

IL-33 promotes airway remodeling in pediatric patients with severe steroid-resistant asthma

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Background: T_H2 cytokines are not responsible for the ongoing symptoms and pathology in children with severe therapy-resistant asthma (STRA). IL-33 induces airway hyperresponsiveness, but its role in airway remodeling and steroid resistance is unknown.

Objective: We sought to investigate the relationship between IL-33 and airway remodeling in pediatric patients with STRA.

Methods: IL-33 levels were quantified in neonatal mice given inhaled house dust mite (HDM), and the effect of blocking IL-13 on remodeling and IL-33 levels was assessed. HDM-induced allergic airways disease (AAD) in neonatal ST2^{-/-} mice lacking the IL-33 receptor was assessed, together with collagen production after IL-33 administration. The effect of steroid therapy on IL-33 levels in patients with neonatal AAD was explored. IL-33 expression was quantified in endobronchial biopsy (EB) specimens from children with STRA and related to remodeling, and collagen production by airway fibroblasts from pediatric patients stimulated with IL-33 and budesonide was quantified.

Results: Blocking IL-13 after AAD was established in neonatal mice and did not reduce remodeling or IL-33 levels; airway hyperresponsiveness was only partially reduced. IL-33 promoted collagen synthesis both from asthmatic fibroblasts from pediatric patients and after intranasal administration in mice. Increased cellular expression of IL-33, but not IL-13, was associated with increased reticular basement membrane thickness in EB specimens from children with STRA, whereas remodeling was absent in HDM-exposed ST2^{-/-} mice. IL-33 levels were maintained, whereas IL-13 levels were abrogated by

steroid treatment in neonatal HDM-exposed mice and in EB specimens from children with STRA.

Conclusion: IL-33 is a relatively steroid-resistant mediator that promotes airway remodeling in patients with STRA and is an important therapeutic target. (*J Allergy Clin Immunol* 2013;■■■:■■■-■■■.)

Key words: Asthma, pediatric, airway remodeling, steroid resistance, IL-33, therapy

Children with severe therapy-resistant asthma (STRA) represent a small number of all asthmatic patients, yet they use a disproportionate amount of health care resources¹ and present a significant clinical challenge because they remain symptomatic despite maximal doses of conventional therapy.

The key pathophysiologic abnormalities of asthma include airway hyperresponsiveness (AHR), inflammation, and tissue remodeling, including increased thickness of the reticular basement membrane (RBM)² and increased smooth muscle mass.³ We have shown that children with STRA have ongoing symptoms, abnormal lung function, and evidence of airway remodeling in the absence of the classical T_H2 cytokines (IL-4, IL-5, and IL-13) that are thought to mediate allergic disease in adults.⁴ However, the high-dose maintenance steroid therapy that is prescribed in patients with severe disease might attenuate these relatively steroid-sensitive cytokines.⁵⁻⁷ Critically, even in adults, severe asthma is recognized as being heterogeneous, with the T_H2 pathway being active in only select subgroups, as demonstrated by the variable efficacy of anti-IL-13 antibody therapy.⁸ A significant limitation of current asthma therapies is their lack of effect on any features of airway remodeling, even though these structural changes contribute to disease chronicity. Specific steroid-resistant mediators driving airway remodeling in patients with STRA therefore require elucidation.

The importance of innate immune responses in asthma pathogenesis has become increasingly apparent,^{9,10} with particular emphasis on the innate cytokine IL-33.¹¹ IL-33 is expressed in the epithelium and smooth muscle of adults with severe asthma.^{12,13} It is also sufficient to initiate allergic airway responses in adult mice lacking T and B cells through the induction of IL-13-producing innate helper cells.^{14,15} At present, little is known about the role of IL-33 in the pathophysiology of childhood asthma. Specifically, the contribution of IL-33 to airway remodeling and steroid resistance has not been investigated, although both are critical features of severe disease. Because the T_H2 cytokines were not predominant in our patients with STRA,⁴ we hypothesized that IL-33 induces airway remodeling and is a steroid-resistant mediator in pediatric patients with STRA. We have investigated this using a neonatal mouse model of house dust mite (HDM)-induced allergic airways disease

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Abbreviations used

AAD: Allergic airways disease
 AHR: Airway hyperresponsiveness
 BAL: Bronchoalveolar lavage
 EB: Endobronchial biopsy
 HDM: House dust mite
 RBM: Reticular basement membrane
 STRA: Severe therapy-resistant asthma

(AAD) and endobronchial biopsy (EB) specimens and cultured airway fibroblasts from children with STRA. For the first time, we demonstrate the relationship between IL-33 levels and airway remodeling and identify IL-33 as a therapeutic target for severe steroid-resistant asthma.

METHODS**Animals and reagents**

Female BALB/c mice and litters were maintained by in-house breeding in specific pathogen-free conditions and given food and water *ad libitum* (see the [Methods](#) section in this article's Online Repository at www.jacionline.org). Mice were matched for age and background strain. All procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986. Recombinant mouse IL-33 (50 µg/kg per mouse) for intranasal administration was purchased from eBioscience (Hatfield, United Kingdom). Anti-IL-13 antibody was a gift¹⁶ and 10 mg/kg was administered intraperitoneally twice weekly. Relevant isotype-matched antibodies were used as controls. Budesonide Respules (Breath Ltd, Stevenage, United Kingdom) (0.25 mg/mL) were used for intranasal administration (0.6 mg/kg).

Allergen challenge

From day 3 of life, pups received either intranasal HDM extract (Greer, Lenoir, NC) or PBS 3 times per week, according to our published protocol.¹⁷ For more information, see the [Methods](#) section in this article's Online Repository.

Measurement of AHR

Airways resistance was measured from 2 weeks of age by using the flexiVent small-animal ventilator (SCIREQ, Montreal, Quebec, Canada) according to our established protocols.¹⁷ For more information, see the [Methods](#) section in this article's Online Repository.

Inflammation and cell recovery

Bronchoalveolar lavage (BAL) was performed with PBS through a tracheal cannula, as previously described.¹⁷ Lavage fluid was centrifuged, and cell pellets were resuspended in 0.5 mL of complete medium. After lavage, 2 lobes from the right lung were dissected and processed, as previously published, to obtain lung cells.¹⁷ The total cell yield of BAL and lung cells was quantified with a hemocytometer (Immune Systems Ltd, Devon, United Kingdom). For more information, see the [Methods](#) section in this article's Online Repository.

Quantification of cytokines

Lung tissue was homogenized, and the supernatant was collected (see the [Methods](#) section in this article's Online Repository). Cytokines were analyzed in lung homogenate supernatants. Paired antibodies for mouse IL-4 and IL-5 (PharMingen, San Jose, Calif), IL-13 and IL-25 (eBioscience), and IL-33 (R&D Systems, Minneapolis, Minn) were used in standardized sandwich ELISAs, according to the manufacturer's protocol.

Airway remodeling

Lung sections stained with Sirius red and Gordon and Sweet's silver stain, as previously described,¹⁷ were used to quantify peribronchiolar collagen and reticulin levels, respectively, by using computer-aided image analysis (QWin version 3; Leica, Wetzlar, Germany). For more information, see the [Methods](#) section in this article's Online Repository.

RNA extraction and real-time PCR

Total RNA was extracted from 50 to 100 mg of lung tissue by using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 1 µg of total RNA and analyzed by using PCR on a CFX96 Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, Calif). Reactions were run with TaqMan probes (Applied Biosystems, Foster City, Calif) and SsoFast Probe Supermix (Bio-Rad Laboratories). For more information, see the [Methods](#) section in this article's Online Repository.

Human data

Subjects. School-aged children (age, 6-16 years) with STRA⁴ who were undergoing a clinically indicated bronchoscopy were recruited. Their clinical details have been published.⁴ They were prescribed 800 µg/d or greater budesonide (or equivalent). Children with mild-to-moderate asthma (inhaled corticosteroids, <800 µg/d) and age-matched nonasthmatic control subjects having a bronchoscopy for another indication were also recruited. The study was approved by the institutional ethics committee, and written informed parental consent and child assent were obtained. For clinical details of all subjects, see the [Methods](#) section in this article's Online Repository.

Histopathology. EB specimens were processed to paraffin. Five-micrometer sections were stained with hematoxylin and eosin and used to assess morphology and quantify RBM thickness⁴ and smooth muscle mass.³ Sections were stained by using immunohistochemistry for IL-13 and IL-33. Human polyclonal rabbit anti-IL-13 antibody (PeproTech, Rocky Hill, NJ; dilution, 1:50) and Human Clone IL3305B mouse mAb (Alexis Biochemicals, Enzo Life Sciences, Farmingdale, NY) used at a dilution of 1:1000 in PBS and 1% BSA by using an avidin/biotin staining. Subepithelial cells were quantified and expressed per square millimeter of tissue.

Fibroblast culture and stimulation. Primary pediatric bronchial fibroblasts were isolated by outgrowth culture and used between passages 3 and 6. Fibroblasts were grown to 80% confluence. Recombinant human IL-33 was used between 10 and 100 ng/mL, and budesonide was used at a final concentration of 10⁻⁸ mol/L. In costimulations budesonide was added to cultures for 2 hours before the addition of rIL-33. Conditioned media were taken, and cell lysates were prepared in RIPA buffer. For more information, see the [Methods](#) section in this article's Online Repository.

Western blotting and densitometry. Cell lysates were cleared by means of centrifugation at 10,000g for 10 minutes. Five micrograms of protein per well in LDS sample buffer (Invitrogen, Carlsbad, Calif) was loaded onto 4-12% Bis-Tris gels (Bio-Rad Laboratories) and run at 150V in a Criterion Cell (Bio-Rad Laboratories). α-Smooth muscle actin primary antibody (ab5694; Abcam, Cambridge, United Kingdom) was added at 1:5000 dilution in blocking buffer and incubated overnight at 4°C. Horseradish peroxidase-conjugated anti-rabbit antibody (Cell Signaling Technology, Danvers, Mass) was used at 1:2000 dilution, and membranes were developed with ECL substrate (Pierce, Cheshire, United Kingdom) for film exposure (Amersham, Little Chalfont, Bucks, United Kingdom). For more information, see the [Methods](#) section in this article's Online Repository.

Soluble collagen measurement. Secreted collagen in conditioned media was assayed with the Sircol kit (Biocolor, Carrickfergus, United Kingdom), according to the manufacturer's instructions.

Statistical analysis

Nonparametric tests (Mann-Whitney *U* tests) were used to detect differences between groups by using GraphPad Prism 4 software (GraphPad Software, La Jolla, Calif), and statistical significance was accepted at a *P* value

of less than .05. Correlations were assessed by using the Spearman correlation coefficient. For more information, see the [Methods](#) section in this article's Online Repository.

