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Regulatory and T Effector Cells Have Overlapping Low to High Ranges in TCR Affinities for Self during Demyelinating Disease

Jennifer D. Hood,* Veronika I. Zarnitsyna,† Cheng Zhu,‡ and Brian D. Evavold*

Having regulatory T cells (Tregs) with the same Ag specificity as the responding conventional T cells is thought to be important in maintaining peripheral tolerance. It has been demonstrated that during experimental autoimmune encephalomyelitis there are myelin oligodendrocyte glycoprotein (MOG)-specific Tregs that infiltrate into the CNS. However, the affinity of naturally occurring polyclonal Tregs for any self-antigen, let alone MOG, has not been analyzed in the periphery or at the site of autoimmune disease. Utilizing the highly sensitive micropipette adhesion frequency assay, which allows one to determine on a single-cell basis the affinity and frequency of polyclonal Ag-specific T cells directly ex vivo, we demonstrate that at peak disease MOG-specific Tregs were progressively enriched in the draining cervical lymph nodes and CNS as compared with spleen. These frequencies were greater than the frequencies measured by tetramer analysis, indicative of the large fraction of lower affinity T cells that comprise the MOG-specific conventional T cell (Tconv) and Treg response. Of interest, the self-reactive CD4+ Tconvs and Tregs displayed overlapping affinities for MOG in the periphery, yet in the CNS, the site of neuroinflammation, Tconvs skew toward higher affinities. Most of the MOG-specific Tregs in the CNS possessed the methylation signature associated with thymic-derived Tregs. These findings indicate that thymic-derived Treg affinity range matches that of their Tconvs in the periphery and suggest a change in TCR affinity as a potential mechanism for autoimmune progression and escape from immune regulation. *The Journal of Immunology, 2015, 195: 000–000.

A constant challenge for the immune system is balancing clearance of foreign pathogens while remaining tolerant of self. To limit overt autoreactivity, central tolerance restricts the number of self-specific cells by the process of negative selection in the thymus, although it has been demonstrated that this process is imperfect (1). In the periphery, self-reactivity can be limited in several different ways, including, but not limited to, anergy, inability to access Ag, and suppression by regulatory T cells (Tregs). One of the earliest examples of Tregs was demonstrated by pMHC tetramers showing that depletion of CD4+CD25+ T cells in mice led to systemic autoimmune (2). Later, it was determined that Foxp3 is a key transcription factor expressed by Tregs, and mutations in this transcription factor lead to multiorgan autoimmunity (3, 4).

Abbreviations used in this article: CLN, cervical lymph node; 2D, two-dimensional; EAE, experimental autoimmune encephalomyelitis; EAS45, experimental additive solution 45; MOG, myelin oligodendrocyte glycoprotein; pMHC, peptide-MHC; pTreg, peripherally induced regulatory T cell; SPL, spleen; Tconv, T conventional cell; Treg, regulatory T cell; TSDR, regulatory T cell–specific demethylated region; tTreg, thymic-derived regulatory T cell; tTreg-Me, thymic-derived regulatory T cell methylation.

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become refractory to the Tregs. The authors demonstrated that this similar ignorance of suppression could be achieved using naïve Tconvs with exogenous IL-6 and TNF-α added to the coculture (18). In a study of relapsing and remitting multiple sclerosis patients, there was a significant correlation between the level of IL-6Rα expression and the ability of the Tconvs to escape suppression by Tregs (19). In addition to the Tconvs escaping regulation by Tregs, the presence of proinflammatory cytokines can actively recruit DNA methyltransferases to the Treg-specific demethylated region (TSDR), leading to de novo methylation at this site (20). The reintroduction of methyl groups at these CpGs makes Foxp3 expression less stable and impairs its inheritability upon division into daughter cells (21). By BrdU labeling, it has been demonstrated that Tregs in the CNS during EAE are capable of dividing, so this could support a conversion of Tregs into memory-like effector T cells (18, 22).

