Induction of gut regulatory CD39$^+$ T cells by teriflunomide protects against EAE

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ABSTRACT

Objective: To determine whether as an orally delivered treatment, teriflunomide, an inhibitor of the mitochondrial enzyme dihydroorotate dehydrogenase approved to treat relapsing forms of multiple sclerosis, could affect gut-associated lymphoid tissue (GALT) immune responses functionally.

Methods: C57BL/6 mice were treated orally with teriflunomide and flow cytometric analysis of immune GALT cells performed ex vivo, and adoptive transfer experiments were used to test the protective effects of GALT regulatory T (Treg) cells.

Results: Teriflunomide reduced the percentages of antigen-presenting cells of Peyer patches when compared to controls. Conversely, a significant increase of the relative frequency of CD39$^+$ Treg cells was observed. In vivo, the protective effect of GALT-derived teriflunomide-induced CD39$^+$ Treg cells was established by adoptive transfer into recipient experimental autoimmune encephalomyelitis mice.

Conclusions: Our results identify specific GALT-derived CD39$^+$ Treg cells as a mechanism of action that may contribute to the efficacy of teriflunomide during CNS inflammatory demyelination and as an oral therapeutic in relapsing multiple sclerosis. ***Neurol Neuroimmunol Neuroinflamm* 2016;3:e291; doi: 10.1212/NXI.0000000000000291**

GLOSSARY

APC = antigen-presenting cell; **ATP** = adenosine 5′-triphosphate; **CLN** = cervical lymph node; **DHODH** = dihydroorotate dehydrogenase; **EAE** = experimental autoimmune encephalomyelitis; **GALT** = gut-associated lymphoid tissue; **JAK** = Janus kinase; **MLN** = mesenteric lymph node; **MOG** = myelin oligodendrocyte glycoprotein; **MS** = multiple sclerosis; **PP** = Peyer patch; **PSA** = polysaccharide A; **SPL** = spleen; **Treg** = regulatory T.

Teriflunomide is an approved oral therapeutic to treat relapsing forms of multiple sclerosis (MS). The mechanism of action is presumed to be the inhibition of de novo pyrimidine synthesis by acting as a reversible, noncompetitive inhibitor of mitochondrial dihydroorotate dehydrogenase (DHODH). The importance of teriflunomide as a therapy against MS relies on the antiproliferative effects of DHODH inhibition, which is preferentially observed in autoreactive T and B cells by blocking cell cycling in the G1 phase.[1] Because of the effects in autoreactive cells and proinflammatory pathways associated with MS and other diseases, teriflunomide is proposed as an immunomodulatory drug.[2,3]

Although the efficacy of teriflunomide in rat models of experimental autoimmune encephalomyelitis (EAE) has been demonstrated,[4–7] its therapeutic protection against EAE in mice is not optimal. Because of its reduced protective effects in mice, little is understood regarding any alternative immunologic mechanisms by which it may regulate CNS demyelinating disease. In this study, we aimed to determine whether teriflunomide, an approved oral therapy against relapsing forms of MS, targets and modifies the phenotype and function of the gut-associated lymphoid tissue (GALT).

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We hypothesized that teriflunomide would significantly affect the GALT. The phenotypic changes could then influence EAE severity since the GALT is a known reservoir for proinflammatory cells with direct function in EAE development.

METHODS **Mice and treatments.** Female 8-week-old C57BL/6 mice were obtained from the Jackson Laboratories (Bar Harbor, ME). All animal care and procedures were in accordance with Dartmouth College Animal Resources Center institutional policies for animal health and well-being.

Mice were treated with either vehicle or teriflunomide (20 mg/kg, supplied by Sanofi Genzyme Corporation, Cambridge, MA) by daily oral gavage during the duration of each experiment. Dartmouth College Animal Resources Center routinely screens for a wide range of infectious agents including *Helicobacter*. Mice were maintained in a restricted, access-controlled environment.

Cell preparation and flow cytometry. Single lymphocyte preparations from Peyer patches (PPs), mesenteric lymph nodes (MLNs), spleens (SPLs), or cervical lymph nodes (CLNs) were stained using conventional methods. A live/dead fixable fluorescence-labeled viability dye (Molecular Probes–Thermo Fisher Scientific Inc., Waltham, MA) was used in all staining protocols for dead/live discrimination and gated only on viable cells for subsequent analysis. Cell subsets were analyzed using fluorochrome-conjugated monoclonal antibodies against T cell antigens (CD3, CD4, CD8, CD39), NK and NKT cells (NK1.1), and B cells (CD19, B220, CD5, CD1d) (BioLegend, San Diego, CA). Intracellular staining for Foxp3 was performed using fluorochrome-labeled anti-Foxp3 monoclonal antibody (clone FJK-16s; eBioscience, San Diego, CA). Monocytes, macrophages, neutrophils, and dendritic cells were analyzed using CD11b, CD11c, Gr-1, and CD103 (BioLegend). Samples were acquired using a Miltenyi Biotec MACSQuant (Miltenyi Biotec, Bergisch Gladbach, Germany). Data were analyzed with FlowJo software (FlowJo LLC, Ashland, OR).

