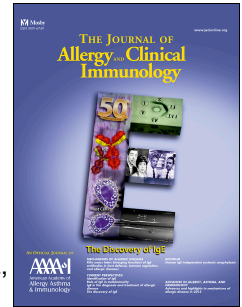


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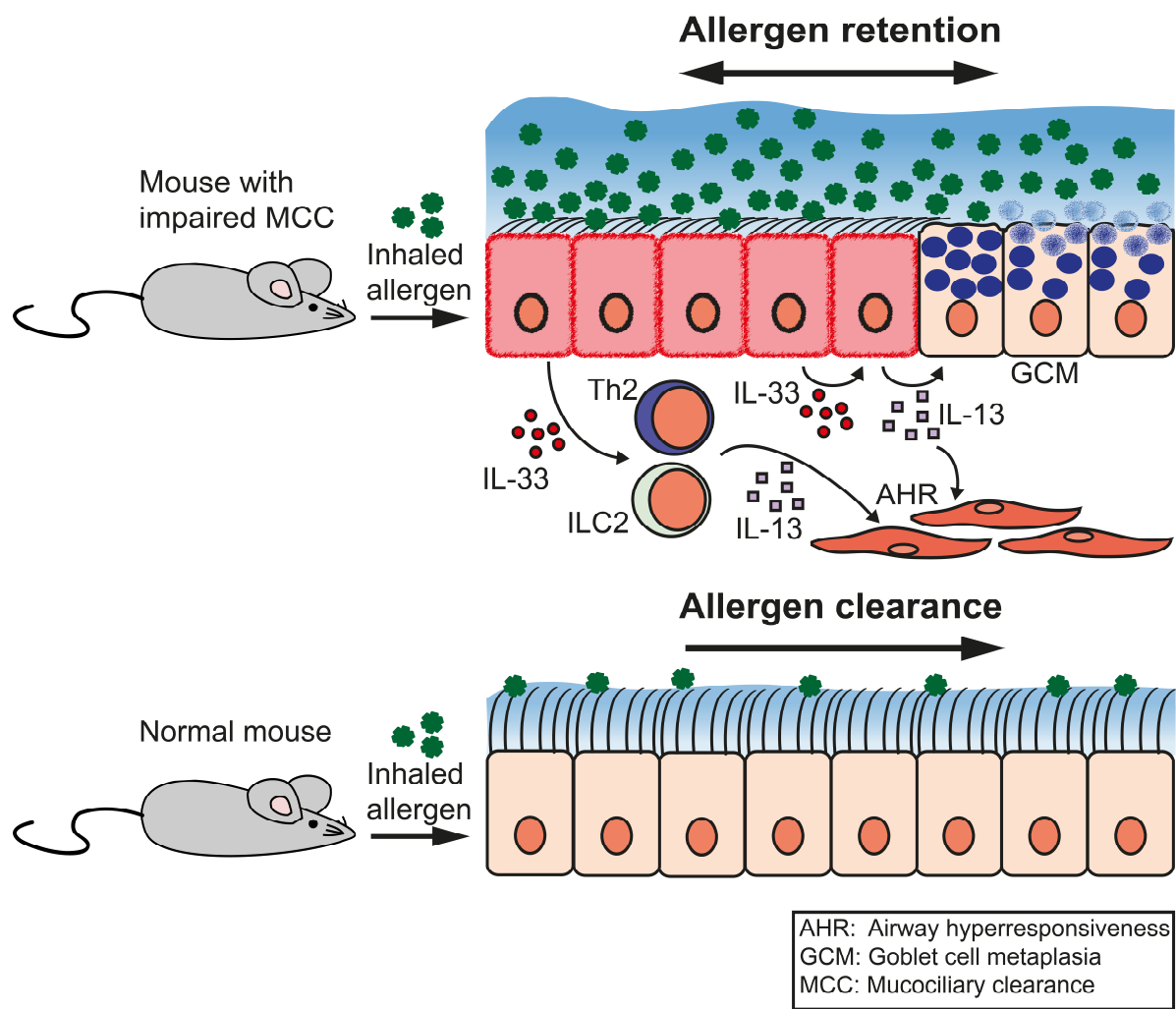
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Impaired mucus clearance exacerbates allergen-induced type 2 airway inflammation in juvenile mice

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Disclosure of potential conflict of interest: M. A. Mall is listed on a patent application filed by the University of North Carolina at Chapel Hill describing the *Scnn1b*-Tg mouse. Of note, the *Scnn1b*-Tg mouse has been deposited at JAX for general disposition. The rest of the authors declared that they have no relevant conflicts of interest.

ABSTRACT

Background: Type 2 airway inflammation plays a central role in the pathogenesis of allergen-induced asthma, but the underlying mechanisms remain poorly understood. We recently demonstrated that reduced mucociliary clearance, a characteristic feature of asthma, produces spontaneous type 2 airway inflammation in juvenile *Scnn1b*-transgenic (*Scnn1b*-Tg) mice.

Objective: To determine the role of impaired mucus clearance in the pathogenesis of allergen-induced type 2 airway inflammation and identify cellular sources of the signature cytokine IL-13.

Methods: We challenged juvenile *Scnn1b*-Tg and wild-type mice with *Aspergillus fumigatus* and house dust mite allergen and compared effects on airway eosinophilia, type 2 cytokine levels, goblet cell metaplasia and airway hyperresponsiveness. Further, we determined cellular sources of IL-13 and effects of genetic deletion of the key type 2 signal transducing molecule STAT6, and evaluated effects of therapeutic improvement of mucus clearance.

Results: Reduced mucociliary allergen clearance exacerbated *Stat6*-dependent secretion of type 2 cytokines, airway eosinophilia and airway hyperresponsiveness in juvenile *Scnn1b*-Tg mice. IL-13 was elevated in airway epithelial cells, macrophages, type 2 innate lymphoid cells and Th2 cells along with increased *Il33* in the airway epithelium of *Scnn1b*-Tg mice. Treatment with the epithelial Na^+ channel blocker amiloride improving airway surface hydration and mucus clearance reduced allergen-induced inflammation in *Scnn1b*-Tg.

Conclusion: Our data support that impaired clearance of inhaled allergen triggering IL-13 production by multiple cell types in the airways plays an important role in the pathogenesis of type 2 airway inflammation and suggest therapeutic improvement of mucociliary clearance as a novel treatment strategy for children with allergen-induced asthma.

Key Messages

- Impaired mucus clearance causing reduced clearance of inhaled allergens is an important risk factor for allergen-induced type 2 airway inflammation in juvenile mice.
- The airway epithelium is a key player in sensing of allergens and transmission of signals to innate and adaptive immune cells that orchestrate the complex in vivo pathogenesis of type 2 inflammation.
- Therapeutic improvement of mucociliary clearance may be a novel treatment strategy for children with allergen-induced asthma.

Capsule summary: This study identifies impaired mucus clearance as a risk factor for allergen-induced type 2 airway inflammation in mice and suggests that pharmacological improvement of mucociliary clearance may be an effective treatment strategy for allergic asthma.

Keywords: Type 2 inflammation, airway epithelium, mucociliary clearance, IL-13, IL-33

Abbreviations used:

AAM, alternatively activated macrophages; *Actb*, gene encoding β -actin; Af, *Aspergillus fumigatus* extract; AHR, airway hyperresponsiveness; ALI, air-liquid interface; AM, bronchoalveolar macrophages; BAL, bronchoalveolar lavage; cDC, conventional DC; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; ENaC, epithelial Na^+ channel; GCM, goblet cell metaplasia; HDM, house dust mite, ILC-2, type 2 innate lymphoid cell; i.t., intratracheal(ly); MCC, mucociliary clearance;; *Scnn1b*, gene encoding the β -subunit of ENaC; Th2, type 2 helper T cell; WT, wild-type.

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INTRODUCTION

Type 2 airway inflammation characterized by the signature type 2 cytokine IL-13 plays a central role in the pathogenesis of asthma and has been associated with disease severity in other chronic inflammatory lung diseases such as chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) (1-6). Emerging evidence suggests that the airway epithelium is a central player in the orchestration of type 2 inflammation in response to inhaled allergens and other environmental stimuli (7-10), but the mechanistic links between epithelial (dys)function and allergic airway inflammation remain poorly understood. Previous studies demonstrated that asthma is associated with impaired mucociliary clearance (MCC) in patients (11-13) and longitudinal studies in *Scnn1b*-Tg mice showed that reduced MCC produces spontaneous type 2 airway inflammation with elevated IL-13, airway eosinophilia, mucus hypersecretion and signatures of alternative macrophage activation at juvenile ages that precedes chronic neutrophilic airway disease observed in adult *Scnn1b*-Tg mice (14-17). In *Scnn1b*-Tg mice impaired MCC is caused by airway surface dehydration (mucus hyperconcentration), an epithelial dysfunction that can either result from defects in epithelial ion/fluid transport as in CF (18, 19), or from mucin hypersecretion under conditions of chronic airway inflammation, and may therefore provide a mechanism underlying mucociliary dysfunction in a spectrum of obstructive lung diseases including asthma and chronic bronchitis (20-24). Because efficient MCC is an important innate defense mechanism that clears inhaled allergens and other noxious stimuli constantly from the lungs (11), and because direct activation of the epithelium by airborne allergens via pattern recognition receptors was shown to be sufficient to induce type 2 airway inflammation (9, 25), we hypothesized that reduced mucus clearance may slow the clearance of aeroallergens, which may trigger innate and/or adaptive immune responses that orchestrate type 2 airway inflammation in juvenile mice with a type 2 biased immune system (14, 26, 27). Second, based on previous studies demonstrating that pharmacological improvement of airway surface hydration by the

epithelial Na⁺ channel (ENaC) blocker amiloride, or by the osmotically active agent hypertonic saline, can enhance reduced MCC in patients with chronic airway diseases including asthma (28-30), we hypothesized that this approach may reduce allergen-induced type 2 inflammation in mucostatic airways in vivo.

To test this hypothesis, we challenged juvenile *Scnn1b*-Tg mice and WT littermates by intratracheal (i.t.) application of *Aspergillus fumigatus* (Af) and house dust mite (HDM) extracts as naturally occurring aeroallergens and compared allergen clearance and effects on type 2 airway inflammation, goblet cell metaplasia (GCM), mucin expression and airway hyperresponsiveness (AHR). To identify cellular sources of the signature type 2 cytokine IL-13 we performed immunohistochemistry, transcript analysis in micro-dissected native airway epithelia and highly differentiated primary tracheal epithelial cultures, and flow cytometry of innate and adaptive immune cells from lungs of naïve and Af-challenged juvenile *Scnn1b*-Tg and WT mice. Further, we determined the role of IL-13/STAT6 signaling in allergen-induced airway inflammation by genetic deletion of the key signal transducing molecule STAT6 in *Scnn1b*-Tg mice (31-33). Finally, we evaluated effects of pharmacological improvement of airway surface hydration and mucus transport by amiloride on allergen-induced airway inflammation and AHR in *Scnn1b*-Tg and WT mice.

METHODS

Further details on methods used in this study can be found in the Methods section in this article's Online Repository at www.jacionline.org.

Experimental animals

All animal studies were approved by the Regierungspräsidium Karlsruhe, Germany. *Scnn1b*-Tg mice were generated as previously described (18). *Stat6*^{-/-} mice (Jackson Laboratories, Bar Harbor, ME) (31) were intercrossed with *Scnn1b*-Tg mice to obtain *Scnn1b*-Tg/*Stat6*^{-/-}, *Scnn1b*-Tg, *Stat6*^{-/-} and WT littermates. Mice were studied at neonatal (10-day-old), juvenile (2 to 3-week-old) and adult (6-week-old) ages.

Allergen exposure

Juvenile mice were challenged with 20 µl and adult mice with 50 µl Af (2 mg/ml in NaCl 0.9%) (Hollister-Stier, Spokane, WA) every 48 h by i.t. instillation for a total of four times as previously described (34). Exposure to HDM (Greer laboratories, Lenoir, NC) was started by i.t. instillation of 10 µg (20 µl of 0.5 mg/ml in NaCl 0.9%) on two consecutive days followed by two i.t. instillation of 1 µg (20 µl of 0.05 mg/ml in NaCl 0.9%) on day 11 and 12 after the first challenge (27). Control groups were instilled with vehicle (NaCl 0.9%) alone.

Amiloride treatment

Mice were treated with intranasal instillation of amiloride (10 mmol/l; 1 µl/g body weight; 3 times per day) or vehicle (ddH₂O) alone for a period of three weeks as previously described (35). In the presence of amiloride treatment, mice were exposed to Af or HDM as detailed in the online supplement.

