

Recombinant basic fibroblast growth factor inhibits the airway hyperresponsiveness, mucus production, and lung inflammation induced by an allergen challenge

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Background: IL-13 is believed to be a central mediator of asthma, and TGF- β 1 is a key downstream mediator in the development of IL-13-mediated asthma phenotypes.

Objective: To evaluate the biological roles of basic fibroblast growth factor (FGF2) in phenotype expression in transgenic (TG) mice overexpressing lung-specific TGF- β 1, and the therapeutic effects of recombinant FGF2 in the development of asthma phenotypes.

Methods: To evaluate the roles of FGF2 in airway hyperresponsiveness (AHR) expression induced by high levels of TGF- β 1, TGF- β 1 TG (+) mice were bred with FGF2-deficient mice. To evaluate the therapeutic effects of recombinant FGF2 (rFGF2) in the development of asthma, mice were given 10 μ g of rFGF2 subcutaneously once a day, 1 hour before the allergen challenge in an asthma mouse model. AHR was evaluated using noninvasive whole-body plethysmography, mucus production by diastase-resistant periodic acid Schiff (DPAS) staining, and lung inflammation using bronchoalveolar lavage (BAL) cellularity and lung histology.

Results: AHR decreased in TGF- β 1 TG (+) mice and was accompanied by the upregulation of FGF2 mRNA expression in lung tissues, when compared with littermate wild-type control mice. Interestingly, AHR was enhanced markedly in TGF- β 1 (+) mice with homozygous FGF2 gene disruption. In an asthma

mouse model, AHR, mucus production, and lung inflammation were inhibited markedly by rFGF2 treatment. This inhibition was accompanied by downregulation of the allergen-induced proliferation of T cells from regional lymph nodes.

Conclusion: FGF2 seems to be a key inhibitor in the development of AHR, and rFGF2 treatment constrains the development of asthma phenotypes. (J Allergy Clin Immunol 2006;117:1233-1241.)

Key words: Asthma, airway hyperresponsiveness, FGF2, mucus production

Asthma is a chronic inflammatory disorder of the airways that is associated with airway hyperresponsiveness (AHR) to nonspecific stimuli, mucus production, and alterations in the structures and functions of the elements involved, for example, fibroblasts and smooth muscle cells.¹ IL-13 is a pleiotropic cytokine produced in large quantities by activated T_H2 lymphocytes, and it is a key cytokine in the pathogenesis of asthma.^{2,3} The chronic inflammatory process in asthma is followed by healing, which results in an altered airway structure, a process referred to as airway remodeling.⁴ It is generally accepted that the modulation of fibroblastic cells toward the myofibroblastic phenotype, in combination with the acquisition of specialized contractile features and the enhanced production of extracellular matrix proteins, is essential for connective-tissue remodeling during wound healing in asthma patients.^{1,5} TGF- β 1 is a key participant in wound healing and in the fibroblast to myofibroblast phenotypic change.⁶ Myofibroblasts are believed to participate in lung fibrosis in asthma and may be more active functionally in terms of producing collagen than resting fibroblasts.⁷ This theory is strongly supported by the finding that transgenic mice overexpressing IL-13 develop subepithelial and lung parenchymal fibrosis mainly via TGF- β 1-dependent pathways.⁸ However, the effect of airway remodeling induced by TGF- β 1 on the expression of AHR is wholly unknown.

During lung development, the local production of cytokines and growth factors by epithelial and mesenchymal cells mediates branching morphogenesis and effectively

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Abbreviations used

AHR:	Airway hyperresponsiveness
BAL:	Bronchoalveolar lavage
dox:	Doxycycline
DPAS:	Diastase-resistant periodic acid Schiff
FGF2:	Basic fibroblast growth factor
L/N:	Lymph nodes
OVA:	LPS-depleted ovalbumin
PBS:	Phosphate-buffered saline
Penh:	Enhanced pause
rFGF2:	Recombinant basic fibroblast growth factor
TG:	Transgenic
WT:	Wild type

creates an epithelial–mesenchymal trophic unit.⁵ Interactions between the fibroblast growth factor (FGF) family and their receptors mediate mesenchymal–epithelial cell interactions during lung branching morphogenesis (lung modeling).⁹ Basic FGF (FGF2) is a member of the large FGF family and plays roles in the proliferation of vascular endothelial and smooth muscle cells during angiogenesis, the migration and proliferation of fibroblasts, the reversal of myofibroblast phenotypes, and the migration and proliferation of airway epithelial cells during wound healing.^{10,11} FGF2 mRNA expression is very low in the fetal lung, but relatively high postnatally, predominantly in the epithelial cells of large airways expressing FGFR1, 2, and 4 and in alveolar cells expressing FGFR2, 3, and 4.¹² However, the role of FGF2 in the pathogenesis of asthma is not known, although FGF2 levels are thought to be elevated in patients with asthma.¹³

