Low-Dose Tolerance Is Mediated By The M Cell Ligand, Reovirus Protein Sigma One[1]

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Abstract

Mucosal tolerance induction generally requires multiple or large antigen (Ag) doses. Since M cells have been implicated as being important for mucosal tolerance induction and because reovirus attachment protein sigma 1 (pσ1) is capable of binding microfold (M) cells, we postulated that targeting a model Ag to M cells via pσ1 could induce a state of unresponsiveness. Accordingly, a genetic fusion between ovalbumin (OVA) and the M cell ligand, reovirus pσ1, termed OVA-pσ1, was developed to enhance tolerogen uptake. When applied nasally, not parenterally, as little as a single dose of OVA-pσ1 failed to induce OVA-specific Abs even in the presence of adjuvant. Moreover, the mice remained unresponsive to peripheral OVA challenge, unlike mice given multiple nasal OVA doses that rendered them responsive to OVA. observed unresponsiveness to OVA-pσ1 could be adoptively transferred using cervical lymph node (CLN) CD4$^+$ T cells, which failed to undergo proliferative or delayed-type hypersensitivity (DTH) responses in recipients. discern the cytokines responsible as a mechanism for this unresponsiveness, restimulation assays revealed increased production of regulatory cytokines, IL-4, IL-10, and TGF-β, with greatly reduced IL-17 and IFN-γ. induced IL-10 was derived predominantly from forkhead box P3 (FoxP3)$^+$ CD25$^+$CD4$^+$ T cells. FoxP3$^+$ CD25$^+$CD4$^+$ T cells were induced in OVA-pσ1-dosed IL-10 deficient (IL-10$^{-/-}$) mice, and despite showing increased TGF-β synthesis, these mice were responsive to OVA. These data demonstrate the feasibility of using pσ1 as a mucosal delivery platform specifically for low-dose tolerance induction.

Keywords

Th1/Th2 cells; tolerance/suppression; mucosa; vaccination

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Disclosures

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Introduction

Mucosal tolerance is a dose-dependent process (1–3) requiring either high doses or multiple administrations of Ag typically applied orally. The former mode induces tolerance by clonal anergy/deletion of effector cells, whereas the latter, based on repeated low-dose administration, causes active suppression of effector cells (3–7). Such suppression occurs via activation of specific regulatory cells, among which $CD25^+CD4^+$ T regulatory (T_{reg})[3] cells have been best described (2,6). Although the phenotypic characteristics of T_{reg} cells are still being investigated, currently, this naturally anergic peripheral $CD25^+CD4^+$ T cell population is thought to normally constitute not more than 5–10 percent of total peripheral $CD4^+$ T cells (8,9). Specific T_{reg} cells are known to express the nuclear forkhead box P3 (FoxP3) transcription factor and suppress the immune response in an IL-10- and/or TGF-β-dependent fashion (10).

Peyer's patches (PPs) are thought to be required for induction of oral tolerance (1,6,11) based upon the findings that treatment of female mice with soluble lymphotoxin-β receptor-Ig fusion protein during gestation results in the disruption of the peripheral lymph nodes (LNs) and PPs in their offspring, leaving the mesenteric, sacral, and cervical LNs intact (12). Offspring of such PP-null mice subjected to a high dose OVA oral tolerance regimen do not respond to peripheral OVA challenge (11). The PPs (1,6,11) and an analogous structure in the upper respiratory tract, the nasopharyngeal - associated lymphoid tissue (NALT; 13, 14), actively facilitate immunity or unresponsiveness by luminal Ag sampling (2,14,15) via a specialized epithelium containing microfold (M) cells (16). Ags are subsequently transported from the luminal surface via M cells, which localize in the follicle-associated epithelium (FAE) to the subepithelial dome area for eventual presentation to mucosal B and T cells (15,17). A number of pathogens, such as reovirus and *Salmonella*, specifically target this specialized FAE to infect the host (18–22). Reovirus infects the host via its cell adhesin, protein sigma one (pσ1), that is responsible for reovirus attachment to M cells (14,18–20). Previous studies demonstrated binding of recombinant fusion pσ1 to NALT, suggesting that a pσ1-based vehicle can be applied for genetic vaccination of mucosal tissues (14,23). A modified version of pσ1 obtained by chemically conjugating pσ1 to poly-L-lysine significantly enhanced immunity to the encoded DNA vaccine following nasal administration (14,24). Due to these targeting capabilities, we queried if recombinant pσ1 could ferry soluble proteins to mucosal tissues, as well.

To test this possibility, a cDNA encoding for the model Ag, OVA, was cloned 5' to pσ1 as a histidine-tag fusion protein and termed OVA-pσ1. We demonstrated here that mice nasally immunized with the OVA-pσ1 fusion protein become tolerogenic to OVA and resistant to peripheral challenge as a result of the generation of Ag-specific T_{reg} cells, which, in turn, actively suppress effector T cells. Immunization with OVA-pσ1 significantly increased the numbers of IL-4-producing $CD25^-CD4^+$ T cells, together with IL-10-producing T_{reg} cells, which inhibited the proliferation of OVA-specific $CD4^+$ T cells *in vivo* and successfully suppressed production of proinflammatory cytokines. In the absence of IL-10, OVA-pσ1-mediated tolerance was lost. Thus, these studies show that nasal pσ1-Ag delivery is an effective means to induce systemic tolerance.

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[3]Abbreviations used in this paper: **Ag**, antigen; **T_{reg}**, T regulatory cells; **FoxP3**, forkhead nuclear transcription factor; **PPs**, Peyer's patches; **LN**s, lymph nodes; **NALT**, nasopharyngeal-associated lymphoid tissue; **M**, microfold; **FAE**, follicle-associated epithelium; **pσ1**, protein sigma one; **CT**, cholera toxin; **DTH**, delayed-type hypersensitivity; **MLN**s, mesenteric lymph nodes; **HNLN**s, head and neck lymph nodes; **CLN**s, cervical lymph nodes; **Tg**, transgenic; **sPBS**, sterile PBS; **p.i.**, post-immunization; **CFC**, cytokine-producing cell.

Materials and Methods

Preparation of OVA-pσ1

Protein σ1 was recloned without its fusion maltose-binding protein partner (23) to allow expression in the yeast *Pichia pastoris* vector pPICB bearing an histag carboxy terminus for protein purification (Invitrogen Corp., Carlsbad, CA), referred to as pσ1. To obtain the fusion protein OVA-pσ1, OVA was amplified with appropriate pairs of primers from an OVA cDNA. The 5′ primer encoded an *EcoR*I site and an ATG initiation codon embedded into an optimal Kozak's sequence. The 3′ primer provided a *Sal*I site. The PCR product was gel-purified and cloned into an intermediate topocloning vector. The insert was excised by cutting with *EcoR*I and *Sal*I and gel-purified again, resulting in *EcoR*I and *Sal*I ends. The upstream primer for pσ1 contained a *Sal*I site designed to frame the OVA *Sal*I end; the downstream primer contained a *Kpn*I primer designed to frame the fused protein to the histag present in the *P. pastoris* expression vector pPICB. The PCR products were gel-purified and cloned into a topocloning vector. The inserts were then excised by cutting with *Sal*I and *Kpn*I and gel-purified again, thus having *Sal*I and *Kpn*I ends. Finally, the yeast expression vector, pPICB, was cut with *EcoR*I and *Kpn*I. A tripartite ligation was set up to join together these components: 1) the "Antigen" (OVA) as an *EcoR*I-*Sal*I fragment; 2) the "Transporter" (pσ1), as a *Sal*I-*Kpn*I fragment; and 3) the vector cut with *EcoR*I and *Kpn*I. The junction between the "passenger Ag" and the "transporter" featured a flexible linker (Gly-Arg-Pro) to minimize steric hindrance between the components. The resulting construct was sequenced and expressed in the yeast *P. pastoris*, according to the manufacturer's directions (Invitrogen Corp.). Recombinant proteins were extracted from yeast cells by a bead-beater (Biospec Products, Bertlesville, OK) and purified on a Talon metal affinity resin (BD Biosciences, Palo Alto, CA), according to manufacturer's instructions. Proteins were assessed for purity and quality by Coomassie-stained polyacrylamide gels and by Western blot analysis using a polyclonal rabbit anti-pσ1 (produced in-house) or a polyclonal rabbit anti-OVA Ab (Sigma-Aldrich, St. Louis, MO). All recombinant proteins migrated as a single band with the expected MW.

Mice

Female BALB/c and C57BL/6N mice (Frederick Cancer Research Facility, National Cancer Institute, Frederick, MD) were used throughout this study. DO11.10 and IL-10$^{-/-}$ breeder pairs were obtained from The Jackson Laboratory (Bar Harbor, ME) to establish our colonies. All mice were maintained in the Montana State University (MSU) Animal Resources Center under pathogen-free conditions in individually ventilated cages under HEPA-filtered barrier conditions and were fed sterile food and water *ad libitum*. The mice were free of bacterial and viral pathogens, as determined by antibody screening and histopathologic analysis of major organs and tissues. All animal studies were approved by the MSU Institutional Animal Care and Use Committee.

Immunizations, tolerance induction, and OVA-specific challenge

For tolerance induction, mice (5 mice/group) were nasally dosed up to three times, as described in the text, with 50 – 100 μg of OVA-pσ1 alone or in combination with cholera toxin (CT; List Biologicals, Campbell, CA): 5 μg of CT for the initial dose and 2.5 μg with each boost (25). OVA-pσ1 was administered nasally in a volume of no more than 20 μl/dose up to four times a day, with no less than 2.5 hour intervals between each administration. Mice were nasally dosed with OVA or OVA-pσ1 plus 25 μg of CpG oligodeoxynucleotide cDNA (CpG-ODN) (Sigma-Aldrich), TCCATGACGTTCCTGACGTT (26). As a tolerance control, a group of age-matched mice received a single oral 25 mg dose of OVA (Grade V, Sigma-Aldrich) in a 200 μl of saline (17). To stimulate anti-OVA immunity, mice were nasally dosed with 100 μg OVA (10 mg/ml) with or without adjuvant (CT or ODN). For peripheral challenge, mice were

given a subcutaneous (s.c.) injection at 1:1 ratio of 100 μg OVA in IFA (Sigma-Aldrich) for a total volume of 100 μl per mouse.

Sample collections and Ab ELISA

Serum (saphenous vein) and fecal samples were collected weekly from each mouse. Fecal extractions (4,14,25) and vaginal washes (25) were performed, as previously described. OVA-specific endpoint Ab titers were measured by ELISA, as previously described (17), using purified OVA (Grade V) as coating Ag. Specific reactivity to OVA was determined using HRP conjugates of goat anti-mouse IgG-, and IgA-specific Abs (1.0 μg/ml; Southern Biotechnology Associates, Birmingham, AL), and ABTS (Moss Inc., Pasadena, CA) enzyme substrate. The absorbences were measured at 415 nm on a Kinetics Reader model ELx808 (Bio-Tek Instruments). Endpoint titers were expressed as the reciprocal dilution of the last sample dilution, giving an absorbence of 0.1 OD units above the OD_{415} of negative controls after 1 h incubation (4,25).

Measurement of delayed-type hypersensitivity (DTH) responses

To measure OVA-specific DTH responses *in vivo* (17), 10 μg of OVA were injected into the left ear pinna, and PBS alone (20 μl) was administered to the right ear pinna as a control. Ear swelling was measured 24 h later with an electronic digital caliper (World Precision Instruments, Sarasota, FL). The DTH response was calculated as the increase in ear swelling after OVA injection following subtraction of swelling in the control site injected with PBS.

Isolation of CD4^{+} T cells and T cell ELISPOT

Lymphocytes were isolated from mesenteric lymph nodes (MLNs), head and neck LNs (HNLNs), and spleens. LNs and spleens were obtained and lymphoid cells isolated, as previously described (4,27). Splenic mononuclear cell suspensions were subjected to Lympholyte-M (Accurate Chemical & Scientific Corporation, Westbury, N.Y.) density gradient centrifugation, and CD4^{+} T cells were isolated by negative selection (Dynal Mouse CD4 Negative Isolation Kit, Invitrogen). An aliquot of 2×10^6 CD4^{+} T cells were cultured for 72 h (37° C, 5 % CO_2) with an equal number of splenic feeder cells (T cell-depleted, mitomycin C-treated) in the presence or absence of 1 mg/ml OVA (17) or 1 μg/ml of $OVA_{323-329}$ peptide. For T cell ELISPOT analysis, CD4^{+} T cells were added to cytokine-coated, nitrocellulose-bottom microtiter plates (Millipore, MutliScreen, MA). For detection of IFN-γ, IL-2, IL-4, IL-10, IL-13, and IL-17, the plates were coated with 5 μg/ml of purified mAbs (BD Pharmingen, San Diego, CA), and the reaction was detected using 0.5 μg/ml of appropriate biotinylated mAbs (BD Pharmingen). For TGF-β1 ELISPOT, an anti-TGF-β1 mAb (10 μg/ml; Clone 1D11; R&D Systems; Minneapolis, MN) was used for coating, and a biotinylated chicken anti-human TGF-β1 Ab (5.0 μg/ml; R&D Systems) was used for detection. The color reaction was developed using a HRP-conjugated goat anti-biotin Ab (Vector Laboratories) and AEC reagent (Moss, Inc.) and enumerated, as previously described (14,27).