Airway hyperresponsiveness

AHR was measured in anesthetized and paralyzed mice by exposure to aerosol containing increasing concentrations of methacholine (range 0.33 mg/ml to 160 mg/ml) using a small animal ventilator (Flexivent, Scireq Inc., Canada) as previously described (36).

Mucociliary clearance, allergen clearance and allergen uptake studies

Mucociliary clearance was determined from the elimination of yellow-green fluorescent microspheres from trachea (Thermo Fisher Scientific, Waltham, MA), and whole lung and airway allergen clearance from the elimination of fluorescently labeled Af (Alexa Fluor 647 Microscale Protein Labeling Kit, Life Technologies, Darmstadt, Germany) from lung homogenates and bronchoalveolar lavage fluid (BAL), respectively, following i.t. instillation (37, 38). Allergen uptake by bronchoalveolar macrophages (AM) and conventional dendritic cells (cDC) in vivo and in vitro was determined from the frequency of cells containing fluorescently labeled Af 4 h after i.t. instillation of mice or incubation of BAL cells, respectively, using a LSRFortessa analyzer and FACSDiva software v8.0.1 (BD Biosciences, Heidelberg, Germany), as previously described (39, 40).

BAL cell counts and type 2 cytokine measurements

BAL and differential cell counts were performed as previously described (14). IL-4, IL-5 and IL-13 were measured in BAL supernatants by ELISA (R&D Systems, Minneapolis, MN) or cytometric bead array enhanced sensitivity flex set (BD Biosciences, Heidelberg, Germany) following manufacturer's instructions.

Flow cytometry

Single cell suspensions were obtained from perfused lungs and restimulated in vitro with 50 ng/ml PMA and 1 µg/ml ionomycin (Sigma-Aldrich, Taufkirchen, Germany). Cells were surface stained with specific fluorescently labeled antibodies to delineate cellular populations

as previously described (41, 42) and detailed in the online supplement. For detection of IL-13, cells were fixed, permeabilized (BD Biosciences, Heidelberg, Germany) and incubated with intracellular antibodies against IL-13 (eBioscience, San Diego, CA). Multiparameter flow cytometry was performed using a LSRFortessa and data were analyzed with FACS Diva software v8.0.1 (BD Biosciences, Heidelberg, Germany) or Flow Jo software v10 (Treestar, Ashland, OR).

Histology and airway morphology

Histology and analyses of goblet cell metaplasia were performed as previously described (14).

Laser micro-dissection

Laser micro-dissection of the airway epithelium was performed in 10 μ m sections of frozen lungs using a 337 nm nitrogen laser (MicroBeam system, PALM Microlaser Technologies, Bernried, Germany) and total RNA was isolated using RNeasy Micro Kit (Qiagen, Hilden, Germany).

Immunohistochemistry

Immunohistochemical staining for IL-13 was performed using a polyclonal goat anti-mouse IL-13 antibody (R&D Systems, Minneapolis, MN) as previously described (43, 44).

Primary tracheal epithelial cultures

Primary tracheal epithelial cells were isolated and cultured on membranes (Costar, Cambridge, MA) under air-liquid interface (ALI) conditions as previously described (45). After reaching confluence (~14 days), IL-33 (10 ng/ml, R&D Systems, Wiesbaden, Germany) or ionomycin (1 μ g/ml, Sigma-Aldrich, Taufkirchen, Germany) were added for 24 h or 4 h, respectively, and total RNA was extracted (RNeasy Plus Mini Kit, Qiagen, Hilden, Germany).

For inhibition of the IL-33/ST2 signaling pathway, cultures were pretreated with anti-ST2 antibody (30 µg/ml, R&D Systems, Wiesbaden, Germany), the MyD88 inhibitory peptide Pepinh-MYD (50 µM, InvivoGen, San Diego, CA) or the p38 MAP kinase inhibitor SB203580 (20 µM InvivoGen, San Diego, CA).

Real-Time RT-PCR

Real-time RT-PCR was performed using TaqMan gene expression assays for *Il13*, *Il33*, *Il25*, *Tslp*, *Postn*, *Mmp12*, *Chia*, *Ym1*, *Ym2*, *Arg1*, *Muc5ac*, *Muc5b*, *Gob5* and *Actb* (Applied Biosystems, Darmstadt, Germany) and analyzed as previously described (14).

Statistics

Data were derived from at least three independent experiments and analyzed with SigmaPlot version 12.5 (Systat Software, Erkrath, Germany) and SPSS Statistics version 22 (IBM, Ehningen, Germany), and are reported as mean ± SEM. Statistical analyses were performed using Student's t-test, Mann-Whitney rank sum test or one-way ANOVA as appropriate and $P < 0.05$ was accepted to indicate statistical significance.

RESULTS

Impaired allergen clearance exacerbates allergen-induced type 2 airway inflammation and airway hyperresponsiveness in juvenile *Scnn1b*-Tg mice

To determine effects of mucociliary dysfunction on allergen clearance and allergen-induced airway disease, we challenged 2 to 3-week-old juvenile *Scnn1b*-Tg and WT mice with i.t. application of the natural aeroallergen Af or vehicle alone and compared allergen clearance, inflammatory cell counts and the type 2 cytokines IL-4, IL-5 and IL-13 in BAL, parameters of mucus hypersecretion (i.e. GCM and mucin expression), and AHR (Figure 1 and Figure E1). MCC and clearance of Af from whole lungs and from the airway lumen were significantly reduced in naïve *Scnn1b*-Tg mice compared to WT littermates (Figure 1 A-C). To test if reduced allergen clearance exacerbates allergen-induced airway disease, we first used a short term challenge protocol consisting of four Af challenges. In WT mice, this challenge induced a modest elevation in BAL macrophages, eosinophils, IL-5 and IL-13 (Figure 1 D-G). In *Scnn1b*-Tg mice, BAL eosinophils, IL-4, IL-5 and IL-13 were spontaneously increased (14, 15) and Af challenge triggered a robust airway eosinophilia and production of IL-4, IL-5 and IL-13 that was significantly increased compared to allergen-induced responses in WT mice (Figure 1 D-G). Type 2 airway inflammation in Af-challenged WT mice was associated with GCM and elevated transcript levels of *Muc5ac*, *Muc5b* and *Gob5* (Figure 1 H and Figure E1). Vehicle-treated *Scnn1b*-Tg mice showed spontaneous GCM and increased expression of *Muc5ac*, *Muc5b* and *Gob5* that was not further increased by Af challenge (Figure E1). Invasive pulmonary function testing demonstrated spontaneous AHR in naïve juvenile *Scnn1b*-Tg vs. WT mice (Figure 1 I). In the challenge model, Af aggravated AHR in juvenile *Scnn1b*-Tg mice, but failed to produce AHR in WT littermates (Figure 1 J).

In contrast to juvenile mice, short term Af challenge did not produce airway eosinophilia and elevated IL-13 levels in 6-week-old adult WT mice (Figure 2 A and B). In adult *Scnn1b*-Tg

mice, consistent with previous studies, eosinophilia and elevated IL-13 in BAL were diminished and airway inflammation was dominated by neutrophils (14), and Af challenge neither induced type 2 inflammation nor AHR (Figure 2 A-C). To determine the relationship between this age-dependent susceptibility to allergen challenge and previously reported skewing towards type 2 immunity in neonatal mice (14, 26, 27), we compared expression levels of a set of type 2 signature genes including *Il13*, *Muc5ac*, *Gob5*, *Arg1*, *Mmp12*, *Ym1*, *Ym2*, and *Chia* (46) in lung tissues from WT and *Scnn1b*-Tg mice at neonatal (10-day-old), juvenile and adult ages (Figure 2 D-K). Consistent with a type 2 biased immune system in the neonatal period, several type 2 signature genes (*Il13*, *Muc5ac*, *Gob5*, *Arg1*, *Ym2*) were significantly increased in neonatal compared to older WT mice (Figure 2 D-K). At all age groups, expression levels of all of these type 2 signature genes were increased in *Scnn1b*-Tg vs. WT mice, however, these differences waned for most signatures at the age of 6 weeks (Figure 2 D-K). These data confirm skewing toward type 2 immunity in neonatal mice and show that this disposition is associated with increased susceptibility to allergen-induced type 2 airway inflammation in juvenile WT mice, as well as exaggerated responses in *Scnn1b*-Tg mice with impaired allergen clearance.

Next, we tested the effects of HDM in juvenile WT and *Scnn1b*-Tg mice using a protocol consisting of i.t. sensitization followed by challenge to determine the role of impaired MCC in an independent allergen model (Figure 3). In WT mice, HDM induced elevated levels of eosinophils, CD4⁺ T cells, IL-4, IL-5 and IL-13, and AHR (Figure 3). In *Scnn1b*-Tg mice, BAL eosinophils, type 2 cytokines and AHR were increased at baseline and, similar to Af, these phenotypes were significantly exacerbated by HDM (Figure 3). Taken together, these results demonstrate that mucociliary dysfunction impairs allergen clearance and aggravates type 2 inflammation and AHR in juvenile *Scnn1b*-Tg mice.

Airway epithelial, innate and adaptive immune cells contribute to exaggerated allergen-induced type 2 inflammation in *Scnn1b*-Tg mice

To determine the cellular sources of IL-13 in type 2 airway inflammation we performed immunohistochemistry in lung sections from juvenile *Scnn1b*-Tg and WT mice challenged with Af or vehicle alone. Distinct IL-13 immunoreactive signals were detected in epithelial cells lining the conducting airways of vehicle-treated *Scnn1b*-Tg mice, but not WT littermates (Figure 4 A and B). Challenge with Af increased the number of IL-13⁺ airway cells in WT and *Scnn1b*-Tg mice slightly, however, this trend did not reach statistical significance (Figure 4 A and B). For independent confirmation of these results, we isolated airway epithelial cells from frozen lung sections by laser micro-dissection and determined transcript levels of the key type 2 cytokine *Il13*. Further, we measured expression levels of *Il33*, *Il25* and *Tslp*, epithelial cytokines that were shown to be secreted in response to allergen exposure and promote type 2 airway inflammation via activation of Th2 cells, type 2 innate lymphoid cells (ILC-2) and cDC (7, 9, 25, 47), and *Postn* that was also shown to be expressed by epithelial cells and implicated in type airway 2 inflammation (48). *Il13*, *Il25* and *Tslp* transcripts were not detectable in micro-dissected airway epithelia from WT mice, whereas *Il33* and *Postn* were readily detected even without Af challenge (Figure 4 C). In naïve *Scnn1b*-Tg mice, epithelial *Il13* and *Il33* were significantly increased, *Il25*, and *Tslp* were not detected and *Postn* mRNA levels were not changed compared to WT mice (Figure 4 C). In airways from Af-challenged *Scnn1b*-Tg mice, *Il13* mRNA levels tended to be ~3-fold higher, however, this difference did not reach statistical significance. Transcript levels of *Il25*, and *Tslp* remained undetectable and *Il33* and *Postn* did not differ in Af-challenged vs. naïve *Scnn1b*-Tg mice (Figure 4 C). To test if airway epithelial cells are capable of expressing *Il13* in vitro, we measured *Il13* transcripts in highly differentiated primary tracheal epithelial cell cultures at baseline and following stimulation with recombinant IL-33 or ionomycin (Figure 4 D). *Il13* was not detected under basal conditions, but was induced in a dose-dependent fashion by IL-

33 and by non-specific stimulation with ionomycin in tracheal epithelial cultures from WT and *Scnn1b*-Tg mice (Figure 4 D and Figure E2). IL-33-induced *Il13* expression did not depend on STAT6 signaling in vitro, but was blocked by inhibitors of the IL-33/ST2 signaling pathway including anti-ST2 antibody, the MyD88 inhibitory peptide Pepinh-MYD and the p38 MAP kinase inhibitor SB203580 (Figure 4 D and E).

To determine if innate or adaptive immune cells contributed to elevated baseline and Af-induced expression of IL-13 in *Scnn1b*-Tg mice (Figure 1 G), we isolated leukocytes from lungs and measured IL-13 expression in different subpopulations by flow cytometry (Figure 4 F). In lungs from WT mice, numbers of IL-13⁺ cells in the different leukocyte subpopulations were low and did not change in response to short term Af challenge. In naïve *Scnn1b*-Tg mice, IL-13⁺ Th2 cells, AM, B cells and ILC-2 were significantly increased compared to WT mice (Figure 4 F), and IL-13⁺ Th2 cells and IL-13⁺ ILC-2 were further increased after Af challenge (Figure 4 F).

Collectively, these data show that airway epithelial cells, innate and adaptive immune cells provide sources of IL-13 in spontaneous and exaggerated allergen-induced type 2 airway inflammation in juvenile *Scnn1b*-Tg mice, and indicate that the release of epithelial IL-33 contributes to this process in vivo.