Based on the idea that FGF2 plays a key role in the development of asthma phenotypes, we evaluated the biological effects of FGF2 on AHR in transgenic (TG) mice overexpressing lung-specific TGF- β 1. In addition, we evaluated the therapeutic effects of recombinant FGF2 in the development of asthma phenotypes in an asthma mouse model.

METHODS

Mice and recombinant FGF2

The generation and breeding of TG (+) mice overexpressing lung-specific TGF- β 1 and their littermate wild-type (WT) control (C57BL/6 background) mice and the administration of water containing doxycycline (dox) were performed under the specific pathogen-free conditions as previously described.¹⁴ FGF2-deficient mice (mixed background) were purchased from the Jackson Laboratory (Bar Harbor, Maine), and their genetic background was changed to a C57BL/6 background by back-crossing with C57BL/6 background WT mice. Briefly, FGF2-deficient mice were cross-bred with WT mice, and their offsprings were then genotyped [PCR primers in the Jackson laboratory homepage (<http://jaxmice.jax.org/strain>)] and identified homozygous FGF2 null mutant (–/–) mice. The back-crossing FGF2^{–/–} mice with WT mice was repeated 10 times. Lung-specific TGF- β 1 overexpressing TG (+) and FGF2-deficient mice (C57BL/6 background) were then cross-bred to obtain TGF- β 1 (–)/FGF2^{+/+}, TGF- β 1 (+)/FGF2^{+/+}, TGF- β 1 (–)/FGF2^{–/–}, and TGF- β 1 (+)/FGF2^{–/–}.

WT mice (BALB/c background) for the asthma model were also purchased from the Jackson Laboratory. Recombinant FGF2 (rFGF2) was generously donated by the Donga Pharmaceutical Company (Suwon, Korea).

Study protocol

The mRNA expression of candidate genes of downstream molecules in 2 TGF- β 1 TG (+) mice lines and their littermate WT controls was assessed 7 days after dox administration. Methacholine AHR was measured in TGF- β 1 TG (+) mice and their littermate WT controls 7 and 14 30 days after dox administration. In addition, methacholine AHR in TGF- β 1 (+)/FGF2^{–/–} mice and their littermate TGF- β 1 (–)/FGF2^{+/+}, TGF- β 1 (+)/FGF2^{+/+}, and TGF- β 1 (–)/FGF2^{–/–} mice was measured 7 and 14 days after dox administration.

As an allergen, LPS-depleted ovalbumin (OVA) was prepared as described previously.¹⁵ To generate an asthma mouse model, 6-week-old BALB/c WT mice were sensitized intraperitoneally twice with 75 μ g of OVA plus 2 mg of alum on days 1 and 7, and then challenged intranasally with 50 μ g of OVA on days 14, 15, 21, and 22. As a negative control group, same-aged WT mice were sensitized intraperitoneally and then challenged intranasally with phosphate-buffered saline (PBS) on the same days. To evaluate the therapeutic effects of rFGF2, 1, 10, and 20 μ g of rFGF2 were injected subcutaneously once a day 1 hour before the allergen challenge on days 14, 15, 21, and 22. As a negative control, PBS was injected in the same manner. This animal study protocol was approved by the IACUC of the Seoul National University (Seoul, Korea).

