

# Transforming Growth Factor- $\beta$ Regulates House Dust Mite–induced Allergic Airway Inflammation but Not Airway Remodeling

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**Rationale:** It is now believed that both chronic airway inflammation and remodeling contribute significantly to airway dysfunction and clinical symptoms in allergic asthma. Transforming growth factor (TGF)- $\beta$  is a powerful regulator of both the tissue repair and inflammatory responses, and numerous experimental and clinical studies suggest that it may play an integral role in the pathogenesis of asthma.

**Objectives:** We investigated the role of TGF- $\beta$  in the regulation of allergic airway inflammation and remodeling using a mouse model of house dust mite (HDM)–induced chronic allergic airway disease.

**Methods:** We have previously shown that intranasal administration of an HDM extract (5 d/wk for 5 wk) elicits robust Th2-polarized airway inflammation and remodeling that is associated with increased airway hyperreactivity. Here, Balb/c mice were similarly exposed to HDM and concurrently treated with a pan-specific TGF- $\beta$  neutralizing antibody.

**Measurements and Main Results:** We observed that anti-TGF- $\beta$  treatment in the context of either continuous or intermittent HDM exposure had no effect on the development of HDM-induced airway remodeling. To further confirm these findings, we also subjected SMAD3 knockout mice to 5 weeks of HDM and observed that knockout mice developed airway remodeling to the same extent as HDM-exposed littermate controls. Notably, TGF- $\beta$  neutralization exacerbated the eosinophilic infiltrate and led to increased airway hyperreactivity.

**Conclusions:** Collectively, these data suggest that TGF- $\beta$  regulates HDM-induced chronic airway inflammation but not remodeling, and furthermore, caution against the use of therapeutic strategies aimed at interfering with TGF- $\beta$  activity in the treatment of this disease.

**Keywords:** immunology; allergic asthma; mouse model; lung

Asthma is a chronic disease that is characterized clinically by variable airflow obstruction and a decline in airway function (1). It is now believed that these outcomes are the consequences of two distinct, although likely connected, processes—namely,

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## AT A GLANCE COMMENTARY

### Scientific Knowledge on the Subject

Transforming growth factor (TGF)- $\beta$  appears to play a prominent role in the development of airway remodeling, a recognized feature of chronic allergic asthma.

### What This Study Adds to the Field

This research suggests that airway remodeling may be independent of TGF- $\beta$ , and that therapeutic strategies to prevent remodeling through TGF- $\beta$  interference may have detrimental effects by increasing eosinophilic infiltration and airway hyperreactivity.

allergic airway inflammation and airway remodeling (2–5). Experimental studies in animal models of allergic airway disease have afforded considerable understanding with respect to the nature and mechanisms that drive allergic inflammation; however, relatively speaking, our knowledge of the mechanistic processes that underlie the development of airway remodeling remains limited and in some cases controversial. Moreover, although it is generally accepted that inflammation-associated damage consequently triggers tissue reparative responses as a means of restoring tissue integrity, whether and how this is coordinately regulated in allergic airway disease are also poorly understood.

In the context of allergic asthma, the pathologic hallmarks of airway remodeling include increased subepithelial extracellular matrix deposition, alterations in the airway epithelium (goblet cell hyperplasia) leading to excessive mucus production, and airway smooth muscle thickening resulting from smooth muscle cell hypertrophy and hyperplasia (6, 7). Among the various factors that are believed to be involved, transforming growth factor (TGF)- $\beta$ , a powerful regulator of the tissue repair response, has been strongly implicated in the development of airway remodeling. Clinical studies have documented increased TGF- $\beta$  expression on both the level of mRNA and protein in bronchial biopsies and in the bronchoalveolar lavage (BAL) of humans with asthma compared with control subjects (8–12). Similarly, TGF- $\beta$  expression has also been shown to be increased in animal models of allergic airway disease (13–17). In addition, the number of TGF- $\beta$ –expressing epithelial or submucosal cells has been correlated with the basement membrane thickness in patients with asthma (12). Direct evidence in support of a role for TGF- $\beta$  in the development of allergic airway remodeling comes from a study by McMillan and colleagues, who showed that TGF- $\beta$  blockade using a neutralizing antibody (Ab) reduced the extent of ovalbumin (OVA)–induced remod-

eling in a chronic mouse model (18). In addition, two very recent studies, one using SMAD3 knockout (KO) animals (19) and the other using blocking Abs (20), have also demonstrated that TGF- $\beta$  contributes to the development of airway remodeling in similar OVA-based systems. Animal studies demonstrating a critical role for TGF- $\beta$  in the pathogenesis of related diseases involving aberrant tissue repair, such as liver, kidney, and lung fibrosis, further support the notion of TGF- $\beta$  involvement in allergic airway remodeling (21–24).

Importantly, it is clear that TGF- $\beta$  is also one of the most potent negative regulators of inflammation (25). Indeed, TGF- $\beta$ -deficient mice die soon after birth due to rampant, multifocal inflammation (26). This activity may also be of particular relevance to allergic airway disease as TGF- $\beta$  is believed to contribute, at least in part, to the regulation of the Th2-polarized airway inflammatory response that is characteristic of this disease. Numerous *in vitro* studies have collectively shown that TGF- $\beta$  suppresses the activity and/or proliferation of various inflammatory cells, including Th2 cells, B cells, macrophages, and eosinophils (25, 27–29), and moreover, TGF- $\beta$  can promote their apoptosis (30–32). Three separate studies have investigated the impact of genetically interfering with TGF- $\beta$  activity on allergic sensitization and airway inflammation in acute models of allergic airway disease using OVA. Their findings consistently demonstrate that abrogation of TGF- $\beta$  or TGF- $\beta$  signaling pathways substantially potentiates the immune response and exacerbates the allergic inflammatory response (33–35).

In light of these reports, we and others have proposed that TGF- $\beta$  may play a dual role in asthma by coordinately regulating both the inflammatory and reparative responses; specifically, that TGF- $\beta$  may dampen the inflammatory response elicited upon aeroallergen exposure in a sensitized individual while subsequently initiating tissue repair processes. In this study, we investigated the role of TGF- $\beta$  in the regulation of chronic allergic airway inflammation and remodeling. To address this issue experimentally, we conducted a series of loss-of-function experiments in a model of chronic allergic airway disease induced by respiratory mucosal exposure to a clinically relevant aeroallergen, house dust mite (HDM). We have previously shown that repeated exposure to an HDM extract elicits robust Th2-polarized airway inflammation and remodeling that are associated with increased bronchial hyperreactivity (36). Here we report that mice exposed to HDM and concurrently treated with a pan-neutralizing anti-TGF- $\beta$  ( $\alpha$ TGF- $\beta$ ) Ab developed airway remodeling comparable to mice exposed to HDM and treated with an irrelevant Ab control. Similarly, we also observed that HDM-exposed SMAD3 KO mice developed remodeling to the same extent as HDM-exposed wild-type (WT) littermate control animals. Interestingly, we further demonstrate that TGF- $\beta$  neutralization exacerbates the eosinophilic inflammatory infiltrate and that this was associated with a significant increase in bronchial hyperreactivity. Collectively, these data suggest that TGF- $\beta$  regulates HDM-induced airway inflammation, but that it may not play a critical role in the generation of HDM-induced airway remodeling.

Some of the results of these studies have been previously reported in the form of an abstract (37).

## METHODS

### Animals

Female Balb/c mice (6–8 wk old) were purchased from Charles River Laboratories (Ottawa, ON, Canada). SMAD3 heterozygous mice (129SV/EV  $\times$  C57B/6 background; courtesy of Dr. A. Roberts, National Institutes of Health, Bethesda, MD) (38) were bred in-house.

The genotypes of both WT littermate and SMAD3 KO mice were determined by polymerase chain reaction analysis on tail DNA obtained from 3-week-old animals. All mice were housed under specific pathogen-free conditions and maintained on a 12-hour light:dark cycle, with food and water *ad libitum*. All experiments described in this study were approved by the Animal Research Ethics Board of McMaster University (Hamilton, ON, Canada).

### Sensitization Protocols

**Allergen administration.** HDM extract (Greer Laboratories, Lenoir, NC) was resuspended in sterile saline at a concentration of 2.5 mg (protein)/ml and 10  $\mu$ l were administered to isoflurane-anesthetized Balb/c mice intranasally. SMAD3 KO and littermate control mice received the same amount of HDM per dose (i.e., 25  $\mu$ g) delivered in 15  $\mu$ l of saline.

