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B Lymphocytes Promote Expansion of Regulatory T Cells in Oral Tolerance: Powerful Induction by Antigen Coupled to Cholera Toxin B Subunit

Jia-Bin Sun,†* Carl-Fredrik Flach,* Cecil Czerkinsky,‡ and Jan Holmgren*

Mucosal administration of Ag conjugated to cholera toxin B subunit (CTB) can efficiently induce peripheral immunologic tolerance, so-called oral tolerance, associated with development of Foxp3\(^+\)CD25\(^+\)CD4\(^+\) regulatory T (Treg) cells. Using an established sublingual tolerization regimen with Ag(OVA)/CTB conjugate, wherein CTB mediates Ag uptake and presentation by most B lymphocytes irrespective of their Ag specificity, we have assessed the importance of B cells for induction of Ag-specific Treg cells and oral tolerance. We found that Treg cells are reduced in μMT\(^−/−\) B cell-deficient mice compared with wild-type (WT) mice. After sublingual Ag/CTB treatment, Treg cells increased much more in WT than in μMT\(^−/−\) mice; however, adoptive transfer of B cells before treatment normalized Treg cell development and functional oral tolerance. B cells from OVA/CTB-treated mice expressed more IL-10 and less CD86 than control B cells. Adoptive transfer of these cells before parenteral immunization with OVA led to efficient suppression of proliferation and to induction of apoptotic depletion of Ag-specific CD25\(^+\)CD4\(^+\) effector T cells associated with the expansion of Treg cells. However, also OVA/CTB-treated μMT\(^−/−\) mice could suppress the immune response to parenteral immunization with OVA, which was associated with a strong increase in Foxp3\(^−/−\)CD4\(^+\) T cells expressing LAP/TGF-β. Our results indicate that mucosal tolerance comprises at least two separate pathways: one being B cell dependent and associated with expansion of Treg cells and Treg-mediated suppression and depletion of effector T cells, and one being B cell independent and associated with development of Foxp3\(^−/−\)LAP\(^−/−\)TGF-β\(^−/−\) regulatory T cells. The Journal of Immunology, 2008, 181: 8278 – 8287.

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Abbreviations used in this paper: Teff, effector T; Treg, CD4\(^+\)Foxp3\(^+\) regulatory T; CMLN, cervicovaginal/balbicular lymph node; CTB, cholera toxin B subunit; DC, dendritic cell; DLN, draining lymph node; DTH, delayed-type hypersensitivity; MT, membrane-bound TGF-β; LAP, latency-associated peptide; MLN, mesenteric lymph node; s.l., sublingual; WT, wild type; Tg, transgenic; PLN, popliteal lymph node; 7AAD, 7-aminoactinomycin D; mTGF-β, membrane-bound TGF-β.

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many cell types (35), dramatically potentiates the mucosal tolerizing effect (27–34) both by facilitating Ag transport across the mucosal barrier and, most important, by increasing Ag uptake and presentation by different APCs more than 100-fold (36). Thus, the majority of all B cells irrespective of their BCR specificity become effective APCs for any contacted Ag coupled to CTB. In the present study, using OVA as model Ag and a well established tolerance-inducing regimen of s.l. administration of OVA/CTB conjugate (33, 34), we have tested the importance of B lymphocytes in the induction of oral tolerance and especially for the development of Ag-specific Treg cells. Our results indicate that B cells play an important role as tolerogenic APCs after mucosal exposure to Ag/CTB conjugate and are especially important for the induction of Ag-specific Foxp3+ Treg cells, which is a hallmark of mucosal tolerance.

Materials and Methods

Mice

Two strains of 6- to 8-wk-old female mice were used: BALB/c and C57BL/6 mice (B&K Universal). In addition, we used μMT−/− mice on a C57BL/6 background (gift from Dr. N. Lycke, University of Gothenburg, Göteborg, Sweden) and also T cells prepared from DO11.10. OVA TCR-transgenic (Tg) mice on a BALB/c background (The Jackson Laboratory), a clone with nearly 50% of the CD4+ T cells expressing a TCR specific for the peptide212–219 fragment of OVA, and OVA TCR Tg T cells from OT-II mice or OT-II (CD45.1+) with the same TCR specificity as DO11.10, but on the C57BL/6 background (gift from Dr. M. J. Wick, University of Gothenburg, Göteborg, Sweden). All mice were kept under specific pathogen-free conditions at the Department of Experimental Biomedicine (University of Gothenburg). Studies were approved by the University of Gothenburg Ethical Committee for Animal Experimentation.

Ag and conjugation of OVA to CTB subunit

OVA protein (grade VII) was purchased from Sigma-Aldrich and OVA233–349 peptide (OVA233–349) (SQQYVAHAAHAEINAGK) of >95% purity was obtained from TAG Copenhagen. Highly purified recombinant CTB was provided by SBL. Vaccin OVA protein was chemically coupled to CTB using N-succinimidyl (3-[2-pyridyl]-dithio)propionate (Pierce Biotechnology) as a bifunctional coupling reagent as previously described (32). The purified conjugate was analyzed by a GM1 ganglioside ELISA and shown to have strong GM1-binding activity as well as high reactivity with both OVA and CTB (32). The conjugate also had strong capacity to induce OVA-specific T cell proliferation when tested on DO11.10 or OT-II splenocytes; furthermore, in the latter assays, the activity of the conjugate was not significantly inhibited by precipitation and coculture with polymyxin but was completely inhibited by precipitation and coculture with highly purified GM1 ganglioside (10 nmol/ml; donated by late Prof. L. Svennerholm). Finally, the capacity of the OVA/CTB conjugate to bind to B cells was documented by comparing the staining of purified CD19+ B cells after incubation with FITC-labeled CTB (10 μg/ml for 30 min; Sigma-Aldrich) in the absence or presence of the conjugate (20 μg/ml added 30 min before the FITC-CTB). Although >90% of cells were stained by FITC-CTB alone as analyzed by flow cytometry, the precipitation with OVA/CTB conjugate almost completely inhibited/blockaded staining (as did also precipitation with 10 μg/ml unlabeled CTB or incubation along with 10 nmol/ml GM1 ganglioside).

