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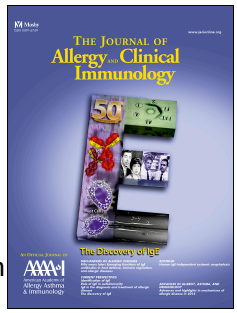

Age dictates a steroid resistant cascade of Wnt5a, transglutaminase-2 and leukotrienes in inflamed airways

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Title page

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

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ABSTRACT

Background

Airway remodeling is a detrimental and refractory process showing age-dependent clinical manifestations, which 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 allergens (HDM) from day 3, 15 or 60 after birth. Remodeling factors in murine bronchoalveolar lavage fluid (BALF), lung or human nasal polyp tissues were analyzed by westernblot, immunoassays or histology. Regulatory mechanisms were studied in cytokine/HDM- stimulated NHBEs and macrophages.

Results

BALF 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, whilst adult mice expressed more secretory phospholipase A2 (sPLA2-X), Wnt5a and transglutaminase 2 (TGM2). Older mice showed co-localization 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 in turn reduced LT release. TGM2, sPLA2-X 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 may represent a therapeutic target for airway inflammation and remodeling.

Key messages

- A steroid resistant cascade of wnt5a, transglutaminase 2 and leukotrienes drives late-onset house dust mite allergic airway inflammation
- Airway epithelial cells secrete wnt5a to promote the production of remodeling factors by macrophages
- Epithelial cells may represent a major source of cysteinyl leukotrienes in inflamed airways even after treatment with glucocorticosteroids.

Capsule summary

Epithelial remodeling factors are differentially regulated during early- and late-onset allergic airway inflammation. Age-specific diagnostic, preventive and therapeutic approaches may

thus be required to improve the clinical outcomes for patients suffering from chronic airway inflammation.

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

Abbreviations:

5-LO	5-lipoxygenase
AERD	aspirin exacerbated respiratory disease
CRSwNP	chronic rhinosinusitis with nasal polyps
cysLTs	cysteinyl leukotrienes
LTA_4H	leukotriene A_4 hydrolase
LTB_4	leukotriene B_4
LTC_4S	leukotriene C_4 synthase
NHBEs	normal human bronchial epithelial cells
MDM	monocyte derived macrophages
sPLA2-X	group 10 secretory phospholipase A2
TGM2	transglutaminase 2

INTRODUCTION

Asthma affects more than 300 million patients worldwide and airway remodeling, characterized by persistent changes in airway structure, airway hyper-responsiveness and lung function impairment, is a major cause of morbidity, which cannot be reversed by currently available therapies.[1,2] Thus, airway remodeling represents a major unmet clinical need, which is however insufficiently addressed as 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 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 TGFβ1 that 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 may be parallel rather than causally related processes.[13,14] This has lead 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 TSLP, which activate type 2 inflammation.[15] In mice sensitized with house dust mite (HDM) allergens from day 3 after birth, IL-33 was shown to increase with age and to be crucial for remodeling, whilst 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 TGFβ1. [19–21] In response to inflammatory stimuli such as bradykinin, LPS or HDM extract, human bronchial epithelial cells (HBEC) upregulate enzymes of the LT biosynthetic cascade (5-lipoxygenase (5-LO), 5-LO activating protein (FLAP), LTC_4 synthase (LTC_4S) and LTA_4 hydrolase (LTA_4H)), 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 asthmatics and to regulate cysLT production via activating group X secretory phospholipase A2 (sPLA2-X), another hallmark enzyme of asthma severity and remodeling.[26–28] Increased expression of a different 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, which has only recently emerged as being involved in asthma is the wingless/ integrase (Wnt) pathway, which in humans 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), potently upregulated by TGFβ1 and promotes collagen and fibronectin expression, thus implicating Wnt5a in remodeling in asthma.[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 NHBEs.[38] It is also known that Wnt signaling is defective in the adipose tissue of TGM2 deficient mice.[39] However, if 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 airway epithelial cells abundantly express these factors in settings of airway inflammation. Finally, we identify a regulatory network of TGM2, Wnt5a and LTs, which may contribute to the steroid resistance of remodeling processes in asthma and nasal polyposis.

METHODS

Animal experiments were performed according to institutional guidelines and to 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 supplement.

Nasal tissue culture

Nasal polyp tissues or inferior turbinates were obtained after written informed consent from patients undergoing functional sinus or turbinoplastic surgery, respectively. The study was approved by the local ethics committee, Technical University of Munich. Detailed tissue culture procedures can be found in the supplement.

Culture of human monocyte derived macrophages

Monocyte derived macrophages (MDM) were generated from $CD14^+$ PBMCs as described previously.[40] Detailed procedures can be found in the supplement.

Culture of normal human bronchial epithelial cells

NHBEs were obtained from Lonza (Visp, Switzerland). A detailed description of culture procedures can be found in the supplement.

Enzyme immunoassays (EIA)

Leukotriene B_4 or cysteinyl leukotrienes in BALF or culture supernatants were quantified using commercially available EIA kits (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer's instructions.

Histology

Fixed lung, nasal polyp tissue or cells were processed and stained as previously published.[41,42] For details refer to the supplement.

Western Blot

Total cell lysates, cell supernatants or BALF were analyzed by western blotting. For details refer to the supplement.

Data analysis

Data were analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA). Data were considered statistically significant if $p<0.05$. Image analysis was performed in ImageJ and Cell Profiler software using previously published macros.[41,42]

RESULTS

Leukotriene 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) as compared to mice sensitized in the pre-weanling or adult period (starting from day 15 or day 60 after birth, respectively).[8] As LTs are central mediators of allergic airway inflammation and remodeling[43,44], we assessed LT levels in the bronchoalveolar lavage fluid (BALF) and LT enzyme expression in lung tissue of mice sensitized at different ages. High levels of cysLTs and LTB_4 were measured in the BALF of mice sensitized as neonates or adults, whereas significantly lower LT levels were found in mice sensitized from day 15 after birth (Figure 1 A, B). HDM-naïve mice showed around ten to fifty fold lower BALF LT levels with no major age differences for LTB_4 and a moderate decrease of cysLTs in adult as compared to younger mice (Figure 1 A, B, E1 A, B).

In line with the high levels of LTs in the BALF, immunohistochemistry of lung tissue from neonatal and adult sensitized mice showed a strong expression of LT enzymes (Figure 1 C-G), with 5-LO and LTC_4S being highly expressed in bronchial epithelial cells. In contrast 12/15-LO was localized to perivascular infiltrates (Figure 1 G), which were abundant in sensitized but absent in naïve mice (Figure E1 G). Numbers of infiltrating 12/15-LO^+ cells were increased in neonates, consistent with previous findings of increased eosinophilia.[8,42] Pre-weanling mice showed reduced levels of 5-LO and LTC_4S compared to neonatal and adult sensitized mice (Figure 1 G), which was however not fully represented in the quantitative data (Figure 1 D, E) as DAB quantification only allows for the assessment of the stained tissue area, rather than the staining intensity. In contrast to 5-LO and LTC_4S, LTA_4H was mostly localized to leukocytes and smooth muscle cells, with a tendency for increased expression in adult mice (Figure 1 F, G). Despite having the highest levels of cysLTs, mice sensitized from day 60 tended to accumulate less 12/15-LO positive eosinophils and to show reduced overall 5-LO expression as compared to neonatally sensitized mice. However, epithelial 5-LO and LTC_4S expression were comparable in neonatal and adult mice (Figure 1 G), suggesting a major contribution of the bronchial epithelium to LT production in inflamed airways.

Levels of TGM2, sPLA2-X and Wnt5a in the lungs of sensitized mice increase with age

As LT production can be regulated by epithelium-derived TGM2 and sPLA2-X[27,28], we further investigated potential age-dependent differences in the 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 of increased sPLA2-X expression in most pre-weanling mice (Figure 2 A). In adult HDM-sensitized mice, sPLA2-X expression was significantly increased (Figure 2 A) with apparent localization in SMCs, infiltrating leukocytes and epithelial cells (Figure 2 B). sPLA2-X expression was however not different between HDM-naïve mice at different ages (Figure E1 D, G). This was consistent with human studies showing increased expression of sPLA2-X in airway epithelial cells 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 (Figure 2 B). However, TGM2 was abundant in BALF and increased with age (Figure 2 C, D), suggesting efficient secretion of this protein by epithelial cells and/or alveolar macrophages. As we previously observed co-regulation of TGM2 and Wnt5a in IL-4 stimulated airway epithelial cells[38], we assessed Wnt5a levels in the lungs and airways of HDM-sensitized mice. Wnt5a was present in ASMCs and BALF (Figure 2 B, D-F) and Wnt5a levels increased with age (Figure 2 D-F). Indeed, epithelial Wnt5a expression could only be observed in adult mice (Figure 2 B).

