

Modulation of Ovalbumin-Induced Airway Inflammation and Hyperreactivity by Tolerogenic APC¹

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Allergic asthma is mediated in part by unregulated Th2 inflammation in response to an allergen. Induction of peripheral tolerance by inoculation of Ags into the anterior chamber of the eye (ocular tolerance) before sensitization blocks Th2 responses. Thus, we proposed that induction of ocular tolerance to the allergen might modulate an ongoing allergen-induced Th2 pathogenesis in the lung. We initiated ocular tolerance in previously immunized mice in a classic mouse model of OVA-induced pulmonary allergic inflammation. In the model of ocular tolerance, the need for inoculation of Ag into the anterior chamber can be bypassed by i.v. inoculation of in vitro-generated tolerogenic (TGF- β 2-treated, Ag-pulsed) APC (tol-APC). We observed that with i.v. inoculation, such tolerogenic APC, but not control APC, reduced eosinophil and lymphocyte pulmonary infiltration in experimental mice. Similarly, production of Th2 cytokines (IL-4, -5, and -13), but not IFN- γ , was reduced. Importantly, airway hyperresponsiveness and mucus production were significantly reduced after treatment with the tol-APC. We also show that in vitro suppression of IL-13 production from OVA-sensitized effector T cells was mediated by CD8⁺, not CD4⁺, T regulatory cells. Thus, i.v. inoculation of the tol-APC induced peripheral tolerance that suppressed Th2-mediated pathogenesis in the lungs of presensitized mice. The ability of the tol-APC to induce peripheral tolerance and suppress existing Th2 immune inflammation may lead to novel therapies for pulmonary allergic inflammation and its related pathology. *The Journal of Immunology*, 2005, 175: 7117–7124.

Allergic asthma is a chronic airway inflammation that leads to airway hyperresponsiveness (AHR)³ and enhanced mucus production in the lung (1–3). Although Th1-associated inflammatory response (the presence of IFN- γ) could exacerbate allergic disease (4–6), the dominant immune disorder is driven by an unregulated Th2 response that leads to increased inflammatory cell infiltration and Th2 cytokine production in the lung and is a cornerstone of the pathogenesis in allergic asthma (5, 7–10). Increased IL-4 in the lung contributes to the recruitment of Th2 cells (11). Overexpression of IL-13 in the lung is associated with AHR and mucus hypersecretion and, together with IL-5, increases eotaxin production and eosinophilia in the lung (3, 8).

Evidence suggests that the lack of proper immune regulation leads to the development of allergic asthma (4, 12). Several types of CD4⁺ T regulatory (Tr) cells, such as Th3, TR1, and CD4⁺CD25⁺ T cells, are able to suppress either airway inflammation or AHR in asthma models by producing TGF- β and/or

IL-10 (4). Previous publications by our laboratory showed that tolerance induced by inoculation of Ag into the anterior chamber (a.c.) of the eye prevents the expression of both Th1 (delayed-type hypersensitivity (DTH) response) and Th2-mediated immune responses through a mechanism of eye-induced tolerance called anterior chamber-associated immune deviation (ACAID) (13–15). The suppression of Th1 responses by the eye-induced peripheral tolerance is mediated by both afferent CD4⁺ and efferent CD8⁺ Tr cells (13, 16, 17). However, the cellular mechanism of the tolerance in suppressing Th2 immune responses has yet to be identified.

It is known that the microenvironment in which APC capture Ags influences the function of APC. Indigenous APC in the eye are exposed to immunosuppressive factors (such as TGF- β 2, vasoactive intestinal peptide, α -melanocyte-stimulating hormone, and calcitonin gene-related peptide) within the local environment and preferentially induce tolerance instead of immune inflammation (18–22). During tolerance induction, eye-derived APC carry tolerogenic signals (enhanced production of TGF- β , IL-10, thrombospondin, and MIP-2) to the spleen and induce the generation of CD8⁺ efferent Tr cells in the marginal zone (MZ) of the spleen with the collaboration of CD4⁺ invariant NKT cells and MZ B cells (23–27).

Importantly, tolerance-inducing eye-derived APC can be mimicked in vitro by TGF- β 2 treatment of APC (thioglycolate-elicited peritoneal exudate cells or in vitro-generated, bone marrow-derived APC) before Ag exposure (28, 29). Intracameral inoculation of Ag to induce tolerance can be bypassed by i.v. inoculation of in vitro-generated, TGF- β 2-treated, Ag-pulsed (tolerogenic (tol-)) APC (14, 15, 29). We reported that i.v. inoculation of TGF- β 2-treated, OVA-pulsed APC before OVA sensitization suppressed OVA-specific IgE and Th2 cytokine (IL-4, -5, and -13) production in a mouse model of allergic pulmonary inflammation (15). In this study, we show that i.v. inoculation of the tol-APC induces CD8⁺ Tr cells that suppress the effector arm of the Th2 immune response in presensitized mice and prevent the expression of the majority of

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³ Abbreviations used in this paper: AHR, airway hyperresponsiveness; a.c., anterior chamber; ACAID, anterior chamber-associated immune deviation; BAL, bronchoalveolar lavage; dLNC, draining lymph node cell; DTH, delayed-type hypersensitivity; HMI, histologic mucus index; i.t., intratracheally; KLH, keyhole limpet hemocyanin; MZ, marginal zone; PAS, periodic acid-Schiff with diastase; Penh, enhanced pause; SFM, serum-free medium; tol, tolerogenic; Tr, T regulatory.

clinical symptoms in the lung associated with OVA-induced airway hyperreactivity and inflammation in the mouse.

Materials and Methods

Animals

Female BALB/c mice (8–16 wk old) were used in the experiments. All mice were purchased from The Jackson Laboratory and maintained in the Schepens Eye Research Institute vivarium. All animals were treated humanely in accordance with National Institutes of Health guidelines and with the approval of the Schepens animal care and use committee.