FACS analysis

Lymphocytes from CLNs, HNLNs, MLNs, PPs, and spleens from different immunization groups following OVA plus IFA challenge were cultured at 5×10^6 cells/ml in medium alone or in the presence of OVA (1 mg/ml). Cells were then stained for FACS analysis with Abs to cell surface molecules: FITC-anti-mouse CD4 (GK1.5; BD Pharmingen), Streptavidin-PE-Cy5 (BD Pharmingen) for biotinylated chicken anti-hTGF-β1 IgY (R&D Systems), and PE-Cy5- or APC-anti-mouse CD25 (both clones PC61) (eBioscience). Intracellular staining was performed following standard protocols with 2 % paraformaldehyde and 0.2 % saponin to stain with PE or APC-anti-mouse/rat FoxP3 (clone FJK-16s; eBioscience) or PE-rat anti-mouse IL-10 (BD Pharmingen), as previously described (27). Fluorochrome-conjugated rat IgG2a

(clone eBR2a), IgG2b (clone A95-1), and IgG1 (clone R3-34) were used as isotype control Abs. FACS analysis was performed on FACSCalibur^{TM} (BD Bioscience). At least 50,000 events were collected for each sample.

Adoptive transfer of CD4$^+$ T cell subsets

Adoptive transfer presented in Fig. 2*A*: aliquots of 1×10^7 CD4$^+$ T cells isolated from spleens of naïve DO11.10 TCR transgenic (Tg) mice were adoptively transferred via i.v. injection into naïve BALB/c mice, which, after 24 h, were dosed nasally with sterile PBS (sPBS), 400 μg OVA, or 80 μg OVA-pσ1 or i.m. (tibialis anterior muscle) with 400 μg of OVA. Three days later, total CLN CD4$^+$ T cells (2×10^5) isolated by cell-sorting were adoptively transferred into naïve BALB/c mice, which, 24 h later, were challenged s.c. with 100 μg of OVA in IFA. CD4$^+$ T cells were isolated from the CLNs and spleens 5 days later and subjected to an *in vitro* proliferation assay.

Adoptive transfer experiments are presented in Figs. 2*B*, *C*, and 3: naïve BALB/c mice were adoptively transferred with Vybrant CM-Dil (Molecular Probes, Eugene, OR) -labeled DO11.10 CD4$^+$ T cells (1×10^7) isolated from naïve OVA-Tg mice and with 6×10^5 of one of the following T cell subsets: CD25$^+$CD4$^+$, CD25$^-$CD4$^+$ or total CD4$^+$ T cells from OVA-pσ1-dosed mice or with total CD4$^+$ T cells from OVA-dosed mice. Control mice received Tg DO11.10 CD4$^+$ T cells and PBS (positive proliferation control). All recipients were challenged 24 h later with OVA, as described above, and four days later, HNLNs, MLNs, and spleens were evaluated by FACS for proliferation of total and Vybrant$^+$ CD4$^+$ T cells.

In vitro T cell assays

Cell-sorted CD4$^+$ T cells isolated from CLNs, HNLNs, MLNs and spleens were cultured, as described elsewhere (27). Cells were pulsed with ^{3}H-TdR (0.5 μCi/well), and ^{3}H-TdR incorporation by proliferating CD4$^+$ T cells (triplicate cultures) was measured and expressed as a stimulation index (SI) (27). To assess cytokine production by T$_{reg}$ cells and effector T cells, CD25$^+$CD4$^+$ and CD25$^-$CD4$^+$ T cells (2×10^5) were stimulated *in vitro* with anti-CD3 mAb-coated wells (10 μg/ml; BD Pharmingen) and a soluble anti-CD28 mAb (5 μg/ml; BD Pharmingen) for 5 days (final volume of 300 μl in a 48-well plate). Capture ELISA was used to quantify triplicate sets of samples to measure cytokine production (27).

Cytokine secretion by DO11.10 T cells cultured for 24 h or 72 h with or without OVA, OVA-pσ1, OVA + pσ1, or pσ1 stimulation was assessed by cytokine ELISA assay. Cytokine ELISA was conducted, as previously described (27), to determine the levels of IFN-γ, IL-4, IL-10, IL-17 in each sample.

Apoptosis assay

Lymphocytes isolated from spleens of naïve DO11.10 mice were cultured with or without stimulation for 24 h or 72 h in 37° C with 5 % CO$_2$. Cells were stimulated with one of the following per 1 ml of culture: 1 mg OVA, OVA-pσ1, pσ1, 1 mg OVA + 1 mg pσ1, 50 μg OVA-pσ1, 50 μg pσ1, or 1 mg OVA + 50 μg pσ1. Percentage of apoptosis of CD4$^+$ T cells was determined by FACS based on the co-staining of CD4$^+$ T cells with Cy-7-AAD (BD Pharmingen), and PE-Annexin V (BD Pharmingen) according to the manufacturer's instructions.

Statistical analysis

One-way analysis of variance (ANOVA) ($p < 0.05$) followed by a post hoc Tukey test was applied to evaluate statistical differences between groups in Fig. 1F, Fig. 4, and Fig. 7*A*. The

remaining groups were analyzed by a Student's *t*-test to evaluate statistical differences between groups or treatments in performed experiments, and *p* values < 0.05 are indicated.

Results

Nasal immunization with OVA-pσ1 induces B and T cell unresponsiveness to OVA

Our past studies (14,23) have shown that chemically modified pσ1 can be successfully used for mucosal delivery of DNA vaccines. To further exploit pσ1 as a mucosal vaccine delivery platform, OVA cDNA was genetically fused 5′ to the pσ1 gene so as not to disrupt pσ1′s binding capacity. The resulting fusion protein was termed OVA-pσ1 (Fig. 1*A*). This modified OVA retained its Ag integrity when detected using an anti-OVA mAb (Fig. 1*B*), and the pσ1 portion retained its binding activity to L cells (Fig. 1*C*). To test its immunogenicity, OVA-pσ1 was applied nasally with or without the potent mucosal adjuvant CT (Fig. 1*D, E,* and *F*). BALB/c mice dosed with OVA-pσ1 or OVA-pσ1 plus CT were unresponsive, as evident by the depressed or lack of OVA-specific IgG and IgA Abs, when compared to mice given OVA alone or OVA plus CT (Fig. 1*D* and *E*). Even immunization with OVA alone, which is a poor mucosal immunogen, elicited greater Ag-specific IgG Ab titers than did OVA-pσ1-dosed mice (Fig. 1*E*). To test unresponsiveness to OVA challenge, mice dosed with OVA, OVA plus CT, or OVA-pσ1 and CT as mucosal adjuvant were peripherally challenged with OVA. Results showed significantly lower OVA-specific DTH responses by OVA and OVA-pσ1 plus CT-dosed mice when compared to mice given OVA plus CT as mucosal adjuvant (Fig. 1*F*). To discern whether OVA-pσ1-induces tolerance to pσ1, serum samples collected from BALB/c mice nasally dosed with OVA-pσ1 or with DNA complexes with pσ1-poly-L-lysine were tested by Ab ELISA. Virtually no pσ1-specific IgG Ab titers were detected in mice dosed with OVA-pσ1 compared to mice given DNA complexes with pσ1-poly-L-lysine, which exhibited IgG titers in excess of 2^{13} (Fig. 1*G*). Thus, OVA-pσ1 tolerizes the host to pσ1- and OVA-specific B and T cell responses, even in the presence of co-administered CT, and these results show the feasibility of using pσ1 as a mucosal vaccine delivery platform for induction of mucosal tolerance, rather than immunity.

Tolerance can be adoptively transferred with OVA-specific CD4^{+} T cells

Using CD4^{+} T cells isolated from OVA-transgenic (Tg) DO11.10 TCR mice, we hypothesized that OVA-pσ1 would induce tolerance in an OVA-specific fashion. Splenic CD4^{+} T cells isolated from DO11.10 Tg mice were adoptively transferred into naïve BALB/c mice. Twenty four h after adoptive transfer, the recipient mice were dosed nasally with 400 μg of OVA to induce tolerance (28) or with only 80 μg of OVA-pσ1. Control mice received an intramuscular (i.m.) injection of 400 μg of OVA as a positive immunized control group (28) or nasal sPBS (negative control group). Since T_{reg} cells are highly enriched in CLNs (28), CD4^{+} T cells were isolated from recipient CLNs and were adoptively transferred 3 d after immunization into a second group of naïve recipient BALB/c mice, which, 24 h later, were challenged s.c. with OVA. Five days post-peripheral challenge, the second recipient mice were evaluated for tolerance induction by an *in vitro* OVA-specific proliferation assay (Fig. 2*A*). Contrary to the i.m. immunized group, CD4^{+} T cells originating from mice dosed nasally with OVA-pσ1 or with a high dose of OVA failed to proliferate following *in vitro* stimulation with OVA (Fig. 2*A*). The CD4^{+} T cells originated from the i.m. OVA-dosed mice showed a ~5-fold increase in OVA-specific proliferation. Thus, these data show that OVA-pσ1 can stimulate OVA-specific tolerance via CD4^{+} T cells.

Dosing with OVA-pσ1 induces FoxP3^{+} T_{reg} cells

To define the possible regulatory T cells induced by tolerization with OVA-pσ1, FACS analysis was performed. Mice given nasal OVA-pσ1 revealed a significant induction of FoxP3^{+} CD25^{+}CD4^{+} T cells (Fig. 2*E*), even following a peripheral OVA challenge (Tables I and II).

More than a 40 % increase in FoxP3^{+} CD25^{+}CD4^{+} T cells in HNLNs (Fig. 2*E*) and in MLNs (data not shown) was observed in mice given nasal OVA-pσ1, when compared to naïve mice (Fig. 2*D* and *E* -upper histograms). In a similar fashion, OVA-pσ1-dosed mice, but not naïve mice, showed a > 60 % increase in FoxP3^{+} CD25^{−}CD4^{+} T cells (Fig. 2*D* and *E* - lower histograms). The effector function of T_{reg} cells is often associated with secretion of regulatory cytokines, such as IL-10 and/or TGF-β1. Subsequent FACS analysis confirmed that increased T_{reg} cells in OVA-pσ1-dosed mice expressed significantly more IL-10, but not TGF-β1, when compared with PBS- dosed mice (Table I). These results further suggest that tolerance induced by OVA-pσ1 is supported by T_{reg} cells, which may act in an IL-10-dependent manner.

OVA-specific T_{reg}, as well as CD25^{−}CD4^{+} T cells, suppress in vivo proliferation of Ag-specific effector CD4^{+} T cells

The combined evidence from the adoptive transfer study and from the phenotypic analysis suggests that OVA-pσ1 induces Ag-specific T_{reg} cells. To investigate the ability of OVA-pσ1-induced T_{reg} cells to inhibit proliferation of OVA-specific effector CD4^{+} T cells *in vivo*, naïve BALB/c mice were adoptively transferred with CD4^{+}, CD25^{+}CD4^{+}, or CD25^{−}CD4^{+} T cells isolated from mice given nasal OVA-pσ1 or with CD4^{+} T cells from mice given only nasal OVA. All recipient mice simultaneously received Vybrant-labeled OVA-specific CD4^{+} T cells isolated from Tg DO11.10 mice. Recipient mice were s.c. challenged with OVA in IFA 24 h after adoptive transfer, and 4 d later, Tg CD4^{+} T cells were evaluated by flow cytometry for *in vivo* proliferation (Fig. 2*B*). Proliferation of the Tg CD4^{+} T cells was significantly reduced in mice receiving OVA-pσ1-specific T_{reg} cells, since only 15 % of these cells underwent cell division (Fig. 2*B*). In contrast, adoptively transferred CD4^{+} T cells from OVA-immunized mice, allowed expansion of the Tg CD4^{+} T cells, as evidenced by > 55 % of them undergoing proliferation (Fig. 2*B*). Significant suppression of the Tg CD4^{+} T cell proliferation was also obtained using CD25^{−}CD4^{+} and total CD4^{+} T cells from OVA-pσ1-dosed mice ($p < 0.001$), but to a lesser extent (20 % proliferation). This study shows that OVA-pσ1-derived T_{reg} cells significantly inhibited the proliferation of Tg CD4^{+} T cells greater than OVA-pσ1-derived CD25^{−} ($p = 0.009$) or OVA-pσ1-derived total CD4^{+} T cells ($p = 0.001$). Collectively, these results suggest that OVA-pσ1-induced tolerance is mediated by CD25^{+}CD4^{+} T cells and, interestingly, may also be contributed in part by CD25^{−}CD4^{+} T cells, which inhibited the proliferation of OVA-specific effector T cells.

