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Regulatory T (Treg) cells show promise for treating autoimmune diseases, but their induction to elevated potency has been problematic when the most optimally derived cells are from diseased animals. To circumvent reliance on autoantigen-reactive Treg cells, stimulation to myelin-independent Ags may offer a viable alternative while maintaining potency to treat experimental autoimmune encephalomyelitis (EAE). The experimental Salmonella vaccine expressing colonization factor Ag I possesses anti-inflammatory properties and, when applied therapeutically, reduces further development of EAE in SJL mice. To ascertain Treg cell dependency, a kinetic analysis was performed showing increased levels of FoxP3+CD25+CD4+ T cells. Inactivation of these Treg cells resulted in loss of protection. Adoptive transfer of the vaccine-induced Treg cells protected mice against EAE with greater potency than naive or Salmonella vector-induced Treg cells, and cytokine analysis revealed enhanced production of TGF-β, not IL-10. The development of these Treg cells in conjunction with immune deviation by Th2 cells optimally induced protective Treg cells when compared those induced in the absence of Th2 cells. These data show that Treg cells can be induced to high potency to non-disease-inducing Ags using a bacterial vaccine. The Journal of Immunology, 2007, 178: 1791–1799.

Multiple sclerosis (MS) is a human inflammatory disease of the CNS that remains problematic because of no curative treatment (1). This neurodegenerative disease is characterized by perivascular inflammatory lesions, demyelination, and axonal damage (2). Experimental autoimmune encephalomyelitis (EAE) has many features similar to MS and can be induced by active immunization with specific myelin Ags, including myelin basic protein (3, 4), proteolipid protein (PLP) (5–7), and myelin oligodendrocyte glycoprotein (8, 9) or passively by adoptive transfer with encephalitogenic CD4+ Th1 cells (10). These encephalitogenic T cells secrete the proinflammatory cytokines, IFN-γ, TNF-α, and IL-2, resulting in macrophage and microglial activation, infiltration of inflammatory cells into the CNS, and eventual demyelination (11, 12). Protection against EAE can be induced by adoptive transfer of myelin-specific Th2 cells or by immunization with altered peptide ligands bearing reactive T cell epitopes that reverse the development of Th1 cells. Thus, promotion of Th2 cell induction can neutralize the development of proinflammatory encephalitogenic T cells.

Regulatory T (Treg) cells were originally described in neonatally thymectomized mice that showed increased susceptibility to autoimmune diseases (13, 14). Consequently, these cells have been shown to be important for maintenance of peripheral tolerance and ultimately protect against colitis (15), arthritis (16), and EAE (17–23). In contrast, some viruses have subverted Treg cells to dampen host responses to permit viral persistence (24, 25). Nonetheless, the expectation is that these Treg cells can serve as a potential therapeutic for treating autoimmunity. In particular, these cells have been shown to effectively reverse EAE, but depending upon their source, innate vs inducible Treg cells (26), variable amounts are required (17, 18, 22, 27). Thus, the issue of potency becomes essential in attempting to stimulate Treg cells with sufficient potency that can be readily induced and not require their expansion from diseased animals (18).

To address this feasibility, we tested whether our Salmonella vaccines could be adapted for stimulating Treg cells, which could readily address the demand for stimulating Treg cells against defined, irrelevant Ags and not against autoantigens. Because Treg cells share anti-inflammatory properties with Th2 cells, it is possible that the Salmonella-colonization factor Ag I (CFA/I) vaccine could possess the ability to elicit Treg cells. Immunization with innocuous or vaccine Ags as subunits or as shown here, with a live vaccine, could provide an alternative for Treg cell stimulation. We hypothesize the presence of a Th2 cell-promoting environment (28–31) favors the development of Treg cells as a consequence of vaccination and can allow further development of myelin-specific Treg cells. In the present work, we tested whether Salmonella-CFA/I could orally treat EAE, and we can demonstrate the critical role of Treg cells induced by Salmonella vaccines. Although only partial protection was observed after adoptive transfer of Treg cells obtained from mice immunized with the Salmonella vector, our results clearly demonstrate that Treg cells induced by immunization with Salmonella-CFA/I provide optimal protection.
Materials and Methods

Mice

Female SJL/J mice (6 wk old) were obtained from The Jackson Laboratory. All mice were maintained at Montana State University Animal Resources Center under pathogen-free conditions in individual ventilated cages under HEPA-filtered barrier conditions and were fed sterile food and water ad libitum. All animal care and procedures were in accordance with institutional policies for animal health and well-being.