EAE induction. EAE was induced in C57BL/6 mice by subcutaneous challenge with 200 μg $MOG_{35–55}$ (Peptides International, Louisville, KY) in 200 μL of complete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO). On days 0 and 2 postchallenge, mice received 400 ng of *Bordetella pertussis* toxin intraperitoneally (List Biological Laboratories, Campbell, CA). Mice were monitored and scored daily for disease progression as previously shown.[8]

Adoptive transfer of T cells. $CD39^+CD4^+$ or $CD39^-CD4^+$ T cell populations were sorted by flow cytometry from pooled GALT (PPs and MLNs) of teriflunomide- or vehicle-treated mice. $CD4^+$ T cells were first enriched with magnetic beads (Dynal Biotech ASA, Oslo, Norway). After sorting, the cells were resuspended in sterile phosphate-buffered saline and injected IV into recipient mice.

Statistical analysis. Parametric and nonparametric *t* tests and 2-way analysis of variance followed by Šidák comparison of multiple groups was applied to show differences in flow cytometric analysis. Two-way analysis of variance followed by Šidák comparison of multiple groups was applied to show differences in EAE scores. The *p* values <0.05, <0.01, and <0.001 are indicated.

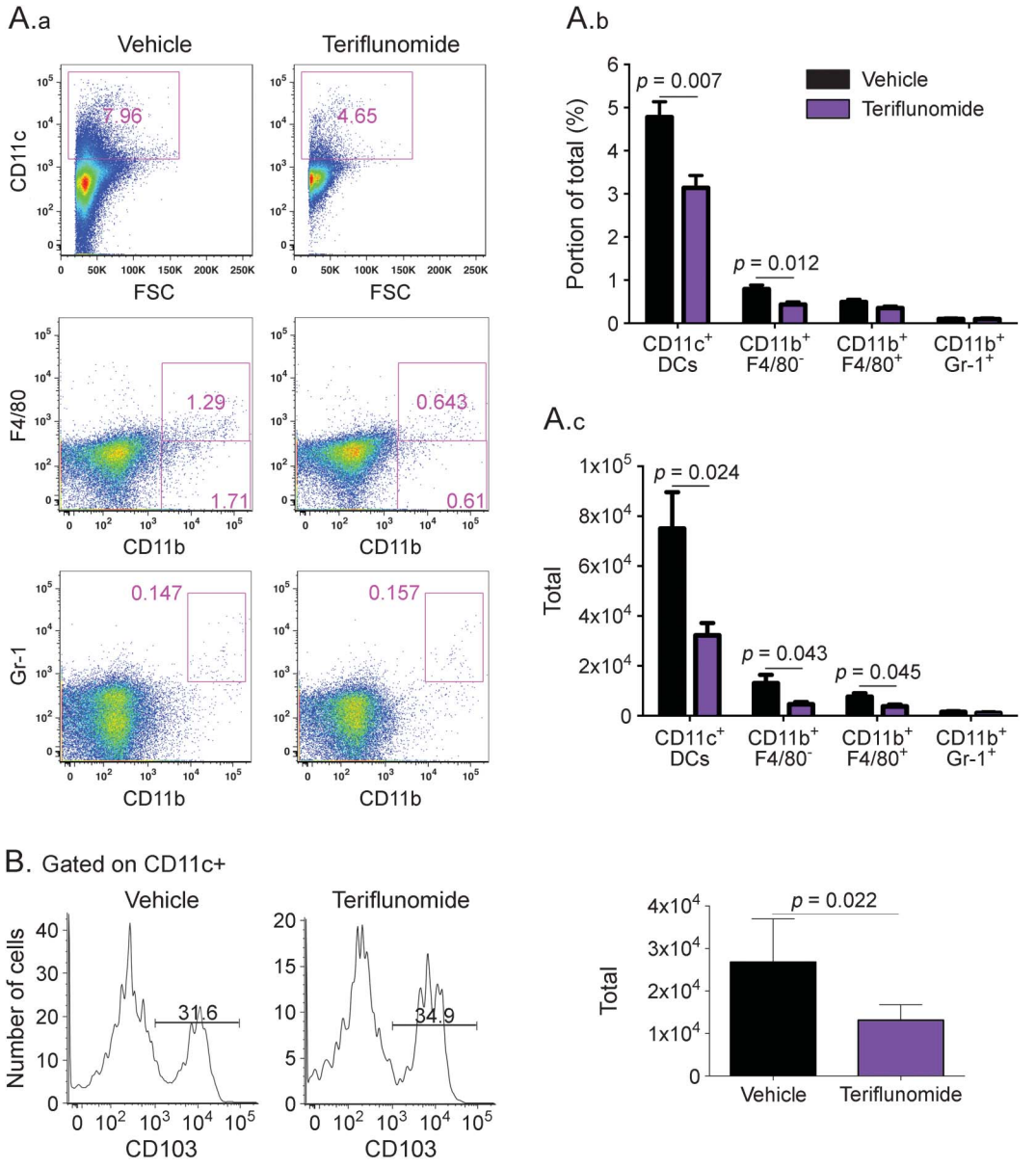
RESULTS **Teriflunomide reduces the frequencies of GALT antigen-presenting cells.** For our study, we treated C57BL/6 mice orally with daily doses of teriflunomide (20 mg/kg). The dosage was selected according to previous studies performed by Sanofi Genzyme Corporation. The proliferation inhibitory capacity of teriflunomide is species-specific, which results in an increased dosage required for protection in mice vs rats.[9] In mice, 20 mg/kg/d is needed to obtain comparable levels of protection to those observed in rats treated with 3 and 10 mg/kg/d teriflunomide.[9] As described later, we did not perform direct protection studies with teriflunomide in mice. We compared the frequencies of common antigen-presenting cell (APC) populations including dendritic cells, monocytes/macrophages, and B cells in the GALT of mice treated orally with teriflunomide or vehicle control. Neutrophil frequencies were also determined. Daily oral gavages with teriflunomide significantly reduced the frequencies and absolute numbers of several important APC phenotypes present in the PPs, including dendritic cells ($CD11c^+$) and $F4/80^-CD11b^+$ monocytes (figure 1A). A reduced trend in the frequencies of macrophages ($CD11b^+F4/80^+$) was also seen in the PPs of teriflunomide-treated mice when compared to controls, significance that was observed when comparing the absolute number of cells (figure 1A). No effects on the neutrophil ($CD11b^+Gr\text{-}1^+$) frequencies were observed. Although a similar trend in the reduction of dendritic cells was observed in the MLNs of teriflunomide-treated mice, no statistical significant was achieved (not shown), suggesting a tissue differential in response to teriflunomide exposure.

