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Oral Tolerance Induction with Antigen Conjugated to Cholera Toxin B Subunit Generates Both Foxp3<sup>+</sup>CD25<sup>+</sup> and Foxp3<sup>+</sup>CD25<sup>−</sup>CD4<sup>+</sup> Regulatory T Cells<sup>1</sup>

Jia-Bin Sun,<sup>2</sup> Sukanya Raghavan, Åsa Sjöling, Samuel Lundin, and Jan Holmgren

Oral administration of Ag coupled to cholera toxin B subunit (CTB) efficiently induces peripheral immunological tolerance. We investigated the extent to which this oral tolerance is mediated by CD25<sup>+</sup>CD4<sup>+</sup> regulatory T cells (T<sub>reg</sub>). We found that total T<sub>reg</sub> KJ1–26<sup>+</sup> T<sub>reg</sub> and CTLA-4<sup>+</sup> T<sub>reg</sub> were all increased in Peyer’s patches, mesenteric lymph nodes, and, to a lesser extent, in spleen of mice after intragastric administration of OVA/CTB conjugate, which also increased TGF-β in serum. This could be abolished by coadministering cholera toxin or by treatment with anti-TGF-β mAb. CD25<sup>+</sup> T<sub>reg</sub> but also CD25<sup>−</sup>CD4<sup>+</sup> T cells from OVA/CTB-treated BALB/c or DO11.10 mice efficiently suppressed effector T cell proliferation and IL-2 production in vitro. Following adoptive transfer, both T cell populations also suppressed OVA-specific T cell and delayed-type hypersensitivity responses in vivo. Foxp3 was strongly expressed by CD25<sup>+</sup> T<sub>reg</sub> from OVA/CTB-treated mice, and treatment also markedly expanded CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub>. Furthermore, in Rag1<sup>−/−</sup> mice that had adoptively received highly purified Foxp3<sup>+</sup>CD25<sup>−</sup>CD4<sup>+</sup> OT-II T cells OVA/CTB feeding efficiently induced CD25<sup>+</sup> T<sub>reg</sub> cells, which expressed Foxp3 more strongly than naturally developing T<sub>reg</sub> and also had stronger ability to suppress effector OT-II T cell proliferation. A remaining CD25<sup>−</sup> T<sub>cell</sub> population, which also became suppressive in response to OVA/CTB treatment, did not express Foxp3. Our results demonstrate that oral tolerance induced by CTB-conjugated Ag is associated with increase in TGF-β and in both the frequency and suppressive capacity of Foxp3<sup>+</sup> and CTLA-4<sup>+</sup> CD25<sup>+</sup> T<sub>reg</sub> together with the generation of both Foxp3<sup>+</sup> and Foxp3<sup>+</sup>CD25<sup>−</sup>CD4<sup>+</sup> T<sub>reg</sub>.

1 Abbreviations used in this paper: T<sub>reg</sub>, regulatory T cell; PP, Peyer’s patch; LN, lymph node; MLN, mesenteric LN; CT, cholera toxin; CTB, CT B subunit; i.g., intragastric; i.d., intraduodenal; T<sub>e</sub>, T effector cell; DTH, delayed-type hypersensitivity; FPLC, fast protein liquid chromatography; HpRt, hypoxanthine phosphoribosyltransferase; MNC, mononuclear cell; int, intermediate.

Department of Microbiology and Immunology, Institute of Biomedicine and Göteborg University Vaccine Research Institute, Sahlgrenska Academy at Göteborg University, Göteborg, Sweden

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2 Address correspondence and reprint requests to Dr. Jia-Bin Sun, Department of Microbiology and Immunology, Institute of Biomedicine and Göteborg University, Göteborg, Sweden. E-mail address: jia-bin.sun@microbio.gu.se

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regulatory CD4+ T cells can strongly suppress effector T cell responses to Ag in vitro and in vivo. Both the CD25+ and the CD25- suppressor T cell populations induced by OVA/CTB feeding expressed the Foxp3 gene, although to a different extent, and oral OVA/CTB treatment efficiently generated Foxp3+CD25+ Treg cells from Foxp3-CD25- CD4+ T cells independent of natural CD25+ Treg cells.

Materials and Methods

Mice

For the experiments we used four strains of 6- to 8-wk-old female mice: 1) BALB/c (B & K Universal); 2) DO11.10 OVA TCR transgenic (Tg) mice on BALB/c background (The Jackson Laboratory), a clone with nearly 50% of the CD4+ T cells expressing a TCR specific for the peptide323–339 fragment of OVA; 3) OVA TCR Tg OT-II mice with the same TCR specificity as DO11.10, but on the C57BL/6 background (a gift from Dr. M. J. Wick, Göteborg University, Göteborg, Sweden); and 4) Rag1-/- mice (The Jackson Laboratory). The mice were kept in ventilated cages under specific pathogen-free conditions at the Department of Experimental Medicine, Göteborg University, Sweden, The Göteborg University Ethical Committee for Animal Experimentation approved these studies.

Antigens

OVA protein (grade VII) was purchased from Sigma-Aldrich, and OVA peptide323–339 (ISQAVHAAHAEINEAGR) was obtained from TAG Copenhagen. CT was purchased from List Biological Laboratories. Highly purified recombinant CTB was provided by SBL Vaccines.

Conjugation of OVA to CTB

OVA protein was chemically coupled to CTB using N-succinimidyl (3- (2-pyridyl)-dithio)propionate (Pierce) as a bifunctional coupling reagent as described (17). Coupled OVA/CTB was purified and quantified by fast protein liquid chromatography (FPLC) gel filtration (Superdex 200 16/60 column; Pharmacia Biotech) using the Biologic Workstation FPLC system (Bio-Rad). After use, the lots of purified conjugate were analyzed by GM1-ELISA using biotinylated anti-CTB mAbs and were shown to have retained GM1-binding activity (22). They were also shown to have similar, strong capacity to induce OVA-specific T cell proliferation when tested on DO11.10 splenocytes. In these assays, they were not significantly inhibited by preincubation and coculture with polymyxin (100 ng/ml). OVA/CTB-treated or PBS-treated BALB/c mice or OVA TCR Tg mice were injected i.v. into BALB/c mice (4 × 10^6 cells/mouse). One day after transfer, the mice were immunized s.c. in the right footpad with 100 μg of OVA in 50 μl of CFA containing 100 μg of M. tuberculosis. Seven days after this immunization, mice received a s.c. challenge injection of 20 μg of OVA in PBS in the left footpad. Left footpad thickness was measured before and 24 h after OVA challenge in a blinded fashion using a caliper (Mitutoyo).

