ T cells were detectable by tetramer (Figure 4.2C). Even fewer NF-M_{15-35} CD4+ T cells (4.1 ± 1.1%) showed specific binding to the NF-M tetramer (Figure 4.2C). Although polyclonal CD4+ T cells to MOG_{35-55} and NF-M_{15-35} showed a minimal amount of functional cross-reactivity (Figure 4.1B-C), we used tetramers conjugated with different fluorophores to compare the cross-specificity of the MOG and NF-M tetramers within both T cell populations *in vitro*. Importantly, co-staining with both tetramers did not markedly differ from single tetramer staining (Figure 4.3). Small, but detectable fractions of polyclonal MOG_{35-55} and NF-M_{15-35} CD4+ T cells showed cross-specific binding to both tetramers (2.5 ± 0.9% and 1.2 ± 0.1%, respectively) (Figure 4.2C). Thus, in contrast to 2D2 T cells, functional and tetramer analyses indicated that polyclonal MOG_{35-55} and NF-M_{15-35} CD4+ T cells were minimally cross-reactive.

MOG- and NF-M-specific CD4+ T cells are partially cross-reactive, but of very low affinity

It was possible that the low degree of tetramer detection of MOG_{35-55} and NF-M_{15-35} CD4+ T cells was due to overall low TCR affinities. Using a highly sensitive micropipette adhesion frequency assay that measures 2D receptor-ligand interactions [67, 371], we have previously shown greatly enhanced detection of low affinity CD4+ T cells below the detection threshold for pMHC II tetramer (Chapter 3). We therefore reasoned

that 2D2 CD4+ T cell specificity for MOG and NF-M might be detectable by micropipette analysis, despite the lack of detection by tetramer. A single T cell was brought in and out of contact with a red blood cell (RBC) coated with pMHC II to derive the percentage of adhesions out of the total number of contacts, termed the adhesion frequency (described in Methods). The adhesion frequency was determined by TCR and pMHC densities and time of contact (until equilibrium) to detect a wide range of affinities (10^{-2} to 10^{-8} μm^4 from highest to lowest affinity) [67, 371].

Although 2D2 CD4+ T cells were undetectable by MOG or NF-M tetramers, the T cells readily bound to NF-M_{18-30}:IA^b by 2D analysis (Figure 4.4A). The 2D kinetics of the 2D2 TCR and NF-M were overall very low (A_cK_a = 9.23 x 10^{-6} μm^4, A_ck_{on} = 1.52 x 10^{-5} μm^4/s, k_{off} = 1.77/s), on-par with that of a weak agonist for a TCR transgenic CD8+ T cell [67]. Moreover, the 2D2 affinity for NF-M was approximately the same overall affinity of polyclonal MOG_{35-55} CD4+ T cells for MOG antigen (Chapter 3), and below the affinity threshold level we have identified as necessary for pMHC II tetramer binding. Strikingly, 2D2 CD4+ T cells showed no specific binding to MOG_{38-49}:IA^b even at very high antigen densities, prolonged contact times (up to 2 minutes), or following activation with MOG_{35-55} or NF-M_{15-35} peptides (Figure 4.4A). The markedly higher 2D kinetics of 2D2 for NF-M compared to MOG was therefore consistent with the functional responsiveness differences (Figure 4.1A).

Although polyclonal MOG_{35-55} and NF-M_{15-35} CD4+ T cells were poorly cross-reactive by the previous analyses, it was possible that there was a larger cross-reactive population with affinity below that needed for detectable functional responses or tetramer binding. To test this, we compared the ability of polyclonal MOG_{35-55} and NF-M_{15-35}

CD4+ T cells to bind to MOG_{38-49}:IA^b and NF-M_{18-30}:IA^b (Figure 4.4B-C, representative experiments). As expected, the majority of both polyclonal CD4+ T cell populations were detected by their specific antigens (71.9 ± 5.6% for MOG_{35-55} and 73.8 ± 4.8% for NF-M_{15-35}) in the 2D assay (Figure 4.4D). Consistent with our previous results, both polyclonal CD4+ T cell populations had low overall 2D affinities for their respective antigens, but NF-M_{15-35} CD4+ T cells had an approximately 4-fold lower 2D affinity than MOG_{35-55} CD4+ T cells (3.15×10^{-6} μm^4 versus 1.06×10^{-5} μm^4). Surprisingly, approximately one-third of polyclonal MOG_{35-55} and NF-M_{15-35} CD4+ T cells showed cross-specific binding to the NF-M and MOG antigens (Figure 4.4D). However, the affinities of cross-reactive MOG_{35-55} and NF-M_{15-35} CD4+ T cells for NF-M and MOG, respectively, was quite low (MOG:NF-M = 1.99×10^{-6} and NF-M:MOG = 1.15×10^{-6}).

Low frequency of MOG and NF-M tetramer-positive CD4+ T cells in the CNS during EAE

We and others have previously used the MOG tetramer to demonstrate a low frequency of tetramer-positive MOG_{35-55} CD4+ T cells in the CNS during EAE [256, 257, Chapter 3]. Because NF-M_{15-35} cannot induce active EAE [129], we compared the kinetics of tetramer-positive MOG_{35-55} and NF-M_{15-35} CD4+ T cells in the CNS during MOG_{35-55}-induced EAE. As shown in polyclonal CD4+ T cells *in vitro*, staining of *ex vivo* CNS CD4+ T cells with both tetramers labeled with different fluorophores allowed the identification of MOG, NF-M, and MOG/NF-M tetramer-positive CD4+ T cells (Figure 4.5A, representative experiment). As expected, the detection of all tetramer-positive CD4+ T cells directly overlapped with the onset of EAE symptoms (approximately day 15). Peak MOG and NF-M tetramer detection was overall low and

occurred five days post-symptom onset (8.6 ± 1.4% and 4.2 ± 1.3%, respectively) (Figure 4.5B). This was followed by a rapid and then a gradual contraction phase with both tetramer populations maintained at approximately 1% of CD4+ T cells at day 75. A very small population of double tetramer-positive CD4+ T cells was detected throughout EAE, though this never exceeded approximately 1% of CD4+ T cells (Figure 4.5B). In order to compare the functional frequencies of MOG_{35-55} and NF-M_{15-35} CD4+ T cells in the CNS, we re-stimulated *ex vivo* CNS-infiltrating CD4+ T cells with both peptides and assessed the frequency of cells producing cytokine (TNFα and IFNγ) (Figure 4.5C, representative experiment). Approximately 20% (19.0 ± 3.6%) of CD4+ T cells produced cytokine in response to MOG_{35-55} stimulation, whereas nearly five times fewer CD4+ T cells produced cytokine in response to NF-M_{15-35} (4.1 ± 1.6%) (Figure 4.5D).

High frequency of MOG and NF-M CD4+ T cells in the CNS during EAE are cross-reactive, but low affinity

In light of the MOG and NF-M cross-specificity observed in polyclonal MOG_{35-55} CD4+ T cells *in vitro*, we used the micropipette assay to determine whether a similar degree of cross-specificity also occurred *ex vivo*. To test this, we isolated CD4+ T cells from the CNS at the peak of EAE and tested their specificity for MOG and NF-M (Figure 4.6A, representative experiment). We observed that the majority of the CD4+ T cells were specific for MOG (71.4 ± 1.7%) and NF-M (58.0 ± 5.4%) (Figure 4.6B), which were more than 8- and 14-fold higher than the frequencies measured by each of the respective tetramers (Figure 4.5B). While the 2D affinities to both antigens were low, the affinity for MOG was approximately 3-fold higher than that of NF-M (7.11×10^{-6} vs 2.72

x 10^{-6}), consistent with the higher frequency of tetramer-positive MOG_{35-55} than NF-M_{15-35} CD4+ T cells.