Using Foxp3 reporter mice to discern the contributions of Tregs from Tconvs, we expanded on our previous studies demonstrating that autoreactivity is dominated by low-affinity T cells (14). Only approximately 10 and 30% of Tregs and Tconvs were Ag reactive in the CNS by tetramer and cytokine production, respectively, during EAE. However, when using the same pMHC monomer that is used for the tetramer, >80% of the Tregs and Tconvs from the CNS are MOG specific when measured by the micropipette adhesion frequency assay. Furthermore, this assay allowed the identification of how many MOG-specific Tregs were in the spleen (SPL), cervical lymph nodes (CLN), and CNS, defining enrichment of MOG-specific Foxp3+ T cells that have infiltrated into the CNS. All T cell responses are comprised of TCRs that span a range from lower to higher affinities. The overall affinity ranges in the periphery are similar between Tregs and Tconvs, with an enrichment of higher affinity Tconvs in the CNS. Based on a methylation signature and Helios expression, the Tregs in the CNS are likely derived from tTregs. Thus, the micropipette adhesion frequency assay allows one to define for the first time the distribution and affinity of polyclonal Tregs during autoimmune disease, revealing new insight into autoimmune disease progression and potential mechanisms of Treg development.

Materials and Methods

Mice

B6.Cg-Foxp3tm2Cdc1 (Foxp3 GFP) mice were obtained from The Jackson Laboratory and were bred in the Emory University Division of Animal Resources facility (23). C57BL/6 mice were obtained from National Cancer Institute. All experiments were approved by the Institutional Animal Care and Use Committee at Emory University.

EAE induction and isolation of cells

EAE was induced in 6- to 10-wk-old male or female mice by injecting s.c. 200 μg MOG35-55 emulsified in CFA containing 5 mg/ml heat-inactivated Mycobacterium tuberculosis (H37 RA, Difco) on days 0 and 7 and i.p. with 250 ng pertussis toxin (List Biologicals) on days 0 and 2 as described (24). Mice are scored and weighed daily starting at day 7 after induction using a 5-point scale: 0, no sign of disease; 1, limp tail; 2, hindlimb weakness; 3, hindlimb paralysis; 4, forelimb weakness; 5, moribund.

Reagents

Cell culture media was composed of RPMI 1640 (CellGro) supplemented with 10% heat-inactivated FBS (Life Technologies), 4 mM L-glutamine (CellGro), 0.01 M HEPES (CellGro), 100 μg/ml gentamicin (Sigma-Aldrich), and 20 μM 2-ME (Sigma-Aldrich). Phenol red–free reagents (RPMI 1640 and HBSS) were used in some experiments because phenol red–containing reagents can decrease enhanced GFP fluorescence by FACS and fluorescence microscopy (25). Experimental additive solution 45 (EAS45) was made as described previously (26). All EAS45 compounds were from Sigma-Aldrich except dibasic sodium phosphate, which was from Fisher Scientific. EAS45 was then further supplemented with 1% BSA (Sigma-Aldrich). FACS wash consisted of 0.1% BSA and 0.05% sodium azide in PBS.

Isolation of CNS-infiltrating cells

Mice were sacrificed using CO2 inhalation and were perfused with 1× Dulbecco’s PBS (CellGro) via the left ventricle after the inferior vena cava was cut. For each mouse, the spinal cord and brain were combined and a single-cell suspension was obtained by pushing cells through a 100-μm cell strainer. Cells were collected from the interface of a 27%/63% discontinuous gradient of Percoll (GE Healthcare), washed twice with R10, and then counted on a hemocytometer to obtain the number of infiltrating cells.

Flow cytometry

Staining of Helios and Foxp3 was accomplished using the Foxp3 staining buffer set from eBioscience according to the manufacturer’s protocol. For intracellular cytokine staining, cells were stimulated with PMA and ionomycin for 4 h in the presence of brefeldin A, as described previously, and then after staining surface markers, the cells were fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences) followed by intracellular staining for cytokines (27). Tetramer frequencies were determined by staining cells with MOG35-55 microbeads (BD Biosciences) for at least 6 h at 37°C in complete media as described previously (28). Data were collected on an LSR II (BD Biosciences) and then analyzed in FlowJo (Tree Star). Monomer-coated RBCs and T cells were stained to determine the absolute numbers of pMHC and TCR, respectively, on their surfaces using PE Quantibrite beads (BD Biosciences) as described previously (29). The following Abs (clones) were purchased from a variety of vendors (BD Biosciences, eBioscience, and BioLegend): Helios (22F6), Foxp3 (FJK-16s), CD8α (53-6.7), CD3ε (145-2C11), CD4 (RM4-5), CD11b (M1/70), CD11c (HL3), CD19 (1D3), CD45.2 (104), TCRβ (H57-597), IA/E (M5/114.15.2), IFN-γ (XMG1.2), IL-17 (eBio17B7), and IL-10 (JES5-16E3).