In an effort to define the mechanism by which OVA-pσ1-derived CD4^{+} T cells inhibit proliferation of these Tg CD4^{+} T cells, we analyzed the phenotypes of the CD4^{+} T cells isolated from the OVA-challenged recipients. Mice receiving OVA-pσ1-derived CD25^{−}CD4^{+} T cells showed significant increases in FoxP3^{+} CD25^{−}CD4^{+} T cells (~ 40 %) and elevated numbers of FoxP3^{+} T_{reg} cells (~ 20 %; Fig. 2C -left panel). On the other hand, mice adoptively transferred with OVA-pσ1-derived CD25^{+}CD4^{+} T cells showed even greater increases in FoxP3^{+} T_{reg} cells (~ 30 %), but FoxP3^{+} CD25^{−}CD4^{+} T cells in these mice were not elevated and, in fact, were equivalent with control CD4^{+} T cells from OVA-immunized or PBS-dosed mice (Fig. 2*C* -left panel). To determine if adoptively transferred OVA- or OVA-pσ1-derived CD4^{+} T cells induce CD4^{+} regulatory T cells among the responder DO11.10 CD4^{+} T cell subset, the percentages of DO11.10 FoxP3^{+} CD25^{−}CD4^{+} and FoxP3^{+} CD25^{+}CD4^{+} T cells in mice given OVA- or OVA-pσ1-derived CD4^{+} T cells were evaluated. FACS analysis revealed that prior to the adoptive transfer, the DO11.10 CD4^{+} T cell population contained ~ 5 % T_{reg} cells and ~ 10 % FoxP3^{+} CD25^{−}CD4^{+} T cells (Fig. 2*C* - right panel). These FoxP3^{+} CD25^{+}CD4^{+} T cells expanded in mice given OVA-pσ1-derived (both CD25^{+} and CD25^{−}) CD4^{+} T cells, although the greatest increase, by nearly 5-fold, was in mice given OVA-pσ1-derived CD25^{+}CD4^{+} T cells (Fig. 2*C* - right panel). In contrast, expansion of DO11.10 FoxP3^{+} CD25^{+}CD4^{+} T cells was not observed in mice dosed with PBS or adoptively transferred with OVA-derived CD4^{+} T cells. Interestingly, more than a 3-fold increase in Tg

FoxP3$^+$ CD25$^-$CD4$^+$ T cells were observed in mice co-transferred with OVA-pσ1-derived CD25$^-$CD4$^+$ T cells. By examining the expansion of Tg FoxP3$^+$ CD25$^-$CD4$^+$ T cells in mice given OVA-pσ1-derived CD25$^+$CD4$^+$ T cells, it was found that these FoxP3$^+$ CD25$^-$CD4$^+$ T cells expanded to a lesser extent. No increases in FoxP3$^+$ CD25$^-$CD4$^+$ T cells were observed in mice given OVA- or PBS-dosed recipients given Tg CD4$^+$ T cells (Fig. 2*C* - right panel). This evidence suggests that OVA-pσ1-induced FoxP3$^+$ CD25$^-$CD4$^+$ T cells do contribute to OVA-pσ1-mediated inhibition of effector T cell proliferation, possibly via expansion of either CD4$^+$ T cell subset or via conversion of FoxP3$^+$ T cells (29).

OVA-pσ1-induced tolerance is mediated by increased regulatory cytokine production

To investigate the specific mechanisms of the observed tolerance, cytokine secretion by CD4$^+$ T cells was measured. Total CD4$^+$ T cells were isolated from recipient HNLNs and spleens given Tg CD4$^+$ T cells and various CD4$^+$ T cell subsets, as described in the adoptive transfer studies in Fig. 2*B* and *C*. After *in vitro* restimulation with OVA$_{323-339}$ peptide, the cytokine profiles of the responding OVA-specific CD4$^+$ T cells were analyzed. Unlike controls, mice receiving OVA-pσ1-derived (CD25$^+$, CD25$^-$, and total CD4$^+$) T cells failed to induce IL-17-secreting CD4$^+$ T cells and showed no or reduced IFN-γ-secreting CD4$^+$ T cells in all tested lymphoid tissues (Fig. 3*A* and *B*). Instead, these mice showed greater anti-inflammatory responses, as evidenced by significant increases in the numbers of IL-10- and IL-4-producing CD4$^+$ T cells. Adoptive transfer of T$_{reg}$ cells resulted in a 15-fold enhancement in IL-10-producing CD4$^+$ T cells, whereas recipient mice given CD25$^-$CD4$^+$ T cells from OVA-pσ1-dosed mice showed a 10-fold enhancement IL-4-producing CD4$^+$ T cells when compared to recipient mice given CD4$^+$ T cells from OVA-vaccinated or PBS-dosed mice (Fig. 3).

To evaluate the specific cytokine profiles for CD25$^+$ and CD25$^-$ T cell subsets from OVA-pσ1-dosed mice, BALB/c mice adoptively transferred with DO11.10 Tg CD4$^+$ T cells were given a single nasal dose of OVA-pσ1, OVA, or PBS. Recipient mice were then challenged with OVA/IFA on day 3 post-immunization (p.i.) and sacrificed on day 7 p.i. Induction of tolerance was confirmed in OVA-pσ1-dosed mice by the absence of an OVA-specific DTH response (data not shown). CD25$^+$CD4$^+$ and CD25$^-$CD4$^+$ T cells from pooled HNLNs, MLNs, and spleens were stimulated with plate-bound anti-CD3 and soluble anti-CD28 mAbs. Analysis of cytokines produced by cultured cells revealed that the majority of IL-10 from OVA-pσ1-dosed mice was derived from T$_{reg}$ cells (Fig. 4*D*), whereas almost all of the IL-4 was produced by CD25-CD4$^+$ T cells (Fig. 4*C*). These data further confirm that OVA-pσ1-induced tolerance depends largely upon IL-10-producing T$_{reg}$ cells and is further supported Th2 cells. Although TGF-β1 was secreted by both CD25$^+$ and CD25$^-$CD4$^+$ T cells, this cytokine was not strikingly elevated in OVA-pσ1-dosed mice when compared to OVA- and PBS-dosed mice (Fig. 4*E*), further implying that TGF-β1 may have a lesser role for OVA-pσ1-induced tolerance. Contrary to control mice, production of proinflammatory cytokines, IFN-γ (Fig. 4*A*) and IL-17 (Fig. 4*B*), was significantly diminished by both T$_{reg}$ and CD25$^-$CD4$^+$ T cell subsets.

IL-10 is necessary for induction of OVA-pσ1-mediated tolerance

Results thus far have shown that mucosal administration of OVA-pσ1 induces secretion of regulatory cytokines, IL-4, IL-10, and TGF-β, although the most pronounced increase was observed in the production of IL-10. Since past studies have shown that IL-10 is not responsible for tolerance induction (30–32), we queried whether tolerance could be induced in IL-10$^{-/-}$ mice given nasal OVA-pσ1. C57BL/6 mice given nasal OVA-pσ1 were unresponsive to OVA, as evidenced by the low plasma IgG and mucosal IgA anti-OVA Ab titers and a lack of DTH responses, when compared with PBS-primed mice (Fig. 5*A* and *B*). In contrast, OVA-pσ1-dosed IL-10$^{-/-}$ mice became immune to OVA after peripheral challenge, as did PBS-primed IL-10$^{-/-}$ mice (Fig. 5*A* and *B*). CD4$^+$ T cells isolated from HNLNs, MLNs, and spleens of IL-10$^{-/-}$ mice dosed with OVA-pσ1 showed significant increases in OVA-specific T cell

proliferation when compared to the same cells obtained from tolerized C57BL/6 mice. As evidenced in OVA-pσ1-dosed C57BL/6 mice, these had at least three-fold less CD4$^+$ T cell proliferative responses to OVA in HNLNs and spleens than their PBS-dosed littermates (Fig. 5C). Interestingly, FACS analysis of FoxP3$^+$ T$_{reg}$ cells revealed no differences in T$_{reg}$ cell numbers between PBS- and OVA-pσ1-dosed IL-10$^{-/-}$ mice (Fig. 5D), and, in fact, they resembled baseline levels of CD25$^+$CD4$^+$ T cells in these mice (Fig. 5E). In contrast, the HNLNs and spleens of OVA-pσ1-dosed C57BL/6 mice contained significantly more OVA-specific T$_{reg}$ cells than the HNLNs and spleens of both PBS-dosed C57BL/6 mice and OVA-pσ1-dosed IL-10$^{-/-}$ mice, the latter result suggesting that T$_{reg}$ cells in IL-10$^{-/-}$ mice were simply not induced (Fig. 5D). Additionally, almost 50 % of the FoxP3$^+$ T$_{reg}$ cells in HNLNs and spleens of OVA-pσ1-dosed C57BL/6 mice showed intracellular expression of IL-10 (Fig. 5F), confirming the supportive role of IL-10 in OVA-pσ1-induced tolerance. Upon OVA restimulation, analysis of cytokine-secreting CD4$^+$ T cells from C57BL/6 (Fig. 6A and C) and IL-10$^{-/-}$ mice (Fig. 6B and D) was performed. As expected, OVA-pσ1-dosed C57BL/6 mice showed a significant increase in CD4$^+$ T cells secreting regulatory cytokines IL-10 and TGF-β1 in HNLNs and MLNs, and IL-4-producing cells in all tested tissues when compared with PBS-dosed mice (Fig. 6C). Numbers of CD4$^+$ T cells producing IL-13 were not significantly different between immunized and control mice (Fig. 6C).

Production of proinflammatory cytokines, IFN-γ, IL-2, and IL-17 (Fig. 6A), was significantly inhibited in all tested tissues of OVA-pσ1-dosed C57BL/6 mice. IL-10$^{-/-}$ mice revealed that, although the IL-4 and IL-13 responses were significantly more elevated in OVA-pσ1- than PBS-dosed mice (Fig. 6D), TGF-β-secreting CD4$^+$ T cells were significantly depressed, except in the spleen (Fig. 6D). No significant differences in numbers of IL-2-producing CD4$^+$ T cells were observed between OVA-pσ1- and PBS-dosed IL-10$^{-/-}$ mice. Consistent with an antagonistic relationship between IFN-γ and IL-17 (33), there was a significant decrease in IFN-γ and a concomitant increase in the IL-17 in OVA-pσ1-dosed IL-10$^{-/-}$ mice when compared to PBS-dosed mice (Fig. 6B).

OVA-pσ1- mediated tolerance can be induced after a single dose

Since the pσ1-based approach offers the possibility of inducing tolerance while using significantly less Ag, the persistence of OVA-pσ1-induced tolerance was investigated after single dose. C57BL/6 mice were nasally given either 100 μg per dose of OVA, OVA-pσ1, or sterile PBS for three consecutive days, or a single 50 μg dose of OVA-pσ1 or OVA alone. Additionally, a group of C57BL/6 mice was given 0.5 mg of OVA nasally for 5 consecutive days. Ten days p.i., all mice were challenged with OVA by the s.c. route. One day prior to challenge, no OVA-specific serum IgG Ab responses in either immunization group were detected (data not shown). By day 18 post-challenge (day 28 p.i.), mice given OVA or PBS revealed significantly elevated OVA-specific Ab titers in both mucosal (not shown) and systemic immune compartments, and these responses were sustained (Fig. 7A). Moreover, we were unable to induce tolerance to OVA, even in mice dosed nasally with as much as 2.5 mg of OVA alone. In contrast, virtually no OVA-specific serum-IgG Abs (Fig. 7A) nor DTH responses (Fig. 7B) were observed in OVA-challenged mice given a single or three nasal doses of OVA-pσ1. These data show that mice given OVA-pσ1 nasally are tolerized to OVA and that this tolerance can be induced by substantially lower doses of pσ1-delivered Ag.

While OVA-pσ1 can induce tolerance to OVA, even in the presence of CT, chemically modified pσ1 is found to be immunogenic. Mucosal delivery of DNA complexed to poly-L-lysine-modified pσ1 results in enhanced transgene-specific immune responses (14). To determine whether there is an adjuvant effect by the plasmid cDNA that can overcome the OVA-pσ1-induced tolerance, BALB/c mice were given a single nasal dose of OVA or OVA-pσ1 alone, or co-administered with CpG-ODN. In contrast to mice dosed with OVA or OVA

+ ODN, mice dosed with OVA-pσ1 alone or OVA-pσ1 + ODN showed significantly reduced OVA-specific serum and mucosal Ab (Fig. 7*C*) and DTH responses (Fig. 7*D*). Therefore, the tolerogenic effect of this pσ1-based delivery system cannot be overridden by co-administration of the immune-modulatory molecule CpG-ODN.

Mucosal administration of OVA-pσ1 is necessary for induction of tolerance

We have shown here that mucosal administration of OVA-pσ1 induces a low-dose tolerance to OVA. In an effort to determine if the pσ1-mediated tolerance can be induced via a non-mucosal route, mice were i.m. immunized on days 0, 7, and 14 with OVA-pσ1 without or with CT and compared to mice similarly immunized with OVA (Fig. 8). On day 21 (one week after the last immunization), mice were evaluated for serum IgG anti-OVA Ab titers by ELISA. As expected, OVA-immunized responded well, but naïve and OVA-pσ1-immunized mice remained OVA unresponsive (Fig. 8*A* and *B* - open bars). All mice were subsequently challenged on day 28 with OVA in IFA, and one week later, serum from individual mice was collected and measured for IgG anti-OVA titers. Even though, mice dosed with OVA-pσ1 + CT (Fig. 8B) showed reduced Ab titers relative to OVA + CT-dosed mice, these anti-OVA Ab titers were greater than those obtained in the OVA-challenged, naïve mice (Fig. 8*A*). Thus, these results show that induction of the optimal tolerance to OVA via the use of pσ1 requires mucosal, not parenteral, administration of OVA-pσ1 (Fig. 8*A* and *B* - black bars).

