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IL-13 Is Pivotal in the Fibro-Obliterative Process of Bronchiolitis Obliterans Syndrome[1]

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Acute allograft rejection is considered to be a predominately type 1 immune mediated response to the donor alloantigen. However, the type 2 immune mediated response has been implicated in multiple fibroproliferative diseases. Based on the fibro-obliterative lesion found during bronchiolitis obliterans syndrome (BOS), we hypothesized that the type 2 immune mediated response is involved in chronic lung allograft rejection. Specifically, whereas acute rejection is, in part, a type 1 immune response, chronic rejection is, in part, a type 2 immune response. We found the type 2 cytokine, IL-13, to be elevated and biologically active in human bronchoalveolar lavage fluid during BOS. Translational studies using a murine model of BOS demonstrated increased expression of IL-13 and its receptors that paralleled fibro-obliteration. In addition, in vivo neutralization of IL-13 reduced airway allograft matrix deposition and murine BOS, by a mechanism that was independent of IL-4. Furthermore, using IL-13Rα2$^{-/-}$ mice, we found increased fibro-obliteration. Moreover, anti-IL-13 therapy in combination with cyclosporin A had profound effects on reducing murine BOS. This supports the notion that IL-13 biological axis plays an important role during the pathogenesis of BOS independent of the IL-4 biological axis. *The Journal of Immunology,* 2007, 178: 511–519.

Bronchiolitis obliterans syndrome (BOS)[3] is the main reason that the 7-year survival postlung transplantation is ~31% (1). The histopathology of BOS is characterized by a peribronchiolar leukocyte infiltration that eventually disrupts the submucosa, basement membrane, and airway epithelium (1). This is followed by an exuberant fibroproliferative process with accumulation of extracellular matrix ultimately ending in fibro-obliteration of the allograft airway (1).

The concept of type 1 immune responses promoting rejection stems from studies involving immunosuppressive regimens that promote allograft survival by inhibiting type 1, while sparing type 2 immune responses (2). This suggests that this type of allograft accommodation may be, in part, due to a type 2 immune response. However, there is evidence indicating that type 2 immune responses can also promote rejection (3–5).

IL-13 is considered to be one of the most potent profibrotic type 2 cytokines. IL-13 is produced by type 1 and 2 polarized mononuclear cells, airway smooth muscle cells, and fibroblasts (6, 7). IL-13 mediates its action via IL-13Rα1, which, in the presence of the IL-4Rα chain, binds IL-13 with high affinity (8). The active IL-13R complex (IL-4R/IL-13Rα1) is expressed on mononuclear phagocytes, B cells, dendritic cells, fibroblasts, epithelial cells, and smooth muscle cells. In contrast, IL-13Rα2 alone binds IL-13 with high affinity; however, it has a short cytoplasmic tail that is devoid of signaling motifs, and the majority of studies suggest that it acts as a decoy receptor (9–13).

We found that bronchoalveolar lavage fluid (BALF) from patients with future BOS (FBOS), BOS, and treated BOS (TBOS) have increased profibrotic activity. Furthermore, this profibrotic activity was predominately due to elevated levels of IL-13. Using a murine model of BOS, we demonstrated elevated levels of IL-13 and its receptors that paralleled fibro-obliteration. Importantly, inhibition of IL-13 interactions with its receptors using an anti-IL-13 Ab inhibited procollagen expression and attenuated airway allograft fibro-obliteration independent of IL-4 biology. In addition, IL-13Rα2$^{-/-}$ mice acting as either donors or recipients of tracheal allografts demonstrated a marked increase in fibro-obliteration. Furthermore, the combination of anti-IL-13 Ab with cyclosporin A (CsA) had profound effects in reducing murine BOS (mBOS). These studies demonstrate the importance of the IL-13 during the pathogenesis of BOS.

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[3] Abbreviations used in this paper: BOS, bronchiolitis obliterans syndrome; BALF, bronchoalveolar lavage fluid; CsA, cyclosporin A; FBOS, future BOS; mBOS, murine BOS; NHLF, normal human lung fibroblast; qPCR, quantitative PCR; C$_T$, threshold cycle; SFM, serum-free medium; TBOS, treated BOS.

Materials and Methods

Patient population

With Institutional Review Board approval and informed written consent, we prospectively enrolled all patients undergoing lung transplantation from

June 1992 to April 2000. Patients were eligible for this study if they survived at least 6 mo posttransplantation. One hundred and sixty-nine patients were evaluated, of which 47 met criteria for being a healthy lung transplantation recipient that never had an episode of acute rejection or BOS, and 30 met criteria for having BOS as well as no clinical or laboratory data demonstrating allograft colonization or infection. All transplantation recipients were routinely followed according to a standard protocol. This protocol included clinical visits weekly for the first 3 wk, then at 6 wk, and then 3, 6, 9, and 12 mo. Subsequently, they were seen every 4 mo for the second year and thereafter annually. The clinic visit involved a history, physical, pulmonary function testing, and a bronchoscopy. Bronchoscopy was also performed at times when either infection or rejection was suspected. We chose the last available BALF in the healthy group to try to ensure an effective comparison of duration posttransplantation, between the healthy transplantation recipients and patients with BOS.

The third group evaluated was a subpopulation of the BOS group called FBOS ($n = 28$). This group consisted of BALF samples taken from the last bronchoscopy that the BOS patients had without colonization, infection, acute rejection, or BOS. The fourth group, TBOS ($n = 10$), were BOS recipients that had undergone treatment with either pulse methylprednisolone, monoclonal OKT3, or anti-thymocyte globulin Ab, and had undergone a clinical evaluation with pulmonary function testing and bronchoscopy within 6 wk of the treatment. Unfortunately, none of the patients treated for BOS had a response, as determined by clinical parameters and by pulmonary function testing criteria.

BALF was obtained from lung transplantation recipients by methods previously described (14–16). The cell-free solution was aliquoted and frozen immediately at 70°C until thawed for cytokine ELISAs (14–17). Throughout the entire study, we excluded any BALF performed at a time when infection and/or colonization were diagnosed with the following criteria: a positive BALF or transbronchial biopsy by cytology or microbiology Gram stain and culture for bacterial, acid fast bacillus, fungus, CMV, other respiratory virus, or *Pneumocystis carinii* pneumonia.

Diagnosis of BOS and healthy lung transplant recipients

Patients were diagnosed with BOS on the basis of an unexplained and sustained decrease in the forced expiratory volume in 1 s (FEV_1) by 20% or more of the peak predicted value after transplantation with or without pathologic evidence of BOS, as previously described (18). The diagnosis of acute rejection was excluded based on pathologic findings by transbronchial biopsy (18). Healthy transplantation recipients were those patients undergoing surveillance bronchoscopy without ever having clinical or biopsy proven evidence of acute rejection or BOS.