An unanswered question, however, was to what extent the CD4+ T cells that bound to MOG and NF-M were the same (i.e. cross-reactive) or distinct T cell populations. Since the total frequency of CNS-infiltrating CD4+ T cells specific for MOG and NF-M exceeded 100%, this implied that at least a portion of the CD4+ T cells were specific for both self-antigens. To test this, we used a sequential binding technique, in which the adhesion frequency for a given T cell was first measured by binding to an RBC coated with one antigen (e.g. MOG), followed by measuring the adhesion frequency of the same T cell to an RBC coated with the other antigen (e.g. NF-M) (Figure 4.6C, representative experiment). The sequence of whether MOG or NF-M was tested first was alternated and was not found to have any significant effect on the adhesion frequency to the subsequent antigen (Figure 4.7). Strikingly, 80.9 ± 4.4% of the CD4+ T cells tested in the CNS were specific for MOG and/or NF-M (Figure 4.6D), indicating that the overwhelming majority of CD4+ T cells during acute EAE were specific for the two CNS auto-antigens. Furthermore, only a small fraction of CD4+ T cells were specific for only MOG or NF-M (17.7 ± 3.0% and 11.8 ± 0.0%, respectively) (Figure 4.6D) and exhibited low 2D affinities (4.02 x 10^{-6} and 2.61 x 10^{-6}, respectively). Thus, '2D2-like' CD4+ T cells that showed specific binding to NF-M, but not to MOG, appeared to be an infrequent population. In contrast, approximately two-thirds of the CD4+ T cells specific for MOG or NF-M were cross-specific for both antigens and were overall of low affinity (5.99 x 10^{-6} and 3.13 x 10^{-6}, respectively).

NF-M stimulation of MOG CD4+ T cells drives expansion of cross-reactive T cells

The percentage of CD4+ T cells specific for NF-M in the CNS during acute EAE (58.0 ± 5.4%) (Figure 4.6B) was significantly higher than the percentage of polyclonal MOG_{35-55} CD4+ T cells that were cross-reactive to NF-M *in vitro* (33.7 ± 7.0%) (Figure 4.4D). It was possible that this was due to the expansion of MOG_{35-55}-primed CD4+ T cells following encounter with NF-M antigen within the CNS. To test this, we expanded polyclonal MOG_{35-55} CD4+ T cells *in vitro* with MOG_{35-55} and/or NF-M_{15-35} peptide and measured the specificity of the T cells for MOG and NF-M using the sequential binding technique as described above (Figure 4.8A-B, representative experiments). Stimulation of polyclonal MOG_{35-55} CD4+ T cells with NF-M_{15-35} (Figure 4.8C) did not alter the frequency of MOG-specific binding compared to those stimulated with MOG_{35-55} alone (Figure 4.4D). In contrast to the relatively low degree of NF-M specificity exhibited by polyclonal MOG_{35-55} CD4+ T cells expanded with MOG_{35-55} alone, expansion of MOG_{35-55} CD4+ T cells with NF-M_{15-35} resulted in approximately three-quarters (72.5 ± 12.5%) of the CD4+ T cells being cross-specific for NF-M (Figure 4.8C) with low affinity for both antigens (7.02×10^{-6} for MOG and 4.37×10^{-6} for NF-M), which was similar to the cross-reactive affinities and frequencies detected in the CNS during EAE. This therefore indicated that exposure to NF-M_{15-35} antigen can expand out NF-M cross-reactive MOG_{35-55} CD4+ T cells. Thus, the high degree of MOG and NF-M CD4+ T cell cross-reactivity observed in the CNS during acute EAE was likely due to the responsiveness of the autoreactive CD4+ T cells to NF-M antigen within the CNS.

Discussion

Cross-reactivity to multiple antigens is a well-known feature of T cells. Autoreactive T cells, in particular, have been noted for their antigen promiscuity, which has been suggested to play an important role in breaking tolerance and the induction of autoimmune disease [278-284]. A critical question is to what extent cross-reactivity occurs within polyclonal autoreactive T cell populations, and whether there is a correlation between T cell receptor affinity and the degree of functional cross-reactivity. Although it has been suggested that there is no simple correlation between TCR affinity and cross-reactivity [374], other studies have suggested that T cells with low affinity for self-antigen were more cross-reactive than higher affinity T cells [375, 376]. To further address this important question, we examined the MOG_{35-55} CD4+ T cell response, which was recently reported to be cross-reactive with the neuronal self-antigen NF-M_{15-35} [129]. In agreement with this report, we also found that MOG_{35-55}-specific 2D2 TCR transgenic CD4+ T cells were more responsive to NF-M_{15-35} than to MOG_{35-55}. Despite their high functional avidity to NF-M_{15-35}, 2D2 T cells were undetectable by NF-M tetramer. This is likely due to their low 2D affinity, which is below our previously reported affinity threshold required for pMHC II tetramer binding (Chapter 3).

In contrast to the monoclonal 2D2 TCR clonotype, *in vitro* polyclonal MOG_{35-55} and NF-M_{15-35} CD4+ T cells demonstrated a low degree of cross-reactivity by functional or pMHC II tetramer analysis. However, approximately one-third of polyclonal CD4+ T cells were cross-reactive to MOG or NF-M by 2D analysis. That the majority of *in vitro* MOG and NF-M cross-specific CD4+ T cells were of low overall affinity likely limited functional responsiveness to higher antigen concentrations and prohibited their detection

by tetramer. Our analyses found that a very low percentage (approximately 12%) of CD4+ T cells generated by priming with MOG_{35-55} demonstrated detectable by binding to NF-M, but not to MOG. Thus, '2D2-like' CD4+ T cells appear to represent a very narrow repertoire of MOG_{35-55} CD4+ T cells. These findings further demonstrate that monoclonal T cell populations fail to faithfully recapitulate polyclonal immune responses.

Strikingly, the polyclonal MOG_{35-55} CD4+ T cell response in the CNS during EAE exhibited a much stronger degree of cross-reactivity to NF-M_{15-35} than was observed *in vitro*. Indeed, the majority of the CD4+ T cells in the CNS during acute EAE were specific for both MOG and NF-M. The cross-reactive CD4+ T cells were of overall low affinity, which likely explains the low degree of cross-reactivity detected functionally and by tetramer. These data suggest that cross-reactivity of low affinity autoreactive CD4+ T cells to multiple self-antigens, as in the case of MOG and NF-M in EAE, provides an additional mechanism for the perpetuation of autoimmunity. Intriguingly, the frequency of NF-M-specific MOG_{35-55} CD4+ T cells was substantially increased by the addition of NF-M_{15-35} peptide *in vitro*. This suggests that although a degree of NF-M cross-reactivity is intrinsic to polyclonal MOG_{35-55} CD4+ T cells, the response can be shifted (e.g. via repertoire skewing or tuning) toward greater cross-reactivity by the presence of NF-M. Thus, the increased prevalence of MOG_{35-55} CD4+ T cells cross-reactive to NF-M_{15-35} is likely due to encounter with NF-M in the CNS during EAE. This is consistent with the findings that 2D2 T cells induce EAE in MOG -/- mice and that inflammatory infiltrates are found in peripheral nerves where MOG is not present [129]. Thus, despite being overall low affinity, cross-recognition of the neuronal antigen is sufficient to drive the

expansion of cross-reactive MOG_{35-55} CD4+ T cells during EAE. Further studies are needed to determine what role this low affinity cross-reactivity plays in breaking tolerance and in EAE pathogenesis.

Materials and Methods

Mice

C57BL/6 mice were purchased from the National Cancer Institute and 2D2 mice were purchased from The Jackson Laboratory. All animals were housed in an Emory University Department of Animal Resources facility and used in accordance with an IACUC-approved protocol.

Peptides

MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK) and NF-M_{15-35} (RRVTETRSSFSRVSGSPSSGF) peptides were synthesized on a Prelude peptide synthesizer (Protein Technologies, Inc.).

Cells and reagents

All cells were cultured in RPMI 1640, supplemented with 10% FBS, 2mM L-glutamine, 0.01 M HEPES, 100 μg/ml gentamicin (all Mediatech), and 20 μM 2-ME (Sigma-Aldrich). Polyclonal T cell lines were generated by footpad priming 2-3 month old male B6 mice with 100 μg of the indicated peptide emulsified in CFA containing 1 mg/ml heat-killed *M. tuberculosis* and draining lymph nodes (LNs) were harvested 10-14 days later. For proliferation assays, 5 x 10^5 LN cells or 2D2 splenocytes were stimulated *ex vivo* with the indicated concentrations of peptides and ^{3}H-thymidine incorporation was as previously described [257]. For pMHC II tetramer and micropipette analysis, *ex vivo* LN cells were stimulated for one week with 1 μM of the priming peptide and IL-2. In a subset of experiments, *ex vivo* MOG_{35-55} LNs were stimulated with 1 μM MOG_{35-55} and/or 10 μM NF-M_{15-35}. CD4 purification was done by positive selection microbeads (Miltenyi Biotec).