OVA-pσ1 induces apoptosis of OVA-Tg CD4+ T cells in vitro

Mucosal delivery of OVA-pσ1 triggers the regulatory T cell response and secretion of anti-inflammatory cytokines. In contrast, parenteral administration of OVA-pσ1 induces anti-OVA Ab response after s.c. challenge with OVA. To evaluate if pσ1 has a direct impact on CD4+ T cell populations, or if the induction of tolerance relies solely on the pσ1's interaction with the mucosal epithelium, an *in vitro* study was performed. Lymphocytes isolated from naïve DO11.10 Tg mice were cultured with high- (1 mg/ml) or low-dose (0.05 mg/ml) of OVA-pσ1 or pσ1 and compared to *in vitro* cultures stimulated with OVA or OVA + pσ1. After 24 or 72 h, cells and culture supernatants were harvested and evaluated for CD4+ T cell apoptosis by FACS, and cytokine production by ELISA, respectively. Only about 13 % of Tg CD4+ T cells underwent apoptosis in response to *in vitro* stimulation with OVA for up to 72 h. In contrast, stimulation with pσ1, OVA-pσ1, or OVA + pσ1 induced much greater apoptosis of Tg CD4+ T cells after 24 and 72 h (Table III), and the extent of this apoptosis was time- and dose- dependent. Less than 31 % of these cells died after 24 h of culture with a low-dose (0.05 mg/ml) stimulation, whereas 1 mg/ml of OVA-pσ1, pσ1 or OVA + pσ1 doubled the number of apoptotic cells at this time point (Table III). After 72 h, low-dose pσ1 exposure resulted in ~ 54 – 62 % of Tg CD4+ T cell undergoing apoptosis unlike high-dose treatment that resulted in ~ 80 % apoptosis (Table III). Concomitant cytokine analysis of cultured supernatants revealed increased production of IL-4 and IL-10 by the cells stimulated for 24 h with 1 mg/ml OVA-pσ1 or 72 h with 0.05 mg/ml OVA-pσ1 (Table IV). Consistently, these OVA-pσ1-stimulated cells showed significant reduction in inflammatory cytokines IFN-γ and IL-17 at both time points, when compared to OVA-stimulated cells. Cells cultured with a high-dose of OVA-pσ1 for 72 h failed to produce any cytokines, presumably due to apoptosis of 80 % of these cells (Table III). Interestingly, cells incubated with unconjugated pσ1 and OVA mimicked the inflammatory response of OVA-stimulated cells, showing elevated IL-17 and IFN-γ secretion after 24 h, and even after 72 h, the production of IL-10 and IL-4 by these cells was significantly reduced in relation to OVA-stimulated cells (Table IV). These results offer an alternative mechanism in which pσ1 may induce apoptosis of OVA-responsive CD4+ T cells if intact pσ1 survives delivery beyond the initial cell binding to the mucosal epithelium or M cells.

In summary, these studies show that OVA-pσ1 can readily accomplish tolerance with a single dose and stimulate the production of FoxP3$^+$ T$_{reg}$ cells producing IL-10, and tolerance induction is IL-10 -dependent.

Discussion

We previously showed that the reovirus adhesin, pσ1, can enhance the efficacy of mucosal DNA vaccination (14). In this work, we investigated if a genetic fusion between pσ1 and a model Ag (OVA) would have the same effect. Unlike the previous findings, it was learned that when pσ1 was fused to a protein Ag, tolerance was induced. In fact, tolerance could be accomplished even with a single dose of OVA-pσ1 delivered mucosally. In contrast, parenteral delivery of OVA-pσ1 failed to induce tolerance to OVA. This pσ1-based mucosal tolerance even resisted co-treatment with potent mucosal adjuvants (CT and ODN), and tolerance was not broken after peripheral challenge with OVA. In contrast to OVA-pσ1, nasal administration of OVA alone, which is known to be a poor mucosal immunogen (34), elicited greater Ag-specific Ab responses than did OVA-pσ1. Moreover, the mechanism for this pσ1-induced tolerance was CD4$^+$ T cell-dependent and could be adoptively transferred into naïve mice. Adoptive transfer of CLN-derived CD4$^+$ T cells from mice tolerized with OVA-pσ1 significantly inhibited OVA-specific proliferation of CD4$^+$ T cells *in vitro*. In subsequent analysis, it was revealed that tolerization via OVA-pσ1 induced significant increases in FoxP3$^+$ CD25$^+$CD4$^+$ T cells, as well as an elevation in FoxP3$^+$ CD25$^-$CD4$^+$ T cells. Adoptive transfer of OVA-pσ1-primed CD25$^+$CD4$^+$ or CD25$^-$CD4$^+$ T cells significantly inhibited Ag-specific proliferation of OVA-Tg CD4$^+$ T cells *in vivo*. This suppression was due to increased production of IL-10 by OVA-pσ1-induced T$_{reg}$ cells, as evidenced by cytokine and FACS analyses, as well as by the lack of OVA-specific tolerance in OVA-pσ1-dosed IL-10$^{-/-}$ mice. Additionally, it was learned from the *in vitro* studies that OVA-pσ1 induces apoptosis of OVA-responsive CD4$^+$ T cells in a time- and a dose- dependent manner, offering an additional potential mechanism for pσ1's action if it can survive delivery beyond the initial cell binding to the mucosal epithelium or M cells.

Induction of peripheral tolerance to a number of mucosally delivered Ags has been demonstrated previously using both high- and low-dose tolerization regimen (2,7,17,28,35); however, we propose that fusion of pσ1 to a protein Ag can significantly improve both mucosal and systemic unresponsiveness to this Ag. Previously, we have shown that chemically modified pσ1 with poly-L-lysine significantly enhances immunity to delivered DNA (14), and because of this modification, Abs to pσ1 are induced, but not to the unmodified pσ1. Thus, genetic fusion of an Ag to pσ1 is instrumental to stimulate tolerance. When OVA-pσ1 was co-administered with potent mucosal adjuvants, CT or CpGODN, the results provided further evidence that immunity to OVA was impaired. This finding was surprising since it is well established that CT given mucosally always induces Ag-specific Abs (36). Although the mechanism by which OVA-pσ1-induced tolerance overrides the immunostimulatory effects of CT is unclear, it is plausible that OVA-pσ1 resists co-treatment with CT due to the pσ1 adhesive properties which, in turn, may act on different receptors than CT-B. Nonetheless, pσ1-induced tolerance to OVA remained effective. Another mucosal immune modulator, the unmethylated CG dinucleotides CpG-containing motifs (CpGODN), was also shown to enhance immunity to OVA after oral administration (26). Here it was shown nasal co-administration of OVA-pσ1 plus CpGODN also resulted in tolerance. In contrast to mice given OVA plus CpGODN, OVA-pσ1 plus CpGODN-dosed mice lacked OVA-specific DTH responses and showed limited OVA-specific systemic and mucosal Ab titers, suggesting that the presence of a plasmid cDNA does not affect tolerogenic properties of genetically modified pσ1.

Despite the reported feasibility to induce low-dose nasal tolerance with OVA (28,37), a sustainable tolerance by a variety of nasal OVA doses (0.05 – 2.5 mg) could not be induced, but a single oral 25 mg dose of OVA was found effective. In contrast, as little as a single 50 μg nasal dose of OVA-pσ1 was sufficient to induce OVA-specific tolerance that was long-lasting and resisted peripheral challenge with OVA in IFA, showing that OVA-pσ1 is at least a 1000-fold more efficient on a molar basis.

Previous studies have shown that the CT-B subunit could be adapted for mucosal tolerance induction (38–41). In those reports, efficiency of Ag-specific tolerance was improved by its conjugation to CT-B, as evidenced by reduced Ag-specific Ab and DTH responses following mucosal delivery. Many of these studies depended upon chemically coupling the tolerogen, which produces a heterogeneous population of carrier-conjugates and may limit only a fraction of these to bind the intended cell surface receptor (42). Additionally, CT-B is also commonly used as a mucosal adjuvant (43,44) because it lacks the toxic moieties associated with native CT (45). To circumvent these potential barriers for using CT-B to induce tolerance, a genetic fusion between CT-B and proteolipid protein (PLP) immunodominant peptide was done to ameliorate the clinical manifestations of EAE (42). Although multiple doses were required, nasal administration of this CT-B-PLP peptide fusion protein was effective for inducing tolerance.

Regulatory T cells consist of a phenotypically diverse group bearing a variety of cell surface receptors, but they commonly share their ability to suppress T cell function (46–48). Induction and maintenance of low-dose mucosal tolerance has been associated with activation of T_{reg} cells acting in an Ag-specific fashion (6,7,49–51). Evidence provided in this study shows that OVA-pσ1-induced tolerance is mediated by Ag-specific $CD4^+$ T cells, since adoptive transfer of these OVA-pσ1-primed $CD4^+$ T cells conferred unresponsiveness in naïve recipients to *in vivo* peripheral OVA challenge and inhibited OVA-specific $CD4^+$ T cell proliferation. Studies have also found that low-dose mucosal tolerance is mediated by T_{reg} cells that express the FoxP3 transcription factor (5,47,52–54). Consistent with these findings, nasal administration of OVA-pσ1 significantly increased the numbers of T_{reg} cells, in which > 97 % were $FoxP3^+$. Along these lines, expression of FoxP3 was also induced in $CD25^-CD4^+$ T cells following tolerization with OVA-pσ1, although the intensity of FoxP3 staining was ~50-fold less than that observed for the $CD25^+CD4^+$ T cells. A low level intensity of FoxP3 and its transient expression on activated human $CD25^-$ T cells has been previously reported (55). These FoxP3-expressing $CD25^-$ T cells are not capable of inhibiting cytokine production nor proliferation, and expression of FoxP3 on these cells is not correlated with the induction of a T_{reg} cell phenotype (55). In contrast to that study, adoptive transfer of OVA-pσ1-primed $CD25^+$ or $CD25^-$ T cells significantly inhibited *in vivo* proliferation of OVA-Tg $CD4^+$ T cells. Additionally, FACS analysis of lymphocytes isolated from mice adoptively transferred with OVA-pσ1-derived $CD25^-$ T cells showed significant enrichment in $FoxP3^+CD25^-CD4^+$ T cells. A significant increase in FoxP3 expression in DO11.10 responder $CD4^+$ T cells (both $CD25^+$ and $CD25^-$) was observed in mice adoptively transferred with OVA-pσ1-derived $CD25^+$ or $CD25^-$ $CD4^+$ T cells, suggesting that OVA-pσ1-derived $CD4^+$ T cells induce $FoxP3^+$ expression by the responder $CD4^+$ T cells. This finding is not surprising, since tolerance induction can occur via conversion of $CD25^-$ to $FoxP3^+$ $CD25^+CD4^+$ T cells, as reported by others (29,56), and these FoxP3-expressing $CD25^-CD4^+$ T cells, as well as converted $CD25^+CD4^+$ T cells, are potent inhibitors of $CD4^+$ T cell expansion *in vivo* (29). Thus, the OVA-pσ1-induced $CD25^-$ T cells also have regulatory properties, as evident by being able to suppress proliferation of Tg $CD4^+$ T cells.

Examination of $OVA_{323-339}$-specific CD4+ T cell cytokine production in recipient mice adoptively transferred with either total $CD25^-$ or $CD25^+$ $CD4^+$ T cells from OVA-pσ1-primed mice revealed greatly reduced numbers of $CD4^+$ T cells secreting the proinflammatory

cytokines, IFN-γ and IL-17, and instead produced IL-4 and IL-10. These primarily segregated with IL-4 coming from recipients given OVA-pσ1-primed $CD25^-CD4^+$ T cells and with IL-10 from recipients given OVA-pσ1-primed T_{reg} cells. The increased production of IL-4 may play a beneficial role in OVA-pσ1-mediated tolerance, perhaps, via the induction of a Th2-type immune bias, which was previously shown to support mucosal tolerance (57,58). Additionally, IL-4 can also facilitate induction of T_{reg} cells from naïve, peripheral $CD25^-CD4^+$ T cells, as recently demonstrated *in vitro* by others (57). The role for IL-10 in maintenance of tolerance is well established (30–32), even though its role in the induction of both mucosal and peripheral unresponsiveness remains controversial (59–61). One report has shown that the early production of IL-10 by $CD4^+$ T cells is important for induction of high-dose oral tolerance (59). Here, we demonstrated that IL-10 is also necessary for induction of a low-dose nasal tolerance mediated by pσ1, since OVA-pσ1-mediated tolerance could not be established in $IL-10^{-/-}$ mice. $IL-10^{-/-}$ mice given OVA-pσ1 nasally developed elevated serum IgG anti-OVA Ab and DTH responses. In contrast to C57BL/6 mice, immunization of $IL-10^{-/-}$ mice with OVA-pσ1 failed to induce Ag-specific T_{reg} cells and failed to inhibit Ag-specific proliferation of $CD4^+$ T cells. Therefore, we conclude that IL-10 is essential for induction and maintenance of pσ1-induced tolerance, and IL-10-producing T_{reg} cells, as well as possibly $CD25^-CD4^+$ T cells, mediate the observed pσ1-induced tolerance.

