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Joseph J. Sabatino, Jr., Emory University
Jun Huang, Georgia Institute of Technology
Cheng Zhu, Georgia Institute of Technology
Brian Evavold, Emory University

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High prevalence of low affinity peptide–MHC II tetramer–negative effectors during polyclonal CD4+ T cell responses

Joseph J. Sabatino Jr.,1 Jun Huang,2 Cheng Zhu,2 and Brian D. Evavold1

1Department of Microbiology and Immunology, Emory University, Atlanta, GA 30322
2Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology, Atlanta, GA 30332

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. In this study, we used a highly sensitive two-dimensional binding assay to identify low affinity cells in polyclonal autoreactive and pathogen–reactive CD4+ T cell populations specific for myelin oligodendrocyte glycoprotein (MOG) and lymphocytic choriomeningitis virus (LCMV) antigens, respectively. Low affinity CD4+ T cells, below detection with peptide–major histocompatibility complex class II tetramers, were at least as frequent as high affinity responders and contributed significant effector cytokines in both primary antigen–specific responses. We further demonstrated that MOG– and LCMV–specific CD4+ T cells possessed similarly broad ranges in their affinities (>100–fold wide), 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.

Determining the affinities of T cells in polyclonal responses is essential for understanding the outcome of cell–mediated immunity directed toward both foreign and self–antigens. The prevailing models of clonal selection and avidity maturation suggest that cells bearing the highest affinity TCRs for antigen are selectively expanded (Savage et al., 1999; Malherbe et al., 2004; Price et al., 2005), 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 contributing to immunity is supported by the findings that monoclonal CD8+ T cells can proliferate to low affinity antigens (Zehn et al., 2009). 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 (Liu et al., 1995; Bouneaud et al., 2000; Zehn and Bevan, 2006). Although low affinity T cells may potentially contribute to responses to both foreign and self–antigens, it has been unclear how extensively low affinity T cells participate in polyclonal T cell responses where high affinity clonotypes are also present.

Insight into TCR affinity for antigen has been provided by three–dimensional and two–dimensional (2D) technologies, such as surface plasmon resonance, Förster resonance energy transfer, or micropipette–based assays (Alam and Gascoigne, 1998; Kersh et al., 1998; Huang et al., 2010; Huppa et al., 2010). However, to date, studies have only considered monoclonal TCRs and cannot reveal T cell frequency and breadth of affinities comprising an antigen–specific polyclonal population (Alam and Gascoigne, 1998; Kersh et al., 1998; Huang et al., 2010; Huppa et al., 2010). Peptide–MHC (pMHC) tetramers based on an enhanced TCR avidity via multivalent interactions provide the most valuable technique for detecting the frequency of antigen–specific T cells (Altman et al., 1996; Moon et al., 2007), yet the extent to which their limited avidity may preclude detection of low affinity

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Polyclonal myelin- and viral-reactive CD4+ T cells (Vollers and Stern, 2008; Wooldridge et al., 2009) is unknown. Developing sensitive and accurate measurements of T cell antigen reactivity is therefore of utmost importance to dissect the nature of the polyclonal T cell response.

Recently, we used a 2D-based affinity analysis, which measures TCR–pMHC binding in the cell membrane–anchored context (Huang et al., 2010; Huppa et al., 2010) to define a 1,000-fold range in affinities corresponding to response levels between a monoclonal CD8+ T cell and a panel of altered peptide ligands (Huang et al., 2010). In this study, we harnessed the sensitivity of the 2D binding assay to define the antigen-specific frequencies and affinities of two polyclonal IAα-restricted CD4+ T cell populations specific for a self-antigen, myelin oligodendrocyte glycoprotein (MOG)35–55, which induces experimental autoimmune encephalomyelitis (EAE; Mendel et al., 1995), and a foreign-antigen, glycoprotein (GP)61–80, the dominant T helper epitope for lymphocytic choriomeningitis virus (LCMV; Oxenius et al., 1995; Homann et al., 2001). The 2D analysis revealed significantly larger frequencies of antigen-reactive CD4+ T cells for both antigens as compared with pMHC II tetramers. Polyclonal T cell affinities were diverse, covering more than a 100-fold range of affinities, with a fraction identified as high affinity and tetramer positive and many as low affinity and tetramer negative. We defined the 2D affinity necessary for pMHC II tetramers to bind CD4+ T cells and found that the low affinity tetramer-negative CD4+ T cells contributed significantly to the effector cytokine response. The presence of low and high affinity T cells greatly expands the previously estimated frequencies of polyclonal CD4+ T cell populations in peak effector responses (Homann et al., 2001; Moon et al., 2007; Williams et al., 2008).