Alternative macrophage activation and allergen challenge in *Scnn1b*-Tg mice

Because previous studies showed signatures of alternative macrophage activation in *Scnn1b*-Tg mice (16, 49) and alternatively activated macrophages (AAM) have been implicated in type 2 inflammation (50, 51), we next determined activation markers of AM and the capacity of Af uptake by AM and cDC in vivo and in vitro using flow cytometry (Figure 5). Compared to WT mice, *Scnn1b*-Tg mice showed significant upregulation of CD11b on AM as defined

by F4/80⁺CD11c^{hi}CD3⁻ indicating macrophage activation (Figure 5 A and B). Upregulation of scavenger receptors CD206 and CD301 confirmed the presence of AAM (Figure 5 C and D). CD206 and CD301 were already elevated in vehicle-treated *Scnn1b*-Tg mice and reduced by ~15-25% after Af challenge (Figure 5 C and D). The uptake of fluorescently labeled Af by AM from naïve *Scnn1b*-Tg mice was significantly reduced compared to WT mice in vivo and in vitro (Figure 5 E and F). Conversely, uptake of Af by lung cDC was significantly increased in *Scnn1b*-Tg mice (Figure 5 G and H). These data demonstrate robust AM activation in mucostatic airways of naïve *Scnn1b*-Tg mice but indicate that AAM do not play a major role in exaggerated allergen-induced type 2 inflammation.

Lack of STAT6 abrogates type 2 airway inflammation in *Scnn1b*-Tg mice

The key type 2 signaling molecule IL-13 exerts its effector functions via STAT6 signaling (31-33). To determine if elevated IL-13 plays a causal role in baseline and/or exaggerated allergen-induced type 2 inflammation, we challenged juvenile *Stat6*-deficient *Scnn1b*-Tg (*Scnn1b*-Tg/*Stat6*^{-/-}) mice and *Scnn1b*-Tg littermates with Af or vehicle alone and compared their pulmonary phenotypes. Spontaneous eosinophilic airway inflammation, IL-13 and GCM observed in naïve *Scnn1b*-Tg mice were significantly reduced in *Scnn1b*-Tg/*Stat6*^{-/-} mice (Figure 6 A-C). Further, *Scnn1b*-Tg/*Stat6*^{-/-} mice were protected from Af-induced airway eosinophilia, elevated expression of IL-13 and GCM (Figure 6 A-C). IL-13 immunoreactive signals detected in lung sections from *Scnn1b*-Tg mice were abrogated in *Scnn1b*-Tg/*Stat6*^{-/-} mice (Figure 6 D). Taken together, these data show that IL-13/STAT6 signaling plays a critical role in the exaggerated type 2 inflammatory response observed upon allergen challenge in juvenile *Scnn1b*-Tg mice.

Preventive amiloride treatment reduces allergen-induced type 2 inflammation and airway hyperresponsiveness in *Scnn1b*-Tg mice

Previous studies demonstrated that improvement of airway surface hydration by preventive treatment with the ENaC blocker amiloride reduced airway mucus obstruction and spontaneous type 2 airway inflammation in juvenile *Scnn1b*-Tg mice (35). To test if allergen-induced inflammation can be prevented by ENaC inhibition, we treated newborn *Scnn1b*-Tg mice and WT controls with airway instillation of amiloride or vehicle alone for three weeks and superimposed an allergen challenge with Af or HDM in the presence of this treatment regime (Figure 7 A and E). Preventive amiloride treatment significantly reduced exacerbated Af- and HDM-induced eosinophilia and IL-13 levels in BAL from *Scnn1b*-Tg mice (Figure 7 A-H). Further, amiloride treatment prevented HDM-induced AHR in *Scnn1b*-Tg mice (Figure 7 J). These data demonstrate that improvement of airway surface hydration by pharmacological inhibition of ENaC protected juvenile *Scnn1b*-Tg mice from allergen-induced type 2 airway inflammation.

DISCUSSION

Type 2 airway inflammation is a key feature of allergen-induced asthma and has recently been implicated in other common chronic inflammatory lung diseases including COPD and CF (1-6). However, the underlying risk factors and mechanisms that trigger and sustain allergic airway inflammation remain poorly understood. Using juvenile *Scnn1b*-Tg mice in combination with two independent models of allergen-induced asthma (Af and HDM), this study demonstrates for the first time that impaired MCC with reduced clearance of inhaled aeroallergen constitutes a risk factor of type 2 inflammation that aggravates allergen-induced secretion of IL-13 and characteristic phenotypes of allergic airway disease including airway eosinophilia and AHR in vivo (Figure 1 and 3). Further, our data yield novel insights into the role of the airway epithelium and cells of the innate and adaptive immune system in the pathogenesis of type 2 inflammation in mucostatic airways (Figure 4 and 5). Analysis of *Scnn1b*-Tg mice with genetic deletion of *Stat6* confirmed a role of the canonical IL-4/IL-13-STAT6 immune axis in the type 2 nature of the exaggerated immune response towards allergen challenge (Figure 6). Finally, we provide a proof-of-concept that pharmacological improvement of mucus clearance is an effective therapeutic strategy to prevent exaggerated allergen-induced type 2 inflammation and AHR in mucostatic airways from *Scnn1b*-Tg mice (Figure 7).

Previous studies demonstrated that IL-13 is a central signaling molecule that is sufficient to produce the key features of type 2 airway inflammation including airway eosinophilia, GCM, mucus hypersecretion and AHR in vivo (1-3) and identified Th2 cells, ILC-2 and AAM as cellular sources of IL-13 production (8, 50). Interestingly, our immunohistochemistry studies in lung sections and transcript analysis of micro-dissected airways detected robust expression of IL-13 in airway epithelial cells from *Scnn1b*-Tg mice at baseline and after Af challenge (Figure 4). In contrast to airway tissues, spontaneous *Il13* expression was not detectable in

396 primary airway epithelial cultures from *Scnn1b*-Tg mice in vitro suggesting that *Il13*
 397 expression required epithelial cell activation in vivo (Figure 4 D). Recent studies
 398 demonstrated that the airway epithelium acts as a sentinel that can sense allergens and other
 399 noxious stimuli such as viruses on airway surfaces through a range of pattern recognition
 400 receptors, and that activation by these stimuli leads to the release of epithelial cytokines such
 401 as IL-33, IL-25 and TSLP, which in turn participate in the activation of innate and adaptive
 402 immune cells that orchestrate type 2 airway inflammation (7, 9, 10, 25). Consistent with its
 403 constitutive expression in epithelial cells (47), *Il33* transcripts were readily detected in micro-
 404 dissected airway tissues from naïve WT mice and were significantly increased (~14-fold) in
 405 *Scnn1b*-Tg littermates (Figure 4 C). In contrast, *Il25* and *Tslp* were neither detected in WT nor
 406 in *Scnn1b*-Tg airway tissues. In vitro, IL-33 induced dose-dependent expression of *Il13* in
 407 primary airway epithelial cultures from WT and *Scnn1b*-Tg mice (Figure 4 D and Figure E2).
 408 Following Af challenge in vivo, *Il33* expression remained elevated and *Il25* and *Tslp*
 409 transcripts remained undetectable in airway epithelia from *Scnn1b*-Tg mice (Figure 4 C). *Il13*
 410 transcript levels tended to be increased (~3-fold) in Af challenged compared to naïve *Scnn1b*-
 411 Tg littermates, however, this difference did not reach statistical significance based on the
 412 number of mice included in our studies (Figure 4 C). Of note, induction of *Il13* by IL-33 in
 413 airway epithelial cultures in vitro was mediated by ST2/MyD88 signaling and independent of
 414 STAT6, whereas allergen-induced airway eosinophilia, elevated IL-13 secretion and GCM in
 415 *Scnn1b*-Tg mice were STAT6-dependent in vivo (Figure 4 and 6). However, these data do not
 416 exclude a role of IL-33 in IL-13 induction in vivo. Recent studies in the intestinal epithelium
 417 showed that in vitro effects of IL-33 were entirely MyD88-dependent and independent of
 418 STAT6, whereas the in vivo effects of IL-33 injected in mice were also dependent on STAT6
 419 and IL-13 (52). These results suggest that IL-13 triggered by IL-33 via the ST2/MyD88
 420 signaling pathway may promote autocrine/paracrine activation of IL-13 secretion by the
 421 epithelium and other cell types, which in turn depends on STAT6 signaling and is necessary

to produce full-blown type 2 inflammation in vivo (52). Collectively, these results identify the airway epithelium as an important source of IL-13 that may contribute to the orchestration of type 2 inflammation at the mucosal interface. Further, our data suggest that activation of airway epithelial cells by IL-33 released in response to allergens or other danger-associated molecular patterns that accumulate in mucostatic airways may be implicated in triggering this process.

Consistent with an important role of epithelial IL-33 in the pathogenesis of type 2 inflammation triggered by mucociliary dysfunction, we identified Th2 cells and ILC-2 as cellular sources of elevated baseline and exaggerated allergen-induced IL-13 production in lungs of *Scnn1b*-Tg mice (Figure 1 and 4 F). As expected from previous studies (16), AM expressed markers of alternative activation (AAM) and contributed to IL-13 production in *Scnn1b*-Tg mice even in the absence of allergen challenge (Figure 4 F and 5). In contrast to Th2 cells and ILC-2, AAM were not further increased in Af-challenged *Scnn1b*-Tg mice (Figure 5). However, allergen uptake by AM was reduced in *Scnn1b*-Tg compared to WT mice. In combination with impaired MCC, this reduced AM-mediated allergen uptake may contribute to impaired clearance and thus overall burden of airborne allergens in the lungs of *Scnn1b*-Tg mice (Figure 5, E and F). Conversely, allergen uptake by cDC was significantly increased in *Scnn1b*-Tg compared to WT mice. This enhanced allergen uptake may be due to activation of cDC in the pro-inflammatory milieu and/or increased allergen load in mucostatic airways, and may contribute to the activation of Th2 cells observed in the lungs of *Scnn1b*-Tg mice (Figure 5, G and H). Taken together, these results demonstrate that mucociliary dysfunction, leading to reduced clearance of inhaled allergens from airway surfaces, triggers activation of innate and adaptive immune cells that contribute to the pathogenesis of type 2 airway inflammation, and that the airway epithelium plays an important role in orchestrating this process.

These results also suggest that improvement of slowed mucus clearance may be an effective therapeutic strategy to reduce allergic airway inflammation. To test this hypothesis, we pretreated juvenile *Scnn1b*-Tg mice and WT littermates with intrapulmonary application of the ENaC blocker amiloride, and superimposed challenges with either Af or HDM on this preventive therapy (Figure 7) (35). ENaC is limiting for the absorption of Na⁺ and fluid from airway surfaces and airway surface dehydration (mucus hyperconcentration), either due to an imbalance between epithelial Cl⁻ secretion and Na⁺ absorption or unbalanced mucin secretion, has been identified as a key disease mechanism of mucociliary dysfunction in a spectrum of chronic airway diseases including asthma (18, 20-24). In this context, ENaC blockade provides a pharmacological strategy to improve airway surface hydration and accelerate mucus clearance (53). Indeed, amiloride and other hydration therapies were shown to improve MCC in patients with CF and other chronic airway diseases (28-30), however, the effects of this intervention on allergen-induced airway inflammation remained unknown. In a previous study, we demonstrated that improving airway surface hydration with preventive amiloride treatment reduced mucus obstruction and spontaneous type 2 inflammation in juvenile *Scnn1b*-Tg mice to near normal levels (35). In the present study, we show that continuous amiloride treatment largely prevented exaggerated airway eosinophilia and IL-13 induction in *Scnn1b*-Tg mice independent of the allergen used (Figure 7 A-H). In addition, preventive amiloride treatment also protected *Scnn1b*-Tg mice from allergen-induced AHR (Figure 7 J). These data provide the first proof-of-concept that pharmacological improvement of airway surface hydration and mucus clearance, in a susceptible host, is an effective therapeutic approach to reduce allergen-induced airway inflammation in chronic airways disease associated with mucociliary dysfunction in vivo.

