CD4+ T helper cells engineered to produce latent TGF-β1 reverse allergen-induced airway hyperreactivity and inflammation

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T helper 2 (Th2) cells play a critical role in the pathogenesis of asthma, but the precise immunological mechanisms that inhibit Th2 cell function in vivo are not well understood. Using gene therapy, we demonstrated that ovalbumin-specific (OVA-specific) Th cells engineered to express latent TGF-β abolished airway hyperreactivity and airway inflammation induced by OVA-specific Th2 effector cells in SCID and BALB/c mice. These effects correlated with increased concentrations of active TGF-β in the bronchoalveolar lavage (BAL) fluid, demonstrating that latent TGF-β was activated in the inflammatory environment. In contrast, OVA-specific Th1 cells failed to inhibit airway hyperreactivity and inflammation in this system. The inhibitory effect of TGF-β–secreting Th cells was antigen-specific and was reversed by neutralization of TGF-β. Our results demonstrate that T cells secreting TGF-β in the respiratory mucosa can indeed regulate Th2-induced airway hyperreactivity and inflammation and suggest that TGF-β–producing T cells play an important regulatory role in asthma.

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Introduction

The worldwide prevalence of asthma has increased dramatically in the last 2 decades, such that asthma now affects as many as 10% of individuals in industrialized nations (1, 2). Asthma is characterized by airway hyperreactivity to a variety of specific and nonspecific stimuli, by chronic airway inflammation with pulmonary eosinophilia, by mucus hypersecretion, and by increased serum IgE levels. The pathology in asthma occurs as a consequence of excessive production of IL-4, IL-5, and IL-13 by CD4+ Th2 cells (3, 4).

Whereas the immunological mechanisms that induce asthma and allergy are relatively well characterized, the specific mechanisms that transpire in vivo to downmodulate Th2 cell–mediated allergic inflammatory responses are not yet clear. Th1 cells, which secrete IFN-γ, have been proposed to protect against asthma and allergic disease by dampening the activity of Th2 effector cells (5); however, the evidence for this is indirect. In an adoptive transfer model, we recently demonstrated that whereas antigen-specific Th1 cells reduced airway mucus production and eosinophilia, they failed to inhibit Th2 cell–induced airway hyperreactivity and inflammation, even when Th1 cells were given in great excess (6). Therefore, we hypothesized that other cell types must be involved in regulating Th2 effector cells in asthma.

TGF-β, which is a pleiotropic cytokine with significant anti-inflammatory and immunosuppressive properties, is a key regulator in the maintenance of immunological homeostasis. Mice deficient in TGF-β1 develop a lethal multiorgan inflammatory disease at 3 weeks of age with increased expression of inflammatory cytokines and mediators, such as INF-γ, TNF-α, and nitric oxide (7–10). 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 (11–13) and in vivo (14–17). Administration of TGF-β diminishes the severity of autoimmune diseases, such as collagen-induced arthritis (18), allergic encephalomyelitis (EAE) (16, 19), and experimental colitis (20), and neutralization of TGF-β adversely affects the course of these diseases. In addition, an important role for TGF-β has been postulated in oral tolerance, which is mediated by regulatory T cells that produce TGF-β preferentially induced at mucosal sites, possibly under the influence of IL-10 and/or IL-4 (21).

Although TGF-β has a well-recognized role in downregulating autoimmune disease and in inducing tolerance, its capacity to modulate Th2-mediated diseases such as asthma has not been well studied. Because TGF-β–producing T cells are found primarily at mucosal sites (22), and because allergic disease and asthma affect pri-

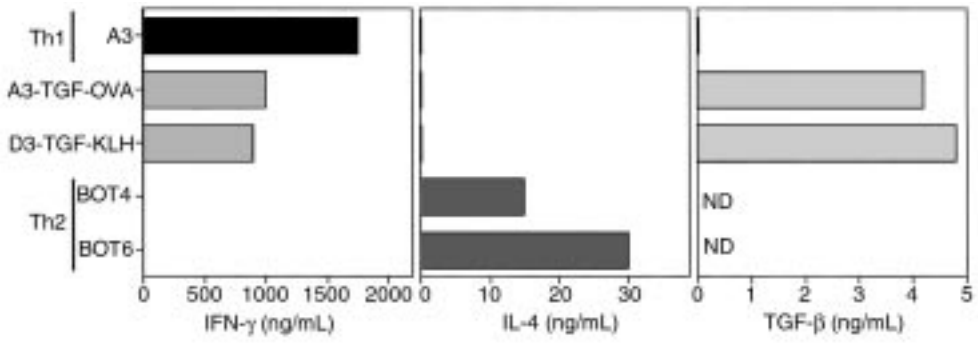

Figure 1
Cytokine profiles of Th cell lines used in these studies. OVA-specific Th1 (A3), Th2 (BOT4, BOT6), and TGF-β–producing cell lines (A3-TGF-OVA), as well as a KLH-specific TGF-β–producing cell line (D3-TGF-KLH) (10^6 cells/mL) were stimulated with concanavalin A (1 μg/mL) for 18 hours in DMEM containing 10% FCS. Supernatants were collected and analyzed by ELISA for IFN-γ, IL-4, and IL-5. Production of active and latent TGF-β in the supernatants was measured 18 hours after culture in HyClone CCM1 serum-free medium using Mv1Lu cells in a bioassay. ND, not done.

marily the respiratory epithelium, we investigated whether TGF-β–producing T cells could reduce Th2-driven inflammation and airway hyperreactivity in asthma and allergy. To directly examine the role of T cells producing latent TGF-β in the respiratory mucosa, we generated Th-cell lines secreting TGF-β by transducing established antigen-specific Th1-cell lines with a retrovirus vector containing the cDNA for latent TGF-β (23). We assessed the capacity of such cells to downmodulate allergic inflammation and airway hyperreactivity in an established adoptive transfer mouse model of asthma. Surprisingly, ovalbumin-specific (OVA-specific) TGF-β–secreting Th cells, but not OVA-specific Th1 cells, profoundly inhibited airway hyperreactivity and airway inflammation induced by established effector Th2 cells in SCID mice or by OVA immunization in BALB/c mice. Our results indicate that T cells secreting TGF-β in the respiratory mucosa can indeed regulate Th2-induced airway hyperreactivity and inflammation and may be key regulatory cells in asthma.