Chloroform was purchased from Sigma-Aldrich and stored at −20°C in single-use aliquots at a concentration of 1 mg/ml until used.

Sublingual Ag administration and s.c. immunization

BALB/c or C57BL/6 or μMT−/− mice were first infused with 5 × 10^6 purified CD4+ DO11.10 or OT-II T cells given i.v. Starting 1 day later, the mice were given two or three 5-μl doses of either 60 μg of OVA/CTB or PBS s.i. at 2-day intervals.

In some experiments, mice were further immunized at the tail base s.c. with 50 μg of OVA emulsified 1:1 in IFA or CFA 1 day after the last s.l. treatment. At indicated times after s.l. treatment only, or s.l. treatment followed by s.c. immunization, the mice were sacrificed and cervicodibular lymph nodes (CMLNs), mesenteric lymph nodes (MLNs), or popliteal/inguinal nodes (PLNs) and spleen were harvested and cells were prepared (32) for further in vitro studies.

Relative mRNA quantification by real-time PCR

Total RNA was prepared from isolated CD19+ or CD4+ CMLN cells by using Qiagen’s RNeasy mini kit. The concentration was measured with the NanoDrop ND-100 spectrophotometer (Thermo Fischer Scientific). For each sample, 500 ng of RNA was converted into cDNA by random hexamer priming and the Omniscript reverse transcriptase (Invitrogen). After termination, the cDNA reactions were diluted with water to a final volume of 50 μl. An Applied Biosystems 7500 Real-Time PCR System was used for the relative quantification, by using SYBR Green as detector, according to the manufacturer’s protocol. For each reaction, set up in triplicates, 2 μl if cDNA was used. The following oligonucleotide primers (Eurofins MWG Operon) were used: IL-10 (5′-cctgtaattcccttggtgta-3′ and 5′-gcgctact gcctggctt-3′), TGF-β (5′-ccagcagaacccgattc-5′ and 5′-ctccaccgaccttcctaa-3′), and β-actin (5′-cctgacaggatcagaggtact-3′ and 5′-gc cacgatccacaggt-3′). The expression of IL-10 and TGF-β was in all samples related to the β-actin expression.

Immunohistochemistry staining and confocal laser scanning microscopy

For immunohistochemical studies of Foxp3+ CD4+ (Treg) T cells, spleens were frozen in Tissue-Tek (Sakura) and sliced into 8-μm-thin sections that were fixed for 10 min in cold (−20°C) acetone and then washed in PBS. Sections were then incubated with polyvalent rabbit anti-mouse Foxp3 (eBioscience) in PBS supplemented with 0.5% Tween 20, 1% normal goat serum, and 1% BSA, followed by incubation with goat anti-rabbit IgG conjugated with Fluor Alexa 488 (Molecular Probes). The sections were then further stained for CD4+ T cells by incubations with purified rat anti-mouse CD4 mAb (BD Biosciences Pharmingen) followed by anti-rat IgG conjugated with Fluor Alexa 647 (Molecular Probes). Sections were carefully washed between all steps, and after final washing the stained sections were placed under coverglass in the presence of Prolong Gold antifade reagent with Dapi (Invitrogen/Molecular Probes) to prevent fading and examined by fluorescence microscopy using a computer-assisted image analysis system (Zeiss software AxioVision 4.5).

The stained sections were further examined by confocal laser-scanning microscopy (>100 objective) with a krypton/argon laser (Bio-Rad MRC 1024) using triple-channel scanning.

T cell proliferation

Single-cell suspensions from MLNs, PLNs, or spleen were prepared and erythrocytes were removed by lysis. For studies of cell proliferation, 4 × 10^5 cells/well were cultured (in a 200-μl volume of IMDM supplemented with 10% FCS, 1% l-glutamine, 1% gentamicin, and 50 μM 2-ME) for 3 days in 96-well plates with or without 0.1 or 1 μg/ml OVA233–349 peptide. [3H]Thymidine (1 μCi/ml) was added for the last 16 h of culture, after which [3H] incorporation was measured as previously described (32).

Isolation and adoptive transfer of cells

CD4+ T cells were prepared from lymph node and spleen cells of C57BL/6, OT-I, or OT-II mice by negative selection using a mixture of MACS microbeads coated with mAbs (Milteny Biotech). Five million CD4+ T cells were i.v. injected into the tail vein of naive BALB/c or C57BL/6 WT or μMT−/− recipient mice. In some experiments, purified (>99%) CD19+ B cells were prepared by positive selection using MACS microbeads coated with Ab-PE against mouse CD19 (Milteny Biotech). These cells were then used for studies in vitro or injected i.v. into the tail vein of naive BALB/c or C57BL/6 WT or μMT−/− recipient mice (1 × 10^6 or 2 × 10^6 were infected) 1 day after the adoptive transfer of CTB Tg T cells (in one experiment a second such B cell infusion was given 1 wk later).