Tetramer and cell surface staining

MOG_{38-49}:IA^b, NF-M_{18-30}:IA^b, $hCLIP_{103-117}$:IA^b monomers and tetramers were provided by the NIAID Tetramer Core Facility at Emory University (Atlanta, GA). CD4+ T cells were incubated with 4 μg/ml of the indicated tetramers for 8-20h in complete RPMI at 37 °C. The following antibodies and stains were used for analysis: CD4 (RM4.5), CD8α (53-6.7), B220 (RA3-6B2), CD11b (M1/70), Vα3.2 (RR3-16), 7-AAD (all BD Biosciences), TCRβ (H57-597; eBioscience), MHC II (M5/114.15.2; BioLegend). All flow cytometric analysis was performed on a FACSCalibur (BD) and data were analyzed using FlowJo (Tree Star).

Intracellular cytokine detection

CD4+ T cells were stimulated with IA^b-expressing fibroblasts (clone FT7.1C6, [372] and 0 or 10 μM of the indicated peptide for 5 h at 37 °C in the presence of 10 μg/ml brefeldin A. T cells were stained with CD4, fixed/permeabilized using the Caltag Laboratories Fix and Perm Cell Permeabilization kit (Invitrogen), and stained for intracellular IFNγ (XMG1.2) and TNFα (MP6-XT22) (both BD Biosciences).

Ex vivo CD4+ T cell analysis in EAE

EAE was induced in 6-8 week old female as previously described [257, 348]. At the indicated time of analysis, all mice were euthanized and perfused with saline. For the tetramer analysis, cells were stained MOG_{38-49}:IA^b PE and NF-M_{18-30}:IA^b APC tetramers (using previously described staining conditions), washed and stained with CD4 FITC and a dump channel cocktail of CD8α, B220, CD11b (all PerCP) and 7-AAD. For the micropipette analysis of *ex vivo* CNS CD4+ T cells, brains and spinal cords were harvested 21 days post-immunization and pooled together from 10-12 mice per

experiment. Infiltrating mononuclear cells were isolated over Percoll (Sigma-Aldrich) CD4-purified as above to 90-95% purity.

Cell preparation for micropipette adhesion frequency assay

Human RBCs were isolated in accordance with the IRB at the Georgia Institute of Technology and prepared as previously described [67, 83]. RBCs coated with various concentrations of Biotin-X-NHS (Calbiochem) were coated with 0.5 mg/ml streptavidin (Pierce), followed by 1-2 μg of pMHC II monomer. The site densities of IA^b monomers per RBC and TCRs per T cell were derived using anti-MHC II FITC and anti-TCR FITC antibodies and FITC MESF beads (Bangs Labs) and normalized for the F/P ratios of the antibodies, as previously described [67, Chapter 3].

2D TCR kinetics analysis

The details of the micropipette adhesion frequency assay are described in detail elsewhere [67, 371]. Briefly, a pMHC-coated RBC and T cell were placed on apposing micropipettes and brought into contact by micromanipulation for a controlled contact area (A_c) and time (t). The T cell was retracted at the end of the contact period and the presence of adhesion (indicating TCR:pMHC ligation) was observed microscopically by elongation of the RBC membrane. This contact-retraction cycle was carried out 50 times per T cell-RBC pair to calculate an adhesion frequency (P_a). The contact area was kept constant for all experiments so it would not affect the affinity comparison. For each experiment, an average P_a was calculated based only on T cells that bound specifically to antigen. The population-averaged 2D affinity (A_cK_a) using the average P_a at equilibrium (where $t \rightarrow \infty$) was calculated using the following equation:

$$A_cK_a = \ln\left[1 - P_a(\infty)\right] / (m_r m_l)$$

where m_r and m_l reflect the receptor (TCR) and ligand (pMHC) densities, respectively. The 2D affinities were derived at 5 seconds, since we have previously demonstrated that polyclonal CD4+ T cells reach equilibrium within seconds of contact (Chapter 3). The 2D off-rate (k_{off}) was calculated by:

$$k_{\text{off}} = \frac{1}{t_{1/2}} \ln\left\{1 - \frac{\ln[1 - \frac{1}{2}P_a(\infty)]}{\ln[1 - P_a(\infty)]}\right\}^{-1}$$

where $t_{1/2}$ is the time to reach the half-maximal P_a at equilibrium (∞). The 2D on-rate (A_cK_{on}) was calculated from $k_{on} = K_a \times k_{off}$.

Determination of T cell frequency by 2D binding analysis

The frequencies of polyclonal antigen-specific CD4+ T cells derived *in vitro* or *ex vivo* were determined by 2D analysis by measuring the percentage of T cells with binding above irrelevant antigen background ($P_a > 0.12$, see $hCLIP_{103\text{-}117}$:IA^b). The frequencies of cross-specific CD4+ T cells were tested using the sequential binding technique, as described in the Results. In these assays, the adhesion frequencies were derived from 25 contacts to $MOG_{38\text{-}49}$:IA^b and NF-$M_{18\text{-}30}$:IA^b-coated RBCs each, which was found not to differ significantly from the adhesion frequencies measured after 50 contacts.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 4. Two-way student's t-tests were used for all statistical comparisons.

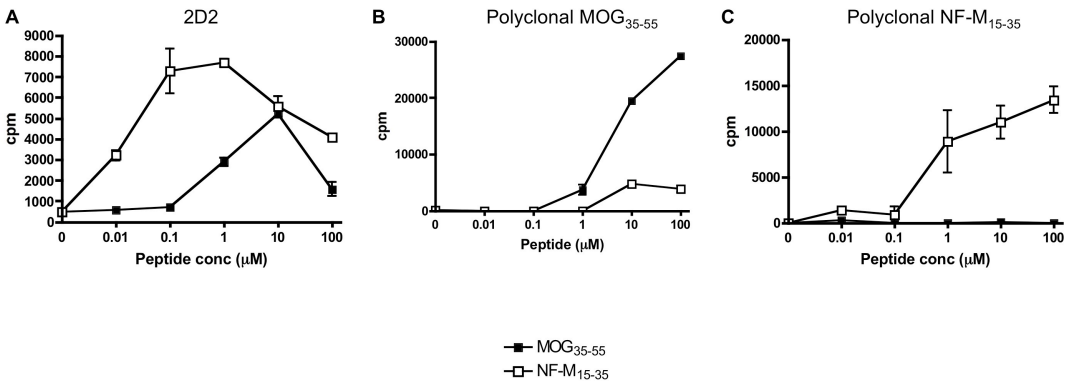

Figure 4.1. Functional cross-reactivity of 2D2 TCR transgenic and polyclonal CD4+ T cells. Naïve 2D2 TCR transgenic splenocytes (A) or *ex vivo* MOG_{35-55}- (B) or NF-M_{15-35}-primed (C) lymph node cells were stimulated with the indicated concentrations of MOG_{35-55} or NF-M_{15-35}. Data were representative of 3-6 independent experiments per CD4+ T cell population.

