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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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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. M. J. Wick, University of Gothenburg, Göteborg, Sweden) and also T cells prepared from DO11.10/10a 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 peptide213–239 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.

Ags and conjugation of OVA to CTB subunit

OVA protein (grade VII) was purchased from Sigma-Aldrich and OVA233–339 peptide (OVA233–339) (IQQVAAHAAHEINAGR) 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 pretreatment and coculture with polymyxin but was completely inhibited by pretreatment and coculture with highly purified GM1 ganglioside (10 μmol/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 pretreatment with OVA/CTB conjugate almost completely inhibited/blockedin stained (as did also preincubation with 10 μg/ml unlabeled CTB or incubation along with 10 μmol/ml GM1 ganglioside).

Chloroform was purchased from Sigma-Aldrich and stored at −70°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 mice or μMT−/− mice were first infused with 5 × 106 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.i. treatment only, or s.i. 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 of cDNA was used. The following oligonucleotide primers (Eurorgen MWG Operon) were used: IL-10 (5′-cttgaatccggcgtgga-3′ and 5′-gctcact gcctgtctt-3′), TGFB- β (5′-caggagcctggata-3′ and 5′-ttcaagccagtcttcaaa-3′), and β-actin β (5′-cagcaagagcagagattact-3′ and 5′-gc gacagatccacagact-3′). The expression of IL-10 and TGFB-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 polyclonal 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).

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 × 106 cells/well were cultured (in a 200-μl volume of IMDMEM 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 OVA233–339 peptide. [3H]Thymidine (1 μCi/ml) was added for the last 16 h of culture, after which 1% mention was incorporated as previously described (32).

Isolation and adoptive transfer of cells

CD4+ T cells were prepared from lymph node and spleen cells of DO11.10, OT-II mice, or OT-II (CD45.1+) 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 Bio Tec). These cells were then used for studies in vitro or injected i.v. into the tail vein of naive BALB/c, C57BL/6 WT, or μMT−/− recipient mice (1 × 107 or 2 × 107 were infused) 1 day after the adoptive transfer of TCR 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 × 107 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 × 106 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, allophyocyanin, and/or phycoerythrin-labeled mAbs (BD Biosciences Pharmingen). NFLs were stained for TCR Tg T cells from 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

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permeabilized with Cytofix/Cytoperm solution (eBioscience) according to the manufacturer’s recommended protocol and then incubated with allophycocyanin-conjugated anti-Foxp3 FLK-16 mAb (1 μg/10^6 cells; Nordic Biosite) at 4°C for 30 min in the dark. Cells were then washed and analyzed by flow cytometry (FACS Calibur machine; BD Pharmingen). Apoptotic cells were detected by staining against 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 peptide 323–339 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 against 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.

Results

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 a 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) cell populations 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).
Deficient Treg response in μMT−/− mice after s.l. OVA/CTB treatment

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 OVA P323–339-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 PBS at 2-day intervals between the doses. Two days after the last dose, mice were sacrificed and their spleens, CMLNs, and MLNs were examined 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 OVA P323–339.

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, OVA P323–339-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 B-deficient Treg response in comparison to PBS-treated mice. The μMT−/− mice show a reduced Treg cell response and a lack of suppression of T cell proliferation. Groups of naïve μMT−/− and WT C57BL/6 mice adoptively received 5 × 106 purified CD4+ OT-II or CD4+ OT-II (CD45.1+) Tg T cells by i.v. injection and then, starting 1 day later, the mice were given three s.l. doses of either OVA/CTB (60 μg/dose) or PBS every second day. Two days after the last s.l. treatment, the mice were sacrificed and their spleens, CMLNs, and MLNs were collected. Spleen tissue sections were prepared for immunohistochemistry examination of Foxp3+ CD4+ Treg cells (A), CMLNs and MLNs were prepared for processing of Foxp3+ CD4+ Treg cells by flow cytometry (B) and MLN cells were used also for T cell proliferation tests (C).

A. For microscopic visualization of Foxp3 and CD4, frozen spleen sections were stained and analyzed by immunofluorescence microscopy as described in Materials and Methods; inset images are representative for cell staining intensity after each type of treatment as analyzed by confocal laser-scanning microscopy. Values in (boxes) show mean ± SD numbers of double-stained Foxp3+ (green, Alexa Fluor 488) and CD4+ (red, Alexa Fluor 647) T cells per square millimeter for each treatment group (as determined from nine randomly selected fields per spleen with the field areas being measured by an imaging computer system). B. The percentages of OVA-specific Foxp3+ 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. OVA P323–339 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 one of two independent experiments giving similar results and show mean ± SD values (six mice per group). In B and C, the initial 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.

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 OVA P323–339. The results
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 Treg 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+ Teff 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 Teff 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 CMLNs 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 WT mice.
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 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 each CMLN. 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 × 107 CD25+ CD4+ T cells isolated from naive DO11.10 mice. Data are from one of two independent experiments giving similar results and show 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. 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. 5A).

