

Reduced expression of transforming growth factor β_1 exacerbates pathology in an experimental asthma model

Wiebke Scherf¹, Stefan Burdach² and Gesine Hansen¹

¹ Department of Pediatrics, Division of Allergy and Pulmonology,
Martin-Luther-University Halle-Wittenberg, Halle, Germany

² Department of Pediatrics, Technical University Munich, Munich, Germany

Allergic asthma is characterized by airway hyperreactivity (AHR), eosinophilic airway inflammation and elevated serum IgE levels. T-helper 2 (Th2) cells play a critical role in the pathogenesis of asthma, but the immunological mechanisms that inhibit Th2 cell function *in vivo* are not well understood. Conflicting results regarding the protective role of Th1 cytokines and TGF- β in asthma have been reported. To further investigate the role of TGF- β_1 in asthma, we examined mice heterozygous for deletion of the TGF- β_1 gene (TGF- $\beta_1^{+/-}$ mice) in a murine asthma model. While TGF- $\beta_1^{+/-}$ mice seem phenotypically normal, they express only about 30% of wild type TGF- β_1 protein levels as shown before. The reduced expression of TGF- β_1 is accompanied by a strikingly increased eosinophilic inflammation and mucus secretion in response to ovalbumin (OVA) sensitization. Moreover, TGF- $\beta_1^{+/-}$ mice develop significantly enhanced Th2-cytokine levels, decreased IFN- γ production and increased levels of OVA-specific IgE in serum. In contrast, AHR in response to methacholine is not altered significantly. Our data demonstrate that reduced expression of TGF- β_1 exacerbates pathology in an experimental asthma model and support the view that the elevated levels of TGF- β_1 in asthmatic airways might be, at least in part, a result of anti-inflammatory compensation by this cytokine.

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Introduction

Allergic asthma is an inflammatory disease dependent on Th2-type immune responses and characterized by airway hyperreactivity (AHR) to specific and nonspecific stimuli. So far therapeutic attempts focused on the reconstitution of the balance between Th1 and Th2 cells, since IFN- γ -secreting Th1 cells have been proposed to protect against asthma and allergic disease by dampening the activity of Th2 effector cells. Evidence for this hypothesis was mainly derived from epidemiological studies, demonstrating a negative correlation between

the asthma incidence and a cleaner environment with fewer Th1-promoting childhood infections in westernized countries [1]. Moreover, recent animal experiments using bacterial components, plasmid DNA, CpG oligonucleotides and other approaches [2–4] demonstrated that induction of Th1 cells is associated with the inhibition of Th2 cell development and effectively downmodulates AHR and airway inflammation.

On the other hand, both Th1 and Th2 cells can be found in the lungs of asthmatic patients [5], and the administration of IL-12 has failed to show significant effects in the early and late phase of the asthmatic response [6]. Moreover, adoptive transfer of antigen-specific Th1 cells into SCID mice and immunized BALB/c mice failed to inhibit Th2 cell-induced AHR and inflammation, even when Th1 cells were given in great excess [7]. Unexpectedly, in this model, Th1 cells were even harmful and increased airway inflammation. These conflicting data underline the need for a better under-

Correspondence: Gesine Hansen, Department of Pediatrics, Division of Allergy and Pulmonology, Martin-Luther-University Halle-Wittenberg, D-06120 Halle, Germany
e-mail: gesine.hansen@medizin.uni-halle.de

Abbreviations: TGF- β_1 : Transforming growth factor- β_1 ·

AHR: Airway hyperreactivity · i.n.: Intranasal

standing of the regulatory mechanisms and cytokines involved in the development of AHR and airway inflammation.

One key regulator in the maintenance of immunological homeostasis is TGF- β , a pleiotropic cytokine with significant anti-inflammatory and immunosuppressive properties. TGF- β inhibits the production of proinflammatory cytokines from macrophages, B cells, and T cells and is a potent inhibitor of T cell-mediated immune responses, both *in vitro* [8–10] and *in vivo* [11–13]. The essential role for TGF- β_1 in the maintenance of immune homeostasis is demonstrated in TGF- $\beta_1^{-/-}$ mice, which develop a severe, multifocal inflammatory response in all of the pups that are born alive [14, 15]. In contrast, mice heterozygous for deletion of TGF- β_1 gene (TGF- $\beta_1^{+/-}$ mice) seem phenotypically normal and show no significant difference in the lifespan compared to wild type (WT) animals [16].

In a previous study with T cells genetically engineered to produce TGF- β , we demonstrated that, in contrast to Th1 cells, these TGF- β -producing cells were able to abolish AHR and airway inflammation induced by OVA-specific Th2 cells [17]. In humans, the levels of TGF- β_1 in the airways of asthmatics are reported to be higher than in normal subjects [18]. So far, it is not clear whether this resembles the profibrotic effects of TGF- β in the airways of asthmatics or a protective anti-inflammatory effect of this cytokine in allergic asthma.

To further investigate the role of TGF- β_1 in asthma, we examined mice heterozygous for deletion of TGF- β_1 gene (TGF- $\beta_1^{+/-}$ mice) in a murine asthma model. While TGF- $\beta_1^{+/-}$ mice seem phenotypically normal [14], Tang et al. [19] have demonstrated that TGF- $\beta_1^{+/-}$ mice express only about 30% of TGF- β_1 protein levels compared to WT mice. This finding was confirmed in our study. In these animals, the inflammatory response to OVA sensitization is clearly increased and mucus secretion is enhanced as compared to WT controls. Moreover, the Th2 cytokines IL-4, IL-5 and IL-13 are strikingly elevated, which is accompanied by an increase in IgE production in TGF- $\beta_1^{+/-}$ mice. In contrast, IFN- γ was significantly decreased. Although AHR in response to methacholine is elevated at high methacholine concentrations, these differences do not reach the level of significance, demonstrating that 30% of TGF- β_1 expression as presented in TGF- $\beta_1^{+/-}$ mice is sufficient to control AHR in response to OVA sensitization, while it is not sufficient to control airway inflammation, mucus secretion and IgE production. Bearing in mind the profibrotic potential of TGF- β_1 , our data support, at least in part, a protective role of TGF- β_1 in asthma.

Results

Decreased TGF- β_1 protein levels in mice heterozygous for deletion of the TGF- β_1 gene (TGF- $\beta_1^{+/-}$ mice)

Although TGF- $\beta_1^{+/-}$ mice seem phenotypically normal, TGF- β_1 protein levels in TGF- $\beta_1^{+/-}$ mice were significantly reduced and ranged from $26 \pm 15\%$ of normal lung, to $29 \pm 18\%$ of normal serum, to $29 \pm 6\%$ of normal spleen (Fig. 1). These data confirm the data of Tang et al. [19], who showed a comparable or even stronger reduction of TGF- β_1 protein levels in mice heterozygous for deletion of the TGF- β_1 gene compared to WT animals (10–30%). OVA sensitization did not significantly change TGF- β_1 protein levels in serum, lung and spleen of TGF- $\beta_1^{+/-}$ or WT mice.

