

Autoregulatory CD8 T cells depend on cognate antigen recognition and CD4/CD8 myelin determinants

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ABSTRACT

Objective: To determine the antigenic determinants and specific molecular requirements for the generation of autoregulatory neuroantigen-specific CD8⁺ T cells in models of multiple sclerosis (MS).

Methods: We have previously shown that MOG35-55-specific CD8⁺ T cells suppress experimental autoimmune encephalomyelitis (EAE) in the C57BL/6 model. In this study, we utilized multiple models of EAE to assess the ability to generate autoregulatory CD8⁺ T cells.

Results: We demonstrate that alternative myelin peptides (PLP178-191) and other susceptible mouse strains (SJL) generated myelin-specific CD8⁺ T cells, which were fully capable of suppressing disease. The disease-ameliorating function of these cells was dependent on the specific cognate myelin antigen. Generation of these autoregulatory CD8⁺ T cells was not affected by thymic selection, but was dependent on the presence of both CD4⁺ and CD8⁺ T-cell epitopes in the immunizing encephalitogenic antigen.

Conclusions: These studies show that the generation of autoregulatory CD8⁺ T cells is a more generalized, antigen-specific phenomenon across multiple neuroantigens and mouse strains, with significant implications in understanding disease regulation. *Neurol Neuroimmunol Neuroinflamm* 2015;2:e170; doi: 10.1212/NXI.0000000000000170

GLOSSARY

CFA = complete Freund's adjuvant; **CFSE** = carboxyfluorescein succinimidyl ester; **EAE** = experimental autoimmune encephalomyelitis; **GA** = glatiramer acetate; **MHC** = major histocompatibility complex; **MOG** = myelin oligodendrocyte glycoprotein; **MS** = multiple sclerosis; **OVA** = ovalbumin; **PLP** = proteolipid protein; **TCR** = T-cell receptor; **UTSW** = UT Southwestern; **WT** = wild-type.

Multiple sclerosis (MS) is a chronic immune-mediated demyelinating disorder of the CNS. Studies utilizing the experimental autoimmune encephalomyelitis (EAE) model of MS have established T cells to be major mediators of CNS pathology. Studies in human MS show that CD8⁺ T cells outnumber CD4⁺ T cells in MS lesions, produce inflammatory interleukin-17, and express cytotoxic markers like granzyme B.¹⁻³ The antigenic specificity of these CNS-infiltrating cells is unclear. In EAE models, there are descriptions of pathogenic as well as disease-ameliorating CD8⁺ T cells. For example, myelin basic protein-specific CD8⁺ T cells in C3H mice and myelin oligodendrocyte glycoprotein (MOG)-reactive CD8⁺ T cells in B6 mice have been shown to be pathogenic in certain settings.⁴⁻⁸ In contrast, β 2-microglobulin and CD8-deficient mice demonstrate more severe EAE,⁹⁻¹² CD8⁺CD28⁻ and CD8⁺CD122⁺ cells modulate pathogenic CD4⁺ T cells,^{11,13} and there are robust descriptions of T-cell receptor (TCR)-specific and Qa-1-restricted¹⁴⁻¹⁶ non-CNS-specific disease-modulating CD8⁺ T cells.

In both MS and EAE, we have observed that neuroantigen-specific CD8⁺ T cells (CNS-CD8) modulate encephalitogenic CD4⁺ T cells.^{12,17-20} Specifically, our EAE studies reveal a disease-regulatory function for CNS-CD8⁺ T cells, which is major histocompatibility complex

Supplemental data
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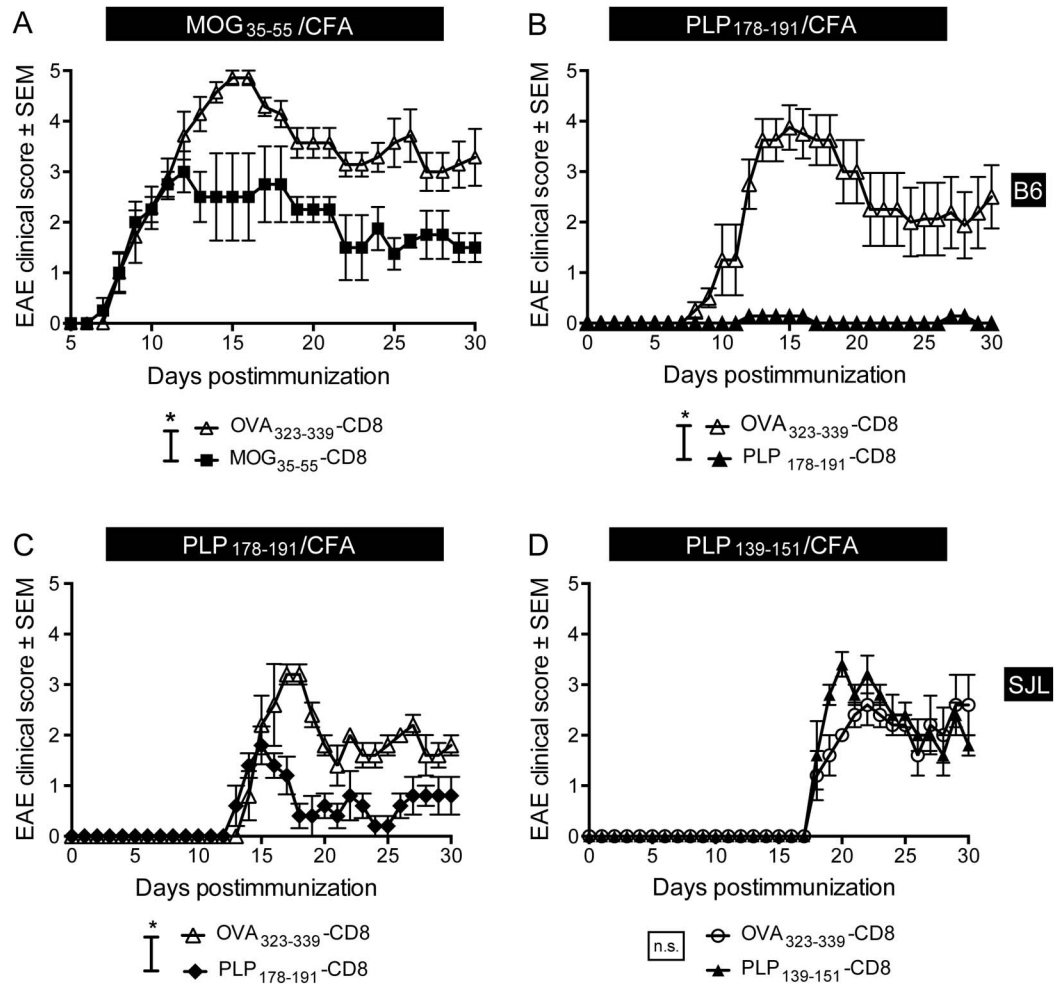
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Funding information and disclosures are provided at the end of the article. Go to Neurology.org/nn for full disclosure forms. The Article Processing Charge was paid by the authors.

