Regulatory T Cells Maintain Long-Term Tolerance to Myelin Basic Protein by Inducing a Novel, Dynamic State of T Cell Tolerance[1]

Sarah E. Cabbage,* Eric S. Huseby,[2†] Blythe D. Sather,[†] Thea Brabb,[‡] Denny Liggitt,[‡] and Joan Goverman[3†]

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.**

The adaptive immune system maintains a delicate balance between generating a sufficiently diverse repertoire of lymphocytes capable of responding to an infinite array of pathogens and preventing these lymphocytes from recognizing self and destroying the organism. For T cells, much of this balance is established in the thymus, where the TCR repertoire is subjected to negative selection to eliminate T cells exhibiting high-avidity interactions with self-Ag/MHC complexes (1). However, thymic selection is imperfect and some self-reactive T cells escape to the periphery, where additional tolerance mechanisms prevent these T cells from mediating pathogenic responses. Regulatory T cells play a critical role in maintaining peripheral T cell tolerance. Different subsets of regulatory T cells have been identified, including $CD4^+CD25^+Foxp3^+$, type I regulatory T cells (Tr1),[4] and Th3 T cells. $CD4^+CD25^+Foxp3^+$ cells are referred to as "natural" regulatory T cells because they arise during thymic maturation. However, human peripheral $CD4^+CD25^-Foxp3^-$ T cells can express Foxp3 upon activation in vitro and peripheral $CD4^+CD25^-Foxp3^-$ T cells in mice can become $CD4^+CD25^+Foxp3^+$ regulatory T cells during chronic exposure to systemic self-Ag (2–6). In vitro, suppression by $CD4^+CD25^+$ $Foxp3^+$ T cells is cell contact dependent and cytokine independent, but in vivo suppression appears to depend on IL-10 and/or TGF-β activity (7). In contrast to $CD4^+CD25^+Foxp3^+$ T cells, Tr1 and Th3 regulatory cells differentiate from mature $Foxp3^-$ T cells and mediate dominant suppression via production of TGF-β and IL-10 (Tr1) or TGF-β alone (Th3) (8–10).

Whether regulatory T cells act directly on responding effector T cells or indirectly via inhibitory effects on APCs has been a subject of intense investigation. Most evidence indicates that a direct effect of regulatory T cells on dendritic cells (DCs) is critical to their suppressive activity. Regulatory T cells can dominantly suppress DC maturation (11) and their suppressive activity can be overcome by APC activation in vitro and in vivo (11–13). Accordingly, stimuli that trigger DC maturation in vivo can initiate autoimmune disease (14, 15). Recently, two-photon laser-scanning microscopy studies have shown that regulatory T cells interact directly with DCs presenting self-Ags in vivo in the absence of effector T cells and that these interactions inhibit subsequent stable interactions between effector T cells and DCs (16, 17). These studies suggest that the ability to prevent DC maturation is central to regulatory T cell-mediated tolerance. However, it is not known how regulatory T cells suppress autoreactive T cell responses when the self-Ag is presented by activated DCs.

Multiple sclerosis (MS) is believed to be an autoimmune disease reflecting a loss of tolerance in myelin-specific T cells (18). To study the tolerance mechanisms that regulate myelin-specific T cells, we previously generated TCR-transgenic mice specific for an MHC class II-restricted epitope of myelin basic protein (MBP), MBP121–140 (19). MBP is a major protein component of CNS

*Molecular and Cellular Biology Program, †Department of Immunology, and ‡Department of Comparative Medicine, University of Washington, Seattle, WA 98195

Received for publication August 4, 2006. Accepted for publication October 30, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants to J.G. from the National Institutes of Health (NS043417) and from the National Multiple Sclerosis Society (3328-A-4). S.E.C. was supported in part by Public Health Service National Research Service Award T32 GM07270 from the National Institute of General Medical Sciences. E.S.H. was supported by a Samuel and Althea Stroum Endowed Diabetes Fellowship.

[2] Current address: Department of Immunology, Howard Hughes Medical Institute, National Jewish Medical Center, Denver, CO.

[3] Address correspondence and reprint requests to Dr. Joan Goverman, Department of Immunology, University of Washington, Box 357650, Seattle, WA 98195-7650. E-mail address: goverman@u.washington.edu

[4] Abbreviations used in this paper: Tr1, type I regulatory T cell; DC, dendritic cell; MBP, myelin basic protein; MS, multiple sclerosis.

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 autoimmunity 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 TCR$\alpha^{-/-}$ mutation was backcrossed onto the B10.PL background for 10 generations before use. MBP121–140 TCR-transgenic, MBP-deficient *shiverer* ($MBP^{-/-}$), RAG-$1^{-/-}$, 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 >20% of their starting weight.

Antibodies

Abs specific for CD4, Vα2, Vβ8, Thy1.1, Thy1.2, IL-2, IFN-γ, TNF-α, and IL-4 were obtained from BD Biosciences. Anti-CD40 (clone 1c10) was obtained from R&D Systems. Anti-IL-10R (clone 1B.1.3), anti-TGF-β (clone 1D11), and isotype control Ab (clone GL113) were gifts from Dr. S. Stohlman, University of Southern California (Los Angeles, CA). FITC-conjugated annexin-V was purchased from Molecular Probes.

Cytokine neutralization

Neutralizing anti-IL-10R and anti-TGF-β (500 μg each) were administered i.p. on days -6, -1, and 4 relative to the day of transfer of MBP-specific T cells. In mice that received the combination of both Abs, or an equal quantity of control IgG, Abs were administered at days 1 and 4 after transgenic T cell transfer.