Reagents

OVA, controlled processed serum replacement factor 3, and tissue culture grade BSA were purchased from Sigma-Aldrich. OVA used in APC pulsing was further processed to remove residual endotoxin by passage over Detoxi-Gel Endotoxin Removing Gel (Pierce). Porcine TGF- β 2 was purchased from R&D Systems, and tissue culture medium (DMEM and RPMI 1640), HBSS, and PBS were obtained from BioWhittaker. Supplements for tissue culture medium (vitamins, L-glutamine, sodium pyruvate, nonessential amino acid, HEPES, and penicillin/streptomycin) were obtained from Invitrogen Life Technologies. FBS and horse serum were purchased from HyClone.

Murine model of allergic pulmonary inflammation

BALB/c mice were sensitized (i.p.) with alum (Imject Alum; Pierce)-precipitated OVA (10 μ g/mouse) on days 0 and 7. Ten to 12 days after the second sensitization, mice were challenged intratracheally (i.t.) with OVA (50 μ g/50 μ l PBS/mouse).

Generation of bone marrow-derived APC

Bone marrow-derived APC were generated by culturing mouse bone marrow cells from femur with L929 cell-conditioned medium for 6 days in petri dishes (30, 31). After culturing, the nonadherent cells and loosely adherent cells were collected by pipetting. Cell viability was checked by the trypan blue exclusion and routinely was >90%.

In vitro preparation of ACAID tol-APC

Bone marrow-derived APC were harvested from culture, washed, and exposed to TGF- β 2 (5 ng/ml) for 6–8 h in serum-free medium (SFM) containing RPMI 1640, 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μ g/ml streptomycin, 0.1% BSA, and insulin-transferrin-sodium selenite supplement (ITS culture supplement; BD Biosciences). Then OVA (5 mg/ml) was added to the culture and incubated (37°C, 5% CO₂) overnight. ACAID tol-APC (5 $\times$ 10⁵/100 μ l HBSS/mouse) were transferred (i.v.) to recipient mice 3–5 days after the second i.p. OVA sensitization and 7 days before OVA challenge (i.t.).

Measurement of AHR

Three days after OVA challenge (i.t.), AHR was measured using whole-body plethysmography (Buxco Electronics) (6, 32). The whole-body plethysmography system measures changes in pressure in the chamber during mouse expiration and inspiration. Peak expiratory and peak inspiratory pressures, expiratory time, and a relaxation time (time of the pressure in the chamber decay to 36% of the peak expiratory pressure) were collected. The enhanced pause (Penh) that directly correlates with airway resistance was calculated ([Penh = peak expiratory pressure/peak inspiratory pressure $\times$ (expiratory time – relaxation time)/relaxation time]). During the experiment, mice were placed in individual chambers for 5 min before measurement. Baseline Penh and Penh after PBS and methacholine (100 mg/ml) aerosol challenge were measured (6, 33). Penh after PBS challenge was averaged over 3 min from the beginning of the nebulization, and Penh after methacholine challenge was averaged over 8 min. The result is presented as the ratio of Penh (PBS or methacholine challenge)/Penh (baseline).

Quantification of airway mucus

Mucus in the lung was quantitated by the histologic mucus index (HMI) (3, 11, 34). Lungs were inflated, fixed in 10% buffered formalin, and paraffin embedded. The fixed lung samples were sectioned (4 μ m) longitudinally across all five lobes. Sections with visible central airways were selected for periodic acid-Schiff with diastase (PAS) staining. The stained slides were examined at $\times$ 100 final magnification on an Olympus BX40 microscope with a 10-mm square reticule grid inserted into one eyepiece. The slide was moved on the microscope stage so that the reticule grid covered consecu-

tively through the entire tissue section, and mucus-containing and mucus-free epithelia in the field of the reticule grid were counted. Two or three sections per lung were examined in a double-blind fashion. The histologic mucus index (HMI) was calculated as a percentage of the number of mucus-containing epithelia/number of total epithelia counted in each tissue section.

Differential cell counts

Differential cell counts were performed on cytopins of bronchoalveolar lavage (BAL) cells collected from the experimental groups. BAL cells were collected from individual mice by washing their lungs with 1 ml of PBS 10 times. Cell counts were determined, and 5 $\times$ 10⁴ cells in 50 μ l of complete medium were centrifuged in a cytocentrifuge (Shandon Southern Products) onto each FBS-coated slide (microslides; VWR Scientific) and stained in HEMA 3 (Fisher Scientific) according to the manufacturer's directions. Stained slides were examined using light microscopy ($\times$ 1000, under oil). A total of 1000 cells/slide were counted in consecutive areas covered by an eyepiece grid and categorized according to the following morphology: alveolar macrophages, large size and large cytoplasm region and single round nucleus; monocytes, kidney-shaped nucleus with light blue granules in the cytoplasm; lymphocytes, intensely blue-stained spherical nucleus with little cytoplasm; neutrophils, either a ring-shaped or two- to five-lobed nucleus with clear cytoplasm; and eosinophils, bilobed nucleus with pink cytoplasm granules.

T cell enrichment and Ab plus complement treatment

T cells were enriched using a T cell column that was packed in the laboratory using goat anti-mouse IgG-coated IMMULAN beads (Biotex Laboratories). After enrichment, the percentages of T cells were monitored by CD4 plus CD8 staining and flow cytometric analyses (EPICS XL; Beckman Coulter). At least 10,000 cells were analyzed in each sample. Together, CD4⁺ and CD8⁺ cells account for ~85% of the cells.