Immunosuppression and prophylactic antimicrobials

Patients were placed on a standard pre- and posttransplantation immunosuppression protocol, including cyclosporine or tacrolimus, azathioprine or mycophenolate mofetil, methylprednisolone, prophylactic antibiotics, and antivirals, as previously described (15, 16). All episodes of acute lung allograft rejection were treated with a 3-day pulse of 1 g/day methylprednisolone without adjustment of daily prednisone.

Reagents

Abs to human IL-13, IL-4, TGF-β, and murine IL-4 were purchased from R&D Systems. Polyclonal rabbit anti-murine IL-13 was produced by the immunization of a rabbit with murine rIL-13 (R&D Systems), as previously described (19). Direct ELISA was used to evaluate antiserum titers, and serum was used when titers had reached greater than 1/1,000,000. The specificity of the anti-IL-13 Ab was assessed by Western blot analysis and ELISA against a panel of other recombinant cytokines. The anti-IL-13 Ab was specific without cross-reactivity to IL-1R antagonist protein, IL-1, IL-2, IL-6, TNF-α, CXCL10, CXCL9, and other members of the CXC and CC chemokine families (20–24). The IgG portion of the serum was purified over a protein A column and used in a sandwich ELISA. Whole serum (0.5 ml) was used in vivo to block IL-13 during mBOS, as previously described (25). The neutralizing capacity of the anti-IL-13 Ab was assessed using a proliferation assay with a premyeloid TF1 cell line (20–24). Neutralization of IL-13 was also confirmed by reversal of IL-13-mediated inhibition of macrophage NO production in vitro, and the anti-IL-13 Ab completely neutralizes 50 ng of murine IL-13 (20–24).

IL-13, IL-4, and TGF-β cytokine ELISAs

Human IL-13 and IL-4 and murine IL-13 and IL-4 protein was quantitated using the double-ligand method, as previously described (14). Human IL-13 and IL-4 had the lowest detectable limit of 14 and 11 pg/ml, respec-

tively, whereas murine IL-13 and IL-4 had the lowest detectable limit of 23 and 9 pg/ml, respectively.

Fibroblast proliferation assay

Normal human lung fibroblast (NHLF) proliferation assay was used, as previously described (26). Briefly, NHLF were isolated and cultured from histologically normal pulmonary parenchymal tissue obtained from patients undergoing a nodule resection (27). Pulmonary fibroblasts were grown to 80% confluence and passaged. During the fourth passage, pulmonary fibroblast purity was >99%, as determined by the absence of nonspecific esterase, factor VIII, cytokeratin, smooth muscle α-actin, and desmin, and positive for vimentin, laminin, and fibronectin by immunostaining (27). Pulmonary fibroblasts were plated out in 96-well plates at a concentration of 7500 cells/well, washed, and cultured for 24 h under serum-free medium (SFM) conditions. At 24 h, SFM with or without IL-13 (1.0 and 10 ng/ml), IL-4 (10 ng/ml), or BALF was added. The BALF was pooled from six randomly selected lung transplantation patients from each of the following categories: healthy lung transplantation recipients, FBOS, BOS, or TBOS. The BALF was filtered and diluted 1/8 in SFM with or without anti-IL-13 (2 μg/ml), anti-IL-4 (2 μg/ml), or appropriate control Ab for 72 h. Seventy-two hours and a 1/8 dilution of the BALF were chosen because the characterization of this assay with BALF at multiple concentrations (neat, 1/4, 1/8, 1/16, and 1/32) at 24, 48, and 72 h demonstrated these (72 h and 1/8 dilution) conditions yielded maximal proliferation. Tritiated [³H]thymidine was added, and the percentage of incorporation was assessed using a scintillation counter (Beckman Coulter).

Fibroblast procollagen type I and III expression assay

NHLF procollagen expression assay was performed using NHLF isolated and cultured, as described above. Pulmonary fibroblasts were plated out in six-well plates at a concentration of 75,000 cells/well, washed, and cultured for 24 h under SFM conditions. At 24 h, SFM with or without IL-13 (1.0 and 10 ng/ml), IL-4 (10 ng/ml), and TGF-β (10 ng/ml) or BALF was added. The BALF was pooled from six randomly selected lung transplantation patients from each of the following categories: healthy lung transplantation recipients, FBOS, BOS, or TBOS. The BALF was filtered and diluted 1/8 in SFM with or without anti-IL-13 (2 μg/ml), anti-IL-4 (2 μg/ml), anti-TGF-β (6 μg/ml), or appropriate control Ab for 48 h. Forty-eight hours and a 1/8 dilution of the BALF were chosen because the characterization of this assay with BALF at multiple concentrations and time points demonstrated (48 h and 1/8 dilution) conditions yielded maximal procollagen type I and III expression. The expression of procollagen type I and III was determined using quantitative PCR (qPCR). Results were expressed as the fold increase of NHLF procollagen expression from BALF, as compared with SFM control.

mBOS model

We used the murine model of BOS involving the heterotopic s.c. trachea transplantation, as previously described (15, 17). The MHC class I- and II-disparate combination was BALB/c (H-2^d) to C57BL/6 (H-2^b) (allografts) and C57BL/6 (H-2^b) to C57BL/6 (H-2^b) (syngeneic control). In separate experiments, animals received either 20 mg of anti-IL-13 Ab or an equivalent quantity of control Ab given on days 0, 1, 3, 5, 7, 9, 11, 13, 15, and 17 by (i.p.) injection. In addition, we performed the murine model of BOS with donor tracheas from IL-4$^{-/-}$ mice on a BALB/c background transplanted heterotopically into IL-4$^{-/-}$ recipients on a C57BL/6 background. Furthermore, we performed the murine model of BOS with donor tracheas from IL-13Rα2$^{-/-}$ mice on a BALB/c background transplanted heterotopically into C57BL/6 recipient mice. The converse experiments also performed were donor tracheas from C57BL/6 mice transplanted heterotopically into IL-13Rα2$^{-/-}$ recipient mice on a BALB/c background. Moreover, to create experiments analogous to the human clinical setting, we used a threshold dose (20 mg/kg/day) of CsA (mixed in Cremophor EL castor oil; Sigma-Aldrich), as compared with control oil (Cremophor EL castor oil) given (i.p.) starting at postoperative day 0 and given every day until sacrifice in combination with either anti-IL-13 or control Ab therapy.

Histopathologic grading of BOS

Three random 3-μm paraffin-embedded tissue sections for five different trachea allografts were stained with H&E at three time points: days 7, 14, and 21. The histopathology was blindly reviewed with a histologic scoring system based on airway lining epithelial loss, deposition of extracellular matrix, leukocyte infiltration, and luminal obliteration, as previously described (15, 17).

