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Regulation of Found in Inflammatory Zone 1 Expression in Bleomycin-Induced Lung Fibrosis: Role of IL-4/IL-13 and Mediation via STAT-6

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Found in inflammatory zone (FIZZ)1, also known as resistin-like molecule α, belongs to a novel class of cysteine-rich secreted proteins, also known as resistin-like molecules (RELMs) (1–3). The FIZZ/RELM family consists of three known members, FIZZ1/RELM-α, FIZZ2/RELM-β, and FIZZ3/resistin. These three members have unique tissue specific distribution. Thus, FIZZ1 is expressed in lung, white adipose tissue, mammary, tongue, and heart (1), and is found lately to be a key marker, along with Ym-1, of alternatively activated macrophages (4, 5). FIZZ2 is detected exclusively in the colon and small bowel (2), whereas FIZZ3 is found in white adipose tissue and circulating mononuclear cells (1, 6). More recently, there is evidence of an additional member of this family of proteins, referred to as RELM-γ (7). This newest member exhibits 69.4 and 72.1% homology with RELM-α/FIZZ1 in the rat and mouse, respectively. This high degree of homology is thought to have made it difficult to distinguish expression of RELM-α/FIZZ1 from RELM-γ expression in white adipose tissue in the previous study (7). The FIZZ/RELM family is characterized by a signature carboxyl terminus sequence containing 10 completely conserved cysteine residues, in which the spacing between cysteines is invariant (1, 2). FIZZ1 lacks one cysteine residue in the amino terminus that is present in both FIZZ2 and FIZZ3 in multiple species. This difference may be responsible for the inability of FIZZ1 to form disulfide-linked homodimers, while both FIZZ2 and FIZZ3 have been found as homodimers (8). However, FIZZ1 has been found to form hetero-oligomers with FIZZ3 but not FIZZ2 (9). These structural signatures of conserved spacing between cysteine residues and small size (between 8 and 12 kDa) indicate that FIZZ1 is likely to be a secreted protein with potential as a signaling molecule. This possibility is supported by a study showing that FIZZ1 is secreted into the culture supernatant of FIZZ1-transfected 293T cells (2).

There is only limited information about the precise biological function or activity of this family of molecules. FIZZ1 is first reported to inhibit the nerve growth factor-mediated gene expression of dorsal root ganglion neurons (1). It has an inhibitory effect on 3T3-L1 preadipocyte differentiation into adipocytes, which is not accompanied by an increase in cell proliferation (9). Recently, a study in a mouse chronic hypoxia model of pulmonary hypertension revealed that FIZZ1 is able to stimulate the proliferation of pulmonary vascular smooth muscle cells, and thus, is referred to also as a hypoxia-induced mitogenic factor (10). FIZZ1 is also highly induced in bleomycin (BLM)-induced lung fibrosis as assessed by cDNA microarray analysis, and found to localize primarily to alveolar epithelial cells by in situ hybridization (11, 12). Furthermore, FIZZ1 can induce myofibroblast differentiation in lung fibroblast cultures, as manifested by increased expression of α-smooth muscle actin (α-SMA) and type I collagen (11). This suggests the potential involvement of FIZZ1 in the fibrotic response in BLM-induced lung injury model. However, the mechanism of induction and regulation of FIZZ1 expression in alveolar epithelial cells in the context of lung injury and fibrosis is undetermined.
However, there is evidence from studies with alternatively activated macrophages that suggest induction of FIZZ1 expression may be under the influence of type 2 cytokines (4, 5). Moreover, there is ample evidence to suggest the involvement of the type 2 cytokines, IL-4 and IL-13, in fibrotic diseases (13–16). For instance, IL-4 gene expression is significantly increased in a murine model of lung injury induced by BLM with primary expression by macrophages and T lymphocytes (14). Moreover, IL-4 and IL-13 are capable of stimulating fibroblast proliferation, as well as α-SMA and collagen expression, thus triggering myofibroblast differentiation (17). A recent study suggests that IL-13 could mediate its fibrogenic effects in the lung and other organs by inducing and activating TGF-β1 (18). Like TGF-β1, IL-4 and IL-13 have similar effects on myofibroblast differentiation by inducing α-SMA production in human synovial fibroblasts (19). These data clearly suggest the profibrotic roles of IL-4 and IL-13 in fibrosis by affecting fibroblast activation and myofibroblast differentiation.

