Online data supplement

Supplemental Methods

Cell culture. Primary aSMCs were isolated from human bronchial biopsies. Tissues were cleaned manually and the muscular layer was further digested for 1 hour with collagenase II (1 mg/mL, Worthington Biochemical, Lakewood, NJ) at 37°C under agitation. Cells grew up in Dulbecco modified Eagle medium (Gibco; Invitrogen ThermoFisher Scientific, Waltham, Mass) containing 10% FBS, 100 units/mL penicillin, and 100 mg/mL streptomycin at 37°C and 5% CO₂. The culture medium was changed every 72 hours. All experiments were performed between passages 1 and 6.

Allergic asthma models. For acute allergic asthma model, mice were sensitized on days D0, D7, D14, and D21 by skin application of 500 µg Der f in 20 µL of dimethyl sulfoxide (Sigma) onto the ears. Control mice were sensitized with dimethyl sulfoxide. Intranasal challenges were performed with 250 µg of Der f in 40 µL of sterile PBS on D27 and D34. For severe allergic asthma model, mice were submitted to the same protocol but were intranasally challenged on days D26, D27, D28 and D33, D34, D35. When indicated, allergic asthma mice were treated by repeated inhalations of NSC23766 (40 µg/kg in 400 µL PBS), formoterol (125 µg/kg in 400 µL PBS) or beclomethasone (150 or 1500 µg/kg in 400 µL PBS) before each challenge. All mice were sacrificed 24 hours after last intranasal challenge for analysis.

Bronchoalveolar lavage (BAL) fluid analysis. Mice were tracheotomized and 1 mL of sterile PBS was administrated intratracheally through a catheter. Cells and supernatants from recovered fluid were separated by centrifugation. Total cell number was counted on Kova slides by optical microscopy. Identification of immune cell subpopulations was performed by
flow cytometry analysis. Acquisition was performed on LSR II (BD Bioscience) and analyzed
with FlowJo software.

Airways reactivity ex vivo. Murine primary bronchi were cleaned, cut in rings, and mounted
on a multichannel isometric myograph in Krebs-Henseleit physiological solution (118.4 mM
NaCl, 4.7 mM KCl, 2 mM CaCl$_2$, 1.2 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$, 25 mM NaHCO$_3$, and 11 mM
glucose) at 37°C and gassed with a mixture of 95%O$_2$/5%CO$_2$. A pretension of 0.5 mN was
applied. We constructed dose-response curves to methacholine (Sigma-Aldrich, Paris, France).

Histology. Paraformaldehyde (4% in PBS, 1 mL) was administered intratracheally in the lungs
through a flexible catheter, trachea was ligatured, and lungs were excised. Lungs were fixed
in 4% paraformaldehyde for 48 h and embedded into paraffin. Sections measuring 6 mm in
size were stained with periodic acid-Schiff or hematoxylin/eosin for morphological studies.

Histological grade (over 12 points) was determined to assess inflammation (0-8) and
pulmonary remodeling (0-4) as previously described.

Immunoblotting. Primary aSMCs were incubated on ice with lysis buffer supplemented with
protease and phosphatase inhibitor cocktails (Sigma Aldrich, Saint Quentin Fallavier, France)
and sodium orthovanadate. Lysates were subjected to SDS-PAGE, transferred to nitrocellulose
membranes, and incubated with specific antibodies: p-Akt (9271), Akt (9272), p-P44/42
(9101), P44/42 (4695), p-Pak (2605), Pak1 (2602), p-STAT3 (9131) and STAT3 (4904) antibodies
were from Cell Signaling Technology (Leiden, The Netherlands). Equal loading was checked by
reprobing of the membrane with an anti-tubulin antibody (Beckman Coulter; Villepinte,
France). Immune complexes were detected with appropriate secondary antibodies and
enhanced chemiluminescence reagent (Clarity ECL BioRad, Marnes la Coquette, France).

Protein band intensities were quantified using ImageJ Software (NIH software, Bethesda, Md).

Supplemental legends

Figure E1. aSMC proliferation is induced by bFGF and PDGFbb. (A) Representative images of aSMC proliferation by indicated factors. Nuclei are detected by DAPI staining (blue) and aSMC proliferation by EdU staining (green). Scale bar, 25 µm. (B) Proliferation of haSMC induced by bFGF, PDGFbb, IL-13, IL-33, IL-17, IL-9 and TSLP. Detection and quantification of haSMC proliferation by EdU staining. Results are expressed as the percentage of EdU positive cells (n=10). Data are presented as mean ± SEM. Kruskal-Wallis test followed by Dunns’ posttest were used. **P<0.01, ***P<0.001 vs control cells.

Figure E2. Activation of Akt and P44/42 in response to bFGF and PDGFbb. (A) Immunoblot analysis of Akt and P44/42 expression and phosphorylation in haSMCs stimulated with bFGF or PDGFbb at different time points. When indicated, EHT1864 was preincubated 30 min before stimulation. (B) Quantification of phosphorylation and expression of Akt and P44/42 (n=4-5). Data are expressed as mean ± SEM. Kruskal-Wallis test followed by Dunns’ posttest were used. *P<0.05, **P<0.01, ***P<0.001.

Figure E3. Effect of beclomethasone on pulmonary inflammatory cells infiltrate in an acute allergic asthma murine model. Infiltrating cells in BAL fluid from DP and HH mice from the acute allergic asthma protocol treated with beclomethasone (150 or 1500 µg/kg) or NaCl (n = 10 mice). Data are expressed as mean ± SEM. Kruskal-Wallis test followed by Dunns’ posttest were used. ***P<0.001 vs DP NaCl; $P<0.05 vs HH NaCl.
Figure E1

A

Control  bFGF  PDGFbb  IL13  IL33  IL17  IL9  TSLP

B

hASMC proliferation (%)

Control  bFGF  PDGFbb  IL13  IL33  IL17  IL9  TSLP

**  ***
Figure E2

A

bFGF (min) 0 5 15 30 0 5 15 30
p-Akt
Akt
p-P44/42
P44/42
EHT1864

PDGFbb (min) 0 5 30 60 0 5 30 60
p-Akt
Akt
p-P44/42
P44/42
EHT1864

B

P-Akt/total Akt
0 5 15 30
bFGF (min)
P-Akt/total P44/42
0 5 15 30

EHT1864

Ctrl

P-Akt/total Akt
0 5 15 30
PDGFbb (min)
P-Akt/total P44/42
0 5 30 60

EHT1864

Ctrl
Figure E3

BAL Cells (nb/mL)

- Lymphocytes
- Macrophages
- Eosinophils
- Neutrophils

- DP
- HH
- HH beclo 150
- HH beclo 1500

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