

Blockade of Transforming Growth Factor β /Smad Signaling in T Cells by Overexpression of Smad7 Enhances Antigen-induced Airway Inflammation and Airway Reactivity

By Atsuhito Nakao,* Satoshi Miike,[†] Masahiko Hatano,[§]
Ko Okumura,* Takeshi Tokuhisa,[§] Chisei Ra,* and Itsuo Iwamoto[‡]

From the *Allergy Research Center, Juntendo University, School of Medicine, Tokyo 113-8421, Japan; and the [†]Department of Medicine II, School of Medicine, and the [§]Department of Developmental Genetics, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan

Abstract

Transforming growth factor (TGF)- β has been implicated in immunosuppression. However, it remains obscure whether regulation of T cells by TGF- β contributes to the immunosuppression in vivo. To address this issue, we developed transgenic mice expressing Smad7, an intracellular antagonist of TGF- β /Smad signaling, selectively in mature T cells using a plasmid construct coding a promoter element (the distal lck promoter) that directs high expression in peripheral T cells. Peripheral T cells were not growth inhibited by TGF- β in Smad7 transgenic mice. Although Smad7 transgenic mice did not spontaneously show a specific phenotype, antigen-induced airway inflammation and airway reactivity were enhanced in Smad7 transgenic mice associated with high production of both T helper cell type 1 (Th1) and Th2 cytokines. Thus, blockade of TGF- β /Smad signaling in mature T cells by expression of Smad7 enhanced airway inflammation and airway reactivity, suggesting that regulation of T cells by TGF- β was crucial for negative regulation of the inflammatory (immune) response. Our findings also implicated TGF- β /Smad signaling in mature T cells as a regulatory component of allergic asthma.

Key words: signaling • inhibitor • asthma • mouse • eosinophils

Introduction

TGF- β is a multifunctional cytokine that has diverse effects on a variety of cell types (1). In the immune system, TGF- β regulates growth, differentiation, and function of macrophages, T cells, B cells, and NK cells (2–4). Importantly, TGF- β null mice developed extensive inflammation in various organs and died shortly after birth, suggesting that TGF- β played a critical role in suppression of the immune system in vivo (5, 6). Downregulation of T cell function by inhibiting T cell proliferation is thought to be an important means for TGF- β -mediated immunosuppression based on in vitro studies. However, since TGF- β acts on multiple targets affecting the immune system (2), it remains obscure whether negative regulation of T cells by TGF- β contributes to the immunosuppression in vivo.

Recent identification of the Smad family of proteins has advanced our understanding of how TGF- β signals from

membrane to nucleus (7–10). The activated TGF- β receptors induce phosphorylation of Smad2 and Smad3, which form heterooligomeric complexes with Smad4. The complexes then translocate to the nucleus and regulate transcriptional responses together with DNA binding cofactors. Recently, Smad7 has been identified as an intracellular antagonist of TGF- β signaling; it inhibits TGF- β -induced transcriptional responses (11–13). Smad7 associates with the activated TGF- β receptors and interferes with the activation of Smad2 and Smad3 by preventing their receptor interaction and phosphorylation.

To determine whether negative regulation of T cells by TGF- β contributes to immunosuppression in vivo, we have developed transgenic (Tg)¹ mice expressing Smad7 preferentially in mature T cells using a plasmid construct coding a promoter element (the distal lck promoter) that directs high expression in peripheral T cells (14). This strat-

A. Nakao and S. Miike contributed equally to this work.

Address correspondence to Atsuhito Nakao, Allergy Research Center, Juntendo University, School of Medicine, 2-1-1, Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. Phone: 81-3-5802-1591; Fax 81-3-3813-5512; E-mail: anakao@med.juntendo.ac.jp

¹Abbreviations used in this paper: AHR, airway hyperresponsiveness; APTI, airway pressure-time index; B6, CB57L/6; BAL, bronchoalveolar lavage; BALF, BAL fluid; hGH, human growth hormone; Tg, transgenic; Wt, wild-type.

egy also has the advantage of avoiding complications that may occur by blocking TGF- β /Smad signaling in T cells during early thymocyte maturation (15). Here, we showed that antigen-induced airway inflammation and airway reactivity were enhanced in Smad7 Tg mice, suggesting that TGF- β /Smad signaling in mature T cells was crucial for negative regulation of the inflammatory (immune) response. Since antigen-induced airway inflammation and airway reactivity in mice is a well-established model of allergic asthma (16), our findings also implicated TGF- β /Smad signaling in mature T cells as a regulatory component of allergic asthma.