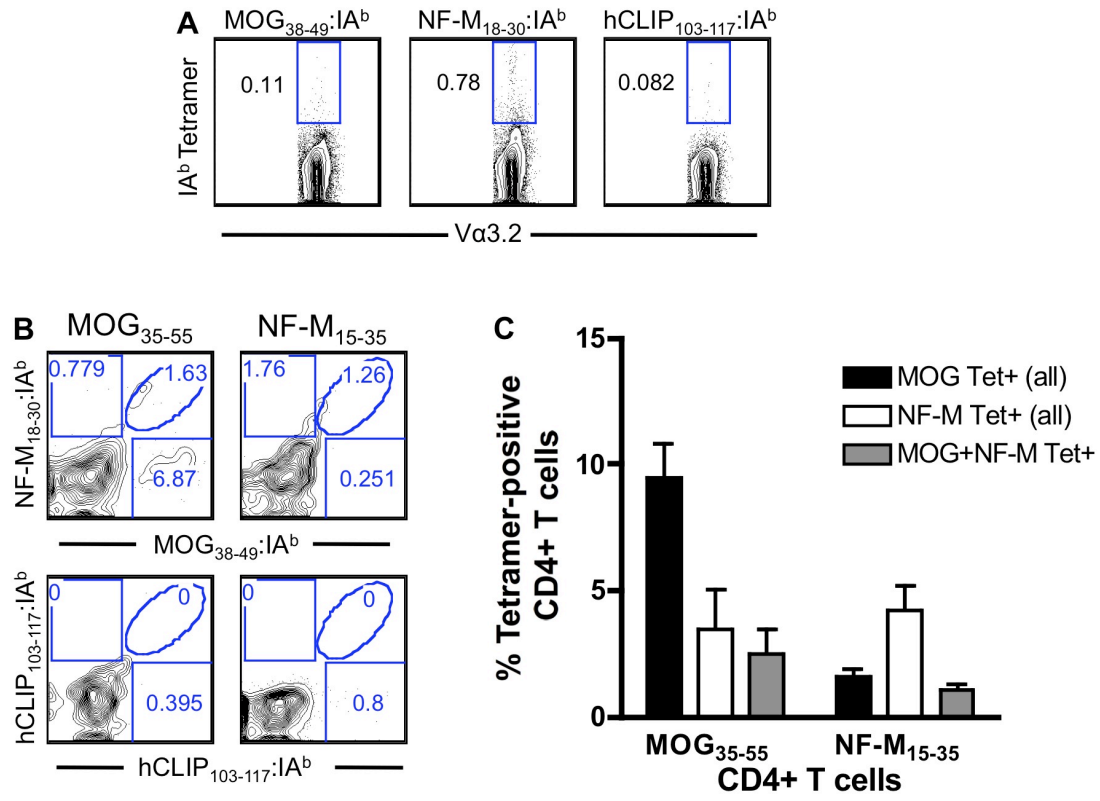

Figure 4.2. pMHC II tetramer detection of MOG_{35-55} and NF-M_{15-35}-specific CD4+ T cells. Naïve 2D2 TCR transgenic splenocytes were stained with MOG, NF-M, or hCLIP tetramers overnight at 37 °C and gated on CD4+ Vα3.2+ T cells; representative of 2 independent experiments (A). Representative tetramer analysis of polyclonal MOG_{35-55} and NF-M_{15-35} CD4+ T cells stained simultaneously with MOG-PE and NF-M APC or hCLIP PE and hCLIP APC tetramers (B). The mean frequency (± SEM) of MOG, NF-M, or MOG-NF-M tetramer-positive MOG_{35-55} and NF-M_{15-35} CD4+ T cells (C) were based on 3-6 independent experiments per group.

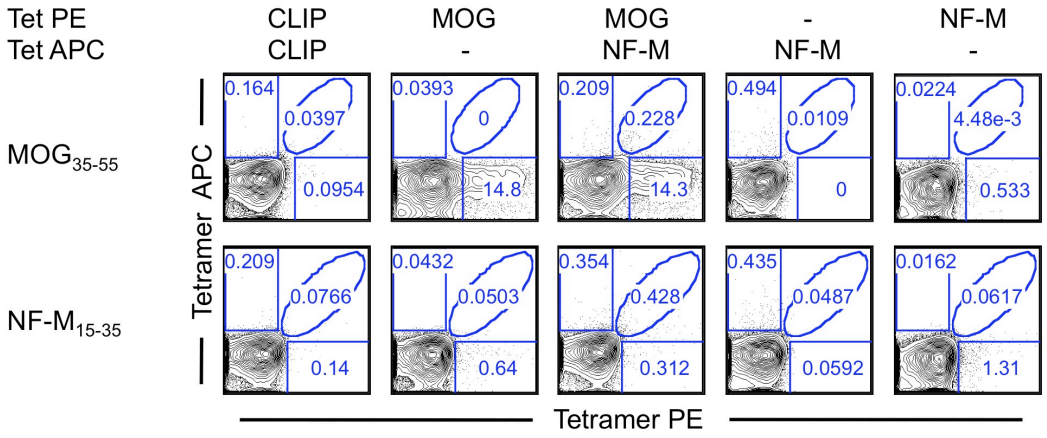

Figure 4.3. MOG and NF-M tetramer co-staining of polyclonal CD4+ T cells.

Polyclonal MOG_{35-55} and $NF\text{-}M_{15-35}$ CD4+ T cells were co-incubated with the indicated PE- and APC-labeled IA^b tetramers. Cells were gated on CD4+ lymphocytes.

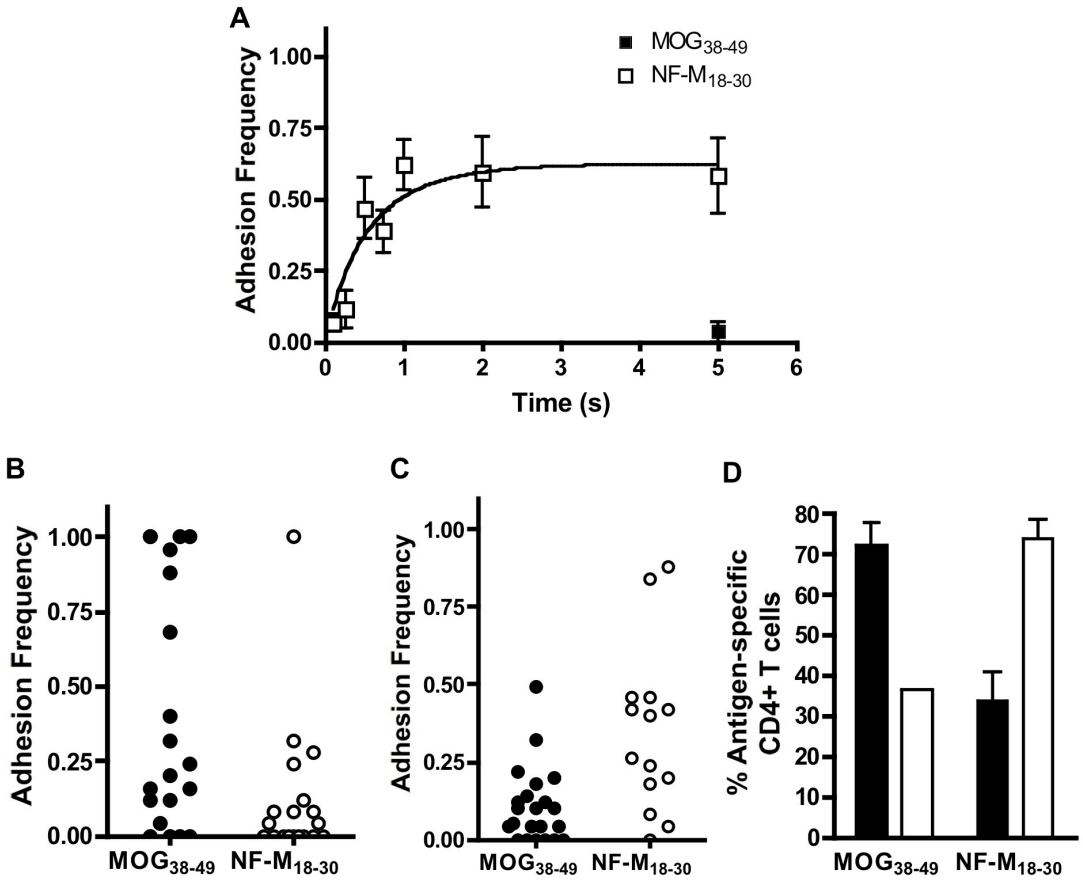

Figure 4.4. 2D binding analysis of 2D2 and polyclonal MOG_{35-55} and NF-M_{15-35} CD4+ T cells. Naïve 2D2 splenocytes were CD4-purfied and tested for binding to MOG_{38-49}:IA^b (■) or NF-M_{18-30}:IA^b (□) monomer-coated RBCs for the indicated periods of contact (A). Data are representative of 2 (NF-M:IA^b) and 4 (MOG:IA^b) independent experiments. Representative adhesion frequencies of polyclonal MOG_{35-55} (B) and NF-M_{15-35} (C) CD4+ T cells to MOG_{38-49}:IA^b (●) or NF-M_{18-30}:IA^b (○) monomer-coated RBCs. The average frequency (± SEM) of MOG- (■) and NF-M-specific (□) 2D binding of polyclonal MOG_{35-55} and NF-M_{15-35} CD4+ T cells (D) was based on 2-6 independent experiments for each group (except the NF-M:NF-M group, based on 1 experiment).