Cells (10⁷/ml) were resuspended in complete medium (RPMI 1640, 10% FBS, 1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μ g/ml streptomycin). Ab ascites (GK1.5 or 2.43; produced in the laboratory; 1/500 dilution) were added to the cell suspensions, which were then incubated for 40 min on ice. The cells were washed, and baby rabbit complement (1/15 dilution; Cedarlane Laboratories) was added to the cell suspensions before 30-min incubation at 37°C. The depletion of CD4⁺ and CD8⁺ T cells was confirmed by staining the cells with either CD4 (RM4-4) or CD8 (53-6.7) recognizing Ab and flow cytometric analyses.

RT-PCR

Total RNA was extracted from cells by TRIzol reagent (Invitrogen Life Technologies). The RNA sample (100 ng for cytokines and $\leq$ 1 ng for β -actin) was used for the one-step RT-PCR amplification using the Access RT-PCR system (Promega) and GeneAmp PCR System 9600 (PerkinElmer). The primer pairs for IL-13 (sense, cccatccatccctacagaa; antisense, tggcagacaggagtgtgtct; deduced from published gene sequence), IL-4 (sense, atgggtctcaacccccagtagt; antisense, gctctttaggtcttcaggaagtc) (35), IL-5 (sense, caccgagctctgttgacaagc; antisense, tctctctgccacactctc) (36), IFN- γ (sense, tgaacgtcacactgcattctgg; antisense, cgactctttccgcttcctgag) (37), CCR6 (sense, actcttctgtctcacctacccg; antisense, atctctgcagctcgattcttg) (38), CCR7 (sense, acagcggcctccagaagaacagcgg; antisense, tgacgtcataggcaatgttgagctg) (39), IL-12p35 (sense, ggctactagagagacttcttc; antisense, gtgaagcaggatgcagagcttc) (40), IL-15 (sense, gtgatgttccccagtggtc; antisense, tcacattcttgcatccaga) (41), and β -actin (sense, gtggccgctctaggccacaa; antisense, ctcttggatgcacgcagatttc) (41) were generated by Oligos Etc. The RT reaction was one cycle of 48°C for 45 min, followed by 94°C for 2 min. The PCR amplification was 40 cycles for the cytokines and β -actin at 94°C for 30 s, 60°C for 1 min, and 68°C for 2 min, followed by one cycle at 68°C for 7 min. The PCR products were separated on a 1% agarose gel and visualized using GelStar nucleic acid gel stain (FMC BioProducts) and UV illumination. The density of the bands on the gel was measured using Gel Doc 2000 (Bio Rad).

Cytokine ELISAs

The concentrations of cytokines in culture supernatants were analyzed by quantitative sandwich ELISA, according to the manufacturer's instructions for IFN- γ (BD Pharmingen) and IL-13 (R&D Systems). The detection levels of the ELISA were 62.5 pg/ml for IL-13 and 290 pg/ml for IFN- γ .

Statistical analyses

ANOVA and post hoc tests were used to evaluate the differences among experimental groups. A value of $p \leq 0.05$ was considered significant. Each

group contained five mice unless the number of mice used is indicated in the graph. Each result shown is a representative of two or three experiments.

Results

Ocular peripheral tolerance induction blocks inflammatory cell infiltration

Ag inoculation into the a.c. of the eye induces peripheral tolerance that suppresses DTH responses and the induction (afferent) phase of the Th2 response in a mouse model of OVA-induced allergic pulmonary inflammation (15, 23). We examined the effect of eye-derived tolerance induction in the efferent phase of the Th2 response on OVA-induced airway inflammation. OVA (50 $\mu\text{g}/2 \mu\text{l}$ of PBS) was injected into the a.c. 5 days after the second i.p. sensitization and 7 days before i.t. challenge with OVA. Three days after i.t. challenge, BAL cells were collected, and differential cell counts were performed to identify the number of various infiltrating inflammatory cells (Fig. 1). Inoculation of OVA into the a.c. of the eye blocked lymphocyte, monocyte, eosinophil, and neutrophil infiltration into the lung in presensitized mice.

As previously shown, tol-APC that mimic eye-derived APC that induce ocular tolerance can be generated in vitro by TGF- β 2 and Ag treatment (14, 42–44). In this study, we examined the effect of i.v. transfer of the tol-APC on cell infiltration in OVA-induced airway inflammation in mice. Injection of OVA-pulsed tol-APC 7 days before i.t. challenge in OVA-presensitized mice blocked lymphocyte, monocyte, eosinophil, and neutrophil infiltration into the lung after OVA pulmonary challenge (Fig. 2*a*). Transfer of OVA-pulsed APC did not alter the ongoing immune response or inflammatory cell infiltration into the lung (Fig. 2*b*). These results show that induction of peripheral tolerance by either a.c. inoculation of Ag or i.v. inoculation of in vitro-generated tol-APC in presensitized mice blocked inflammatory cell infiltration in the lung. When irrelevant Ag keyhole limpet hemocyanin (KLH)-pulsed tol-APC were injected (i.v.) into presensitized mice, they failed to suppress lymphocyte and monocyte infiltration but selectively blocked eosinophil infiltration in the lung after i.t. challenge with OVA (data not shown).