Stronger inflammatory response and mucus production in OVA-immunized TGF- $\beta_1^{+/-}$ mice as compared to WT mice

After having demonstrated that loss of one TGF- β_1 allele causes significantly reduced TGF- β_1 protein levels, we tested the effect of reduced TGF- β_1 expression in OVA-immunized animals in a murine asthma model. First, TGF- β_1 WT and TGF- $\beta_1^{+/-}$ mice were sensitized twice, i.p. to OVA protein adsorbed to Alum, and then challenged intranasally (i.n.) with OVA in PBS six times over a course of 24 days (see Sect. 4). Two days after the last i.n. challenge with OVA, mice were killed, and bronchoalveolar lavage (BAL) was performed. The inflammatory response to OVA sensitization was strikingly increased in the TGF- $\beta_1^{+/-}$ animals with lower TGF- β_1 levels as compared to WT controls (Fig. 2). Cell

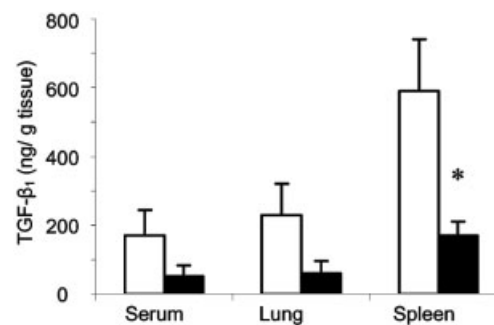


Fig. 1. Expression of TGF- β_1 in WT and TGF- $\beta_1^{+/-}$ mice. TGF- β_1 protein levels were determined from acid ethanol extracts of freshly isolated tissues of 8-week-old mice using isoform-specific sandwich ELISA. TGF- β_1 protein levels in TGF- $\beta_1^{+/-}$ mice (black columns) are significantly reduced compared to WT mice (white columns) and range from $26 \pm 15\%$ of normal lung, to $29 \pm 18\%$ of normal serum, to $29 \pm 6\%$ of normal spleen. All data are expressed as mean $\pm$ SEM of six mice. * $p < 0.05$, OVA WT vs. OVA TGF- $\beta_1^{+/-}$ by Student's t-test.

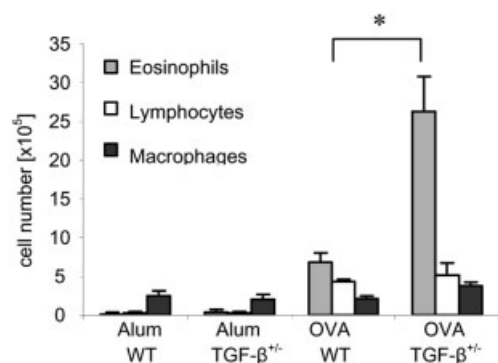


Fig. 2. Cell differentials in BAL fluid from TGF- $\beta_1^{+/-}$ and WT mice. TGF- $\beta_1^{+/-}$ and WT mice were immunized i.p. with OVA in Alum twice and i.n. with OVA in NaCl 0.9% six times within 24 days. BAL was performed 2 days after the last i.n. challenge with three aliquots of 400 μ l PBS with 1% BSA. The total number of different types of leukocytes was determined on slide preparations of BAL fluid stained with May-Grünwald-Giemsa. Cell numbers in the BAL fluid of OVA-sensitized TGF- $\beta_1^{+/-}$ animals exceed those of WT animals almost threefold, and the percentage of eosinophils in the BAL is increased almost fourfold in TGF- $\beta_1^{+/-}$ animals as compared to controls. The data are expressed as mean $\pm$ SEM ($n=8$ for each group). For eosinophils, $*p<0.05$ OVA WT vs. OVA TGF- $\beta_1^{+/-}$ by Student's *t*-test.

numbers in the BAL fluid of OVA-sensitized TGF- $\beta_1^{+/-}$ animals exceeded those of WT animals almost threefold ($35 \pm 6.1 \times 10^5$ vs. $13 \pm 1.4 \times 10^5$, $p<0.01$) and the percentage of eosinophils in the BAL was increased almost fourfold in TGF- $\beta_1^{+/-}$ animals as compared to controls ($26.3 \pm 4.5 \times 10^5$ vs. $6.8 \pm 1.2 \times 10^5$, $p<0.01$).

In addition to examination of the BAL fluid, we compared lung histology of OVA-sensitized TGF- $\beta_1^{+/-}$ animals and OVA-sensitized WT controls. For this, mice were killed 2 days after the last i.n. OVA challenge, lungs were removed and lung histology was examined after fixation and staining of one part of the lung with hematoxylin and eosin (H&E), and another part with periodic acid-Schiff's reagent (PAS) for better mucus demonstration. Lung sections from WT mice that were immunized with OVA showed strong airway inflammation with dense peribronchiolar and perivascular infiltrates, consisting of lymphocytes, eosinophils and some neutrophils (Fig. 3). The inflammatory response to OVA sensitization was stronger in TGF- $\beta_1^{+/-}$ animals with lower TGF- β_1 protein levels as compared to WT controls. Interestingly, lungs of naive TGF- $\beta_1^{+/-}$ control mice revealed a slight cellular infiltrate that was not seen in WT controls. PAS staining demonstrated strong mucus