Induction of oral tolerance and immunization

BALB/c mice, DO11.10 OVA TCR Tg mice, BALB/c mice adoptively transferred with 1.5 × 10^5 DO11.10 CD4+ T cells, Rag1-/- mice adoptively transferred with 2.5 × 10^5 or 5 × 10^5 CD4+ T cells from OT-II Tg mice were given intragastrically (i.g.) either 20 mg of OVA, 200 μg of OVA/CTB conjugate, 200 μg of OVA/CTB mixed with 4 μg of CT, or only PBS up to three times at 2-day intervals. In some experiments, mice were also immunized once s.c. in a footpad with 50 or 100 μg of OVA emulsified 1/1 in CFA containing 100 μg of Mycobacterium tuberculosis H37Ra (Difco). At indicated days after i.g. treatment or s.c. immunization, the mice were sacrificed, and FP, draining LN (DLN; axillary, inguinal, MLN, or popliteal), and spleen were harvested for further in vitro studies.

Cell purification and sorting by FACS

Spleens and LN were pressed through nylon nets, and single-cell suspensions were prepared. Purified CD4+ T cells were isolated by negative selection using MACS microbeads labeled with various mAbs (Miltenyi Biotech) according to the manufacturer’s suggested protocol. To separate CD25+ CD4+ T cells and CD25- CD4+ T cells, the isolated CD4+ T cells were further incubated with PE-conjugated anti-CD25 (10 μl/10^6 cells) at 4°C for 10 min, whereafter anti-PE coated microbeads (Miltenyi Biotec) were added and incubated another 15 min at 4°C. Magnetic separation was performed with a positive selection column according to the manufacturer’s suggested protocol. CD25+ CD4+ Tg T cells purified from LN or spleen of DO11.10 OT-II mice were treated with 0.1 EU/mg of endotoxin.

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CFSE labeling of T cells

CD4+ T cells purified from DO11.10 mice were labeled at 3 × 10^5 cells/ml with 5 μM CFSE (Molecular Probes) in PBS for 5 min followed by incubation with 5% FCS-PBS (5 mM EDTA) for 10 min at 37°C. After two washes, 5 × 10^6 of the labeled cells in 200 μl of PBS were injected i.v. into the tail vein of recipient mice.

T cell proliferation and cytokine assays

Spleen cells or the draining popliteal or inguinal LN cells were collected at indicated days after immunization. Cells were isolated and RBC were removed by lysis and then cultured. For proliferation of spleen and LN cells, 2.5 × 10^5 or 5 × 10^5 cells/well (in 200 μl) were cultured for 3 days in 96-well plates with or without 1–10 μg/ml OVA. [3H]Thymidine was added (1 μCi/well) for the last 16 h of culture. To test the regulatory function of isolated CD4+ T cells or separated CD25+ CD4+ or CD25- CD4+ T cells from mice with different treatments, these cells were cocultured in different numbers with 1 × 10^5 DO11.10 Tg effector cells together with 5 × 10^5 of bone marrow-derived dendritic cells (DC) prepared as described in Ref. 23 and with 1 μg/ml OVA323–339 peptide. These tests were performed using 96-well round-bottom plates (Nunc) for 3 days with [3H]thymidine added during the last 16 h. IL-2 production by cultured cells were assayed in 2-day culture supernatants using the cytokine cytometric bead array method (BD Biosciences). TGF-β in serum was measured by ELISA according to the manufacturer’s instructions (Duoset kit; R&D Systems).

FACS analyses

Freshly isolated splenic or LN cells were incubated with FITC-, or PE-, or allophycocyanin-labeled mAbs to mouse CD4, CD8α, CD25, CD62L, CD69, or KJ1–26 (BD Biosciences). For analysis of intracellular CTLA-4 and Foxp3, stained CD25-CD4+ T cells or CD25+CD4+ T cells were fixed and permeablized with Cytofix/Cytoperm solution (BD Pharmingen) according to the manufacturer’s suggested protocol and then incubated with PE- or allophycocyanin-conjugated anti-CTLA-4 (BD Biosciences) or anti-Foxp3 FLK-16 (Nordic BioSite) (0.5–1 μg/10^6 cells) at 4°C for 30 min in the dark. Cells were then washed and analyzed by flow cytometry (FACSCalibur; BD Biosciences), which was also used to assess cell division in CFSE-labeled cells.

Adaptive transfer of cells, delayed-type hypersensitivity (DTH) testing, and in vivo depletion of TGF-β

Total CD4+ T cells or CD25-CD4+ or CD25+ CD4+ T cells from i.g. OVA/CTB-treated or PBS-treated BALB/c mice or OVA TCR Tg mice were injected i.v. into BALB/c mice (4 × 10^6 cells/mouse). One day after transfer, the mice were immunized s.c. in the right footpad with 100 μg of OVA in 50 μl of CFA containing 100 μg of M. tuberculosis. Seven days after this immunization, mice received a s.c. challenge injection of 20 μg of OVA in PBS in the left footpad. Left footpad thickness was measured before and 24 h after OVA challenge in a blinded fashion using a caliper (Mitutoyo).

In separate experiments, purified CD25-CD4+ and CD25+CD4+ T cells from OT-II mice were injected i.v. into Rag1-/- mice which were then treated i.g. with different regimens before receiving a s.c. immunization with OVA in CFA. One or 3 wk later, spleen CD25-CD4+ and CD25+CD4+ T cells were purified by FACS cell sorting and used for Foxp3 gene expression studies.

In some experiments, we first adoptively transferred CD25-CD4+ OVA Tg DO11.10 T cells to normal BALB/c mice. Then, starting 4 days later, we gave groups of mice three i.v. injections every second day with either anti-mouse TGF-β mAb (1D11.16.8) or sham treatment (normal mouse IgG). One day after each injection, mice in the different treatment groups were given either OVA/CTB conjugate (200 μg/dose) or PBS i.g. The anti-mouse TGF-β mAb was purified from a hybridoma supernatant by affinity chromatography and shown to give a single peak in FPLC and to contain <0.1 EU/mg of endotoxin.

Analysis of Foxp3 expression

In addition to the intracellular staining of Foxp3 protein (scurfin) as described above, Foxp3 gene expression was also studied by RT-PCR and real-time PCR methods.