Methods

Animals. BALB/cBy mice were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA), and histocompatible C.B-17Icr$^{scid/scid}$ mice (24) were obtained from the Stanford Medical Center Division of Laboratory Animal Medicine (Stanford, California, USA). DO11.10 mice, which are transgenic for T cell receptor (TCR) recognizing OVA peptide 323–339 (pOVA$^{323–339}$) and backcrossed to BALB/c (25) were kindly provided by Dennis Loh and were bred in our facilities. Animals were used between 6 and 10 weeks of age and were age and sex matched within each experiment. The Stanford University Committee on Animal Welfare approved all animal protocols.

mAbs. mAbs were purified from ascites fluid by ammonium sulfate precipitation and ion-exchange chromatography. We used the following hybridomas: R46A2 (anti–IFN-γ mAb), obtained from American Type Culture Collection (ATCC; Rockville, Maryland, USA); 11B11 (anti–IL-4 mAb), generously provided by Bill Paul (National Institutes of Health, Bethesda, Maryland, USA); XMG1.2 (anti–IFN-γ antibody) and TRFK-4 and TRFK-5 (anti–IL5 mAbs), generously provided by Tim Mosmann (University of Alberta, Edmonton, Canada); BVD4-1D11 and BVD6-24G2 (anti–IL-4 mAb), generously provided by M. Howard (DNAX Research Institute, Palo Alto, California, USA); C17.8 (anti–IL12 mAb), generously provided by G. Trinchieri (Wistar Institute, Philadelphia, Pennsylvania, USA); anti-clonotypic antibody KJ1-26.1, generously provided by Philippa Marrack (National Jewish Medical Center, Denver, Colorado, USA); and 2G7 (anti–TGF-β1), generously provided by B.M. Fendly (Genentech Inc., San Francisco, California, USA).

Preparation of OVA-specific Th1 and Th2 Cells. OVA-specific Th1 and Th2 lines were generated from spleen and lymph node cells from DO11.10 (OVA TCR Tg) mice, as described previously (6). Positively selected OVA-specific TCR$^+$ T cells were cultured at 5×10^5/well with 4×10^6 irradiated spleen cells as APC and OVA (50 μg/mL; ICN Biomedicals, Costa Mesa, California, USA). To generate Th2 lines, rIL4 (40 units/mL) and anti–IL-12 mAb (10 μg/mL) were added to the cultures. Th1 lines were generated by culturing the purified T cells in the presence of anti–IL-4 mAb and rIL12 (0.1 ng/mL). The derivation of the keyhole limpet hemocyanin–specific (KLH-specific) Th1 clone D3 was described previously (26). T-cell lines were maintained by weekly stimulation with APC and OVA (or KLH) without addition of anti-cytokine antibodies and were used after the T-cell lines achieved a stable, committed phenotype (by the third cycle of stimulation). The cytokine profiles of the Th1 and Th2 lines were confirmed, by direct examination of supernatants generated from these lines, using ELISA for IL4, IL-5, and IFN-γ, as described previously (26).

Establishment of antigen-specific T-cell lines producing latent TGF-β using gene transfer. T-cell lines producing TGF-β were generated by transferring the cDNA for latent TGF-β into T cells using a retroviral packaging

cell line capable of producing high titers of recombinant Moloney murine leukemia virus particles that have incorporated the cDNA for latent TGF-β, as reported previously (23). The cDNA encoding murine latent TGF-β1 was first subcloned into the pMFG retroviral vector and into CRIP–TGF-β packaging cells, which produce replication-defective retrovirus, as reported previously (27). The CRIP-TGF-β packaging cell was γ-irradiated (28 Gy) and cultured with activated Th1-cell lines (10^6/well) for 48 hours. The T cells were then cloned by limiting dilution in 96-well plates, using γ-irradiated BALB/c spleen cells (5×10^5/mL) as feeder cells. Clones were expanded and screened by PCR for the presence of TGF-β cDNA, using the primers FP, 5′: GCCCTGGACACCAACTATTGCT and RP, 3′: AGGCTCCAAATGTAGGGGCAGG.

Transfer of cells into SCID mice. Th-cell lines (2.5×10^6 cells of each line per mouse) were transferred intravenously into histocompatible SCID mice, as described previously (6). To facilitate homing of the T-cell lines to the lungs, mice were given OVA, 50 μg in 50 μL PBS intranasally, 1 day before intravenous transfer of cells. To facilitate pulmonary aspiration of OVA, mice were anesthetized intraperitoneally with 0.25–0.35 mL of ketamine (0.44 mg/mL)/xylazine (6.3 mg/mL) in normal saline. Seventy-five percent of the intranasally administered antigen could be detected subsequently in the lungs (28). Control mice immunized with OVA received intravenous saline rather than T cells, whereas other control mice that received T cells intravenously were treated with saline rather than OVA intranasally. One and 2 days after the adoptive transfer of cells, OVA was again administered intranasally. Airway hyperreactivity was determined 24 hours after the last intranasal dose of antigen was administered. Lung fixation and collection of bronchoalveolar lavage (BAL) for cytology and TGF-β determinations was performed 18–24 hours after airway testing. In a subset of the animals, BAL samples for IL-4 and IL-5 were collected 4.5–6.5 hours after the first post-transfer antigen administrations.

Immunization protocol of normal BALB/c mice to induce airway hyperreactivity. TGF-β–producing and Th1 cells were also transferred into OVA-immunized BALB/c mice. BALB/c mice were immunized with OVA (50 μg) intraperitoneally complexed with aluminum potassium sulfate (alum) on day 0 and intranasally (50 μg OVA in 50 μL of PBS) after light anesthesia on days 7, 8, and 9.

