The Disease-Ameliorating Function of Autoregulatory CD8 T Cells Is Mediated by Targeting of Encephalitogenic CD4 T Cells in Experimental Autoimmune Encephalomyelitis

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Multiple sclerosis (MS) is an immune-mediated demyelinating disease of the CNS, and CD8 T cells are the predominant T cell population in MS lesions. Given that transfer of CNS-specific CD8 T cells results in an attenuated clinical demyelinating disease in C57BL/6 mice with immunization-induced experimental autoimmune encephalomyelitis (EAE), we investigated the cellular targets and mechanisms of autoreactive regulatory CD8 T cells. In this study we report that myelin oligodendrocyte glycoprotein peptide (MOG\textsubscript{35-55})-induced CD8 T cells could also attenuate adoptively transferred, CD4 T cell-mediated EAE. Whereas CD8\textsuperscript{+/-} mice exhibited more severe EAE associated with increased autoreactivity and inflammatory cytokine production by myelin-specific CD4 T cells, this was reversed by adoptive transfer of MOG-specific CD8 T cells. These autoregulatory CD8 T cells required in vivo MHC class Ia (K\textsubscript{D}B\textsuperscript{+}) presentation. Interestingly, MOG-specific CD8 T cells could also suppress adoptively induced disease using wild-type MOG\textsubscript{35-55}-specific CD4 T cells transferred into K\textsubscript{D}B\textsuperscript{+/-} recipient mice, suggesting direct targeting of encephalitogenic CD4 T cells. In vivo trafficking analysis revealed that autoregulatory CD8 T cells are dependent on neuroinflammation for CNS infiltration, and their suppression/cytotoxicity of MOG-specific CD4 T cells is observed both in the periphery and in the CNS. These studies provide important insights into the mechanism of disease suppression mediated by autoreactive CD8 T cells in EAE. The Journal of Immunology, 2013, 191: 000–000.

Studies focusing on CNS-specific CD8 T cells have shown their ability to mediate disease pathogenesis, as demonstrated in the C3H mouse strain using myelin basic protein--specific CD8 T cell clones (11), myelin oligodendrocyte glycoprotein (MOG)--specific CD8 T cells in C57BL/6 mice (10, 21), and MOG\textsubscript{35-55}-specific transgenic TCR-bearing (1C6) CD8 T cells from NOD mice (22). We have recently demonstrated the unexpected disease regulating ability of CNS-specific CD8 T cells in wild-type (WT) EAE (23). MOG-specific CD8 T cells could significantly ameliorate EAE in C57BL/6 (B6) mice (23). Moreover, our recent studies in human MS also suggest an important, clinically relevant immune regulatory function for CNS-targeted autoreactive CD8 T cells (24). Both MS patients and healthy subjects harbor neuroantigen-specific CD8 T cell responses (8) with TCR homology to published sequences from CNS-infiltrating T cells in MS lesions (25). However, the suppressive ability of these cells is dramatically reduced during an acute disease relapse (24). Thus, it is critical to understand the biology of these cells and dissect their mechanism of action.

In this study, we demonstrate that CNS-specific autoreactive regulatory CD8 T cells are restricted by classical MHC class Ia molecules and are capable of directly targeting and suppressing previously activated pathogenic CD4 T cells. Thus, these studies demonstrate a novel population of disease-modulating CD8 T cells that could be harnessed for adoptive immunotherapy in the future.
β2-microglobulin (β2m)/−/−, B6.D9 IL-4−/−, B6.D129 IFN-γ−/−, B6-D129 IL-10−/−, C57BL/6-Tg(Tcr2D2,Ctcr2D2)1Kuch/J, and C57BL/6 Prf−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.D129 IFN−/− mice were purchased from The Jackson Laboratory and provided by Dr. Jerry Niederkorn (University of Texas Southwestern Medical Center, Dallas, TX). B6.D129 Tap−/− mice were purchased from The Jackson Laboratory and provided by Dr. Jerry Forman (University of Texas Southwestern Medical Center). C57BL/J TCR−/− mice were purchased from Taconic (Hudson, NY). WT B6 mice were purchased from Taconic and the University of Texas Southwestern Mouse Breeding Core Facility (Dallas, TX). B6 Ly5.2/Cr mice were purchased from the National Cancer Institute (Bethesda, MD).