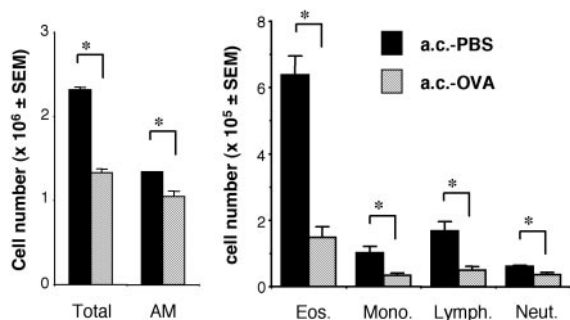


FIGURE 1. Differential cell count of BAL cells from mice with allergic pulmonary inflammation that received a.c. inoculation of OVA. Histograms show the number of total BAL cells and the various types of infiltrating inflammatory cells in the BAL of experimental mice 3 days after i.t. challenge with OVA. OVA or vehicle control PBS were inoculated into the a.c. of the mouse eye 5 days after the second OVA sensitization and 7 days before i.t. challenge with OVA. BAL cells were fixed to the slides by cytopspin. A blinded differential cell count was performed on HEMA3-stained slides. The absolute number of alveolar macrophages (AM), eosinophils (Eos), monocytes (Mono), lymphocytes (Lymph), and neutrophils (Neut) equals the total number of BAL cells per lung $\times$ percentage of that cell type by differential cell count. *, Statistically significant ($p \leq 0.05$) difference between mice that received a.c. inoculation of OVA (a.c.-OVA) and PBS (a.c.-PBS).

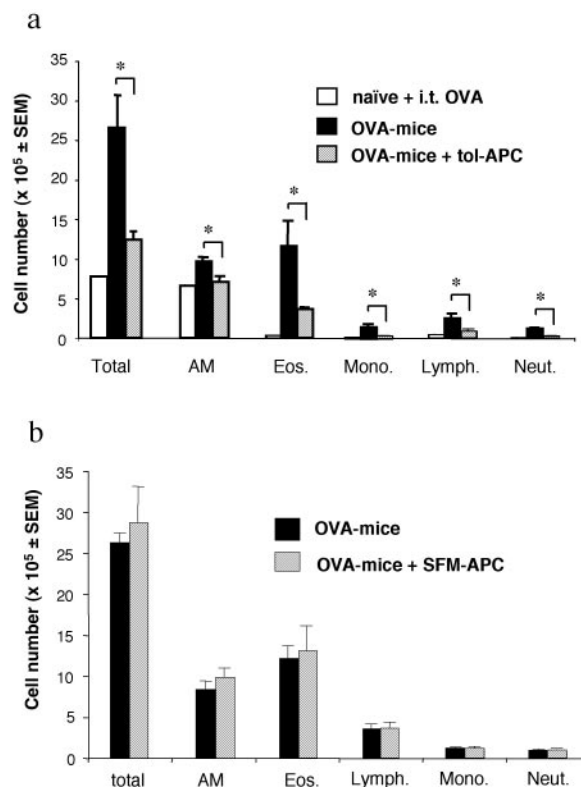


FIGURE 2. Differential cell count of BAL cells from mice with allergic pulmonary inflammation that received (i.v.) tol-APC. Histograms show the absolute number of total BAL cells and the various types of infiltrating inflammatory cells in BAL from experimental mice 3 days after i.t. challenge. *a*, Number of different cells in BAL from mice with allergic pulmonary inflammation that were treated with or without OVA-pulsed tolerogenic APC. *b*, Number of cells in BAL from mice with allergic pulmonary inflammation that were treated with or without OVA-pulsed control APC (APC in SFM without TGF- β 2 treatment). Tol-APC were delivered (i.v.; 5×10^5 /mouse) 5 days after the second sensitization and 7 days before i.t. challenge with OVA. Naive mice that received i.t. OVA inoculation were used as controls for mice with allergic pulmonary inflammation. The differential cell count and the calculation of absolute numbers of each cell type are the same as in Fig. 1. *, Statistically significant ($p \leq 0.05$) difference between the two groups indicated.

Tol-APC treatment reduces AHR and mucus production in the airway

AHR and enhanced airway mucus production result from chronic airway inflammation and are hallmarks of allergic asthma. Thus, mechanisms that suppress chronic immune responses in the lung could result in suppression of AHR and mucus production. The effect of tol-APC in blocking inflammation-mediated AHR and mucus production in the lung was analyzed. AHR was measured 3 days after i.t. challenge in experimental mice that were treated with the tol-APC using the Buxco whole-body plethysmography system. Mice that received the tol-APC showed a 50% reduction of AHR, compared with mice that received no treatment (Fig. 3). These results indicate that adoptively transferred tol-APC that suppress the recruitment of immune cells to the lung also suppress AHR, a pathological consequence of airway inflammation.

Another consequence of chronic immune inflammation in the lung is increased mucus production. To measure mucus production in airways, lungs were harvested from the experimental mice 3 days after i.t. challenge, and lung sections were stained with PAS. Mucus production in the lung was quantitated immunohistologically by counting mucus-positive epithelia. Experimental mice

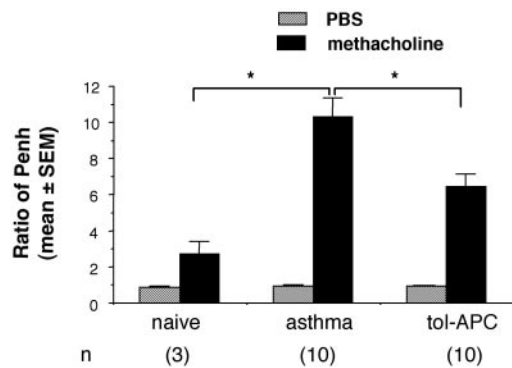


FIGURE 3. Whole-body plethysmography measurement of AHR in mice with allergic pulmonary inflammation that were treated with tol-APC. The graph shows the ratio of Penh (a relative unit that correlates with airway resistance) of mice after either PBS (vehicle) or methacholine aerosol challenge. The ratio of Penh equals Penh after PBS or methacholine challenge/Penh at baseline. The experimental groups consisted of naive mice, mice with allergic pulmonary inflammation induced by OVA sensitization and challenge (OVA-mice), and mice with OVA-induced allergic pulmonary inflammation that received OVA-pulsed tol-APC. AHR was measured 3 days after i.t. challenge (described in *Materials and Methods*). The results are pooled from two separate experiments, and *n* is the number of mice used in each group. *, Statistically significant ($p \leq 0.05$) difference between the two groups indicated.

treated with the tol-APC showed a one-third reduction in the number of mucus-containing epithelia, compared with untreated experimental mice (Fig. 4). These results suggest that the tol-APC modulated Th2-mediated pathogenesis in presensitized mice.

