

IL-5-Induced Hypereosinophilia Suppresses the Antigen-Induced Immune Response via a TGF- β -Dependent Mechanism¹

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Although eosinophils play an essential role in allergic inflammation, their role has recently been under controversy. Epidemic studies suggest that hypereosinophilia induced by parasite infection could suppress subsequent Ag sensitization, although the mechanism has not been fully clarified. In this study, we investigated whether eosinophils could suppress the Ag-specific immune response in the airway. BALB/c mice were sensitized and airway challenged with OVA. Systemic hypereosinophilia was induced by delivery of an IL-5-producing plasmid. IL-5 gene delivery suppressed the Ag-specific proliferation and cytokine production of CD4⁺ T cells in the spleen. IL-5 gene delivery before OVA sensitization significantly suppressed airway eosinophilia and hyperresponsiveness provoked by subsequent OVA airway challenge, while delivery during the OVA challenge did not suppress them. This IL-5-induced immune suppression was abolished in eosinophil-ablated mice, suggesting an essential role of eosinophils. IL-5 treatment increased the production of TGF- β 1 in the spleen, and we demonstrated that the main cellular source of TGF- β 1 production was eosinophils, using eosinophil-ablated mice and depletion study. TGF- β 1, but not IL-5 itself, suppressed the Ag-specific immune response of CD4⁺ T cells in vitro. Furthermore, IL-5 treatment enhanced phosphorylation of Smad2 in CD4⁺ T cells. Finally, a TGF- β type I receptor kinase inhibitor restored this IL-5-induced immune suppression both in vitro and in vivo. These results suggest that IL-5-induced hypereosinophilia could suppress sensitization to Ag via a TGF- β -dependent mechanism, thus suppressed allergic airway inflammation. Therefore, hypereosinophilia could reveal an immunosuppressive effect in the early stage of Ag-induced immune response. *The Journal of Immunology*, 2007, 179: 284–294.

Over the past several decades, the prevalence of allergic diseases such as bronchial asthma has increased in industrialized countries (1). Bronchial asthma is a chronic disorder characterized by eosinophilic airway inflammation, mucus hypersecretion, partly reversible airway obstruction, heightened airway hyperresponsiveness (AHR),³ and airway remodeling (2). Th2 cell-type immune responses play a critical role in the development of bronchial asthma.

Eosinophils are thought to be one of the principal inflammatory cells in the pathophysiology of asthma. They release various lipid mediators, cytokines, and growth factors involved in the patho-

genesis of asthma (3, 4). In eosinophil-deficient PHIL mice, both mucus hypersecretion and AHR were abolished (5), although these findings were not confirmed in another eosinophil-ablated strain, Δ dbl GATA mice (6). In addition, several clinical studies found a close correlation between the number of blood or airway eosinophils and the intensity of AHR or airway remodeling in asthmatic patients (7–11). These findings strongly indicate that eosinophils play a central role in the effector phase of allergic inflammation.

In contrast, the role of eosinophils in allergic inflammation has recently come under great scrutiny. For example, administration of humanized IL-5-neutralizing mAb does not decrease AHR despite this treatment's depletion of blood and sputum eosinophils (12), although another study demonstrated that administration of this neutralizing anti-IL-5 mAb does not completely exclude eosinophils from the lung (13). These results suggest that eosinophils may not be the only factor in the induction and maintenance of AHR. In addition, epidemic studies demonstrate that children infected with the helminth parasite present a diminished skin test reactivity to other Ags as well as a decreased risk of wheezing (14–18), although these findings remain controversial (19). In animal studies, a passive helminth infection before systemic OVA sensitization suppresses subsequent airway eosinophilia induced by OVA inhalation (20, 21). As parasite infections are generally accompanied by hypereosinophilia, these findings beg the question of whether eosinophils play a protective role in the Ag-induced immune response under certain circumstances. In a study by Kobayashi et al. (22), in IL-5 transgenic mice, AHR induced by Ag sensitization was reduced despite marked eosinophil infiltration in the airway. This finding supports our speculation that eosinophils might play a protective role. However, a precise mechanism was

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³ Abbreviations used in this paper: AHR, airway hyperresponsiveness; alum, aluminum hydroxide; SA, physiologic saline; AR, airway responsiveness; Mch, methacholine; Penh, enhanced pause; Raw, airway resistance; BALF, bronchoalveolar lavage fluid; PAS, periodic acid-Schiff; cysLT, cysteinyl leukotriene; EPO, eosinophil peroxidase; CTAB, cetyltrimethylammonium bromide; T β R-I inhibitor, TGF- β type I receptor kinase inhibitor; Treg, regulatory T.

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neither proposed nor investigated in their report, leaving the association between eosinophils and immune suppression unresolved to this day.

The purpose of the present study was to investigate whether eosinophils could suppress Ag-specific immune response in the airway and to clarify the mechanism of this suppression. We delivered *IL-5* gene into mice to induce systemic eosinophilia, and then examined its effect on allergic airway inflammation. We found that hypereosinophilia induced during Ag sensitization suppressed subsequent airway eosinophilia and AHR provoked by Ag inhalation, whereas that induced during the effector phase did not. Furthermore, we found that TGF- β 1 produced by eosinophils in the spleen played a critical role.

Materials and Methods

Mice

Male BALB/c mice were obtained from Charles River Japan. OVA TCR-transgenic DO11.10 mice and Δ dbl GATA mice (6, 23) were obtained from The Jackson Laboratory. All animal experiments were approved by the Animal Research Ethics Board of the Department of Allergy and Rheumatology, University of Tokyo.

Delivery of *IL-5* into mice

In this study, we delivered *IL-5* in vivo using a hydrodynamic-based method through the i.v. injection of plasmid DNA from previously reported methods (24–26). The plasmid pCAGGS-*IL-5* (27) was amplified in *Escherichia coli*, and purified with a Qiagen Endo Free plasmid Giga kit (Qiagen). The empty plasmid pCAGGS was used as a control. Plasmid DNA in lactated Ringer's solution (0.1 ml/g body weight) was injected i.v. from the tail, and injection was completed within 5 s. Some mice received plasmid DNA (100 μ g; pCAGGS-*IL-5* or control pCAGGS) i.v. on day –3 (before systemic immunization: pre) or on day 17 (during aerosol challenge; after systemic immunization: post).

Immunization of mice and evaluation of allergic airway inflammation

Mice were immunized as reported previously (24, 28, 29). Seven-week-old animals were sensitized with an i.p. injection of 2 μ g of OVA (Sigma-Aldrich) plus 2 mg of aluminum hydroxide (alum) on days 0 and 11. Control mice received injections of physiologic saline (SA) on days 0 and 11. Mice were challenged with an aerosolized solution of 3% OVA or PBS for 10 min from day 18 to day 20. On day 21, airway responsiveness (AR) to methacholine (Mch) was measured with the enhanced pause (Penh) system or assessed by measuring airway resistance (Raw) as described previously (24, 29). Mch concentration that induced a 100% increase in Penh or Raw was expressed as PC₂₀₀Mch (μ g/ml) or PC₂₀₀Mch Raw (μ g/ml) as an indicator of AHR. Samples of serum and bronchoalveolar lavage fluid (BALF) were then obtained. The lungs were cut out and fixed with 10% neutralized buffered formalin (Wako). Three-micrometer-thick sections were prepared and subjected to H&E or periodic acid-Schiff (PAS) staining to evaluate mucus hypersecretion.

Measurement of cytokines and Ig

IL-4, *IL-5*, *IL-10*, *IL-13*, IFN- γ , TGF- β 1, cysteinyl leukotrienes (cysLTs), IgE, and IgG concentrations were measured using ELISA kits (*IL-4*, *IL-5*, *IL-10*, IFN- γ , and IgE from BD Pharmingen; *IL-13* and TGF- β 1 from R&D Systems; cysLTs from Cayman Chemical; and IgG from Bethyl Laboratories). OVA-specific IgE or IgG was measured using an ELISA kit for IgE or IgG, although the plate was coated with OVA (1000 μ g/ml) at 4°C overnight instead of anti-mouse IgE or IgG Ab. The OVA-specific IgE or IgG standard was derived by pooling sera from five OVA-sensitized mice. Results are expressed as a percentage of the value of the standard.