**RESULTS**

**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 experiments. To compare their responses, we generated in vitro, three times as many GP61–80 CD4+ T cells were identified as compared with MOG35–55 CD4+ T cells bound only with their respective antigens but not RBCs alone or irrelevant antigen (Fig. 1, C and 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 hCLIP103–117-IAα in Fig. 1, C and D). Surprisingly, the majority of the MOG35–55 CD4+ T cells were antigen specific (69.2 ± 6.0%), eightfold higher than the tetramer-based frequencies measured in parallel (8.7 ± 1.6%; Fig. 1 E). Moreover, the micropipette assay identified a similar wide range in adhesion frequencies and a 2.5-fold increase over tetramer-positive GP61–80 CD4+ T cells (76.9 ± 3.4% vs. 30.9 ± 8.8%; Fig. 1 E). 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 (Fig. 1 B).

**pMHC 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 (Crawford et al., 1998; Savage et al., 1999; Fassò et al., 2000; Slifka and Whittington, 2001), a parameter determined by intrinsic TCR affinity (Vollers and Stern, 2008; Wooldridge et al., 2009) and TCR levels (Crawford et al., 1998; Mallone et al., 2005). However, MOG35–55 and GP61–80 CD4+ T cells expressed similar levels of surface TCRs (Fig. S1). 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 (AK, 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 (A) and 2D affinity (K). The 2D affinities were derived at 5 s because binding of both T cell populations reached equilibrium within seconds of contact (Fig. S2). 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^-2–10^-8 μm^4; Huang et al., 2010), with lower affinity T cells requiring increased pMHC densities.

For comparable adhesion frequencies, GP61–80 CD4+ T cells (Fig. 1 D) required lower levels of antigen than many MOG35–55 CD4+ T cells (Fig. 1 C), which is indicative of overall higher affinity. Indeed, the population-averaged effective 2D affinity of GP61–80 CD4+ T cells was 26-fold higher than MOG35–55 CD4+ T cells (4.21 ± 1.48 × 10^-4 μm^4 and 1.63 ± 0.48 × 10^-5 μm^4, respectively; Fig. 2 A). Moreover, the effective 2D affinities paralleled the functional avidities (inverse of peptide interactions (Chesla et al., 1998; Huang et al., 2010; Mallone et al., 2005). However, MOG35–55 and GP61–80 CD4+ T cells were antigen specific (69.2 ± 6.0%), eightfold higher than the tetramer-based frequencies measured in parallel (8.7 ± 1.6%; Fig. 1 E). Moreover, the micropipette assay identified a similar wide range in adhesion frequencies and a 2.5-fold increase over tetramer-positive GP61–80 CD4+ T cells (76.9 ± 3.4% vs. 30.9 ± 8.8%; Fig. 1 E). 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 (Fig. 1 B).

