Characterization of smooth-muscle-derived wnt-5a in allergic asthma: modulating effects on th2-cell activation

Research manuscript
Abstract

Asthma is a heterogeneous disease characterized by chronic inflammation and progressive development of persistent structural changes in the airways. The airway smooth muscle (ASM) is centrally involved in the pathology of asthma. It is primarily responsible for airway narrowing and airway hyperresponsiveness. In addition, the ASM is an important source of inflammatory mediators and is closely involved with infiltrating inflammatory cells. We and others have previously shown that WNT-5A is increasingly expressed in the ASM of asthmatics and in airway biopsies of Th2-high asthma patients. We observed that WNT-5A mediates both contractile maturation and extracellular matrix deposition in ASM cells. Thus, we extended on these findings by generating a tet-ON smooth-muscle-specific WNT-5A transgenic mouse model, enabling in vivo characterization of smooth-muscle-derived WNT-5A in an allergic asthmatic context, using chronic ovalbumin exposure to drive asthmatic changes. Contrary to previous reports from our lab in human ASM cells, induced WNT-5A did not increase collagen deposition and smooth muscle thickness in ovalbumin-treated mice, nor affect methacholine-induced contraction. However, WNT-5A significantly enhanced the production of Th2-cytokines IL-4, IL-5 and IL-13. In line with this, WNT-5A increased mucus production in ovalbumin-treated animals, and enhanced eosinophilic infiltration and serum IgE production. In conclusion, rather than being involved in airway remodelling, induced WNT-5A drives parameters of Th2 immunity in mice. These results highlight non-canonical WNT signalling as a new potential therapeutic target in allergic asthma.

Keywords

Asthma, allergic inflammation, Th2, airway smooth muscle, WNT
Introduction

Asthma is a heterogeneous disease characterized by chronic inflammation of the large and small airways. 235 million people worldwide are estimated to be affected by asthma, and prevalence is still rising. Asthma is characterized by episodic changes in respiratory symptoms resulting from airway hyperresponsiveness (AHR), such as breathlessness, wheezing, chest tightness and coughing. Apart from this variable component of AHR, asthma is characterized by the progressive development of persistent structural changes in the airways, termed airway remodelling, which contribute to persistent AHR. The airway smooth muscle (ASM) is a central player in the pathology of asthma, as evidenced by several findings. Airway smooth muscle thickness correlates with asthma severity and lung function. In line with this, bronchial thermoplasty (BT) results in diminished ASM mass in severe asthmatics for up to at least two years, which is associated with improvements in quality of life, and a reduction in symptoms and number of exacerbations. The ASM has a widespread impact on asthma pathology. They are the major effector cells and proximate cause of excessive airway narrowing. Their increased thickness in asthma due to hypertrophy and hyperplasia, as well enhanced deposition of extracellular matrix (ECM) proteins further aggravates airway narrowing and bronchoconstriction. In addition, the ASM is an important source of inflammation, is both responsive to, and produces a variety of pro-inflammatory cytokines and chemokines that are induced through para- or autocrine actions in asthma. As a direct result of this, ASM in asthmatics closely interacts with infiltrating inflammatory cells, such as mast cells and lymphocytes.

The WNT (wingless-integrase-1) signalling pathway consists of a family of secreted glycoproteins that are heavily modified before entering the extracellular space. WNT proteins are crucially involved in embryonic development and maintenance of adult tissue homeostasis and are broadly categorized into β-catenin-dependent (canonical) and -independent signalling (non-canonical). We have previously shown that the non-canonical WNT ligand WNT-5A is actively utilized by human ASM and contributes to airway remodelling on multiple levels. TGF-β increases ECM turnover in human ASM through activation of WNT-5A. Similarly, human ASM cells require WNT-5A for the TGF-β-mediated
induction of alpha-smooth-muscle-actin (α-SMA) \(^{15}\). Treatment of human ASM with recombinant WNT-5A promotes formation of actin filaments and increases contractility \(^{15}\). In addition, we have shown that WNT-5A is increasingly expressed in ASM cells isolated from mild to moderate asthmatics \(^{14}\). Additionally, WNT-5A expression in bronchial biopsies has been strongly associated with Th2-high asthma \(^{16}\), and peripheral blood mononuclear cells (PBMCs) stimulated with IL-4 or IL-13 show elevated levels of WNT-5A \(^{17}\). While these results highly suggest that WNT-5A drives airway inflammation and remodelling in asthma, conclusive evidence is still lacking to confirm this. To that end, we have generated a tetracycline-based (tet-ON) smooth-muscle-specific WNT-5A transgenic mouse model, enabling in vivo characterization of the relevance of smooth-muscle derived WNT-5A in an allergic asthmatic context, using chronic ovalbumin exposure to drive asthma-like changes.

