Cardiovascular, Pulmonary and Renal Pathology

Obligatory Role for Interleukin-13 in Obstructive Lesion Development in Airway Allografts

Vibha N. Lama,* Hiroaki Harada,[†] Linda N. Badri,* Andrew Flint,[‡] Cory M. Hogaboam,[‡] Andrew McKenzie,[§] Fernando J. Martinez,* Galen B. Toews,* Bethany B. Moore,* and David J. Pinsky[†]

From the Division of Pulmonary and Critical Care Medicine,* Division of Cardiovascular Medicine,[†] and the Department of Pathology,[‡] University of Michigan Health System, Ann Arbor, Michigan; and the Laboratory of Molecular Biology,[§] Medical Research Council, Cambridge, United Kingdom

The pathogenesis of bronchiolitis obliterans (BO), a common and devastating obliterative disorder of small airways following lung transplantation, remains poorly understood. Lesions are characterized in their early stages by lymphocyte influx that evolves into dense fibrotic infiltrates. Airway specimens taken from patients with histological BO revealed infiltrating myofibroblasts, which strongly expressed the signaling chain of the high affinity interleukin-13 (IL-13) receptor IL-13R α 1. Because IL-13 has proinflammatory and profibrotic actions, a contributory role for IL-13 in BO development was examined using murine models of orthotopic and heterotopic tracheal transplantation. Compared with airway isografts, allografts exhibited a significant increase in relative IL-13 mRNA and protein levels. Allogeneic tracheas transplanted into IL-13-deficient mice were protected from BO in both transplant models. Flow cytometric analysis of orthotopic transplant tissue digests revealed markedly fewer infiltrating mononuclear phagocytes and CD3⁺ T lymphocytes in IL-13-deficient recipients. Furthermore, protection from luminal obliteration, collagen deposition, and myofibroblast infiltration was observed in heterotopic airways transplanted into the IL-13^{-/-} recipients. Transforming growth factor- β 1 expression was significantly decreased in tracheal allografts into IL-13^{-/-} recipients, compared to wild-type counterparts. These human and murine data implicate IL-13 as a critical effector cytokine driving cellular recruitment and subsequent fibrosis in clinical and ex-

perimental BO. (Am J Pathol 2006, 169:47–60; DOI: 10.2353/ajpath.2006.050975)

Bronchiolitis obliterans syndrome (BOS) is the major factor limiting quality of life as well as long-term survival after lung transplantation. BO is a histological diagnosis, characterized by fibrosis and obliteration of the small airways.¹ BOS, the clinical correlate of BO, is diagnosed by irreversible deterioration of pulmonary function in the absence of other causes.² BOS is seen in up to 60% of lung transplant recipients by 5 years after transplantation. There are currently no meaningful therapeutic interventions, providing a compelling rationale to understand fully BOS pathogenesis as this may lead to development of new therapeutic approaches.

Data from human as well as animal studies suggest associations between inflammatory cells/mediators and BOS. Lymphocytic bronchitis is considered a risk factor for development of BOS in humans,³ and lymphocytic infiltration precedes development of luminal obliteration in animal models of tracheal transplantation.^{4,5} Similarly, several markers of inflammation (eg, neutrophilia,⁶ IL-8,⁷ and monocyte chemoattractant protein-1 (MCP-1/CCL2)^{8,9}) are predictive of development of BO in humans. However, the failure of immunosuppressive therapy and anti-inflammatory agents to demonstrate efficacy in preventing or treating this disease has focused attention on BOS as a fibroproliferative response to airway epithelial injury. Evidence for this is provided by studies suggesting a role for transforming growth factor- β (TGF- β), a major profibrotic factor, in the pathogenesis of BOS.^{7,10,11}

Supported by grants from the National Institutes of Health (K23 HL077719 to V.N.L.; R01 HL55397 to D.J.P.; R01HL69448 to D.J.P.; HL071586 to B.B.M.; and P50HL56402 to G.B.T., C.M.H., F.J.M., and B.B.M.) and the American Lung Association (RG-1059-N to V.N.L.).

V.N.L. and H.H. contributed equally to this work.

Accepted for publication April 3, 2006.

Address reprint requests to Vibha N. Lama, M.D., M.S., Division of Pulmonary and Critical Care Medicine, University of Michigan Health System, 1150 W. Medical Center Drive, Ann Arbor, MI 48109-0644. E-mail: vlama@umich.edu.

Table 1. Characteristics of Patients Analyzed in this Study

Characteristic	Mean $\pm$ SD	Range
Age at transplant (years)	48 $\pm$ 11	19 to 63
Transplant to BOS (months)	23.9 $\pm$ 26.7	3.4 to 110
BOS to biopsy (months)	5.6 $\pm$ 9.4	-7.8 to 30.1
Transplant to biopsy (months)	29.9 $\pm$ 29.4	3 to 123

Interleukin-13 (IL-13), a Th2 cytokine produced by multiple cell types, including T cells and natural killer cells, has been implicated in both inflammatory cell recruitment and remodeling in the lung.^{12,13} IL-13 exerts a strong profibrotic effect by promoting fibroblast proliferation, extracellular matrix deposition, adhesion molecule expression, profibrotic cytokine secretion, and contraction of collagen gels^{14–20} and has been associated with both airway^{12,21} and alveolar fibrosis.²² Neutralization of IL-13 can ameliorate *Schistosoma mansoni*-induced hepatic fibrosis²³ as well as bleomycin-induced alveolar fibrosis.²⁴

To investigate the role of IL-13 in the development of BO, human specimens with BO were evaluated for expression of the IL-13 receptor, and the causal role for IL-13 in BO was studied in two well-established murine tracheal transplant models. These experiments used a strategy wherein airways were implanted into IL-13 gene-deleted recipients, and the effect on the inflammatory and fibrotic components of BO were studied. The rationale for these experiments is that IL-13 has been implicated in inflammation, airway remodeling, and tissue fibrosis, conditions that are replicated in the growing obliterative lesion of BO.

Materials and Methods

Human Lung Biopsy Specimens

Fifteen transbronchial biopsy (TBBx) samples with a pathological diagnosis of BO, defined by the standardized criteria of the International Society of Heart and Lung Transplantation (ISHLT),²⁵ were identified under an Institutional Review Board-approved protocol. Patients from whom these biopsies were obtained included 13 females and two males with a median age of 48 $\pm$ 11 years (range, 19–63 years) at the time of transplantation. Primary indications for transplantation in these cases included emphysema with or without α -1 antitrypsin deficiency (n = 12), primary pulmonary hypertension (n = 2), and interstitial lung diseases (n = 1). Although all patients met the criteria of BOS as defined by the ISHLT guidelines,² they were at different stages of BOS at the time of biopsy (BOS 0 (n = 1), BOS 0p (n = 1), BOS 1 (n = 5), BOS 2 (n = 3), and BOS 3 (n = 5)). Further characteristics of the cases analyzed are shown in Table 1. Controls included 14 TBBx specimens demonstrating terminal bronchioles but no evidence of BO. These biopsies were obtained from patients who had no evidence of clinical BOS and were matched to BO cases by time after transplantation. Time-matched control for one biopsy case could not be obtained. TBBx specimens used in the

present study were processed and paraffin-embedded according to standard clinical laboratory procedures.