Active EAE and evaluation

Neuropetide MOG35–55 (MEVGWYRSPFSRVVHYLRNGK) and control peptide OVA323–339 (ISQAVHAAHAEINEAGR) were synthesized by the University of Texas Southwestern Protein Chemistry Technology Center. On day 0, B6 mice were s.c. immunized with 100 μg MOG35–55 in CFA supplemented with 4 mg/ml Mycobacterium tuberculosis (H37Ra; Difco, Detroit, MI). Additionally, at days 0 and 2, mice were administered 250 ng pertussis toxin (List Biological Laboratories, Campbell, CA) via i.p. injection. Clinical EAE disease was assessed using the following criteria: 0, no paralysis; 1, loss of tone in the tail; 2, mild hind limb weakness; 3, significant hind limb paralysis; 4, complete hind limb paralysis; 5, hind limb paralysis with urinary incontinence/dead. Mice that showed grade 5 disease were sacrificed as part of the protocol and were counted as grade 5 through the remainder of the disease course. When appropriate, each experimental condition was represented across multiple cages and the evaluator was blinded to experimental condition, that is, two-blind way blinded EAE scoring.

Adaptive EAE

Lymph node cells from day 10 post-MOG35–55–immunized B6 mice were harvested and incubated for 72 h at 37°C in EAE culture media (RPMI 1640 medium supplemented with 10% FCS, l-glutamine, penicillin, streptomycin, HEPES buffer, nonessential amino acids, sodium pyruvate, and 2-ME) containing 20 μg MOG35–55 and murine rIL-12 (10 ng/ml). CD4 T cells were obtained using anti-CD4 (L3T4) microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and a total of 5 × 10^6 live CD4 T cells were injected i.p. into naive, WT B6 mice at day 0. Pertussis toxin was administered on days 0 and 2 and EAE disease was monitored daily.

Autoregulatory CD8 T cell adoptive transfer experiment

Lymph nodes and splenocytes were harvested from 20- to 25-d-postimmunized mice, and viable lymphocytes were isolated using Lympholyte-M (Cedarlane Laboratories, Burlington, NC) treatment as per the manufacturer’s instructions. Next, cells were stimulated with cognate Ag and murine rIL-2 (10 pg/ml) for 72 h at 37°C in a culture flask at 7.5 × 10^6/ml concentration. Highly purified (TRC9)CD4−/−CD8+ T cells were obtained using anti-CD8 (Ly-2) microbeads (Miltenyi Biotec), and a total of 5 × 10^6 live CD8 T cells were injected i.p. into naive, WT B6 mice at day 0. Pertussis toxin was administered on days 0 and 2 and EAE disease was monitored daily.

CFSE-based proliferation and cytokine assay

Ag-specific responses were evaluated using the CFSE-based dilution assay using bulk splenocyte and lymph node cells from myelin peptide–immunized mice. Bulk cells were harvested and incubated for 72 h at 37°C in EAE culture media (RPMI 1640 medium supplemented with 10% FCS, l-glutamine, penicillin, streptomycin, HEPES buffer, nonessential amino acids, sodium pyruvate, and 2-ME) containing 20 μg MOG35–55 and murine rIL-12 (10 ng/ml). CD4 T cells were obtained using anti-CD4 (L3T4) microbeads (Miltenyi Biotec), and a total of 5 × 10^6 live CD4 T cells were injected i.p. into naive, WT B6 mice at day 0. Pertussis toxin was administered on days 0 and 2 and EAE disease was monitored daily.