**Ab administration.** Murine pan-neutralizing  $\alpha$ TGF- $\beta$  specific for all forms of murine TGF- $\beta$  or an irrelevant murine isotype-matched control Ab (IgG) were administered to anesthetized mice intraperitoneally at a dose of 10 or 100  $\mu$ g (corresponding to  $\sim$ 0.5 and 5 mg/kg, respectively) in 0.5 ml of sterile phosphate-buffered saline immediately before HDM administration.

**Continuous HDM exposure protocol.** Separate groups of mice were exposed daily to HDM or saline for 5 consecutive days a week followed by 2 days of rest for a total of 5 weeks. Depending on the experiment,  $\alpha$ TGF- $\beta$  or control Ab was concurrently administered either at the beginning of the third week (Day 14) or on the fourth day of HDM exposure (Figures 1A and 1B). Ab was administered every other day until the mice were killed, 72 hours after the last challenge.

**Intermittent HDM exposure protocol.** Separate groups of mice were exposed daily to HDM or saline for 10 consecutive days followed by approximately 30 days of rest. We have previously shown that 10 days of HDM exposure elicits robust Th2-polarized allergic sensitization and airway inflammation (39). Saline- and HDM-sensitized mice were subsequently subjected to six cycles of saline or HDM reexposure, respectively; each cycle consisted of daily intranasal exposure for 3 consecutive days followed by 12 days of rest. In addition, mice were concurrently treated every other day with  $\alpha$ TGF- $\beta$  or control IgG over each of the six reexposure cycles beginning 2 days before the first saline/HDM exposure and ending on the second day after the last (i.e., third) saline/HDM exposure, for a total of four Ab administrations per cycle. Mice were killed 72 hours after the last challenge (Figure 1C).

### Collection and Measurement of Specimens

BAL fluid, lungs, blood, and spleen were collected at the time of killing. BAL was performed as previously described (40). Total cell counts were determined using a hemocytometer. Differential cell counts of BAL cells were determined from at least 500 leukocytes using standard hemocytologic criteria to classify the cells as neutrophils, eosinophils, or mononuclear cells. Where applicable, after BAL, the right lung was dissected for tissue homogenate preparation; the left lung was inflated with 10% formalin at a constant pressure of 20 cm H<sub>2</sub>O, and then fixed in 10% formalin for 48 to 72 hours. Peripheral blood was collected by retro-orbital bleeding and serum was obtained and stored at  $-20^{\circ}\text{C}$ . Harvested spleens were placed in sterile tubes containing sterile Hanks' balanced salt solution. See the online supplement for additional details on the methods used to make these measurements.

### Preparation of Lung Tissue Homogenate and Hydroxyproline Measurement

See the online supplement for details on the methods used to make these measurements.

### Histology and Immunohistochemistry

After formalin fixation, tissues were embedded in paraffin, and 3- $\mu$ m-thick cross-sections of the left lung were cut and stained with hematoxylin and eosin, Picro Sirius red (PSR), and periodic acid-Schiff (PAS). Immunohistochemistry for  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) was also performed. See the online supplement for additional details on the methods used to make these measurements.

### Morphometric Analysis

Images for morphometric analysis were captured with OpenLab software (version 3.0.3; Improvision, Guelph, ON, Canada) via a Leica

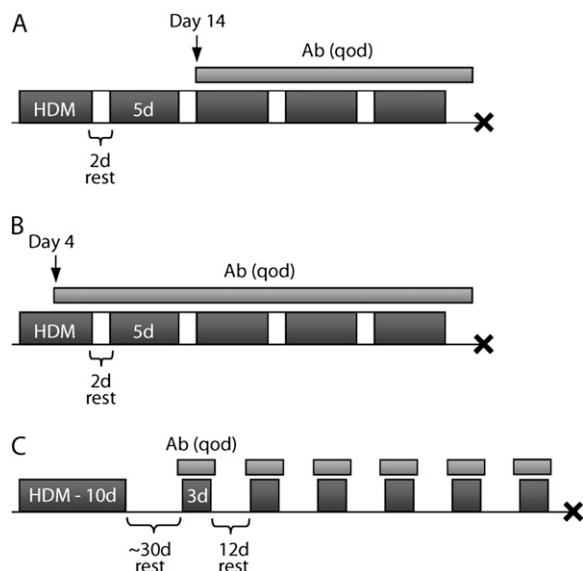


**Figure 1.** Schematic diagrams of the experimental protocols used. (A) Continuous house dust mite (HDM) exposure protocol: Separate groups of mice were exposed to saline or HDM for 5 weeks and concurrently treated (every other day) during the last 3 weeks of exposure (i.e., starting on Day 14) with either a pan-neutralizing anti-transforming growth factor- $\beta$  ( $\alpha$ TGF- $\beta$ ; 10 or 100  $\mu$ g/dose) antibody (Ab), or the control IgG Ab (100  $\mu$ g/dose). (B) Continuous HDM exposure protocol with extended treatment: Separate groups of mice were exposed to saline or HDM for 5 weeks and concurrently treated (every other day) beginning at Day 4 of exposure with either  $\alpha$ TGF- $\beta$  (100  $\mu$ g/dose) or the control IgG Ab (100  $\mu$ g/dose). (C) Intermittent HDM exposure protocol: Separate groups of mice were exposed to saline or HDM for a period of 10 consecutive days and then rested for approximately 30 days. Mice were subsequently subjected to six cycles of saline or HDM reexposure; each cycle consisted of daily exposure for 3 consecutive days followed by 12 days of rest. In addition, mice were concurrently treated (every other day) with either  $\alpha$ TGF- $\beta$  (100  $\mu$ g/dose) or the control IgG Ab (100  $\mu$ g/dose), over each 3-day reexposure to saline or HDM as described in METHODS. In all protocols, mice were killed 72 hours after the last challenge unless otherwise stated.

camera and microscope (Leica Microsystems, Richmond Hill, ON, Canada). Image analysis was performed using a custom computerized analysis system (Northern Eclipse software version 5; Empix Imaging, Mississauga, ON, Canada). Analysis of sections stained for  $\alpha$ -SMA, PSR, and PAS were performed as previously described (41). See the online supplement for additional details on the methods used to make these measurements.

### Splenocyte Culture

Splenocytes were isolated, resuspended in complete RPMI (Roswell Park Memorial Institute) at a concentration of  $8 \times 10^6$  cells/ml and cultured in medium alone, or with medium supplemented with HDM (31.25  $\mu$ g/ml) in a flat-bottom, 96-well plate (Becton Dickinson, Mississauga, ON, Canada), in triplicate. After 5 days of culture, supernatants were harvested and triplicates were pooled for cytokine measurements. See the online supplement for additional details on the methods used to make these measurements.

### Bioassay for TGF- $\beta$

See the online supplement for details on the methods used to make these measurements.

### Cytokine and Immunoglobulin Measurements by ELISA

Levels of IL-4, IL-5, IL-13, and active TGF- $\beta$ 1 were measured by ELISA using DuoSet kits purchased from R&D Systems (Minneapolis,

MN) according to the manufacturer's instructions. Levels of  $\alpha$ TGF- $\beta$  murine monoclonal Ab and HDM-specific IgE and IgG<sub>1</sub> were measured by sandwich ELISA. See the online supplement for additional details on the methods used to make these measurements.

### Airway Responsiveness Measurements

Airway responsiveness was measured on the basis of the response of total respiratory system resistance to increasing intravenous (internal jugular vein) doses of methacholine as previously described (42, 43). See the online supplement for additional details on the methods used to make these measurements.

### Data Analysis

Data were analyzed using SigmaStat version 2.03 (SPSS, Inc., Chicago, IL). Data are expressed as mean  $\pm$  SEM. Results were interpreted using analysis of variance with Fisher's least significant difference post hoc test, unless otherwise indicated. Differences were considered statistically significant when *P* values were less than 0.05.