Micropipette adhesion frequency assay

To determine the two-dimensional (2D) affinities and frequencies of MOG-specific cells, the micropipette adhesion frequency assay was conducted as previously described (14, 15, 27, 29–33). Human RBCs were coated with biotinylated pMHC monomers (National Institutes of Health Tetramer Core Facility). Binding events are scored manually by visualizing distension of the RBC membrane upon retraction of the T cell. After 25 or 50 touches, average adhesion frequencies are calculated and cells are considered to be Ag specific above the background of 0.1 binding frequency. The background binding frequency was set by using hCLIP-I-Å coated RBCs as a nonspecific binding control for the cells that had also been tested for binding to MOG-I-Å. RBC pMHC density is manipulated to obtain adhesion frequencies between 0.1 and 1.0, which could be used to calculate an affinity. For example, cells that had an adhesion frequency of 1 were tested with a second RBC coated with a lower density of MOG-I-Å to decrease the adhesion frequency and allow for measurement of the affinity. The number of Ag-specific cells divided by the total number of cells tested gives the frequency of MOG-I-Å. Effective 2D affinities (Am,Km) are calculated using the following equation:

\[ A_m = \left( \frac{-\ln(1 - P_r(n))}{m, n} \right) \]

where \( P_r \) is the adhesion frequency measured at an equilibrium contact time (≥ 2 s) and \( m \) and \( n \) are the densities of TCR and pMHC ligands, respectively. TCR densities are calculated for each T cell individually using the TCR level for the population, as determined by flow cytometry, and that cell’s diameter, measured during the micropipette assay. For each independent experiment at least 14 cells were tested.

Bisulfite sequencing

Cells from male mice were used for methylation studies to avoid confounding results due to random X inactivation because Foxp3 is an X-linked gene. CD4+ Tconvs and CD4+ Foxp3+ Tregs were sorted from the indicated sources on a FACSAria II (BD Biosciences). DNA was extracted from cell pellets and bisulfite converted using a DNaseasy blood and tissue kit (Qiagen) followed by an EpiTect bisulfite kit (Qiagen) or with the EZ DNA Methylation-Direct kit (Zymo Research) as per the manufacturers’ protocols. Regions of interest were amplified using published primers and luminated pMHC monomers (National Institutes of Health Tetramer Core Facility) to determine the Treg methylation (tReg-Me) signature (34). Gel-purified PCR products were subcloned using the TOPO TA cloning kit (Invitrogen), followed by blue/white screening with X-gal. Single-pass

OVERLAPPING AFFINITIES FOR (Tregs AND CD4+ Tconvs)

Mice were sacrificed using CO2 inhalation and were perfused with 1× Dulbecco’s PBS (CellGro) via the left ventricle after the inferior vena cava was cut. For each mouse, the spinal cord and brain were combined and a single-cell suspension was obtained by pushing cells through a 100-μm cell strainer. Cells were collected from the interface of a 27%/63% discontinuous gradient of Percoll (GE Healthcare), washed twice with R10, and then counted on a hemocytometer to obtain the number of infiltrating cells.
DNA sequencing was done by Beckman Coulter Genomics. Sequences were aligned with their in silico bisulfite-converted genomic sequences using a custom R script in Bioconductor Biostings R package, and only those that contained all of the CpGs and had a bisulfite conversion rate >95% were included in the results (35).

**Statistical analysis**

Prism version 6 (GraphPad Software) was used for t tests and all ANOVAs with multiplicity-adjusted p values. Testing of normality via Q–Q plots, Shapiro–Wilk, and Jarque–Bera normality tests and comparison of affinity distributions using Kolmogorov–Smirnov were done using R script. A two-sided Fisher exact test was used to determine significance in the methylation patterns.

**Results**

**Enrichment of MOG-specific Tregs and Tconvs in the CNS**

At the peak of myelin destruction during EAE (day 21), approximately one in five CNS-infiltrating CD4+ T cells expresses Foxp3, the transcription factor that marks Tregs. This frequency is increased as compared with the Foxp3+ frequencies found in the draining CLN and SPL (Fig. 1A, 14% each). Although a 1.5-fold enrichment in the frequency of Tregs from CNS over periphery occurred, this increase reflects changes in the total Treg population and not the frequency of MOG-specific Tregs. Additionally, there was a slight increase in the frequency of Tregs in the CLN and SPL during EAE compared with age-matched naive controls. Because Ag-specific Tregs are more effective than polyclonal Tregs at suppression, we sought to reveal the frequency of MOG-specific Tregs (36, 37).