As an alternative measure of determining the frequency of antigen-specific CD4+ T cells, we used the micropipette adhesion frequency assay, which measures the 2D interactions of receptor–ligand interactions (Chesla et al., 1998; Huang et al., 2007, 2010). In this assay, a single T cell was brought in and out of contact with an RBC coated with pMHC II to yield an adhesion frequency (the percentage of adhesions out of the total number of contacts, as described in Materials and methods). After stimulation with antigen for 1 wk in vitro, polyclonal MOG35–55 and GP61–80 CD4+ T cells bound only with their respective antigens but not RBCs alone or irrelevant antigen (Fig. 1, C and 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 hCLIP103–117-IAα in Fig. 1, C and D). Surprisingly, the majority of the MOG35–55 CD4+ T cells were antigen specific (69.2 ± 6.0%), eightfold higher than the tetramer-based frequencies measured in parallel (8.7 ± 1.6%; Fig. 1 E). Moreover, the micropipette assay identified a similar wide range in adhesion frequencies and a 2.5-fold increase over tetramer-positive GP61–80 CD4+ T cells (76.9 ± 3.4% vs. 30.9 ± 8.8%; Fig. 1 E). 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 (Fig. 1 B).

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whole, all tetramer-positive CD4+ T cells had an affinity \( >1.10 \times 10^{-4} \, \text{µm}^4 \), and all tetramer-negative CD4+ T cells had an affinity \( <1.52 \times 10^{-5} \, \text{µm}^4 \) (Fig. 2 F). We were unable to more precisely pinpoint the affinity threshold for tetramer binding as the measurements were based on the MOG35–55 and GP61–80 experimental means and because the intermediate tetramer-binding CD4+ T cells were excluded during cell sorting (Fig. S3, A and B). The presence of high and low affinity (henceforth broadly distinguished by the 2D affinity cutoff for tetramer binding) MOG35–55 and GP61–80 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 \( \times 10^{-7} \) to 2.01 \( \times 10^{-4} \, \text{µm}^4 \) for MOG35–55 and 5.37 \( \times 10^{-6} \) to 5.53 \( \times 10^{-4} \, \text{µm}^4 \) for GP61–80; Fig. 2 F).

Despite their lower affinity, the tetramer-negative CD4+ T cells elicited robust effector functions as similar percentages of tetramer-positive and -negative MOG35–55 concentration for half-maximal proliferation, 1/EC\(_{50}\) as GP61–80 CD4+ T cells had a 61-fold higher functional avidity than MOG35–55 CD4+ T cells (Fig. 2 B).

pMHC tetramers provide a useful tool for separating the polyclonal MOG35–55- and GP61–80-specific CD4+ T cells in two distinct populations based on reactivity to the tetramer (Fig. S3, A and B). Essentially all tetramer-positive CD4+ T cells (>94%) were detected by 2D binding, showing high adhesion frequencies at low antigen densities (Fig. 2, C–E). Importantly, the majority of tetramer-negative CD4+ T cells (68.6 \( \pm \) 6.2% of MOG35–55 and 75.0 \( \pm \) 0.0% of GP61–80) also bound to antigen in the micropipette assay (Fig. 2, C–E). Nevertheless, tetramer-negative MOG35–55 and GP61–80 CD4+ T cells had lower effective 2D affinities (6.67 \( \pm \) 3.14 \( \times \) \( 10^{-6} \, \text{µm}^4 \) and 8.36 \( \pm \) 2.49 \( \times \) \( 10^{-6} \, \text{µm}^4 \), respectively) than their tetramer-positive counterparts (1.48 \( \pm \) 0.27 \( \times \) \( 10^{-4} \, \text{µm}^4 \) and 3.91 \( \pm \) 0.83 \( \times \) \( 10^{-4} \, \text{µm}^4 \), respectively; Fig. 2 F).
(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 TNF and/or IFN-$\gamma$ after antigen stimulation (Fig. 2 G). Considering that tetramer-negative cells were not exclusively antigen reactive by 2D binding analysis, cytokine was produced at a higher frequency than in the tetramer-positive populations.