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Figure 1 EAE regulation by myelin-specific CD8⁺ T cells across various models



Either B6 (A, B) or SJL (C, D) were used as donors of myelin-specific (indicated) or control (ovalbumin [OVA]₃₂₃₋₃₃₉)-specific CD8⁺ T cells, which were adoptively transferred IV into naive mice (same strains) on day 1. On day 0, experimental autoimmune encephalomyelitis (EAE) was induced in all mice by immunization with the appropriate cognate antigens in complete Freund's adjuvant (CFA) (A: 100 μg of myelin oligodendrocyte glycoprotein [MOG]₃₅₋₅₅; B and C: 100 μg of proteolipid protein [PLP]₁₇₈₋₁₉₁; and D: 100 μg of PLP₁₃₉₋₁₅₁). Graphs represent mean clinical scores ± SEM. Data are representative of 3-5 independent experiments, 7-10 mice per condition per experiment. **p* < 0.05. n.s. = not significant.

(MHC) Class I restricted, requires interferon-γ and perforin, and modulates antigen-presenting cells and CD4⁺ T cells. These studies were performed predominantly in the B6 model of EAE induced by MOG₃₅₋₅₅ antigen. Moreover, the antigenic requirements of these interactions and the conditions needed to generate autoregulatory CNS-CD8⁺ T-cell responses have not been elucidated and this is the focus of the current report.

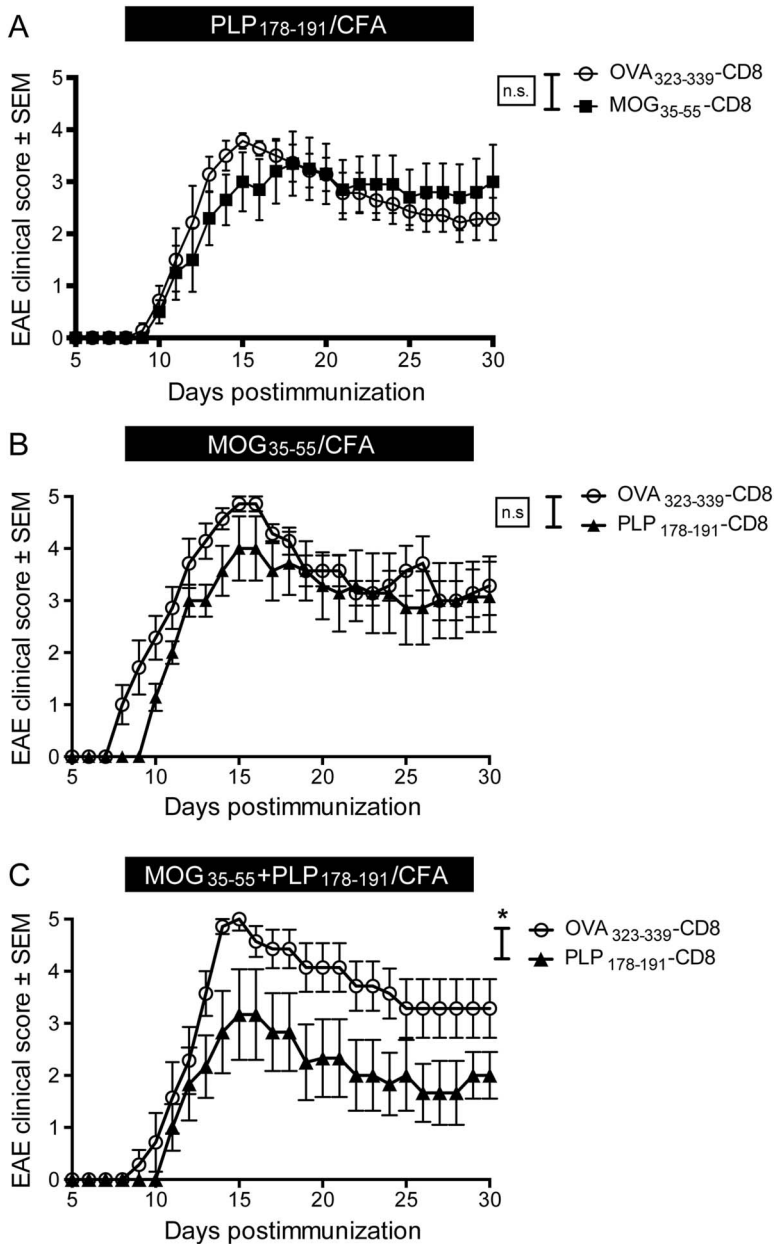
METHODS Mice. All mouse protocols were approved by the UT Southwestern (UTSW) Medical Center Institutional Animal Care and Use Committee. Mice were housed in the UTSW Animal Resource Center. Wild-type (WT) C57BL/6 (B6) mice were purchased from Taconic (Hudson, NY) and UTSW Breeding Core. SJL/J (SJL) mice were purchased from the NCI Mouse Repository (Frederick, MD). MOG-deficient mice²¹ were a gift from Dr. Eric Huseby.