Magnetic bead cell separation

Magnetic cell separation was performed on an autoMACS (Miltenyi Biotec) using biotin-conjugated Abs specific for CD3, CD25, Vα2, or Thy1.1 (BD Biosciences) and either streptavidin- or anti-biotin-conjugated microbeads (Miltenyi Biotec).

Histology

CNS sections were fixed and stained with H&E as previously described (23). For H&E staining of peripheral tissue, formalin-fixed mice were decalcified in formic acid (Cal-Rite). Sections were cut through the pelvis, perpendicular to the spine.

Transgenic T cell transfers

Spleen and lymph nodes were harvested from $MBP^{-/-}$ TCR-transgenic mice and an aliquot was stained with Abs specific for Vα2, Vβ8, and CD4 to determine the percentage of transgenic T cells. Lymphocytes containing 3×10^6 transgenic T cells were injected i.v. into recipient mice, except for Fig. 1*B*, where lymphocytes were first Vα2 enriched by magnetic bead selection and then 3×10^5 transgenic T cells were injected per recipient. For retransfer experiments, transgenic T cells were purified from recipients by magnetic bead separation using anti-Thy1.1; 3×10^6 purified T cells were injected i.v. into the second recipients.

Transgenic T cell quantitation in recipient mice

Mice were perfused and single-cell suspensions were prepared from the CNS and spleen as previously described (24). The percentage of transgenic MBP121–140-specific T cells in the tissues was determined by flow cytometry using Abs against CD4, Vα2, and Vβ8.

In vivo T cell proliferation

Proliferation in response to endogenous MBP was measured by maintaining mice on drinking water containing 0.8 mg/ml BrdU (Sigma-Aldrich) for 2 wk beginning either 1 wk before transfer of MBP-specific T cells (early recipients) or 3–4 wk posttransfer (late recipients). Splenocytes were stained with Abs specific for CD4 and Thy1 genetic markers, and the FITC BrdU Flow kit (BD Biosciences) was used to determine BrdU incorporation. Transgenic T cells were differentiated from nontransgenic T cells by expression of Thy1.1 and Thy1.2 genetic markers. Proliferation in response to an in vivo MBP peptide pulse was measured following i.v. injection of 0.4 μmoles MBP121–140 or MBPAc1–11 (control) peptide (Genemed Synthesis). A total of 1 mg of BrdU was given i.p. at 0, 24, and 48 h later. Mice were sacrificed 24 h after the final BrdU injection and splenocytes were analyzed as described above.

Intracellular cytokine staining

Cytokine production was measured in response to an in vivo peptide pulse according to a protocol modified from Pape et al. (25). Mice were administered 0.4 μmoles MBP121–140 peptide i.v. and sacrificed 2 h later. Splenocytes were prepared in medium supplemented with GolgiStop (4 μl/6 ml) and GolgiPlug (1 μl/ml) (BD Biosciences) and stained with anti-CD4 and anti-Thy1.1 Abs. Cells were then permeabilized with the BD Cytofix/Cytoperm kit, stained with PE-conjugated Abs specific for IL-2, IFN-γ, TNF-α, IL-4, or isotype controls and analyzed by flow cytometry gating on $CD4^+Thy1.1^+$ cells. Positive staining with isotype control Abs was subtracted as background. "N" indicates the number of mice analyzed per group.

Real-time RT-PCR

Genetically marked transgenic T cells were sorted to >90% purity on a FACSAria (BD Biosciences). RNA extraction was performed using either the Absolutely RNA RT-PCR Miniprep kit (Stratagene) or RNAqueous reagents (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 Th1- and Th2-skewed splenocyte controls were a gift from Dr. A. Weinmann (University of Washington). The following primers were used: β-actin, 5′-GATCTG GCACCACACCTTCT-3′, 5′-GGGGTGTTGAAGGTCTCAAA-3′; T-bet (Tbx21), 5′-CAACAACCCCTTTGCCAAAG-3′, 5′-TCCCCCAAGCAG TTGACAGT-3′; Foxp3, 5′-TCCTTCCCAGAGTTCTTCCA-3′, 5′-AA GTAGGCGAACATGCGAGT-3′; IL-10, 5′-CCTCAGGATGCGGCTG AG-3′, 5′-CCACTGCCTTGCTCTTATTTT-3′; TGFβ-1, 5′-TTGCTTCA GCTCCACAGAGA-3′, 5′-TGGTTGTAGAGGGCAAGGAC-3′.

Anti-CD40/LPS administration

Agonistic 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 1×10^6 to 5×10^6 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 CD4$^+$Thy1.1$^+$ or CD4$^+$Thy1.2$^+$ 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 in the same mice, a paired Student's t test was used (see Fig. 3*A*). 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 TCR$\alpha^{-/-}$ 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. 1*A*) 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. 1*B*). 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. 1*B*). 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. 1*C*). However, wild-type mice that received anti-IL-10R and anti-TGF-β 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.

