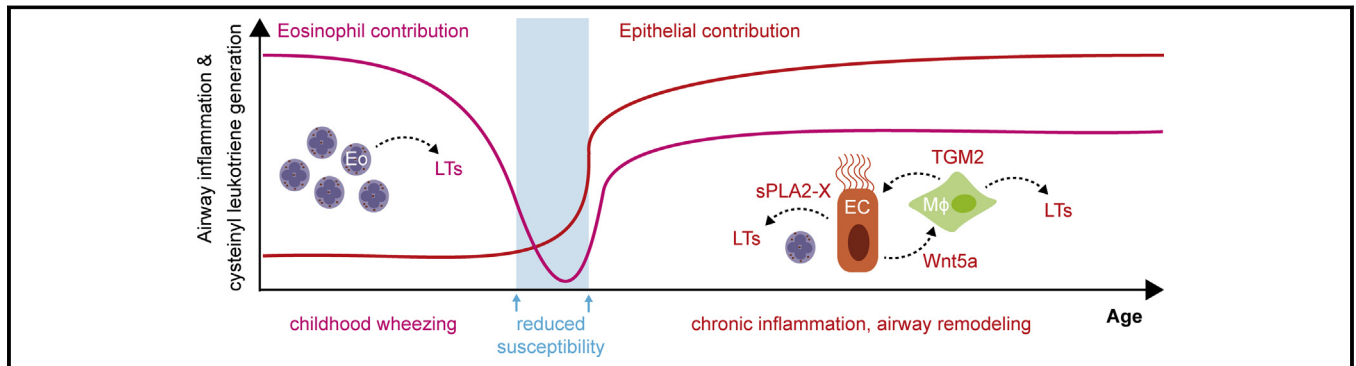


Age dictates a steroid-resistant cascade of Wnt5a, transglutaminase 2, and leukotrienes in inflamed airways

Katharina Dietz, MSc,^a Marta de los Reyes Jiménez, MSc,^a Eva S. Gollwitzer, PhD,^b Adam M. Chaker, MD,^{a,c} Ulrich M. Zissler, PhD,^a Olof P. Rådmark, PhD,^d Hoeke A. Baarsma, PhD,^e Melanie Königshoff, MD, PhD,^e Carsten B. Schmidt-Weber, PhD,^a Benjamin J. Marsland, PhD,^b and Julia Esser-von Bieren, PhD^a *Munich, German, Lausanne, Switzerland, and Stockholm, Sweden*

GRAPHICAL ABSTRACT



Background: Airway remodeling is a detrimental and refractory process showing age-dependent clinical manifestations that are mechanistically undefined. The leukotriene (LT) and wingless/integrase (Wnt) pathways have been implicated in remodeling, but age-specific expression profiles and common regulators remained elusive. **Objective:** We sought to study the activation of the LT and Wnt pathways during early- or late-onset allergic airway inflammation and to address regulatory mechanisms and clinical relevance in normal human bronchial epithelial cells (NHBEs) and nasal polyp tissues. **Methods:** Mice were sensitized with house dust mite (HDM) allergens from days 3, 15, or 60 after birth. Remodeling factors

in murine bronchoalveolar lavage fluid, lung tissue, or human nasal polyp tissue were analyzed by means of Western blotting, immunoassays, or histology. Regulatory mechanisms were studied in cytokine/HDM-stimulated NHBEs and macrophages. **Results:** Bronchoalveolar lavage fluid LT levels were increased in neonatal and adult but reduced in juvenile HDM-sensitized mice. Lungs of neonatally sensitized mice showed increased 5-lipoxygenase levels, whereas adult mice expressed more group 10 secretory phospholipase A₂, Wnt5a, and transglutaminase 2 (Tgm2). Older mice showed colocalization of Wnt5a and LT enzymes in the epithelium, a pattern also observed in human nasal polyps. IL-4 promoted epithelial Wnt5a secretion, which upregulated macrophage Tgm2 expression, and Tgm2 inhibition

From ^athe Center of Allergy and Environment (ZAUM), Member of the German Center for Lung Research (DZL), Technical University of Munich and Helmholtz Center Munich; ^bthe Faculty of Biology and Medicine, University of Lausanne, Service de Pneumologie, Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne; ^cthe Department of Otolaryngology, Allergy Section, Klinikum Rechts der Isar, Technische Universität München, Munich; ^dthe Division of Physiological Chemistry II, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm; and ^ethe Comprehensive Pneumology Center (CPC), Helmholtz Zentrum München, Member of the German Center for Lung Research (DZL) and Ludwig-Maximilians-Universität, University Hospital Grosshadern, Munich.

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Corresponding author: Julia Esser-von Bieren, PhD, Center of Allergy and Environment (ZAUM), Technische Universität München and Helmholtz Zentrum München, 80802 Munich, Germany. E-mail: Julia.esser@tum.de.

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in turn reduced LT release. Tgm2, group 10 secretory phospholipase A₂, and LT enzymes in NHBEs and nasal polyps were refractory to corticosteroids.

Conclusion: Our findings reveal age differences in LT and Wnt pathways during airway inflammation and identify a steroid-resistant cascade of Wnt5a, Tgm2, and LTs, which might represent a therapeutic target for airway inflammation and remodeling. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: Age, airway remodeling, airway inflammation, allergy, asthma, bronchial epithelial cells, house dust mite, leukotrienes, lipid mediators, nasal polyps, secretory phospholipase A₂, transglutaminase 2, Wnt5a

Asthma affects more than 300 million patients worldwide, and airway remodeling, characterized by persistent changes in airway structure, airway hyperresponsiveness, and lung function impairment, is a major cause of morbidity that cannot be reversed by currently available therapies.^{1,2} Thus airway remodeling represents a major unmet clinical need, which is insufficiently addressed because underlying mechanisms are incompletely understood.³ Recently, age differences in the susceptibility and clinical picture of airway inflammation and remodeling have emerged.^{4,5} Consistent with the increase in markers of airway eosinophilia in human neonates,⁶ rodent models of airway inflammation have shown that airway eosinophilia is increased in neonates.⁷⁻⁹ However, despite increased eosinophilia at a young age, changes in airway structural cells are more severe later in life.^{7,9}

Leukocytes, which infiltrate the respiratory tissue of allergic and asthmatic patients, produce cytokines, such as IL-4, IL-13, IL-5 and TGF-β1, which promote inflammation and remodeling.¹⁰ In addition, myeloid cells produce lipid mediators, such as leukotrienes (LTs), which perpetuate inflammatory cell recruitment and remodeling.^{11,12} Although cell infiltration was traditionally assumed to correlate with airway remodeling, recent evidence suggests that these might be parallel rather than causally related processes.^{13,14} This has led to an increasing appreciation of airway epithelial cells (AECs) as central players in airway inflammation and remodeling.^{15,16} AECs can produce IL-25, IL-33, and thymic stromal lymphopoietin, which activate type 2 inflammation.¹⁵ In mice sensitized with house dust mite (HDM) allergens from day 3 after birth, IL-33 levels were shown to increase with age and to be crucial for remodeling, whereas lung IL-13 levels peaked early after HDM sensitization, suggesting an early neonatal T_H2 bias, followed by increased production of epithelial remodeling factors during persistent allergen exposure.^{17,18} AECs also respond to type 2 cytokines and bioactive lipids, such as cysteinyl leukotrienes (cysLTs), by producing eotaxins, matrix metalloproteinases, and TGF-β1.¹⁹⁻²¹ In response to inflammatory stimuli, such as bradykinin, LPS, or HDM extract, human bronchial epithelial cells upregulate enzymes of the LT biosynthetic cascade (5-lipoxygenase [5-LO], 5-lipoxygenase activating protein, leukotriene C₄ synthase [LTC₄S], and LTA₄ hydrolase), resulting in the capacity to produce LTs.²²⁻²⁴

Transglutaminase 2 (Tgm2) has been identified as another epithelium-expressed enzyme that was crucial for the development of airway inflammation in mice.²⁵ Tgm2 was found to be overexpressed in the airways of asthmatic patients and to regulate cysLT production through activating group 10 secretory phospholipase A₂ (sPLA2-X), another hallmark enzyme of asthma severity and remodeling.²⁶⁻²⁸ Increased expression of a different

Abbreviations used

AEC:	Airway epithelial cell
ASMC:	Airway smooth muscle cell
BALF:	Bronchoalveolar lavage fluid
CRSwNP:	Chronic rhinosinusitis with nasal polyps
cysLT:	Cysteinyl leukotriene
DMSO:	Dimethyl sulfoxide
FZD:	Frizzled
GC:	Glucocorticosteroid
HDM:	House dust mite
5-LO:	5-Lipoxygenase
LTC ₄ S:	Leukotriene C ₄ synthase
MDM:	Monocyte-derived macrophage
NHBE:	Normal human bronchial epithelial cell
αSMA:	α Smooth muscle actin sPLA2-X, Group 10 secretory phospholipase A ₂
STAT6:	Signal transducer and activator of transcription 6
Tgm2:	Transglutaminase 2
Wnt:	Wingless/integrase
ZO-1:	Zonula occludens 1

sPLA2 (group IID) has recently been shown to confer increased susceptibility to respiratory tract infection associated with aging,²⁹ but age-dependent regulation of lipid mediator pathways in allergic airway inflammation has remained elusive.