These findings suggest the possibility that FIZZ1 expression in alveolar epithelial cells may be regulated by cytokines, especially type 2 cytokines. IL-13 shares 30% homology with IL-4 and appears to have certain overlapping biological activities in Th2 type responses. Both cytokines bind to the IL-4R α-chain (20). In response to treatment with IL-4 or IL-13, receptor multimerization is accompanied with binding and activation of the downstream signaling molecules, JAKs, resulting in their transphosphorylation, as well as receptor phosphorylation. The phosphorylated receptor creates docking sites for STAT6, which can then be phosphorylated by JAKs, leading to STAT6 dissociation from the receptor, homodimerization and translocation to the nucleus. These STAT6 homodimers can then interact with specific DNA sites for STAT6, which can then be phosphorylated by JAKs, and then platted on 6-well tissue culture dishes precoated with fibronectin (R&D Systems, Minneapolis, MN). Isolated cells were evaluated by immunofluorescence after staining with anti-cytokeratin 5/8 Abs (BD Biosciences, San Diego, CA), which recognized the cytokeratins found in AECs, but not present in macrophages, fibroblasts, or endothelial cells (28, 29). After 2 days in culture, the adherent cells were consistently >90% epithelial cells. Primary cultured AECs were used without passaging.

When ready to use, AECs were cultured in DMEM supplemented with 10% newborn calf serum (Sigma-Aldrich, St. Louis, MO), which were suspended in DMEM supplemented with 10% newborn calf serum (Sigma-Aldrich), and then plated onto 6-well tissue culture dishes precoated with fibronectin (R&D Systems, Minneapolis, MN).

Materials and Methods

Animals and induction of pulmonary fibrosis

Male specific pathogen-free Fisher 344 rats (6–8 wk old) were purchased from Charles River Breeding Laboratories (Wilmington, MA). BALB/c, C57BL/6, and IL-4–deficient (IL-4−/−) mice on a C57BL/6 background (6–8 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-13-deficient (IL-13−/−) mice, IL-4 and IL-13 doubly deficient (IL-4/13−/−) mice, and STAT6-deficient mice, all on BALB/c background (7–10 wk old) were produced as previously described (24, 25), and bred at the University of Michigan (Ann Arbor, MI) for these studies. To induce pulmonary fibrosis, BLM (Blenoxane; Mead Johnson, Princeton, NJ) was dissolved in sterile PBS and instilled endotracheally on day 0 as previously described (26). Due to the strain-dependent differences in sensitivity to BLM (27), the doses of BLM were 0.0015 U/g and 0.01 U/g body weight for C57BL/6 and BALB/c mice, respectively. Control groups received the same volume of sterile PBS only. Mice (n = 3–5) were randomly assigned to each of the indicated treatment groups. At indicated time points after BLM treatment, the mice were sacrificed and the lungs were harvested rapidly. Where indicated, the lung tissue samples were immediately placed in TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) for total RNA isolation, or in lysis buffer (50 nM Tris·Cl, pH 7.5, 1% Nonidet P-40) for Western blotting analysis.

Regulation of FIZZ1 expression in lung fibrosis

AECs were isolated by elastase digestion and IgG panning as previously described (28). Briefly, aortic ring whole lung lavage with 5 mg/mL EDTA in balanced salt solution, porcine pancreatic elastase (4.3 U/ml; Worthington Biochemical, Lakewood, NJ) was instilled via the trachea to release type II cells. Contaminating cells bearing Fcs were removed from the cell suspension by panning on plates coated with rat IgG (Sigma-Aldrich, St. Louis, MO). The cells were suspended in DMEM supplemented with 10% newborn calf serum (Sigma-Aldrich), and then plated onto 6-well tissue culture dishes precoated with fibronectin (R&D Systems, Minneapolis, MN). Isolated cells were evaluated by immunofluorescence after staining with anti-cytokeratin 5/8 Abs (BD Biosciences, San Diego, CA), which recognized the cytokeratins found in AECs, but not present in macrophages, fibroblasts, or endothelial cells (28, 29). After 2 days in culture, the adherent cells were consistently >90% epithelial cells. Primary cultured AECs were used without passaging.