Low affinity tetramer-negative CD4$^+$ T cells in the central nervous system (CNS) dominate during peak EAE

An added strength of the micropipette assay is that its increased sensitivity to antigen allows assessment of ex vivo polyclonal T cell responses. CD4$^+$ T cells isolated from the CNS after EAE induction bound specifically to MOG$_{38-49}$-IAb but not irrelevant antigen by both tetramer (Fig. 3 A) and 2D analysis (Fig. 3 B). The mean frequency of tetramer-positive MOG$_{35-55}$ CD4$^+$ T cells isolated from the CNS of mice with peak EAE symptoms was 8.2 ± 1.3% (Fig. 3 C), similar to previous findings (Korn et al., 2007; Sabatino et al., 2008). In parallel with the in vitro data, eightfold more CNS-infiltrating MOG$_{35-55}$ CD4$^+$ T cells were detected by 2D binding (63.6 ± 6.9%; Fig. 3 C). Moreover, the population-averaged effective 2D affinity of MOG$_{35-55}$-specific CD4$^+$ T cells from the CNS was 7.95 ± 2.77 × 10$^{-6}$ µm$^4$, which was comparable (2.1-fold lower) with the in vitro analysis (Fig. 2 A). This affinity level explains the low degree of tetramer staining as it was below the threshold for tetramer binding (Fig. 2 F). Thus, the majority of

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Figure 2. 2D affinity and antigen specificity of tetramer-positive and -negative CD4$^+$ T cells. (A) The mean 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 seven different antigen densities; *, P = 0.03). (B) The mean ± SEM functional avidities (1/EC$_{50}$, based on proliferation) of polyclonal MOG$_{35-55}$ (1.8 ± 1.3 × 10$^6$ M$^{-1}$) and GP$_{61-80}$ (1.1 ± 0.6 × 10$^8$ M$^{-1}$) CD4$^+$ T cells were based on six and four independent experiments, respectively (*, P = 0.04). (C and D) Representative adhesion frequencies of tetramer-positive and -negative sorted MOG$_{35-55}$ and GP$_{61-80}$ CD4$^+$ T cells were performed at the indicated antigen densities. (E) The mean percentage ± SEM of antigen-specific 2D binding of tetramer-positive and -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. (F) The mean 2D affinities of tetramer-positive and -negative MOG$_{35-55}$ and GP$_{61-80}$ CD4$^+$ T cells were based on at least three independent experiments per group. The affinity threshold for tetramer binding is shown by the dotted lines, representing the lowest affinity for tetramer-positive CD4$^+$ T cells (>1.10$^{-4}$ µm$^4$) and the highest affinity for tetramer-negative CD4$^+$ T cells (<1.52 × 10$^{-5}$ µm$^4$). (G) The mean percentage ± SEM of cytokine-producing (IFN-$\gamma$ and TNF) tetramer-positive and -negative MOG$_{35-55}$ and GP$_{61-80}$ CD4$^+$ T cells was based on three and two independent experiments, respectively.
High frequency of LCMV-specific tetramer-negative CD4+ T cells at the peak effector phase

To measure ex vivo antiviral CD4+ T cells, we analyzed the GP61-80 LCMV-specific CD4+ T cell response at its peak, 8 d postinfection with LCMV Armstrong (Homann et al., 2001; Whitmire et al., 2006). CD4+ T cells from the spleen bound specifically to GP66-77-IAb by tetramer (Fig. 4 A) and 2D analysis (Fig. 4 B). Interestingly, although 8.9 ± 0.4% of CD4+ T cells from the spleen were detected by pMHC II tetramer, 33.7 ± 4.8% were in fact GP61-80 specific by 2D adhesion analysis (Fig. 4 C). This frequency closely matched the percentage (37.2%) of CD44+ CD4+ T cells that were induced by LCMV at day 8 postinfection. The mean effective 2D affinities (1.65 ± 0.79 × 10^{-4} µm^4) of the GP61-80 CD4+ T cells in LCMV infection were again 2.6-fold less than in vitro GP61-80 CD4+ T cells (Fig. 2 A). 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 fourfold.