Tol-APC treatment reduces Th2 cytokine production

The cellular mechanisms of suppression initiated by the in vitro-generated tol-APC in the mouse model of OVA-induced allergic

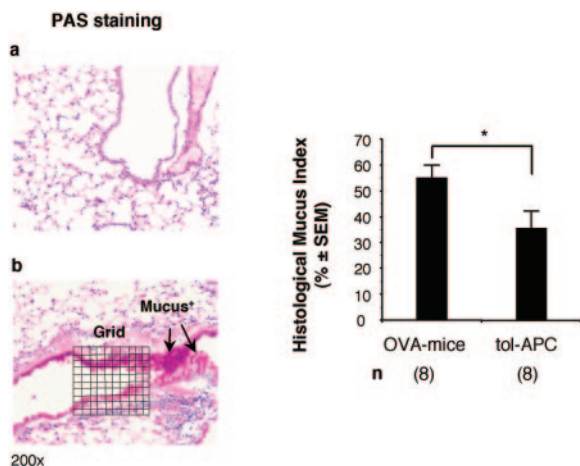


FIGURE 4. Quantitative measurement of airway mucus production in mice with allergic pulmonary inflammation that were treated with tol-APC. *Left*, Examples of PAS staining on lung sections from naive mice (*a*) and mice that were i.p. sensitized and pulmonary challenged with OVA (*b*). Mucus-producing goblet cells (bright purple) along the airway are indicated by arrows. The graph on the *right* shows HMI in mice with allergic pulmonary inflammation that were treated with tol-APC 7 days before i.t. challenge. Lungs were harvested from experimental mice 3 days after i.t. challenge and processed as described in *Materials and Methods*. HMI, Number of mucus-containing epithelium/total epithelium counted $\times 100$. The results are pooled from two separate experiments. A total of eight mice were used in each group. *, Statistically significant ($p \leq 0.05$) difference between the two groups indicated.

pulmonary inflammation were examined. BAL cells and lung-draining lymph node (hilar and mediastinal) cells (dLNCs) were collected from experimental mice 3 days after i.t. challenge. The mRNA levels of Th2 cytokines (IL-4, IL-5, and IL-13) were measured by RT-PCR analyses and compared in the experimental mice with or without tol-APC treatment. The results showed that lung dLNCs (data not shown) and BAL cells from tol-APC-treated disease mice had reduced IL-4, IL-5, and IL-13 mRNA levels, compared with cells in diseased mice without the treatment (Fig. 5). However, there was no difference in IFN- γ mRNA level between these two groups of mice. Because IL-13 is a major Th2 cytokine in allergic pulmonary inflammation and IFN- γ is an indicator for a Th1 response, the production of cytokines IL-13 and IFN- γ from lung dLNCs in an Ag recall assay was measured using cytokine ELISA (Fig. 6). We observed that tol-APC treatment reduced Th2 cytokine (IL-13) production in experimental mice but had no effect on IFN- γ production by lung dLNCs. Control APC (APC in SFM pulsed with OVA) did not change the IL-13 production. Mice that received irrelevant Ag KLH-tol-APC had partially reduced IL-13 production (data not shown). However, the difference in IL-13 production was not statistically different when KLH-tol-APC-treated mice were compared with either OVA-tol-APC-treated or control APC-treated mice. These results, in combination with the effect of KLH-tol-APC on inflammatory cell infiltration in the lung, suggest the possibility of bystander suppression in certain aspects of the Th2 response by tol-APC.

Tol-APC generate CD8⁺ Tr cells to suppress effluent Th2 immune response

Many studies have been published showing that CD8⁺ Tr cells generated after a.c. injection of Ag suppress the effluent phase of a Th1 response. We asked whether effluent Tr cells generated by the tol-APC suppressed the Th2 effector cell response in this allergic pulmonary inflammation model as well. We enriched T cells from spleens of OVA-presensitized mice that were treated with the tol-APC or control APC (APC pulsed with OVA without TGF- $\beta 2$ treatment). The total enriched T cells, CD4-depleted T cells (mAb GK1.5 and complement), and CD8-depleted T cells (mAb 2.43 and complement) were cocultured with sensitized spleen cells in the presence of Ag OVA. The culture supernatant was collected at 48

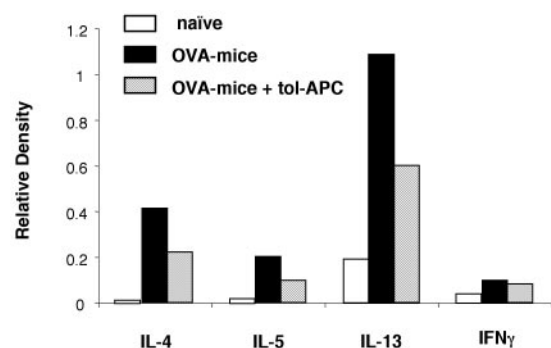


FIGURE 5. RT-PCR and densitometric analysis of IL-4, IL-5, IL-13, and IFN- γ mRNA levels in BAL cells from mice with allergic pulmonary inflammation that were treated with tol-APC. The histogram shows IL-4, IL-5, IL-13, and IFN- γ mRNA levels in BAL cells collected from naive mice (naive), OVA-sensitized and -challenged mice (OVA-mice), or tol-APC-treated OVA-mice (OVA-mice + tol-APC). BAL cells were collected 3 days after i.t. challenge. The PCR product was separated on a 1% agarose gel and visualized using GelStar nucleic acid gel stain and UV illumination. The density of the bands on the gel was measured by Gel Doc 2000 and Quantity I software (Bio Rad), and the relative density to β -actin was calculated.