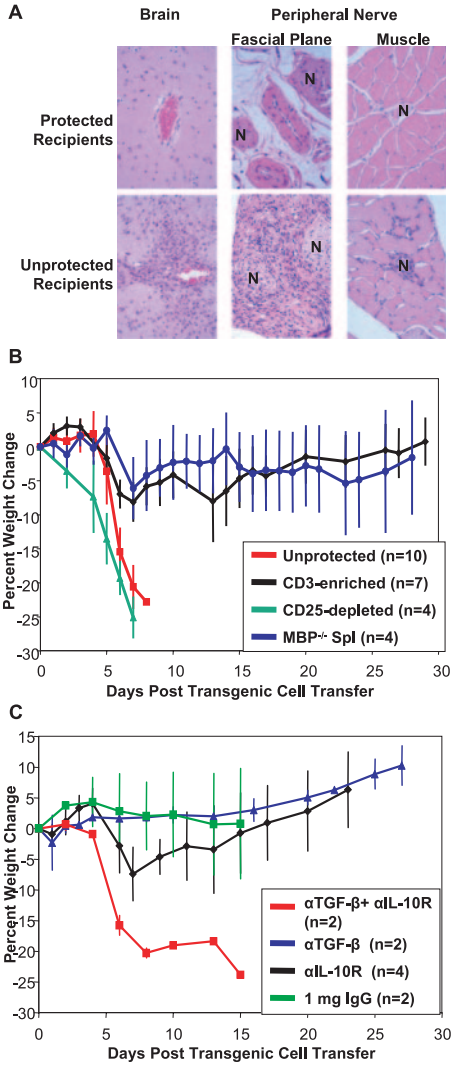

FIGURE 1. MBP121–140-specific T cells induce autoimmunity in regulatory T cell-deficient recipients. *A*, H&E-stained tissue sections from TCR$\alpha^{-/-}$ recipients that either received (protected) or did not receive (unprotected) 2×10^7 wild-type splenocytes 7 days before transfer of 3×10^6 transgenic T cells. N indicates peripheral nerves. Clusters of dark blue staining inflammatory cells are present within and adjacent to brain and peripheral nervous tissues of unprotected recipients. *B*, RAG$^{-/-}$ mice received either no splenocytes (red), 2×10^7 bulk MBP$^{-/-}$ splenocytes (blue), or splenocyte populations purified by magnetic bead separation: 7×10^6 CD3$^+$ cells (97.6% CD3$^+$, black) or 2×10^7 CD25-depleted splenocytes (0.6% CD4$^+$CD25$^+$, green). Seven days later, all mice were injected with 3×10^5 transgenic T cells and monitored for weight loss. *C*, A total of 500 μg of either anti-IL-10R Ab (black), anti-TGF-β neutralizing Ab (blue), anti-IL-10R, and TGF-β together (500 μg each, red), or an equal quantity of control IgG (green) were administered to mice receiving 3×10^6 transgenic T cells as described in *Materials and Methods.*

Regulatory T cells do not prevent expansion of MBP121–140-specific T cells in the spleen or CNS

We previously showed that MBP121–140-specific T cells proliferate in response to endogenous MBP in lymphoid tissues immediately after transfer into wild-type mice (19). To determine whether regulatory T cells prevent the accumulation or trafficking of MBP121–140-specific T cells, we analyzed the number of TCR-transgenic T cells in the spleen and CNS after transfer into either TCR$\alpha^{-/-}$ or wild-type mice. The CD4$^+$Vα2$^+$Vβ8$^+$ T cell number increased significantly in the spleens of wild-type recipients 4

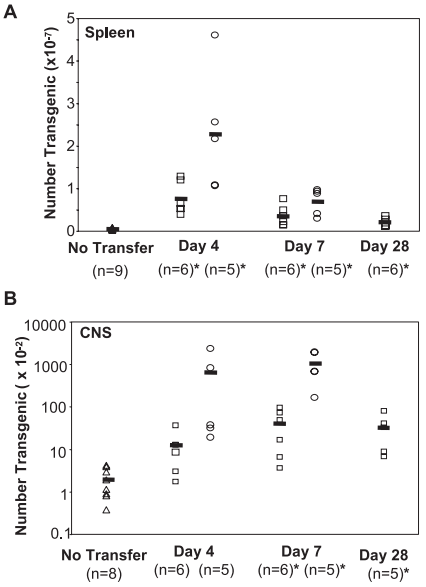

FIGURE 2. MBP-specific T cells expand in the spleen and CNS of wild-type and TCR$\alpha^{-/-}$ recipients. In *A* and *B*, wild-type (□) and TCR$\alpha^{-/-}$ (○) mice were injected with 3×10^6 transgenic T cells. At the indicated times posttransfer, recipients were perfused and cells collected from the spleen (*A*) and CNS (*B*). Percentages of $CD4^+V\alpha2^+V\beta8^+$ cells were determined by flow cytometry and used to calculate the number of transgenic T cells present in each tissue. The number of endogenous $CD4^+V\alpha2^+V\beta8^+$ cells present in each tissue was determined using uninjected wild-type mice (△). For splenocytes, the average number was 5.45×10^5. ∗, Statistically significant differences ($p \leq 0.01$) compared with uninjected mice.

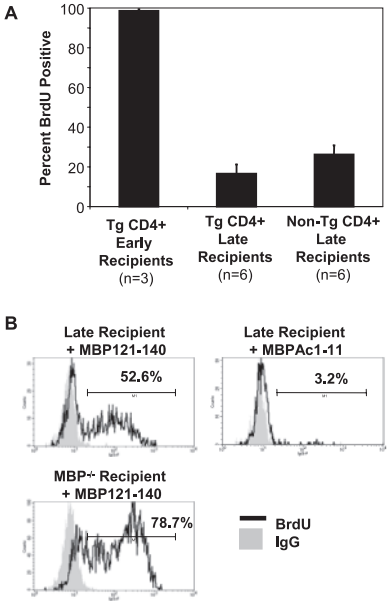

FIGURE 3. MBP121–140-specific T cells proliferate slowly in long-term recipients but are not anergic. *A*, T cell proliferation in response to endogenous MBP was measured in early and late recipients by measuring BrdU incorporation in donor transgenic (Tg) and host nontransgenic (Non-Tg) splenocytes as described in *Materials and Methods. B*, Proliferation of transgenic T cells in late recipients in response to MBP peptide in vivo was measured as described in *Materials and Methods.* For proliferation in $MBP^{-/-}$ mice, MBP121–140 was injected 4 days after T cell transfer.