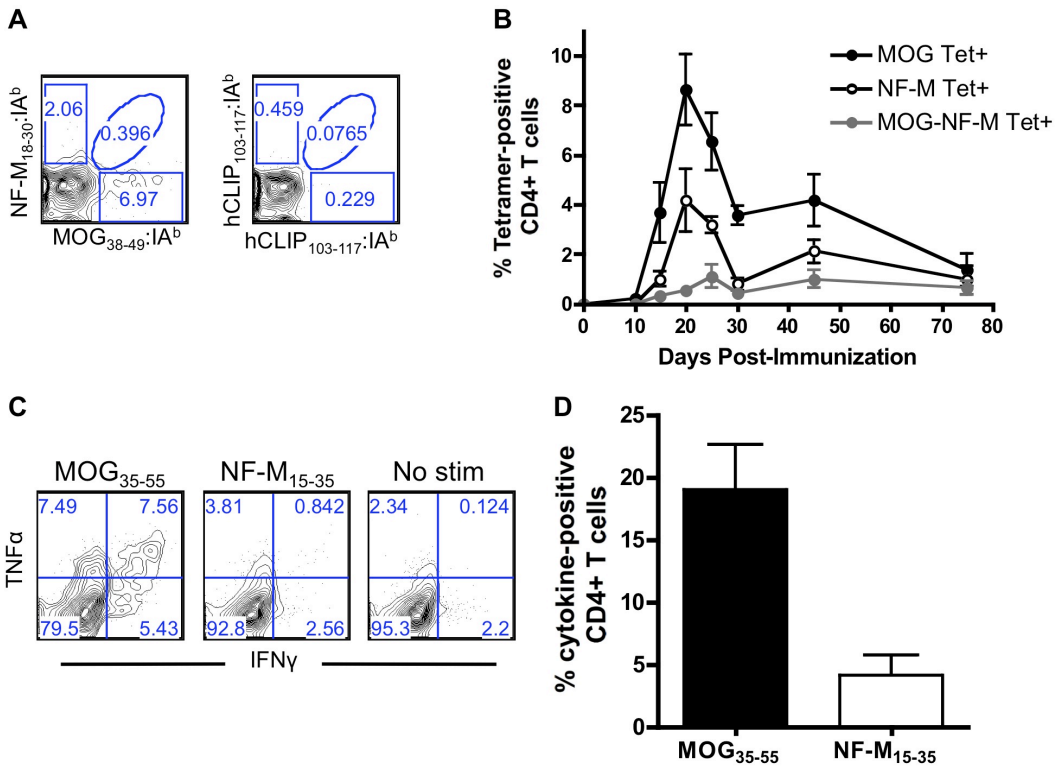

Figure 4.5. pMHC II tetramer and functional reactivity of MOG_{35-55} and $NF\text{-}M_{15-35}$ CD4+ T cells in the CNS during EAE. Representative pMHC II tetramer analysis of CD4+ T cells isolated from day 20 of EAE following co-staining with MOG_{38-49}:IA^b PE and $NF\text{-}M_{18-30}$:IA^b APC tetramers or $hCLIP_{103-117}$:IA^b PE or $hCLIP_{103-117}$:IA^b APC tetramers (A). The average percentages (± SEM) of CNS-infiltrating MOG_{38-49}:IA^b and or $NF\text{-}M_{18-30}$:IA^b tetramer-positive CD4+ T cells at the indicated time points of EAE (B). Data are based on 6-8 mice per group from 2 independent experiments per day of analysis. Representative cytokine production of CD4+ T cells isolated from the CNS during acute EAE with 10 μM of MOG_{35-55}, 10 μM $NF\text{-}M_{15-35}$ or media alone (no stim) (C). The average frequency (± SEM) of cytokine producing (TNFα and/or IFNγ) CD4+ T cells from the CNS at peak EAE are based on two independent experiments where the CNS was pooled from 10-12 mice per experiment (D).

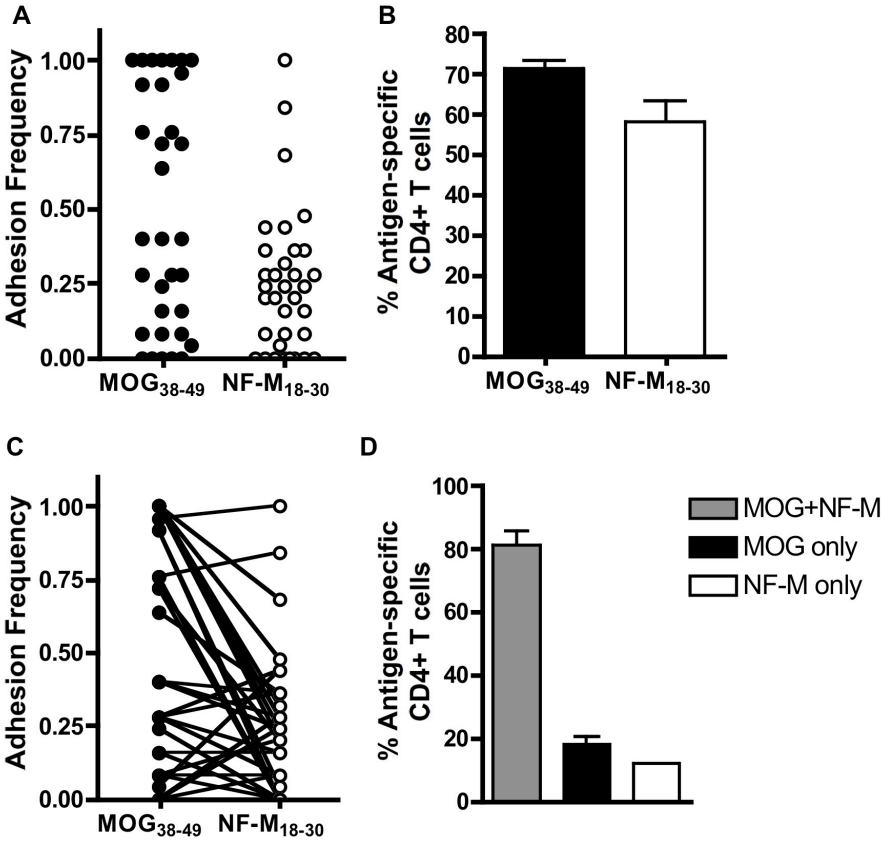

Figure 4.6. High frequency of low affinity MOG and NF-M cross-reactive CD4+ T cells in the CNS during peak EAE. CD4+ T cells isolated from the CNS at the peak of EAE were measured for binding to MOG_{38-49}:IA^b (●) and NF-M_{18-30}:IA^b (O) by 2D analysis in a representative experiment (A). The average percentage (± SEM) of MOG- and NF-M-specific CD4+ T cells measured by 2D binding is based on 90 and 87 CD4+ T cells, respectively, from 3 independent experiments with 8-12 mice per experiment (B). CD4+ T cells cross-specificity for MOG_{38-49}:IA^b (●) and NF-M_{18-30}:IA^b (O) was measured using a sequential binding technique on the micropipette assay (see Methods) in a representative experiment (C) and the average frequency (± SEM) of CD4+ T cells specific for MOG and/or NF-M (n), MOG only (■), or NF-M alone (□) was based on 68 CD4+ T cells from 10-12 mice per experiment from 2 independent experiments (D).