A pathway that has only recently emerged as being involved in asthma is the wingless/integrase (Wnt) pathway, which in human subjects and mice comprises 19 secreted glycolipoproteins.^{30,31} Wnt5a in particular is essential for lung development and expressed in the epithelial and mesenchymal compartment in the neonatal lung.^{32,33} Wnt5a is abundant in airway smooth muscle cells (ASMCs), is potently upregulated by TGF-β1, and promotes collagen and fibronectin expression, thus implicating Wnt5a in remodeling in asthmatic patients.³⁴⁻³⁶ In hematopoietic stem cells Wnt5a expression was observed to increase with age,³⁷ but potential age-dependent fluctuations of Wnt5a levels during airway inflammation are unclear.

Recent work from our own group has shown that Tgm2 and Wnt5a are among the most prominently IL-4-induced genes in normal human bronchial epithelial cells (NHBEs).³⁸ It is also known that Wnt signaling is defective in the adipose tissue of Tgm2-deficient mice.³⁹ However, whether regulatory loops between Tgm2 and Wnt signaling occur in the respiratory tract was unknown. Moreover, potential links between the LT and Wnt pathways during airway inflammation and remodeling have not been investigated.

Here we show that airway LT, Tgm2, Wnt5a, and sPLA2-X profiles show age-dependent alterations during HDM-induced allergic airway inflammation and that AECs abundantly express these factors in settings of airway inflammation. Finally, we identify a regulatory network of Tgm2, Wnt5a, and LTs, which might contribute to the steroid resistance of remodeling processes in the setting of asthma and nasal polyposis.

METHODS

Animal experiments were performed according to institutional guidelines and Swiss federal and cantonal laws on animal protection.

Mouse model of allergic airway inflammation

Allergic airway sensitization was induced, as previously published.⁸ Additional information can be found in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

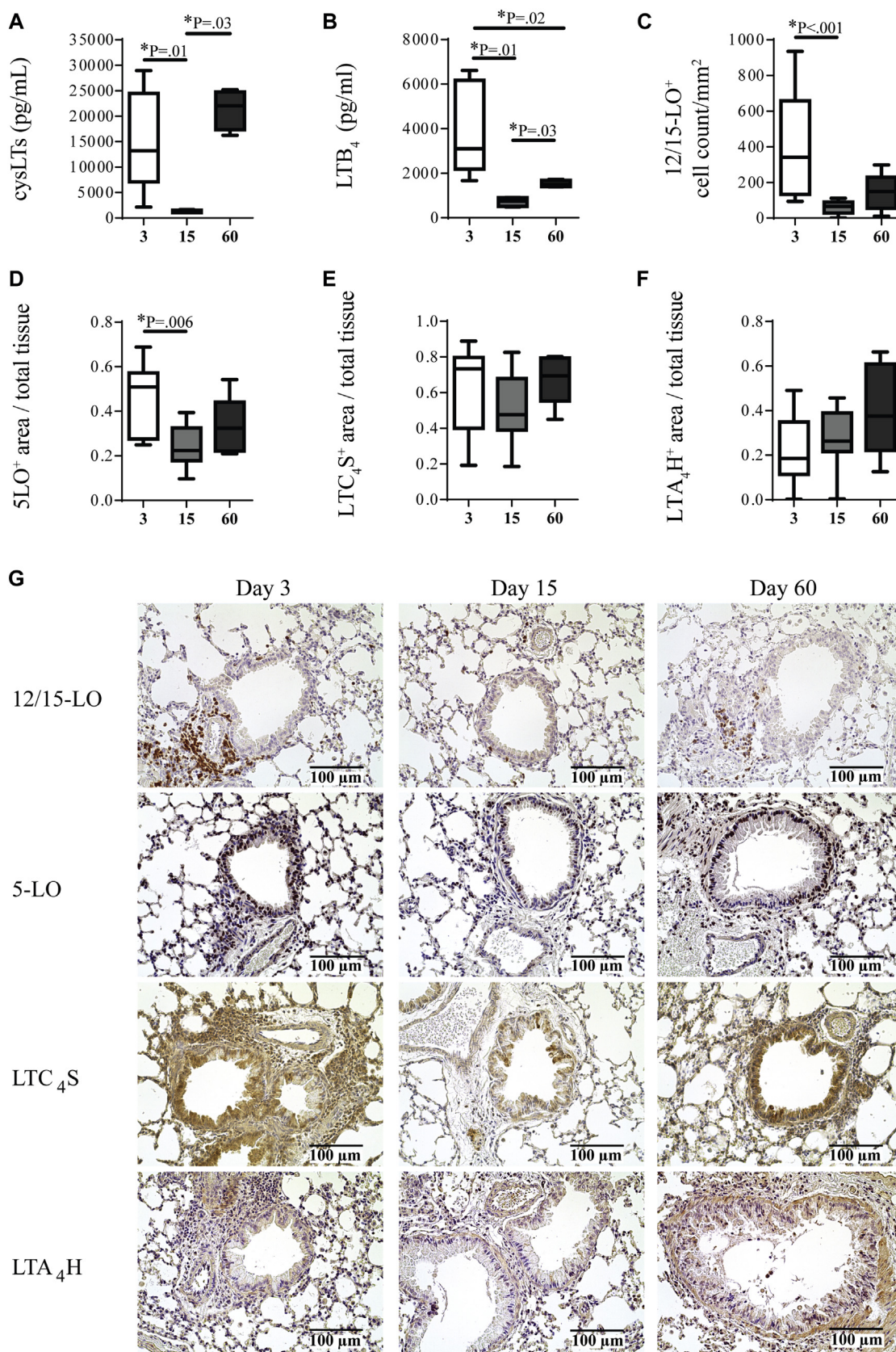


FIG 1. LT production is age dependent, and LT biosynthetic enzymes are abundant in the bronchial epithelium during allergic airway inflammation *in vivo*. **A** and **B**, Levels of cysLTs and leukotriene B₄ (LTB_4) measured in BALF from sensitized mice (n = 4-6; Mann-Whitney test). **C**, Counts of 12/15-LO⁺ cells per square millimeter lung section (n = 5-9; Mann-Whitney test). **D-F**, Quantification of 5-LO, LTC₄S, and leukotriene A₄ hydrolase (LTA_4H) expression in lung sections (n = 4-8; Mann-Whitney test). **G**, Representative immunohistochemistry staining images of lung sections.

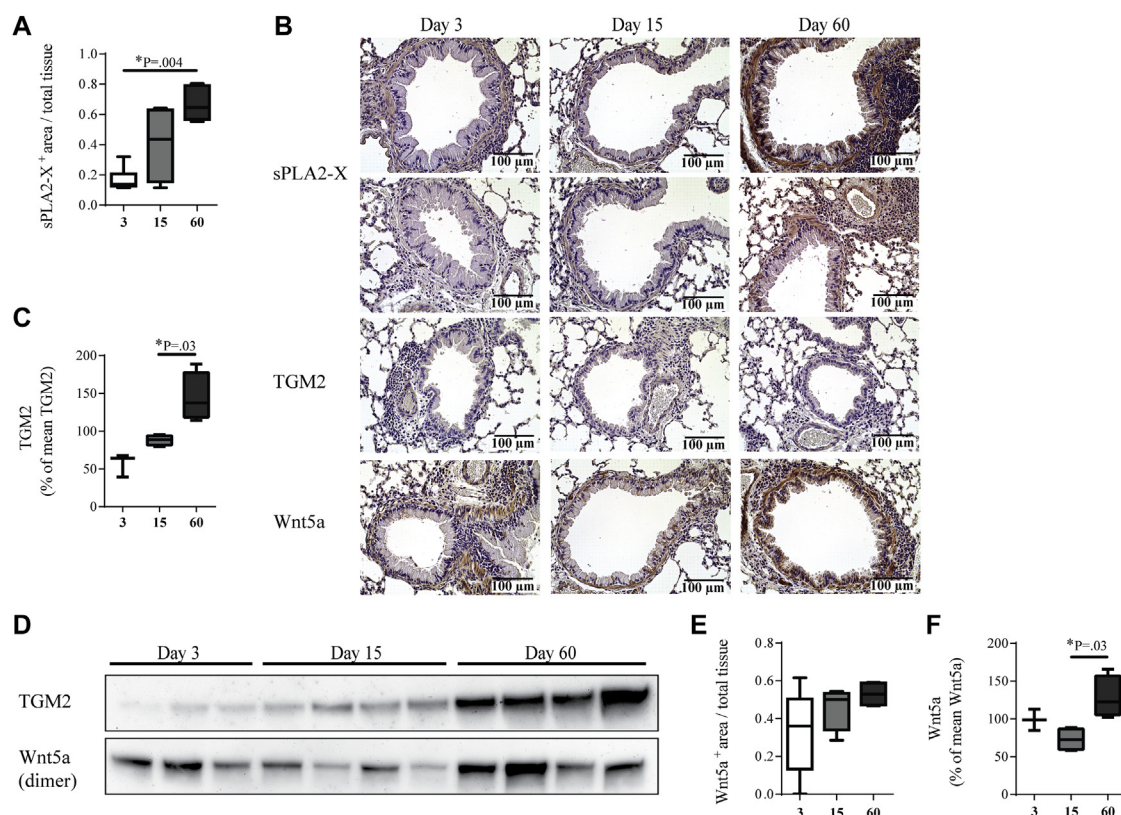


FIG 2. Age-dependent increases in sPLA2-X, Tgm2, and Wnt5a levels in the lungs of sensitized mice. **A**, Quantification of positive area of sPLA2-X. **B**, Representative immunohistochemistry staining of lung sections for sPLA2-X, Tgm2, and Wnt5a. **C**, Percentage of Tgm2 (Western blot) of BALF from sensitized mice. **D**, Western blot corresponding to Fig 2, C and F. **E**, Quantification of positive area of Wnt5a. **F**, Percentage of Wnt5a (Western blot) of BALF from sensitized mice ($n = 3-6$; Mann-Whitney test).