days after transfer compared with the endogenous $CD4^+V\alpha2^+V\beta8^+$ T cell number in uninjected wild-type mice ($p = 0.006$, Fig. 2*A*). The MBP-specific T cell number was higher in T cell-deficient recipients than in wild-type recipients ($p = 0.03$), although there was more variation in T cell-deficient mice. By 7 days posttransfer, the number of transgenic T cells in the spleen had decreased in both wild-type and T cell-deficient recipients relative to day 4, but still remained above background. Between 7 and 10 days posttransfer, T cell-deficient recipients typically exhibited severe weight loss requiring euthanasia. In contrast, wild-type recipients remained healthy, with no signs of autoimmunity, even though the MBP-specific T cell number in the spleen remained ~4-fold above background at 28 days posttransfer.

The number of $CD4^+V\alpha2^+V\beta8^+$ T cells also increased in the CNS of wild-type and T cell-deficient recipients by 4 days posttransfer, indicating that regulatory T cells do not prevent trafficking of MBP-specific T cells to the CNS (Fig. 2*B*). The number of transgenic T cells in the CNS of wild-type mice was similar at days 4 and 7 posttransfer. At 28 days posttransfer, the number of $CD4^+V\alpha2^+V\beta8^+$ T cells in the CNS of healthy, wild-type recipients was still 10-fold higher than the number in uninjected mice. Thus, elevated numbers of MBP-specific T cells can be sustained in the CNS of mice containing regulatory T cells without inducing disease.

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. 3*A*). 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 ($p = 0.1$). The transgenic T cells in long-term recipients exhibited an activated/memory phenotype ($CD45Rb^{low}CD44^{high}$, 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. 3*B*). 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.

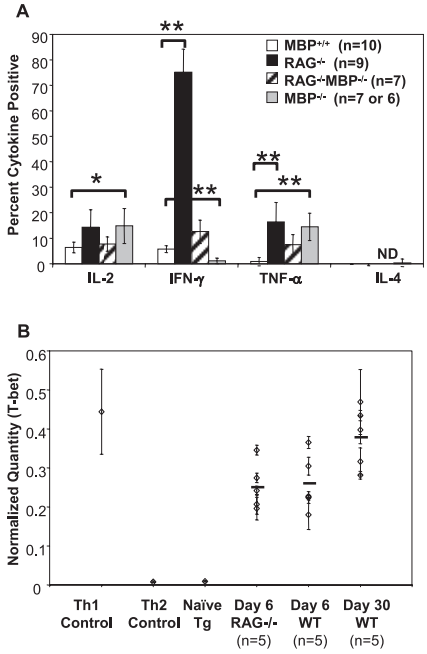

FIGURE 4. MBP-specific T cells suppress Th1 cytokines without decreasing T-bet in wild-type recipients. *A*, A total of $1.5–3 \times 10^6$ transgenic T cells were transferred into wild-type (□), $RAG^{-/-}$ (■), $RAG^{-/-}MBP^{-/-}$ (▨), or $RAG^{+/+}MBP^{-/-}$ (▩) recipients. Cytokine production by genetically marked transgenic splenocytes was measured directly ex vivo 6 days after transfer. ND indicates IL-4 was not determined for $RAG^{-/-}MBP^{-/-}$ recipients. *B*, T-bet expression by transgenic T cells purified from the indicated recipients was measured by real-time RT-PCR. Naive transgenic T cells were obtained from $MBP^{-/-}$ mice. T-bet expression is normalized to β-actin. *, $p = 0.05$. **, $p \leq 0.004$.

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 $RAG^{-/-}$ recipients (Fig. 4*A*). 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 $RAG^{-/-}$ 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 $RAG^{-/-}$ 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 $RAG^{-/-}$ 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 $RAG^{-/-}$ recipients compared with $RAG^{-/-}$ 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 $MBP^{-/-}$ mice reflect their naive phenotype in that low levels of TNF-α and IL-2 but no IFN-γ are produced (Fig. 4*A*). Compared with $MBP^{-/-}$ 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 $RAG^{-/-}$ recipients affected the cytokine profile of MBP-specific T cells, we analyzed cytokines produced by transgenic T cells after transfer into $RAG^{-/-}MBP^{-/-}$ mice. The percentage of TNF-α^+ and IL-2^+ MBP-specific T cells in $RAG^{-/-}MBP^{-/-}$ recipients was comparable to the percentages seen in both $RAG^{-/-}$ and $MBP^{-/-}$ recipients. A higher percentage of transgenic T cells produced IFN-γ in $RAG^{-/-}MBP^{-/-}$ compared with $RAG^{+/+}MBP^{-/-}$ mice, indicating that homeostatic expansion influences production of IFN-γ. However, the percentage of IFN-γ-producing transgenic T cells in $RAG^{-/-}MBP^{-/-}$ recipients is dramatically reduced compared with $RAG^{-/-}MBP^{+/+}$ recipients (12.6 vs 75.2%, respectively), demonstrating that the high percentage of IFN-γ-producing T cells in $RAG^{-/-}MBP^{+/+}$ 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 $RAG^{-/-}$ mice. T-bet is considered a master regulator of IFN-γ production (26). RNA isolated from transgenic T cells purified from $RAG^{-/-}$ recipients (6 days posttransfer), wild-type recipients (6 and 30 days posttransfer) and a $MBP^{-/-}$ 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. 4*B*). No difference in T-bet expression was seen in MBP-specific T cells isolated from $RAG^{-/-}$ 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. 5*A*). 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. 5*A*).