Active EAE induction and evaluation. MOG₃₅₋₅₅ (MEVGWYRSPFSRVVHLYRNGK), proteolipid protein (PLP)₁₇₈₋₁₉₁ (NTWTTCQSIAPFSK), PLP₁₃₉₋₁₅₁ (HSLGKWLGHDPDKF), MOG₃₇₋₄₆, MOG₄₀₋₄₉, MOG₄₄₋₅₄, MOG₃₇₋₅₀, and ovalbumin (OVA)₃₂₃₋₃₃₉ (ISQAVHAAHAEINEAGR) were synthesized by UTSW Chemistry Technology Center. EAE was induced as described previously.^{12,20} Briefly, female mice were immunized with 100 μg of antigens/complete Freund's adjuvant (CFA), followed by 250 ng of pertussis toxin IP on days 0 and 2, except the PLP₁₃₉₋₁₅₁-induced model. EAE severity was assessed in a blinded manner on a 0 to 5 scale.

Adoptive transfer of antigen-specific CD8⁺ T cells. Naive mice were immunized with 100 μg of antigen. At day 20, lymph node cells and splenocytes were harvested and stimulated with cognate antigen and rmIL-2 (10 pg/mL) for 72 hours. Highly enriched CD8⁺ T cells (purity ~95%) were obtained using anti-CD8 Miltenyi microbeads; 5 × 10⁶ cells were injected IV. After 24 hours, primary EAE was induced.

CFSE-based proliferation. Antigen-specific responses were evaluated using the carboxyfluorescein succinimidyl ester

Figure 2 In vivo requirement of cognate antigen for autoreactive CD8⁺ T-cell disease regulation



(A) Myelin oligodendrocyte glycoprotein (MOG)₃₅₋₅₅-specific CD8⁺ T cells cannot inhibit proteolipid protein (PLP)₁₇₈₋₁₉₁ experimental autoimmune encephalomyelitis (EAE). MOG₃₅₋₅₅-specific (A), PLP₁₇₈₋₁₉₁-specific (B, C), or ovalbumin (OVA)₃₂₃₋₃₃₉-specific (controls in all panels) CD8⁺ T cells were transferred into naive B6 mice at day 1. On day 0, EAE was induced by immunization with PLP₁₇₈₋₁₉₁ (A), MOG₃₅₋₅₅ (B), and a combination of both PLP₁₇₈₋₁₉₁ and MOG₃₅₋₅₅ (C). Representative of 2 independent experiments each. **p* < 0.05. CFA = complete Freund's adjuvant; n.s. = not significant.

(CFSE)-based proliferation assay as described.^{12,20} Cells were activated with cognate antigen at 20 μg/mL for 5 days, washed, incubated with FcR blocking reagent, stained for CD8/CD4/TCRβ, and fixed in paraformaldehyde, followed by acquisition of flow data.

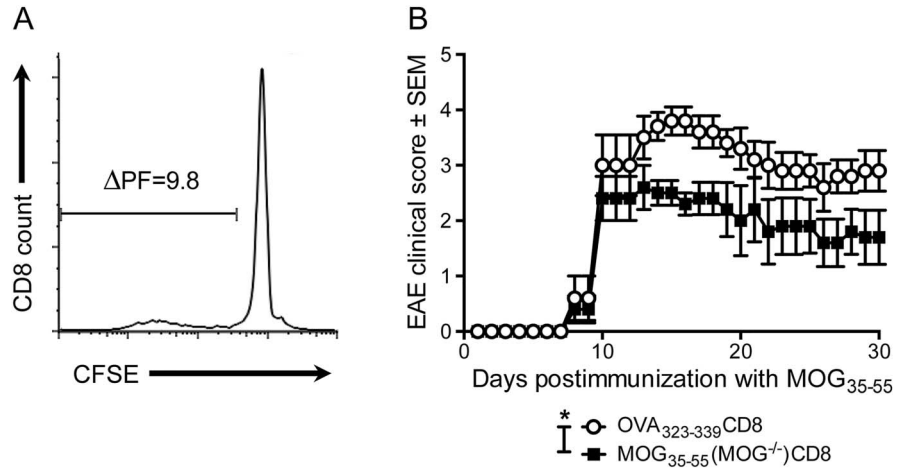
Statistics. Statistical analyses between groups (2-tailed Student *t* tests) were performed using GraphPad (La Jolla, CA) Prism 5.0c. A *p* value ≤ 0.05 was considered statistically significant.