Materials and Methods

Generation of Tg Mice. A 1.4-kb fragment including the full-length mouse Flag-tagged Smad7 cDNA was removed from the Smad7-pcDNA3 construct (13) by digestion with HindIII and XhoI. This fragment was subcloned into a pGEM-4Z vector that had been cut with HindIII-SalI. This pGEM-4Z vector containing Smad7 was cut with BamHI, and then ligated into the BamHI site of the pUC19 vector containing the lck distal promoter (14). In the pUC19 vector, the lck distal promoter sequences were fused at the BamHI site to the human growth hormone (hGH) intronic and polyadenylation sequences, the presence of which has been found to improve the expression of cDNA transgenes in a nonspecific fashion. The diagrammatic representation of the lck distal promoter-Smad7 transgene is shown in Fig. 1 A. The SpeI fragment of these lck distal promoter-Smad7 constructions was used for microinjection. The transgenes were injected into the pronuclei of the fertilized eggs of BDF1 mice. The Tg mice were identified by PCR and slot-blot hybridization of genomic DNA to confirm the presence of hGH sequences. C57BL/6 (B6) and BDF1 mice were purchased from Clea, Inc. Transgene-positive founders were backcrossed to B6 mice to establish lines. Animal experiments were approved by the Institutional Review Boards of Chiba and Juntendo Universities.

Purification of T Cells. Mouse CD3⁺ T cells were purified from spleens harvested from Smad7 Tg mice or wild-type (Wt) littermates by Ficoll-Paque (Amersham Pharmacia Biotech) gradient centrifugation of lymphocytes and magnetic cell sorting using MACS anti-CD3 microbeads (Miltenyi Biotec) following the manufacturer's recommendations. The purity of mouse CD3⁺ T cells was confirmed by FACScanTM (Becton Dickinson) and was consistently >98%.

In Vitro Stimulation of T Lymphocytes. For proliferation assays, 96-well microtiter plates were coated with 2 μ g/ml of anti-CD3 ϵ mAb (2C11; BD PharMingen). Splenocytes (200 μ l/well) were added at a density of 10⁶ cells/ml and incubated in the presence or absence of TGF- β (1 ng/ml) at 37°C in 5% CO₂ for 72 h. 1 μ Ci of [³H]thymidine (Amersham Pharmacia Biotech) was added to the culture for the last 12 h. Thymidine incorporation was assayed by harvesting cells with a PHD cell harvester (Cambridge Technologies). For detection of phosphorylation of Smad2 in T cells, purified CD3⁺ T cells (10⁶ cells) from mouse spleen were stimulated with TGF- β (5 ng/ml) for 30 min followed by immunoblotting. For cytokine assays, spleen cells (5 \times 10⁶ cells) isolated from mice sensitized with OVA at 2-wk intervals were restimulated with OVA (100 μ g/ml) 7 d after the second sensitization. 72 h after the culture, supernatants were collected for measurement of cytokines.

Western Blots. Immunoblotting with anti-Flag antibody (Sigma-Aldrich), anti-Smad2 antibody (Transduction Laboratories), antiphosphorylated Smad2 antibody (17), or anti-Smad7 antibody (KAF) (17) was performed as described previously (18).

Flow Cytometry. FACS[®] analysis was performed as described previously (19). In brief, thymus or spleen cells (10⁶) suspended in PBS containing 0.1% NaN₃ and 1% FCS were incubated on ice with a mixture of FITC-labeled anti-CD4, PE-labeled anti-CD8, PE-labeled anti-B220, and PE-labeled anti-CD25 antibodies (BD PharMingen). For intracellular cytokine staining, bronchoalveolar lavage (BAL) cells were stained with a mixture of Cy-Chrome-labeled anti-CD4, fixed and permeabilized using a Cytofix/Cytoperm kit (BD PharMingen), and then stained using FITC-labeled anti-IFN- γ antibody and PE-labeled anti-IL-4 antibody (BD PharMingen) according to the manufacturer's recommendations. After gating on CD4 T cells, results were analyzed on a FACSCaliburTM (Becton Dickinson) using CellQUESTTM software (Becton Dickinson).

Immunization and Exposure of Mice. 5–8-wk-old Tg mice or Wt littermates were immunized intraperitoneally with 1 μ g of chicken OVA (Sigma-Aldrich) in 4 mg of aluminum hydroxide at 2-wk intervals. 10 d after the second immunization, the sensitized mice inhaled aerosolized OVA (50 mg/ml) dissolved in 0.9% saline by a DeVilbiss 646 nebulizer for 30 min for three consecutive days as described previously (20). As a control, 0.9% saline alone was administered by the nebulizer.

BAL. BAL was performed as described previously (21). In brief, 48 h after OVA inhalation, the mice were killed by cervical dislocation, the trachea was cannulated, and the airway lumen was washed three times with 0.3 ml of PBS. The BAL fluid (BALF) was centrifuged at 400 *g* for 10 min at 4°C, and the amount of IL-5, IFN- γ , and TGF- β in the supernatant was measured by enzyme immunoassay as described below. The BAL cells were resuspended in 0.2 ml of PBS and counted using a hemocytometer. For differential cell counts, 2–4 \times 10³ cells were spun onto glass slides (Cytospin), air dried, fixed with ethanol, and stained with Giemsa solution. The number of eosinophils, neutrophils, lymphocytes, and macrophages in 200 cells was counted based on morphology.