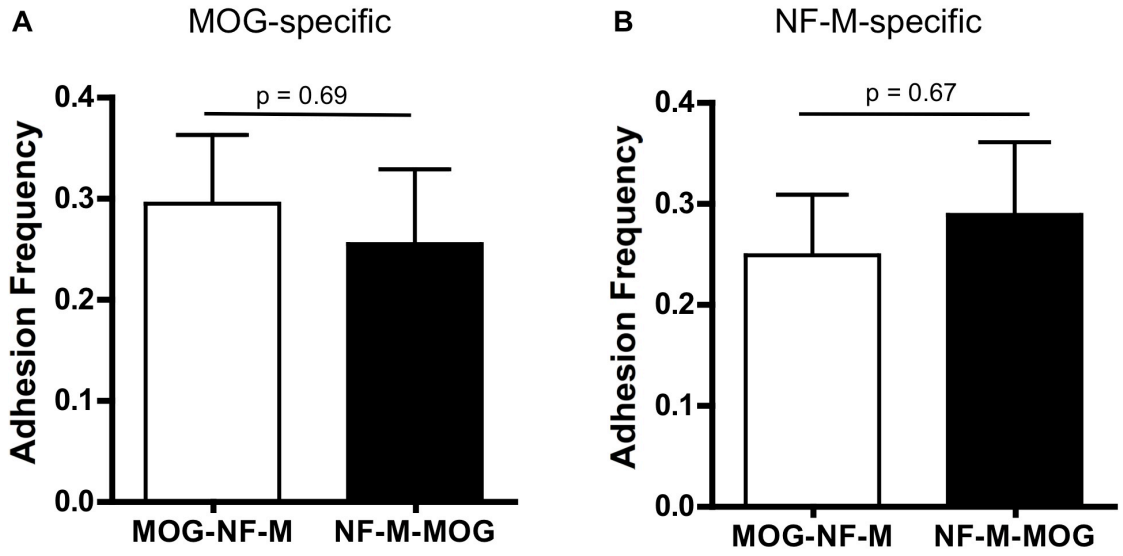

Figure 4.7. Effect of sequential MOG:IA^b and NF-M:IA^b binding on the adhesion frequency of CD4+ T cells. CD4+ T cells were isolated from the CNS at the peak of EAE and measured for specificity for MOG_{38-49}:IA^b and NF-M_{18-30}:IA^b using the sequential binding technique described in the Results and Methods. The average adhesion frequencies of CD4+ T cells specific for MOG (A) and NF-M (B) were derived from binding to MOG-coated RBCs, followed by NF-M-coated RBCs, or vice versa.

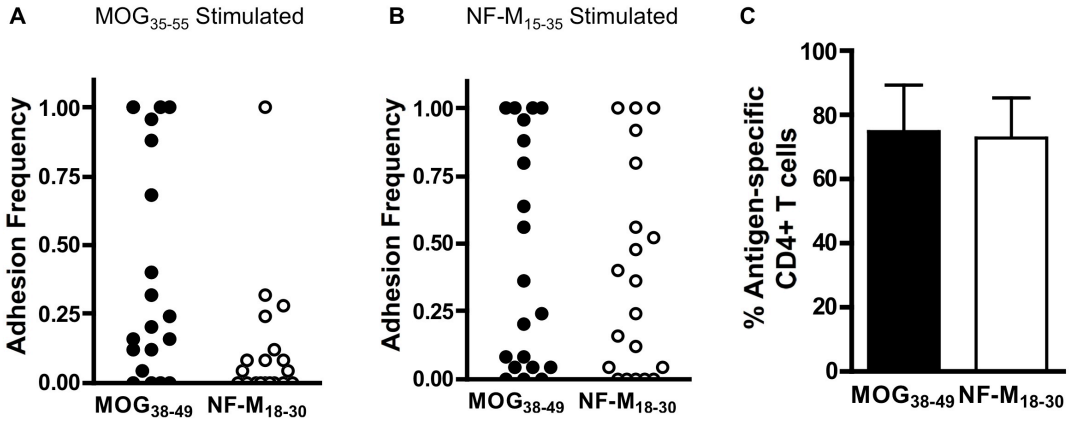

Figure 4.8. Stimulation of MOG_{35-55} CD4+ T cells *in vitro* increases cross-reactivity to NF-M. Representative 2D binding of polyclonal MOG_{35-55} CD4+ T cells to MOG_{38-49}:IA^b (●) and NF-M_{18-30}:IA^b (O) following stimulation with MOG_{35-55} alone (A) and/or with NF-M_{15-35} (B). The average frequency (± SEM) of MOG_{38-49}:IA^b - (■) and NF-M_{18-30}:IA^b- (□) specific binding of polyclonal MOG_{35-55} CD4+ T cells re-stimulated with MOG_{35-55} and/or NF-M_{15-35} were based on 2 independent experiments (C).

Chapter 5: Discussion

An essential question in T cell immunology is what is the role of affinity in cell-mediated immunity. Based on the existing models of clonal selection and affinity maturation, it is likely that T cell receptor affinity is involved in the induction, duration, and regulation of T cell responses. However, it has not been possible to precisely determine the role of affinity in T cell immunity because it has been unclear what is the minimal affinity required for T cell activation and what are the affinity ranges of the responders within a polyclonal antigen-specific population. Although numerous studies have sought to address this question, they have been hampered by a variety of technical limitations.

Monoclonal T cell models (i.e. T cell clones, TCR transgenic/retrogenic) are highly valuable for their ability to provide large quantities of antigen-reactive T cells, whose responsiveness can be reliably reproduced. However, monoclonal T cells fail to take into account complex T cell dynamics that are likely to occur in a polyclonal population comprised of various T cell clonotypes with variable affinities and frequencies. Thus, although a given T cell clone may respond (i.e. proliferation, cytokine profile, cytolysis) in one way when in isolation, its responsiveness may be profoundly altered when in the presence of other clonotypes (e.g. due to space/growth factor limitations or extrinsic suppression/activation exerted by one clonotype over another). For instance, although a previous report demonstrated that T cells could proliferate to low affinity antigens *in vivo* [111], the study did not address whether a similar expansion occurred in the presence of T cells responding to higher affinity antigens.

Polyclonal T cell populations must therefore be directly studied in order to

understand the complex interactions among different T cell clonotypes. While functional avidity is undoubtedly shaped by TCR affinity, it represents an average affinity of the most dominant clones within the response, and therefore does not provide insight into the array of T cell affinities that are present. Flow cytometric analysis of antigen-specific T cells detected by pMHC tetramers represent an advantage by demonstrating the ranges of affinities of various T cells within tetramer-positive populations. However, it has been unclear whether tetramers faithfully detect the full range of the antigen-specific repertoire.

The limitations of these methods were circumvented by employing a 2D-based micropipette adhesion frequency to measure the frequencies and affinity ranges of polyclonal myelin-specific (MOG_{35-55}) and viral-specific (GP_{61-80}) CD4+ T cells in EAE and LCMV, respectively. These studies provided the first demonstration that polyclonal autoreactive and pathogen-specific CD4+ T cell populations differ markedly in their overall affinities (Chapter 3), consistent with the purported negative selection of myelin-reactive CD4+ T cells. The overall affinity difference between these two CD4+ T cell populations was the basis for the large discrepancy in their detection by pMHC II tetramers (Chapters 2 and 3). Despite these overall affinity differences, both MOG_{35-55} and GP_{61-80} CD4+ T cell responses were comprised of a wide array of T cell affinities. As a result, both populations were composed of significant frequencies of low affinity antigen-specific CD4+ T cells that were undetectable by pMHC II tetramer, yet elicited sizeable effector cytokine responses. These results indicate that not only are the MOG_{35-55} and GP_{61-80} CD4+ T cell populations larger than indicated by pMHC II tetramer or functional measurements, but also that low affinity antigen-specific CD4+ T cells are

able to successfully expand in the presence of their higher affinity counterparts. These results therefore reveal nuances in clonal selection, whereby low and high affinity T cells appear to be a hallmark of polyclonal CD4+ T cell responses.

Using 2D analysis, it was further demonstrated that the majority of MOG_{35-55} CD4+ T cells in EAE were also cross-reactive for the neuronal antigen NF-M_{15-35} (Chapter 4). Moreover, the majority of cross-reactive CD4+ T cells were of overall low affinity and thus escaped detection by pMHC II tetramer. We also expanded on the previous finding that the increased responsiveness of MOG_{35-55}-specific 2D2 TCR transgenic CD4+ T cells to NF-M compared to MOG [129] is reflected by differences in the 2D affinity to both antigens. However, only a small fraction of CD4+ T cells generated to MOG_{35-55} showed an increased affinity for NF-M_{15-35}, indicating that the '2D2-like' phenotype is an exception of the polyclonal MOG_{35-55} CD4+ T cell response, rather than a prototypic T cell clonotype (Chapter 4). Once again, these findings highlight the limitations of extrapolating to polyclonal T cell populations based on findings in monoclonal T cell models.