## RESULTS

### Kinetic Analysis of HDM-induced Responses in the Lung

Consistent with our previous report, we observed that Balb/c mice exposed via the respiratory mucosa to HDM for 5 weeks develop robust Th2-polarized airway inflammation (36). Kinetic analysis of the BAL revealed that an increase in the total cell number is evident after just 1 week of HDM, peaks at 3 weeks, and is maintained at this level throughout the course of allergen exposure (Figure 2A). Furthermore, we observed that this process is associated with a progressive airway reparative response that results in altered airway structure (Figure 2A and Reference 36). These structural changes are incipient after 3 weeks of HDM exposure and overt after 5 weeks, and include increased subepithelial collagen deposition and smooth muscle thickening. The concept that inflammation and repair are related strongly implicates TGF- $\beta$ . Thus, we examined the expression of TGF- $\beta$  in the BAL of mice exposed to 1, 3, or 5 weeks of HDM. We observed that the concentration of active TGF- $\beta$ 1 was substantially elevated after 3 and 5 weeks of HDM compared with untreated (0 wk) and saline exposed control animals (data not shown), and appeared to be increased after just 1 week of HDM (Figure 2B). Interestingly, we noted that the increase in active TGF- $\beta$ 1 at 3 and 5 weeks corresponded with both the plateau in inflammation and the airway remodeling response. These findings supported our hypothesis that TGF- $\beta$  may be dampening the chronic allergic inflammatory response while at the same initiating reparative processes.

To directly investigate this *in vivo* we used a pan-neutralizing  $\alpha$ TGF- $\beta$  Ab that is specific for the active form of all three mammalian TGF- $\beta$  isoforms. This Ab has been previously shown to reduce the extent of OVA-induced allergic airway remodeling (18). Although the characteristics and functional properties of this Ab have been extensively assessed, we reconfirmed the functionality of each batch of Ab by testing its ability to block TGF- $\beta$ 1 detection by ELISA (Figure 2C) and TGF- $\beta$ -induced luciferase expression in the plasminogen activator inhibitor-1 luciferase-based (PAI-L) bioassay (data not shown). To determine whether the Ab is able to reach the airway walls and subepithelial space, we also tracked its kinetics and distribution *in vivo*, in three separate compartments: the serum, lung tissue, and airway lumen. Separate groups of mice were given a single intraperitoneal administration of  $\alpha$ TGF- $\beta$  and then killed 2, 6, and 12 hours postinjection. We observed that the  $\alpha$ TGF- $\beta$  Ab was already present at considerable levels in the serum and lung tissue 2 hours postinjection, could be detected in the airway lumen in as little as 6 hours, and that

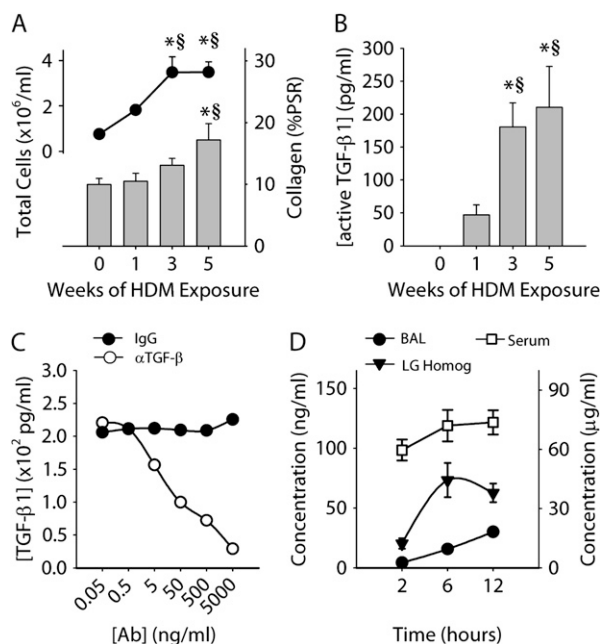


**Figure 2.** Kinetic analysis of house dust mite (HDM)-induced responses and pan-neutralizing anti-transforming growth factor- $\beta$  ( $\alpha$ TGF- $\beta$ ) antibody (Ab) distribution. (A) Kinetic analysis of HDM-induced airway inflammation and remodeling showing the total cell number in the bronchoalveolar lavage (BAL) (solid circles) and the extent of collagen deposition (shaded bars) based on morphometric analysis of Picro Sirius red-stained lung sections from mice exposed to 0 (naive), 1, 3, or 5 weeks of HDM. (B) Concentration of active TGF- $\beta$ 1 in the BAL of mice exposed to HDM for 0 (naive), 1, 3, or 5 weeks. (C) Functionality of  $\alpha$ TGF- $\beta$  Ab. A fixed amount of active TGF- $\beta$ 1 (200 pg/ml) was incubated with varying amounts of  $\alpha$ TGF- $\beta$  (open circles) or the isotype-matched control Ab (IgG; solid circles) for 2 hours and the amount of active TGF- $\beta$ 1 was then measured by commercial ELISA. (D) Concentration of  $\alpha$ TGF- $\beta$  Ab in the BAL (solid circles), lung homogenate (LG Homog; solid triangles), and serum (open squares) of mice killed at 2, 6, and 12 hours after one 100- $\mu$ g injection. Where applicable,  $n = 3$ –5/group. Data are expressed as mean  $\pm$  SEM.  $P < 0.05$  compared with \*0- and §1- week HDM-exposed groups, respectively.

concentrations steadily increased or were maintained thereafter (Figure 2D). This demonstrated that  $\alpha$ TGF- $\beta$  was indeed able to reach critical target regions in the lung.

### Impact of TGF- $\beta$ Blockade on the Development of HDM-induced Allergic Airway Remodeling

To investigate the role of TGF- $\beta$  in the development of allergic airway remodeling, we exposed Balb/c mice to daily administrations of HDM (5 d/wk) for 5 weeks (continuous HDM exposure protocol) and concurrently treated them with 10  $\mu$ g ( $\sim 0.5$  mg/kg) of  $\alpha$ TGF- $\beta$  Ab every other day during the last 3 weeks of exposure; this dose was shown to be efficacious in a previous study (18). Our intent was to begin TGF- $\beta$  neutralization when levels of active TGF- $\beta$ 1 in the BAL are low (Figure 2B) and airway remodeling is not yet apparent (Figure 2A), but at a time when allergic sensitization is fully established. We evaluated the impact of TGF- $\beta$  blockade on the development of HDM-induced airway remodeling both qualitatively and by quantitative morphometric analysis of three prominent remodeling-associated events: subepithelial collagen deposition, smooth muscle thickness, and mucus production (as a marker of goblet cell hyperplasia). We observed significant increases in each of the three above parameters in HDM-exposed animals,

regardless of whether they were treated with  $\alpha$ TGF- $\beta$  or the control IgG, compared with saline-exposed control animals (Figure 3). In fact, treatment with  $\alpha$ TGF- $\beta$  had absolutely no impact on the development of HDM-induced remodeling, because no differences were observed between HDM-exposed  $\alpha$ TGF- $\beta$ -treated and HDM-exposed IgG-treated mice (Figure 3). Saline- and HDM-alone-exposed groups (i.e., receiving no Ab treatment) did not differ from saline-exposed  $\alpha$ TGF- $\beta$ -treated and HDM-exposed IgG-treated control animals, respectively (data not shown). That  $\alpha$ TGF- $\beta$  treatment had no effect on the remodeling response is unlikely to be a consequence of inadequate dosing because HDM-exposed mice treated with a 10-fold higher dose of  $\alpha$ TGF- $\beta$  Ab (100  $\mu$ g/dose every other day corresponding to  $\sim 5$  mg/kg) displayed increases in collagen deposition, mucus production, and smooth muscle thickening that were virtually identical to HDM-exposed IgG-treated control animals (Figure 3). To confirm that TGF- $\beta$  was being neutralized under these experimental conditions, we assessed the levels of bioactive TGF- $\beta$  in the BAL fluid using the PAI/L bioassay for TGF- $\beta$ . We observed that  $\alpha$ TGF- $\beta$  treatment decreased the levels of bioactive TGF- $\beta$  in the BAL of HDM-exposed animals by approximately 80% compared with the HDM-exposed IgG-treated control group (Figure E1 of the online supplement), demonstrating that TGF- $\beta$  was being effectively neutralized in our system.