We also studied the expression of alpha smooth muscle actin (aSMA) and the tight junction protein ZO-1 as signs of airway remodeling. HDM-sensitized mice showed increased aSMA levels as compared to HDM-naïve mice with a tendency of increased airway smooth muscle

thickening in older mice (Figure E1 E, G). In addition, levels of ZO-1 were reduced in mice sensitized from day 60 as compared to day 15 after birth (Figure E1 F, G). Together, these findings supported a key role for the adult epithelium in the production of remodeling factors during airway inflammation.

Wnt5a is selectively upregulated by IL-4 treatment of NHBEs

In order to investigate a potential relevance of these findings for airway inflammation in humans, we studied the regulation of the Wnt and LT pathways in normal human bronchial epithelial cells (NHBEs) exposed to pro-inflammatory stimuli. We first confirmed the responsiveness of NHBEs to IL-4 by showing IL-4- induced STAT6 phosphorylation (Figure E2 A, B) and enhanced expression of the pSTAT6 target GATA3 (Figure E2 C).

Next we assessed global Wnt mRNA expression in IL-4 stimulated epithelial cells and potential transcriptional changes in response to IL-4. Even if some Wnts were only expressed at low levels ($\Delta C_t > 30$), we detected mRNA transcripts for most Wnt ligands and all FZD receptors, with Wnt7a, Wnt7b and FZD6 being the most expressed (Figure 3 A, Figure E2 D). In addition, we found that IL-4 selectively regulated particular Wnt ligands and FZD receptors (Figure 3 B, Figure E2 E). The expression of Wnt5a was increased within 6h of IL-4 stimulation, whilst mRNA levels of Wnt7a, Wnt8b, Wnt5b and Wnt4 were decreased (Figure 3 B). We also observed increased expression of the Wnt receptors FZD10 and FZD9 after 6h of IL-4 stimulation (Figure E2 E).

As Wnt5a was recently shown to be induced by TGFβ in ASMC[29,30], we additionally assessed the effect of TGFβ1 on Wnt5a expression in NHBEs. Indeed, TGFβ1 was able to induce the expression of Wnt5a, but only to comparable levels as IL-4 (Figure 3 C, D, F). Also the stimulation with both cytokines did not result in an additive upregulation (Figure 3 D, F). Whilst the upregulation of Wnt5a expression was only moderate, IL-4 clearly increased Wnt5a secretion (Figure 3 D-G).

LT production by HDM-stimulated NHBEs partially depends on TGM2

Next we assessed the effect of IL-4, TGFβ1 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, B), thus confirming previous findings.[24] As TGM2 has been suggested to regulate cysLT production[27], we assessed the effect of TGM2 inhibitors (monodansylcadaverine (MDC) and cystamine (Cys)) 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.

Whilst TGM2 was induced by IL-4, HDM stimulation did not further alter TGM2 expression as demonstrated by western blotting (Figure 4 C, D, E). Most NHBEs donors were middle-aged, thus precluding the investigation of age effects. However, NHBEs from one teenage donor (14 years old) showed the lowest induction of TGM2 in response to IL-4.

TGM2 and sPLA2-X expression in NHBEs are steroid resistant

As airway remodeling is largely resistant to steroid treatment, we assessed the effect of fluticasone propionate, one of the most widely used inhaled glucocorticosteroids (ICS)[46], on TGM2 and sPLA2-X expression in NHBEs. To induce maximal expression of both enzymes, NHBEs were stimulated with IL-4, TGFβ1 and HDM extract. Fluticasone propionate (1 µM, 24h) caused an increase rather than a decrease of TGM2 and sPLA2-X expression in NHBEs of most donors (Figure 4 F, G), thus implicating TGM2 and sPLA2-X in steroid resistant airway remodeling.

Epithelial-produced Wnt5a increases TGM2 expression in human macrophages

As we could not detect intracellular TGM2 in the lung tissue of sensitized mice, we next assessed if macrophages could be a source of TGM2 in the airways and if epithelial secretions could affect macrophage TGM2 expression. Monocyte derived macrophages

(MDM) in our study expressed high basal levels of TGM2 due to differentiation in the presence of TGFβ1[47], 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 (pre-incubated at 37°C for 24h) did not further increase TGM2 expression (Figure 5 A, B). However, conditioned medium from IL-4-stimulated NHBEs upregulated macrophage TGM2 expression as compared to conditioned medium from untreated NHBEs (Figure 5 C, D). This effect was abrogated upon addition of a Wnt5a blocking antibody (Figure 5 C, D). Thus, Wnt5a secreted by the airway epithelium can likely act on myeloid cells in the airways to promote TGM2 expression. Next, we treated MDM with TGM2 inhibitors to study potential effects on LT production by these cells. Both inhibitors showed a tendency to decrease cysLT production in IL-4-treated MDM (Figure 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 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 (prednisolone 40 mg daily for 5 days) prior to surgery (table E2). As shown in Figure 6 A, 5-LO, LTC_4S, Wnt5a and sPLA2-X were highly abundant in nasal polyp tissues with predominant localization in the nasal epithelium. In contrast, only LTC_4S was present in normal nasal turbinate tissue (Figure E3). In line with expression patterns of 5-LO, LTC_4S, Wnt5a and sPLA2-X, nasal polyp, but not normal nasal turbinate tissues released considerable levels of cysLTs, when cultured overnight in epithelial cell culture medium (Figure 6 B). TGM2 was not detected within the epithelium, but appeared to be secreted basolaterally and to co-localize with collagen in some, but not all patients (Figure 6 C).

Further stratification showed that TGM2 was particularly abundant in nasal polyp tissue of HDM-sensitized individuals, followed by a grass pollen allergic individual and non-allergic, aspirin tolerant (AT) patients (Figure 6 D). In contrast, TGM2 was largely absent from nasal polyps of patients suffering from aspirin exacerbated respiratory disease (AERD), a distinct non-allergic endotype of chronic airway inflammation (Figure 6 C, D). As all nasal polyp patients 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 pathological cascade consisting of TGM2, Wnt5a, sPLA2-X and LTs (Figure 7 A) 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 epithelial-expressed cascade of remodeling factors, which is age-dependent and steroid resistant. This cascade involves transglutaminase-2, 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 may underlie the differential development of airway remodeling in adults, school children and neonates.

In view of the previously reported increase in type 2 cytokines and airway eosinophilia in HDM-sensitized neonatal as compared to adult mice, it was unexpected to find a tendency of higher cysLT levels in adult airways. Similar airway resistance was reported for neonatal and adult mice in response to moderate metacholine 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 of increased smooth muscle thickening and a loss of the tight junction protein ZO-1 in adult as compared to 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 of hallmark type 2 cytokines in neonates. We show that these include TGM2, Wnt5a and sPLA2-X, which regulate macrophage activation and ECM crosslinking.[35,53,54]

As our 3-week HDM model involved both infiltration of eosinophils due to 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 via innate receptors, can indeed contribute to cysLT production during airway inflammation *in vivo*.[24] The abundance of 5LO and LTC_4S in the airway epithelium, observed in our study, may 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 (Figure 7 B). Indeed, our data on human macrophages support the view that airway epithelial cells may communicate with myeloid cells via 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 (sPLA-IID), which contributed to immune suppression and lung damage during viral infection.[29] Thus, the increase of sPLA2 expression in response to lung inflammation during adulthood may be a universal event, which may explain age-dependent differences in the susceptibility to infection, fibrosis and asthma. As sPLA2-X expression in the lung has been linked to asthma severity[55], future studies should investigate factors, which 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 lead to the development of TGM2 inhibitors for the treatment of these diseases.[54,56–59] Thus, airway TGM2 levels should be assessed in asthmatic and

CRSwNP patients and correlated to disease severity to investigate if TGM2 might represent a therapeutic target in asthma or nasal polyposis. As TGM2 expression can be modulated by DNA methylation[60] 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 of TGM2 and its regulatory targets in airway inflammation.

Mechanistically, TGM2-activated sPLA2-X exerts its effects via increasing intracellular Ca^{2+} and MAPK activation, two events, which enhance cytosolic PLA2 (cPLA2) and 5-LO activity and thus LT production.[28] Increased expression of sPLA2-X might therefore 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 as we observed strong expression of sPLA2-X in ASMCs. However, these earlier studies used OVA as an allergen, which might explain this discrepancy as expression of sPLA2-X may 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 glucocorticosteroid (GC) resistant airway inflammation as 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 steroid resistant forms of airway inflammation. As 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 severe airway inflammation.[70–72]

Of note, the most commonly used LT-modifying drug (cysLT1R antagonist Montelukast) does not suppress the formation of cysLTs, which may thus bind to alternative receptors such as cysLT2R, recently shown to promote platelet-driven allergic airway inflammation.[73] Thus, targeting the epithelial TGM2-Wnt5a-sPLA2-X pathway by topically administered compounds may prove advantageous over current LT modifiers and should be considered as add-on therapy in patients with difficult to treat asthma and/ or nasal polyps.