RESULTS

IL-13 and IL-33 show divergent responses with increasing duration of intranasal HDM exposure in neonatal mice

Because IL-13 has been shown to be necessary and sufficient to induce all features of AAD in adult mice,¹⁸ we investigated the relationship between IL-13 and IL-33 during the development of neonatal AAD (Fig 1, A). The longitudinal production of IL-13 and IL-33 in response to allergen in neonatal mice of increasing age (Fig 1, B and C) was strikingly different. IL-13 production started at week 1, peaked during week 2, and then decreased with increasing age (Fig 1, B). IL-33 production was similar in allergen-exposed and control neonatal mice at week 1 but increased with age in response to HDM exposure (Fig 1, C). IL-33 levels remained increased in HDM-exposed mice after 4 weeks without allergen challenge (see Fig E1 in this article's Online Repository at www.jacionline.org). In contrast, the T_H2 cytokines IL-4, IL-5, and IL-13 reduced to baseline levels after 4 weeks without allergen challenge.¹⁷ A single rechallenge with HDM after 4 weeks without allergen exposure in neonatal mice (Fig 1, D) re-established AHR (Fig 1, E), a significant further increase in IL-33 levels (Fig 1, F), and increased levels of the T_H2 cytokines IL-13 (Fig 1, G), IL-4, and IL-5 (see Fig E1). These data suggest that IL-33 levels remain increased and might mediate the persistent airway remodeling observed in the absence of continued allergen exposure.¹⁷

Therapeutic blocking of IL-13 in neonatal HDM-induced AAD does not reduce airway remodeling or IL-33 levels and only partly abrogates AHR

We have previously shown persistent airway pathology in pediatric patients with STRA, despite the absence of IL-13.⁴ Blocking the action of IL-13 in our neonatal mouse model of HDM-induced AAD after disease was established (Fig 2, A) only partly reduced AHR (Fig 2, B and C) and had no effect on airway inflammation (see Fig E2 in this article's Online Repository at www.jacionline.org) or airway remodeling, as demonstrated based on peribronchiolar reticulin or collagen deposition (Fig 2, D and E). We next found that lung IL-33 levels remained increased despite blocking IL-13 therapeutically (Fig 2, G). In contrast, blocking IL-13 with a prevention regimen before AAD was established completely abrogated AHR to control levels and significantly reduced airway remodeling (see Fig E2). This suggests IL-33 might contribute toward maintaining the critical features of asthma, including AHR, inflammation, and airway remodeling, in the absence of IL-13.

Allergen challenge in neonatal ST2^{-/-} mice does not generate AHR or airway remodeling, whereas intranasal IL-33 induces AHR and promotes pulmonary collagen secretion

The relationship between IL-33 levels, AHR, and airway remodeling was investigated by administration of high-dose allergen to neonatal mice lacking a functional receptor for

IL-33 (ST2^{-/-} mice) for 5 weeks (Fig 3, A). HDM exposure from day 3 of life did not cause AHR (Fig 3, B), increased goblet cell hyperplasia (data not shown), or increased peribronchiolar collagen deposition in ST2^{-/-} mice (Fig 3, C). However, there was increased total BAL inflammation (see Fig E3 in this article's Online Repository at www.jacionline.org), suggesting this is not IL-33 dependent.

Intranasal IL-33 was administered to 6-week-old BALB/c mice for 2 weeks to assess whether IL-33 might contribute to AHR and airway remodeling (Fig 3, D). AHR was significantly increased compared with control values and was significantly greater at week 2 than week 1 (Fig 3, E). Lung collagen levels were significantly increased after 2 weeks of IL-33 administration (Fig 3, F), and at the same time point, fibronectin mRNA was significantly upregulated (Fig 3, G). Eosinophilic inflammation (see Fig E3) and levels of the T_H2 cytokines IL-5, IL-4, and IL-13 also increased after rIL-33 (see Fig E3). These data suggest that IL-33 promotes AHR and that it contributes to collagen production (which is the main component of the thickened subepithelial RBM in asthmatic patients¹⁹) either directly or through the T_H2 cytokines and eosinophilic inflammation.

IL-33 expression is significantly increased in submucosal inflammatory cells in pediatric patients with STRA

We determined IL-33 and IL-13 expression in EB specimens from children with severe asthma. Although IL-13⁺ cells were similar in the submucosa of asthmatic patients and control subjects (Fig 4, A, C, and E), IL-33⁺ cell numbers were significantly increased in the submucosa of asthmatic patients compared with control subjects (Fig 4, B, D, and F). In contrast, IL-33 expression was similar in the epithelium and smooth muscle (see Fig E4 in this article's Online Repository at www.jacionline.org) of asthmatic patients and control subjects. These data suggest children with STRA who are receiving high-dose steroid therapy have persistent symptoms and poor control despite an absence of tissue IL-13 but have increased IL-33 expression, which might be a more important mediator of disease pathophysiology.

IL-33 promotes collagen secretion from asthmatic airway fibroblasts of pediatric patients

Because children with STRA had increased IL-33 levels and airway remodeling is a key feature of the disease,⁴ we explored the relationship between IL-33 expression and airway remodeling in children with STRA. There was a positive correlation between mucosal IL-33 expression and RBM thickness in children with STRA (Fig 5, A); there were too few nonasthmatic control subjects to explore any relationship in this group. To investigate the functional relationship between IL-33 and remodeling, we assessed the response of airway fibroblasts from children with STRA to IL-33 *in vitro*. Cultured fibroblasts produced increased collagen levels after stimulation with IL-33. Although the addition of budesonide significantly decreased collagen secretion, increased secretion was maintained with both budesonide and IL-33 (Fig 5, B). The time at which the maximal effects of IL-33 were seen in asthmatic children varied from 24 up to 72 hours after stimulation (see Fig E5 in this article's Online Repository at www.jacionline.org). Collagen secretion in response to IL-33 was only apparent in fibroblasts cultured from asthmatic patients,

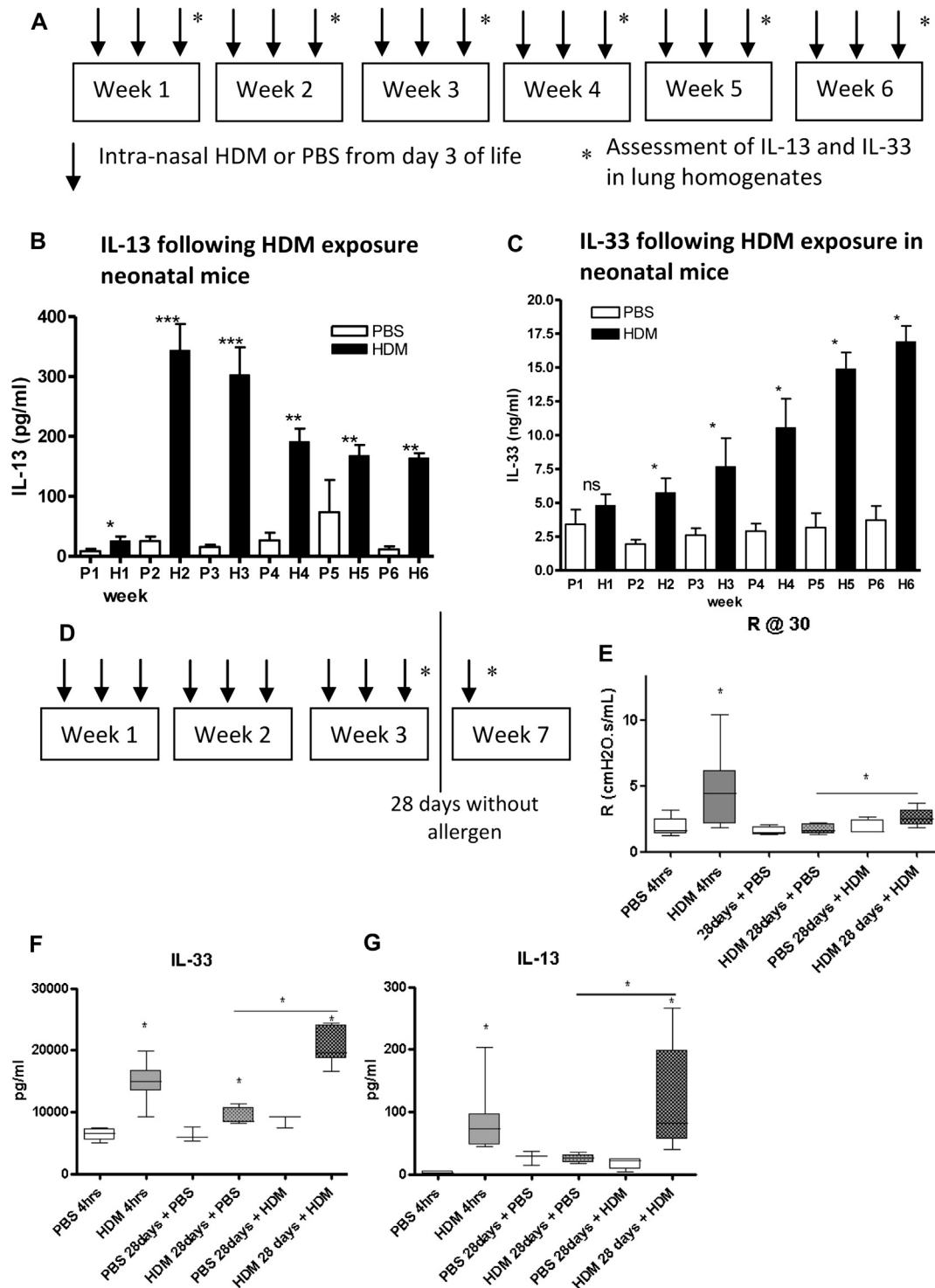


FIG 1. A, Experimental plan of intermittent HDM or saline exposure. B and C, IL-13 and IL-33 levels in lung homogenates from HDM-exposed and control mice. D, Experimental plan to assess the effects of allergen exposure for 3 weeks, followed by the absence of allergen challenge for 28 days, in neonatal mice. E, Airway resistance at 30 mg/mL methacholine in neonatal mice after 3 weeks of allergen exposure (4 hours after last challenge) and 28 days without allergen challenge. F and G, Lung IL-33 and IL-13 levels after 3 weeks of allergen exposure (4 hours after last dose) and 28 days without allergen. Data are representative of 2 experiments (n = 4-6 for control mice and n = 6-8 for HDM-exposed mice). **P* < .05, ***P* < .01, and ****P* < .001.

whereas fibroblasts from healthy adults did not show any response to IL-33 (Fig 5, C). The relationship between IL-33 and remodeling only applied to a specific feature of airway remodeling,

increased RBM thickness, and not smooth muscle mass (see Fig E5). Fibroblasts from pediatric asthmatic patients stimulated with IL-33 did not express α -smooth muscle actin, showing