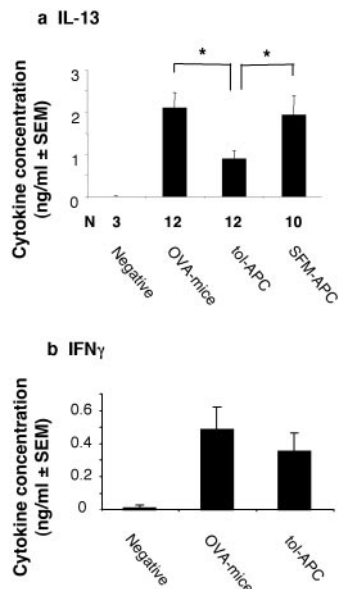


FIGURE 6. ELISA analysis of IL-13 and IFN- γ production by lung dLNCs. Histograms show the concentrations of IL-13 (*a*) and IFN- γ (*b*) in the culture supernatant of lung dLNCs that were restimulated with OVA in vitro. Lung dLNCs (2×10^6 /ml) were harvested from experimental mice 3 days after i.t. challenge and incubated in complete medium with OVA (1 mg/ml) for 48 h. Negative, Mice received i.t. inoculation of OVA only without sensitization; OVA-mice, mice received OVA i.p. sensitization and i.t. challenge; OVA-SFM-APC, OVA-mice received i.v. inoculation of control APC (APC in SFM pulsed with OVA, but without TGF- β 2 treatment); OVA-tol-APC, OVA-mice received i.v. inoculation of tol-APC (OVA pulsed and TGF- β 2 treated in SFM). The IL-13 result is pooled from three experiments. IFN- γ production is from a separate experiment. *, Significant ($p \leq 0.05$) difference between the two groups compared.

or 72 h, and the IL-13 concentration was measured (Fig. 7). The results show that enriched splenic T cells and CD4-depleted T cells, but not CD8-depleted T cells from mice that received i.v. tol-APC, partially suppressed IL-13 (Th2 cytokine) production in an Ag recall assay, suggesting that CD8 $^+$ T cells mediated the suppression of the Th2 response in sensitized mice. Cultures that received total T cells or CD4-depleted T cells from tol-APC-treated mice had a 35% reduction in IL-13 production, compared with cultures that received T cells from control APC-treated mice.

Characteristics of gene expression in tol-APC

The categories of genes that are down-regulated in TGF- β 2-treated APC include critical cosignaling molecules such as CD40 and Th1 cytokine IL-12 (45). Increases are noted in genes that are associated with suppression or regulation of activation (IL-10, TGF- β 1, TGF- β 2, and thrombospondin) (27).

To support the idea that the transferred tol-APC generated Tr cells in this model, we analyzed the unique genes that might be associated with the function of the tol-APC. Gene array analyses were performed at the Bauer Center for Genomics Research (Harvard University, Boston, MA) using an Affymetrix chip (murine MG-U74A) that detects 12,000 mouse genes so that we could compare the genetic phenotype of the tolerogenic APC to that of immune and immature APC. We considered that bone marrow-derived APC cultured in SFM without TGF- β 2 treatment might be similar to the APC described in the literature as immature, and that APC treated with LPS (1 μ g/ml) in SFM overnight might represent mature-activated or immune APC. The array results were analyzed and compared among the three groups of APC using Rosetta Re-

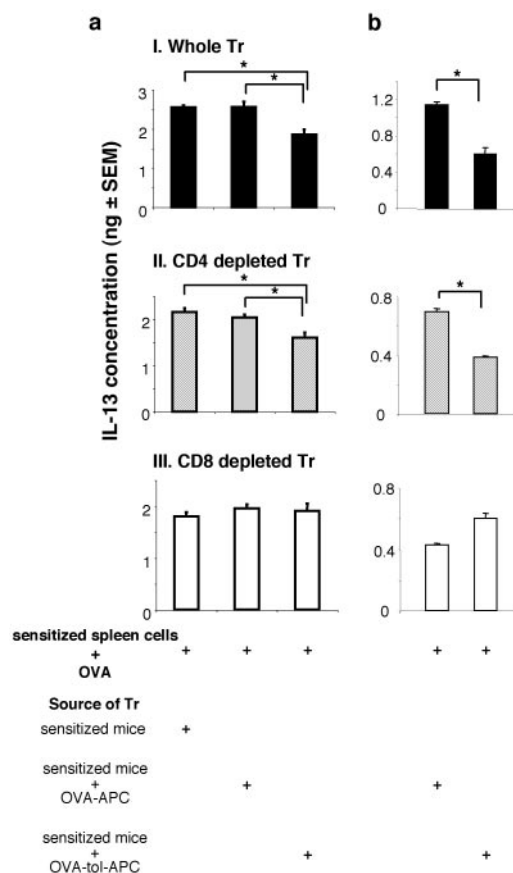


FIGURE 7. ELISA analysis of IL-13 production by sensitized spleen cells cocultured with Tr cells from tol-APC-treated presensitized mice. Results from two separate experiments are shown in *a* and *b*. Sensitized spleen cells (2×10^6 /ml; 4×10^5 /well) were harvested from mice that received OVA i.p. (twice) and were restimulated in vitro with OVA (1 mg/ml) in SFM. Tr cells were enriched splenic T cells that were harvested from presensitized mice treated with OVA-pulsed tol-APC (OVA-tol-APC) or OVA-pulsed APC (OVA-APC) 7 days earlier. CD8 $^+$ or CD4 $^+$ T cells were further depleted in Tr cells by Ab (2.43 and GK1.5, respectively) plus complement treatment. Tr cells (1×10^5 /well; I), CD4-depleted Tr cells (II), and CD8-depleted Tr cells (III; equivalent to 1×10^5 T cells/well) were added to the culture. Supernatant was harvested 72 h after stimulation and analyzed by ELISA. *, The concentration of IL-13 in the OVA-tol-APC-treated group is significantly ($p \leq 0.05$) reduced compared with that in the control group.