FIGURE LEGENDS

Figure 1: Leukotriene 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 LTB_4 measured in the BALF from sensitized mice (n=4-6; Mann-Whitney test). C, Counts of 12/15-LO positive cells per mm^2 lung section (n=5-9; Mann-Whitney test). D, E and F, Quantification of 5-LO, LTC_4S and LTA_4H expression in lung sections (n=4-8; Mann-Whitney test). G, Representative IHC staining images of lung sections.

Figure 2: Age-dependent increases of sPLA2-X, TGM2 and Wnt5a levels in the lung of sensitized mice

A, Quantification of positive area of sPLA2-X, B, Representative IHC 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 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)

Figure 3: IL-4 exposure of NHBEs selectively upregulates Wnt5a expression

A and B, Wnt mRNA expression in IL-4 stimulated NHBEs (n=6; One-sample t-test). C, Relative expression of Wnt5a mRNA in stimulated NHBEs (n=4-6). D and E, normalized expression of Wnt5a determined by western blot of stimulated NHBEs or NHBE supernatants (n=5; Wilcoxon test) F and G, Representative western blots of D (lysates (F)) or E (supernatants (G)).

Figure 4: TGM2 contributes to HDM-stimulated LT production in NHBEs; TGM2 and sPLA2-X expression in NHBEs are steroid resistant

A, Representative immunofluorescence images of 5-LO expression in NHBEs. B, Levels of cysLTs secreted by NHBEs +/- TGM2 inhibitors (n=6-7). C and D, Normalized expression of TGM2 in NHBEs (n=5; Wilcoxon test). E, Representative western blots of C and D. F and G, Mean fluorescence intensity of TGM2 or sPLA2-X in NHBEs (+/- fluticasone propionate (FP)), representative immunofluorescence images (n=4-5).

Figure 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 MDM treated with IL-4 containing media or NHBE conditioned medium (CM) +/- Wnt5a neutralizing antibody (n=8-9; Wilcoxon test). B and D, Representative immunofluorescence images of TGM2 expression in MDM. E, Levels of cysLTs secreted by MDM stimulated with IL-4 and treated with TGM2 inhibitors (n=4).

Figure 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 IHC staining for 5-LO, LTC_4S, 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). C, Representative IHC staining for TGM2 or Col1A1 in polyps of aspirin tolerant (AT) or AERD patients. D, Quantification of TGM2 positive area (n=6 (AT), n=4 (AERD), Mann-Whitney test).

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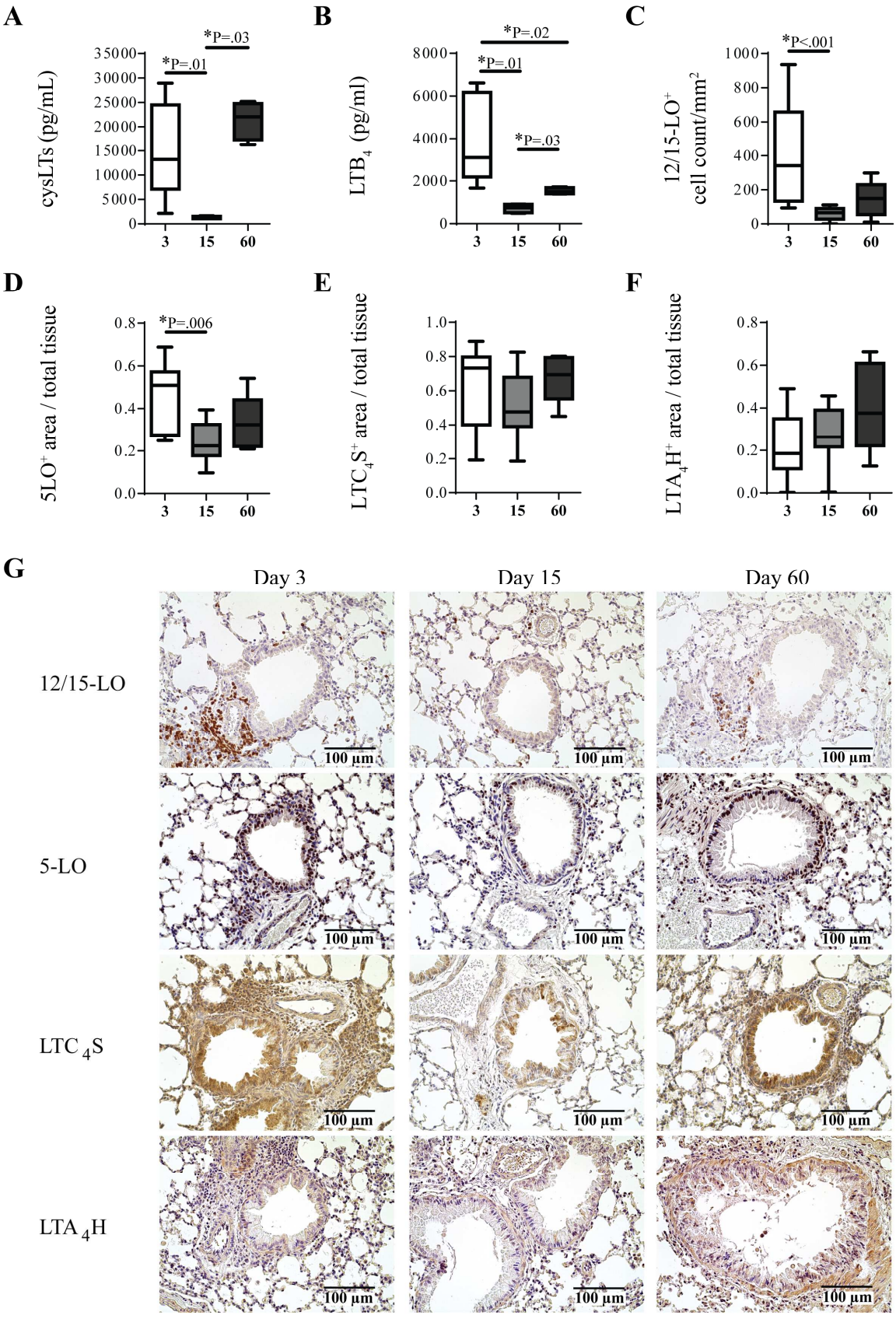
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*P=.03
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*P=.01
*P=.03
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*P<.001
D
5LO+ area / total tissue
*P=.006
E
LTC4S+ area / total tissue
F
LTA4H+ area / total tissue
3
15
60
G
Day 3
Day 15
Day 60
12/15-LO
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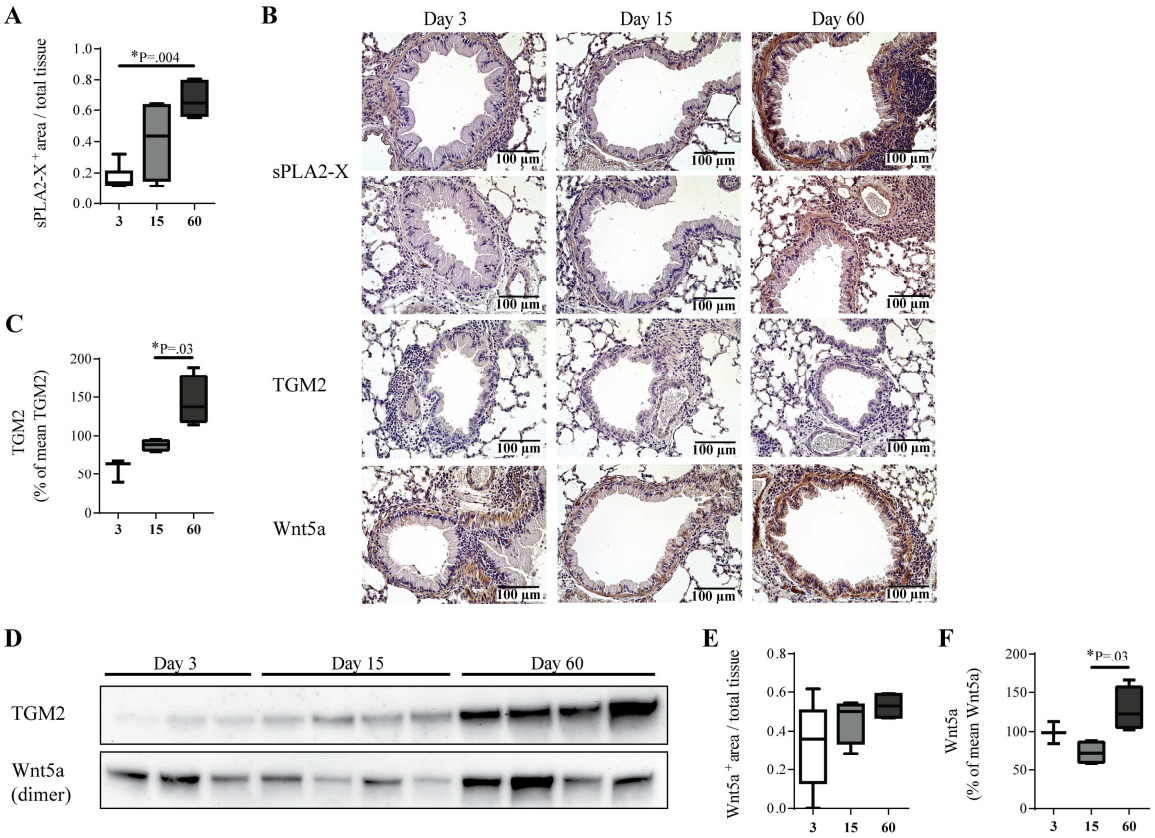