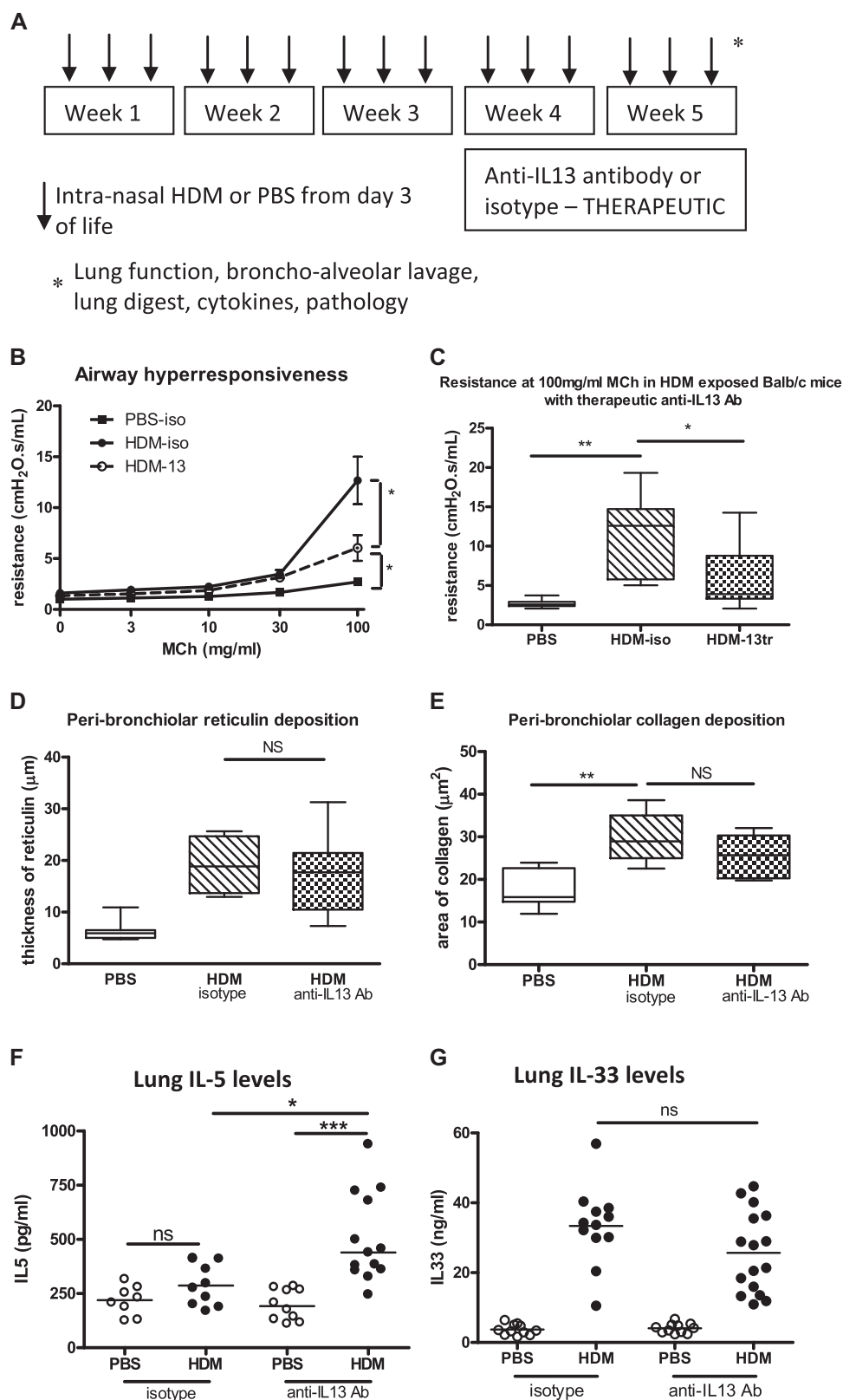


FIG 2. A, IL-13 was blocked in HDM-exposed neonatal mice from weeks 3 to 5. B and C, Airway resistance in HDM-exposed mice after blocking with anti-IL-13 antibody. D and E, Peribronchiolar reticulin and collagen deposition. F and G, IL-5 and IL-33 levels after blocking IL-13. Data are representative of 2 experiments ($n = 4-6$ for control mice and $n = 6-8$ for HDM-exposed mice). Horizontal bars represent medians. MCh, Methacholine; NS, not significant. * $P < .05$, ** $P < .01$, and *** $P < .001$.

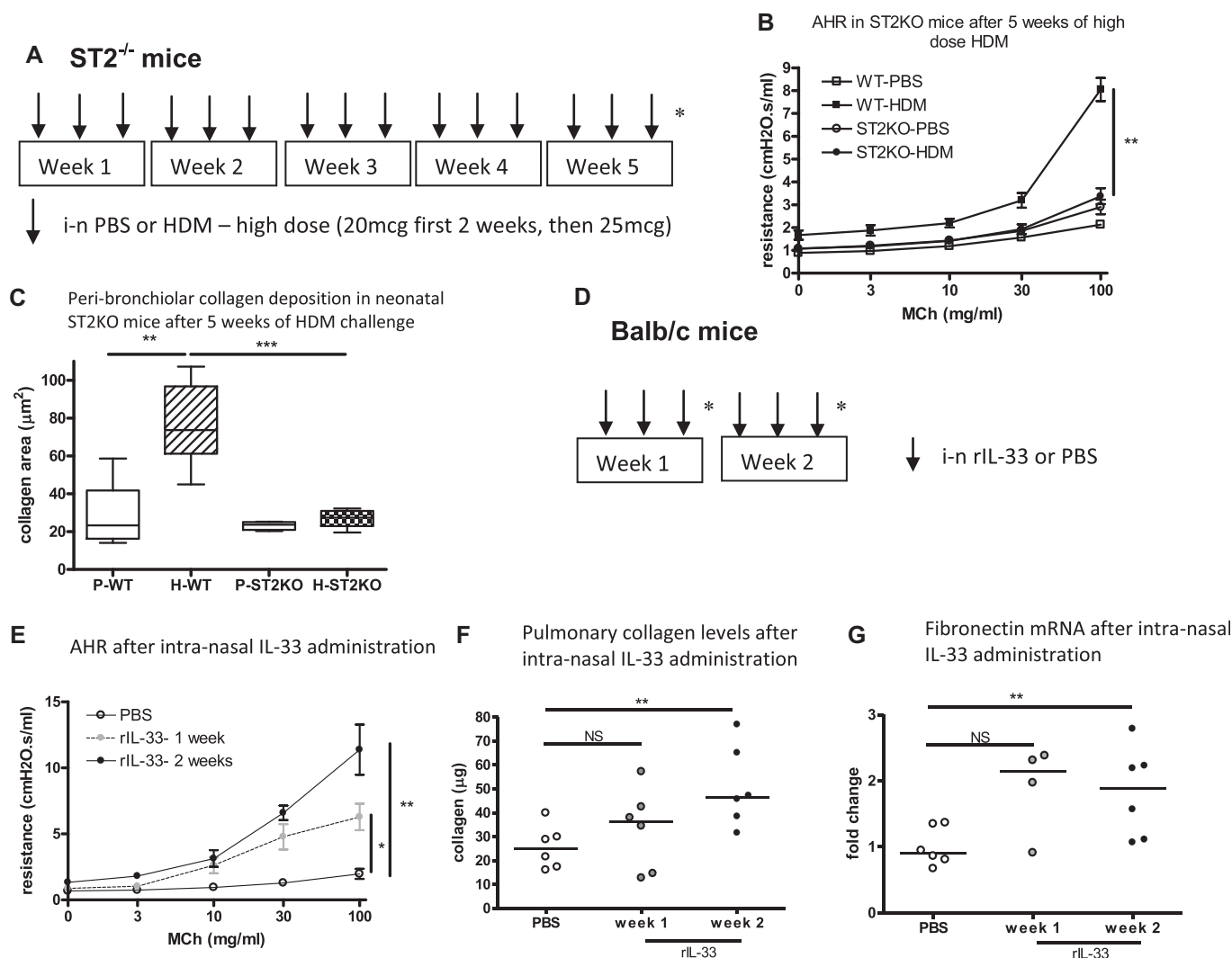


FIG 3. A, High-dose intranasal HDM or saline exposure from day 3 of life for 5 weeks in ST2^{-/-} or wild-type (WT) BALB/c mice. B, AHR in WT and ST2^{-/-} mice exposed to HDM. C, Peribronchiolar collagen deposition in WT and ST2^{-/-} mice exposed to HDM. Data are representative of 2 experiments (n = 4-8 per group). D, Intranasal administration of IL-33 to 6-week-old mice for 2 weeks (n = 4-6 per group). E, Airway resistance at week 1 and 2 weeks after IL-33 administration. F, Collagen levels in lung homogenates. G, Fibronectin mRNA after 2 weeks of rIL-33. MCh, Methacholine; NS, not significant. *P < .05, **P < .01, and ***P < .001.

they had not developed a myofibroblast phenotype (see Fig E5). In addition, cultured asthmatic fibroblasts expressed the receptor for IL-33 (Fig 5, D). These data show that IL-33 promotes increased RBM thickness in pediatric patients with STRA by causing collagen secretion from fibroblasts of pediatric asthmatic patients and that IL-33 is relatively steroid resistant because induction of collagen secretion is maintained despite the addition of budesonide.

IL-33 is less steroid sensitive than IL-13 in pediatric patients with STRA

IL-13 expression was not increased in biopsy specimens from children with STRA (Fig 4, A), possibly because of high-dose steroid therapy. To determine how IL-13 and IL-33 levels were affected by steroids, budesonide was administered intranasally to HDM-exposed neonatal mice before AAD was established (see

Fig E6 in this article's Online Repository at www.jacionline.org). This resulted in a complete reduction in AHR and inflammation (see Fig E6). IL-13 levels were significantly reduced whereas IL-33 levels remained increased in HDM-exposed mice treated with budesonide compared with control mice (see Fig E6). In contrast, when budesonide was administered therapeutically after AAD was established (Fig 6, A), AHR was only partially reduced (Fig 6, B), although inflammation was still attenuated (Fig 6, C). Critically, levels of IL-13 were significantly reduced in budesonide-treated mice (Fig 6, D), whereas levels of IL-33 remained increased (Fig 6, E). In addition, peribronchiolar collagen deposition was unchanged in HDM-exposed mice treated with budesonide (Fig 6, F). For the first time, these data show that IL-33 is less steroid sensitive than IL-13. There was tissue IL-33 expression in children with STRA despite high-dose inhaled steroid therapy, and expression was even higher in those additionally prescribed oral corticosteroids (see Fig E6).

