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Regulatory T Cells Maintain Long-Term Tolerance to Myelin Basic Protein by Inducing a Novel, Dynamic State of T Cell Tolerance

Sarah E. Cabbage,* Eric S. Huseby,** Blythe D. Sather,† Thea Brabb,‡ Denny Liggitt,§ and Joan Goverman3†

The pathogenesis of multiple sclerosis involves a breakdown in T cell tolerance to myelin proteins like myelin basic protein (MBP). Most MBP-specific T cells are eliminated by central tolerance in adult mice, however, the developmentally regulated expression of MBP allows MBP-specific thymocytes in young mice to escape negative selection. It is not known how these T cells that encounter MBP for the first time in the periphery are regulated. We show that naive MBP-specific T cells transferred into T cell-deficient mice induce severe autoimmunity. Regulatory T cells prevent disease, however, suppression of the newly transferred MBP-specific T cells is abrogated by activating APCs in vivo. Without APC activation, MBP-specific T cells persist in the periphery of protected mice but do not become anergic, raising the question of how long-term tolerance can be maintained if APCs presenting endogenous MBP become activated. Our results demonstrate that regulatory T cells induce naive MBP-specific T cells responding to nonactivated APCs to differentiate into a unique, tolerized state with the ability to produce IL-10 and TGF-β1 in response to activated, but not nonactivated, APCs presenting MBP. This tolerant response depends on continuous activity of regulatory T cells because, in their absence, these uniquely tolerized MBP-specific T cells can again induce autoimmunity. The Journal of Immunology, 2007, 178: 887–896.
myelin and a minor component of peripheral myelin (20). Our studies showed that most MBP121–140-specific thymocytes generated in adult animals are eliminated by bone marrow-derived cells presenting MBP acquired from peripheral myelin (19). However, MBP121–140-specific T cells in young (<3 wk old) mice escape central tolerance (19), reflecting the developmentally regulated expression of MBP that generates only low levels of the protein during the first 2–3 wk of life (21). Transgenic MBP-specific T cells found in the periphery of 4-wk-old mice are capable of responding to endogenous MBP, as administration of pertussis toxin alone induces autoimmune disease (19). However, no spontaneous autoimmune develops in the transgenic mice, indicating that peripheral tolerance mechanisms suppress MBP121–140-specific T cell responses.

We investigated peripheral tolerance mechanisms that regulate MBP-specific T cells using an experimental system in which TCR-transgenic MBP121–140-specific T cells from MBP-deficient (MBP−/−) mice were adoptively transferred into wild-type MBP+/+ recipients. This approach allowed us to analyze the regulation of naive MBP-specific T cells that were not subjected to central tolerance as they encounter MBP in the periphery. Our results show that CD4+CD25+ regulatory T cells are required to suppress Th1 cytokine production by the transferred MBP121–140-specific T cells and protect recipient mice from autoimmunity. However, activation of APCs in vivo 5 days after transfer of naive T cells abrogated protection from disease. Surprisingly, APC activation 30 days after T cell transfer did not induce disease and instead triggered an increase in transcription of IL-10 and TGF-β1 by MBP-specific T cells. Our studies indicate that regulatory T cells maintain long-term peripheral tolerance by inducing naive self-reactive T cells responding to nonactivated APCs to differentiate into a novel state of tolerance in which production of Th1 cytokines is decreased and transcription of suppressive cytokines is increased in response to MBP presented by activated APCs. Both the initial suppression and the subsequent tolerant state of self-reactive T cells require the presence of regulatory T cells, underscoring their importance in maintaining self-tolerance, even under inflammatory conditions.

Materials and Methods

Mice

Wild-type B10.PL(73 NS)/Sn mice purchased from The Jackson Laboratory were maintained in our breeding colony. The TCRα−/− mutation was backcrossed onto the B10.PL background for 10 generations before use. MBP121–140 TCR-transgenic, MBP-deficient shiverer (MBP−/−), RAG−/−, and Thy1.1 B10.PL mice have been described previously (19, 22). All mice were bred and maintained in a specific pathogen-free facility and all procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Washington (Seattle, WA). Mice in disease induction experiments were euthanized upon losing 90% purity on a FACSARia (BD Biosciences). RNA extraction was performed using either the Absolutely RNA RT-PCR Miniprep kit (Stratagene) or RNAqueous kit (Ambion). cDNA was prepared using the SuperScript III First-Strand Synthesis kit (Stratagene) with oligo(dt) primers. Real-time RT-PCR was performed with Brilliant SYBR Green QPCR reagents (Stratagene) on a DNA Engine Opticon system (MJ Research). Relative quantities of target genes were determined using relative standard curves as described in ABI User Bulletin No. 2 and are presented as unit quantity normalized to β-actin expression. cDNA from Thy1- and Th2-skewed splenocyte controls was a gift from Dr. A. Weinmann (University of Washington). The following primers were used: β-actin, 5′-GATCTG GCACCAACACCTTCT-3′, 5′-GGGGTGTTGAAGGCTTAAA-3′; H-2b (Thy1.1), 5′-CAACACACCTTGGCAGAG-3′, 5′-TCCCCCAACGAGCTGTTTCA-3′, 5′-AA GTAGGCGAACATCGGAG-3′; IL-10, 5′-CCATTGGCTGCTTATATT-3′; IFN-γ, 5′-CTGCCACAGAGGCAGTATCC-3′, 5′-AA GAGCAGAGGCAGTATCC-3′.
Anti-CD40/LPS administration

