

Sublingual 'Oral Tolerance' Induction with Antigen Conjugated to Cholera Toxin B Subunit Generates Regulatory T Cells that Induce Apoptosis and Depletion of Effector T Cells

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Abstract

Sublingual (s.l.) immunotherapy has in the last decade emerged as an effective approach to desensitize patients with pollen, food and insect sting allergies. This treatment has recently also attracted interest as a potential modality to control self-reactive T-cell responses associated with autoimmune disorders. Here, we show that s.l. administration of ovalbumin (OVA) conjugated to cholera toxin B subunit (CTB) (OVA/CTB) can efficiently suppress peripheral effector T (Teff) cell responses to OVA in mice that had adoptively received OVA-specific T-cell receptor (TCR) transgenic CD4⁺ T cells, and that the suppression was associated with the development of OVA-specific Foxp3⁺CD25⁺CD4⁺ regulatory T (Treg) cells as well as with apoptosis (Annexin V⁺) and depletion of OVA-specific Teff cells in peripheral lymph nodes. The induction of Teff cell apoptosis by s.l. OVA/CTB administration was found to be critically dependent on CD25⁺ Treg cells but independent of IL-10 production. Our results suggest that s.l. administration of a CTB-conjugated antigen can efficiently induce peripheral Teff cell tolerance through the induction of antigen-specific Treg cells that both inhibit Teff cell proliferation and cytokine production and induce Teff cell apoptosis and depletion.

Introduction

Induction of peripheral immunological tolerance by mucosal administration of antigen (Ag) has been proposed as a promising way to prevent or treat allergic, autoimmune or infection-induced immunopathological reactions [1, 2]. Most studies of the mechanisms of mucosally induced tolerance in animal models have shown that oral administration of very large dosages of Ag can induce Ag-specific T-cell anergy or depletion. When lesser Ag dosages are given, oral tolerance is instead largely mediated by the mucosal induction of regulatory CD4⁺ T cells, which produce immunomodulating cytokines such as interleukin (IL)-10 and tumour growth factor (TGF)- β [1–4]. Several types of regulatory CD4⁺ T cells have been described after oral administration of Ags [5–8]: Type 1 regulatory T (Tr1) cells secreting large amounts of IL-10 (and TGF- β); Th3 cells secreting predominantly TGF- β (and IL-10); and most recently and perhaps of the greatest

importance CD25⁺CD4⁺ regulatory T (Treg) cells expressing the transcription factor Foxp3 [1, 2, 9] as well as both secreted and cell-associated forms of TGF- β [10, 11]. Expression of Foxp3 appears to be critical for the generation and suppressive function of both natural and Ag-induced CD25⁺ Treg cells [10–14].

We and others have shown that oral or nasal administration of relevant Ags conjugated to the non-toxic B subunit protein of cholera toxin (CTB) can induce peripheral tolerance and suppress autoimmune diseases in experimental models with an efficiency that greatly exceeds that achieved by administration of Ag alone [15–20]. The tolerance induced by Ag conjugated to CTB, which was recently found to extend also to humans in a proof-of-concept clinical trial in patients with Behcet's disease [20] is associated with increased IL-10 and TGF- β cytokine production by mucosally induced regulatory T cells [16, 20, 21]. Consistent with this, oral tolerization by Ag/CTB conjugate treatment has resulted in pronounced increases in

Ag-specific Foxp3⁺CD25⁺CD4⁺ Treg cells in both draining and peripheral lymph nodes together with a strong induction of TGF- β and also in the Ag/CTB induced development of such cells from Foxp3⁻CD25⁻CD4⁺ cells in a TGF- β dependent process [21].

Recently, tolerance induction by sublingual (s.l.) administration of allergens has emerged as an especially promising form of non-invasive Ag-specific immunotherapy for the treatment of patients with allergic problems. Especially for the treatment of allergic rhinitis s.l. immunotherapy (SLIT) has been shown to be both more efficacious than subcutaneous (s.c.) immunotherapy [22–24]. Whilst mainly used in allergic patients for generating blocking IgG and/or IgA antibodies to allergens and for inducing a Th2 to Th1 cytokine shift [24], it was recently shown that s.l. Ag administration can also efficiently induce peripheral effector T (Teff) (Th1) cell tolerance and Foxp3⁺ Treg cells [25]. Thus, when we investigated the development of Ag specific tolerance and Treg cells in response to s.l. administered ovalbumin (OVA) given either conjugated to CTB (OVA/CTB) or alone to mice that had adoptively received OVA T-cell receptor (TCR) transgenic (Tg) T cells as a means of increasing the frequency of Ag-specific T cells, the s.l. OVA/CTB treatment was much more efficient than OVA alone and even more efficient than oral OVA/CTB treatment in inducing peripheral tolerance [25]. The s.l. OVA/CTB treatment effectively suppressed Ag-specific T-cell responses and delayed-type hypersensitivity (DTH) inflammation, and this was associated with a strong increase in Ag-specific Foxp3⁺CD25⁺ Treg cells in both draining and peripheral lymph nodes, and with increased TGF- β 1 levels in serum [25].