Among the GALT dendritic cells that have been shown to promote immune tolerance by inducing regulatory T (Treg) cell conversion, the $CD103^+$ subpopulation is perhaps the most studied. When comparing the frequency of the CD103-expressing subset within the $CD11c^+$ cells of the PPs (figure 1B) and MLNs (not shown) following teriflunomide treatment, we observed no reduction, yet a slight enhancement was noted (not significant). The total numbers of $CD103^+$ dendritic cells were significantly reduced after teriflunomide treatment, which could correspond with the reduced size of the lymph nodes observed in these mice (not shown).

A significant reduction in the absolute frequencies of B cells ($CD19^+B220^+$) was observed in the PPs of mice treated with teriflunomide (figure 2A). The absolute numbers were not significantly reduced.

Similarly, when B cells were gated on CD5 and CD1d double-positive ($CD5^+CD1d^+$) cells, a significant reduction was observed compared to controls (figure 2B). These B cell subpopulations have been previously documented as regulatory and protective during murine EAE.[10,11] In the MLNs, no differences were observed in the frequencies of B cells (figure 2A); however, the absolute numbers were found to be significantly lower in teriflunomide-treated mice (figure 2A). The discrepancies might reflect the

Figure 1 Teriflunomide affects the frequencies and numbers of antigen-presenting cell populations in PPs



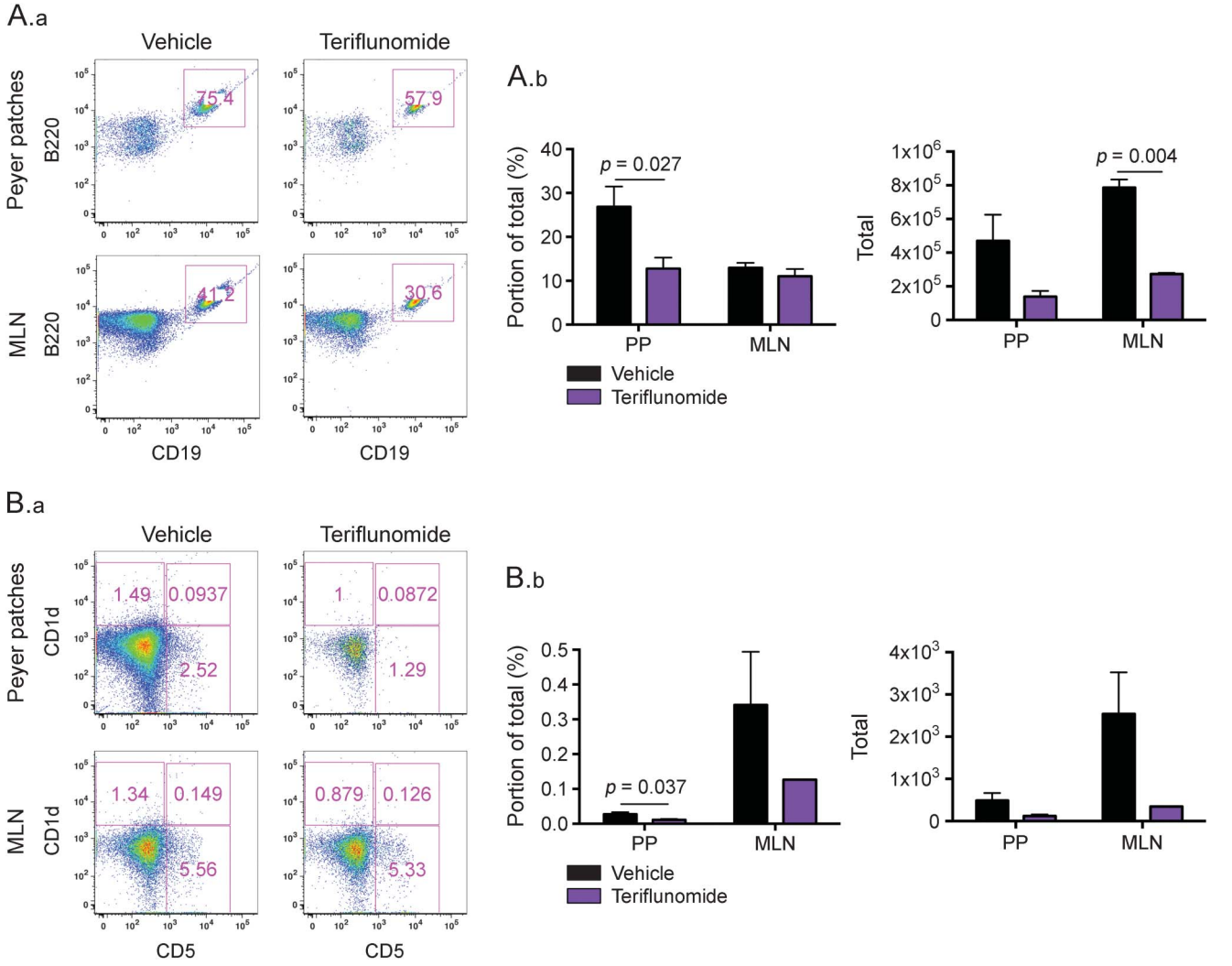
C57BL/6 mice were treated with teriflunomide (20 mg/kg) or vehicle for 14 days. After euthanasia, PPs of mice were isolated and flow cytometric analysis of $CD11c^+$ dendritic cells and $CD11b^+$ monocytes, also expressing either F4/80 or Gr-1, was performed (A) (frequencies [A.b] and absolute numbers [A.c] shown). Flow cytometric analysis of CD103 expression was performed on the $CD11c^+$ dendritic cells in the PPs (frequencies and absolute numbers shown) (B). Bar graphs show the mean ± standard errors of 1 of 2 repeats (n = 5, per group). PP = Peyer patch.