RESULTS Disease-ameliorating ability of various myelin-specific CD8⁺ T cells in EAE. We have previously shown that myelin-specific CD8⁺ T-cell responses (myelin-CD8) are generated in multiple EAE models.²⁰ However, we have predominantly utilized the MOG₃₅₋₅₅-induced model of EAE in B6 mice to dissect the regulatory role of these cells.^{12,19,20} In this study, we asked whether this phenomenon of myelin-CD8-mediated downregulation of EAE was more generalized. First, we confirmed our prior findings that immunization with MOG₃₅₋₅₅ in B6, PLP₁₇₈₋₁₉₁ in B6 and SJL, and PLP₁₃₉₋₁₅₁ in SJL mice resulted in appropriate EAE disease (figure e-1A at Neurology.org/nn), with induction of robust CD8⁺ T-cell responses (figure e-1B).

We next asked if myelin-CD8 plays a pathogenic/regulatory role in EAE. Similar to our previous reports,^{12,20} myelin-CD8 obtained from donor mice (figure e-2) was transferred to naive mice (day -1), followed by EAE induction with the corresponding cognate antigen (day 0). Similar to disease amelioration by MOG₃₅₋₅₅-CD8⁺ T cells (figure 1A),^{12,19} PLP₁₇₈₋₁₉₁-CD8 were capable of suppressing PLP₁₇₈₋₁₉₁-induced EAE in B6 mice (figure 1B). Additionally, PLP₁₇₈₋₁₉₁-CD8 also suppressed PLP₁₇₈₋₁₉₁-induced EAE in SJL (figure 1C). Interestingly, PLP₁₃₉₋₁₅₁-CD8 from SJL neither suppressed nor exacerbated PLP₁₃₉₋₁₅₁-induced EAE (figure 1D).

Cognate antigen presentation is required for the regulatory function of CD8⁺ T cells. We observed that CD8⁺ T cells with 2 separate antigenic specificities (MOG₃₅₋₅₅ and PLP₁₇₈₋₁₉₁) from the same strain of mouse (B6) had the ability to downregulate disease (figure 1, A and B). This gave us the tools to address antigenic specificity of this regulation. We asked whether MOG₃₅₋₅₅-specific CD8⁺ T cells were capable of suppressing PLP₁₇₈₋₁₉₁-induced EAE (and vice versa). PLP₁₇₈₋₁₉₁, MOG₃₅₋₅₅, or OVA₃₂₃₋₃₃₉-specific CD8⁺ T cells were generated and transferred on day -1. On day 0, EAE was induced using noncognate encephalitogenic peptide antigen (PLP₁₇₈₋₁₉₁/CFA in case of MOG₃₅₋₅₅-CD8 [figure 2A] and MOG₃₅₋₅₅/CFA in case of PLP-CD8 [figure 2B]). We observed that MOG₃₅₋₅₅-CD8⁺ T cells were unable to protect mice from PLP₁₇₈₋₁₉₁-induced EAE disease (figure 2A). Similarly, PLP₁₇₈₋₁₉₁-specific CD8 T cells were unable to modulate MOG₃₅₋₅₅-induced EAE (figure 2B). This was despite the ability of the same cells (in the same experiments) to regulate disease induced by their corresponding cognate antigens (figure 1). Interestingly, PLP₁₇₈₋₁₉₁-CD8 T cells were able to suppress disease symptoms when EAE was induced by MOG₃₅₋₅₅/PLP₁₇₈₋₁₉₁ coimmunization (figure 2C). These data reveal that autoregulatory neuroantigen-specific CD8⁺

Figure 3 Inhibition of EAE by MOG₃₅₋₅₅-specific CD8⁺ T cells derived from MOG^{-/-} mice



(A) Following the immunization of myelin oligodendrocyte glycoprotein (MOG)^{-/-} B6 mice with MOG₃₅₋₅₅, lymph node cells and splenocytes were harvested and carboxyfluorescein succinimidyl ester (CFSE) stained. Cells were then cultured in vitro with MOG₃₅₋₅₅ and 5 days later CFSE dilution measured. Δ Proliferating fraction (PF) is the % of CFSE low CD8⁺ T cells in the MOG₃₅₋₅₅ stimulated condition minus the no antigen (background) condition. Data are representative of 3 independent experiments. (B) MOG-CD8⁺ T cells generated from MOG^{-/-} mice were injected IV into naive B6 mice and experimental autoimmune encephalomyelitis (EAE) was induced with MOG₃₅₋₅₅/complete Freund's adjuvant (CFA) immunization on day 0. Representative data of 2 experiments are shown, n = 5-7 per condition. *p < 0.05.

T cells are dependent on cognate antigen presentation during in vivo disease suppression.