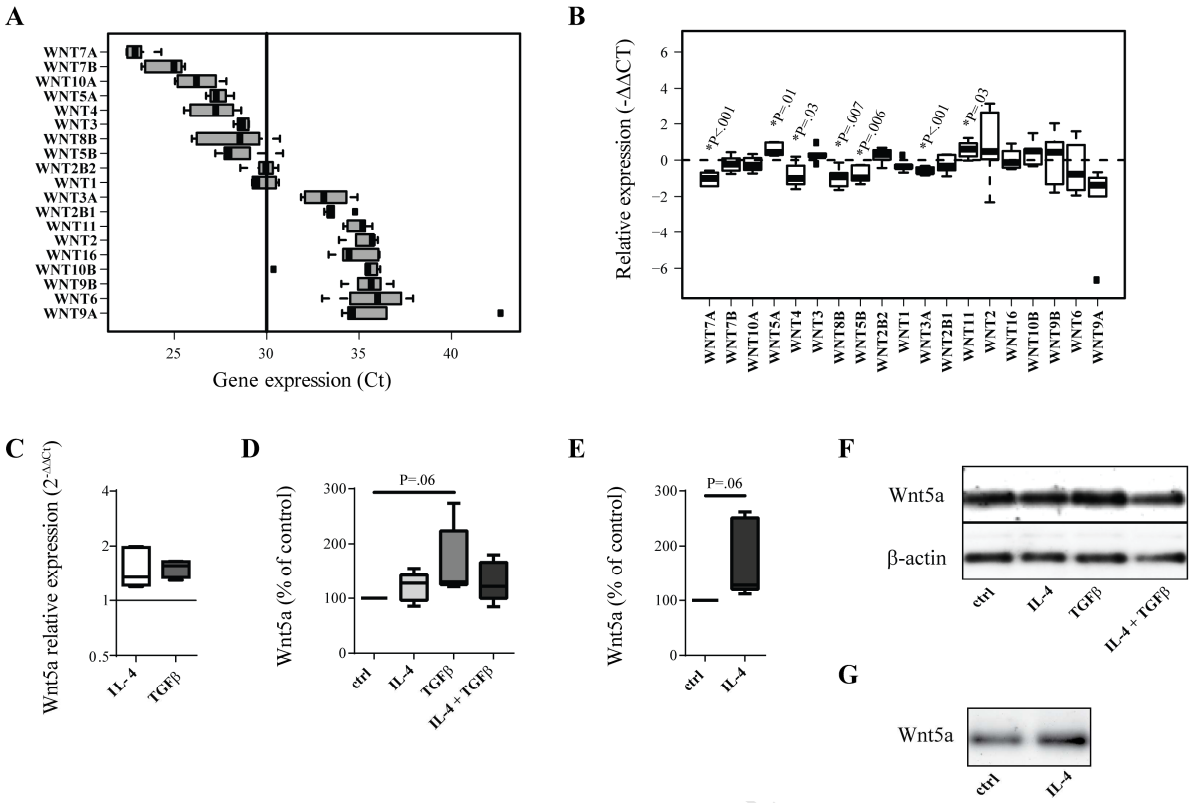

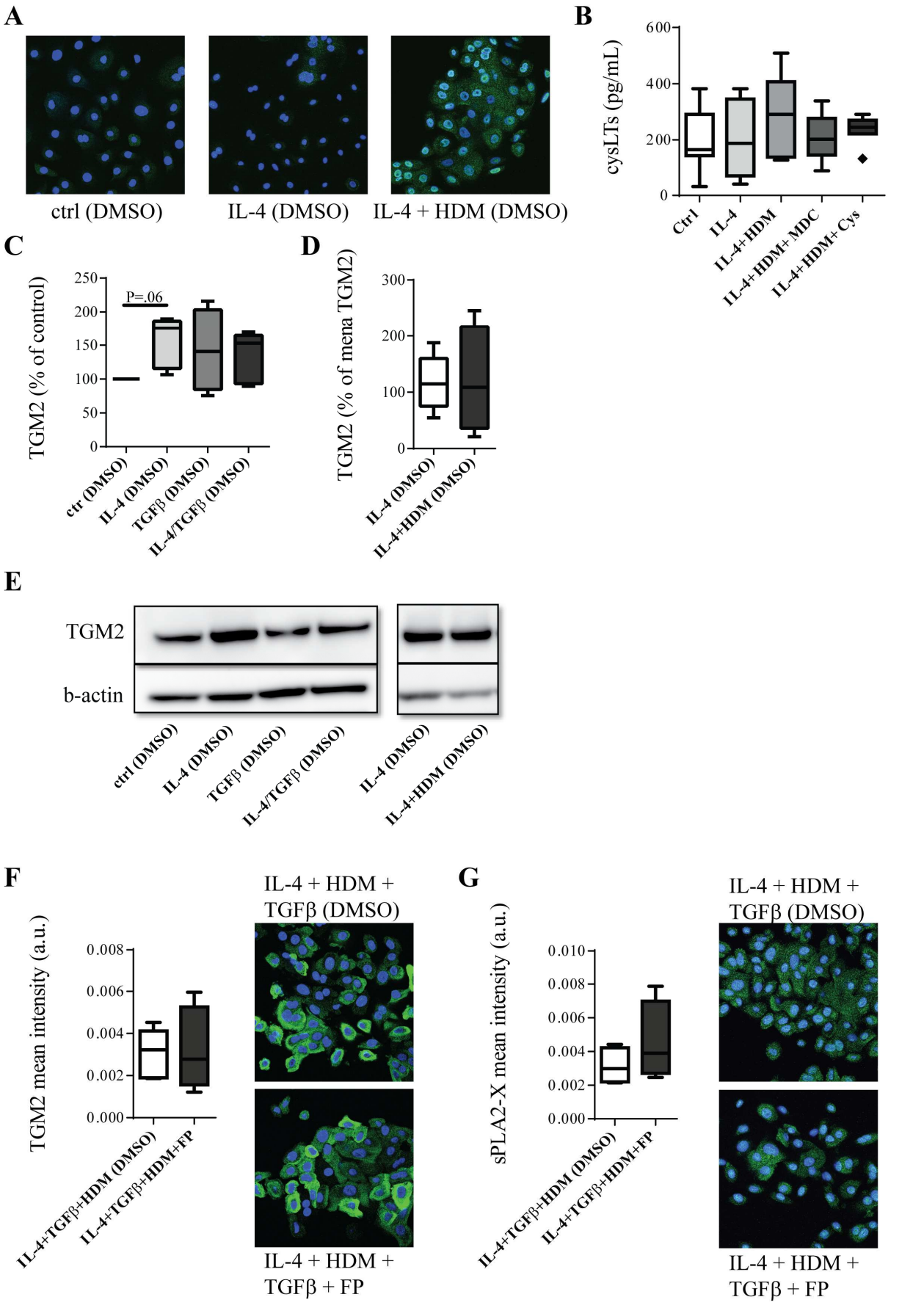
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ctrl (DMSO)
IL-4 (DMSO)
IL-4 + HDM (DMSO)
B
cysLTs (pg/mL)
600
400
200
0
Ctrl
IL-4
IL-4+HDM
IL-4+HDM+MDC
IL-4+HDM+ Cys
C
TGM2 (% of control)
250
200
150
100
50
0
P=.06
ctr (DMSO)
IL-4 (DMSO)
TGFβ (DMSO)
IL-4/TGFβ (DMSO)
D
TGM2 (% of mena TGM2)
300
200
100
0
IL-4 (DMSO)
IL-4+HDM (DMSO)
E
TGM2
b-actin
ctrl (DMSO)
IL-4 (DMSO)
TGFβ (DMSO)
IL-4/TGFβ (DMSO)
IL-4 (DMSO)
IL-4+HDM (DMSO)
F
TGM2 mean intensity (a.u.)
0.008
0.006
0.004
0.002
0.000
IL-4+TGFβ+HDM (DMSO)
IL-4+TGFβ+HDM+FP
IL-4 + HDM + TGFβ (DMSO)
IL-4 + HDM + TGFβ + FP
G
sPLA2-X mean intensity (a.u.)
0.010
0.008
0.006
0.004
0.002
0.000
IL-4+TGFβ+HDM (DMSO)
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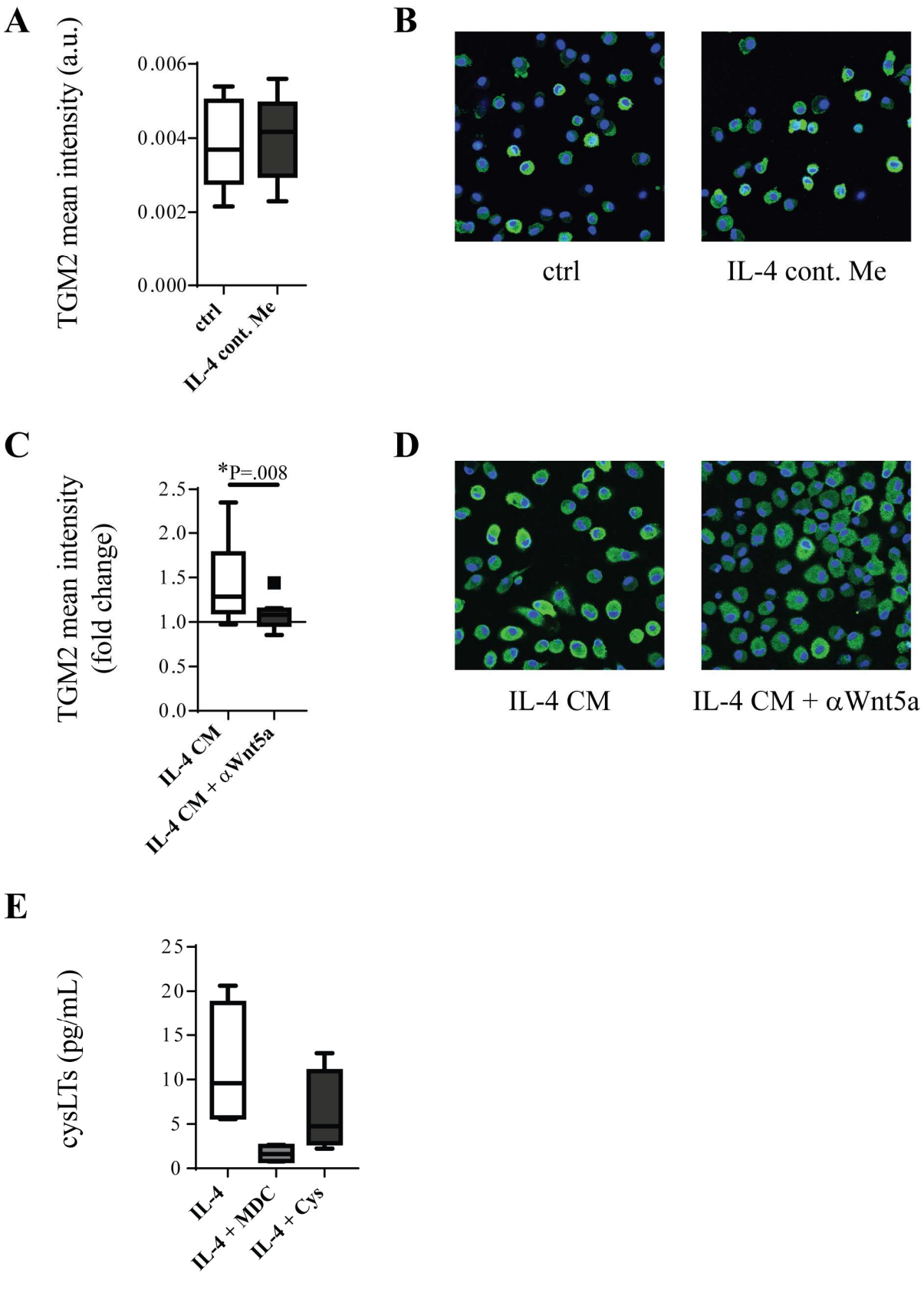