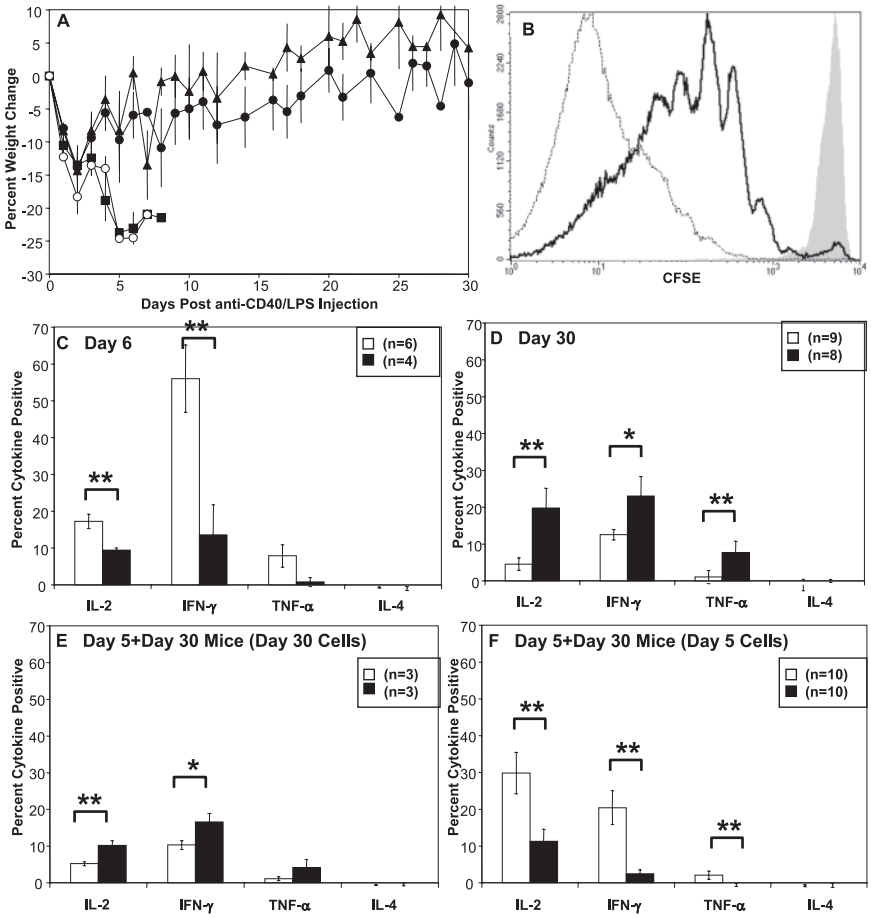

FIGURE 5. MBP121–140-specific T cells alter their response to activated APCs. *A*, Anti-CD40/LPS was injected into wild-type mice either 5 days (■) or 30 days (▲) after transfer of 3×10^6 transgenic T cells and weight loss was monitored after anti-CD40/LPS injection. Anti-CD40/LPS was also injected into day 5 plus day 30 recipients 5 days after the second injection of transgenic T cells; ●, day 5 plus day 30 recipients that did not develop disease ($n = 10$); ○, day 5 plus day 30 recipients that developed disease ($n = 3$). *B*, Naive transgenic T cells from $MBP^{-/-}$ mice were CFSE-labeled and injected into either wild-type recipients (thin line), day 30 recipients (thick line), or $MBP^{-/-}$ recipients (filled). Splenocytes from the recipients were analyzed 5 days later gating on $CD4^+$ Thy1-genetically marked donor T cells or on $CFSE^+$ cells in the $MBP^{-/-}$ recipient. *C* and *D*, Cytokine production was analyzed by gating on genetically marked $CD4^+$ transgenic T cells in day 6 and day 30 wild-type recipients 2 days after administration of anti-CD40/LPS (□). Control mice (■) did not receive anti-CD40/LPS. *E* and *F*, Cytokine production in day 5 plus day 30 recipients was analyzed as in *C* and *D*. Day 5 T cells were differentiated by allelic Thy1 expression and day 30 T cells within the same mice were differentiated by Thy1, CD4, Vα2, and Vβ8 expression. *, $p \leq 0.01$. **, $p \leq 0.003$.

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. 2*A*), or because MBP-specific T cells are proliferating much more rapidly at 5 compared with 30 days posttransfer into wild-type mice (see Fig. 3*A*). 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. 5*A*). 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. 5*B*). 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 $MBP^{+/+}$ recipients are hyporesponsive 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. 5*C*). The percentage of transgenic T cells producing IFN-γ 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-γ, IL-2, and TNF-α after receiving anti-CD40/LPS compared with day 30 recipients in which APCs were not activated (Fig. 5*D*). Thus, while APC activation triggers increased Th1 cytokine production by MBP-specific T cells 5 days after transfer into $MBP^{+/+}$ 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. 5*E*), 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. 5*F*). 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 TGFβ-1 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. 6*A*, 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. 6*A*). 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 TGFβ-1 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. 7*A*), although the rate of proliferation was somewhat slower than that of naive transgenic T cells transferred into wild-type mice (Fig. 5*B*), 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.