CFSE labeling of T cells and infusion of labeled cells

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

Flow cytometry staining and analyses

Freshly isolated spleen or lymph node cells were incubated with FITC-PE, FITC- Con A, allophycocyanin-Alexa488 (BD Biosciences Pharmingen) to mouse CD4, CD25, CD45.1 (Ly5.1), or OVA233–349-specific clonotypic TCR (for DO11.10 (BALB/c) cells to KJ1-26 and for OT-II (C57BL/6) cells to Vn2). For detection of intracellular Foxp3, cells were fixed and
permeabilized with Cytofix/Cytoperm solution (eBioscience) according to the manufacturer’s recommended protocol and then incubated with allopurinol-conjugated anti-Foxp3 FLK-16 mAb (1 μg/10⁶ cells; Nordic Biosite) at 4°C for 30 min in the dark. Cells were then washed and analyzed by flow cytometry (FACSCalibur machine; BD Pharmingen). Apoptotic cells were detected by staining with anti-mouse annexin V-allophycocyanin and 7-aminoactinomycin D (7AAD)-PerCP Abs (BD Biosciences Pharmingen) according to the manufacturer’s protocol. For analysis of cells expressing latency-associated peptide (LAP) (and indirectly membrane-bound TGF-β (mTGF-β) bound to LAP), cells were stained with biotinylated anti-LAP Ab (R&D Systems) followed by streptavidin-allophycocyanin and other labeled Abs against surface markers before FACS analysis. For analysis of intracellular staining of IL-10 in OVA-specific T cells, cells were first incubated for 3 days with OVA p323–339 and then after addition of IL-2 (5 ng/ml) and IL-4 (2 ng/ml) for another 3 days; the cells were then finally transferred to a plate coated with anti-CD3 (5 μg/ml) and incubated for an additional 5 h with the addition of monensin (2 μM) to the medium for the last 2 h. Cells were finally stained with Abs to surface markers and then fixed and permeabilized with Cytofix/Cytoperm solution (eBioscience and further incubated with FITC-conjugated anti-IL-10 mAb (BD Biosciences Pharmingen) at 4°C for 30 min in the dark before FACS analysis.

After adoptive transfer of OVA-specific OT-II cells, carrying either the CD45.1 marker or being labeled with CFSE, we analyzed the percentages of OVA-specific Treg cells among total CD45.1⁺ or CFSE⁺ cells and also estimated the total numbers of such cells in the examined tissues.

**Statistical analysis**
Results are expressed as mean ± or ± SD. When not specified otherwise, we used the unpaired Student t test for calculating statistical differences between experimental and control groups. Values of p < 0.05, <0.01, and <0.001 are referred to in the figures with symbols *, **, and ***, respectively.

**Results**
We have previously shown that for the induction of Ag-specific peripheral T cell tolerance (“mucosal tolerance”) using OVA as model Ag, oral administration of OVA/CTB conjugate is superior to administration of either OVA alone or OVA mixed with CTB (32, 33). We have also found that s.l. administration of OVA/CTB is at least as efficient as oral administration in inducing mucosal tolerance and that tolerance is associated with the generation of Ag-specific Foxp3⁺CD25⁺CD4⁺ Treg cells and apoptotic depletion of Ag-specific Foxp3⁺CD25⁻CD4⁻ effector T (Teff) cells (34). A contributory role of B cells in the induction of oral tolerance has been described previously (17–26), but the role of B cells for the generation of Ag-specific Treg cells after mucosal Ag administration is poorly understood. In this study, we wished to further address this question as well as to assess the quantitative importance of B cells in mucosal tolerance induction.

**Treg cells are reduced and Teff cells are increased in μMT⁻/⁻ mice**
We examined whether the frequency of Treg cells in lymphoid organs differed between μMT⁻/⁻ and WT mice, since this might be assumed to be the case if B cells are important in the generation of Treg cells. We collected spleen, CMLNs, and MLNs from naive μMT⁻/⁻ mice or WT mice and analyzed the expression of Foxp3 among the CD4⁺ T cells by flow cytometry. The results show that the frequencies of Foxp3⁺ cells in both the total CD4⁺ (Fig. 1A) and the gated CD25⁺CD4⁺ (T Fig. 1B) population from the spleen are reduced by ~50% in μMT⁻/⁻ mice compared with age- and sex-matched WT mice. In contrast, spleen cells from the μMT⁻/⁻ mice had a substantially increased level of Foxp3⁺CD25⁺CD4⁺ T cells (largely Teff cells) (Fig. 1C). A similar pattern was also found in CMLNs and MLNs although differences were less striking, approximately one-half of those found in the spleen (data not shown). Since lymphoid organs in naive μMT⁻/⁻ mice have significantly fewer CD4⁺ T cells than WT mice, which is especially marked in the spleen, the observed reductions in frequencies of Treg cells in μMT⁻/⁻ mice compared with WT mice represent even bigger differences in the total numbers of Treg cells in the different organs: a 13-fold difference in spleen and 38 and 33% reductions in total Treg cells in CMLNs and MLNs, respectively, in μMT⁻/⁻ compared with WT mice (data not shown).