To identify Ag-specific cells, pMHC tetramers were used to stain cells from Foxp3 GFP reporter mice that were sick with EAE. We observed approximately 4 and 8% of the Tconvs and Tregs, respectively, staining with tetramer in the CNS compared with ≤1% in the CLN and SPL at peak disease (Fig. 1B, Supplemental Fig. 1). On average, MOG-I-A<sup>B</sup> tetramers only detected <10% of the cells infiltrating the CNS as being specific for the inducing Ag (14, 18, 28). Another more sensitive method to enumerate polyclonal MOG-specific Tconvs and Tregs is the micropipette adhesion frequency assay. Individual cells within the polyclonal response were interrogated for their ability to specifically bind MOG-I-A<sup>B</sup>-coated RBCs while not binding hCLIP-I-A<sup>B</sup>-coated RBCs from the CNS of sick mice. Adhesion frequencies for MOG-I-A<sup>B</sup> ranged from 0.1 to 1.0. However, an adhesion frequency of 100% (1.0) cannot be used to derive affinity from the equation, so those T cells must be retested on RBCs coated with progressively lower Ag densities.

The adhesion frequency assay demonstrates specificity for the target Ag, as none of the cells that bound MOG-I-A<sup>B</sup> displayed any adhesion >0.1 for hCLIP-I-A<sup>B</sup> (Fig. 1C). Our previous work with monoclonal and polyclonal T cells has defined the 0.1 frequency as an operational cutoff for nonspecific binding, and it is considered the background level of the assay (14, 29, 32). Analysis of several tissues for Ag-specific T cells revealed similar adhesion frequencies and ranges in those frequencies for Tconvs and Tregs in the same tissue (Fig. 1D). Based on the adhesion frequencies, 80% of the CNS-infiltrating CD4+Foxp3<sup>T</sup> T cells were specific for MOG, whereas the draining CLN and SPL displayed 70 and 15% reactivity for MOG, respectively (Fig. 1E). We also observed similar frequencies of MOG-specific Tconvs in the same tissues. These data demonstrate an additional specificity control of the assay, as not all T cells are able to interact with the MOG-I-A<sup>B</sup>-coated RBCs (Fig. 1D).

These results parallel the increased numbers of Ag-reactive T cells that one would identify with specific tetramer but inclusive of the lower affinity T cells missed by all MHC class II tetramer staining with tetramer in the CNS compared with naive mice (open box plots) and at peak disease (filled box plots). Data are from five experiments. **p < 0.01 (one-way repeated measures ANOVA; CNS versus CLN, p = 0.0070; CNS versus SPL, p = 0.0051). (B) Tetramer staining frequencies by organ during peak EAE for Tconvs and Tregs, with representative FACS plots shown in Supplemental Fig. 1. Data are from at least five independent experiments. ***p < 0.0001 (two-way ANOVA repeated measures). (C) Representative adhesion frequencies for individual CNS cells for MOG-I-A<sup>B</sup>- and hCLIP-I-A<sup>B</sup>-coated RBCs. All cells were tested for binding to both Ags, and data are from two independent experiments, with 10 cells per each experiment tested. (D) MOG-I-A<sup>B</sup> adhesion frequencies for T cells isolated from indicated organs at peak EAE, where each dot represents an individual cell tested. Number of cells tested: CNS Tconvs, 65; CNS Tregs, 107; CLN Tconvs, 41; CLN Tregs, 40; SPL Tconvs, 55; SPL Tregs, 55. Overall frequency of cells that recognize MOG from the indicated organs for (D) and (E), data are from at least three, three, and four experiments for CNS, CLN, and SPL, respectively. For (E), two-way ANOVA comparing means for indicated cell type between tissues are shown: ****p < 0.0001 (CNS Tconvs versus SPL Tconvs, CNS Tconvs versus SPL Tregs, CNS Tregs versus SPL Tconvs, CNS Tregs versus SPL Tregs, CNS Tregs versus SPL Tconvs, CLN Tregs versus SPL Tconvs), ***p < 0.001 (CLN Tconvs versus SPL Tconvs, p = 0.0004; CLN Tregs versus SPL Tconvs, p = 0.0003), **p < 0.01, CLN Tconvs versus SPL Tconvs, p = 0.0025, ns (Tconvs, CNS versus CLN; Tregs, CNS versus CLN). (F) The frequency of MOG-specific Tregs as a percentage of total CD4<sup>T</sup> cells was determined by multiplying the average frequency of Foxp3<sup>T</sup> cells in each location, one-way ANOVA: ****p < 0.0001 (CNS versus SPL), **p < 0.01 (CNS versus CLN, p = 0.0078; CLN versus SPL, p = 0.0200). Box plots with minimum to maximum whiskers for all panels except (C) and (D), where each dot represents an individual cell, and for CLN in (E) and (F), where each dot represents an individual experiment because n = 3, n.d., not determined.
ramers (18). As measured by the micropipette assay, there are 8-fold more cells that are capable of recognizing MOG:1–11. The frequency of MOG-specific Tregs as a percentage of the total CD4+ T cell compartment revealed a striking difference among the tissues, with MOG-specific Foxp3+ cells comprising ∼18, 11, and 2% within CNS, CLN, and SPL, respectively (Fig. 1F). As expected from other reports, the intracellular cytokine assay also greatly underestimated the number of Ag-reactive T cells (Fig. 2) (14, 15, 38–41).