Generation of autoregulatory CD8⁺ T cells is not dependent on central tolerance but requires presence of CD4⁺ and CD8⁺ determinants in immunizing antigen. Next, we wanted to determine the antigenic requirements for the generation of autoregulatory CD8⁺ T cells. In human MS, we have previously observed that foreign antigen-CD8⁺ T cells lack immunosuppressive ability.¹⁷ In this study, we observed a difference between disease regulatory ability of MOG₃₅₋₅₅ and PLP₁₇₈₋₁₉₁-CD8⁺ cells (both autoregulatory in nature) vs that of PLP₁₃₉₋₁₅₁ (figure 1). We hypothesized that this difference could relate to thymic presentation of certain epitopes (such as MOG₃₅₋₅₅ and PLP₁₇₈₋₁₉₁ in mice and other autoantigens in humans) and lack of presentation of specific epitopes (such as PLP₁₃₉₋₁₅₁),²² leading to qualitatively different responses. We therefore investigated whether central tolerance might play a role in autoregulatory CD8⁺ T-cell generation by utilizing MOG^{-/-} B6 mice

for this purpose (with the notion that these mice would not have central tolerance to MOG). We immunized these mice with MOG₃₅₋₅₅ peptide and used them as donors of CD8⁺ T cells. Parallel CFSE assays confirmed the presence of MOG₃₅₋₅₅-specific CD8⁺ T-cell responses in these mice (figure 3A). Activated CD8⁺ T cells derived from such cultures were transferred to naive WT B6 recipient mice at day -1, followed by induction of EAE on day 0. As shown in figure 3B, MOG₃₅₋₅₅-CD8⁺ T cells derived from MOG^{-/-} mice showed the ability to suppress EAE, similar to those from WT mice (figure 1A). Thus, lack of central tolerance to MOG did not affect the disease-ameliorating ability of myelin-CD8 T cells in this model.

In these experiments, MOG₃₅₋₅₅ peptide generated both pathogenic CD4⁺ T-cell responses and regulatory CD8⁺ T-cell responses. Within the MOG₃₅₋₅₅ peptide, there are 2 previously characterized CD8 epitopes: MOG₃₇₋₄₆⁶ and MOG₄₄₋₅₄,²³ and one CD4 epitope: MOG₄₀₋₄₉.²⁴ Using this information, we wanted to determine what portion of the MOG₃₅₋₅₅ molecule was required to generate the disease-ameliorating myelin-specific CD8⁺ T cells. First, we immunized mice with several truncated peptides (table 1) and determined whether there was an antigen-specific CD8⁺ T-cell response and if the generated CD8⁺ T cells were capable of ameliorating MOG₃₅₋₅₅-induced EAE.

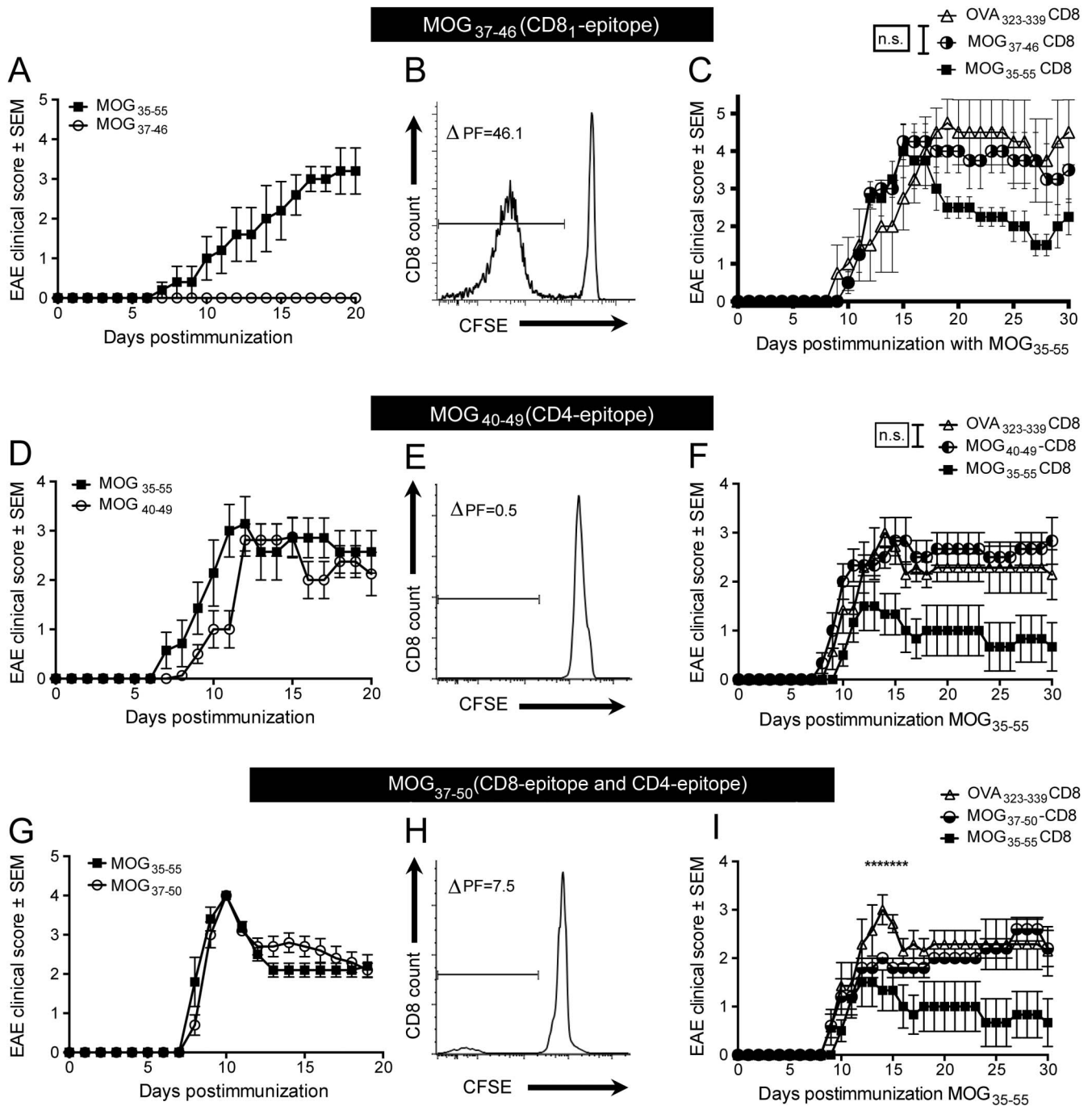
Although MOG₃₇₋₄₆ and MOG₄₄₋₅₄ immunizations did not induce EAE, robust CD8 responses were observed (figures 4, A and B, and e-3). Adoptive transfer of CD8⁺ T cells specific to these epitopes, however, failed to suppress MOG₃₅₋₅₅-induced EAE (figures 4C