reduced size of the lymph nodes observed in mice treated with the drug (not shown). When MLN $CD5^+CD1d^+$ B cells were compared, we observed a reduction in both frequencies and total numbers in teriflunomide-treated mice, although no statistical significance was observed (figure 2B).

Treatment with teriflunomide did not affect the frequencies of NK and NKT cells in either PPs or MLNs (not shown). Collectively, the flow cytometric analysis of the PPs and MLNs of mice subjected to teriflunomide suggests that oral treatment with teriflunomide reduces the frequencies and numbers of the major APC populations of the PPs. Similar trends, although not significant, were also observed in the MLNs.

Teriflunomide increases the frequency of a $CD39^+$ phenotype in GALT $CD4^+$ T cells. We next aimed to determine whether teriflunomide treatment would increase the populations of Treg cells in the GALT. The numbers of $CD4^+$ and $CD8^+$ T cells isolated from the PPs were unaffected by treatment with teriflunomide (figure 3A). A significant increase in the total percentage of $CD39^+Foxp3^+$ Treg cells was observed when compared to controls (figure 3A). A similar, but

Figure 2 Teriflunomide reduces the frequencies of PP B cells and regulatory B cell subsets

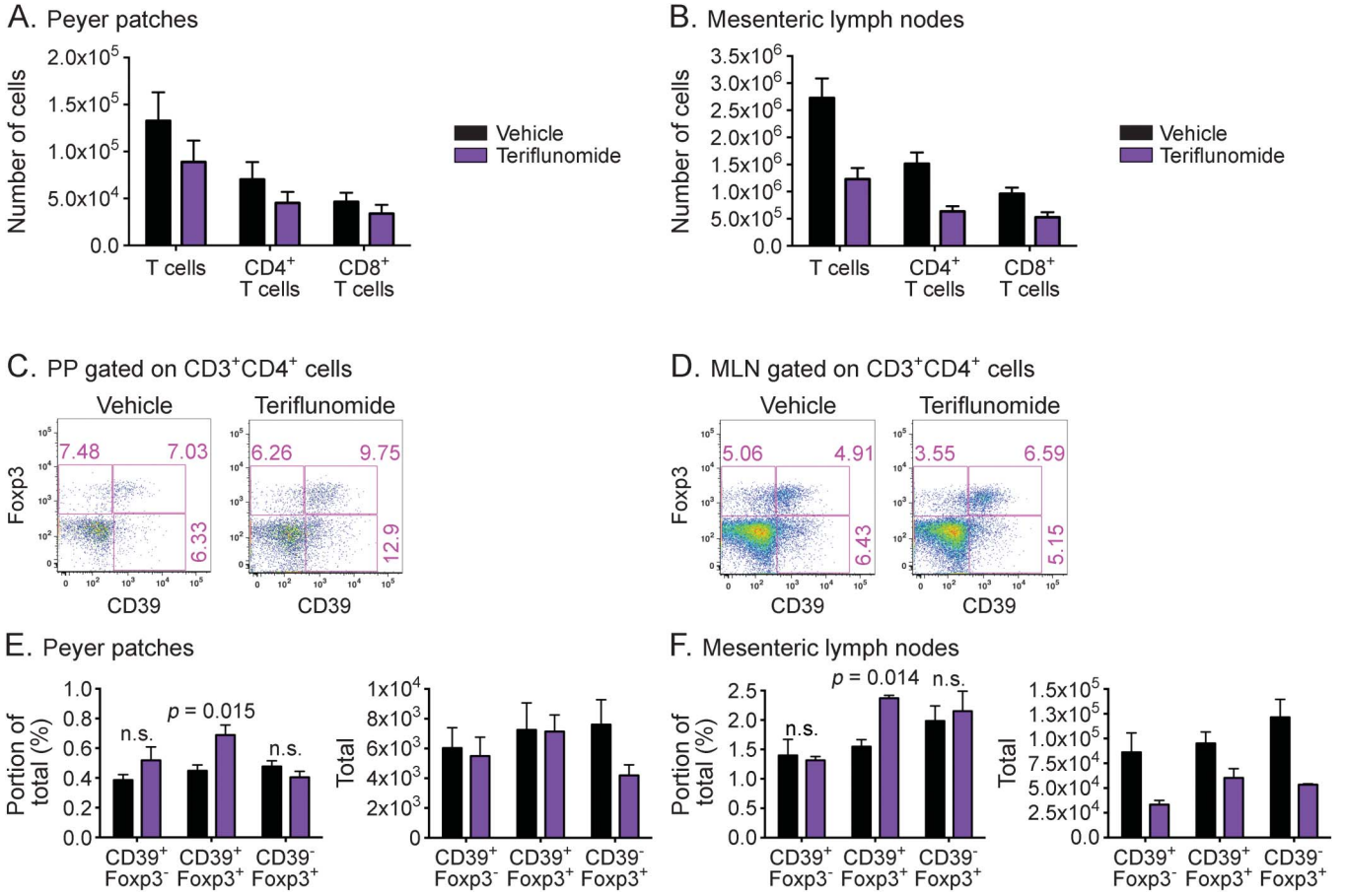


Flow cytometric analysis of B220$^+$CD19$^+$ B cells was performed in PPs and MLNs as described in figure 1 (A.a). Bar graphs show the mean ± standard errors of 1 of 2 repeats (n = 5, per group) (A). CD5$^+$ and CD1d$^+$ double-positive subset of gated B220$^+$CD19$^+$ cells were also compared (B.a). For both B cells (A.b) and CD5$^+$Cd1d$^+$ B cells (B.b), frequencies on tissue and absolute numbers are shown. Analysis as described in figure 1 (n = 5, per group). MLN = mesenteric lymph node; PP = Peyer patch.