**Results**

**TetO-WNT-5A;SM-22-rtTA mice produce active WNT-5A in smooth muscle cells**

TetO-WNT-5A mouse founder lines carrying the mouse WNT-5A gene under the control of a Tet-inducible promoter were generated as described previously \(^{18}\). This promoter is inducible by the rtTA transcription activator in a doxycyline (DOX) dependent manner. TetO-WNT-5A founders were crossed with the SM-22-rtTA transgenic mouse line, which drives ubiquitous expression of the rtTA gene under control of the SM-22 promoter (specific for smooth muscle). This allows transgenic WNT-5A induction only in smooth muscle cells through administration of DOX (Figure 1A). WNT-5A expressing mice were identified by staining frozen lung tissue slices with WNT-5A antibody. While the airway smooth muscle bundle surrounding the airway lumen already displayed high endogenous levels of WNT-5A, it was significantly more abundant in the transgenic mouse line that received DOX (Figure 1B). Endogenous expression of WNT-5A in the smooth muscle layer as part of the elastic arteries was very high, and we were not able to detect a difference between wild-type and transgenic mice (Figure 1C). For the muscular arteries, which had much lower endogenous WNT-5A expression, smooth-muscle-specific WNT-5A was increasingly expressed in the transgenic animals (Figure 1D).
**Induced WNT-5A expression does not increase contraction**

A defining feature of asthma is increased bronchial reactivity and constriction. We have previously shown that WNT-5A increases maximum contractile force generation in bovine smooth muscle strips and human ASM cells through modulation of the actin cytoskeleton by increasing polymerization of actin filaments. Thus, we tested the hypothesis that increased WNT-5A expression in our transgenic mouse model would yield similar results through autocrine actions of WNT-5A. To test this, we prepared precision cut lung slices from naïve WT and transgenic mice that received DOX seven days prior to the onset of the experiment. *Ex vivo* lung slices were treated with a methacholine (MCh) titration curve ($10^{-10}$-$10^{-4}$ M) to induce airway constriction. Contrary to what we reported before in bovine and human smooth muscle, increased WNT-5A expression in transgenic mice did not alter airway contractility in response to MCh. There were no differences in both sensitivity to MCh and degree of airway narrowing (Figure 2).

**Mucus production is enhanced in OVA-treated TetO-WNT-5A;SM-22-rtTA mice**

To assess the effects of smooth-muscle-derived WNT-5A on airway remodelling within the context of allergic asthma, we chronically exposed mice to ovalbumin (OVA). The OVA model induces a robust pulmonary inflammatory response and is widely used to reproduce the pathophysiology of clinical (allergic) asthma and displays many features of airway remodelling similar to the disease (Figure 3A). DOX was administered after the sensitisation phase to OVA and prior to the OVA challenge phase. As we have previously shown a role for WNT-5A in the TGF-β-mediated production of α-SMA as well as deposition of matrix proteins in human ASM, we initially focused our attention on these parameters of airway remodelling. Airway smooth muscle thickness was assessed with an α-SMA antibody staining on paraffin-embedded lung tissue slices. While ovalbumin treatment was associated with an elevation in WNT-5A in whole lung homogenates (Figure 3C), increased expression of α-SMA induced by OVA was not enhanced by WNT-5A expression in the transgenic (Figure 3B). In addition, whole lung homogenates subjected to SDS-PAGE and probed for α-SMA yielded similar
results (Figure 3D). Next we determined ECM production adjacent to the airway wall with a Picro Sirius Red histological staining, indicative of the total collagen abundance. OVA-treated animals showed enhanced deposition of collagen around the airways, however, TetO-WNT-5A;SM-22-rtTA mice did not further increase peribronchial production of collagen (Figure 3E), which was corroborated with a Western blot for Collagen 1α1 on whole lung homogenates (Figure 3F). Because WNT-5A is associated with Th2-high asthma we next quantified mucus production with a periodic acid-Schiff staining, which is a typical Th2-driven event in allergic asthma. Mucus production was significantly enhanced in OVA-treated animals. Interestingly, DOX-treated transgenic animals displayed an even higher production of mucus (Figure 3G). To corroborate these findings, we looked at gene expression levels of MUC5A in whole lung homogenates. OVA-treated TetO-WNT-5A;SM-22-rtTA mice showed significantly enhanced MUC5A mRNA levels compared to OVA-treated WT mice (Figure 3H). Taken together, transgenic mice producing smooth-muscle-specific WNT-5A display increased mucus production when exposed to ovalbumin, without affecting smooth muscle thickness and matrix deposition.