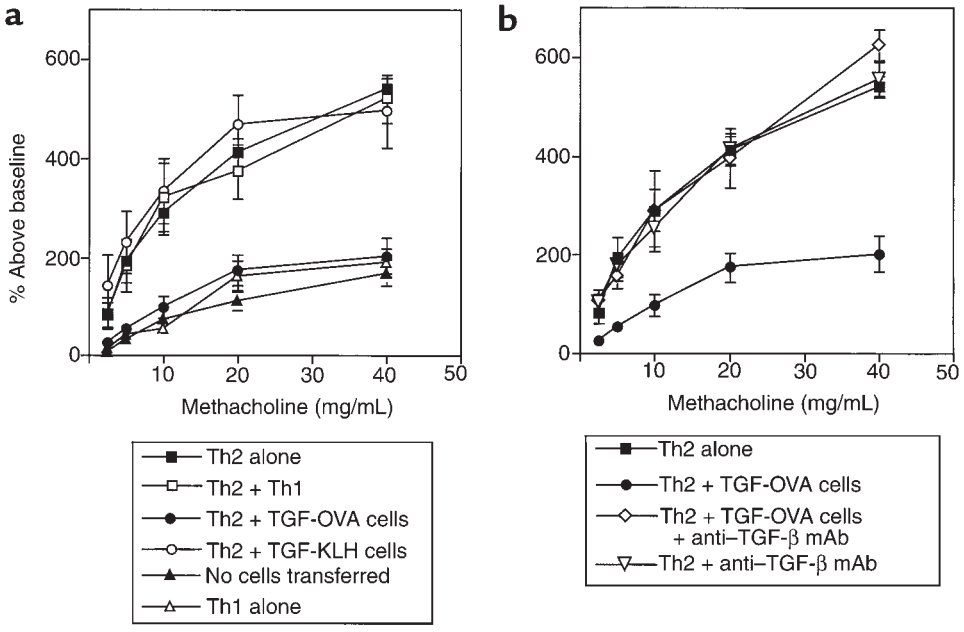

Figure 2

(**a**) TGF-β–producing cells, but not Th1 cells, inhibit Th2 cell–induced airway hyperreactivity in SCID mice. SCID mice received OVA-specific Th1, Th2, or TGF-β–producing cells (2.5×10^6 cells/mouse) intravenously plus intranasal OVA (50 μg) 18 hours before cell transfer. Other SCID mice received a mixture of OVA-specific Th1 and Th2 cells (2.5×10^6 cells/mouse) or a mixture of OVA-specific TGF-β–producing cells and Th2 cells (2.5×10^6 cells/mouse). To examine the specificity of the effect, some mice received KLH-specific TGF-β–producing cells and OVA-specific Th2 cells. Control mice received either OVA only (no cells transferred) or cells only without antigen (data not shown). One and 2 days after the adoptive cell transfer, OVA was again administered intranasally. Three days after adoptive cell transfer, airway hyperreactivity in response to increasing concentrations of inhaled methacholine was measured in a whole-body plethysmograph. Data are expressed as mean percent above baseline (± SEM, $n \geq 6$ for each data point). Cell transfer without intranasal administration of OVA resulted in minimal airway hyperreactivity (data not shown). (**b**) Anti–TGF-β mAb abolishes the inhibitory effect of TGF-β–producing cells on Th2 cell–induced airway hyperreactivity. Neutralizing mAb 2G7 (500 μg/mouse) specific for active TGF-β was given intraperitoneally to SCID mice that received either a mixture of TGF-β–producing cells and Th2 cells (2.5×10^6 cells/mouse) intravenously or Th2 cells alone intravenously plus intranasal OVA on the day of cell transfer. Results are provided as mean percent above baseline (± SEM, $n \geq 5$ for each data point).

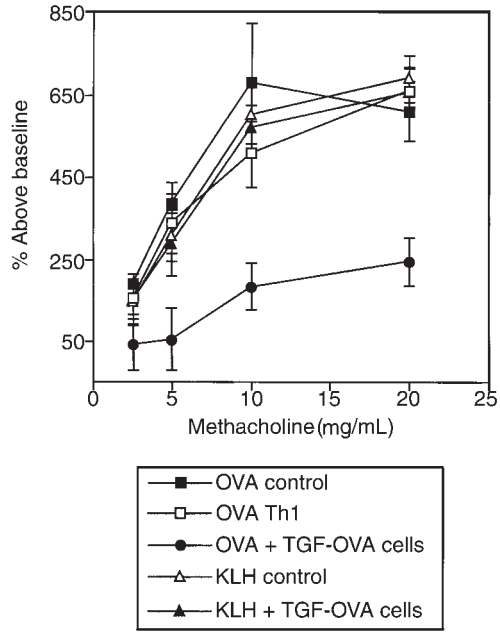

Figure 3
TGF-β–producing cells inhibit airway hyperreactivity in OVA-immunized BALB/c mice. BALB/c mice were immunized with OVA (50 μg) in alum intraperitoneally on day 0 and intranasally (50 μg OVA in 50 μL PBS) on days 7, 8, and 9. Another group of mice was immunized with KLH rather than OVA (KLH, 25 μg intraperitoneally in alum; and KLH, 25 μg in PBS, 50 μL, intranasally). Mice from both groups received OVA-specific TGF-β–producing cells (2.5×10^6 cells/mouse) intravenously on day 7. Some of the OVA-immunized mice received OVA-specific Th1 cells (2.5×10^6 cells/mouse) intravenously on day 7. Airway hyperreactivity in response to inhaled methacholine was measured in a whole-body plethysmograph on day 10 ($n = 5$ for each data point). Results are demonstrated as mean percent above baseline (± SEM).

Control mice received intraperitoneal injections of alum alone and PBS intranasally. Some mice received either TGF-β–producing cells or Th1 cells intravenously (2.5×10^6 cells/mouse) on day 7. Airway hyperreactivity to inhaled methacholine was measured 24 hours after the last intranasal dose of OVA (day 10). BAL and lung fixation was performed the following day (day 11).