Tregs and Tconvs have overlapping ranges of affinity for MOG but unique distributions in the CNS

We next determined how Treg affinities for MOG compared with Tconvs. In the CLN, the geometric mean 2D affinities of Tconvs and Tregs were the same (Fig. 3A; CLN Tconvs, 1.446 [0.04940–11.42] and CLN Tregs, 1.349 [0.04670–14.35] × 10^{-5} μm^2 (range, minimum to maximum)). The range in affinities was >100-fold for both cell types in the CLN. The greatest range in affinities was in the CNS with 560- and 2450-fold for Tconvs and Tregs, respectively (Fig. 3A). This increased breadth in the CNS populations is due to enrichment of higher affinity cells. The CNS Tregs had a similar geometric mean affinity as did the CLN cells, but the CNS showed enrichment for higher affinity cells as depicted by the increase in range (1.204 [0.09270–227.1] × 10^{-5}). Even though the CLN and CNS Tregs had the same mean affinity, the presence of high-affinity MOG-specific Tregs in the CNS was countered by an increase in low-affinity cells. However, the CNS Tconvs had a significant increase in the mean 2D affinity (4.631 [0.2159–121] × 10^{-5}) compared with CNS Tregs and cells in the periphery. Thus, in the CNS at peak disease, Tconv affinity is expected from other reports, the intracellular cytokine assay also displayed the skewing and kurtosis expected of normal distributions as determined by the Jarque–Bera test (CLN Tconvs, p = 0.1484, Tregs, p = 0.1971). However, they do deviate from normality, as can be seen by the data on either end of the range not falling on the line in the Q–Q plots (Fig. 4C, 4D, Shapiro–Wilk CLN Tconvs, p = 0.02409, Tregs, p = 0.002664). Of interest, the CNS Treg affinity distribution deviated from normality, as the corresponding Q–Q plot shows that the CNS Tregs are right-skewed (Fig. 4B) with poor Shapiro–Wilk and Jarque–Bera p values (p values of 0.002404 and 0.005418, respectively). Although we do not know the mechanism for this difference in Treg distribution at this time, it is interesting to speculate that Treg affinities are skewed at the site of active autoimmune disease.

To determine how similar two distributions are to each other, the Kolmogorov–Smirnov test can be used along with Q–Q plots. Comparison of Tconv and Treg distributions from the CNS using the Kolmogorov–Smirnov test demonstrated a significant difference (p = 6.978 × 10^{-5}) that can be visualized in the Q–Q plot where none of the points falls on the line (Fig. 4E). This shift is seen in the Gaussian curves where the CNS Treg curve is shifted to the left of the CNS Tconv curve (Fig. 3C). Although the CNS Treg affinity range is as wide as the CNS Tconv range, their distributions are significantly different (Figs. 3A, 4E). This difference in distribution for Tconvs and Tregs was specific to the CNS, as the same comparison of the peripheral CLN populations revealed overlapping distributions (p = 0.7724, Fig. 4F).

CNS Tregs are of thymic origin

We next wanted to determine the developmental origins of the CNS Tregs at peak disease. It has been published that adoptive transfer of highly purified naive Tconvs from Foxp3+ GFP reporter mice followed by EAE induction led to no pTregs generated (18). Helios was used as a marker of iTregs because it has been shown to be highly expressed by Foxp3+ recent thymic emigrants whereas pTregs induced during oral tolerance lack Helios (42). Our data show >90% of the Foxp3+ Tregs expressed Helios in the CNS (Fig. 5A, 5B). It has been reported that Helios expression can be induced under certain circumstances (43, 44); however, we did not observe any ex vivo staining of Tconvs >6% in any organ during EAE (Supplemental Fig. 2). To provide further evidence of the CNS Tregs being of thymic origin, a iTreg-Me signature was used. This methylation signature consists of key regions in foxp3, tnfrsf18, clo4, and ikzf4, which are hypomethylated in iTregs compared with naive Tconvs (34). Ohkura et al. (34) and others (45–49) have demonstrated that this methylation signature was not induced merely by the expression of Foxp3, because TGF-β induced Tregs failed to demethylate these regions, and their methylation was consistent with Tconvs.