not significant, increase was observed among the total frequency of CD39$^+$Foxp3$^-$ T cells harvested from the PPs of teriflunomide-treated mice (figure 3A). No differences in the frequencies of CD39$^-$Foxp3$^+$ Treg cells were observed in the PPs of teriflunomide- vs vehicle-treated mice. As observed in the analysis of the PPs, no statistically significant differences in the CD4$^+$ and CD8$^+$ T cells were seen in the MLNs (figure 3B), although the absolute numbers of both T cell subpopulations were diminished when compared with controls. A statistically significant increase in the CD39$^+$Foxp3$^+$ Treg population was also quantified in the MLNs of mice treated with teriflunomide when compared with controls (figure 3B). Because the overall numbers of T cells appeared reduced, or at least not increased after teriflunomide treatment, the numbers of Treg subsets were mostly reduced in PPs (figure 3A) and MLNs (figure 3B), although no significance was observed in the analysis of any of the subpopulations compared.

Although increases in the frequencies of both Foxp3$^+$ and Foxp3$^-$CD39$^+$ T cells were observed (figure 3), 14 days of treatment with teriflunomide did not affect the CD39$^+$ percentages within CD4$^+$ T cells of PPs and MLNs (not shown). To determine whether more prolonged treatment would affect these frequencies, we next performed a kinetic analysis of the frequencies of CD39 expression within CD4$^+$ T cells. The PPs, MLNs, SPLs, and CLNs of mice treated with teriflunomide or vehicle, daily for a total of 28 days, were isolated and analyzed using flow cytometry (figure 4). Of interest, the increase in the relative frequency of CD39$^+$ T cells was not observed in the SPLs and CLNs of mice treated with

Figure 3 Teriflunomide enhances the frequencies of gut-associated lymphoid tissue CD39⁺ regulatory T cells