**FIGURE 1.** Reduced frequency of Treg cells (A and B) and increased frequency of Teff cells (C) in spleens of B cell-deficient μMT⁻/⁻ mice. Spleen cells were collected from groups (six mice per group) of naïve μMT⁻/⁻ and WT C57BL/6 mice and examined by flow cytometry after surface staining with anti-CD4-FITC and anti-CD25-PE followed by intracellular staining with anti-Foxp3-allophycocyanin. Results show proportions of Foxp3⁺ cells among gated total CD4⁺ T lymphocytes (A) and gated CD4⁺CD25⁺ cells (B), respectively, and in C the frequencies of CD4⁺ cells among gated CD25⁺Foxp3⁺ (Teff) cells. Values are percentages of indicated cell populations and show a reduction by ~50% of Teff cells (48.9 ± 9.4% (mean ± SD) for CD4⁺Foxp3⁺ cells, p < 0.05) and a similar increase in Teff cells (96 ± 12.4%, p < 0.05) in μMT⁻/⁻ mice compared with WT mice.
We next wished to determine whether the lack of B cells in μMT−/− mice would affect the ability of these mice in comparison to WT mice to generate functional Ag-specific Treg cells after s.l. tolerization with OVA/CTB conjugate. We first adoptively transferred OVAp323–339-specific Tg CD4+ T cells from OT-II or OT-II (CD45.1+) mice into either μMT−/− or WT mice, and then starting 1 day later gave the mice three s.l. administrations of OVA/CTB (60 μg/dose) or for control purposes PBS at 2-day intervals between the doses. Two days after the last dose, mice were sacrificed and their MLN cells were tested for the presence of cells expressing Foxp3 and/or CD4 by immunohistochemistry or flow cytometry. In addition, MLN cells were tested for their capacity to mount a proliferative response when cultured in vitro along with OVAp323–339.

The results are shown in Fig. 2. Consistent with the flow cytometric data in Fig. 1, the frequency of Foxp3+ CD4+ T (Treg) cells was markedly reduced in the spleen from PBS-treated μMT−/− mice compared with WT mice (Fig. 2A). Interestingly, however, in response to s.l. treatment with OVA/CTB, there was a similar 2- to 3-fold increase in the expression of such Treg cells in the spleen of both WT and μMT−/− mice, but the absolute numbers of Treg cells in the μMT−/− mice after treatment with OVA/CTB did not exceed those in the PBS-treated WT mice and thus were much lower than in the OVA/CTB-treated WT mice (Fig. 2A). Furthermore, as indicated by confocal microscopy, the Foxp3 staining intensity in CD4+ T cells was higher in PBS-treated WT mice compared with PBS-treated μMT−/− mice even after OVA/CTB treatment (Fig. 2A, insets).

This picture was confirmed by flow cytometric examinations of OVA-specific Treg cells in CMLNs and MLNs of WT and μMT−/− mice in response to s.l. OVA/CTB treatment. The results show that only the WT mice had a significant expansion of OVA-specific Foxp3+CD4+ cells in CMLNs and MLNs in response to s.l. OVA/CTB treatment (Fig. 2B).

Finally, OVAp323–339-specific MLN T cell proliferation was compared in μMT−/− mice and WT mice after s.l. treatment with OVA/CTB. Although such responses were markedly suppressed in the spleen of μMT−/− mice compared with WT mice (Fig. 2A), it should be noted that the overall T cell responses are much lower in μMT−/− mice compared with WT mice.

Experiments in which WT and μMT−/− mice were s.l. treated with three doses of corresponding amounts of OVA alone showed only a marginal increase in Treg numbers and suppression of OVAp323–339-specific T cell proliferation, and the same was true for mice treated with OVA/CTB given in combination with 2 μg of cholera toxin/dose (data not shown).

We conclude that after mucosal treatment with OVA/CTB, μMT−/− mice had a reduced ability compared with WT mice to generate a Foxp3+ Treg cell response and to suppress Ag-specific Teff cell reactivity in peripheral lymph nodes.

**Substitution with B cells normalizes the response to s.l. OVA/CTB treatment in μMT−/− mice**

To further validate the role of B cells in the induction of Treg cell expansion and tolerance, we conducted experiments in which purified CD19+ B cells were adoptively transferred along with purified CD4+ OT-II T cells into μMT−/− or WT mice. Then, 1 day later, recipient mice were treated with OVA/CTB or PBS. Two days after the last s.l. treatment, animals were sacrificed and their MLN cells were tested for in vitro T cell proliferation in response to OVAp323–339. The results showed that the number of OVA-specific Teff cells was much lower in μMT−/− mice than in the OVA/CTB-treated WT mice (Fig. 2A). Further analysis of Foxp3+ T cells among total CD4+ Treg cells among total OT-II (CD45.1+) cells in CMLN and MLN cells from μMT−/− and WT mice (dotted and open bars, respectively) as well as the total numbers of such cells in these organs (striped and filled bars) in response to the different treatments. C, OVAp323–339 stimulated T cell proliferation in MLN cells from μMT−/− and WT mice in response to s.l. treatment with OVA/CTB or PBS. Data in B and C are from experiments with a single strain of mice and are representative of similar results obtained in LSstrain mice. Values (in boxes) show mean ± SD values (six mice per group). (B) and (C), the percentages of transferred total OVA-specific OT-II (CD45.1+) cells among total CD4+ cells were 1.1 ± 0.1% and 1.7 ± 0.2% in CMLNs for μMT−/− mice treated with OVA/CTB and PBS, respectively, and 2.6 ± 0.7% and 1.8 ± 0.4% for WT mice; in MLNs, the relative numbers were 1.1 ± 0.1% and 1.6 ± 0.3% in μMT−/− mice and 1.6 ± 0.2% and 2.0 ± 0.2% in WT mice. *p < 0.05; **p < 0.01; and ***p < 0.001 for the indicated OVA/CTB treatment groups in comparison to PBS-treated mice.
show that the MLN T cells from μMT−/− mice that had received B cells before s.l. tolerization showed a significantly reduced (>50% inhibition) proliferative response, although they were still less strongly suppressed than MLN T cells from similarly OVA/CTB-treated WT mice (Fig. 3A). This suppression of Ag-specific MLN T cell proliferation by mucosal OVA/CTB treatment in μMT−/− mice after B cell substitution contrasts sharply with the results seen with MLN T cells from μMT−/− mice that had not been substituted with B cells; in these mice, no inhibition but rather an increase in their proliferation to OVAp323–339 was found after the s.l. treatment with OVA/CTB (see Fig. 2C).