Agnostic anti-CD40 (30 μg) and LPS (50 μg, Sigma-Aldrich) were administered i.v. in 200 μl of sterile PBS.

CFSE analysis

Thy1 genetically marked cells from TCR-transgenic MBP-/− mice (naive transgenic T cells), and transgenic T cells purified from day 30 wild-type recipients by magnetic bead separation, were labeled with 4.2 μM CFSE (Molecular Probes) for 20 min. A total of 1 × 10⁶ to 5 × 10⁶ labeled transgenic T cells were transferred to either wild-type, MBP-/−, or day 30 wild-type recipients of transgenic T cells. Splenocytes were analyzed 5 days later gating on CD4Thy1.1+ or CD4Thy1.2+ cells.

Statistical analyses

All p values are derived from two-tailed Student’s t tests, with the following exceptions. To compare BrdU incorporation in nontransgenic vs transgenic CD4+ T cells, we used the Wilcoxon rank-sum test. For all real-time RT-PCR data, groups were compared by random permutation analysis.

Results

MBP121–140-specific T cells are pathogenic in the absence of regulatory T cells

To investigate how naive MBP-specific T cells that were not subjected to central tolerance respond to MBP presented in the periphery, CD4+ T cells isolated from MBP−/− TCR-transgenic mice were transferred into either T cell-deficient or wild-type mice. In both RAG−/− and TCRα−/− recipients, the MBP-specific T cells induced severe autoimmune disease characterized by acute weight loss and inflammatory cell infiltrates in the CNS, peripheral nerves, and tissues surrounding peripheral nerves (Fig. 1), consistent with constitutive presentation of MBP in the periphery (19). No signs of disease were observed in wild-type recipients (data not shown). Reconstitution of T cell-deficient mice with bulk splenocytes 7 days before transfer of the MBP-specific T cells protected mice from both histological (Fig. 1A) and clinical signs (data not shown) of disease.

The phenotype of cells that prevented MBP121–140-targeted autoimmunity was determined by transferring different subsets of splenocytes into RAG−/− recipients before transfer of naive MBP121–140-specific T cells. Transfer of purified CD3+ T cells was sufficient to protect mice from disease, however, depletion of CD25+ cells abrogated all protection (Fig. 1B). These data, in conjunction with observed lack of protection by CD4-depleted splenocytes and successful protection by CD8-depleted splenocytes (data not shown), indicate that CD4+CD25+ T cells are required to prevent MBP121–140-specific T cell-mediated autoimmune disease. Interestingly, splenocytes from MBP−/− mice completely protected RAG−/− recipients from disease (Fig. 1B). Thus, regulatory T cells that did not mature in an animal synthesizing MBP can suppress pathogenic MBP-specific T cells.

We investigated whether IL-10 and/or TGF-β activity were required in vivo for regulatory T cell-mediated protection. Neither administration of anti-IL-10R nor anti-TGF-β Ab alone abrogated protection from disease (Fig. 1C). However, wild-type mice that received anti-IL-10R and anti-TGF-β Ab together lost >20% of their body weight −1 wk after transfer of MBP-specific cells. These mice showed focal lesions in the brain, spinal cord, and periphery, comparable to unprotected recipients. In contrast, control mice that received an equal quantity of isotype-matched Ab did not lose weight and had few to no lesions in the periphery. One control had some lesions in the brain but minimal involvement of the spinal cord.

