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Central Nervous System Demyelinating Disease Protection by the Human Commensal Bacteroides fragilis Depends on Polysaccharide A Expression

Javier Ochoa-Reparaz,* Daniel W. Mielcarz,† Lauren E. Ditrio,† Ashley R. Burroughs,‡ Sakhina Begum-Haque,* Suryasarathi Dasgupta,‡ Dennis L. Kasper,‡ and Lloyd H. Kasper*

The importance of gut commensal bacteria in maintaining immune homeostasis is increasingly understood. We recently described that alteration of the gut microflora can affect a population of Foxp3+Treg cells that regulate demyelination in experimental autoimmune encephalomyelitis (EAE), the experimental model of human multiple sclerosis. We now extend our previous observations on the role of commensal bacteria in CNS demyelination, and we demonstrate that Bacteroides fragilis producing a bacterial capsular polysaccharide Ag can protect against EAE. Recolonization with wild type B. fragilis maintained resistance to EAE, whereas reconstitution with polysaccharide A-deficient B. fragilis restored EAE susceptibility. Enhanced numbers of Foxp3+Treg cells in the cervical lymph nodes were observed after intestinal recolonization with either strain of B. fragilis. Ex vivo, CD4+T cells obtained from mice reconstituted with wild type B. fragilis had significantly enhanced rates of conversion into IL-10–producing Foxp3+Treg cells and offered greater protection against disease. Our results suggest an important role for commensal bacterial Ags, in particular B. fragilis expressing polysaccharide A, in protecting against CNS demyelination in EAE and perhaps human multiple sclerosis. The Journal of Immunology, 2010, 185: 4101-4108.

Multiple sclerosis (MS) is a human disease of the CNS that is characterized by an inflammatory process followed by demyelination and axonal loss (1). In MS, autoreactive cells respond to self-Ags by inducing inflammation and attack the CNS, causing the axonal damage. Experimental autoimmune encephalomyelitis (EAE), the most widely used animal model for human MS, has provided significant information suggesting the role of T cells in the induction of this autoimmune disorder (2). It has been hypothesized that encephalitogenic T cells are activated in the periphery and then cross the blood-brain barrier into the CNS, where they encounter the self-Ag, and become reactivated. This reactivation could induce the release of proinflammatory cytokines, provoking the activation of resident macrophage–microglia cells to release NO that is directly involved in the demyelination of the neuronal myelin sheath (3). There is substantial evidence collected in EAE suggesting that Th1- and balanced immune responses might be polarized toward Th1 and Th17 (1).

Recent findings have further supported a primary role for IL-17 in the pathogenesis of human MS (4, 5).

Gut commensal microorganisms can modulate immune homeostasis (6-13). Studies in germ-free animals, born and raised in sterile conditions, showed that monocolonization with Bacteroides fragilis was sufficient to stimulate early development of the GALT, to induce normal organogenesis in the spleen and thymus, and balanced immune development (14). Bacteroides species are gram-negative bacteria that compose ~25% of the microbiota in both humans and other mammals. B. fragilis is symbiotic with the host, but if it reaches sterile then extraluminal sites can be responsible for tissue infection, bacteremia, and abscess formation in the peritoneal cavity, brain, liver, pelvis, or lungs (15, 16).

The administration of a mixture of gut commensal bacteria can protect against EAE (17). The protection observed was found to be IL-10 dependent. We (18) and others (19) recently demonstrated that modification of the bacterial populations of the gut alters the clinical outcome of EAE in mice. Oral treatment of mice with antibiotics reduced EAE severity by diminishing proinflammatory responses and the enhancement of Foxp3+Treg cells that significantly accumulated in mesenteric and cervical lymph nodes (LNs). Adoptive transfer of these IL-10–producing T regulatory (Treg) cells conferred protection against EAE. In this study, we investigate the effect of oral antibiotic treatment followed by gut reconstitution with a human isolate of B. fragilis that produces the zwitterionic capsular polysaccharide A (PSA), or with an isogenic mutant of B. fragilis deficient in the production of PSA in the development and protection against EAE. PSA has been found to be determinant in the regulatory effect of B. fragilis, restoring the default Th2-immune bias of germ-free animals (14, 16, 20). Moreover, IL-10–producing CD4+CD45RBlow T cells induced in response to PSA administration were protective in a Helicobacter hepaticus model of experimental colitis (21), and IL-10–producing Treg cells pro-
ective against EAE (22). In this study, we demonstrate that the absence of PSA production by a human isolate of \( B. \) \textit{fragilis} used to reconstitute disease-resistant mice restores clinical disease susceptibility, whereas the reconstitution with PSA-producing \( B. \) \textit{fragilis} maintains resistance by the induction of highly potent IL-10–producing T\(_{\text{reg}}\) cells. Our results suggest a potent regulatory role for this specific bacterial Ag in the control of CNS demyelination in this experimental model of human MS.