Total RNA isolation and real-time qPCR

Total cellular RNA was isolated, as previously described (28). Total RNA was determined, reversed transcribed into cDNA, and amplified using TaqMan reverse-transcription reagents (Applied Biosystems). qPCR was performed using specific TaqMan primers and probes, using the ABI Prism 7700 Sequence Detector and SDS analysis software (Applied Biosystems). Negative controls were performed, as follows: qPCR was performed without reverse transcription to exclude contamination and amplification of genomic DNA, and without cDNA template to exclude reagent contamination with DNA. TaqMan murine IL-4, murine IL-13, and 18S Pre Developed Assay Reagent (Applied Biosystems) were used, and human procollagen type I, human procollagen type III, murine procollagen type I, murine procollagen type III, murine IL-4Rα, murine IL-13Rα1, and murine IL-13Rα2 primers and probe sequence (forward primer, reverse primer, TaqMan probe) were as follows: human procollagen type I, TCA CCTACAGCGTCACTGTCG, CACTGTCTTGCCCCAGGC, TGGCTGC ACGAGTCACACCGG; human procollagen type III, GCCAGGCATGG TGGCA, CCCCGGGTTCTAGTGATTCTC, CCTAGCTACCCAGGAGG CTGAGGCA; murine procollagen type I, CAAGGTTTCCAAGGCCCC, GGCATGGAGGACTAACTTACTGAA, CTGGTGAACCTGGCGAGCC TGG; murine procollagen type III, TGGACCAGCAGGAACTAATGG, CACCGTTCTTGCCGGGT, CCCGGAACACGAGGTCCTTCAGG; murine IL-4Rα, CAAACACAGTTGCCGGGTG, AGGTATCGCCTTGACT GCCA, AGGGCCCAGCGGGCACGATA; murine IL-13Rα1, TGAAAA AGTGCATCTCACCCC, GCCAAATGCACTTGAGCTCA, TGAAGGT GATCCTGAGTCCGCTGTCA; and murine IL-13Rα2, CACACCTGGA GGACCCATTC, GCAGACTCCCAGGAAATATCGT, CCAAGGTGTT ACACTTATGAAATTGTGATCCGA.

Quantitative analysis of gene expression was done using the comparative C_T (ΔC_T) methods, in which C_T is the threshold cycle number (17). The arithmetic formula for the ΔC_T method is described as the difference in C_T for a target (i.e., murine IL-13) and an endogenous reference (i.e., housekeeping gene 18S). The amount of target normalized to an endogenous reference (i.e., murine IL-13 in allografts at day 3) and relative to a calibration normalized to an endogenous reference (i.e., murine IL-13 in naive controls at day 3) is given by $2^{-\Delta\Delta CT}$, which is the murine IL-13 fold increase of the allograft, as compared with the naive control, as previously described (28).

Hydroxyproline assay

Before removal, heterotopically transplanted tracheas were dissected free of surrounding tissue, and total tracheal collagen was determined by analysis of hydroxyproline, as previously described (15). The range of the hydroxyproline assay is between 0 and 400 ng/ml, with the lowest detectable limit of 12.5 ng/ml.

Statistical analysis

Data were analyzed on a computer using the Statview 4.5 statistical package (Abacus Concepts). All cytokine levels in human BALF from different groups were evaluated by the nonparametric Kruskal-Wallis test with the post hoc analysis, Dunn. Data were displayed using a box plot summary. The human BALF fibroblast proliferation assay and fibroblast procollagen type I and III expression assay from different groups were evaluated by the ANOVA test with the post hoc analysis, Bonferroni/Dunn, and data are expressed as mean ± SEM. All animal group comparisons were evaluated by the ANOVA test with the post hoc analysis, Bonferroni/Dunn, and data are expressed as mean ± SEM.

Results

Human BALF from lung transplantation recipients with BOS has increased fibrotic activity

Previous studies have demonstrated that the obliterative bronchiolitis lesion found in BOS has fibrous scarring with dense collagen deposition (29). Based on these data, we characterized the BALF from lung transplantation recipients with BOS to determine its fibrotic potential. We used the fibroblast proliferation assay in which we placed human BALF from lung transplantation recipients with FBOS, BOS, TBOS, and healthy lung transplantation recipients on NHLF for 72 h and measured fibroblast proliferation by the incorporation of tritiated [³H]thymidine. Pooled samples of six human BALF normalized to total protein were used in a four-group comparison among FBOS, BOS, TBOS, and healthy lung transplantation recipient groups. We found that BALF from lung
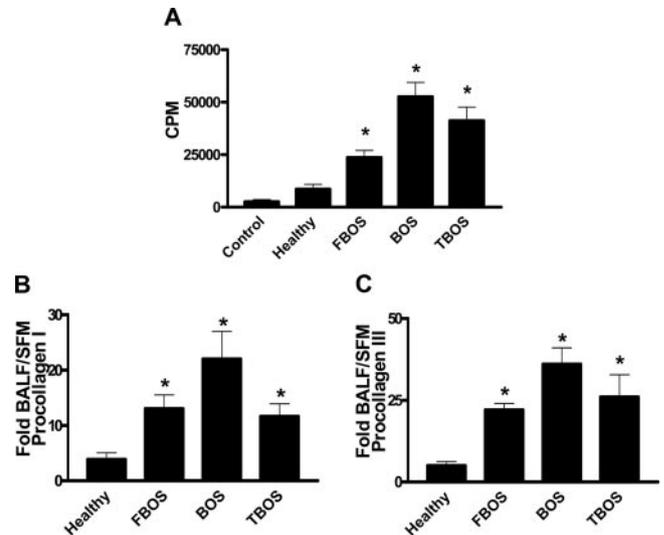

FIGURE 1. Increased fibrotic activity in human BALF from lung transplant recipients with BOS. *A*, NHLF proliferation cpm at 72 h in response to FBOS, BOS, TBOS, and healthy lung transplantation recipient BALF specimens. BALF from each group (FBOS, BOS, and TBOS) resulted in significant fibroblast proliferation, as compared with the healthy lung transplantation recipient group. *, $p < 0.008$. Procollagen type I (*B*) and procollagen type III (*C*) expression from NHLF at 48 h in response to FBOS, BOS, TBOS, and healthy lung transplantation recipient BALF specimens presented as a fold increase of NHLF procollagen expression from BALF, as compared with SFM control. BALF from each group (FBOS, BOS, and TBOS) resulted in significant fibroblast procollagen expression, as compared with the healthy lung transplantation recipient group. *, $p < 0.008$.

transplantation recipients with BOS ($n = 6$) induced a greater fibroblast proliferative response, as compared with samples of BALF from healthy lung transplantation recipients ($n = 6$) (Fig. 1A). Moreover, samples of BALF from lung transplantation recipients with FBOS ($n = 6$) and TBOS ($n = 6$) also induced a greater fibroblast proliferative response than the healthy lung transplantation recipients (Fig. 1A).