Antigen-induced Eosinophil Infiltration in Mouse Trachea. Antigen-induced eosinophil infiltration in mouse trachea was evaluated as described previously (20). In brief, at 48 h after the inhalation, the trachea was excised, fixed in 10% formalin, and stained with Luna solution. The number of eosinophils in the submucosal tissue of trachea was counted in Luna-stained sections and expressed as the number of eosinophils per the length of the basement membrane of trachea, which was measured with a digital curvimeter.

Cytokine ELISA. The amount of IL-4, IL-5, and IFN- γ in the culture supernatant of spleen cells or BALF was determined using murine IL-4 and IL-5 ELISA kits (Endogen) and an IFN- γ ELISA kit (Nalgen). The amount of TGF- β in the supernatant of BALF or plasma was measured using a human TGF- β 1 ELISA kit (R&D Systems) that detects mouse TGF- β 1 protein because of the high homology of TGF- β 1 across species. The assay detects only the active form of TGF- β 1. Samples were activated before measuring according to the manufacturer's recommendations. The minimum significant value of IL-4, IL-5, IFN- γ , and TGF- β 1 was 5, 5, 1, and 5 pg/ml, respectively.

Measurement of Airway Responsiveness. Airway responsiveness to acetylcholine challenge was measured as described (21). In brief, at 24 h after OVA or saline inhalation, mice were anesthetized with pentobarbital (50 mg/kg; Abbott Laboratories), intu-

bated with a 20-gauge tracheal cannula, and ventilated at a rate of 120 breaths/min with a constant tidal volume of air (0.2 ml). Airway pressure was measured with a pressure transducer via a port of the tracheal cannula. Muscle paralysis was provided by intravenous administration of pancuronium bromide (0.1 mg/kg; Tocris Cookson). After establishment of a stable airway pressure recording, acetylcholine (500 mg/kg; Wako) was injected intravenously, and the changes in airway pressure were recorded. Airway responsiveness was defined by the time-integrated change in peak airway pressure (airway pressure–time index [APTI]; $\text{cmH}_2\text{O} \cdot \text{s}$).

Data Analysis. Data are summarized as mean \pm SD. The statistical analysis of the results was performed by analysis of variance using Fisher's least significant difference test for multiple comparisons. $P < 0.05$ was considered significant.

Results

Generation of Smad7 Tg Mice. To express the Smad7 transgene preferentially in peripheral T cells, we used a plasmid construct expressing Flag-tagged mouse Smad7 (13) under the control of a promoter element (the distal lck promoter) that directed high expression in mature thymocytes and peripheral T cells (14; Fig. 1 A). The strategy also has the advantage of avoiding complications that may occur by blocking TGF- β /Smad signaling in T cells during early thymocyte maturation (15). We obtained five transgenic lines, out of which two lines displayed relatively high levels of transgene expression in the spleen (Fig. 1 B). Some level of expression was detected in the thymus (Fig. 1 B), and little expression was observed in the lungs or other organs that were not enriched in T cells (data not shown). We also confirmed expression of the Smad7 trans-

gene in peripheral blood lymphocytes and CD3⁺ T cells purified from the spleens of Smad7 Tg mice (data not shown). In addition, endogenous Smad7 protein was not detected in CD3⁺ T cells purified from the spleens of Wt mice and Smad7 Tg mice by Western blotting with anti-Smad7 antibody (data not shown).

Lack of Responsiveness to TGF- β in Splenic T Cells from Smad7 Tg Mice. To determine whether peripheral T cells expressing the Smad7 transgene were resistant to TGF- β action, we examined the proliferation of splenocytes isolated from Smad7 Tg mice and Wt littermates when activated with mAb to CD3 in the presence and absence of TGF- β . Without stimulation of mAb to CD3, splenocytes from Smad7 Tg mice and Wt littermates did not proliferate (data not shown). The growth-inhibitory effect of TGF- β was largely lost in splenocytes from Smad7 Tg mice when activated with mAb to CD3, although similarly treated Wt splenocytes were growth inhibited $\sim 60\%$ by TGF- β (Fig. 2 A). The purified population of CD3⁺ T cells isolated from the spleens of Smad7 Tg mice and activated by anti-CD3 was also insensitive to the inhibitory effect of TGF- β (data not shown). Interestingly, the non-TGF- β -treated levels of proliferation were about threefold higher in splenocytes from Smad7 Tg mice than in those from Wt littermates (Fig. 2 A).