An interesting finding of our study was the age-dependent susceptibility of mice to develop type 2 airway inflammation in response to allergen challenge in the context of impaired MCC. We show that even short term exposure to Af that failed to induce airway inflammation in adult mice produced robust type 2 inflammation with airway eosinophilia, elevated IL-13 and GCM in juvenile WT mice, and that allergen-induced inflammation was exacerbated and associated with AHR in juvenile, but not adult *Scnn1b*-Tg mice (Figure 1 and 2). We speculate that this enhanced sensitivity originates from a type 2 skewed immunity in early life that renders the host susceptible to develop type 2 inflammation upon allergen challenge (26). This notion is supported by previous studies in WT mice exposed to HDM from the neonatal period showing that allergen-induced airway eosinophilia and type 2 cytokine levels peaked in juvenile mice and waned in adult mice (27), as well as previous longitudinal studies in naïve WT and *Scnn1b*-Tg mice showing spontaneous elevation of eosinophils, IL-13 and goblet cells in juvenile WT mice that were enhanced in *Scnn1b*-Tg mice and waned in adult mice of both genotypes (14). In our present study, this concept was further corroborated by comprehensive transcripts analyses of a spectrum of type 2 signature genes including *Il13*, *Muc5ac*, *Gob5*, *Arg1*, *Mmp12*, *Ym1*, *Ym2*, and *Chia*. These studies demonstrated that expression levels of *Il13* as well as most of the other genes were increased in lungs from neonatal compared to older WT mice, and that this age-dependent type 2 pattern was exacerbated in *Scnn1b*-Tg mice (Figure 2 D-K). Interestingly, this pattern included genes exclusively expressed in epithelial cells such as *Muc5ac* and *Gob5* supporting an important role of the airway epithelium in this age-dependent process. A similar type 2 skewing in early life was reported in humans (54) and this bias persists in many older children and adults with “Th2-high” asthma (55, 56). We therefore speculate that our findings in juvenile mice may be applicable to a broader age range in patients with this common asthma endotype. However, future studies will be required to determine the relationship between reduced MCC, allergen-

induced airway inflammation and disease severity in patients with “Th2-high” vs. “Th2-low” asthma (12, 13, 55, 56).

In summary, we demonstrate for the first time that mucociliary dysfunction causing reduced clearance of inhaled allergens from airway surfaces represents an important risk factor and attractive therapeutic target of allergen-induced type 2 airway inflammation in a susceptible host in vivo. Further, our results support the emerging concept that the airway epithelium is not only an important effector tissue, but also an important player in sensing of allergens and transmission of pro-inflammatory signals to innate and adaptive immune cells that orchestrate the complex in vivo pathogenesis of type 2 inflammation. These results suggest therapeutic improvement of airway mucus clearance as a novel treatment strategy for patients with allergen-induced asthma and potentially other chronic inflammatory airway diseases associated with mucociliary dysfunction.

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FIGURE LEGENDS

Figure 1. Mucociliary dysfunction impairs allergen clearance and exacerbates type 2 airway inflammation and AHR in juvenile *Scnn1b*-Tg mice. (A-C) MCC (A), whole lung (B) and airway (C) allergen clearance in juvenile (2 to 3-week-old) WT and *Scnn1b*-Tg mice. $n = 4-13$ for each group. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ compared to WT mice. (D-H) Juvenile WT and *Scnn1b*-Tg mice were challenged with Af or vehicle alone. (D) BAL cell counts (AM; Eos., eosinophils; PMN, neutrophils; Lymph., lymphocytes). $n = 20-28$ mice for each group. (E-G) IL-4, IL-5 and IL-13 in BAL. $n = 9-11$ for each group. (H) Goblet cells. $n = 17-25$ for each group. $*P < 0.05$ and $***P \leq 0.001$ compared to vehicle-treated mice of same genotype. $\dagger P < 0.05$, $\ddagger P < 0.01$ and $\S P < 0.001$ compared to mice with same treatment but different genotype. (I and J) Assessment of AHR in naïve juvenile WT and *Scnn1b*-Tg mice (I) and after Af exposure (J). $n = 7-14$ for each group. (I) $*P < 0.05$ and $**P < 0.01$ compared to WT mice. (J) $*P < 0.05$ and $**P < 0.01$ compared to vehicle-treated mice of same genotype. $\dagger P < 0.05$, $\ddagger P < 0.01$ and $\S P < 0.001$ compared to mice with same treatment but different genotype.

Figure 2. Allergen-induced type 2 airway inflammation and AHR is age-dependent and correlates with expression levels of type 2 signature genes in mouse lung. (A-C) Adult (6-week-old) WT and *Scnn1b*-Tg mice were challenged with Af or vehicle alone and BAL cell counts (AM; Eos., eosinophils; PMN, neutrophils; Lymph., lymphocytes) (A), IL-13 levels in BAL (B) and AHR (C) were determined. $n = 7-17$ mice for each group. $\dagger P < 0.05$ and $\ddagger P < 0.01$ compared to mice with same treatment but different genotype. (D-K) Expression of type 2 signature genes *Il13* (D), *Muc5ac* (E), *Gob5* (F), *Arg1* (G), *Mmp12* (H), *Ym1* (I), *Ym2* (J), and *Chia* (K) in naïve neonatal (10-day-old), juvenile (3-week-old) and adult (6-week-old) WT and *Scnn1b*-Tg mice. $n = 7-16$ mice for each group. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ compared to mice with same age but different genotype. $\dagger P < 0.05$, $\ddagger P < 0.01$ and $\S P < 0.001$ compared to mice with same age but different genotype.

0.001 compared to neonatal mice with same genotype. # $P < 0.05$ and ¶ $P < 0.01$ compared to juvenile mice with same genotype.

Figure 3. Impaired mucus clearance exacerbates HDM-induced type 2 airway inflammation and AHR in juvenile *Scnn1b*-Tg mice. (A-F) Juvenile (2 to 3-week-old) WT and *Scnn1b*-Tg mice were sensitized and challenged with HDM or vehicle alone. (A) BAL cell counts (AM; Eos., eosinophils; PMN, neutrophils; Lymph., lymphocytes). (B) Airway CD4⁺ T cells. (C-E) IL-4, IL-5 and IL-13 in BAL. (F) Assessment of AHR. In HDM-exposed *Scnn1b*-Tg mice severe airway obstruction and mucus plugging of cannulae impeded ventilation and measurements had to be discontinued at methacholine concentrations > 32 mg/ml. n = 13-23 for each group. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to vehicle-treated mice of same genotype. † $P < 0.05$, ‡ $P < 0.01$ and § $P < 0.001$ compared to mice with same treatment but different genotype.

Figure 4. Airway epithelial cells, innate and adaptive immune cells express IL-13 and contribute to type 2 airway inflammation in *Scnn1b*-Tg mice. Juvenile (2 to 3-week-old) WT and *Scnn1b*-Tg mice were challenged with Af or vehicle alone. (A) Representative immunohistochemical detection of IL-13 in lung sections. Staining was not detected in sections incubated with secondary antibody only (control). Scale bars = 100 µm. (B) IL-13⁺ cells in airway epithelium per mm basal membrane (BM). (C) Expression of *Il13*, *Il33*, *Il25*, *Tslp* and *Postn* transcripts in laser micro-dissected airway epithelium. n = 7-11 for each group. † $P < 0.05$ compared to WT mice with same treatment. (D and E) Induction of *Il13* expression in ALI primary tracheal epithelial cell cultures of WT, *Scnn1b*-Tg and *Stat6*^{-/-} mice by IL-33 and ionomycin (D) and effects of inhibitors of the IL-33/ST2 signaling pathway in WT cultures (E). n = 4-10 for each group. * $P < 0.05$ and ** $P < 0.01$ compared to vehicle-treated cultures of same genotype (D) or compared to IL-33 treated WT cultures (E). (F) IL-13

expression by leukocyte subpopulations (T cells; AM; B cells; ILC-2; Eos., eosinophils; PMN, neutrophils; cDC) isolated from lungs determined by flow cytometry after in vitro stimulation. n = 3-8 for each group. * $P < 0.05$ compared to vehicle-treated mice of same genotype. † $P < 0.05$ and ‡ $P < 0.01$ compared to mice with same treatment but different genotype.

Figure 5. Alternative macrophage activation and altered allergen uptake by bronchoalveolar macrophages and cDC in *Scnn1b*-Tg mice. (A-D) Juvenile (2 to 3-week-old) WT and *Scnn1b*-Tg mice were challenged with Af allergen or vehicle alone, BAL was performed and bronchoalveolar macrophages (AM) were characterized by flow cytometry. (A) Dot plots were gated for CD45⁺F4/80⁺CD3⁻CD19⁻ leukocytes. Numbers in gates show percentage of CD11b⁺ AM. Scale indicates log₁₀ fluorescence. (B-D) Percentage of CD11b⁺ AM (B) and mean fluorescence intensity (MFI) for fluorescently-labeled antibody bound to CD206 (C) and CD301 (D) on AM. n = 14-20 for each group. * $P < 0.05$ compared to vehicle-treated mice with same genotype. † $P < 0.001$ compared to mice with same treatment but different genotype. (E-H) Percentage of BAL derived AM and cDC from WT and *Scnn1b*-Tg mice positive for fluorescently labeled Af after i.t. application in vivo (E and G) or incubation in vitro (F and H). n = 5-6 for each group. * $P < 0.05$ and *** $P < 0.001$ compared to WT mice.

Figure 6. Lack of STAT6 abrogates type 2 airway inflammation in *Scnn1b*-Tg mice. (A-D) Juvenile (2 to 3-week-old) *Scnn1b*-Tg and *Scnn1b*-Tg mice with genetic deletion of *Stat6* (*Scnn1b*-Tg/*Stat6*^{-/-} mice) were challenged with Af or vehicle alone. (A) BAL cell counts (AM; Eos., eosinophils; PMN, neutrophils; Lymph., lymphocytes. (B) IL-13 levels in BAL. (C) Goblet cells. n = 5-15 for each group. * $P < 0.05$ and ** $P \leq 0.01$ compared to vehicle-treated mice of same genotype. † $P < 0.05$ and ‡ $P \leq 0.01$ compared to mice with same treatment but different genotype. (D) Representative immunohistochemical detection of IL-13 in main axial airways. Scale bars = 100 μ m.

Figure 7. Preventive treatment with amiloride reduces allergen-induced type 2 airway inflammation and AHR in *Scnn1b*-Tg mice. Newborn WT and *Scnn1b*-Tg mice were treated with airway instillation of amiloride or vehicle (ddH₂O) alone from the first day of life for a period of three weeks. (A-D) Challenge with Af or vehicle (NaCl 0.9%) alone was superimposed to amiloride treatment in juvenile mice (A) and BAL total cells (B), eosinophils (C) and IL-13 levels (D) were determined. n = 14-49 in WT groups and n = 4-9 in *Scnn1b*-Tg groups. (E-J) Alternatively, sensitization and challenge with HDM or vehicle (NaCl 0.9%) alone was superimposed to amiloride (E) and BAL total inflammatory cells (F), eosinophils (G), IL-13 levels (H), and AHR (I, WT mice; J, *Scnn1b*-Tg mice) were determined. n = 13-23 in WT groups and n = 16-28 in *Scnn1b*-Tg groups. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 compared to vehicle-treated (ddH₂O and NaCl 0.9%) mice of same genotype. †*P* < 0.05, ‡*P* < 0.01 and §*P* < 0.001 compared to vehicle-treated (H₂O) Af or HDM-induced mice of same genotype.