In the present study, we have focused our efforts on investigating the effects of SLIT on Teff cells and the relation of these effects to the SLIT-induced increase in Ag-specific Treg. We found that s.l. administration of OVA/CTB conjugate caused a pronounced depletion of OVA-specific Teff cells in peripheral lymph nodes associated with the expression of Annexin V (an apoptosis marker molecule) in the Teff cells. In contrast, induction of apoptosis in Teff cells was not seen in mice depleted in CD25⁺ Treg whilst it was fully developed in IL-10^{-/-} deficient mice in response to s.l. OVA/CTB treatment. Our findings suggest that s.l. administration of a CTB-conjugated Ag can efficiently induce peripheral Teff cell tolerance which mechanistically comprises both the induction of Ag-specific Treg cells and the Treg-mediated induction of apoptotic deletion of Teff cells in addition to Treg cell inhibition of Teff cells.

Materials and methods

Mice. For the experiments two 'wild-type' (WT) strains of 6- to 8-week-old female mice were used: BALB/c

and C57BL/6 mice (B & K Universal AB, Stockholm, Sweden). In addition we used T cells from DO11.10 OVA TCR Tg mice on BALB/c background (Jackson Laboratory, Bar Harbor, ME, USA), a clone with nearly 50% of the CD4⁺ T cells expressing a TCR specific for the peptide_{323–339} fragment of OVA, and OVA TCR Tg T cells from OT-II mice with the same TCR specificity as DO11.10, but on the C57BL/6 background (kind gift from Dr Mary Jo Wick, Göteborg). Finally, in one set of experiment we used C57BL/6 IL-10^{-/-} mice (kind gift from Dr Nils Lycke, Göteborg): All mice were kept in ventilated cages under specific pathogen-free conditions at the Department of Experimental Biomedicine, Göteborg University. Studies were approved by the Göteborg University Ethical Committee for Animal Experimentation.

Antigens and conjugation of OVA to CTB subunit. Ovalbumin protein (grade VII) was purchased from Sigma (St Louis, MO, USA), and OVA peptide_{323–339} (ISQAVHAAHAEINEAGR) of >95% purity was obtained from TAG Copenhagen A/S (Klampenborg, Denmark). Highly purified recombinant CTB was kindly provided by SBL Vaccine AB (Stockholm, Sweden). OVA protein was chemically coupled to CTB using *N*-succinimidyl (3-[2-pyridyl]-dithio) propionate (Pierce Biotechnology, Inc., Rockford, IL, USA) as a bifunctional coupling reagent as described [19]. Coupled OVA/CTB was purified by FPLC gel filtration [Superdex 200 16/60 column (Pharmacia Biotech, Uppsala, Sweden) using the Biologic Workstation FPLC system (Bio-Rad Laboratories, Richmond, CA, USA)]. The purified conjugate was analysed by a GM1-ganglioside enzyme-linked immunosorbent assay (GM1-ELISA) and shown to have retained GM1-binding activity as well as strong reactivity with antibodies to both OVA and CTB [21]. The conjugate also had strong capacity to induce OVA-specific T-cell proliferation when tested on DO11.10 splenocytes; further, in the latter assay, the activity of the conjugate was not significantly inhibited by pre-incubation and co-culture with polymyxin (10 μ g/ml; Sigma) but was completely inhibited by pre-incubation and co-culture with highly purified GM1-ganglioside (10 nmol/ml; donated by late Prof. Lars Svennerholm).

S.l. Ag administration and s.c. immunization. BALB/c or C57BL/6 mice were first adoptively transferred with 1.5×10^7 DO11.10 or OT-II T cells given intravenously (i.v.). Starting 1 day later, the mice were given two or three 5- μ l doses of either 40 μ g or 60 μ g of OVA/CTB or PBS s.l. at 2-day intervals.

In some experiments, mice were further immunized s.c. at the tail base with 50 μ g of OVA emulsified 1:1 in complete Freund's adjuvant (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 cervical lymph nodes (CLN),

mesenteric lymph nodes (MLN) or popliteal/inguinal nodes (PLN) and spleen were harvested and cells were prepared for further *in vitro* studies.

T-cell proliferation and cytokine assays and adoptive transfer. Cells from MLN, PLN or spleen were prepared as single cell suspensions at indicated days after s.i. or s.c. immunization and erythrocytes were removed by lysis. For studies of cell proliferation, 4×10^5 cells/well (in a 200- μ l volume of Iscove's medium supplemented with 10% FCS, 1% L-glutamine, 1% gentamicin and 50 μ M mercaptoethanol) were cultured for 3 days in 96-well plates with or without 0.1 or 1 μ g/ml of OVA_{323–339} peptide. [³H]thymidine was added (1 μ Ci/well) for the last 16 h of culture, cells were harvested and [³H] incorporation was measured as described [21]. Interferon- γ production by cultured cells was assayed in 3-day culture supernatants by ELISA.

For adoptive transfer, T cells were prepared from lymph node and spleen cells of DO11.10 or OT-II mice by using a T-cell column (R & D Systems, Minneapolis, MN, USA). We then i.v. injected 1.5×10^7 cells into the tail vein of naive BALB/c or C57Bl/6 WT recipient mice. For some experiments, we first purified the CD4⁺ T cells (>99%) and/or further removed >99% of the CD25⁺ cells from the later cells by negative selection using MACS microbeads labelled with various mAbs (Miltenyi Biotec, Auburn, CA, USA) before injecting 5×10^6 of these further prepared T cells i.v. into recipient mice.