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MBP-specific T cells persisting in the periphery of MBP+/- mice are not anergic

The proliferation rate of MBP-specific T cells was measured after transfer into wild-type recipients by maintaining mice on drinking water containing BrdU for 2 wk beginning either 1 wk before (“early recipients”) or 3–4 wk after (“long-term recipients”) transgenic T cell transfer. Mice were sacrificed after the 2-wk period and BrdU incorporation in transgenic and nontransgenic splenocytes was analyzed. Virtually 100% of the transgenic T cells incorporated BrdU in the early recipients, reflecting the rapid rate of proliferation triggered by their initial exposure to endogenous MBP (Fig. 3A). In contrast, only ~20% of the transgenic T cells in long-term recipients were BrdU+, comparable to the percentage of BrdU+ nontransgenic CD4+ splenocytes in the same mice (p = 0.01). The transgenic T cells in long-term recipients exhibited an activated/memory phenotype (CD45RBlowCD44high, data not shown), indicating that they had initially responded to self-Ag. Thus, the proliferation of MBP-specific T cells slows dramatically over time in long-term recipients, even though the T cells are exposed to the same amount of endogenous MBP.

To determine whether MBP121–140-specific T cells become unresponsive to Ag, long-term recipients were injected with either MBP121–140 or irrelevant (MBPAc1-11) peptide 3–4 wk after transfer into wild-type mice. Mice were sacrificed 12 h after peptide injection and splenocytes were analyzed for expression of activation markers. The majority of MBP-specific T cells in the MBP+/- recipients expressed CD25 (69.5%) and CD69 (92.1%) in response to MBP121–140 peptide but not to MBPAc1-11 (2.9% CD25+ and 16.6% CD69+). Analyses of BrdU incorporation over a 72-h period following peptide administration showed that this single dose of MBP121–140 peptide triggered approximately half of the MBP-specific T cells residing in long-term MBP+/- recipients to proliferate (Fig. 3B). The percentage of BrdU+ transgenic T cells in MBP+/– recipients following a MBP121–140 peptide pulse was somewhat higher, suggesting that long-term exposure to endogenous MBP in the MBP+/- recipients may impair the proliferative response of some of the MBP-specific T cells. Nevertheless, at least half of the transgenic T cells were not anergic in MBP+/- hosts.
Regulatory T cells suppress Th1 cytokine production by MBP-specific T cells

The fact that T cell-deficient recipients of MBP121–140-specific T cells succumb to autoimmune disease within 7–10 days indicates that regulatory T cell activity is required shortly after transfer. Because regulatory T cells do not prevent proliferation, expansion, or trafficking of the MBP-specific T cells, we analyzed cytokine production by MBP-specific T cells in response to an in vivo MBP peptide pulse 6 days after transfer into either wild-type or RAG-1−/− recipients (Fig. 4A). In contrast to assays involving in vitro restimulation, this method allows assessment of the T cell response triggered in the in vivo environment. MBP-specific transgenic T cells transferred into RAG-1−/− mice exhibited a Th1 phenotype in which most transgenic T cells produced IFN-γ and some produced IL-2 and TNF-α. In contrast, IFN-γ production was strongly suppressed in transgenic T cells transferred into wild-type recipients. The percentages of transgenic T cells producing TNF-α and IL-2 were also reduced in wild-type recipients compared with RAG-1−/− recipients. We have previously shown that naive B cells in wild-type mice present endogenous MBP (22), raising the possibility that interactions with B cells may contribute to the decrease in the percentage of Th1 cytokine-producing MBP-specific T cells seen in wild-type vs RAG-1−/− recipients. This does not appear to be the case, however, as no differences in cytokine production were observed when MBP-specific T cells were transferred into unmanipulated RAG-1−/− recipients compared with RAG-1−/− recipients that were reconstituted with highly purified B cells 7 days before transgenic T cell transfer (data not shown). No IL-4 production was detected in any recipients, indicating that the decrease in Th1 cytokine production in wild-type recipients did not reflect a shift to a Th2 effector phenotype. Cytokines produced by MBP-specific T cells transferred into MBP-1−/− mice reflect their naive phenotype in that low levels of TNF-α and IL-2 but no IFN-γ are produced (Fig. 4A). Compared with MBP-1−/− recipients, a lower percentage of transgenic T cells in MBP+−−− recipients produced TNF-α and IL-2 and a higher percentage produced IFN-γ, demonstrating that regulation of the transgenic T cells in MBP+−−− hosts does not simply maintain a naive phenotype. No cytokine production was observed in mice injected with MBPAc1-11 (data not shown). To determine whether the lymphopenic environment in RAG-1−/− recipients affected the cytokine profile of MBP-specific T cells, we analyzed cytokines produced by transgenic T cells after transfer into RAG-1−/−/MBP-1−/− mice. The percentage of TNF-α+ and IL-2+ MBP-specific T cells in RAG-1−/−/MBP-1−/− recipients was comparable to the percentages seen in both RAG-1−/− and MBP-1−/− recipients. A higher percentage of transgenic T cells produced IFN-γ in RAG-1−/−/MBP-1−/− compared with RAG+−/+MBP-1−/− mice, indicating that homeostatic expansion influences production of IFN-γ. However, the percentage of IFN-γ-producing transgenic T cells in RAG-1−/−/MBP-1−/− recipients is dramatically reduced compared with RAG-1−/−/MBP+−−− recipients (12.6 vs 75.2%, respectively), demonstrating that the high percentage of IFN-γ-producing T cells in RAG-1−/−/MBP+−−− recipients reflects an Ag-specific response to endogenous MBP.