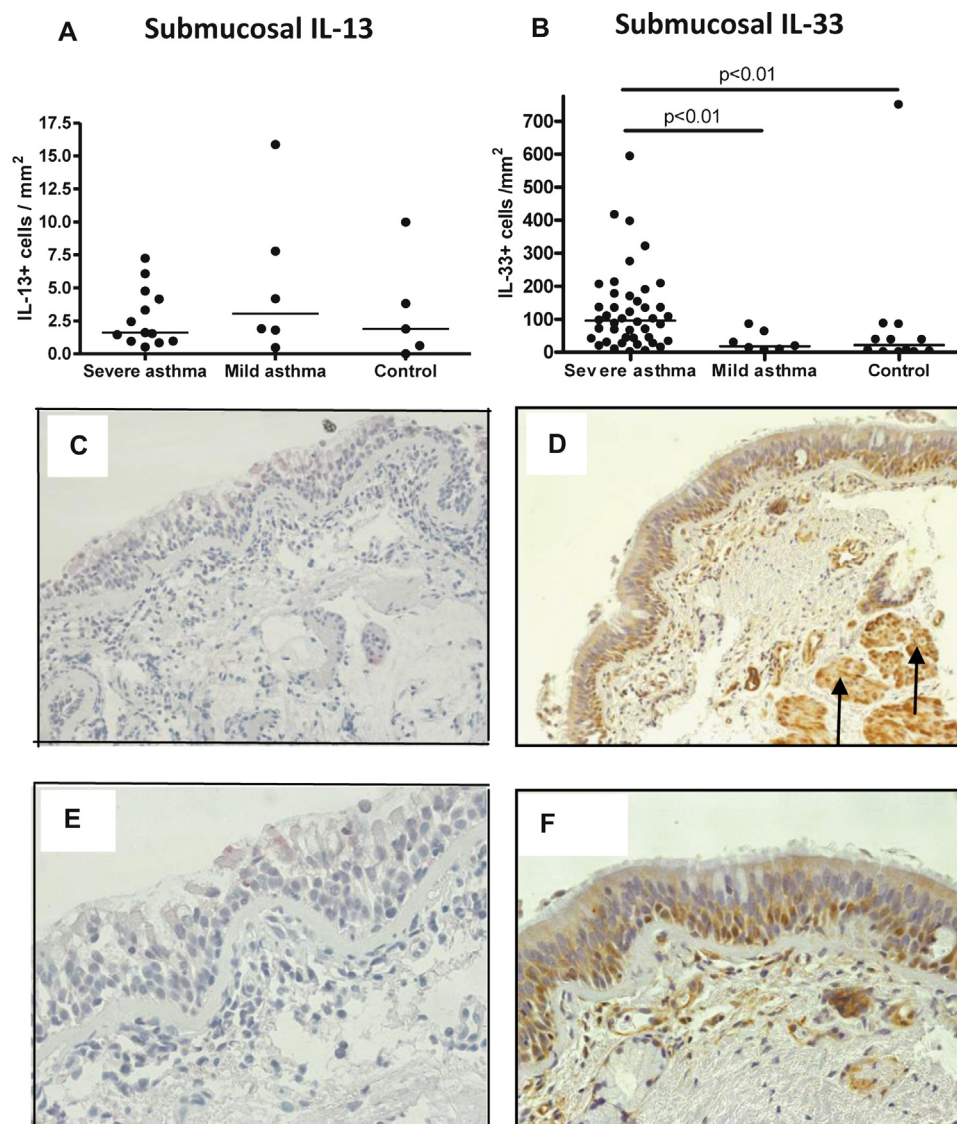


FIG 4. **A**, Subepithelial IL-13⁺ cells in EB specimens from children with severe asthma compared with those with mild-to-moderate asthma and age-matched control subjects ($n = 13$ for severe asthma, $n = 6$ for mild asthma, and $n = 5$ for control subjects). **B**, Subepithelial IL-33⁺ cells in EB specimens from school-aged children with severe asthma compared with children with mild asthma and nonasthmatic control subjects ($n = 50$ for severe asthma, $n = 7$ for mild asthma, and $n = 11$ for control subjects). **C** and **E**, EB specimens from a school-aged asthmatic patient stained for IL-13 (magnification $\times 200$ and $\times 400$). **D** and **F**, EB specimens from a school-aged asthmatic patient stained for IL-33, including smooth muscle (black arrows; magnification $\times 200$ and $\times 400$).

DISCUSSION

For the first time, we have shown that IL-33 levels are increased in a neonatal mouse model of HDM-induced AAD and in biopsy specimens from children with STRA. Moreover, IL-33 promotes a specific feature of airway remodeling, increased RBM thickness, through collagen secretion from airway fibroblasts of asthmatic patients *in vitro* and after direct *in vivo* administration of IL-33 to mice. Of note, we have shown that AHR and airway remodeling are unaffected when IL-13 is blocked therapeutically in the neonatal mouse model, whereas levels of IL-33 remain increased. Critically, the role of IL-33 is of particular importance in patients with severe asthma because we have shown, for the first time, that its expression in biopsy specimens from pediatric

asthmatic patients and function in the neonatal mouse model are less sensitive to steroid therapy than IL-13.

Although there has been evidence from experimental models that allergic asthma is mediated by T_H2 cytokines, asthma is now recognized as being a heterogeneous disease.²⁰ This is particularly important for pediatric asthma because we have previously found little evidence of T_H2 mediators in children with STRA after careful exclusion of modifiable factors, such as poor adherence and persistent allergen exposure, that might contribute to severe disease.²¹ Therefore we explored the role of the innate cytokine IL-33 in mediating severe disease. IL-33 is a member of the IL-1 family, is the ligand of ST2,²² and has been shown to induce the production of innate helper cells, which in turn secrete IL-13

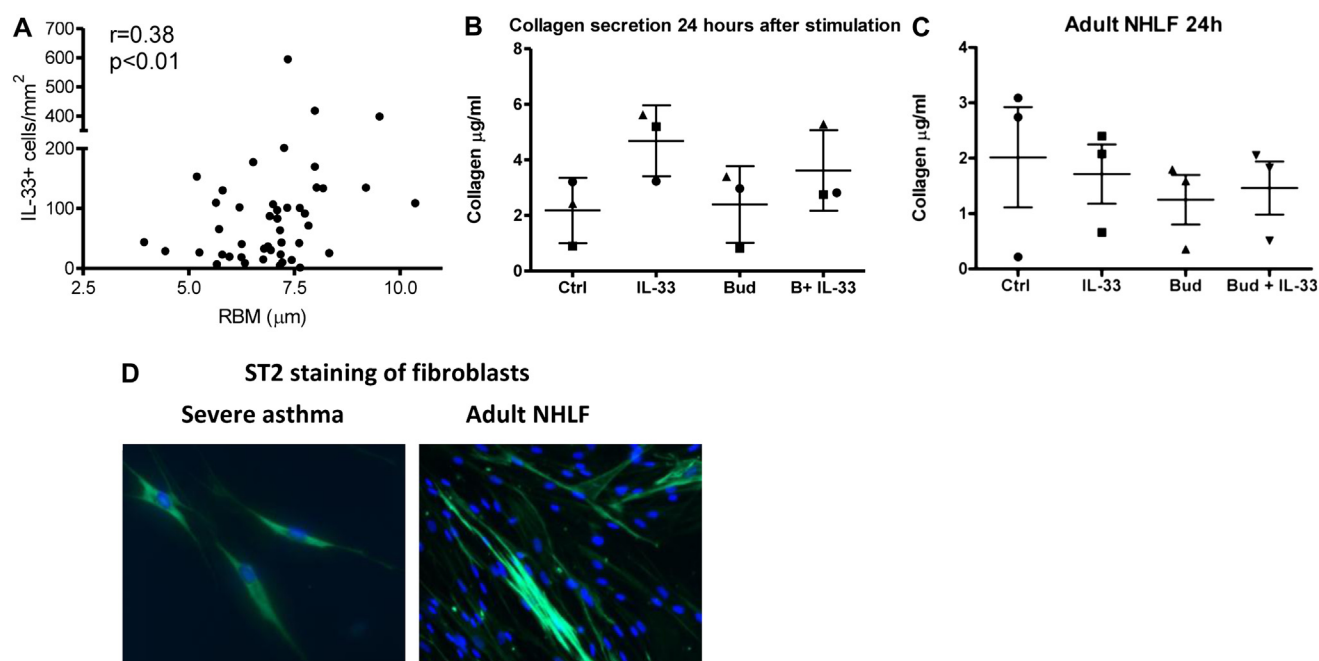


FIG 5. **A**, Correlation between IL-33⁺ cells per area of submucosa and RBM thickness in EB specimens from children with STRA ($n = 50$; Spearman correlation coefficient, $r = 0.38$). **B**, *In vitro* collagen secretion from pediatric airway fibroblasts at baseline and after stimulation with rIL-33 and budesonide alone and together. Each dot represents 1 child (circle and triangle, severe asthma; square, mild asthma). **C**, Collagen secretion from adult normal human lung fibroblasts 24 hours after stimulation (each dot represents average data from $n = 2$ subjects). **D**, ST2 staining of pediatric and normal (Fig 5, D and E) adult lung fibroblasts. NHLF, Normal human lung fibroblasts.

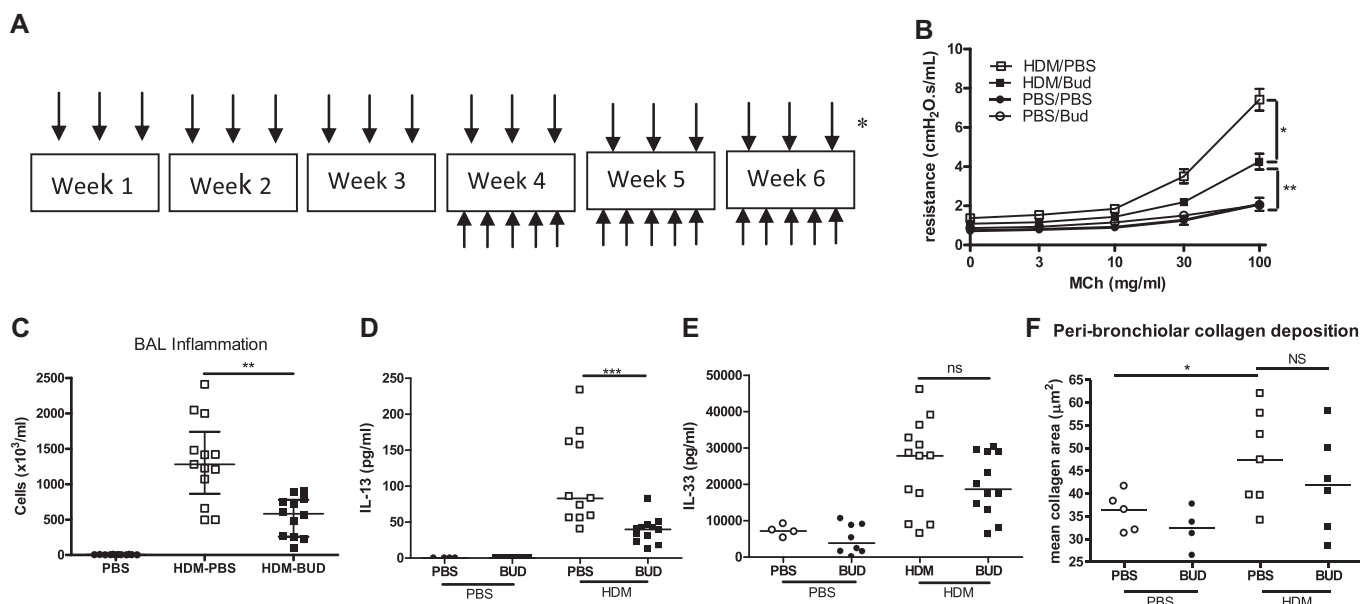


FIG 6. **A**, Administration of HDM or saline from day 3 of life ($\downarrow$) and intranasal budesonide ($\uparrow$); (0.6 mg/kg per dose) between weeks 3 and 6. **B-F**, AHR (Fig 6, B), inflammation in BAL fluid (Fig 6, C), IL-13 levels (Fig 6, D), IL-33 levels (Fig 6, E), and peribronchiolar collagen deposition (Fig 6, F) in budesonide-treated and HDM-exposed mice. Data are representative of 2 experiments ($n = 4-6$ for PBS and $n = 6-8$ for HDM). Horizontal bars represent medians, and vertical bars represent interquartile ranges. NS, Not significant. * $P < .05$, ** $P < .01$, and *** $P < .001$.

