

TGF- β Regulates Airway Responses Via T Cells¹

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Allergic asthma is characterized by airway hyperreactivity, inflammation, and a Th2-type cytokine profile favoring IgE production. Beneficial effects of TGF- β and conflicting results regarding the role of Th1 cytokines have been reported from murine asthma models. In this study, we examined the T cell as a target cell of TGF- β -mediated immune regulation in a mouse model of asthma. We demonstrate that impairment of TGF- β signaling in T cells of transgenic mice expressing a dominant-negative TGF- β type II receptor leads to a decrease in airway reactivity in a non-Ag-dependent model. Increased serum levels of IFN- γ can be detected in these animals. In contrast, after injection of OVA adsorbed to alum and challenge with OVA aerosol, transgenic animals show an increased airway reactivity and inflammation compared with those of wild-type animals. IL-13 levels in bronchoalveolar lavage fluid and serum as well as the number of inducible NO synthase-expressing cells in lung infiltrates were increased in transgenic animals. These results demonstrate an important role for TGF- β signaling in T cells in the regulation of airway responses and suggest that the beneficial effects observed for TGF- β in airway hyperreactivity and inflammation may be due to its regulatory effects on T cells. *The Journal of Immunology*, 2003, 170: 1313–1319.

The prevalence of allergic airway diseases such as asthma is increasing in industrialized nations (1). Allergic asthma is characterized by airway hyperreactivity (AHR),³ inflammatory infiltrates with eosinophilic leukocytes, lymphocytes, and mast cells, increased mucus secretion, and elevated serum IgE levels (2). It is well recognized that Th2 cytokines, such as IL-4, IL-5, IL-9, and IL-13, are important for the phenotype observed (3). IL-4 has been considered the main mediator of AHR, but it has recently been shown that IL-13 alone is sufficient for the development of AHR, while having less influence on airway inflammation (4–6).

The role of Th1 cytokines for the development of allergic airway disease is poorly understood. It has been described that Th1 cytokines, such as IL-12 or IFN- γ , can antagonize the actions of Th2 cytokines in asthma models (7–10). However, there seems to be no clear distinction between beneficial Th1 cytokines and more detrimental Th2 cytokines in the pathogenesis of asthma. Recent evidence suggests that Th1 cytokines, such as IFN- γ , can influence the development of Th2 subpopulations depending on the level of IFN- γ at the time of stimulation (11). IFN- γ even augments some of the effects of IL-13 in a T cell transfer model for asthma (12).

Moreover, cotransfer of Th1 cells failed to counteract Th2-mediated AHR but induced airway inflammation in another transfer model (13). In humans, there is indirect evidence for protective effects of a Th1 cytokine shift in patients with multiple sclerosis or infected with *Mycobacterium tuberculosis* (14, 15), and Th1 cytokine production in infancy appeared to protect from development of atopy in childhood (16). In contrast, both Th1 and Th2 T cells can be found in the lungs of asthmatic patients (17), and the administration of IL-12 has failed to show significant effects in the early and late asthmatic response (18). These conflicting data underline the need for a better understanding of the regulatory mechanisms and cytokines involved in the development of AHR and airway inflammation.

TGF- β is a pleiotropic factor produced by a variety of cells, with actions depending on the context of its production (19). The immunoregulatory effects of TGF- β have been demonstrated in TGF- β -null mice, which die by 4 wk of age due to multifocal inflammatory lesions mainly in the lung and heart (20, 21). It has been recently shown that TGF- β -producing T cells are able to ameliorate Th2-induced AHR and airway inflammation (22). The effector cells and the modulation of the cytokine response by TGF- β in Ag-specific AHR are largely unknown.

The three isoforms of TGF- β (TGF- β 1, - β 2, and - β 3) expressed in mammals bind to the same receptor complex (23). The receptor consists of a type II chain that is phosphorylated upon binding of the TGF- β dimer. Subsequently, the type I receptor is recruited and itself phosphorylated by the intracellular serine/threonine kinase domain of the type II receptor (24–26). The signal is then forwarded to the nucleus via activated Smad proteins (27).

We have generated transgenic mice that overexpress a truncated version of the type II receptor lacking the intracellular kinase domain under control of the human CD2 promoter in T cells (47). In these mice, the regulatory effects TGF- β exerts on T cells are impaired.

We performed this study to determine the effects of TGF- β on T cells in the regulation of AHR and inflammation in a well-established murine asthma model. Our results show that TGF- β regulates airway responses via its effects on T cells. Impairment of

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³ Abbreviations used in this paper: AHR, airway hyperreactivity; BALF, bronchoalveolar lavage fluid; iNOS, inducible NO synthase; alum, aluminum potassium sulfate; Penh, enhanced pause; MCh₅₀, concentration of methacholine that causes a 50% reduction in midexpiratory airflow.