In parallel experiments, we also tested the effect of B cell transfer to μMT−/− mice on the in vivo cell division and generation of Foxp3+ cells among transferred CFSE-labeled CD4+ OT-II cells in response to s.l. treatment with OVA/CTB or PBS. We found that after adoptive transfer of B cells the frequency of OVA-specific (i.e., divided) Foxp3+CD4+ Treg cells among the total CFSE-labeled CD4+ OT-II T cells in μMT−/− mice in response to s.l. OVA/CTB treatment was much higher than in μMT−/− mice that had not received any B cells and similar to the level in OVA/CTB-treated WT mice (Fig. 3B). Consistent with this, in μMT−/− mice that received B cells along with CFSE-labeled CD4+ cells and then were treated twice s.l. with OVA/CTB, 50–80% of CFSE-labeled Foxp3+CD4+ Treg cells had undergone division (Fig. 3C). These frequencies were similar to the proportion (67%) of divided CFSE-labeled Foxp3+CD4+ cells in OVA/CTB-treated WT mice (Fig. 3C). We conclude that substitution with B cells could fully compensate the μMT−/− mice for their normally deficient Treg response to mucosal treatment with OVA/CTB.

Sublingual tolerization with OVA/CTB enhances the tolerogenic activity of B cells and their production of IL-10.

Next, we wished to test whether the induction of Ag-specific Treg cells could be related to the Ag-presenting capacity, expression of costimulatory molecules, and/or production of cytokines by B cells. This was tested in BALB/c mice after adoptive transfer of DO11.10 T cells to allow us to directly identify Ag-specific T cells by staining with the clonotype-specific KJ1-26 Ab. We treated BALB/c WT mice that had adoptively received CD4+ DO11.10 T cells three times s.l. at 2-day intervals with either OVA/CTB conjugate or PBS. One day after the last treatment, the mice were sacrificed and CD19+ B cells from CMLNs and spleen were isolated and cocultured with purified DO11.10 CD25−CD4+ T effector cells in the presence of OVAp323–339. We found that B cells isolated from mice tolerized with s.l. OVA/CTB had a significantly reduced capacity to induce T cell proliferation compared with B cells from PBS-treated mice (Fig. 4A). Furthermore, CMLN B cells from the OVA/CTB-treated mice had significantly less expression of CD86 but not CD80 compared with B cells from PBS-treated mice (Fig. 4B). Our results indicate that the mucosal OVA/CTB treatment reduced the Ag-presenting capacity of B cells in DLNs or more likely made these cells partly tolerogenic as APC for Teff cells.

Based on this, we examined whether s.l. OVA/CTB treatment could also alter the capacity of B cells to produce regulatory-suppressive cytokines such as IL-10 and TGF-β and/or the production of these cytokines in CD4+ T cells. After adoptive transfer of DO11.10 CD4+ T cells, WT mice were treated with OVA/CTB as described above and 1 day later CD19+ B cells and CD4+ T cells were isolated from draining CMLNs. Isolated B cells were tested for IL-10 and TGF-β mRNA expression by real-time PCR. Furthermore, CD4+ T cells were examined for expression of these cytokines by flow cytometry after in vitro stimulation with OVAp323–339. The results show a much stronger expression of IL-10 in B cells from CMLNs of WT mice treated with OVA/CTB compared with B cells from PBS-treated
Role of B cells in induction of Ag-specific apoptosis in Teff cells after s.l. OVA/CTB treatment

As mentioned, we recently described the induction of Ag-specific apoptosis in Teff cells associated with and probably mediated by Treg cells in mice treated s.l. with OVA/CTB (34). Consistent with this, we found that 2 days after the last of three s.l. treatments with OVA/CTB Ag-specific CFSE-labeled OT-II Teff cells from CMLNs of WT mice had a much higher proportion of apoptotic (annexin V+) dividing cells than found in similarly treated μMT−/− mice (data not shown). We wished to further define to which extent OVA/CTB-treated B cells are important for these effects and, therefore, in the same experiments as described in Fig. 5b, the transfer of B cells from mice treated s.l. with OVA/CTB significantly suppressed the Ag-specific DTH response compared with transfer of B cells from PBS-treated mice. Likewise, the PLN T cell proliferative response to OVAp323–339 was significantly reduced in mice that had received B cells from OVA/CTB-treated mice compared with animals that received B cells from PBS-treated animals or did not receive any B cells (Fig. 5b).