In vitro killing assay

As described previously (26) but adapted for murine cells, cytokotoxic MOG-specific CD8 T cells were obtained using splenocytes from day 12–immunized mice, which were in vitro activated and expanded for 7 d in MOG35–55 media with stimulating Ag (MOG35–55 or OVA323–339) at 20 μg/ml and murine rIL-2 (10 pg/ml). After in vitro stimulation, dead cells were removed using the Dead Cell Removal Kit (Miltenyi Biotec), and CD4+ or CD8+ T cells were isolated using CD4 (L3T4) or CD8 (L2–) microbeads (Miltenyi Biotec). The purity of T cells was consistently >95%. A total of 5 × 10^6 CD4 or CD8 T cells were injected i.v. into naive, WT B6 mice at day 0. Subsequently, at days 10 and 20, mice were anesthetized with 400 μl 1.5% Avertin and perfused with 20 ml cold PBS via left ventricular puncture. Brain and spinal cord tissue was harvested and processed via 30% Percoll (GE Healthcare, Piscataway, NJ) gradient. Cervical and lumbar lymph nodes and spleens were harvested and processed using EAE culture media with washing media, followed by RBC lysis buffer and subsequent wash. Once all tissues where processed into single-cell suspensions, cells were washed with FACS buffer and incubated for 5 min at 4°C with mouse FcR blocking reagent (Miltenyi Biotec). Cells were then stained with allophycocyanin-anti-45.1, PerCP5.5-anti-CD45.2, Pacific Blue-anti-CD4, and PE-Cy7-anti-CD8 (BD Biosciences), incubated for 45 min at 4°C, washed with staining buffer, and fixed with 1% paraformaldehyde.

Data analysis

Statistical analyses between groups were performed using GraphPad Prism 5.0. Differences in disease severity, peak, and onset were evaluated using a two-tailed Student t test. A p value ≤0.05 was considered statistically significant.

Results

Autoregulatory CD8 T cells suppress CD4 T cell–mediated autoimmune disease

We have recently demonstrated the unexpected finding that autoreactive MOG35–55-induced CD8 T cells could attenuate EAE in MOG35–55-immunized B6 mice (23). To dissect the mechanisms of this suppression, we first asked whether this attenuation was restricted to actively induced EAE or could be seen in EAE transferred by preprimed CD4 T cells. Similar to our prior findings, the transfer of MOG35–55-induced CD8 (MOG-CD8) T cells 1 d prior to active EAE induction resulted in significant reduction of disease severity compared with mice receiving OVA323–339-induced CD8 (OVA-CD8) T cells as controls (Fig. 1A).
In this setting, we wanted to test whether transfer of MOG-specific CD8 T cells had an effect on MOG35–55-specific CD4 T cell responses. We addressed this by adoptively transferring transgenic MOG-specific (2D2) CFSE-stained naive CD4 T cells (CD45.2+) into CD45.1+ congenic mice treated with either MOG- or OVA-specific CD8 T cells (CD45.1+) and subsequent immunization with MOG35–55/CFA on day 0. At days 5, 7, and 10 postimmunization, cervical and inguinal lymph nodes and spleens were harvested and CFSE dilution of CD45.2+CD8+ CD4+ T cells was measured. Representative histograms of 2D2 CD4 T cell CFSE dilution at day 10 in control CD8 T cell recipient mice (top panel) and MOG-specific CD8 T cells (bottom panel) are shown. Numbers in the histograms indicate the percentage of gated cells that were either in the CFSElow or CFSEhigh fractions in the OVA-CD8 and MOG-CD8 T cell recipients. (C) The graph indicates percentage suppression, calculated as 100% – [(test condition /control condition) × 100]. Data are representative of three independent experiments. Cumulative graphs from days 5, 7, and 10 are shown. (D) Mice were immunized with MOG35–55/CFA, followed by transfer of MOG-specific (or control) CD8 T cells at day 12 postimmunization. Data are representative of two independent (n = 20/condition). (E) MOG or OVA CD8 T cells were transferred into naive mice. The next day, adoptive EAE was induced using purified activated CD4 from MOG35–55-immunized mice. Data are representative of five experimental replicates (n = 25/condition). **p < 0.05.

CD8<sup>−/−</sup> mice exhibit augmented CD4 T cell autoreactivity, which can be reversed by autoregulatory CD8 T cells

To study the effect of transferred CD8 T cells without contribution from endogenous CD8 T cells, we used CD8<sup>−/−</sup> mice, which are known to have an increase in disease severity in this model (15). We hypothesized that in CD8-deficient mice, EAE severity increases owing to the unfettered activation of encephalitogenic CD4 T cells. First, we induced EAE in CD8<sup>−/−</sup> B6 mice using a range of immunizing doses of MOG35–55 (200, 100, and 50 μg/mouse). As expected, disease was significantly more severe with greater incidence in CD8<sup>−/−</sup> mice compared with littermate controls, with the differences more obvious when suboptimal doses were used (Table I). Disease curves at the 100-μg dose demonstrated a significant effect at the recovery/chronic stages of disease (Fig. 2A). These observations were also confirmed in experiments where CD8 T cells were depleted using anti-CD8 Ab.
versus IgG control injections (Supplemental Fig. 1A, 1B). CD8-depleted mice showed an increase in mean maximum score and decrease in mean day of onset (Supplemental Fig. 1C).