CD4+ T cells penetrating the CNS at the peak of EAE were in fact MOG35-55 specific.

The presence of a large frequency of predominantly tetramer-negative MOG35-55 CD4+ T cells in the CNS during EAE suggested that low affinity myelin-reactive CD4+ T cells contributed to disease pathogenesis. To define the effector function of low affinity MOG35-55 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 IFN-γ and TNF (Fig. 3 D). Approximately 2.4-fold more CD4+ T cells produced cytokine in response to MOG35-55 peptide stimulation than were detected by tetramer (19.0 ± 3.6% vs. 8.0 ± 0.7%, in parallel experiments; Fig. 3 E). Because not all antigen-specific CD4+ T cells produced cytokine (Fig. 2 G) and because the tetramer-negative MOG35-55 CD4+ T cells outnumbered their tetramer-positive counterparts by ~8:1 (Fig. 3 C), this indicated that the majority of proinflammatory cytokine-secreting MOG35-55-specific CD4+ T cells were of low affinity.

Figure 3. Dominance of proinflammatory low affinity myelin-reactive CD4+ T cells during EAE. (A and B) Representative tetramer (A) and 2D binding (B) of CNS-infiltrating CD4+ T cells to MOG38-49-IAb and hCLIP103-117-IAb. (C) The mean frequency ± SEM of MOG35-55-specific binding by tetramer and 2D analysis was based on three experiments performed in parallel, and CNS tissue was pooled together from 6–10 mice per experiment (*, P = 0.004). (D) Representative frequency of cytokine-producing (IFN-γ and TNF) CD4+ T cells isolated from the CNS during acute EAE after stimulation with MOG35-55 or no peptide (no stimulation [stim]). (E) The mean percentage ± SEM of CNS-infiltrating MOG35-55 CD4+ T cells was compared in parallel by MOG38-49-IAb tetramer and the percentage producing cytokine (IFN-γ and TNF) upon stimulation with MOG35-55 (no stimulation background subtracted) from two independent experiments (CNS tissue pooled from 12–24 mice; *, P = 0.04).
To determine the contribution of low affinity T cells to overall effector function, splenocytes 8 d postinfection were sorted into GP66–77-IAb tetramer-positive and -negative CD4+ T cells and assessed for cytokine production (TNF and IFN-γ) in response to GP61–80 stimulation. In the absence of the GP66–77-IAb tetramer-positive CD4+ T cells, the tetramer-negative population produced approximately half of the total cytokines detected at all doses of antigen (Fig. 4 D). There was no significant difference (P = 0.80) in the EC50 values for cytokine production from the LCMV tetramer-positive or -negative or intact (i.e., unsorted) CD44+ CD4+ T cell populations (Fig. 4 E). The data were displayed as a percentage of maximal response because essentially all of the tetramer-positive CD4+ T cells were antigen reactive, whereas LCMV-specific CD4+ T cells comprised a third or less of the total and tetramer-negative CD4+ T cell populations. Thus, LCMV-specific, tetramer-negative CD4+ T cells were present at a greater frequency than the tetramer-positive population and produced a substantial fraction of the overall effector cytokine response.

DISCUSSION

T cells mount a response against antigens that is typically clonally diverse, but often experiments analyzing cell-mediated immunity must use monoclonal cells to facilitate analysis of cell fate, location, frequency, and affinity. Biophysical analyses of T cell affinity have almost exclusively focused on a single TCR species complicating extrapolation to polyclonal populations. The focus on one clonal TCR very likely limits our understanding into the range of affinities comprising the antigen-activated T cell population and prevents investigation into the distinct possibility that low affinity T cells appreciably participate in immunity (Gronski et al., 2004; Zehn et al., 2009). In this study, 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 (Huang et al., 2010). Beyond allowing analysis of polyclonal CD4+ T cell affinity, 2D analysis revealed significantly larger numbers of antigen-specific CD4+ T cells than can be quantified using pMHC II tetramers.