Table 1 Truncated MOG peptides

Truncated peptide	Sequence	Described epitope
MOG ₃₅₋₅₅	MEVGWYRSPFSRVVHLYRNGK	CD8 ₁ and CD8 ₂ and CD4 (parent peptide)
MOG ₃₇₋₄₆	MEVGWYRSPFSRVVHLYRNGK	CD8 ₁
MOG ₄₄₋₅₄	MEVGWYRSPFSRVVHLYRNGK	CD8 ₂
MOG ₄₀₋₄₉	MEVGWYRSPFSRVVHLYRNGK	CD4
MOG ₃₇₋₅₀	MEVGWYRSPFSRVVHLYRNGK	CD8 ₁ & CD4

Abbreviation: MOG = myelin oligodendrocyte glycoprotein.

Figure 4 Generation of autoregulatory CD8⁺ T cells requires immunization with CD4 and CD8 epitopes



Naive B6 mice were immunized with 100 μg of myelin oligodendrocyte glycoprotein (MOG)₃₅₋₅₅ or indicated peptides and disease was monitored for 20 days (A, D, G). CD8⁺ T-cell responses were assayed to the indicated peptides using carboxyfluorescein succinimidyl ester (CFSE) dilution assays (B, E, H). Post in vitro activated donor CD8⁺ T cells specific to indicated peptides were transferred to naive mice, followed by induction of experimental autoimmune encephalomyelitis (EAE) using MOG₃₅₋₅₅ in complete Freund's adjuvant (C, F, I). Data are representative of 3-5 independent experiments each, 7-10 mice per condition per experiment. ΔPF = Δ proliferating fraction; n.s. = not significant; OVA = ovalbumin.

and e-3). We next immunized a separate group of mice with the CD4⁺ T-cell epitope MOG₄₀₋₄₉, which resulted in robust disease similar to MOG₃₅₋₅₅ immunization (figure 4D) with an absence of any CD8⁺ T-cell response (figure 4E). Expectedly, transfer of these cultured CD8⁺ T cells (likely representing naive and nonspecific CD8⁺ T cells) did not result in

any disease modulation (figure 4F). Next, to test if immunization with both the CD8 epitopes contained within MOG₃₅₋₅₅ might be required to induce a fully functional regulatory CD8⁺ T-cell population, we generated CD8⁺ T cells by coimmunizing donor B6 mice with MOG₄₄₋₅₄ and MOG₃₇₋₄₆. Again, while MOG₃₅₋₅₅-induced CD8⁺ T cells

suppressed disease, (MOG₃₇₋₄₆ + MOG₄₄₋₅₄)–induced CD8⁺ T cells failed to modulate EAE (figure e-4).

We finally hypothesized that immunization with an encephalitogenic CD4 T-cell epitope along with a CD8 T-cell epitope may be important to generate autoregulatory MOG-CD8 T cells. To test this, we immunized mice with MOG₃₇₋₅₀, which encompasses a CD4 and a CD8 epitope. MOG₃₇₋₅₀ immunization resulted in primary EAE disease similar to MOG₃₅₋₅₅ (figure 4G) and a CD8 response (figure 4H). Transfer of MOG₃₇₋₅₀-induced CD8⁺ T cells resulted in partial protection from EAE (figure 4I) that was not as robust as protection using MOG₃₅₋₅₅-CD8 (peptide containing 2 CD8 and one CD4 determinant). Collectively, these data suggest that generation of autoregulatory CD8⁺ T cells requires the presence of the encephalitogenic CD4 epitope and at least one CD8 epitope in the MOG₃₅₋₅₅-EAE model (table 2).

DISCUSSION Our understanding of autoreactive T cells is undergoing a paradigm shift. Until recently, cells capable of responding to self were viewed as effectors of pathology and this was understandable in light of the many endogenous central and peripheral mechanisms involved in tolerance induction. However, there is ample evidence that CNS-specific T-cell autoreactivity does not always equate to pathology and may, in fact, provide essential functions during health and serve a neuroprotective role in various settings,^{25–27} a concept labeled as protective autoimmunity.

There are several lines of evidence demonstrating the presence of autoantigen-specific immunoregulatory (autoregulatory) CD8⁺ T cells (reviewed in reference 28). In several reports by others and us, autoregulatory CD8⁺ T cells have been described in various human autoimmune diseases and models.^{29–34} In human MS, we have demonstrated the clinical relevance of this autoregulatory function that resides in the terminally differentiated subset,¹⁸ is significantly deficient during an acute relapse of disease, but recovers during the quiescent stage of MS.¹⁷ In addition, we have shown that myelin-specific CD8⁺ T cells modulate both active and adoptive EAE,^{12,20} by targeting encephalitogenic CD4⁺

T cells¹² and modulating dendritic cell function.¹⁹ Since our prior EAE studies predominantly focused on the MOG₃₅₋₅₅-induced EAE model in B6 mice, we wanted to determine the finer antigenic specificity of CD8-mediated regulation, the generalizability of this observation, and the antigenic requirements for the generation of this regulatory response.