Figure 1

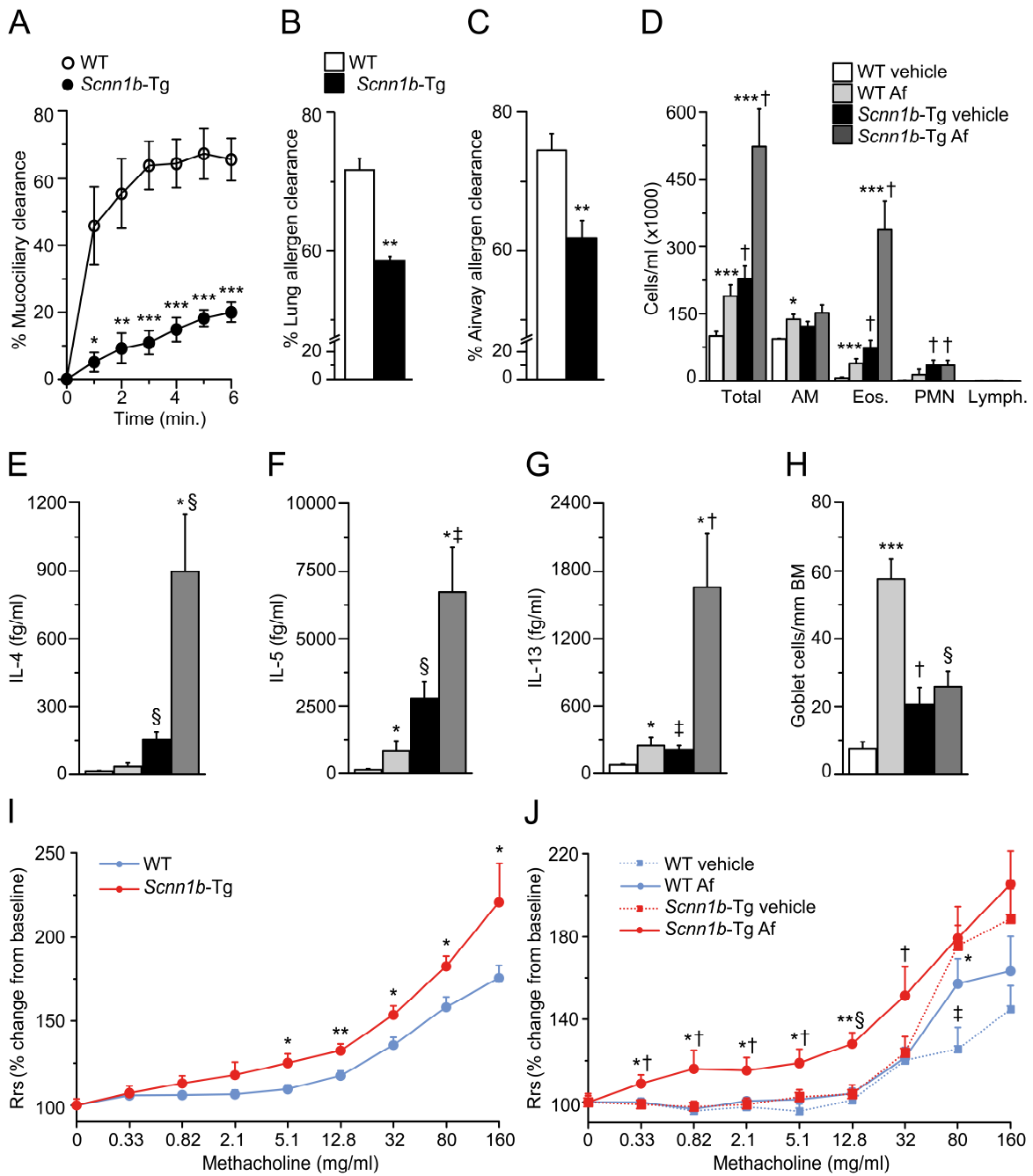


Figure 2

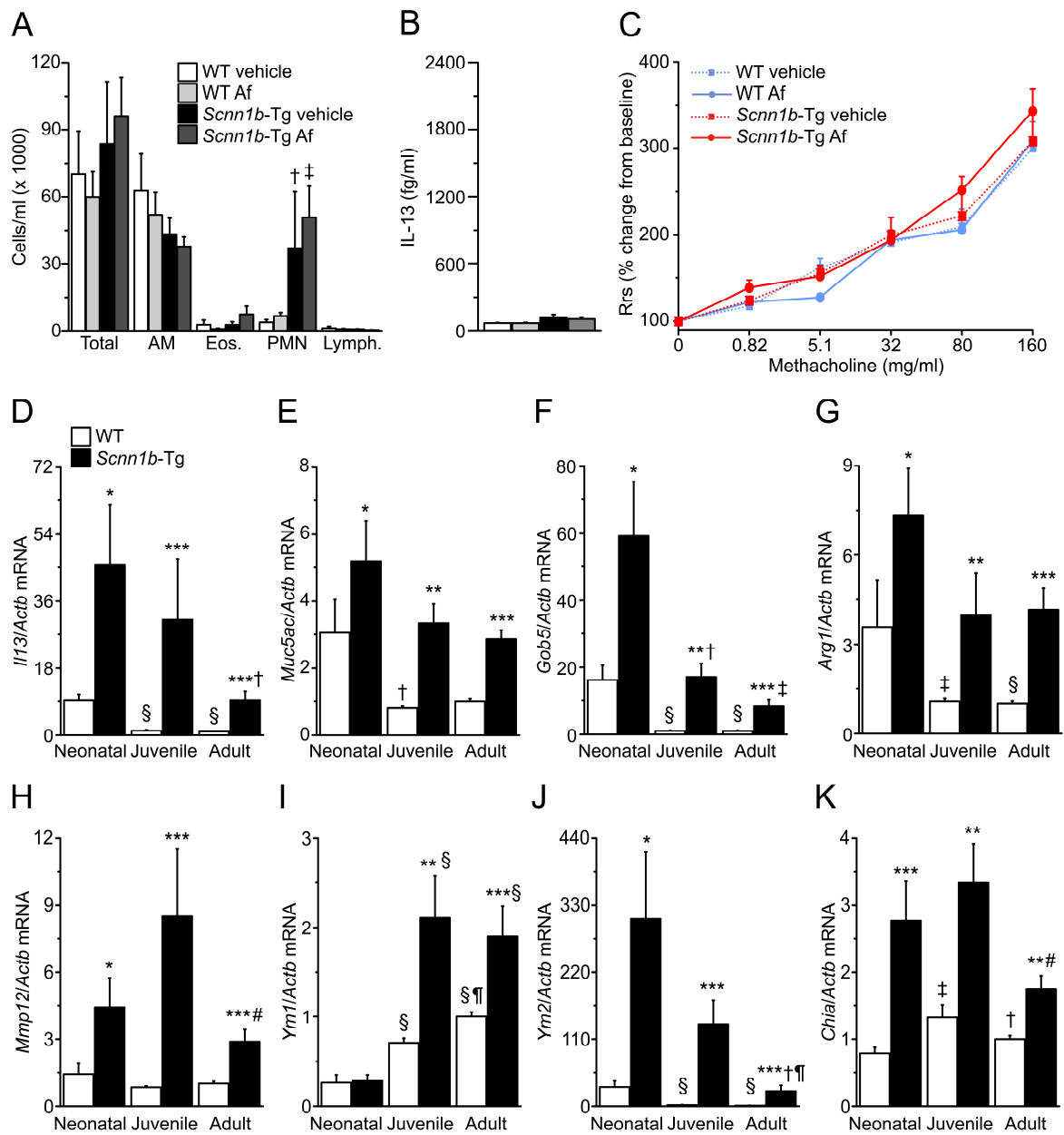


Figure 3

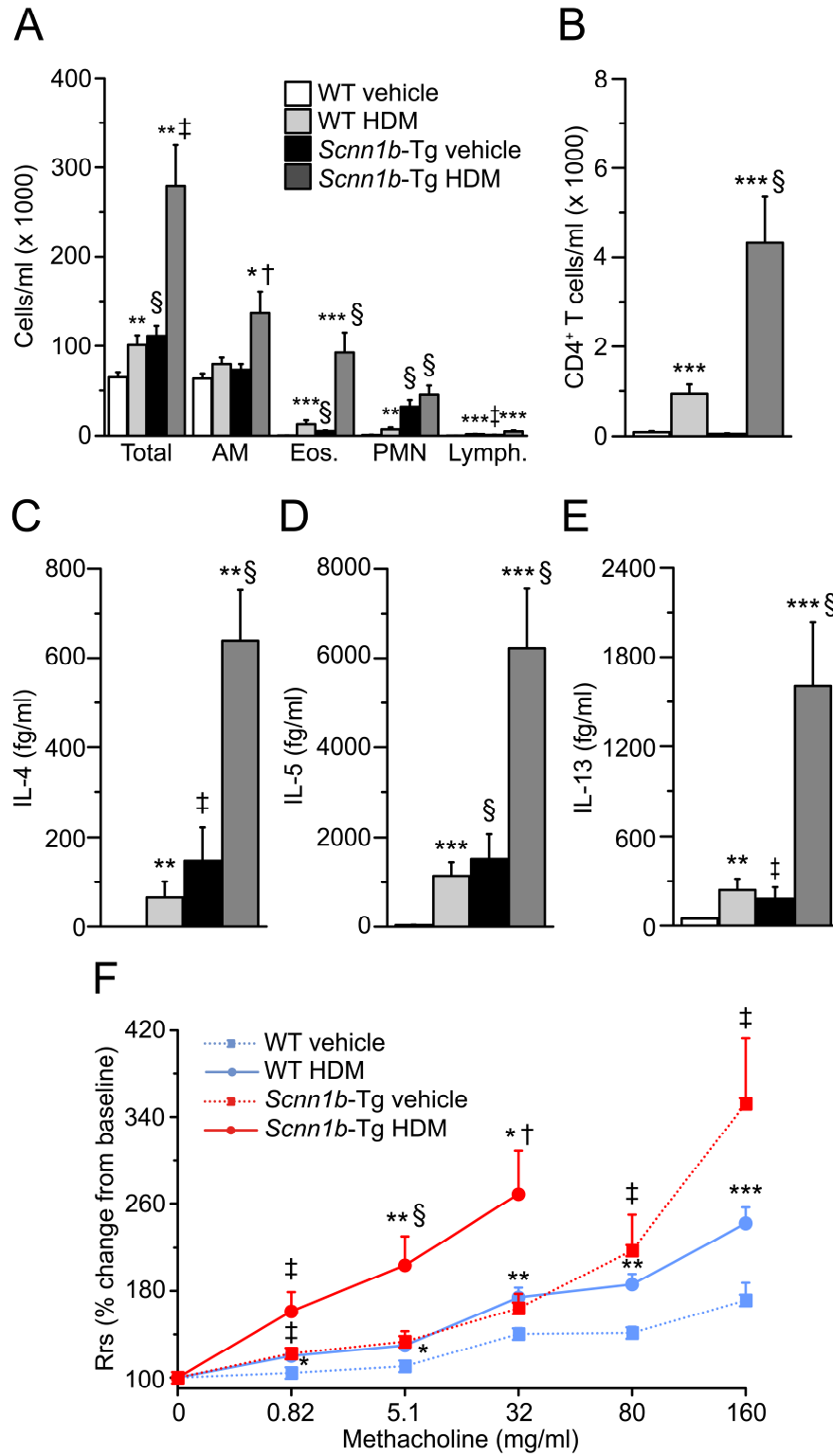


Figure 4

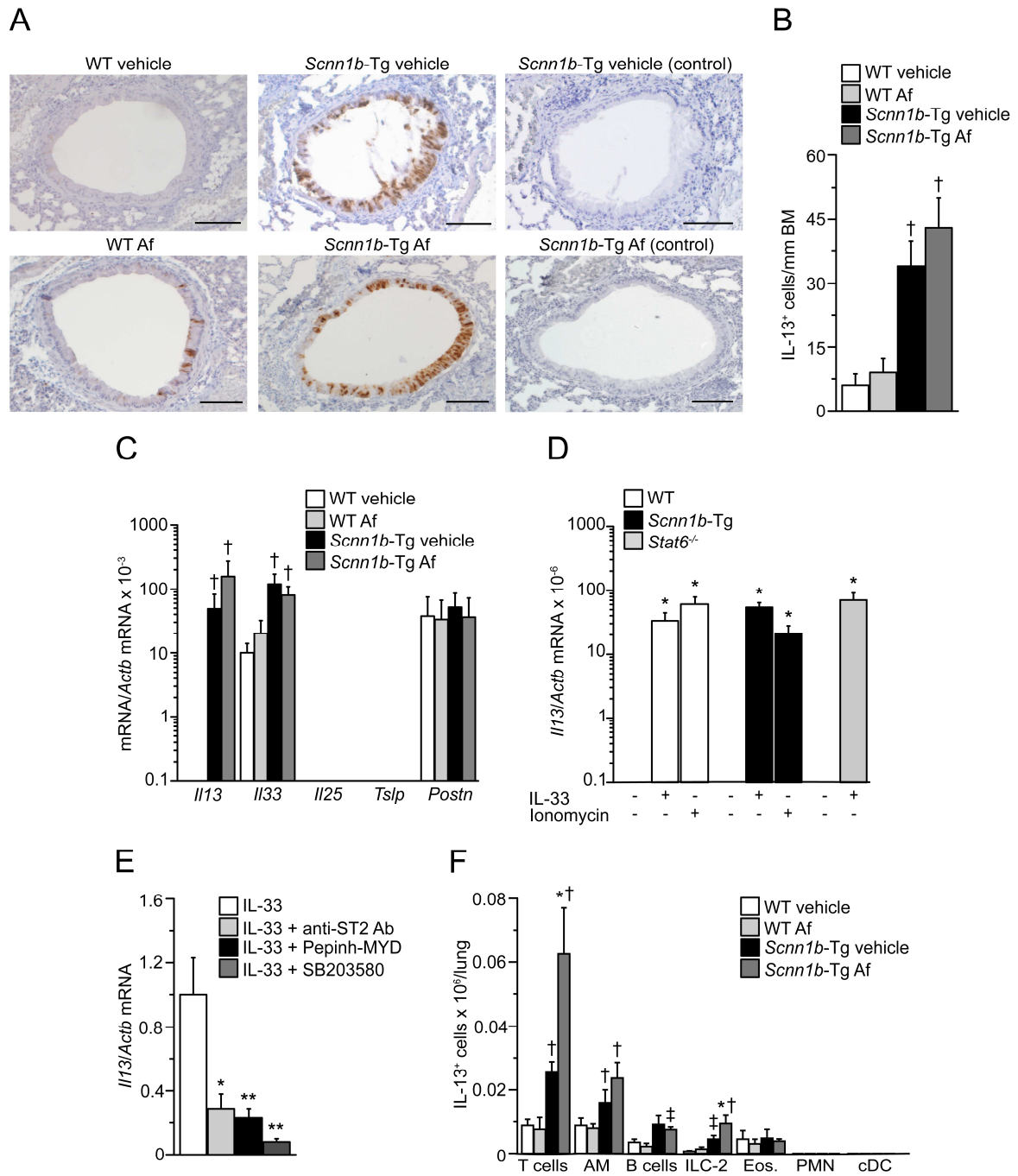


Figure 6

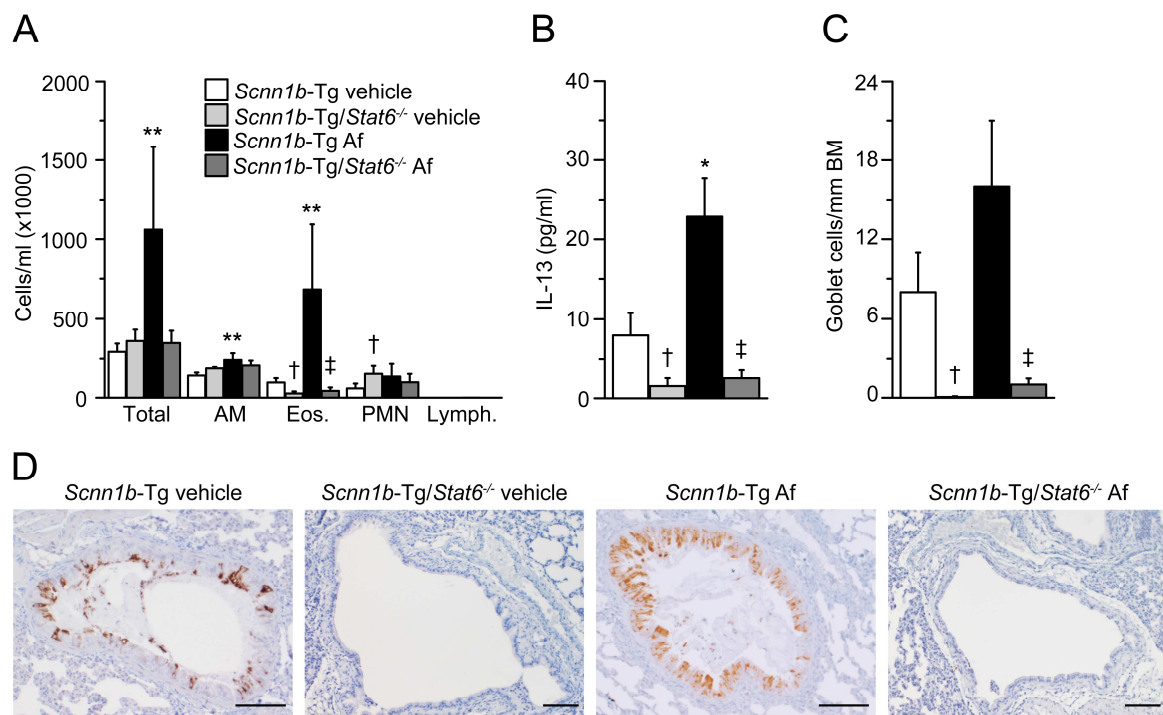
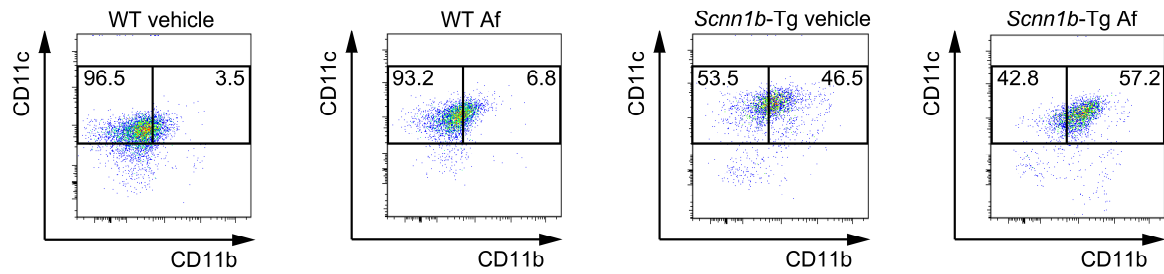
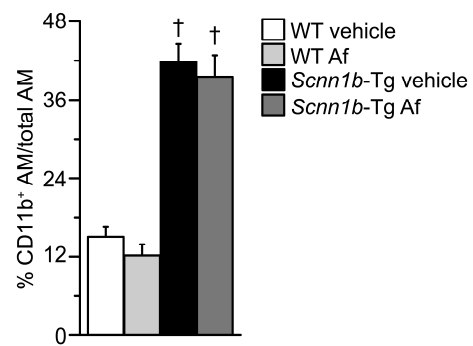


Figure 5

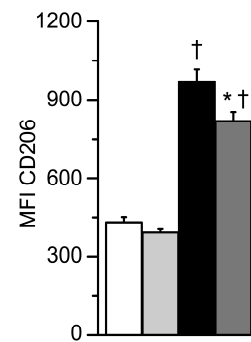
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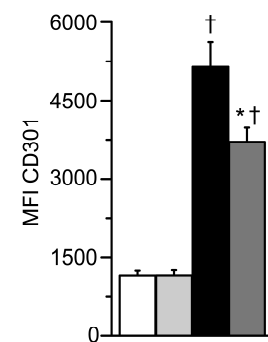
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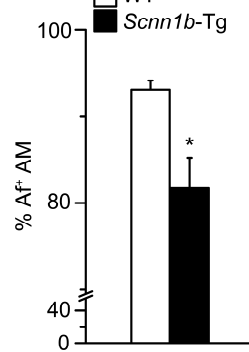
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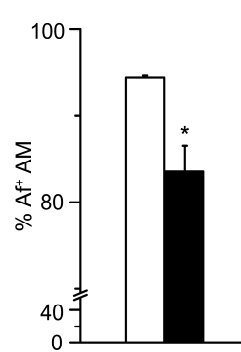
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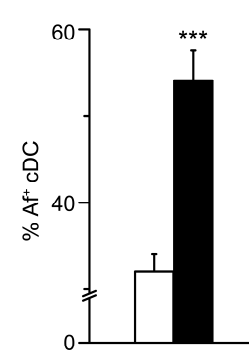
E



F



G



H

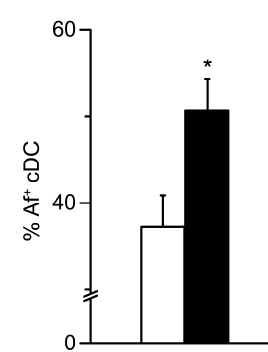
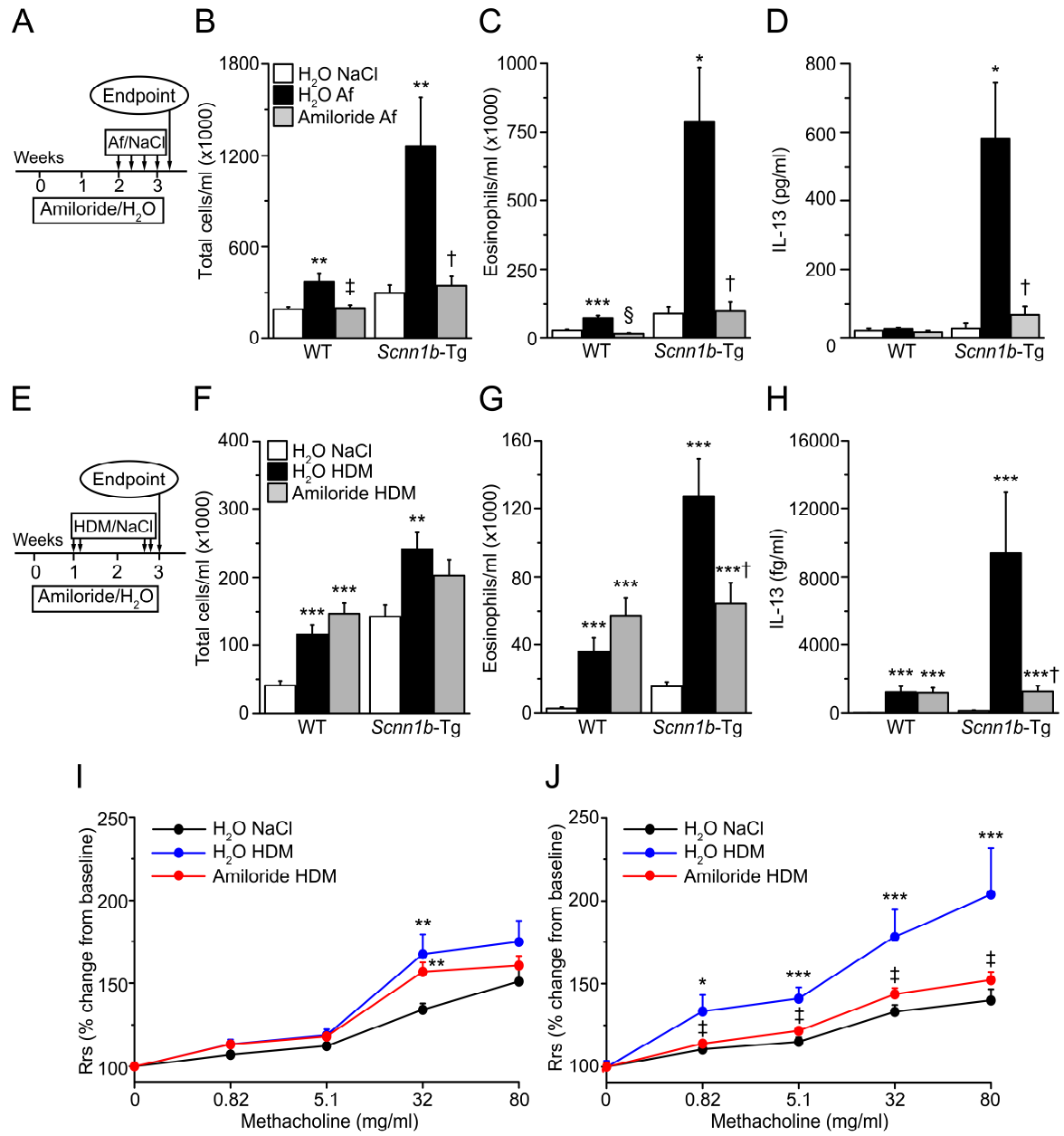


Figure 7



Impaired mucus clearance exacerbates allergen-induced type 2 airway inflammation in juvenile mice

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SUPPLEMENTAL METHODS

Experimental animals