Next, we determined whether the increase in disease susceptibility in CD8−/− mice correlated with an altered functional profile of the neuroantigen-specific CD4 T cells. First, we compared MOG35–55-specific CD4 T cell recall responses between CD8−/− and WT mice using the CFSE dilution assay. Briefly, WT and CD8−/− mice were immunized with MOG35–55, followed by harvesting of draining lymph node cells, spleen cells, and CNS-infiltrating cells at day 20. Cells were stained with CFSE and cultured in vitro for 5 d in the presence or absence of cognate Ag. Subsequently, cells were stained with fluorophore-conjugated anti-TCRβ, CD4, and CD8 Abs, and CFSE dilution was measured within the TCRβ+CD8+CD4+ population. Replicate cultures were stimulated on day 5 with a PMA/ionomycin/brefeldin A mixture for 4 h for characterization of cytokine profiles. Both peripheral (Fig. 2B) and CNS (Supplemental Fig. 2) CD4 T cells from CD8−/− mice were found to have a significantly higher recall response to MOG35–55 peptide, as compared with WT mice. Moreover, evaluation of intracellular cytokine production within the MOG35–55-responding (CFSElow) CD4 T cells revealed an increase in IL-17A+, IFN-γ+, and TNF-α–producing MOG35–55-specific CD4 T cells in CD8−/− mice as compared with WT mice (Fig. 2C). Interestingly, IL-10+ MOG35–55-specific CD4 T cells were found to be increased in CD8−/− mice. Foxp3+ MOG35–55-specific CD4 T cells were found to be comparable between CD8−/− and WT cohorts.

We next determined whether autoregulatory CD8 T cells were sufficient in reversing the increased EAE severity in CD8−/− mice. We performed a rescue experiment by reconstituting CD8−/− mice with IgG isotype control, anti-KbDb Ab, or anti-Qa-1b Ab and transferred CD8−/− mice with MOG35–55 autoregulatory CD8 T cells (versus OVA-specific controls), followed by induction of primary EAE. In CD8−/− mice, transfer of MOG-specific CD8 T cells was sufficient to significantly suppress EAE (Fig. 2D). At the same time, recall CD4 T cell responses from “protected” mice revealed a decrease in TNF-α+, IFN-γ+, IL-17A+, and GM-CSF–producing encephalitogenic MOG35–55-specific CD4 T cells (Fig. 2E).

**Myelin Ag-specific CD8 T cell activation, as well as disease suppression, is MHC class Ia-dependent**

Next, we delineated the requirement of MHC class I molecules in CD8 T cell–mediated suppression of autoimmune demyelinating disease. First, we performed an in vitro blocking assay where CFSE-stained bulk splenocytes from MOG35–55-immunized mice were co-cultured with IgG isotype control, anti-KbDb Ab, or anti-Qa-1b Ab and stimulated with either MOG35–55 peptide or Con A for 5 d. Relative to the isotype control, KbDb blockade (but not Qa-1b blockade) showed a reduction in MOG-specific CD8 T cell response (Fig. 3A). Con A stimulation revealed no appreciable loss of responses with either KbDb or Qa-1b blockade (data not shown).

To confirm our Ab blockade findings, we ascertained whether MHC class I–deficient APCs could stimulate autoregulatory CD8 T cells. In vitro stimulation with cognate Ag revealed a significant response from MOG-specific CD8 T cells in the presence of WT APCs, which was absent in KbDb−/− APC culture conditions (Supplemental Fig. 3A).