Immunohistochemistry for Human Samples

Contiguous sections of paraffin wax-embedded TBBx specimens were analyzed immunohistochemically for localization of α -smooth muscle actin (α -SMA) and IL-13 receptor α 1 (IL-13R α 1). Immunohistochemical staining with human IL-13R α 1 antibody (R&D Systems, Minneapolis, MN) was performed as previously described.²⁶ Staining for α -SMA (Sigma-Aldrich, St. Louis, MO) was done according to standard clinical laboratory procedures.

Mice

BALB/c and C57Bl/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). The generation of IL-13^{-/-} mice has been previously described.²⁷ This genotype, backcrossed onto the BALB/c background for eight generations, was bred at the University of Michigan. All mice were used at 7 to 15 weeks of age (25 to 30 g). All experiments were performed according to protocols approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Tracheal Transplant Models

Two well-established tracheal transplant models were used to study the effect of IL-13 on the pathogenesis of allograft rejection. The first model was the previously described double lumen airway (orthotopic) transplant model for studying chronic airway rejection.^{28,29} Briefly, after anesthesia, donor mice were exsanguinated, and whole trachea was harvested by transecting below the cricoid cartilage distal to the carinal bifurcation under sterile conditions. Recipient mice were similarly anesthetized, and the whole trachea was exposed. Distal (the seventh intercartilaginous space) and proximal (immediately subjacent to the cricoid cartilage area) orifices positioned on the recipient trachea were anastomosed with both ends of the tracheal graft. This air permissive model mimics lymphocytic bronchitis and allows studies focusing on inflammation. Isogeneic tracheal transplants were performed using C57Bl/6 mice as both recipients and donors or BALB/c mice as both recipients and donors. Allogeneic tracheal grafts were performed using donor (C57Bl/6) airway tracheas and transplanting them into BALB/c or IL-13^{-/-} recipient mice. Mixed lymphocyte reactions confirmed that splenocyte responses to C57Bl/6 stimulators were similar between BALB/c and IL-13^{-/-} mice. Experiments were also performed using reverse donor/recipient transplantation protocols, using BALB/c or IL-13^{-/-} mice as donors and C57Bl/6 mice as recipients. All surgeries were performed using a Leitz-Wild surgical microscope (Urban Engineering, Burbank, CA) under $\times$ 16 magnification.

A heterotopic model, characterized by complete epithelial loss and fibroproliferative plug formation by 4

weeks after transplant as previously described,^{4,11,30} was also used. Donor tracheas were harvested as described above. The trachea was inserted in a subcutaneous pouch created after a 3-mm incision and blunt dissection in the back of the neck. The skin pocket was closed with a size 5.0 nylon suture.

Histopathological Evaluation of Tracheal Transplants

Grafts were harvested at the indicated time points and embedded en bloc in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC) in Disposable Base Molds (Richard-Allan Scientific, Kalamazoo, MI) in liquid nitrogen and stored at -80°C until the time of analysis or were formalin-fixed and paraffin-embedded. For the orthotopic model histochemical staining was performed for elastin (Accustain; Sigma-Aldrich) to determine graft luminal occlusion on 5- μm -thick sections. Immunostaining was also performed using serial adjacent sections from each group, with primary antibodies directed against a pan-T-cell marker (hamster anti-mouse CD3; BD Pharmingen, San Diego, CA) and a macrophage marker (anti-mouse F4/80; BD Pharmingen). Morphometric measurements of cross-sectional areas were performed by blindly tracing both epithelial and subepithelial areas using a computer-assisted image analysis system (AxiCamHR; Carl Zeiss Microimaging, Thornwood, NY). All samples were analyzed at 1 week after transplantation, because that was the time of maximal lumen narrowing and cellular infiltration in allografts.

For the heterotopic model, transplants were harvested for evaluation at 7, 14, 21, and 28 days after implantation ($n = 4-6$ at each time point). Contiguous sections, 6 μm thick, obtained from the center of each tracheal segment were stained with hematoxylin and eosin and elastic trichrome. Four histological features (airway lining epithelial loss, leukocyte infiltration, luminal obliteration, and extracellular matrix deposition) were assessed semiquantitatively, in a blinded fashion, using a modified scoring system based on those previously described by authors in this field.^{5,31} The scoring used was as follows: 1) airway lining epithelial loss (0, no change; 1+, <25% circumference loss; 2+, 25 to 50% circumference loss; 3+, 50 to 75% circumference loss; 4+, >75% circumference loss); 2) leukocyte infiltration (0, no change; 1+, sparse perivascular infiltrates involving some vessels; 2+, perivascular infiltrates involving most vessels with extension into adjacent connective tissue; 3+, diffuse but mild to moderate panmural infiltration; 4+, diffuse marked panmural infiltration with striking subepithelial and intraepithelial component); 3) luminal obliteration (0, no change; 1+, <25% obliteration; 2+, 25 to 50% obliteration; 3+, 50 to 75% obliteration; 4+, >75% obliteration); and 4) extracellular matrix deposition (0, no change; 1+, <25% deposition; 2+, 25 to 50% deposition; 3+, 50 to 75% deposition; 4+, >75% deposition).

mRNA Isolation and PCR Analysis

Total RNA was isolated from mouse tracheas using the RNeasy mini kit (Qiagen, Inc., Valencia, CA) as per the manufacturer's instructions. The TaqMan real-time polymerase chain reaction (PCR) primers for IL-13, TGF- β 1, MCP-1, and β -actin were purchased from Applied Biosystems (Foster City, CA). Reverse transcriptase (RT)-PCR and real-time PCR were performed using the one-step QuantiTect Probe RT-PCR kit (Qiagen) as per the manufacturer's instructions. Results were normalized to β -actin amplified from the same RNA mix in each sample.

Protein Analysis by ELISA

Harvested grafts were homogenized in 500 μl of Cell LyticTM mammalian tissue lysis reagent (Sigma-Aldrich) containing protease inhibitors (Complete Protease tablets; Roche, Mannheim, Germany). Cellular debris was removed by centrifugation at 10,000 rpm for 10 minutes. Aliquots of tracheal homogenate were then assayed by specific ELISA for cytokines. Measurements of MCP-1, regulated on activation normal T cell expressed and secreted (RANTES), and IL-13 were performed on tracheal homogenates using ELISA kits from R&D Systems (Minneapolis, MN).