**Materials and Methods**

**Mice and treatments**

Female 6-wk old SJL/J mice were obtained from The Jackson Laboratories (Bar Harbor, ME). All animal care and procedures were in accordance with protocols and animal health and well-being. The animal supplier confirmed that all mice used in the studies were free of exposure to \textit{Helicobacter}. Dartmouth College Animal Resources Center institutional policies for animal health and well-being. All water, feed, and cages were used after sterilization and were not available, mice were maintained in a restricted, access-controlled environment.

**Bacteriologic analysis**

Serial dilutions of intestinal and fecal samples were collected 1 wk after the end of treatment with antibiotics and/or bacterial reconstitution and cultured in general bacteriologic agar plates (CDC blood agar; BD Diagnostic Systems, Sparks, MD) and \textit{Bacteroides} selective media plates (\textit{Bacteroides} Bile Esulin Agar; BD Diagnostics Systems) for 48 h at 37°C. Plates were cultured in aerobic and anaerobic conditions. Total bacteria per gram of sample was calculated based on the colony forming units counted in each serial dilution.

**EAE induction**

SJL mice were challenged s.c. with 200 \( \mu \text{g} \) PLP\(_{139–151}\) (Peptides International, Louisville, KY) in 200 \( \mu \text{M} \) CFA (Sigma-Aldrich, St. Louis, MO). On days 0 and 2 after challenge, mice received 200 ng \textit{Borrelia pernix} toxin i.p. (List Biological Laboratories, Campbell, CA) (24). Mice were monitored and scored daily for disease progression (24).

**Cytokine detection by Luminex and cytokine ELISA**

Luminex and specific cytokine ELISA were used to quantify triplicate sets of supernatants. Cells were cultured in 24-well tissue plates at 2 \( \times 10^6 \) cells/mL in the presence of anti-CD3/CD28 and IL-2 at different concentrations of TGF-\( \beta \) (0, 0.1, 0.5, and 5 ng/mL). To compare the role of CD11c\(^{\text{high}}\)/CD103\(^{+}\) cells in the conversion of CD4\(^{+}\)CD25\(^{+}\)T cells into Foxp3\(^{+}\)T cells, 1 \( \times 10^3 \) sorted CD103\(^{+}\)CD11c\(^{\text{high}}\) cells were cocultured with 5 \( \times 10^5 \) splenic naive CD4\(^{+}\)CD25\(^{+}\)T cells in the presence of anti-CD3 Ab (plates were precoated with 10 \( \mu \text{g}/\text{ml} \) anti-CD3 Ab [BD Biosciences]). Cultures were set in the presence of PBS, purified PSA (100 nmol/\( \mu \text{L} \)), or 4 nm retinoic acid (RA) and 5 ng/mL TGF-\( \beta \). Foxp3 acquisition by CD4\(^{+}\)T cells was measured by flow cytometry. For adoptive transfer experiments, 1 \( \times 10^5 \) in vitro Foxp3\(^{+}\)T cells were injected i.v. into naive recipients. EAE was induced 1 d after adoptive transfers.

**In vivo inactivation of CD25\(^{+}\) cells**

To inactivate CD25\(^{+}\)CD4\(^{+}\)T cells, mice were given 0.3 mg anti-CD25 mAb (ATCC No. TIB-222, clone PC 61.5.3) on days 4 and 2 before EAE challenge (24). As a control group, treated and naive mice received 0.3 mg purified rat IgG Ab. A separate control group was immunized with PBS 7 d prior to EAE challenge.

**Statistical analysis**

Kruskal-Wallis followed by Dunn’s comparison of multiple groups was applied to show differences in EAE clinical scores, cumulative clinical scores, luminex and ELISA detection of cytokines as well as in the flow

**FACS analysis**

Single cervical LN lymphocyte preparations were stained using conventional methods. T cell subsets were analyzed using fluorochrome-conjugated mAbs (BD Biosciences) for CD4 and CD25. Intracellular staining for Foxp3 was performed using fluorochrome-labeled anti-Foxp3 mAb (clone FJK-16s; eBioscience, San Diego, CA). Fluorochrome-labeled anti-rat IgG2a (eBioscience) isotype control for Foxp3 expression was used. Dendritic cells (DCs) were analyzed using CD11c, CD11b, and CD103 (BD Biosciences). Bound fluorescence was analyzed with an FACS Canto (BD Biosciences).

**Cell purifications**

CD11c\(^{+}\) cells were enriched with magnetic beads (StemCell Technologies, Vancouver, British Columbia, Canada) and then sorted (FACS Vantage with Turbo-Sort; BD Biosciences) after staining with FITC-anti-CD103 into CD11c\(^{+}\)/CD103\(^{+}\) cells. CD4\(^{+}\) T cells were obtained with magnetic beads (Dynal Biotech ASA, Oslo, Norway). The enriched CD4\(^{+}\) T cells were cell-sorted for CD4\(^{+}\)CD25\(^{+}\) T cells using FITC-anti-CD4 and PE-anti-CD25 mAbs (BD Biosciences).