To confirm that there was increased fibrotic activity in BALF from patients with BOS, we used the fibroblast procollagen type I and III assay in which we placed human BALF from lung transplantation recipients on NHLF for 48 h and measured fibroblast procollagen type I and III expression using qPCR. Pooled samples of six human BALF normalized to total protein were used in a four-group comparison among FBOS, BOS, TBOS, and healthy lung transplantation recipient groups. We found that BALF from lung transplantation recipients with BOS ($n = 6$) induced a greater procollagen type I and III expression, as compared with samples of BALF from healthy lung transplantation recipients ($n = 6$) (Fig. 1, *B* and *C*). Moreover, BALF from lung transplantation recipients with FBOS ($n = 6$) and TBOS ($n = 6$) also induced a greater fibroblast procollagen type I and III expression than the healthy lung transplantation recipients ($n = 6$) (Fig. 1, *B* and *C*).

Elevated levels of IL-13 are biologically active and contribute to the fibrotic activity of the BALF from patients with BOS

With the finding that BALF from lung transplantation recipients with BOS have the ability to modulate fibroblast proliferation and procollagen expression, we determined whether there were any significant elevations in the type 2 profibrotic cytokine, IL-13, in BALF. Using a four-group comparison among lung transplantation recipients with BOS ($n = 30$), FBOS ($n = 28$), TBOS ($n = 10$),
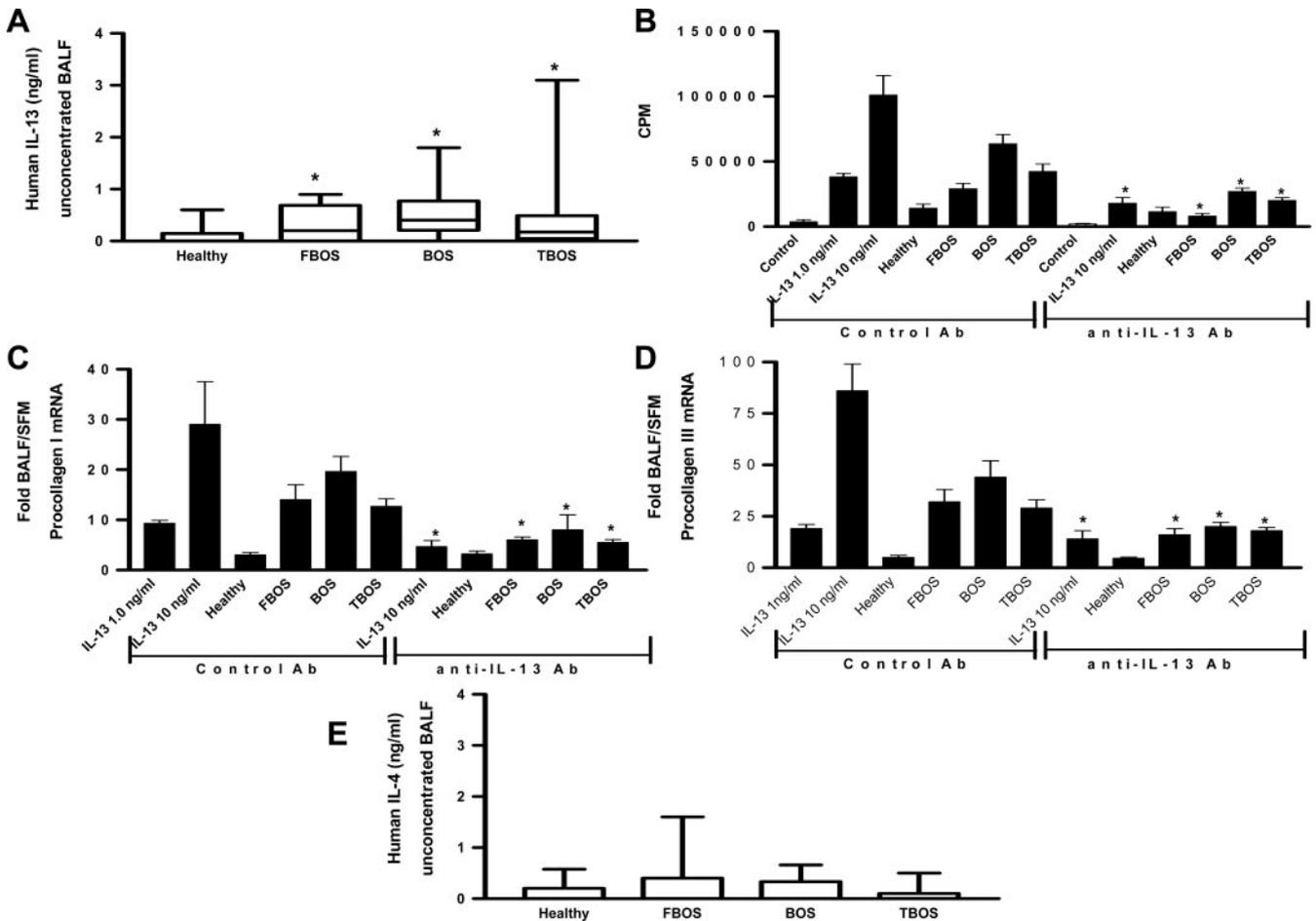
FIGURE 2. Elevated BALF IL-13 levels are profibrotic from patients with BOS. *A,* Human IL-13 protein levels in unconcentrated BALF from recipients with FBOS, BOS, and TBOS groups were significantly elevated, as compared with healthy lung transplantation recipients. ∗, $p < 0.05$. *B,* Neutralizing Ab to IL-13, as compared with control Ab inhibition of NHLF proliferation at 72 h from the BALF of the FBOS, BOS, and TBOS groups. ∗, $p < 0.05$. Procollagen type I (*C*) and procollagen type III (*D*) expression from NHLF with neutralizing Ab to IL-13, as compared with control Ab at 48 h from the BALF of the FBOS, BOS, and TBOS groups presented as a fold increase of NHLF procollagen expression from BALF, as compared with SFM control. ∗, $p < 0.05$. *E,* Human IL-4 protein levels in unconcentrated BALF from recipients with FBOS, BOS, TBOS, and healthy lung transplant recipients.

and healthy lung transplantation recipients ($n = 47$), we found significantly elevated levels of IL-13 in the FBOS, BOS, and TBOS groups, as compared with the healthy transplantation recipients (Fig. 2*A*). To substantiate the elevated levels of IL-13 were biologically active, we performed fibroblast proliferation assays using NHLF stimulated with BALF from our healthy lung transplantation recipients ($n = 6$), FBOS ($n = 6$), BOS ($n = 6$), and TBOS ($n = 6$) groups in the presence of neutralizing Ab to IL-13 or control Ab. We found a significant reduction in fibroblast proliferation by BALF from the FBOS, BOS, and TBOS groups in the presence of anti-IL-13, as compared with control Ab (Fig. 2*B*). Similarly, we performed the fibroblast procollagen expression assays using NHLF stimulated with BALF from our four different groups in the presence of anti-IL-13 or control Ab. We found significant reductions in procollagen type I and III expression by BALF from the FBOS, BOS, and TBOS groups in the presence of anti-IL-13 Ab, as compared with control Ab (Fig. 2, *C* and *D*).