CFSE labelling of T cells. Purified CD4⁺ T cells from DO11.10 or OT-II mice were labelled at 3×10^7 cells/ml with 5 μ M CFSE (Molecular Probes, Eugene, OR, USA), 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 labelled cells in 200 μ l of PBS were injected i.v. into the tail vein of recipient mice.

Adoptive transfer. Either 1.5×10^7 total lymph node cells or 5×10^6 purified CD4⁺ T cells or CD25⁺ T cell depleted (CD25⁺CD4⁺) T cells from naive DO11.10 OVA TCR Tg or OT-II mice were injected i.v. into BALB/c mice, C57BL/6 mice or C57/Bl IL-10^{-/-} mice. One day after transfer, the mice were s.i. treated with OVA/CTB conjugate or PBS as described earlier.

In some experiments, 1.5×10^7 total DO11.10 or OT-II T cells were injected i.v. into either BALB/c mice or C57BL/6 mice who were then similarly treated s.i. with OVA/CTB conjugate or PBS before receiving an s.c. immunization with OVA in CFA (see above). One or 2 weeks later, CLN, MLN, spleen and PLN cells were collected and used for phenotype expression and proliferation studies.

Flow cytometry analyses. Freshly isolated spleen or lymph node cells were incubated with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)- or allophycocyanin (APC)-labelled mAbs to mouse CD4, CD25, or DO11.10 clonotypic TCR (KJ1-26) or Va2 for OT-II clonotypic TCR

(BD Biosciences PharMingen, San Diego, CA, USA). For analysis of intracellular Foxp3, stained freshly isolated cells were fixed and permeabilized with Cytofix/Cytoperm solution (eBioscience, San Diego, CA, USA) according to the manufacturer's suggested protocol and then incubated with APC-conjugated anti-Foxp3 FLK-16 mAb (Nordic Biosite, Taby, Sweden) (1 μ g/10⁶ cells) at 4 °C for 30 min in the dark. Cells were then washed and analysed by flow cytometry (Calibur FACS machine; BD PharMingen, San Diego, CA, USA). For analysis of cell apoptosis, cells were stained with anti-mouse Annexin V-APC and 7AAD-PerCP antibodies according to the manufacturer's suggested protocol before FACS analysis.

Results

'Oral tolerance' induction by sublingual administration of OVA/CTB conjugate is associated with a decrease in effector T cells in peripheral lymph nodes

Peripheral immunological tolerance after mucosal administration of Ag is characterized by a decreased ability to mount a systemic immune response, especially a Th1 type immune response, to a parenteral immunization with the same Ag [1, 2]. We have recently shown that oral or s.i. administration of OVA/CTB conjugate can efficiently induce peripheral immunological tolerance and increased numbers of regulatory T (Treg) cells [21, 25]. In this study, we wished to examine how the induction of peripheral tolerance by s.i. treatment would affect Treg and effector T (Teff) cells *in vivo* in response to antigen exposure. To this end, we first adoptively transferred 1.5×10^7 OVA-specific (TCR Tg DO11.10) T cells to BALB/c mice. One day later, these mice were given three 60- μ g doses s.i. of OVA/CTB conjugate or PBS every second day, and the day after the last s.i. treatment they were injected s.c. at the tail base with 50 μ g of OVA in CFA. Seven or 14 days after the s.c. immunization, PLN cells of the mice were collected and examined for (1) T-cell proliferation and IFN- γ production in response to OVA_{323–339} stimulation *in vitro*, and (2) the frequency of OVA-specific Treg and Teff cells by flow cytometry.

The results show that there was a marked suppression of the T-cell proliferative response as well as of IFN- γ production to OVA_{323–339} peptide *in vitro* in cultures of PLN cells from mice treated s.i. with OVA/CTB compared with mice treated with PBS after s.c. immunization with OVA in CFA both at 1 week after the s.c. immunization (Fig. 1A) and even further pronounced after 2 weeks (Fig. 1B). Suppression was apparently Ag-specific, as PLN cell cultures responded equally well to Con A stimulation whether the cells came from mice given s.i. treatments with OVA/CTB or PBS (not shown). Strikingly, this functional suppression of T-cell responses in