**In vitro conversion assays and adoptive transfer experiments**

Cervical LN CD4\(^{+}\)CD25\(^{+}\) T cells were and cultured for 4 d in the presence of anti-CD3/CD28 and IL-2 at different concentrations of TGF-\( \beta \) (0, 0.1, 0.5, and 5 ng/mL). To compare the role of CD11c\(^{\text{high}}\)/CD103\(^{+}\) cells in the conversion of CD4\(^{+}\)CD25\(^{+}\) T cells into Foxp3\(^{+}\)T cells, 1 \( \times 10^7 \) sorted CD103\(^{+}\)CD11c\(^{\text{high}}\) cells were cocultured with 5 \( \times 10^5 \) splenic naive CD4\(^{+}\)CD25\(^{+}\) T cells in the presence of anti-CD3 Ab (plates were precoated with 10 \( \mu \text{g}/\text{ml} \) anti-CD3 Ab [BD Biosciences]). Cultures were set in the presence of PBS, purified PSA (100 \( \mu \text{g}/\text{ml} \)), or 4 nm retinoic acid (RA) and 5 ng/mL TGF-\( \beta \). Foxp3 acquisition by CD4\(^{+}\)T cells was measured by flow cytometry. For adoptive transfer experiments, 1 \( \times 10^5 \) in vitro Foxp3\(^{+}\)T cells converted cells were injected i.v. into naive recipients. EAE was induced 1 d after adoptive transfers.

**FIGURE 1.** Oral treatment with antibiotics significantly altered the numbers of gut bacteria. Reconstitution with both \( B. \) \textit{fragilis} strains restored the numbers. Oral treatment with antibiotics reduced the total numbers of bacteria present in the gut, compared with naive mice. Reconstitution with WT or PSA \( B. \) \textit{fragilis} restored those numbers (A). Depicted are the means ± SD from three separate experiments (\( n = 9 \) per group). \textit{Bacteroides}-selective media plates were used to confirm the colonization of the guts with \( B. \) \textit{fragilis} (B).
cytometry of Treg cell and DC experiments. The p values <0.05 and <0.01 are indicated.

Results
Reconstitution with PSA deficient B. fragilis restores the susceptibility to EAE

We have reported that oral antibiotic treatment can protect against CNS demyelination in a murine model of EAE (18). Four experimental groups were used throughout the studies: 1) mice treated orally with antibiotics for 1 wk, 2) mice treated with antibiotics and subsequently reconstituted with WT B. fragilis, 3) mice treated with antibiotics and subsequently reconstituted with ΔPSA B. fragilis, and 4) mice sham-treated with PBS. EAE was induced 1 wk after bacterial reconstitution or PBS sham-treated control. Oral antibiotic treatment significantly reduced the total bacterial numbers recovered (Fig. 1A). WT or ΔPSA B. fragilis persist and replicate similarly in vivo (14). The reconstitution of mice with either WT or ΔPSA B. fragilis restored the number of detectable bacteria (Fig. 1A). Bacteriologic culture of fecal samples in Bacteroides selective media showed that the treatment with antibiotics reduced the Bacteroides spp. counts when compared with naive mice whereas oral administration of WT or ΔPSA B. fragilis resulted in effective reconstitution of the gut (Fig. 1B).

We examined whether reconstitution of mice with WT or ΔPSA B. fragilis confers protection against EAE (18, 19). Oral treatment with antibiotics reduces EAE severity and cumulative scores, and it delays the clinical onset (18) (Fig. 2A, Table I). Reconstitution of antibiotic-treated mice with ΔPSA B. fragilis reversed protection by restoring susceptibility to disease. Mice reconstituted with the intact strain of B. fragilis were protected against disease and demonstrated reduced clinical severity when compared with the PBS control group or ΔPSA B. fragilis-reconstituted mice (Table I). There was no significant difference in the protection between treatment with antibiotics and antibiotic treatment followed by reconstitution with WT B. fragilis (Table I). In contrast, oral antibiotic treatment followed by reconstitution with ΔPSA B. fragilis resulted in clinically significant disease severity consistent with that observed in the PBS control group.