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. 5 above, we also analyzed the isolated PLN cells for Ag-specific (KJ1-26+) annexin V+CD4+ cells and Treg (Foxp3+) cells, respectively, as well as for IL-10-producing B cells. The results show a strong, 3-fold increase in early apoptotic Ag-specific (KJ1-26+ annexin V+7AAD−CD4+) T cells in PLNs from mice that...
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 \(\mu\)MT\(^{-/-}\) 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 T 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 WT and \(\mu\)MT\(^{-/-}\) 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 \(\mu\)MT\(^{-/-}\) 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 \(\mu\)MT\(^{-/-}\) mice had no significant increase in this type of cells. Furthermore, after s.l. OVA/CTB treatment, there was a significant reduction of Ag-specific DTH and T cell proliferation in recipient mice immunized parenterally. A, Effects on DTH. Purified CD19\(^{+}\) B cells were prepared from pooled CMLN and splenic cells of BALB/c mice 2 days after the last of three s.l. administrations every second day with 60-\(\mu\)g doses of OVA/CTB or PBS, and the cells were then injected i.v. (8 x 10\(^{6}\) cells/mouse) into the tail vein of BALB/c WT mice, which had 1 day before received 5 x 10\(^{5}\) CD4\(^{+}\) DO11.10 T cells/mouse; 1 wk later this was followed by a second adoptive transfer of CD19\(^{+}\) B cells from mice that had been treated in the same way as for the first B cell transfer. Yet 1 wk later, these mice (and controls given no B cell transfer, No Bc) were immunized s.c. with 50 \(\mu\)g of OVA/IFA in the right foot back and another 2 wk later the mice were DTH challenged by a s.c. injection of 10 \(\mu\)g of OVA in 20 \(\mu\)l of PBS in the left ear. Ear thickness was measured before and 24 h after challenge and differences/increments were calculated as measures of DTH responses. B, Effects on T cell proliferative responses. PLN cells from mice treated as in A (\(\bigcirc\)), no Bc; (\(\blacklozenge\)), Bc (PBS); and (\(\bullet\)), Bc (OVA/CTB)) but not challenged for DTH, were isolated and cultured with 0.1 or 0.5 \(\mu\)g/ml OVAp323–339. Results show mean and SD values for six mice per group and statistical analyses showed that both the DTH (Student's t test) and T cell proliferation (ANOVA) in the Bc (OVA/CTB) group differed significantly (***, p < 0.001) from either of the control groups.

FIGURE 5. Adoptive transfer of B cells from in vivo s.l.-treated mice suppresses Ag-specific DTH and T cell proliferation in recipient mice immunized parenterally. A, Effects on DTH. Purified CD19\(^{+}\) B cells were prepared from pooled CMLN and splenic cells of BALB/c mice 2 days after the last of three s.l. administrations every second day with 60-\(\mu\)g doses of OVA/CTB or PBS, and the cells were then injected i.v. (8 x 10\(^{6}\) cells/mouse) into the tail vein of BALB/c WT mice, which had 1 day before received 5 x 10\(^{5}\) CD4\(^{+}\) DO11.10 T cells/mouse; 1 wk later this was followed by a second adoptive transfer of CD19\(^{+}\) B cells from mice that had been treated in the same way as for the first B cell transfer. Yet 1 wk later, these mice (and controls given no B cell transfer, No Bc) were immunized s.c. with 50 \(\mu\)g of OVA/IFA in the right foot back and another 2 wk later the mice were DTH challenged by a s.c. injection of 10 \(\mu\)g of OVA in 20 \(\mu\)l of PBS in the left ear. Ear thickness was measured before and 24 h after challenge and differences/increments were calculated as measures of DTH responses. B, Effects on T cell proliferative responses. PLN cells from mice treated as in A (\(\bigcirc\)), no Bc; (\(\blacklozenge\)), Bc (PBS); and (\(\bullet\)), Bc (OVA/CTB)) but not challenged for DTH, were isolated and cultured without Ag or with 0.1 or 0.5 \(\mu\)g/ml OVAp323–339. Results show mean and SD values for six mice per group and statistical analyses showed that both the DTH (Student's t test) and T cell proliferation (ANOVA) in the Bc (OVA/CTB) group differed significantly (***, p < 0.001) from either of the control groups.