We examined 4 EAE models from 2 mouse strains, and observed that 3 of 4 conditions resulted in the generation of autoregulatory CD8⁺ T cells. PLP₁₇₈₋₁₉₁-CD8 from both B6 and SJL mice were capable of suppressing EAE induced by the specific cognate antigen. Interestingly, PLP₁₃₉₋₁₅₁-CD8 neither suppressed nor worsened cognate antigen-induced EAE. Thus, while we did not find evidence of a pathogenic role for these cells, these data also suggest that autoregulatory function may not be inherent to all CNS-CD8⁺ T cells. The nature of the difference between autoantigen-specific nonregulatory vs autoregulatory CD8⁺ T cells is unclear at this point. Overall, the magnitude of CD8⁺ T-cell responses to various autoantigens was comparable across the models, with no statistically significant differences over multiple experiments. It is plausible that the pool of PLP₁₃₉₋₁₅₁-CD8⁺ T cells may be functionally different. One explanation may be the lack of pertussis in the PLP₁₃₉₋₁₅₁-induced EAE model, although this is not borne out in preliminary studies (not shown). We have also observed in other experiments that CNS-specific autoregulatory CD8⁺ T cells are induced in systems devoid of pertussis (unpublished observations, 2015). Also, CD8⁺ T cells generated to MOG₃₇₋₄₆, MOG₄₀₋₄₉, and MOG₄₄₋₅₄ with pertussis failed to suppress EAE. Therefore, pertussis does not completely explain these functional differences and future studies focused on the detailed evaluation of various functional aspects of these nonregulatory autoreactive CD8⁺ T cells are essential.

We have previously shown that, in humans, myelin antigen-specific CD8⁺ T cells exhibit an autoregulatory function, whereas foreign antigen-specific CD8⁺ T cells do not show consistent suppressive potential.¹⁷ We hypothesized that this may be based on thymic selection, where only the low avidity autoantigen-specific T cells would escape negative selection.³⁵ Interestingly, in mice, PLP expression in the thymus is restricted to the DM20 splice variant,²² which does not include the PLP₁₃₉₋₁₅₁ region. This may potentially explain the differences between the CD8⁺ T-cell populations. To address this hypothesis, we utilized MOG^{-/-} mice as donors of MOG₃₅₋₅₅-CD8⁺ T cells (figure 3). These mice have higher avidity MOG-specific CD8⁺ T cells³⁶ and an expansion of both CD4⁺ and CD8⁺ T cells in response to MOG immunization. Again, we did not see any evidence of CD8-mediated pathogenicity using MOG₃₅₋₅₅-CD8⁺ T cells from these mice. However,

Table 2 Antigen response and disease suppression of autoreactive CD8⁺ T cells

Epitope	CD4 response	CD8 response	Primary EAE	Disease suppression
MOG ₃₅₋₅₅	+	+	++	++
MOG ₃₇₋₄₆	–	+	–	–
MOG ₄₄₋₅₄	–	+	–	–
MOG ₄₀₋₄₉	+	–	++	–
MOG ₃₇₋₅₀	+	+/-	++	+/-

Abbreviations: EAE = experimental autoimmune encephalomyelitis; MOG = myelin oligodendrocyte glycoprotein.

neither did we observe any significant deficiency in their ability to protect from EAE, suggesting that lack of thymic selection did not affect the regulatory repertoire in the MOG/B6 model.

As mentioned above, we found that PLP₁₇₈₋₁₉₁-CD8 T cells were suppressive in nature. Most of the well-characterized immunosuppressive CD8⁺ T-cell populations, where antigenic specificity has been dissected, are targeted against antigens expressed on pathogenic CD4⁺ T cells, such as Qa-1-restricted heat shock proteins or fragments of TCR sequences.^{15,37,38} In these settings, suppressor CD8⁺ T cells raised against one autoreactive CD4⁺ T cell can, in turn, target other autoreactive CD4⁺ T cells that express the same TCR or heat shock proteins. We have also described regulatory CD8⁺ T-cell responses, induced in mice and patients with MS following treatment with glatiramer acetate (GA), which appear to be MHC Class Ib restricted and are capable of functioning in vivo without the need for additional GA administration.^{39,40} In contrast to these situations, the regulatory populations of MOG- and PLP-induced CD8⁺ T cells appear to be specific to the CNS target antigens. To address this issue in greater detail, we took advantage of our observation that 2 different specificities of CNS-CD8 were disease suppressive in B6 mice. Thus, we asked whether PLP₁₇₈₋₁₉₁-CD8 could suppress MOG₃₅₋₅₅-induced disease and vice versa. These experiments revealed that MOG₃₅₋₅₅-CD8 could not inhibit PLP₁₇₈₋₁₉₁ disease (figure 2A). Similarly, PLP₁₇₈₋₁₉₁-CD8 could not suppress MOG₃₅₋₅₅ disease (figure 2B). These findings support 2 important interpretations: (1) the CD8⁺ T cells derived from these cultures do not contain significant populations of cells targeted against non-CNS antigens expressed generally on autoreactive CD4⁺ T cells (such as heat shock proteins or TCR peptides), and (2) the presence of their cognate antigens is required in vivo for their disease suppressive effects. The latter is also corroborated by our prior observations that CNS-CD8 T cells require in vivo MHC Class I¹² and that they do not modulate dendritic cells in mice that do not receive cognate antigens.¹⁹ Moreover, PLP-CD8 significantly suppressed disease symptoms when EAE was induced by coimmunization with MOG₃₅₋₅₅ and PLP₁₇₈₋₁₉₁, further indicating that immunization with cognate antigen supported their disease-ameliorating role. In the coimmunization setting, it is unclear whether PLP-CD8 only affected the PLP-CD4 response or were able to have an antigen nonspecific effect on MOG-CD4 response (e.g., through antigen-presenting cell modulation). This dissection will require future studies and will be important for the clinical context where multiple CNS specificities are involved.