Next, we evaluated the requirement of in vivo MHC class I presentation during CD8 T cell–mediated disease regulation. WT B6 MOG-specific CD8 T cells were transferred into β2m−/− (MHC class I–deficient) or WT mice, followed by MOG35–55 EAE induction. Compared with the protection seen when WT MOG-specific CD8 T cells were transferred into WT recipient mice, these same cells had no effect on EAE disease in β2m−/− recipients (Fig. 3B). Similarly, WT MOG-specific CD8 T cells were incapable of suppressing disease when transferred into Tap−/− (MHC class I–deficient) mice (Fig. 3C). However, in β2m−/− and Tap−/− mice there is loss of CD8 T cells over time, which may explain these findings. Therefore, we further elucidated the requirement of classical (class Ia) versus nonclassical (class Ib) MHC class I by using the KbDb−/− (MHC class Ia–deficient and class Ib–competent) mice, where we observed >50% survival of transferred CD8 T cells, compared with WT hosts (data not shown). Even in this setting, the transferred CD8 T cells were ineffective in disease amelioration (Fig. 3D), indicating an in vivo requirement of classical MHC class Ia–restricted presentation. Similar to prior reports (16), we also observed an augmentation of disease in MHC class I–deficient mice when comparing the control CD8 T cell–treated groups (Fig. 3B, 3D).

Thus, having observed that autoregulatory CD8 T cells could suppress CD4 T cell–mediated adoptive disease (Fig. 1E) and that disease suppression by these cells was MHC class Ia–dependent (Fig. 3D), we next investigated whether targeting of MHC class I–replete CD4 T cells by autoregulatory CD8 T cells may be a sufficient mechanism for EAE amelioration. For this, we used the adoptively transferred EAE model, such that only the inducing CD4 T cells were the source of MHC class Ia molecules. This was done by modifying our adoptive EAE protocol and transferring purified WT MOG35–55-specific CD4 T cells into KbDb−/− host mice. Interestingly, autoregulatory CD8 T cells were capable of suppressing WT adoptively transferred EAE when the recipient host was devoid of MHC class Ia molecules (Fig. 3E). Hence, we concluded that focused targeting of encephalitogenic CD4 T cells was sufficient in modulating disease severity.

**Suppression of EAE by autoregulatory CD8 T cells is dependent on IFN-γ and perforin but not on IL-4 or IL-10**

The phenotypic characteristics of autoregulatory CD8 T cells were examined by combining the CFSE dilution assay and evaluating the expression of various functional molecules on MOG-specific (proliferating) CD8 T cells. A significant proportion of autoregulatory CD8 T cells was found to produce IFN-γ, TNF-α, and perforin, whereas a negligible fraction showed IL-17A, IL-10, Foxp3, or IL-4 positivity (Supplemental Fig. 4A). To test potential cytotoxic function, we directly determined whether autoregulatory CD8 T cells could be cytotoxic by performing an in vitro killing assay. Con A–stimulated MOG-loaded splenocytes

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### Table I. Increased EAE disease susceptibility in CD8−/− B6 mice

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<th>MOG35–55 (µg)</th>
<th>Incidence (%)</th>
<th>Mean Day of Onset</th>
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<tr>
<td></td>
<td>WT</td>
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<tr>
<td>200</td>
<td>10/10 (100)</td>
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<td>9.5 ± 0.2</td>
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<tr>
<td>100</td>
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<td>50</td>
<td>8/10 (80)</td>
<td>10/10 (100)</td>
<td>15.3 ± 2.8</td>
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*Clinical disease data statistically compared between WT and CD8−/− mice. p < 0.05.*
were used as target cells and cultured with an increasing number of MOG-specific CD8 T cells in media alone or with cognate Ag for 48 h. Killing was measured by evaluating the number of target cells normalized to control beads. For control purposes, MOG-specific CD8 T cells were cultured with P815 cells decorated with murine anti-CD3 (redirected lysis). Autoregulatory CD8 T cell killing increased as the E:T ratio increased (Supplemental Fig. 4B).