Measuring methacholine AHR

Pulmonary function testing was assessed in conscious, unrestrained mice using noninvasive whole-body plethysmography (Allmedicus, Seoul, Korea). Measurements were made as described previously.¹⁶ Briefly, mice were placed in the plethysmograph chamber and exposed to an aerosol of PBS (basal readings) and then aerosol doses of methacholine at 6.25, 12.5, 25, and 50 mg/mL. The aerosol was generated with an ultrasonic nebulizer and drawn through the chamber for 3 minutes. Enhanced pause (Penh) readings were taken for 3 minutes and averaged. A direct correlation exists between Penh and airway resistance in response to methacholine challenge.¹⁷

Evaluating lung inflammation and mucus production

Lung and bronchoalveolar lavage (BAL) samples were obtained as described previously.¹⁸ Briefly, the mice were anesthetized, and the trachea was isolated by blunt dissection. A small-caliber tube was inserted and secured in the airway. Two successive volumes of 0.75 mL PBS were instilled and aspirated gently, and these 2 volumes were pooled. Each BAL sample was centrifuged, and the supernatants were stored at –70°C until use. The total numbers of inflammatory cells were counted after dilution of cell pellets with 50 μ L of PBS. After Diff-quick staining (Dade Behring, Dudingon, Switzerland) of BAL pellets in a cytospin preparation, the types of inflammatory cells were determined by counting 300 cells, which were classified as macrophages, lymphocytes, neutrophils, or eosinophils. The lung was perfused with cold PBS through the right ventricle until the pulmonary vasculature was clean. Then, the entire lung was inflated with fixatives for histology. Hematoxylin and eosin and diastase-resistant periodic acid Schiff (DPAS) stains were used on lung sections after pressure fixation with Streck solution (Streck Laboratories, La Vista, Nebr). The same microscopic magnification was used in all sample slides for comparison. Lung inflammation was assessed by the degree of peribronchiol and perivascular inflammation, which were evaluated on a subjective scale of 0 to 3 as previously described.¹⁹ Briefly, a value of 1 was assigned when no