We proceeded to examine whether TGF- $\beta$  blockade was ineffective in the above experimental setting due to the timing of treatment initiation. Given that the levels of active TGF- $\beta$ 1 are increased early on during HDM exposure (1–2 wk; Figure 2B), albeit to a lesser extent than at later time points, we questioned whether this early expression may have been sufficient to trigger a cascade of events that subsequently drove the remodeling response in a manner that was then independent of TGF- $\beta$ . To address this, we subjected mice to the continuous HDM exposure protocol and treated them with  $\alpha$ TGF- $\beta$  or the control IgG over nearly the entire course of exposure. Even under this extended treatment regimen and using the 100- $\mu$ g dose, we observed that HDM-exposed  $\alpha$ TGF- $\beta$ -treated mice developed equivalent increases in subepithelial collagen, mucus production, and smooth muscle thickening as HDM-exposed IgG-treated mice (Figure 4). The similarity in the extent of collagen accumulation between HDM-exposed  $\alpha$ TGF- $\beta$ - and IgG-treated groups was further confirmed by a hydroxyproline assay (Figure E2).

Next, we ascertained whether the inability of TGF- $\beta$  neutralization to inhibit airway remodeling was due to the severity of the inflammatory response that is elicited by this protocol. To this end, we developed a model of intermittent HDM exposure that was based on short, recurrent HDM rechallenges separated by 12-day rest periods (Figure 1C), rather than continuous HDM exposure. In this model, we observed that each 3-day HDM rechallenge elicited an eosinophilic inflammatory response, which then partially resolved over the subsequent rest period; total cells and eosinophils were decreased by approximately 60 and 65%, respectively, before subsequent rechallenge. Thus, the structural alterations that develop in this experimental protocol are associated with a less severe inflammatory response. We subjected mice to this intermittent exposure protocol and treated them with four 100- $\mu$ g administrations of  $\alpha$ TGF- $\beta$  or the IgG control Ab during each HDM rechallenge (Figure 1C). We documented that  $\alpha$ TGF- $\beta$ -treated HDM-exposed mice developed identical increases in subepithelial collagen deposition as HDM-exposed IgG-treated control animals (Figures 5A–5D), demonstrating that, even under these less severe conditions, TGF- $\beta$  blockade had no effect on the development of HDM-induced airway remodeling.

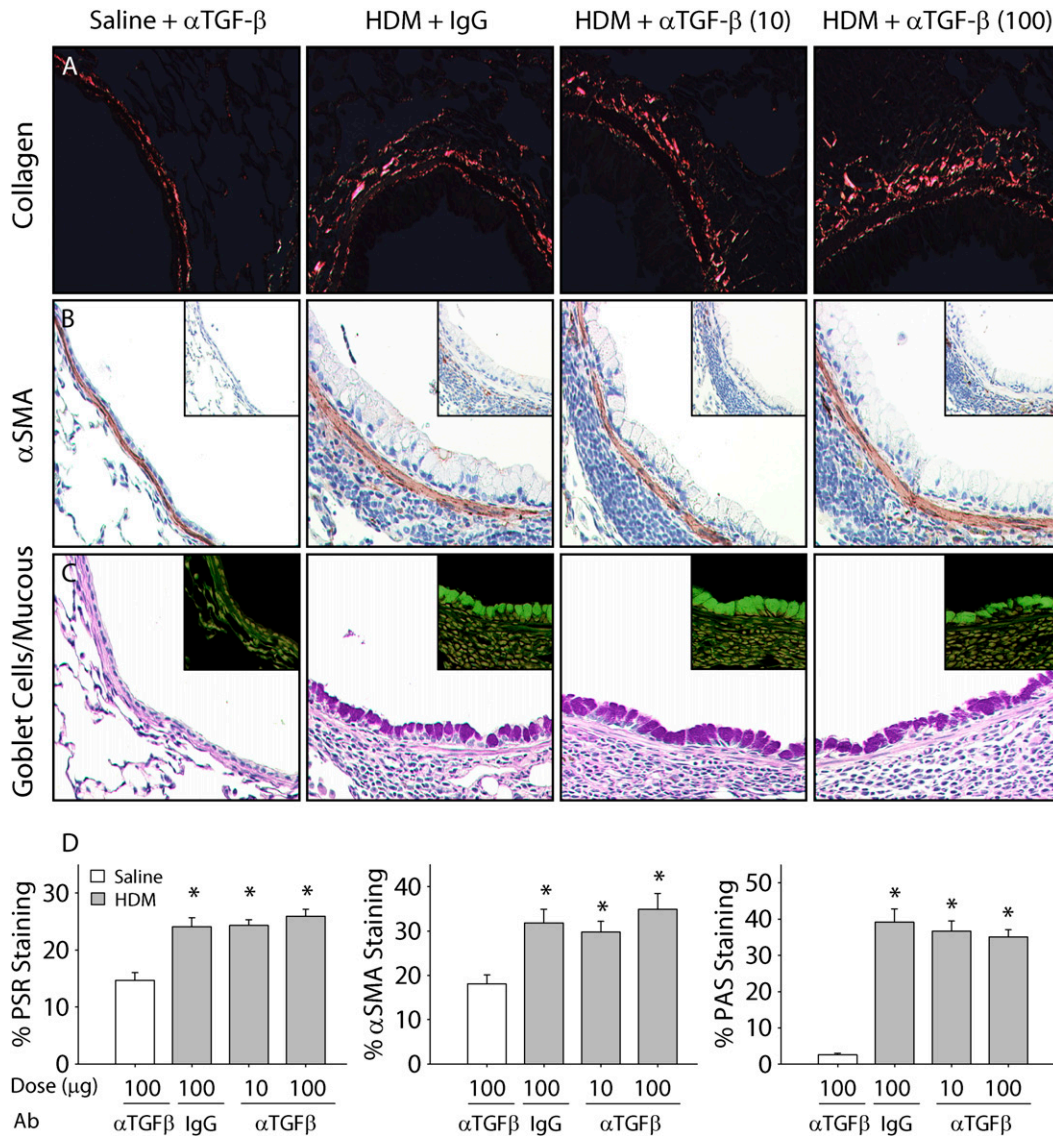


**Figure 3.** Impact of transforming growth factor (TGF)- $\beta$  blockade on the development of house dust mite (HDM)-induced airway remodeling. Separate groups of mice were exposed to saline or HDM for 5 weeks and concurrently treated (every other day) during the last 3 weeks of exposure with either anti-TGF- $\beta$  ( $\alpha$ TGF $\beta$ ) (10 or 100  $\mu$ g/dose) or the control IgG (100  $\mu$ g/dose) (Ab). Pictures show representative light photomicrographs of paraffin-embedded cross-sections of lung tissue obtained 72 hours after the last HDM exposure. (A) Picro Sirius red (PSR) staining visualized under polarized light indicating subepithelial collagen deposition; (B) immunohistochemistry for  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) indicating contractile elements in the airway wall (brown; insets show non-specific staining in the corresponding negative control section); and (C) periodic acid-Schiff (PAS) staining indicating mucus production by epithelial goblet cells (magenta; insets show color inverted image used for morphometric analysis). (D) Morphometric analysis of lung histology; data represent the percentage of the area of interest that is stained with PSR,  $\alpha$ -SMA, or PAS. All pictures were taken at  $\times 20$  original magnification except insets in (C), which were at  $\times 40$ ;  $n = 5-7$ /group. Data are expressed

as mean  $\pm$  SEM and are from one of three independent experiments that yielded similar results. \* $P < 0.05$  compared with the saline +  $\alpha$ TGF- $\beta$ -treated group. Ab = antibody.

Latent TGF- $\beta$  protein is embedded throughout the extracellular matrix of most organs, especially the lungs, and it is believed that, once activated, TGF- $\beta$  acts rapidly on only those cells that are in direct contact. Thus, we questioned whether such a mechanism would allow at least some TGF- $\beta$  to escape neutralization by the blocking Ab. Importantly, we note that, in other experimental models of fibrosis, in which such a mechanism is believed to mediate fibrogenesis (44), treatment with the  $\alpha$ TGF- $\beta$  Ab used here significantly abrogated the development of fibrosis (Reference 45 and S. Lanning, personal communication). To confirm our results given this possibility, we used a genetically based approach to interfere with TGF- $\beta$  activity. The use of TGF- $\beta$ -deficient mice, although ideal, is effectively impossible given their extremely short life spans. As an alternative, we used SMAD3 KO mice because SMAD3 is a signaling molecule that has been shown to be a critical mediator of TGF- $\beta$ -induced remodeling-associated gene expression (46). We subjected SMAD3 KO mice and their WT littermate controls to the continuous exposure protocol. After 5 weeks of HDM administration, we evaluated the development of airway remodeling in these mice. Morphometric analysis showed signif-

icant increases in collagen deposition (Figure 6A) and smooth muscle thickening (Figure 6B) in both WT and KO animals exposed to HDM compared with their respective saline-treated controls. Moreover, we found that the structural alterations observed in HDM-exposed SMAD3 KO mice were equivalent in magnitude to HDM-exposed WT control animals (Figure 6).