IL-4 and TGF-β do not significantly contribute to the fibrotic activity of the BALF from patients with BOS

Because IL-4 is also a profibrotic type 2 cytokine that can induce fibroplasia similar to IL-13, we assessed whether IL-4 contributed to the fibrotic activity in BALF during human BOS. The protein levels

of IL-4 in BALF from patients with FBOS ($n = 28$), BOS ($n = 30$), TBOS ($n = 10$), and healthy lung transplantation recipients ($n = 47$) were not statistically different using a four-group comparison (Fig. 2*E*). In addition, we assessed the contribution of IL-4 to the fibrotic activity. We performed the human fibroblast proliferation and procollagen type I and III expression assays using NHLF stimulated with BALF from our four groups in the presence of neutralizing Ab to IL-4 or control Ab and found no significant changes (data not shown). However, anti-IL-4 Ab could inhibit exogenously added IL-4-mediated fibroblast proliferation and procollagen type I and III expression, as compared with control Ab (data not shown).

TGF-β is another pleiotropic profibrotic cytokine that is known to be involved in many fibrotic disorders. Previously, we have demonstrated that there were no differences in TGF-β BALF levels from patients with FBOS, BOS, TBOS, and healthy lung transplantation recipients (16). We now assessed whether TGF-β contributed to the fibrotic activity in BALF during human BOS. We performed the human fibroblast procollagen type I and III expression assay using NHLF stimulated with BALF from our four groups in the presence of neutralizing Ab to TGF-β or control Ab and found no significant changes. However, anti-TGF-β Ab could inhibit exogenously added TGF-β-mediated fibroblast procollagen type I and III expression, as compared with control Ab (data not shown).

FIGURE 3. Increased fibroplasia during mBOS. Procollagen type I (*A*) and procollagen type III (*B*) mRNA expression presented as a fold increase of procollagen expressed from the allografts, as compared with syngeneic controls. ∗, *p* < 0.05. *C*, Hydroxyproline levels in whole tracheal homogenates from allografts, as compared with syngeneic controls. ∗, *p* < 0.05.
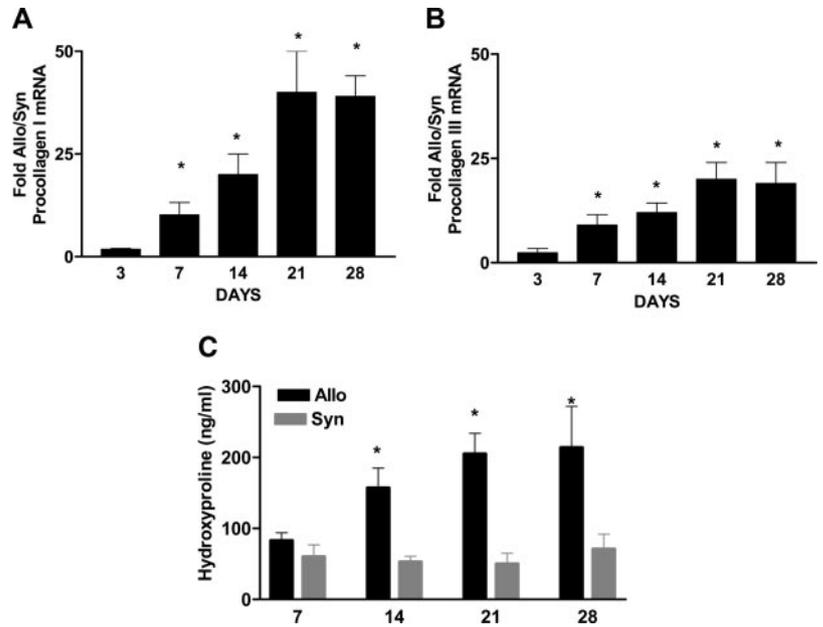
IL-13 is elevated during mBOS and is associated with allograft airway fibroplasia

The above human findings support the notion that IL-13 can modulate a fibrotic response during the pathogenesis of BOS. To ascertain whether this biology contributes to fibro-obliteration of the allograft airway, we used an animal model of BOS. We characterized the allograft airway fibroproliferative events during mBOS by determining the kinetics of procollagen type I and III mRNA expression using qPCR on tracheal homogenates. We found a temporal increase in the expression of both procollagens from whole tracheal allografts, as compared with syngeneic controls over a 28-day time course (Fig. 3, *A* and *B*). We confirmed these results using the hydroxyproline assay (Fig. 3*C*).

IL-13 is a potent inducer of fibroplasia; thus, the kinetics of IL-13 expression during mBOS was assessed. qPCR of whole tracheal homogenates demonstrated markedly greater mRNA expression of IL-13 from allografts, as compared with the syngeneic controls (Fig. 4*A*). Specifically, IL-13 expression from allografts was up-regulated by day 7 and remained persistently elevated throughout day 28 (Fig. 4*A*). These results were confirmed by measuring whole tracheal homogenate protein levels of IL-13, by ELISA (Fig. 4, *A* and *B*).

Expression of specific components of the IL-13R from the tracheal allografts parallels its ligand, IL-13, during mBOS

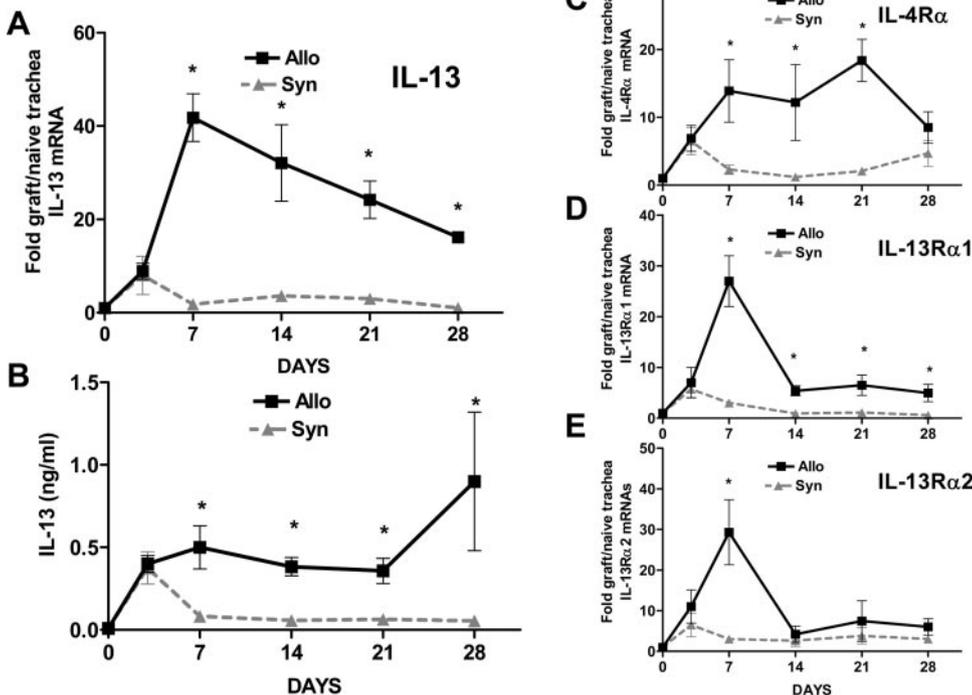We evaluated the IL-13Rs by performing qPCR on tracheal homogenates for mRNA expression of IL-4Rα, IL-13Rα1, and