All animal studies were approved by the Regierungspräsidium Karlsruhe, Germany (35-9185.81/G-26/14). *Scnn1b*-Tg mice were studied on a mixed genetic background (C3H/HeN x C57BL/6N) (E1, E2) or on the C57BL/6N background (E3). In the colony with mixed genetic background, *Scnn1b*-Tg mice were bred with C3B6 F1 WT mice (Charles River Laboratories, Sulzfeld, Germany) to prevent a genetic drift as previously described (E1, E2). *Stat6*^{-/-} mice (E4) obtained on the BALB/c background (Jackson Laboratories, Bar Harbor, ME) were intercrossed with *Scnn1b*-Tg mice on the mixed (C3H/HeN x C57BL/6N) background to obtain *Scnn1b*-Tg/*Stat6*^{-/-}, *Scnn1b*-Tg, *Stat6*^{-/-} and WT littermates in the F2 generation. Mice were genotyped as previously described (E1) or as recommended by Jackson Laboratories (Bar Harbor, ME, USA). Experiments were performed at neonatal (10-day-old), juvenile (2 to 3-week-old) and adult (6-week-old) ages as indicated in the figure legends and WT littermates with the respective genetic background served as controls. All animals were housed in specific pathogen-free animal facilities and had free access to chow and water.