Cytokine analysis of CNS tissue derived from WT B. fragilis-reconstituted mice versus either control PBS-treated or ΔPSA B. fragilis-reconstituted mice was compared (Fig. 2B). T-box transcription factor Tbx21 (T-bet) and IFN-γ, both indicators of Th1 polarization, were reduced in mice treated with antibiotics compared with PBS treated mice, whereas GATA-3 and IL-13 expression were enhanced. There was a reduction in RORγt and IL-17–relative expression in mice treated with antibiotics, and in

Table I. Reconstitution with ΔPSA B. fragilis restores EAE susceptibility in mice previously treated with antibiotics

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>EAE (Mice/Total)</th>
<th>Day of Clinical Onset</th>
<th>Maximum Clinical Score</th>
<th>Cumulative Score*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>12/12</td>
<td>8.1 ± 0.9</td>
<td>5</td>
<td>64.2</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>10/12</td>
<td>9.9 ± 0.9*</td>
<td>2</td>
<td>12.5**</td>
</tr>
<tr>
<td>WT B. fragilis reconstituted</td>
<td>11/12</td>
<td>9.7 ± 1.1*</td>
<td>3</td>
<td>17.1**</td>
</tr>
<tr>
<td>ΔPSA B. fragilis reconstituted</td>
<td>12/12</td>
<td>8.2 ± 0.8***</td>
<td>5</td>
<td>59.3****</td>
</tr>
</tbody>
</table>

*SJL mice were treated with PBS, antibiotics, antibiotics and reconstitution with WT B. fragilis, and antibiotics and reconstitution with ΔPSA B. fragilis by oral gavage. Seven days after treatment, EAE was induced.

*The cumulative scores were calculated as the sum of all EAE clinical scores divided by the total number of mice per group (three experiments are combined).

*p < 0.05; Kruskal-Wallis test followed by Dunn’s multiple comparison test, for PBS versus antibiotics and PBS versus WT B. fragilis; **p < 0.01, Kruskal-Wallis test followed by Dunn’s multiple comparison test, for PBS versus antibiotics and PBS versus WT B. fragilis; ***p < 0.05, for antibiotics versus ΔPSA B. fragilis and WT B. fragilis versus ΔPSA B. fragilis; ****p < 0.05, for antibiotics versus ΔPSA B. fragilis and WT B. fragilis versus ΔPSA B. fragilis.
WT *B. fragilis*-reconstituted mice when compared with PBS-treated mice. Reconstitution with WT *B. fragilis* in EAE mice enhanced GATA-3, SMAD3, and IL-10 compared with PBS control mice. The profile of mice reconstituted with ΔPSA *B. fragilis* showed no alterations of cytokine relative expression compared with PBS control mice. Enhanced ROR*γt*, IL-17, and T-bet levels and reduced GATA-3, IL-10, and IL-13 levels of expression were detected in ΔPSA *B. fragilis*-reconstituted mice versus mice treated with antibiotics. When compared with WT *B. fragilis*, ΔPSA *B. fragilis*-reconstituted mice showed enhanced ROR*γt* and IL-17 as well as reduced SMAD-3 and IL-10.

Previous studies from our laboratory have demonstrated changes in cytokine production by cells in the cervical LNs of mice after oral antibiotic treatment (18). IL-13 levels were increased in mice treated with antibiotics compared with mice reconstituted with WT or ΔPSA *B. fragilis* (Fig. 3). The reconstitution with either

**FIGURE 3.** PSA-deficient *B. fragilis* reconstitution enhances production of IL-17 and IL-6 in cervical LN compared with mice treated with antibiotics. By contrast, colonization with WT *B. fragilis* enhanced the levels of IL-10 and IL-12 (p40). Both strains increased the levels of IFN-γ compared with mice treated with antibiotics. IL-10, GATA3, and SMAD3 levels were increased in WT *B. fragilis* compared with ΔPSA *B. fragilis*-reconstituted mice, whereas IL-6 was reduced. IL-13 levels were enhanced in mice treated with antibiotics compared with the rest of experimental groups. Cytokines released to the culture media were detected by Luminex (A), and relative expressions of GATA3, SMAD3, and IL-12 (p40) were detected by PCR (B). Depicted are the means ± SD from all data combined of three experiments (*n* = 8 per group). *p* < 0.05; **p** < 0.01.

**FIGURE 4.** *Bacteroides* reconstitution maintains the enhanced frequencies of Treg cells found in mice treated orally with antibiotics. Flow cytometry was used to compare the frequencies of Foxp3⁺CD25⁺ among total CD4⁺ T cells (A, B) gated on cervical LNs in PBS-treated mice, mice treated with antibiotics, and mice reconstituted with either WT or ΔPSA *B. fragilis*, 0, 3, and 7 d after treatments, and CD25⁺ T cells or CD25highCD4⁺ T cells (C, D) 7 d after bacterial reconstitution. Depicted are results for a representative experiment (A, C) and the means for a representative experiment from two separate experiments (*n* = 8 per group; B, D).
FIGURE 5. Bacteroides reconstitution depends on Treg cells to induce protection. In vivo depletion of CD25+ cells restores the susceptibility to EAE in animals reconstituted with WT B. fragilis. To inactivate CD25+ T cells, mice were given 0.3 mg anti-CD25 mAb (clone PC 61.5.3) on days 4 and 2 before EAE induction. As a control group, treated and naive mice received 0.3 mg purified rat IgG Ab. When EAE was induced, protection observed in mice treated with antibiotics and in mice reconstituted with WT B. fragilis was lost. Depicted are the means for a representative experiment from two separate experiments (n = 4 per group); the combination of all data [n = 8] from the experimental groups are represented in Table II. \( p < 0.01 \) for PBS/IgG versus WT B. fragilis-reconstituted IgG, PBS/IgG versus antibiotic-treated IgG, and PBS/IgG versus PBS/oCD25. \( p < 0.01 \), for WT B. fragilis reconstituted/IgG versus WT B. fragilis reconstituted/oCD25, and \( \Delta \)PSA B. fragilis reconstituted/IgG versus \( \Delta \)PSA B. fragilis reconstituted/oCD25.