RT-PCR. RNA was purified by the total RNA extraction kit for mammalian tissue (Sigma-Aldrich) and murine RNA was treated by the DNase I Amp grade kit (Invitrogen Life Technologies) to remove residual genomic DNA. cDNA was synthesized by using an oligo(dT) primer and the Sensiscrypt RT-PCR Kit (Qiagen) as described by the manufacturer. Primers for

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FIGURE 1. Intragastric administration of OVA/CTB conjugate efficiently suppresses in vivo T cell proliferative responses to s.c. immunization with OVA. Groups of BALB/c mice were given three 200-μg doses i.g. of OVA/CTB conjugate alone or mixed with 4-μg CT or PBS every second day. One day after the last i.g. administration, the mice were immunized s.c. with 50 μg of OVA in CFA at the tail base, and 1 wk later the mice were killed and their T cell proliferative responses in peripheral LN (PLN) to OVA were examined. Cells (5 × 10^5 cells/well) were incubated for 72 h in the presence or absence of indicated concentrations of OVA; [3H]thymidine was added to the cell cultures 16 h before the cells were harvested and examined for [3H]thymidine incorporation in DNA by scintillation. **, Values of p < 0.001 by ANOVA test for indicated OVA/CTB-treated mice in comparison with corresponding mice treated with PBS. Values shown are means and SDs for four mice per group, and are representative of three independent experiments.

mouse hypoxanthine phosphoribosyltransferase (Hprt), designed by primer3 software (http://frodo.wi.mit.edu/cgi-bin/prime3prime3 www.cgi) and the Foxp3 primers described by Jason et al. (24) were from MGW-Bio tech. Semiquantitative RT-PCR was performed using standard PCR conditions (1.5 mM MgCl₂ and annealing at 55°C) and forward and reverse primers (Foxp3, 5’-ggcccttctccaggacaga-3’ and 5’-agaggtccttttcaccagca-3’; Hprt, 5’-atcagtcaacgggggacata-3’; and 5’-agaggtccttttcaccagca-3’). The reactions were amplified for 37 cycles and analyzed on 2% agarose gels stained with ethidium bromide.

CTB CONJUGATE-INDUCED Treg

Real-time PCR. Real-time quantitative PCR was performed on an ABI Prism 7500 thermal cycler (PerkinElmer) using SYBR Green master mix (Applied Biosystems) and the Foxp3 and Hprt primers, both at a final concentration of 200 nM. The specificity of the PCR was confirmed by the appearance of a single peak in the analysis of the dissociation curve, showing the predictable melting temperature of the primer pairs. No amplification was seen in the nontemplate control. A standard curve was generated from serial dilutions of purified primer product of Hprt and Foxp3. Normalized values for Foxp3 mRNA expression were calculated as the relative quantity of Foxp3 divided by the relative quantity of Hprt extrapolated from the standard curve calculated by the ABI Prism 7500 software (Applied Biosystems).

Statistical analysis

Results are expressed as mean ± SD. When not specified otherwise, we used the Student t test for determining statistical differences between experimental and control groups. Values of p < 0.05 or p < 0.01 are referred to as significant or highly significant, respectively.

Results

Oral tolerance induction by i.g. administration of OVA/CTB conjugate efficiently induces CD25⁺ CD4⁺ Treg and coadministration of CT abrogates this effect

In preliminary work, we found that i.g. administration to BALB/c (or C57BL/6) mice of a low-dose OVA/CTB conjugate, given either as a single 200-μg dose or as three 200-μg doses every second day, efficiently induced regulatory CD4⁺ T cells in MLN and spleen that could completely suppress OVA-specific proliferation of OVA TCR Tg DO11.10 or C57BL/6 OT-II T cells in coculture. These regimens of OVA/CTB also efficiently prevented the immunogenic effect of a s.c. OVA/CFA immunization 1 day after the i.g. treatment as manifested in a practically complete suppression of spleen or DLN T cells to OVA or OVA323–339 peptide 7 or 21 days after immunization (Fig. 1 and data not shown). In contrast, i.g. administration of the same dose of OVA, whether alone or

FIGURE 2. Strong Ag-specific suppression of T effector cells (Teff; DO11.10 CD25⁺ CD4⁺ OVA TCR Tg T cells) by CD25⁺ CD4⁺ T cells purified from OVA/CTB-treated mice, but not by cells from mice treated with the OVA/CTB conjugate plus CT. CD25⁺ and CD25⁻ CD4⁺ T cells were purified from pooled MLN and spleen MNC of BALB/c mice after three i.g. administrations of either PBS (A), 20 μg of OVA (B), 200 μg of OVA/CTB (C), or 200 μg of OVA/CTB mixed with 4 μg of CT (D) in 0.3 ml of a 6% sodium bicarbonate solution. E. Direct comparison of the suppressive capacity of the CD25⁺ CD4⁺ T cells from the different treatment groups. The purified cells (10⁶ cells/well) or a 1:1 mixture of both cell populations were incubated with T eff (10⁵/well) and DC (5 × 10⁵ cell/well) in the presence of 1 μg/ml OVA323-339 peptide. After 48 h, 0.5 μCi of [3H]thymidine was added to each well and the cells were further incubated for 16 h before harvest and [3H]thymidine incorporation measurement. Results are means ± SDs of triplicate cultures from one of three independent experiments with similar results. *, **, and ***, Values of p < 0.05, p < 0.01, and p < 0.001, respectively, for indicated cocultures in comparison with the proliferative response of T eff alone.
mixed with 100 μg of CTB, did not induce cells with any detectable regulatory activity in coculture with Tg T cells (data not shown), whereas cells with such suppressive activity could be found after three i.g. doses of a 100-fold higher amount of OVA (20 mg/feeding).

We wished to compare the relative efficiency of OVA/CTB conjugate and the high-dose unconjugated OVA to induce CD4+ T cells capable of suppressing OVA-specific stimulation of CD25−CD4+ Tg T eff cells in vitro and to determine whether the suppressive cells were CD25+ or CD25−. We therefore gave either 200 μg of OVA/CTB or 20 mg of OVA or PBS i.g. on three occasions every second day to BALB/c mice. Three days after the last i.g. treatment, CD25+ and CD25−CD4+ T cells were isolated from pooled MLN and spleen mononuclear cells (MNC) using MACS microbeads, and their in vitro suppressive activity was tested in coculture experiments. In these experiments, we used isolated naive CD25−CD4+ DO11.10 cells as T eff cells, DC as APC, and OVA323−339 peptide for Ag-specific stimulation. As expected, CD25−CD4+ T cells isolated from the PBS-treated control mice had no suppressive effect on the proliferative response of DO11.10 T eff cells to OVA323−339 peptide. In contrast, CD25+CD4+ T cells or a 1:1 mixture of CD25+ and CD25−CD4+ T cells from these mice could significantly suppress the T eff response to OVA peptide (Fig. 2A). A stronger suppressive effect was achieved using CD25+CD4+ T cells isolated from mice treated i.g. with high-dose OVA (Fig. 2B) and was even further pronounced using CD25+CD4+ T cells isolated from the low-dose OVA/CTB-treated mice (Fig. 2, C and E). CD25+ cells from OVA- or OVA/CTB-treated mice in contrast had only a marginal suppressive effect under these conditions (Fig. 2, B and C).