To investigate the mechanism of Th1 cytokine suppression in wild-type recipients, we analyzed expression of the transcription factor T-bet (Tbx21) in MBP-specific T cells transferred into either wild-type or RAG-1−/− mice. T-bet is considered a master regulator of IFN-γ production (26). RNA isolated from transgenic T cells purified from RAG-1−/− recipients (6 days posttransfer), wild-type recipients (6 and 30 days posttransfer) and a MBP-1−/−TCR-transgenic mouse was analyzed by real-time RT-PCR. Surprisingly, T-bet expression by MBP-specific T cells was increased in all recipient mice compared with naive transgenic T cells (Fig. 4B). No difference in T-bet expression was seen in MBP-specific T cells isolated from RAG-1−/− vs wild-type recipients 6 days posttransfer (p = 0.834), despite the fact that IFN-γ production is strongly suppressed at this time point in wild-type recipients. T-bet expression was actually elevated in MBP-specific T cells residing in wild-type recipients for 30 compared with 6 days (p = 0.034). These data indicate that the suppression of IFN-γ in MBP-specific T cells transferred into wild-type mice occurs at a point downstream of T-bet expression.

APC activation in vivo abrogates protection at 5 but not at 30 days after MBP-specific T cell transfer

To determine the importance of preventing APC maturation in this model of autoimmunity, we administered agonistic anti-CD40 Ab and LPS (anti-CD40/LPS) to wild-type recipients at both 5 and 30 days after transfer of MBP-specific T cells, as well as to control mice that had not received transgenic T cells. All mice exhibited an initial weight loss in the first 2 days after injection of anti-CD40/LPS (10–15% of starting weight). However, wild-type mice injected with anti-CD40/LPS 5 days after MBP-specific T cell transfer continued to lose weight and exhibited neurological symptoms (Fig. 5A). Histological analyses revealed inflammatory infiltrates in the CNS and in tissues within and surrounding peripheral nerves (data not shown). Administration of either anti-CD40 Ab or LPS alone at day 5 posttransfer also induced disease (data not shown). In contrast, both control mice (data not shown) and mice that received MBP-specific T cells 30 days earlier recovered their initial weight loss and exhibited no clinical signs up to 30 days after anti-CD40/LPS injection (Fig. 5A).
We hypothesized that APC activation did not induce disease in day 30 recipients either because there are fewer MBP-specific T cells in the periphery of day 30 vs day 5 recipients (Fig. 2A), or because MBP-specific T cells are proliferating much more rapidly at 5 compared with 30 days posttransfer into wild-type mice (see Fig. 3A). To investigate these possibilities, a second dose of naive MBP-specific T cells was injected into mice that had received a first dose of MBP-specific T cells 30 days earlier (referred to as day 5 plus day 30 recipients). Anti-CD40/LPS was administered 5 days after injection of the second dose of MBP-specific T cells. Surprisingly, administration of anti-CD40/LPS to day 5 plus day 30 recipients did not induce disease in the majority of mice (10 of 13 remained healthy, Fig. 5A). Analyses of CFSE-labeled transgenic T cells injected into day 30 recipients confirmed that the second set of MBP-specific T cells proliferated rapidly (Fig. 5B). Together these data indicate that day 30 recipients of MBP-specific T cells differ from naive wild-type mice in that they can regulate the APC activation-induced pathogenicity of newly transferred, rapidly proliferating MBP-specific T cells.