WT or \( \Delta \)PSA B. fragilis induced the production of IFN-\( \gamma \), compared with mice treated with PBS and with antibiotics. Whereas treatment with antibiotics significantly reduced the levels of IL-17 and IL-6 produced compared with PBS treatment (18), re-treatment with antibiotics significantly augmented those levels. WT B. fragilis reconstitution induced enhanced levels of IL-10, IFN-\( \gamma \), IL-12 (p40) in cervical LN cells when compared with mice treated with antibiotics. IL-10 production and GATA3- and SMAD3-relative expressions in cells sorted from the cervical LNs of mice treated with antibiotics and in mice treated with antibiotics. IL-10 induced enhanced levels of IL-10, IFN-\( \gamma \), IL-12 (p40) in cervical LN cells when compared with mice treated with antibiotics.

WT B. fragilis reconstituted mice were also enhanced, compared with mice reconstituted with \( \Delta \)PSA B. fragilis and PBS-treated mice. Although reductions in TNF-\( \alpha \) and MCP-1 were observed in mice treated with antibiotics and reconstituted with WT B. fragilis, the differences were found to be not significant compared with PBS control mice and mice reconstituted with \( \Delta \)PSA B. fragilis (not shown). No significant changes were observed in IL-4, IL-5, MIP-1x, or MIP-1\( \beta \) levels (not shown).

**EAE regulation by PSA exposure depends on Treg cells**

We assessed whether PSA expression in B. fragilis could enhance the percentages of Foxp3+ Treg cells. Oral treatment with antibiotics increased the frequency of Foxp3+CD25+ in CD4+ T cells isolated from the cervical LN (18). No significant differences were observed in the frequency of Foxp3+CD25+ Treg cells detected in mice reconstituted with either WT or \( \Delta \)PSA B. fragilis, compared with mice treated with antibiotics (Fig. 4A, 4B). The frequency of Foxp3+ was enhanced in the CD25high versus the total CD25+ fraction of CD4+ T cells in all groups (Fig. 4C, 4D). Mice subjected to bacterial reconstitution showed similar frequencies of Foxp3+ Treg cell populations in the GALT (Peyer’s patches and mesenteric LN) and spleens to those observed in antibiotic treated mice (Supplemental Fig. 1). In vivo CD25+ cell depletion demonstrated a significant effect on the protection after reconstitution with WT B. fragilis and in mice treated with antibiotics, as shown before (18) (Fig. 5, Table II). Disease susceptibility after reconstitution with WT B. fragilis or in mice rendered susceptible after oral antibiotic treatment was enhanced when CD25+ T cells were depleted. Of interest was the increased severity of disease following reconstitution with WT B. fragilis compared with mice treated with antibiotics (Table II). As shown in both Fig. 2 and Table I, no significant differences were observed in the severity of EAE in mice reconstituted with \( \Delta \)PSA B. fragilis and in PBS-treated mice. Depletion of CD25+ cells induced equivalent increases in the cumulative disease indexes and mortality of both experimental groups.