Consistent with our previous work that coadministration of CT can abrogate oral tolerance induction (19–21), and in sharp contrast to the effects after feeding OVA/CTB conjugate without CT,
Table I. Intragastric treatment of DO11.10 mice with OVA/CTB conjugate increases total and Ag clonotype-specific CD25+ CD4+ T cells and CTLA-4 expression in PP, MLN, and spleen, but which is abrogated by coadministration of CT

<table>
<thead>
<tr>
<th>DO11.10 Mice</th>
<th>CD25+ CD4+ (% change vs PBS)</th>
<th>CD25+ CD4+/KJ1–26+ T Cells (% change vs PBS)</th>
<th>CTLA-4+ CD25+ CD4+ T Cells (% change vs PBS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1: OVA/CTB</td>
<td>7.95 ± 0.2 (+176)b</td>
<td>8.9 ± 0.4 (+69)b</td>
<td>7.83 ± 1.22 (+58)c</td>
</tr>
<tr>
<td>G2: OVA</td>
<td>3.62 ± 0.9</td>
<td>6.1 ± 0.4</td>
<td>4.43 ± 0.62</td>
</tr>
<tr>
<td>G3: PBS</td>
<td>2.88 ± 0.3</td>
<td>5.4 ± 0.9</td>
<td>4.90 ± 0.1</td>
</tr>
<tr>
<td>G4: OVA/CTB+CT</td>
<td>3.38 ± 0.3</td>
<td>5.2 ± 0.2</td>
<td>4.93 ± 0.42</td>
</tr>
<tr>
<td>MLN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1: OVA/CTB</td>
<td>4.46 ± 0.18 (+97)b</td>
<td>5.2 ± 0.06 (+58)c</td>
<td>10.1 ± 1.16 (+6)c</td>
</tr>
<tr>
<td>G2: OVA</td>
<td>2.05 ± 0.8</td>
<td>4.3 ± 0.7</td>
<td>7.54 ± 2.87</td>
</tr>
<tr>
<td>G3: PBS</td>
<td>2.26 ± 1.68</td>
<td>3.3 ± 0.06</td>
<td>6.90 ± 0.21</td>
</tr>
<tr>
<td>G4: OVA/CTB+CT</td>
<td>1.95 ± 0.1</td>
<td>4.4 ± 0.5</td>
<td>6.50 ± 0.94</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1: OVA/CTB</td>
<td>3.70 ± 0.38 (+71)b</td>
<td>4.0 ± 0.2 (+48)c</td>
<td>2.58 ± 0.1 (+33)</td>
</tr>
<tr>
<td>G2: OVA</td>
<td>3.16 ± 0.07</td>
<td>2.8 ± 0.1</td>
<td>1.73 ± 0.21</td>
</tr>
<tr>
<td>G3: PBS</td>
<td>2.16 ± 0.03</td>
<td>2.7 ± 0.1</td>
<td>1.80 ± 0.5</td>
</tr>
<tr>
<td>G4: OVA/CTB+CT</td>
<td>1.61 ± 0.4</td>
<td>2.9 ± 0.07</td>
<td>2.20 ± 0.1</td>
</tr>
</tbody>
</table>

a DO11.10 OVA TCR Tg mice (6/group) were treated three times i.g. every second day with 200 μg of OVA/CTB conjugate, 20 mg of OVA, PBS, or a mixture of 200 μg of OVA/CTB and 4 μg of CT. Three days later, lymphocytes were prepared from PP, MLN, and spleen, and analyzed by FACS after staining with mAbs against mouse CD25, CD4, KJ1–26, and CTLA-4. Results are from one of four independent experiments giving similar results. Values shown are percentages (mean ± SD) of CD25+ CD4+ cells among total and KJ1–26+ lymphocytes, and percentages of CTLA-4+ cells among the CD25+ CD4+ cells.
b and c Values of p < 0.05 and p < 0.01 were determined by Student’s t test for statistical differences between treatments with OVA/CTB and PBS.

Neither CD25− nor CD25+ CD4+ T cells isolated from BALB/c mice after feeding with a mixture of OVA/CTB and CT could effectively suppress the proliferative response of DO11.10 T eff cells (Fig. 2D).

Titration experiments using unfractionated pooled MLN and splenic CD4+ T cells from the different mouse treatment groups confirmed the findings with the isolated CD25+ and CD25− cells. The CD4+ T cells from the OVA/CTB-fed mice were suppressive even when added to the T eff cells in a ratio between 1:8 and 1:16; in contrast, the CD4− cells from OVA-treated mice only worked in a 1:1 ratio, and the PBS-treated CD4− cells did not work at all even when tested in a 1:4 ratio with T eff cells (data not shown).

Corresponding experiments in DO11.10 Tg mice confirmed the findings in normal BALB/c mice. Thus, three i.g. doses of OVA/CTB conjugate induced CD4+ CD25+ T cells in MLN, which strongly suppressed T eff cells in coculture (Fig. 4). Titration experiments also confirmed that the induced CD25+ CD4+ suppressor T cells from OVA/CTB-fed Tg mice (Fig. 4A) were far more potent in their suppressive capacity than CD25− CD4+ T cells (natural Treg) from PBS-treated Tg mice (Fig. 4C). Although the latter cells lost their suppressive effect when diluted to a Treg:T eff cell ratio of 1:3, Treg from the OVA/CTB-fed mice significantly suppressed T eff cells, even when used at a ratio of 1:8.1. Coadministration of CT with the OVA/CTB conjugate resulted in a loss of detectable suppressive activity of the CD4+ CD25+ MLN T cells (Fig. 3B).

In additional experiments, these results were also confirmed in BALB/c mice that had adoptively received DO11.10 Tg T cells. These mice were treated and tested as described in Fig. 1. The results (shown in Fig. 4A) demonstrate that in much the same way as described for the normal and Tg mice, MLN cells from OVA/CTB-treated animals were strongly, almost completely, suppressed in their proliferative response to OVA323–339 peptide. These cells could also strongly suppress the response of DO11.10 T eff cells in coculture, and titration experiments showed that the isolated CD4− CD25+ Treg (Fig. 4B) or CD4+ MLN (Fig. 4C) cells from the OVA/CTB-fed mice were much more potent in their suppressive capacity than the corresponding cells from PBS-treated mice.

Furthermore, in these mice, coadministration of CT together with OVA/CTB practically completely abrogated the oral tolerance induction (Fig. 4A, last panel). In a separate experiment, we examined whether CT could break already established tolerance induced by prior i.g. treatment with OVA/CTB. To this end, we gave groups of BALB/c mice that had adoptively received DO11.10 Tg T cells, three i.g. doses of either 200 μg of OVA/CTB or PBS every second day, and 1 day after the last treatment we gave to half of these mice 50 μg of OVA in CFA or 50 μg of OVA together with 2 μg of CT. One week later, we examined the spleen T cell proliferative response to stimulation with 1 μg/ml OVA323–339 peptide. The results showed that while in the OVA/CFA-challenged group the feeding with OVA/CTB suppressed proliferation by 88±11%, it only gave 45±6% suppression in the OVA/CT-challenged mice (data not shown).