Nasal tissue culture

Nasal polyp tissues or inferior turbinates were obtained after written informed consent was obtained from patients undergoing functional sinus or turbinoplasty surgery, respectively. The study was approved by the local ethics committee of the Technical University of Munich. Detailed tissue culture procedures can be found in the [Methods](#) section in this article's Online Repository.

Culture of human monocyte-derived macrophages

Monocyte-derived macrophages (MDMs) were generated from CD14⁺ PBMCs, as described previously.⁴⁰ Detailed procedures can be found in the [Methods](#) section in this article's Online Repository.

Culture of NHBEs

NHBEs were obtained from Lonza (Visp, Switzerland). A detailed description of culture procedures can be found in the [Methods](#) section in this article's Online Repository.

Enzyme immunoassays

LTB₄ or cysLTs in bronchoalveolar lavage fluid (BALF) or culture supernatants were quantified by using commercially available enzyme immunoassay (EIA) kits (Cayman Chemicals, Ann Arbor, Mich), according to the manufacturer's instructions.

Histology

Fixed lung or nasal polyp tissue or cells were processed and stained, as previously published.^{41,42} For details, refer to the [Methods](#) section in this article's Online Repository.

Western blot

Total cell lysates, cell supernatants, or BALF were analyzed by means of Western blotting. For details, refer to the [Methods](#) section in this article's Online Repository.

Data analysis

Data were analyzed with GraphPad Prism software (GraphPad Software, La Jolla, Calif). Data were considered statistically significant if P values were less than .05. Image analysis was performed in ImageJ (National Institutes of Health, Bethesda, Md) and CellProfiler software (Broad Institute, Cambridge, Mass) by using previously published macros.^{41,42}

RESULTS

LT production is age dependent and LT biosynthetic enzymes are abundant in the bronchial epithelium during allergic airway inflammation *in vivo*

We previously reported increased type 2 cytokine secretion in the airways of mice that were sensitized immediately after birth (day 3) compared with mice sensitized in the preweanling or adult period (starting from day 15 or day 60 after birth, respectively).⁸ Because LTs are central mediators of allergic airway inflammation and remodeling,^{43,44} we assessed LT levels in BALF and LT enzyme expression in lung tissue of mice sensitized at different ages. High cysLT and LTB₄ levels were measured in the BALF of mice sensitized as neonates or adults, whereas

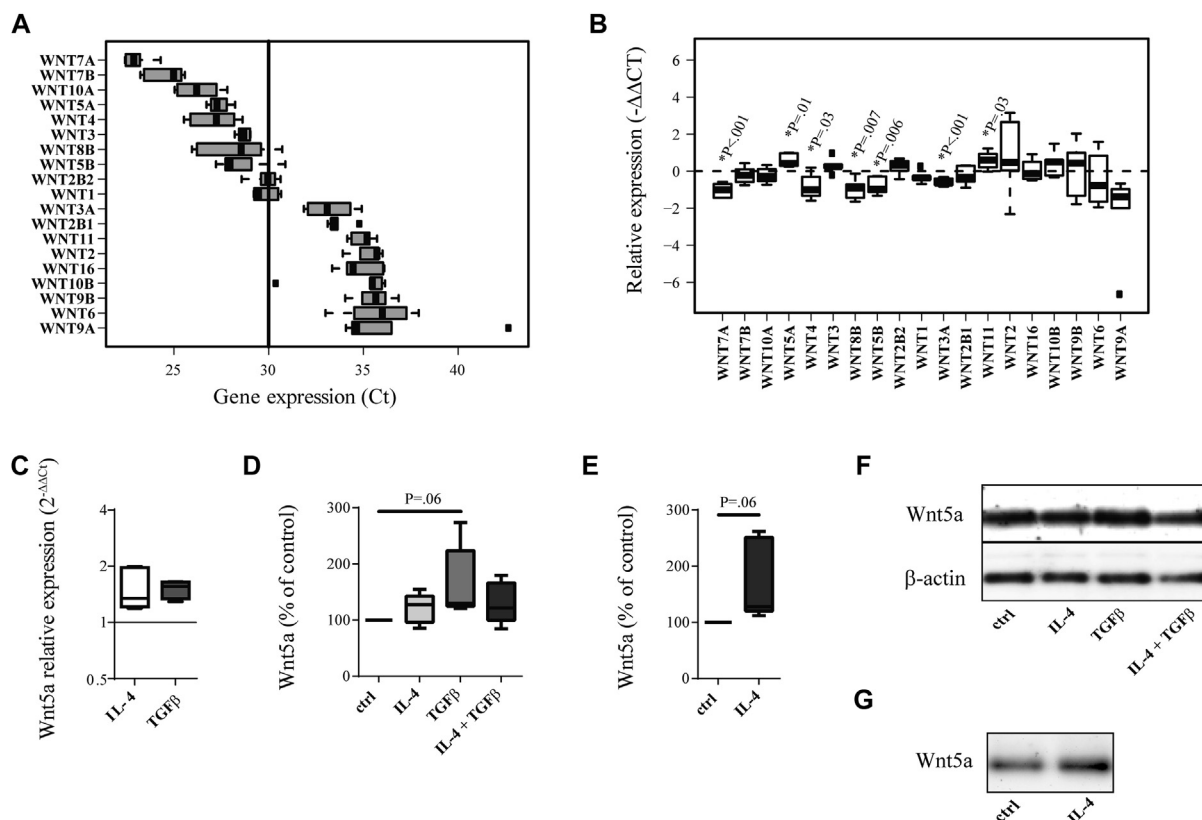


FIG 3. IL-4 exposure of NHBEs selectively upregulates Wnt5a expression. **A** and **B**, Wnt mRNA expression in IL-4-stimulated NHBEs ($n = 6$; 1-sample t test). **C**, Relative expression of Wnt5a mRNA in stimulated NHBEs ($n = 4-6$). **D** and **E**, normalized expression of Wnt5a determined by means of Western blotting of stimulated NHBEs or NHBE supernatants ($n = 5$; Wilcoxon test). **F** and **G**, Representative Western blots of Fig 3, **D** (lysates = Fig 3, **F**) or **E** (supernatants = Fig 3, **G**).

significantly lower LT levels were found in mice sensitized from day 15 after birth (Fig 1, **A** and **B**). HDM-naïve mice showed around 10- to 50-fold lower BALF LT levels, with no major age differences for LTB₄ levels and a moderate decrease in cysLT levels in adult compared with younger mice (Fig 1, **A** and **B**, and see Fig E1, **A** and **B**, in this article's Online Repository at www.jacionline.org).

In line with the high LT levels in BALF, immunohistochemistry of lung tissue from neonatal and adult sensitized mice showed a strong expression of LT enzymes (Fig 1, **C-G**), with 5-LO and LTC₄S being highly expressed in bronchial epithelial cells. In contrast, 12/15-LO was localized to perivascular infiltrates (Fig 1, **G**), which were abundant in sensitized but absent in naïve mice (see Fig E1, **G**). Numbers of infiltrating 12/15-LO⁺ cells were increased in neonates, which is consistent with previous findings of increased eosinophilia.^{8,42} Prewanling mice showed reduced levels of 5-LO and LTC₄S compared with neonatal and adult sensitized mice (Fig 1, **G**), which was not fully represented in the quantitative data (Fig 1, **D** and **E**) because 3,3'-diaminobenzidine quantification only allows for assessment of the stained tissue area rather than staining intensity. In contrast to 5-LO and LTC₄S, LTA₄ hydrolase was mostly localized to leukocytes and smooth muscle cells, with a tendency for increased expression in adult mice (Fig 1, **F** and **G**). Despite having the highest cysLT levels, mice sensitized from day 60 tended to accumulate less 12/15-LO⁺ eosinophils and to show reduced overall 5-LO expression compared with neonatally sensitized mice. However, epithelial

5-LO and LTC₄S expression was comparable in neonatal and adult mice (Fig 1, **G**), suggesting a major contribution of the bronchial epithelium to LT production in inflamed airways.

Tgm2, sPLA2-X, and Wnt5a levels in lungs of sensitized mice increase with age

Because LT production can be regulated by epithelium-derived Tgm2 and sPLA2-X,^{27,28} we investigated potential age-dependent differences in expression of these enzymes in the airways of HDM-sensitized mice. Immunohistochemistry of lung tissues showed low expression of sPLA2-X in neonatally sensitized mice and a tendency for increased sPLA2-X expression in most preweanling mice (Fig 2, **A**). In adult HDM-sensitized mice sPLA2-X expression was significantly increased (Fig 2, **A**), with apparent localization in smooth muscle cells, infiltrating leukocytes, and epithelial cells (Fig 2, **B**). However, sPLA2-X expression was not different between HDM-naïve mice at different ages (see Fig E1, **D** and **G**). This was consistent with human studies showing increased expression of sPLA2-X in AECs and macrophages of asthmatic patients and abundant expression in eosinophils.^{28,45}

In contrast to sPLA2-X, we could not detect Tgm2 expression in lung tissue (Fig 2, **B**). However, Tgm2 was abundant in BALF and increased with age (Fig 2, **C** and **D**), suggesting efficient secretion of this protein by epithelial cells, alveolar macrophages, or both.