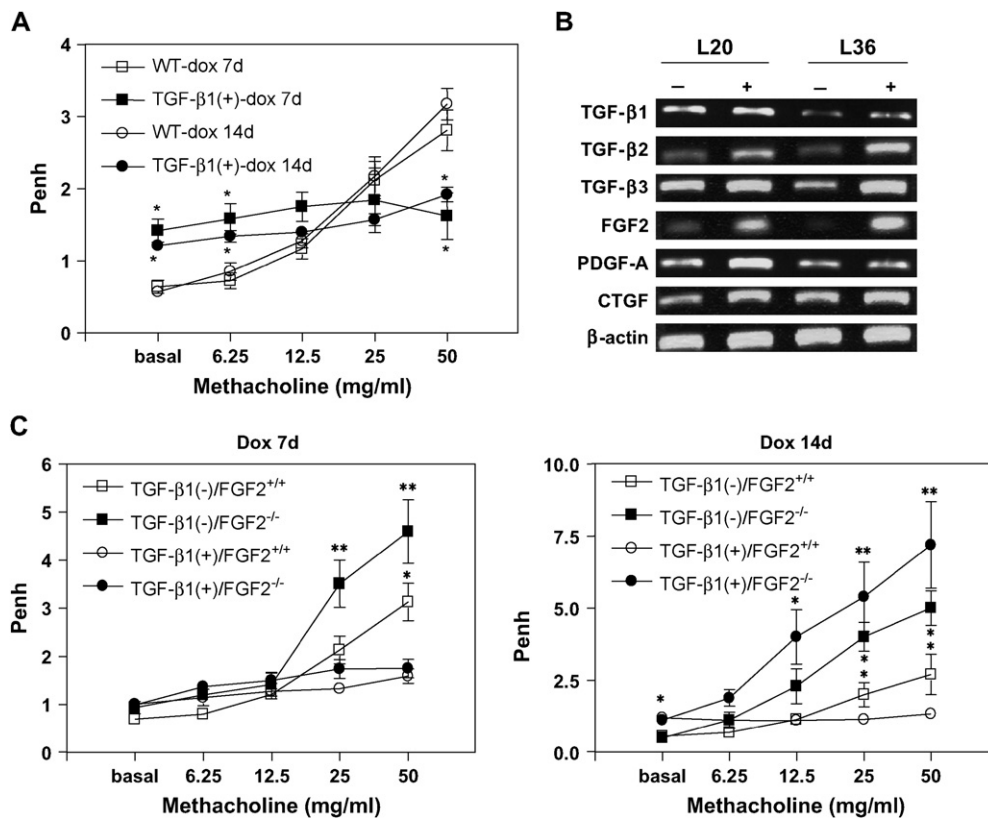


FIG 1. AHR kinetics in lung-specific TGF-β1 TG (+) and FGF2-deficient mice after administering water containing doxycycline. **A** and **C** show a representative experiment out of 5 replicates. **A**, Methacholine AHR was lower in TGF-β1 (+) mice than in littermate WT controls 7 and 14 days after dox administration. **P* < .05 compared with WT. **B**, Enhanced FGF2 mRNA expression in the TGF-β1 TG (+) lungs of different lines (Lines 20 and 36). **C**, Enhanced methacholine AHR in TGF-β1 (+) mice with homozygous disruption of the FGF2 gene 14 days after dox administration. **P* < .05 compared with TGF-β1 (+)/FGF2^{+/+}; ***P* < .05 compared with the other groups.

inflammation was detectable, a value 1 for occasional cuffing with inflammatory cells, a value of 2 when most bronchi or vessels were surrounded by a thin layer (1 to 5 cells) of inflammatory cells, and a value of 3 when most bronchi or vessels were surrounded by a thick layer (more than 5 cells deep) of inflammatory cells. As for the evaluation of mucus production, the percentage of cells staining positive for mucin was enumerated in the small airway. To semiquantify mucin-positive cells, each lung tissue was categorized according to scale as grade 0 = 0–25% positive cells; grade 1 = 25–50%; grade 2 = 50–75%; and grade 3 = 75–100% of each bronchiolar epithelial area, and this was counted and summarized from 5 bronchiolar areas.

Evaluating immunologic parameters

BAL protein quantification was performed using an ELISA kit according to manufacturer's protocol (R&D systems, Minneapolis, Minn). As for RT-PCR from lung tissues, the gene-specific primers used for RT-PCR were as follows: TGF-β1, CTACTACGCCAAGG AGGTCAC and TTGCTGAGGTATCGCCAGGAA; TGF-β2, GAT GCCATCCCGCCCACTTTC and TCCCAGGTTCTGTCTTTA TG; TGF-β3, CACCCAGGAAAACACCGAGTC and CTCATTG TCCCACGCCTTTGAA; FGF2, GCCAGCGGCATCACCTCGCT and TATGGCCTTCTGTCCAGGTCCCGT; PDGF-A, CCTGCCC ATTCCGAGGAAGAG and TTGGCCACCTTGACGCTGCG; CTGF, CTAAGACCTGTGGAATGGGC and CTCAAAGATGTC ATTGCCCCC; fibromodulin, AACCATCACACACACACAC and AGCATTTGACCTTCTCTCC; LTBP-2, GGACGCGGAGT

GTGTGAATACC and GGGGTAGCAGAAGCAGCAGTAGG; and β-actin, TGGAATCCTGTGGCATCCATGAAAC and TAAA ACGCAGCTCAGTAACAGTCCG.

T cells from regional lymph nodes (L/N) were subject to the cell proliferation assay with and without OVA incubation. Briefly, L/N cells were harvested in RPMI 1640 media (GIBCO BRL, Gaithersburg, Md) and incubated (2×10^5 cells/well) in 96-well flat-bottom plates at 37°C for 96 hours in medium alone or with 300 μg OVA/mL (60 μg/well). We assessed the cellular thymidine incorporation after 18 hours of culture with 1 μCi [^{3} H]thymidine.

Statistics

Significant differences among groups were assessed using the Student *t* test, analysis of variance, or the Wilcoxon rank-sum test. For multiple comparisons, analysis of variance was used initially, and if significant differences were found, individual *t* tests or the Wilcoxon rank-sum tests between pairs of groups were used.