Plasminogen Activator Inhibitor-1/Luciferase (PAIL) Assay for TGF- β 1

Tracheal homogenates were prepared as described above, and 250 μl of the tracheal homogenate was acid-treated, then neutralized to activate latent TGF- β 1 within the sample. These tracheal homogenates were then analyzed in a bioassay for TGF- β 1 activity (PAIL cell assay) as described previously.²²

Sircol Assay for Collagen Measurements

Tracheal collagen levels were determined by harvesting heterotopic tracheal transplants at day 28. Animals were euthanized, and tracheas were harvested and snap-frozen in liquid nitrogen. Before analysis, two tracheas were homogenized in 500 μl of normal saline and spun at 2000 rpm for 10 minutes. Aliquots of homogenate (100 μl) were then assayed for total tracheal collagen levels and compared with a standard curve prepared from rat tail collagen using the Sircol collagen dye binding assay (Accurate Chemical and Scientific Corp., Westbury, NY) according to the manufacturer's instructions.

FACS Analysis

Infiltrating inflammatory cells were isolated from transplanted airway graft tissue by using the Medimachine (DakoCytomation, Glostrup, Denmark) to isolate cells as per the manufacturer's protocol. Immunophenotyping of graft-infiltrating leukocytes was accomplished by flow cytometric analysis. Isolated cells were stained for multipa-

parameter fluorescence-activated cell sorting (FACS) analysis using phycoerythrin-labeled anti-mouse CD3, and fluorescein isothiocyanate-labeled anti-mouse macrophage (F4/80; Caltag Laboratories, Burlingame, CA). Cells were prepared in staining buffer consisting of Ca^{2+} - and Mg^{2+} -free Dulbecco's phosphate-buffered saline (Mediatech, Herndon, VA) with 0.05% sodium azide and 1% fetal bovine serum. To reduce nonspecific binding of FcII/III receptors, 5-minute incubation at 4°C with purified anti-2.4G2 (BD Biosciences, San Diego, CA) was performed. After incubation for 30 minutes at 4°C in the dark with the indicated fluorescent antibodies (1:100), cells were washed twice in staining buffer, and populations of lymphocytes and macrophages were then identified using a FACSCalibur system (BD Biosciences). Cell detector voltages (gating) were set using unstained lymphocytes isolated from the spleen. Absolute numbers of specific cell populations were derived from percentages of cells determined by FACS analysis, multiplied by the total number of cells isolated from grafts (determined by manual cell counts performed using a hemocytometer).

Neutralization of IL-13

Anti-murine IL-13 polyclonal antibodies were raised by immunizing New Zealand White rabbits with recombinant murine IL-13 (R&D Systems) as described before³² and were a generous gift of Dr. Nick Luckacs (University of Michigan). IL-13 immunoneutralization studies were performed by daily intraperitoneal injection of anti-murine IL-13 (0.2 ml) in the wild-type recipients of orthotopic tracheal transplantation from days 1 to 6 after surgery.

Statistics

Student's *t*-tests were used to determine *P* values when comparing two groups. When comparing three or more groups, analysis of variance was performed with a posthoc Bonferroni test to determine which groups showed significant differences. A value of *P* < 0.05 was considered significant.

Results

Detection of Myofibroblasts in Human BO Lesions

Myofibroblasts are recognized as key effector cells in tissue fibrosis because of their enhanced ability to synthesize interstitial collagen. The composition of the fibrotic lesions in human BO has not been fully characterized. Tissue sections from 15 biopsy samples with pathologist-confirmed diagnosis of BO were stained with a monoclonal antibody to α -SMA, a marker of myofibroblasts. The characteristics of the cases analyzed are shown in Table 1. Bronchial tissue was identified by the presence of characteristic bronchial epithelium and/or presence of smooth muscle bundles. Mesenchymal cells expressing α -SMA were identified in all 15 samples of

BO. Myofibroblasts were identified predominantly in the lamina propria (Figure 1A) and varied in number from a few cells to marked infiltration. These cells were not seen in the lung airway tissue of biopsies in which BO was not present (Figure 1B, inset). In cases of BO marked by intraluminal obstruction, myofibroblasts were identified in the polypoid obstructing lesions (Figure 1C). This finding supports the involvement of myofibroblast accumulation in the fibroproliferative lesion of BOS.

Expression of IL-13R α 1 in Myofibroblasts in BO

We next determined whether the myofibroblasts seen in lesions of BO demonstrate expression of IL-13R α 1, the signaling chain of the high affinity IL-13 receptor. Immunohistochemical analysis indicated that IL-13R α 1 was expressed abundantly in myofibroblasts seen in terminal bronchi in transbronchial biopsy specimens demonstrating BO. Representative staining to identify myofibroblasts with α -SMA is shown in Figure 1D. IL-13R α 1 and control staining is shown in a serial section (Figure 1, E and F). In addition IL-13R α 1 expression was seen on epithelial cells and smooth muscle bundles.

IL-13 mRNA and Protein Expression in Murine Tracheal Allograft Models

To determine whether IL-13 expression is altered in the setting of BO, IL-13 mRNA was measured at various time points after orthotopic (Figure 2, A and B) and heterotopic (Figure 2, C and D) tracheal transplantation. IL-13 expression peaked at day 7 after transplantation in both models (Figure 2, A and C). Furthermore, protein expression of IL-13 by specific ELISA at day 7 confirmed significantly increased levels of IL-13 in allografts compared to isografts (Figure 2, E and F). To differentiate the contribution of donor graft versus infiltrating recipient cells to the up-regulation of IL-13 seen in allografts, we measured IL-13 mRNA expression in allografts implanted into IL-13^{-/-} recipients. Allografts placed into IL-13^{-/-} recipients showed no IL-13 mRNA elevation in either model (Figure 2, G and H). These data suggest that infiltrating recipient cells comprise the dominant source of the elevated IL-13 mRNA expressed in tracheal allografts. To determine whether expression of another important Th2 cytokine, IL-4, was modulated in IL-13^{-/-} recipients, we measured graft expression of IL-4 at day 7, the time of maximal IL-13 up-regulation. No statistically significant difference was noted in levels of IL-4 mRNA between transplanted tracheal allografts placed into IL-13^{-/-} and wild-type recipients in both orthotopic and heterotopic models (*P* = 0.46 and 0.35, respectively).