 FIGURE 4. Sublingual treatment with OVA/CTB conjugate induces increased in vitro tolerogenic activity in CMLN B cells associated with reduced CD86 expression and increased IL-10 expression and also increases the production of IL-10 and TGF-β in CD4+ T cells from CMLNs. Groups of naive BALB/c mice were adoptively transferred with 5 × 106 purified CD4+ DO11.10 Tg T cells by i.v. injection and, starting 1 day later, the mice were given three s.l. doses of either 60 μg of OVA/CTB (filled symbols) or PBS (open symbols) every second day. One day after the last s.l. treatment, the mice were sacrificed and CD19+ B cells were isolated from their CMLNs. A, These B cells were then cocultured in triplicates for 3 days in the absence or presence of 0.1–1.0 μg/ml OVAp323–339 with 1 × 105 CD25+ CD4+ T (Teff) cells isolated from naive DO11.10 mice. Data are from one of two independent experiments giving similar results and showing mean ± SD values (five mice per group). Statistical analysis by ANOVA indicate a significant difference, p < 0.05 (⁎), between the group that received B cells from OVA/CTB-treated mice and the group that received B cells from PBS-treated mice. B and C, Similarly prepared B cells were tested for percentages of CD19+ B220− cells (mean ± SD, five animals per group) expressing CD86 or CD80 by flow cytometry (B) and for production of IL-10 and TGF-β mRNAs by real-time PCR (mean ± SEM values of triplicate assays, ×10−4 for IL-10 and ×10−2 for TGF-β) (C). Furthermore, unfractionated CMLN cells isolated from similarly treated mice were examined by flow cytometry for mean ± SD percentages of cells showing surface expression of LAP/mTGF-β or intracellular expression of IL-10 among gated CD4+ T cells after culture in vitro as described (D). (The percentages of Ag-specific (KJ1-26+) cells among total CD4+ cells before culture were 1.1 ± 0.4% and 2.6 ± 0.6% for PBS- and OVA/CTB-treated mice, respectively.)

DOSAGE OF OVAp323–339 TREATMENT

mice, whereas no difference was seen in the expression of TGF-β mRNA (Fig. 4C); B cells isolated 2 days instead of 1 day after the last treatment showed the same pattern as did B cells from mice given a single s.l. 120 μg of OVA/CTB (data not shown). In contrast, when CD4+ T cells from CMLNs of the same animals were examined by FACS, both IL-10 and LAP+ mTGF-β+ cells were greatly increased in response to OVA/CTB treatment (Fig. 4D); no such effects were seen in μMT−/− mice tested in a separate experiment (data not shown). We conclude that the mucosal treatment with OVA/CTB reduced CD86 but not CD80 expression and also induced increased IL-10 but not TGF-β production by B cells in DLN and also led to an increased frequency of both IL-10+ and LAP+ mTGF-β+ CD4+ T cells in these lymph nodes.

To further examine to which extent the generation of tolerogenic B cells could be responsible for the induction of not only Treg cell expansion but also functional mucosal tolerance, adoptive transfer experiments were conducted. WT BALB/c mice were given three s.l. doses of OVA/CTB or PBS every second day, and 2 days after the last treatment CD19+ B cells were isolated from CMLNs and spleens. These B cells were transferred along with OVA-specific (DO11.10) CD4+ T cells into WT BALB/c mice and 1 wk later recipient mice were given a second infusion of identically prepared B cells (without DO11.10 CD4+ T cells). One week after the last B cell transfer, the mice were s.c. immunized with OVA in IFA and 2 wk later mice were tested for their in vivo DTH response to OVA Ag challenge. A separate set of mice was sacrificed and their PLN cells examined for in vitro T cell proliferation in response to OVAp323–339 stimulation. As shown in Fig. 5A, the transfer of B cells from mice treated s.l. with OVA/CTB significantly suppressed the Ag-specific DTH response compared with transfer of B cells from PBS-treated mice. Likewise, the PLN T cell proliferative response to OVAp323–339 was significantly reduced in mice that had received B cells from OVA/CTB-treated mice compared with animals that received B cells from PBS-treated animals or did not receive any B cells (Fig. 5B).
had received B cells from s.l. OVA/CTB-treated mice before being s.c. immunized with OVA/IFA, compared with mice that had received B cells from PBS-treated mice (Fig. 6A). In agreement with the results described above for spleens, CMLNs, and MLNs of WT mice or B cell-supplemented μMT−/− mice treated s.l. with OVA/CTB (see Fig. 2 above), the frequency of Ag-specific Foxp3+ Treg cells was also increased in mice that had received B cells from mice treated s.l. with OVA/CTB compared with mice that had received B cells from PBS-treated mice (Fig. 6B). Furthermore, PLN cells from the OVA/CTB B cell-treated mice showed a significant increase in IL-10-producing B cells (Fig. 6C).

B cells are dispensable for oral tolerance induction and for development of LAP/TGF-β-expressing Foxp3+ Treg cells

Given the importance in the experiments described above of B cells for the induction of Treg cells and suppression of Teff cells both in vivo and in vitro after s.l. treatment with OVA/CTB, we wished to test whether B cells are truly critical for the development of effective T cell tolerance after mucosal Ag treatment. We therefore gave WT and μMT−/− mice three s.l. treatments with OVA/CTB at 2-day intervals between doses and then, 1 day after the last treatment, immunized the mice s.c. with OVA along with CFA. Two weeks after the s.c. immunization, PLN cells were examined for Ag-specific Teff cell proliferation and the contents of both Treg (Foxp3+CD25+CD4+ cells and Teff (Foxp3−CD25+CD4+) cells. The results are seen in Table I and show that both in μMT−/− and WT mice s.l. treatment with OVA/CTB induced a strong suppression of proliferation of cultured PLN T cells. In contrast, there was a significant difference in Foxp3+ Treg development in the WT and μMT−/− mice, inasmuch as the WT mice showed a strong significant increase in Foxp3+ Treg cells in PLNs after s.l. treatment with OVA/CTB, whereas the μMT−/− mice had no significant increase in this type of cells. Furthermore, after s.l. OVA/CTB treatment, there was a significant reduction of
Foxp3− CD25− CD4+ T (Teff) cells in the WT but not in the μMT−/− mice (Table I). Thus, it is clear that effective Ag-specific suppression of the peripheral Teff cell responsiveness can be achieved by prior mucosal treatment with Ag/CTB conjugate also in μMT−/− mice which are lacking B cells, even though these mice are unable to mount a significant Foxp3+ CD25+ CD4+ Treg cell response.