For each experiment, female SJL mice (five per group) were challenged s.c. with 200 μl of PLP139–151 (200 μg; Ref. 28). On days 0 and 2 postchallenge, mice received i.p. 200 ng of Bordetella pertussis toxin (PT; List Biological Laboratories). Six days after PLP139–151 challenge, mice were given a single oral dose of 5 × 10^6 CFU of the Salmonella-CFA/I vaccine (Arthrobacter salmoni enterica serovar Typhimurium-CFA/I vector vaccine, strain H696, expressing functional CFA/I fimbriae from Escherichia coli; Ref. 32) or its isogenic control strain H647 (Salmonella vaccine; Ref. 32). Fimbriate expression was maintained by a plasmid bearing a functional asd gene to complement the lethal chromosomal asd mutation in the parent Salmonella strain. Control groups were treated with PBS. Mice were monitored and scored daily for disease progression (28, 33): 0, normal; 1, a limp tail; 2, hind limb weakness; 3, hind limb paresis; and 4, quadriplegia; and 5, death.

Histological evaluation of spinal cords

For histological evaluation of tissue pathology, spinal cords were removed 14 days after challenge and fixed with neutral-buffered formalin (VWR International), embedded into paraffin, and sectioned at 5 μm. Transverse sections of spinal cords were stained with H&E for pathological changes and inflammatory cell infiltration. Adjacent sections were stained with luxol fast blue (LFB) and examined for loss of myelin. Pathological manifestations were scored separately for cell infiltrates and demyelination. Each H&E section was scored from 0 to 4: 0, normal; 1, cell infiltrate into the meninges; 2, one to four small focal perivascular infiltrates; 3, five or more small focal perivascular infiltrates and/or one or more large infiltrates invading the parenchyma; and 4, extensive cell infiltrates involving ≥20% of the spinal cord matter (28).

Cytokine ELISA

Spleens and cervical lymph nodes (CLN) were aseptically removed 14 days after challenge from PBS-, H647-, and H696-treated groups of mice. Lymphocytes were prepared, as previously described, and resuspended in complete medium (CM): RPMI 1640 medium supplemented with 1 mM sodium pyruvate, 1 mM nonessential amino acids, penicillin/streptomycin (100 U/ml), and 10% FBS (Atlanta Biologicals). Lymphocytes were cultured in 24-well tissue culture plates at 5 × 10^5 cells/ml in CM alone or in the presence of OVA (10 μg/ml; Sigma-Aldrich), CFA/I fimbriae (10 μg/ml), or PLP139–151 peptide (30 μg/ml) in a total volume of 1 ml for 60 h at 37°C. The supernatants were collected by centrifugation and stored at −80°C. Captured ELISA was used to quantify, on duplicate sets of samples, the levels of INF-γ, IL-4, IL-10, and IL-13 produced by lymphocytes, as described previously (28). For the TGF-β ELISA, wells were coated with 10 μg/ml anti-TGF-β mAb (clone 1D11; R&D Systems) and, for IL-17 ELISA, 2 μg/ml anti-IL-17 mAb (clone TNC11-18H10; BD Pharmingen) overnight at 4°C. After blocking with PBS plus 1% BSA for 2 h at 37°C, washed wells were incubated with cell culture supernatants at 4°C for 24 h. After washing, 5.0 μg/ml biotinylated chicken anti-human TGF-β1 Ab (BD Systems) or 0.5 μg/ml biotinylated rat anti-mouse IL-17 Ab (clone TNC11-8H4; BD Pharmingen) were added for 2 h at 37°C. Following washing, 1:1000 HRP-goat anti-biotin Ab (Vector Laboratories) was added for 1 h at room temperature. After washing, ABTS peroxidase substrate (Moss) was added to develop the reaction.

FACS analysis

Lymphocytes from the CLN, submaxillary gland LN (SMLN), Peyer’s patches (PP), mesenteric LN (MLN), and spleens were isolated 14 days after challenge and single cell preparations were prepared, as described previously (28). To obtain lymphocytes from spinal cords, mice were perfused through the left ventricle with 20 ml of cold PBS, and spinal cords were removed by flushing the vertebral canal with medium. Spinal cords were forced through 70-μm nylon mesh (BD Falcon). The single suspensions were incubated 75 min (37°C) in HEPES-buffered medium containing collagenase (300 U/ml; Sigma-Aldrich). The homogenates were resuspended in 20% Percoll (Sigma-Aldrich) and underlaid with 80% Percoll. The gradients were centrifuged at 2500 × g at 20°C for 20 min. Lymphocytes were collected from the Percoll interface, washed, and resuspended in FACS buffer.