IL-4 + HDM + TGFβ (DMSO)
IL-4 + HDM + TGFβ + FP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0.002
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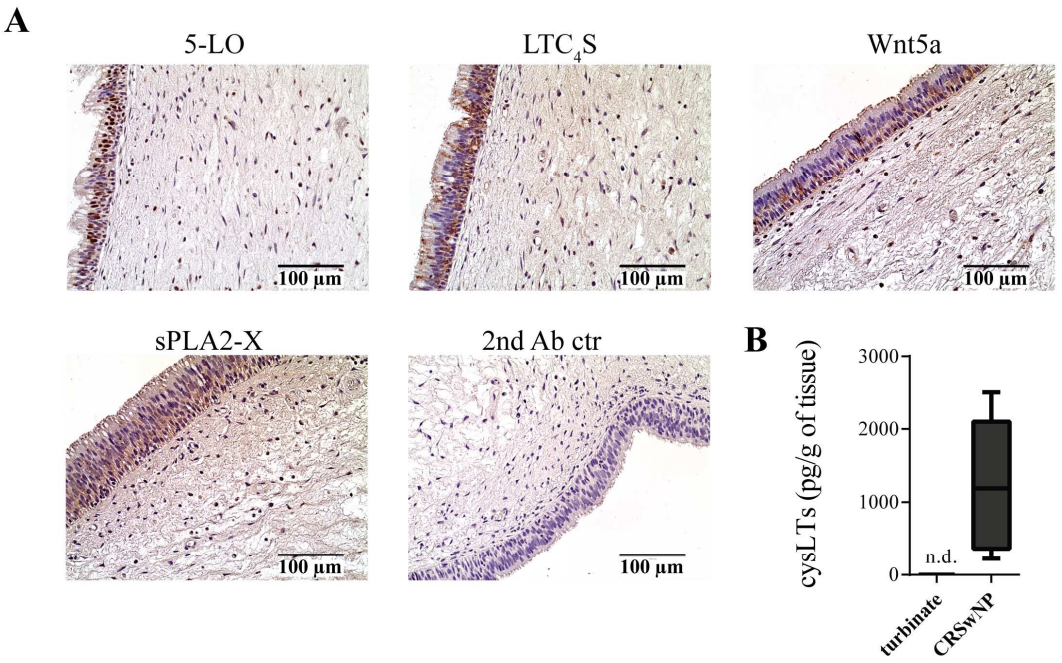
B
ctrl
IL-4 cont. Me
C
TGM2 mean intensity (fold change)
*P=.008
2.5
2.0
1.5
1.0
0.5
0.0
IL-4 CM
IL-4 CM + αWnt5a
D
IL-4 CM
IL-4 CM + αWnt5a
E
cysLTs (pg/mL)
25
20
15
10
5
0
IL-4
IL-4 + MDC
IL-4 + Cys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5-LO
LTC4S
Wnt5a
sPLA2-X
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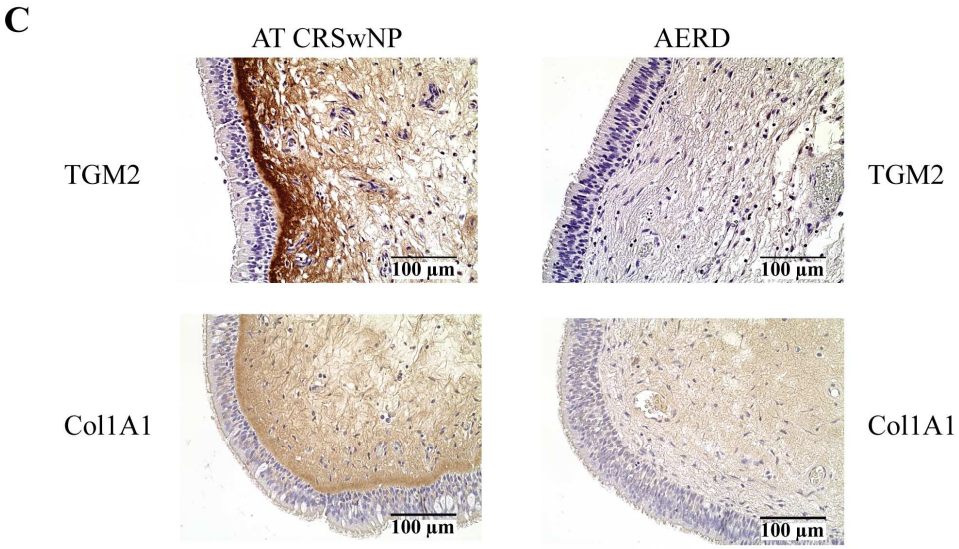
B
cysLTs (pg/g of tissue)
3000
2000
1000
0
n.d.
turbinate
CRSwNP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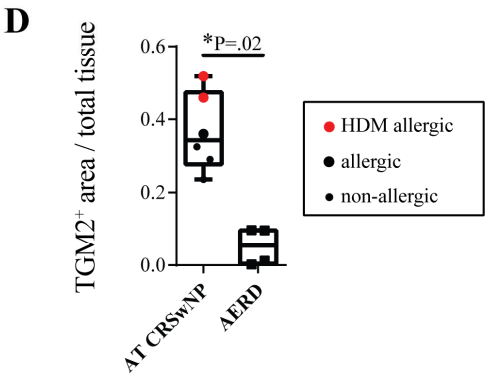
AT CRSwNP
AERD
TGM2
TGM2
Col1A1
Col1A1
100 µm