Since previous work has shown that in addition to Treg cells also TGF-β-expressing Foxp3+ CD4+ T cells with regulatory-suppressive function on Teff cells are induced by mucosal (oral) OVA/CTB administration (32), we examined the PLN T cells from the s.i. OVA/CTB- and PBS-treated WT and μMT−/− mice also for LAP+ TGF-β+ T cells to see whether such cells could explain the effective oral tolerance induction also in the B cell-deficient μMT−/− mice, despite the absence of a significant Foxp3+ Treg cell response to OVA/CTB treatment in these mice. The results show that in both WT and even stronger in the μMT−/− mice the OVA/CTB treatment induced an increase in LAP+ TGF-β+ Foxp3− CD4+ T cells with presumed regulatory-suppressive function (Table I). Such cells were increased by 115% in μMT−/− mice treated with OVA/CTB compared with mice treated with PBS, which was significantly more than seen in WT mice (p < 0.05).

Discussion

Mucosal administration of Ag conjugated to CTB can efficiently induce peripheral tolerance, so-called oral tolerance, which is associated with the induction of various types of regulatory T cells including as a prominent type Foxp3+ CD25+ CD4+ Treg cells (27–34). Using OVA as model Ag and a well-established s.i. tolerization regimen with OVA/CTB conjugate (33, 34), we have tested the role of B lymphocytes for the induction of oral tolerance. Our results indicate that B cells are efficient tolerogenic APCs after mucosal treatment with Ag/CTB conjugate, especially for the development of Ag-specific Foxp3+ Treg cells. The conjugation of Ag to CTB very much enhances the tolerogenic effects, including the Ag-specific expansion of Treg cells. Nevertheless, B cells are dispensable for induction of functional peripheral T cell tolerance after mucosal treatment with OVA/CTB conjugate. Thus, μMT−/− B cell-deficient mice could be tolerized by s.i. treatment with OVA/CTB associated with a strong increase in Foxp3− CD4+ T cells expressing LAP/TGF-β.

In previous work describing a role of B cells in T cell tolerance, several mechanisms have been described, including both B cell-induced T cell deletion and/or anergy and the induction of regulatory CD4 or CD8 T cells producing IL-10 and/or TGF-β (19–22, 25, 26, 37). A direct effect of B cells on Treg cells was also recently reported by Chen and Jensen (38) who, using primary B cells as APCs and allogeneic CD4 T cells as responder cells in MLRs, found that B cells in the absence of exogenous cytokines preferentially expanded Foxp3+ over Foxp3− CD4 T cells. Our results are in general agreement with these findings and to our knowledge we are the first to show a potent role in vivo for primary B cells in inducing and/or expanding Ag-specific Treg cells in oral tolerance. We found that Foxp3 expression among naive CD4+ T cells was markedly reduced in μMT−/− mice compared with WT mice, and after s.i. OVA/CTB treatment Treg cells increased to much higher levels in WT than in μMT−/− mice. The transfer of B cells to μMT−/− mice before s.i. OVA/CTB treatment on the other hand made these mice equally as able as WT mice to develop OVA-specific Treg cells and functional oral tolerance. Notably, B cells from OVA/CTB-treated mice had substantially reduced expression of CD86 and decreased Ag-presenting capacity to activate Teff cells compared with B cells from PBS-treated mice, and they also produced much more IL-10. When adoptively transferred to normal naive mice, these cells could both efficiently suppress the T cell proliferative response and restore the induction of apoptosis and depletion in Ag-specific CD25+ CD4+ effector T cells by promoting the expansion of Foxp3+ Treg cells. Importantly, adoptive transfer of such in vivo “educated” B cells increased not only KJ1-26 Foxp3+ Treg but also IL-10-producing CD19+ B cells in DLNs of inflamed sites in recipient mice. This implies that mucosal treatment with Ag/CTB not only induced B cell-dependent Ag-specific Treg cell development in the mucosal-inductive DLNs (i.e., CMLNs after s.i. OVA/CTB treatment), but also generated or expanded Ag-specific IL-10+ B cells with capacity (at least after i.v. transfer) to migrate to inflamed effector sites and/or DLNs where they may contribute to the peripheral suppression of Teff cells. Yet, despite the important role of B cells indicated by these results not only for mucosal Treg cell induction but also for functional tolerance, it was clear that effective Ag-specific suppression of the peripheral Teff cell response to parenteral immunization by prior mucosal treatment with Ag/CTB conjugate could be achieved also in μMT−/− mice lacking B cells, even though these mice were unable to mount a significant level of Foxp3+ CD25+ CD4+ Treg cell response. Others have also described effective oral tolerance induction in μMT−/− mice (17, 18). Our previous work has shown that besides Foxp3+ Treg cells also Foxp3− CD4+ T cells with regulatory-suppressive

### Table I

**Sublingual administration of OVA/CTB conjugate suppresses OVA-specific T cell responses in vivo to a subsequent parenteral immunization with OVA in both WT and μMT−/− mice**