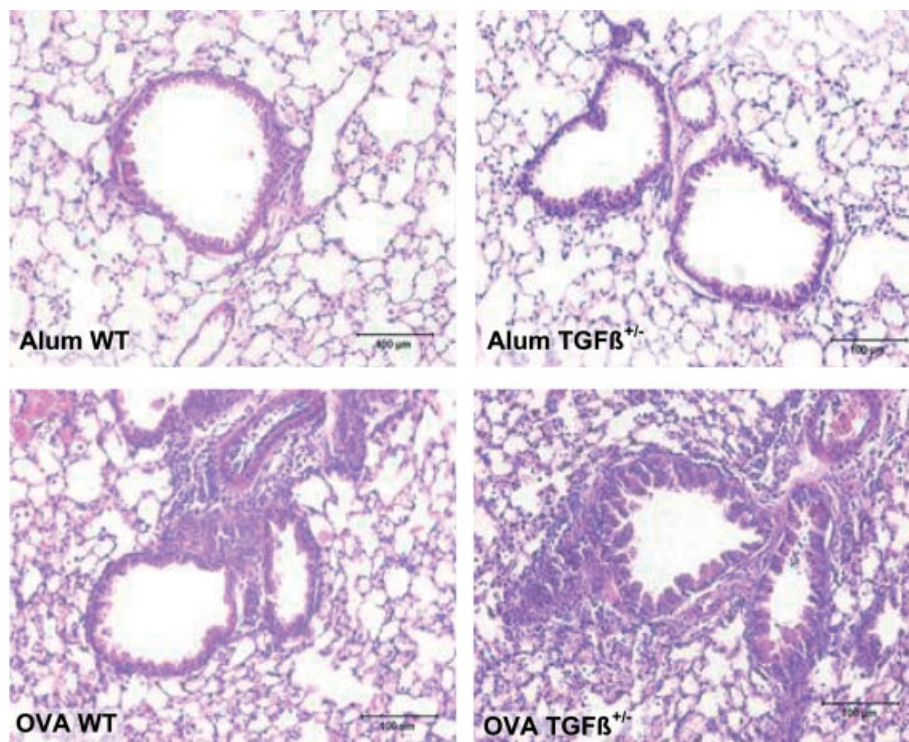


Fig. 3. Lung histology of OVA-immunized TGF- $\beta_1^{+/-}$ and WT mice or control animals (H&E). TGF- β_1 WT and TGF- $\beta_1^{+/-}$ mice were sensitized to OVA in Alum twice i.p., and challenged i.n. with OVA in NaCl 0.9% six times during the 24-day protocol. Control animals received Alum i.p. and NaCl 0.9% i.n. Two days after the last i.n. challenge with OVA, mice were killed; lungs were removed, fixed in formalin and stained with H&E. Lung tissue from WT mice that had received Alum i.p. and NaCl i.n. reveals normal lung histology without inflammation or eosinophilia (Alum WT), while lung tissue from naive TGF- $\beta_1^{+/-}$ mice shows some cellular infiltrates (Alum TGF- $\beta_1^{+/-}$). OVA immunization of WT mice results in dense peribronchiolar mononuclear cell infiltrates consisting of lymphocytes, eosinophils, and some neutrophils (OVA WT). The inflammatory response to OVA is clearly stronger in TGF- $\beta_1^{+/-}$ mice.

production in OVA-immunized animals as quantified by the mucus index described in Sect. 4 (Fig. 4). Mucus production was increased in the TGF- β_1 ^{+/-} animals as compared to WT controls. Our data demonstrate that reduced expression of TGF- β_1 leads to increased airway inflammation, airway eosinophilia and mucus production after OVA sensitization.

No significant differences in airway hyperreactivity in TGF- β_1 ^{+/-} mice as compared to WT mice

The effect of TGF- β_1 expression on AHR in response to methacholine was tested in a whole-body-plethysmograph for mice 1 day after the last i.n. challenge with OVA in the 24-day immunization protocol. The response to methacholine was similar in OVA-sensitized TGF- β_1 ^{+/-} animals as in WT animals, although there was a slight increase in AHR at 80 mg/ml methacholine (Fig. 5). This difference did not reach the level of significance.

Reduced TGF- β_1 expression leads to strikingly increased Th2 cytokine levels and decreased IFN- γ levels after OVA sensitization

Allergic asthma is a Th2 cytokine-dominated disease, and the pathology in asthma is closely related to the Th2 cytokines IL-4, IL-5 and IL-13 that all play a crucial role for the development of AHR and inflammation in this disease. To investigate the effect of reduced TGF- β_1 protein levels on cytokine production, cells from OVA-sensitized TGF- β_1 ^{+/-} and WT animals were isolated from spleen and restimulated *in vitro* with OVA. Levels of IL-4,

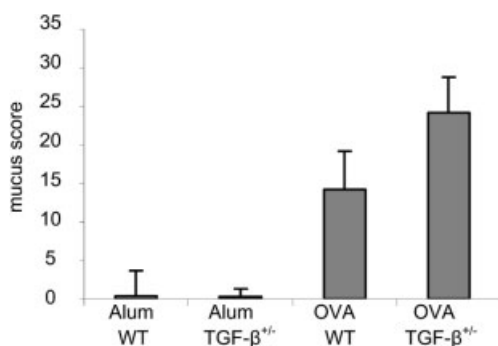


Fig. 4. Mucus production of TGF- β_1 ^{+/-} and WT mice. TGF- β_1 ^{+/-} and WT mice were immunized twice i.p. with OVA in Alum and six times i.n. with OVA in NaCl 0.9% within 24 days. On day 26, mice were killed; the left lung was tied off for histology and formalin-fixed lung tissue was stained with PAS. The degree of mucus production in the lungs was assessed by a mucus score described in Sect. 4.6. Mucus production is increased in the TGF- β_1 ^{+/-} animals as compared to WT controls. Data are derived from eight mice per group of two independent experiments in a blinded fashion.

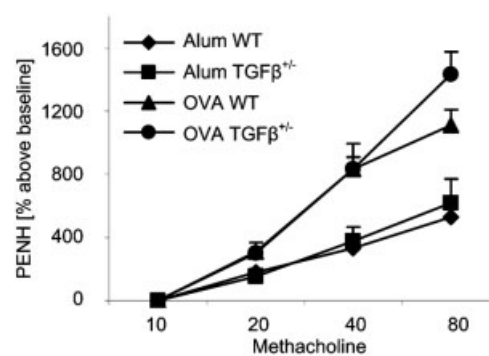


Fig. 5. Lung function test in TGF- β_1 ^{+/-} and WT mice. AHR was measured in response to methacholine in conscious mice placed in a whole body plethysmograph 1 day after the last i.n. OVA challenge of a 24-day lasting immunization protocol. Mean P_{enh} values were calculated and data are expressed as percent above baseline (NaCl-induced AHR). No significant difference is seen between OVA-sensitized TGF- β_1 ^{+/-} animals and WT animals, although bronchoconstriction is slightly increased at 80 mg/ml methacholine in heterozygous animals. Error bars represent SEM of eight animals per group and data point.

IL-5, IL-10, IL-13 and IFN- γ in culture supernatants were determined by ELISA. IL-4, IL-5 and IL-13 were strikingly increased in TGF- β_1 ^{+/-} mice compared to WT controls, while IFN- γ levels were significantly decreased. These data demonstrate a Th2 shift in TGF- β_1 ^{+/-} mice and show that in our system Th1 and Th2 responses are not equally suppressed by TGF- β_1 . IL-10 concentrations are increased in WT mice compared to TGF- β_1 ^{+/-} mice, although these differences were not significant (Fig. 6).

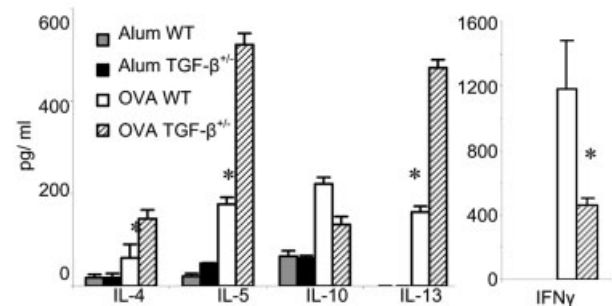


Fig. 6. Cytokine levels in culture supernatants. After OVA immunization of TGF- β_1 ^{+/-} and WT mice by two i.p. and six i.n. applications of OVA, spleens were removed and splenocytes cultured in medium with 200 μ g/ml OVA. ELISA for IL-4, IL-5, IL-10, IL-13 and IFN- γ were performed. Levels for the Th2 cytokines IL-4, IL-5 and IL-13 were strikingly increased and IFN- γ levels were decreased in OVA-sensitized TGF- β_1 ^{+/-} mice compared to WT mice. IL-10 production was decreased in TGF- β_1 ^{+/-} mice, although this effect was not significant. Error bars represent SEM of triplicates from pooled lymphocyte cultures of four animals per group. Representative data from one of two independent experiments are shown. * p < 0.05, OVA WT vs. OVA TGF- β_1 ^{+/-} by Student's *t*-test.