#### Impact of TGF- $\beta$ Blockade on the Regulation of Chronic Allergic Airway Inflammation Induced by HDM

Given that TGF- $\beta$  is a potent negative regulator of inflammation and has been shown to inhibit the activity, differentiation, and proliferation of various Th2-associated inflammatory cell types *in vitro*, we explored whether TGF- $\beta$  blockade had any impact on the regulation of established chronic allergic airway inflammation. We exposed mice to the continuous HDM protocol and blocked TGF- $\beta$  during the last 3 weeks of exposure using the neutralizing  $\alpha$ TGF- $\beta$  Ab. Treatment was initiated at this time to allow sensitization and the inflammatory response to become fully established. Analysis of the cellular infiltrate in the BAL revealed that mice exposed to HDM and treated with

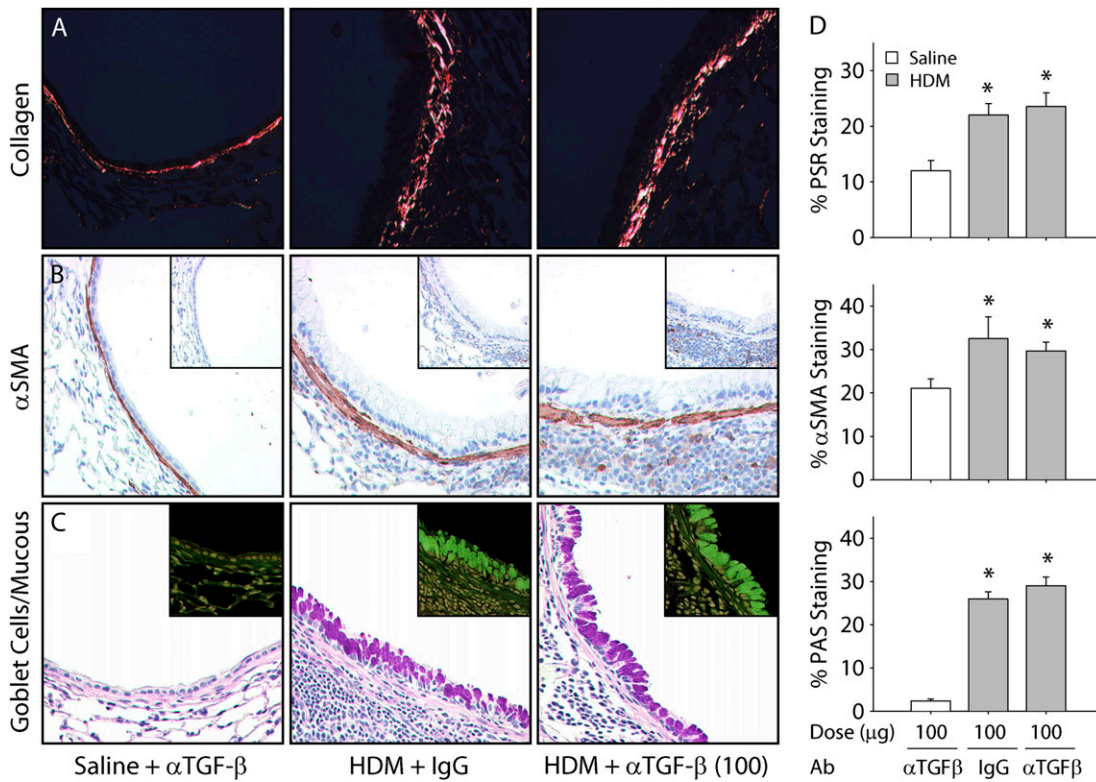


**Figure 4.** Impact of extended transforming growth factor (TGF)- $\beta$  blockade on the development of house dust mite (HDM)-induced airway remodeling. Separate groups of mice were exposed to saline or HDM for 5 weeks and concurrently treated (every other day) beginning at Day 4 of exposure with either a pan-neutralizing anti-transforming growth factor- $\beta$  ( $\alpha$ TGF- $\beta$ ; 100  $\mu$ g/dose) antibody (Ab) or the control IgG Ab (100  $\mu$ g/dose). Panels show representative light photomicrographs of paraffin-embedded cross-sections of lung tissue obtained 72 hours after the last HDM exposure. (A) Picro Sirius red (PSR) staining visualized under polarized light indicating subepithelial collagen deposition; (B) immunohistochemistry for  $\alpha$ -smooth muscle

actin ( $\alpha$ -SMA) indicating contractile elements in the airway wall (brown; insets show nonspecific staining in the corresponding negative control section); and (C) periodic acid-Schiff (PAS) staining indicating mucus production by epithelial goblet cells (magenta; insets show color inverted image used for morphometric analysis). (D) Morphometric analysis of lung histology; data represent the percentage of the area of interest that is stained with PSR,  $\alpha$ -SMA, or PAS. All pictures were taken at  $\times 20$  original magnification except insets in (C), which were at  $\times 40$ ;  $n = 6-7$ /group. Data are expressed as mean  $\pm$  SEM. \* $P < 0.05$  compared with the saline +  $\alpha$ TGF- $\beta$ -treated group.

10  $\mu$ g of  $\alpha$ TGF- $\beta$  had a slight, but nonsignificant, increase in the number of total cells and eosinophils compared with IgG-treated control animals (Figure 7A). Treatment with a higher dose of  $\alpha$ TGF- $\beta$  Ab (100  $\mu$ g/dose) significantly increased the number of total cells present in the BAL compared with HDM-exposed IgG-treated mice and, interestingly, this increase was due exclusively to a greater number of eosinophils (Figure 7A). Indeed, the proportion of eosinophils present in the BAL of mice exposed to HDM and treated with 100  $\mu$ g of  $\alpha$ TGF- $\beta$  was

nearly double that of IgG control animals. We further examined the effect of TGF- $\beta$  neutralization on the inflammatory response in the context of extended Ab treatment and intermittent HDM exposure. We observed that treatment with  $\alpha$ TGF- $\beta$  over nearly the entire 5-week course of HDM administration also exacerbated the proportion of eosinophils in the BAL (Figure 7B). Notably, this extended treatment also resulted in a considerable decrease in the number of mononuclear cells (data not shown); this was not unexpected because TGF- $\beta$  is

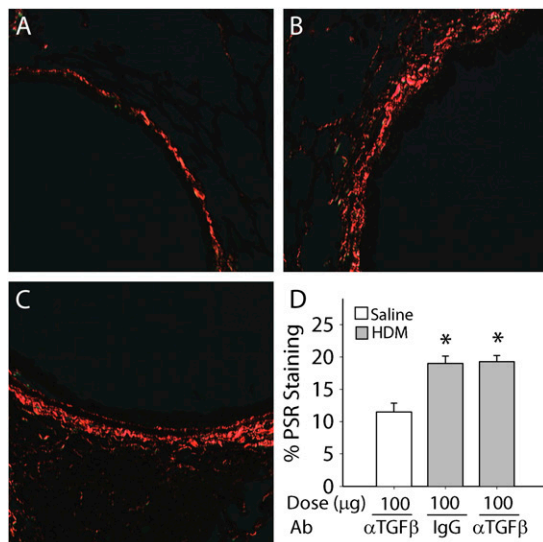


**Figure 5.** Impact of transforming growth factor (TGF)- $\beta$  blockade on the development of house dust mite (HDM)-induced airway remodeling in a model of intermittent allergen exposure. Separate groups of mice were exposed to saline or HDM for a period of 10 consecutive days and then rested for approximately 30 days. Mice were subsequently subjected to six cycles of saline or HDM reexposure; each cycle consisted of daily exposure for 3 consecutive days followed by 12 days of rest. Mice were concurrently treated (every other day) with either anti-TGF $\beta$  ( $\alpha$ TGF $\beta$ ) (100  $\mu$ g/dose) or the control IgG antibody (Ab) (100  $\mu$ g/dose), over each 3-day reexposure to saline or HDM as described in METHODS. (A–C) Representative light photomicrographs of paraffin-embedded cross-sections of lung tissue obtained 72 hours after the last HDM exposure. All panels show Picro Sirius red (PSR)-stained sections visualized under polarized light indicating subepithelial collagen deposition. (D) Morphometric analysis of lung histology; data represent the percentage of the area of interest that is stained with PSR. All images were taken at  $\times 20$  original magnification;  $n = 6-7$ /group. Data are expressed as mean  $\pm$  SEM. \* $P < 0.05$  compared with the saline +  $\alpha$ TGF- $\beta$ -treated group.