Measurement of the biological activity of TGF-β1. The activity of TGF-β was assayed in a bioassay using Mv1Lu mink cells (ATCC) (29). T-cell supernatants, prepared by culture of 10^6 cells/mL in serum-free medium (HYQ-CCM1; HyClone Laboratories, Logan, Utah, USA) for 24 hours, and BAL fluid, performed with serum-free medium, taken from SCID and BALB/c mice 4 days after cell transfer, were assayed for the presence of latent and active TGF-β. Latent TGF-β was converted to the active form by acidification with 1 N HCl to pH 2 at 4°C for 15 minutes. TGF-β1 activity was inversely proportional to Mv1Lu cell growth, as determined by ^{3}[H]-thymidine incorporation. The sensitivity of the assay is about 80 pg/mL. The mink cell assay is very specific for active TGF-β, although TNF-α at very high concentrations (> 10 μg/mL) can inhibit Mv1Lu cell proliferation (30).

Cytokine ELISA. ELISAs were performed as described previously (31). The antibody pairs used were as follows, listed by capture/biotinylated detection: IFN-γ, R4-6A2/XMG1.2; IL-4, 11B11/BVD6-24G2; IL-5, TRFK-5/TRFK-4. The standards were recombinant cytokine curves generated in 1:2 dilutions from 20 to 0.156 ng/mL for IFN-γ, 500 to 7.5 pg/mL for IL-4, and 5000 to 78.2 pg/mL for IL-5.

Measurement of airway responsiveness. Airway responsiveness was assessed by methacholine-induced airflow obstruction from conscious mice placed in a whole-body plethysmograph (model PLY 3211; Buxco Electronics Inc., Troy, New York, USA), as described previously (6). Pulmonary airflow obstruction was measured by enhanced pause (Penh) using the following formula:

Equation 1

$$\text{Penh} = \left(\frac{\text{Te}}{\text{RT}} - 1\right) \times \left(\frac{\text{PEF}}{\text{PIF}}\right)$$

where Penh = enhanced pause (dimensionless), Te = expiratory time, RT = relaxation time, PEF = peak expiratory flow (mL/s), and PIF = peak inspiratory flow (mL/s), measured with a transducer (model TRD5100) and analyzed by system XA software (model SFT 1810; Buxco Electronics Inc.). Measurements of methacholine responsiveness were obtained by exposing mice for 2 minutes to nebulized NaCl (0.9%), followed by incremental doses (2.5–40 mg/mL) of nebulized methacholine and monitoring Penh. Results were expressed for each methacholine concentration as the percentage of baseline Penh values after NaCl exposure.

Collection of BAL fluid and lung histology. Animals were injected intraperitoneally with a lethal dose of phenobarbital (450 mg/kg), as described previously (6). After ligating the left main stem bronchus, the trachea was cannulated, and the right lung was then lavaged with 0.4 mL of serum-free medium 3 times, and the fluid was pooled. Cells in the lavage fluid were counted, and BAL cell differentials were determined on slide preparations stained with Hansel stain (Lide Laboratories, Florissant, Missouri, USA). At least 200 cells were differentiated by light microscopy based on conventional morphologic criteria. After washing with PBS, 1 part of the left lung was fixed in 10% formalin, routinely processed, and embedded in paraffin wax. Five micrometer sections were prepared and stained with hematoxylin and eosin (H&E). Some of the samples were stained with Masson's trichrome to assess pulmonary fibrosis. In some experiments, a section of the left lung was embedded in OCT compound (Tissue Tec; Miles Inc, Elkhart, Indiana, USA), snap-frozen in liquid nitrogen, and stored at –80°C until sectioning. Five micrometer sections were fixed in acetone and stained with the biotinylated anti-clonotypic antibody, KJ1-26.1, and streptavidin-FITC (PharMingen, San Diego, California, USA). A biotinylated anti-human IL-10 antibody (JES-312G8-biotinylated) was used as control antibody.

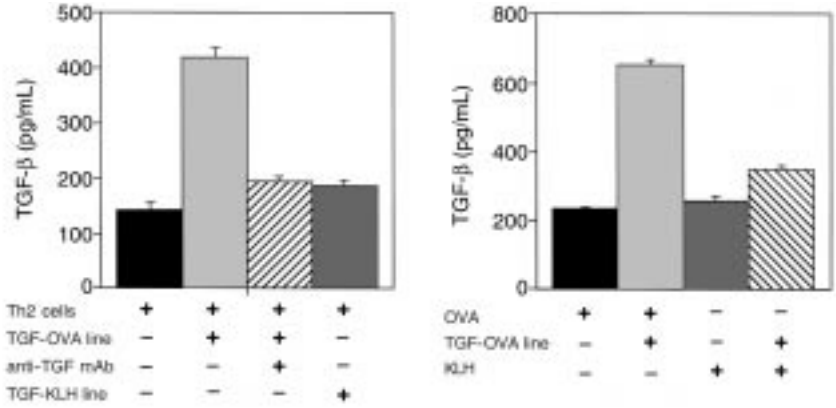

Figure 4
Increased levels of active TGF-β are present in BAL of SCID and BALB/c mice receiving latent TGF-β–producing T cells. (**a**) SCID mice received OVA-specific Th2 cells (2.5×10^6 cells/mouse) or a mixture of OVA-specific TGF-β–producing cells and Th2 cells (2.5×10^6 cells /mouse) intravenously, plus intranasal OVA. Some mice were treated intraperitoneally with neutralizing mAb 2G7.5A9 (500 μg/mouse), specific for active TGF-β, at the day of the cell transfer. BAL was performed 4 days after cell transfer with 3 aliquots of 0.4 mL serum-free DMEM. Active TGF-β was measured in the supernatant of the BAL-fluid using Mv1Lu cells in a bioassay. Data are shown as mean ± SEM. (**b**) BALB/c mice were immunized with OVA (50 μg) or KLH (50 μg) intraperitoneally in alum on day 0 and intranasally as described in Figure 3. Mice from both groups received OVA-specific TGF-β–producing cells intravenously on day 7. BAL was performed 4 days after cell transfer with 3 aliquots of 0.4 mL serum-free medium. Active TGF-β was measured in the supernatant of the BAL-fluid using Mv1Lu cells in a bioassay. Data are shown as mean ± SEM.