Effects of reduced TGF- β_1 expression on IgE, IgG₁ and IgG_{2A} levels after OVA sensitization

One hallmark of allergic asthma is the production of allergen-specific IgE. To investigate the effect of reduced TGF- β_1 expression on OVA-specific IgE, IgG₁ and IgG_{2A} production, blood was taken 2 days after the last i.n. challenge with OVA and OVA-specific IgE levels were measured by ELISA. We found that IgE levels in OVA-sensitized TGF- β_1 ^{+/-} mice were significantly higher than those in OVA-sensitized TGF- β_1 WT controls ($p \leq 0.05$). Furthermore, we observed increased IgG₁ levels and significantly reduced IgG_{2A} levels in OVA-sensitized TGF- β_1 ^{+/-} mice, also indicating a stronger Th2 response in TGF- β_1 ^{+/-} mice compared to WT controls (Fig. 7). These results confirm the selective increase of Th2 cytokines in TGF- β_1 ^{+/-} mice.

Discussion

TGF- β_1 is a member of a large family of evolutionarily conserved proteins with highly pleiotropic properties [20]. A loss-of-function mutation in TGF- β_1 mice results in a severe, multifocal inflammatory response in all of the pups that are born alive [14, 15], which demonstrates the essential role for TGF- β_1 in maintenance of immune homeostasis. While TGF- β_1 ^{+/-} mice seem phenotypically normal, we and others [19] have demonstrated that TGF- β_1 ^{+/-} mice express only about 30% of WT TGF- β_1 protein levels. In our study, lower expression of TGF- β_1 in OVA-sensitized TGF- β_1 ^{+/-} animals led to strikingly increased levels of the Th2 cytokines IL-4, IL-5 and IL-13, which all play a crucial role in the pathogenesis of allergic asthma. The increased Th2 cytokine levels were accompanied by

an increased inflammatory response in the lung, enhanced mucus production and significantly elevated serum IgE and reduced IgG_{2A} levels. At the same time, IFN- γ , which is thought to counterbalance Th2 cytokine effects, was significantly decreased. These data demonstrate a Th2 shift in TGF- β_1 ^{+/-} mice and show that, in our system, Th1 and Th2 responses are not equally suppressed by TGF- β_1 .

IL-10 concentrations are decreased in TGF- β_1 ^{+/-} mice compared to WT animals. The production and action of IL-10 and TGF- β , which both are able to down-regulate immune responses, likely involve a positive feedback loop in which TGF- β enhances IL-10 production and vice versa. For example, treatment of colitis by administration of a plasmid encoding TGF- β provides a beneficial effect via stimulation of IL-10 production [21]. This effect may be due to the ability of Smad3, a transcription factor required for signaling downstream of the TGF- β R, to interact with GATA3 and form a complex contributing to the transcriptional activation of the IL-10 promoter [22]. Intestinal epithelial cells transgenic for IL-10 display an increase in TGF- β production [23], supporting previous *in vitro* data demonstrating that exogenous IL-10 stimulates the production of TGF- β [24]. In contrast, lymphocytes from mice deficient for IL-10 produce less TGF- β than cells from WT mice [23]. Similarly, reduced expression of TGF- β in TGF- β_1 ^{+/-} mice might lead to the reduced production of IL-10 in TGF- β_1 ^{+/-} mice observed in this study.

Our data support the hypothesis that the elevated levels of TGF- β_1 in asthmatic airways [18] might be, at least in part, a result of anti-inflammatory compensation.

In addition to the data from this study, our previous study clearly supports a protective role of TGF- β_1 in allergic asthma. Here we have demonstrated that transfer of OVA-specific T cells engineered *in vitro* to express latent TGF- β abolished AHR and airway inflammation induced by OVA-specific Th2 cells in severe combined immunodeficient (SCID) and BALB/c mice [17]. In this model, the inhibitory effect on AHR was abolished when mice were treated with a neutralizing monoclonal antibody to TGF- β .

Similarly, a recent study identified the T cells as a central effector cell of TGF- β_1 -mediated regulation of AHR [25]. Upon antigen-specific challenge, mice with impaired TGF- β_1 signaling in T cells demonstrated increased AHR and lung inflammation dominated by eosinophils compared with WT mice. In contrast to these data, we did not see significant changes in AHR in TGF- β_1 ^{+/-} mice with reduced expression of TGF- β_1 . This finding demonstrates that 30% of TGF- β_1 expression, as presented in TGF- β_1 ^{+/-} mice, is sufficient to control AHR in response to OVA sensitization. In contrast, it does not

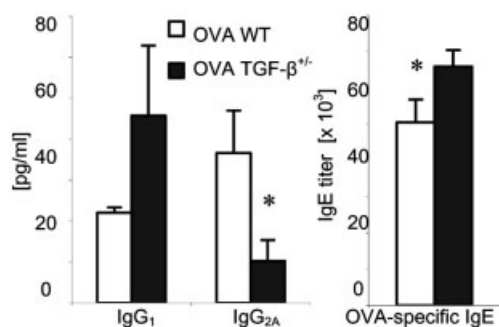


Fig. 7. Immunoglobulin levels in serum measured by ELISA. Blood was taken 2 days after the last i.n. challenge with OVA and OVA-specific IgE levels, IgG₁ and IgG_{2A} levels were measured by ELISA. Serum IgE and IgG₁ were increased, while IgG_{2A} was decreased in OVA-sensitized TGF- β_1 ^{+/-} mice compared to WT animals. Error bars represent SEM of six animals per group and data point. * $p < 0.05$, OVA WT vs. OVA TGF- β_1 ^{+/-} by Student's *t*-test.

appear to be sufficient for controlling airway inflammation, mucus secretion, and cytokine and IgE production.