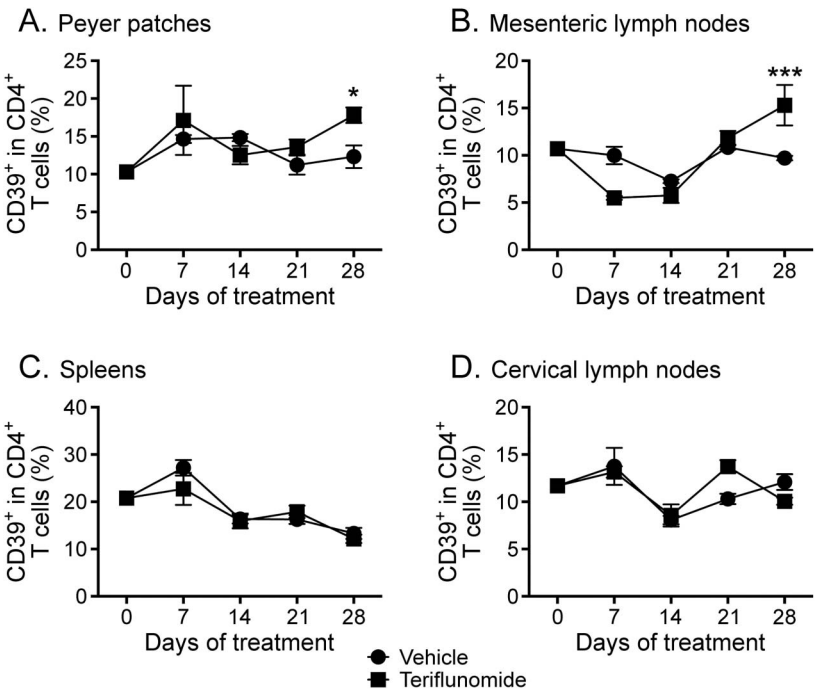
Flow cytometric analysis was performed to examine T cells (total T cells, CD4⁺, and CD8⁺ T cell numbers are shown) and CD39⁺Foxp3⁻, CD39⁺Foxp3⁺, and CD39⁻Foxp3⁺ subpopulations (after gating on CD4⁺CD3⁺ T cells) in the PPs (A, C, E) and MLNs (B, D, F). Bar graphs show the mean ± standard errors of frequencies and absolute numbers of 1 of 2 repeats (n = 5, per group). MLN = mesenteric lymph node; PP = Peyer patch.

teriflunomide (figure 4). These results suggest that the effects are restricted to the GALT, at least in naive mice lacking any overt autoimmune-driven inflammation.

Teriflunomide-induced GALT CD39⁺ T cells are sufficient to reduce EAE severity, prophylactically and therapeutically. Our flow cytometric analysis of the GALT compartments of mice treated with teriflunomide showed an increase in GALT T cells that express CD39. We next hypothesized that this cellular subpopulation would be active in vivo and could diminish EAE severity. Thus, CD39⁺CD4⁺ T cell populations were sorted by flow cytometry from pooled GALT tissues (PPs and MLNs) of teriflunomide- or vehicle-treated mice and transferred into untreated recipient mice with EAE. We first evaluated the prophylactic effects of a Treg cell transfer. For that, 100,000 CD39⁺CD4⁺ cells from teriflunomide- or vehicle-treated mice were adoptively transferred into naive mice 1 day before the induction of EAE (figure 5A). An additional group receiving no cells was used as a positive control for EAE disease. As shown in figure 5A, the adoptive transfer of CD39⁺CD4⁺ T cells was able to significantly reduce the severity of EAE in vivo. No differences in the EAE clinical scores were observed in mice receiving CD39⁺CD4⁺ GALT cells from vehicle- vs teriflunomide-treated donors.

We next evaluated the potential therapeutic effects of CD39⁻CD4⁺ T cells and CD39⁺CD4⁺ T cells both isolated from the GALT of teriflunomide-treated mice. At the onset of CNS disease (day 17), 60,000 cells per recipient mouse were transferred (figure 5B). Of note, despite the low number of cells transferred into the recipient mice, CD39⁺CD4⁺ T cells significantly reduced the severity of EAE disease for approximately 1 week past the injection. Together, these results confirm the protective effects of CD39⁺ T cells isolated from the GALT demonstrating an additional mechanism of action for teriflunomide during CNS inflammatory demyelination, based on the induction of protective CD39⁺ T cells in the GALT.

Figure 4 **CD39⁺ T cells increase following sustained treatment with teriflunomide**

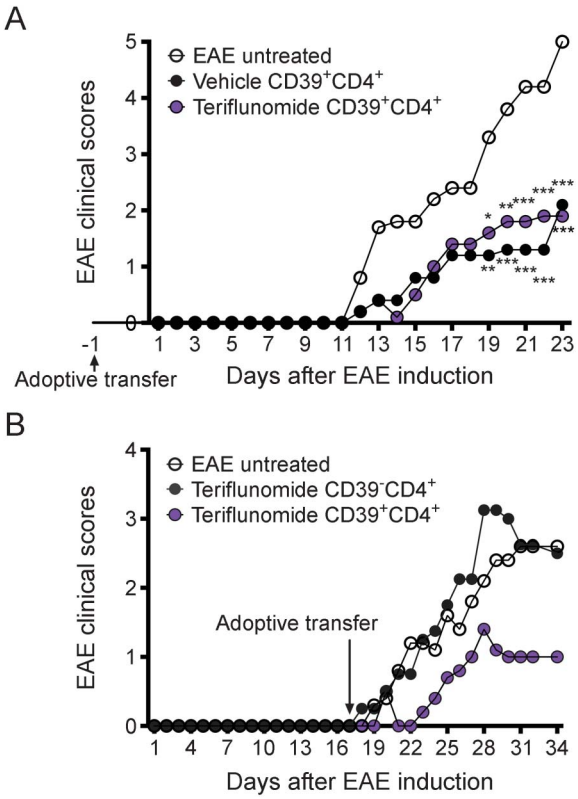


Gut-associated lymphoid tissue A flow cytometric-based kinetics analysis of $CD4^+CD39^+$ T cells was performed in Peyer patches (A), mesenteric lymph nodes (B), spleens (C), and cervical lymph nodes (D) of mice treated with teriflunomide (20 mg/kg) or vehicle for 28 days. Tissues were harvested from mice weekly (days 0, 7, 14, and 28) and stained for flow cytometry (n = 3, per group). A 2-way analysis of variance followed by Šidák multiple comparison test was performed. *$p < 0.05$; ***$p < 0.01$.