Preparation of spleen cells

Spleen cells were prepared as reported previously (24). The number of total splenic cells and eosinophils was counted 1, 3, 5, 8, 12, 18, and 24 days after plasmid injection. To clearly distinguish eosinophils from the neutrophils, three different stains were applied: Diff-Quick stain, May-Grünwald-Giemsa stain, and Eosino (Hansel) stain (30). On the basis of the findings with these stainings, cell differentials were counted with at least 300 leukocytes in each sample. The cell types were judged according to standard hemocytologic procedures. We also confirmed the reliability of

our manual eosinophil counts by flow cytometric analysis with anti-Siglec F mAb (31) (BD Pharmingen). For the preparation of splenic CD4⁺ T cells, spleen cells were incubated with anti-CD4 mAb-coated microbeads (Miltenyi Biotec). The bead-bound cells were then isolated using magnetic separation columns. The purities of the enriched CD4⁺ cell populations were 95% (data not shown). Splenic eosinophils or eosinophil-depleted cells were prepared using previously reported methods with a slight modification (32–34). Briefly, spleen cells were incubated with anti-Thy1 mAb-coated microbeads (Miltenyi Biotec), anti-B220 mAb-coated microbeads (Miltenyi Biotec), and biotinylated anti-MHC class II mAb (anti I-A/I-E mAb; 2G9; BD Pharmingen), all of which were then incubated with streptavidin-microbeads (Miltenyi Biotec). The bead-bound cells were isolated for eosinophil-depleted cells using magnetic separation columns. To obtain purified eosinophils, the bead-bound cells were depleted, and the depleted cells were incubated with PE anti-CCR3 mAb (R&D Systems) and were then incubated with PE-microbeads (Miltenyi Biotec). The bead-bound cells were isolated for eosinophils using magnetic separation columns. The purities of each subset were found to be greater than 94% according to the morphologic criteria.

In vitro proliferation and cytokine assays

Spleen cells were cultured (5×10^5 cells/well) with or without OVA (20 μ g/ml) in complete DMEM. In some experiments, positively selected CD4⁺ T cells (2.5×10^5 cells/well) were cultured with freshly isolated mitomycin C (Sigma)-treated splenocytes (2.5×10^5 cells/well) and OVA (20 μ g/ml). After 72 or 96 h, the proliferation was assessed with a cell proliferation ELISA BrdU kit (Roche Applied Science). After 120 h, cytokine concentrations in the supernatants were measured using ELISA kits. For measurement of TGF- β 1 concentrations, we used serum-free medium X-vivo 15 (Cambrex BioScience) instead of complete DMEM. We measured total TGF- β 1 concentrations after complete activation by acidification. In some experiments, positively selected CD4⁺ T cells (1.25×10^5 cells/well) were incubated in Ag-nonspecific manner with plate-bound anti-CD3 Ab (10 μ g/ml; BD Pharmingen) for 48 h or PMA (1 ng/ml; Sigma-Aldrich) and ionomycin (1 μ g/ml; Sigma-Aldrich) for 24 h, after which the proliferation was assessed. To examine the direct effect of TGF- β 1 or *IL-5* on CD4⁺ T cell functions in vitro, OVA-sensitized CD4⁺ T cells (1.25×10^5 cells/well) were incubated with plate-bound anti-CD3 Ab (10 μ g/ml) for 48 h or PMA (1 ng/ml) and ionomycin (1 μ g/ml) for 24 h with TGF- β 1 (2 or 10 ng/ml; R&D Systems) or *IL-5* (5 or 20 ng/ml; R&D Systems), after which the proliferation was assessed. To examine the effect of coinubation with TGF- β 1 or *IL-5* on Ag-specific immune response of CD4⁺ T cells, OVA-sensitized CD4⁺ T cells (2.5×10^5 cells/well) were incubated with freshly isolated mitomycin C-treated splenocytes (2.5×10^5 cells/well) and OVA (20 μ g/ml) for 72 h with TGF- β 1 (2 or 10 ng/ml) or *IL-5* (5 or 20 ng/ml). The proliferation was then assessed and the *IL-4* concentration measured.

Effect of anti-*IL-5R* mAb on Ag-specific immune response of CD4⁺ T cells

Mice were sensitized with OVA or SA on day 0 and received plasmid (pCAGGS-*IL-5* or control pCAGGS) on day –3. Some pCAGGS-*IL-5*-injected mice received anti-*IL-5R* mAb (35) (H7; 1 mg) or control IgG i.p. on days –2, –1, and 1. On day 11, splenic CD4⁺ T cells were obtained and the proliferation was examined as described previously.

Immunohistochemistry

Spleens were directly frozen in dry-iced acetone. Frozen samples were further sectioned at 10- μ m thickness in a cryostat, fixed with 10% formalin, and then incubated with primary and secondary Abs. Anti-phospho Smad2 Ab (a gift from A. Moustakas and C.-H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden)) and anti-CD4 Ab (2 μ g/ml; Santa Cruz Biotechnology) were used as primary Abs. Alexa 488-conjugated anti-rabbit IgG Ab (Molecular Probes) and Alexa 594-conjugated anti-rat IgG Ab (Molecular Probes) were used as secondary Abs. Samples were observed using a Zeiss LSM510 Meta confocal microscope.

Eosinophil peroxidase (EPO) activity

EPO activity was measured in the cell-free supernatants using previously reported methods (36). Briefly, cell-free supernatants (75 μ l) were transferred to a 96-well microplate, and the reaction was initiated by the addition of substrate solution (75 μ l). The substrate solution consisted of 12 mM *o*-phenylenediamine (Sigma-Aldrich), 0.005% H₂O₂ (Wako), 10 mM HEPES, and 0.22% cetyltrimethylammonium bromide (CTAB; Sigma-Aldrich). The reaction was stopped with 50 μ l of 4 N sulfuric acid, and absorbance was measured at 490 nm. As a positive control, eosinophil

extract was obtained from lysed eosinophils using previously reported methods (37). Briefly, eosinophils were suspended in 10 mM HEPES buffer containing 0.22% CTAB. After vortexing for 1 min, the suspensions were freeze-thawed once, spun at $10,000 \times g$ for 10 min, and the supernatant was used as eosinophil extract.

CFSE proliferation assay

CD4⁺ T cells obtained from DO11.10 mice were labeled with the mitosis-sensitive dye CFSE (2.5 μ M; Molecular Probes). CFSE-labeled CD4⁺ T cells (1×10^6 cells/well) were incubated with mitomycin C-treated splenocytes from naive mice (1×10^6 cells/well) and OVA (20 μ g/ml) in the presence or absence of eosinophils (1×10^6 cells/well). After 48 or 96 h, the cells were harvested from the well and stained with CyChrome anti-CD4 mAb (BD Pharmingen). Proliferation of CD4⁺ T cells was assessed for dilution of the CFSE label by flow cytometry.

Effect of TGF- β type I receptor kinase inhibitor (T β R-I inhibitor) on Ag-specific immune response of CD4⁺ T cells and Ag-induced eosinophilic airway inflammation

Mice were sensitized with OVA or SA on day 0 and received plasmid (pCAGGS-IL-5 or control pCAGGS) on day -3. Some plasmid-injected mice (pCAGGS-IL-5 or control pCAGGS) received T β R-I inhibitor ([3-(Pyridin-2-yl)-4-(4-quinonyl)]-1H-pyrazole; 20 μ g; Calbiochem (catalog no. 616451)) or DMSO i.p. on days -1, 1, 3, 6, 8, and 10. On day 11, splenic CD4⁺ T cells were obtained, and the proliferation was examined as described previously. To examine the effect of T β R-I inhibitor on eosinophilic airway inflammation, mice were sensitized with OVA or SA on days 0 and 11 and received plasmid on day -3. Some plasmid-injected mice received T β R-I inhibitor or DMSO i.p. on days -1, 1, 3, 6, 8, 10, 13, 15, and 17. The mice were then challenged with OVA or PBS from day 18 to day 20. On day 21, the mice were analyzed.

Statistics

Values are expressed as means $\pm$ SEM. Statistical analysis was performed by one-way ANOVA followed, when differences were significant, by appropriate post hoc tests using Turkey-Kramer test. For analysis of the differences between two groups, we used Student's *t* test. Values of *p* < 0.05 were considered statistically significant.

Results

IL-5 expression in serum and in BALF after hydrodynamic-based gene delivery of plasmid DNA by i.v. injection

To induce systemic hyper eosinophilia, we delivered the IL-5 gene by i.v. injecting the plasmid DNA (24–26). First, we examined the kinetics of IL-5. Samples were collected at predetermined time intervals following the injection of IL-5-expressing plasmid (pCAGGS-IL-5) or control plasmid (control pCAGGS). The temporal expression of the IL-5 protein in serum (Fig. 1A) and in BALF (Fig. 1B) was confirmed. The level of IL-5 peaked 1 day after the injection, and gradually decreased thereafter.