Since TGF- β_1 has been shown to be produced by a variety of cells within the lungs of asthmatic patients [26–28], the higher levels of TGF- β_1 in the airways of asthmatics as compared to normal subjects [18] are very likely produced by different kinds of cells, not only the T cells. TGF- β_1 is produced by T and B lymphocytes, macrophages, dendritic cells (DC) and platelets [29–31]. In the lung of mice, TGF- β_1 mRNA is found in bronchiolar epithelium, Clara cells, mesenchymal cells, vascular endothelium, and alveolar cells, including macrophages [32]. Additionally, a very high expression of TGF- β_1 messenger RNA transcripts is found in the smooth muscle cells of the large vessels [33]. In humans, bronchial epithelial cells contain a large amount of TGF- β_1 protein [34]. The TGF- β isoforms and their receptors have also been demonstrated in macrophages [32, 34–36], mesenchymal cells [32], vascular and airway smooth muscle cells [34–36] and bronchial glands [37]. While some studies also detected TGF- β_1 in normal epithelium [32, 35, 37], others [36] reported epithelial presence of TGF- β_1 only in association with fibrosis.

The presence of TGF- β in the normal lung suggests its participation in the normal regulation of physiological processes to maintain lung homeostasis. These functions may include local immunomodulation, regulation of cell proliferation and differentiation, as well as the control of normal tissue repair. On this background, the investigation of TGF- $\beta_1^{+/-}$ mice with reduced TGF- β_1 expression in the lung caused by different cell types resembles the natural situation more than an investigation of transgenic mice with impaired TGF- β signaling.

In our study, lower TGF- β_1 levels were accompanied by increased mucus secretion. Low levels of mucus are produced in the normal lung and mucus metaplasia with mucus hypersecretion is a characteristic feature of asthma. While the role that TGF- β_1 plays in these responses has not yet been evaluated, a variety of inflammatory mediators, including the Th2 cytokines IL-4, IL-5, IL-13 and IL-9, are believed to contribute to the pathogenesis of the observed mucus abnormalities in this disorder [38–41]. Therefore, it is likely that the strikingly increased Th2 cytokine levels in TGF- $\beta_1^{+/-}$ mice are responsible for the increased mucus production, so that TGF- β_1 indirectly controls mucus production via the control of other cytokines.

Another key feature of allergic asthma is the elevated level of allergen-specific IgE. In this study, we have shown that reduced TGF- β_1 levels lead to increased antigen-specific IgE levels in antigen-sensitized mice. The mechanism for this phenomenon is not clear, but increased levels of IL-4 and IL-13 in TGF- $\beta_1^{+/-}$ mice might be responsible, since IL-4 and IL-13 stimulate B cells to produce IgE [42]. Another possible mechanism

is the regulation via Id2. Recently, evidence has been provided for the inhibitory and selective role of Id2 in IgE class switch recombination in response to TGF- β_1 . Id2 might act as a molecular safeguard to suppress the IgE class switch recombination to prevent serious complications such as allergic hypersensitivity during the normal course of immune responses [43].

To our knowledge, so far no data have been published on the immune response of TGF- $\beta_1^{+/-}$ mice in asthma or other allergic diseases. Moreover, only very little is known about the immune response of TGF- $\beta_1^{+/-}$ mice. One study on cardiac transplant arteriosclerosis showed that TGF- $\beta_1^{+/-}$ transplant recipients had an increased expression of the transcription factor STAT 4, IFN- γ and IL-2 and unaltered or reduced expression of the transcription factor STAT 6, IL-4, and IL-10 [44]. In contrast to the sparse data on the immune response of TGF- $\beta_1^{+/-}$ mice, several tumor studies have been done with TGF- $\beta_1^{+/-}$ mice, which have demonstrated a significant role of TGF- β_1 as tumor suppressor protein [19], and in the carcinogenesis of lung [45–47] or other carcinomas [48, 49]. Although TGF- $\beta_1^{+/-}$ mice have also been used in different studies with varying disease models [50–52], the immune response of TGF- $\beta_1^{+/-}$ mice has not been in the focus of these studies.

On the basis of the data from our animal study with TGF- $\beta_1^{+/-}$ mice, the elevated levels of TGF- β_1 in asthmatic airways might be, at least in part, a result of protective, anti-inflammatory compensation in asthmatic airway inflammation. In addition, as TGF- β has been implicated in tissue repair [53], the high levels of TGF- β in asthmatic airways might be involved in repair processes of asthmatic airways that are damaged by inflammation. TGF- β_1 in the airways of asthmatics could thus function as a healing molecule, promoting the process of tissue repair and diminishing airway inflammation. Under ideal circumstances the actions of TGF- β might lead to the restoration of normal tissue architecture. On the other hand, TGF- β_1 is clearly a profibrotic cytokine [54]. Therefore, under circumstances such as persistent activity of TGF- β_1 induced by chronic inflammation, which might be caused by repeated stimulation with allergens, TGF- β_1 expression might lead to the detrimental effect of TGF- β_1 known as tissue fibrosis or airway remodeling, which results in chronic airway obstruction.

Although TGF- β_1 might have damaging effects, probably depending on concentration and duration of tissue contact, our data and the data from previous studies clearly support the protective potential of TGF- β_1 in allergic asthma.

Materials and methods

Animals

B6.129S2-TGF- β 1^{tm1Doe} were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred and maintained in our animal facility at the BioCenter in Halle/Saale (Germany). These mice have been backcrossed to C57BL/6J for at least 22 generations. C57BL/6J were used as WT mice. For all experiments sex-matched 8–10-week-old animals were used; experiments involved groups of four to five mice and were performed according to institutional and state guidelines. The local Committee on Animal Welfare (Bezirksregierung Halle) approved animal protocols used in this study.

Screening of mice by PCR

Neonates from heterozygote mice were screened for the TGF- β 1 WT and KO allele by genomic PCR. Briefly, DNA was extracted from tail biopsies of 4-week-old mice. The DNA was examined by PCR analysis using two primer pairs (A: 5'-GAGAAGAACTGCTGTGTGCG-3' and 5'-GTGTCCAGGCTCCAAATATAGG-3'; and B: 5'-AGACAATCGGCTGCTCTGAT-3' and 5'-GTGTCCAGGCTCCAAATATAGG-3'), the first pairing to exon 6 sequences of the TGF- β 1, the second amplifying neomycin-specific sequences in the mutant mice. PCR synthesis was run for 35 cycles (94°C for 1 min, 60°C for 30 s and 72°C for 2 min) followed by final extension at 72°C for 10 min. PCR products were visualized on agarose gels.

Measurement of TGF- β 1 protein levels in mice heterozygous for TGF- β 1 gene and WT animals

Freshly isolated tissues or serum were homogenized and extracted with acid-ethanol solution as described [55]. ELISA for TGF- β was performed with DuoSet® (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The detection limit was 8 pg/ml.

Immunization of mice with OVA

Mice were sensitized to OVA protein for the induction of AHR and airway inflammation using the following protocol: OVA (20 μ g) adsorbed to 2 mg aluminium potassium sulfate (Alum) was administered i.p. on days 1 and 14, followed by 20 μ g OVA in 40 μ l normal saline given i.n. on days 14, 16, 17 and 20, 21, 24. Control mice received i.p. injections of alum alone and normal saline i.n. One day after the last i.n. challenge (day 25), AHR was measured in conscious mice after inhalation of increasing concentrations of methacholine (see below). On day 26, mice were killed; blood was taken, the left lung was tied off for histology and BAL was performed in the right lung.