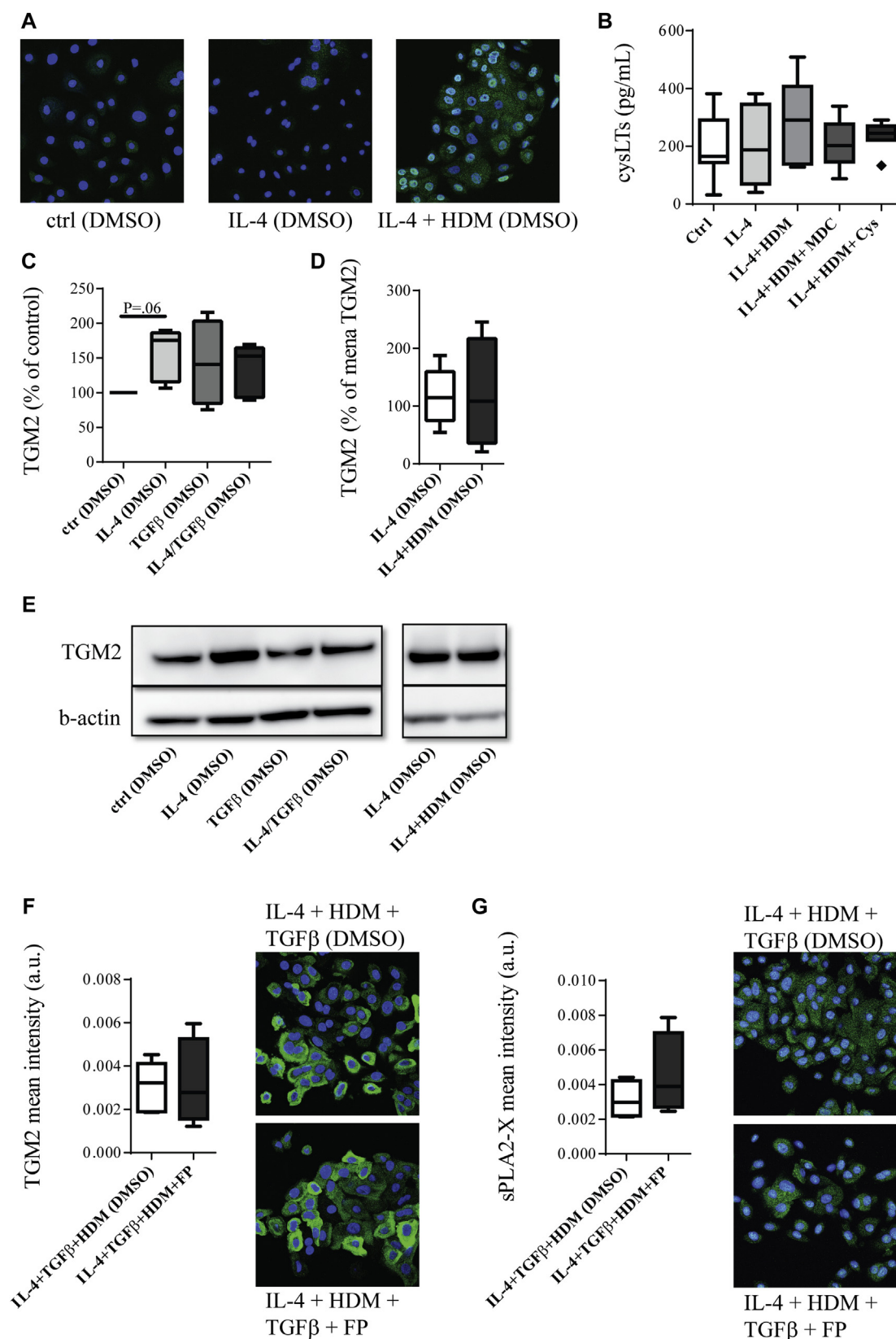


FIG 4. Tgm2 contributes to HDM-stimulated LT production in NHBes. Tgm2 and sPLA2-X expression in NHBes is steroid resistant. **A**, Representative immunofluorescence images of 5-LO expression in NHBes. **B**, Levels of cysLTs secreted by NHBes $\pm$ Tgm2 inhibitors ($n = 6-7$). **C** and **D**, Normalized expression of Tgm2 in NHBes ($n = 5$; Wilcoxon test). **E**, Representative western blots of Fig 4, **C** and **D**. **F** and **G**, Mean fluorescence intensity of Tgm2 or sPLA2-X in NHBes ($\pm$ fluticasone propionate [FP]). Representative immunofluorescence images are shown ($n = 4-5$).

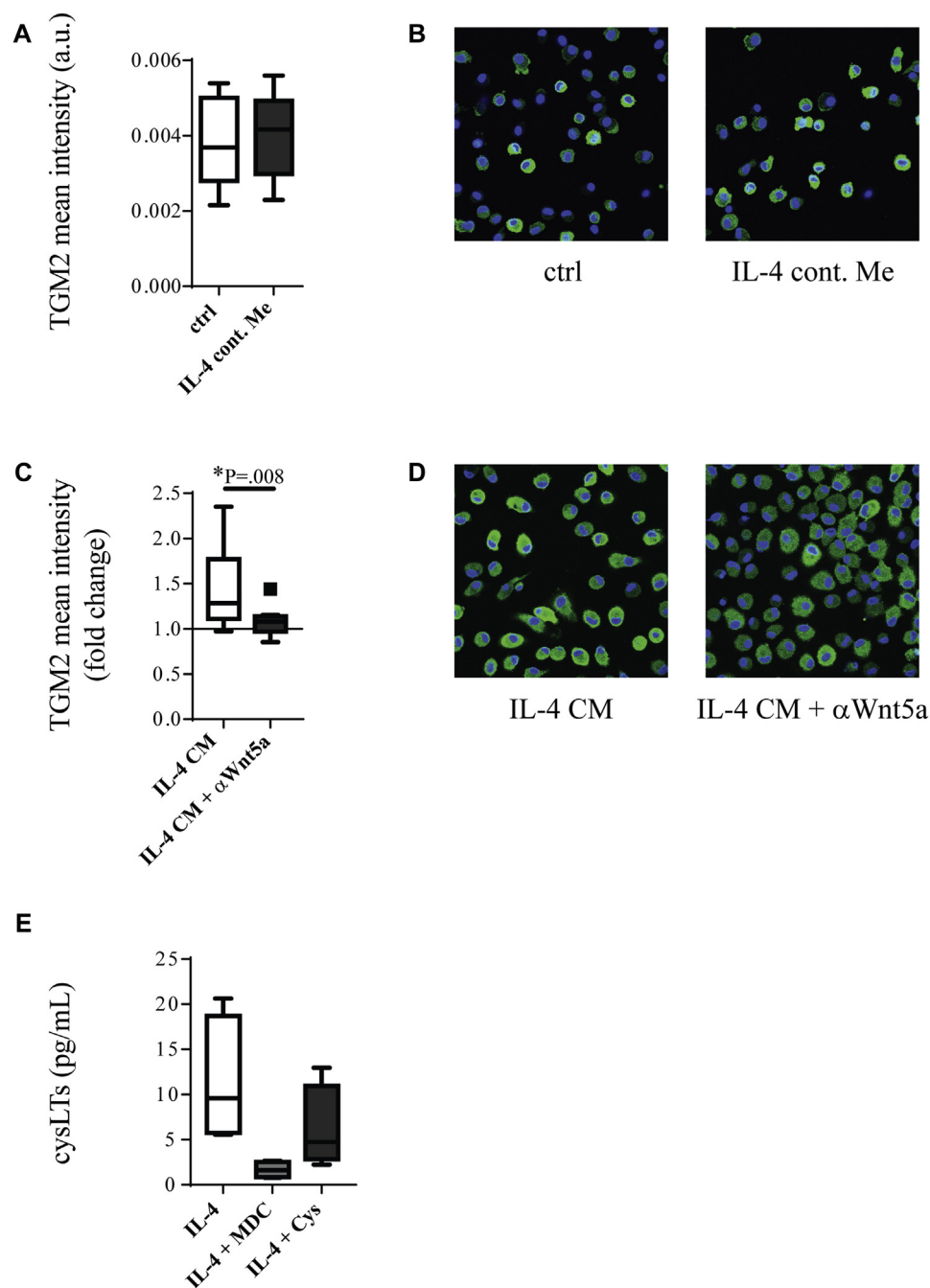


FIG 5. Secretions from IL-4-stimulated NHBEs promote Tgm2 expression in human macrophages in a Wnt5a-dependent fashion. **A** and **C**, Mean fluorescence intensity of Tgm2 in MDMs treated with IL-4-containing medium or NHBE-conditioned medium (CM) $\pm$ Wnt5a neutralizing antibody ($n = 8-9$; Wilcoxon test). **B** and **D**, Representative immunofluorescence images of Tgm2 expression in MDMs. **E**, Levels of cysLTs secreted by MDMs stimulated with IL-4 and treated with Tgm2 inhibitors ($n = 4$).

Because we previously observed coregulation of Tgm2 and Wnt5a in IL-4-stimulated AECs,³⁸ we assessed Wnt5a levels in the lungs and airways of HDM-sensitized mice. Wnt5a was present in ASMCs and BALF (Fig 2, B and D-F), and Wnt5a levels increased with age (Fig 2, D-F). Indeed, epithelial Wnt5a expression could only be observed in adult mice (Fig 2, B).

We also studied the expression of α smooth muscle actin (α SMA) and the tight junction protein zonula occludens 1 (ZO-1)

as signs of airway remodeling. HDM-sensitized mice had increased α SMA levels compared with HDM-naïve mice with a tendency toward increased airway smooth muscle thickening in older mice (see Fig E1, E and G). In addition, ZO-1 levels were reduced in mice sensitized from day 60 compared with day 15 after birth (see Fig E1, F and G). Together, these findings supported a key role for the adult epithelium in the production of remodeling factors during airway inflammation.