Day 30 transgenic T cells in long-term MBP \textsuperscript{+/+} recipients are hypo-responsive when triggered by activated APCs

To investigate why anti-CD40/LPS abrogates protection when administered early but not late after transfer of MBP-specific T cells, we analyzed the cytokine responses of transgenic T cells in day 6 and day 30 recipients after anti-CD40/LPS administration. Anti-CD40/LPS was administered 4 days after transfer of MBP-specific T cells and cytokine production in response to an in vivo MBP peptide pulse was determined 2 days later (Fig. 5C). The percentage of transgenic T cells producing IFN-\( \gamma \) increased dramatically in recipients that received anti-CD40/LPS compared with those that did not (56.0 and 13.6%, respectively). A small but significant increase was also seen in the percentage of IL-2-producing transgenic T cells after anti-CD40/LPS administration. Surprisingly, the opposite result was observed in day 30 recipients. A smaller percentage of transgenic T cells in day 30 recipients produced IFN-\( \gamma \), IL-2, and TNF-\( \alpha \) after receiving anti-CD40/LPS compared with day 30 recipients in which APCs were not activated (Fig. 5D). Thus, while APC activation triggers increased Th1 cytokine production by MBP-specific T cells 5 days after transfer into MBP \textsuperscript{+/+} mice, there is less Th1 cytokine production by MBP-specific T cells in day 30 recipients when APCs are activated than when they are not activated. The absolute number of transgenic T cells present in day 30 recipients that had received anti-CD40/LPS was equivalent to the number in untreated mice. Furthermore, no increase in apoptosis, as detected by annexin V staining, was observed for transgenic cells in anti-CD40/LPS-treated vs untreated mice. Therefore, the lower percentage of Th1 cytokine-producing transgenic T cells in anti-CD40/LPS-treated day 30 recipients does not reflect increased susceptibility to activation-induced cell death.

A possible explanation for these findings is that APCs presenting endogenous MBP during the initial regulation of MBP-specific T cells are altered such that, upon subsequent activation, they exert a suppressive effect on MBP-specific T cells. In this case, cytokine production by all MBP-specific T cells in day 5 plus day 30 recipients should be decreased when anti-CD40/LPS is administered compared with untreated recipients. Activated APCs in day 5 plus day 30 recipients triggered a smaller percentage of the original (day 30) MBP-specific T cells to produce Th1 cytokines in response to an in vivo MBP peptide pulse (Fig. 5E), as was observed
in day 30 recipients that did not receive a second dose of MBP-specific T cells. However, the second dose of MBP-specific T cells (day 5) in day 5 plus day 30 recipients increased IL-2, IFN-γ, and TNF-α production in response to the same activated APCs (Fig. 5F). Thus, the APCs in mice that have regulated MBP-specific T cells have not acquired a dominant suppressive phenotype, rather the day 30 T cells appear to have altered their response to MBP presented by activated APCs, compared with the day 5 T cells. Although day 5 T cells increased Th1 cytokine production in response to activated APCs in these day 5 plus day 30 recipients, they produced less IFN-γ after anti-CD40/LPS injection in the presence of day 30 MBP-specific T cells than they did in naive recipients (Fig. 5, F vs C), which may account for the lack of disease observed in day 5 plus day 30 mice that received APC-activating stimuli.

**MBP-specific T cells in long-term recipient mice express suppressive cytokines in response to APC activation but do not become Foxp3⁺**

To determine whether MBP-specific T cells residing in wild-type recipients for 30 days acquired a regulatory phenotype, the expression levels of Foxp3, IL-10, and TGF-β1 were analyzed in MBP-specific T cells before and after transfer into MBP−/− recipients. A low level of Foxp3 expression was detected in transgenic T cells before transfer (Fig. 6A, naive transgenic), which presumably reflects the presence of a small number of CD25⁺ T cells in RAG−/− MBP−/− TCR-transgenic mice (data not shown). RAG−/−MBP−/− TCR-transgenic mice could not be used as T cell donors in our experiments because MBP−/− RAG−/− mice have poor viability on the B10.PL background (data not shown). However, the Foxp3⁺ T cells present in the T cell population isolated from MBP−/− RAG−/− TCR-transgenic mice were not sufficient to protect T cell-deficient adoptive transfer recipients from autoimmune disease (Fig. 1). No increase in Foxp3 expression was observed in MBP-specific transgenic T cells 6 days after transfer into RAG−/− or wild-type recipients. Foxp3 expression was also not increased in MBP-specific T cells 30 days after transfer into wild-type mice, with or without administration of anti-CD40/LPS (Fig. 6A). These data indicate that MBP-specific T cells in long-term recipients do not convert to a Foxp3⁺ phenotype, nor is there a selective outgrowth of Foxp3⁺ T cells that were initially transferred. Interestingly, APC activation in long-term recipients induced MBP-specific T cells to respond to a MBP peptide pulse by expressing increased levels of both IL-10 and TGF-β1 mRNA (Fig. 6, B and C), indicating that these T cells have altered their programmed effector response to Ag presented by activated APCs without becoming Foxp3⁺.