solver software. We observed that >2000 genes showed a change (≥ 2 -fold increase or decrease) in expression levels in the tol-APC, compared with genes expressed in APC without TGF- β 2 treatment or LPS-treated APC (data not shown). Among these genes, we chose to investigate two chemokine receptor (CCR6 and CCR7) and two cytokine (IL-12 p35 and IL-15) genes. The profile of CCR6 and CCR7 expression is critical for the migration of APC into secondary lymphoid organs (46, 47). APC in the tissue are usually CCR6 $^{\text{high}}$ CCR7 $^{\text{low}}$. During an inflammatory immune response, they up-regulate CCR7 and down-regulate CCR6 expression after they take up Ags. CCR7 enables APC to migrate into the T cell area of the draining lymphoid tissues, where they present Ags to T cells and initiate an immune response. Our previous report showed that ACAID-inducing F4/80 $^+$ APC localized in the MZ of the spleen to induce tolerance and suppress Th1 responses (25). Similar to mature APC, tol-APC showed a decrease in their expression of CCR6, but, unlike their mature counterpart, the tolerogenic APC did not show a dramatic up-regulation of CCR7

(Table I). The changes in the expression levels of selected chemokine receptor genes (CCR6 and CCR7) seen in the gene array analyses were confirmed by RT-PCR (Fig. 8). This pattern of an inverse relationship of the expression of chemokine receptors CCR6 and CCR7 supports our *in vivo* functional studies, which showed that F4/80⁺ tol-APC leave the eye after a.c. inoculation of Ag and do not migrate to the T cell area (white pulp) of the spleen as inflammatory APC would. Instead, they accumulate in the MZ of the spleen, where they settle in close contact with T cells, MZ B cells, and NKT cells to generate Tr cells (23–25).

Both IL-12 and IL-15 are proinflammatory cytokines (48). IL-12 is critical for the priming of Th1 cells, and IL-15 is critical for elicitation of a DTH response in a sensitized host (48). In addition, IL-15 promotes proliferation and activation of CD8⁺ memory cells (49–51), and IL-15 expression is associated with exacerbation of rheumatoid arthritis, sarcoidosis, and inflammatory bowel disease as well as allograft rejection (52). In this study, our RT-PCR analyses confirm previous reports (27, 45) that the expression of IL-12 is low in tol-APC (Fig. 8). Furthermore, IL-15, a pivotal cytokine for CD8 memory T cells, is expressed less in TGF- β 2-treated APC than in LPS-treated inflammatory APC.

We conclude that the differences in gene expressed in tol-APC, compared with untreated or immune inflammatory APC, were compatible with their being a unique and stable subpopulation of APC, which supports the idea that TGF- β 2-treated, Ag-pulsed APC may orchestrate the generation of efferent Tr cells that suppress both Th1 and Th2 effector T cells.

Discussion

In summary, we showed for the first time that the induction of peripheral tolerance either by a.c. inoculation of Ag or by i.v. transfer of TGF- β 2-treated and Ag-pulsed APC suppressed Th2 responses and subsequent pulmonary pathogenesis in presensitized mice in a model of allergic pulmonary inflammation. The partial suppression of Th2 cytokine IL-13 production of T effector cells *in vitro* was mediated by a CD8⁺ T cell, not a CD4⁺ T cell. The suppression of inflammatory cell infiltration into the lung by the tol-APC was >75%, and Th2 cytokines and pulmonary pathogenesis were consistently reduced. Analyses of genes expressed in TGF- β 2-treated and OVA-pulsed APC showed that they were genetically different from either untreated or LPS-activated inflammatory APC.

The APC that are modulated by TGF- β 2 in eye-derived peripheral tolerance are unique and are phenotypically and functionally different from other subpopulations of APC that are known to induce tolerance, such as immature dendritic cells and B220⁺Gr-1⁺ pulmonary plasmacytoid dendritic cells (53–56). The specific chemokine receptor and cytokine profile (CCR6^{low}CCR7^{low}IL-12^{low}IL-15^{low}IL-10^{high}) in the *in vitro*-generated tol-APC that suppress Th2 responses is compatible with their trafficking to the MZ, where they may set up a unique tolerogenic environment with the cells for Ag presentation (25). In addition, TGF- β 2 is able to mod-