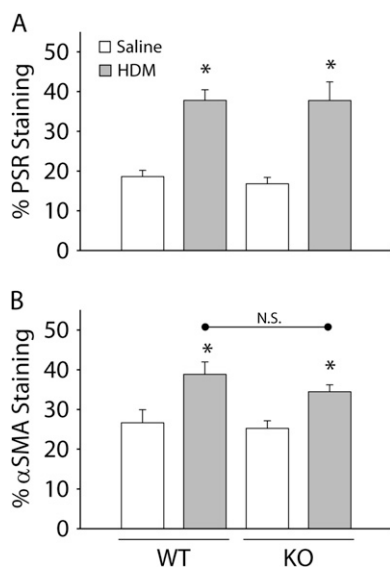


**Figure 6.** Impact of SMAD3 deletion on the development of house dust mite (HDM)-induced airway remodeling. Separate groups of SMAD3 knockout (KO) mice and wild-type (WT) littermate controls were exposed to saline or HDM for 5 weeks. Lung tissues were obtained 72 hours after the last exposure. Morphometric analysis of lung cross-sections (A) stained with Picro Sirius red (PSR), indicating subepithelial collagen deposition, and (B) immunohistochemistry for  $\alpha$ -SMA, indicating contractile elements in the airway wall. Data represent the percentage of the area

of interest that is stained with either PSR or  $\alpha$ -SMA;  $n = 4-8$ /group. Data are expressed as mean  $\pm$  SEM. \* $P < 0.05$  compared with the corresponding saline-exposed control group. N.S. = not significant.

known to be a potent monocyte chemoattractant factor (47). Finally, consistent with the findings above, TGF- $\beta$  blockade also noticeably increased the number of total cells (data not shown) and the extent of eosinophilia in the BAL of mice subjected to the intermittent HDM exposure protocol (Figure 7C), although these differences did not reach statistical significance.

#### Impact of TGF- $\beta$ Blockade on HDM-specific Adaptive Immunity

Because TGF- $\beta$  blockade led to increased airway eosinophilic inflammation, we explored whether this may have been a consequence of altered T-cell responsiveness. To evaluate T-cell function, we harvested splenocytes and measured the production of Th2-associated cytokines after stimulation with HDM *in vitro*. We observed a significant increase in the production of IL-5 by splenocytes taken from mice exposed to HDM for 5 weeks and concurrently treated with  $\alpha$ TGF- $\beta$  (100- $\mu$ g dose) during the final 3 weeks compared with HDM-exposed IgG-treated control animals (Figure 8A). We also documented significantly greater IL-5 production when the duration of  $\alpha$ TGF- $\beta$  administration was increased (Figure 8B). In addition, we further noted a trend toward augmented IL-13 production in both of these experimental protocols; however, this did not reach significance (Figures 8A and 8B). No differences were observed in HDM-specific IL-4 production.

We also examined the impact of TGF- $\beta$  blockade on humoral immunity. Similar to the findings above, mice treated with  $\alpha$ TGF- $\beta$  Ab during the last 3 weeks or over almost the entire course of the continuous HDM exposure protocol possessed significantly elevated serum levels of HDM-specific IgE and IgG<sub>1</sub> compared with the HDM-exposed IgG-treated group (Figures 8C and 8D).

#### Impact of TGF- $\beta$ Neutralization on Lung Function

Compelled by the findings that TGF- $\beta$  neutralization led to an exacerbated inflammatory response and to increased systemic immunity, we assessed the physiologic impact of TGF- $\beta$  blockade on airway function. We evaluated respiratory resistance after increasing doses of methacholine in mice subjected to the continuous HDM exposure protocol and treated with  $\alpha$ TGF- $\beta$  or IgG during the last 3 weeks of HDM. Importantly, we

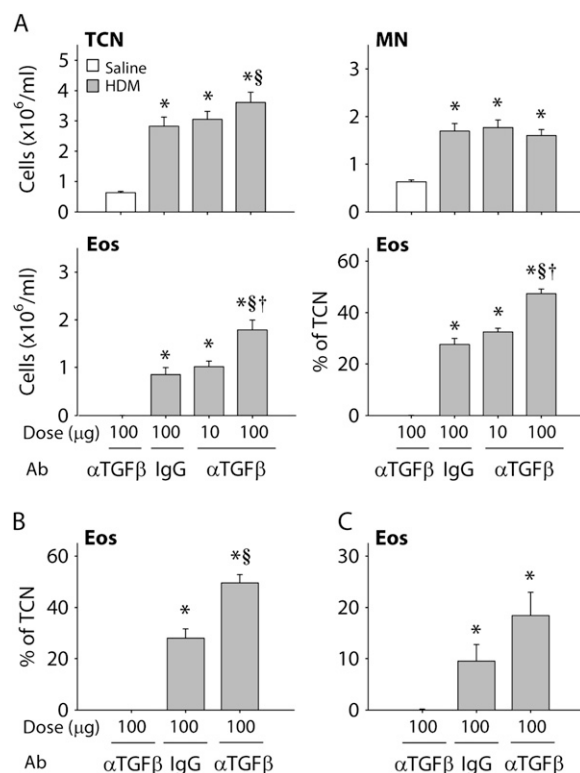


**Figure 7.** Impact of transforming growth factor (TGF)- $\beta$  blockade on house dust mite (HDM)-induced allergic airway inflammation. (A) Differential cell analysis showing the number of total cells (TCN), mononuclear cells (MN), eosinophils (Eos), and the percentage of eosinophils in the bronchoalveolar lavage fluid of mice exposed to saline or HDM for 5 weeks and concurrently treated during the last 3 weeks of exposure with either anti-TGF $\beta$  ( $\alpha$ TGF- $\beta$ ) (10 or 100  $\mu$ g/dose) antibody (Ab) or the control IgG (100  $\mu$ g/dose). (B) Percentage of eosinophils in the BAL fluid of mice exposed to saline or HDM for 5 weeks and concurrently treated beginning at Day 4 of exposure with either  $\alpha$ TGF- $\beta$  (100  $\mu$ g/dose) or the control IgG (100  $\mu$ g/dose). (C) Percentage of eosinophils in the BAL fluid of mice subjected to the intermittent allergen exposure and treated with either  $\alpha$ TGF- $\beta$  (100  $\mu$ g/dose) or the control IgG (100  $\mu$ g/dose) according to the protocol shown in Figure 1C.  $n = 14-21$ /group in (A) and  $n = 5-7$ /group in (B) and (C). Data are expressed as mean  $\pm$  SEM.  $P < 0.05$  compared with \*saline +  $\alpha$ TGF- $\beta$ , §HDM + IgG-, and †HDM +  $\alpha$ TGF- $\beta$  (10  $\mu$ g/dose)-treated groups, respectively.

observed that HDM-exposed  $\alpha$ TGF- $\beta$ -treated mice exhibited a trend toward increased maximum resistance (Figures 9A and 9B) and significantly greater airway reactivity versus HDM-exposed IgG-treated mice (Figures 9A and 9C).