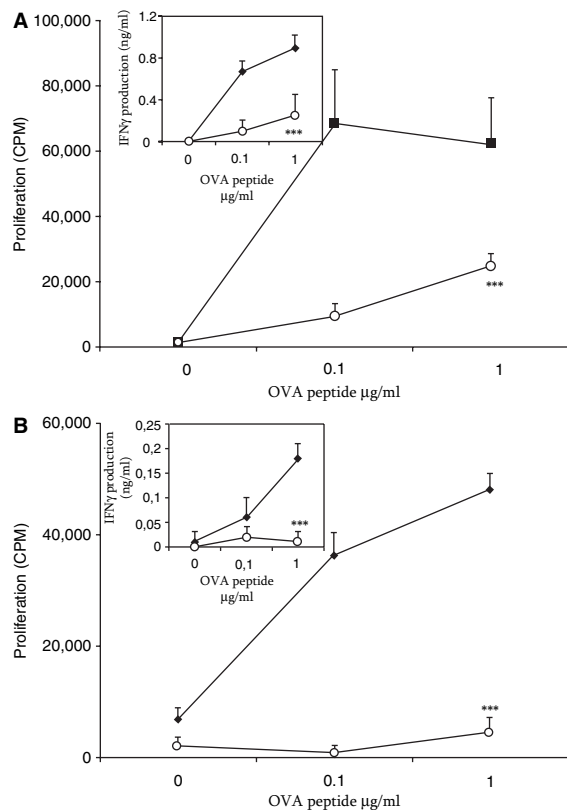


Figure 1 Sublingual (S.L.) administration of OVA/CTB conjugate suppresses *in vivo* T cell responses to s.c. immunization with OVA. Groups of BALB/c mice (six mice per group) were adoptively transferred with 1.5×10^7 purified DO11.10 Tg T cells injected i.v. and starting 1 day later the mice were given three s.l. doses of either 60 μg OVA/CTB or PBS. One day after last s.l. treatment, the mice were immunized s.c. with 50 μg of OVA in CFA at the tail base and 7 days (A) or 14 (B) days later PLN cells were examined for Ag-specific T cell proliferative response and IFN-γ production. Data are from one of two independent experiments giving similar results. Results (mean + SD) for OVA/CTB treated mice are shown as open circles, and those for PBS-treated controls as filled diamonds. *** denotes $P < 0.001$ by ANOVA for indicated treatment groups in comparison with mice treated with PBS.

PLN by s.l. treatment with OVA/CTB was associated with a marked, progressive decrease in the frequency of OVA-specific CD4⁺Foxp3⁻ T (presumed Teff) cells in PLN (Fig. 2A). At 1 week after the s.c. immunization the frequency of OVA-specific Teff cells in PLN was reduced by 60–65% (range) in mice treated s.l. with OVA/CTB compared to PBS, and at 2 weeks the reduction was 70–81% (data not shown). Similar to the functional suppression, the reduction in Teff cells in PLN was antigen-specific: neither the total number of lymphocytes nor KJ1-26⁻ CD4⁺ T cells in PLN differed between the OVA/CTB and the PBS-treated groups at either 1 or 2 weeks after the s.c. immunization (data not shown).

The picture for Treg frequencies in PLN was more complex. Even though the frequency of Treg cells in

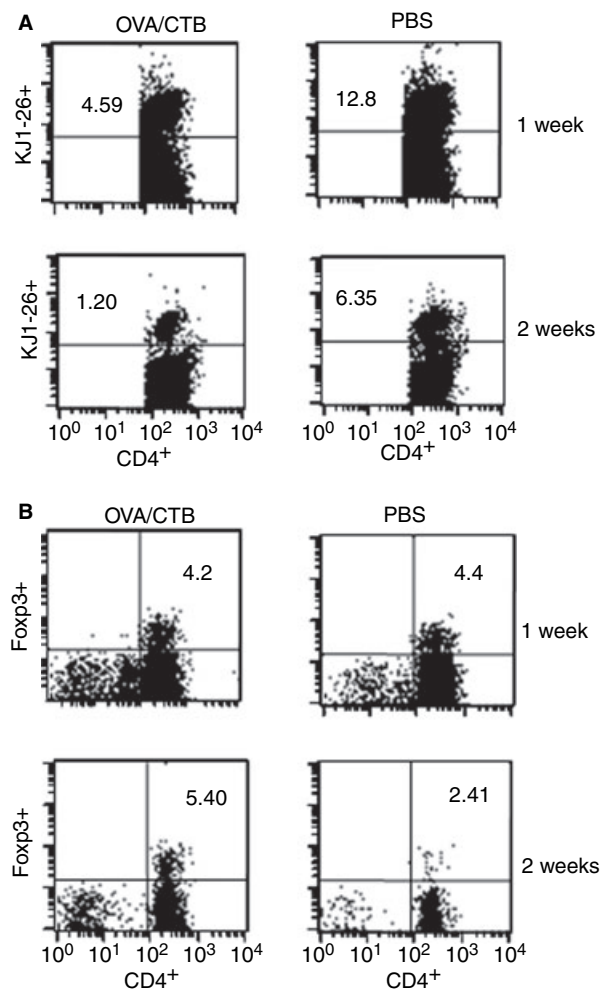


Figure 2 Sublingual (S.L.) administration of OVA/CTB conjugate increases Ag-specific Foxp3⁺ regulatory T cells and depletes Foxp3⁻ effector T cells. Groups of BALB/c mice were treated as described in the legend to Fig. 1. After adoptive transfer, s.l. treatment and s.c. immunization, PLN cells of the mice were collected and examined for the frequency of OVA-specific effector T (Teff) cells (A) and regulatory T (Treg) cells (B) by flow cytometry. Ag-specific Teff cells were defined as KJ1-26⁺Foxp3⁻CD4⁺ T cells and identified as KJ1-26⁺ and CD4⁺ cells after gating on Foxp3⁻CD4⁺ T cells (A). Treg cells were instead defined as KJ1-26⁺Foxp3⁺CD4⁺ T cells and identified as Foxp3⁺CD4⁺ cells after gating on KJ1-26⁺ cells (B). Values given are the percentages of indicated populations among examined gated cells and are representative for the respective cell populations in each treatment group.