DISCUSSION Teriflunomide inhibits the action of DHODH in proliferating cells. This inhibition reduces DNA synthesis and affects both B and T cell proliferation, which requires de novo synthesis of pyrimidine, as opposed to those resting cells that utilize the salvage pathway for pyrimidine requirements. By blocking DHODH, teriflunomide reduces the rate of proliferation by reducing the supply of de novo synthesized pyrimidine.[12] Furthermore, other proposed mechanisms suggest that teriflunomide inhibits the Janus tyrosine kinase (JAK) enzymes JAK1 and JAK3[13] and reduces transforming growth factor α–induced nuclear factor κB activation.[14] Teriflunomide is the active metabolite of leflunomide, a drug approved by the Food and Drug Administration for the treatment of rheumatoid arthritis.[15] In patients with rheumatoid arthritis, treatment with leflunomide also reduces the transcription of cell adhesion molecules and matrix metalloproteinases.[16] Teriflunomide is partially metabolized by the CYP enzyme family, and recycled by ABCG2, an adenosine 5′-triphosphate (ATP)-binding "cassette transmembrane transporter protein" present in the liver and gut. The half-life of teriflunomide in humans is 2 weeks, and it is cleared in the liver. When the enterohepatic recirculation is blocked with cholestyramine, the half-life is reduced to 2 days.[17] Teriflunomide is protective in rat models of EAE.[4–7] However, its therapeutic protection against EAE in mice is not optimal. As opposed to active EAE induced in mice using a standard MOG protocol, EAE was induced in Dark Agouti rats by injection with frozen Dark Agouti rat spinal cord homogenate in combination with complete Freund's adjuvant.[6] Whether the differences in the experimental protocols between mouse and rat EAE models account for the observed differences in the protection by teriflunomide remains uncertain.[7,9] Nevertheless, because of its reduced protective effects in mice, little is understood regarding any alternative immunologic mechanisms by which it may regulate CNS demyelinating disease.

In recent years, the importance of gut microbiota as an immunomodulatory entity has emerged. Although more and larger studies are needed, experimental studies suggest the relevance of the microbiota in regulating human MS.[8,18–27] More directly relevant to MS, a recent study has now demonstrated that approved therapies and those currently under evaluation for the treatment of MS significantly modify the gut microbiome.[28] Fewer studies have focused on the potential effects of drugs that target the immune system on the microbiota or the GALT when drugs are administered orally. Treatment with teriflunomide reduced the overall size of PPs and MLNs (not shown), resulting in reduced numbers of cells. This reduction affected the main and most common APCs (dendritic cells, B cells, monocytes, and macrophages) (figures 1 and 2). It is remarkable that we did not observe a reduction in the expression levels of the $CD103^+$ subset in $CD11c^+$ cells in PPs (figure 1B), although the total numbers were reduced, and MLNs (not shown). $CD103^+$ dendritic cells have a tolerogenic phenotype with a previously described role in the induction of $Foxp3^+$ Treg cells.[29] Of interest is the significant reduction that treatment with teriflunomide induced in the frequencies and numbers of B cell populations, including the $CD5^+CD1d^+$ subset, of the PPs (figure 2, A and B). In the MLNs, no changes in the frequencies of B cells were observed, although the absolute numbers of B cells were reduced, possibly because of the overall reduction in the size of lymph nodes (figure 2A). The role of B cells in MS is increasingly understood primarily as an effector cell population. B cells also act as presenting cells, and significant reductions in monocytes and mainly dendritic cells were also observed in the PPs when mice were orally treated with teriflunomide. The mechanisms by which GALT APCs appear to be significantly affected by treatment with teriflunomide remain unknown. The systemic immunomodulatory effects of teriflunomide might account

Figure 5 Teriflunomide-induced CD39⁺CD4⁺ T cells are protective against EAE



Peyer patches and mesenteric lymph nodes were isolated from mice treated with teriflunomide or vehicle for 2 weeks, pooled, and CD39⁺ and CD39⁻CD4⁺ T cells were sorted by flow cytometry. One day before EAE induction, 50,000 to 100,000 CD39⁺CD4⁺ T cells from either teriflunomide- or vehicle-treated mice were transferred to recipient mice (A). Similarly, CD39⁺ or CD39⁻CD4⁺ T cells from teriflunomide-treated mice were next adoptively transferred at the onset of disease (B) (depicted is 1 of 2 experiments performed, for n = 5 per group, and n = 6 per group, respectively). Two-way analysis of variance followed by multiple comparison tests: *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$. EAE = experimental autoimmune encephalomyelitis.

for such reductions; however, our results show that in MLNs, teriflunomide effects in APC populations are already diminished (not shown). We speculate and are currently evaluating whether teriflunomide may have a direct effect on the microbiota composition of the gut. We will determine whether potential changes in the gut microbial populations of mice treated with teriflunomide influence the percentages, and more importantly, the functions of these APC populations of the PPs, necessary for the appropriate sampling of the gut content.