## DISCUSSION

In light of the mounting evidence implicating TGF- $\beta$  in the pathogenesis of asthma, we investigated the impact of interfering with TGF- $\beta$  activity on the regulation of chronic allergic airway inflammation and remodeling induced by exposure to HDM. We observed that treatment with a neutralizing  $\alpha$ TGF- $\beta$  Ab had no effect on the development of airway remodeling, even if the dose of Ab was increased 10-fold and treatment was extended over nearly the entire course of HDM exposure. These observations suggested to us that the remodeling response, at least when triggered by continuous HDM exposure, could occur independently of TGF- $\beta$  activity. Similarly, treatment with

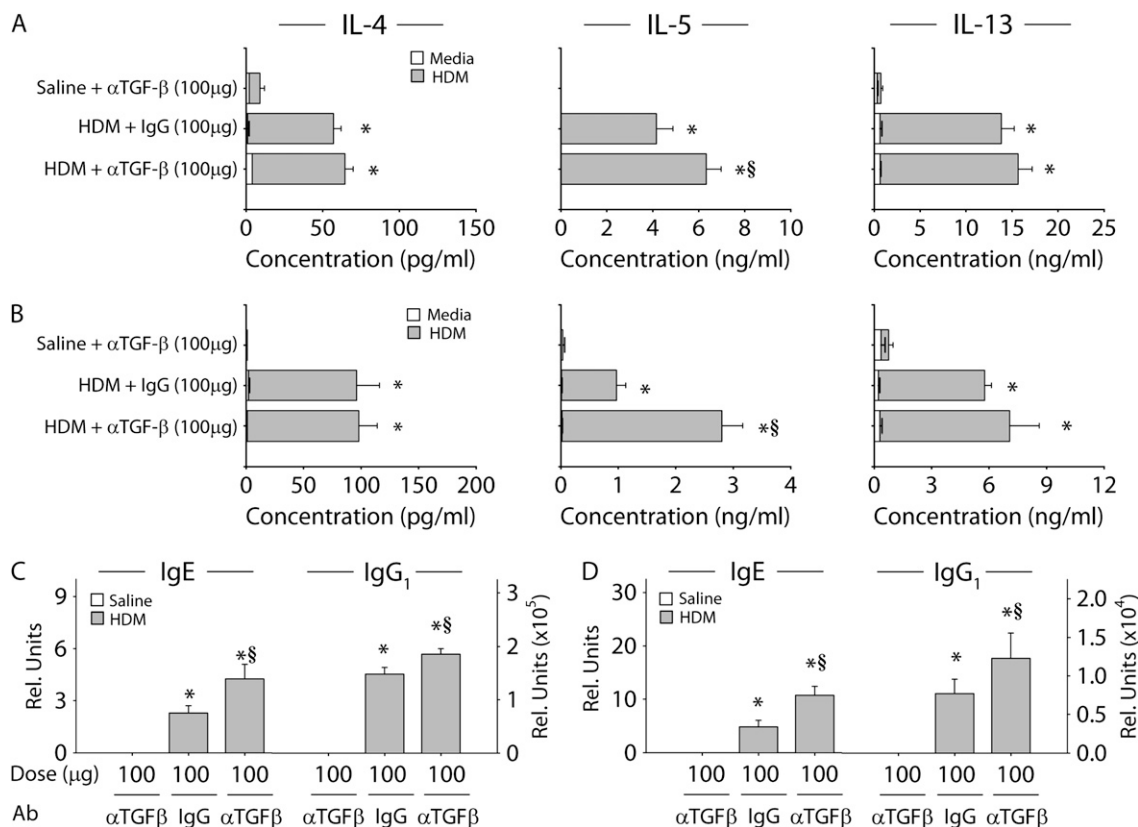


**Figure 8.** Impact of transforming growth factor (TGF)- $\beta$  blockade on house dust mite (HDM)-specific Th2-associated immune responses. HDM-specific Th2-associated splenocyte cytokine production (A and B) and serum immunoglobulin levels (C and D) from mice exposed to saline or HDM for 5 weeks and concurrently treated during the last 3 weeks of exposure (A and C) or beginning at Day 4 of exposure (B and D) with either anti-TGF $\beta$  ( $\alpha$ TGF- $\beta$ ) (100  $\mu$ g/dose) antibody (Ab) or the control IgG Ab (100  $\mu$ g/dose). Splenocytes from individual mice were cultured in medium alone (open bars) or stimulated with HDM (shaded bars) *in vitro*.  $n = 5-7$ /group. Data are expressed as mean  $\pm$  SEM.  $P < 0.05$  compared with \*saline +  $\alpha$ TGF- $\beta$ - and §HDM + IgG-treated groups, respectively.

$\alpha$ TGF- $\beta$  in the context of an intermittent HDM exposure protocol, in which the ensuing inflammatory response is recurrent and therefore considerably lower, also had no effect. This argued against the possibility that TGF- $\beta$  was dispensable in the continuous HDM exposure protocol simply because that protocol elicits a robust inflammatory response that is sustained over several weeks. Moreover, these findings suggest that other factors, such as the nature of the eliciting agent and/or the type of the inflammatory response, may ultimately influence whether TGF- $\beta$  is required in a given reparative/fibrotic response. Indeed, that the type of inflammation impacts whether a reparative/fibrotic response will develop has been previously demonstrated (14, 48, 49).

To further confirm the observations made with the blocking Ab, we used a second approach, the use of SMAD3 KO mice, to evaluate the role of TGF- $\beta$  in HDM-induced remodeling. The SMAD signaling pathway is not the only pathway by which the fibrogenic effects of TGF- $\beta$  may be mediated (50); however, SMAD3 has been clearly shown to play a fundamental role in the signal transduction pathways associated with TGF- $\beta$ -mediated wound healing and fibrosis (46). In agreement with the findings above, we observed that HDM-exposed SMAD3 KO mice developed airway remodeling to the same extent as WT littermate controls. Thus, when taken together, the findings we report here demonstrate that HDM-induced allergic airway remodeling can develop independently of TGF- $\beta$ .

Although our data may initially appear to be at variance with the prevailing notion that TGF- $\beta$  mediates, in all circumstances,

tissue repair, careful consideration of the differences between the various systems used may in fact account for these seemingly divergent findings. In the case of fibrogenesis, a common feature of models of inflammation and fibrosis is that there is a *single* eliciting event (e.g., bleomycin, radiation, administration of TGF- $\beta$ ). This generates an acute and self-limited inflammatory process that is associated with the development of fibrosis. These approaches remarkably contrast with our model in that the eliciting agent (HDM) is delivered continuously for a considerable period of time. In addition, the type of inflammation (Th2 polarized) is yet another central difference between our model and most models of fibrosis.

The findings by McMillan and colleagues warrant particular consideration as they investigated the impact of TGF- $\beta$  neutralization, using the same Ab as the one used here, in a model of chronic allergic airway disease (18). They showed that TGF- $\beta$  neutralization prevented the progression of airway remodeling after repeated OVA challenge. However, there are several important considerations that must be taken into account. First, one should note that, whereas this treatment indeed prevented airway remodeling at an early time point (Day 35) of their protocol, the effects of TGF- $\beta$  neutralization on the extent of remodeling at a later time point (Day 55) were substantially reduced, suggesting, as the authors noted, that additional factors are likely contributing to the development of airway remodeling in their model. Second, as is well known in models using OVA, repeated OVA challenge leads to a diminution of the inflammatory response, as certainly occurred in McMillan and

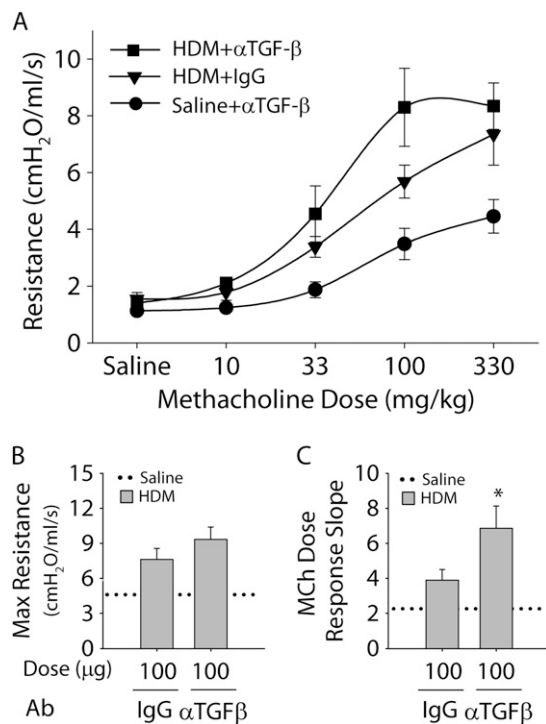


**Figure 9.** Impact of transforming growth factor (TGF)- $\beta$  blockade on house dust mite (HDM)-induced airway responsiveness to methacholine (MCh). (A) Total respiratory resistance was measured at increasing doses of MCh in separate groups of mice exposed to saline or HDM for 5 weeks and concurrently treated during the last 3 weeks of exposure with either anti-TGF $\beta$  ( $\alpha$ TGF- $\beta$ ) (100  $\mu$ g/dose) antibody (Ab) or the control IgG Ab (100  $\mu$ g/dose). (B) Maximum respiratory resistance values and (C) airway reactivity as measured by maximum resistance and MCh dose-response slope, respectively, for mice exposed to saline (dotted line) or HDM (shaded bars) and concurrently treated during the last 3 weeks of exposure with either  $\alpha$ TGF- $\beta$  (100  $\mu$ g/dose) or the control IgG Ab (100  $\mu$ g/dose).  $n = 6$ –7/group. Data are expressed as mean  $\pm$  SEM.  $P < 0.05$  compared with \*HDM + IgG-treated group.