RESULTS

High TGF-β1 levels in lung induce decreased airway responses to methacholine mainly via the FGF2-dependent pathway

IL-13-induced airway remodeling, such as subepithelial fibrosis, is mainly dependent on TGF-β1-mediated

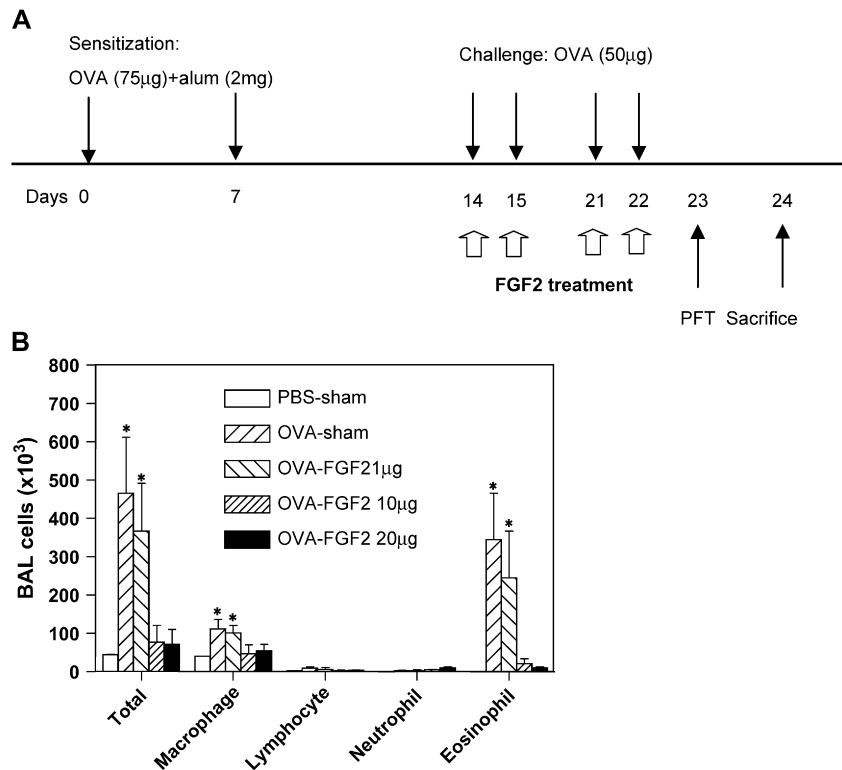


FIG 2. Study protocol and determination of an optimal dose of rFGF2. **A**, Study protocol. **B**, BAL cellularity in asthma mice treated with different doses of rFGF2 ($n = 5$ each group): BAL cellularity was significantly decreased in asthma mice treated with 10 or 20 μg of rFGF2, but not with 1 μg of rFGF2, than in asthma mice treated with sham.

pathways.⁸ Previous data also showed that lung-specific TGF- β 1 TG (+) mice nicely demonstrated the biological functions of TGF- β 1 in airway remodeling, especially subepithelial and lung parenchymal fibrosis.²⁰ Here we show that methacholine AHR was decreased significantly in TGF- β 1 TG (+) mice 7 and 14 days after dox administration compared with littermate WT controls (Fig 1, A).

Many gene families are regulated by TGF- β 1 in lung-specific TGF- β 1 (+) mice, as determined by changes in mRNA expression using a GeneChip (Affymetrix, Santa Clara, Calif) analysis (data not shown). Of those genes regulated by TGF- β 1, FGF2 was over-expressed in the 2 lines of TGF- β 1 TG (+) mice 7 days after dox administration compared with littermate WT controls (Fig 1, B). Here, we evaluated the role of FGF2 in the maintenance of decreased airway responses to methacholine induced by high TGF- β 1 levels. To test this, we cross-bred FGF2-deficient mice with TGF- β 1 TG (+) mice. Our data showed that the methacholine AHR was similar in TGF- β 1 (+)/FGF2^{-/-} and TGF- β 1 (+)/FGF2^{+/-} mice 7 days after dox administration (Fig 1, C). However, the AHR was markedly more enhanced in TGF- β 1 (+)/FGF2^{-/-} mice 14 days after dox administration compared with TGF- β 1 (+)/FGF2^{+/-} mice (Fig 1, C).

rFGF2 treatment inhibits AHR, mucus production, and lung inflammation in an asthma mouse model