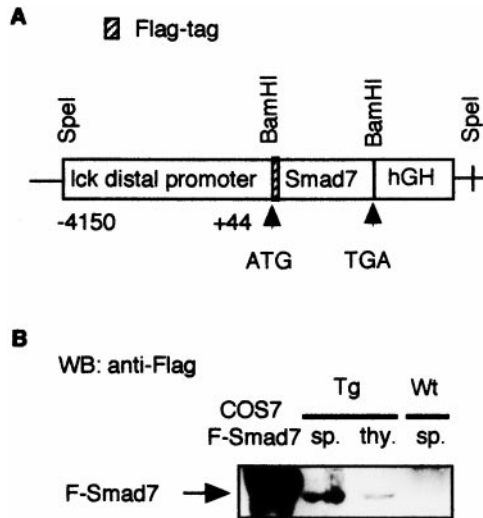


Figure 1. Generation of Smad7 Tg mice. (A) Schematic diagram of constructs used for peripheral T cell-specific expression of the mouse Flag-tagged Smad7 transgene. (B) Expression of the Flag-tagged Smad7 transgene in Smad7 Tg mice. Whole cell lysates were prepared from thymus (thy.) or spleen (sp.) from Smad7 Tg mice or Wt littermates, and subjected to Western blot (WB) analysis using anti-Flag antibody to detect protein expression of the Flag-tagged Smad7 transgene. As a positive control, cell lysate from COS7 cells transfected with Flag-tagged Smad7 (F-Smad7) cDNA was used.

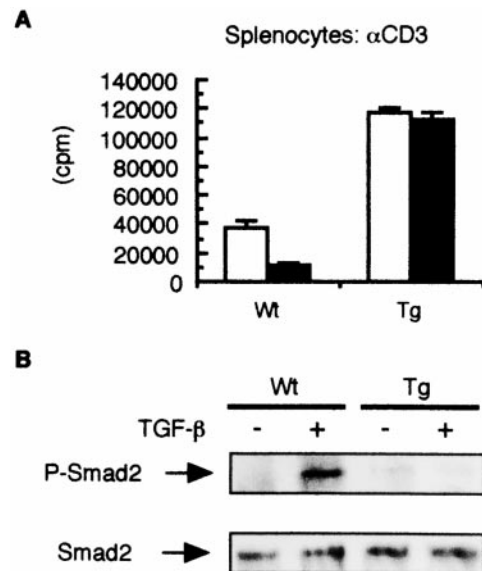


Figure 2. Altered responsiveness of splenic T cells from Smad7 Tg mice to TGF- β . (A) Lack of responsiveness to TGF- β in splenocytes from Smad7 Tg mice. Splenocytes from Smad7 Tg mice or Wt littermates were stimulated with anti-CD3 mAb in the presence or absence (control) of TGF- β (1 ng/ml). Aliquots of cells were pulsed with [³H]thymidine, and incorporated counts (in cpm) were determined. Shown are means \pm SD of [³H]thymidine incorporation from triplicate cultures. The results are from one representative experiment out of three. (B) Inhibition of TGF- β -mediated phosphorylation of Smad2 in purified CD3⁺ T cells from Smad7 Tg mice or Wt littermates, and then immunoblotted with antiphosphorylated Smad2 antibody (top, P-Smad2) or anti-Smad2 antibody (bottom). The results are from one representative experiment out of three.

Phosphorylation of Smad2 and Smad3 by activated TGF- β receptors is a key event for the initiation of TGF- β signal transduction (18, 22), and Smad7 antagonizes TGF- β signaling by preventing TGF- β receptor-mediated phosphorylation of Smad2 and Smad3 (13). Thus, we next determined whether phosphorylation of Smad2 by TGF- β was inhibited in peripheral T cells from Smad7 Tg mice. As shown in Fig. 2 B, we found that TGF- β -mediated phosphorylation of Smad2 was suppressed in CD3⁺ T cells purified from the spleens of Smad7 Tg mice in contrast to those from Wt littermates (Fig. 2 B). These findings indicated that peripheral T cells from Smad7 Tg mice were insensitive to TGF- β .

Normal T Cell and B Cell Development in Smad7 Tg Mice. To determine whether any developmental defect in the immune system was observed in Smad7 Tg mice, we examined thymic and splenic T and B cell populations by performing FACS[®] analysis. As shown in Fig. 3 A, the thymi of Smad7 Tg mice contained normal proportions of CD4 and CD8 single- and double-positive T cells, suggesting that thymic T cell maturation is normal in Smad7 Tg mice. Similarly, the spleens from Smad7 Tg mice contained normal numbers and percentages of B cells and CD4 and CD8 single-positive cells, suggesting that there was not an abnormal expansion of lymphocytes in the spleens of Smad7 Tg mice (Fig. 3 B). These findings indicated that