We observed hypomethylation of these regions in Tregs but not Tconvs when cells from SPL of naive mice were analyzed (Fig. 5C, 5D, Supplemental Table I). Because our data validated the iTreg-Me signature, we assessed the methylation status of the CNS-infiltrating Tregs during EAE. We observed the same pattern of significant demethylation for the CNS CD4+Foxp3+ Tregs.

FIGURE 2. Production of cytokines by Tconvs and Tregs during EAE. (A) Representative flow plots showing IL-17A and IFN-γ production by CNS Tconvs. (B) Frequencies of single and double cytokine producing Tconvs in various organs at peak EAE upon stimulation with PMA and ionomycin. Data are from three independent experiments. (C) Percentage of Tregs producing IL-10 or IFN-γ in the various organs in response to PMA/ionomycin stimulation. Data are from three independent experiments, except for SPL, which is from two experiments.
whereas CNS Tconvs were mostly methylated (Fig. 5E, 5F, Supplemental Table I). As an internal control for each experiment, CD25 intron 1a was included because in resting Tconvs that lack CD25 expression, this region is methylated and it is demethylated in cells that express CD25-like Tregs and activated Tconvs (34). As expected, CD25 was mostly methylated for Tconvs from naive mice, whereas this region was predominately demethylated for Tregs (naive and EAE mice) and Tconvs from the CNS of EAE mice (Fig. 5C–F). Based on these methylation data, we conclude that most Tregs in the CNS at peak EAE are of thymic origin. The region in foxp3 analyzed was the TSDR that is required for stable foxp3 expression and maintenance upon cell division (21). We observed high levels of methylation at the TSDR in the CNS Tregs (Fig. 5E–F, foxp3). There was still a significant difference in the methylation pattern of the TSDR between the CNS Tconvs and CNS Tregs (Supplemental Table I).

Discussion
For EAE, tetramers and cytokines greatly underrepresented the frequency of MOG-specific Tconvs and Tregs present in the CNS when compared with the ability of those cells to specifically bind MOG:I-Ab–coated RBCs in a micropipette adhesion frequency assay. We and others have demonstrated that especially for self-specific CD4+ T cells in multiple autoimmune models, tetramers miss most Ag-specific cells (14–17, 33). If the cells have low affinity for their cognate pMHC, then they are unlikely to interact with tetramer. For example, we have shown that 2D2 transgenic cells do not stain with MOG:I-Ab tetramer due to their extremely low affinity (15). Cytokine production in response to cognate peptide is able to identify more Ag-specific cells than tetramer (14, 16, 17, 33). In a parallel comparison, we showed that there are ∼2.4-fold more CNS Tconvs capable of producing cytokines (IFN-γ and TNF-α) in response to MOG35–55 peptide compared with tetramer staining (14). During viral infection we also demonstrated that sorted tetramer CD44+ T cells could produce cytokines after stimulation with the same Ag specificity as was in the tetramer (14). However, it is known that even cytokine production does not faithfully identify all of the Ag-specific cells as has been shown with cell lines and transgenic cells (38–41).

Therefore, to better assess the total number of Ag-specific cells in a polyclonal response, a more sensitive assay such as the micropipette adhesion frequency assay is needed. This assay allows for the biophysical interactions of proteins of interest to be measured as the probability of an adhesion event occurring when the receptor and ligand are allowed to interact (29, 30). Unlike surface plasmon resonance, which is considered a three-dimensional measure of affinity, 2D measures such as the micropipette adhesion frequency assay were shown to better correlate with functional data (29, 50). Another advantage of the micropipette assay is the ability to rapidly measure the Ag
specificity of a polyclonal response directly ex vivo, because intact T cells can be used to probe various pMHC monomer–coated RBCs. As shown in Fig. 1C, all of the cells that bound MOG:I-Ab did not have any adhesions for hCLIP specificity control, demonstrating the exquisite discrimination of Ags in this assay. Additionally, analysis of a polyclonal response provides an internal control for Ag specificity in that not all of the cells tested will in fact bind the pMHC of interest, especially in sites such as the SPL, which contains a diverse repertoire of Ag specificities.