In view of these functional observations, we next evaluated the relevance of these cytokines to in vivo disease suppression. It has been previously shown that IFN-γ-producing CD8 T cells may act in a suppressive manner (17, 27, 28). Thus, we evaluated the role of IFN-γ in autoregulatory CD8 T cell disease inhibition. We obtained IFN-γ−deficient MOG-specific CD8 T cells from MOG35–55-immunized IFN-γ−/− B6 mice. We transferred these}
CD8 T cells into WT naive recipient mice at day 1. As controls, we also transferred MOG- and OVA-specific CD8 T cells from WT mice. At day 0, EAE was induced in all three groups and clinical disease was evaluated. Recipients of IFN-γ-deficient MOG-specific CD8 T cells showed no protection from disease, exhibiting significantly more severe disease at the acute and chronic phases, compared with the WT MOG-specific CD8 T cell recipients (Fig. 4A). Tajima et al. (29) and others (30) have shown that IL-12 can augment the activation of CD8 T cells. Thus, we supplemented our cultures with IL-12 and observed both an increase in IFN-γ+ autoreactive CD8 T cells, as well as an increase in IFN-γ production per cell (Supplemental Fig. 4C). These increases in IFN-γ production and IFN-γ+ cells correlated with an enhanced disease-suppressing role (Supplemental Fig. 4D). We also evaluated the role of the cytotoxic molecule perforin, cytokines IL-4 and IL-10, and the requirement of IFN-γR on MOG-specific CD8 T cells. We observed that in vivo protection by these cells was also dependent on perforin (Fig. 4B), but not the other molecules (Fig. 4C–E).

MOG-specific CD8 T cells decrease both peripheral and CNS MOG-specific CD4 T cell numbers

We next wanted to ascertain the in vivo location of CD8 T cell–mediated immune regulation (i.e., peripheral immune compartment versus CNS). First, we evaluated whether MOG-CD8 T cells could traffic to the CNS. After transferring congenic CD45.1+ MOG-CD8 donor T cells i.v., we attempted to detect them in the CNS at days 5, 10, and 20. Whereas we could consistently detect cells in the cervical and inguinal lymph nodes, as well as in the spleen, we could not detect MOG-specific CD8 T cells in the CNS (Fig. 5A). In contrast (and as expected), CD45.1+ MOG-CD8 T cells were transferred into K^d^{−/−} mice, followed by induction of adoptive EAE using WT MOG-specific CD4 T cells. These data demonstrate

FIGURE 3. Autoregulatory CD8 T cells require MHC class Ia in vivo for disease amelioration. (A) In vitro blocking experiments using Abs to classical and nonclassical MHC class I molecules were performed to evaluate the role MHC class I in CD8 T cell–mediated disease suppression. Representative CFSE dilution histograms of gated TCRβ^+CD4^+CD8^+ T cells are shown (left panels) as well as the cumulative data (right panel). Numbers indicate CFSE^{low} fraction of CD8 T cells in the presence of IgG control, anti-K^d^{+}, or anti-Qa-1B Ab. (B) In vivo MHC requirements were evaluated by transferring WT MOG (or OVA-reactive) CD8 T cells into either WT or β2m^{−/−} mice. MOG35–55 EAE was induced the following day and clinical disease was evaluated in all four groups. Results are representative of two independent experiments (n = 20/condition). (C) Disease curves for MHC class I-deficient (Tap^{−/−}) versus control group (WT). Both groups received WT MOG-specific CD8 T cells at day −1 and were immunized with MOG35–55/CFA at day 0. Results are representative of two independent experiments (10 mice/condition). (D) MOG-CD8 or OVA-CD8 T cells were transferred into either WT or K^d^{−/−} mice, followed by disease induction. Data are representative of two independent experiments (n = 20/condition). (E) WT MOG or OVA-CD8 T cells were transferred into K^d^{−/−} mice, followed by induction of adoptive EAE using WT MOG35–55-specific CD4 T cells. Results are representative EAE data of two independent experiments (n = 14/condition). **p < 0.05.
CNS infiltration by autoregulatory CD8 T cells only during CNS pathology, further reducing the possibility that they could be independently pathogenic and strongly suggesting that they may potentially have their regulatory effect at the site of pathology.

To address the effect of autoregulatory CD8 T cells on CNS-infiltrating CD4 T cells, we transferred MOG-CD8 or OVA-CD8 T cells into CD45.1+ B6 mice, followed by transfer of MOG35–55-specific transgenic (2D2) CD45.2+ CD4 T cells, with subsequent induction of primary EAE. 2D2 CD4 T cell numbers were evaluated in the CNS and the periphery. We observed reduction in the numbers of MOG35–55-specific CD4 T cells both in the periphery (inguinal lymph node and spleen) as well as in the CNS (Fig. 5C), suggesting that the disease suppression could be explained by either a peripheral mechanism alone or a combination.