FIGURE 6. Adoptive transfer of B cells from in vivo s.l.-treated mice followed by parenteral immunization of recipient mice leads to a strong increase in Ag-specific Teff cell apoptosis along with increased frequencies of both Foxp3\(^{+}\) Treg cells and IL-10-producing B cells in PLNs. Groups of BALB/c mice were given B cells from OVA/CTB- or PBS-treated BALB/c mice on two occasions (the first time in combination with DO11.10 CD4 T cells) followed by s.c. immunization with OVA in IFA as described in Fig. 5. Two weeks later, PLN cells were examined by flow cytometry for signs of apoptosis among OVA-specific Teff cells (\(\bigcirc\)), induction of OVA-specific Treg cells (\(\blacklozenge\)), and presence of IL-10- producing B cells (\(\bullet\)) after staining with fluorochrome-labeled Abs against CD4 and KJ1-26, annexin V and 7AAD, intracellular Foxp3, and CD19 and intracellular IL-10. A, Percentages of annexin V\(^{+}\)7AAD\(^{-}\) cells among gated CD4\(^{+}\) T cells and percentages of Foxp3\(^{+}\) cells among gated CD4\(^{+}\) T cells. B, Percentages of Foxp3\(^{+}\) cells among gated CD11-26\(^{+}\)CD4\(^{+}\) cells and percentages of Foxp3\(^{+}\) cells among gated CD11-26\(^{+}\)CD4\(^{+}\) T cells. Total CD11-26\(^{+}\) cells in PLNs were 2.6 \(\pm\) 0.4% and 2.9 \(\pm\) 0.5% of total CD4\(^{+}\) cells in mice that had received B cells isolated from mice s.l. treated with OVA/CTB and PBS, respectively, as compared with 3.3 \(\pm\) 0.2% in mice that did not receive any B cell transfer.) C, Percentages of IL-10\(^{+}\) cells among gated CD19\(^{+}\) B cells. Data are from one of two independent experiments giving similar results and show mean values \(\pm\) SDs for six animals per group; *, p < 0.05 and **, p < 0.01, respectively, for group differences calculated by Student’s t test.
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 T 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 treatment (32), we examined the PLN T cells from the s.l. OVA/CTB- and PBS-treated WT and μMT$^{-/-}$ mice also for LAP$^+$/mTGF-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$^+$/mTGF-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.l. 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 tolerantized by s.l. treatment with OVA/CTB associated with a strong increase in Foxp3$^+$ CD4$^+$ T cells expressing LAP/mTGF-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.l. 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.l. 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 Treg 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.l. 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
function on Teff cells are induced by oral OVA/CTB administration in mice (32). Our current results show that in the μMT−/− mice, OVA/CTB treatment induced a significantly stronger increase in LAP+CD4+ T cells with presumed regulatory-suppressive 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-βcomplexing with LAP on the cell surface (40). Therefore, we believe that the efficient development of regulatory Foxp3+ LAP+TGF-β+CD4+ T cells in μMT−/− mice may at least in part compensate for their weak Foxp3+ Treg cell response. Such increased TGF-β+Foxp3+ regulatory T cells could explain the observed suppression of Ag-specific T cell proliferation in PLNs of μMT−/− 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 Teff cells (41), in contrast to LAP+TGF-β+ cells which specifically appear to suppress activated (not resting) T eff cells (40, 42).

It is also notable that only the WT mice showed signs of increased apoptosis among the Teff cells after OVA/CTB treatment. This agrees with our previous findings (34) that Teff 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 mucosal suppression of the peripheral immune response comprises at least two separate pathways: one being B cell dependent and characterized by induction of Foxp3− cells leading to Teff cell suppression, apoptosis, and depletion and one being B cell independent and characterized by the dominant induction of LAP+ TGF-β+ Treg cells that can functionally suppress but not kill Teff cells (14, 18, 40). The B cell-dependent pathway activated through mucosal Ag/CTB possibly also exert direct regulatory activity on Teff cells peripherally in inflamed or immunized tissues or DLNs. 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-β 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 administration 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, cholera toxin abrogates tolerance induction most likely by providing a strong “danger signal” to local mucosal APCs (27, 28, 32). Our present findings show that B cells from mice mucosally treated with OVA/CTB were tolerogenic and had acquired a capacity to educate naïve T cells to develop into Foxp3+, LAP+TGF-β+, and/or IL-10+ regulatory T cells. We speculate that the high-affinity binding of CTB-Ag complexes to B cell (or other APC) rafts via the GM1 receptor and the exceptional persistence of such complexes on the cell surface (46, 47) may be important both for inducing tolerogenic effects in the B cells and possibly also immune synapse formation of B cells with Ag-specific T cells promoting the development and expansion of Treg cells (48–50).

The recent findings in several systems of a role for B cells in tolerance induction has led to an interest in exploring the potential of using primary B cells in cell therapy of autoimmune or allergic reactions and for preventing transplant rejection (21–26, 38, 43–45). Our work in progress has shown that also in vitro the treatment of B cells with Ag/CTB conjugate or fusion protein can efficiently generate and expand Foxp3+CD25+CD4+ Treg cells having a strong suppressive function on the proliferation of Teff cells (J.-B. Sun and J. Holmgren, unpublished data). Furthermore and most important, such Ag/CTB-induced in vitro “education” renders the B cells extremely efficient for use in cell therapy to suppress the development of DTH reactions as well as autoimmune disease in animal models in an Ag-specific manner (J.-B. Sun and J. Holmgren, unpublished data). Based on this and the findings in the present study, we propose that in vitro treatment of autologous B cells with relevant Ag/CTB conjugate or fusion protein followed by reinfusion of the treated B cells should be further explored as a method for inducing Ag-specific suppression of certain harmful immune responses, such as those associated with severe autoimmune diseases and graft rejection reactions.

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Disclosures
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