When ready to use, AECs were cultured in DMEM supplemented with 10% FBS. When they reached ~90% confluence, the cells were made quiescent by culturing in DMEM containing 0.5% FBS for 4–6 h. Where indicated, recombinant rat IL-4 and/or recombinant mouse IL-13 (R&D Systems) were then added at the indicated doses, and further incubated for 4, 8, 12, and 24 h before harvesting for total RNA isolation.

Analysis of rat AECs and treatment with cytokines

Primers and probe for GAPDH were purchased from Applied Biosystems. For each assay, 100 ng of total RNA was used as template. GAPDH mRNA was used as internal control to normalize the amount of input RNA. One-step real time RT-PCR (48°C 30 min, 95°C 10 s, followed by 45 cycles of 95°C 10 s, 60°C 1 min) was undertaken with TaqMan One Step RT-PCR Master Mix (Applied Biosystems) using a GeneAmp 5700 Sequence Detection System (Applied Biosystems). Results were expressed as 2−ΔΔCT as previously described (30).

Western blotting analysis for STAT6 and JAK1

Western blotting to detect STAT6 or JAK1 protein expression was performed as described previously (11). Briefly, 20 μg of cell extract protein was loaded and separated by SDS-PAGE (4–10% gels). Rat STAT6 and Jak1 Abs (Santa Cruz Biotechnology, Santa Cruz, CA) were used as primary Abs, and immunostained bands were visualized with HRP-labeled anti-mouse or anti-rabbit IgG (Amersham Biosciences, Buckinghamshire, U.K.) as appropriate, followed by exposure to ECL Hyperfilm (Amersham Biosciences). The film was then scanned and quantitated using 1D Image Analysis software (Kodak, Rochester, NY).

Transfection of STAT6 and antisense oligonucleotides

PKroytic expression plasmid pusbk-STAT6 was a kind gift of Dr. J. N. Ihle (St. Jude Children’s Research Hospital, Memphis, TN). STAT6 cDNA was digested with EcoRI and Norf from pBSK-STAT6, and then subcloned into pEGFP-C1 (BD Clontech, Palo Alto, CA) using T4 DNA ligase (Promega, Madison, WI) in accordance with the manufacturer’s instructions to form mammalian expression plasmid pEGFP-STAT6. The identity of the construct was confirmed by sequencing.

Where indicated, AECs were transiently transfected with pEGFP-STAT6 plasmid. Test plates were incubated in 6-well plates for 24 h and the next day transfected cells were incubated with 25 μg of STAT6 antisense oligonucleotides or control (BD Biosciences). At 24 h after transfection, 10 μg/ml IL-4 or IL-13 were added and incubation was continued for an additional 4 h before harvesting for RNA purification. Empty plasmid pEGFP-C1 was also transfected under identical conditions and used as negative control. Where indicated, transfected cells were also transfected with antisense STAT6 phosphorothioate-derivated oligonucleotides (5′CCCCACAGAGACATGATCTG3′) at a final concentration...
of 500 nM to study the effects of specific inhibition of induced FIZZ1 expression. The corresponding sense oligonucleotide was used as control.

**Hydroxyproline assay**

Lung collagen deposition was estimated by measuring the hydroxyproline content of whole lung homogenates as previously described (17). Briefly, the lungs were excised, homogenized in 0.5 M acetic acid, and hydrolyzed in 6 N HCl overnight at 110°C. Hydroxyproline was assessed by colorimetric assay and the results were expressed as micrograms of hydroxyproline per lung.

**Statistic analysis**

All data were expressed as mean ± SE unless otherwise indicated. Differences between means of various treatment and control groups were assessed for statistical significance by ANOVA followed by post hoc analysis using Scheffe’s test for comparison between any two groups. A p value < 0.05 was considered to indicate statistical significance.