Figure 4. Low affinity viral-specific CD4+ T cells contribute significant effector responses during LCMV infection. (A and B) Representative tetramer (A) and 2D binding (B) of CD4+ T cells from the spleen at day 8 LCMV to GP66–77-IAb and hCLIP 103–117-IAb. (C) The mean frequency ± SEM of GP61–80 CD4+ T cells in the spleen during LCMV infection by tetramer and 2D analysis was performed in parallel in three independent experiments (two spleens pooled per experiment; *, P = 0.007). (D and E) At day 8 postinfection, total CD44+ CD4+, tetramer-positive CD44+ CD4+, or tetramer-negative CD44+ CD4+ T cells were stimulated with the indicated concentrations of GP61–80 peptide, and the mean total ± SEM percentages of (D) and percentage of maximal ± SEM (E) cytokine-producing (IFN-γ and TNF) cells were assessed in four independent experiments (two to four spleens pooled per experiment).
pMHC tetramers provide an important technique for quantifying the immune response and can be used to define a polyclonal population of antigen-specific precursor or effector T cells, yet their limited avidity inherently restricts the range of affinities that can be analyzed. Indeed, the ex vivo GP<sub>61-80</sub> and MOG<sub>35-55</sub> CD4<sup>+</sup> T cell populations were four- and eightfold larger, respectively, than indicated by pMHC II tetramer. These differences between GP<sub>61-80</sub> and MOG<sub>35-55</sub>-specific CD4<sup>+</sup> T cells as prototypical foreign- and self-specific responses mirror other studies in which CD4<sup>+</sup> T cells specific for foreign antigens (Homann et al., 2001; Moon et al., 2007) were more prevalent and easier to detect by tetramer than those specific for self-antigens (Gebe et al., 2003; Bischof et al., 2004; Falta et al., 2005; Korn et al., 2007). Regardless, pMHC II tetramer underestimated the frequency of MOG<sub>35-55</sub> and GP<sub>61-80</sub> CD4<sup>+</sup> T cells, as the pMHC II tetramer staining was only accurate to the extent that the underlying T cell responses were dominated by high affinity T cells. It is currently unknown whether pMHC II tetramers are a sufficient surrogate for tracking CD4<sup>+</sup> T cell responses. Interestingly, the CD4<sup>+</sup> T cells possessed similar overlapping distributions of low to high affinities for MOG<sub>35-55</sub> and GP<sub>61-80</sub>. In fact, the effective 2D affinities of tetramer-negative or positive cells were essentially the same regardless of T cell specificity for the myelin or viral antigens.

Previous work in CD4<sup>+</sup> T cells suggested rapid dominance of high affinity T cell clonotypes (Savage et al., 1999; Malherbe et al., 2004), yet these studies were based on pMHC II tetramer measurements. By including tetramer-negative CD4<sup>+</sup> T cells in our analysis, a 2D affinity range &gt;100-fold wide was present at the peak immune response for both foreign and self-antigens. This indicates heterogeneity in TCR affinity regardless of antigen specificity during the polyclonal primary immune responses and may raise issues over the accuracy that a monoclonal T cell population possessing a single affinity completely reflects the ongoing immune processes. For example, monoclonal MOG<sub>35-55</sub>-specific CD4<sup>+</sup> T cells may differ because of limited affinity diversity from polyclonal populations of cells. In fact, the MOG<sub>35-55</sub>-specific 2D2 TCR transgenic, while a valuable model to highlight many features associated with demyelinating disease, generates a variant form of EAE (Bettelli et al., 2003, 2006; Wasserman and Evavold, 2008). Interestingly, 2D2 T cells are undetectable by the MOG-IA<sup>b</sup> tetramer (unpublished data) yet pathogenic (Bettelli et al., 2003, Wasserman and Evavold, 2008), demonstrating that low affinity MOG<sub>35-55</sub> CD4<sup>+</sup> T cells can induce EAE. We are in the process of carrying out experiments to test the concept that autoimmune disease outcome relates to the affinity range and diversity of the response.