To evaluate the therapeutic effects of rFGF2, rFGF2 (1, 10, and 20 μg) was injected subcutaneously during the allergen challenge in an asthma mouse model (Fig 2, A). As shown in Fig 2, B, BAL cellularity was significantly lower in asthma mice treated with 10 or 20 μg of rFGF2 than in asthma mice treated with sham or 1 μg of rFGF2. From this finding, we injected subcutaneously 10 μg of rFGF2 in an asthma mouse model to further evaluate therapeutic and immunologic effects of rFGF2. We demonstrated that the methacholine AHR was significantly inhibited in OVA-challenged WT BALB/c mice treated with rFGF2 (10 μg) compared with OVA-challenged mice treated with PBS (Fig 3, A). Moreover, the inflammatory cell counts in BAL fluids decreased significantly in OVA-challenged mice treated with rFGF2 compared with OVA-challenged mice treated with PBS (Fig 3, B). Lung histology showed that lung inflammation disappeared in OVA-challenged mice treated with rFGF2 (Fig 3, C and D). Moreover, DPAS staining of lung tissues indicated that mucus production was also completely inhibited in the former group compared with the latter group (Fig 3, C and E).

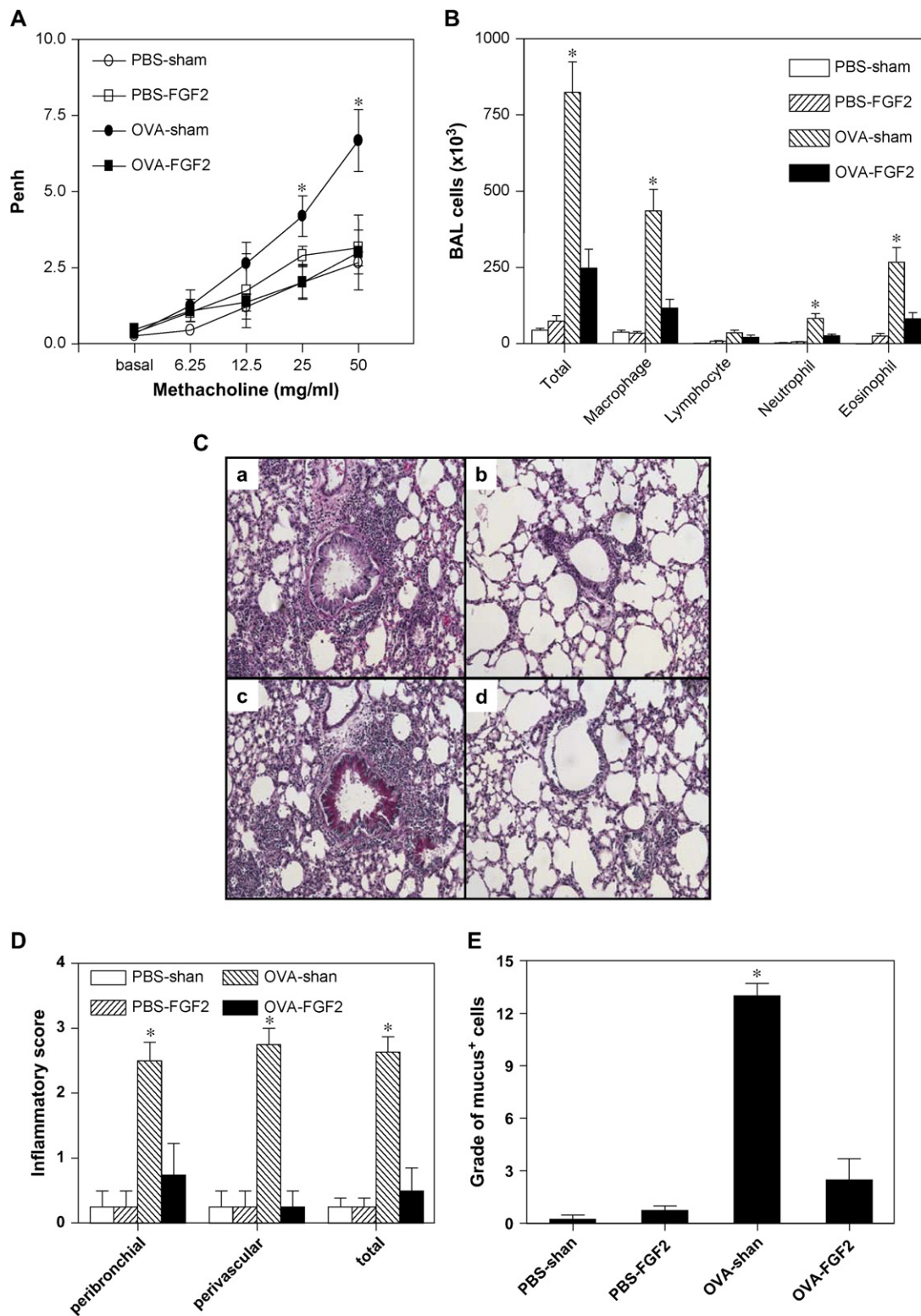


FIG 3. Therapeutic effects of recombinant FGF2 (10 μ g) in an asthma mouse model ($n = 5$ each group). * $P < .05$ compared with the other groups. **A**, Methacholine AHR. **B**, BAL cellularity. **C**, Lung histology. (**A** and **C**: OVA-challenged mice without FGF2 treatment; **B** and **D**: OVA-challenged mice treated with FGF2; **A** and **B**: Hematoxylin and eosin stain; **C** and **D**: DPAS stain, $\times 200$.) **D**, Inflammatory scores. **E**, Grades of mucus staining cells.