and IL-5 in adult mouse models of AAD.¹⁴ We have previously shown early release of IL-13 in a neonatal model of AAD before development of an adaptive immune response, as demonstrated

by the presence of either increased serum total or HDM-specific IgE levels.¹⁷ This reinforces the idea that innate immune mechanisms are important in the initiation of early-onset allergic

disease. We measured both IL-33 and IL-25 levels because both have been shown to induce innate helper cells and AHR in adult mice.¹⁴ IL-25 levels were unchanged after neonatal HDM exposure (see Fig E7 in this article's Online Repository at www.jacionline.org); however, IL-33 levels rapidly increased. This finding is supported by reports of the association between IL-33 levels and childhood asthma in genome-wide association studies.²³ However, unlike IL-13, levels of which peaked at 2 to 3 weeks after allergen exposure in neonatal mice, IL-33 production increased with age and allergen exposure. Although the role of IL-13 in initiating AHR in patients with AAD has been widely investigated, its role in disease maintenance has been questioned.^{16,20} We confirmed that disease maintenance was not IL-13 dependent because AHR was only partially abrogated when IL-13 was neutralized during established disease. This has also been shown after exposure of adult mice to inhaled HDM.¹⁶ In addition, expression of IL-13 in airway biopsy specimens from children with STRA was not increased compared with that seen in age-matched nonasthmatic control subjects. In contrast, IL-33 levels were maintained during therapeutic blockade of IL-13 in our neonatal model, and tissue expression of IL-33 in pediatric patients with STRA was increased compared with that seen in nonasthmatic control subjects. Persistence of IL-33 during established disease was further supported in our neonatal mouse model because increased IL-33 levels were maintained in HDM-exposed neonatal mice up to 4 weeks without allergen challenge, and this coincided with a persistence of peribronchiolar collagen deposition at 4 weeks without allergen challenge.¹⁷ We have also shown that mucosal infiltrating inflammatory cells are the likely source of IL-33 in patients with severe pediatric asthma rather than epithelial cells or smooth muscle, as has been reported in adults with severe asthma.^{12,13}

Although IL-33 has been shown to induce AHR in models of acute allergen exposure^{14,15} and infection²⁴ and the IL-33/ST2 signaling pathway has been shown to activate airway eosinophils in an autocrine and paracrine manner,²⁵ this is the first report of a relationship between IL-33 and airway remodeling in asthmatic patients. Increased thickness of the RBM is a specific feature of airway remodeling in asthmatic patients that is independent of disease severity² and is present in childhood (median age, 10 years)^{2,26} and preschool wheezers (median age, 3 years).²⁷⁻²⁹ RBM thickening results from increased deposition of collagen fibrils in the subepithelial basement membrane, which ultrastructurally resemble collagen III.¹⁹ Critically, there are currently no available therapies that target airway remodeling in asthmatic patients.³⁰ This is a significant limitation specifically in patients with STRA, in whom persistent lung function abnormalities and symptoms might be due to structural airway changes. We have shown that IL-33 induces collagen release from airway fibroblasts cultured from pediatric asthmatic patients, and this likely explains the association between increased IL-33 expression in submucosal cells and increased RBM thickness in biopsy specimens from children with STRA. Importantly, IL-33 also resulted in increased pulmonary collagen production after intranasal administration in mice. Interestingly, the distribution of collagen deposition in the lung after intranasal rIL-33 was between the bronchovascular bundle, whereas collagen deposition after HDM exposure is peribronchiolar. This suggests the distribution of the cells promoting fibrosis dependent on IL-33 might be predominantly in that region. It has been argued that increased RBM thickness might be protective in asthmatic patients because

it can cause increased airways stiffness, which in turn prevents excessive AHR.³¹ However, in our pediatric cohort RBM thickness was not associated with bronchodilator responsiveness,⁴ and in adults increased RBM thickness has been reported as a predictor of poor steroid response,³² suggesting RBM thickness might be adverse.

An important finding from our study was the lack of a response to IL-33 from healthy adult lung fibroblasts. Although an inherent functional abnormality of the asthmatic airway epithelium has been reported,³³ these novel data suggest other structural airway cells, including fibroblasts, might also have different functional characteristics that are specific to asthma. An acknowledged limitation is the absence of fibroblast cultures from healthy children. However, performance of bronchoscopy and EB for research in healthy children is not ethically acceptable. IL-33 has been associated with fibrosis in patients with other lung diseases, such as pulmonary fibrosis in systemic sclerosis,³⁴ and with fibrosis in other organs, such as cardiac fibrosis.³⁵ IL-33 has also been shown to be a critical regulator/enhancer of TNF- α -induced functions in synovial fibroblasts from patients with rheumatoid arthritis.³⁶

We have shown that IL-33 promotes increased collagen production, but in contrast to adult studies,¹² we have not shown an association between IL-33 expression and smooth muscle in airway biopsy specimens. The absence of α -smooth muscle actin release after IL-33 stimulation of asthmatic fibroblast cultures suggests they were unlikely to have a myofibroblast phenotype.

Increased IL-33 expression was present in our patients with STRA despite high-dose steroid therapy. In contrast, IL-13 was not detectable, suggesting IL-13 is steroid sensitive, whereas IL-33 might be a relatively more steroid-resistant mediator. Of note, IL-33 is not known to have a classical steroid response element. Hence we administered steroids to neonatal mice exposed to HDM. We used inhaled budesonide at a high dose to optimally reflect treatment in patients with severe asthma. IL-13 was completely abrogated after budesonide administration when administered both prophylactically (before AAD was established) and therapeutically (after fully established AAD). In contrast, IL-33 levels remained higher in HDM-exposed, budesonide-treated mice than in control animals in both regimens, and in the therapeutic regimen AHR was only partially reduced, whereas peribronchiolar collagen deposition was unaffected by budesonide administration. Because only partial effects of budesonide were seen in the mice when used therapeutically, this suggests the neonatal model of HDM exposure is closely aligned with human severe asthma. In further support of IL-33 being less steroid sensitive than IL-13, we found that children who were prescribed regular oral steroids and high-dose inhaled steroids had significantly higher mucosal IL-33 expression than those just prescribed high-dose inhaled steroids. Therefore we provide compelling data *in vitro* and *in vivo* in patients and mouse models that IL-33 is a relatively steroid-resistant cytokine involved in airway remodeling.

In summary, for the first time, we have shown that IL-33 promotes increased RBM thickness and that IL-33 alone, in the absence of IL-13, seems sufficient to maintain remodeling despite steroid treatment. IL-33 might be the first therapeutic target identified that specifically attenuates airway remodeling in the severe, steroid-resistant asthma phenotype, and it seems directly applicable as a target for children.

We thank Lorraine Lawrence for histology support. Finally, we thank Dr Andrew McKenzie for the ST2 knockout mice.

Key messages

- IL-33 promotes a specific feature of airway remodeling, increased RBM thickness, in pediatric patients with STRA.
- IL-33 is a less steroid-sensitive cytokine than IL-13 and persists in pediatric patients with STRA.

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METHODS

Animals and reagents

Female BALB/c wild-type mice with 2-day-old litters were obtained commercially from Harlan (Bicester, United Kingdom) and maintained by in-house breeding. Two breeding pairs of ST2 knockout (ST2^{-/-}) mice were obtained (Dr Andrew McKenzie, Cambridge, United Kingdom) and bred in house. Each mother with its litter was housed separately until weaned at 3 weeks. Mice were maintained in specific pathogen-free conditions and given food and water *ad libitum*. In individual experiments all mice were matched for age and background strain. ST2^{-/-} mice were also on a BALB/c background. All procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986. Recombinant mouse IL-33 (50 µg/kg per mouse) for intranasal administration was purchased from eBioscience. Anti-IL-13 antibody was a gift from UCB Celltech, and 10 mg/kg was administered intraperitoneally twice weekly. Relevant isotype-matched antibodies were used as controls for injection. Budesonide Respules (0.25 mg/mL) were used for intranasal administration (0.6 mg/kg) 5 times per week.

Allergen challenge

From 3 days of age, pups received intranasal administration of either 10 µg (10 µL of 1 mg/mL protein weight solution dissolved in saline [PBS]) of HDM extract (Greer) or 10 µL of PBS 3 times a week for the first 2 weeks. The HDM dose was increased to 15 µg from week 3 onward.^{E1} Intranasal administration was performed by using inhaled recovery anesthesia (isoflurane) from 2 weeks of age. All outputs were assessed at 4 hours after allergen challenge.^{E2}

Measurement of AHR

Airways resistance was measured from 2 weeks of age by using the flexiVent small-animal ventilator (SCIREQ) according to our established protocols.^{E1} Mice were anesthetized with pentobarbital sodium (intraperitoneal) and ketamine (intramuscular), tracheostomized, and connected to the flexiVent ventilator through a blunt-ended 21-gauge (2-3 weeks of age) or 19-gauge (4-6 weeks of age) needle. The mice were ventilated with an average breathing frequency of 150 breaths/min, a tidal volume of 10 mL/kg body weight, and a positive end-expiratory pressure of approximately 2 cm H₂O. Measurements of resistance and compliance were determined from a user-defined protocol by using the snapshot-150 perturbation, which is a single-frequency sinusoidal wave at a frequency equivalent to the ventilation rate (ie, 2.5 Hz). Resultant data were fitted by using multiple linear regression to the single-compartment model in the following form:

$$\text{Pressure} = \text{Resistance} \times \text{Flow} + \text{Elastance} \times \text{Volume} + \text{Fitting constant.}$$

Fitting the data to this model provides readings of resistance and compliance. Changes in resistance to increasing concentrations of nebulized methacholine (3-100 mg/mL) were measured from snapshot perturbation measurements taken by using the forced oscillation technique.

Inflammation and cell recovery

BAL was performed with PBS through a tracheal cannula, recovered volume was recorded, and cell counts were adjusted accordingly. The volume of BAL fluid instilled was increased with the age of the mouse as follows: week 1, 3 × 150-µL aliquots of PBS; week 2, 3 × 200-µL aliquots; week 3, 3 × 300-µL aliquots; and week 4 onward, 3 × 400-µL aliquots. Lavage fluid was collected and centrifuged (200g for 5 minutes at 4°C), and cell pellets were resuspended in 0.5 mL of complete medium (RPMI + 10% FCS, 2 mmol/L L-glutamine, and 100 U/mL penicillin/streptomycin). After lavage, 2 lobes from the right lung were mechanically chopped and incubated at 37°C for 1 hour in complete media containing 0.15 mg/mL collagenase (Type D; Roche Diagnostics, Mannheim, Germany) and 25 mg/mL DNase (Type 1, Roche Diagnostics). Cells were recovered by means of filtration through a 70-µm nylon sieve (Falcon, BD Biosciences), washed twice, resuspended in 1 mL of complete medium, and counted in a hemocytometer (Immune Systems). The total cell yield of BAL and lung cells was quantified

by using a hemocytometer. Differential cell counts were performed on glass slides prepared by means of cytocentrifugation (5 × 10⁴ cells per slide). These slides were fixed in methanol and stained with modified Wright-Giemsa stain (Thermo Fisher Scientific, Uppsala, Sweden), and 300 cells were counted for each sample by using microscopy. Macrophages, lymphocytes, neutrophils, and eosinophils were enumerated. All cell counts were performed blind by the same observer.