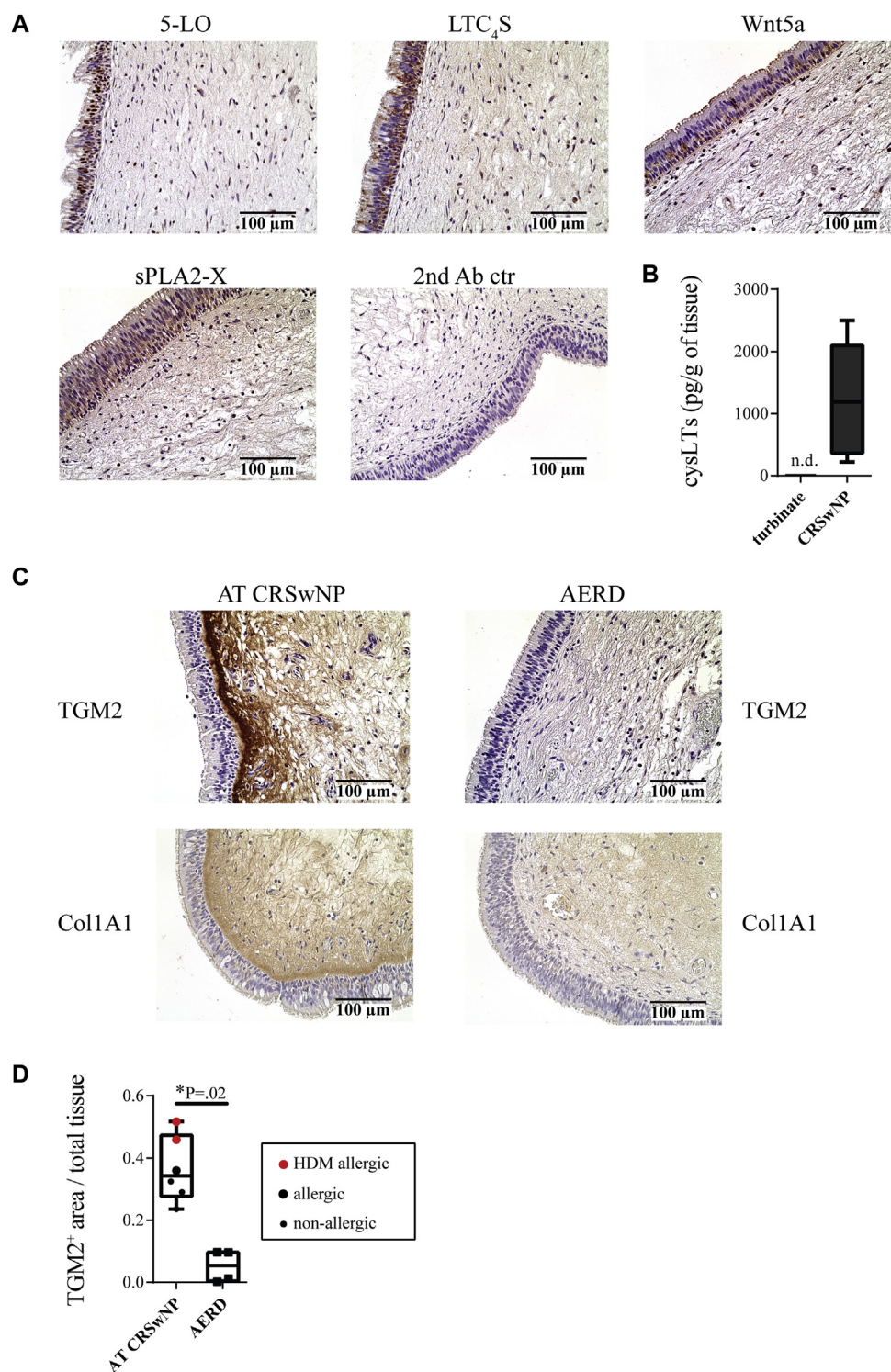


FIG 6. LT enzymes, sPLA2-X, and Wnt5a are abundant in human nasal polyps despite systemic steroid treatment, and Tgm2 levels are highest for HDM-sensitized patients. **A**, Representative immunohistochemistry staining for 5-LO, LTC₄S, Wnt5a, or sPLA2-X or control staining in nasal polyps. **B**, Levels of cysLTs in tissue culture supernatants of nasal polyps (CRSwNP; n = 7) or turbinates (n = 2). n.d., Not determined. **C**, Representative immunohistochemistry staining for Tgm2 or Col1A1 in polyps of patient with aspirin-tolerant (AT) or aspirin-exacerbated respiratory disease (AERD). **D**, Quantification of Tgm2⁺ area (n = 6 [AT] and n = 4 [AERD], Mann-Whitney test).

Wnt5a is selectively upregulated by IL-4 treatment of NHBEs

To investigate the potential relevance of these findings for airway inflammation in human subjects, we studied the regulation of the Wnt and LT pathways in NHBEs exposed to proinflammatory stimuli. We first confirmed the responsiveness of NHBEs to IL-4 by showing IL-4-induced signal transducer and activator of transcription 6 (STAT6) phosphorylation (see Fig E2, A and B, in this article's Online Repository at www.jacionline.org) and enhanced expression of the phospho-STAT6 target GATA3 (see Fig E2, C).

Next, we assessed global WNT mRNA expression in IL-4-stimulated epithelial cells and potential transcriptional changes in response to IL-4 (for a list of primers see Table E1 in this article's Online Repository at www.jacionline.org). Even if some Wnts were only expressed at low levels ($\Delta Ct > 30$), we detected mRNA transcripts for most Wnt ligands and all frizzled (FZD) receptors, with WNT7a, WNT7b, and FZD6 being the most expressed (Fig 3, A, and see Fig E2, D). In addition, we found that IL-4 selectively regulated particular WNT ligands and FZD receptors (Fig 3, B, and see Fig E2, E). The expression of Wnt5a was increased within 6 hours of IL-4 stimulation, whereas mRNA levels of WNT7a, WNT8b, WNT5b, and WNT4 were decreased (Fig 3, B). We also observed increased expression of the Wnt receptors FZD10 and FZD9 after 6 hours of IL-4 stimulation (see Fig E2, E).

Because Wnt5a was recently shown to be induced by TGF- β in ASMCs,^{29,30} we additionally assessed the effect of TGF- β 1 on Wnt5a expression in NHBEs. Indeed, TGF- β 1 was able to induce the expression of Wnt5a but only to levels comparable to those seen with IL-4 (Fig 3, C, D, and F). Also, stimulation with both cytokines did not result in an additive upregulation (Fig 3, D and F). Although upregulation of Wnt5a expression was only moderate, IL-4 clearly increased Wnt5a secretion (Fig 3, D-G).

LT production by HDM-stimulated NHBEs partially depends on Tgm2

Next, we assessed the effect of IL-4, TGF- β 1, and/or HDM on the LT pathway and the LT regulatory factors Tgm2 and sPLA2-X in NHBEs. Basal expression of 5-LO strongly depended on the donor, and no changes were observed after IL-4 stimulation (Fig 4, A). In contrast, HDM induced 5-LO expression and increased LT production in NHBEs (Fig 4, A and B), thus confirming previous findings.²⁴ Because Tgm2 has been suggested to regulate cysLT production,²⁷ we assessed the effect of Tgm2 inhibitors (monodansylcadaverine and cystamine) on cysLT production in cytokine/HDM-stimulated NHBEs. As shown in Fig 4, B, Tgm2 inhibition only had a weak effect on cysLT production by HDM-stimulated NHBEs, suggesting a minor contribution of Tgm2 to epithelial-intrinsic HDM-triggered LT release.

Although Tgm2 was induced by IL-4, HDM stimulation did not further alter Tgm2 expression, as demonstrated by means of Western blotting (Fig 4, C-E). Most NHBE donors were middle-aged, thus precluding the investigation of age effects. However, NHBEs from 1 teenage donor (14 years old) showed the lowest induction of Tgm2 in response to IL-4.

Expression of Tgm2 and sPLA2-X in NHBEs is steroid resistant

Because airway remodeling is largely resistant to steroid treatment, we assessed the effect of fluticasone propionate, one

of the most widely used inhaled glucocorticosteroids (GCs),⁴⁶ on Tgm2 and sPLA2-X expression in NHBEs. NHBEs were stimulated with IL-4, TGF- β 1, and HDM extract to induce maximal expression of both enzymes. Fluticasone propionate (1 μ mol/L for 24 hours) caused an increase rather than a decrease of Tgm2 and sPLA2-X expression in NHBEs of most donors (Fig 4, F and G), thus implicating Tgm2 and sPLA2-X in steroid-resistant airway remodeling.

Epithelially produced Wnt5a increases Tgm2 expression in human macrophages

Because we could not detect intracellular Tgm2 in the lung tissue of sensitized mice, we next assessed whether macrophages could be a source of Tgm2 in the airways and whether epithelial secretions could affect macrophage Tgm2 expression. MDMs in our study expressed high basal levels of Tgm2 because of differentiation in the presence of TGF- β 1,⁴⁷ which was performed to mimic a setting of allergic airway inflammation and to confer the high LT-producing capacity typical for alveolar macrophages.^{48,49} IL-4-containing medium (preincubated at 37°C for 24 hours) did not further increase Tgm2 expression (Fig 5, A and B). However, conditioned medium from IL-4-stimulated NHBEs upregulated macrophage Tgm2 expression compared with conditioned medium from untreated NHBEs (Fig 5, C and D). This effect was abrogated on addition of a Wnt5a blocking antibody (Fig 5, C and D). Thus Wnt5a secreted by the airway epithelium can likely act on myeloid cells in the airways to promote Tgm2 expression.