FIGURE 4. Increased IL-13 and receptor expression from allografts during mBOS. *A*, IL-13 mRNA expression presented as a fold increase of IL-13 expressed from the allografts or syngeneic controls, as compared with naive tracheas. ∗, *p* < 0.05. *B*, Protein levels of IL-13 from whole tracheal allografts and syngeneic controls. ∗, *p* < 0.05. IL-4Rα (*C*), IL-13Rα1 (*D*), and IL-13Rα2 (*E*) mRNA expression presented as a fold increase of IL-13R component expressed from the allografts or syngeneic controls, as compared with naive tracheas. ∗, *p* < 0.05.
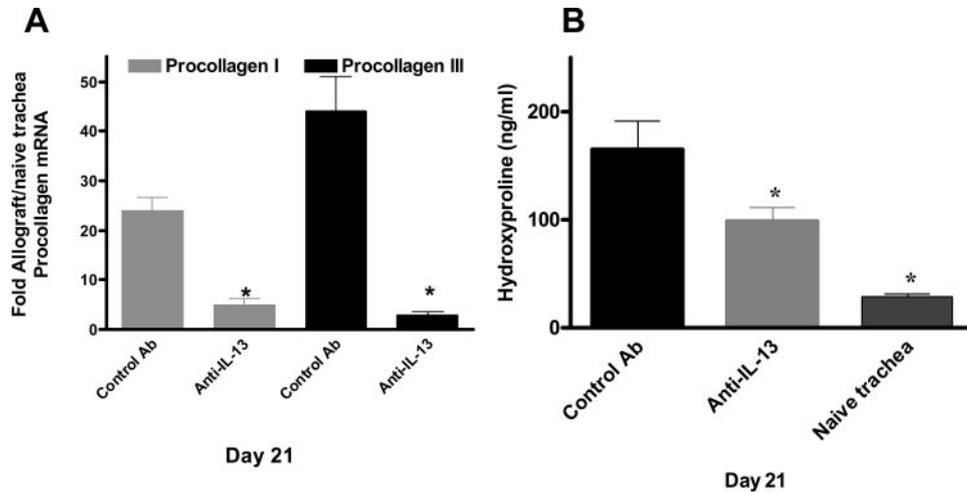
FIGURE 5. In vivo neutralization of IL-13 decreases allograft fibroplasia. *A*, Procollagen type I and III mRNA expression presented as a fold increase of procollagen expressed in allografts from anti-IL-13 Ab- or control Ab-treated groups, as compared with naive tracheas. *∗, $p < 0.05$. B*, Hydroxyproline levels of tracheal allografts from animals treated with anti-IL-13 Ab, as compared with control Ab, and naive tracheas. Hydroxyproline levels in tracheal allografts from anti-IL-13 Ab-treated animals were significantly reduced, as compared with control Ab-treated animals. Hydroxyproline levels in naive tracheas were significantly reduced, as compared with tracheal allografts from anti-IL-13 Ab-treated animals. *∗, $p < 0.0167$.

IL-13Rα2 chains, individually. We found a significant increase in IL-4Rα mRNA expression from tracheal allografts that peaked on day 21, as compared with syngeneic controls (Fig. 4*C*). Additionally, allografts had significant increases in IL-13Rα1 mRNA expression that peaked at day 7 and remained markedly elevated throughout the rest of the time course, as compared with syngeneic controls (Fig. 4*D*). In contrast, allograft IL-13Rα2 mRNA expression peaked at day 7 and then returned to levels similar to the syngeneic controls (Fig. 4*E*).

Fibroplasia during mBOS is attributable to IL-13 interactions with its receptors

The effects of the interactions of IL-13 and its receptors were evaluated during allograft airway fibro-obliteration by performed in vivo neutralization studies of IL-13 during mBOS. Recipient animals received specific anti-IL-13 or control Ab every 48 h until day 17. Tracheal allografts from animals that received in vivo neutralizing IL-13 Ab had significantly less fibroplasia at day 21, as accessed by qPCR of whole tracheal allograft mRNA expression of procollagen type I and III, as compared with allografts from recipient animals that received control Ab (Fig. 5*A*). These findings were further substantiated using the hydroxyproline assay (Fig. 5*B*). However, hydroxyproline levels in the anti-IL-13 Ab group, whereas albeit reduced, were still elevated as compared with naive tracheas (Fig. 5*B*).

IL-13-deficient mice can develop high levels of IL-4 (30). In addition, IL-4 has a controversial role in promoting neonatal tolerance (31, 32). Therefore, we determined whether inhibiting IL-13 biology had any effect on allograft expression of IL-4. We found no significant change in IL-4 expression, using qPCR, in tracheal allografts from recipient animals treated with anti-IL-13, as compared with control Ab at day 21 or earlier time points (data not shown). Furthermore, we found no difference in IL-4 mRNA or protein between the allografts and syngeneic controls, over a 28-day time course (data not shown).