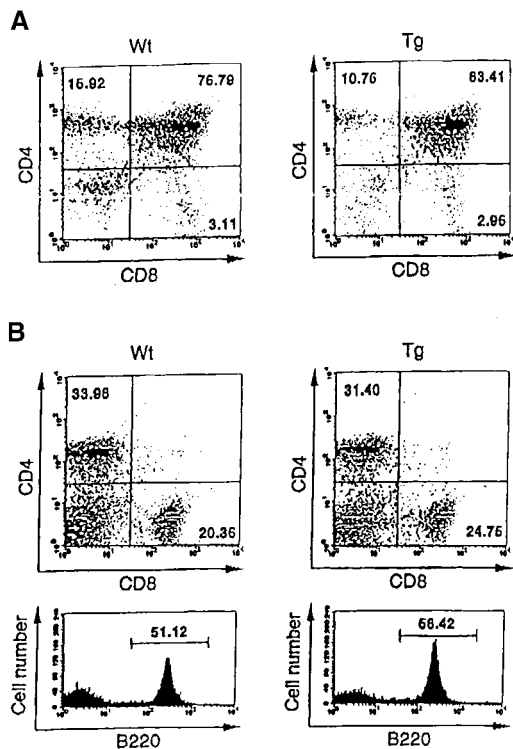


Figure 3. Normal T cell and B cell development in Smad7 Tg mice. FACS[®] analysis of thymocytes (A) or splenocytes (B) using the indicated conjugated antibodies. All data were gated for viable cells. Percentages represent proportions of viable cells in each region or quadrant. The results are from one representative experiment out of three.

Smad7 Tg mice did not have any developmental defect in their immune system.

Normal Phenotype of Smad7 Tg Mice. The Smad7 Tg mice we developed were viable, survived to adulthood, and were phenotypically indistinguishable from their littermate controls at birth and at least until 6 mo of age. Histological examination of lungs or other organs did not show any morphological abnormalities or inflammatory lesions in Smad7 Tg mice (data not shown).

Enhanced Antigen-induced Airway Inflammation in Smad7 Tg Mice. Since Smad7 Tg mice showed a normal phenotype, contrary to our expectations, we asked whether Smad7 Tg mice were any different in pathological situations, particularly in inflammation. Thus, to determine whether TGF- β /Smad signaling in T cells contributes to regulation of the inflammatory response, we developed airway inflammation in Smad7 Tg mice by inhalation of antigen (OVA) after antigen sensitization as described previously (20). 48 h after the inhaled OVA challenge, BALF was recovered from the animal, and the total cell number in the BALF was counted by cell differentiation analysis. As shown in Fig. 4 A, the total cell count in the BALF recovered from Smad7 Tg mice upon OVA challenge was threefold higher than that in the BALF derived from Wt littermates. Cell differentiation analysis revealed that the number of eosinophils, neutrophils, and lymphocytes in the BALF was significantly increased in Smad7 Tg mice compared with Wt littermates, indicating that OVA-induced airway inflammation was enhanced in Smad7 Tg mice in general (Fig. 4 A). In addition, eosinophil infiltration into the mouse trachea after OVA inhalation was also two- to fourfold higher in Smad7 Tg mice than in Wt littermates (Fig. 4 B). These findings indicated that antigen-induced airway inflammation was enhanced in Smad7 Tg mice, suggesting that TGF- β /Smad signaling in mature T cells had a negative regulatory role in the development of antigen-induced airway inflammation.

Cytokine Production in Smad7 Tg Mice. Since antigen-induced airway inflammation was enhanced in Smad7 Tg mice, we focused on the mechanisms underlying these observations. Because we have previously shown that IL-5 and IFN- γ regulate antigen-induced airway inflammation positively and negatively, respectively (20, 23), we examined antigen-induced IL-5 and IFN- γ production in the airways of sensitized mice. IL-5 and IFN- γ levels in the BALF of Smad7 Tg mice were significantly higher at 48 h after OVA inhalation than those of Wt littermates (Fig. 5 A). Little IL-5 or IFN- γ was detected in the BALF of Smad7 Tg mice or Wt littermates after saline inhalation (data not shown). In addition, *in vitro* OVA-induced IL-4, IL-5, and IFN- γ production was significantly higher in the spleen cells of Smad7 Tg mice than those of Wt littermates (Fig. 5 B). TGF- β levels in the BALF of Smad7 Tg mice at 48 h after OVA inhalation were equivalent to those of Wt littermates (Fig. 5 C). In addition, there was no significant difference in plasma TGF- β levels between Smad7 Tg mice and Wt littermates (Smad7 Tg mice: 1.4 ± 0.4 ng/ml; Wt littermates: 1.2 ± 0.5 ng/ml; $n = 5$, $P = 0.49$). These findings indicated that antigen-induced production