**Both MBP-specific T cells and the host environment are changed during prevention of MBP-specific autoimmunity**

The unusual response of day 30 but not day 5 MBP-specific T cells to activated APCs suggested that some intrinsic properties of these T cells may have changed as a result of regulation in wild-type recipients. To investigate this possibility, MBP-specific T cells were isolated from day 30 recipients and retransferred into new wild-type mice. The retransferred T cells proliferated in the new recipients (Fig. 7A), although the rate of proliferation was somewhat slower than that of naive transgenic T cells transferred into wild-type mice (Fig. 5B), suggesting that MBP-specific T cells in long-term recipients have adapted to their environment by decreasing their responsiveness to endogenous MBP over time. Interestingly, transgenic T cells isolated from day 30 recipients and retransferred into different day 30 recipients did not divide after 5 days in the new recipients, even though the amount of endogenous MBP epitopes is presumably the same.

![FIGURE 6. Tolerized MBP-specific T cells are Foxp3low but express suppressive cytokines in response to APC activation. Genetically marked transgenic T cells were purified from RAG−/− or wild-type recipients at the indicated times after transfer and Foxp3 (A), IL-10 (B), and TGF-β1 (C) expression was analyzed by real-time RT-PCR. Anti-CD40/LPS was injected 2 days before harvesting T cells where indicated. Naive transgenic T cells were isolated from an MBP−/− TCR-transgenic mouse. Each point represents data obtained from an individual mouse, error bars are the SD of triplicate wells. In A, CD25-enriched (57.5% CD4⁺CD25⁺) and CD25-depleted (0.06% CD4⁺CD25⁺) splenocytes were isolated from nontransgenic mice. In B and C, day 30 recipients were injected with MBP121–140 1 h before harvesting cells. All data are normalized to β-actin expression levels. * p ≤ 0.04.](http://www.jimmunol.org/)
Day 30 MBP121–140-specific T cells regain pathogenicity in the absence of regulatory T cells

In light of the intrinsic ability of day 30 MBP-specific T cells to suppress Th1 cytokines in response to activated APCs, we investigated whether the pathogenic potential of day 30 T cells was permanently silenced. As shown in Fig. 8, MBP-specific T cells isolated from day 30 wild-type recipients and retransferred into RAG<sup>−/−</sup> mice induced autoimmune disease. The number of MBP-specific T cells retransferred in these experiments (3 × 10⁵) represents ~14% of the average number of MBP-specific T cells persisting in the spleen of day 30 recipients. The onset of disease induced by retransfer of day 30 T cells into RAG<sup>−/−</sup> mice was delayed by 2–3 days compared with disease induced by transfer of naive MBP-specific T cells. This delay may reflect the slower proliferative response of retransferred T cells compared with naive T cells (Fig. 5B). Thus, the tolerance exhibited by MBP-specific T cells in day 30 recipients is a dynamic, rather than terminally differentiated, phenotype.

Discussion

Our previous studies indicated that MBP-specific T cells that escape central tolerance early in life require peripheral tolerance mechanisms to prevent autoimmunity (19). In this study, we have defined the peripheral tolerance mechanisms that prevent naive MBP-specific T cells from inducing autoimmune disease when they encounter MBP in the periphery. We show that naive MBP<sup>121–140</sup>-specific T cells transferred into MBP<sup>−/−</sup> mice lacking CD4<sup>+</sup>CD25<sup>+</sup> T cells induced autoimmunity that targeted virtually all innervated peripheral tissues, reflecting the ubiquitous presentation of MBP derived from peripheral myelin (19, 22, 27).

The fact that T cell-deficient mice were not protected from disease when they were reconstituted with CD25-depleted splenocytes 7 days before transferring MBP-specific T cells suggests that the induction of autoimmunity was not dependent on a highly lymphopenic environment. Interestingly, splenocytes from MBP<sup>−/−</sup> mice were as effective in protecting RAG<sup>−/−</sup> mice from disease as splenocytes from wild-type mice, indicating that the regulatory T cells did not need to mature in an animal synthesizing endogenous MBP. This result suggests that either MBP-specific regulatory T cells can be generated in the absence of MBP expression in the thymus, MBP-specific nonregulatory T cells present in the spleen of mutant mice acquire regulatory activity during the first week after transfer to MBP<sup>−/−</sup> hosts, or regulatory T cells specific for other self-Ags that are released by low levels of inflammation protect the recipients from disease via bystander suppression. Multiple organs are affected by MBP<sup>121–140</sup>-targeted autoimmunity, so the activity of the MBP-specific T cells may be suppressed by regulatory T cells specific for numerous non-MBP self-Ags. Our data do not distinguish between these possibilities. Recent studies in experimental autoimmune uveitis demonstrated that regulatory T cells specific for bacterial components in CFA can suppress uveitis induced by immunization with interphotoreceptor retinoid-binding protein in CFA (28). Although our experimental system does not involve immunization in CFA or exposure to any foreign Ag, the results in uveitis support the idea that autoimmunity can be prevented via bystander suppression.