Intragastric administration of OVA/CTB conjugate increases the frequency of CD25+ and CTLA-4+ CD4+ T cells in PPs, MLNs, and spleen

Our next goal was to determine whether a tolerizing i.g. administration regimen of OVA/CTB conjugate would enhance suppressive activity and expand the number of CD25+ CD4+ T cells in different organs. We, therefore, again gave three i.g. doses of OVA/CTB, OVA alone, PBS, or OVA/CTB plus CT to groups of DO11.10 OVA TCR Tg mice as described in Fig. 2. Three days after the last treatment, PP, MLN, and spleen cells were collected, and we examined the frequency of total and OVA323–339 peptide-specific (KJ1–26+) CD25+ CD4+ T cells by flow cytometry. As shown in Table I, there was a consistent and significant, albeit quantitatively modest, increase in both total and KJ1–26+ CD25+ CD4+ T cells in PP in mice treated with OVA/CTB compared with PBS-treated control mice. Mice fed a high dose of OVA had a much smaller increase in CD25+ CD4+ T cells (Table I). CD25+ CD4+ cells in MLN and spleen were also significantly increased in response to oral OVA/CTB treatment, although to a lesser extent than in PP. Coadministration of CT with the OVA/CTB conjugate abrogated the increase in both total and KJ1–26+ CD25+ CD4+ cells in PP, MLN, and spleen (Table I).
CTLA-4 is considered to be a negative signaling molecule expressed on activated T cells and has been associated with suppressive capacity of natural Treg cells (12). We found that CTLA-4 expression on CD25+CD4+ T cells was significantly increased in PP and MLN of OVA/CTB-treated mice compared with PBS-treated control mice, irrespective of whether they were DO11.10 Tg mice (Table I) or BALB/c mice that had adoptively received DO11.10 T cells (data not shown). Interestingly, in the latter group, expression of CTLA-4 was also increased on CD25+CD4+ T cells isolated from MLN cells after i.g. OVA/CTB-treatment compared with PBS treatment resulting in a doubling of CTLA-4 expression in the CD25+ T cells (data not shown). In contrast, CTLA-4 expression on either CD25+ or CD25−CD4+ T cells did not increase appreciably in either PP or MLN or spleen after treatment with a high dose of OVA or coadministration of CT together with OVA/CTB (Table I).

Expression of Foxp3 gene and protein is increased in CD25+CD4+ T cells from mice treated with OVA/CTB conjugate

Expression of Foxp3 is a key marker for identification of functional Treg cells in the CD4+CD25+ T cell population (14–16). By using the RT-PCR assay, we found that the Foxp3 gene was strongly expressed in purified CD25+CD4+ T cells from pooled MLNs and spleen from mice treated i.g. with OVA/CTB, OVA, PBS, or OVA/CTB plus CT (Fig. 5A). More surprisingly, Foxp3 was also expressed in highly purified CD25+CD4+ T cells from the OVA/CTB-fed mice, although to a much lesser extent than in the CD25+ cells (Fig. 5A). In contrast, no Foxp3 expression was seen in the CD25− cells from mice fed with either the conjugate mixed with CT, with high-dose OVA, or with PBS alone (Fig. 5A). Quantitative analyses by real-time PCR confirmed these findings (Fig. 5B) and showed that Foxp3 expression in CD25−CD4+ T cells from OVA/CTB-treated mice was more than twice that of cells from PBS-treated mice and also higher than in cells from OVA-only treated mice. A very small increase in Foxp3 expression in CD25−CD4+ T cells after the OVA/CTB feeding was also confirmed (Fig. 5B). The addition of CT to the OVA/CTB treatment completely abrogated the increase in Foxp3 activity and, in fact, led to even lesser activity than in the PBS-fed controls (Fig. 5B). The increase in expression of Foxp3 was further confirmed by intracellular staining of Foxp3 protein in KJ1−26+ cells using FACS analyses (Fig. 5C).

Intragastric treatment with OVA/CTB induces cell division in CD4+ T cells associated with expansion of Foxp3+ Treg

We tested whether i.g. administration of OVA/CTB could induce cell division in adoptively transferred CFSE-labeled CD4+
CD25+ regulatory CD4+ T cells efficiently suppresses in vivo T cell responses to s.c. immunization with OVA. A and B. Effects of BALB/c Treg on DTH and T cell proliferative responses. Highly purified CD25+ (●) or CD25− (□) CD4+ T cells were prepared by flow cytometric sorting from pooled MLN and splenic cells of BALB/c mice fed three times every second day with 200-μg doses of OVA/CTB (O/CTB) or PBS and then injected i.v. into BALB/c mice (4 × 10^5 cells/mouse). These mice (and controls given no transfer; No Tr.) were immunized s.c. with 100 μg of OVA/CFA in the right footpad on the following day, and challenged 7 days later by s.c. injection of 20 μg of OVA in 20 μl of PBS in the left footpad. Footpad thickness was measured before and 24 h after challenge and differences were calculated (6 mice/group). Spleen cells from mice treated as in A, but not challenged and staged for DTH, were isolated and cultured at 5 × 10^6 cells/well with 10 μg/ml OVA. The cells were incubated for 72 h; 0.5 μCi of [3H]thymidine was added to the cell cultures 16 h before cell harvest. C and D. Effects of DO11.10 Treg on DTH and T cell proliferative responses. Highly purified CD25+ (●) or CD25− (□) CD4+ T cells were prepared by flow cytometric sorting from pooled MLN and splenic cells of DO11.10 mice after three i.g. treatments with OVA/CTB, or OVA/CTB plus CT (O/B+CT), or PBS and immediately injected i.v. into BALB/c mice (4 × 10^5 cells/mouse), which were then treated and staged and spleen cell proliferation tested as in A and B. Results are mean ± SD values of individual mice (6 mice/group) and are from one of three independent experiments giving similar results. * and **, Values of p < 0.05 and p < 0.01, respectively, for indicated treatment groups in comparison with mice receiving CD25+ or CD25− T cells from PBS-treated controls.

DO11.10 T cells in wild-type BALB/c mice. The results, illustrated in Fig. 6, show that 3 days after a single 200-μg OVA/CTB feeding CD25+CD4+ T cells in MLN had undergone intense cell division, in sharp contrast to MLN cells from PBS-treated mice (Fig. 6A). Consistent with this, it was further found that the OVA/CTB treatment also induced the expansion of Foxp3+ cells (Fig. 6B).