D
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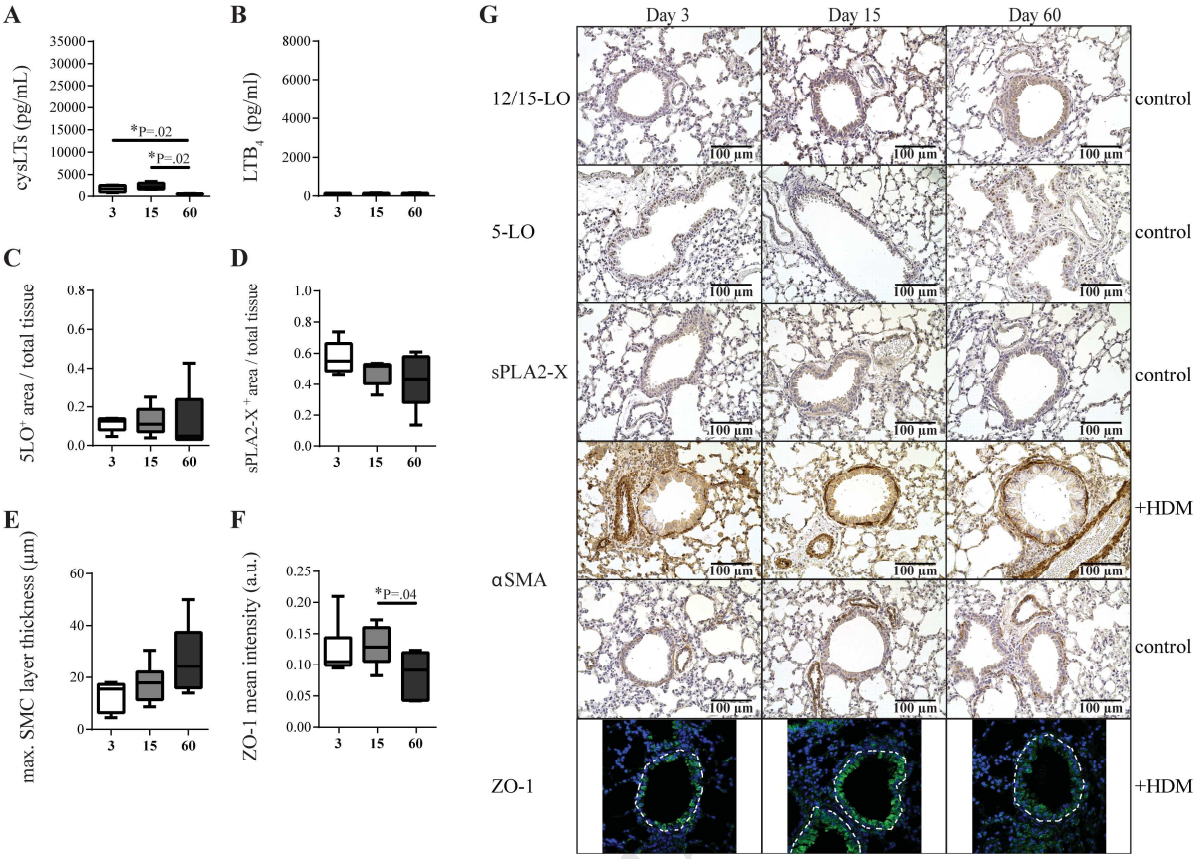
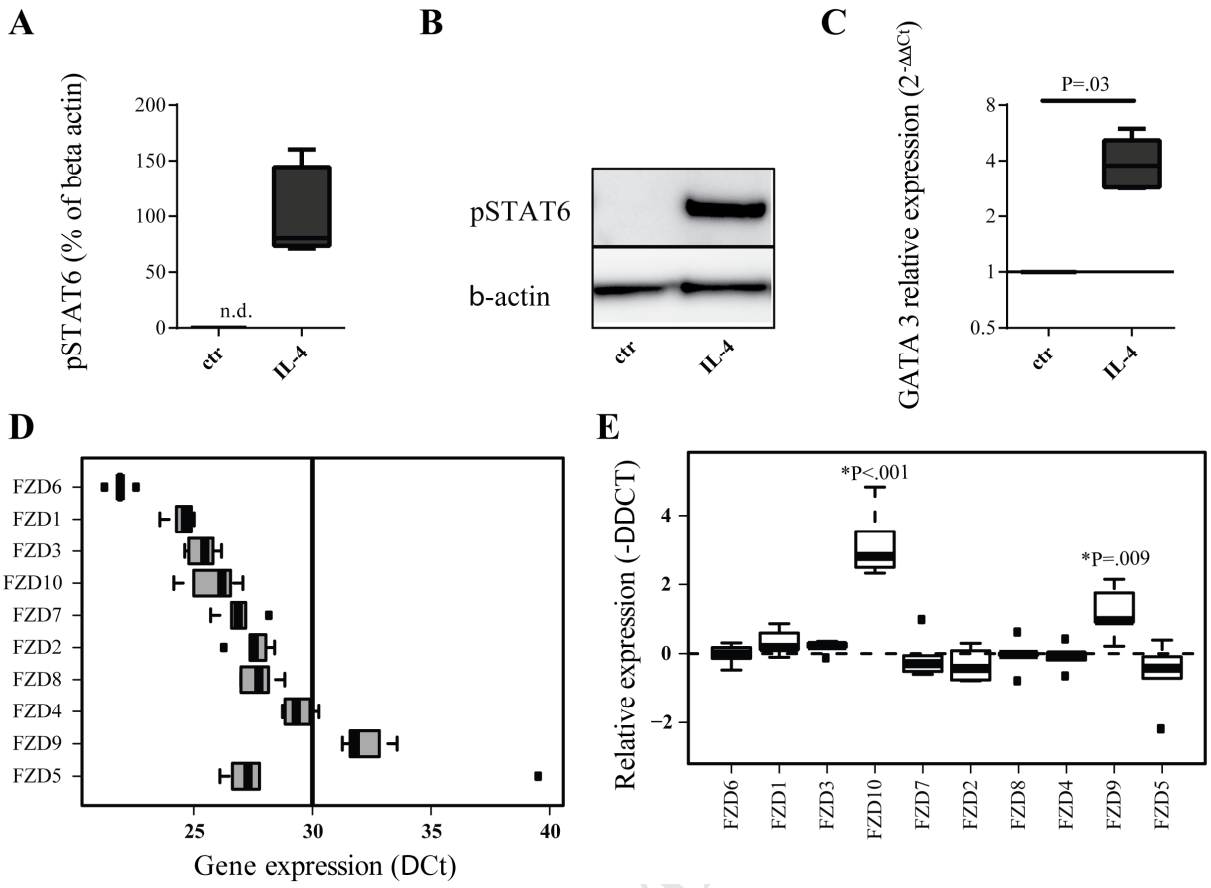
0.4
0.2
0.0
HDM allergic
allergic
non-allergic
AT CRSwNP
AERD





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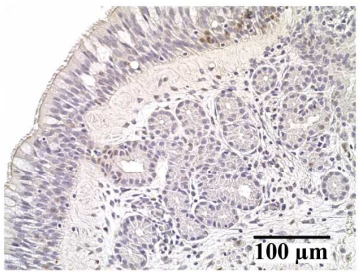

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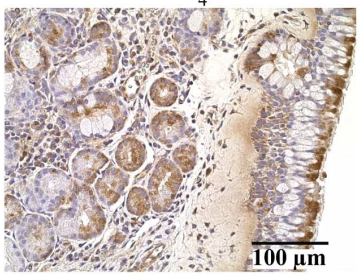

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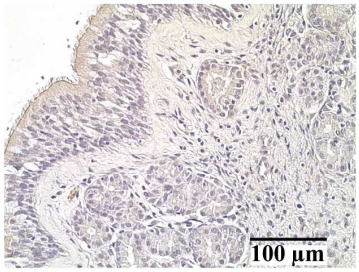
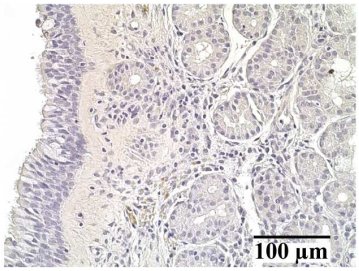
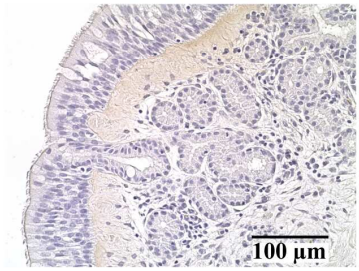


sPLA2-X



TGM2



Online supplement

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

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Supplemental Methods

Mouse model of allergic airway inflammation

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

Nasal tissue culture

Nasal polyp tissues or inferior turbinates were obtained after written informed consent from patients undergoing functional sinus or turbinoplastic surgery, respectively. All patients with chronic rhinosinusitis with nasal polyposis (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 24h (5% CO_2, 37℃) under air-liquid interface conditions.