Regulatory T cells did not prevent the expansion of MBP-specific T cells in lymphoid tissues or their trafficking into the CNS. The greater expansion of MBP-specific T cells in the periphery and CNS of T cell-deficient mice may reflect combined effects of lymphopenic conditions and/or the inflammatory milieu associated with the onset of autoimmunity, in addition to the Ag-specific proliferation that occurs in both T cell-deficient and wild-type recipients. Interestingly, the MBP-specific T cell number in the CNS of wild-type mice remained as high at 28 days posttransfer as at 7 days posttransfer of naive T cells.
days posttransfer, even though these mice remain healthy. MBP-specific T cells in the CNS of wild-type recipients down-regulated the expression of the transgenic TCR (data not shown), consistent with recognition of their cognate Ag in situ. Thus, the population of transgenic T cells persisting in the CNS 28 days after transfer into wild-type mice could be due to retention of Ag-specific T cells in the target organ that has the highest concentration of MBP.

The most striking effect of regulatory T cells on MBP121–140-specific T cells during their initial encounter with endogenous MBP is suppression of their Th1 cytokine production, particularly IFN-γ. The majority of MBP-specific T cells differentiate into IFN-γ-producing cells in RAG-2−/− recipients due to interaction with endogenous MBP and not due to lymphopenia-induced expansion (Fig. 3). Regulatory T cells strongly suppressed this Th1 response without inducing a shift toward a Th2 phenotype. Suppression of IFN-γ production by self-reactive T cells in vivo is consistent with some (29, 30) but not all (31, 32) previous studies of the in vivo effects of regulatory T cells on effector cytokine production. In a diabetes model, Sarween et al. (29) found that regulatory T cells inhibited both IFN-γ production by effector T cells and target organ infiltration, which was attributed to lack of IFN-γ-mediated up-regulation of CXCRR3 expression. In our model, MBP-specific T cells infiltrate the CNS, consistent with the observation that CXCRR3 is not required for trafficking across the blood brain barrier (33). DiPaolo et al. (30) also found that regulatory T cells suppress IFN-γ production by effector T cells without inhibiting Ag-specific proliferation or target organ infiltration in an animal model of autoimmune gastritis. However, IFN-γ suppression was associated with down-regulation of T-bet expression in this model. Surprisingly, we found that the level of T-bet mRNA is as high in MBP-specific T cells in wild-type recipients as it is in MBP-specific T cells in RAG-2−/− recipients, which produce high levels of IFN-γ. These results demonstrate that regulatory T cells can mediate down-regulation of IFN-γ expression independent of changes in T-bet transcription. A recent study that examined patterns of gene expression in MBP-specific regulatory T cells generated via chronic peptide administration found elevated levels of T-bet expressed in these cells, despite reduced IFN-γ production (34). This study suggested that T-bet expression was required to suppress IL-2 production in tolerized T cells. Although we have not examined the function of T-bet in MBP-specific T cells undergoing tolerance, our data are consistent with this hypothesis.

This initial suppression of Th1 cytokines by regulatory T cells prevents pathogenicity while the MBP121–140-specific T cells appear to adapt to the levels of endogenous MBP by slowing proliferation without becoming anergic (Fig. 3). Our results suggest that the proliferative rate of MBP-specific T cells reflects the amount of perceived cognate Ag when presented by nonactivated APCs. Day 30 T cells proliferate more rapidly after retransfer into naive recipients than after retransfer into day 30 recipients, perhaps because the number of retransferred T cells is smaller than the number of resident MBP-specific T cells in day 30 recipients, resulting in less competition for endogenous MBP and thus a functionally higher level of available Ag. Alternatively, the context of Ag presentation may differ in day 30 recipients vs naive mice, such that day 30 mice are less conducive to the expansion of Ag-experienced, MBP-specific T cells (a scenario originally suggested by Tanchot et al. (35) in their studies of adaptive T cell tolerance). Although day 30 T cells increased proliferation after retransfer into naive wild-type mice, the rate was slower than the rate of proliferation observed for naive MBP-specific T cells transferred into wild-type recipients (compare Figs. Sb to 7a). This result indicates that the day 30 MBP-specific T cells have become intrinsically less responsive to endogenous MBP than naive MBP121–140-specific T cells. A similar system of adaptive tolerance has been described before in the absence of regulatory T cells, suggesting that adaptation may be a consequence of persistent antigenic stimulation (35). However, regulatory T cells are required in our system to prevent the initial onset of autoimmunity disease and allow adaptive tolerance to occur.