TGF- β signaling in T cells leads to decreased airway reactivity associated with increased serum levels of IFN- γ in an Ag-independent context. In contrast, upon Ag-specific challenge, increased AHR and inflammation develop in transgenic animals. Elevated IL-13 levels in bronchoalveolar lavage fluid (BALF) and sera and a marked increase in inducible NO synthase (iNOS)-expressing cells are detected in lung tissue from transgenic mice. This suggests an important role for TGF- β in regulating IL-13 and iNOS in AHR and lung inflammation.

Materials and Methods

Animals

The generation and characterization of transgenic hCD2- Δ kt β RII mice is described elsewhere (47). All transgenic lines were established and maintained as heterozygotes on a FVB/N background. Susceptibility to Ag-induced AHR and inflammation in FVB/N mice has been previously assessed (28). Animals from line 2 were used between 6 and 12 wk of age and were age and sex matched for each experiment. Nontransgenic littermates were used as controls. Animal care was in accordance with the governmental and institutional guidelines.

Sensitization protocol and measurement of airway reactivity

Wild-type and transgenic mice were injected i.p. with 100 μ g of OVA (0.2 ml of 500 μ g/ml in normal saline; Calbiochem, Bad Soden, Germany) adsorbed to aluminum potassium sulfate (alum; Sigma-Aldrich, Deisenhofen, Germany) or with saline only and alum on days 0 and 14. On days 25, 26, and 27, mice were challenged with a 1% (w/v) OVA/PBS aerosol for 20 min. Twenty-four hours after the last aerosol challenge, four mice at a time were placed in a head-out body plethysmograph and exposed to increasing concentrations of nebulized methacholine essentially as described (29). The concentration of methacholine that causes a 50% reduction in midexpiratory airflow (MCh₅₀; mg/ml) was determined as a measure of airway reactivity. Eleven wild-type mice injected with saline, 13 wild-type mice injected with OVA, 11 transgenic mice injected with saline, and 7 transgenic mice injected with OVA were included in two separate experiments (including 13 and 29 mice, respectively). For the analysis of prolonged airway reactivity, AHR to methacholine was assessed 10 days after the last aerosol challenge in 10 wild-type and 6 transgenic mice.

Baseline airway reactivity in untreated wild-type and transgenic mice was assessed by analyzing enhanced pause (Penh) responses at baseline and after exposure to 50 and 100 mg/ml aerosolized methacholine for 3 min in a body plethysmograph (model PLY 3211; Buxco Electronics, Birmingham, U.K.) (30). Results are expressed as the mean Penh over 5 min at rest and as the mean fold increase in Penh within 5 min after methacholine treatment. Eight wild-type and eight transgenic mice were included in this experiment.

ELISA

Cytokine levels of IL-4, IL-13, and IFN- γ in BALF and serum were measured using Mouse BD OptEIA ELISA sets (BD PharMingen, Heidelberg, Germany) according to the manufacturer's instructions.

For detection of OVA-specific IgE in mice sera, plates were coated for 12 h with 20 μ g/ml OVA in PBS at 4°C. After overnight incubation with sera from mice at 4°C, HRP-conjugated rat anti-mouse IgE (Southern Biotechnology, Birmingham, AL) was used for detection of specific IgE.

Assessment of leukocyte distribution in BALF

After measuring airway reactivity, mice were sacrificed, the trachea cannulated, and bronchoalveolar lavage was performed twice with 0.8 ml of ice-cold PBS. The two BALF aliquots of each animal were pooled, and the recovered volume and total cell number were recorded. Cytospins were prepared from each sample by centrifugation of 50 μ l of BALF. After fixation, cytopins were stained with May-Grünwald Giemsa. Cells were classified as macrophages, neutrophils, eosinophils, and lymphocytes using standard morphological criteria. Cell-free BALF was stored at -70°C until further use.

Histological assessment of inflammation

The lungs were inflated and fixed in formalin and embedded in paraffin. Five-micrometer sections were stained with H&E or May-Grünwald Giemsa. For the determination of the degree of histological inflammation, H&E-stained lung sections were scanned with a digital camera (Olympus, Hamburg, Germany) and the areas of inflammation measured using analy-

SIS 3.0 software (Software Imaging Systems, Münster, Germany). The percentage of inflamed area in relation to the lung area scanned is given. A representative area of the lung was chosen for measurement, and 20 mm² of lung tissue was analyzed per animal.