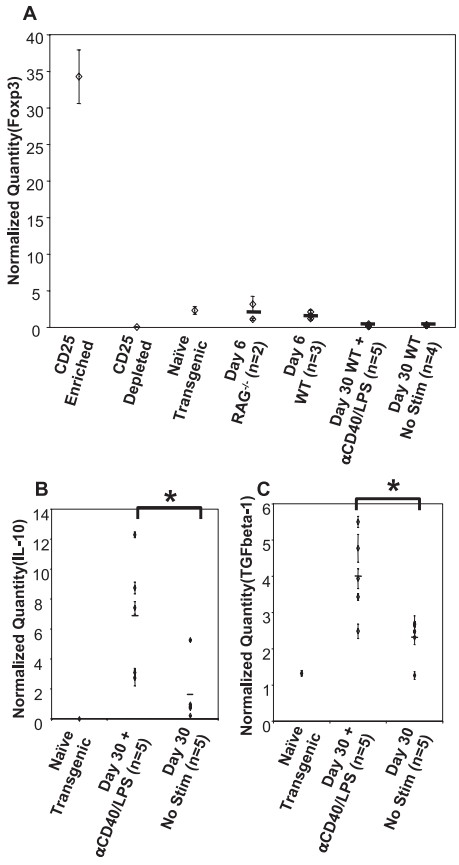

FIGURE 6. Tolerized MBP-specific T cells are Foxp3^{low} but express suppressive cytokines in response to APC activation. Genetically marked transgenic T cells were purified from RAG$^{-/-}$ or wild-type recipients at the indicated times after transfer and Foxp3 (*A*), IL-10 (*B*), and TGFβ-1 (*C*) expression 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. $*$, $p \leq 0.04$.

To determine whether the decreased production of Th1 cytokines in response to activated APCs was an intrinsic property of MBP-specific T cells that had undergone regulation, day 30 T cells were retransferred into new wild-type recipients and cytokine production in response to anti-CD40/LPS was analyzed. Despite their de novo proliferation in new wild-type mice, a smaller percentage of the retransferred day 30 transgenic T cells produced Th1 cytokines after APC activation compared with when APCs were not activated (Fig. 7*B*), as was observed in the original day 30 recipients. Thus, the Th1 cytokine hyporesponsiveness is an intrinsic property acquired by MBP-specific T cells during their initial regulation. Consistent with this, APC activation did not induce disease in the hosts of the retransferred T cells (Fig. 7*C*). However, the retransferred day 30 T cells did not prevent disease triggered by anti-CD40/LPS administration when a second dose of naive MBP-specific T cells was transferred into the same recipients 5 days later (Fig. 7*C*). Thus, lack of disease following APC activation in the original day 5 plus day 30 mice requires factors that develop in the host that has regulated MBP-specific T cells but that are separate from the day 30 T cells themselves.

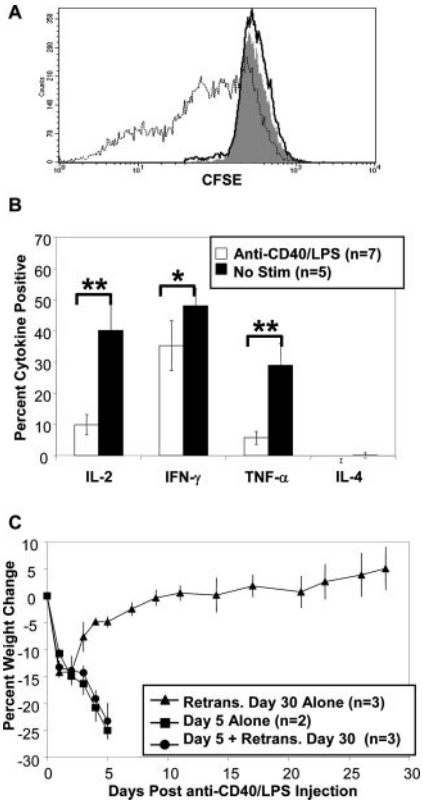

FIGURE 7. MBP-specific T cells retransferred into wild-type mice proliferate and suppress cytokine responses to APC activation. *A*, Day 30 MBP-specific T cells were CFSE labeled and retransferred into either wild-type mice (thin line), separate day 30 recipients (thick line), or $MBP^{-/-}$ mice (filled). T cell proliferation was analyzed 5 days later. Representative data from two experiments with two mice per group. *B*, Day 30 transgenic T cells were retransferred into wild-type mice and anti-CD40/LPS was injected 5 days later (□). Control recipients received no anti-CD40/LPS (■). Cytokine production by the retransferred T cells was analyzed 2 days after anti-CD40/LPS injection. *, $p < 0.04$. **, $p < 0.002$. *C*, Day 30 transgenic T cells (3×10^6/mouse) were retransferred into naive recipients. Five days later, naive MBP-specific T cells from $MBP^{-/-}$ mice were injected (3×10^6/mouse) into half of the recipients of the retransferred T cells (●) as well as into naive wild-type recipients (■) as controls. Half of the recipients of the retransferred day 30 cells did not receive naive transgenic T cells (▲). All mice were injected with anti-CD40/LPS 5 days after transfer of naive T cells and monitored for weight loss.

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 $RAG^{-/-}$ mice induced autoimmune disease. The number of MBP-specific T cells retransferred in these experiments (3×10^5) 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 $RAG^{-/-}$ 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 proliferation observed for day 30 T cells retransferred into wild-type mice compared with naive T cells (Fig. 5*B*). Thus, the tolerance exhibited by MBP-specific T cells in day 30 recipients is a dynamic, rather than terminally differentiated, phenotype.

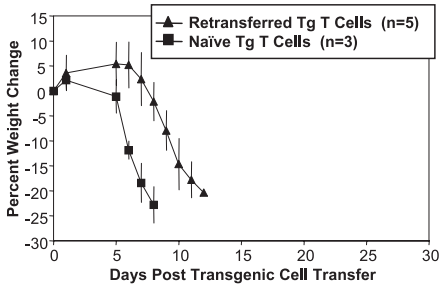

FIGURE 8. Day 30 MBP-specific T cells retransferred into $RAG^{-/-}$ mice induce autoimmunity. Purified day 30 transgenic T cells (3×10^5/mouse) were retransferred into $RAG^{-/-}$ recipients (▲). Control $RAG^{-/-}$ recipients were injected with equal numbers of naive MBP-specific T cells isolated from $MBP^{-/-}$ TCR-transgenic mice (■). Recipients were monitored for weight loss.