<table>
<thead>
<tr>
<th>Type of Mice and s.i. Treatment</th>
<th>PLN T Cell Proliferation (cpm)</th>
<th>% Cells with Indicated Staining among PLN Mononuclear Cells (% changes vs treatment with PBS)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>μMT−/−</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>28.927 ± 5.430</td>
<td></td>
</tr>
<tr>
<td>OVA/CTB</td>
<td>4.380 ± 5.45 (−85%)</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>45.018 ± 12.844</td>
<td></td>
</tr>
<tr>
<td>OVA/CTB</td>
<td>9.690 ± 5.022 (−78%)</td>
<td></td>
</tr>
</tbody>
</table>

% Cells with Indicated Staining among PLN Mononuclear Cells (% changes vs treatment with PBS)

- **Foxp3+ CD25+ CD4+**
  - PBS: 3.9 ± 0.7
  - OVA/CTB: 5.4 ± 0.3 (+38%)
  - WT: 42 ± 5.5
  - OVA/CTB: 43 ± 5.1 (+28%)

- **Foxp3+ CD25+ CD4+**
  - PBS: 6.6 ± 0.5
  - OVA/CTB: 12 ± 0.7 (+82%)
  - WT: 22 ± 3.2
  - OVA/CTB: 9.8 ± 2.1 (+55%)

- **LAP+ Foxp3+ CD4+**
  - PBS: 6.0 ± 0.8 (−50%)

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In μMT−/− mice, this is associated with a strong increase in Foxp3+ CD4+ T cells expressing LAP/TGF-β rather than as in the WT mice a strong increase in Foxp3+ Treg cells. Groups of μMT−/− and WT C57BL/6 mice (six mice per group) were given 5 × 105 purified CD4+ OT-II T cells i.v. followed by three s.i. doses (60 μg/dose) of either OVA/CTB or PBS. One day after the last s.i. treatment, the mice were immunized s.c. with OVA in CFA at the tail base and 14 days later PLN cells were examined for OVA/p323–339-specific T cell proliferative responses and after appropriate staining also by flow cytometry for relative frequencies of indicated cells. Data (means ± SDs) are from one of two independent experiments giving similar results.

b−dValues of p < 0.01, p < 0.001, and p < 0.05, respectively, are for statistical differences between groups treated with OVA/CTB and PBS.
function on Teff cells are induced by oral OVA/CTB administration in mice (32). Our current results show that in the \( \mu M \) mice, OVA/CTB treatment induced a significantly stronger increase in LAP\(^+\) CD4\(^+\) T cells with presumed regulatory-suppres- sive function than in the WT mice. Although recently LAP was described to have anti-inflammatory immunomodulating activity in its own right (39), the main immunomodulatory activity of LAP\(^+\) cells is usually ascribed to TGF-\( \beta \) complexing with LAP on the cell surface (40). Therefore, we believe that the efficient development of regulatory Foxp3\(^+\) LAP\(^+\)/TGF-\( \beta \)/CD4\(^+\) T cells in \( \mu M \) mice may at least in part compensate for their weak Foxp3\(^+\) Treg cell response. Such increased TGF-\( \beta \)-binding Foxp3\(^+\) regu- latory T cells could explain the observed suppression of Ag-specific T cell proliferation in PLNs of \( \mu M \) mice after s.c. injection of OVA in combination with (proinflammatory) CFA. In fact, under inflammatory conditions, Foxp3\(^+\) Treg cells are reported to be relatively inefficient to suppress T cells (41), in contrast to LAP\(^+\)/TGF-\( \beta \)-binding cells which specifically appear to sup- press activated (not resting) T cells (40, 42).

It is also notable that only the WT mice showed signs of in- creased apoptosis among the T cells after OVA/CTB treatment. This agrees with our previous findings (34) that T cell depletion is dependent on Foxp3\(^+\) cells, which as shown in the present study in turn is B cell dependent. Thus, our findings indicate that mu- cosal suppression of the peripheral immune response comprises at least two separate pathways: one being B cell dependent and char- acterized by induction of Foxp3\(^+\) cells leading to T cell sup- pression, apoptosis, and depletion and one being B cell indepen- dent and characterized by the dominant induction of LAP\(^+\)/TGF-\( \beta \)-binding Treg cells that can functionally suppress but not kill T cells (14, 18, 40). The B cell-dependent pathway activated through mucosal Ag/CTB possibly also exert direct regulatory activity on T eff cells peripherally in inflamed or immunized tissues or DLTNs. The relationship of either of these types of B cells to the various phenotypes of regulatory B cells recently described (43– 45) remains to be determined.

The s.i. treatment with OVA/CTB induced increased IL-10 but not TGF-\( \beta \) production by B cells in draining CMLNs. However, the precise means by which B cells respond to the s.i. administered Ag/CTB conjugates are not well understood. Our previous studies have established that in vitro, CTB-mediated binding to GM1 ganglioside receptors, which are mainly located in lipid rafts on the plasma membrane, Ag/CTB complexes can efficiently be taken up by B cells irrespective of their BCR specificity and significantly increase their Ag-presenting capacity for T cells in vitro (36). This is in sharp contrast to the outcome after similar mucosal adminis- tration of Ag alone or even Ag/CTB conjugate given along with toxic-active cholera toxin, which is one of the strongest mucosal adjuvants known for inducing immune responses; in the latter case, toxic-active cholera toxin, which is one of the strongest mucosal

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Disclosures

The authors have no financial conflict of interest.

References