In the final set of experiments, we dissected the fine specificity of MOG-CD8 and delineated the

immunization requirements for generating MOG-specific suppressor CD8⁺ T cells. The encephalitogenic MOG₃₅₋₅₅ sequence has been shown to contain one CD4 determinant (MOG₄₀₋₄₉)²⁴ and 2 CD8 determinants (MOG₃₇₋₄₆ and MOG₄₄₋₅₄),^{6,23} as outlined in table 1. We utilized these peptides to assess their encephalitogenicity and ability to induce suppressor CD8⁺ T cells. As expected, the CD4 determinant induced EAE, but did not induce any regulatory CD8⁺ T-cell responses (figure 4, D–F). The CD8⁺ T-cell epitopes, either individually or in combination, showed good induction of CD8⁺ T-cell responses, but no induction of EAE (figure 4, A and B, and not shown). This is in contrast to previous studies showing pathogenicity of MOG-induced CD8⁺ T cells^{6,8,23}; however, this corroborates findings from us and others, showing no EAE induction using MOG₃₅₋₅₅-induced CD8⁺ T cells.^{12,19,20} In keeping with this observation, CD8⁺ T cells induced by immunization with individual CD8 epitopes could not transfer EAE to naive mice and did not worsen EAE induced by MOG₃₅₋₅₅ immunization, providing no evidence of their pathogenic role. Thus, at least in our hands, we have been unable to demonstrate pathogenic function for any of these MOG- or PLP-specific CD8⁺ T cells in the B6 or SJL systems.

Interestingly, CD8⁺ T cells induced by either MOG₃₇₋₄₆ or MOG₄₄₋₅₄ were also not capable of suppressing EAE, in contrast to MOG₃₅₋₅₅-induced CD8⁺ T cells. We asked whether this lack of suppressive activity could simply be a result of not having representation of CD8 responses to both of the CD8 determinants within the MOG₃₅₋₅₅ peptide sequence. We therefore obtained CD8⁺ T cells from mice immunized with both CD8 epitopes. However, these CD8 responses were incapable of suppressing EAE (figure e-4). We thus hypothesized that it may be important to have a concomitant CD4 response to induce fully functional suppressor CD8⁺ T cells and addressed this hypothesis by utilizing the MOG₃₇₋₅₀ peptide, containing one CD4 and one CD8 epitope. MOG₃₇₋₅₀-derived CD8⁺ T cells were partially suppressive and decreased the severity of the acute phase of the disease, but did not replicate the full suppressive ability exhibited by MOG₃₅₋₅₅-derived CD8 cells. An obvious reason might be the lack of CD8 responses to the other CD8 epitope. It is also possible that the full MOG₃₅₋₅₅ sequence contains other CD8 epitopes that have not been characterized thus far or are represented by lower avidity responses that are important in the overall suppressive ability. There may also exist specific functional deficits in the MOG₃₇₋₅₀-derived CD8⁺ T cells, compared to the complete complement, and these would need to be dissected through future studies. Overall, these findings suggest that generation of autoregulatory CD8⁺ T cells is dependent on the combination of CD4 and CD8 epitopes available and the context in which these responses are induced.

Taken together, in this study we provide evidence of the presence of myelin-specific regulatory CD8⁺ T cells across different models of EAE. These cells require interaction with their cognate antigen in vivo for suppressive functionality and their generation is dependent on the availability of CD4 and CD8 specificities. These findings significantly augment our understanding of autoregulatory CD8⁺ T-cell biology and have important implications in harnessing these cells to develop immunotherapeutic approaches for MS and other immune-mediated diseases.

AUTHOR CONTRIBUTIONS

S.B.O. designed and performed experiments, analyzed data, and wrote the manuscript. V.P.K. designed and performed experiments, analyzed data, and cowrote the manuscript. K.C. and J.F. performed some of the experiments and critically evaluated the manuscript. N.J.K. designed and supervised the study and cowrote the manuscript.

ACKNOWLEDGMENT

The authors thank Thomas Lee and Wallace Baldwin for technical assistance; and Ibrahim Noorbhai, Katie Poinsette, and Drs. Ethan Baughman, Todd Eagar, Mihail Firan, Sushmita Sinha, and Ann M. Stowe for discussions and critical reading of this manuscript.

STUDY FUNDING

These studies were supported, in part, by grants (to N.J.K.) from the NIH and the National Multiple Sclerosis Society.

DISCLOSURE

S. Ortega reports no disclosures. V. Kashi is an associate editor for *Journal of Human Virology & Retrovirology*. K. Cunnusamy and J. Franco report no disclosures. N.J. Karandikar received research support from NIH/NIAD and National Multiple Sclerosis Society. Go to Neurology.org/nn for full disclosures.

Received April 17, 2015. Accepted in final form September 10, 2015.

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