Although our experiments do not directly address the effect of thymocyte negative selection or peripheral events in shaping T cell affinity as we examined expanded cell populations, the mere presence of high affinity MOG<sub>35-55</sub> CD4<sup>+</sup> T cells suggests 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 (Savage et al., 1999; Malherbe et al., 2004; Price et al., 2005) because they did not dominate over their low affinity counterparts at the peak of the primary immune response or on extended culture in vitro. Similarly, the affinities of the GP<sub>61-80</sub> CD4<sup>+</sup> T cells were broadly distributed and contained a significant population of low affinity LCMV-reactive CD4<sup>+</sup> T cells detectable by 2D binding but not pMHC II tetramer. The presence of low affinity GP<sub>61-80</sub> CD4<sup>+</sup> T cells further demonstrates 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<sup>+</sup> polyclonal population, at least during the peak effector response.

To detect antigen-reactive CD4<sup>+</sup> T cells by 2D analysis, the T cells had to recognize and proliferate to antigen encountered in vivo, regardless of low or high affinity. Moreover, we identified low and high affinity T cells within the immune-relevant tissues. In the case of the CNS, its immune privileged status would be presumed to allow primarily the myelin-reactive CD4<sup>+</sup> T cells, which we demonstrated by 2D analysis. Further demonstrating the active role of tetramer-negative CD4<sup>+</sup> T cells in immunity was their ability to contribute substantially to the overall cytokine responses. We found that the relative cytokine contribution of tetramer-negative CD4<sup>+</sup> T cells was approximately equal to or greater than the high affinity responders in viral immunity. Moreover, the high and low affinity LCMV-specific CD4<sup>+</sup> T cells had the same functional avidities despite their inherent 2D affinity differences, which is likely the result of tuning to the same level of antigen in vivo (Grossman and Paul, 1992; Slifka and Whitton, 2001). The prevalence of cytokine-positive low affinity myelin-reactive CD4<sup>+</sup> T cells clearly outnumbered the small frequency of tetramer-positive MOG<sub>35-55</sub> CD4<sup>+</sup> T cells, demonstrating the importance of low affinity effectors in CNS autoimmunity.

However, cytokine analyses of CD4<sup>+</sup> T cell effector function are limited because we and others have shown that not all antigen-specific CD4<sup>+</sup> T cells produce cytokine (Weaver et al., 1998; Bettelli et al., 2006; Whitmire et al., 2006; Williams et al., 2008). Because tetramer and intracellular cytokine staining usually cannot be performed concurrently, they are often performed in parallel. Although we confirm in this study what others have shown, that pMHC II tetramer and cytokine responses were approximately equal in LCMV infection (Homann et al., 2001; Williams et al., 2008), we demonstrated that the tetramer-negative CD4<sup>+</sup> T cells were major contributors to the effector cytokine response. Thus, an identical frequency of antigen-specific CD4<sup>+</sup> T cells by cytokine and tetramer is fortuitous as opposed to being an indication that they are the same high affinity responding T cell population.

In conclusion, 2D analysis resolved the affinity of polyclonal T cell responses and revealed that low affinity CD4<sup>+</sup> T cells participated during autoimmunity and viral infection, although they were accompanied by high affinity counterparts in both cases. Characterization of low affinity cell frequency
therefore allows for increased understanding of the polyclonal T cell response and ultimately enables defining how the affinity range impacts the extent of 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 (Atlanta, GA) and used in accordance with an Institutional Animal Care and Use Committee–approved protocol.