Cells were stained for FACS analysis using conventional methods. To distinguish between neutrophils, monocytes/macrophages, and lymphocytes, staining for CD45 and MHC class II was done as described previously (34–37). Leukocyte gates were set within the forward and side scatter profiles to exclude resting microglia cells from the spinal cord preparations. To detect neutrophils, cells were stained with SK208 mAb (28), followed by FITC-concanavalin acid-R-phycoerythrin (BD Pharmingen) and, for macrophages, fluorochrome-conjugated anti-CD11b (BD Pharmingen) and, for chondocytes, fluorochrome-conjugated CD45 (clone 30-F11; BD Pharmingen), fluorochrome-conjugated I-A^+ (clone 10-3.6; BD Pharmingen), and FITC-anti-CD11b mAb and PE-F4/80 mAb (Serotec). T cells were analyzed using fluorochrome-conjugated mAbs (BD Pharmingen) for CD4, CD25, TCRβ, and CD8. Intracellular staining for FoxP3 was accomplished using PE-anti-FoxP3 mAb (clone FJK-16s; eBioscience). Bound fluorescence was analyzed with a FACSCalibur (BD Biosciences).

In vivo inactivation of Treg cells

Mice were orally immunized 7 days before EAE challenge with PLP139–151 and PT. To deplete/inactivate CD25+CD4+ T cells, the same mice were given i.p. 1.0 mg of anti-CD25 mAb (clone PC 61.5.3; ATCC TIB-222) on days 0 and 2 before EAE challenge. As a control group, vaccinated mice received 1.0 mg of purified rat IgG Ab on the same days before EAE challenge. A separate control group was immunized with PBS 7 days before EAE challenge. All mice were monitored daily for development of EAE.

Adoptive transfer studies

Fourteen days after oral immunization with H696 or H647, CD4+ T cells from spleens, head and neck LN (HNLN), and MLN were obtained (negative CD4+ T cell isolation kit; Dynal Biotech). CD25+CD4+ cells and CD25–CD4+ T cells were isolated to >94 and 99%, respectively, by cell sorting (FACSVerse; BD Biosciences) of stained T cells. To test Treg cell efficacy, 6 × 10^5 CD25+CD4+ T cells or CD25+CD4– T cells were i.v. injected into naive recipients. Separate groups of mice were orally dosed with Salmonella-CFA/I, the Salmonella vaccine vector, or PBS 7 days before PLP139–151 challenge. One day after the adoptive transfer of T cells, mice were challenged with PLP139–151. Naive CD25+CD4+ and CD25–CD4+ T cells were also tested in a PLP139–151 challenge.

In vitro T cell assays

To test Treg cell activity, 1 × 10^6 responder (CD25+CD4+) T cells were cocultured in triplicate with 5 × 10^5 Treg cells. Feeder cells (T cell-depleted mitomycin C-treated), splenocytes prepared from naive SJL mice (32), were added at 1 × 10^5 cells/well. Cells were cocultured with or without 10 μg/ml purified CFA/I fimbriae (32). Cells were incubated at 37°C in 5% of CO2 for 72 h before pulsed with 1 μCi of [3H]Tdr for 18 h at 37°C with a 5% of C2. [3H]Tdr incorporated was measured by scintillation counting.

To assess cytokine production by Treg and effector T cells, CD25+CD4+ T cells and CD25–CD4+ T cells (2 × 10^5) were stimulated in vitro with anti-CD3 mAb-coated wells (10 μg/ml; BD Pharmingen) plus a soluble anti-CD28 mAb (5.0 μg/ml; BD Pharmingen) for 5 days in CM (final volume of 300 μl in a 48-well plate). Capture ELISA was used to quantify triplicate sets of samples to measure cytokines.

Statistical analysis

The ANOVA followed by a post hoc Tukey test was applied to show differences in clinical scores in treated vs PBS mice and in the Treg cell kinetic experiments. The Student t test was used to evaluate the differences between variations in cytokine level production.
Table I. Therapeutic treatment with Salmonella vaccines after PLP139-151 challenge protects SJL/J mice from EAE