In vivo IL-5 gene delivery increases the number of splenic eosinophils

Next, we examined the kinetics of the number of total cells as well as eosinophils in the spleen after plasmid injection. Injection of pCAGGS-IL-5 (p-IL-5) induced an increase in the number of total splenic cells and eosinophils, whereas injection of control pCAGGS (p-Cont) did not (Fig. 1, C and D). On day 3, the number of splenic eosinophils had already increased significantly in the pCAGGS-IL-5-injected mice (p-IL-5 mice) (Fig. 1D). On day 11, number of total splenic cells from p-IL-5 mice was 6-fold greater than that of control pCAGGS-injected mice (p-Cont mice) or naive mice. Over 60% of splenocytes in the p-IL-5 mice were eosinophils at this point. We also confirmed the reliability of our manual eosinophil counts by flow cytometric analysis with anti-Siglec F mAb (31) (data not shown).

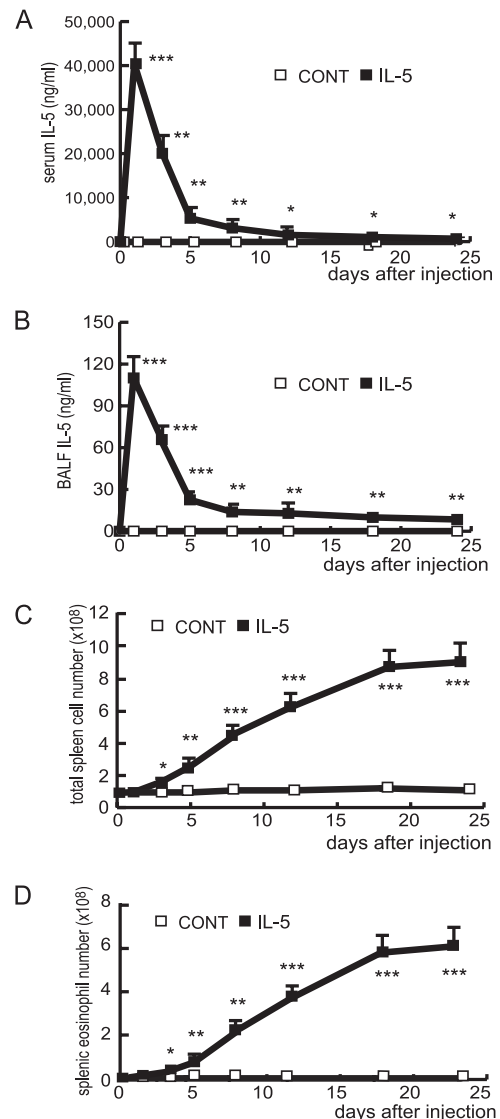
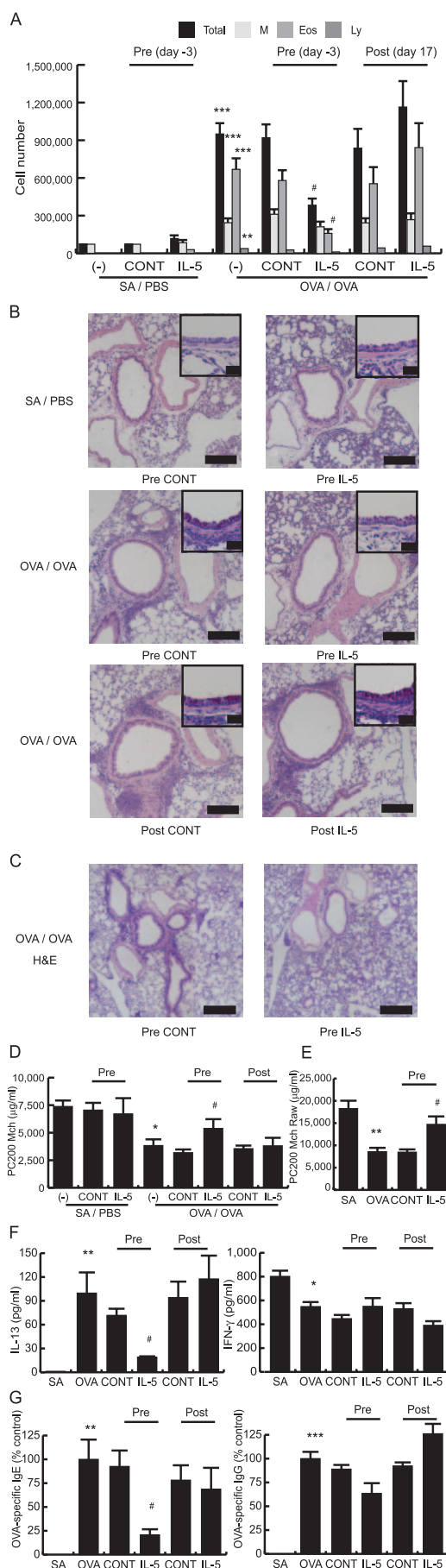


FIGURE 1. Expression of IL-5 and number of splenic eosinophils after a hydrodynamic-based gene delivery of plasmid DNA via i.v. injection. Mice received an i.v. injection of pCAGGS-IL-5 (IL-5) or control pCAGGS (CONT) on day 0. A and B, Concentrations of IL-5 in serum (A) and in BALF (B) were measured at the indicated times postinjection using ELISA. Values are presented as means $\pm$ SEM for six mice per group. C, Total splenic cell count after plasmid injection. D, Number of splenic eosinophils after plasmid injection. *, *p* < 0.05, **, *p* < 0.01, and ***, *p* < 0.001 compared with the value of CONT.

In vivo IL-5 gene delivery before systemic Ag sensitization suppresses Ag-induced eosinophilic airway inflammation, mucus hypersecretion, and AHR

We next elucidated the effect of IL-5 gene delivery on Th2-mediated allergic inflammation using an experimental model of allergic airway inflammation (Fig. 2). Mice were sensitized with either OVA or SA, and then challenged with nebulized OVA or PBS. Injection of plasmid (p-IL-5 or p-Cont) was performed before the systemic Ag sensitization (Pre, on day -3) or during the aerosol challenge (after systemic Ag sensitization; Post, on day 17). In vivo IL-5 delivery alone (with SA i.p. injection) induced mild infiltration of eosinophils into BALF (Fig. 2A). OVA-sensitization and nebulization markedly increased eosinophils in BALF (Fig. 2A). IL-5 gene delivery before sensitization significantly diminished the infiltration of eosinophils (Fig. 2A). Histologically, in



vivo IL-5 delivery alone induced moderate infiltration of eosinophils in the alveolar walls, whereas infiltration of eosinophils in peribronchial area was mild (Fig. 2B). These findings were consistent with those observed in the IL-5 transgenic mice (22). The histology of OVA-sensitized and OVA-challenged mice revealed a prominent infiltration of eosinophils into the peribronchial interstitial area and mucus hypersecretion by bronchial epithelial cells (Fig. 2B). In the mice that received p-IL-5 injection before sensitization (pre-p-IL-5 mice), infiltration of inflammatory cells in peribronchial area and mucus hypersecretion both decreased (Fig. 2, B and C). Although there was a moderate infiltration of eosinophils in the alveolar walls in the pre-p-IL-5 mice, the intensity was similar to the baseline level observed in the mice that received in vivo IL-5 delivery alone (Fig. 2, B and C). In contrast, IL-5 gene delivery during the aerosol challenge deteriorated the infiltration of eosinophils and mucus hypersecretion compared with mice that had received the p-Cont during the aerosol challenge (Fig. 2B). AHR to Mch decreased in the pre-p-IL-5 mice, as was measured by the Penh system (Fig. 2D) and by Raw (Fig. 2E). BALF IL-13 concentration also decreased in the pre-p-IL-5 mice, whereas IFN- γ did not (Fig. 2F), suggesting that IL-5 gene delivery had suppressed the Th2-mediated immune response in the airway. IL-5 delivery before sensitization suppressed OVA-specific IgE and total IgE production (Fig. 2G and data not shown). It slightly suppressed OVA-specific IgG and total IgG production, although there was not significant (Fig. 2G and data not shown). These results indicate that in vivo IL-5 gene delivery during the initial stage of sensitization, but not during the effector phase, suppressed allergic airway inflammation.