The advantages of the 2D micropipette assay are multiple and greatly explain its increased sensitivity for identification of Ag-specific T cells. Many incorrectly assume that the RBC provides a simple increase in avidity extrapolating from work with pMHC tetramers. However, in the case of the RBCs, it is not a measure of avidity but an increase of valency (density of proteins) presented in their proper membrane context that increase the sensitivity of the assay. Avidity requires one binding event to alter the kinetic rates of subsequent binding events as occurs with pMHC tetramers and Abs. However, the increased number of pMHC molecules on the
RBC only increases the probability of a binding event occurring, as demonstrated by multiple pMHC densities yielding different adhesion frequencies but the same affinity (29). Using monoclonal cells, it has been shown that when multiple densities of ligands are used for the same density of receptor, plots confirm that the assay is measuring interactions between a single receptor and ligand (29–31). This monovalent interaction measured by the micropipette assay has been observed for Fcγ receptors with IgG, TCR with pMHC, and CD8 with pMHC. Note also that surface plasmon resonance–based assays that measure affinity of purified proteins (three-dimensional measurements) require the measurement of multiple molecules interacting to detect their signal and are not considered to be an avidity interaction. To solve for 2D affinity, the adhesion frequency must be measured at an equilibrium time point. The time required to reach equilibrium is determined by generating a curve where adhesion frequency is plotted against time. As time increases, the probability of binding events also increases before attaining a plateau level. The equilibrium frequency at the plateau level allows for derivation of the 2D affinity, as the equation is defined by the adhesion frequency at equilibrium, density of the TCR, density of the pMHC, and surface area of the interaction (see Materials and Methods). All parameters except the surface contact area can be accurately measured. Because one cannot get an accurate assessment of the contact area, we report our data as relative 2D affinities that contain the contact area term. The density of the TCR takes into account differences in TCR numbers and cell size that may differ among responding cells at a distinct point in their cell cycle, allowing measurement of the base 2D affinity for the TCR.

As expected, there were fewer MOG-specific cells in the SPL, with increased frequencies in the draining CLN for Tconvs and Tregs. We also determined that most of the CNS-infiltrating T cells (~80%) were in fact specific for MOG1-17, in contrast to less than half and ~10% by cytokine and tetramer staining, respectively (Figs. 1E, 1B, 2), which emphasizes the matched Ag specificities between Tregs and Tconvs. There were significantly more Tregs than Tconvs that stained with tetramer in the CNS, which could result from differences in the clustering or kinetics of TCR reorganization between Tregs and Tconvs. Because tetramer is an avidity-based reagent, these differences could greatly affect staining. We have shown using bulk polyclonal cells that there appears to be an affinity cut-off that allows for tetramer staining; however, future studies are needed to determine whether these boundaries are different for Tregs and Tconvs or other Th cell subsets (14). In fact, we would argue that even a higher percentage of the CNS T cells are MOG specific, given that the micropipette assay will also miss extremely low-affinity cells represented by the 2D TCR transgenic cells (15). The high frequency of MOG-specific cells in the CNS is not surprising because it is the site of Ag expression and inflammation. Similarly, the CLN collects APCs loaded with CNS Ags to prime T cell responses (51). Although these data cannot completely rule out the possibility of epitope spread, it would comprise a relatively small number of T cells at peak disease. We have also recently shown in a relapsing/remitting secondary progressive EAE model in NOD mice that >70% of the CD4+ T cells are still specific for the inducing Ag (27), again suggesting that additional Ag specificities are not a required factor for chronicity.

Based on the methylation signature and Helios expression, we contend that the CNS Tregs are of thymic origin. They had similar hypomethylation of key regions previously identified to be only demethylated in Tregs and not in Tconvs or induced Tregs (34). This conclusion is also supported by the inability to identify pTregs generated during EAE (18). The TSDR region was mostly methylated in the CNS Tregs compared with naive Tregs; however, all other gene regions were still hypomethylated. There is the possibility that these cells could be pTregs, but the origin of Tregs, whether thymic or peripherally induced, is a difficult question to answer, and to date there has not been a discriminating difference identified that faithfully and consistently discriminates this distinction. It has been shown that in the presence of proinflammatory cytokines, STAT6 and STAT3 bind to the TSDR, recruiting DNA methyltransferases to this region, which could explain the high levels of methylation that we observed (20). During acute EAE, the TSDR is demethylated during disease recovery when the inflammatory milieu would be waning, which could provide further support for the reason we saw methylation of this region during peak response in the chronic model (52).