**Results**

**Effects of IL-4 and IL-13 on FIZZ1 expression in AECs**

BLM-induced lung injury in rats is known to induce FIZZ1 gene expression, which is localized to airway epithelial cells and AECs, but not to fibroblasts (11, 12). To seek out a possible mechanism for the regulation of FIZZ1 gene expression, a number of cytokines were investigated for their ability to regulate FIZZ1 gene expression in isolated AECs using RT-PCR. Initial screening showed that FIZZ1 expression was not significantly affected by treatment with a number of cytokines, including IL-1, TNF-α, IFN-γ, and IL-12 (data not shown). However, FIZZ1 gene expression was significantly up-regulated in a dose-dependent manner by the Th2 cytokines, IL-4 and IL-13 (Fig. 1A). Stimulation was not significantly enhanced beyond 10 ng/ml for either cytokine at the 4-h time point (Fig. 1A). Stimulation by IL-4 was rapid with detectable increase as early as 2 h and peak increase at 4 h after addition of cytokine (Fig. 1B). Significantly stimulated levels were sustained up to as long as 20 h of treatment. Similar kinetics was noted with IL-13 treatment (data not shown). These results clearly indicated the ability of both IL-4 and IL-13 to significantly stimulate FIZZ1 gene expression by AECs in a dose- and time-dependent manner.

**Effects of IL-4 and/or IL-13 deficiency on lung FIZZ1 gene expression and fibrosis**

To confirm the in vivo significance of the in vitro observation using isolated AECs, the effects of IL-4 and/or IL-13 deficiency on lung FIZZ1 gene expression and fibrosis were examined using the BLM model. For these studies, the responses of IL-4−/−, IL-13−/−, and IL-4 and IL-13 doubly deficient (IL-4/13−/−) mice to BLM-induced lung injury were compared with those in their respective wild-type controls. In the wild-type strain, lung FIZZ1 mRNA was significantly increased over 6-fold that in saline-treated control lungs at the day-14 (after BLM administration) time point (Fig. 2A). This increase was significantly reduced to <3-fold in IL-4−/− mice. Thus, IL-4 deficiency resulted in a >50% decrease in BLM-induced stimulation of lung FIZZ1 mRNA levels. A slight decrease in lung FIZZ1 mRNA was also observed in saline-injected IL-4−/− mice in comparison with IL-4+/− saline controls. Similar reductions in BLM-induced stimulation of lung FIZZ1 mRNA levels were noted in IL-13−/− mice when compared with their respective wild-type controls (Fig. 2B). Remarkably, this BLM-induced increase in lung FIZZ1 mRNA levels was completely abolished in the doubly deficient IL-4/13−/− mice. Thus, the BLM-induced stimulation of lung FIZZ1 expression appeared to be dependent entirely on IL-4 and IL-13, which would confirm the in vivo significance of the in vitro data obtained above using isolated AECs in tissue culture. It appears that in the absence of one of these Th2 cytokines, stimulation of FIZZ1 expression could be maintained partially by the unaffected cytokine.

Previously, IL-4 deficiency was shown to cause reduced BLM-induced lung fibrosis at a dose of BLM used in this study (17). Thus, reduced lung FIZZ1 expression in IL-4−/− mice appears to correlate with reduced lung fibrosis. To examine whether such a link also existed between lung FIZZ1 expression and BLM-induced lung fibrosis in IL-13-deficient mice or IL-4/IL-13 doubly deficient mice, the amplitude of lung fibrosis was compared in IL-13−/− and IL-4/13−/− mice to their respective wild-type control mice. Analysis of lung hydroxyproline at day 21 after BLM administration showed significant reduction in both IL-13−/− and IL-4/13−/− mice, relative to that in wild-type controls (Fig. 3). Thus, similar to that in IL-4 deficiency, decreased levels of BLM-induced increase in lung FIZZ1 expression in IL-13-deficient or IL-4/IL-13 doubly deficient mice were associated with significant reduction in pulmonary fibrosis.