Next, we treated MDMs with Tgm2 inhibitors to study the potential effects on LT production by these cells. Both inhibitors showed a tendency to decrease cysLT production in IL-4-treated MDMs (Fig 5, E), suggesting a role for Tgm2 in regulating LT production in macrophages, which represent a potent source of these mediators in the airways.⁴⁸

LT enzymes, sPLA2-X, and Wnt5a are abundant in human nasal polyps despite systemic steroid treatment, and Tgm2 expression is highest in HDM-sensitized patients

To address the clinical relevance of the identified network of remodeling factors, we studied the expression of LT enzymes, Tgm2, sPLA2-X, and Wnt5a in human nasal polyp specimens as an accessible clinical sample from an adult setting of chronic airway inflammation associated with tissue remodeling. Of note, all patients received oral systemic treatment with steroids (40 mg/d prednisolone for 5 days) before surgery (see Table E2 in this article's Online Repository at www.jacionline.org). As shown in Fig 6, A, 5-LO, LTC₄S, Wnt5a, and sPLA2-X were highly abundant in nasal polyp tissues, with predominant localization in the nasal epithelium. In contrast, only LTC₄S was present in normal nasal turbinate tissue (see Fig E3 in this article's Online Repository at www.jacionline.org). In line with expression patterns of 5-LO, LTC₄S, Wnt5a, and sPLA2-X, nasal polyps, but not normal nasal turbinate tissue, released considerable levels of cysLTs when cultured overnight in epithelial cell culture medium (Fig 6, B). Tgm2 was not detected within the epithelium but appeared to be secreted basolaterally and to colocalize with collagen in some but not all patients (Fig 6, C).

Further stratification showed that Tgm2 was particularly abundant in nasal polyp tissue of HDM-sensitized subjects, followed by a patient with grass pollen allergy and nonallergic, aspirin-tolerant patients (Fig 6, D). In contrast, Tgm2 was largely absent from nasal polyps of patients with aspirin-exacerbated respiratory disease, a distinct nonallergic endotype of chronic airway inflammation (Fig 6, C and D). Because all patients with nasal polyps were middle-aged (41–68 years old), we could not compare Tgm2, sPLA2-X, or cysLT levels in nasal polyp tissue from children, teenagers, and adults. However, during allergic airway inflammation in adults, the pathologic cascade consisting of Tgm2, Wnt5a, sPLA2-X, and LTs (see the graphical abstract) appears to be conserved across different respiratory compartments, disease settings, and species.

DISCUSSION

Age-specific discrepancies in airway eosinophilia and remodeling are increasingly recognized,^{4,14,50} but explanatory mechanisms have been lacking. Combining a murine model of allergic airway inflammation induced by HDM sensitization at different ages and human *in vitro* studies, we identify an epithelially expressed cascade of remodeling factors, which is age dependent and steroid resistant. This cascade involves Tgm2, secretory phospholipase A₂, and Wnt5a and likely governs cysLT production in inflamed airways, particularly during adult-onset disease. Thus the present study describes a mechanism that might underlie the differential development of airway remodeling in adults, schoolchildren, and neonates.

In view of the previously reported increase in type 2 cytokine levels and airway eosinophilia in HDM-sensitized neonatal compared with adult mice, it was unexpected to find a tendency toward higher cysLT levels in adult airways. Similar airway resistance was reported for neonatal and adult mice in response to moderate methacholine doses, but airway resistance in neonates was increased at a higher dose.⁸ This suggests that type 2 cytokines, such as IL-33,⁵¹ rather than cysLTs are driving increased airway inflammation in neonates. In our acute model of allergic airway inflammation, we did not observe major structural changes, such as collagen deposition. However, there was a tendency toward increased smooth muscle thickening and a loss of the tight junction protein ZO-1 in adult compared with younger mice, which might be explained by high cysLT production.^{24,52} Thus our findings suggest that adults upregulate remodeling mechanisms that are distinct from the increase in levels of hallmark type 2 cytokines in neonates. We show that these include Tgm2, Wnt5a, and sPLA2-X, which regulate macrophage activation and extracellular matrix crosslinking.^{35,53,54}

Because our 3-week HDM model involved both infiltration of eosinophils caused by an adaptive T_H2 response and innate upregulation of LT biosynthetic enzymes in bronchial epithelial cells, we cannot discriminate the major source of cysLTs in our experiments. In a shorter (3-day) HDM model BALF cysLT levels were increased despite the absence of infiltrating eosinophils, indicating that resident cells, which recognize components of HDM through innate receptors, can contribute to cysLT production during airway inflammation *in vivo*.²⁴ The abundance of 5-LO and LTC₄S in the airway epithelium, as observed in our study, might suggest a major contribution of epithelial cells to cysLT production not only during the early sensitization phase but also during subsequent inflammation and remodeling. The

high production of cysLTs in adult mice despite lower numbers of infiltrating granulocytes further supported a role for epithelial cells and macrophages in cysLT production in adult inflamed airways. Indeed, our data on human macrophages support the view that AECs might communicate with myeloid cells through Wnt5a, Tgm2, and sPLA2 to perpetuate cysLT production and remodeling.^{27,28,53}

The most striking age differences were observed for Tgm2 and sPLA2-X expression, which were both increased when mice were sensitized with HDM as adults instead of as neonates. Although age-dependent regulation of Tgm2 and sPLA2-X in lung inflammation has not been reported previously, a recent study has shown that aging increased the expression of another phospholipase (sPLA2-IIID), which contributed to immune suppression and lung damage during viral infection.²⁹ Thus the increase in sPLA2 expression in response to lung inflammation during adulthood might be a universal event, which could explain age-dependent differences in the susceptibility to infection, fibrosis, and asthma. Because sPLA2-X expression in the lung has been linked to asthma severity,⁵⁵ future studies should investigate factors that govern differential sPLA2 induction at different ages.

To our knowledge, our study is the first to implicate Tgm2 as an age-dependent regulator of remodeling in allergic airway inflammation. However, studies on other airway diseases, such as cystic fibrosis, idiopathic pulmonary fibrosis, and lung cancer, support a central role for Tgm2 in aberrant pulmonary wound healing and have led to the development of Tgm2 inhibitors for the treatment of these diseases.^{54,56–59} Thus airway Tgm2 levels should be assessed in asthmatic patients and patients with chronic rhinosinusitis with nasal polyps (CRSwNP) and correlated to disease severity to investigate whether Tgm2 might represent a therapeutic target in asthma or nasal polyposis. Because Tgm2 expression can be modulated by DNA methylation⁶⁰ or oxidative stress,⁶¹ which are both strongly influenced by age,^{62,63} future studies should clarify the role of these factors in age differences in levels of Tgm2 and its regulatory targets in the setting of airway inflammation.

Mechanistically, Tgm2-activated sPLA2-X exerts its effects through increasing intracellular Ca²⁺ and mitogen-activated protein kinase activation, 2 events that enhance cytosolic PLA₂ and 5-LO activity and thus LT production.²⁸ Therefore increased sPLA2-X expression might explain the increased BALF cysLT levels in the lungs of adult HDM-sensitized mice. Previous studies identifying sPLA2-X as a key driver of allergic airway inflammation and remodeling^{64,65} reported sPLA2-X expression in bronchial epithelial cells and alveolar macrophages but not in ASMCs. This is in partial contrast to our data because we observed strong expression of sPLA2-X in ASMCs. However, these earlier studies used ovalbumin as an allergen, which might explain this discrepancy because expression of sPLA2-X can vary depending on the cytokine milieu.⁶⁶ Another recent study reported the sPLA2-V–dependent regulation of Tgm2 in human IL-4–activated macrophages, thus supporting a role for sPLA2-Tgm2 interactions during type 2 inflammation.⁶⁷

Tgm2 might also represent an important driver of GC-resistant airway inflammation because GC treatment increased Tgm2 activity and expression in brain and kidney cells.^{68,69} We observed a similar resistance of Tgm2 and its regulatory target sPLA2-X to GC treatment in NHBEs and nasal polyps of allergic patients, suggesting that Tgm2 is an attractive drug target, particularly in patients with steroid-resistant forms of airway inflammation.

Because GCs can induce epithelial cell apoptosis and thereby contribute to airway remodeling, it would be interesting to determine the role of Tgm2 and sPLA2-X during GC-driven epithelial damage in patients with severe airway inflammation.⁷⁰⁻⁷²

Of note, the most commonly used LT-modifying drug (the *cysLT1R* antagonist montelukast) does not suppress the formation of *cysLTs*, which might thus bind to alternative receptors, such as *cysLT2R*, which was recently shown to promote platelet-driven allergic airway inflammation.⁷³ Thus targeting the epithelial Tgm2–Wnt5a–sPLA2-X pathway by using topically administered compounds might prove advantageous over current LT modifiers and should be considered as add-on therapy in patients with difficult-to-treat asthma, nasal polyps, or both.

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Key messages

- A steroid-resistant cascade of Wnt5a, Tgm2, and LTs drives late-onset HDM-induced allergic airway inflammation.
- AECs secrete Wnt5a to promote the production of remodeling factors by macrophages.
- Epithelial cells might represent a major source of *cysLTs* in inflamed airways, even after treatment with GCs.

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METHODS

Mouse model of allergic airway inflammation

BALB/c mice, which were obtained from Charles River Breeding Laboratories (l'Arbresle Cedex, France), were housed under specific pathogen-free conditions. Briefly, mice were sensitized intranasally with 5 μ g of HDM extract (Greer Laboratories, Lenoir, NC) from days 3, 15, or 60 of life every second day for a total of 6 exposures. BALF and lung tissue samples were collected 4 days after the last challenge.