Though the focus of the data presented in this body of work was only on CD4+ T cells, there is no reason to doubt that a similar diversity of T cell affinities is likewise present in polyclonal CD8+ T cell responses. Indeed, this is supported by the finding that T cells of lower affinity are present in polyclonal CD8+ T cell responses, as measured by pMHC I tetramers or functional avidity [79, 91, 377-379]. However, given the known differences in the affinities of CD8 and CD4 for their respective MHC molecules, it is possible that the fidelity of pMHC I tetramers in detecting the scope of responding antigen-specific CD8+ T cells is significantly higher than that of pMHC II tetramers for

CD4+ T cells. It is also possible that lower affinity CD8+ T cells, which may nevertheless be tetramer-positive, are in fact more prevalent than in CD4+ T cell responses due to the ability of comparatively higher affinity of CD8 to compensate for a lower affinity TCR. Despite greater sensitivity of pMHC I tetramers, at least several studies have suggested that antigen-specific CD8+ T cells can escape pMHC I tetramer detection [379-381]. Once again, this highlights the tremendous diversity of T cell affinities that appears to be a feature of T cell immunity.

The demonstration that a similar range of low to high affinity antigen-specific CD4+ T cells are found in polyclonal myelin- and viral-specific responses begs a critical question: why do polyclonal T cell responses maintain a broad array of T cell affinities? More broadly, the diversity of T cell affinities in polyclonal CD4+ T cell responses raises the question of how the affinity profile, broken down in simplest form in the ratio of tetramer-negative and tetramer-positive T cells, affects the outcome of autoimmunity and viral infection. The role of affinity on the outcome of T cell responses, whether in autoimmunity or against a pathogen, is ultimately dictated by the balance between its effect on effector functions and susceptibility to tolerance (Figure 5.1). Therefore the answer to this fundamental question involves considering the potential implications of T cell receptor affinity on both the initiation of the immune response as well as its modulation.

If TCR affinity directly impacts T cell effector function, then it is likely that low and high affinity T cells will differ regardless of antigen specificity. Thus, it would be expected that effectors of a particular affinity within self- and pathogen-reactive CD4+ T cell populations would be similarly affected with comparable functional outcomes.

Because of the correlation observed between T cell affinity and functional responsiveness, it is expected that higher affinity T cells would respond more readily (e.g. enhanced proliferation, cytokine secretion) to lower doses of antigen than lower affinity counterparts. Thus, higher affinity effectors CD4+ T cells may represent the initiator or driver clonotypes that respond during the early phases of viral infection or autoimmunity, where the amount of antigen available is initially low. As antigen dose increases (e.g. due to increased viral replication or progressive demyelination), low affinity responders may be subsequently recruited. Given their predominance in EAE and LCMV, low affinity CD4+ T cells appear to undergo a substantial number of cell divisions, albeit potentially with a delayed response or fewer divisions compared to high affinity responders. Moreover, a large fraction of the low affinity CD4+ T cells in both model immune responses produce cytokine in response to antigen stimulation. However, it is possible they may receive lower TCR signal strength and thus may produce less cytokine or be skewed somewhat to alternate cytokine profiles.

In cases where tolerance is broken leading to the activation of autoreactive T cells and the induction of autoimmune disease, regulatory mechanisms must be engaged to turn off these aberrantly activated T cells. The persistence of an ongoing immune response by self-reactive CD4+ T cells during autoimmune disease leads to the question: why are autoreactive CD4+ T cells persistently recruited and/or activated during chronic autoimmune disease, while pathogen-specific CD4+ T cells responses are shut off or become dysfunctional during chronic infection? Clearly, the regulatory processes that downmodulate the responsiveness of pathogen-specific T cells either fail to regulate autoreactive T cells or these regulatory mechanisms fail to be induced.

In a manner similar to that of effector function, TCR affinity likely plays a role in the susceptibility of T cells to peripheral tolerance (Figure 5.1). The expression of negative regulators (e.g. PD-1 and CTLA-4) and proteins mediating cell death (e.g. Fas) may be dependent on the strength of signal mediated via the TCR [382]. It is therefore possible that these methods of regulation are suboptimal or not operative in autoreactive T cells. On one hand, peripheral tolerance does appear to keep low affinity autoreactive T cells at least somewhat in check [383]. Nonetheless, several studies demonstrated that low affinity self-reactive T cells were less prone to central and peripheral tolerance, which enabled them to generate an autoimmune response [52, 212]. Thus, while the degree of expansion of low affinity T cells may be less than higher affinity T cells, they may be less susceptible to tolerance as a result of less TCR stimulation. The end result could lead to a substantial number of low affinity T cells contributing to chronic inflammation. Based on this model, differences in the affinity composition between autoreactive and pathogen-reactive T cell populations can potentially explain why T cell responses persist during autoimmunity, but become exhausted during chronic infection. That is, because low affinity T cells dominate autoreactive responses, they may avoid peripheral tolerance. In contrast, a larger proportion of high affinity T cells amongst pathogen-specific responses may render the response more prone to down-modulation. It is possible that lower affinity pathogen-reactive T cells may escape exhaustion, yet are not high enough in frequency or lack sufficiently potent effector responses to eliminate the infectious agent.

It is intriguing to speculate that a consequence of T cell affinity diversity is that clones of differing affinities may actually influence the fate of other T cell clonotypes.

For example, production of IL-2 by high affinity T cells could improve the viability of lower affinity T cells. Conversely, low affinity T cells could serve to preserve the functionality of more pro-inflammatory high affinity clones thereby perpetuating the inflammatory process. It is therefore possible that chronic T cell responses require a mixture of low and high affinity T cells, but with an overall low affinity in order to escape from tolerance. Although a mixture of TCR affinities appears to be a hallmark of polyclonal CD4+ T cell responses, it is interesting to consider how the responses in EAE and LCMV may be impacted by altering the affinity toward one direction or the other. That is, how are the outcomes of EAE and LCMV altered by the antigen-reactive CD4+ T cells that are purely low affinity (i.e. tetramer-negative) or purely high affinity (i.e. tetramer-positive)?

In the case of EAE, do low and high affinity myelin-reactive CD4+ T cells induce EAE with similar onset, severity, and duration? It is possible that high affinity myelin-reactive CD4+ T cells can induce severe EAE rapidly due to enhanced functional avidity. However, it might also be expected that the disease course would be quickly attenuated (i.e. acute EAE in the absence of the chronic phase), as the high affinity autoreactive T cells succumbed to the previously mentioned mechanisms of peripheral tolerance. Likewise, it is predicted that purely low affinity myelin-reactive CD4+ T cells can also induce some form of EAE independently since they already overwhelmingly predominate in acute EAE. However, it is possible that a higher frequency of low affinity T cells might be needed to induce EAE or that onset might be delayed due to lower functional avidity. Alternatively, low affinity myelin-reactive CD4+ T cells may induce no or weak EAE (e.g. optic neuritis in the absence of paralysis), which would indicate the need for at

least a small number of high affinity autoreactive clonotypes to break tolerance and initiate CNS autoimmune disease. Furthermore, the EAE phenotype could be additionally impacted by differences in the infiltration of low and high affinity myelin-reactive CD4+ T cells into distinct areas of the CNS with differing degrees of myelin and neuronal damage. Such a scenario is plausible, given that the density of MOG can differ among different areas of the CNS [236] and that the neuronal antigen NF-M is found in the grey matter of the CNS as well as found in the peripheral nervous system [384, 385].