Teriflunomide induced a significant increase in the frequencies of CD39⁺ cells specifically within the CD4⁺ subset in murine GALT (PPs and MLNs). CD39 (ENTPD1) is an ectoenzyme that mediates immune suppression of Treg cells by hydrolysis of ATP and ADP (adenosine 5′-diphosphate) into 5′AMP (adenosine monophosphate).[30] CD39⁺ Treg cells can suppress the proliferation of autoreactive Th17 cells and their production of interleukin 17.[31] The oral immunization of mice with an attenuated strain of *Salmonella enterica* serovar Typhimurium that expresses on its surface the colonization factor antigen 1 fimbriae of the enterotoxigenic *Escherichia coli*, the causative agent of the traveler's diarrhea, promotes CD39⁺ T cells that mediate protection against the collagen-induced experimental model of arthritis.[32–34] Furthermore, the suppressive function of CD39⁺ Treg cells from patients with MS is reduced when compared to those obtained from healthy individuals.[35] We showed that the immunomodulatory effect of polysaccharide A (PSA) produced by the gut commensal *Bacteroides fragilis* is dependent on interleukin 10–producing CD39⁺ T cells.[26,27] In the context of EAE, exposure to PSA promoted the accumulation of CD39⁺ cells in the CLNs with a regulatory and a migratory phenotype. More recently, we reported that the oral treatment of EAE with the murine-specific anti-CD52 antibody increases in CD39⁺ regulatory T cells.[36] Treatment of patients with relapsing MS with the humanized anti-CD52 also promoted an enrichment of the CD39⁺ subset in circulating Treg cells.[37] Fingolimod, a sphingosine-1-phosphate receptor 1 (S1P1) antagonizer approved for the treatment of MS, therapeutically enhances the levels of CD39 messenger RNA and CD39⁺ Treg cell frequencies in the circulating blood of patients treated for MS.[38] Based on these findings and our previous reports with PSA, we propose that the induction of CD39⁺ Treg cells is not drug-specific. Additional studies are needed to test this hypothesis.

An increase in the relative frequency of CD39⁺ T cells was not observed in the SPLs and CLNs of mice treated with teriflunomide (figure 4). These results suggest that the effects seen are restricted to the GALT. It is important to note, however, that these mice were not subjected to EAE. Our previous work using PSA, which is also administered orally and induces a CD39⁺ Treg phenotype in the gut, showed that EAE induction is required to observe these tolerogenic changes beyond the gut. In naive mice, PSA did not increase the frequencies of CD39⁺ Treg cells except in the GALT.[27] By contrast, in mice with EAE, Treg cells and CD39⁺ Treg cells were enhanced in relative frequencies (of total CD4⁺ T cells) and absolute frequencies in CLNs[27] and even the CNS of diseased mice.[26]

Despite the modest, although significant, increase in the frequencies of GALT CD39⁺ T cells after 3 to 4 weeks of treatment, these cells were protective in low numbers. Furthermore, we observed that CD39⁺ T cells isolated from teriflunomide-treated mice were protective when adoptively transferred 1 day before EAE onset (figure 5A), and also at clinical onset (figure 5B). Of interest is the observation that GALT CD39⁺ T cells isolated from vehicle-treated mice were also protective (figure 5A). Our results suggest an immunomodulatory role for CD39⁺ T cells in the protection conferred by teriflunomide. We propose

that the immunoregulatory effects provide an additional mechanism to the already established antiproliferative effects of teriflunomide. The adoptive transfer of $CD39^+CD4^+$ T cells, independent of Foxp3 expression, was previously shown to be protective against murine rheumatoid arthritis after oral treatment with gut living vaccine vectors *Salmonella* Typhimurium[33] and with *Lactococcus lactis*.[39] Based on our results, and although the effect of teriflunomide on the proliferation of lymphocytes in MS is well established, we postulate that, in addition, teriflunomide as an orally delivered treatment alters the GALT immune response due to changes in the colonization of gut microbial populations. The increase in the frequencies of inducible Treg cells in patients with relapsing-remitting MS treated with teriflunomide has been recently reported, as part of the TERIDYNAMIC study that focused on the immunomodulatory effects of the drug.[40] Investigators showed that after treatment with teriflunomide, the frequencies of B and T cells in circulating blood are reduced compared to control individuals, but inducible Treg cells are increased. Our findings show that in non-EAE mice, teriflunomide increases a $CD39^+$ Treg population in the gut. Studies to evaluate the effect of teriflunomide on the gut microbiota are being explored.

AUTHOR CONTRIBUTIONS

Javier Ochoa-Repáraz: conception and design of the study, acquisition and analysis of data, and drafting of the manuscript and figures. Sara L. Colpitts: acquisition and analysis of data. Christopher Kircher: acquisition and analysis of data. Eli J. Kasper: acquisition and analysis of data. Kiel M. Telesford: acquisition and analysis of data. Sakhina Begum-Haque: acquisition and analysis of data. Anudeep Pant: acquisition and analysis of data. Lloyd H. Kasper: conception and design of the study and drafting of the manuscript.

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DISCLOSURE

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