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 MBP121–140-specific T cells transferred into $MBP^{+/+}$ mice lacking $CD4^+CD25^+$ 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 $MBP^{-/-}$ mice were as effective in protecting $RAG^{-/-}$ 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 $MBP^{-/-}$ mice acquire regulatory activity during the first week after transfer to $MBP^{+/+}$ 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 MBP121–140-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, 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$^{-/-}$ 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 CXCR3 expression. In our model, MBP-specific T cells infiltrate the CNS, consistent with the observation that CXCR3 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$^{-/-}$ 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. 5*B* to 7*A*). 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 autoimmune 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 activated, 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 TGFβ-1 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 tolerized if regulatory T cell numbers decrease or their activity is impaired.

Acknowledgments

We thank James Dai for statistical analysis of real-time RT-PCR data, Dr. Amy Weinmann for her gift of T-bet control RNA, Dr. Stephen Stohlman for neutralizing anti-IL-10R and anti-TGF-β Abs, Molly Ottele, Hannah Simkins, and Neal Mausolf for expert technical assistance, and Ingunn Stromnes and Qingyong Ji for critical reading of this manuscript.

Disclosures

The authors have no financial conflict of interest.

References

1. Kyewski, B., and L. Klein. 2006. A central role for central tolerance. *Annu. Rev. Immunol.* 24: 571–606.
2. Walker, M. R., D. J. Kasprowicz, V. H. Gersuk, A. Benard, M. Van Landeghen, J. H. Buckner, and S. F. Ziegler. 2003. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human $CD4^+CD25^-$ T cells. *J. Clin. Invest.* 112: 1437–1443.
3. Chen, T. C., S. P. Cobbold, P. J. Fairchild, and H. Waldmann. 2004. Generation of anergic and regulatory T cells following prolonged exposure to a harmless antigen. *J. Immunol.* 172: 5900–5907.
4. Apostolou, I., and H. von Boehmer. 2004. In vivo instruction of suppressor commitment in naive T cells. *J. Exp. Med.* 199: 1401–1408.
5. Curotto de Lafaille, M. A., A. C. Lino, N. Kutchukhidze, and J. J. Lafaille. 2004. $CD25^-$ T cells generate $CD25^+Foxp3^+$ regulatory T cells by peripheral expansion. *J. Immunol.* 173: 7259–7268.
6. Knoechel, B., J. Lohr, E. Kahn, J. A. Bluestone, and A. K. Abbas. 2005. Sequential development of interleukin 2-dependent effector and regulatory T cells in response to endogenous systemic antigen. *J. Exp. Med.* 202: 1375–1386.
7. von Boehmer, H. 2005. Mechanisms of suppression by suppressor T cells. *Nat. Immunol.* 6: 338–344.
8. Battaglia, M., S. Gregori, R. Bacchetta, and M. G. Roncarolo. 2006. Tr1 cells: from discovery to their clinical application. *Semin. Immunol.* 18: 120–127.
9. Buckner, J. H., and S. F. Ziegler. 2004. Regulating the immune system: the induction of regulatory T cells in the periphery. *Arthritis Res. Ther.* 6: 215–222.
10. Weiner, H. L. 2001. Induction and mechanism of action of transforming growth factor-β-secreting Th3 regulatory cells. *Immunol. Rev.* 182: 207–214.
11. Serra, P., A. Amrani, J. Yamanouchi, B. Han, S. Thiessen, T. Utsugi, J. Verdaguer, and P. Santamaria. 2003. CD40 ligation releases immature dendritic cells from the control of regulatory $CD4^+CD25^+$ T cells. *Immunity* 19: 877–889.
12. Pasare, C., and R. Medzhitov. 2003. Toll pathway-dependent blockade of $CD4^+CD25^+$ T cell-mediated suppression by dendritic cells. *Science* 299: 1033–1036.
13. George, T. C., J. Bilsborough, J. L. Viney, and A. M. Norment. 2003. High antigen dose and activated dendritic cells enable Th cells to escape regulatory T cell-mediated suppression in vitro. *Eur. J. Immunol.* 33: 502–511.
14. Waldner, H., M. Collins, and V. K. Kuchroo. 2004. Activation of antigen-presenting cells by microbial products breaks self tolerance and induces autoimmune disease. *J. Clin. Invest.* 113: 990–997.
15. Ichikawa, H. T., L. P. Williams, and B. M. Segal. 2002. Activation of APCs through CD40 or Toll-like receptor 9 overcomes tolerance and precipitates autoimmune disease. *J. Immunol.* 169: 2781–2787.
16. Tang, Q., J. Y. Adams, A. J. Tooley, M. Bi, B. T. Fife, P. Serra, P. Santamaria, R. M. Locksley, M. F. Krummel, and J. A. Bluestone. 2006. Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. *Nat. Immunol.* 7: 83–92.