Immunohistochemistry

For immunohistochemistry, 2- μ m sections were cut. Immunohistochemical staining was performed after blocking endogenous peroxidase with methanol and hydrogen peroxide. For the staining of T cells, rat anti-mouse CD3 mAb (NovoCastra, Newcastle, U.K.), biotinylated anti-rat Ab (BD PharMingen), and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) were used. Staining was enhanced with ammonium nickel sulfate hexahydrate. For the staining of B cells, anti-CD45R/B220 mAb (BD PharMingen) was used as primary Ab. For the detection of iNOS, anti-iNOS Ab (Biomol, Hamburg, Germany) and biotinylated anti-rabbit Ab (Sigma-Aldrich) were used. Granulocytes were stained using the specific naphthol AS-D chloroacetate esterase kit (Sigma-Aldrich) according to the instructions of the supplier. Immunohistochemical analysis was performed on at least three animals per group. For the analysis of iNOS expression, three transverse sections through the right and left lung were scored per animal (four wild-type and three transgenic mice injected with OVA and alum and an equal number of wild-type and transgenic mice injected with saline and alum). The number of iNOS-expressing cells per 10 mm² was counted in each section.

Statistical analysis

Mean results $\pm$ SEM (and for $n < 8$, median and range) are given. For comparison of groups, the two-sided Wilcoxon rank sum test was applied. A value of $p < 0.05$ was considered significant.

Results

hCD2- Δ kt β RII mice show decreased airway reactivity after Ag-independent immune activation with alum

We have previously described that the T cells in transgenic hCD2- Δ kt β RII mice were shifted toward a Th1 phenotype upon activation (47). Therefore, it was of interest to analyze the baseline airway reactivity in wild-type and transgenic mice with impaired TGF- β signaling in T cells. Baseline Penh showed no significant difference between untreated wild-type and transgenic mice at rest (wild-type, 0.87 ± 0.08 , vs transgenic, 0.83 ± 0.07) and a trend toward decreased airway reactivity after exposure to methacholine (fold increase in Penh: wild-type, 1.49 ± 0.21 , vs transgenic, 1.21 ± 0.16 ; Fig. 1A). Airway reactivity to methacholine was then determined after immunization with saline and alum and challenge with OVA aerosol. Interestingly, transgenic mice were significantly less susceptible to AHR induced by methacholine than were their wild-type littermates (MCh₅₀: wild-type-saline, 61.7 ± 3.9 mg/ml, vs transgenic-saline, 103.0 ± 6.5 mg/ml; $p < 0.001$; Fig. 1B) after this Ag-independent immune activation with alum. This might indicate a beneficial role for a Th1 environment in improving AHR in the setting of impaired TGF- β signaling in T cells.

Ag-specific aerosol challenge increases AHR in hCD2- Δ kt β RII mice

After immunization with OVA adsorbed to alum and Ag-specific aerosol challenge, transgenic mice increased in AHR from their reduced levels of responsiveness to levels similar to those of wild-type mice (MCh₅₀: wild-type-OVA, 45 ± 3 mg/ml, vs 48 ± 4 mg/ml; Fig. 1B). Compared with airway reactivity after Ag-independent immune activation, Ag-specific immunization therefore led to a significant increase in AHR to methacholine in transgenic mice (wild-type-OVA, $73 \pm 5\%$, vs transgenic-OVA, $48 \pm 4\%$, in comparison to mice injected with saline and alum; $p = 0.001$).

In these experiments, AHR was analyzed on the day after the last aerosol challenge. To assess whether transgenic mice maintain AHR for a longer period of time, mice were measured 10 days after the last aerosol challenge with OVA. AHR was found to be