In vivo IL-5 gene delivery suppresses the Ag-induced immune response of CD4⁺ T cells ex vivo

Next, we examined the effect of in vivo IL-5 gene delivery on the Ag-induced immune response by conducting ex vivo analyses. Mice received either p-IL-5 or p-Cont before OVA sensitization. On day 11, whole spleen cells or CD4⁺ cells were subjected to analyses. In the p-IL-5 mice, the splenic cells showed a diminished proliferation (Fig. 3A) and cytokine production (Fig. 3B) in response to OVA compared with mice that had received OVA-sensitization without plasmid injection (OVA mice), or to p-Cont

FIGURE 2. In vivo IL-5 gene delivery before systemic sensitization (pre) suppresses eosinophilic airway inflammation, mucus hypersecretion, and AHR. Mice were sensitized with OVA or SA on days 0 and 11. Some mice received an i.v. injection of pCAGGS-IL-5 (IL-5) or control pCAGGS (CONT) on day -3 (pre) or on day 17 (post). The mice were nebulized with OVA or PBS from day 18 to day 20. On day 21, the mice were analyzed. *A*, In vivo IL-5 delivery before sensitization suppresses eosinophil count in BALF. BALF analyses were performed ($n = 12$). Leukocytes were identified by morphologic criteria. *B*, Histological findings. Lungs were excised and subjected to H&E staining. Scale bar, 200 μ m. *Insets*, PAS staining. Scale bar, 40 μ m. *C*, Histological findings (low power field, H&E staining). Lung sections from mice that received control pCAGGS before sensitization and from mice that received pCAGGS-IL-5 before sensitization were shown. Scale bar, 400 μ m. *D*, In vivo IL-5 gene delivery before sensitization suppresses AHR. Airway responsiveness (AR) to Mch was measured with a Penh system ($n = 12$). *E*, AR was assessed by measuring Raw ($n = 12$). *F*, BALF cytokine concentrations. Supernatant of BALF was assayed for IL-13 and IFN- γ concentrations by ELISA ($n = 12$). *G*, OVA-specific IgE and IgG concentrations. Blood samples were obtained from the mice. OVA-specific IgE and IgG concentrations were measured by ELISA ($n = 12$). Pooled sera from five OVA-sensitized mice were set as a control (OVA; 100%). *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$ compared with the value of SA/PBS or SA. #, $p < 0.05$ compared with the value of CONT.

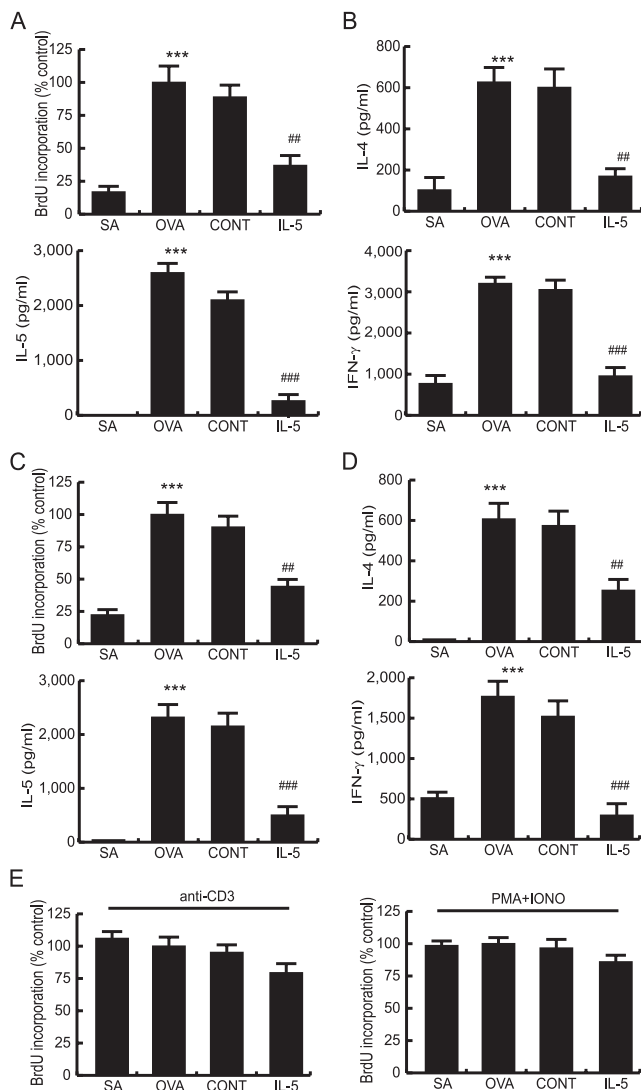


FIGURE 3. In vivo IL-5 gene delivery suppresses the Ag-induced immune response of CD4⁺ T cells. Mice were sensitized with OVA or SA on day 0. Some mice received plasmid (pCAGGS-IL-5 or control pCAGGS) on day -3. On day 11, the mice were analyzed. **A** and **B**, Proliferation and cytokine production of whole spleen cells in response to OVA. Whole spleen cells (5×10^5 cells/well) were incubated with OVA (20 μ g/ml). **A**, After 72 h, the proliferation was assessed based on BrdU incorporation ($n = 6$). The maximum proliferation observed in response to OVA for spleen cells from OVA-sensitized mice was set as a control (OVA; 100%). **B**, After 120 h, cytokine levels of the supernatants were measured ($n = 6$). **C** and **D**, Proliferation and cytokine production of CD4⁺ T cells in response to OVA. Splenic CD4⁺ T cells (2.5×10^5 cells/well) were positively selected by magnetic cell sorting and cultured with freshly isolated mitomycin C-treated splenocytes (2.5×10^5 cells/well) and OVA (20 μ g/ml). **C**, After 96 h, the proliferation was assessed ($n = 6$). The maximum proliferation observed in response to OVA for splenic CD4⁺ T cells from OVA-sensitized mice was set as a control (OVA; 100%). **D**, After 120 h, cytokine levels of the supernatants were measured ($n = 6$). **E**, Proliferation of CD4⁺ T cells in response to plate-bound anti-CD3 Ab or PMA/ionomycin. Splenic CD4⁺ T cells (1.25×10^5 cells/well) were positively selected and incubated with plate-bound anti-CD3 Ab (10 μ g/ml) for 48 h or PMA (1 ng/ml) and ionomycin (1 μ g/ml) for 24 h. The proliferation was assessed ($n = 6$). The maximum proliferation observed in response to plate-bound anti-CD3 Ab or PMA/ionomycin for splenic CD4⁺ T cells from OVA-sensitized mice was set as a control (OVA; 100%). ***, $p < 0.001$ compared with the value of SA. ##, $p < 0.01$ and ###, $p < 0.001$ compared with the value of CONT.

mice. As the physiological ratio of CD4⁺ T cells in the spleen had differed between p-IL-5-mice and p-Cont mice, we then examined the effect of IL-5 on purified CD4⁺ T cells, and found that similar results were obtained (Fig. 3, *C* and *D*). These results indicated that in vivo IL-5 gene delivery before sensitization suppressed the Ag-induced overall immune response of CD4⁺ T cells. Next, to examine whether the suppression would be Ag specific or not, purified CD4⁺ T cells were stimulated with anti-CD3 Ab or PMA/ionomycin. In the p-IL-5-mice, proliferation and cytokine production of CD4⁺ T cells were slightly suppressed, however, they were not significant (Fig. 3*E*). Therefore, IL-5 gene delivery suppressed immune response mainly by Ag-specific mechanism, although Ag-nonspecific mechanism for suppression might have existed.

Eosinophils and IL-5 protein play a critical role in the suppression of the immune response of CD4⁺ T cells induced by in vivo IL-5 gene delivery

Next, we examined whether eosinophils would play a critical role in the IL-5-induced immune suppression. To examine an essential role of eosinophils, we used the eosinophil-ablated mice (Δ dbl GATA mice) for analyses. IL-5 gene delivery did not increase the number of eosinophils in the spleen of the Δ dbl GATA mice (data not shown). In the Δ dbl GATA mice, suppression of Ag-induced immune response of CD4⁺ T cells by IL-5 gene delivery, observed in wild-type mice (Fig. 3, *C* and *D*), was completely abrogated (Fig. 4*A*). These results suggested that eosinophils played an essential role in the IL-5-induced immune suppression. We then confirmed that the effect of in vivo IL-5 gene delivery was indeed mediated by the IL-5 protein. In vivo IL-5 gene delivery suppressed the Ag-specific proliferation of CD4⁺ T cells in a plasmid dose-dependent manner (Fig. 4*B*). We also examined the effect of anti-IL-5R mAb. Administration of anti-IL-5R mAb decreased the number of eosinophils in the spleen of p-IL-5 mice to $\sim 20\%$ of the eosinophil count in the control IgG-treated mice (on day 11; $4.13 \pm 0.32 \times 10^6$ (IL-5 gene delivery + control IgG); $0.78 \pm 0.03 \times 10^6$ (IL-5 gene delivery + anti-IL-5R mAb)). This treatment restored the suppression of Ag-specific proliferation of CD4⁺ T cells induced by p-IL-5 injection (Fig. 4*C*). These results confirmed that the IL-5 protein itself played an essential role in triggering the hyporesponsiveness of CD4⁺ T cells induced by in vivo IL-5 gene delivery.