Role for IL-13 in Orthotopic Tracheal Transplants

We next sought to determine the influence of IL-13 on the luminal narrowing of orthotopic tracheal allografts (Figure 3). Tracheal isografts (BALB/c into BALB/c) maintained their normal architecture and demonstrated no significant

Figure 1. Immunohistochemistry staining for α -SMA and IL-13R α 1 in TBBx specimens demonstrating evidence of BO. **A:** α -SMA-positive (stained brown) mesenchymal cells (myofibroblasts) are demonstrated in the submucosa of bronchi in a patient with BOS. **B:** TBBx specimen from a patient without BOS showing normal bronchial epithelium with underlying smooth muscle bundle and absence of myofibroblast infiltration in the lamina propria and the lumen. **C:** α -SMA staining (brown) of a TBBx specimen demonstrates bronchi cut tangentially as evidenced by smooth muscle bundles (arrows). The lumen is filled with mesenchymal cells that stain positively for α -SMA, demonstrating the presence of myofibroblasts. **D–F:** Contiguous sections from **C** (marked by the rectangle) are shown at higher magnification stained with α -SMA (**D**), IL-13R α 1 (stained red) (**E**), and the negative control for IL-13R α 1 (**F**). Original magnifications: $\times 100$ (**A** and **C**); $\times 200$ (**B**); $\times 400$ (**D–F**). These findings are representative of all 15 patients examined.

inflammatory infiltrates or luminal encroachment (Figure 3, A and B). Allografted tracheal transplants (C57Bl/6 into BALB/c) demonstrated partial concentric graft luminal occlusion ($55.43 \pm 6.07\%$ compared to $25.30 \pm 5.32\%$ in isografts, $P = <0.001$; Figure 3, C and D) secondary to characteristic thickening of the epithelial and subepithelial layer.^{28,29} To study the role of IL-13, C57Bl/6 tracheas were transplanted into allogeneic IL-13^{-/-} recipients. These experiments showed a significant decrease in luminal occlusion in allografts transplanted into IL-13^{-/-} recipients when compared to allografts placed into IL-13-sufficient, BALB/c mice with diminution in both the epithelial and subepithelial components (total occlusion =

$37.37 \pm 4.30\%$, $P = 0.004$; subepithelial layer = $24.28 \pm 6.07\%$, $P < 0.001$ compared to allograft) (Figure 3, E and F). In contrast to these data, no protection was seen when IL-13^{-/-} donor tracheas were implanted into allogeneic, C57Bl/6 recipients ($48.49 \pm 3.71\%$; Figure 3, G and H). These data demonstrate that recipient, but not donor, IL-13 gene-deleted mice are protected from airway luminal obliteration following allograft implantation. To definitively implicate IL-13 in the rejection process, IL-13 immunoneutralization studies were performed by injecting anti-murine IL-13 in the allograft recipient (C57Bl/6 into BALB/c). Immunoneutralization of IL-13 between days 1 and 6 of transplantation significantly decreased graft lu-

A

B

C

D

E

F

G

H

minimal occlusion with decreased epithelial and subepithelial infiltration of leukocytes (total occlusion = $43.35 \pm 5.96\%$, $P = 0.006$; subepithelial layer = $27.11 \pm 2.83\%$, $P < 0.03$ compared to allograft) (Figure 3, I and J).

Effect of IL-13 on Cellular Recruitment in Orthotopic Tracheal Allografts

Luminal occlusion in the tracheal allografts was characterized by epithelial hypertrophy and cellular infiltration of both the epithelium and the subepithelium. To study cellular infiltration into the orthotopic allografts and the effect of IL-13 on this rejection feature, immunohistochemical staining to identify F4/80+ macrophages and CD3+ T cells was performed in allografts transplanted into wild-type and IL-13^{-/-} mice. Additionally, F4/80+ macrophages and CD3+ T cells were quantified by FACS in leukocytes isolated from pooled tracheal allografts. A significant reduction in infiltrating mononuclear phagocytes and CD3+ lymphocytes was demonstrated in allografts placed in IL-13^{-/-} recipients as compared to BALB/c recipients (Figure 4). To investigate whether this reduction in allograft leukocyte infiltration was due to differences in the chemokines regulated by IL-13, we studied MCP-1 and RANTES mRNA and protein expression in allografts transplanted into BALB/c versus IL-13^{-/-} recipients, by real-time quantitative PCR and ELISA. Both RANTES and MCP-1 demonstrated significant up-regulation of both mRNA as well as protein expression in the wild-type allograft as compared to isografts. However, no significant differences were seen in MCP-1 or RANTES mRNA and protein levels between allografts placed in IL-13^{-/-} recipients as compared to BALB/c recipients (data not shown).

Causal Role for IL-13 in Airway Obliteration in Heterotopic Tracheal Transplants

The effect of IL-13 on fibroproliferation was next studied in the heterotopic tracheal transplant model. This allograft model demonstrates complete airway obliteration with loss of epithelium and development of a fibrous plug by day 28 after transplantation.^{4,11,30} Allografts from C57Bl/6 mice transplanted into BALB/c mice demonstrated loss of epithelium with complete obliteration of the lumen by day 28 (Figure 5, A and B). In contrast, allografts from C57Bl/6 mice transplanted into IL-13^{-/-} recipients demonstrated maintenance of epithelial integrity, and luminal fibroproliferation was absent (Figure 5, C and D). This protection was not seen when IL-13^{-/-} mice were used as donors (Figure 5, E and F). To quantify the

changes seen over time, allografts transplanted into IL-13^{-/-} recipients and their wild-type counterparts were scored at various time points (days 7, 14, 21, and 28) after transplantation (Figure 5G). Complete loss of epithelium was seen in the allografts by day 21. Allografts transplanted into IL-13^{-/-} recipients demonstrated preserved epithelium at day 21, but by day 28 the epithelium, though intact, appeared metaplastic. Similarly, although intraluminal airway occlusion was significantly reduced in IL-13-deficient recipients, there was expansion of the subepithelial layer suggestive of subluminal matrix deposition.

Effect of IL-13 on Myofibroblast Accumulation and Collagen Deposition in Heterotopic Tracheal Allografts

Trichrome staining demonstrated collagen deposition in the lumen of allografts from C57Bl/6 mice placed into BALB/c recipients (Figure 6, A and B). In contrast, intraluminal collagen deposition in allografts placed into IL-13^{-/-} recipients was absent (Figure 6, C and D). When allografts from IL-13^{-/-} donors were implanted into C57Bl/6 recipients, there was no diminution of intraluminal collagen accumulation demonstrated (Figure 6, E and F).

On histopathology scoring, significant differences in intraluminal collagen deposition were seen in the allografts transplanted into IL-13 intact and deficient mice at days 21 and 28 (Figure 5G). This was subsequently confirmed using the Sircol assay, which quantitates the presence of soluble collagen (Figure 6G). Collagen deposition was significantly greater in allografts transplanted into BALB/c mice than in allografts transplanted into IL-13^{-/-} recipients ($P < 0.001$) or control isografts ($P < 0.01$).