Discussion
As thymic negative selection is not perfect, the presence of peripheral autoreactive CD4 and CD8 T cells is the norm rather than the exception. As a corollary, peripheral control of potentially pathogenic autoreactive T cells is important in controlling immune-mediated disease. It is becoming clear that several immune-mediated diseases, such as MS, are characterized by perturbation of immune regulation (26, 31–37). In the murine model of MS (EAE), there is ample evidence that neuroantigen-specific CD4 T cells can initiate and sustain neuroinflammation and pathology. In contrast, the role of CD8 T cells, particularly CNS-specific CD8 T cells, is still poorly understood and somewhat controversial. Some studies have demonstrated a potential pathogenic role for CNS-targeted CD8 T cells (10, 11, 21, 22, 38), and this makes intuitive sense in that CNS Ags would be presented to these cells by resident CNS cells in the context of MHC class I. In that regard, recent mouse models based on transgenically expressed CNS-sequestered Ags combined with TCR-transgenic CD8 T cells or HLA-transgenic mice also suggest that these cells may have proinflammatory potential (39–41). Although these studies do demonstrate the pathogenic potential of certain CD8 T cells, many of them involved either induced homeostatic expansion of CD8 T cells in T cell–deficient mice or the use of transgenic manipulation, potentially selecting for either a rare self-reactive clone or a clone of T cells that does not evolve through usual thymic selection.

Using WT B6 mice, we have recently demonstrated the unexpected disease regulatory role of CNS-targeted CD8 T cells (23). As a population, MOG-specific CD8 T cells are able to suppress MOG35–55-induced primary EAE. We have observed similar suppressive function for CD8 T cells of other CNS specificities as well (unpublished data and S. Ortega, V. Kashi, and N. Karandikar, manuscript in preparation). In the present study, we further confirm that these autoregulatory CD8 T cells can suppress ongoing primary EAE, as well as EAE induced with adoptively transferred, preactivated CD4 T cells. Using different variants of MHC class I–deficient mice, we demonstrate that these cells are restricted by classical MHC class I molecules (K\textsuperscript{D}\textsuperscript{D}), and their function is mediated through a combination of IFN-γ–mediated regulation and perforin-mediated cytotoxicity. We further show that in the absence of CD8 T cells, CD4 T cells show an enhanced proinflammatory phenotype, which is reversed by the transfer of CNS-specific CD8 T cells. Importantly, using the adoptive transfer model where only the transferred CD4 T cells had MHC class Ia, we provide evidence that these autoregulatory CD8 T cells are capable of directly targeting and suppressing encephalitogenic CD4 T cells.

CNS-specific autoregulatory CD8 T cells appear to be a unique class of T cells that have some features of cytotoxic CD4-targeting Qa-1–restricted regulatory CD8 T cells (19, 20), at the same time being classical MHC class I restricted, much like effector CD8 T cells. Thus, they are distinct from therapeutically induced regulatory CD8 T cells (20, 26, 32, 42, 43). Moreover, they are
specific for target tissue Ag (CNS), requiring the in vivo priming with their cognate antigenic peptide (unpublished data). In that regard, they appear to be similar to other autoantigen-specific CD8 T cells described in diabetes models (44). It is noteworthy that tissue-specific CD8 T cells can target CD4 T cells in an MHC class I–restricted manner. This requires that CNS Ag get presented on the CD4 T cells. Several mechanisms may result in this ability, including passive loading of digested myelin components at the site of pathology or trogocytosis (i.e., membrane exchange) between APCs and CD4 T cells, as proposed in prior studies (45–49). Future studies are needed to elucidate these mechanisms in the context of EAE. However, it is notable that effects on CD4 T cell numbers and function were noted both in the periphery as well as in the CNS, suggesting that the mechanism of suppression could be operative at both sites.