Allergen exposure

Airway allergen exposure was performed with *Aspergillus fumigatus* extract (Af; Hollister-Stier laboratories, Spokane, WA) or house dust mite (*Dermatophagoides farina*) extract (HDM, Greer laboratories, Lenoir, NC) according to previously established protocols (E5-E9). In brief, mice were anesthetized with 3% isoflurane (Baxter, Deerfield, IL) in oxygen and either Af or HDM dissolved in 0.9% NaCl, or 0.9% NaCl alone were administered by i.t. instillation. In the Af model, 20 µl (juvenile mice) or 50 µl (adult mice) of Af (2 mg/ml) or 0.9% NaCl alone was administered i.t. every 48 h for a total of four times and endpoint studies were performed 24 h after the last allergen exposure. In the HDM model, juvenile mice were i.t. sensitized with 20 µl of HDM (0.5 mg/ml) or 0.9% NaCl alone on two

consecutive days followed by two i.t. challenges with 20 μ l of HDM (0.05 mg/ml) or 0.9% NaCl alone on day 11 and 12 after the first HDM exposure, and endpoint studies were performed 48 h after the last challenge dose. For HDM exposure during amiloride treatment neonatal mice were sensitized intranasally (i.n.) with 10 μ l HDM (1 mg/ml) or 0.9% NaCl alone and challenged as described above.

Amiloride treatment

Amiloride treatment studies were performed as previously described (E10). In brief, amiloride hydrochloride (Sigma-Aldrich, Taufkirchen, Germany) was dissolved in ddH₂O and newborn *Scnn1b*-Tg mice and WT littermates were anesthetized with isoflurane 3% in oxygen and treated by i.n. instillation of amiloride (10 mmol/l; 1 μ l/g body weight; 3 times per day) or equal volumes of vehicle (ddH₂O) alone for a period of three weeks. During treatment, mice were weighed three times per day to monitor for loss of body mass due to increased diuresis caused by renal effects of systemically absorbed amiloride, and volume deficits were replaced by subcutaneous injections of isotonic NaCl 0.9% as previously described (E10). In the third week of treatment, i.n. instillation was replaced by i.t. instillation, and mice were additionally exposed to Af or HDM as described above and shown in Figure 7. Endpoint studies were performed 12 h after the last amiloride treatment (Figure 7).

Airway hyperresponsiveness

Mice were anesthetized by i.p. injection of 80 mg/kg sodium pentobarbital (Sigma-Aldrich, Taufkirchen, Germany), tracheostomized and connected to a computer-controlled small animal ventilator (Flexivent, Scireq Inc., Montreal, QC, Canada) via a 19-gauge i.t. cannula. After initiating mechanical ventilation, mice were paralyzed by i.p. injection of 1.6 mg/kg pancuronium bromide (Sigma-Aldrich, Taufkirchen, Germany). Mice were exposed to PBS aerosol followed by aerosols containing increasing concentrations of methacholine (range

0.33 mg/ml to 160 mg/ml; Sigma-Aldrich, Taufkirchen, Germany) and respiratory resistance (Rrs) was determined as previously described (E11, E12). Previous studies demonstrated that absolute Rrs is age- and weight-dependent in developing mice and mean values drop from ~4 cmH₂O.s/ml to ~2 cmH₂O.s/ml (i.e. ~50%) between 2 and 4 weeks of age (E7). To control for this age- and weight-dependent variability, AHR at different methacholine concentrations was determined from the percent change of Rrs from baseline. In HDM-induced *Scnn1b*-Tg mice severe airway obstruction and plugging of cannulae with mucus impeded ventilation, and experiments had to be discontinued at high methacholine concentrations in this experimental group (>32 mg/ml when *Scnn1b*-Tg mice were challenged with HDM alone [Figure 3] and >80 mg/ml in studies with concurrent instillation of ddH₂O 3 times per day [Figure 7]).

BAL cell counts and cytokine measurements

Mice were deeply anesthetized via i.p. injection of 120 mg/kg ketamine and 16 mg/kg xylazine (Sigma-Aldrich, Taufkirchen, Germany), exsanguinated and the trachea was cannulated. Lungs were lavaged with PBS using a weight-adjusted volume of 0.035 ml/g body weight. This weight-adjusted lavage volume approximates the vital capacity of the developing rodent lung and was used to account for differences in lung size of the age populations of mice included in our study (E13, E14). BAL was repeated twice and both fractions were pooled, centrifuged at 300 × g for 5 min at 4°C and the cell-free supernatant was collected and stored at -80°C. BAL total and differential cell counts were performed and cell numbers were determined per ml lavage fluid to improve comparability of results from developing mice with different lung sizes as previously described (E2, E10). Concentrations of type 2 cytokines IL-4, IL-5 and IL-13 were measured in BAL supernatants using ELISA (R&D Systems, Minneapolis, MN) or cytometric bead array enhanced sensitivity flex set (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions.

Mucociliary clearance

Mice were anesthetized by i.p. injection of 80 mg/kg sodium pentobarbital (Sigma-Aldrich, Taufkirchen, Germany), the trachea was exposed by a median anterior neck incision and the anterior wall was opened longitudinally to expose the mucosal surface. Yellow-green fluorescent microspheres (Thermo Fisher Scientific, Waltham, MA) with a diameter of 1.1 μm from a 2% solids solution (approximately 3.6×10^{10} microspheres/ml) were applied to the mucosal surface of the pars membranacea of the distal trachea. MCC was determined from imaging the elimination of fluorescent beads towards the larynx using a DFC3000 G camera coupled to a MZ10 F modular stereo microscope and analyzed using Application Suite X software (Leica, Wetzlar, Germany). For quantification of mucociliary clearance, the decrease in bead-derived fluorescent intensity was measured at the area of bead application and % clearance at $t = i$ min was calculated by $[1 - \text{fluorescence intensity at } t = i / \text{fluorescence intensity at } t = 0] \times 100$.

Allergen clearance

Allergen clearance from the whole lung and the airway lumen was assessed by measuring the elimination of fluorescently labeled Af extract following i.t. instillation. Af extract was fluorescently labeled with Alexa Fluor 647 Microscale Protein Labeling Kit (Life Technologies, Darmstadt, Germany) according to the manufacturer's instructions. Mice were anesthetized with isoflurane 3% in oxygen and 2.5 μg of fluorescently labeled Af extract was administered in a volume of 20 μl dissolved in PBS by i.t. instillation. To determine whole lung allergen clearance mice were killed by exsanguination after deep anesthesia via i.p. injection of 120 mg/kg ketamine and 16 mg/kg xylazine (Sigma-Aldrich, Taufkirchen, Germany) either immediately ($t = 0$) or 1 h after allergen instillation. Lungs and tracheae were immediately excised en bloc, placed in PBS with proteinase inhibitor (Roche, Basel, Switzerland), homogenized and fluorescence intensity was measured in a plate reader (Perkin

Elmer, Waltham, MA). Baseline ($t = 0$) fluorescence intensity (arbitrary units) did not differ between WT (10525 ± 953 , $n = 6$) and *Scnn1b*-Tg mice (10896 ± 877 , $n = 5$, $P = 0.78$). To determine allergen clearance from the airway lumen, BAL was performed and fluorescence intensity measured in BAL supernatant. Baseline ($t = 0$) fluorescence intensity (arbitrary units) did not differ between WT (17658 ± 1949 , $n = 6$) and *Scnn1b*-Tg mice (17492 ± 1677 , $n = 7$, $P = 0.87$). For quantification of whole lung and airway allergen clearance, the decrease in allergen-derived fluorescent intensity was measured in lung homogenates and BAL supernatants, respectively, and the % clearance after $t = 1$ h was calculated by $[1 - \text{fluorescence intensity at } t = 1 \text{ h} / \text{fluorescence intensity at } t = 0] \times 100$.

Allergen uptake

Uptake of Af extract by AM and cDC in vivo was determined as previously described (E15, E16). In brief, mice were anesthetized with isoflurane 3% in oxygen, 2.5 μg of Af extract labeled with Alexa Fluor 647 was administered in a volume of 20 μl dissolved in PBS by i.t. instillation, and BAL was performed 4 h after instillation and processed as described below for flow cytometry. Allergen uptake by alveolar macrophages and cDC was assessed by determining the frequency of Af-Alexa Fluor 647 positive cells using a BD LSRFortessa cell analyzer and FACSDiva software v8.0.1 (BD Biosciences, Heidelberg, Germany). For in vitro allergen uptake BAL was performed, BAL cells were immediately washed with PBS, 50000 cells were suspended in 100 μl RPMI-1640 medium (GE Healthcare Life Science, South Logan, UT), and 1.25 μg labeled Af extract was added in a volume of 10 μl PBS. Cells were incubated for 4 h and allergen uptake by AM and cDC was assessed as described for in vivo allergen uptake.

Flow cytometry

Multiparameter flow cytometry was performed using a LSRFortessa flow cytometer (BD Biosciences, Heidelberg, Germany). For isolation of leukocytes from the lungs, mice were deeply anesthetized via i.p. injection of 120 mg/kg ketamine and 16 mg/kg xylazine, killed by exsanguination, the lungs were perfused with PBS, removed and placed in RPMI-1640 medium (GE Healthcare Life Science, South Logan, UT) on ice for immediate processing. Each lung was removed from RPMI-1640 medium, placed into a petri dish, minced with scissors into pieces no larger than 2-3 mm, transferred to a 50 ml conical tube with 10 ml of PBS containing 300 U/ml collagenase type II (Worthington Biochemical Corp., Lakewood, NJ) and 0.15 mg/ml DNase I (Sigma-Aldrich, Taufkirchen, Germany), and incubated on a shaker with an orbital speed of 125 rpm for 30 min at 37°C as previously described (E17-E19). Digested lung solutions were passed through a 100 μ m mesh and subsequently a 40 μ m mesh to obtain single-cell suspensions. Red blood cells were lysed with red blood cell lysis buffer (BD Pharm Lyse, BD Biosciences, Heidelberg, Germany) and single cells were finally resuspended in RPMI-1640 medium. For detection of intracellular IL-13 expression aliquots of single cell suspensions containing 1 to 1.5×10^6 cells were loaded into 96-well plates and restimulated in vitro with 50 ng/ml PMA and 1 μ g/ml ionomycin (Sigma-Aldrich, Taufkirchen, Germany) in the presence of BD Golgi-Stop (BD Biosciences, Heidelberg, Germany) for 4 h according to the manufacturer's instructions. Next, cells were washed with PBS containing 1% inactivated FBS (Gibco Life Technologies, Darmstadt, Germany) and 0.09% sodium azide (Sigma-Aldrich, Taufkirchen, Germany). Prior to surface staining with specific monoclonal fluorochrome-conjugated antibodies or respective isotype control antibodies, cells were incubated with FcBlock (BD Biosciences, Heidelberg, Germany). For detection of IL-13⁺ T cells (CD45⁺CD3⁺CD19⁻), B cells (CD45⁺CD3⁻CD19⁺), AM (CD45⁺F4/80⁺CD11c^{hi}CD3⁻CD19⁻) and cDC (CD45⁺F4/80⁻CD11c⁺MHCII^{hi}CD3⁻CD19⁻), cells were stained with CD45-PE-CF594, CD3-V500, CD19-FITC, CD11c-PE-Cy7, CD11b-BV605 and F4/80-APC-Cy7, Gr-1-PerCP-Cy5.5 and MHCII-eFluor 450, CD301-Alexa Fluor

647 and CD206-PE or IL-13-PE antibodies. For detection of IL-13⁺ eosinophils (CD45⁺CD11c^{low}SiglecF⁺CD3⁻CD19⁻) and neutrophils (CD45⁺Gr1^{hi}CD11b⁺CD3⁻CD19⁻), cells were stained with CD45-PE-CF594, CD3-V500, CD19-FITC, CD11c-PE-Cy7, Siglec-F-PE, CD11b-BV605 and F4/80-APC-Cy7, Gr-1-PerCP-Cy5.5, MHCII-eFluor 450 and IL-13-APC antibodies. For detection of IL-13⁺ ILC-2 (CD45⁺LIN⁻CD25⁺CD117⁺CD127⁺Sca-1⁺T1/ST2⁺), cells were stained with antibodies against lineage markers CD11c-APC, CD3ε-V500, F4/80-APC, CD19-eVolve605 and CD49b-APC, FcεRIα-APC and CD11b-BV605 and CD25-PerCP-Cy5.5, CD45-V450, CD117-PE-Cy7, CD127-PE-CF594, T1/ST2-FITC, Sca1-Alexa Fluor 700 and IL-13-PE antibodies. Cell surface staining was performed for 30 min at 4°C as previously described (E17, E18). For intracellular detection of IL-13, BD cytofix/cytoperm solution and BD perm/wash solution (BD Biosciences, Heidelberg, Germany) were applied according to the manufacturer's instructions and cells incubated for 30 min at 4°C with antibody against IL-13. For isolation of leukocytes from BAL, cells were incubated in PBS containing 1.5% sputolysin (Merck, Darmstadt, Germany) for 15 min, repeatedly resuspended and washed in PBS to release cells from mucus prior to staining. Total cell numbers and viability were assessed by trypan blue exclusion test. Cell surface staining for AM and cDC was performed as described above. For detection of CD4⁺ T cells (CD3⁺,CD4⁺, CD8⁻) in BAL, cells were stained with CD3-V500, CD4-FITC, and CD8-Alexa Fluor 700. All antibodies were obtained from BD Biosciences (Heidelberg, Germany), Biolegend (San Diego, CA), eBioscience (San Diego, CA), Bio-Rad (Munich, Germany) and mdbioproducts (St. Paul, MN). Flow cytometry was performed immediately after cellular staining with exclusion of non-viable cells and doublets, and data were analyzed with FACS Diva software v8.0.1 (BD Biosciences, Heidelberg, Germany) or Flow Jo software v10 (Treestar, Ashland, Oregon).