Nasal tissue culture

Nasal polyp tissues or inferior turbinates were obtained after obtaining written informed consent from patients undergoing functional sinus or turbinoplastic surgery, respectively. All patients with CRSwNP received systemic steroids preoperatively. Nasal tissues were divided and either fixed in 10% formalin for histology or placed in cell culture medium for tissue culture experiments. Fresh tissues were washed twice with medium for removal of blood and mucus and cultured in supplemented Airway Epithelial Cell Growth Medium (PromoCell, Heidelberg, Germany) plus antibiotics/antimycotics for 24 hours (5% CO₂ at 37°C) under air-liquid interface conditions.

Culture of human MDMs

All MDM cultures were performed in the presence of GM-CSF (10 ng/mL) and TGF- β 1 (2 ng/mL) during the complete differentiation protocol. Stimulation was performed according to NHBE stimulation.

NHBE culture

NHBEs from nonsmoking subjects were obtained from Lonza (Visp, Switzerland) and expanded in bronchial epithelium growth medium (Lonza) before they were stored in liquid nitrogen in passage 2. Before experimentation, cells were further expanded and then seeded in 6-well plates at a density of 1.5 to 2.5 $\times$ 10⁵ cells/well. Cells in passage 4 were grown to 80% to 85% confluency and serum deprived in basal medium (BEBM, Lonza) before stimulation overnight. Rested cells were then stimulated with 50 ng/mL human recombinant IL-4 (PromoKine, PromoCell), 5 ng/mL TGF- β 1 (PeproTech, Rocky Hill, NJ), and/or 10 μ g/mL HDM (Stallergenes, Antony, France) or treated with 100 μ mol/L (in dimethyl sulfoxide [DMSO]) cystamine dihydrochloride (Tocris Bioscience, Bristol, United Kingdom), 25 μ mol/L (in DMSO) Dansylcadaverine (Sigma-Aldrich, Munich, Germany), or 1 μ mol/L (in DMSO) fluticasone propionate (Sigma-Aldrich) for 6 hours to analyze mRNA expression or 24 hours. All experiments were performed at passage 4.

Immunohistochemistry and immunofluorescence

Left lobes of lung or nasal polyp tissues were fixed in 10% formalin at 4°C and paraffin embedded. Sections (4 μ m) were stained after blocking unspecific binding with 5% BSA plus 10% donkey serum by using the following primary antibodies: rabbit anti-human 5-LO (a kind gift of Professor Olof Rådmark, Karolinska Institute, Stockholm, Sweden), Tgm2 (Cell Signaling Technology, Danvers, Mass), 12/15-LO, α SMA (both from Abcam, Cambridge, United Kingdom), Wnt5a (Lifespan Bioscience, Seattle, Wash), LTC₄S, sPLA2-X, and LTA₄ hydrolase (all from Santa Cruz, Dallas, Tex), followed by secondary biotinylated donkey-anti rabbit antibody and a horseradish peroxidase-based

detection kit (ABC, Life Technologies, Thermo Fisher) by using 3,3'-diaminobenzidine detection reagents (Sigma-Aldrich). For immunofluorescent staining, cells were fixed for 15 minutes with 4% paraformaldehyde, followed by permeabilization with acetone (10 minutes at -20°C). After blocking (5% BSA and 10% donkey serum), cells or tissues were incubated with rabbit primary antibodies (see above) or with rabbit anti-human ZO-1 (Life Technologies, Thermo Fisher). Fluorescence-conjugated donkey anti-rabbit (Alexa Fluor 488) antibodies (Life Technologies, Thermo Fisher) were used for detection. Images were recorded on an EVOS system (Life Technologies, Thermo Fisher) or a Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Quantitative real-time PCR

In brief, isolated total RNA from NHBEs was subjected to reverse transcription by using a high-capacity cDNA kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, Mass) after real-time PCR with the ViiA7 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific) and the FastStart Universal SYBR Green Master Mix (Roche, Mannheim, Germany). Expression of genes of interest was analyzed by using the 2^{- $\Delta\Delta$ Ct} equation. The specific primers are listed in [Table E1](#).

Western blotting

Total cell lysates were obtained by means of direct lysis of cells in RIPA buffer (Pierce, Thermo Fischer) with 2% EDTA-free complete protease inhibitor mixture (Roche). Equal volumes of cell-free supernatants were 10 $\times$ concentrated by using Amicon Ultra-0.5 ml Centrifugal Filters 3K (EMD Millipore, Merck, Darmstadt, Germany). NuPAGE LDS sample buffer was added to total lysates, equal volumes of cell-free supernatants, or equal volumes of BALF, according to the manufacturer's recommendations, and heated at 70°C for 10 minutes before loading. Expression of phospho-STAT6 (Cell Signaling, Danvers, Mass), Wnt5a (R&D Systems, Minneapolis, Minn), and Tgm2 (Cell Signaling) was analyzed by means of Western blotting. Hypoxanthine phosphoribosyltransferase (HPRT) or β -actin (both from Santa Cruz) was used as a loading control. Samples were loaded on 4-12% or 10% Bis-Tris protein gels (NuPAGE Novex, Thermo Fisher) and transferred to a polyvinylidene difluoride membrane (Merck Millipore, Darmstadt, Germany) by using the Mini Blot Module (Thermo Fisher) at 20 V for 90 minutes. The polyvinylidene difluoride membrane was blocked in 5% nonfat dry milk in 1 $\times$ TBS containing 0.02% Tween to prevent unspecific binding. The primary antibodies (both 1:1000) were incubated with the membrane overnight in blocking solution at 4°C, and after subsequent washing, the membrane was incubated with the corresponding secondary horseradish peroxidase-conjugated antibody in blocking solution for 1 hour at room temperature. Detection was performed using enhanced chemiluminescence (Amersham ECL Prime, GE Healthcare, Little Chalfont, United Kingdom, or SuperSignal West Femto Maximum Sensitive Substrate, Pierce, Thermo Fisher), according to the manufacturer's instructions, and recorded with the ECL ChemoCam Imager (Intas Science Imaging Instruments, Göttingen, Germany). Protein levels were quantified by using LabImage 1D (Intas Science Imaging Instruments, Göttingen, Germany) by means of normalization to unstimulated control or mean expression and corrected for the amount of HPRT or β -actin in the samples (for lysates).

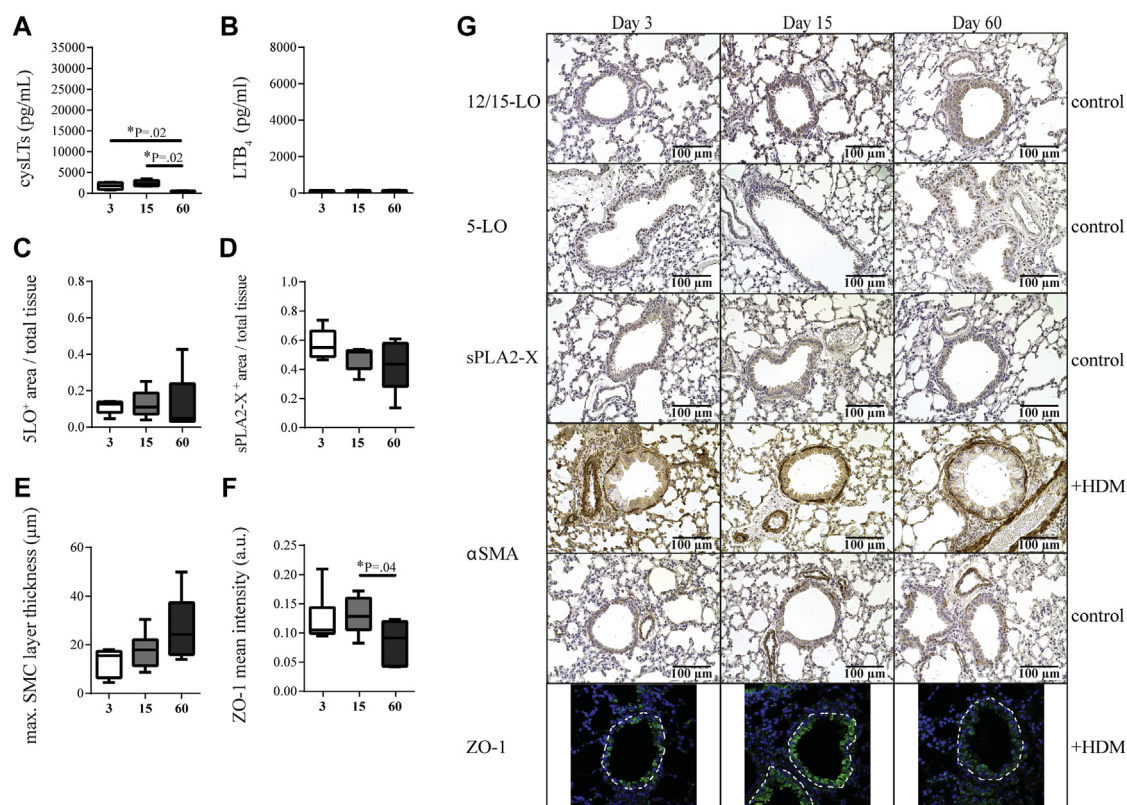


FIG E1. Age differences in airway LT levels are absent at baseline, and early signs of airway remodeling increase with age. **A** and **B**, Levels of cysLTs and leukotriene B_4 (LTB_4) measured in BALF from control mice using the same scale as for HDM-sensitized mice in Fig 1 ($n = 4-5$ per group; Mann-Whitney test). **C** and **D**, Quantification of 5-LO and sPLA2-X staining in lung sections from control mice ($n = 5$ per group). **E**, Quantification of maximal smooth muscle cells layer thickness in HDM-sensitized mice ($n = 6-8$). **F**, Mean fluorescence intensity of ZO-1 in HDM-sensitized mice ($n = 6$; Mann-Whitney test). **G**, Representative immunohistochemistry staining and immunofluorescence images of lung sections.