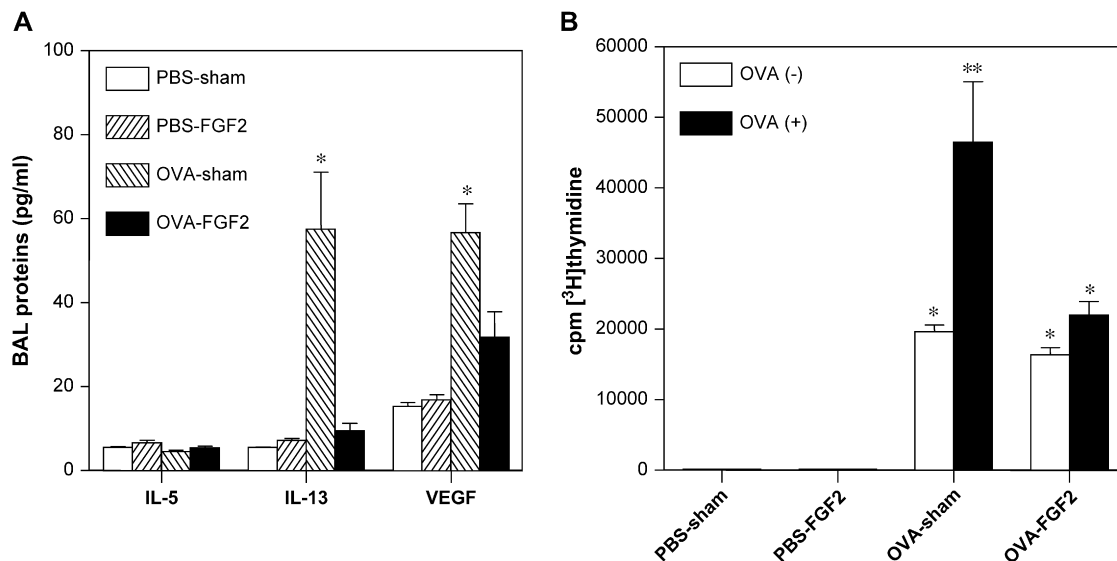


FIG 4. Immunomodulating effects of recombinant FGF2 in an asthma mouse model. **A** and **B** show a representative experiment out of 5 replicates. **A**, BAL cytokine levels: rFGF2 treatment significantly inhibited BAL IL-13 and VEGF production after OVA challenge. * $P < .05$ compared with the other groups. **B**, Proliferation assay of T cells derived from regional L/N. For OVA-challenged mice, OVA-induced T-cell proliferation was significantly inhibited in mice treated with rFGF2 compared with untreated mice. * $P < .05$ compared with PBS-challenged mice; ** $P < .05$ compared with the other groups.

rFGF2 treatment inhibits IL-13 production, which is associated with the downregulation of allergen-induced T-cell proliferation

Finally, we evaluated the immunomodulating effects of rFGF2 in an asthma mouse model. This study showed that BAL IL-13 and vascular endothelial growth factor (VEGF) levels were significantly inhibited in OVA-challenged mice treated with rFGF2 compared with OVA-challenged mice treated with PBS (Fig 4, A). The regional L/N-derived T-cell proliferation without OVA incubation was higher in OVA-challenged mice, regardless of rFGF2 treatment, than in PBS-challenged mice. In the OVA-challenged mice, however, T-cell proliferation with OVA incubation was significantly inhibited in mice treated with rFGF2 compared with those treated with PBS (Fig 3, B).