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EAE/Total</th>
<th>Onset</th>
<th>Maximum Score</th>
<th>Cumulative Scores</th>
<th>Inflammation</th>
<th>Demyelination</th>
<th>Percentage of CD4+ T Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>20/20</td>
<td>10.4 ± 0.6</td>
<td>5</td>
<td>30.6</td>
<td>2.2 ± 0.8</td>
<td>3.3 ± 0.3</td>
<td>4.9 ± 1.3</td>
</tr>
<tr>
<td>H647</td>
<td>19/19</td>
<td>11.1 ± 0.1</td>
<td>3</td>
<td>11.75*</td>
<td>2.0 ± 0.4</td>
<td>2.7 ± 0.2</td>
<td>0.6 ± 0.1*</td>
</tr>
<tr>
<td>H696</td>
<td>19/19</td>
<td>11.2 ± 1.2</td>
<td>2</td>
<td>6.6**</td>
<td>0.7 ± 0.6**</td>
<td>1.1 ± 0.6**</td>
<td>0.0 ± 0.0**</td>
</tr>
</tbody>
</table>

a SJL/J mice were challenged s.c. with 200 μg of PLP139-151 in CFA plus 200 ng of PT i.p. on days 0 and 2.

b Mice were immunized 6 days postchallenge with PBS or 5 × 10⁶ CFU of S. enterica Typhimurium H647 (vector) or H696 (CFA/I fimbrae).

c Number of mice with EAE/total in group.

d Mean day ± SD of clinical disease onset.

e Cumulative scores were calculated as the sum of all scores from disease onset to day 25 postchallenge and divided by the number of mice in each group.

f Mean day onset among the three treatment groups was not different, peaking between days 13 and 15 after PLP139-151 challenge.

g Mean score ± SEM of clinical disease onset.

h Mean score ± SEM of inflammation: the infiltration of nucleated cells into spinal cords was scored from 0 to 4 in each mouse separately, and the mean score and SEM were calculated. * p < 0.001 for PBS vs H696, PBS vs H647, and H647 vs H696.

i Mean score ± SEM of demyelination: the demyelination in spinal cords was scored from 0 to 4 in each mouse separately, and the mean score and SEM were calculated. * p < 0.001 for PBS vs H696 and H647 vs H696.

j Percentage of CD4+ T cells from the total cells in spinal cords and analyzed by FACS (Fig. 1C). * p < 0.001 for PBS vs H647, PBS vs H696, and H647 vs H696.

Results
Resolution of EAE after oral treatment with Salmonella-CFA/I (H696) in SJL mice

SJL mice were subjected to conventional PLP139-151 challenge, and 6 days later, mice were either treated with PBS, the isogenic Salmonella vaccine vector (strain H647), or the anti-inflammatory vaccine, Salmonella-CFA/I (strain H696). While the mean day onset among the three treatment groups was not different, peaking between days 13 and 15 after PLP139-151 challenge (Table I), mice treated with strain H696 showed reduced clinical scores and reduced disease duration (p < 0.001; Table I and Fig. 1A) when compared with the PBS-treated group. To a lesser degree, the H647 also showed a reduction in the maximum clinical score (Table I) and significant differences (p < 0.001) in the disease kinetics (Fig. 1A). Yet, disease in these mice was more severe than in the H696-treated group, as evidenced by the cumulative clinical scores: 28.5, 11.3, and 6.8 for PBS-, H647-, and H696-treated groups, respectively (Table I).

Treatment with Salmonella-CFA/I reduces inflammatory cell infiltration into the CNS

During EAE, autoaggressive myelin-reactive T lymphocytes migrate into the CNS where they recognize their target Ag and initiate an inflammatory response, thus provoking tissue damage. Histopathological analyses performed on spinal cords from the different treatment groups revealed that mice treated with Salmonella-CFA/I showed minimal inflammatory cell infiltration and demyelination when compared with the PBS-treated group (p < 0.001; Fig. 1B and Table I). Mice treated with the Salmonella vector showed no significant differences when compared with the PBS-treated group, suggesting that treatment with the Salmonella vector was insufficient to diminish inflammation and prevent tissue damage.
FACS analysis of inflammatory cells obtained from spinal cords from treated mice was performed. Consistent with the histopathology analysis, Salmonella-CFA/I-treated mice at 14 days after challenge (8 days after treatment) failed to show inflammatory cell infiltration (Fig. 1C). In contrast, both PBS- and Salmonella vector-treated mice showed marked infiltration by neutrophils (CD11b$^+$ SK208$^+$), macrophages (CD11b$^+$ F4/80$^+$), and T cells (CD4$^+$ TCR$^+$ and CD4$^+$ TCR$^+$). Although the H647 showed a reduced clinical score, it failed to prevent inflammatory cell infiltration and demyelination.