Measurement of airway responsiveness

Airway responsiveness was assessed by methacholine-induced airflow obstruction from conscious mice placed in a whole body plethysmograph (model PLT UNR MS, Emka Technologies, Paris). Pulmonary airflow obstruction was measured by

P_{enh} using the following formula: $P_{\text{enh}} = [(Te/RT-1) \times (PEF/PIF)]$, where P_{enh} = enhanced pause (dimensionless), Te = expiratory time, RT = relaxation time, PEF = peak expiratory flow (ml/s), and PIF = peak inspiratory flow (ml/s) [56]. Enhanced pause (P_{enh}), minute volume, tidal volume, and breathing frequency were obtained from chamber pressure, measured with a transducer connected to amplifier module (model AC264), and analyzed by system XA software (version 1.565). Mice were exposed for 2 min to aerosolized 0.9% NaCl produced by a sonicator (model LS 290–990N), followed by increasing amounts of methacholine. Data are expressed as the percentage of baseline P_{enh} values after 0.9% NaCl exposure.

Collection of BAL fluid and lung histology

Animals were killed by CO₂ asphyxiation. Left lung was tied off for histology. The trachea was cannulated and the right lung was lavaged three times with 400 μ l PBS with 1% BSA. Cells in the lavage fluid were counted using a hemocytometer, and BAL cell differentials were determined on slide preparations stained with May-Grünwald-Giemsa in a blinded fashion (Merck, Darmstadt, Germany). At least 200 cells were differentiated by light microscopy based on conventional morphological criteria. Left lung was fixed in 10% formalin; one part was stained with H&E (Merck) and the other part with Alcian blue-PAS (Sigma, Taufkirchen, Germany). The degree of mucus production in the lungs was assessed by a mucus score, which is a product of PAS-positive bronchi and a factor correlating to the number of positive goblet cells (<30% PAS-positive goblet cells factor 1; 30–60% factor 2; >60% factor 3).

To detect or exclude fibrosis, lung tissue of WT and TGF- β 1^{+/-} mice was stained with methyl violet (Merck) in annilin alcohol and treated with Lugol's solution (iodine-potassium iodide solution, Merck). Fibrosis was not detectable in any of the investigated lungs.

Splenocyte culture and assay for cytokines

One day after the airway function test, spleens were removed and passed through a nylon mesh. Single-cell suspensions of splenocytes (5 × 10⁶ cells/ml) were stimulated *in vitro* by incubation with 200 μ g/ml OVA in culture medium (RPMI medium supplemented with 10% FCS, 100 U/ml penicillin, 100 U/ml streptomycin). Supernatants were collected in triplicate after 36 h and ELISA for IL-4, IL-5, IL-10, IL-13 and IFN- γ were done using DuoSet® (R&D Systems) according to manufacturer's instructions.

Immunoglobulin assay

Serum levels of OVA-specific IgE, IgG₁ and IgG_{2A} were measured with a sandwich ELISA according to a standard protocol. Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight with 10 μ g/ml OVA diluted in bicarbonate buffer (pH 9.6). After washing and blocking plates, samples were incubated for 2 h. Subsequently, plates were washed and horseradish peroxidase-conjugated goat anti-mouse IgE antibody (Bethyl Laboratories, Inc., Montgomery, TX), or IgG₁ or IgG_{2A} antibody (Southern Biotechnology Associates, Inc. Birmingham, AL) was added. Tetramethyl-

benzidine was used as substrate, and the absorbance was determined at 450 nm using a Bio-Rad microplate reader (Bio-Rad, Munich, Germany). The titer was calculated by logarithmical regression defined as the reciprocal dilution of the sera, where the extinction was twofold the background extinction. The background extinction was defined by the use of sera from non-immunized mice, diluted 1:100.

Statistical analysis

All statistics were done using an unpaired Student's two-tailed *t*-test. Error bars represent SEM.