Culture of human monocyte derived macrophages

All MDM cultures were performed in the presence of GM-CSF (10 ng/ml) and TGFβ1 (2 ng/ml) during the complete differentiation protocol. Stimulation was performed according to the stimulation of normal human bronchial epithelial cells.

Culture of normal human bronchial epithelial cells

Normal human bronchial epithelial cells (NHBEs) from nonsmoking individuals were obtained from Lonza (Visp, Switzerland) and expanded in Bronchial Epithelium Growth Medium (BEGM, Lonza), before they were stored in liquid nitrogen in passage (p) 2. Before experimentation cells were further expanded and then seeded in 6-well plates at a density of 1.5 – 2.5 x 10^5 cells/well. Cells in p4 were grown to 80-85% confluency and before stimulation serum-deprived in basal medium (BEBM, Lonza) overnight. Rested cells were then stimulated with 50 ng/ml human recombinant IL-4 (Promokine, Promocell), 5 ng/ml TGFβ1 (Peprotech, Rocky Hill, NJ) and/or 10 µg/ml HDM (Stallergenes, Antony, France) or treated with 100 µM (in DMSO) cystamine dihydrochloride (Tocris Bioscience, Bristol, United Kingdom), 25 µM (in DMSO) Dansylcadaverine (Sigma-Aldrich, Munich, Germany) or 1µM (in DMSO) Fluticasone propionate (Sigma-Aldrich) for 6h to analyze mRNA expression or 24h. All experiments were performed at p 4.

Immunohistochemistry and immunofluorescence

Left lobes of lung or nasal polyp tissues were fixed in 10% formalin at 4 ℃ and paraffin embedded. Sections (4 µm) were stained after blocking unspecific binding with 5% BSA + 10% donkey serum, using the following primary antibodies: rabbit

anti-human 5-lipoxygenase (a kind gift of Prof. Olof Rådmark, Karolinska Institute Stockholm), transglutaminase 2 (Cell signaling technology, Danvers, MA), 12/15-lipoxygenase, alpha smooth muscle actin (both Abcam, Cambridge, United Kingdom), Wnt5a (Lifespan Bioscience, Seattle, WA), LTC_4 synthase, sPLA2-X, and LTA_4 hydrolase (all Santa Cruz, Dallas Texas), followed by secondary biotinylated donkey-anti rabbit antibody and HRP-based detection kit (ABC, Lifetechnologies, Thermo Fisher) using DAB detection reagents (Sigma Aldrich). For immunofluorescent staining, cells were fixed for 15 min with 4% PFA followed by permeabilization with acetone (10 min, -20°C). After blocking (5% BSA, 10% donkey serum), cells or tissues were incubated with rabbit primary antibodies (see above) or with rabbit anti-human ZO-1 (Lifetechnologies, Thermo Fisher). Fluorescence conjugated donkey anti-rabbit (AF488) antibody (Lifetechnologies, Thermo Fisher) were used for detection. Images were recorded on an EVOS system (Lifetechnologies, 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 using a high-capacity cDNA kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA), following real-time PCR using the ViiA7 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific) and the FastStart Universal SYBR Green Mastermix (Roche, Mannheim, Germany). Expression of the genes of interest was analyzed using the equation $2^{-\Delta\Delta Ct}$. The specific primers are listed in supplemental Table E1.

Western Blot

Total cell lysates were obtained by direct lysis of cells in RIPA buffer (Pierce, ThermoFischer) with 2% EDTA-free complete protease inhibitor mixture (Roche,

Mannheim, Germany). Equal volumes of cell-free supernatants were 10 times concentrated using Amicon Ultra-0.5ml 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 manufacturer's recommendation and heated at 70℃ for 10 min before loading. Expression of pSTAT6 (Cell signaling technology), Wnt5a (R&D systems, Minneapolis, MN) and TGM2 (Cell signaling technology) was analyzed by Western blotting. HPRT or βactin (both Santa Cruz) was used as loading control. Samples were loaded on 4-12% or 10% Bis-Tris protein gels (NuPAGE Novex, Thermo Fisher) and transferred to a PVDF membrane (Merck Millipore, Darmstadt, Germany) using Mini Blot Module (Thermo Fisher) at 20 Volt for 90 min. The PVDF membrane was blocked in 5% nonfat dry milk in 1x 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℃ and after subsequent washing the membrane was incubated with the corresponding secondary HRP-conjugated antibody in blocking solution for 1h at RT. 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 the manufacturer's instructions and recorded using the ECL ChemoCam Imager (Intas Science Imaging Instruments, Göttingen, Germany). Protein levels were quantified using LabImage 1D (Intas Science Imaging Instruments) by normalization to unstimulated control or mean expression and corrected for the amount of HPRT or beta actin in the samples (for lysates).

Supplemental Figures and Tables

Supplemental Figure and Table Legends

Figure 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 LTB_4 measured in BALF from control mice, same scale as for HDM-sensitized mice in Figure 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 SMC 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 IHC staining and immunofluorescence images of lung sections.

Figure E2: NHBEs are IL-4 responsive and show a distinct regulation pattern of Wnt and FZD genes

A Normalized expression of pSTAT6 determined by western blot of IL-4 stimulated NHBEs (n=5), L-4 stimulated NHBEs (n=6; Wilcoxon test). D and E, FZD mRNA expression was determined by qPCR and is shown as gene expression of FZDs in IL-4 stimulated NHBEs and relative expression to control, corrected for housekeeper genes (n=6; One-sample t-test).

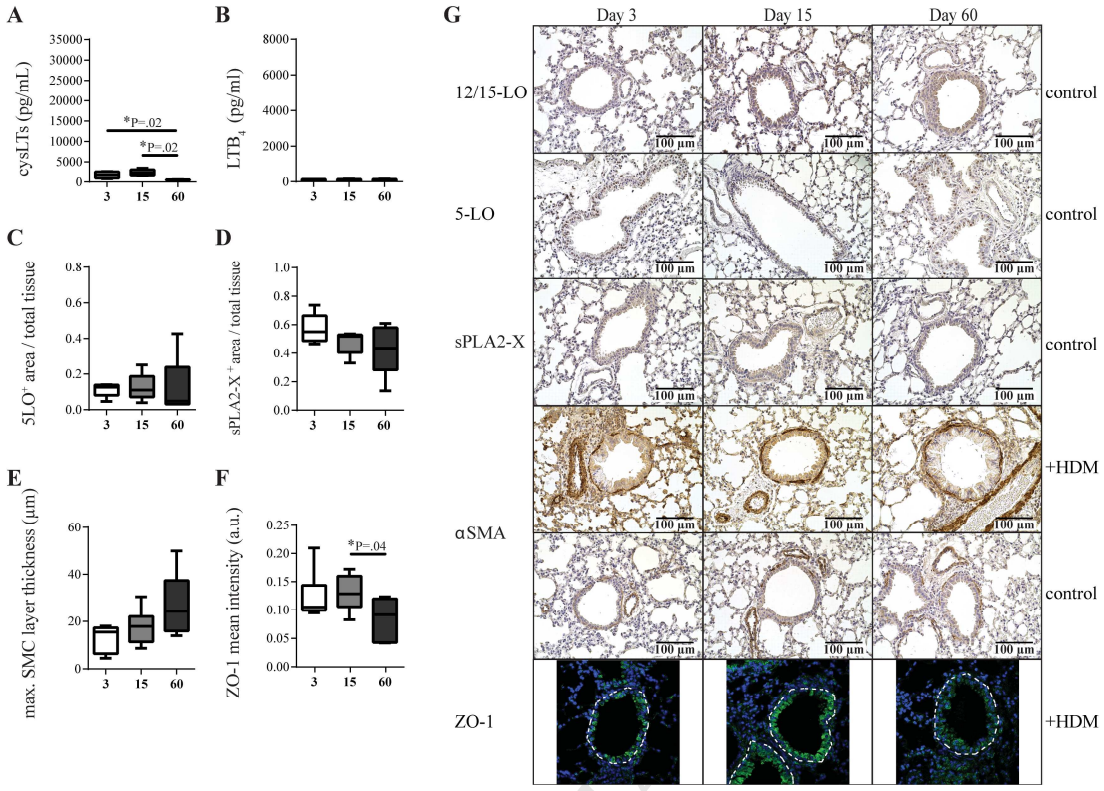
Figure E3: Nasal turbinates from healthy controls show low levels of 5-LO, Wnt5a, sPLA2-X and TGM2

Representative IHC images of turbinate sections for 5-LO, LTC_4S, Wnt5a, sPLA2-X and TGM2.