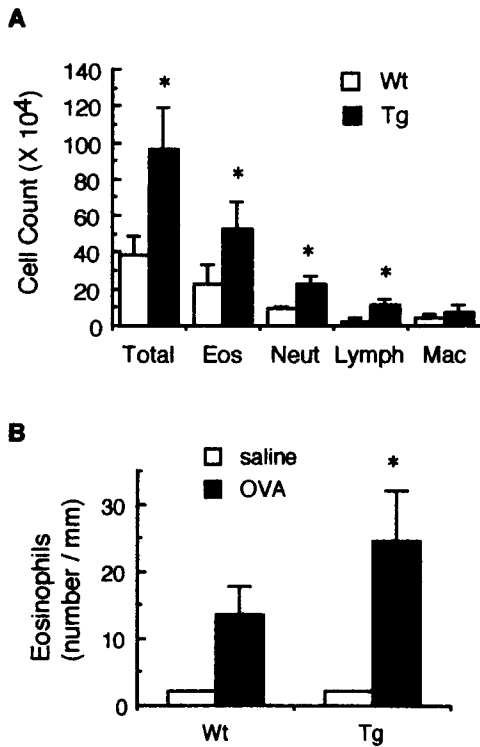


Figure 4. Antigen-induced airway inflammation in Smad7 Tg mice. OVA-sensitized mice were challenged with inhalation of OVA as described in Materials and Methods. Leukocyte infiltration in the BALF (A) and eosinophil infiltration into the trachea (B) of the sensitized mice were then examined at 48 h after OVA inhalation. Data are means \pm SD for five mice in each group. The results are from one representative experiment out of three. Eos, eosinophils; Neut, neutrophils; Lymph, lymphocytes; Mac, macrophages. * $P < 0.05$, significantly different from the mean value of the corresponding control response (Wt).

of Th1 and Th2 cytokines was higher in Smad7 Tg mice than in Wt littermates.

Activated Phenotype of T Lymphocytes in the Airways of Smad7 Tg Mice. Since we found enhanced IL-5 and IFN- γ levels in the BALF of Smad7 Tg mice upon OVA challenge (Fig. 5 A), we further assessed T cell phenotype in the BALF of Smad7 Tg mice. As shown in Fig. 6, the number of CD4 T cells, CD8 T cells, and CD25⁺ CD4 T cells (activated phenotype) in the BALF of Smad7 Tg mice was higher than that of Wt littermates upon OVA challenge. Little T cell infiltration into the BALF of Smad7 Tg mice and Wt littermates was observed upon saline challenge (data not shown). In addition, IL-4 and IFN- γ production in CD4 T cells in the BALF was enhanced in Smad7 Tg mice (Fig. 6 C). These findings indicated that T cell infiltration into the airways of Smad7 Tg mice was enhanced and that the infiltrating T cells were more activated in Smad7 Tg mice than in Wt littermates.

Airway Hyperresponsiveness in Smad7 Tg Mice. Airway hyperresponsiveness (AHR) is associated with airway inflammation and Th2 cytokines, and is a central feature of asthma (16). Thus, we examined antigen-induced AHR in Smad7 Tg mice. Inhaled OVA challenge of sensitized Wt mice resulted in a significant increase in airway responsive-

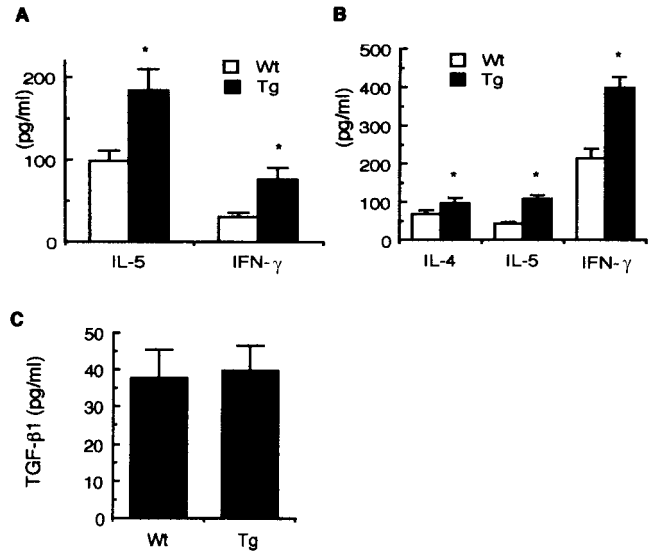


Figure 5. Cytokine production in Smad7 Tg mice. OVA-sensitized mice were challenged with inhalation of OVA. At 48 h after the inhalation, BALF was collected as described in Materials and Methods. For detection of cytokines in the culture supernatant, spleen cells were isolated from the mice sensitized with OVA/alum and stimulated with OVA (100 μ g/ml) in vitro. 72 h after the stimulation, the culture supernatants were collected. IL-5 and IFN- γ levels in the BALF (A), OVA-induced IL-4, IL-5, and IFN- γ production in the supernatant of spleen cells from the sensitized mice (B), and TGF- β 1 levels in the BALF (C) were determined by ELISA. Data are means \pm SD for five mice in each group. The results are from one representative experiment out of three. * $P < 0.05$, significantly different from the mean value of the corresponding control response (Wt).

ness to acetylcholine (APTI: control $1,812 \pm 64$ vs. OVA $2,246 \pm 98$; $n = 5$, $P < 0.005$; Fig. 7) as described previously (21). OVA-induced AHR in sensitized Smad7 Tg mice was significantly higher than that in Wt littermates (APTI: Wt $2,246 \pm 98$ vs. Tg $2,742 \pm 109$; $n = 5$, $P < 0.005$; Fig. 7). These results indicated that overexpression of Smad7 in mature T cells enhanced antigen-induced AHR, suggesting that TGF- β /Smad signaling in mature T cells played a negative role in antigen-induced AHR in sensitized mice.