The role of TGF-β1 appears to be less essential for OVA-pσ1-induced tolerance. TGF-β1 was only modestly induced in OVA-pσ1 tolerized BALB/c mice; however, TGF-β1 was induced in OVA-pσ1 tolerized C57BL/6 mice and greatly reduced in OVA-pσ1-dosed $IL-10^{-/-}$ mice. The PBS-dosed, OVA-challenged $IL-10^{-/-}$ mice also showed elevated numbers of TGF-β1-producing $CD4^+$ T cells. This suggests that TGF-β1 may play a supportive role in OVA-pσ1-induced tolerance, and secretion of TGF-β1 in OVA-pσ1-tolerized mice depends upon the presence of IL-10, as previously suggested (62). Alternatively, the production of TGF-β1 may contribute to the conversion of $CD25^-CD4^+$ to $CD25^+CD4^+$ T cells in tolerized mice, as implicated by recent studies demonstrating that TGF-β1 induces conversion of naïve peripheral $CD25^-CD4^+$ T cells into T_{reg} cells by enhancing FoxP3 expression (29,35,56). Further evidence for the supportive role of TGF-β1 in OVA-pσ1-mediated tolerance is suggested by the depressed levels of $TGF-β1^+ CD4^+$ T cells in the OVA-pσ1-dosed $IL-10^{-/-}$ mice.

We have demonstrated, here and elsewhere (14,23), that reovirus adhesin, pσ1, can be engineered to induce either immunity or tolerance, although the exact mechanism of pσ1-mediated modulation of an immune response remains undefined. Pσ1, aside from binding M cells in murine NALT (14), interacts with a variety of rodent and human cell types, including murine L929 (L) cells, RFL-6 cells, and Caco-2 cells (23). Additionally, pσ1 binds to mammalian erythrocytes (63), as well as to intestinal epithelial cells (64). It is known that pσ1 has two distinct binding domains (64,65). One domain, located in pσ1's head structure, has been shown to interact with cells expressing the junctional adhesion molecule 1 (JAM1), whereas a second binding domain in pσ1's tail is thought to be responsible for binding to ubiquitously expressed sialic acid (64). Interaction of pσ1 with host cells may be JAM1-mediated, since some of them, including primary human DCs and epithelial cells, express this molecule (64). A possible mechanism for pσ1-induced modulation of an immune response could be a well-described ability of pσ1 to induce apoptosis (65,66). Interestingly, the efficiency of pσ1-mediated apoptosis has been linked to the presence of sialic acid binding domain, since pσ1 mutants, unable to bind sialic acid, are insufficient inducers of apoptosis (65). Given these findings, we showed here that OVA-pσ1 and pσ1 trigger apoptosis of OVA-Tg $CD4^+$ T cells *in vitro*. However, in contrast to pσ1 alone, or unconjugated pσ1 + OVA, only OVA-pσ1 was capable of stimulating these cells to produce increased levels of regulatory cytokines. The means by which pσ1 induces tolerance are still being investigated, and our results suggested that more than one mechanism may be associated with this process. Nonetheless, mucosal delivery of OVA-pσ1 is obviously crucial for the induction of OVA-

specific unresponsiveness. It is known that stimulation of mucosal tolerance is dose-dependent, resulting from active suppression, induction of anergy, or clonal deletion of effector cells (2, 5). Results presented here suggest that a low dose of OVA-pσ1 delivered mucosally induces active suppression of anti-OVA immune responses by regulatory CD4$^+$ T cells. While the *in vitro* studies suggested possible clonal deletion because of the accompanied pσ1-induced apoptosis of OVA-specific effector CD4$^+$ T cells, it remains to be determined if such events occur *in vivo* following mucosal delivery.

In summary, we showed that pσ1-mediated tolerance to OVA can be established with a minimal amount of tolerogen when genetically fused to pσ1 and when applied mucosally. In some instances, a single nasal administration using ~1000-fold less OVA was sufficient to induce tolerance to OVA. Tolerance induced by OVA-pσ1 resisted co-treatment with CT, as well as peripheral challenge with OVA and IFA. Although IL-4 and TGF-β seemed to have supportive roles, pσ1-delivered tolerance relied largely on activation of specific FoxP3$^+$ T$_{reg}$ cells that acted in an IL-10-dependent manner. The induced tolerance was IL-10-dependent since IL-10$^{-/-}$ mice were unable to undergo tolerance by OVA-pσ1 presumably via the failure to produce T$_{reg}$ cells. The impact by the OVA-pσ1-induced T$_{reg}$ cells was enhanced by regulatory CD25$^-$CD4$^+$ T cells since a subset of these was FoxP3$^+$ and produced predominantly IL-4 in addition to IL-10. These CD25$^-$CD4$^+$ T cells could also inhibit the proliferation of OVA-Tg CD4$^+$ T cells. TGF-β1 appeared to have a supportive role in tolerance induction by OVA-pσ1. Depression of TGF-β1-producing CD4$^+$ T cells in OVA-pσ1-dosed IL-10$^{-/-}$ mice was observed suggesting that the production of this cytokine may be triggered by IL-10. These collective data show that IL-10 is pivotal in OVA-pσ1-induced tolerance. This finding is relevant because of the low-dose of Ag required for tolerance induction and its potential applicability to treat or prevent a variety of autoimmune diseases and allergies. The opportunity to deliver pσ1-based tolerogens via the nasal route offers a safer, easier, and more cost effective alternative for tolerization.

Acknowledgements

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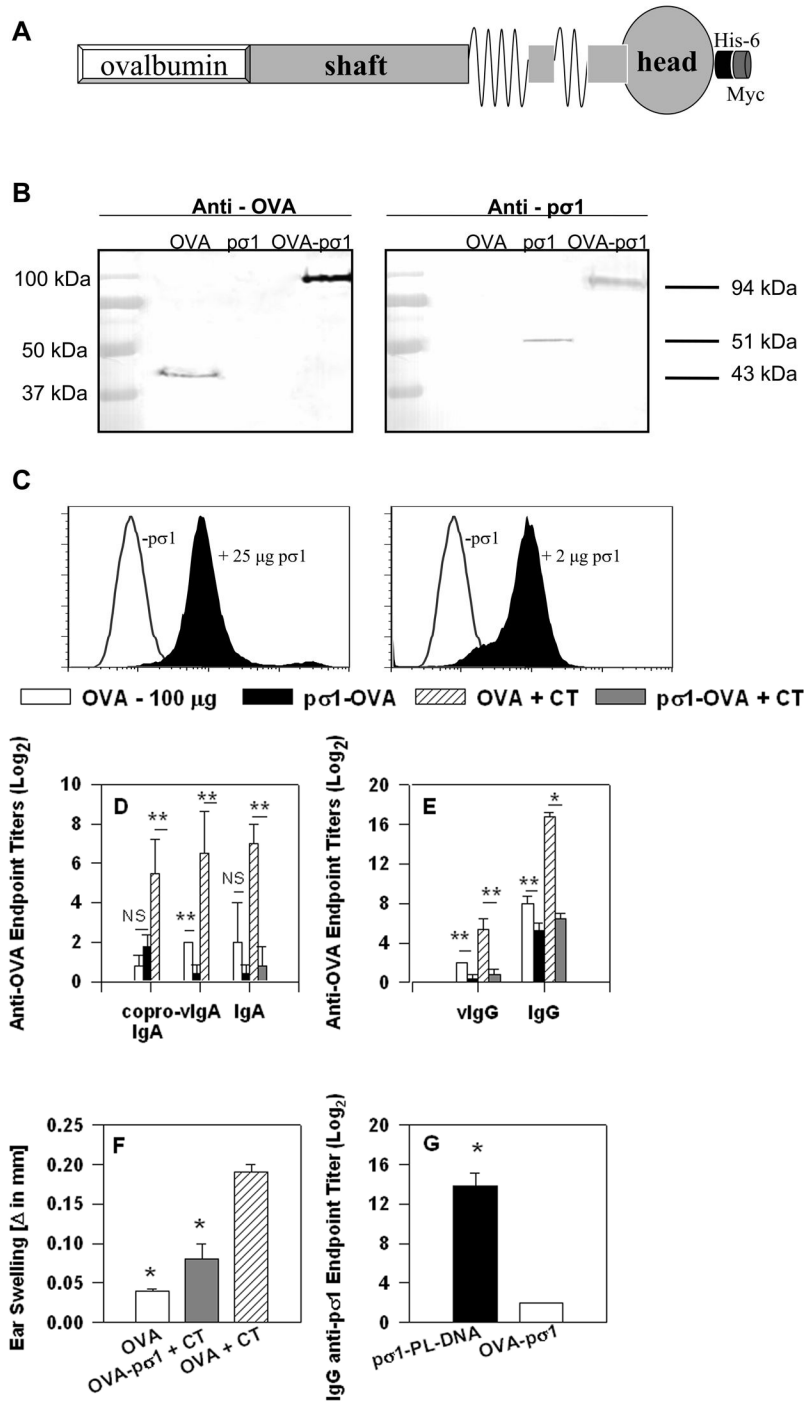
FIGURE 1.
Nasal administration of OVA-pσ1 suppresses OVA-specific immune responses. (*A*) Schematic representation of OVA-pσ1 protein: tolerogen, OVA and pσ1 (shaft and head), 6 histidine-tag, and Myc Ag-tag (components are not drawn to scale). (*B*) Western immunoblot of OVA-pσ1, pσ1, and OVA probed with mouse IgG anti-OVA mAb (left panel), or rabbit anti-pσ1 polyclonal Ab (right panel). (*C*) Recombinant pσ1 binds to L cells. L cells were incubated with 2 μg (left panel) or 25 μg (right panel) of his/myc tagged pσ1, followed by an addition of a

biotinylated anti-pσ1 mAb and streptavidin-PE to detect pσ1 by FACS; thus, his/myc tag on pσ1's C terminus does not interfere with L cell binding. (*D, E, F*) BALB/c mice were nasally dosed with OVA or OVA-pσ1, with or without CT. (*D, E*) Serum, vaginal, and fecal samples collected on day 28 post-immunization (p.i.) were analyzed for IgA and IgG OVA-specific titers by ELISA. Mean of 10 mice ± SEM is depicted. * $p < 0.001$, ** $p < 0.05$ for OVA-pσ1- vs. OVA-, and OVA-pσ1 + CT- vs. OVA + CT- dosed mice, calculated by Student's *t* test. (*F*) DTH reactions to OVA were measured at day 35 p.i. Mean ± SEM of 6 mice per group. Statistical significance between groups was calculated by one-way ANOVA followed by post hoc Tukey test. * $p < 0.05$ vs. OVA + CT; OVA vs. OVA-pσ1 were not significantly different. (*G*) Anti-pσ1 Ab ELISA was performed on serum samples collected from BALB/c mice dosed nasally with OVA-pσ1 or pσ1-poly-L-lysine-DNA. Mice dosed with pσ1-PL-DNA, but not OVA-pσ1, showed the pσ1-specific IgG response. * $p < 0.001$ for OVA-pσ1 vs. pσ1-poly-L-lysine-DNA was calculated by Student's *t* test.
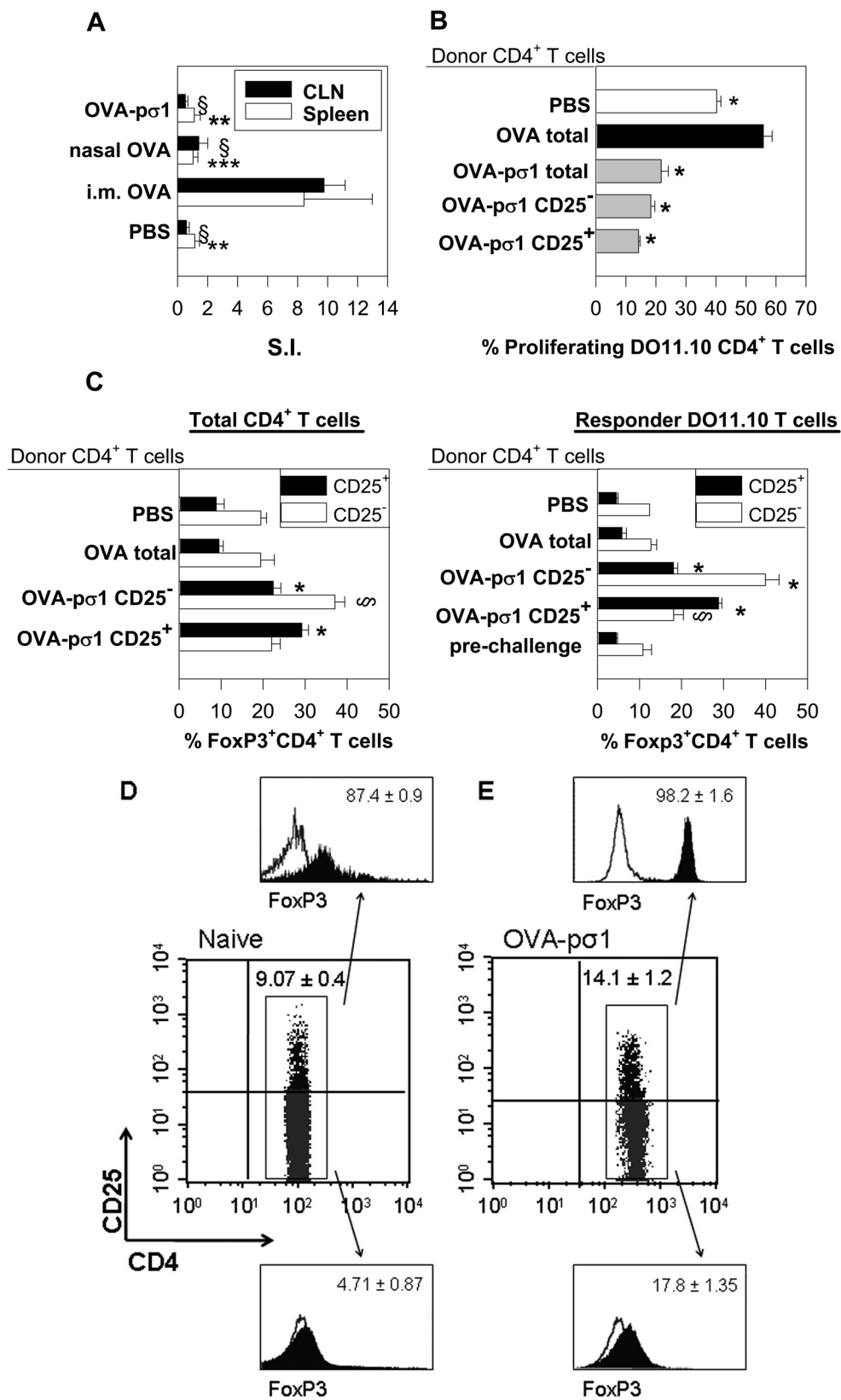
A