To further characterize the composition of the intraluminal cellular infiltrates, immunohistochemical staining for α -SMA was performed. This antigen is characteristically restricted to smooth muscle cells and myofibroblasts, which can be further discriminated by their histological appearance. In isografts, no subepithelial or intraluminal myofibroblast infiltration was noted (Figure 7, A and B). In contrast, C57Bl/6 allografts implanted into BALB/c recipients demonstrated widespread infiltration of the lumen with a meshwork of myofibroblasts (Figure 7, C and D). It is also apparent that there is complete destruction of the epithelial layer in these transplants. C57Bl/6 tracheal allografts implanted into IL-13^{-/-} recipients demonstrated an intact epithelial layer, with no significant intraluminal myofi-

Figure 2. IL-13 up-regulation in the pathogenesis of murine BOS. **A–D:** Time-course analysis of up-regulation of IL-13 in BO. Real-time quantitative PCR of IL-13 in orthotopic (**A**) and heterotopic (**C**) tracheal transplants at 3, 7, 14, 21, and 28 days after transplantation. IL-13 mRNA was markedly elevated in allografts transplanted into BALB/c recipients compared to isografts at day 7 in both orthotopic ($P = 0.0001$) and heterotopic ($P < 0.0001$) models and continued to be elevated at day 14 in the heterotopic model ($P < 0.0001$). Representative band for IL-13 mRNA and β -actin, generated by RT-PCR, comparing isografts to allografts at day 7 are demonstrated for both the orthotopic (**B**) and the heterotopic (**D**) transplant models. **E and F:** Protein expression by ELISA in orthotopic (**E**) and heterotopic (**F**) tracheal transplant models demonstrates increased IL-13 protein in allografts compared to isografts. **G and H:** IL-13 mRNA expression in tracheal allografts transplanted into IL-13^{-/-} animals, by real-time quantitative PCR, demonstrated no up-regulation of IL-13 at day 7 in both orthotopic (**G**) and heterotopic (**H**) models. Data shown represent mean $\pm$ SE for each group. * $P < 0.05$ compared to isografts. $n = 4$ tracheas per group for mRNA analysis. $n = 4$ tracheal homogenates of 2 tracheas per group for ELISA.

Figure 3. Analysis of graft luminal narrowing 1 week after transplantation in the orthotopic tracheal transplant model. Representative histology and morphometric analysis for each of the indicated conditions is shown. **A** and **B**: BALB/c into BALB/c isografts. **C** and **D**: C57Bl/6 allografts into BALB/c. **E** and **F**: C57Bl/6 allografts into IL-13^{-/-} recipients. **G** and **H**: IL-13^{-/-} allografts into C57Bl/6 recipients. **I** and **J**: C57Bl/6 allografts into BALB/c treated with IL-13 antibody. In **A**, **C**, **E**, **G**, and **I** the native trachea is shown on the left, and the orthotopic transplant is shown on the right. Data represent the analysis of at least four transplants per group. **K**: Quantitative morphometric analysis of tissue sections. Allografts treated with IL-13 antibody and allografts placed in IL-13^{-/-} recipients were significantly protected compared to BALB/c recipients. **P* < 0.05 compared to allografts. All allograft conditions were statistically different from the isograft.

Figure 4. FACS analysis and immunohistochemical staining for macrophages and T cells in orthotopic tracheal transplant model. **A:** FACS analysis of F4/80+ cells (macrophages) identified in cell isolations from allografts transplanted into BALB/c or IL-13^{-/-} recipients. Markedly fewer infiltrating mononuclear phagocytes were identified in IL-13^{-/-} recipients ($P < 0.05$). **B:** Immunohistochemical staining for F4/80+ in allografts transplanted into BALB/c mice. **C:** Staining for F4/80+ in an allograft transplanted into an IL-13^{-/-} recipient. **D:** FACS analysis for CD3⁺ cells in cell isolations from allografts into BALB/c or IL-13^{-/-} mice. Significantly decreased infiltration by CD3-positive lymphocytes was demonstrated in IL-13^{-/-} allograft recipients as compared to wild-type recipients ($P < 0.05$). **E:** Staining for CD3 in allografts transplanted into BALB/c mice. **F:** Staining for CD3 in allografts transplanted into IL-13^{-/-} recipients. All data represent analysis at 1 week after transplant. IL-13^{-/-} recipients demonstrate significantly reduced recruitment of both macrophages and T cells ($n = 4$).

broblasts infiltration. However, a modest subepithelial myofibroblast accumulation was noted underneath the intact epithelium (Figure 7, E and F).

Effect of IL-13 in the Production of TGF- β 1 in Heterotopic Tracheal Transplants

TGF- β 1, an important profibrotic mediator and a promoter of myofibroblast differentiation,^{33,34} has been demonstrated to be up-regulated during fibroproliferation in tracheal transplant models.^{11,35–37} Recent work using IL-13-overexpressing mice suggests that IL-13 might exert its profibrotic effects via increasing both the transcription and the activation of TGF- β 1.³⁸ We studied the level of TGF- β expression in the heterotopic tracheal allografts placed into BALB/c (IL-13^{+/+}) recipients or IL-13^{-/-} recipients. On day 28 after tracheal transplantation, grafts were harvested for mRNA analysis of TGF- β 1 or were homogenized and assayed for TGF- β 1 bioactivity. Significant increase in expression of TGF- β 1 mRNA was demonstrated in tracheal allografts ($P < 0.001$, Figure 8A) placed into BALB/c (IL-13^{+/+}) recipients. However, allografts placed into IL-13^{-/-} recipients did not show any significant TGF- β 1 up-regulation as compared to isografts. Further, bioassay of total TGF- β 1 protein demonstrated a statistically significant difference between the allografts transplanted into IL-13^{+/+} versus IL-13^{-/-} recipients (Figure 8B). Thus, similar to the results seen in the orthotopic transplant model, a lack of IL-13 in recipient mice appears to be protective in heterotopic tracheal transplants.

Discussion

BO following lung transplantation is characterized by narrowing and obliteration of terminal airways. Histologically, typical lesions of BO evolve from an early lymphocytic bronchitis to a more chronic, fibroproliferative phenotype that eventually results in luminal encroachment. Identifying the specific drivers of the fibroproliferative response in BO is an area of active investigation. Current concepts suggest that cellular infiltration and local mediator release after airway injury creates a profibrotic milieu, which promotes subsequent fibroproliferation and airway obliteration. However, the pathogenic evolution from inflammation to fibrosis is not well understood.

In this study, data substantiate the crucial role of fibroproliferation in the pathogenesis of human BO by demonstrating for the first time the presence of myofibroblasts in histological sections of BO derived from multiple lung transplant recipients. Although a pleiotropic array of cytokines has been associated with fibroproliferation, expression of IL-13R α 1, the signaling chain of the high affinity IL-13 receptor, on these myofibroblasts pointed to the likely role for the profibrotic Th2 cytokine IL-13 in the development of fibroproliferation in BO. Translational studies using mice deficient in IL-13 in two murine models of BO reveal that absence of this cytokine in allograft recipients leads to marked protection from intraluminal

Figure 5. Histopathology (hematoxylin and eosin) of luminal fibrosis 28 days after transplantation in the heterotopic tracheal transplant model. **A** and **B**: Representative photomicrographs of heterotopic trachea allografts (C57Bl/6 allografts into BALB/c-recipient mice) demonstrate epithelial injury, complete airway obliteration, and matrix deposition. **C** and **D**: Allografts into IL-13^{-/-} recipient mice show intact epithelium and no intraluminal cellular infiltration or airway obliteration. The deposit in the center represents acellular debris as this is not an air-permissive model. **E** and **F**: IL-13^{-/-} tracheas used as donors into C57Bl/6 mice do not demonstrate any protection from airway obliteration. **G**: Quantitative analysis of histopathological sections of tracheal allografts transplanted into IL-13^{+/+} and IL-13^{-/-} recipients. Histology shown is representative of *n* = 4–6 per group. Original magnifications: ×200 (**A**, **C**, and **E**); ×400 (**B**, **D**, and **F**). **P* < 0.05.

obliteration, mononuclear cell infiltration, and fibrosis. In contrast, deficiency of IL-13 in donor tissue was not associated with a reduction in either fibroproliferation or BO. These data demonstrate that IL-13 secreted by infiltrating inflammatory cells contributes to the development of fibrosis in BO.