![FIGURE 1. Effects of IL-4 or IL-13 on FIZZ1 expression in AECs. AECs were treated with the indicated concentrations of IL-4 or IL-13, and then harvested for total cell RNA to analyze for FIZZ1 mRNA by RT-PCR. The amount of FIZZ1 mRNA was normalized to GAPDH signals and expressed as 2^{-△△CT}, using the untreated control sample as reference. Mean ± SE of triplicate samples are shown. A dose-response curve is shown in A, which revealed significant increases (p < 0.01) in FIZZ1 mRNA levels in cells treated with 10 and 20 ng/ml either cytokine. B, The kinetics of IL-4 stimulation of FIZZ1 expression is shown. Stimulation by IL-4 was statistically significant (p < 0.001) at 4, 8, and 20 h after treatment. The same dose of IL-13 showed similar kinetics of stimulation (data not shown).](http://www.jimmunol.org/Download)
FIZZ1 mRNA levels, but the increase was significantly less (p < 0.001) in both IL-13-deficient (IL-13 ko) and IL-4/IL-13 doubly deficient (IL-4/IL-13 ko) mice. The levels of lung hydroxyproline in both BLM-treated knockout murine strains were not significantly different from their respective saline-treated controls. For each murine strain, mean ± SE of five animals per treatment group are shown.

Effects of transduced STAT-6 overexpression on FIZZ1 expression in AECs

To confirm the importance of STAT6 in induction of FIZZ1 expression, the effect of transfecting a STAT6 expression plasmid, pEGFP-STAT6, on AEC FIZZ1 expression was examined. Transfection of pEGFP-STAT6 caused a marked (>16-fold over empty vector only) increase in cellular STAT6 mRNA levels by RT-PCR analysis (Fig. 5A). IL-4 treatment caused an increase in STAT6 mRNA levels in control cells treated with the empty vector, but failed to affect the expression in cells transfected with pEGFP-STAT6. Thus, the transduced level of STAT-6 overexpression could not be stimulated further by IL-4 treatment. Examination of FIZZ1 expression by RT-PCR in similarly treated cells revealed significant stimulation of FIZZ1 expression by the STAT6 expression plasmid compared with untreated cells, or cells transfected with the empty vector (Fig. 5B). Furthermore, the increase induced by the STAT6 expression plasmid was abrogated by transfection with an antisense STAT6 oligonucleotide, indicating that the stimulation in FIZZ1 expression was specifically due to an increase in STAT6 expression in the pEGFP-STAT6-transfected cells. These results strongly suggest that IL-4 stimulation of AEC FIZZ1 expression was mediated by STAT6.

Role of STAT6 in regulation of FIZZ1 expression in vivo

To explore the potential in vivo relevance of these in vitro effects of STAT6 on FIZZ1 expression, the levels of STAT6 mRNA were determined in the lung tissue of mice treated with BLM. As previously noted, lung FIZZ1 expression was markedly induced in
alveolar epithelial cells (11, 12). This novel peptide mediator is found to stimulate type I collagen and 
pro-SMA expression in lung broblasts (33). Formation of these foci is thought to be due to cross-talk between alveolar epithelial cells, a primary source of profibrotic cytokines, and underlying or adjacent proliferating fibroblastic cells. FIZZ1 is recently found to be highly induced in BLM-induced lung fibrosis, and primarily expressed by airway and alveolar epithelial cells (11, 12). This novel peptide mediator is found to stimulate type I collagen and α-SMA expression in lung fibroblasts, two key parameters of fibroblast activation and myofibroblast differentiation that are known to occur in lung fibrosis (11). These properties indicate that FIZZ1 might play a potent role in mediating the cross-talk between epithelial cells and fibroblastic cells that is postulated as being important in formation of fibroblastic foci (33). Given this potentially important role in the pathogenesis of pulmonary fibrosis, elucidation of the mechanisms responsible for the induction of lung FIZZ1 expression should contribute to further understanding of key processes involved in progressive fibrosis. In this study, we present evidence that IL-4 and IL-13 could induce FIZZ1 gene expression in AECs in vitro and in vivo during BLM-induced lung fibrosis, and that this induction was mediated by STAT6 with the possible involvement of JAK1.