CLN, MLN and spleen were increased by 200–300% by s.l. OVA/CTB treatment at the time for the s.c. immunization [25 and data not shown], there was no significant difference in PLN Treg cells between the OVA/CTB and PBS-treated mice at 1 week after the s.c. immunization (Fig. 2B, upper panel). After 2 weeks, on the other hand, there was a more than twofold increase in OVA-specific Treg cells in the OVA/CTB treated group (Fig. 2B, lower panel).

S.I. treatment with OVA/CTB induces strong antigen-specific *in vivo* T cell division comprising both effector and regulatory T cells

To further dissect the immunological response induced by s.l. treatment with O/CTB, we tested whether s.l. administration of OVA/CTB could induce *in vivo* division of adoptively transferred CFSE-labelled CD4⁺ DO11.10 T cells in WT BALB/*c* mice. The results, illustrated in Fig. 3, show that 2 days after the last of two or three consecutive s.l. doses of OVA/CTB, both CD25⁻ (Fig. 3A) and CD25⁺ (Fig. 3B) or Foxp3⁺ (Fig. 3C) CD4⁺ cells in CLN and MLN as well as in spleen (data not shown) had undergone intense division in sharp contrast to cells from PBS-treated mice.

In parallel experiments, we also examined the frequency of OVA-specific T_{eff} and T_{reg} cells in CLN and MLN by FACS analysis 2 days after the last of 3 s.l. treatments with either OVA/CTB, OVA alone or PBS. As shown in Table 1, the frequency of OVA-specific T_{eff} (Foxp3⁻ KJ1-26⁺CD4⁺ T cells) was increased in CLN but decreased by circa 40% among lymphocytes from MLN after s.l. administration of O/CTB conjugate compared with PBS treatment. OVA-specific T_{reg} cells (Foxp3⁺ KJ1-26⁺CD4⁺ T cells) on the other hand were markedly increased among lymphocytes from both MLN and CLN in the animals treated with OVA/CTB. S.l. treatment with OVA alone gave much weaker effects than those achieved with OVA/CTB (Table 1).

Sublingual administration of OVA/CTB conjugate induces apoptosis of OVA-specific effector T cells

To examine if the progressive strong reduction in the number of OVA-specific T_{eff} cells (Foxp3⁻ KJ1-26⁺CD4⁺ cells) in peripheral lymph nodes after s.l. treatment with OVA/CTB, despite the vigorous cell division in both these and Ag-specific T_{reg} cells, could be explained by the induction of apoptosis of the T_{eff} cells, we isolated OVA-specific DO11.10 or OT-II CD4⁺ Tg T cells and then i.v. adoptively transferred them into naive BALB/*c* or C57Bl/6 mice. One day later, the mice received three s.l. treatments with 60- μ g OVA/CTB conjugate or with PBS every consecutive day. Two days after last s.l. treatment the mice were sacrificed, CLN and MLN were collected and their T cells examined for signs of the apoptosis by staining for Annexin V and 7AAD (a marker for cells in late stage apoptosis). The results, being similar in both mouse strains, show that the s.l. OVA/CTB treatment indeed gave rise to a significant increase in the frequency of Annexin V⁺ cells in both 7AAD⁻ (Fig. 4A and Table 2 groups 1 versus 2) and 7AAD⁺ T cells (data not shown).