Because of the primary role of LCMV-reactive CD8+ T cells in the clearance of virus during acute infection, the role of CD4+ T cell affinity may be more complex in this disease model. If CD4+ T cell affinity plays an important role, it would likely be in the maintenance of memory CD8+ T cells and/or in the rescue of exhausted CD8+ T cells during chronic infection. The ability of CD4+ T cells to help CD8+ T cell memory is typically ascribed to an APC licensing mechanism, in which antigen-specific CD4+ T cell interaction with an APC subsequently enables the APC to induce changes in antigen-specific CD8+ T cells [386]. In support of this, CD40L defects in LCMV-specific CD4+ T cells impair the induction of memory CD8+ T cells [191]. Thus, it is conceivable that higher affinity LCMV-specific CD4+ T cells would express higher levels of CD40L (or surface proteins with similar effects) and therefore facilitate greater APC licensing of CD8+ T cell memory. On the other hand, CD4+ T cells can help CD8+ T cells clear the virus during persistent infection, yet are likewise susceptible to exhaustion in chronic LCMV. It is possible that the propensity for exhaustion is directly related to the CD4+ T cell affinity. Thus, low affinity CD4+ T cells in LCMV may retain their functional

responsiveness in chronic infection and thereby help CD8+ T cells (and possibly also their higher affinity CD4+ T cell brethren) to eventually clear the virus.

The heterogeneity of T cell affinities in polyclonal immune responses likely also has important consequences on T cell cross-reactivity, both in autoimmunity and anti-pathogen responses (Figure 5.1). The mere presence of lower affinity T cells would substantially increase the diversity of the responding T cell repertoire so as to enhance the probability of cross-reactivity. In the case of autoimmune disease, cross-reactivity in the form of molecular mimicry provides a clear mechanism for infectious agents to aberrantly activate cross-reactive self-specific T cells and thereby initiate the autoimmune response. Of particular importance is whether T cell cross-reactivity is related to TCR affinity. Although it has been suggested that there is no simple correlation between TCR affinity and cross-reactivity [374], both peptide library and computational modeling studies have both suggested that T cells with low affinity for self-antigen were more cross-reactive than higher affinity T cells [375, 376]. It is clear that low affinity CD4+ T cells are cross-reactive to MOG and NF-M, though not at the complete exclusion of higher affinity CD4+ T cells (Chapter 4). Moreover, cross-reactivity of low affinity autoreactive T cells to multiple self-antigens, as in the case of MOG and NF-M in EAE, provides an additional mechanism for the perpetuation of autoimmunity.

In instances of infection, T cell cross-reactivity could provide enhanced protection in the form of heterologous immunity (Figure 5.1) and preventing the emergence of pathogen escape variants. However, it is not clear whether lower affinity T cells would be more likely to be more cross-reactive, as is suggested to be the case among autoreactive T cells. Indeed, one study has suggested the opposite, as T cells with higher

functional avidity had greater cross-reactivity to viral escape epitopes and were associated with clearance of chronic infections [387]. It is quite conceivable that there is no relationship between T cell affinity and cross-reactivity, and that cross-reactivity is simply more prevalent in the most abundant affinity population.

If T cell affinity is a critical determinant of the outcome of polyclonal T cell responses, then a central strategy of immune therapies, both in autoimmunity and infection, should focus on the impact on the overall affinity of the antigen-specific T cells as well as their diversity of affinities. The development of antigen-specific therapies for the selective tolerization of autoreactive T cells remains the ultimate goal for the treatment of T cell-mediated autoimmune diseases, such as MS. Although numerous studies have sought to target low affinity myelin-reactive CD4+ T cells in EAE, as outlined in Tables 1.3 and 1.4, the difficulty has been to effectively downmodulate all myelin-reactive CD4+ T cells, while also leaving the non-autoreactive T cell repertoire intact. This complexity is probably due to the wide range of responding autoreactive T cells, accompanied by varying degrees of susceptibility to tolerance.

An added complexity is the finding that IFNγ appears to play a fundamental role in setting the T cell activation threshold by allowing the expansion of myelin-reactive CD4+ T cells to low affinity antigens [257]. Thus, the IFNγ pathway appears to alter peripheral tolerance to regulate the affinity composition of the responding MOG_{35-55} CD4+ T cell repertoire. Whether IFNγ signaling plays a role in thymic selection or has a similar regulatory role in other T cell populations is of significant interest, but is unclear at the present time. Furthermore, deficiency of IFNγ or IFNγR reverses the tolerizing effects of 45D [257], a phenotype remarkably similar to the increased encephalitogenicity

of 45D by loss of the inhibitory phosphatase SHP-1 [361]. This suggests that T cell signaling and cytokine deficiencies may have important consequences on myelin-specific T cell functional responsiveness and can affect the efficacy of antigen-based therapies. Thus, effective therapies will require complex strategies targeting all ranges of T cell affinities, with a focus on the lower affinity T cells that dominate, in order to successfully bring the persistent autoreactive T cell response to a halt.

Therapies in infection by pathogens involve vaccines in order to prevent infection or treatments to clear chronic infections. In general, it is likely that higher affinity antigen-specific T cells are better at clearing a particular foreign-antigen because of more potent effector responses. However, due to the ability of pathogens to evolve, as well as the constant threat of subsequent infections, the presence of lower affinity T cells is likely beneficial so as to prevent pathogen escape and ensure heterologous immunity. Thus, vaccine design for cell-mediate immunity should include focusing on the proper balance of T cell affinity diversity that optimally protects against all of these possible outcomes. In the case of chronic infection, it is predicted that the higher affinity T cells become exhausted, whereas the lower affinity pathogen-specific T cells retain their responsiveness. Thus, strategies that boost the frequency and effector potency of lower affinity T cells may serve to restore functionality of higher affinity responders and to eliminate intractable, persistent infections.

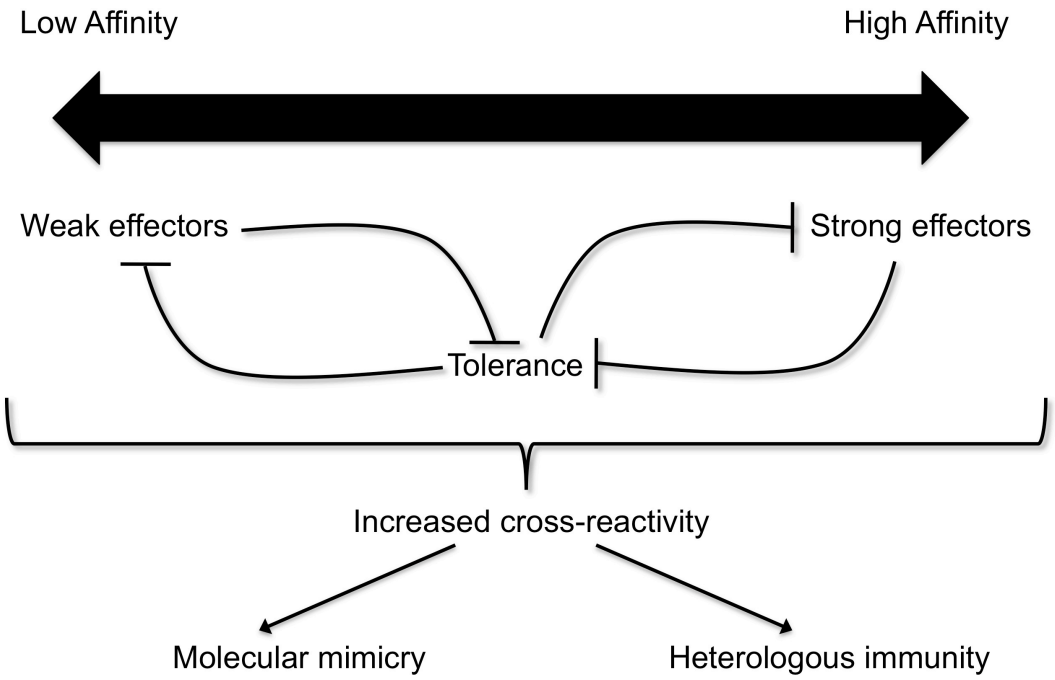

Figure 5.1. Proposed model of the consequences of T cell affinity diversity in polyclonal immune responses. The repertoires of naïve antigen-specific T cells are postulated to be comprised of a range of low to high TCR affinities. Upon antigen exposure, high affinity T cells are initially activated, while lower affinity T cells may be held in check by tolerance. Over time, lower affinity T cells are also recruited to the immune response. Repeated antigen exposure may lead to the downregulation of higher affinity T cells while lower affinity T cells may retain effector responses. The presence of a range of low to high affinity T cells also functions to increase the size of the antigen-specific repertoire. This increases the likelihood of cross-reactivity, which may underlie molecular mimicry and heterologous immunity.

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