Quantification of cytokines

Lung tissue was homogenized at 50 mg/mL in Tissue Protein Extraction Reagent (Perbio Science, Cramlington, United Kingdom) containing protease inhibitor tablets (Roche Diagnostics) and centrifuged at 800g for 10 minutes, and the supernatant was collected. Cytokines were analyzed in lung homogenate supernatants. Paired antibodies for mouse IL-4 and IL-5 (PharMingen), IL-13 and IL-25 (eBioscience), and IL-33 (R&D Systems) were used in standardized sandwich ELISAs, according to the manufacturer's protocol.

Airway remodeling

Whole-lung sections of the large left lobe stained with Sirius red or Gordon and Sweet's silver stain for reticulin^{E1} were used to quantify peribronchiolar collagen and reticulin levels, respectively. Color images of a single airway were acquired at ×400 magnification (with a polarizing lens for Sirius red-stained sections; Leica QWin version 3). Subsequently, images were inverted to grayscale, and the area of positive staining around the airway was determined at a preset pixel intensity by using the Leica QWin software. The mean area of collagen or reticulin around 4 airways per lobe was used to represent the area of collagen or reticulin for each animal.

RNA extraction and real-time PCR

Total RNA was extracted from 50 to 100 mg of lung tissue by using the Qiagen RNeasy Mini Kit. cDNA was synthesized from 1 µg of total RNA prepared with the M-MLV reverse transcriptase mix (Promega, Madison, Wis) and analyzed by means of PCR on a CFX96 Real-Time PCR detection system (Bio-Rad Laboratories). Reactions were run in duplicate with TaqMan probes (Applied Biosystems) and SsoFast Probe Supermix (Bio-Rad Laboratories). The expression of the fibronectin gene (Applied Biosystems probe Mm01256744_m1) was normalized against that of HPRT1 (hypoxanthine phosphoribosyltransferase) (Applied Biosystems probe Mm01545399_m1).

Pediatric fibroblast culture and stimulation

Primary bronchial fibroblasts from pediatric patients were isolated by using outgrowth culture and used between passages 3 and 6. For experimental stimulations, fibroblasts were plated in 6-well tissue culture plates (Corning Laboratories, Corning, NY) and grown to 80% confluence in Dulbecco modified Eagle medium (Invitrogen) supplemented with 10% FCS and penicillin/streptomycin. Cells were serum starved overnight, and all challenges were carried out under serum-free conditions at 37°C in a 5% CO₂ incubator. Recombinant human IL-33 was used between 10 and 100 ng/mL, and budesonide was used at a final concentration of 10⁻⁸ mol/L. In costimulations budesonide was added to cell cultures for 2 hours before addition of rIL-33. Conditioned media were taken at the time points indicated, and cell lysates were prepared in RIPA buffer. All samples were stored at -80°C before analysis.

Western blotting and densitometry

Cell lysates were cleared by using centrifugation at 10,000g for 10 minutes. Five micrograms of protein per well in LDS sample buffer (Invitrogen) was loaded onto 4-12% Bis-Tris gels (Bio-Rad Laboratories) and run at 150 V in a Criterion Cell (Bio-Rad Laboratories). Gels were transferred onto polyvinylidene difluoride membrane (Thermo Scientific) for 30 minutes at 100 V by using a Criterion Blotter (Bio-Rad Laboratories) and blocked for 1 hour in 5% nonfat dry milk in TBS-Tween. α-Smooth muscle actin primary antibody (ab5694, Abcam) was added at 1:5000 dilution in blocking buffer and incubated overnight at 4°C. Membrane washes were carried out in TBS-Tween. Horseradish peroxidase-conjugated anti-rabbit antibody (Cell Signaling

Technology) was used at 1:2000 dilution, and membranes were developed with ECL substrate (Pierce) for film exposure (Amersham). Developed films were scanned, and band densitometry was carried out with ImageJ software (<http://imagej.nih.gov/ij/>).

Human data

Subjects. School-aged children (age, 6–16 years) with STRA who were undergoing a clinically indicated bronchoscopy were recruited from the Royal Brompton Hospital between 2005 and 2009. Their clinical details have been published.^{E3} The symptoms of patients with severe asthma remained poorly controlled despite being prescribed 800 $\mu\text{g}/\text{d}$ or more budesonide (or equivalent) and receiving additional long-acting β -agonists, and these patients either had a previous failed trial or received montelukast. Almost half of the patients with severe asthma were also taking regular oral steroids. Clinical details of the children with STRA are shown in Table E1. Children with mild-to-moderate asthma (inhaled corticosteroids $<800 \mu\text{g}/\text{d}$) having a bronchoscopy for another indication were also included (see Table E2 for clinical details). Age-matched nonasthmatic control subjects were either (1) having a bronchoscopy to investigate upper airway symptoms and agreed to extra research samples being taken or (2) were undergoing general anesthesia for cardiac catheterization and agreed to have a research bronchoscopy at the same time (see Table E3 for details). The study was approved by the Brompton

and Harefield National Heart and Lung Institute Ethics Committee, and informed parental consent and child assent were obtained.

Statistical analysis

All results were expressed as medians $\pm$ SEMs, and data were analyzed with GraphPad Prism 4 software. Nonparametric tests (Mann-Whitney *U* test) were used to detect differences between groups, and statistical significance was accepted at a *P* value of less than .05. Corrections for multiple-group comparisons when 3 or more groups were present were made by using the Bonferroni correction. Correlations were assessed with the Spearman correlation coefficient. All data shown, except those in Fig 3, *F*, are representative of at least 2 experiments.

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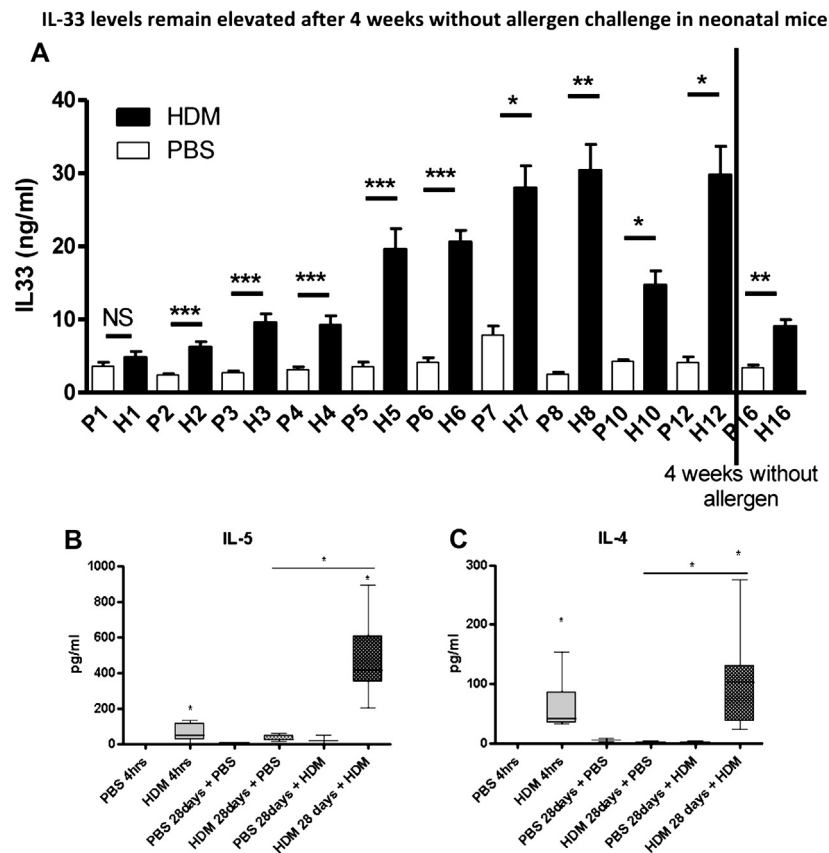


FIG E1. **A**, IL-33 levels during 12 weeks of HDM or saline (PBS) exposure, followed by reassessment after 4 weeks without challenge. **B** and **C**, Lung IL-5 and IL-4 levels after 3 weeks of allergen exposure (4 hours after the last dose) and 28 days without allergen. *NS*, Not significant. * $P < .05$, ** $P < .01$, and *** $P < .001$.

FIG E2. A-D, Experimental plan showing time of administration of anti-IL-13 antibody (Fig E2, A), reduced AHR in mice that received anti-IL-13 antibody (Fig E2, B), reduced peribronchiolar reticulin deposition in mice that received anti-IL-13 antibody (Fig E2, C), and similar total BAL inflammation with and without anti-IL-13 antibody (Fig E2, D). **E**, Total BAL inflammation after blocking IL-13 by using a therapeutic regimen (from weeks 3-6) in HDM-exposed mice. **F**, Differential BAL cell counts after therapeutic blocking of IL-13 in neonatal HDM-exposed mice. *Eos*, Eosinophils; *LMN*, lympho-mononuclear cells; *Mac*, macrophages; *MCh*, methacholine; *Neut*, neutrophils; *ns*, not significant. **P* < .05.