DISCUSSION

Airway hyperresponsiveness and airway inflammation and the resultant remodeling are the hallmarks of asthma.¹ Knowledge of the origin of AHR is central to understanding the origin of asthma, and subjects with or without asthma differ in terms of both the ease with which airway narrowing is produced by the inhalation of a nonspecific stimuli and their ability to demonstrate a maximal response to these agents.²¹ Airway remodeling in asthma refers to a collection of airway structural changes, including mucus production, subepithelial fibrosis, and myofibroblast hypertrophy and hyperplasia.²² However, the direct relationships between AHR and airway remodeling remain controversial.

Rather, the degree of AHR is suggested to be determined by enhancing phenotypes such as increased hyperplasia and contractility phenotypes, and by inhibiting phenotypes such as subepithelial and lung parenchymal fibrosis.²¹ In terms of the effects of high levels of TGF- β 1 on AHR expression, this study showed that AHR significantly decreased in lung-specific TGF- β 1 TG (+) mice after 7 days of dox administration compared with littermate WT control mice. A previous study found that airway structural changes, such as subepithelial myofibroblast hyperplasia and fibrosis, were induced in TGF- β 1 TG (+) mice 7 days after dox administration.²⁰ These findings suggest that airway remodeling induced by high levels of TGF- β 1 is negatively associated with AHR, possibly by shifting the determinants of AHR expression toward inhibitory phenotypes, such as subepithelial and lung parenchymal fibrosis.

Another study showed that lung-specific IL-13 TG (+) mice demonstrated AHR and the airway inflammation and remodeling that characterize asthma.¹⁷ In addition, significant VEGF production was induced in IL-13 TG (+) mice.¹⁸ Interestingly, our previous study showed that AHR significantly increased in lung-specific VEGF TG (+) mice compared with littermate WT controls.^{16,23} These findings suggest that AHR enhancement by IL-13 is mediated via pathways other than TGF- β 1, one of which involves VEGF-mediated effects.

FGF2 plays an important role in wound healing by promoting fibroblast migration and proliferation, reversing myofibroblast phenotypes, and regenerating airway epithelial cells.^{10,11} Recent *in vitro* evidence suggests that TGF- β 1 induces human bronchial smooth muscle proliferation via synergism with FGF2.²⁴ However, this study

showed that the airway responsiveness to methacholine was decreased by high levels of TGF- β 1, and that the decreased airway responsiveness is reversed by the homozygous null mutation of the FGF2 gene. Interestingly, previous data showed that the decreased airway responsiveness in TGF- β 1 TG (+) mice was accompanied by proliferation of subepithelial α -sm (+) cells and by lung fibrosis.^{20,25} These findings suggest that the decreased airway responsiveness in TGF- β 1 TG (+) mice may be determined by factors other than bronchial smooth muscle proliferation. Taken together, the decreased airway responsiveness induced by high levels of TGF- β 1 may be maintained by the presence of FGF2, possibly via reversal of contracting myofibroblast phenotypes.

As the AHR induced by high levels of TGF- β 1 was reversed by FGF2 deficiency, we hypothesized that rFGF2 treatment inhibits AHR induced by an allergen challenge. Indeed, this study showed that rFGF2 treatment significantly inhibited AHR enhancement by such a challenge. Moreover, we showed that rFGF2 treatment inhibits lung inflammation and mucus production induced by an allergen challenge. Interestingly, this inhibition of rFGF2 was accompanied by the downregulation of IL-13 production. With respect to the immunological pathogenesis of asthma, IL-13 is thought to be a central mediator in the development of asthma phenotypes in an asthma mouse model.^{2,3} This study showed that rFGF2 treatment inhibits the allergen-induced proliferation of T cells in regional L/Ns. These findings suggest that FGF2 has immunomodulating effects, one of which is downregulating IL-13 production from T cells.

In summary, this study demonstrated that FGF2 plays a critical role in the expression of AHR mediated by high levels of TGF- β 1, and that rFGF2 has therapeutic effects, inhibiting AHR, lung inflammation, and mucus production in an asthma mouse model.

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