Even though BO has been recognized as a fibrotic disorder of the small airways for a number of years, this is the first study to demonstrate that myofibroblasts are present in human histological sections demonstrating evidence of BO. The presence of myofibroblasts helps explain the excessive deposition of extracellular matrix seen in these patients, as myofibroblasts are known to be

potent producers of collagen and other extracellular matrix proteins.^{39,40} In fact, the data shown here demonstrate that in the murine models there is a strong correlation between the presence of myofibroblasts and the accumulation of collagen. In humans, myofibroblasts have previously been associated with airway remodeling in asthma⁴¹ as well as alveolar fibrosis in idiopathic pulmonary fibrosis.^{39,40} Furthermore, early myofibroblast infiltration predicts chronic renal allograft dysfunction in renal transplant recipients.⁴² Additional studies to determine whether the degree of myofibroblast infiltration in the bronchial tissue correlates with clinical course of the disease would be useful in making clinical predictions.

G

Figure 6. Trichrome staining and quantification of collagen deposition in heterotopic tracheal allografts transplanted into IL-13^{-/-} and wild-type mice. **A** and **D**: Representative photomicrographs of Masson's trichrome staining (blue color) for collagen demonstrates early intraluminal collagen deposition in mouse allografts. **B** and **E**: No significant intraluminal collagen was seen in allografts transplanted into IL-13^{-/-}-recipient mice. **C** and **F**: IL-13^{-/-} mice as donors were indistinguishable from control allografts. Histology shown is representative of $n = 4-6$ per group. **G**: Quantification of collagen deposition by the Sircol assay at day 28 demonstrates significantly increased level of collagen in allografts as compared to isografts ($P < 0.01$). However, tracheal allografts placed into IL-13^{-/-} mice were significantly protected from collagen deposition ($P < 0.001$ for allografts into IL-13^{-/-} mice compared with allografts into BALB/c mice). No significant difference in collagen levels was seen between isografts and IL-13^{-/-} allografts ($P > 0.05$). Values represent the mean $\pm$ SE of six tracheal transplants per group. * $P < 0.05$ versus isograft.

Our studies used two different murine models of BO to evaluate the role of IL-13 in the pathogenesis of this disease. The orthotopic model has the advantage of being airflow-permissive and thus more accurately simulates lung transplantation in humans. This model is useful for modeling the lymphocytic bronchitis that often precedes or accompanies BOS in humans. T lymphocytes play an important role in the pathogenesis of BOS. Both CD4 and CD8 T cells have been demonstrated to be critical in the development of obstructive airway lesion in murine models of tracheal transplantation.^{43,44} However in the orthotopic model, the ability of airway epithelial cells from the native recipient trachea to quickly migrate and repair the allograft appears to limit the development of frank fibrosis.⁴⁵ In contrast, the heterotopic tracheal transplant model, while having the disadvantage of being located in an anatomically irrelevant site, does model the airway obliteration, fibroproliferation, and extracellular

matrix deposition seen in human BOS. Thus, by using both of these model systems, we have been able to analyze the contribution of IL-13 to both the inflammatory (day 7 orthotopic) and fibroproliferative (day 28 heterotopic) phases of BO.

Studies using IL-13^{-/-} mice as both donors and recipients in murine models of BO revealed that recipient-derived inflammatory cells were responsible for the up-regulation of IL-13mRNA seen in both models. Absence of IL-13 in the recipient, but not the donor, trachea was protective for allograft damage in both models. It is likely that the IL-13 is being secreted by inflammatory CD3⁺ T cells in response to immune allostimulation. The presence of IL-13 in the recipients modulates both cellular recruitment and the subsequent development of fibrosis. It has been shown that constitutive overexpression of IL-13 causes a macrophage-, lymphocyte-, and eosinophil-rich pulmonary inflammatory response.^{12,46} These

Figure 7. Immunohistochemical staining for α -SMA in tracheal transplants. **A** and **B**: α -SMA staining (brown) in a representative isograft demonstrates intact epithelium and only smooth muscle cell staining. The intraluminal brown staining represents acellular debris. **C** and **D**: Allografts demonstrate intraluminal mesenchymal cells, which stain intensely for α -SMA obliterating the lumen, thus demonstrating myofibroblast accumulation. **E** and **F**: Allografts into IL-13^{-/-} mice demonstrate myofibroblast infiltration restricted to lamina propria with intact epithelium. Acellular amorphous debris is seen in the lumen. Histology shown is representative of $n = 4$ per group. Original magnifications: $\times 200$ (**A**, **C**, and **E**); $\times 400$ (**B**, **D**, and **F**).

effects of IL-13 are thought to be mediated via its ability to regulate expression of downstream genes rather than as a direct consequence of cytokine-receptor binding.¹³ Although IL-13 has been shown to induce expression of

Figure 8. TGF- β 1 mRNA expression and bioactivity in heterotopic tracheal allografts. **A**: TGF- β 1 mRNA expression by real-time quantitative PCR in tracheal transplants at day 28 demonstrates a 32-fold increase in TGF- β 1 in control allografts as compared to isografts ($P < 0.001$). However, no significant increase in TGF- β 1 mRNA expression was seen in allograft tracheas transplanted into IL-13^{-/-} mice as compared to isografts. **B**: Tracheal homogenates from isografts and allografts transplanted into BALB/c or IL-13^{-/-} recipients were analyzed for TGF- β 1 bioactivity by PAIL assay at day 28. TGF- β 1 bioactivity was significantly lower in allografts transplanted into IL-13^{+/+} compared to IL-13^{-/-} recipients ($P < 0.001$). Data represent the mean $\pm$ SE for four tracheas per group measured in duplicate. * $P < 0.05$.

chemokines such as MCP-1/CCL2⁴⁶ and RANTES,⁴⁷ no significant difference in these chemokines was seen in allografts implanted into IL-13-deficient recipients. Recently it has been demonstrated that IL-13 can up-regulate expression of the 5-lipoxygenase enzyme responsible for leukotriene synthesis as well as the leukotriene receptors.^{48,49} Furthermore, 5-lipoxygenase inhibitors and cysteinyl leukotriene receptor antagonists decrease the ability of IL-13 to mediate tissue inflammatory responses and remodeling.⁴⁹ Thus, it is possible that the effects we have noted on inflammatory cell recruitment in our models are the result of IL-13-induced leukotriene expression. This will be the focus of future experiments.