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References

- Umetsu, D. T., McIntire, J. J., Akbari, O., Macaubas, C. and DeKruyff, R. H., Asthma: an epidemic of dysregulated immunity. *Nat. Immunol.* 2002. **3**: 715–720.
- Hansen, G., Yeung, V. P., Berry, G., Umetsu, D. T. and DeKruyff, R. H., Vaccination with heat-killed *Listeria* as adjuvant reverses established allergen-induced airway hyperreactivity and inflammation: role of CD8⁺ T cells and IL-18. *J. Immunol.* 2000. **164**: 223–230.
- Maecker, H. T., Hansen, G., Walter, D. M., DeKruyff, R. H., Levy, S. and Umetsu, D. T., Vaccination with allergen-IL-18 fusion DNA protects against, and reverses established, airway hyperreactivity in a murine asthma model. *J. Immunol.* 2001. **166**: 959–965.
- Erb, K. J. and Wöhleben, G., Novel vaccines protecting against the development of allergic disorders: a double-edged sword? *Curr. Opin. Immunol.* 2002. **14**: 633–643.
- Krug, N., Madden, J., Redington, A. E., Lackie, P., Djukanovic, R., Schauer, U., Holgate, S. T., Frew, A. J. and Howarth, P. H., T cell cytokine profile evaluated at the single cell level in BAL and blood in allergic asthma. *Am. J. Respir. Cell. Mol. Biol.* 1996. **14**: 319–326.
- Bryan, S. A., O'Connor, B. J., Matti, S., Leckie, M. J., Kanabar, V., Khan, J., Warrington, S. J., Renzetti, L., Rames, A., Bock, J. A. et al., Effects of recombinant human interleukin-12 on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 2000. **356**: 2149–2153.
- Hansen, G., Berry, G., DeKruyff, R. H. and Umetsu, D. T., Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J. Clin. Invest.* 1999. **103**: 175–183.
- Kehrl, J. H., Roberts, A. B., Wakefield, L. M., Jakowlew, S., Sporn, M. B. and Fauci, A. S., Transforming growth factor beta is an important immunomodulatory protein for human B lymphocytes. *J. Immunol.* 1986. **137**: 3855–3860.
- Wahl, S. M., Hunt, D. A., Wong, H. L., Dougherty, S., McCartney-Francis, N., Wahl, L. M., Ellingsworth, L., Schmidt, J. A., Hall, G., Roberts, A. B. et al., Transforming growth factor beta is a potent immunosuppressive agent that inhibits IL-1-dependent lymphocyte proliferation. *J. Immunol.* 1988. **140**: 3026–3032.
- Espevik, T., Figari, I. S., Shalaby, M. R., Lackides, G. A., Lewis, G. D., Shepard, H. M. and Palladino, M. A., Jr., Inhibition of cytokine production by cyclosporin A and transforming growth factor beta. *J. Exp. Med.* 1987. **166**: 571–576.
- Barral-Netto, M., Barral, A., Brownell, C. E., Skeiky, Y. A., Ellingsworth, L. R., Twardzik, D. R. and Reed, S. G., Transforming growth factor-beta in leishmanial infection: a parasite escape mechanism. *Science* 1992. **257**: 545–548.
- Brandes, M. E., Allen, J. B., Ogawa, Y. and Wahl, S. M., Transforming growth factor beta 1 suppresses acute and chronic arthritis in experimental animals. *J. Clin. Invest.* 1991. **87**: 1108–1113.
- Kuruvilla, A. P., Shah, R., Hochwald, G. M., Liggitt, H. D., Palladino, M. A. and Thorbecke, G. J., Protective effect of transforming growth factor beta 1 on experimental autoimmune diseases in mice. *Proc. Natl. Acad. Sci. USA* 1991. **88**: 2918–2921.
- Shull, M. M., Ormsby, I., Kier, A. B., Pawlowski, S., Diebold, R. J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D. et al., Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 1992. **359**: 693–699.
- Kulkarni, A. B. and Karlsson, S., Transforming growth factor-beta 1 knockout mice. A mutation in one cytokine gene causes a dramatic inflammatory disease. *Am. J. Pathol.* 1993. **143**: 3–9.
- Boivin, G. P., Molina, J. R., Ormsby, I., Stemmermann, G. and Doetschman, T., Gastric lesions in transforming growth factor beta-1 heterozygous mice. *Lab. Invest.* 1996. **74**: 513–518.
- Hansen, G., McIntire, J. J., Yeung, V. P., Berry, G., Thorbecke, G. J., Chen, L., DeKruyff, R. H. and Umetsu, D. T., CD4(+) T helper cells engineered to produce latent TGF-beta1 reverse allergen-induced airway hyperreactivity and inflammation. *J. Clin. Invest.* 2000. **105**: 61–70.
- Redington, A. E., Madden, J., Frew, A. J., Djukanovic, R., Roche, W. R., Holgate, S. T. and Howarth, P. H., Transforming growth factor-beta 1 in asthma. Measurement in bronchoalveolar lavage fluid. *Am. J. Respir. Crit. Care Med.* 1997. **156**: 642–647.
- Tang, B., Bottinger, E. P., Jakowlew, S. B., Bagnall, K. M., Mariano, J., Anver, M. R., Letterio, J. J. and Wakefield, L. M., Transforming growth factor-beta1 is a new form of tumor suppressor with true haploid insufficiency. *Nat. Med.* 1998. **4**: 802–807.
- Gorelik, L. and Flavell, R. A., Transforming growth factor-beta in T cell biology. *Nat. Rev. Immunol.* 2002. **2**: 46–53.
- Kitani, A., Fuss, I. J., Nakamura, K., Schwartz, O. M., Usui, T. and Strober, W., Treatment of experimental (trinitrobenzene sulfonic acid) colitis by intranasal administration of transforming growth factor (TGF)-beta1 plasmid: TGF-beta1-mediated suppression of T helper cell type 1 response occurs by interleukin (IL)-10 induction and IL-12 receptor beta2 chain downregulation. *J. Exp. Med.* 2000. **192**: 41–52.
- Blokzijl, A., ten Dijke, P. and Ibanez, C. F., Physical and functional interaction between GATA-3 and Smad3 allows TGF-beta regulation of GATA target genes. *Curr. Biol.* 2002. **12**: 35–45.
- De Winter, H., Elewaut, D., Turovskaya, O., Huflejt, M., Shimeld, C., Hagenbaugh, A., Binder, S., Takahashi, I., Kronenberg, M. and Cheroute, H., Regulation of mucosal immune responses by recombinant interleukin 10 produced by intestinal epithelial cells in mice. *Gastroenterology* 2002. **122**: 1829–1841.
- Seder, R. A., Marth, T., Sieve, M. C., Strober, W., Letterio, J. J., Roberts, A. B. and Kelsall, B., Factors involved in the differentiation of TGF-beta-producing cells from naive CD4⁺ T cells: IL-4 and IFN-gamma have opposing effects, while TGF-beta positively regulates its own production. *J. Immunol.* 1998. **160**: 5719–5728.
- Schramm, C., Herz, U., Podlech, J., Protschka, M., Finotto, S., Reddehase, M. J., Kohler, H., Galle, P. R., Lohse, A. W. and Blessing, M., TGF-beta regulates airway responses via T cells. *J. Immunol.* 2003. **170**: 1313–1319.
- Vignola, A. M., Chanez, P., Chiappara, G., Merendino, A., Pace, E., Rizzo, A., la Rocca, A. M., Bellia, V., Bonsignore, G. and Bousquet, J., Transforming growth factor-beta expression in mucosal biopsies in asthma and chronic bronchitis. *Am. J. Respir. Crit. Care Med.* 1997. **156**: 591–599.
- Minshall, E. M., Leung, D. Y., Martin, R. J., Song, Y. L., Cameron, L., Ernst, P. and Hamid, Q., Eosinophil-associated TGF-beta1 mRNA expression and airways fibrosis in bronchial asthma. *Am. J. Respir. Cell. Mol. Biol.* 1997. **17**: 326–333.