\( \Delta \)PSA B. fragilis reduces T cell conversion into Foxp3+ Treg cells

We further analyzed the role of PSA in the acquisition of regulatory phenotypes by CD4+ T cells. We previously showed that alteration of the gut flora with antibiotics educated CD103+DCs to enhance the conversion of CD4+ T cells in Foxp3+ Treg cells (18). To compare the effect of B. fragilis reorganization in the induction of regulatory phenotypes of T cells, CD103-/+CD11c+DCs DCS were sorted from the cervical LNs of mice treated with antibiotics and from mice reconstituted with either WT or \( \Delta \)PSA B. fragilis. DCS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EAE (Mice/Total)</th>
<th>Day of Clinical Onset</th>
<th>Maximum Clinical Score</th>
<th>Mortality (Dead/Total)</th>
<th>Cumulative Score</th>
</tr>
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<tr>
<td>PBS/IgG</td>
<td>8/8</td>
<td>9.1 ± 0.6</td>
<td>5</td>
<td>2/8</td>
<td>56.2*</td>
</tr>
<tr>
<td>PBS/oCD25</td>
<td>8/8</td>
<td>7.8 ± 0.6**</td>
<td>5</td>
<td>6/8</td>
<td>95.2</td>
</tr>
<tr>
<td>Antibiotics/IgG</td>
<td>8/8</td>
<td>10.6 ± 0.5</td>
<td>2</td>
<td>0/8</td>
<td>11.7*</td>
</tr>
<tr>
<td>Antibiotics/oCD25</td>
<td>8/8</td>
<td>8.8 ± 0.6**</td>
<td>5</td>
<td>4/8</td>
<td>65.3</td>
</tr>
<tr>
<td>WT B. fragilis/IgG</td>
<td>8/8</td>
<td>11.1 ± 0.4</td>
<td>3</td>
<td>0/8</td>
<td>15.4*</td>
</tr>
<tr>
<td>WT B. fragilis/oCD25</td>
<td>8/8</td>
<td>8.5 ± 0.5**</td>
<td>5</td>
<td>4/8</td>
<td>82.5***</td>
</tr>
<tr>
<td>( \Delta )PSA B. fragilis/IgG</td>
<td>8/8</td>
<td>8.6 ± 0.5**</td>
<td>5</td>
<td>3/8</td>
<td>61.2</td>
</tr>
<tr>
<td>( \Delta )PSA B. fragilis/oCD25</td>
<td>8/8</td>
<td>8.1 ± 0.9</td>
<td>5</td>
<td>6/8</td>
<td>98.7***</td>
</tr>
</tbody>
</table>

*SJL mice were treated with PBS, antibiotics, antibiotics and reconstitution with WT B. fragilis, and antibiotics and reconstitution with \( \Delta \)PSA B. fragilis by oral gavage. Seven days after treatment, EAE was induced. Four and 2 d before prior disease induction, mice were treated with 0.3 mg anti-CD25 Ab or rat IgG isotype control.

The cumulative scores were calculated as the sum of all EAE clinical scores divided by the total number of mice per group (two experiments are combined).

\( * p < 0.01 \), Kruskal-Wallis test followed by Dunn’s multiple comparison test, for PBS/IgG versus PBS/oCD25, PBS/IgG versus antibiotics/IgG, PBS/IgG versus Antibiotics/oCD25; and PBS/IgG versus WT B. fragilis/IgG. \( ** p < 0.05 \), Kruskal-Wallis test followed by Dunn’s multiple comparison test, for PBS/IgG versus PBS/oCD25, antibiotics/IgG versus antibiotics/oCD25, and WT B. fragilis/IgG versus WT B. fragilis/oCD25. \( *** p < 0.05 \), for WT B. fragilis/IgG versus \( \Delta \)PSA B. fragilis/IgG. \( **** p < 0.01 \), for antibiotics/oCD25 versus WT B. fragilis/oCD25 and antibiotics/oCD25 versus \( \Delta \)PSA B. fragilis/oCD25.
were cocultured in anti-CD3 Ab precoated plates with splenic CD4+CD25+ (∼10% Foxp3+) T cells in the presence of PBS or purified PSA (100 μg/ml), or with RA (4 nM) and TGF-β (5 ng/ml). Foxp3 acquisition by CD4+ T cells was measured by flow cytometry. Depicted are (A) the results from a representative experiment and (B) the combination of all data (mean ± SD; n = 6 per group). *p < 0.05; **p < 0.01.

FIGURE 6. CD103+ DCs of PSA-deficient B. fragilis have reduced ability to induce Foxp3+ Treg cell conversion in vitro. DCs were harvested from cervical LNs of mice treated with antibiotics, and mice reconstituted with either WT or ΔPSA B. fragilis and sorted into CD103+CD11c+ DCs. Cells were cocultured with CD4+CD25+ (~10% Foxp3+) T cells sorted from spleens of naive mice in the presence of PBS, purified PSA (100 μg/ml), or with RA (4 nM) and TGF-β (5 ng/ml). Foxp3 acquisition by CD4+ T cells was measured by flow cytometry. Depicted are (A) the results from a representative experiment and (B) the combination of all data (mean ± SD; n = 6 per group). *p < 0.05; **p < 0.01.