Results

Characterization of T-cell lines. OVA-specific Th1- and Th2-cell lines were generated by in vitro stimulation of lymph node or spleen cells from OVA-specific TCR DO11.10 transgenic mice, as described previously (6). The Th1 and Th2 lines were used after at least 3 cycles of stimulation, after the T-cell lines achieved a stable committed phenotype (32). OVA-specific Th-cell lines constitutively producing latent TGF-β were produced by transfecting established OVA-specific Th1-cell lines with a retrovirus vector containing the gene for latent TGF-β1 (33). A KLH-specific T-cell line producing latent TGF-β was also generated by transfecting an established KLH-specific Th1 cloned line (34) with the same retrovirus vector. The cytokine profiles of the Th-cell lines are shown in Figure 1. The Th1-cell line produced high levels of IFN-γ, but no detectable IL-4, whereas the Th2-cell lines produced high levels of IL-4 but very low levels of IFN-γ. The TGF-β–transduced cell lines produced large quantities of latent TGF-β, as well as IFN-γ on antigen stimulation.

TGF-β–producing T cells, but not Th1 cells, inhibit airway hyperreactivity induced by Th2 cells. To determine the effects of Th1 cells and TGF-β–producing Th cells on Th2 cell–induced airway hyperreactivity and inflammation, we adoptively transferred the different OVA-specific cell lines into histocompatible, immunodeficient SCID mice. By choosing SCID mice we excluded the contribution of endogenous lymphocytes, which could inhibit the function of transferred Th cells or could contribute to the development of airway hyperreactivity. The cell transfer was performed using an established protocol (6). Localization of the transferred T-cell lines into the lungs was enhanced by intranasal administration of OVA one day before adoptive transfer of the T-cell lines. One and 2 days later, OVA was again administered intranasally. Immunohistochemical staining of lung sections with the anti-TCR clonotypic antibody KJ1.26.1 confirmed that the transferred cell lines homed to the lungs of recipients (data not shown).

Adoptive transfer of OVA-specific Th2 cells resulted in significant airway hyperreactivity, as assessed by responsiveness to inhaled methacholine (Figure 2a). Whereas cotransfer of OVA-specific Th1 cells failed to inhibit Th2 cell–induced airway hyperreactivity, cotransfer of OVA-specific TGF-β–secreting Th cells abolished Th2 cell–induced airway hyperreactivity. This effect was antigen specific, because little inhibition of airway hyperreactivity was observed after transfer of KLH-specific TGF-β–secreting cells with OVA-specific Th2 cells and immunization with intranasal OVA. The inhibitory effect of the OVA-specific TGF-β–secreting cells was dependent on the presence of TGF-β because administration of a neutralizing mAb 2G7, specific for active TGF-β, blocked the protective effect of TGF-β–producing T cells against Th2 cell–induced airway hyperreactivity (Figure 2b). The anti–TGF-β mAb had no effect in control mice receiving OVA-specific Th2 cells but no TGF-β–secreting cells.

We asked if the TGF-β–transduced cells mitigated the airway hyperreactivity induced by Th2 cells by inhibiting localization of the transferred Th2 cells into the lungs or by inhibiting the function of Th2 cells after they had migrated into the lungs. Because significant levels of IL-4 and IL-5 were present in BAL fluid not only of mice receiving Th2 cells (IL-4: 107 ± 60 pg/mL; IL-5: 839 ± 334 pg /mL) at 24 hours after transfer but