Salmonella-CFA/I reduces inflammatory PLP$^{139–151}$-specific CNS damage

PLP$^{139–151}$-specific immune deviation from Th1-type to Th2-type responses by Salmonella-CFA/I was previously shown for prophylactic vaccination of SJL mice against EAE (28). We tested whether the reduction of the EAE clinical scores in H696-treated mice was relevant to the observed protection (Fig. 1 and Table I). Ag restimulation cultures were conducted to ascertain the cytokine profiles in response to PLP$^{139–151}$ peptide (Fig. 2). Following in vitro Ag pulsing, lymphocytes from H696-treated mice spleens and CLN showed an enhanced production of IL-4 and IL-13 with concomitant reduction in IFN-γ when compared with PBS ($p < 0.001$)- or H647-treated mice ($p < 0.001$). The CLN from H696-treated mice showed significant reduction in the production of proinflammatory cytokine, IL-17 ($p < 0.001$), when compared with PBS-treated mice (Fig. 2B). An increase in IL-10 production was only evident in the CLN from H696 ($p < 0.001$)- and to a lesser degree by H647-treated mice ($p < 0.05$; Fig. 2B). PBS- and H647-treated mice showed mostly a Th1-type dominant response with minimal Th2-type cytokines. Thus, the oral Salmonella-CFA/I vaccine can cause immune deviation, particularly in the CLN, a major site for the production of PLP$^{139–151}$-specific T cell activation.

Salmonella-CFA/I vaccination elicits T$_{reg}$ cells and protect against EAE

To ascertain whether T$_{reg}$ cells are induced as a result of Salmonella-CFA/I vaccination, CLN from challenged, then vaccinated, mice were evaluated for the presence of T$_{reg}$ cells. Although it was
evident that all treatment groups stimulated Treg cell induction (Fig. 3A), the percentages of Treg cells in nearly all tissues examined showed an enhanced number of Treg cells in the H696-treated mice when compared with PBS-treated mice (Fig. 3B). Subsequent analysis revealed that >90% of the Treg cells were FoxP3+, and these FoxP3+ Treg cells increased after PLP<sub>139–151</sub> challenge (Fig. 3A). The observed increase of Treg cells in the PBS-treated group was similar to that previously reported (18) and might explain the recovery from EAE observed in SJL mice. In addition, we analyzed whether the Salmonella-CFA/I vaccine by itself could induce Treg cells independent of PLP<sub>139–151</sub> challenge (Fig. 3, C and D). Although the magnitude of induced Treg cells of the total lymphocytes was less, the percentage of FoxP3+ remained elevated, >90%. Thus, oral vaccination with H696 can elicit Treg cells.

To assess the functionality of the observed Treg cells and whether these could suppress CD4<sup>+</sup> T cell responses, CD25<sup>+</sup> CD4<sup>+</sup> and CD25<sup>-</sup> CD4<sup>+</sup> T cells were purified from spleens, MLN, and HNLN 14 days after oral immunization with Salmonella-CFA/I. An anti-CFA/I proliferation assay was performed and showed the inhibitory effect of the CD25<sup>+</sup> CD4<sup>+</sup> T cells in each of the tissues, and 60, 75, and 50% inhibition was observed with Treg cells isolated from spleen, MLN, and HNLN, respectively (Fig. 4A). These results demonstrated that indeed oral immunization with the Salmonella-CFA/I vaccine induces the expansion of CD25<sup>+</sup> CD4<sup>+</sup> T cells, and these Treg cells could contribute to the observed recovery from EAE.

The in vivo role of CD25<sup>+</sup> CD4<sup>+</sup> T cells in the recovery of EAE after the immunization with Salmonella-CFA/I was analyzed in CD25<sup>-</sup>-blocked mice. SJL mice were orally immunized with H696 vaccine 7 days before PLP<sub>139–151</sub> challenge (day 0). On days −5 and −2, they were treated with anti-CD25 mAb or rat IgG. This treatment paradigm with the anti-CD25 mAb was designed to ascertain how the loss of the induced Treg cell function subsequent to
oral immunization with *Salmonella*-CFA/I would impact the recovery from EAE. As a result, the inactivation of CD25+ T cells provoked an increased disease severity in which all of the mice succumbed to EAE within 14 days after challenge (Fig. 4B). The clinical scores of the treated mice were significantly greater (p < 0.001) than mice treated with the rat IgG or the PBS-treated mice, suggesting that Treg cells are essential in the recovery from EAE, and these cells supercede or contribute to the immune deviation by *Salmonella*-CFA/I vaccination.