Our experiments demonstrate that IL-13^{-/-} recipients are significantly protected from the development of fibrosis and luminal obliteration in the heterotopic model. The protection from airway obliteration was accompanied by decreased collagen deposition and reduced myofibroblast infiltration. Although profibrotic effects of IL-13 have been studied in other pulmonary models of airway remodeling and fibrosis, this is the first study showing an association between this Th2 cytokine and the development of fibroproliferation in BO. However, the metaplastic changes in epithelium at 4 weeks after transplantation and subluminal myofibroblast infiltration in the allografts implanted into IL-13-deficient recipients suggests that IL-13 might be associated with delayed kinetic of luminal obliteration rather than complete prevention.

In summary, our data demonstrate that IL-13, produced by recipient-derived inflammatory cells, plays a significant role in the pathogenesis of BO by regulating further recruitment of macrophages and lymphocytes during the allo-immune response and promoting the development of myofibroblast accumulation and extracellular matrix deposition. The findings in this study highlight IL-13 as a key endogenous regulator of inflammation and fibroproliferation in BO.

Acknowledgments

We thank Carol Wilke for technical assistance with the heterotopic tracheal transplants and Marc Peters-Golden for useful comments on the manuscript.

References

- Boehler A, Kesten S, Weder W, Speich R: Bronchiolitis obliterans after lung transplantation: a review. *Chest* 1998, 114:1411–1426
- Estenne M, Maurer JR, Boehler A, Egan JJ, Frost A, Hertz M, Mallory GB, Snell GI, Yousem S: Bronchiolitis obliterans syndrome 2001: an update of the diagnostic criteria. *J Heart Lung Transplant* 2002, 21:297–310
- Sharples LD, McNeil K, Stewart S, Wallwork J: Risk factors for bronchiolitis obliterans: a systematic review of recent publications. *J Heart Lung Transplant* 2002, 21:271–281
- Hertz M, Jessurun J, King MB, Savik SK, Murray JJ: Reproduction of the obliterative bronchiolitis lesion after heterotopic transplantation of mouse airways. *Am J Pathol* 1993, 142:1945–1951
- Boehler A, Chamberlain D, Kesten S, Slutsky AS, Liu M, Keshavjee S: Lymphocytic airway infiltration as a precursor to fibrous obliteration in a rat model of bronchiolitis obliterans. *Transplantation* 1997, 64:311–317
- DiGiovine B, Lynch JP 3rd, Martinez FJ, Flint A, Whyte RI, Iannettoni MD, Arenberg DA, Burdick MD, Glass MC, Wilke CA, Morris SB, Kunkel SL, Strieter RM: Bronchoalveolar lavage neutrophilia is associated with obliterative bronchiolitis after lung transplantation: role of IL-8. *J Immunol* 1996, 157:4194–4202
- Elssner A, Jaumann F, Dobmann S, Behr J, Schwaiblmair M, Reichen-spurner H, Furst H, Briegel J, Vogelmeier C: Elevated levels of interleukin-8 and transforming growth factor-beta in bronchoalveolar lavage fluid from patients with bronchiolitis obliterans syndrome: proinflammatory role of bronchial epithelial cells. *Munich Lung Transplant Group. Transplantation* 2000, 70:362–367
- Reynaud-Gaubert M, Marin V, Thirion X, Farnarier C, Thomas P, Badier M, Bongrand P, Giudicelli R, Fuentes P: Upregulation of chemokines in bronchoalveolar lavage fluid as a predictive marker of post-transplant airway obliteration. *J Heart Lung Transplant* 2002, 21:721–730
- Belperio JA, Keane MP, Burdick MD, Lynch JP 3rd, Xue YY, Berlin A, Ross DJ, Kunkel SL, Charo IF, Strieter RM: Critical role for the chemokine MCP-1/CCR2 in the pathogenesis of bronchiolitis obliterans syndrome. *J Clin Invest* 2001, 108:547–556
- El-Gamel A, Sim E, Hasleton P, Hutchinson J, Yonan N, Egan J, Campbell C, Rahman A, Sheldon S, Deiraniya A, Hutchinson IV: Transforming growth factor beta (TGF-beta) and obliterative bronchiolitis following pulmonary transplantation. *J Heart Lung Transplant* 1999, 18:828–837
- Ramirez AM, Takagawa S, Sekosan M, Jaffe HA, Varga J, Roman J: Smad3 deficiency ameliorates experimental obliterative bronchiolitis in a heterotopic tracheal transplantation model. *Am J Pathol* 2004, 165:1223–1232
- Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, Zhang Y, Elias JA: Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* 1999, 103:779–788
- Wills-Karp M, Chiaramonte M: Interleukin-13 in asthma. *Curr Opin Pulm Med* 2003, 9:21–27
- Kraft M, Lewis C, Pham D, Chu HW: IL-4, IL-13, and dexamethasone augment fibroblast proliferation in asthma. *J Allergy Clin Immunol* 2001, 107:602–606
- Saito A, Okazaki H, Sugawara I, Yamamoto K, Takizawa H: Potential action of IL-4 and IL-13 as fibrogenic factors on lung fibroblasts in vitro. *Int Arch Allergy Immunol* 2003, 132:168–176
- Oriente A, Fedarko NS, Pacocha SE, Huang SK, Lichtenstein LM, Essayan DM: Interleukin-13 modulates collagen homeostasis in human skin and keloid fibroblasts. *J Pharmacol Exp Ther* 2000, 292:988–994
- Leonardi A, Cortivo R, Fregona I, Plebani M, Secchi AG, Abatangelo G: Effects of Th2 cytokines on expression of collagen, MMP-1, and TIMP-1 in conjunctival fibroblasts. *Invest Ophthalmol Vis Sci* 2003, 44:183–189
- Doucet C, Brouty-Boye D, Pottin-Clemenceau C, Jasmin C, Canonica GW, Azzarone B: IL-4 and IL-13 specifically increase adhesion molecule and inflammatory cytokine expression in human lung fibroblasts. *Int Immunol* 1998, 10:1421–1433
- Ingram JL, Rice AB, Geisenhoffer K, Madtes DK, Bonner JC: IL-13 and IL-1beta promote lung fibroblast growth through coordinated up-regulation of PDGF-AA and PDGF-Ralpha. *FASEB J* 2004, 18:1132–1134
- Liu X, Kohyama T, Wang H, Zhu YK, Wen FQ, Kim HJ, Romberger DJ, Rennard SI: Th2 cytokine regulation of type I collagen gel contraction mediated by human lung mesenchymal cells. *Am J Physiol* 2002, 282:L1049–L1056
- Kumar RK, Herbert C, Yang M, Koskinen AM, McKenzie AN, Foster PS: Role of interleukin-13 in eosinophil accumulation and airway remodelling in a mouse model of chronic asthma. *Clin Exp Allergy* 2002, 32:1104–1111
- Kolodnick J, Toews G, Jakubzik C, Hogaboam C, Moore T, McKenzie A, Wilke C, Chrisman C, Moore B: Protection from fluorescein isothiocyanate-induced fibrosis in IL-13 deficient, but not IL-4 deficient mice results from impaired collagen synthesis by fibroblasts. *J Immunol* 2004, 172:4068–4076
- Chiaramonte MG, Donaldson DD, Cheever AW, Wynn TA: An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2-dominated inflammatory response. *J Clin Invest* 1999, 104:777–785
- Belperio JA, Dy M, Burdick MD, Xue YY, Li K, Elias JA, Keane MP: Interaction of IL-13 and C10 in the pathogenesis of bleomycin-induced pulmonary fibrosis. *Am J Respir Cell Mol Biol* 2002, 27:419–427
- Yousem SA, Berry GJ, Cagle PT, Chamberlain D, Husain AN, Hruban RH, Marchevsky A, Ofori NP, Ritter J, Stewart S, Tazelaar HD: Revision of the 1990 working formulation for the classification of pulmonary allograft rejection: Lung Rejection Study Group. *J Heart Lung Transplant* 1996, 15:1–15
- Jakubzik C, Choi ES, Kunkel SL, Evanoff H, Martinez FJ, Puri RK, Flaherty KR, Toews GB, Colby TV, Kazerooni EA, Gross BH, Travis WD, Hogaboam CM: Augmented pulmonary IL-4 and IL-13 receptor subunit expression in idiopathic interstitial pneumonia. *J Clin Pathol* 2004, 57:477–486
- McKenzie GJ, Emson CL, Bell SE, Anderson S, Fallon P, Zurawski G,