Histology and airway morphology

Lungs were removed through a median sternotomy, fixed in 4% buffered formalin, and embedded in paraffin. To determine goblet cell numbers, left lungs were sectioned transversally at the level of the proximal intrapulmonary main axial airway and 1.5 μ m sections were stained with Alcian blue periodic acid-Schiff (Sigma-Aldrich, Taufkirchen, Germany). Goblet cells were identified by the presence of intracellular Alcian blue periodic acid-Schiff positive material, and numeric densities were quantified by counting the number of goblet cells per mm of the basement membrane using cell F analysis software (Olympus, Hamburg, Germany) as described (E2).

Laser micro-dissection

For laser micro-dissection, lungs were frozen in liquid nitrogen after inflation with tissue-tek O.C.T. compound (Sakura Finetek, Torrance, CA), the right superior lobes were sectioned transversally at 10 μ m thickness and mounted on MembraneSlides 1.0 PEN (Zeiss, Jena, Germany). Airway epithelium was micro-dissected and catapulted in an adhesive cap of a microfuge tube (Zeiss, Jena, Germany) using the MicroBeam system (PALM Microlaser Technologies, Bernried, Germany) consisting of a 337 nm nitrogen laser integrated into an inverted Axiovert 200 microscope (Zeiss, Jena, Germany). Micro-dissected tissue was incubated in lysis buffer and total RNA was isolated using RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Immunohistochemistry

Immunohistochemical staining for IL-13 was performed on formalin-fixed, paraffin embedded lung sections using a polyclonal goat anti-mouse IL-13 antibody (R&D Systems, Minneapolis, MN) (E20) at a dilution of 1:1000. Unstained and hydrated paraffin sections were pretreated for 5 min with 3% hydrogen peroxide, followed by antigen retrieval and incubation with a non-specific protein-blocking solution containing animal serum (Vector

Laboratories Inc., Burlingame, CA) as previously described (E21). Lung sections were incubated over night at 4°C with the primary antibody. A biotinylated rabbit anti-goat IgG was used as a secondary antibody and immunoreactivity of IL-13 was visualized with Vectastain Elite Kit ABC Reagent followed by DAB (Vector Laboratories Inc., Burlingame, CA) and hematoxylin (Sigma-Aldrich, Taufkirchen, Germany) counterstaining. As negative control, each experiment included lung sections stained with secondary antibody only. Numeric densities of IL-13⁺ cells within the airway epithelium per mm of the basement membrane were determined on images taken with an Olympus IX71 microscope interfaced with a DP73 camera using cell F analysis software (Olympus, Hamburg, Germany) as described (E2).

Primary tracheal epithelial cultures

Mice were deeply anesthetized via i.p. injection of ketamine (120 mg/kg) and xylazine (16 mg/kg), killed via exsanguination and the tracheae were removed, and epithelial cells were isolated and cultured on membranes (Costar, Cambridge, MA) under ALI conditions as previously described (E1). In brief, excised tracheae were cut open, transferred to collection medium containing DMEM/F-12 (1:1) (Gibco Life Technologies, Darmstadt, Germany) and 1% Penicillin-Streptomycin, washed and transferred to 20 ml buffered dissociation medium containing 1.4 mg/ml protease E and 0.1 mg/ml DNase I (Sigma-Aldrich, Taufkirchen, Germany), and incubated overnight at 4°C. After stopping enzymatic digestion with heat-inactivated FBS (Gibco Life Technologies, Darmstadt, Germany) cell suspensions were filtered through a 100 µm cell strainer and resuspended in DMEM/F-12 (1:1) medium supplemented with heat-inactivated FBS, insulin (human recombinant zinc) (Gibco Life Technologies, Darmstadt, Germany) and primocin (Invivogen, Toulouse, France) and incubated at 37°C for 2 h. Epithelial cells were seeded on collagen IV-coated Costar transwell filters (Corning, Glendale, AZ) and ALI conditions were established. After reaching

confluency, recombinant mouse IL-33 (10 ng/ml, R&D Systems, Wiesbaden, Germany) or ionomycin (1 µg/ml, Sigma-Aldrich, Taufkirchen, Germany) were added to the basolateral side for 24 h or 4 h, respectively. For pharmacological inhibition of the IL-33/ST2/Myd88 signaling pathway ALI cultures were pre-incubated for 1 h with anti-ST2 antibody (30 µg/ml; R&D systems, Wiesbaden, Germany), 6 h with the MyD88 inhibitory peptide Pepinh-MYD (50 µM; InvivoGen, San Diego, CA), or 1 h with the p38 MAP kinase inhibitor SB203580 (20 µM; InvivoGen, San Diego, CA), and subsequently stimulated with IL-33 (10 ng/ml) for 3 h. Before RNA isolation, cultures were washed with PBS and total RNA was extracted using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Real-Time RT-PCR

Lungs were stored in RNAlater (Applied Biosystems, Darmstadt, Germany) and micro-dissected airway epithelial cells and primary tracheal epithelial cultures in lysis buffer (Qiagen, Hilden, Germany), and total RNA was isolated using Trizol reagent (Gibco Life Technologies, Darmstadt, Germany), RNeasy Micro Kit or RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), respectively. RNA integrity was verified by agarose gel electrophoresis, and cDNA was obtained by reverse transcription (Superscript III RT; Invitrogen, Karlsruhe, Germany). Real-time qPCR was performed on an Applied Biosystems 7500 Real Time PCR system using TaqMan Universal PCR master mix and inventoried TaqMan gene expression assays (Applied Biosystems, Darmstadt, Germany) for *Muc5ac* (Mm01276718_m1), *Muc5b* (Mm00466391_m1), *Gob5* (Mm01320697_m1), *Il13* (Mm00434204_m1), *Il33* (Mm00505403_m1), *Il25* (Mm0049822_m1), *Tslp* (Mm00498739_m1), *Postn* (Mm00450111_m1), *Mmp12* (Mm00500554_m1), *Chia* (Mm00458221_m1), *Ym1* (Mm00657889_mH), *Ym2* (Mm00840870_m1), *Arg1* (Mm00475988_m1), and *Actb* (Mm00607939_s1). Relative fold changes of target gene expression were calculated from the

efficiency of the PCR reactions, the crossing point deviation between samples from the different experimental groups and vehicle-treated WT controls, and normalization to the expression of the reference gene *Actb* as previously described (E2, E22). For analysis of micro-dissected airway epithelium, gene specific preamplification was performed prior to qPCR using Platinum Taq DNA Polymerase and the following intron-spanning primers:

Il13, 5'-CTGTGTCTCTCCCTCTGACCC-3' (sense)

and 5'-CCAGGGCTACACAGAACCCG-3' (antisense);

Il33, 5'-ACACATTGAGCATCCAAGGAAC-3' (sense)

and 5'-AAGAAGGCCTGTTCCGGAGG-3' (antisense);

Il25, 5'-TGTACCAGGCTGTTGCATTCT-3' (sense)

and 5'-TAAAGTGGGACGGAGTTGCC-3' (antisense)

Tslp, 5'-GGACTGTGAGAGCAAGCCAG-3' (sense)

and 5'-GGTCCTCTAAGTGTACACCC-3' (antisense)

Postn, 5'-TGTGCCCTCCAGCAAATTCT-3' (sense)

and 5'-TGCGAATGTCAGAATCCAGGT-3' (antisense)

Actb, 5'-AGATCAAGATCATTGCTCCTCCT-3' (sense)

and 5'-TGCTCCAACCAACTGCTGTC-3' (antisense).

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SUPPLEMENTAL FIGURES

Figure E1

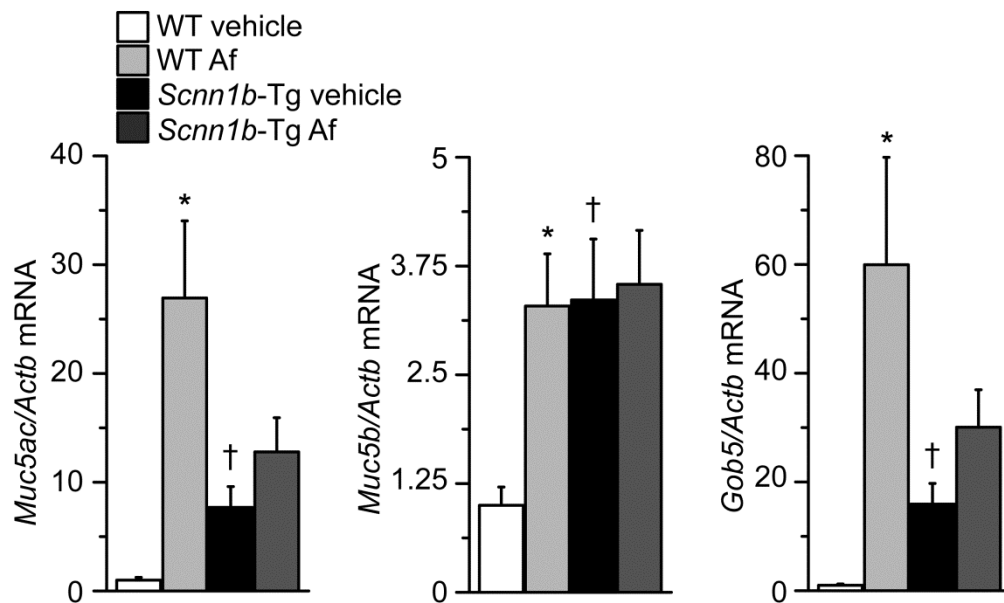


Figure E1. Effects of Af challenge on mucin expression in lungs of juvenile WT and *Scnn1b*-Tg mice. Expression of *Muc5ac*, *Muc5b* and *Gob5* in lungs of juvenile (2 to 3-week-old) WT and *Scnn1b*-Tg mice challenged with Af or vehicle alone. $n = 8$ for each group. * $P < 0.001$ compared to vehicle-treated mice of same genotype and † $P < 0.001$ compared to mice with same treatment but different genotype.

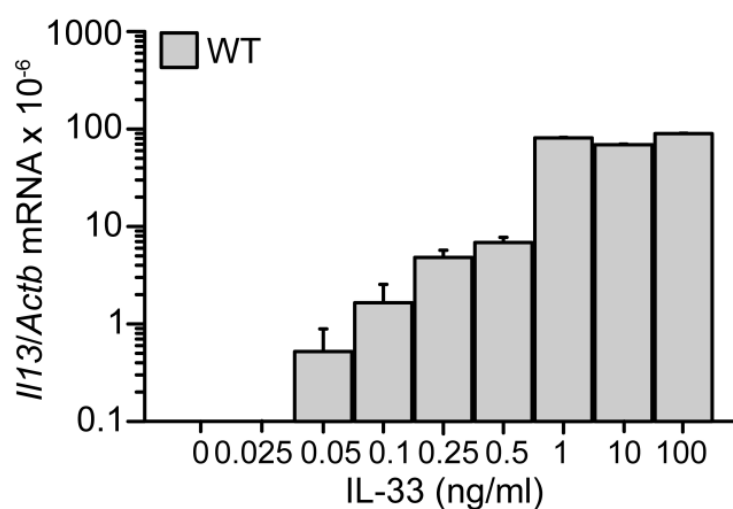
Figure E2

Figure E2. Dose-dependent induction of *Il13* expression by IL-33 in primary tracheal epithelial cultures from WT mice. Primary tracheal epithelial cultures were grown under air-liquid interface (ALI) conditions and IL-33 was added to the basolateral side. $n = 3-10$ for each concentration.