Adoptive transfer of either CD25+ or CD25− CD4+ T cells from mice treated i.g. with OVA/CTB conjugate suppresses OVA-specific DTH reactivity and T cell response in vivo

We isolated total CD4+ T cells and separated the CD25+ and CD25− CD4+ T cells from pooled MLN and spleen MNC of OVA/CTB-treated BALB/c mice 3 days after the three-dose i.g. treatment regimen described above and transferred 4 × 10^5 of
Table II. Intra gastric treatment with OVA/CTB conjugate efficiently suppresses T cell proliferative response and increases the frequency of CD25\(^+\)CD4\(^+\) T cells in Rag1\(^{−/−}\) mice transferred with either highly purified CD25\(^+\)CD4\(^+\) T cells alone or a mixture of CD25\(^+\) and CD25\(^−\) T cells (mean ± SD)\(^{a}\)

<table>
<thead>
<tr>
<th>T Cell Proliferative Response to OVA(_{323-339})</th>
<th>CD25(^+)CD4(^+) T Cells (% change vs PBS)</th>
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<tbody>
<tr>
<td></td>
<td>Spleen</td>
</tr>
<tr>
<td>G1: CD25(^−), OVA/CTB</td>
<td>2430 ± 1456(^{b})</td>
</tr>
<tr>
<td>G2: CD25(^−), PBS</td>
<td>9852 ± 1762</td>
</tr>
<tr>
<td>G3: CD25(^+/−), OVA/CTB</td>
<td>50 ± 512(^{c})</td>
</tr>
<tr>
<td>G4: CD25(^+/−), PBS</td>
<td>12136 ± 2476</td>
</tr>
</tbody>
</table>

\(^{a}\) CD25\(^+\) (>99% pure) and CD25\(^−\) T cells (85% pure) were first isolated from OVA Tg mice by MACS microbead assay and flow cytometry cell sorting as described in Materials and Methods. A total of 2 \(×\) 10\(^5\) of CD25\(^+\) or a mixture of 2 \(×\) 10\(^5\) CD25\(^−\) with 4 \(×\) 10\(^5\) CD25\(^+\) T cells were adoptively transferred by i.v. injection into different groups of Rag1\(^{−/−}\) mice (6/group). After 1 day of rest, mice were treated i.g. every other day with 200 \(μg\) of OVA/CTB three times followed by s.c. immunization with OVA/CFA 1 day after last i.g. administration. Mice were killed 7 days later, and their inguinal DLN, MLN, and spleen were collected for examination of T cell proliferative response to OVA\(_{323-339}\), and FACS analysis of total CD25\(^+\) and OVA-specific clonotypic T cells. Data are from one of three independent experiments with similar results.

\(^{b}\) and \(^{c}\) Values of \(p < 0.05\) and \(p < 0.01\) were determined by Student’s t test for statistical differences between treatments with OVA/CTB and PBS.

Intragastric administration of OVA/CTB conjugate efficiently generates OVA-specific CD25\(^+\) T cells which is associated with increased TGF-β production

Next, we tested to see whether suppressive T cells can be generated from natural CD25\(^+\) CD4\(^+\) T cells after i.g. treatment with OVA/CTB. To this end, we isolated both CD25\(^−\) (>99% purity by FACS testing as shown in Fig. 8A) and CD25\(^+\) CD4\(^+\) T cells from naive OVA TCR Tg OT-II mice and adoptively transferred the CD25\(^−\) cells or a mixture of CD25\(^−\) and CD25\(^+\) cells (5:1 ratio) into Rag1\(^{−/−}\) mice. We then gave three doses of OVA/CTB conjugate (200 \(μg\)/dose) or PBS i.g. every second day, and then 1 day after the last administration, an immunization s.c. with OVA/CFA. Seven or 21 days after immunization, mice were sacrificed, and DLN, MLN, and spleen cells were collected, and MNCs were prepared and used for T cell proliferation tests and/or for FACS analysis of the frequency of total and Ag-specific CD25\(^+\)CD4\(^+\) T cells. As shown in Table II, 7 days after the s.c. immunization, splenic T cell proliferation was completely suppressed in Rag1\(^{−/−}\) mice that had received mixed CD25\(^−\)/CD25\(^+\) CD4\(^+\) T cells and been fed with OVA/CTB compared with corresponding PBS-treated mice. Strikingly, however, the T cell proliferation was also markedly suppressed in Rag1\(^{−/−}\) mice that had received highly purified CD25\(^−\) OVA Tg T cells and then been treated i.g. with OVA/CTB before immunization (Table II). DLN T cell proliferation showed a similar pattern (Table II). Furthermore, the proliferation of OT-II CD25\(^+\) CD4\(^+\) T cells stimulated with OVA\(_{323-339}\) peptide was also markedly suppressed when these cells were cocultured with splenic T cells obtained from Rag1\(^{−/−}\) mice that had received purified CD25\(^−\) CD4\(^+\) OVA Tg T cells and then i.g. treatment with OVA/CTB whether followed by immunization with OVA/CFA or no immunization (data not shown).

Flow cytometric analyses revealed that mice that had received mixed CD25\(^−\)/CD25\(^+\) CD4\(^+\) T cells and mice given highly purified CD25\(^−\) CD4\(^+\) OVA Tg T cells had significantly more CD25\(^−\) CD4\(^−\) T cells than PBS (Table II). Corresponding T cell proliferation and CD25 FACS staining analyses of MNC collected 21 days after immunization showed results similar to those described for the 7-day MNC (data not shown).

To test whether the CD25\(^+\) and/or the CD25\(^−\) T cells generated from the adoptively transferred CD25\(^+\) CD4\(^+\) T cells by oral treatment with OVA/CTB expressed the Foxp3 gene, the spleen cells were stained with anti-CD4-FITC and anti-CD25-PE and further sorted into CD25\(^+\) and CD25\(^−\) CD4\(^+\) T cells by a FACS sorter before examination for Foxp3 expression by RT-PCR. In the OVA Tg naive mice that provided donor cells, Foxp3, as expected, was only expressed in the CD25\(^−\) population and not in the CD25\(^+\) T cells. Of interest, the purified CD25\(^−\) population isolated from mice that had been recipients of Foxp3\(^+\) CD25\(^−\) T cells and treated with OVA/CTB strongly expressed Foxp3 (Fig. 8B) and also strongly suppressed the proliferation of CD25\(^−\) CD4\(^+\) T cells (Fig. 8C). In contrast, the CD25\(^−\) population isolated from mice given CD25\(^−\) cells and treated with PBS, did not express Foxp3 gene and were also much less suppressive (Fig. 8C); however, when the latter type of cells were examined 3 wk after immunization, they too expressed detectable Foxp3 (data not shown).
cells, in contrast, did not express any Foxp3 whether isolated from the OVA/CTB- or PBS-treated groups even though the CD25+ cells from the OVA/CTB-treated group, although being slightly less effective than the CD25+ cells, efficiently suppressed OT-II T eff Ag-specific proliferation when tested in coculture (Fig. 8C).

Finally, we sought to examine whether TGF-β levels in serum are increased and whether TGF-β is important in the generation of Treg in response to i.g. administration of OVA/CTB conjugate. Therefore, we adoptively transferred CD25+ CD4+ OVA Tg DO11.10 T cells to normal BALB/c mice. Then, starting 4 days later, we gave groups of mice three i.v. injections every second day of either anti-mouse TGF-β mAb or control IgG (0.5 mg/dose). Starting 1 day after the first mAb or IgG injection, mice received (every second day) either three 200-μg i.d. doses of OVA/CTB conjugate or PBS. Serum and MLN were collected 3 days later for assay of TGF-β levels in serum (values shown are the levels in serum diluted 1/30) by ELISA. FACs analysis of total and OVA clonotype-specific CD25+ CD4+ T cells in MLN, and proliferative response of MLN cells to OVA peptide. Stimulation index. Data in parentheses are percentage change vs corresponding PBS-fed mice.