OVA-pσ1 §
**

nasal OVA §
***

i.m. OVA

PBS §
**

Legend: ■ CLN   □ Spleen

0 2 4 6 8 10 12 14

S.I.

B

Donor CD4⁺ T cells

PBS *
OVA total
OVA-pσ1 total *
OVA-pσ1 CD25⁻ *
OVA-pσ1 CD25⁺ *

0 10 20 30 40 50 60 70

% Proliferating DO11.10 CD4⁺ T cells

C

Total CD4⁺ T cells

Donor CD4⁺ T cells

PBS
OVA total
OVA-pσ1 CD25⁻ * §
OVA-pσ1 CD25⁺ *

Legend: ■ CD25⁺   □ CD25⁻

0 10 20 30 40 50

% FoxP3⁺CD4⁺ T cells

Responder DO11.10 T cells

Donor CD4⁺ T cells

PBS
OVA total
OVA-pσ1 CD25⁻ * *
OVA-pσ1 CD25⁺ § *
pre-challenge

Legend: ■ CD25⁺   □ CD25⁻

0 10 20 30 40 50

% Foxp3⁺CD4⁺ T cells

D

87.4 ± 0.9

FoxP3

Naive

9.07 ± 0.4

CD25 / CD4

4.71 ± 0.87

FoxP3

E

98.2 ± 1.6

FoxP3

OVA-pσ1

14.1 ± 1.2

17.8 ± 1.35

FoxP3

FIGURE 2.
OVA-pσ1-induced unresponsiveness is mediated by CD4$^+$ T cells. (*A*) CD4$^+$ T cells isolated from CLNs and spleens of mice adoptively transferred with CLN-derived CD4$^+$ T cells from OVA-pσ1- or OVA-dosed mice were cultured *in vitro* without or with 1 mg of OVA for 5 days. ^{3}H-TdR incorporation was measured and expressed as a stimulation index (SI). For CLN, § $p \leq 0.001$ versus i.m. OVA; for spleen** $p = 0.003$, ***$p = 0.006$ vs. i.m. OVA, calculated by Student's *t* test. (*B, C*) BALB/c mice were adoptively transferred with Vybrant-labeled DO11.10 Tg CD4$^+$ T (responder) cells and with the designated T cell population (donor CD4$^+$ T cells) isolated from OVA-pσ1- or OVA-dosed mice. (*B*) *In vivo* suppression of proliferation of DO11.10 Tg CD4$^+$ T cells following challenge with OVA was measured by FACS. * $p \leq 0.001$ vs. mice given CD4$^+$ T cells from OVA-dosed mice, calculated by Student's *t* test. (*C*) Lymphocytes pooled from HNLNs, MLNs, and spleens from recipient mice were stained with anti-CD4, anti-CD25, and anti-FoxP3 mAbs and analyzed by FACS. The mean percentage ($\pm$ SD) of FoxP3$^+$ CD25$^-$CD4$^+$ T cells and FoxP3$^+$ CD25$^+$CD4$^+$ T cells as a (left panel) proportion of total CD4$^+$ T cells, or (right panel) DO11.10 CD4$^+$ T cells is depicted. * $p \leq 0.001$ vs. CD25$^+$CD4$^+$ T cells from mice given CD4$^+$ T cells from OVA-dosed mice; § $p \leq 0.001$, ** $p \leq 0.05$ vs. CD25$^-$CD4$^+$ T cells from mice given CD4$^+$ T cells from OVA-dosed mice, calculated by Student's *t* test. (*D, E*) FACS analysis was performed on lymphocytes isolated from C57BL/6 mice (*D*) naïve or (*E*) nasally dosed with OVA-pσ1 three times at weekly intervals. Depicted are percentages of unstimulated T$_{reg}$ cells isolated from HNLNs. Percentage of FoxP3$^+$ T$_{reg}$ cells (upper filled histogram) and FoxP3$^+$ CD25$^-$CD4$^+$ T cells (lower filled histograms) as proportion of CD4$^+$ T cells are plotted versus isotype control for FoxP3 (empty histogram). Results are presented as an average $\pm$ SD of 5 animals per tissue.
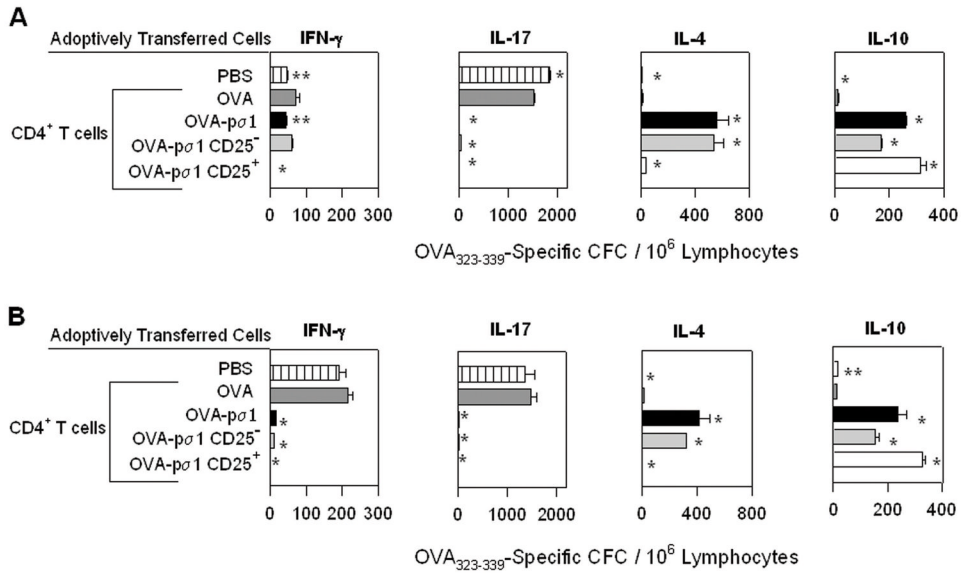
FIGURE 3.
OVA-pσ1-induced tolerance is dependent upon increased production of anti-inflammatory/ regulatory cytokines and depression of proinflammatory cytokines. CD4$^+$ T cells were isolated from (*A*) HNLNs and (*B*) spleens of recipient mice given the various CD4$^+$ T cell subsets plus responding Tg CD4$^+$ T cells in Fig. 2*B* and *C*. To evaluate differences in cytokine production by the responder (DO11.10) CD4$^+$ T cells influenced to become tolerogenic or immunogenic, CD4$^+$ T cells, CD4$^+$ T cells were isolated from recipient mice and subjected to T cell ELISPOT assay after *in vitro* re-stimulation with OVA$_{323-339}$ peptide. Results are depicted as cytokine-forming cells (CFC) per 10^6 CD4$^+$ T cells corrected for unstimulated responses. Adoptive transfer of OVA-pσ1-specific CD25$^+$CD4$^+$ T cells or CD25$^-$ CD4$^+$ T cells significantly enhanced numbers of IL-10- and IL-4-producing CD4$^+$ T cells, respectively, in recipient mice with concomitant reductions in IFN-γ and IL-17 CFC responses. Adoptive transfer of OVA-stimulated CD4$^+$ T cells showed enhanced IFN-γ and IL-17 CFCs with little to no IL-4 or IL-10 CFCs. * $p < 0.001$, ** $p \leq 0.05$ vs. mice given CD4$^+$ T cells from OVA-dosed mice, calculated by Student's *t* test.
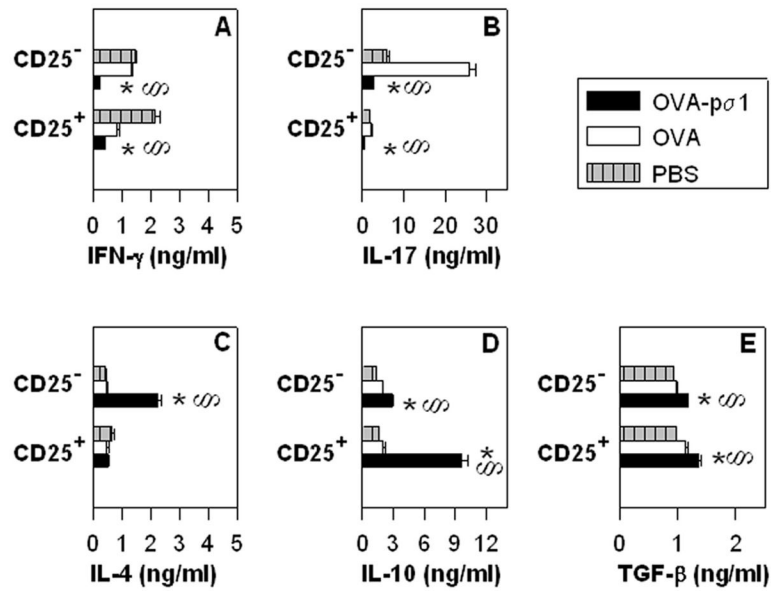
FIGURE 4.
OVA-pσ1-induced tolerance is mediated by IL-10-producing CD25$^+$CD4$^+$ T cells and supported by IL-4-producing CD25$^-$CD4$^+$ T cells. BALB/c mice were adoptively transferred with 1 x 10^7 DO11.10 Tg CD4$^+$ T cells and 24 h later nasally dosed with 50 μg OVA-pσ1 or OVA only. Mice were challenged s.c. on d 3 after transfer and sacrificed 4 d later. CD25$^+$CD4$^+$ T cells and CD25$^-$CD4$^+$ T cells were isolated from HNLNs, MLNs, and spleens of these mice and cultured *in vitro*. Presence of (*A, B*) proinflammatory and (*C, D, E*) regulatory cytokines in cultured supernatants was measured by ELISA. CD25$^+$CD4$^+$ T cells from OVA-pσ1-dosed mice produced significantly more (*D*) IL-10, whereas the majority of (*C*) IL-4 was secreted by CD25$^-$CD4$^+$ T cells. Mean ± SEM of 5 mice per group is shown. Statistical significance for cytokine -producing CD25$^-$ or CD25$^+$ CD4$^+$ T cells was calculated by one-way ANOVA followed by a post hoc Tukey test. § *p* < 0.05 for OVA-pσ1- versus PBS-dosed mice, * *p* < 0.05 for OVA-pσ1- versus OVA-dosed mice.
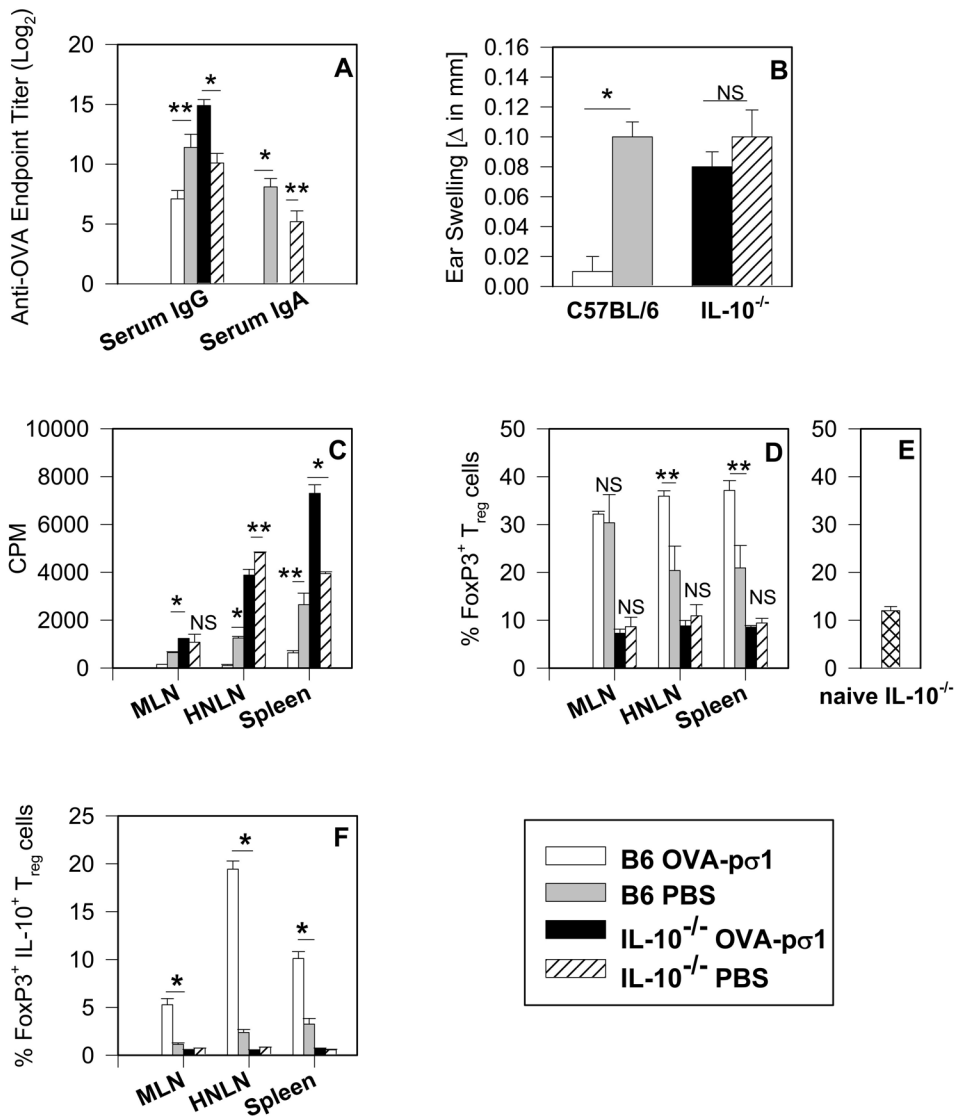
FIGURE 5.
IL-10 is required for induction and maintenance of OVA-pσ1-mediated tolerance. IL-10$^{-/-}$ and C57BL/6 (IL-10$^{+/+}$) (B6) mice were given 100 μg of OVA-pσ1 or PBS nasally on days 0, 1, and 2 and were then challenged s.c. on day 10 p.i. with OVA in IFA. (*A*) OVA-specific ELISA was performed on serum samples collected from OVA-pσ1- and PBS-dosed C57BL/6 and IL-10$^{-/-}$ mice on day 17 p.i. OVA-pσ1-dosed IL-10$^{-/-}$ mice elicited a serum IgG anti-OVA response. (*B*) OVA-specific DTH responses measured on day 15 p.i. OVA-pσ1-dosed IL-10$^{-/-}$ mice were responsive to OVA. (*C*) CD4$^+$ T cells isolated from HNLNs, MLNs, and spleens of OVA-pσ1- or PBS-dosed C57BL/6 (IL-10$^{+/+}$) and IL-10$^{-/-}$ mice were measured for their proliferative responses after *in vitro* stimulation with OVA. CD4$^+$ T cells from IL-10$^{-/-}$ mice dosed with OVA-pσ1 or PBS, and PBS-dosed C57BL/6 mice proliferated in response to OVA. Depicted values are corrected for ^{3}H-TdR uptake by unstimulated cells. (*D*) Percentages of FoxP3$^+$ CD25$^+$CD4$^+$ T cells were measured by FACS after *in vitro* stimulation with OVA, and depicted are values corrected for unstimulated cells. IL-10$^{-/-}$ mice failed to show induction of T$_{reg}$ cells independent of treatment. The mean of two experiments ± SEM per group is shown. * $p < 0.001$, ** $p < 0.05$ for OVA-pσ1- vs. PBS-dosed C57BL/6