- Murray R, Grecis R, McKenzie AN: Impaired development of Th2 cells in IL-13-deficient mice. *Immunity* 1998, 9:423–432
28. Minamoto K, Pinsky DJ: Recipient iNOS but not eNOS deficiency reduces luminal narrowing in tracheal allografts. *J Exp Med* 2002, 196:1321–1333
29. Minamoto K, Harada H, Lama VN, Fedarau MA, Pinsky DJ: Reciprocal regulation of airway rejection by the inducible gas-forming enzymes heme oxygenase and nitric oxide synthase. *J Exp Med* 2005, 202:283–294
30. Medoff BD, Seung E, Wain JC, Means TK, Campanella GS, Islam SA, Thomas SY, Ginns LC, Grabie N, Lichtman AH, Tager AM, Luster AD: BLT1-mediated T cell trafficking is critical for rejection and obliterative bronchiolitis after lung transplantation. *J Exp Med* 2005, 202:97–110
31. Belperio JA, Keane MP, Burdick MD, Lynch JP 3rd, Xue YY, Li K, Ross DJ, Strieter RM: Critical role for CXCR3 chemokine biology in the pathogenesis of bronchiolitis obliterans syndrome. *J Immunol* 2002, 169:1037–1049
32. Matsukawa A, Hogaboam CM, Lukacs NW, Lincoln PM, Evanoff HL, Strieter RM, Kunkel SL: Expression and contribution of endogenous IL-13 in an experimental model of sepsis. *J Immunol* 2000, 164:2738–2744
33. Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J: Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. *J Clin Invest* 1997, 100:768–776
34. Thannickal VJ, Lee DY, White ES, Cui Z, Larios JM, Chacon R, Horowitz JC, Day RM, Thomas PE: Myofibroblast differentiation by transforming growth factor-beta1 is dependent on cell adhesion and integrin signaling via focal adhesion kinase. *J Biol Chem* 2003, 278:12384–12389
35. Aris RM, Walsh S, Chalermkulrat W, Hathwar V, Neuringer IP: Growth factor upregulation during obliterative bronchiolitis in the mouse model. *Am J Respir Crit Care Med* 2002, 166:417–422
36. Liu M, Suga M, Maclean AA, St George JA, Souza DW, Keshavjee S: Soluble transforming growth factor-beta type III receptor gene transfection inhibits fibrous airway obliteration in a rat model of Bronchiolitis obliterans. *Am J Respir Crit Care Med* 2002, 165:419–423
37. Zhou H, Latham CW, Zander DS, Margolin SB, Visner GA: Pirfenidone inhibits obliterative airway disease in mouse tracheal allografts. *J Heart Lung Transplant* 2005, 24:1577–1585
38. Lee CG, Homer RJ, Zhu Z, Lanone S, Wang X, Kotliansky V, Shipley JM, Gotwals P, Noble P, Chen Q, Senior RM, Elias JA: Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor beta(1). *J Exp Med* 2001, 194:809–821
39. Phan SH: The myofibroblast in pulmonary fibrosis. *Chest* 2002, 122:286S–289S
40. Gabbiani G: The myofibroblast: a key cell for wound healing and fibrocontractile diseases. *Prog Clin Biol Res* 1981, 54:183–194
41. Brewster CE, Howarth PH, Djukanovic R, Wilson J, Holgate ST, Roche WR: Myofibroblasts and subepithelial fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol* 1990, 3:507–511
42. Badid C, Desmouliere A, Babici D, Hadj-Aissa A, McGregor B, Le-francois N, Touraine JL, Laville M: Interstitial expression of alpha-SMA: an early marker of chronic renal allograft dysfunction. *Nephrol Dial Transplant* 2002, 17:1993–1998
43. Higuchi T, Jaramillo A, Kaleem Z, Patterson GA, Mohanakumar T: Different kinetics of obliterative airway disease development in heterotopic murine tracheal allografts induced by CD4+ and CD8+ T cells. *Transplantation* 2002, 74:646–651
44. Higuchi T, Maruyama T, Jaramillo A, Mohanakumar T: Induction of obliterative airway disease in murine tracheal allografts by CD8+ CTLs recognizing a single minor histocompatibility antigen. *J Immunol* 2005, 174:1871–1878
45. Fernandez FG, Jaramillo A, Chen C, Liu DZ, Tung T, Patterson GA, Mohanakumar T: Airway epithelium is the primary target of allograft rejection in murine obliterative airway disease. *Am J Transplant* 2004, 4:319–325
46. Zhu Z, Ma B, Zheng T, Homer RJ, Lee CG, Charo IF, Noble P, Elias JA: IL-13-induced chemokine responses in the lung: role of CCR2 in the pathogenesis of IL-13-induced inflammation and remodeling. *J Immunol* 2002, 168:2953–2962
47. Lee PJ, Zhang X, Shan P, Ma B, Lee CG, Homer RJ, Zhu Z, Rincon M, Mossman BT, Elias JA: ERK1/2 mitogen-activated protein kinase selectively mediates IL-13-induced lung inflammation and remodeling in vivo. *J Clin Invest* 2006, 116:163–173
48. Thivierge M, Stankova J, Rola-Pleszczynski M: IL-13 and IL-4 up-regulate cysteinyl leukotriene 1 receptor expression in human monocytes and macrophages. *J Immunol* 2001, 167:2855–2860
49. Vargaftig BB, Singer M: Leukotrienes mediate murine bronchopulmonary hyperreactivity, inflammation, and part of mucosal metaplasia and tissue injury induced by recombinant murine interleukin-13. *Am J Respir Cell Mol Biol* 2003, 28:410–419