<table>
<thead>
<tr>
<th></th>
<th>TGF-β1 Levels in Serum (ng/ml) (%)</th>
<th>CD25+ CD4+ in MLN (%)</th>
<th>KJ1–26+CD25+ CD4+ in MLN (%)</th>
<th>MLN Cell Proliferation (SI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1: anti-TGF-β, OVA/CTB</td>
<td>0.422 ± 0.19 (+63)b</td>
<td>4.90 ± 4.44 (−15)</td>
<td>8.25 ± 5.70 (−31)</td>
<td>3.9 ± 1.3</td>
</tr>
<tr>
<td>G2: anti-TGF-β, PBS</td>
<td>0.260 ± 0.08</td>
<td>5.80 ± 1.20</td>
<td>13.4 ± 0.38</td>
<td>5.7 ± 1.5</td>
</tr>
<tr>
<td>G3: control IgG, OVA/CTB</td>
<td>2.170 ± 0.09 (+92)c</td>
<td>15.9 ± 3.7 (+194)c</td>
<td>19.0 ± 3.6 (+90)c</td>
<td>1.9 ± 0.2c</td>
</tr>
<tr>
<td>G4: control IgG, PBS</td>
<td>1.132 ± 0.02</td>
<td>5.40 ± 2.37</td>
<td>10.5 ± 0.5</td>
<td>3.8 ± 0.4</td>
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</table>

b, c Values of p < 0.05 and p < 0.01 were determined by Student’s t test for statistical differences between treatments with OVA/CTB and PBS.

| Treg cells | CD25+ CD4+ (>99%) pure T cells were first isolated from OVA Tg mice by MACS microbeads as described in Materials and Methods. CD25+ Tg T cells (2 × 10⁶) were then i.v. adoptively transferred into BALB/c mice, which then (every other day) received i.v. injection of anti-mouse TGF-β mAb or control IgG (0.5 mg/dose). Starting 1 day after the first mAb or IgG injection, mice received (every second day) either three 200-μg i.d. doses of OVA/CTB conjugate or PBS. Serum and MLN were collected 3 days later for assay of TGF-β levels in serum (values shown are the levels in serum diluted 1/30) by ELISA. FACs analysis of total and OVA clonotype-specific CD25+ CD4+ T cells in MLN, and proliferative response of MLN cells to OVA peptide. Stimulation index. Data in parentheses are percentage change vs corresponding PBS-fed mice.

Discussion

Our results show that i.g. administration of OVA/CTB conjugate conjugatively induces OVA-specific peripheral immunological tolerance, whether tested in normal BALB/c mice, OVA TCR Tg (DO11.10) mice, or in either BALB/c mice or Rag1−/− mice transferred with OVA TCR Tg T cells. Consistent with our previous findings in other systems (17–22), oral tolerance induction with Ag/CTB conjugate was superior to that induced with free Ag both in markedly increasing the efficacy of tolerization and in dramatically decreasing the effective amounts of Ag. The oral tolerance by i.g. Ag/CTB is associated with strong induction of CD25+ Treg as well as to a lesser extent CD25+ CD4+ Treg that independently can transfer tolerance adoptively to naive recipients. Co-administration of CT, in contrast, effectively abrogates both the induction of oral tolerance and of Treg by i.g. OVA/CTB. The CD25+ Treg cells induced by i.g. administration of OVA/CTB in the absence of CT strongly express the Treg cell-controlling gene Foxp3 as well as CTLA-4, and depletion of TGF-β by specific Ab treatment completely inhibits the generation of CD25+ CD4+ Treg cells in response to i.g. administration of OVA/CTB.

In both OVA Tg and normal BALB/c mice, OVA/CTB-induced CD25+ cells efficiently suppressed OVA-specific CD25+ Treg cell proliferative responses in vitro as well as DTH response in vivo. Consistent with recent reports showing that food Ag could induce mucosal CD25+ Treg cells (6–8, 25), these CD25+ cells in PPs or MLNs were significantly increased after i.g. treatment with OVA/CTB, and studies with adoptively transferred CFSE-labeled CD4+ T cells confirmed the expansion of CD25+ CD4+ Treg cells. However, although the increase in Treg cell number was generally modest, at most 2- or 3-fold, the suppressive activity of these cells on Ag-specific T cell proliferation in coculture experiments in contrast increased >20-fold as determined in cell titration studies. Our results indicate that tolerance induced by OVA/CTB is not only associated with an increase in the number of CD25+ Treg cells, but also and mainly with increased suppressive activity and Foxp3 activation on a per cell basis.

TGF-β plays an important role in the induction and suppressive action of natural Treg (11, 14, 26), and also in the generation of Ag-induced peripheral Treg cells (7, 13, 27), but it is not known whether TGF-β is required for oral tolerance generation of mucosal Treg cells. In our study, oral tolerance induction by i.g. administration of OVA/CTB led to a markedly elevated level of TGF-β in serum associated with an increase in MLN Treg cells. Conversely, in vivo depletion of TGF-β by specific mAb treatment
completely suppressed the generation of CD25⁺ Treg cells in response to the i.g. OVA/CTB treatment and effectively removed most, if not all, of the ability of i.g. OVA/CTB treatment to induce functional suppression of T cell responses. It is notable that mucosal Ag/CTB treatment not only increases TGF-β production, as shown in this study and in previous reports (19, 22), but also completely suppresses proinflammatory IL-6 production (21). In light of the recent finding by Bettelli et al. (27) that TGF-β in the absence of IL-6 strongly promotes Treg generation but when combined with IL-6 instead, induces IL-17-producing, so-called Th17 effector T cells, the combined increase in TGF-β and decrease in IL-6 is put forward as an important explanation for the remarkably efficient oral tolerance induction and Treg generation by Ag/CTB treatment.

Of importance, and consistent with a recent report by Haueter-Broere et al. (25), we found that in addition to the strong induction of CD25⁺CD4⁺ Treg, i.g. treatment with OVA/CTB conjugate also induced CD25⁺CD4⁺ T cells that displayed suppressive function both in vitro and in vivo after adoptive transfer of these highly purified cells in recipients immunized s.c. with OVA/CFA. It is notable that the latter cell population also expressed the Foxp3 gene, although to a much lesser extent than the CD25⁺ cells, and that these cells, at least in part, may therefore be under differentiation toward increased expression of Foxp3 as well as expression of the IL-2rα receptor (CD25). In support of this kind of differentiation pathway, Zelenay et al. (28) recently reported that Foxp3⁺ CD25⁺ CD4⁺ T cells constitute a reservoir of committed regulatory cells that retain CD25 expression upon homeostatic expansion. Of note, CTLA-4, which plays an essential role in the function of CD25⁺ T cells in controlling inflammation (12), was found to be increased not only on OVA/CTB-induced CD25⁺ but to a lesser extent also on OVA/CTB-induced CD25⁺ T cells that had been sorted by FACS to rule out any contamination by CD25⁻ T cells.