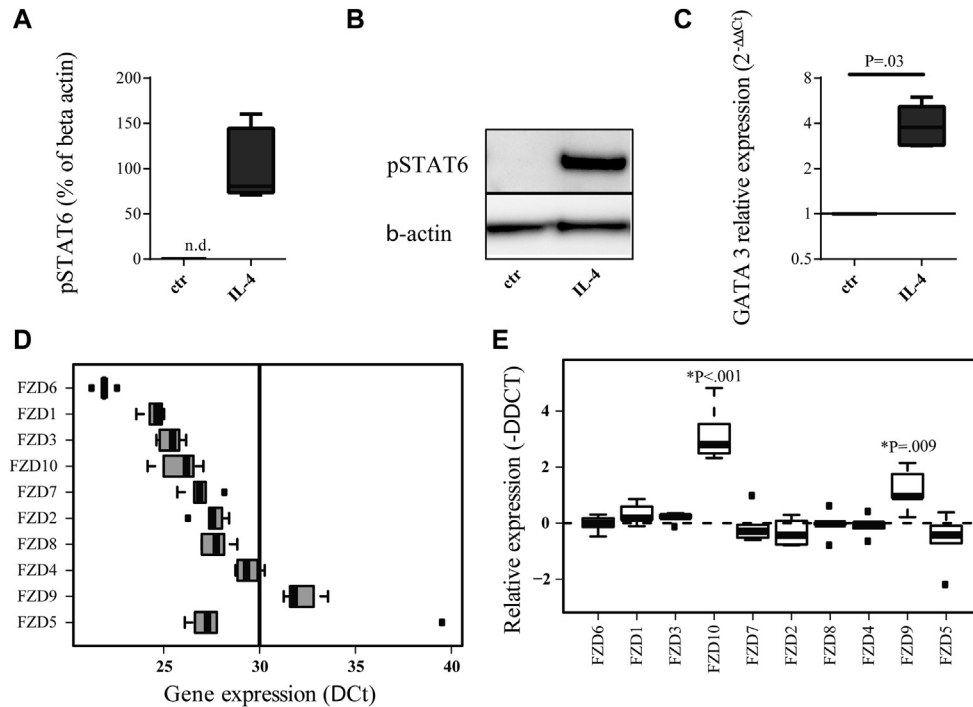


FIG E2. NHBes are IL-4 responsive and show a distinct regulation pattern of Wnt and FZD genes. **A** and **B**, Normalized expression of phospho-STAT6 determined by means of Western blotting of IL-4-stimulated NHBes ($n = 5$) and IL-4-stimulated NHBes ($n = 6$; Wilcoxon test). **C**, Normalized mRNA expression of GATA3 determined by quantitative PCR. **D** and **E**, FZD mRNA expression was determined by means of quantitative PCR and is shown as gene expression of FZDs in IL-4-stimulated NHBes and relative expression to control values corrected for housekeeping genes ($n = 6$; 1-sample t test).

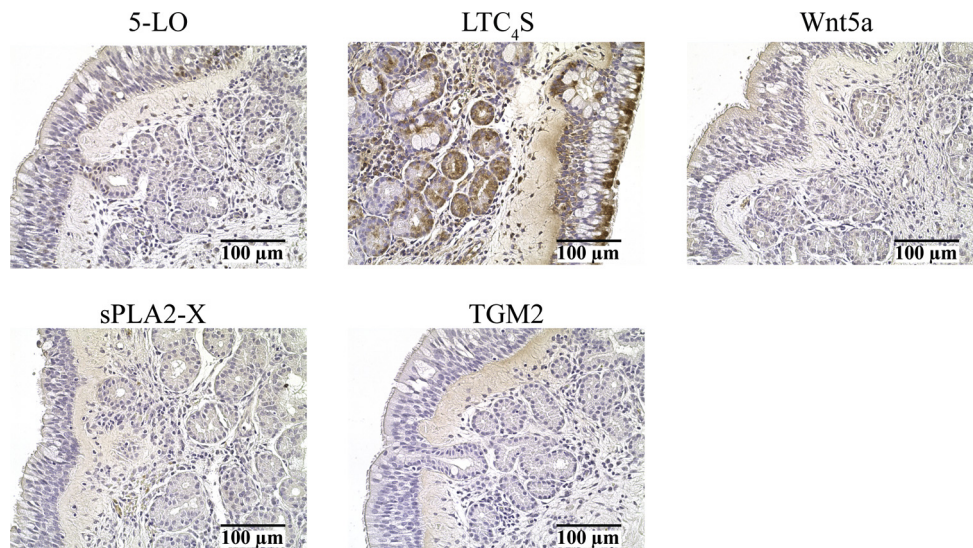


FIG E3. Nasal turbinates from healthy control subjects show low levels of 5-LO, Wnt5a, sPLA2-X, and Tgm2. Representative immunohistochemistry images of turbinate sections for 5-LO, LTC₄S, Wnt5a, sPLA2-X, and Tgm2 are shown.

TABLE E1. Quantitative PCR primers for housekeeping, Wnt, and FZD genes

	Gene name	FWD	RV
Housekeeping gene	β -Actin	TGAGAGGGAAATGTGCGTG	TGCTTGCTGATCCACATCTGC
	<i>HPRT</i>	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
	<i>TFR</i>	TGTGGGGAAGGGGCTGT	CCACCAACAAGTTAGAGAATGC
Wnt gene	<i>Wnt1</i>	GGTTTCTGCTACGCTGCTG	TAAGCAGGTTCTGTGGAGGAG
	<i>Wnt2</i>	GGGCTGGCCTTTATCGCTC	GAGCCAGATTCCACCGAGAG
	<i>Wnt2b1</i>	GATCCTTGAGGACGGCAGTA	GCTACCAAAACCAGGATATT
	<i>Wnt2b2</i>	TAGGTCTTGCTGCCTTCTG	TTGTCAAGATCACTCGTGC
	<i>Wnt3</i>	ACTTTTGTGAGCCCAACCCA	TTCTCCGTCCTCGTGTGTG
	<i>Wnt3a</i>	GTGGAAGTGCACACCGT	ATGAGCGTGTCACTGCAAAG
	<i>Wnt4</i>	CTCGTCTTCGCCGTCTTCT	GATCAGGCCCTTGAGTTTCTC
	<i>Wnt5a</i>	GCTCGGATTCTCGGCT	CAAAGCAACTCCTGGGCTTA
	<i>Wnt5b</i>	AACGCATCTGTCTTTGGGAG	GCTGATGGCGTTGACCA
	<i>Wnt6</i>	TGCTGCTGCTGCTGCTC	CAGATGCTGGTAGGGTCCAT
	<i>Wnt7a</i>	GAAGTTGCACAACAACGAGG	AGTGTGGTCCAGCACGTCTT
	<i>Wnt7b</i>	AGCCAACATCATCTGCAACA	CTGGTACTGGCACTCGTTGA
	<i>Wnt8b</i>	CACCTGTGTCCTCCAACCTCA	TGCCACACTGCTGGAGTAAA
	<i>Wnt9a</i>	CTTCGGCCGCCTACTTC	GTCGCAGGCCTTGTAAGTGC
	<i>Wnt9b</i>	GTGCAGTGGTGCTGCTACG	GCACACATGCCGGTTTATGC
	<i>Wnt10a</i>	AACACCAATTCAGGGACCAG	CAAAAGCGCTCTCTCGGAA
	<i>Wnt10b</i>	GGTCCACGAGTGTCAGCAC	CCAGCATGGAGAAGGAAAAA
	<i>Wnt11</i>	CGTGTGCTATGGCATCAAGT	GTGTGCATGAGCTCCAGGTT
	<i>Wnt16</i>	CACGGGCAAAGAAAACAAAG	GCATGTTTTACAGCACAGG
FZD gene	<i>FZD1</i>	AGCTTTGTGTGGGTTGGAAG	CGGTAAATCTAAGCGCAGG
	<i>FZD2</i>	ACATCGCCTACAACCAGACC	CCTTCACCAGCGGATAGAAC
	<i>FZD3</i>	GCATCTGGGAAACAACGTG	TCAAGTCTGGACGACTCATTG
	<i>FZD4</i>	AAGTTTACACCGCTCATCC	TGACTGAAAGACACATGCCG
	<i>FZD5</i>	TGGGGACTGTCTGCTCTTCT	CAAAGATAAACTGCTTCGGGA
	<i>FZD6</i>	AAAATGGCCTACAACATGACG	ATTCCAGATTGCGAGAGGA
	<i>FZD7</i>	CGCCTCTGTTCGTCTACCTC	CCATGAGCTTCTCCAGCTTC
	<i>FZD8</i>	AAGACAGGCCAGATCGCTAA	CGATAAGGAAGGTGGAGACG
	<i>FZD9</i>	GAAGCTGGAGAAGCTCATGG	AAGTCCATGTTGAGCGGTTT
	<i>FZD10</i>	TATGAGATCCCTGCCCAGTC	CAACCAAGAAAAGCACCACA

TABLE E2. Characterization of patients with CRSwNP

No. (male/female)	10 (6/4)
Age (y)	52.9 ± 9.2
Systemic steroids	10/10
Asthma	6/10
Allergy	3/10
AERD	4/10
HDM sensitized	2/10

AERD, Aspirin-exacerbated respiratory disease.