Discussion

In this study, we developed Tg mice expressing Smad7 under the control of a distal Ick promoter (14), so that we were able to block the TGF- β /Smad signaling pathway preferentially in peripheral T cells. The Tg mice enabled us to determine whether TGF- β /Smad signaling in mature T cells contributed to regulation of the immune system in vivo. We found that Smad7 Tg mice did not spontaneously show a specific phenotype, but showed enhanced antigen-induced airway inflammation and airway reactivity (Figs. 4 and 7). The findings suggested that TGF- β /Smad signaling in mature T cells contributed to negative regulation of the inflammatory (immune) response, but not to T cell homeostasis under normal circumstances.

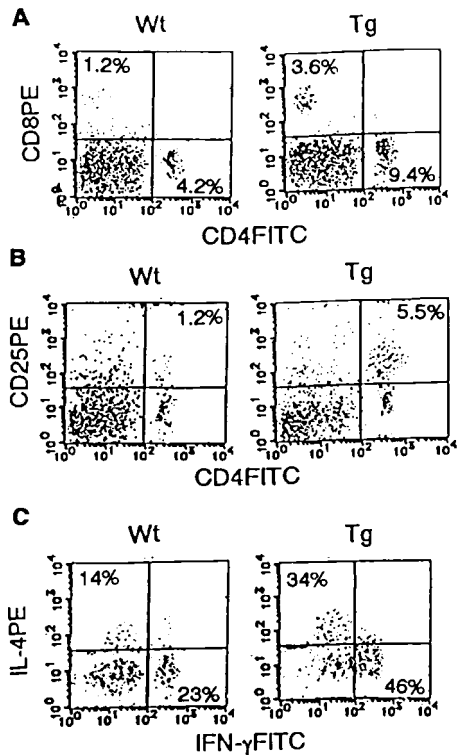


Figure 6. Activated phenotype in T lymphocytes in the airways of Smad7 Tg mice. Representative FACS[®] profiles of CD4 versus CD8 staining (A), CD4 versus CD25 staining (B), and intracellular staining of IL-4 versus IFN- γ in CD4 T cells (C), on BALF cells using anti-CD4-FITC, anti-CD8-PE, anti-CD25-PE, anti-IFN- γ -FITC, and anti-IL-4-PE antibodies ($n = 5$ mice in each group). Percentages represent proportions of viable cells in each region or quadrant.

Antigen-induced airway inflammation and airway reactivity in mice is a well-established model of allergic asthma (16). Thus, the finding that overexpression of Smad7 in mature T cells enhanced antigen-induced airway inflammation and airway reactivity (Figs. 4 and 7) also implicated TGF- β /Smad signaling as a regulatory component of allergic asthma. The role of TGF- β /Smad signaling in the pathophysiology of asthma remains unclear. Since our results suggested the importance of TGF- β /Smad signaling in the suppression of allergic airway inflammation, the pathological role of TGF- β /Smad signaling in asthmatic airways should be investigated in the future.

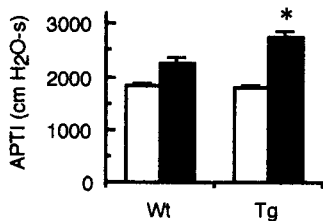


Figure 7. Airway responsiveness to acetylcholine of Smad7 Tg mice. OVA-sensitized mice were challenged with inhalation of OVA (black bars) or saline (white bars). At 24 h after the inhalation, airway responsiveness was assessed by the time-integrated change in peak airway pressure (APTI; $\text{cmH}_2\text{O} \cdot \text{s}$) after intravenous acetylcholine challenge. Data are means \pm SD for five mice in each group. The results are from one representative experiment out of three. * $P < 0.05$, significantly different from the mean value of the corresponding control response (Wt).

Figure 7. Airway responsiveness to acetylcholine of Smad7 Tg mice. OVA-sensitized mice were challenged with inhalation of OVA (black bars) or saline (white bars). At 24 h after the inhalation, airway responsiveness was assessed by the time-integrated change in peak airway pressure (APTI; $\text{cmH}_2\text{O} \cdot \text{s}$) after intravenous acetylcholine challenge. Data are means \pm SD for five mice in each group. The results are from one representative experiment out of three. * $P < 0.05$, significantly different from the mean value of the corresponding control response (Wt).