colleagues' protocol. This is in sharp contrast with the model that we used in which we maintain robust inflammation (30–35% eosinophils in BAL) for several weeks. Third, it may be particularly important that the antigen used in this model was OVA, a prototypic innocuous antigen with fundamental biochemical differences to HDM. Indeed, whereas OVA preparations are essentially pure, HDM is, in stark contrast, a complex material consisting of numerous protein and nonprotein components, with considerable proteolytic activity. This added complexity may be of considerable relevance because the biochemical and immunogenic profile of HDM will likely draw on a distinct network of molecular responses. Therefore, we argue that, whereas OVA can, under certain conditions (e.g., intraperitoneal sensitization with intermittent exposure) lead to remodeling, achieving the same outcome using HDM and a route of mucosal sensitization does not imply the same underlying biochemical or immunologic process; nor can this knowledge be universally applied to the responses elicited by other aeroallergen families. Thus, from this perspective, we do not view our findings to be in contrast with those described above but instead to be a reflection of the differences in the contexts in which the role of TGF- $\beta$  is being explored.

Particular attention should also be drawn to the study by Kaviratne and coworkers (51). There they comprehensively investigated the requirement for TGF- $\beta$  in the generation of

liver fibrosis induced by *Schistosoma mansoni* infection. Most interesting, this fibrotic response develops as a consequence of a Th2-polarized inflammatory response directed against schistosome eggs, which get trapped in the liver. Using an exhaustive array of methodologies, the authors conclusively demonstrated that the fibrotic response induced by schistosome infection was TGF- $\beta$  independent. Notably, IL-13, a prototypical Th2-associated cytokine, was shown to be an indispensable mediator of hepatic fibrosis in this model (52). Interestingly, constitutive IL-13 overexpression in the lung has been shown to trigger a Th2-like response involving airway eosinophilia and hyperactivity that was furthermore associated with the development of subepithelial airway fibrosis (53). However, in contrast to the observations made in the schistosome model, the fibrotic response that develops as a consequence of IL-13 overexpression was found to be mediated by TGF- $\beta$ , because administration of a soluble TGF- $\beta$  receptor-Fc molecule ameliorated IL-13-induced fibrosis (54). The difference in the requirement for TGF- $\beta$  between these two models may reflect the fact that IL-13 overexpression does not fully recapitulate a Th2-polarized immune response. Indeed, many of the other hallmark Th2-associated cell types and molecules (B cells, Th2 cells, IL-4, IL-5, IL-9, and IgE) were absent in the transgenic IL-13 model. These additional components are likely to critically influence the lung microenvironment and therefore may alter the requirement for TGF- $\beta$  in the generation of a fibrotic response that develops in the context of an antigen-dependent Th2 immune response. Taken together, these studies support the concept that some Th2-associated airway reparative responses occur independently of TGF- $\beta$ , and moreover, that the molecular signatures underlying the development of a "fibrotic event" may be critically influenced by the type of inflammatory response, and therefore by the nature of the eliciting agent (e.g., bleomycin, radiation, schistosoma, OVA, HDM). Thus, it follows that there may be several distinct pathways leading to fibrosis—that is, a TGF- $\beta$ -dependent pathway and an IL-13-dependent TGF- $\beta$ -independent pathway, possibly among others. In addition, there exists a panoply of other molecules that display powerful fibrogenic activity. These include platelet-derived growth factor, vascular endothelial growth factor, connective tissue growth factor, and oncostatin M, among several others (55, 56). The precise contribution of these molecules, either alone or in concert, in mediating tissue reparative responses associated with Th2-polarized airway inflammation remains to be fully dissected.

We also investigated the impact of interfering with TGF- $\beta$  activity on the allergic inflammatory response. We observed that TGF- $\beta$  neutralization, when initiated at a time when the inflammatory response was already ongoing (2 wk of HDM exposure), led to a significant increase in inflammation that was characterized exclusively by an increase in eosinophils; similar effects were also evident when TGF- $\beta$  neutralization was initiated early on during continuous HDM exposure (Day 4) and in the intermittent exposure protocol, although the increase did not reach statistical significance in the latter. These increases could be the result of augmented IL-5 production, although it is also plausible that  $\alpha$ TGF- $\beta$  treatment interfered with TGF- $\beta$ -induced eosinophil apoptosis, which has been previously observed *in vitro* (30). We surmise that the increases in eosinophilia likely underlie the enhanced airway reactivity we observed. Similar to our findings, TGF- $\beta$  neutralization was recently reported to result in increased airway hyperresponsiveness in an OVA-based model (20). Although this occurred in the absence of any noticeable effects on inflammation, this study (in agreement with others) showed that TGF- $\beta$  neutralization led to increased production of Th2 cytokines. Thus, it is also plausible that the increase in airway hyperresponsiveness may be a result of increased levels of IL-5

and/or IL-13, among others, via some direct effect(s) on, for example, airway smooth muscle cells. Ultimately, as elegantly discussed by Alcorn and colleagues (20), a number of intertwined mechanisms could account for this observation. Importantly, TGF- $\beta$  blockade, at least in the context of continuous HDM exposure, carried significant functional consequences.

Several related studies using acute conventional OVA models (33–35) have also documented increases in airway inflammation attributable in large part to the following: enhanced eosinophilia; elevated levels of Th2-associated cytokines, including IL-4, IL-5, and IL-13; and increased serum IgE and IgG<sub>1</sub> in mice in which TGF- $\beta$  levels or activity had been impaired. Although it was not possible to clearly identify the effects on established inflammation alone given the genetically based methods these authors used, their data implicate TGF- $\beta$  as an important regulator of allergic airway inflammation. Interestingly, in both the McMillan and Alcorn studies, TGF- $\beta$  neutralization in their models had no effect on established inflammation. We suspect that this observation may be related to the dose of Ab used and/or the system used and not necessarily a reflection of the role of TGF- $\beta$  in the regulation of chronic allergic inflammation. Using a similar dose of Ab used in McMillan and colleagues' protocol, we observed a clear trend toward enhanced eosinophilia. Administration of a higher dose of Ab conclusively demonstrated, at least in our system, that TGF- $\beta$  is a critical regulator of HDM-induced chronic allergic airway inflammation.

In our view, the findings we present here do not question the importance of TGF- $\beta$  to the tissue repair response. Rather, they compel us to consider the notion that the role of a given molecule in a process is contextual. In the context of robust airway inflammation elicited by persistent exposure to a common aeroallergen (i.e., HDM), the research we present here demonstrates that TGF- $\beta$  does not play a critical role in airway remodeling, which, we understand, may be divergent from the prevailing dogma. In addition, our data show that interference with TGF- $\beta$  leads to worsened airway inflammation and function. We surmise that these findings are, at least teleologically, in accord with the principal goal of the immune response: survival of the host. That is, that the key role of TGF- $\beta$  under these conditions may be to protect the host from devastating inflammation and tissue damage. From this perspective, an important concern arises with respect to the proposition of therapeutic interference with TGF- $\beta$  as a means of limiting the progression of airway remodeling, not only that it may not work but also that the treatment may lead to a loss of control of the inflammatory response and, ultimately, the disease.

**Conflict of Interest Statement:** R.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. N.G.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.R.J. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.D.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.P.S. is an employee of Genzyme Corporation and owns shares of Genzyme Corporation common stock. R.C.G. is an employee of Genzyme Corporation and owns shares of Genzyme Corporation common stock. S.L. is a salaried employee of Genzyme Corporation and has shares of Genzyme Corporation common stock. J.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.J. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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