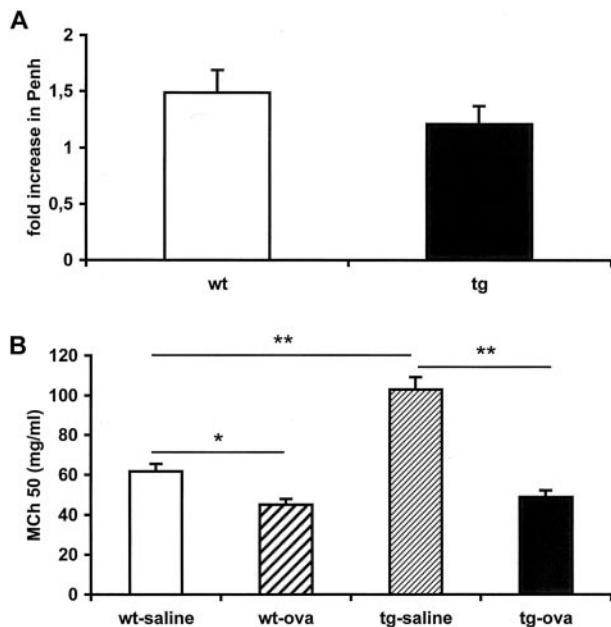


FIGURE 1. TGF- β signaling in T cells regulates airway reactivity. *A*, Baseline airway reactivity is not significantly different between wild-type and hCD2- Δ KT β RII FVB/N mice. Eight animals per group were analyzed in a body plethysmograph after exposure to 50 mg/ml methacholine for 3 min. *B*, Wild-type and transgenic mice were injected i.p. with saline and alum and challenged with OVA aerosol in an Ag-independent model. Airway reactivity to methacholine was measured in a head-out body plethysmograph. The results of two separate experiments including 11 wild-type and 11 transgenic mice are reported. Transgenic mice show significantly decreased airway reactivity to methacholine compared with that of wild-type mice after injection with saline and alum; *, $p = 0.003$; **, $p = 0.001$. By contrast, after sensitization with OVA and challenge with OVA, transgenic mice increase their airway responsiveness to the level of wild-type mice. Therefore, compared with the injection with saline, transgenic mice demonstrate a significantly greater increase in airway reactivity than do wild-type mice. Thirteen wild-type and seven transgenic mice were included in two separate experiments.

increased compared with the day following aerosol challenge, because exposure to 50 mg/ml methacholine led to a 75% mortality due to bronchial spasm in transgenic mice. Therefore, MCh₅₀ could not be determined in these experiments. Exposure to 25 mg/ml methacholine led to a decrease in expiratory flow to 71% in transgenic mice, whereas wild-type mice remained at 96% (data not shown).

Increased inflammation in the lungs of hCD2- Δ KT β RII mice upon Ag-specific challenge

Analysis of BALF. Because AHR appears to be differently regulated compared with lung inflammation in various other models, we determined whether impaired TGF- β signaling in T cells leads to differences in inflammation in the lungs of transgenic mice. After aerosol challenge and measurement of AHR, bronchoalveolar lavage was performed. The recovered volume did not differ significantly between the groups (wild-type-saline, 1.37 ± 0.08 ml; transgenic-saline, 1.26 ± 0.1 ml; wild-type-OVA, 1.41 ± 0.04 ml; and transgenic-OVA, 1.35 ± 0.06 ml). Total cell number was determined and cells differentiated according to morphology (Fig. 2). In animals injected with saline and alum, very few cells could be recovered in BALF (wild-type-saline, $5.3 \pm 1.6 \times 10^3$, and transgenic-saline, $6 \pm 1.5 \times 10^3$). The cell population in BALF of these animals consisted almost exclusively of macrophages (wild-

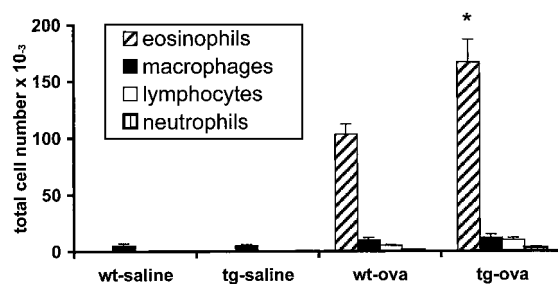


FIGURE 2. Increased airway inflammation in hCD2- Δ KT β RII mice. Eosinophils, macrophages, lymphocytes, and neutrophils in BALF were differentiated using cytopins and the total cell number of each cell population was determined. Mice sensitized and challenged with OVA demonstrated a marked increase in cell sequestration into the bronchial lumen compared with that of mice injected with saline and alum. The number of eosinophils was significantly increased in transgenic as compared with wild-type mice specifically challenged; *, $p = 0.022$.

type-saline, 99% (96–100%), and transgenic-saline, 99% (93–100%); median and range).

After injection with OVA adsorbed to alum and Ag-specific aerosol challenge, a strong inflammatory response was induced as demonstrated by a massive increase in inflammatory cells in BALF (Fig. 2). Significantly more inflammatory cells were counted in BALF of transgenic mice, demonstrating the importance of TGF- β signaling in T cells for the regulation of lung inflammation in addition to AHR (wild-type-OVA, $105 \pm 11 \times 10^3$ per mouse, vs transgenic-OVA, $191 \pm 22 \times 10^3$ per mouse; $p < 0.01$). The predominant cell type shifted from macrophages to eosinophils, and the proportion of different cell types detected was similar between wild-type and transgenic animals (wild-type-OVA, macrophages, $7.8 \pm 0.8\%$, eosinophils, $86.7 \pm 1\%$, neutrophils, $1.2 \pm 0.3\%$, and lymphocytes, $4.5 \pm 0.6\%$, vs transgenic-OVA, macrophages, $6.1 \pm 0.9\%$, eosinophils, $87.1 \pm 1.8\%$, neutrophils, $1.6 \pm 0.8\%$, and lymphocytes, $5.4 \pm 1.1\%$; for absolute numbers, see Fig. 2).