In vivo IL-5 gene delivery up-regulates the production of TGF- β 1 from spleen cells

We speculated that IL-5 exhibited its immunosuppressive effect on CD4⁺ T cells indirectly, probably by affecting immunosuppressive cytokines. We then examined the production of IL-10 and TGF- β 1. IL-5 gene delivery did not induce IL-10 production in whole spleen cells (Fig. 5*A*). By contrast, it significantly up-regulated total amounts of TGF- β 1 production (Fig. 5*B*). We subsequently examined these cellular productions using CD4⁺ T cell. CD4⁺ T cells from p-IL-5 mice produced much less IL-10 compared with OVA mice or p-Cont mice (Fig. 5*C*). In contrast, TGF- β 1 production by CD4⁺ T cells in the p-IL-5 mice did not differ from that in the OVA mice nor from the p-Cont mice (Fig. 5*D*). In addition, in a preliminary study, the ratio of CD4⁺CD25⁺ cells to CD4⁺CD25⁻ cells did not increase in the p-IL-5 mice (data not shown). Furthermore, although Foxp3 mRNA expression in CD4⁺ T cells was slightly up-regulated in the p-IL-5 mice, the CD4⁺ T cells did not manifest suppressive activity (data not shown). Therefore, IL-5 gene delivery would not induce regulatory T (Treg) cells in our system. These results suggested that TGF- β 1-producing

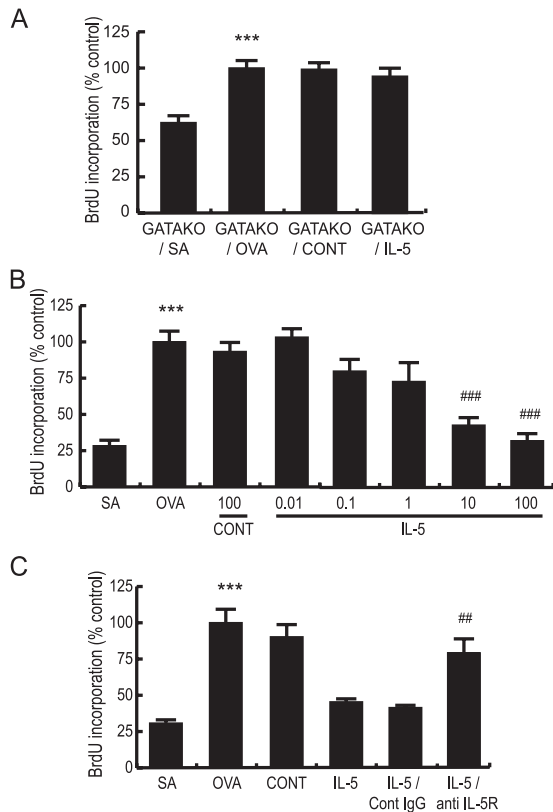


FIGURE 4. Eosinophils and IL-5 protein play a critical role in the suppression of CD4⁺ T cells induced by in vivo IL-5 gene delivery. *A*, Effect of deletion of eosinophils on the IL-5-induced immune suppression. Eosinophil-ablated Δ dbl GATA mice (GATAKO) received plasmid (pCAGGS-IL-5 or control pCAGGS; 100 μ g) on day -3 and were sensitized with OVA or SA on day 0. On day 11, the proliferation of splenic CD4⁺ T cells was examined based on BrdU incorporation ($n = 6$). The maximum proliferation observed in response to OVA for splenic CD4⁺ T cells from OVA-sensitized Δ dbl GATA mice was set as a control (GATAKO/OVA; 100%). ***, $p < 0.001$ compared with the value of GATAKO/SA. *B*, Effect of dose of IL-5 plasmid on the proliferation of CD4⁺ T cells. Wild-type mice received plasmid (pCAGGS-IL-5; 0.01, 0.1, 1, 10, 100 μ g, or control pCAGGS; 100 μ g) on day -3 and were sensitized with OVA or SA on day 0. On day 11, the proliferation of splenic CD4⁺ T cells was examined based on BrdU incorporation ($n = 6$). The maximum proliferation observed in response to OVA for splenic CD4⁺ T cells from OVA-sensitized mice was set as a control (OVA; 100%). ***, $p < 0.001$ compared with the value of SA. ###, $p < 0.001$ compared with the value of CONT. *C*, Effect of anti-IL-5R mAb on the proliferation of CD4⁺ T cells. Wild-type mice received plasmid on day -3 and were sensitized with OVA or SA on day 0. Some pCAGGS-IL-5-injected mice received anti-IL-5R mAb (1 mg) or control IgG i.p. on days -2, -1, and 1. On day 11, the proliferation of splenic CD4⁺ T cells was examined based on BrdU incorporation ($n = 6$). ***, $p < 0.001$ compared with the value of SA. ##, $p < 0.01$ compared with the value of IL-5/Cont IgG.

cells other than CD4⁺ T cells might play a critical role in the suppression of OVA-induced immune response. As almost 60% of spleen cells were eosinophils at this point in time (Fig. 1), these results strongly suggested that eosinophils might be the main cellular sources of TGF- β 1 production.

In vivo IL-5 gene delivery increases spontaneous TGF- β 1 production from eosinophils in the spleen

Next, we examined TGF- β 1 production from spleen cells. There was a significant increase in TGF- β 1 production of p-IL-5 mice

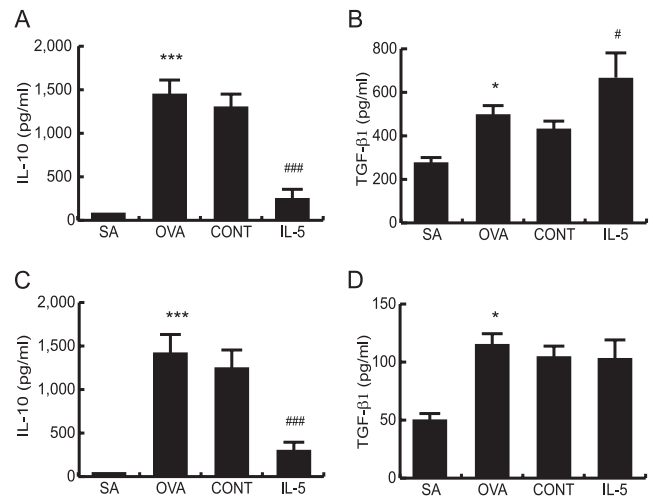


FIGURE 5. In vivo IL-5 gene delivery increases TGF- β 1 production from whole spleen cells, but not from CD4⁺ T cells. Mice were treated as described in Fig. 3. *A* and *B*, IL-10 or TGF- β 1 production of whole spleen cells in response to OVA. On day 11, whole spleen cells (5×10^5 cells/well) were incubated with OVA (20 μ g/ml). After 120 h, the concentration of IL-10 (*A*) or TGF- β 1 (*B*) of the supernatants was assayed ($n = 6$). *C* and *D*, IL-10 or TGF- β 1 production of CD4⁺ T cells in response to OVA. On day 11, splenic CD4⁺ T cells (2.5×10^5 cells/well) were positively selected by magnetic cell sorting and cultured with freshly isolated mitomycin C-treated splenocytes (2.5×10^5 cells/well) and OVA (20 μ g/ml). After 120 h, the concentration of IL-10 (*C*) or TGF- β 1 (*D*) of the supernatants was assayed ($n = 6$). *, $p < 0.05$ and ***, $p < 0.001$ compared with the value of SA. #, $p < 0.05$ and ###, $p < 0.001$ compared with the value of CONT.

compared with p-Cont mice on day 3 (Fig. 6A). The production was higher on day 11 (data not shown). We also confirmed by immunohistochemistry that IL-5 treatment up-regulated TGF- β 1 expression in the spleen (data not shown). In contrast, in the eosinophil-ablated Δ dbl GATA mice, IL-5 gene delivery did not increase TGF- β 1 production from whole spleen cells (Fig. 6A), which suggested that increase in TGF- β 1 production was mainly achieved by eosinophils in our system. Next, to confirm the TGF- β 1 production by eosinophils, we separated eosinophils from whole spleen cell suspension. Eosinophils produced a significantly higher amount of TGF- β 1 than other cells both on days 3 and 11 (Fig. 6, *B* and *C*), supporting the finding above that eosinophils were the main cellular source of TGF- β 1.