Naturally occurring CD25⁺CD4⁺ Treg play crucial roles in the maintenance of immunological self-tolerance and negative control of various immune responses (9, 10). A recent study (29) also demonstrated a strong impairment in oral tolerance in naive mice depleted in vivo of natural CD25⁺CD4⁺ cells by Ab treatment. This raised a question as to whether tolerance induction by i.g. OVA/CTB would be critically dependent on natural CD25⁺CD4⁺ cells. To address this question, we adoptively transferred highly purified CD25⁻ OT-II T cells and, for comparison, a mixture of CD25⁺ and CD25⁻ OT-II T cells to Rag⁻/⁻ mice and then tested the induction of oral tolerance after i.g. treatment of the recipient mice with OVA/CTB conjugate. We found that this treatment strongly suppressed both splenic and DLN T cell proliferation to subsequent systemic immunization with OVA/CFA in both groups. When the CD4⁺ T cell populations of the treated mice were further examined, it was evident that in Rag⁻/⁻ mice that had received only the highly purified CD25⁻ population, the i.g. treatment with OVA/CTB had induced a significant generation of CD25⁺ T cells in MLN, spleen, and DLN similar to that seen in OVA/CTB-treated recipients of CD25⁻/CD25⁺ cells and significantly above the levels in PBS-treated controls. Furthermore, highly purified (cell sorted) OVA/CTB-induced CD25⁺ T cells isolated at the end of the experiment from mice that initially received CD25⁺ cells strongly expressed Foxp3 in contrast to the CD25⁻ population from corresponding PBS-treated mice. However, when the cells were isolated and examined at 3 wk rather than 7 days after treatment, the CD25⁺ cells from the PBS-treated animals also had detectable expressed Foxp3. Our results indicate that mucosal treatment with OVA/CTB independent of natural CD25⁺ Treg cells can promote Ag-induced generation of at least two populations of Treg from CD25⁻ precursors: one comprising strongly suppressive Foxp3⁺CD25⁺ Treg, and one comprising CD25⁺CD4⁺ T cells that have acquired regulatory-suppressive function without activation of the Foxp3 gene system.

The efficient generation and functional activation of CD25⁺ Treg by i.g. treatment with OVA/CTB conjugate is probably largely explained by the effects of the conjugate on gut mucosal APC. Previous studies have shown that conjugation of Ag to CTB, a molecule that binds with high affinity to the GM1 ganglioside receptors present on most cells (30), greatly facilitates Ag uptake and MHC class II-restricted Ag presentation by CD11c⁺DC as well as other types of APC, such as B cells and macrophages (23). One possibility is that mucosally administered Ag/CTB conjugate preferentially binds to and is taken up by tolerogenic subsets of mucosal DC or other APC. Consistent with this, we found that the increase in frequency of CD25⁺CD4⁺ T cells in PP and MLN correlated closely with an increase of CD11c⁺CD8α⁺B220⁺ DC between 2 h and 2 days after i.g. treatment with OVA/CTB (our unpublished data). This agrees fully with the recent findings by Anjua et al. (31) of a selective increase in this DC subset in MLN following CTB feeding, and the ability of this DC population from CTB-fed mice to support the differentiation of CD4⁺ Ag-specific Treg, producing TGF-β and IL-10.

CT is a powerful mucosal adjuvant for most coadministered protein Ags including OVA and stimulates strongly enhanced Th1, Th2, and CTL responses (2, 32). Oral administration of CT can in parallel with its adjuvant action prevent induction of oral tolerance to a coadministered Ag (17–19, 33), although CT has also been reported (34) to promote the induction of Treg (Tr1 cells) in vitro. In this study, we found that coadministration of even very small amounts of CT together with the OVA/CTB conjugate effectively prevented the induction of CD25⁺CD4⁺ suppressor T cells. Furthermore, CT also inhibited the normal suppressive function and Foxp3 gene expression of the mucosal CD25⁺ Treg cells in the OVA/CTB-treated mice in comparison with PBS-fed mice. However, in line with the previously reported inability of CT to abrogate already established oral tolerance (35), our findings showed only a relatively small effect of CT given after, rather than together, with a tolerizing OVA/CTB feeding regimen.

The mechanism(s) by which CT exerts these effects remains to be defined. One obvious mechanism could be that CT inhibits the generation or function of a normally predominant tolerogenic mucosal APC population. In support of this, Anjua et al. (36) found that oral administration of CT to mice results in a marked increase in PP and MLN of CD11c⁺CD8α⁺B220⁺ DC with potent immunological APC capacity together with inhibition of the normal development of tolerogenic CD11c⁺CD8α⁺B220⁺ (plasmacytoid) DC in response to CTB administration. We have also seen that i.g. coadministration of CT with OVA/CTB strongly decreases the frequency of tolerogenic CD11c⁺CD8α⁺B220⁺ DC compared with both OVA/CTB-fed mice and unfed controls (our unpublished data). Of note, CT, different from CTB, is a potent inducer for production of both IL-1β and IL-6 by both DC and other APC (32); these cytokines have recently been reported to activate Foxp3⁺ effector/memory T cells and attenuate Foxp3⁺CD4⁺CD25⁺ Treg cell function (27, 37). In addition, CT down-regulates expression of TGF-β (19) as well as IL-2 and IL-2/CD25 (38). Such effects may also be important, because TGF-β and IL-2 are essential for maintenance of CD25⁺ Treg cells in vivo (11, 14, 26, 27, 39).

In conclusion, our findings show that the exceptionally efficient induction of peripheral tolerance by orally administered Ag coupled to CTB is associated with increases in both the frequency and suppressive activity of Ag-specific CD25⁺CD4⁺ Treg cells that express Foxp3 as well as CTLA-4 proteins, and also leads to the additional generation of both Foxp3⁺ and Foxp3⁺CD25⁺CD4⁺
T\textsubscript{reg}  The predominant induction and activation of CD25\textsuperscript{+} T\textsubscript{reg} cells by oral Ag/CTB conjugate treatment is associated with increased induction of TGF-β1. Co-administration of CT with normally tolerizing Ag/CTB treatment regimens completely blocks the induction of oral tolerance and T\textsubscript{reg}, probably largely through the differential effects of CT compared with CTB on APC that can then translate opposing effects on T\textsubscript{reg} and T\textsubscript{eff} cells.

Note. While this manuscript was undergoing the revision process, another study from this laboratory also described the induction of oral tolerance associated with but not critically dependent on CD25\textsuperscript{+} cell expansion after oral administration of an influenza virus hemagglutinin-derived peptide/CTB gene fusion protein (40).

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Disclosures

J. Holmgren is an inventor on a patent which is titled, “Immunological expertly preparing and characterizing OVA/CTB conjugate lots.

References