Extent of infiltration. To obtain an objective measure not only for sequestration of inflammatory cells in the alveolar and bronchiolar lumen but also for cellular infiltrates within the lung tissue, we measured the areas of tissue infiltration using digital scanning of H&E lung sections. The infiltrated lung area was calculated in relation to the total lung area examined. There was a marked increase in infiltrated lung area after injection with OVA adsorbed to alum and Ag-specific aerosol challenge in transgenic mice compared with that of wild-type mice (transgenic-OVA, 11.95% (9.69–19.95%), vs 7.36% (3.99–7.49%); median and range). In contrast, after injection of saline and alum, transgenic mice showed less infiltrated area compared with that of wild-type mice, keeping in mind that the area of infiltration was very small in both transgenic and wild-type mice in this Ag-independent model (transgenic-saline, 0.19% (0.12–0.48%), vs wild-type-saline, 0.62% (0.61–0.79%); median and range).

Histological characterization of inflammatory infiltrates

Using standard and immunohistochemical staining of lung sections, the inflammatory infiltrates were characterized. The lungs of animals injected with saline and alum showed only minimal inflammation in wild-type (not shown) as well as in transgenic mice (Fig. 3A, H&E). After injection with OVA adsorbed to alum and Ag-specific challenge, an inflammatory reaction was seen in the lungs of wild-type and transgenic animals with a stronger inflammation in the lungs of transgenic animals (Fig. 3B, transgenic,

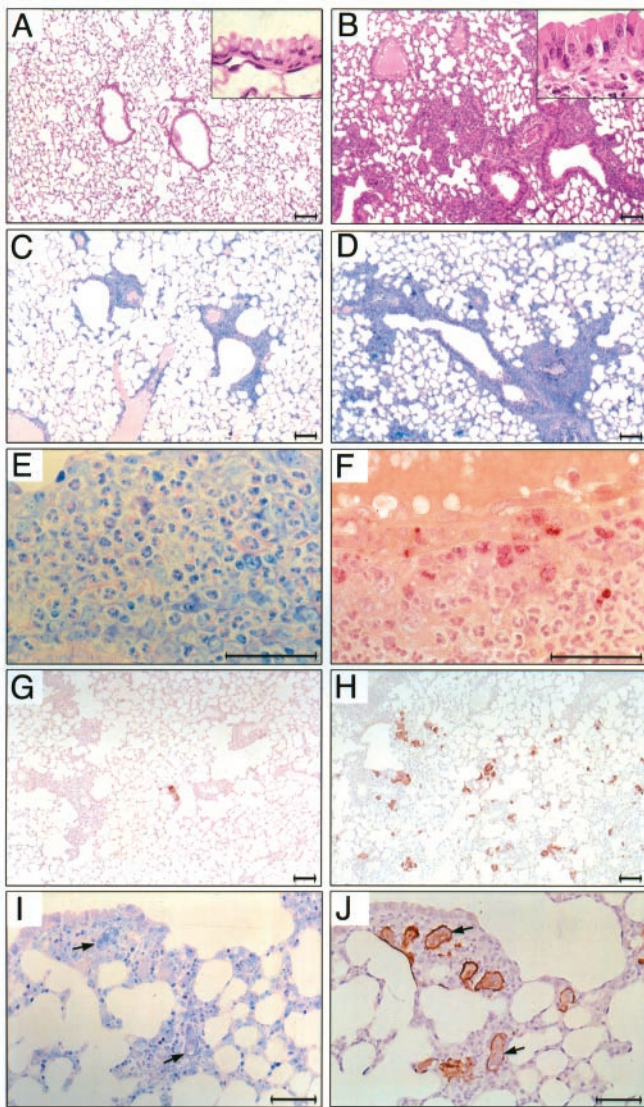


FIGURE 3. Histological examination of lungs. Lung sections were stained with H&E or Giemsa, or immunohistochemically for the analysis of inflammatory infiltrates. Bars represent 100 μ m. **A**, Transgenic mice show almost no inflammatory activity after injection with saline and alum and challenge with OVA aerosol. *Inset*, High-power magnification showing normal bronchiolar epithelium (H&E). **B**, After injection with OVA adsorbed to alum and Ag-specific aerosol challenge, transgenic mice show a massive increase in inflammatory infiltrates. Smaller airways are obstructed by mucus. *Inset*, High-power magnification showing tall columnar cells rich in mucin and subepithelial infiltration with leukocytes (H&E). **C–E**, Mice were injected with OVA adsorbed to alum and challenged with OVA aerosol (Giemsa). **C**, Wild-type mice show moderate inflammatory infiltrates with mucus obstruction of small airways. **D**, Transgenic mice develop marked peribronchial infiltrates with thickening of alveolar walls. **E**, Higher magnification of infiltrates in transgenic mice demonstrating the predominance of eosinophils. **F**, Immunohistochemical staining showing infiltration of bronchial epithelium with neutrophils in transgenic mice after injection with OVA adsorbed to alum and Ag-specific aerosol challenge (naphthol AS-D chloroacetate esterase stain). **G–J**, Immunohistochemical staining for expression of iNOS was performed in animals injected with OVA adsorbed to alum and challenged with OVA aerosol. **G**, Very few iNOS-expressing cells were detected in wild-type lungs. **H**, Transgenic mice show a marked increase in iNOS-expressing cells within infiltrates. **I** and **J**, Serial lung sections were performed in transgenic animals and staining was performed in an alternating way with Giemsa and for iNOS. The majority of cells expressing iNOS were multinucleated giant cells (black arrows).