In vivo IL-5 gene delivery does not activate eosinophils

We next clarified the activation state of eosinophils from p-IL-5 mice. First, we analyzed the expression of CD69, one of the markers for eosinophil activation, but could not confirm up-regulation in eosinophils from p-IL-5 mice (data not shown). Then, we measured secretion of EPO. Eosinophils from p-IL-5 mice did not release EPO (Fig. 6D). These eosinophils did not release cysLTs either (data not shown). These results suggested that eosinophils from p-IL-5 mice were not activated. We also measured production of other cytokines than TGF- β . Purified eosinophils obtained from p-IL-5 mice did not increase cytokine production such as IL-5 and IFN- γ (Fig. 6E and data not shown).

Eosinophils could suppress the Ag-specific immune response of CD4⁺ T cells in vitro

Next, to examine a suppressive role of eosinophils, coculture of eosinophils with APCs and CD4⁺ T cells was performed. Proliferation of CD4⁺ T cells was assessed by CFSE staining.

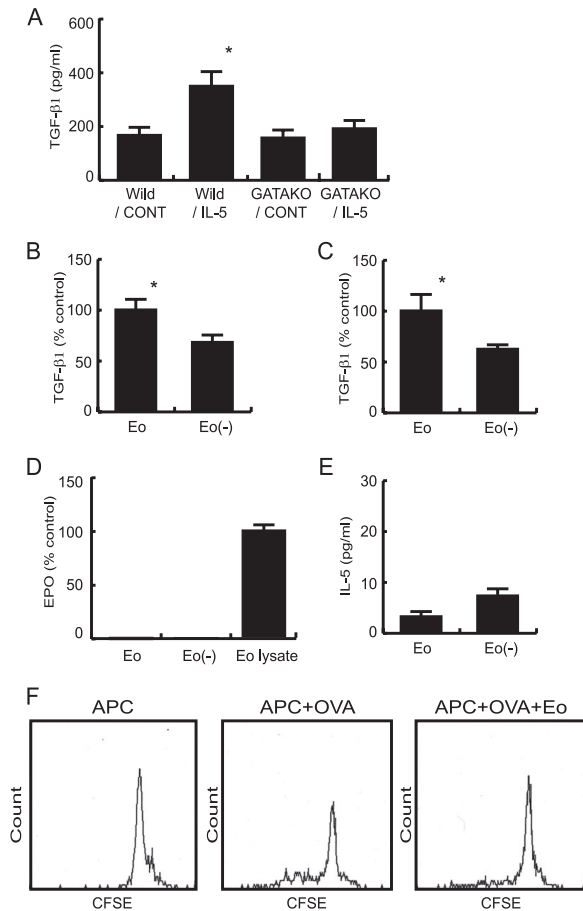


FIGURE 6. In vivo IL-5 gene delivery increases spontaneous TGF- β 1 production from splenic eosinophils. **A**, In vivo IL-5 gene delivery increases spontaneous TGF- β 1 production from spleen cells in wild-type mice, but not in eosinophil-ablated mice. Wild-type mice (Wild) or eosinophil-ablated Δ dbl GATA mice (GATAKO) received plasmid (pCAGGS-IL-5 or control pCAGGS) on day 0. On day 3, whole spleen cells were obtained and spontaneous production of TGF- β 1 for 24 h from spleen cells (1×10^6 cells/well) was examined using ELISA ($n = 6$). *, $p < 0.05$ compared with the value of CONT. **B** and **C**, The major cellular source for TGF- β 1 is eosinophils in the IL-5-treated mice. Wild-type mice received plasmid (pCAGGS-IL-5 or control pCAGGS) on day 0. Splenic eosinophils (Eo) or eosinophil-depleted cells (Eo (-)) were obtained on day 3 (**B**) or on day 11 (**C**) as described in *Materials and Methods*. Spontaneous TGF- β 1 production for 24 h from each subset (1×10^6 cells/well, respectively) was examined using ELISA ($n = 6$). TGF- β 1 production by eosinophils was set as a control (100%). *, $p < 0.05$ compared with the value of Eo (-). **D**, EPO activity. EPO activity of the cell-free supernatants of each subset (1×10^6 cells/well) was measured as described in *Materials and Methods* ($n = 6$). As a positive control, eosinophil extract was obtained from lysed eosinophils (Eo lysate; 100%; 1×10^6 cells/well). **E**, IL-5 production by eosinophils. IL-5 production from each subset (1×10^6 cells/well) was examined using ELISA ($n = 6$). **F**, Eosinophils could suppress the Ag-specific immune response of CD4 $^+$ T cells in vitro. CD4 $^+$ T cells obtained from DO11.10 mice were labeled with CFSE, and then incubated with mitomycin C-treated splenocytes from naive mice in the presence or absence of eosinophils. After 48 h, proliferation of CD4 $^+$ T cells was assessed by flow cytometry. Representative figures were shown.

Addition of eosinophils in coculture suppressed Ag-specific proliferation of CD4 $^+$ T cells (Fig. 6F), although this suppressive effect was mild. These results suggested that eosinophils could directly suppress the Ag-specific immune response of CD4 $^+$ T cells in vitro.