FIGURE 7. IL-10 producing Foxp3+ Treg cells induced by PSA-producing B. fragilis reconstitution protect against EAE. Cervical LN CD4+CD25+ (∼10% Foxp3+) T cells from mice treated with PBS, mice treated with antibiotics, and mice reconstituted with either WT or ΔPSA B. fragilis were cultured in the presence of anti-CD3/anti-CD28 Abs, IL-2, and increasing concentrations of TGF-β (0, 0.1, 0.5, and 5 ng/ml). Flow cytometry was used to compare the conversion rates of CD4+CD25+ (~10% Foxp3+) T cells into Foxp3+ Treg cells. Cells obtained from mice reconstituted with WT B. fragilis and cultured with RA (4 nM) and TGF-β (5 ng/ml) are used to show the acquisition of CD25+ by CD4+ T cells, the isotype control for Foxp3 intracellular staining, and the gating representing the frequency of CD4+ cells that acquired a Foxp3+CD25+ phenotype (A). Supplemental Fig. 2 shows representative results of Foxp3+CD25+ cells gated on CD4+ T cells for all experimental groups. Cervical LN CD4+CD25+ (∼10% Foxp3+) T cells obtained from mice reconstituted with WT B. fragilis showed enhanced rates of conversion into CD25+Foxp3+ cells versus cells sorted from PBS treated mice, mice treated with antibiotics, and mice reconstituted with ΔPSA B. fragilis, when cultured with 0.5 and 5 ng/ml of TGF-β. B. The column graph represents the combined results (n = 8 per group) for the frequency of Foxp3+CD25+ cells. The TGF-β concentrations are represented in the horizontal axes. *p < 0.01. C, IFN-γ, IL-17, IL-10, and IL-13 cytokines were measured by specific ELISA in the supernatants of Foxp3+ converted cells upon stimulation with 5 ng/ml of TGF-β (n = 8 per group). *p < 0.01. D, Foxp3+ converted cells after stimulation with 5 ng/ml of TGF-β were adoptively transferred (1 × 10^6 cells per mouse) into naive recipient mice, and EAE was induced one day later. Depicted are the combined results from two separate experiments (n = 8, per group). *p < 0.01.
reconstituted mice showed enhanced T\textsubscript{reg} conversion rates compared with cells obtained from mice reconstituted with ΔPSA \textit{B. fragilis}, mice treated with antibiotics, and PBS-treated mice, when cultured with 0.5 and 5 ng/ml TGF-β. Conversion rates were significantly enhanced in all groups when TGF-β and RA approached the optimal concentration (Supplemental Fig. 2) (25).

We compared the capacity of these converted Foxp3\textsuperscript{+}T\textsubscript{reg} cells to protect against EAE (Fig. 7D). Cells cultured with 5 ng/ml TGF-β were collected after 4 d and adoptively transferred into naïve recipient mice. Cells converted from WT \textit{B. fragilis}-reconstituted mice protected against EAE induction, whereas no protection was observed in cells converted from PBS, mice treated with antibiotics, or ΔPSA \textit{B. fragilis} reconstituted mice. Cells converted from WT \textit{B. fragilis}-reconstituted mice produced significantly increased levels of IL-10 compared with PBS-treated mice, mice treated with antibiotics, and ΔPSA \textit{B. fragilis} reconstituted mice; they produced a modest but significant increase in TGF-β compared with ΔPSA \textit{B. fragilis}-reconstituted mice (Fig. 7C). No significant differences in the production of IFN-γ, IL-17, IL-6, and IL-13 were observed.

### Discussion

Alterations of the gut commensal bacteria populations by oral treatment with antibiotics can influence the development of EAE (18, 19). We now demonstrate that the reconstitution of mice with \textit{B. fragilis} deficient in the production of the zwitterionic capsular PSA restores disease susceptibility in mice that had been rendered resistant to disease after treatment with oral antibiotics. Reconstitution with both \textit{B. fragilis} strains similarly restored the numbers of detectable bacteria, which were significantly reduced after oral treatment with antibiotics. It has been shown that both WT and ΔPSA \textit{B. fragilis} persist and replicate equally in vivo (14), suggesting that it was PSA and not the number nor strain of \textit{B. fragilis} that was responsible for disease protection or susceptibility.

Oral treatment with antibiotics enhanced the frequency of Foxp3\textsuperscript{+}T\textsubscript{reg} cells within the cervical LN and reduced Th17 responses (18). The alterations of Th17 responses upon oral treatment of antibiotics have been recently confirmed by others (26, 27). After antibiotic treatment, reconstitution with either WT or ΔPSA \textit{B. fragilis} resulted in a similar number and frequency of T\textsubscript{reg} cells. In MS, the in vitro conversion rates of CD4\textsuperscript{+} T cells into T\textsubscript{reg} cells are significantly reduced in MS patients compared with healthy controls (28). Moreover, functional suppression appears to be impaired (29). CD4\textsuperscript{+} T cells obtained from PSA-producing \textit{B. fragilis}-reconstituted mice more efficiently converted into Foxp3\textsuperscript{+}T\textsubscript{reg} cells, with increased IL-10 production and enhanced protective potency after adoptive transfer.