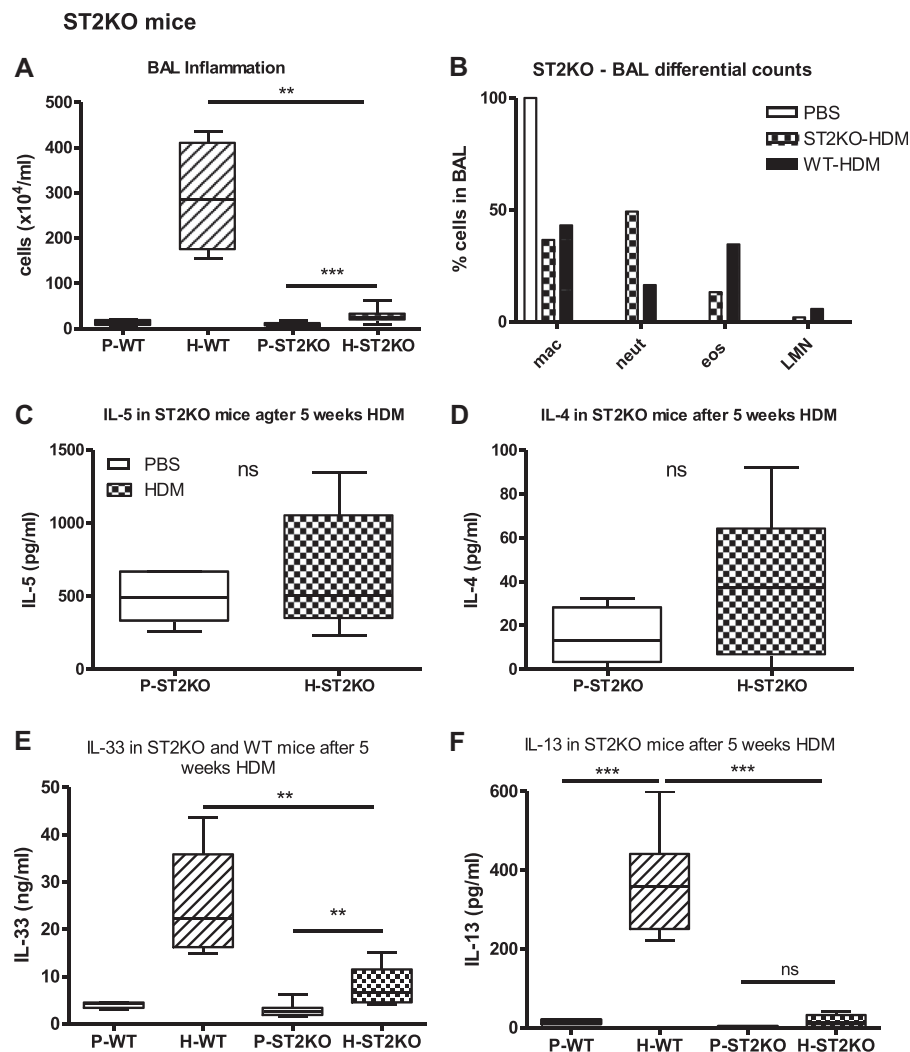


FIG E3. A-D, Total BAL inflammation (Fig E3, A), differential BAL cell counts (Fig E3, B), IL-5 levels (Fig E3, C), and IL-4 levels (Fig E3, D) in lung homogenates. Lung IL-33 and IL-13 levels in wild-type (WT) and $\text{ST2}^{-/-}$ mice are shown. E and F, Data are representative of 2 experiments ($n = 6-8$ per group). $**P < .01$. G and H, Total and differential cell counts in BAL fluid. I, IL-5, IL-4, and IL-13 levels in lung homogenates. Fig E3, A through F, show $\text{ST2}^{-/-}$ mice, and Fig E3, G through I, show BALB/c mice. Eos, Eosinophils; LMN, lymphomononuclear cells; Mac, macrophages; Neut, neutrophils; ns, not significant. $*P < .05$, $**P < .01$, and $***P < .001$.

IL-33 administration

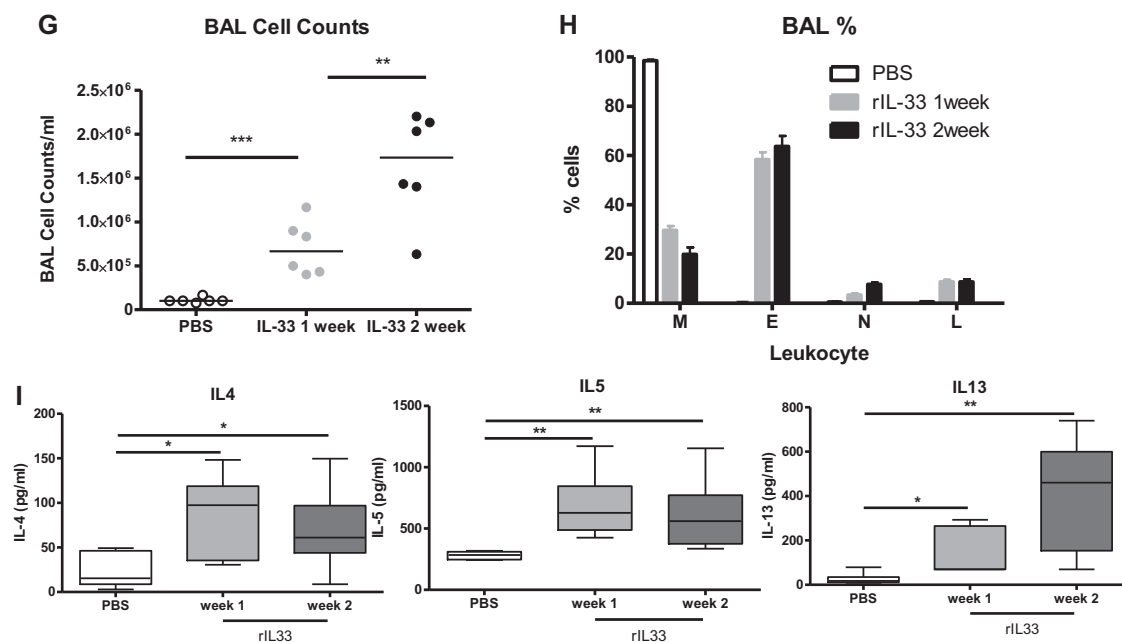


FIG E3. (Continued)

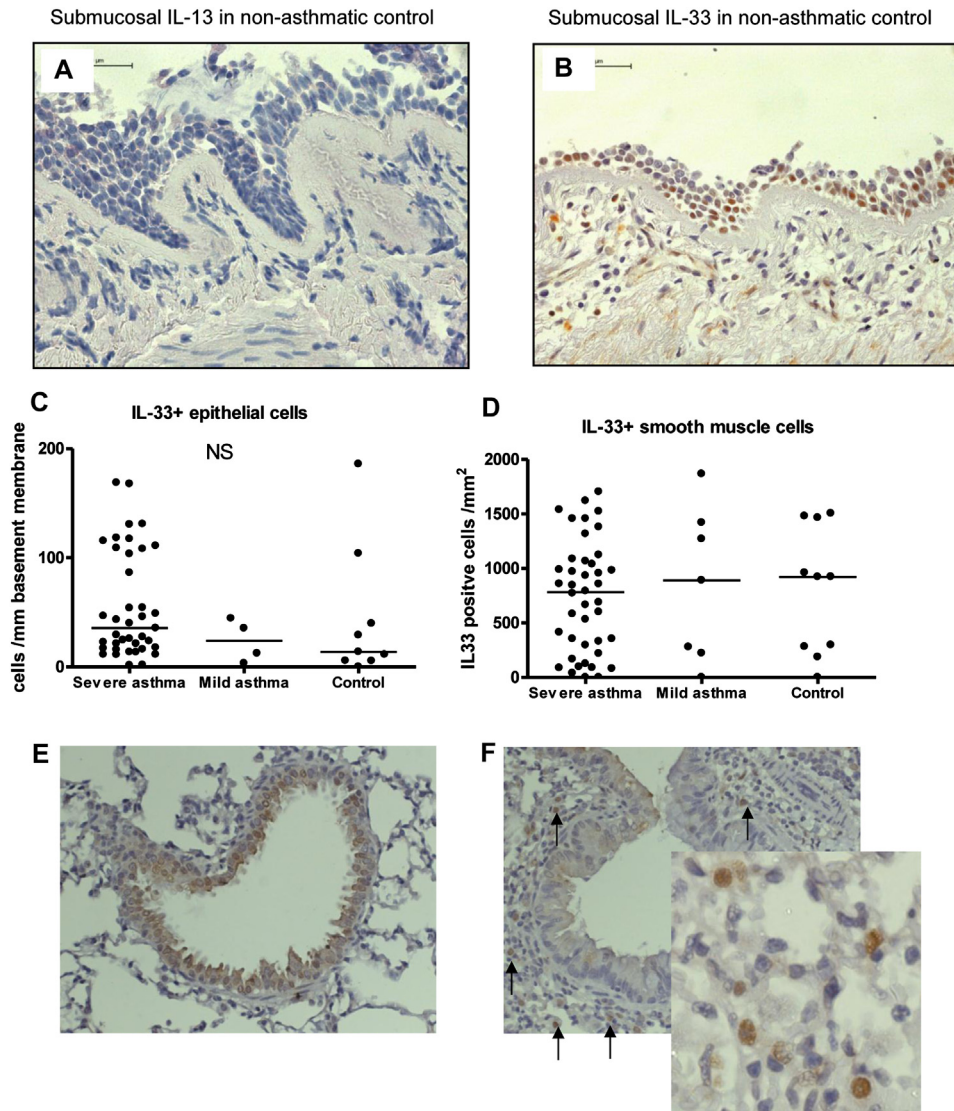


FIG E4. **A** and **B**, EB specimens stained for IL-13 and IL-33 from a nonasthmatic control subject (magnification $\times 400$). **C**, IL-33⁺ epithelial cells in EB specimens from children with severe asthma compared with patients with mild-to-moderate asthma and nonasthmatic control subjects ($n = 39$ for severe asthma, $n = 4$ for mild asthma, and $n = 9$ for control subjects). **D**, IL-33⁺ smooth muscle cells in EB specimens from children with severe asthma, children with mild asthma, and nonasthmatic control subjects ($n = 42$ for severe asthma, $n = 7$ for mild asthma, and $n = 10$ for control subjects). **E**, Epithelial IL-33 expression in a 3-week-old mouse after saline exposure. **F**, IL-33 expression and IL-33⁺ infiltrating inflammatory cells (black arrows and inset) in a 3-week-old mouse after HDM exposure. NS, Not significant.