Contrary to our prediction based on the previous finding that TGF- β null mice developed severe inflammation in various organs and an autoimmune phenotype (5, 6), Smad7 Tg mice did not spontaneously show a specific phenotype. We also found that development of T cells and B cells was normal in Smad7 Tg mice (Fig. 3). These findings suggested that TGF- β /Smad signaling in mature T cells was not critical for T cell homeostasis under normal circumstances.

The spontaneous phenotype presented here was also distinct from those recently reported by three groups in Smad3 null mice in which splenic T cells were insensitive to TGF- β (19, 24, 25). Intestinal malignancy (24), a progressive illness associated with opportunistic infection (19), and an early phenotype of forelimb malformation (25) were not observed in our Smad7 Tg mice. In addition, impaired local inflammation in the process of wound healing was recently reported in Smad3 null mice (26). Since TGF- β acts on multiple targets including epithelial cells, neutrophils, and macrophages/monocytes (1), Smad3 null mice should have defects in a variety of TGF- β -responsive cells. For instance, the reduced inflammation during the process of wound healing could be attributed to a blunted chemotactic response of monocytes and neutrophils to TGF- β (26). In our Smad7 Tg mice, those cells are intact in the TGF- β response. Thus, the T cell-selective inhibition of TGF- β /Smad signaling could explain at least in part the phenotypic differences observed between Smad7 Tg mice and Smad3 null mice.

Antigen-induced airway inflammation was enhanced in Smad7 Tg mice (Fig. 4). Since CD4 T cells and IL-5 play a central role in regulating antigen-induced airway inflammation (20) and antigen-induced IL-5 production was enhanced in Smad7 Tg mice (Fig. 5), the enhanced antigen-induced airway inflammation could be attributed to the enhanced IL-5 production in Smad7 Tg mice. CD4 T cells were thought to be a major source of IL-5 production in the airways because we had preliminary data that IL-5 (and also INF- γ) production in the BALF of sensitized mice after OVA challenge was not detected after depletion of CD4 T cells with anti-CD4 antibody immediately before the OVA challenge (data not shown).

Recently, a cooperative role of Th1 cells with Th2 was reported (27, 28), indicating that INF- γ at physiologically relevant levels was insufficient to neutralize the effects of IL-5 in vivo. Thus, although we observed enhanced INF- γ production in Smad7 Tg mice (Fig. 5), relative contributions of INF- γ to the airway inflammation might be small.

We found that T cells infiltrating into the airways were increased and more activated in Smad7 Tg mice (Fig. 6). In addition, production of TGF- β , which was shown to inhibit the production of and response to cytokines in T cells (4), in the airways of Smad7 Tg mice was equivalent in Wt littermates (Fig. 5 C). Taken together, these findings suggested that enhanced IL-5 and INF- γ production in the airways of Smad7 Tg mice might be due to the enhanced activation of T cells that could have escaped from the inhibitory action of TGF- β .

Another reason for the enhanced cytokine production in Smad7 Tg mice might be that blockade of TGF- β /Smad signaling by Smad7 in T cells augmented differentiation of naive T cells into both Th1 and Th2 cells, resulting in enhanced cytokine production. The role of TGF- β /Smad signaling in T cell differentiation towards Th1 and/or Th2 subsets is still unclear (4). Our findings of higher production of IL-4, IL-5, and IFN- γ in Smad7 Tg mice than in Wt littermates (Figs. 5 and 6) suggested that generation of Th1 and Th2 cells was enhanced under the conditions in which differentiation of T cells was not influenced by TGF- β . However, more defined experiments using a purified population of CD4⁺ T cells are clearly needed to claim that generation of Th1 and Th2 cells was enhanced in Smad7 Tg mice.

Furthermore, most recently Gorelik et al. showed that abrogation of TGF- β signaling in T cells led to a spontaneous autoimmune phenotype using Tg mice expressing the dominant-negative TGF- β type II receptor selectively in T cells (29). Their Tg mice developed inflammatory infiltration in several organs and circulating autoimmune antibodies after 3–4 mo of age, which were not seen in our Smad7 Tg mice. The different mouse backgrounds may explain this difference, since their Tg mice were backcrossed onto a B10.BR background whereas our Tg mice were backcrossed onto a B6 background. Another explanation is that there may be TGF- β signals that are not blocked by overexpression of Smad7 in T cells, which would lead to the largely normal phenotype in our Smad7 Tg mice. The latter possibility should be investigated in the future.

In summary, we showed that blockade of TGF- β /Smad signaling in mature T cells by overexpression of Smad7 enhanced antigen-induced airway inflammation and airway reactivity. Our findings suggested that TGF- β /Smad signaling in mature T cells was crucial for negative regulation of the inflammatory (immune) response. Our findings also implicated TGF- β /Smad signaling in mature T cells as a regulatory component of allergic asthma.

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