Based on previous data showing that induction of lung FIZZ1 in BLM-induced lung fibrosis was mainly observed in AECs, but not in fibroblasts, a number of candidate cytokines were screened for their ability to regulate FIZZ1 expression in isolated AECs. Of the cytokines tested, only IL-4 and IL-13 had consistent and significant effects on FIZZ1 gene expression. Other cytokines such as IL-1, TNF-α, and IFN-γ, failed to induce FIZZ1 gene expression. This is consistent with previous reports of the inability of LPS, TNF-α, or IL-6 to significantly affect FIZZ1/RELM-α expression (34). The Th2 cytokines, IL-4 and IL-13, are known to be important in BLM-induced lung fibrogenesis (13, 16, 35, 36). IL-4 plays....

FIGURE 4. Effects of IL-4 or IL-13 on STAT6 and JAK1 expression. AECs were treated with buffer only (None) or 10 ng/ml IL-4 or IL-13, respectively. Cell extracts were harvested at the indicated time points for analysis of the indicated proteins by Western blotting. Equal amounts (20 μg) of protein were loaded for analysis. Both IL-4 and IL-13 caused phosphorylation of STAT6 at both time points examined. Total STAT6 levels were not affected by these cytokine treatments, while JAK-1 levels were increased by both cytokines. A representative blot from three independent experiments is shown.

FIGURE 5. Effect of forced STAT6 expression on AEC FIZZ1 expression. STAT6 expression plasmid pEGFP-STAT6 (pSTAT6) or empty vector pEGFP-C1 (Vector) was transfected into rat AECs. The cells were then stimulated for 4 h with 10 ng/ml IL-4. Total cell RNA was then harvested and analyzed for FIZZ1 mRNA level by RT-PCR, and the results were expressed as described in Fig. 1. Mean ± SE of triplicate samples are shown. A, STAT6 mRNA levels were examined to confirm successful transfection, which revealed over 16-fold (relative to empty vector) induction in cells transfected with pSTAT6, which was not further stimulated by treatment with IL-4. The untreated vector control was used as a reference. B, FIZZ1 mRNA levels were significantly (p < 0.001) increased in cells transfected with pSTAT6 relative to both untreated and vector-transfected controls. Cotransfection with antisense STAT6 oligonucleotides abolished this increase induced by pSTAT6 transfection (p < 0.05).
a pivotal role in the extension of pulmonary fibrosis by enabling and/or enhancing fibroblast activation and myofibroblast differentiation (17). IL-13 can activate TGF-β1 in vivo with mediation by a serine protease/plasmin and MMP-9-dependent mechanism (18). Markedly diminished eosinophil recruitment and airway remodeling were observed in IL-4/13 double-deficient mice (37). In our present and previous studies (17), IL-4 and IL-13 were also shown to be important for the development of BLM-induced lung fibrosis by hydroxyproline estimation. In this study, we show that IL-4 and IL-13 were able to up-regulate FIZZ1 expression in vitro in AECs in a dose-dependent manner. This in vitro effect appears to be important in vivo, as well as in the BLM-induced pulmonary fibrosis model. Thus, substantial reduction in lung FIZZ1 expression was noted in BLM-treated IL-4- or IL-13-deficient mice when compared with their respective wild-type controls. Furthermore, there was essentially complete suppression of BLM-induced lung FIZZ1 expression in IL-4/IL-13 doubly deficient mice, indicating that induction of FIZZ1 expression in this animal model was completely dependent on these two Th2 cytokines. This reduced FIZZ1 expression in the deficient mice was accompanied by significant reduction in pulmonary fibrosis as assessed by lung hydroxyproline content. Thus, an additional role for these two cytokines in fibrosis is the ability to up-regulate FIZZ1 expression, which could in turn promote fibroblast activation and myofibroblast differentiation, two key elements in fibroelastic foci formation.