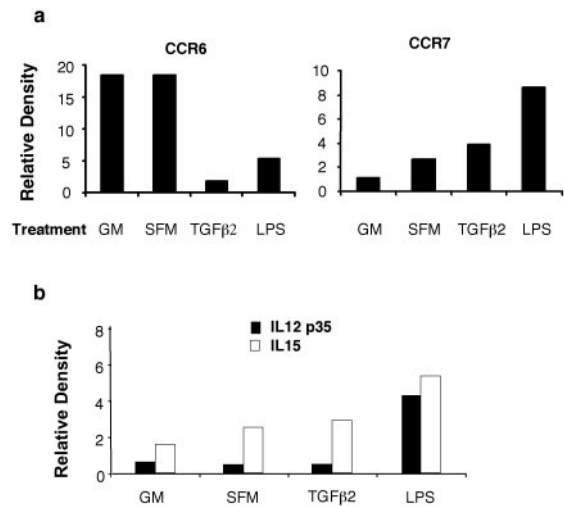


FIGURE 8. RT-PCR and densitometric analysis of mouse chemokine receptor (CCR6 and CCR7) and cytokine (IL-12 p35 and IL-15) mRNA levels in APC. The graph shows the relative levels of CCR6, CCR7 (a) and IL-12p35, IL-15 (b) mRNA in various groups of APC. The treatment was APC pulsed with OVA (5 mg/ml) under different incubation conditions: GM, mouse bone marrow-derived APC grown in growth medium that contains M-CSF; SFM, APC incubated in SFM overnight; TGF- β 2, APC treated with TGF- β 2 (5 ng/ml) in SFM overnight; and LPS, APC treated with LPS (1 μ g/ml) in SFM overnight. The PCR products were separated on a 1% agarose gel and visualized using GelStar nucleic acid gel stain and UV illumination. The densities of the bands on the gel were measured using a Gel Doc 2000 (Bio Rad) and Quantity I software, and relative density to β -actin was calculated.

ulate the function of APC and induce their production of TGF- β 1 and IL-10.

It is possible that the ability of the *in vitro*-generated tol-APC to induce Tr cells in allergic pulmonary inflammation is a direct consequence of TGF- β 1 or -2 modifying the expression of various cytokines and transcription factors that are critical for the activity of T cells, NKT cells, and MZ B cells. TGF- β 2 reduces IL-12 and IL-15 and increases IL-10, TGF- β 1, and thrombospondin production in APC. TGF- β 1 reduces Th1 transcription factor T-bet and IL-12R expression in Th1 cells (57) and blocks the expression of GATA3 in Th2 cells (58, 59). Furthermore, TGF- β suppresses transcription factor NFAT signaling pathway (60). TGF- β has autocrine effects, thus amplifying the tolerance signal initiated by TGF- β treatment and production in eye-derived peripheral tolerance.

It is interesting that, similar to the eye, the resting lung contains a high concentration of TGF- β . Thus, the possibility is raised that the ability of pulmonary dendritic cells to generate Tr cells is related to their exposure to suppressive molecules in airway-lining fluid in a fashion similar to the aqueous humor on APC in the eye.

It has been reported that Th2 responses are suppressed by Th1 deviation. Injection of a CpG motif containing oligonucleotides or vaccination with allergen-IL-18 fusion DNA induces IFN- γ -producing T cells that counteract Th2 inflammation in a mouse asthma model (61, 62). Our model, however, shows no enhancement of Th1 cytokine (IFN- γ) levels and is not a mere deviation from a Th2 to a Th1 response.

Several reports show that other Tr cells suppress Th2 response in allergic pulmonary inflammation. Intratracheal inoculation of a high dose of OVA induces the generation of TGF- β -producing CD4⁺ T cells in mediastinal lymph nodes that suppress Th2-mediated eosinophilic inflammation (63). Akbari et al. (64) show that

Table I. Gene array analysis of tol-APC^a

	tol-APC vs SFM-APC	tol-APC vs LPS-APC
	(Fold change)	
CCR6	-6.58	1.59
CCR7	2.99	-4.86

^a The expression of CCR6 and CCR7 mRNA levels in the tol-APC was determined by gene array analyses using Affymetrix chip and compared with expression levels of these genes in APC that were in SFM (SFM-APC) or treated with LPS in SFM (LPS-APC).

intranasal inoculation of Ag results in the production of CD4⁺ Tr cells by pulmonary dendritic cells that interfere with the priming of a Th2 immune response in the lung. The cellular mechanism of Th2 suppression by eye-derived peripheral tolerance is unique and distinct from either a Th1 deviation or a CD4⁺ Tr cell generated by airway exposure of Ags and is mediated by a regulatory CD8⁺ T cell.

The molecular mechanism used by the CD8⁺ Tr in this model to suppress is unknown. It is known that T cells mediate suppression by multiple mechanisms, such as killing, cytokine modulation, and direct contact through cosignaling molecules. Zinkernagel and colleagues (65) showed that CD8⁺ T cells are capable of killing virus-infected APC, leading to immune suppression. T cell-derived immune regulatory cytokines, such as IL-10 and TGF- β , suppress both Th1 and Th2 responses either by direct modulation of T effector cells or by suppression of APC function. Recent publications showed that the TCR and cosignaling pathways are directly linked to Tr suppression. The B7/CTLA-4 pathway is not only important for the generation of Tr cells, but is also critical for the suppressive effect of Tr cells generated by eye-derived APC (66). These Tr cells seem to obtain the ability to express B7 and CTLA-4 molecules on their surface after they interact with tolerance-inducing APC and interfere with T effector function. Hu et al. (67) showed that the TCR on CD8⁺ Tr cells interacts with Qa-1 (MHC class Ib molecule) on CD4⁺ T effector cells to mediate Ag-specific efferent suppression. Interestingly in the eye-derived peripheral tolerance model ACAID, Niederkorn and colleagues (68) showed that the Qa-1 molecule is required during tolerance induction.

This is not the first report to show that the in vitro-generated APC could suppress an established immune response. Transferred Ag-specific tol-APC suppressed Th1 responses in an autoimmune-mediated pulmonary fibrosis model and an experimental autoimmune encephalomyelitis model (29, 69). However, this is the first report that in vitro modulation of APC by TGF- β 2 produces a unique tolerance-inducing APC that can be used therapeutically to modify ongoing Th2 responses in the mouse model of allergic pulmonary inflammation. The possibility is raised that, in the future, we may be able to modulate/re-educate human APC from an asthma patient in vitro, give them back to modify the response to the inciting Ag, and reduce pulmonary pathology.

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Disclosures

J. Stein-Streilein holds a patent with the name "Tolerogenic antigen presenting cells in treating immune inflammatory conditions."

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