Salmonella-induced, not innate Treg cells are protective against EAE

Studies have shown that Treg cells obtained from EAE-diseased mice and adoptively transferred into naive mice protected against subsequent EAE induction (17, 18, 23). Such results suggest that Treg cells can reduce the severity in an inflammatory disease. While these past studies focused on anti-encephalitogenic Treg cells, the studies from Fig. 3 suggest that the protective Treg cells are independent of myelin T cell epitopes. To directly determine the protective capacity of the vaccine-induced Treg cells, CD25+ CD4+ T cells were allowed to develop 14 days after oral vaccination with *Salmonella*-CFA/I or the Salmonella vaccine vector to become activated and expanded in vivo. CD25+ CD4+ T cells and CD25− CD4+ T cells were isolated by cell sorting from the H696- and H647-vaccinated mice and adoptively transferred (6 × 10^5 cells/mouse) into naive recipients. On day one after the adoptive transfer, mice were challenged with PLP139−151, and mice were monitored for normal course of disease. Beginning with adoptive transfers with cells from H696-dosed mice, the PBS-treated group developed the expected EAE disease (Fig. 5A). The mice receiving the CD25+ CD4+ T cells showed considerable potency with minimal to no EAE developing when compared with PBS-treated mice (p < 0.001) and even compared with control mice vaccinated with H696 (p < 0.001; Fig. 5A and Table II). Mice adoptively transferred with CD25+ CD4+ T cells developed a more severe EAE, but these mice still exhibited a substantially reduced EAE when compared with the PBS-treated mice. These data suggest that the H696 confers protection via both immune deviation by Th2 cells and via the induction of Treg cells. Adoptive transfer of immune CD4+ T cells obtained from *Salmonella* vector-vaccinated mice showed a different outcome. The induced Treg cells were protective against EAE challenge, but these mice still developed EAE, unlike those Treg cells induced with the *Salmonella*-CFA/I vaccine (Fig. 5B and Table II). Similar to H696-induced Treg cells, all the mice recovered from EAE (Fig. 5B). However, adoptive transfer of CD25− CD4+ T cells from H647-vaccinated mice failed to confer any protection, suggesting a requirement for Th2 cells either for the development or coinduction. Moreover, for these studies, it was essential that the Treg cells come from vaccinated mice because the adoptive transfer of naive CD25+ CD4+ T cells had minimal impact. Naive CD25− CD4+ T cells and CD25− CD4+ T cells were sorted from normal SJL mice and adoptively transferred into naive SJL mice. Mice were challenged with PLP139−151 and monitored for disease course (Fig. 5C and Table II). The CD25− CD4+ T cells failed to delay EAE onset and showed a similar disease course as did the PBS-treated mice. While there was a 4-day delay of EAE onset (Table II), the mice given the Treg cells still eventually developed EAE with similar maximum clinical scores, suggesting that their potency is not as great as the vaccine-induced Treg cells. These data show that innate Treg cells are insufficient for protection against EAE.

To discern what attributes contributed to protection, cytokine production for the immune CD25+ CD4+ T cells and CD25− CD4+ T cells was analyzed (Fig. 6). Following in vitro restimulation of the purified CD25+ CD4+ T cells and CD25− CD4+ T cells from H696- and H647-vaccinated mice, the IFN-γ production was mostly derived from CD25+ CD4+ T cells rather than CD25+ CD4+ T cells (p < 0.001), and in fact, the amount of IFN-γ was greater from CD25+ CD4+ T cells obtained from H647-vaccinated mice (p < 0.001; Fig. 6). IL-4, IL-10, and IL-13 production was enhanced in H696-induced CD25+ CD4+ T cells (p < 0.001). As expected, based on results shown in Fig. 2 and in previous reports (28), CD25− CD4+ T cells from of H647-vaccinated mice produced minimal to no Th2-type cytokines. Notably, production of TGF-β was enhanced in H696-induced CD25+ CD4+ T cells when compared with CD25− CD4+ T cells (p < 0.001), as well as when compared with production by H647-induced CD25+ CD4+ or CD25− CD4+ T cells by (p < 0.001), suggesting that Treg cells mediated their suppression via TGF-β. The observed protective effect by H647-induced Treg cells may come from the combined protection of regulatory cytokines, IL-4, IL-10, IL-13, and...
Salmonella-CFA/I-vaccinated mice resulted in a more severe producing Treg cells and IL-4-, IL-10-, and IL-13-producing Teff cells in ease. In fact, in vivo depletion/inactivation (38) of Treg cells in induced Treg cells, which may contribute or lessen the impact by upon vaccination with Salmonella nated mice did not produce any Th2-type cytokines but rather produced the regulatory cytokines (Fig. 6).