The kinetics of cytokine-induced FIZZ1 expression in AECs indicated a rapid induction of FIZZ1 mRNA, starting at 2 h after addition of cytokine. This is consistent with IL-4 or IL-13 stimulation of FIZZ1 expression in alternatively activated peritoneal macrophages (4) and the BMnot cell line isolated from bone marrow of temperature-sensitive SV40 T-Ag transgenic mice (38). In the latter study, FIZZ1 mRNA is elevated starting as early as 1 h after IL-4 stimulation and increased stably for up to 24 h.

The involvement of JAK1 and STAT6 in IL-4- and IL-13-induced signaling pathways is well elucidated (39, 40). Upon IL-4 or IL-13 stimulation, JAK1 activation is followed by activation of STAT6 by tyrosine phosphorylation, leading to homodimerization and translocation of the protein into the nucleus. There, STAT6 homodimers can interact with promoters of IL-4 or IL-13 responsive genes to regulate gene expression. Indeed, a functional STAT6-binding element has been identified in the murine FIZZ1 promoter in studies using the BMnot cell line (38), which could cooperate with C/EBP, a transcription factor known to bind to the FIZZ3/resistin promoter and found to be sufficient for activation of this promoter (41). Consistent with these findings, our data indicated a similar regulatory role for STAT6 in rat AECs. First there is the evidence showing activation of STAT6 in cells treated with IL-4 or IL-13 that was associated with increased expression of JAK-1. Moreover, transfection of a STAT6 expression plasmid into rat AECs stimulated the expression of FIZZ1, which was inhibited by antisense STAT6 oligonucleotides. These findings confirmed that the observed effect on FIZZ1 was specifically due to mediation by STAT6. Furthermore, our findings revealed that STAT6 gene expression was markedly increased in BLM-induced lung fibrosis in a mouse model and its deficiency abolished lung FIZZ1 induction. One study recently showed that peribronchial inflammation was markedly diminished and fibrosis was also significantly reduced in STAT6-deficient mice in contrast to corresponding wild-type mice in a model of airway disease (42). Given the activity of FIZZ1 on myofibroblast differentiation, the STAT6 mediated up-regulation in FIZZ1 expression would be expected to promote fibrosis. Thus, taken together, these observations strongly suggest that STAT6 may be involved in FIZZ1 induction in lung fibrosis and remodeling.

Finally, the relevance of these findings to human pulmonary fibrosis remains to be elucidated. Establishing such relevance is handicapped by the lack of information concerning a human homologue of rodent FIZZ1 (2). Although the human counterparts for FIZZ2 and FIZZ3 have been reported and analyzed, human FIZZ1 has yet to be identified. Recently, two additional members of this family of molecules have been reported, namely murine RELM-γ (7, 43) and human/murine 10 cysteine protein 1 (44). Although RELM-γ shows extensive homology to FIZZ1, its human counterpart is unknown. As with RELM-γ, the in vivo function of 10 cysteine protein 1 is uncertain, and its potential relationship to a human FIZZ1 remains to be determined. Thus, it is unclear at this time whether a human FIZZ1 homologue exists, and/or whether these additional novel members have similar function as rodent FIZZ1. Elucidation of the in vivo role of FIZZ1 in

FIGURE 6. STAT6 expression and effect on FIZZ1 expression in BLM-induced lung fibrosis. A, Lung tissue total RNA was isolated from wild-type mice 14 days after intratracheal BLM instillation for STAT6 mRNA analysis by RT-PCR. Similarly, in B, lung RNA was isolated from wild-type (WT) or STAT6 knockout mice (STAT6-KO) 7 days after BLM treatment, but analyzed for FIZZ1 mRNA by RT-PCR. The results were expressed as described in Fig. 1. The differences in STAT6 (A) as well as FIZZ1 (B) expression between BLM- and saline-treated wild-type mice were significant ($p < 0.05$), but FIZZ1 expression was not significantly increased in BLM-treated STAT6 knockout mice (B). Mean ± SE of five BLM-treated and three saline-treated control animals are shown.
human disease must await identification of a human FIZZ1 and/or further functional characterization of the various members of this gene family.

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