Epidemiological studies suggest that an infectious agent is involved in the pathogenesis of MS (36–38). Therefore, we analyzed the ability of regulatory T cells to suppress MBP121–140-specific T cells when they encounter endogenous MBP on activated APCs, as might occur during an infection. The inability of regulatory T cells to prevent disease when APCs were activated 5 days after MBP-specific T cell transfer is consistent with the notion that suppression by regulatory T cells may depend on preventing DC maturation. Unexpectedly, APC activation 30 days after MBP-specific T cell transfer did not trigger disease, even though the T cells are not anergic at this later time point. Investigation of the basis for this lack of pathogenicity led to the discovery that day 30 MBP-specific T cells have differentiated into a novel phenotype in which they express elevated levels of IL-10 and TGF-β1 RNA and produce lower levels of Th1 cytokines in response to activation, but not nonactivated, APCs presenting MBP. This pattern of cytokine expression represents a signature for MBP-specific T cells that have adapted to an environment containing endogenous MBP. Day 30 MBP-specific T cells did not increase expression of Foxp3, in contrast to another study in which Ag-specific T cells persisting in mice recovering from a systemic autoimmune disease became Foxp3+ (6). MBP-specific T cells in day 30 recipients instead resemble Tr1 regulatory T cells that are induced to express TGF-β1 and IL-10 in the absence of Foxp3 (39, 40). Because experiments that neutralize the activity of IL-10 cannot be conducted in mice that receive LPS in vivo (41), we could not investigate a functional role for TGF-β and IL-10 production by day 30 T cells in suppressing their own pathogenicity in response to APC activation. Interestingly, unlike Tr1 cells, day 30 MBP-specific T cells do not represent a terminally differentiated phenotype. Instead, their tolerant state depends on the continuous presence of regulatory T cells, as illustrated by the ability of day 30 T cells to mediate autoimmune disease when transferred into regulatory T cell-deficient hosts.

Further insights into the long-term tolerant state of MBP-specific T cells were obtained using day 5 plus day 30 MBP-specific T cell recipients. These experiments showed that the unusual suppressive response of day 30 MBP-specific T cells to activated APCs was an intrinsic property that MBP-specific T cells acquired over time, because day 5 MBP-specific T cells increased production of IFN-γ and IL-2 after anti-CD40/LPS administration, while day 30 T cells decreased production of these cytokines in response to the same activated APCs (Fig. 5, E and F). Retransfer of day 30 MBP-specific T cells into naive wild-type mice followed by anti-CD40/LPS treatment confirmed that the suppressive response to APC activation by day 30 MBP-specific T cells was T cell intrinsic. This result suggested that the suppressive response of the day 30 T cells may represent a form of infectious tolerance that could explain the lack of disease observed in day 5 plus day 30 recipients following APC activation. However, our results do not support this hypothesis as day 30 T cells retransferred into new wild-type recipients did not prevent disease when a second set of naive MBP-specific T cells was injected and the APCs were activated. Thus, the protection from autoimmunity following APC activation in the original day 5 plus day 30 recipients must depend in part on as yet unidentified changes in the host environment that occur during regulation of the initial set of MBP-specific T cells.
Our results provide a mechanistic explanation for how regulatory T cells can suppress autoreactive T cells under both noninflammatory and inflammatory conditions. Typically, self-reactive T cells that escape central tolerance will encounter their Ag in the periphery on nonactivated APCs. If the avidity of this interaction is sufficiently high to trigger proliferation, regulatory T cells prevent the self-reactive T cells from differentiating into Th1 effectors and allow them to become tolerant to the level of endogenous Ag presented. At the same time, regulatory T cells "mark" these T cells as self-reactive by inducing a phenotype that is hyporesponsive with respect to Th1 cytokines and associated with transcription of suppressive cytokines when Ag is encountered on activated APCs. This tolerant state allows regulatory T cells to retain control specifically over T cells that have previously demonstrated self-reactivity even when they subsequently encounter their Ag under inflammatory conditions. Although this system has many advantages, it leaves the organism vulnerable to autoimmunity mediated by T cells that had been previously tolerated if regulatory T cell numbers decrease or their activity is impaired.

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Disclosures

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References