TGF-β. Interestingly, IL-17 was only produced by these H647-induced Treg cells, which may contribute or lessen the impact by the regulatory cytokines (Fig. 6).

Discussion

Encephalitogenic T cells secrete Th1-type cytokines inducing the activation of macrophages and microglial cells and the infiltration of inflammatory cells from peripheral lymphoid tissues into CNS (11, 12). Studies to date have explored the potential for developing anti-encephalitogenic Treg cells as a therapeutic for autoimmune diseases (17, 18, 23). Cells producing anti-inflammatory cytokines, IL-4, IL-10 (23, 24, 30), or IL-13 (28), down-regulate these in-flammatory responses, inhibiting autoimmune damage. Such ef-forts involve the stimulation of myelin-based epitopes by vacci-nating with altered peptide ligands (23, 31) or isolating Treg cells from mice orally immunized with H696 or H647 were evaluated for cytokine production following anti-CD3 and anti-CD28 costimulation. Treg cells had reduced IFN-γ production but elevated TGF-β when compared with Treg cells. IL-4, IL-10, and IL-13 segregated with the Treg cells induced by vaccination with H696. Treg cells from H696-vaccinated mice did not produce any Th2-type cytokines but rather produced elevated levels of IFN-γ. H647-induced Treg cells did produce TGF-β (although less than H696-vaccinated mice) and IL-17. Thus, protection conferred by Salmonella-CFA/I-induced Treg cells is because of reduced IFN-γ production and increased TGF-β, as well as in part supported by immune deviation by the Treg cells. *p < 0.001 represents differences in cytokine production between CD25CD4+ T cells and CD25CD4+ T cells, and †, p < 0.001 represents differences in cytokine production between H696- and H647-sorted cells.

FIGURE 6. Oral vaccination with Salmonella-CFA/I elicits TGF-β-producing Treg cells and IL-4-, IL-10-, and IL-13-producing Teff cells in contrast to IL-17-producing, but no Th2-type cytokine-producing, Treg cells upon vaccination with Salmonella vector. Cell-sorted CD25CD4+ and CD25CD4+ T cells from mice orally immunized with H696 or H647 were evaluated for cytokine production following anti-CD3 and anti-CD28 costimulation. Treg cells had reduced IFN-γ production but elevated TGF-β when compared with Treg cells. IL-4, IL-10, and IL-13 segregated with the Treg cells induced by vaccination with H696. Treg cells from H696-vaccinated mice did not produce any Th2-type cytokines but rather produced elevated levels of IFN-γ. H647-induced Treg cells did produce TGF-β (although less than H696-vaccinated mice) and IL-17. Thus, protection conferred by Salmonella-CFA/I-induced Treg cells is because of reduced IFN-γ production and increased TGF-β, as well as in part supported by immune deviation by the Treg cells. *p < 0.001 represents differences in cytokine production between CD25CD4+ T cells and CD25CD4+ T cells, and †, p < 0.001 represents differences in cytokine production between H696- and H647-sorted cells.

What became evident from this study is the importance of Th2 cells upon Treg cell development as implicated from the adoptive transfer of CD25+ and CD25−CD4+ T cells. Partial protection was observed, as denoted by the reduction in EAE after adoptive transfer of Salmonella vector-induced Treg cells, yet this was unmatched to the potency obtained with the Salmonella-CFA/I-induced Treg cells in which minimal to no disease was observed. Moreover, adoptive transfer of effector CD4+ T cells induced by the Salmonella vaccine vector did not protect against EAE, whereas adoptive transfer of the H696-induced effector Treg cells conferred partial protection of similar magnitude to that obtained with the Salmonella vector-induced Treg cells. Not only were there clear distinctions in the functional outcomes of these collective CD4+ T cells from the two vaccine groups, differences in the cytokine profiles for both the CD25+CD4+ and CD25−CD4+ T
cells were observed. Examination of the effector CD25^-CD4^+ T cells revealed that the mice vaccinated with H696 showed lower IFN-γ production, but greater IL-10 and IL-13 when compared with *Salmonella* vector-immunized effector CD25^+CD4^+ T cells. Interestingly, the H696-induced T_{reg} cells, for the most part, produced TGF-β whereas the CD25^+CD4^+ T cells did not. The role of IL-10 in this present study was less prominent than TGF-β. In contrast, the H647-induced CD25^+CD4^+ T cells produced less TGF-β but more IL-4 and IL-17. This latter observation is of interest because IL-17 development is TGF-β dependent and has an antagonist properties to Th1 and Th2 cells (46), suggesting that perhaps neutralization of this cytokine may make the H647-induced T_{reg} cells more potent. The effector CD25^+CD4^+ T cells from the H647-vaccinated mice showed no Th2- nor Th17-type cytokine production.