H&E; **C**, wild-type, Giemsa; and **D**, transgenic, Giemsa). Giemsa staining revealed eosinophils as the predominant cell type of inflammatory infiltrates (transgenic, Fig. 3E). Neutrophils were detected within inflammatory infiltrates but also within alveolar walls by staining with naphthol AS-D chloroacetate (Fig. 3F). Immunohistochemical staining using anti-CD3 and anti-CD45R/B220 Abs was performed to detect the number of T and B cells within the infiltrates. There were few lymphocytes within the infiltrates with no significant difference between transgenic and wild-type animals (data not shown).

Increased iNOS expression in the lungs of hCD2- Δ kt β RII mice

NO produced predominantly by iNOS has been implicated in immune regulation and specifically in the pathogenesis of asthma including the inhibition of Th1 responses. Therefore, we determined whether impaired TGF- β signaling in T cells influenced iNOS expression in peribronchiolar infiltrates. Immunohistochemical staining for iNOS revealed a marked increase in the number of iNOS-expressing cells in transgenic animals compared with that of wild-type animals after sensitization and challenge with OVA (Fig. 3, **G** and **H**, wild-type and transgenic, respectively). The number of iNOS-expressing cells per 10 mm² was counted on three separate sections per animal (wild-type-OVA, 3 ± 1 , vs transgenic-OVA, 72 ± 35 ; $p < 0.02$). Examination of serial sections showed that iNOS expression could be mainly attributed to multinucleated giant cells (Fig. 3, **I** and **J**, Giemsa and iNOS, respectively). No iNOS expression could be detected in transgenic or wild-type animals after injection with alum and saline only.

Increased levels of IL-13 in BALF and sera of transgenic hCD2- Δ kt β RII mice after Ag-specific challenge

IL-13 levels were determined in sera and BALF after challenge with OVA aerosol. Significantly increased levels of IL-13 could be detected in BALF of transgenic mice (transgenic-OVA, 388 (267–868) pg/ml, vs wild-type-OVA, 316 (169–451) pg/ml; $p = 0.02$) as well as sera of transgenic mice (transgenic-OVA, 290 (211–362) pg/ml, vs wild-type-OVA, 189 (126–282) pg/ml; median and range; $p < 0.005$; Fig. 4A) compared with wild-type mice after injection with OVA adsorbed to alum and subsequent Ag-specific challenge. No IL-4 could be detected in either group in BALF, indicating that IL-13 was the predominant Th2 cytokine that is regulated by the action of TGF- β on T cells in the model we used. In animals injected with alum and saline, no IL-13 was detected in BALF of wild-type or transgenic animals after challenge with OVA aerosol, and little IL-13 was measured in sera with no significant difference between wild-type and control animals.

Inverse relation between IFN- γ serum levels and airway reactivity

To determine whether a Th1 environment might play a role in protection from AHR, IFN- γ levels were determined in sera and BALF of mice after injection with alum and saline and challenge with OVA aerosol. Increased levels of IFN- γ were detected in sera of transgenic mice (transgenic-saline, 2625 (1060–8570) pg/ml, vs wild-type-saline, 1420 (1120–2550) pg/ml; median and range; Fig. 4B), indicating a role for IFN- γ in protection from AHR. In contrast, after injection with OVA adsorbed to alum and Ag-specific aerosol challenge, serum levels were decreased in transgenic animals, paralleling the increased AHR observed (transgenic-OVA, 1905 (507–2800) pg/ml, vs wild-type-OVA, 3075 (778–7680) pg/ml). IFN- γ -levels in BALF were low and no difference between transgenic and wild-type mice could be detected after Ag-specific challenge (transgenic-OVA, 63 (0–221) pg/ml, vs wild-type-OVA, 78 (0–139) pg/ml; median and range).