- 28 Chu, H. W., Trudeau, J. B., Balzar, S. and Wenzel, S. E., Peripheral blood and airway tissue expression of transforming growth factor beta by neutrophils in asthmatic subjects and normal control subjects. *J. Allergy Clin. Immunol.* 2000. **106**: 1115–1123.
- 29 Luethviksson, B. R. and Gunnlaugsdottir, B., Transforming growth factor-beta as a regulator of site-specific T cell inflammatory response. *Scand. J. Immunol.* 2003. **58**: 129–138.
- 30 Bartram, U. and Speer, C. P., The role of transforming growth factor beta in lung development and disease. *Chest* 2004. **125**: 754–765.
- 31 Letterio, J. J. and Roberts, A. B., Regulation of immune responses by TGF-beta. *Annu. Rev. Immunol.* 1998. **16**: 137–161.
- 32 Coker, R. K., Laurent, G. J., Shahzeidi, S., Hernandez-Rodriguez, N. A., Pantelidis, P., du Bois, R. M., Jeffery, P. K. and McAnulty, R. J., Diverse cellular TGF-beta 1 and TGF-beta 3 gene expression in normal human and murine lung. *Eur. Respir. J.* 1996. **9**: 2501–2507.
- 33 Pelton, R. W., Johnson, M. D., Perkett, E. A., Gold, L. I. and Moses, H. L., Expression of transforming growth factor-beta 1, -beta 2, and -beta 3 mRNA and protein in the murine lung. *Am. J. Respir. Cell. Mol. Biol.* 1991. **5**: 522–530.
- 34 Magnan, A., Frachon, I., Rain, B., Peuchmaur, M., Monti, G., Lenot, B., Fattal, M., Simonneau, G., Galanaud, P. and Emilie, D., Transforming growth factor beta in normal human lung: preferential location in bronchial epithelial cells. *Thorax* 1994. **49**: 789–792.
- 35 de Boer, W. I., van Schadewijk, A., Sont, J. K., Sharma, H. S., Stolk, J., Hiemstra, P. S. and van Krieken, J. H., Transforming growth factor beta1 and recruitment of macrophages and mast cells in airways in chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 1998. **158**: 1951–1957.
- 36 Khalil, N., O'Connor, R. N., Flanders, K. C. and Unruh, H., TGF-beta 1, but not TGF-beta 2 or TGF-beta 3, is differentially present in epithelial cells of advanced pulmonary fibrosis: an immunohistochemical study. *Am. J. Respir. Cell. Mol. Biol.* 1996. **14**: 131–138.
- 37 Jagirdar, J., Lee, T. C., Reibman, J., Gold, L. I., Aston, C., Begin, R. and Rom, W. N., Immunohistochemical Localization of Transforming Growth-Factor Beta Isoforms in Asbestos-Related Diseases. *Environ. Health Perspect.* 1997. **105**: 1197–1203.
- 38 Zhu, Z., Homer, R. J., Wang, Z., Chen, Q., Geba, G. P., Wang, J., Zhang, Y. and Elias, J. A., Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J. Clin. Invest.* 1999. **103**: 779–788.
- 39 Rankin, J. A., Picarella, D. E., Geba, G. P., Temann, U. A., Prasad, B., DiCosmo, B., Tarallo, A., Stripp, B., Whitsett, J. and Flavell, R. A., Phenotypic and physiologic characterization of transgenic mice expressing interleukin 4 in the lung: lymphocytic and eosinophilic inflammation without airway hyperreactivity. *Proc. Natl. Acad. Sci. USA* 1996. **93**: 7821–7825.
- 40 Longphre, M., Li, D., Gallup, M., Drori, E., Ordonez, C. L., Redman, T., Wenzel, S., Bice, D. E., Fahy, J. V. and Basbaum, C., Allergen-induced IL-9 directly stimulates mucin transcription in respiratory epithelial cells. *J. Clin. Invest.* 1999. **104**: 1375–1382.
- 41 Lee, J. J., McGarry, M. P., Farmer, S. C., Denzler, K. L., Larson, K. A., Carrigan, P. E., Brenneise, I. E., Horton, M. A., Haczku, A., Gelfand, E. W. et al., Interleukin-5 expression in the lung epithelium of transgenic mice leads to pulmonary changes pathognomonic of asthma. *J. Exp. Med.* 1997. **185**: 2143–2156.
- 42 Grunig, G., Warnock, M., Wakil, A. E., Venkayya, R., Brombacher, F., Rennick, D. M., Sheppard, D., Mohrs, M., Donaldson, D. D., Locksley, R. M. and Corry, D. B., Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 1998. **282**: 2261–2263.
- 43 Sugai, M., Gonda, H., Kusunoki, T., Katakai, T., Yokota, Y. and Shimizu, A., Essential role of Id2 in negative regulation of IgE class switching. *Nat. Immunol.* 2003. **4**: 25–30.
- 44 Koglin, J., Glysing-Jensen, T., Raisanen-Sokolowski, A. and Russell, M. E., Immune sources of transforming growth factor-beta1 reduce transplant arteriosclerosis: insight derived from a knockout mouse model. *Circ. Res.* 1998. **83**: 652–660.
- 45 Kang, Y., Mariano, J. M., Angdisen, J., Moody, T. W., Diwan, B. A., Wakefield, L. M. and Jakowlew, S. B., Enhanced tumorigenesis and reduced transforming growth factor-beta type II receptor in lung tumors from mice with reduced gene dosage of transforming growth factor-beta1. *Mol. Carcinog.* 2000. **29**: 112–126.
- 46 McKenna, I. M., Ramakrishna, G., Diwan, B. A., Kang, Y., Shiao, Y. H., Wakefield, L. M., Powell, D. A., Anderson, L. M. and Jakowlew, S. B., Heterozygous inactivation of TGF-beta1 increases the susceptibility to chemically induced mouse lung tumorigenesis independently of mutational activation of K-ras. *Toxicol. Lett.* 2001. **123**: 151–158.
- 47 Kang, Y., Ozbun, L. L., Angdisen, J., Moody, T. W., Prentice, M., Diwan, B. A. and Jakowlew, S. B., Altered expression of G1/S regulatory genes occurs early and frequently in lung carcinogenesis in transforming growth factor-beta1 heterozygous mice. *Carcinogenesis* 2002. **23**: 1217–1227.
- 48 Im, Y. H., Kim, H. T., Kim, I. Y., Factor, V. M., Hahm, K. B., Anzano, M., Jang, J. J., Flanders, K., Haines, D. C., Thorgeirsson, S. S. et al., Heterozygous mice for the transforming growth factor-beta type II receptor gene have increased susceptibility to hepatocellular carcinogenesis. *Cancer Res.* 2001. **61**: 6665–6668.
- 49 Quintanilla, M., Ramirez, J. R., Perez-Gomez, E., Romero, D., Velasco, B., Letarte, M., Lopez-Novoa, J. M. and Bernabeu, C., Expression of the TGF-beta coreceptor endoglin in epidermal keratinocytes and its dual role in multistage mouse skin carcinogenesis. *Oncogene* 2003. **22**: 5976–5985.
- 50 Brooks, W. W. and Conrad, C. H., Myocardial fibrosis in transforming growth factor beta(1)heterozygous mice. *J. Mol. Cell. Cardiol.* 2000. **32**: 187–195.
- 51 Geiser, A. G., Zeng, Q. Q., Sato, M., Helvering, L. M., Hirano, T. and Turner, C. H., Decreased bone mass and bone elasticity in mice lacking the transforming growth factor-beta1 gene. *Bone* 1998. **23**: 87–93.
- 52 Krause, T. J., Katz, D., Wheeler, C. J., Ebner, S. and McKinnon, R. D., Increased levels of surgical adhesions in TGFbeta1 heterozygous mice. *J. Invest. Surg.* 1999. **12**: 31–38.
- 53 Singer, A. J. and Clark, R. A., Cutaneous wound healing. *N. Engl. J. Med.* 1999. **341**: 738–746.
- 54 Branton, M. H. and Kopp, J. B., TGF-beta and fibrosis. *Microbes Infect.* 1999. **1**: 1349–1365.
- 55 Danielpour, D., Dart, L. L., Flanders, K. C., Roberts, A. B. and Sporn, M. B., Immunodetection and quantitation of the two forms of transforming growth factor-beta (TGF-beta 1 and TGF-beta 2) secreted by cells in culture. *J. Cell. Physiol.* 1989. **138**: 79–86.
- 56 Hamelmann, E., Schwarze, J., Takeda, K., Oshiba, A., Larsen, G. L., Irvin, C. G. and Gelfand, E. W., Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care Med.* 1997. **156**: 766–775.