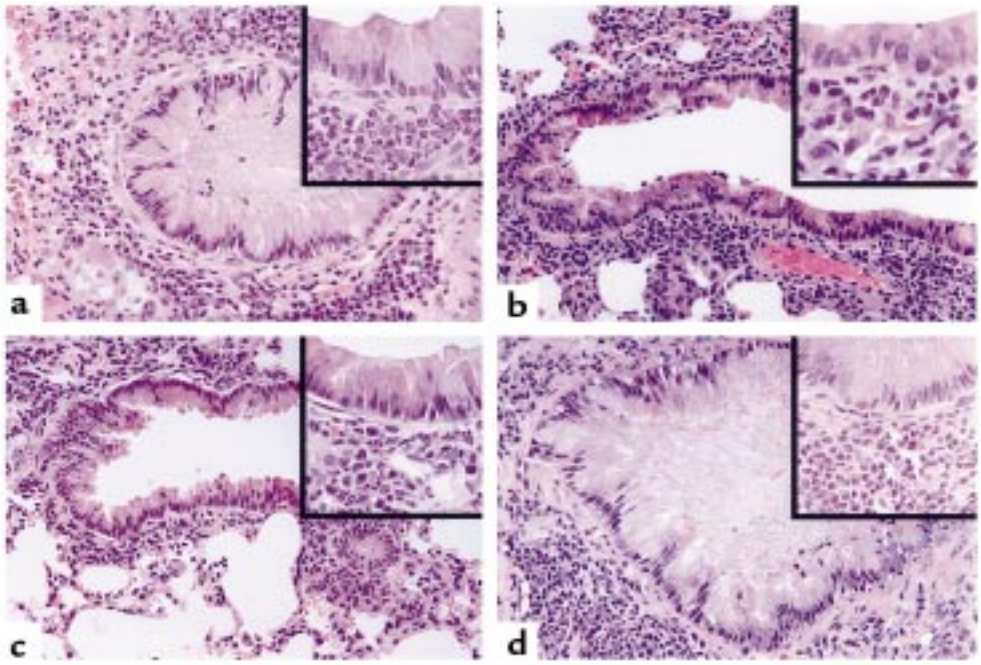

Figure 5
Lung histology of OVA- or KLH-immunized BALB/c mice after transfer of TGF-β–producing Th cells or Th1 cells. (**a**) Lung tissue from a control BALB/c mouse immunized with OVA intraperitoneally and intranasally, as described in Figure 3. Lung tissue was removed on day 11. An intense peribronchiolar mononuclear cell infiltrate is present, consisting of large numbers of eosinophils, lymphocytes, and a few neutrophils. H&E, ×300. Insert: The airway lumen is filled and expanded by thick mucus. The airway epithelium shows tall columnar cells exhibiting abundant cytoplasmic mucin. H&E, ×400. (**b**) Lung tissue from an OVA-immunized BALB/c mouse that received OVA-specific Th1 cells (2.5×10^6 cells/mouse) intravenously on day 7. Lung tissue was removed on day 11, 3 days after cell transfer. A dense peribronchiolar inflammatory infiltrate is present, consisting of lymphocytes, neutrophils, and few eosinophils. H&E, ×300. Insert: Lymphocytes are penetrating the airway epithelium and surrounding tissue spaces, but the airway lumen is free of mucus plugs. H&E, ×400. (**c**) Lung tissue from an OVA-immunized BALB/c mouse that received OVA-specific TGF-β–producing cells (2.5×10^6 cells/mouse) intravenously on day 7. H&E, ×300. Insert: There is a significant reduction of peribronchiolar inflammatory cells and a significant reduction in the number of eosinophils as compared with Figures 5, **a** and **b**. In addition, the airway epithelium is almost normal, and almost no mucus is present in the airway lumen. H&E, ×400. (**d**) The inflammatory response in lung tissue from a KLH-immunized BALB/c mouse is not reduced by transfer of OVA-specific TGF-β–producing cells (2.5×10^6 cells/mouse). Intense peribronchiolar mononuclear cell infiltrates are present, consisting of eosinophils and lymphocytes, and the airway lumen is filled and expanded by thick mucus, similar to that seen in Figure 5a. H&E, ×300. Insert: The airway epithelium shows tall columnar cells exhibiting abundant cytoplasmic mucin and inflammatory cells, including eosinophils and lymphocytes. H&E, ×400.

also of mice receiving both Th2 cells and TGF-β–transduced cells (IL-4: 86 ± 43 pg /mL; IL-5: 801 ± 99 pg/mL), it is unlikely that the homing of Th2 cells was affected by the presence of the TGF-β–transduced cells. At 96 hours after transfer, IL-4 and IL-5 levels in BAL from mice receiving both TGF-β–transduced cells and Th2 cells were undetectable, but the levels were similarly low in BAL from mice receiving only Th2 cells. Furthermore, because the TGF-β–transduced cells inhibited airway hyperreactivity even when transferred 6 hours after injection of the Th2 cells (after the Th2 cells had migrated into the lungs; data not shown), it appears that the TGF-β–transduced cells functioned by inhibiting the activity of Th2 effector cells in the microenvironment of the lung.

TGF-β–producing T cells, but not Th1 cells, inhibit the development of airway hyperreactivity in OVA-immunized BALB/c mice. Because TGF-β–producing Th cells clearly reversed the function of established Th2 effector cells when adoptively transferred into SCID mice, we wished to determine whether the TGF-β–producing Th cells could function in normal mice and inhibit the development of Th2-driven allergen-induced airway hyperreactivity. Figure 3 shows that intraperitoneal and intranasal immunization of BALB/c mice with OVA resulted in the development of significant airway hyperreactivity. Adoptive transfer of OVA-specific TGF-β–producing Th cells, but not Th1 cells, significantly reduced this airway hyperreactivity. The effect was antigen specific, because OVA-specific TGF-β–producing Th cells failed to reduce airway hyperreactivity induced in BALB/c mice immunized with KLH rather than OVA.

Increased concentration of active TGF-β in BAL fluid of SCID and BALB/c mice receiving TGF-β–producing Th cells. One day after measurement of airway hyperreactivity, the lungs of the mice were lavaged, and the BAL fluid was examined for the presence of active TGF-β using a bioassay. BAL fluid from SCID mice that received both TGF-β–producing T cells and Th2 cells contained significant levels of active TGF-β, which was abolished by the in vivo administration of anti–TGF-β mAb (Figure

4a). In contrast, BAL fluid contained only low levels of active TGF-β when SCID mice received only Th2 cells or when KLH-specific, rather than OVA-specific, TGF-β–producing cells were transferred with OVA-specific Th2 cells into OVA-immunized SCID mice. Transfer of the OVA-specific TGF-β–producing cells into OVA-immunized BALB/c mice also resulted in increased levels of active TGF-β in BAL fluid (Figure 4b). The effect was antigen specific because only baseline levels of active TGF-β were found in BAL fluid from mice immunized with KLH and given OVA-specific TGF-β–producing cells. Thus, the reversal of Th2 cell–induced airway hyperreactivity by TGF-β–secreting T cells was associated with the presence of active TGF-β in BAL fluid. These data indicate that the transferred TGF-β–producing cells migrated to the lungs of recipients and suggest that conversion of T cell-derived latent TGF-β into the active form occurred in the inflammatory environment created by OVA-specific Th2 cells.

Airway histology in BALB/c recipients of Th1 and TGF-β–producing cells. The TGF-β–secreting cell line not only reduced airway hyperreactivity, but also reduced lung inflammation, as assessed by histological examination of lung tissue and examination of cells in BAL fluid. As expected, intraperitoneal and intranasal immunization of BALB/c mice with OVA induced copious intrabronchial mucus and intense peribronchiolar and perivascular infiltrates, consisting of eosinophils, lymphocytes, and some neutrophils (Figure 5a). Adoptive transfer of OVA-specific Th1 cells into these mice during the 10-day immunization protocol decreased mucus secretion and decreased the number of eosinophils, but had little effect on the density of the peribronchiolar and perivascular infiltrates (Figure 5b). In contrast, adoptive transfer of TGF-β–producing Th cells significantly reduced not only bronchial mucus production, but also greatly decreased the number of inflammatory cells, and especially the number of eosinophils, in the respiratory epithelium and around most bronchi (Figure 5c). The reduction of the inflammatory infiltrates correlated with low airway hyperreactivity (Figure 3). The effect was antigen specific, because transfer of OVA-specific TGF-β–producing Th cells did not reduce airway mucus production or peribronchiolar inflammation in KLH-immunized BALB/c mice (Figure 5d). Special stains for collagen deposition (Masson's trichrome stain) did not show any fibrosis in the lungs of mice receiving the TGF-β–producing cell lines (data not shown). Because the TGF-β–transduced cell line produced both IFN-γ and TGF-β, it is possible that the reduction in airway inflammation and airway hyperreactivity was due to the presence of both Th1 cytokines and TGF-β. However, transfer of the TGF-β–transduced cells produced an effect that was qualitatively different from that observed when Th1 cells were transferred; there was a major reduction in the cellularity in the lungs when TGF-β–transduced cells were transferred. This reduction indicated that the additional presence of TGF-β in the lungs resulted in a significant anti-inflammatory effect not observed with untransduced Th1 cells.