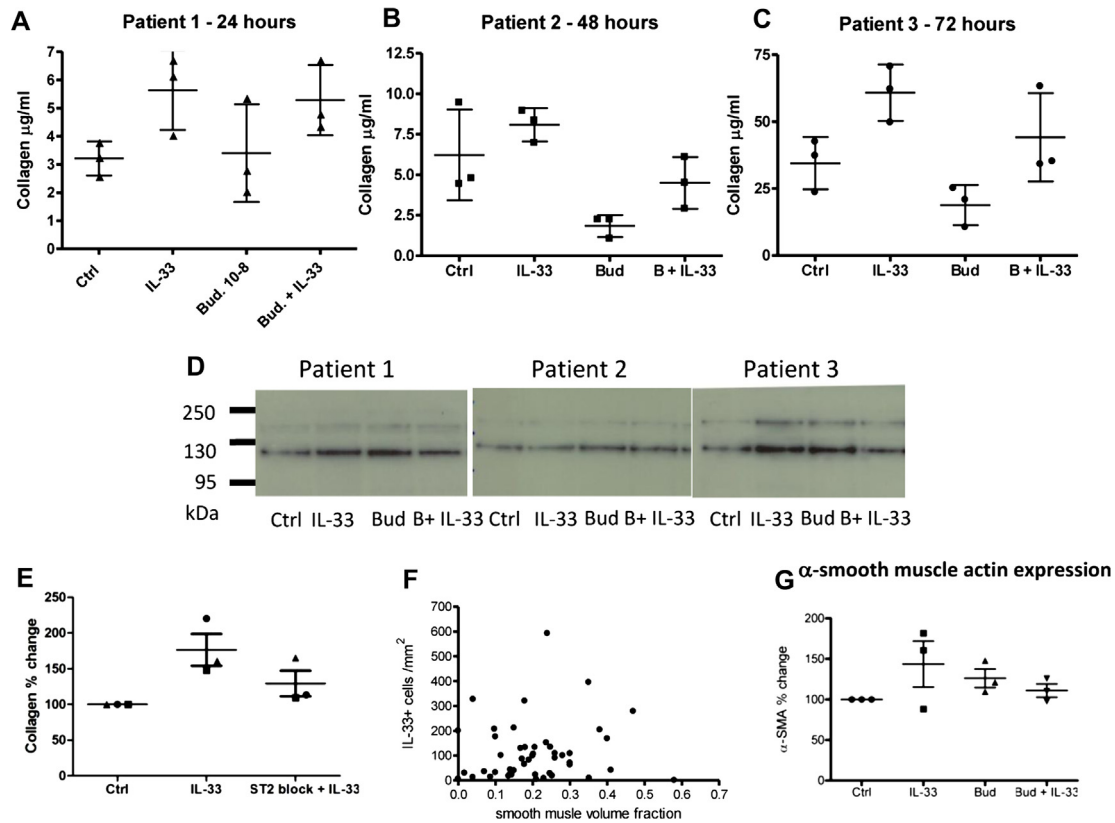


FIG E5. A-C, Collagen secretion from pediatric cultured fibroblasts at 24, 48, and 72 hours after stimulation with rIL-33, budesonide, or both. Each *graph* represents 1 child, and each *dot* represents a culture well. **D,** Western blots for the ST2 ligand showing ST2 expression on fibroblasts from the asthmatic children. The fibroblasts were harvested 24 hours after challenge, and 20 μg of cell lysate was loaded per lane. Positive bands for ST2 ligand were seen for all 3 patients. **E,** Release of collagen from fibroblast cultures from EB specimens from pediatric patients stimulated with rIL-33 with and without preincubation with ST2 blocking antibody for 2 hours before addition of IL-33. Sircol assay of conditioned media for soluble collagen release (ST2 blocking antibody from R&D Systems at 2 $\mu\text{g/ml}$) incubated for 2 hours before addition of IL-33. **F,** Correlation between IL-33⁺ cells in the submucosa in EB specimens from children with STRA and smooth muscle volume fraction ($n = 42$, Spearman correlation coefficient = 0.06, $P = .73$). **G,** Percentage change in α -smooth muscle actin expression from baseline from fibroblasts cultured from children with asthma. Each *dot* represents 1 child.

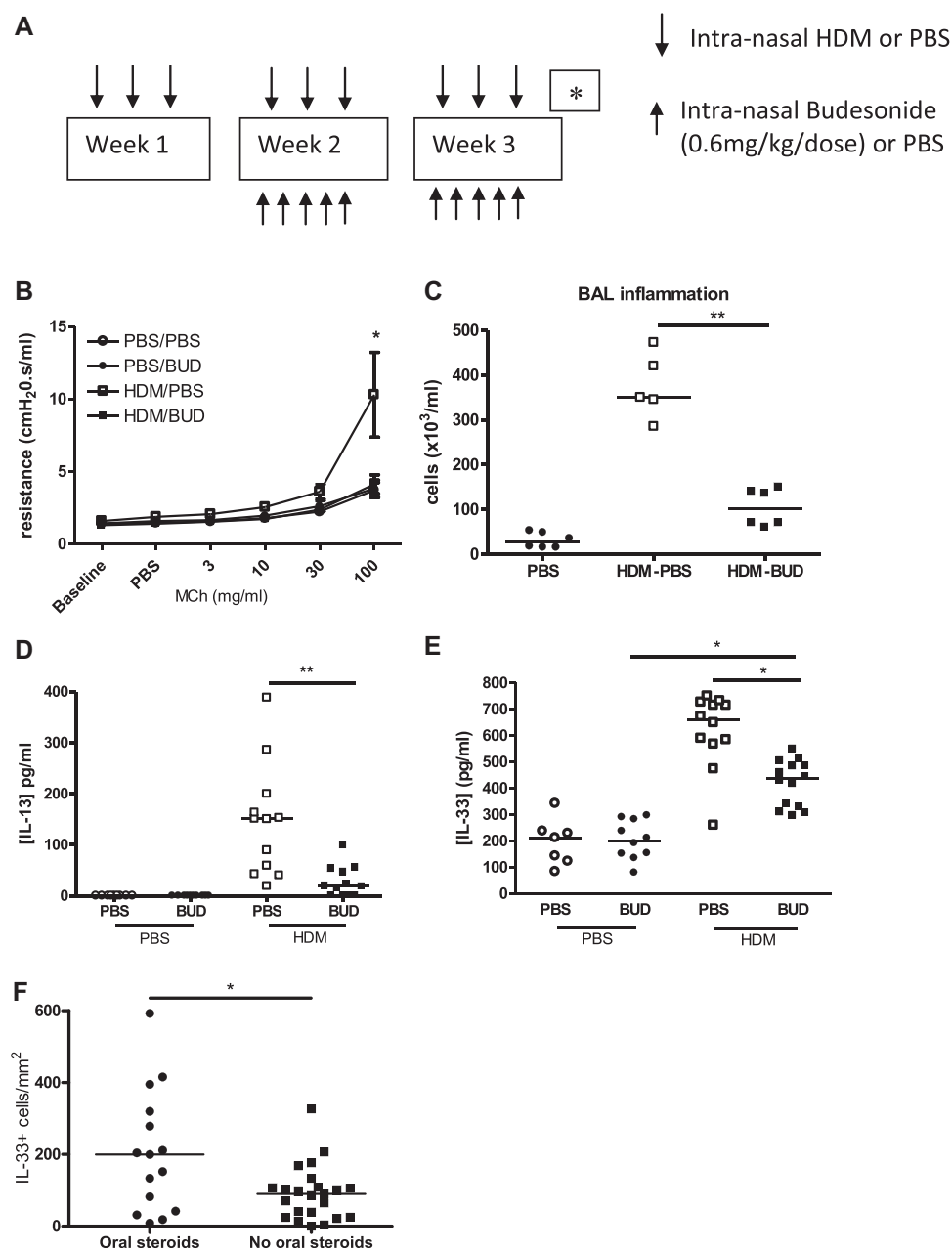


FIG E6. **A**, Administration of intranasal budesonide (0.6 mg/kg) between 1 and 3 weeks of age to neonatal mice exposed to HDM or saline. **B-E**, AHR (Fig E6, **B**), inflammation in BAL fluid (Fig E6, **C**), and lung IL-13 and IL-33 levels (Fig E6, **D** and **E**). **F**, IL-33⁺ cells in the bronchial submucosa of children with STRA prescribed maintenance oral and inhaled steroid therapy ($n = 15$) and those only prescribed inhaled steroids ($n = 23$). * $P < .05$ and ** $P < .01$.

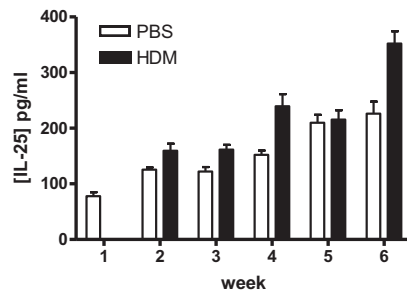


FIG E7. Similar IL-25 levels in lung homogenates from neonatal mice that had received intranasal HDM from day 3 of life for 6 weeks compared with saline control animals.

TABLE E1. Clinical characteristics of asthmatic and control children

	Patients with STRA (n = 53)	Control subjects (n = 16)
Age (y)	11.97 (9.97-14.4)	10.65 (7.54-12.96)
Male sex	33/53 (62%)	8/16 (50%)
Atopy	45/53 (85%)	7/15 (47%)*
Age at first symptoms	1 (0.25-2.25)	—
Duration of symptoms	10.1 (8.3-12.7)	—
Previous intubation for asthma	11/53 (21%)	—
Parental smoking	13/53 (25%)	2/9 (22%)
FEV ₁ (% predicted)	68.5 (54.8-86.5)	94 (85-106)*
Bronchodilator reversibility (%)	15.6 (5.5-23.4)	2.5 (0-8.5)
Persistent airflow limitation	13/53 (25%)	—
Total IgE	386 (115-1286)	40 (14-130)
Medications		
Inhaled corticosteroid (mg/d)	1.6 (0.8-1.6)	—
Maintenance oral prednisolone	24/53 (45%)	—
Long-acting β -agonist	53/53 (100%)	—
Leukotriene receptor antagonist	31/53 (58%)	—
Theophylline	12/53 (23%)	—
ACT score (/25)	13 (9-17)	—
FENO ₅₀ (ppb; normal, <24 ppb)	50.3 (29.3-69.7)	—
Sputum eosinophil (%; normal, <2.5%)	7.5 (3.2-30.4)	—

ACT, Asthma Control Test; FENO₅₀, fraction of exhaled nitric oxide at 50 mL/s.**P* < .05

TABLE E2. Clinical characteristics of patients with mild-to-moderate asthma

Patient no.	Age (y)	ICS dose (μ g/d)	Atopic	Reason for FOB	Findings at FOB
1	8.9	0	No	Wheeze	Gram-positive cocci, normal BAL fluid neutrophils
2	10.4	200	Yes	Wheeze and hemoptysis	Normal
3	6.7	200	Yes	Wheeze	Normal
4	11.2	200	No	Wheeze	Normal
5	13.3	0	Yes	Hemoptysis (history of asthma)	Normal
6	15.6	500	Yes	Wheeze	Normal
7	13.1	400	Yes	Recurrent cough	Laryngomalacia

FOB, Fiberoptic bronchoscopy.

TABLE E3. Clinical details of nonasthmatic control subjects

Age (y)	Atopic status	Indication	Findings at FOB and outcome	Biopsy quality
11.2	Nonatopic	Recurrent barking cough	Large adenoids; human metapneumovirus in BAL fluid	Adequate
16.4	Atopic	Change of pacemaker and wire	Normal	Adequate
5.6	Atopic	Recurrent pneumonia	Upper airway bleeding; neutrophilia (11.7%) in BAL fluid	Adequate
6.4	Nonatopic	PDA repair	Normal, PDA repaired	Insufficient
9.1	Atopic	Recurrent croup	<i>Haemophilus influenzae</i> and <i>Streptococcus pneumoniae</i> in BAL fluid and neutrophilia (14%), antibiotics administered	Adequate
11.0	Nonatopic	Recurrent croup and barking cough	Moderate adenoids, <i>Streptococcus pneumoniae</i> in BAL fluid and neutrophilia (2%); antibiotics administered	Adequate
13	Nonatopic	Exertional stridor	Moderately large adenoids and tonsils	Adequate
12.4	Atopic	Stridor, cardiac history	Vascular ring, referred for cardiac surgery	Adequate
10.7	Atopic	Recurrent episodes of shortness of breath	Pharyngomalacia, tracheomalacia, laryngomalacia	Adequate
14.2	Nonatopic	Hemoptysis	No cause found; symptoms resolved	Adequate
16.4		Hemoptysis	Enlarged adenoids and tonsils otherwise normal, symptoms resolved	Adequate
7.5	Atopic	Recurrent croup	Pharyngomalacia, laryngomalacia	Adequate
5.9	Nonatopic	Recurrent croup	No cause found, symptoms resolved	H&E section only
10.37	Nonatopic	Recurrent croup	No cause found, symptoms resolved	H&E section only
10.13	Nonatopic	Hemoptysis	No cause found, symptoms resolved	H&E section only
6.67	Atopic	Noisy breathing	Tracheomalacia	H&E section only

FOB, Fiberoptic bronchoscopy; H&E, hematoxylin and eosin; PDA, patent ductus arteriosus.