17. Tadokoro, C. E., G. Shakhar, S. Shen, Y. Ding, A. C. Lino, A. Maraver, J. J. Lafaille, and M. L. Dustin. 2006. Regulatory T cells inhibit stable contacts between $CD4^+$ T cells and dendritic cells in vivo. *J. Exp. Med.* 203: 505–511.
18. Sospedra, M., and R. Martin. 2005. Immunology of multiple sclerosis. *Annu. Rev. Immunol.* 23: 683–747.
19. Huseby, E. S., B. Sather, P. G. Huseby, and J. Goverman. 2001. Age-dependent T cell tolerance and autoimmunity to myelin basic protein. *Immunity* 14: 471–481.
20. Lemke, G. 1988. Unwrapping the genes of myelin. *Neuron* 1: 535–543.
21. Barbarese, E., J. H. Carson, and P. E. Braun. 1978. Accumulation of the four myelin basic proteins in mouse brain during development. *J. Neurochem.* 31: 779–782.
22. Seamons, A., A. Perchellet, and J. Goverman. 2006. Endogenous myelin basic protein is presented in the periphery by both dendritic cells and resting B cells with different functional consequences. *J. Immunol.* 177: 2097–2106.
23. Huseby, E. S., D. Liggitt, T. Brabb, B. Schnabel, C. Ohlen, and J. Goverman. 2001. A pathogenic role for myelin-specific $CD8^+$ T cells in a model for multiple sclerosis. *J. Exp. Med.* 194: 669–676.
24. Brabb, T., P. von Dassow, N. Ordonez, B. Schnabel, B. Duke, and J. Goverman. 2000. In situ tolerance within the central nervous system as a mechanism for preventing autoimmunity. *J. Exp. Med.* 192: 871–880.
25. Pape, K. A., R. Merica, A. Mondino, A. Khoruts, and M. K. Jenkins. 1998. Direct evidence that functionally impaired $CD4^+$ T cells persist in vivo following induction of peripheral tolerance. *J. Immunol.* 160: 4719–4729.
26. Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100: 655–669.
27. Perchellet, A., I. Stromnes, J. M. Pang, and J. Goverman. 2004. $CD8^+$ T cells maintain tolerance to myelin basic protein by "epitope theft." *Nat. Immunol.* 5: 606–614.
28. Grajewski, R. S., P. B. Silver, R. K. Agarwal, S. B. Su, C. C. Chan, G. I. Liou, and R. R. Caspi. 2006. Endogenous IRBP can be dispensable for generation of natural $CD4^+CD25^+$ regulatory T cells that protect from IRBP-induced retinal autoimmunity. *J. Exp. Med.* 203: 851–856.
29. Sarween, N., A. Chodos, C. Raykundalia, M. Khan, A. K. Abbas, and L. S. Walker. 2004. $CD4^+CD25^+$ cells controlling a pathogenic CD4 response inhibit cytokine differentiation, CXCR-3 expression, and tissue invasion. *J. Immunol.* 173: 2942–2951.
30. DiPaolo, R. J., D. D. Glass, K. E. Bijwaard, and E. M. Shevach. 2005. $CD4^+CD25^+$ T cells prevent the development of organ-specific autoimmune disease by inhibiting the differentiation of autoreactive effector T cells. *J. Immunol.* 175: 7135–7142.
31. Kohm, A. P., P. A. Carpentier, H. A. Anger, and S. D. Miller. 2002. Cutting edge: $CD4^+CD25^+$ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis. *J. Immunol.* 169: 4712–4716.
32. Klein, L., K. Khazaie, and H. von Boehmer. 2003. In vivo dynamics of antigen-specific regulatory T cells not predicted from behavior in vitro. *Proc. Natl. Acad. Sci. USA* 100: 8886–8891.
33. Liu, L., D. Huang, M. Matsui, T. T. He, T. Hu, J. Demartino, B. Lu, C. Gerard, and R. M. Ransohoff. 2006. Severe disease, unaltered leukocyte migration, and reduced IFN-γ production in $CXCR3^{-/-}$ mice with experimental autoimmune encephalomyelitis. *J. Immunol.* 176: 4399–4409.
34. Anderson, P. O., B. A. Manzo, A. Sundstedt, S. Minaee, A. Symonds, S. Khalid, M. E. Rodriguez-Cabezas, K. Nicolson, S. Li, D. C. Wraith, and P. Wang. 2006. Persistent antigenic stimulation alters the transcription program in T cells, resulting in antigen-specific tolerance. *Eur. J. Immunol.* 36: 1374–1385.
35. Tanchot, C., D. L. Barber, L. Chiodetti, and R. H. Schwartz. 2001. Adaptive tolerance of $CD4^+$ T cells in vivo: multiple thresholds in response to a constant level of antigen presentation. *J. Immunol.* 167: 2030–2039.
36. Kurtzke, J. F. 2002. Epidemiology and multiple sclerosis. *Rev. Neurol.* 35: 1177.
37. Marrie, R. A. 2004. Environmental risk factors in multiple sclerosis aetiology. *Lancet Neurol.* 3: 709–718.
38. Granieri, E., I. Casetta, M. R. Tola, and P. Ferrante. 2001. Multiple sclerosis: infectious hypothesis. *Neurol. Sci.* 22: 179–185.
39. Roncarolo, M. G., R. Bacchetta, C. Bordignon, S. Narula, and M. K. Levings. 2001. Type 1 T regulatory cells. *Immunol. Rev.* 182: 68–79.
40. Levings, M. K., S. Gregori, E. Tresoldi, S. Cazzaniga, C. Bonini, and M. G. Roncarolo. 2005. Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not $CD25^+CD4^+$ Tr cells. *Blood* 105: 1162–1169.
41. Berg, D. J., R. Kuhn, K. Rajewsky, W. Muller, S. Menon, N. Davidson, G. Grunig, and D. Rennick. 1995. Interleukin-10 is a central regulator of the response to LPS in murine models of endotoxic shock and the Shwartzman reaction but not endotoxin tolerance. *J. Clin. Invest.* 96: 2339–2347.