One possible consequence of the TSDR being remethylated is lineage instability leading to the generation of pathogenic ex-Foxp3 cells (22). Mice that lack this region of DNA develop thymic Tregs that are capable of preventing the overt autoimmunity seen in mice deficient in Foxp3, but upon transfer to a lymphopenic host and subsequent homeostatic proliferation they lose Foxp3 expression (21). It has been demonstrated that Tregs in the CNS are capable of proliferation on the basis of BrdU incorporation (18). However, it was recently shown that the lineage instability due to lacking the TSDR requires multiple rounds of division before the loss of Foxp3 expression (20). Therefore, if the cells are not actively dividing, then they still could be stable, and upon the withdrawal of the inflammatory cytokines the TSDR could become demethylated again. In fact, a recent study showed that the TSDR is actively demethylated in Foxp3+ Treg thymocytes even in the presence of a late G1 inhibitor, which would allow for nondividing Tregs to demethylate this region to maintain lineage stability (53). To rule out the possibility of misidentification of Tregs due to differences in protein turnover for Foxp3 and GFP in the reporter mice, we repeated the experiments using Ab staining for Foxp3 to separate Tregs from Tconvs and obtained similar results. Future work will be done to determine the extent of any contribution to affinity by ex-Foxp3 cells.

At peak disease, there is a 1.5-fold enrichment of Tregs present in the CNS compared with peripheral sites. Additionally, there was an increase in the overall frequency of Tregs present in the periphery of EAE mice compared with age-matched naive control mice. This result is consistent with a study that showed that peptide immunization could lead to de novo Treg development of MBP-specific TCR Tg mice on a RAG-deficient background, which normally lack Tregs (54). It was shown that when the mice were thymectomized prior to peptide administration, there was only a slight delay in disease kinetics and the generation of Tregs was greatly impaired. Therefore, during EAE induction, it is possible that the MOG peptide required to induce disease is also leading to de novo Treg development. The authors did note that when two doses of pertussis toxin were given, there was a marked decrease in the numbers of Tregs generated in their transgenic RAG−/− mice, which were not sufficient for disease prevention. When the frequency of MOG-specific Tregs as a percentage of total CD4+ T cells is calculated, there is an enrichment of MOG-specific Tregs in the draining lymph node and at the site of autoimmunity, which is not appreciated when just the total percentage of Foxp3+ cells is shown.

To our knowledge, this study is the first to report the affinities of Treg cells for a self-antigen. The CNS Tregs had the largest range of affinity for MOG, but on average it was lower than for CNS Tconvs. It is intriguing to speculate that this deviation in affinities between Tconvs and Tregs at the site of tissue inflammation is causative for chronic disease and escape from Treg mechanisms of
peripheral tolerance. Although it has been hypothesized that Ag-specific Tregs would have high affinity for their cognate Ags, this question is extremely difficult to answer (5, 6, 55–58). The reason for this is the uncertainty of whether the self-specific Tregs that developed in the thymus selected on the same cognate Ag. For a cytotoxic c-specific transgenic, a positive selecting ligand has been identified, and based on the fact that it is unable to induce proliferation of mature T cells on its own, we assume that it is of lower affinity than the cognate agonist (12). Although we do not know the selecting peptide for the MOG-reactive T cells, the outcome is Tregs and Tconvs spanning an entire range of affinities. The concept that higher affinity TCRs are selected and survive to become iTregs for each self-selecting peptide is possible, but the identification of lower affinity TCRs for MOG and the wide range of affinity raise questions about this model of iTreg selection. Proof would have to come from analysis of polyclonal populations of T cells for which the self-selecting peptides are known, which is currently not possible. What we can take from these studies is that for the same self-antigen, Tconvs and Tregs have a range of affinities comprised of predominately low-affinity cells below the detection level of pMHC class II tetramers.

In conclusion, to our knowledge these data are the first to identify the frequency and affinity of iTregs for an endogenous self-antigen during autoimmune disease. We show that Ag-specific Tregs span a range of affinities for self that overlaps with Tconvs in the periphery, although in the CNS at the site of autoimmune disease the two populations have unique affinity distributions shifted to favor high-affinity Tconvs. The functional consequences for higher and lower affinity TCRs on iTregs need to be determined, but the altered Treg distribution pattern in the CNS potentially suggests mismatched TCR affinities as a mechanism for disease progression.

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