or IL-10$^{-/-}$ mice; NS, not significant, calculated by Student's *t* test. (*E*) Depicted is the percentage of CD25$^+$CD4$^+$ T cells for the combined MLNs, PPs, and spleens from 5 naïve IL-10$^{-/-}$ mice. Results determined by FACS show the mean ± SD. (*F*) Percentages of FoxP3$^+$IL-10$^+$ T$_{reg}$ cells, as a proportion of CD25$^+$CD4$^+$ T cells, were measured by FACS. T$_{reg}$ cells induced in C57BL/6 mice given nasally OVA-pσ1 expressed IL-10, whereas significantly less IL-10 was expressed by PBS-dosed C57BL/6 mice. Average ± SEM of 4 mice is shown. * *p* < 0.001 for OVA-pσ1-versus PBS dosed C57BL/6 mice, calculated by Student's *t* test; NS = not significant.
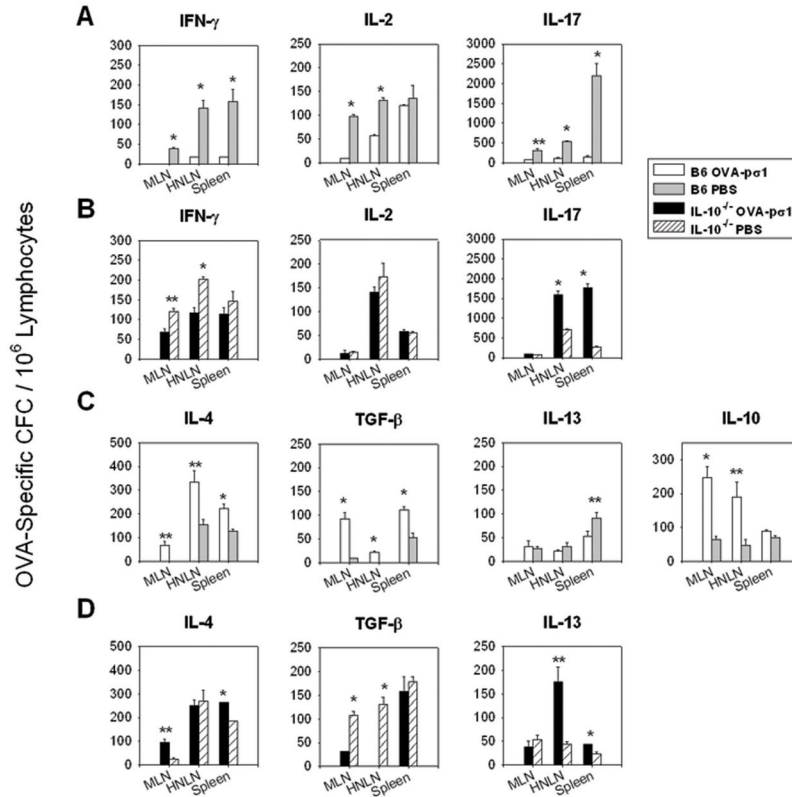
FIGURE 6.
Immunization of IL-10$^{-/-}$ mice with OVA-pσ1 increases IL-17- and IFN-γ producing CD4$^+$ T cells with concomitant increases in anti-inflammatory cytokines, (IL-4 and IL-13) responses when compared to similarly treated C57BL/6 mice. C57BL/6 (IL-10$^{+/+}$) (B6) and IL-10$^{-/-}$ mice were immunized and challenged, as described in Fig. 5 legend. CD4$^+$ T cells isolated from HNLNs, MLNs, and spleens were restimulated with OVA, and cytokine-specific T cell ELISPOT was performed. CFC/10^6 CD4$^+$ T cells in (*A, C*) C57BL/6 and (*B, D*) IL-10$^{-/-}$ mice are depicted. For OVA-pσ1-dosed C57BL/6 mice, CD4$^+$ T cells producing anti-inflammatory cytokines in MLNs and HNLNs were significantly elevated when compared with (*C*) PBS-dosed mice. The numbers of CD4$^+$ T cells producing (*A*) proinflammatory cytokines were significantly reduced in OVA-pσ1-dosed mice versus PBS-dosed C57BL/6 mice. OVA-pσ1-dosed IL-10$^{-/-}$ mice showed significant increases when compared with PBS-dosed mice in (*B, D*) IL-4-, IL-13-, and IL-17-producing CD4$^+$ T cells, but not TGF-β-producing CD4$^+$ T cells. IFN-γ- and IL-17-producing CD4$^+$ T cells were significantly elevated in IL-10$^{-/-}$ mice given OVA-pσ1 when compared to OVA-pσ1-dosed C57BL/6 mice (*A, B*). Depicted is the mean of two experiments ± SEM. * $p < 0.001$, ** $p < 0.05$ for OVA-pσ1- versus PBS-dosed C57BL/6 or IL-10$^{-/-}$ mice, calculated by Student's *t* test.
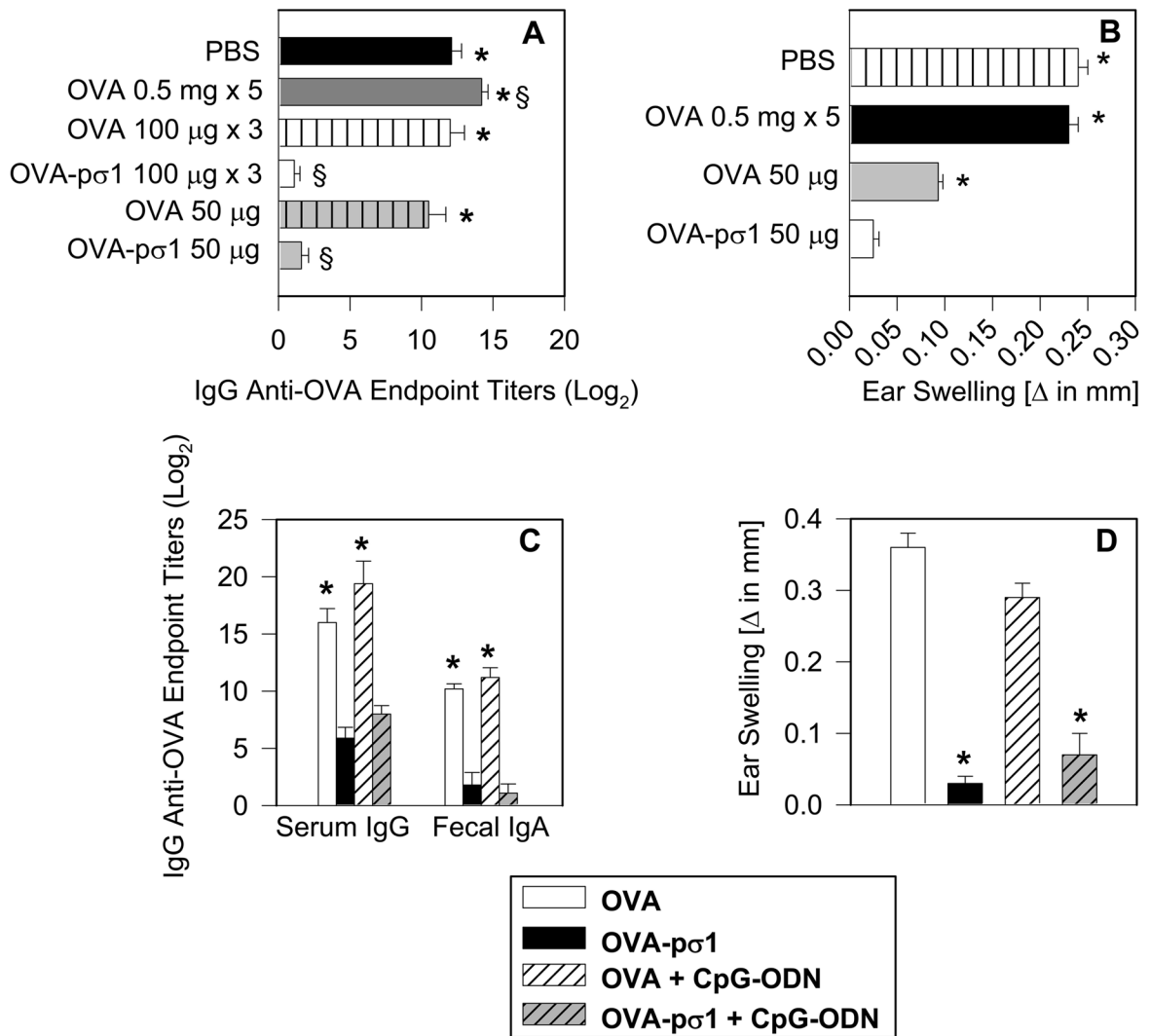
FIGURE 7.
A single nasal dose of OVA-pσ1 is sufficient to induce tolerance to OVA. (*A, B*) C57BL/6 mice were given OVA-pσ1, OVA, or sterile PBS and were subsequently challenged s.c. with OVA/IFA. (*A*) Data depict serum IgG anti-OVA Ab titers at 18 d post-challenge (28 d p.i.). Statistical significance was calculated by one-way ANOVA followed by post hoc Tukey test. * $p < 0.05$ versus mice nasally given 50 μg of OVA-pσ1, § $p < 0.05$ versus. mice given nasally 50 μg of OVA. (*B*) OVA-specific DTH responses were measured at day 15 p.i. (day 5 post-challenge). A single 50 μg dose of OVA-pσ1, but not OVA alone, was sufficient for tolerance induction. * $p < 0.001$ for OVA-pσ1-dosed vs. control mice, calculated by Student's *t* test. Data are representative of three separate experiments. (*C, D*) BALB/c mice were nasally given 100 μg of OVA-pσ1 or OVA, with or without 25 μg of CpG. Ten days p.i., mice were challenged s.c. with OVA/IFA. Data depict (*C*) plasma IgG and fecal IgA anti-OVA Ab titers 18 d post-challenge (28 d p.i.), and (*D*) OVA-specific DTH responses at day 15 p.i. (day 5 post-challenge). Statistical significance * $p < 0.001$ for OVA-pσ1 versus OVA-dosed mice, or for OVA-pσ1 + CpG versus. OVA + CpG -dosed mice, calculated by Student's *t* test.
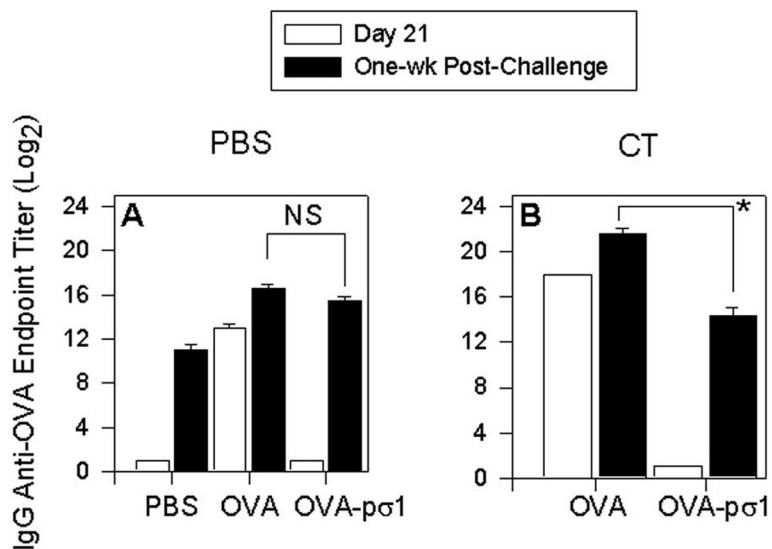
FIGURE 8.
Parenteral immunization with OVA-pσ1 induces anti-OVA Ab response following OVA
challenge. C57BL/6 mice were i.m. immunized with (*A*) PBS, 50 μg OVA, or OVA-pσ1, or
(*B*) with 50 μg OVA or OVA-pσ1 plus 1 μg CT, on days 0, 7, and 14. On day 21, sera were
collected and analyzed for the presence of anti-OVA IgG Abs by ELISA (open bars), and
showed unresponsiveness by PBS-, OVA-pσ1-, and OVA-pσ1 + CT-dosed mice. On day 28,
mice were s.c. challenged with OVA in IFA. Sera collected from mice one week post-challenge
(day 35) revealed the presence of IgG anti-OVA Abs (black bars) in all tested groups, although
the anti-OVA response was higher in mice dosed with OVA + CT compared to OVA-pσ1 +
CT-dosed mice. NS = not significant; * $p < 0.001$ for OVA vs. OVA-pσ1 and OVA + CT vs.
OVA-pσ1 + CT -dosed mice, calculated by Student's *t* test.