Th1 and TGF-β–producing cells reduce airway eosinophilia. The number of eosinophils recovered in BAL fluid was greatly reduced by transfer of OVA-specific TGF-β–producing cells as well as by Th1 cells to OVA-immunized BALB/c recipients. The number of eosinophils in BAL fluid was reduced 16-fold after transfer of TGF-β–producing cells and 5-fold after transfer of Th1 cells (Figure 6). The reduction was antigen specific, because the number of eosinophils in BAL fluid of KLH-immunized BALB/c mice that received OVA-specific TGF-β–producing cells was not significantly reduced. However, the total number of cells recovered in BAL was not changed by transfer of TGF-β–producing T cells or OVA-specific Th1 cells. The number of BAL cells recovered per mouse was: 13×10^5 in control mice immunized with OVA; 12×10^5 in mice given OVA + Th1 cells; 9×10^5 in mice receiving OVA plus TGF-β–producing cells; 12×10^5 in control mice immunized with KLH; and 11×10^5 in mice receiving KLH + TGF-β–producing cells.

Discussion

In this study we demonstrated in a murine model of asthma that $CD4^+$ T cells engineered to produce latent TGF-β effectively inhibited the capacity of Th2 effector cells to induce airway hyperreactivity and airway inflammation, whereas Th1 cells failed to counterbalance the proasthmatic effects of Th2 cells. The function of TGF-β–producing cells was evaluated by adoptive transfer of well-defined antigen-specific T

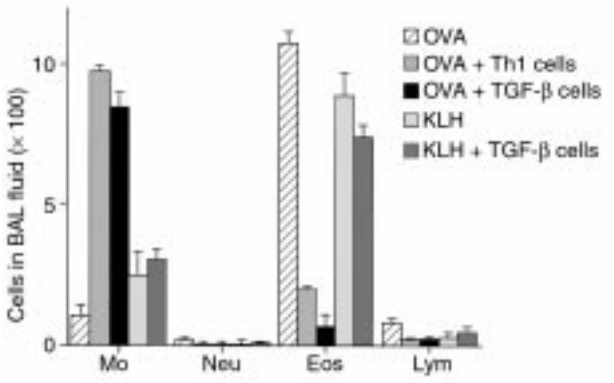

Figure 6
TGF-β–producing cells and Th1 cells significantly reduce the number of eosinophils in the BAL fluid of OVA-immunized BALB/c mice. BALB/c mice were immunized intraperitoneally and intranasally either with OVA or KLH, as described in Figure 3. OVA-specific TGF-β–producing cells or Th1 cells were adoptively transferred into these animals, and BAL was performed 4 days after cell transfer with 3 aliquots of 0.4 mL serum-free medium. The total number of different types of leukocytes was determined from Hansel stain slide preparations of BAL fluid. The data are expressed as cells recovered ± SD per mouse of each cell type, based on differentials of 200 cells ($n = 5$ for each data point). Mo, macrophages; Lym, lymphocytes; Eos, eosinophils; Neu, neutrophils.

cells expressing identical TCRs into lymphocyte-deficient SCID mice. Because the recipient SCID mice had no T cells of their own, we can conclude that the transferred TGF-β–producing T cells, and not other T cells, directly inhibited airway hyperreactivity and inflammation. The effect of TGF-β–producing T cells was antigen specific and was dependent on secretion of TGF-β because a neutralizing mAb to TGF-β abolished the inhibitory effect, and directly downregulated the function of Th2 cells rather than inhibited homing of Th2 cells into the lungs. Moreover, the effect was also observed by transfer of the TGF-β–producing T cells into OVA-immunized immunocompetent BALB/c mice, establishing that such cells can not only inhibit the function of effector Th2 cells but can also prevent the development of airway hyperreactivity in immunocompetent animals.

To our knowledge, this is the first study to show directly that TGF-β produced by allergen-specific T cells effectively downregulates Th2-driven airway hyperreactivity. By contrast, the conventional wisdom indicates that Th1 cells, by inhibiting the function of Th2 cells, are the primary cell type that provides protective immunity in asthma. This paradigm, which maintains that Th1 cells are beneficial in allergy and asthma, is supported by several studies providing indirect evidence (2, 35–43). However, direct examination of the role of antigen-specific Th1 effector cells in asthma demonstrated that whereas Th1 cells were able to reduce mucus production and airway eosinophilia, they failed to inhibit Th2 cell–induced airway hyperreactivity, and, in fact, exacerbated airway inflammation (6, 44). These studies indicate that the Th1/Th2 paradigm may be more complex than initially appreciated and that suppression of allergic inflammation and Th2 activity in vivo may depend, at least in part, on cells other than Th1 lymphocytes. Therefore, we sought to identify other regulatory cell types that modulate Th2 effector cells.