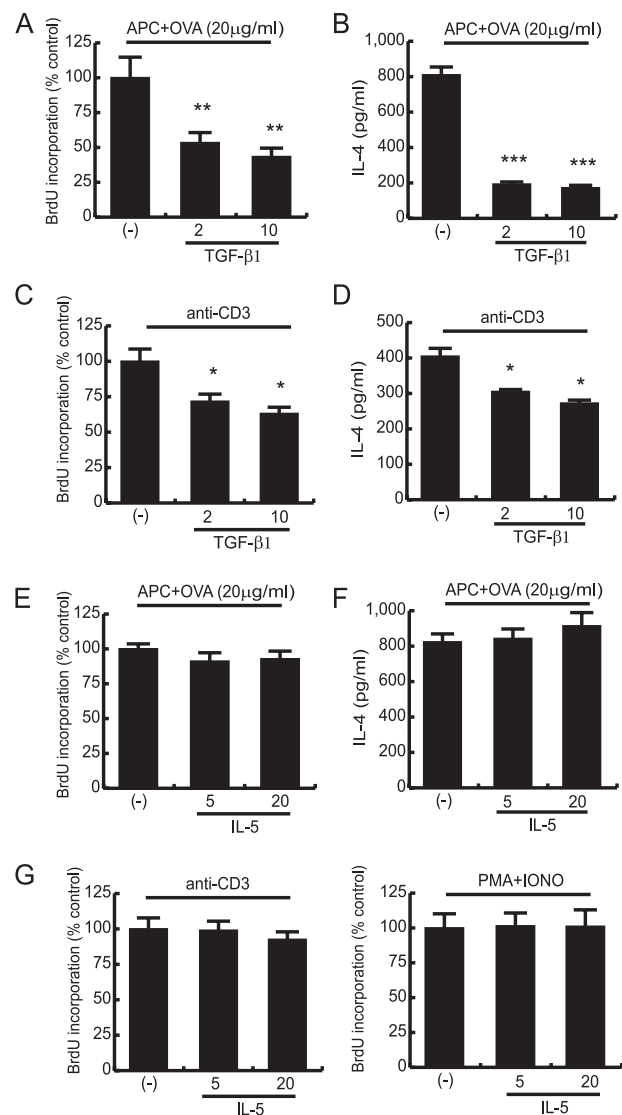


FIGURE 7. TGF- β 1, but not IL-5, suppresses the immune response of CD4 $^+$ T cells in vitro. **A** and **B**, Effect of TGF- β 1 on Ag-specific immune response of CD4 $^+$ T cells. OVA-sensitized splenic CD4 $^+$ T cells (2.5×10^5 cells/well) were incubated with freshly isolated mitomycin C-treated splenocytes (2.5×10^5 cells/well) and OVA (20 μ g/ml) for 72 h with or without TGF- β 1 (2 or 10 ng/ml). **A**, The proliferation was assessed ($n = 6$). The proliferation of CD4 $^+$ T cells in response to OVA without TGF- β was set as a control (100%). **B**, IL-4 concentration in the supernatants ($n = 6$). **, $p < 0.01$ and ***, $p < 0.001$ compared with the value without TGF- β 1. **C** and **D**, Direct effect of TGF- β 1 on CD4 $^+$ T cells. OVA-sensitized splenic CD4 $^+$ T cells (1.25×10^5 cells/well) were incubated with plate-bound anti-CD3 Ab (10 μ g/ml) for 48 h with or without TGF- β 1 (2 or 10 ng/ml). **C**, The proliferation was assessed ($n = 6$). The proliferation of CD4 $^+$ T cells in response to plate-bound anti-CD3 Ab without TGF- β was set as a control (100%). **D**, IL-4 concentration ($n = 6$). *, $p < 0.05$ compared with the value without TGF- β 1. **E** and **F**, Effect of IL-5 on Ag-specific immune response of CD4 $^+$ T cells. OVA-sensitized splenic CD4 $^+$ T cells were incubated with freshly isolated mitomycin C-treated splenocytes and OVA for 72 h with or without IL-5 (5 or 20 ng/ml). **E**, The proliferation was assessed ($n = 6$). The proliferation of CD4 $^+$ T cells in response to OVA without IL-5 was set as a control (100%). **F**, IL-4 concentration ($n = 6$). **G**, Direct effect of IL-5 on CD4 $^+$ T cells. OVA-sensitized splenic CD4 $^+$ T cells were incubated with plate-bound anti-CD3 Ab for 48 h or PMA and ionomycin for 24 h with or without IL-5 (5 or 20 ng/ml). The proliferation was assessed ($n = 6$). The proliferation of CD4 $^+$ T cells in response to plate-bound anti-CD3-Ab or PMA/ionomycin without IL-5 was set as a control (100%).

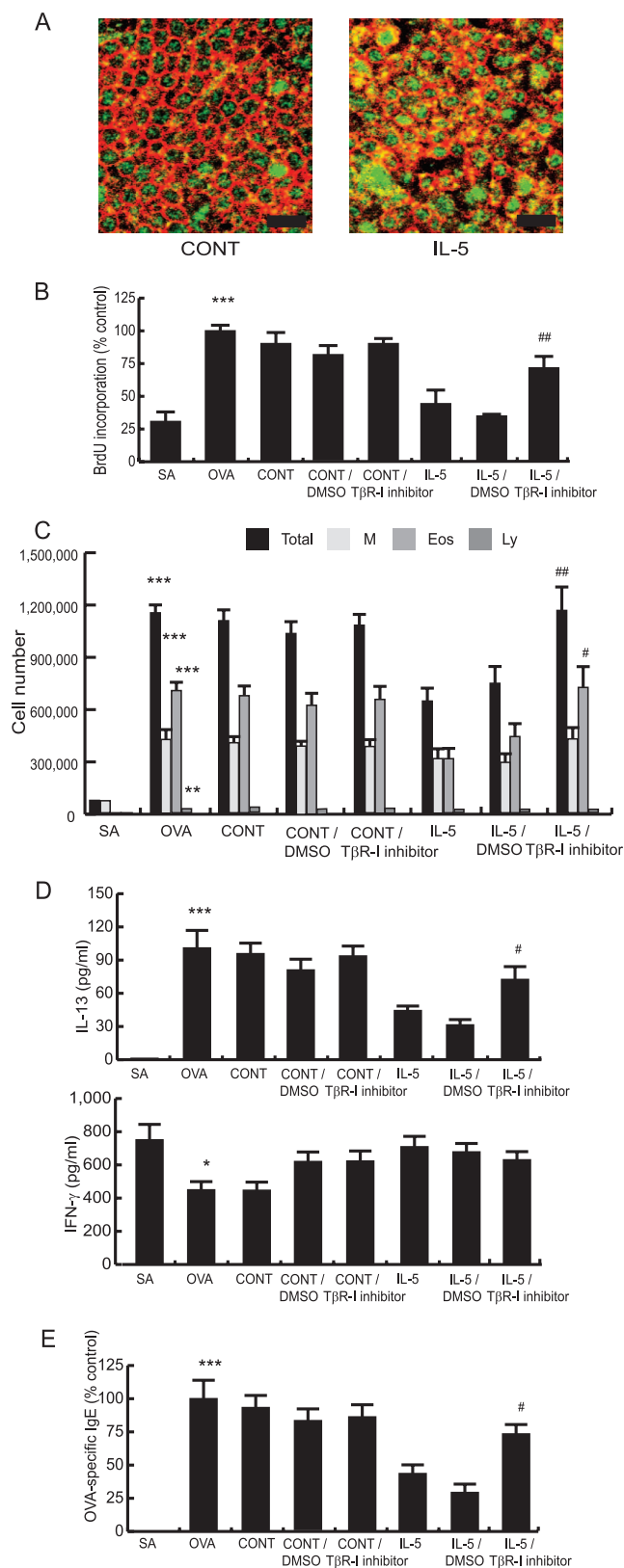


FIGURE 8. TGF- β plays an important role in the suppression of CD4⁺ T cell-mediated immune response and the suppression of eosinophilic airway inflammation. *A*, IL-5 gene delivery up-regulated TGF- β signaling of CD4⁺ T cells. Mice received plasmid (pCAGGS-IL-5 or control pCAGGS) on day -3 and were sensitized with OVA on day 0. On day 11, spleens were excised, and stained with anti-phospho Smad2 Ab (green) and anti-CD4 Ab (red). Double-positive cells indicated up-regulated TGF- β signaling in CD4⁺ T cells. Scale bar, 10 μ m. *B*, Effect of T β R-I inhibitor

TGF- β 1 suppresses the immune response of CD4⁺ T cell in vitro

Next, we examined whether TGF- β 1 or IL-5 could suppress the immune response in vitro. When OVA-sensitized splenic CD4⁺ T cells were stimulated with APCs and OVA, TGF- β 1 suppressed the proliferation of CD4⁺ T cells and IL-4 production (Fig. 7, *A* and *B*). When the cells were stimulated with plate-bound anti-CD3 Ab, TGF- β 1 again suppressed the CD4⁺ T cell proliferation and IL-4 production (Fig. 7, *C* and *D*). In contrast, IL-5 did not suppress the OVA Ag-specific immune response of CD4⁺ T cells directly (Fig. 7, *E* and *F*). When the cells were stimulated with plate-bound anti-CD3 Ab or PMA/ionomycin, IL-5 did not suppress this proliferation either (Fig. 7*G*). Therefore, TGF- β 1, but not IL-5, played an essential role in the immunosuppressive response of CD4⁺ T cells induced by in vivo IL-5 gene delivery. In another experiment, we examined whether supernatant of IL-5-induced eosinophils could suppress the proliferation of CD4⁺ T cells. It did not suppress the proliferation probably because most of TGF- β 1 in the supernatant was inactive (data not shown).

IL-5 gene delivery actually up-regulates TGF- β signaling of CD4⁺ T cells

Next, we examined whether TGF- β signaling was actually transduced in CD4⁺ T cells of p-IL-5 mice. We analyzed the phosphorylation status of Smad2, a downstream effector for TGF- β and an indicator of active TGF- β signaling, in CD4⁺ T cells. Phosphorylated Smad2 expression in CD4⁺ T cells of the spleen strongly increased in the p-IL-5 mice as compared with that in the p-Cont mice (Fig. 8*A*), which suggested that IL-5 gene delivery up-regulated TGF- β signaling of CD4⁺ T cells.

TGF- β plays an important role in the suppression of CD4⁺ T cell-mediated immune response and the suppression of eosinophilic airway inflammation

Finally, we examined the effect of T β R-I inhibitor on the IL-5-induced immune suppression. In the p-Cont mice, administration of T β R-I inhibitor did not affect the immune response of CD4⁺ T cells (Fig. 8*B*). CD4⁺ T cells from p-IL-5 mice proliferated in response to OVA when treated with the T β R-I inhibitor (Fig. 8*B*). Furthermore, administration of the T β R-I inhibitor restored the IL-5-induced suppression of eosinophilic airway inflammation although it did not affect the inflammation in the p-Cont-mice

on Ag-specific proliferation of CD4⁺ T cells. Mice received plasmid (pCAGGS-IL-5 or control pCAGGS) on day -3 and were sensitized with OVA or SA on day 0. Some plasmid-injected mice (pCAGGS-IL-5 or control pCAGGS) received T β R-I inhibitor (20 μ g) or DMSO i.p. on days -1, 1, 3, 6, 8, and 10. On day 11, the proliferation of splenic CD4⁺ T cells was examined based on BrdU incorporation ($n = 6$). The maximum proliferation observed in response to OVA for splenic CD4⁺ T cells from OVA-sensitized mice was set as a control (OVA; 100%). *C*, Effect of T β R-I inhibitor on eosinophilic airway inflammation. Mice received plasmid on day -3 and were sensitized with OVA or SA on days 0 and 11. Some plasmid-injected mice received T β R-I inhibitor (20 μ g) or DMSO i.p. on days -1, 1, 3, 6, 8, 10, 13, 15, and 17. The mice were then challenged with OVA or PBS from day 18 to day 20. On day 21, the mice were sacrificed. BALF analyses were performed ($n = 12$). *D*, BALF cytokine concentrations. Supernatant of BALF was assayed for IL-13 and IFN- γ concentrations by ELISA ($n = 12$). *E*, OVA-specific IgE concentration. OVA-specific IgE concentration was measured by ELISA ($n = 12$). Pooled sera from five OVA-sensitized mice were set as a control (OVA; 100%). *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$ compared with the value of SA. #, $p < 0.05$ and ##, $p < 0.01$ compared with the value of IL-5/DMSO.