FIGURE 6. In vivo neutralization of endogenous IL-13 attenuates mBOS. *A*, Histopathologic quantitative analysis, and *B*, representative H&E-stained histopathology of tracheal allografts from animals treated with anti-IL-13, demonstrating decreased airway lining epithelial loss, deposition of extracellular matrix, leukocyte infiltration, and airway obliteration, as compared with control Ab. *∗, $p < 0.05$.
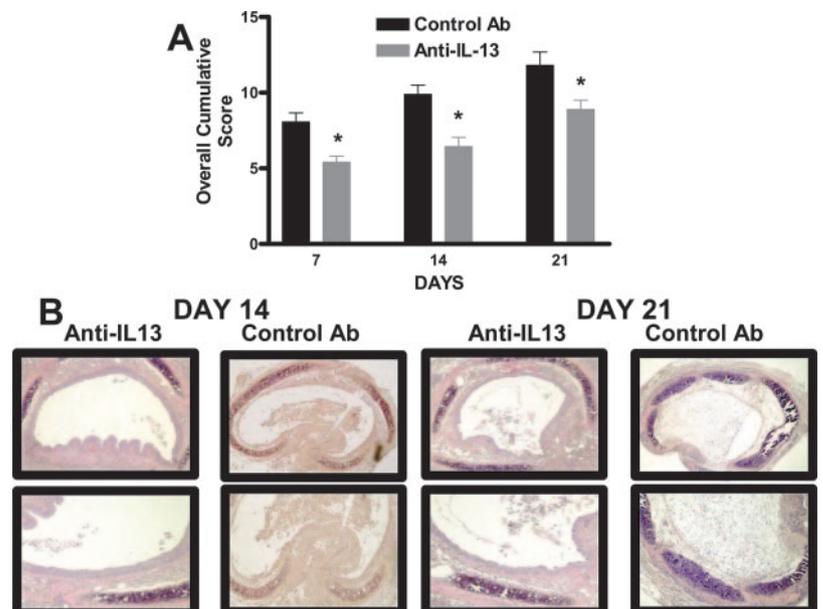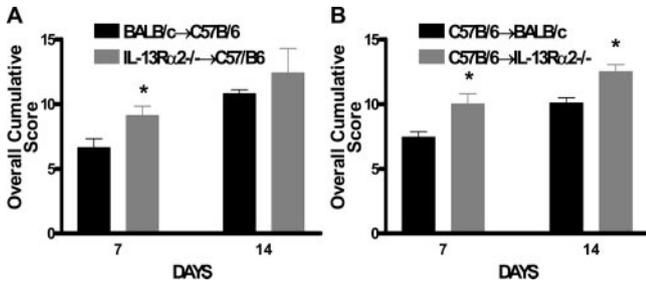
FIGURE 7. IL-13Rα2$^{-/-}$ mice demonstrate increased mBOS. Histopathologic quantitative analysis of *A*, donor IL-13Rα2$^{-/-}$ tracheas on a BALB/c background transplanted into C57BL/6 recipients, as compared with donor IL-13Rα2$^{+/+}$ tracheas on a BALB/c background transplanted into C57BL/6 recipients. ∗, $p < 0.05$. *B*, Donor C57BL/6 tracheas transplanted into IL-13Rα2$^{-/-}$ recipients on a BALB/c background, as compared with C57BL/6 donor tracheas transplanted into IL-13Rα2$^{+/+}$ recipients on a BALB/c background. ∗, $p < 0.05$.

Moreover, to exclude any significant role for IL-4 during the pathogenesis of mBOS, we performed experiments with donor IL-4$^{-/-}$ tracheas on a BALB/c background transplanted into IL-4$^{-/-}$ recipients on a C57BL/6 background, as compared with IL-4$^{+/+}$ donor tracheas on a BALB/c background transplanted into IL-4$^{+/+}$ recipient mice on a C57BL/6 background. Using a quantitative histopathological analysis of overall cumulative BOS score based on four easily identifiable pathologic processes, airway lining epithelial loss, leukocyte infiltration, extracellular matrix deposition, and luminal obliteration, we did not find any significant difference between the IL-4$^{-/-}$ group, as compared with the IL-4$^{+/+}$ group at either early or late time points, days 7 and 21, respectively (data not shown).

Inhibition of IL-13 interactions with its receptors results in the attenuation of mBOS

With a significant reduction in collagen deposition in the allografts from animals treated with anti-IL-13 Ab, we next assessed whether there was any effect on mBOS. We found that tracheal allografts from recipient animals treated with anti-IL-13 Ab demonstrated marked reductions in overall cumulative BOS scores throughout day 21, as compared with animals treated with control Ab (Fig. 6).

To further confirm our in vivo neutralization studies of IL-13, we performed the converse experiments. Based on substantial data demonstrating IL-13Rα2 is a decoy receptor that inhibits the activity of IL-13 (6–8), we used a genetic approach, in which we transplanted donor tracheas from IL-13Rα2$^{-/-}$ mice on a BALB/c background into recipient C57BL/6 mice. Because our initial characterization of the expression of IL-13Rα2 in our allografts, as compared with syngeneic controls, demonstrated a marked increase in IL-13Rα2 only at the early time point day 7 during mBOS (Fig. 4*E*), we evaluated the effect of the donor IL-13Rα2$^{-/-}$ tracheal allografts at the early time points, days 7 and 14. Histopathologic analysis of donor IL-13Rα2$^{-/-}$ tracheal allografts in C57BL/6 recipients demonstrated a significant increase in overall cumulative BOS scores at day 7, but not at day 14, as compared with IL-13Rα2$^{+/+}$ tracheal allografts transplanted into recipient C57BL/6 mice (Fig. 7*A*). Moreover, when donor C57BL/6 (IL-13Rα2$^{+/+}$) tracheas were transplanted into recipient IL-13Rα2$^{-/-}$ mice on a BALB/c background, histopathologic analysis of tracheal allografts demonstrated a significant increase in overall cumulative BOS scores at both days 7 and 14, as compared with C57BL/6 (IL-13Rα2$^{+/+}$) tracheas transplanted into IL-13Rα2$^{+/+}$ recipient mice on a BALB/c background (Fig. 7*B*).

In vivo administration of anti-IL-13 + CsA has profound effects on attenuating mBOS

Using a threshold dose of CsA (20 mg/kg/day) that inhibits BOS at day 7, but not days 14 or 21, in combination with anti-IL-13 Ab, demonstrated a significant effect on fibro-obliteration with a marked reduction in hydroxyproline levels of allografts, as compared with the combination of CsA + control Ab at day 28 (Fig. 8, *A* and *B*). In addition, using a four-group comparison among control Ab + CsA, anti-IL-13 Ab + CsA, anti-IL-13 Ab + control oil, and control Ab + control oil, we found the combination of anti-IL-13 Ab + CsA caused a significant reduction in cumulative BOS scores, as compared with control Ab + CsA and control Ab + control oil (Fig. 8*C*). Furthermore, using a subgroup analysis, we found that there was a significant reduction in mBOS from animals treated with anti-IL-13 + CsA, as compared with anti-IL-13 + control oil.
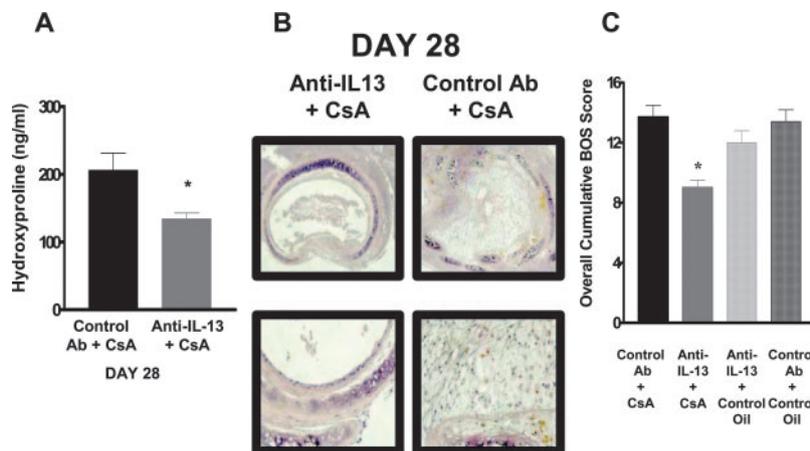

FIGURE 8. In vivo neutralization of endogenous IL-13 combined with CsA causes a profound reduction in mBOS. *A*, Hydroxyproline levels of tracheal allografts from animals treated with anti-IL-13 + CsA, as compared with control Ab + CsA. ∗, $p < 0.05$. *B*, Representative H&E-stained histopathology of tracheal allografts from animals treated with anti-IL-13 + CsA, demonstrating decreased airway lining epithelial loss, deposition of extracellular matrix, leukocyte infiltration, and airway obliteration, as compared with control Ab + CsA. *C*, Histopathologic quantitative analysis of allografts from animals treated with control Ab + CsA, anti-IL-13 Ab + CsA, anti-IL-13 Ab + control oil, and control Ab + control oil. Overall cumulative BOS scores of tracheal allografts from anti-IL-13 Ab + CsA-treated animals were significantly reduced, as compared with control Ab + CsA- and control Ab + control oil-treated animals. ∗, $p < 0.008$.