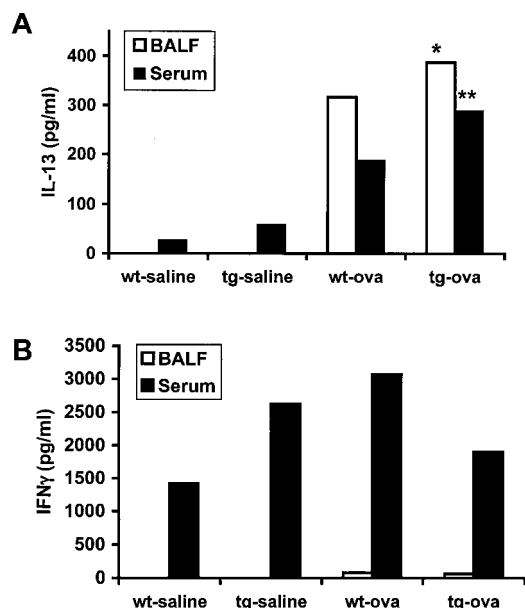


FIGURE 4. Analysis of cytokine production in BALF and sera. Cytokine levels were determined in BALF and sera harvested after aerosol challenge using ELISA. **A**, IL-13 levels in BALF and sera of transgenic mice were significantly increased in comparison to those of wild-type mice after injection with OVA adsorbed to alum and Ag-specific aerosol challenge; *, $p = 0.02$; **, $p < 0.005$. **B**, In transgenic mice, increased levels of IFN- γ could be detected in sera after injection with saline and alum and challenge with OVA aerosol, whereas levels were decreased after injection with OVA adsorbed to alum and Ag-specific aerosol challenge. Low levels of IFN- γ could be detected in BALF of these mice.

No significant difference in OVA-specific IgE Ab serum levels

As increased Th2 cytokine levels influence IgE production, we determined whether OVA-specific IgE serum levels differed in wild-type and transgenic animals. We found a strong increase in OVA-specific IgE levels in sera of mice injected with OVA adsorbed to alum with a small and nonsignificant difference between specific IgE serum levels in transgenic and wild-type mice (transgenic-OVA, 0.35 (0.23–0.6), vs wild-type-OVA, 0.29 (0.12–0.63); OD, median and range). Levels of OVA-specific IgE after injection with alum and saline and subsequent aerosol challenge were very low.

Discussion

Our results demonstrate that, in a murine asthma model, TGF- β regulates airway responses via its effects on T cells. The importance of TGF- β in maintaining immune homeostasis has been impressively demonstrated in TGF- β -knockout mice, which die within the first 4 wk of life due to multifocal inflammatory lesions, especially in heart and lungs (20, 21). TGF- β is a pleiotropic cytokine, which is produced by a variety of cells and exerts its effects depending on the effector cell and the context of production (19). The role of TGF- β signaling in T cells in immune regulation has been studied by impairing TGF- β signaling using dominant-negative TGF- β type II receptors (31, 32) or overexpression of Smad7, an inhibitory Smad protein (33). The phenotype of these transgenic mice turned out to be different, probably depending on the strain of mice used, the promoter specificity, expression pattern, and the strength of transgene expression. The mice we described exhibit a normal morphological phenotype similar to the mice overexpressing Smad7 (33). These mice showed increased airway inflammation and reactivity, which suggested that signaling

via Smad proteins has a role in regulating airway responses. Smad7 is an inhibitory molecule not only for TGF- β signaling but also for other members of the TGF- β family, such as activins and bone morphogenetic proteins (27). Therefore, it is difficult to correlate the phenotype observed in these mice to the missing influence of a distinct member of the TGF- β superfamily on T cells. Activins have been implicated in inflammatory processes, such as inflammatory bowel disease, wound repair, and inflammatory arthropathies (34). Activins also appear to be involved in the modulation of pulmonary fibrosis (35). It has recently been shown in an animal model that phosphorylation of Smad2 as a marker for an activated TGF- β or activin signaling pathway is increased in allergen-challenged lungs and that mRNA for activin showed a stronger induction than did mRNA for TGF- β isoforms (36). In addition, the interaction of different receptors and inhibitory Smads is poorly understood, which makes an interpretation of the actual role of TGF- β in the regulation of airway responses even more difficult (27). The dominant-negative TGF- β type II receptor specifically blocks TGF- β 1, - β 2, and - β 3 signaling. We can thus attribute the changes observed in airway reactivity, inflammation, and cytokine production specifically to the impaired effects of TGF- β on T cells.

TGF- β has been shown to be produced by a variety of cells within the lungs of asthmatic patients (37, 38, 39) and might therefore be an important regulator in allergic airway disease. Beneficial effects for TGF- β -producing T cells on airway inflammation were recently demonstrated using retrovirally transfected T cells secreting TGF- β (22). In addition, the effects of oral tolerization in eosinophilic tracheitis have been attributed to TGF- β -producing T cells (40). In this study, we have identified the T cell as a central effector cell of TGF- β -mediated regulation of airway responses. Upon Ag-specific challenge, mice with impaired TGF- β signaling in T cells demonstrated increased AHR compared with wild-type mice. An increase in lung inflammation predominated by eosinophils was detected in transgenic animals.