(Fig. 8C). It also restored the suppression of other Th2-mediated immune response such as IL-13 production in BALF (Fig. 8D) and OVA-specific IgE production in serum (Fig. 8E). These results strongly indicated that in vivo IL-5 gene delivery suppressed Ag-induced immune response of CD4⁺ T cells and eosinophilic airway inflammation through a TGF- β -dependent mechanism.

Discussion

In this study, we have demonstrated that eosinophils could suppress the Ag-specific immune response via a TGF- β -dependent mechanism. Hypereosinophilia induced by in vivo IL-5 gene delivery before systemic sensitization suppressed the Ag-specific proliferation of CD4⁺ T cells in the spleen, eosinophilic airway inflammation, and AHR. IL-5 gene delivery increased TGF- β production by spleen cells and the TGF- β actually worked on CD4⁺ T cells. Eosinophils were the main cellular source of TGF- β 1 produced in the spleen. T β R-I inhibitor abolished this IL-5-induced immune suppression.

A study by Kobayashi et al. (22) demonstrated that in IL-5 transgenic mice, AHR induced by Ag sensitization was reduced despite a marked eosinophil infiltration in the airway. They also found an increase in TGF- β 1 in the lung in these mice. Treating the mice with IL-5 Ab diminished airway eosinophilia and TGF- β 1 whereas AHR increased. They therefore speculated that TGF- β might have played an important role as an immunosuppressive cytokine in the suppression of AHR. However, in their report, airway inflammation was neither suppressed nor its mechanism elucidated. By contrast, in the current study, hypereosinophilia induced during sensitization inhibited both airway eosinophilia and AHR. We have clarified that suppression of AHR was achieved by suppressing Ag sensitization and the consequent airway inflammation. Furthermore, we found that TGF- β 1, which was mainly produced by eosinophils, played a key role in this suppression. A reason for the discrepancy in the results would be due to a difference in the expression of IL-5 protein (e.g., 2–40 μ g/ml in our study (Fig. 1) vs 2–20 ng/ml in transgenic mice (38)). The finding that a lower dose of IL-5-expressing plasmid could not have suppressed the proliferation of CD4⁺ T cells (Fig. 4B; 0.01–1 μ g) would support our speculation.

IL-5 is a cytokine that induces eosinophil proliferation, differentiation, and migration from bone marrow (39–41). It is generally considered an aggravating factor in the Ag-induced eosinophilic airway inflammation (42–46). However, in the current study, we demonstrated that in vivo IL-5 gene delivery before sensitization suppressed eosinophilic airway inflammation (Fig. 2) and the Ag-specific proliferation of CD4⁺ T cells (Fig. 3). Administration of anti-IL-5R mAb restored the suppression of the Ag-specific proliferation of CD4⁺ T cells induced by IL-5 gene delivery (Fig. 4C). These results suggested that in our system, IL-5 suppressed the Ag-specific proliferation of CD4⁺ T cells, thus suppressing eosinophilic airway inflammation. In contrast, it is known that T cells rarely express the IL-5R (39). We also confirmed through in vitro studies that IL-5 neither suppressed the Ag-specific immune response of CD4⁺ T cells nor did it directly suppress the proliferation of CD4⁺ T cells (Fig. 7). We believe that IL-5 contributed indirectly to the immunosuppressive response. We then extended our research to examine the expression of immunosuppressive cytokines, and found that TGF- β 1, and not IL-10, played a pivotal role.

TGF- β is an immunosuppressive cytokine (47, 48). Generally, it suppresses the proliferation of CD4⁺ T cells and also blocks the differentiation of Th1 and Th2 cells (47–50). In this

study, TGF- β 1 suppressed the proliferation and cytokine production of CD4⁺ T cells in vitro (Fig. 7). In addition, in vivo IL-5 gene delivery increased OVA-induced TGF- β 1 production by spleen cells (Fig. 5B), and spontaneous TGF- β 1 production by spleen cells showed $\sim$ 2-fold increase (Fig. 6A). Moreover, phosphorylated Smad2 expression in CD4⁺ T cells of the spleen strongly increased in the IL-5-treated mice (Fig. 8A), suggesting that IL-5 gene delivery up-regulated TGF- β signaling of CD4⁺ T cells. Furthermore, T β R-I inhibitor restored the suppression of the proliferation of CD4⁺ T cells of IL-5-treated mice (Fig. 8B). Taken together, TGF- β 1 worked to suppresses the immune response in our system as well. Some reports have indicated that TGF- β converts CD4⁺CD25⁺ T cells to Treg cells in vitro though the induction of Foxp3 (51, 52). So it is likely that in vivo IL-5 gene delivery could have induced Treg cells in our system. However, our preliminary experiments suggested that Treg cells would not be induced by our IL-5 gene delivery. Instead, in our experimental system, TGF- β 1 could well have suppressed the overall Ag-induced immune response of CD4⁺ T cells.

Generally, eosinophils produce TGF- β 1 (9–11, 53–55). In this study, in vivo IL-5 gene delivery induced a marked increase in the number of splenic eosinophils (Fig. 1). In the eosinophil-ablated mice, IL-5 gene delivery did not suppress the Ag-induced immune response of CD4⁺ T cells (Fig. 4A). Although IL-5 treatment increased TGF- β 1 production by spleen cells in wild-type mice, it did not increase TGF- β 1 production in eosinophil-ablated Δ dbl GATA mice (Fig. 6A), which suggested that TGF- β 1 was mainly produced by eosinophils in our system. We also confirmed by depletion study that the main cellular source producing TGF- β 1 in the spleen were eosinophils (Fig. 6, B and C). Moreover, CFSE analyses suggested that eosinophils could suppress Ag-specific proliferation of CD4⁺ T cells in vitro (Fig. 6F), although the suppressive effect was mild. Therefore, in our system, TGF- β 1-producing eosinophils, induced by IL-5 gene delivery, played a central role in regulating the immune response of CD4⁺ T cells. An in vitro study demonstrating that IL-5 increased TGF- β 1 production from eosinophils (22) supports our speculation.

In this study, we demonstrated that eosinophils from IL-5-treated mice were not activated. Eosinophils from IL-5-treated mice did not express CD69, one of the markers for eosinophil activation (data not shown). Moreover, these eosinophils did not release eosinophil granule protein such as EPO (Fig. 6D). They did not release cysLTs either (data not shown). It is well known that mouse eosinophils do not routinely degranulate in vivo under any known circumstances (22, 56–58). Moreover, Lee et al. (56) recently suggested the possibility that eosinophil granule proteins might be an evolutionary vestige and they might not have effector function. So, in the current study, eosinophils would have produced TGF- β 1 without being activated or degranulated.

Recently, many epidemic studies have indicated that parasite infections inducing eosinophilia suppressed further sensitization to other Ags (14–18). Serum IL-5 concentration is higher in patients infected with parasites than in healthy subjects (59). Moreover, in animal studies, helminth infection before systemic OVA sensitization suppressed Ag-induced eosinophilic airway inflammation, whereas helminth infection after sensitization did not (20, 21). These findings are both similar to and in support of our current results obtained by IL-5 gene delivery. So far, one of the major mechanisms for suppression by parasite infection is considered the induction of parasite-mediating Treg cells (14, 15). In this study, we propose another mechanism

where eosinophils, induced by parasite infection, might play an important role in the suppression of Th2 immune response through TGF- β .

In summary, IL-5 gene delivery increased the TGF- β 1 production from spleen cells, thus suppressing the Ag-specific immune response of CD4⁺ T cells and Ag-induced eosinophilic airway inflammation. The major cellular source of TGF- β 1 in the IL-5 delivered mice was eosinophils, suggesting an important role of TGF- β 1-producing eosinophils in the early stage of Ag-specific immune response. This mechanism of immunosuppression would play a specific role in a possible suppression of asthma in instances such as parasite infection.

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Disclosures

The authors have no financial conflict of interest.

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