Table E1: qPCR primers

Table E2: Patient characteristics

145 **FIGURE E1.**



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148 **FIGURE E2.**

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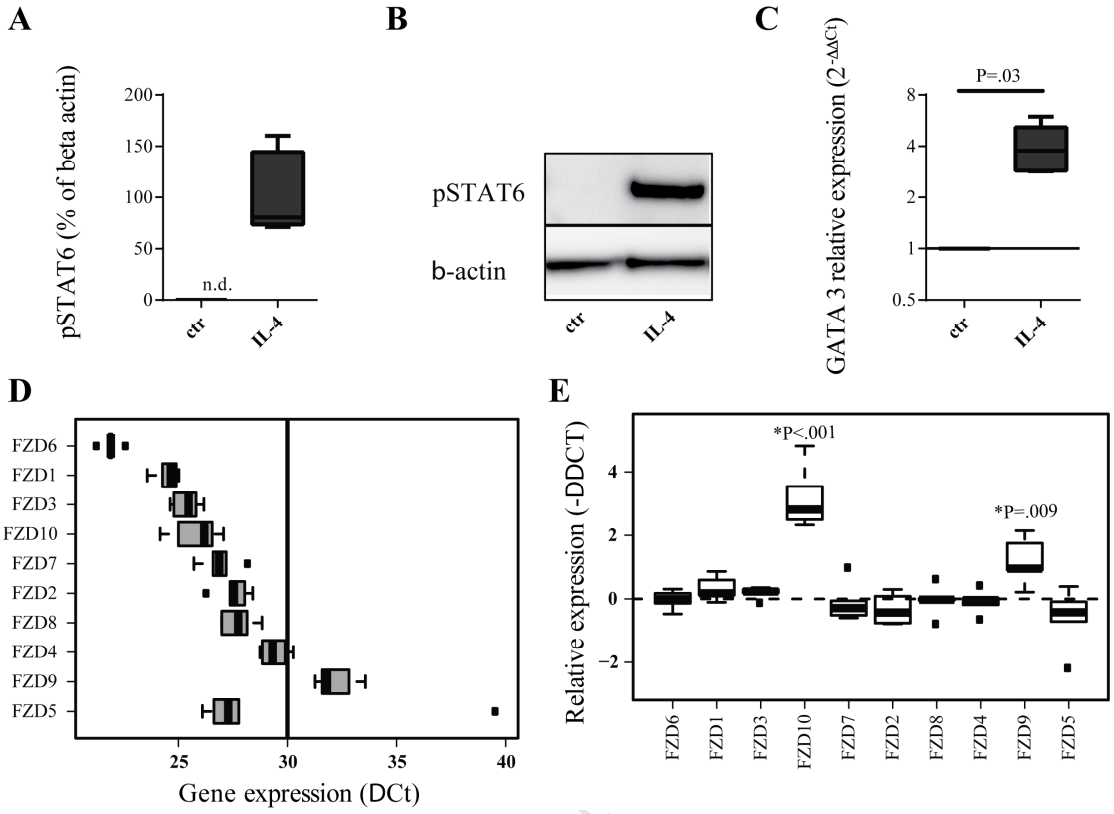

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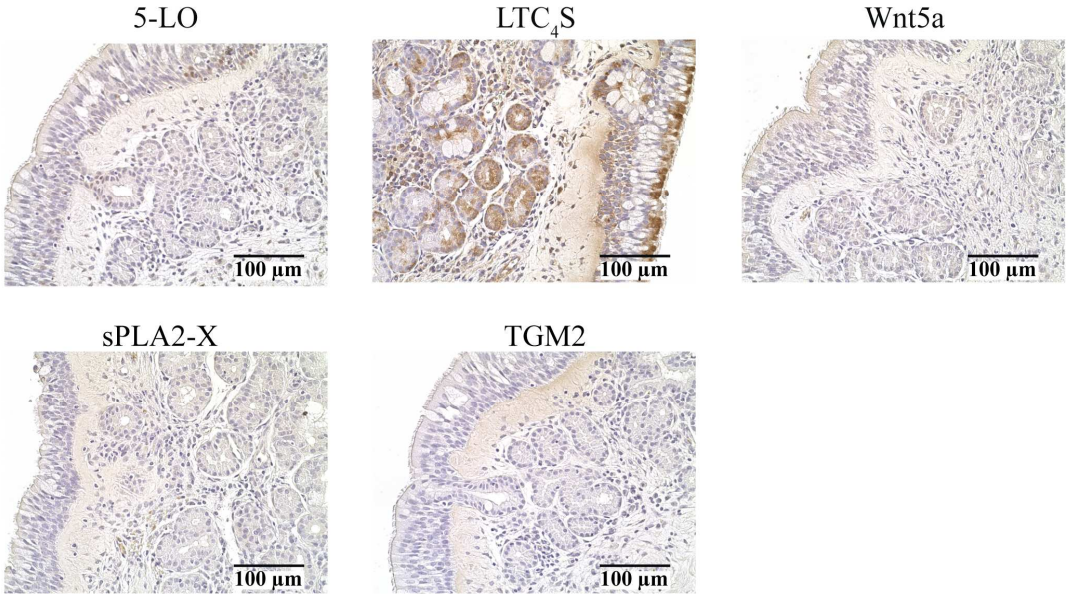
151 **FIGURE E3.**



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153 **TABLE E1.**

154 Primer for housekeeper, Wnt and FZD genes

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	GeneName	FWD	RV
Housekeeper	β-Actin	TGAGAGGGAAATGTGCGTG	TGCTTGCTGATCCACATCTGC
	HPRT	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
	TFRC	TGTGGGGAAGGGGCTGT	CCACCAAACAAGTTAGAGAATGC
Wnt	Wnt1	GGTTTCTGCTACGCTGCTG	TAAGCAGGTTCGTGGAGGAG
	Wnt2	GGGCTGGCCTTTATCGCTC	GAGCCAGATTCCACCGAGAG
	Wnt2b1	GATCCTTGAGGACGGCAGTA	GCTCACCAAACCAGGGATATT
	Wnt2b2	TAGGTCTTGCCTGCCTTCTG	TTGTCACAGATCACTCGTGC
	Wnt3	ACTTTTGTGAGCCCAACCCA	TTCTCCGTCCTCGTGTTGTG
	Wnt3a	GTGGAACTGCACCACCGT	ATGAGCGTGTCACTGCAAAG
	Wnt4	CTCGTCTTCGCCGTCTTCT	GATCAGGCCCTTGAGTTTCTC
	Wnt5a	GCTCGGATTCCTCGGCT	CAAAGCAACTCCTGGGCTTA
	Wnt5b	AACGCATCTGTCTTTGGGAG	GCTGATGGCGTTGACCA
	Wnt6	TGCTGCTGCTGCTGCTC	CAGATGCTGGTAGGGTCCAT
	Wnt7a	GAACTTGCACAACAACGAGG	AGTGTGGTCCAGCACGTCTT
	Wnt7b	AGCCAACATCATCTGCAACA	CTGGTACTGGCACTCGTTGA
	Wnt8b	CACCTGTGTCCTCCAACTCA	TGCCACACTGCTGGAGTAAA
	Wnt9a	CTTCGGCCGCCTACTTC	GTCGCAGGCCTTGTAGTGC
	Wnt9b	GTGCAGTGGTGCTGCTACG	GCACACATGCCGGTTTATGC
	Wnt10a	AACACCAATTCAGGGACCAG	CAAAAGCGCTCTCTCGGAA
	Wnt10b	GGTCCACGAGTGTCAGCAC	CCAGCATGGAGAAGGAAAAA
	Wnt11	CGTGTGCTATGGCATCAAGT	GTGTGCATGAGCTCCAGGTT
	Wnt16	CACGGGCAAAGAAAACAAAG	GCATGTTTTCACAGCACAGG
FZD	FZD1	AGCTTTGTGTGGGTTGGAAG	CGGTAAAATCTAAGCGCAGG

	FZD2	ACATCGCCTACAACCAGACC	CCTTCACCAGCGGATAGAAC
	FZD3	GCATCTGGGAAACAACGTG	TCAAGTCTGGACGACTCATTTG
	FZD4	AACTTTCACACCGCTCATCC	TGACTGAAAGACACATGCCG
	FZD5	TGGGGACTGTCTGCTCTTCT	CAAAGATAAACTGCTTCGGGA
	FZD6	AAAATGGCCTACAACATGACG	ATTCCAGATTTGCGAGAGGA
	FZD7	CGCCTCTGTTCGTCTACCTC	CCATGAGCTTCTCCAGCTTC
	FZD8	AAGACAGGCCAGATCGCTAA	CGATAAGGAAGGTGGAGACG
	FZD9	GAAGCTGGAGAAGCTCATGG	AAGTCCATGTTGAGGCGTTC
	FZD10	TATGAGATCCCTGCCCAGTC	CAACCAAGAAAAGCACCACA

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157 **TABLE E2.**

158 Characterization of CRSwNP patients

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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

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