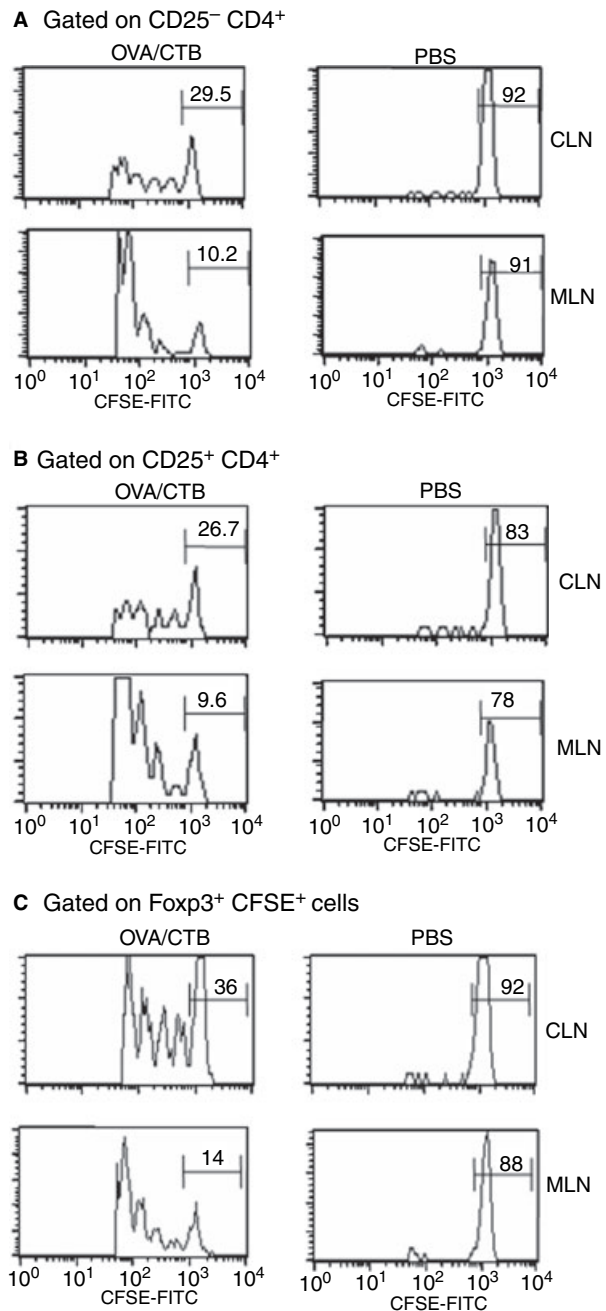


Figure 3 Sublingual (S.l.) administration of OVA/CTB conjugate induces early *in vivo* expansion of both T_{eff} and T_{reg} cells. Purified OVA TCR Tg DO11.10 CD4⁺ T cells were labelled with CFSE and 5×10^6 labelled cells were then i.v. injected into the tail vein of naive BALB/*c* recipient mice, and starting 1 day later, two 60- μ g doses of OVA/CTB conjugate or PBS were administered s.l. with a 2-day interval. After another 2 days CLN and MLN cells were prepared, and CFSE-labelled cells were examined for cell proliferation after staining also with PE- or APC-labelled Abs against CD4, CD25 or KJ1-26. A, B and C show histograms of CFSE-labelled cell divisions among gated CD25⁻CD4⁺ (A) and CD25⁺CD4⁺ (B) or Foxp3⁺ CD4⁺ (C) cells; the percentages of gated cells that have remained undivided are shown.

Table 1 Effects of sublingual administration of OVA/CTB conjugate or OVA alone on the frequencies of Ag-specific regulatory and effector T cells in draining (cervical) and peripheral (mesenteric) lymph nodes.

		Foxp3 ⁺ /KJ1-26 ⁺	Foxp3 ⁻ /KJ1-26 ⁺
In CLN			
Group 1	OVA/CTB × 3	8.31 ± 1.0 ^b (+83%)	4.72 ± 0.57 ^a (+87%)
Group 2	OVA × 3	5.48 ± 0.74	2.41 ± 0.26
Group 3	PBS	4.54 ± 1.2	2.52 ± 0.86
In MLN			
Group 1	OVA/CTB × 3	18.5 ± 3.2 ^b (+117%)	1.20 ± 0.02 ^a (-40%)
Group 2	OVA × 3	11.9 ± 2.68	1.70 ± 0.01
Group 3	PBS	8.53 ± 0.76	1.93 ± 0.07

Groups of BALB/c mice were adoptively transferred with 1.5×10^7 DO11.10 T cells. Starting 1 day later, these mice were given three 60- μ g doses s.l. of OVA/CTB conjugate, OVA alone or PBS every second day, and 2 days after the last s.l. treatment CLN and MLN cells were collected and examined for the frequency of OVA-specific T_{eff} and T_{reg} cells by flow cytometry. OVA-specific T_{eff} cells were defined as Foxp3⁻ KJ1-26⁺CD4⁺ cells and T_{reg} cells as Foxp3⁺KJ1-26⁺CD4⁺ cells. Results are from 1 of 3 independent experiments giving similar results and show percentages (mean ± SD for six mice in each group) of Foxp3⁺CD4⁺ cells among KJ1-26⁺ lymphocytes (the proportion of T_{reg} among the total Ag-specific CD4⁺ T cells) or KJ1-26⁺CD4⁺ cells among gated Foxp3⁺CD4⁺ T cells (the proportion of Ag-specific Foxp3⁺CD4⁺ 'effector' T cells among total Foxp3⁺CD4⁺ T cells).

^{a,b}*P* < 0.05 and *P* < 0.01 by Student's *t*-test for statistical differences between treatments with OVA/CTB and PBS.

Apoptosis induction in peripheral T_{eff} cells by s.l. OVA/CTB treatment is dependent on T_{reg} cells, but independent of IL-10

Strikingly, in mice who had received CD25⁻CD4⁺ OT-II T cells (i.e. CD4⁺ cells specifically depleted in CD25⁺ T_{reg} cells), the s.l. OVA/CTB treatment did not induce any notable apoptosis in T_{eff} cells compared with corresponding mice treated with PBS (Fig. 4B and Table 2 groups 3 versus 4). The results indicate that the s.l. induction of apoptosis was dependent on the presence of Ag-specific CD25⁺CD4⁺ T_{reg}.

To also test the role of IL-10 production in T cell apoptosis induced by OVA/CTB, we i.v. adoptively transferred 5×10^6 isolated CD4⁺ OT-II T cells into naive IL-10^{-/-} mice or WT C57Bl/6 mice followed by s.l. treatment with OVA/CTB or PBS. The OVA/CTB s.l. treatment in the IL-10^{-/-} mice increased the frequency of apoptotic T_{eff} cells (Fig. 4C, and Table 2 groups 5 versus 6) to a similar extent as in the WT mice (Fig. 4A and Table 2, groups 1 and 2).

Consistent with these results, the MLN T cell proliferative response to OVA₃₂₃₋₃₃₉ peptide was significantly suppressed in mice who had received CD4⁺ OT-II T cells and then been treated s.l. with OVA/CTB compared with PBS-treated mice (Table 2, group 1 versus 2). The results were similar in IL-10^{-/-} mice (Table 2, groups 5 versus 6). In contrast, the suppression was much less in WT mice who had received only CD25⁻CD4⁺ OT-II T cells (which lack CD25⁺CD4⁺ T_{reg} cells) before the s.l. treatment with OVA/CTB or PBS (Table 2, groups 3 versus 4).