Other works have shown that protective T_{reg} cells preferentially produce IL-10 (23, 47–49). Clearly, this was not the case here, although some IL-10 was produced by CD25^-CD4^+ T cells. Our finding with TGF-β^-CD25^+CD4^+ T cells was consistent with what others have found for T_{reg} cells' role in oral tolerance (50, 51) and protection to autoimmune diseases (52, 53). In this regard, TGF-β appears to be critical for the expression of FoxP3 in CD25^-CD4^+ T cells (54), conferring the regulatory role to these cells (15, 55). TGF-β has important immunosuppressive properties on lymphocytes and has also been implicated in the conversion of CD25^-CD4^+ T cells into CD25^+CD4^+ T cells by the induction of FoxP3. Moreover, it seems to be an important factor for the expansion of CD25^-CD4^+ T cells in inflammatory diseases, such as colitis (15), and has been associated with the recovery from experimental colitis in mice (53). These reports and our data suggest that TGF-β plays a critical role in the recovery from autoimmune diseases.

It was interesting to learn that infection with an attenuated *Salmonella* strain could elicit T_{reg} cells. Such a finding had not been reported previously for *Salmonella* infections. The difference in the mechanism of action between the two *Salmonella* strains may be due to the absence of inducing immune deviation by the *Salmonella* vector, as affirmed by the lack of effector Th2 cells by H647-vaccinated mice. While the induced Th2 cells by *Salmonella*-CFA/I vaccination were not as effective as purified T_{reg} cells (but this effect may be dose dependent) from the same vaccinated mice, the addition of immune deviation may enhance T_{reg} cell development (56) and ultimately its potency. This became further evident upon the evaluation of the relative potency among the induced T_{reg} cells compared with naive CD25^-CD4^+ T cells. When the naive CD25^-CD4^+ T cells were isolated, adoptively transferred, and tested for their ability to inhibit EAE, these failed to prevent EAE development. Previous studies have shown that at least 2–3 million naive T_{reg} cells are required to show some level of protection (29, 31, 55, 57). Since we used a lesser amount, the absence of protection may be because of insufficient numbers. Nonetheless, the *Salmonella*-CFA/I vaccine-induced T_{reg} cells were clearly more potent.

The feasibility of using adoptively transferred T_{reg} cells remains problematic because of insufficient levels. While others have addressed this issue by in vitro activation with cognate Ag (22), which clearly expands T_{reg} cell numbers, such an approach may be hampered if the TCR specificity is unknown. Alternatively, polyclonal activation methods using anti-CD3 and IL-2 (57–59) can successfully increase T_{reg} cell numbers, but not knowing the specificity of polyclonally activated T_{reg} cells may have other unknown or adverse consequences (60). To address these concerns, one option could be the induction of T_{reg} cells with a defined specificity but unrelated to autoantigens. As in the case for *Salmonella*-CFA/I, vaccination can be readily accomplished to elicit T_{reg} cells that ultimately protect or reduce EAE. To our knowledge, this is the first work describing such an approach of vaccinating against an irrelevant Ag, thus not relying on naive T_{reg} cells and, to a lesser extent, relying on disease-induced T_{reg} cells.

In summary, we demonstrated that therapeutic treatment with *Salmonella*-CFA/I is able to protect SJL mice against EAE by preventing encephalitogenic T cells to enter the CNS. Although immune deviation plays a significant role in diminishing EAE, its participation may also enhance the development of T_{reg} cells. Eliminating T_{reg} cell function in mice immunized with *Salmonella*-CFA/I provoked a more severe EAE, clearly indicating that vaccination stimulates T_{reg} cell formation. Adoptive transfer with *Salmonella*-CFA/I-induced T_{reg} cells, but not naive T_{reg} cells, and to a lesser degree with *Salmonella* vaccine-induced T_{reg} cells, protected against EAE challenge. The suppressive FoxP3^- T_{reg} cells producing TGF-β conferred protection. To our knowledge, this is the first work that reports the protective effects of immune T_{reg} cells induced and expanded by immunization with an irrelevant, non-self Ag, such as CFA/I/limbriae.

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**Disclosures**

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**References**