Our results suggest that deficient PSA production could influence the functional role of DCs and Foxp3\textsuperscript{+}T\textsubscript{reg} cells induced by \textit{B. fragilis}. Prior studies have demonstrated that CD4\textsuperscript{+} T cell activation by PSA is dependent on the presentation of the Ag by CD11c\textsuperscript{+} DCs (30). Foxp3\textsuperscript{+}T\textsubscript{reg} cell conversion by CD103\textsuperscript{+} DCs purified from PSA-deficient \textit{B. fragilis}-reconstituted mice was significantly reduced compared with DCs from PSA-producing \textit{B. fragilis}. Foxp3\textsuperscript{+} T\textsubscript{reg} cell conversion studies showed enhanced conversion rates of CD4\textsuperscript{+} T cells obtained from WT \textit{B. fragilis}-reconstituted mice. SMAD3 was significantly increased in WT \textit{B. fragilis}-reconstituted mice, and IL-6 was substantially reduced compared with ΔPSA \textit{B. fragilis}-reconstituted mice. Differentiation of Th17 cells requires TGF-β and IL-6, whereas TGF-β is also required for T\textsubscript{reg} cell induction in the absence of IL-6 (31). The differences observed in the conversion rates could be due to the potential capability of WT \textit{B. fragilis} CD4\textsuperscript{+} T cells to enhance TGF-β in cultures that could facilitate their conversion into Foxp3\textsuperscript{+}T\textsubscript{reg} cells.

PSA may influence a distinct pathway involved in disease protection, as suggested by our experiments of T\textsubscript{reg} neutralization. CD27\textsuperscript{+} cell depletion exacerbated EAE in all groups, but the enhancement of disease severity was significantly higher in mice treated with antibiotics compared with WT \textit{B. fragilis}-reconstituted mice. Other subpopulations of regulatory cells such as NKT cells may participate in the protection against disease-induced treatment with oral antibiotics (19). Recent observations from our laboratory suggest that B cells could be important in this protective response (32). IL-13 production was reduced in mice reconstituted with WT \textit{B. fragilis} compared with mice treated with antibiotics, and only IL-10–producing T\textsubscript{reg} cells converted from Foxp3\textsuperscript{+}CD4\textsuperscript{+} T cells of WT \textit{B. fragilis}-reconstituted mice protected against EAE. Foxp3\textsuperscript{+}T\textsubscript{reg} cells that were derived from CD4\textsuperscript{+} T cells of PSA-deficient \textit{B. fragilis}-reconstituted mice failed to protect against the disease. We recently demonstrated that a highly purified preparation of PSA is protective against EAE in conventional mice and that this protection is completely abrogated in IL-10–deficient mice, suggesting an important role of this cytokine in the PSA-induced control of the disease (22).

The presence or absence of PSA could determine protective or pathogenic outcomes in EAE. WT or ΔPSA \textit{B. fragilis} reconstitution induced production of IFN-γ, when compared with mice treated with PBS and with antibiotics; however, IL-10 production was enhanced only after reconstitution with WT \textit{B. fragilis}, whereas PSA-deficient \textit{B. fragilis} reconstitution induced enhanced levels of IL-6. The imbalance created by alterations of PSA expression within the gut lumen may lead to peripheral systemic autoimmune disorders, such as EAE or human MS. In the absence of PSA, the human commensal \textit{B. fragilis} can no longer regulate immune homeostasis, leading to autoimmune disease of the intestine, as described recently (21) and as reported in this study in the CNS. Our previous (18, 22, 32) and present studies suggest that differing compositions of gut microbiota could regulate the balance between protection and disease induction in MS and may offer a novel therapeutic approach for disease intervention.

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### Disclosures

The authors have no financial conflicts of interest.

### References

Supplemental Figure 1: Flow cytometry analysis of Peyer’s patches, mesenteric LN and spleens of mice treated with PBS, mice treated with antibiotics, and mice treated with antibiotics and reconstituted with WT B. fragilis or ΔPSA B. fragilis.

Supplemental Figure 2: Treg conversion assay shown in the figure 6. CD4+CD25- T cells were sorted from the cervical LN of mice treated with PBS, mice treated with antibiotics, and mice treated with antibiotics and reconstituted with WT B. fragilis or ΔPSA B. fragilis. Cells were cultured with anti-CD3/anti-CD28 antibodies, IL-2. Transforming-growth factor beta (TGF-β; none, 0.1, 0.5 and 5 ng/mg) and retinoic acid (RA; none or 4nM) were added to the cultures. After four days of culture, cells were collected and flow cytometry was performed to compare the acquisition of a FoxP3+ phenotype in CD4+CD25+ T cells. Results show the frequency of FoxP3+CD25+ on the gated CD4+ T cells.
Supplemental Fig. 2

Gated on CD4+

0 nM retinoic acid

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4 nM retinoic acid

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TGF-β (ng/μl)

FoxP3

CD25

IgG2a

CD25