Discussion

We have recently reported that s.l. administration of an Ag (OVA)/CTB conjugate even more efficiently than oral Ag administration induces peripheral Ag-specific suppression of Th1 cell and DTH responses and that this was associated with a strong increase in T_{reg} cells and production of TGF- β . In this study, beyond confirming these findings, we show that the suppression of T cell responses induced by s.l. treatment with Ag/CTB *in vivo* comprises a progressive, pronounced depletion of T_{eff} cells in addition to the strong Ag-specific regulatory inhibition of such cells in peripheral lymph nodes. Our data further indicate that not only the functional inhibition of T_{eff} cell responses but also the Ag-specific T_{eff} cell depletion are dependent on and probably caused by T_{reg} cells induced by the mucosal Ag/CTB treatment.

By studying adoptively transferred CFSE-labelled OVA TCR Tg T cells isolated from various lymph nodes after s.l. OVA/CTB administration by flow cytometry, we found that the s.l. treatment stimulated a rapid cell division and expansion of both T_{reg} cells (defined as Foxp3⁺CD25⁺CD4⁺ T cells) and T_{eff} cells (defined as Foxp3⁻CD25⁻CD4⁺ cells) in the draining CLN. In peripheral lymph nodes, such as MLN or PLN, the T_{eff} cells already a few days after the s.l. OVA/CTB treatment showed signs of OVA-specific apoptosis and cell depletion. Adoptive transfer experiments indicated that the induction of apoptosis was dependent on Ag-specific T_{reg} cells (CD25⁺CD4⁺ T cells) but not on the ability of the recipient mice to produce IL-10. After s.c. immunization

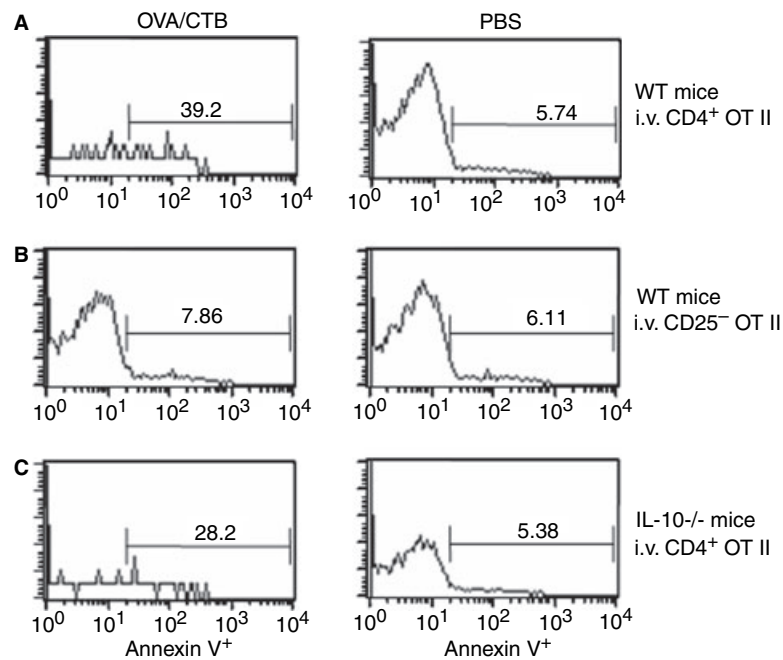


Figure 4 Sublingual (S.l.) administration of OVA/CTB conjugate induces apoptosis in peripheral Teff cells which is dependent on Treg cells, but independent of IL-10 production. OVA-specific total OT-II CD4⁺ T cells (comprising both CD25⁺ and CD25⁻ cells) or CD25⁻CD4⁺ Tg T cells were isolated and then i.v. adoptively transferred into groups of naive C57Bl/6 WT or IL-10^{-/-} mice. One day later, the mice received three s.l. treatments with 60-μg OVA/CTB conjugate or PBS every second day. Two days after the last s.l. treatment the mice were sacrificed, CLN and MLN were collected and their T cells examined for signs of apoptosis by staining for Annexin V and 7AAD. The data show histograms of Annexin V⁺ cells among 7AAD⁻CD4⁺ OT-II cells in: (A) WT mice who had adoptively received CD4⁺ OT II cells, (B) WT mice who had received CD25⁻CD4⁺ OT II cells; and (C) IL-10^{-/-} mice who had received CD4⁺ OT II cells. Gated CD4⁺Vα2⁺7AAD⁻ cells expressing Annexin V are shown, and values given are percentages of Annexin V⁺ cells in representative animals (see further Table 2).

Table 2 Sublingual administration of OVA/CTB conjugate induces CD25⁺ Treg cell-dependent, IL-10 independent apoptosis of OVA-specific Teff cells and functionally inhibits T cell responses.