In our murine model of asthma, transfer of OVA-specific TGF-β–producing T cells produced much greater salutary effects than transfer of OVA-specific Th1 cells. The TGF-β–producing cells inhibited the major features of asthma, including airway hyperreactivity, airway inflammation, eosinophilia, and mucus production. Moreover, the TGF-β–transduced cells produced an effect that was qualitatively different from that observed when Th1 cells were transferred: there was a major reduction in the cellularity in the lungs when TGF-β–transduced cells were transferred. In addition, the TGF-β–transduced cells significantly reduced airway hyperreactivity whereas Th1 cells had no inhibitory effect on this parameter. Our results therefore demonstrate that TGF-β has the capacity to reverse ongoing allergic inflammation and airway hyperreactivity and suggest that TGF-β–producing T cells play a significant regulatory role in asthma. It is possible, however, that although the presence of TGF-β resulted in a significant anti-inflammatory effect in the lungs not observed with untransduced Th1 cells, the anti-inflammatory effect observed with TGF-β–transduced Th1 cells may depend on the presence of TGF-β in combination with Th1 cytokines.

Our interest was drawn to TGF-β because it has been shown previously to be a key immunoregulatory factor in the development of unresponsiveness to antigens in the gastrointestinal tract, an anatomic site that is closely related developmentally to the respiratory tract. For example, several investigators demonstrated that TGF-β–producing Th3 cells and Tr1 cells inhibited experimental colitis (17, 45), and TGF-β induced by oral tolerance inhibited subsequent tracheal eosinophilia (46). Furthermore, TGF-β production controlled Th2-driven inflammation in chemical-induced autoimmunity (47). These studies, as well as those showing that TGF-β regulates Th1-driven autoimmune diseases such as EAE, uveoretinitis, and collagen-induced arthritis (16, 18, 20, 48–50), indicate that TGF-β inhibits both Th2- as well as Th1-driven inflammation. The modulation by TGF-β of both Th2 and Th1 responses is consistent with the fact that the same TGF-β–containing retrovirus vector used in our studies to reverse allergic asthma, reversed the symptoms of EAE when transfected into myelin-specific Th1 cells subsequently transferred into mice (23). Thus, T cells activated to produce TGF-β may regulate both allergen-induced airway hyperreactivity as well as autoimmune diseases.

Although TGF-β production in the lungs may significantly reduce undesirable inflammation, potentially, TGF-β could also cause severe pulmonary fibrosis because of mitogenic and chemotactic activity on fibroblasts (51). However, TGF-β is normally produced in a latent form, attached to a latency-associated protein, which is removed during an activation process that uncovers the receptor-binding region (52). This activation process requires an inflammatory environment, occurs in an autoregulatory manner that prevents overexpression of active TGF-β, and may allow TGF-β–secreting T cells to effectively attenuate airway inflammation, but minimize tissue damage. Our current and previous studies (23) are unique, we believe, in that we used TGF-β produced in its natural latent form by antigen-specific T cells. The latent TGF-β was activated in the inflammatory environment of the lung as demonstrated by increased concentrations of active TGF-β in the BAL fluid of mice that received antigen-specific TGF-β–producing T cells, resulting in a significant reduction of pulmonary inflammation. Although we cannot exclude the possibility that pulmonary fibrosis might occur if TGF-β–producing T cells were present for an extended period of time, in this study, over a 4-day period, antigen-specific T cells delivering latent TGF-β locally to the lungs effectively reversed airway hyperreactivity and reduced airway inflammation without inducing pulmonary fibrosis.

If TGF-β–producing Th cells indeed regulate airway hyperreactivity and inflammation in the lung mucosa, what is the role of allergen-specific Th1 cells in asthma?

What of the observations demonstrating that allergen immunotherapy improves clinical status by inducing a switch from a Th2 to a Th1 cytokine production in allergen-specific cells (36–38)? What of the observations or that administration of IL-12 intratracheally, of IL-12 fusion proteins, or of plasmids containing cDNA for allergens reverses Th2-dominated immune responses and reduces airway hyperreactivity (39, 41–43), or that individuals predisposed toward the production of Th1 cytokines (e.g., patients with multiple sclerosis or those infected with *Mycobacteria tuberculosis*) have a reduced likelihood of developing allergic disease (2, 35)? We speculate that both Th1 cells as well as TGF-β–secreting T cells play pivotal roles in regulating protective immunity in asthma, but each may function in a different compartment and at a different stage of T-cell activation. Th1 cells might act in peripheral lymphoid organs, such as lymph nodes and the spleen, and, when present early during the development of an immune response, may inhibit the development of Th2 effector cells from naïve or resting memory cells. It is unlikely that large numbers of Th1 cells function normally in the lung mucosa itself, because Th1 cells could exacerbate inflammation and cause tissue damage (6). In contrast, we suggest that T cells producing high levels of TGF-β (e.g., allergen-specific Th3 cells; ref. 17) act preferentially in the respiratory epithelium to counterbalance effector Th2 cells.

The site at which TGF-β–producing cells function may be related to specific conditions that induce TGF-β production in T cells. Such mechanisms are as yet not fully understood, but appear to be related to local mucosal rather than systemic exposure to antigen (32, 53, 54). For example, low antigen concentrations at mucosal sites (22) or high local concentrations of IL-4 (53), IL-10 (45), or TGF-β (55) may enhance TGF-β production in T cells. In addition, it is possible that antigen-presenting cells in mucosal tissues express low levels of costimulatory molecules such as B7 (CD80 and CD86) (56), which may result in preferential cross-linking of CTLA-4 and in the induction of TGF-β production in T cells (33). Thus, we believe that Th1 and TGF-β–producing T cells complement each other in modulating allergic inflammation and asthma. Although active TGF-β is present at low levels in the lungs of patients with status asthmaticus (57), it is possible that higher levels are transiently present in nonasthmatic individuals, preventing the development of airway inflammation. Thus, further study of levels of TGF-β present during allergen stimulation in vivo in both allergic and nonallergic individuals is required to understand its regulation of airway inflammation.

In summary, our data demonstrate that TGF-β–producing T cells in the respiratory mucosa downregulate airway inflammation and hyperreactivity induced by Th2 effector cells. Studies to further define the role of TGF-β–producing T cells in the regulation of asthma and allergies and to elucidate the mechanisms that enhance the expansion of allergen-specific TGF-β–producing T cells are ongoing. Such studies are particularly important as prerequisites for the development of therapies focused on immunomodulation of deleterious Th2-polarized responses in asthma and allergic diseases.

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