Peptides. MOG35-55 (MEGVYWRSPFSRVHHLRNGK) and GP34-40 (GLNGPDIYKVQF Kensington) 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, 2 mM 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-mo-old male mice with 100 µg of the indicated peptide emulsified in CFA containing 1 mg/ml heat-killed Mycobacterium tuberculosis. Draining lymph nodes were harvested 10–14 d later and stimulated for 1 wk with 1 µM of the priming peptide and IL-2. Cells were CD4 purified by positive selection microbeads (Miltenyi Biotec). CD4+ T cells were restimulated weekly with irradiated syngeneic splenocytes (3,000 rad) and 1 µM peptide and IL-2. Proliferation was measured by [3H]thymidine incorporation as previously described (Sabatino et al., 2008).

Tetramer and cell surface staining. MOG35-55-IAb (generated from a previously described construct; Sabatino et al., 2008), GP34-40-IAb, and hCLIP103-117-IAb monomers and tetramers were provided by the National Institute of Allergy and Infectious Diseases Tetramer Core Facility at Emory University; CD4+ T cells were incubated with 4 µg/ml MOG35-55-IAb (8–20 h), GP34-40-IAb tetramers (3–4 h), or hCLIP103-117-IAb in complete RPMI at 37°C. The following antibodies and stains were used for analysis: CD4 (RM4.5), CD8α (XMG1.2) and TNF (MP6-XT22; both BD). T o calculate the percent frequency (f) of cytokine-producing CD4+ T cell stimulated with IAb-coated RBCs, the following equation was used: $f = \ln[1-P(\delta)]\times|\text{mean}|$, where $m$ and $m'$ reflect the receptor (TCR) and ligand (pMHC) densities, respectively.

2D TCR affinity analysis. The details of the microcippette adhesion frequency assay are described in detail elsewhere (Huang et al., 2007, 2010). In brief, a pMHC-coated RBC and T cell were placed on contact by microinjection for a controlled contact area (A) 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 performed 50 times per T cell–RBC pair to calculate an adhesion frequency ($P_2$). The contact area was kept constant for all experiments so it would not affect the affinity comparison. For each experiment, a mean $P_2$ was calculated based only on T cells that bound specifically to antigen. The population-averaged 2D affinity ($A_2K_2$) using the mean $P_2$ at equilibrium (where $t \rightarrow \infty$) was calculated using the following equation: $A_2K_2 = \ln[1-P_2(\delta)\times|\text{mean}|]$, where $m$ and $m'$ reflect the receptor (TCR) and ligand (pMHC) densities, respectively.

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Statistical analysis. Statistical analyses were performed using Prism 4 (GraphPad Software, Inc.). Two-way Student’s t tests were used for all statistical comparisons, except in the comparison of the EC50 values of cytokine-producing CD4+ T cells in LCMV, where a one-way analysis of variance was used.

Online supplemental material. Fig S1 shows that MOG35-55 and GP34-40 CD4+ T cells do not differ in their TCR levels. Fig S2 demonstrates that the TC concentration that was elicited at any peptide stimulation.

Ex vivo CD4+ T cell analysis in EAE and LCMV. CD4+ T cells purified from the spleen at day 8 postinfection were sorted into GP34-40-IAb tetramer-positive and -negative CD4+ CD4+ T cells. Cell preparation for micropipette adhesion frequency assay. Human RBCs were isolated in accordance with the Institutional Review Board at the Georgia Institute of Technology and prepared as previously described (Huang et al., 2007, 2010). RBCs coated with various concentrations of Biotin-X-NHS (EMD) were coated with 0.5 mg/ml streptavidin (Thermo Fisher Scientific), followed by 1–2 µg of pMHC II monomer. The pMHC-coated RBCs were stained with anti-MHC II FITC antibody, and T cells were stained with anti-TCR FITC antibody. The site densities of IAb monomers per RBC and TCRs per T cell were derived using FITC MESF beads (Bangs Laboratories) as previously described (Huang et al., 2007, 2010) and normalized for the F/P ratios of the antibodies.

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