Recipient mice	Adoptively transferred OT II cells	Sublingual treatments	% Annexin V ⁺ /Vα2 ⁺ 7AAD ⁻	T-cell proliferation (CPM)
Gr1: WT	CD4 ⁺ (CD25 ⁺ /CD25 ⁻)	OVA/CTB	39.7 ± 3.4 ^a	56 ± 101 ^b
Gr2: WT	CD4 ⁺ (CD25 ⁺ /CD25 ⁻)	PBS	5.9 ± 1.2	6220 ± 2053
Gr3: WT	CD25 ⁻ CD4 ⁺	OVA/CTB	8.2 ± 1.5	2776 ± 1699 ^c
Gr4: WT	CD25 ⁻ CD4 ⁺	PBS	6.3 ± 1.1	6087 ± 488
Gr5: IL-10 ^{-/-}	CD4 ⁺ (CD25 ⁺ /CD25 ⁻)	OVA/CTB	29.2 ± 2.6 ^a	80 ± 144 ^b
Gr6: IL-10 ^{-/-}	CD4 ⁺ (CD25 ⁺ /CD25 ⁻)	PBS	5.9 ± 0.9	14855 ± 3772

Total CD4⁺ (CD25⁺/CD25⁻) or CD25⁻CD4⁺ T cells were isolated from OVA TCR transgenic OT-II mice and then i.v. adoptively transferred (5×10^6 cells per mouse) into groups of naive C57Bl/6 WT or IL-10^{-/-} mice. Starting 1 day later, the mice received three s.l. treatments with 60-μg OVA/CTB conjugate or with PBS every second day. Two days after last s.l. treatment the mice were sacrificed, MLN were collected and their T cells examined by FACS for signs of apoptosis after staining for Annexin V and 7AAD as well as tested for proliferative responses to OVA₃₂₃₋₃₃₉ peptide. Values shown are percentages of Annexin V⁺ cells among gated 7AAD⁻Vα2⁺CD4⁺OT-II cells and OVA-peptide specific MLN T-cell proliferative responses (mean ± SD for six mice per group).

^{a,b} $P < 0.01$ and $P < 0.001$ by Student's *t*-test for statistical differences between treatments with OVA/CTB and PBS.

^cNo significant difference ($P > 0.05$).

with OVA together with CFA subsequent to the s.l. OVA/CTB treatment, a progressive, pronounced Teff cell depletion was found in PLN with a loss of 60–65% of all Teff cells after 1 week and 70–81% after 2 weeks. The frequencies of Treg cells on the other hand were markedly increased by the s.l. OVA/CTB treatment both in MLN and, even more pronounced, in PLN 2 weeks

after systemic Ag re-exposure. In PLN, at 1 week after s.c. immunization the Treg frequency did not differ much from that in PBS-treated controls, which we interpret as suggestive evidence of a partial depletion also of Ag-specific Treg cells at that time although to a much lesser extent than the depletion of Teff cells. It has been described that apoptotic T cells release TGF-β which

both contributes to the suppression of Teff cells and to the further expansion of the local Treg cell population [26, 27]. In consequence with this, after another week the Treg frequency again had increased and the depletion of Teff cells had progressed even further in evidence of the definitive win by the Treg over the Teff cells in the apoptosis battle at that time.

Despite the extensive research that has been undertaken on Treg cells in the last decade, the mechanism(s) by which these cells inhibit Teff cells has remained elusive. TGF- β has been found to be critical for both the induction and the suppressive action of Treg cells [11, 12]; however, it has remained controversial whether the cell contact dependant inhibition of Teff cells is mediated by membrane-bound or secreted TGF- β and how the inhibition occurs [28]. Recently, evidence has been reported indicating that the induction of apoptosis in Teff cells is an important mechanism in the Teff cell inhibition by Treg cells [26, 27, 29], possibly through a granzyme B-dependent, perforin-independent Treg–Teff cell contact mechanism [30]. In addition, Treg cells have been found to express a latent form of TGF- β (latency-associated peptide, LAP) which may also be involved in the cell contact dependent Treg-mediated inhibition of Teff cells [31].

Early studies of oral tolerance suggest that there are two primary effector mechanisms: oral administration of low doses of Ag favour the generation of regulatory cell-driven tolerance whereas high Ag doses favour anergy and /or deletion reviewed in [28]. However, recent studies suggest that these forms of tolerance after mucosal administration of Ag are not mutually exclusive and may indeed overlap [28]. Further, NKT cells may play an important role in both of these oral tolerance processes. Thus, as recently studied in NKT^{-/-} mice and in such mice after transfer of NKT cells, NKT cells have been found to be critical for both the proper development of mucosal dendritic cells with a propensity for inducing Treg cells in response to mucosal Ag administration and for the clonal deletion of Ag-specific CD4⁺ Teff cells [32]. Thus, anergy/deletion and active regulation may be different aspects of the same tolerogenic processes that are triggered by antigenic contacts in the intestine.

The strong induction of Ag-specific Treg-dependent Teff cell apoptosis and depletion after SLIT are fully consistent with apoptosis being an important mechanism by which Ag-induced Treg can suppress Teff responses. We propose that this could be an important mechanism in the peripheral 'oral tolerance' induction that may be achieved by mucosal administration of selected types of Ag, in the present case by s.l. administration of OVA Ag linked to CTB. Thus, in conclusion, our results suggest that s.l. administration of a CTB-conjugated Ag can efficiently induce peripheral Teff cell tolerance which

mechanistically comprises the induction of Ag-specific Treg cells that both inhibit Teff cell proliferation and cytokine production and induce progressive apoptosis and depletion of Teff cells.

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