Discussion

Chronic lung allograft rejection, like other solid organ rejection, is commonly thought of as a type 1-mediated immune response generated toward alloantigens of the donor lung. However, specific animal studies of rejection have demonstrated that the type 2-mediated immune response may be just as injurious to the allograft as a type 1-mediated immune response (3–5). Based on these findings and the limited data on the role of type 2 cytokines during the pathogenesis of BOS, we hypothesized that the IL-13 biological axis has an important role during BOS.

Although histopathologic studies have demonstrated a significant amount of matrix deposition in postlung transplantation obliterative bronchiolitis (29), we have now expanded upon these data by demonstrating that the BALF from patients with BOS has increased fibrotic activity. In addition, we found marked elevations in IL-13 in the BALF from patients with FBOS, BOS, and TBOS that were biologically active in promoting fibrotic activity. These data are supported by several studies demonstrating that interstitial lung disease BALF has increased IL-13 expression and fibrotic activity, and the inhibition of IL-13 interactions with interstitial lung disease fibroblasts decreases fibroplasia (33, 34). Collectively, this suggests that IL-13 is contributing to the fibroplasia involved in BOS. Furthermore, the elevated levels of IL-13 in the FBOS group suggest that a persistent low grade allospecific injury to the allograft airways may be promoting the production of IL-13, which eventually contributes to allograft fibro-obliteration. Moreover, the elevated levels of IL-13 in the TBOS group suggest that our current treatment failure of BOS may be due, in part, to its inability to suppress IL-13 expression.

The biological activity of another closely related type 2 cytokine, IL-4, as well as the pleiotropic/profibrotic cytokine TGF-β was also evaluated in the BALF from our lung transplantation populations. We found no elevation in IL-4, and we have demonstrated previously no significant elevation of TGF-β in BALF during human BOS (16). We confirmed these findings, as there was no effective reduction of BALF fibrotic activity by inhibiting either cytokine. Similarly, IL-13, not IL-4, has been implicated in the fibrosis, occurring in a variety of human disorders, including obstructive and restrictive lung disorders (35). Overall, these data suggest a more important role for IL-13, as compared with IL-4 or TGF-β in propagating the fibroplasia involved in the continuum of FBOS to BOS.

The above findings support the notion that IL-13 is a critical factor involved in human BOS. To determine whether IL-13 biology contributes to fibro-obliteration, we used an animal model of BOS. Analogous to our human BOS data, we found an increase in procollagen type I and III expression from allografts. In addition, we found elevated levels of IL-13 and increased expression of individual components of the active IL-13R complex (IL-4Rα/IL-13Rα1), which paralleled allograft airway fibro-obliteration. In contrast, IL-13Rα2 expression was only elevated during the early inflammatory phase and not during the fibro-obliterative phase of mBOS. This is similar to what has been shown in the schistosoma granuloma models of liver fibrosis (12). Interestingly, we also found elevated expression of IL-13 in both allografts and syngeneic controls at day 3, a time point when ischemia-reperfusion injury occurs. This suggests that IL-13 may play a role during postlung transplantation ischemia-reperfusion injury.

We next determined the effects of IL-13 on allograft airway fibroplasia by in vivo neutralization of endogenous IL-13. Allografts from animals treated with anti-IL-13 Ab had marked reductions in procollagen type I and III expression and collagen deposition. These data are supported by studies demonstrating that

ablation of IL-13 inhibits the fibrosis involved in animal models of pulmonary and liver schistosoma, asthma, and pulmonary fibrosis (12, 30, 35, 36).

Previous animal models of neonatal tolerance have demonstrated an association with increased IL-4 expression (31), and anti-IL-4 therapy reversed this tolerance (32). Furthermore, IL-13-deficient mice can develop high levels of IL-4. Therefore, we determined whether there was a contribution of IL-4 biology to mBOS (30–32). Surprisingly, we did not find any significant up-regulation of IL-4. In addition, anti-IL-13 therapy during mBOS had no significant effect on IL-4 expression. Furthermore, the use of IL-4$^{-/-}$ donor tracheas transplanted into completely mismatched IL-4$^{-/-}$ recipients had no effect on fibro-obliteration, indicating no significant role for IL-4 during mBOS. These results are supported by studies demonstrating that the overexpression of IL-13, but not the IL-4 gene, in the murine lung results in subepithelial fibrosis (30, 35, 36). Collectively, these data suggest that IL-13 is a more important regulator of airway fibrosis than IL-4, which has important implications for human BOS.

Although one study has suggested that the interactions of IL-13 with IL-13Rα2 cause cell signaling (37), the majority of data suggests that IL-13Rα2 is a decoy receptor that inhibits the activity of IL-13 (6–8). Thus, to further confirm our in vivo neutralization studies showing that IL-13 attenuates mBOS, we preformed the converse experiments. We transplanted donor tracheas from IL-13Rα2$^{-/-}$ mice into fully mismatched recipient mice and found an early increase in mBOS. Moreover, when donor tracheas were placed into fully mismatched recipient IL-13Rα2$^{-/-}$ mice, allografts demonstrated a significant increase in mBOS that was more durable then that seen from the IL-13Rα2$^{-/-}$ tracheal donors. This suggests that in the absence of IL-13Rα2, there is excess free IL-13, which is able to bind to its active receptor complex (IL-4Rα/IL-13Rα1), ultimately causing fibrosis.

We also determined the clinically relevant effects of combining CsA with anti-IL-13 therapy and found a profound reduction in collagen deposition and mBOS that lasted out to day 28. Studies have demonstrated that CsA not only affects T cell function, but can also down-regulate both IL-13 and IgG production in vivo (38, 39). Therefore, the addition of CsA to our anti-IL-13 treatment regimen is most likely working at multiple levels.

In conclusion, we have demonstrated that elevated levels of biologically active IL-13 are associated with human BOS. In addition, IL-13 is the important inducer of fibrotic activity, as compared with IL-4 and TGF-β during human BOS. Proof of concept studies using the murine model of BOS demonstrated that IL-13 and its receptor play a critical role in the fibro-obliterative process of BOS that was independent of any effects on IL-4. The findings of this study may ultimately result in novel therapies designed to attenuate the IL-13 biological axis and prevent/treat BOS postlung transplantation.

Disclosures

The authors have no financial conflict of interest.

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