Two important mechanisms of these autoregulatory CD8 T cells are noteworthy. First, these cells produce IFN-γ and this production is necessary for in vivo disease amelioration, and we hypothesize that its primary role is to target pathogenic CD4 T cells. Again, unlike the regulatory Qa-1–restricted CD8 T cells, which secrete IL-10 to exert their inhibitory activity, EAE-generated autoregulatory CD8 T cells did not need to produce IL-10 to mediate their effects. Although we have shown that CD4 T cell targeting by autoregulatory CD8 T cells is sufficient for some of their function in a reductionist approach, this does not rule out their effects on APCs, either through IFN-γ–mediated modulation or cytotoxic killing of activated, proinflammatory APCs. This question is being actively addressed in ongoing studies.

The clinically relevant role of these autoregulatory CD8 T cells is revealed by the observations that these T cells infiltrate their target organ only during ongoing CNS inflammation. These temporal dynamics explain the somewhat delayed effect on EAE, as evidenced in CD8-deficient mice. Moreover, lack of CD8 T cell infiltration in nonimmunized mice also strongly argues against a predominant pathogenic role for these cells. The importance of CNS-specific CD8 T cells in human MS was highlighted by our recent observations, where we showed that CNS-specific CD8 T cells showed immune suppressive properties, which were significantly deficient during an active disease relapse, but were recovered during disease quiescence (24). Thus, it is tempting to
speculate that the adoptive transfer of a selected population of nonpathogenic, autoregulatory CD8 T cells may offer a novel immune intervention for these patients. It is encouraging to note that these cells were able to ameliorate ongoing/established EAE. As further preclinical evidence, it would be important to delineate methods to enhance the in vitro propagation of the most effective subpopulation of these cells, perhaps using selection based on functional molecules and demonstration of the most effective disease reversal.

In summary, our studies demonstrate a novel autoregulatory population of CNS-specific CD8 T cells that ameliorates EAE using suppressor/cytotoxic mechanisms. Although distinct from their MHC class 1b-restricted siblings, these autoregulatory CD8 T cells reveal an additional potent immunomodulatory arm of the adaptive immune system, which may in a concerted effort try to ameliorate autoimmune demyelinating disease and may be harnessed for immunotherapeutic intervention.

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Disclosures

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References

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Figure S1. Increase of EAE severity in antibody-mediated CD8 depleted mice. (A) Flow cytometric data showing the successful depletion of CD8 T-cells from the peripheral immune compartment. (B) Clinical disease severity comparison between anti-CD8 depleted versus IgG control treated mice, post EAE induction. (C) Mean maximum EAE score and mean day of onset of CD8-depleted (vs. control) WT mice. *=p<0.05. Representative data from three independent experiments (5-7 mice per condition).
Figure S2. Augmented CNS-specific CD4 T-cells autoreactivity within the CNS of CD8\(^{-/-}\) mice. CFSE-based proliferation assays were performed on cells isolated from brain and spinal cord of CD8\(^{-/-}\) and WT mice at day 20 post-immunization. CFSE histograms are shown. ΔPF represents difference between proliferation in the presence of cognate antigen minus background. Representative of two independent experiments (n=10 per condition).
Figure S3. Autoregulatory CD8 T-cell are ineffective in the absence of MHC Ia. (A) Dependence of MHC I for CD8 T-cell activation was determined by performing an in vitro culture where MOG-specific CD8 T-cells were cultured with WT or MHC Ia deficient APC.
Figure S4

(A) Intracellular cytokine stain of MOG-specific (i.e., proliferating/CFSE Low) TCRβ+CD4-CD8+ T-cells. Representative histogram and cumulative graph of five independent experiments. (B) In vitro cytotoxicity assay is shown where MOG-loaded, splenic ConA blasts were co-cultured with increasing numbers of MOG-specific CD8 T-cells. Anti-CD3-decorated P815 target cells were used as positive controls. Percent killing was defined as live CFSE target cells for a particular ratio divided by live CFSE target cells in the no effector condition. (C) Intracellular cytokine stain of MOG-specific (i.e. proliferating /CFSE Low) TCRβ+CD4-CD8+ T-cells following IL12 modulation. Interval numbers indicate the percentage of IFN-γ+CD8 T-cells within the responding population. Mean fluorescence intensity (MFI) within the IFN-γ+ population is shown. (D) Clinical disease severity comparison between control, standard and IL12 modulated MOG-specific CD8 T-cells is shown. Representative of three independent experiments (5-7 mice per condition).