Table I

Nasal immunization with OVA-pσ1 induces IL-10-secreting $CD25^+CD4^+$ T_{reg} cells

Immunization Group[a]	OVA-pσ1[b]	sPBS[b]
% $CD25^+CD4^+$ T_{reg}	15.4 ± 4.5[c]	9.7 ± 3.1
% $IL-10^+$ T_{reg}	63.2 ± 6.4[d]	45.1 ± 6.6
% of $TGF-\beta^+$ T_{reg}	49.4 ± 3	46 ± 3.2

[a]C57BL/6 mice were given 100 μg of OVA-pσ1 or PBS nasally on days 0, 1, and 2, and were challenged s.c. on day 10 p.i. with OVA in IFA.

[b]FACS analysis was performed on lymphocytes isolated from HNLNs and *in vitro* stimulated for 72 h with 1 mg OVA. Mean ± SEM of 5 mice per group is presented. Mice nasally dosed

with OVA-pσ1 showed significant increases in OVA-specific T_{reg} cells that were $IL-10^+$.

[c, d]Statistical significance was calculated by Student's *t* test.

[c]Differences between OVA-pσ1- versus PBS-dosed mice. $p = 0.031$.

[d]Differences between OVA-pσ1- versus PBS-dosed mice. $p = 0.008$.

Table II

Nasal immunization with OVA-pσ1-induces FoxP3^{+} CD25^{+} and FoxP3^{+} CD25^{−} CD4^{+} T cells

Immunization Group[a]	OVA-pσ1[b]	OVA oral[b]	OVA nasal[b]	sPBS[b]
% FoxP3^{+} CD25^{+}CD4^{+} T cells				
CLN	19.15 ± 1.13[c, d]	11.6 ± 1.07	6.92 ± 0.8	5.92 ± 1.44
HNLN	18.04 ± 1.5[c, d]	11.98 ± 1.71	7.29 ± 1.4	7.5 ± 1.03
MLN	17.46 ± 2.8[d]	17.45 ± 0.5	8.07 ± 1.01	7.3 ± 1.4
PP	18.35 ± 1.48[c, d]	14.45 ± 1.4	6.91 ± 0.9	6.7 ± 1.84
Spleen	15.95 ± 2.69[c, d]	9.49 ± 2.57	5.56 ± 1.16	7.33 ± 2.08
% FoxP3^{+} CD25^{−} CD4^{+} T cells				
CLN	22 ± 1.42[c, d]	16.7 ± 2.4	14 ± 2.55	14.5 ± 3.39
HNLN	21.88 ± 2.71[c, d]	15.6 ± 1.5	14.13 ± 1.05	10.5 ± 1.14
MLN	19.83 ± 2.4[c, d]	10.02 ± 1.7	13 ± 0.95	12.9 ± 1
PP	19.93 ± 2.16[c, d]	12.03 ± 3.36	12.05 ± 1.64	12.25 ± 2.19
Spleen	21.73 ± 2.81[c, d]	10.5 ± 1.42	10.2 ± 3.01	9.62 ± 3.51

[a] C57BL/6 mice were nasally dosed with 100 μg OVA-pσ1, OVA or PBS, or orally with 25 mg OVA and challenged, as described in Table I.

[b] FACS analysis was performed on lymphocytes isolated from CLNs, HNLNs, MLNs, PPs, and spleens and *in vitro* stimulated for 72 h with 1 mg of OVA. Mean ± SD of 5 mice per group is presented. Mice nasally dosed with OVA-pσ1 showed significant increases in OVA-specific FoxP3^{+} CD25^{+}CD4^{+} T cells and FoxP3^{+} CD25^{−} CD4^{+} T cells.

[c, d] Statistical significance was calculated by Student's *t* test.

[c] $p < 0.04$, differences between OVA-pσ1- versus OVA oral-dosed mice.

[d] $p < 0.005$, differences between OVA-pσ1- versus i.n. OVA- and PBS-dosed mice.

Table III

Apoptosis of Tg DO11.10 CD4^{+} T cells

Treatment [a]			% Apoptosis of DO11.10 Tg CD4^{+} T Cells [b]			
Ag	Dose	24 hours [c]	P_1 [d]	72 hours [e]	P_2 [f]
media		7.06 ± 0.6		10.37 ± 0.92	
OVA	1 mg/ml	10.96 ± 2.82		12.73 ± 6.53	
OVA-pσ1	1 mg/ml	67.35 ± 7.97	< 0.001	83.80 ± 7.10	< 0.001
OVA-pσ1	0.05 mg/ml	30.99 ± 3.98	< 0.001	54.41 ± 11.6	0.003
pσ1	1 mg/ml	77.82 ± 4.82	< 0.001	86.52 ± 11.8	< 0.001
pσ1	0.05 mg/ml	32.43 ± 7.26	0.005	62.58 ± 10.6	< 0.001
OVA + pσ1	1 mg/ml, 1 mg/ml	77.56 ± 8.42	< 0.001	80.34 ± 8.44	< 0.001
OVA + pσ1	1mg/ml, 0.05 mg/ml	29.83 ± 9.86	0.025	62.29 ± 10.13	< 0.001

[a] Lymphocytes were isolated from spleens of naïve DO11.10 Tg mice. Cells were stimulated *in vitro* with designated concentrations of Ags and analyzed by FACS.

[b] Apoptosis was determined by co-staining with Annexin-V and 7-AAD.

[c, e] Mean ± SD of three to four replicates are presented after 24 and 72 h of culture.

[d, f] *p*-values for DO11.10 CD4^{+} T cells stimulated with OVA versus OVA-pσ1, pσ1, or OVA + pσ1 for 24 or 72 hours.

Table IV

Cytokine production by in vitro cultured DO11.10 lymphocytes

Treatment [a]		Cytokine Secretion after 24 Hours [b]			
Ag	Dose	IL-4 (ng/ml) [d]	IL-10 (ng/ml) [e]	IL-17 (ng/ml) [f]	IFN-γ (ng/ml) [g]
OVA	1 mg/ml	0.58 ± 0.03	0.66 ± 0.04 *	5.59 ± 0.54 *	4.06 ± 0.52
OVA-pσ1	1 mg/ml	1.20 ± 0.1 §	2.76 ± 0.14 *	0.37 ± 0.11 *	0.31 ± 0.08 §
OVA-pσ1	0.05 mg/ml	1.67 ± 0.2 §	4.73 ± 0.29 *	0 ± 0 *	0 ± 0 *
pσ1	1 mg/ml	0 ± 0 *	0.26 ± 0.04 §	0.63 ± 0.17 *	0.22 ± 0.07 §
pσ1	0.05 mg/ml	0 ± 0 *	0 ± 0 *	0.58 ± 0.14 *	0 ± 0 *
OVA + pσ1	1 mg/ml, 1 mg/ml	0 ± 0 *	0.56 ± 0.02	4.17 ± 0.6	2.6 ± 0.45
OVA + pσ1	1mg/ml, 0.05 mg/ml	0 ± 0	0.42 ± 0.17	1 ± 0.14 §	0.97 ± 0.08 §
		Cytokine Secretion after 72 Hours [c]			
OVA	1 mg/ml	2.21 ± 0.13	1.82 ± 0.12	6.22 ± 0.46	10.1 ± 0.98
OVA-pσ1	1 mg/ml)	0 ± 0 *	0 ± 0 *	0 ± 0 *	0 ± 0 *
OVA-pσ1	0.05 mg/ml	3.54 ± 0.15 §	2.2 ± 0.03 §	0.52 ± 0.13 *	0.38 ± 0.03 *
pσ1	1 mg/ml	0 ± 0 *	0 ± 0 *	0.58 ± 0.14 *	0.72 ± 0.05 *
pσ1	0.05 mg/ml	0 ± 0 *	0.29 ± 0.02 *	0 ± 0 *	0.94 ± 0.09 *
OVA + pσ1	1 mg/ml, 1 mg/ml	0 ± 0 *	0.37 ± 0.19 §	0.72 ± 0.18 *	0.77 ± 0.2 *
OVA + pσ1	1mg/ml, 0.05 mg/ml	1.22 ± 0.18 *	0.46 ± 0.16 §	2.87 ± 0.47 §	2.3 ± 0.29 §

[a] Lymphocytes were isolated from spleens of naïve DO11.10 Tg mice.

[a] Cultured supernatants from cells stimulated in vitro with designated concentrations of Ags were analyzed by cytokine ELISA.

[b, c] cells were cultured with or without Ag for 24 or 72 hours.

[d-g] Cytokine concentration was measured in triplicates and is depicted as an average ± SEM. Presented cytokine concentration is corrected over the cytokine production by unstimulated cells.

Statistical significance was calculated by Student's t test;

*
p ≤ 0.001

§
p < 0.05 for the differences between cells stimulated with OVA vs. OVA-pσ1, pσ1 or OVA + pσ1 - stimulated cells, for each time point.