It is well known that T cells play a central role in airway inflammation by production of Th2 cytokines, such as IL-4, IL-5 and IL-13 (3). We were able to show a significant increase in IL-13 levels in BALF and sera of transgenic mice, suggesting that TGF- β signaling in T cells might be important for the regulation of IL-13 production. In various studies, the contribution of the different Th2 cytokines for the asthmatic phenotype has been examined, and it became evident that IL-4 is particularly important for Th2 lineage development and for IgE production by B cells (3). Using IL-13-knockout mice, it has recently been shown that IL-13 alone is sufficient for the development of AHR, while having less influence on airway inflammation (4). We did not detect IL-4 in either BALF or sera, suggesting that IL-13 might be more important than other Th2 cytokines for the phenotype observed.

The role of Th1 cytokines in the modulation of allergic responses is unclear. In humans, increased Th1 cytokine production in infancy has been reported to protect from atopic disorders in later life (16). A decreased incidence of asthma has been described in *Mycobacterium tuberculosis* infection (15) and in patients with multiple sclerosis (14). In contrast, Th1 cytokine-producing cells have been detected in the lungs of asthmatics (17) and the administration of rIL-12 in patients has failed to show significant benefit in the immediate and late asthmatic response (18). In mice, beneficial effects for the administration of Th1 cytokines were described. Application of IFN- γ and IL-12 were shown to inhibit Th2-mediated allergic responses (7–10). Still, the Th1/Th2 paradigm does not seem to hold true for IFN- γ in asthma models. Recently, it has been shown that IFN- γ inhibits certain effects like eosinophil count in BALF but potentiates others like IL-6 levels

and NK cell count in BALF when coadministered with IL-13 (12). Cotransfer of Th1 cells failed to counteract Th2-mediated AHR and even induced airway inflammation (13). In this study, we demonstrate that transgenic mice with impaired TGF- β signaling in T cells are less susceptible to AHR in a Th2-mediated asthma model after unspecific immunization with alum and saline. Together with the increased IL-2 and IFN- γ production by T cells and splenocytes previously described in the hCD2- Δ kT β R11 mice (47), in this study, we show increased levels of IFN- γ in sera of transgenic mice injected with alum and saline only. These mice had slightly less inflammatory infiltrates in lung tissue, but the area infiltrated was small compared with that of mice after injection with OVA adsorbed to alum and Ag-specific challenge. These results indicate a protective role for IFN- γ in the setting of an unspecific immune stimulation with alum. Interestingly, it has recently been shown that the level of IFN- γ at the time of stimulation influences the pattern of cytokine production by Th2 cells (11). It has been postulated that T cells modulate genetically determined airway responsiveness (41). It may be that TGF- β signaling in T cells is one of the factors involved in genetic susceptibility to AHR.

Upon Ag-specific challenge, IFN- γ serum levels were decreased in transgenic animals, and IL-13 was the predominant cytokine observed. In addition to a decreased inhibition of Th2 cytokines, it is possible that the remaining IFN- γ adds to the effects of IL-13 by increasing airway inflammation as has been described in an asthma model (12). Thus, it seems that IFN- γ has modulatory effects on Th2-mediated airway responses depending on the context of immune activation. This clearly warrants further investigation. Only minor differences could be detected in the level of OVA-specific IgE in our experiments, indicating that differential humoral responses play a minor role in the model we used.

In addition to Th1 and Th2 cytokines, NO has been implicated in the modulation of allergic airway disease (42). Increased amounts of NO were detected in exhaled air of animals and humans (43) and the expression of iNOS has been demonstrated in various cell types in the lung (44). It was postulated that NO enhances airway inflammation by suppression of Th1 cells and a lack of suppression of Th2 cytokine production (42). In accordance with this hypothesis, we found a markedly increased number of iNOS-expressing cells in the lungs of transgenic mice after injection with OVA adsorbed to alum and Ag-specific aerosol challenge. IFN- γ serum levels were reduced in these animals. The inhibition of iNOS expression by TGF- β has been examined in LPS-stimulated macrophages (45) and in cardiac myocytes after hypoxic stress (46). To our knowledge, the role of TGF- β signaling in T cells for the expression of iNOS has not been examined so far. In serial cuts, we were able to show that iNOS expression could be mainly attributed to multinucleated giant cells. Whether this is a specific effect of transgenic T cells on macrophages or a secondary effect due to increased inflammation remains to be elucidated.

Taken together, our results demonstrate that TGF- β acts on T cells to regulate airway reactivity and inflammation. Depending on the mode of immune stimulation, impaired TGF- β signaling in T cells modulates airway reactivity by the interaction of Th1 and Th2 cytokines. IL-13 and iNOS expression appears to play a role in the increased airway response observed after Ag-specific airway challenge of mice with impaired TGF- β signaling in T cells.

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