**WNT-5A induces eosinophilic infiltration in OVA-treated animals**

Eosinophilic infiltration in the airways of asthmatics has classically been associated with allergic sensitization and a Th2-dominant inflammatory response. As we observed increased mucus production typical of Th2-inflammation, we tested the hypothesis that induced WNT-5A expression in DOX-treated transgenic animals increased eosinophilic influx after OVA-challenge. Endogenous peroxidase activity of infiltrating eosinophils allowed for their quantification around the airways using 3,3’-diaminobenzidine (DAB). OVA-treated animals had significantly more eosinophils surrounding the airways, while TetO-WNT-5A;SM-22-rtTA mice showed an even higher abundance (Figure 4A). Next we investigated the effects of WNT-5A on tissue resident macrophages. Although macrophages are well appreciated for their critical role in host defense, tissue homeostasis and repair, an important role for macrophages in Th2 immunity is becoming more clearly defined. However, while ovalbumin induced an increased number of alveolar macrophages, as determined with a CD68
antibody staining, we could not detect any differences between WT and transgenic mice (Figure 4B).

**Induced WNT-5A expression increases production of Th2-cytokines**

To further confirm the role of Th2-inflammation our model, we studied the expression of the Th2 cytokines IL-4, IL-5 and IL-13 in whole lung homogenates. As we expected, induced WNT-5A expression enhanced the production of these cytokines in OVA-treated animals, on both mRNA and protein level, as determined by RT-qPCR and Luminex assays (Figure 5A/B/C). As IL-4, IL-13, and IL-5 results in enhanced production of IgE by B cells, we used ELISA to investigate whether transgenic WNT-5A-producing mice showed enhanced antigen-specific IgE in blood serum. Indeed, OVA-treated animals had significantly higher serum IgE, which was enhanced even more in the transgenic mice (Figure 5D). To summarize, induced smooth-muscle-specific WNT-5A drives Th2 inflammation through production of IL-4, IL-5, IL-13 and serum IgE.
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Figure 1: tetO-WNT-5A;SM-22-rtTA mice produce active WNT-5A in smooth muscle cells. (A) Southern blot of mouse tail samples lysed and subjected to PCR to detect WT (left) and transgenic mice (right) carrying TetO-WNT-5A and rtTA positive founders. (B) Representative immunohistochemistry image (left) and the quantification (right) of WNT-5A visualised with DAB, from frozen lung tissue sections of wild type (WT) and transgenic (Tg) mouse airways. Alv is alveoli, Ep is epithelium, SM is smooth muscle. (C) Immunohistochemistry image as in (B) of mouse elastic arteries. (D) Immunohistochemistry image as in (B) of mouse muscular arteries. En is endothelium. Data represents eight independent experiments. Data is expressed as the mean ± SEM. * vs WT.
Figure 2: induced WNT-5A expression does not increase contraction. Precision cut lung slices prepared from wild type (WT) and transgenic (Tg) mice, and exposed to increasing doses of methacholine (MCh).
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Figure 3: mucus production is enhanced in OVA-treated TetO-WNT-5A;SM-22-rtTA mice. (A) Time overview of the ovalbumin (OVA) protocol to induce allergic asthmatic-like changes. Mice were initially sensitised to OVA on days 1, 14 and 21 and subsequently challenged with saline or OVA aerosols for four consecutive weeks. Doxycycline (DOX) was administered through the drinking water from day 23 onwards. (B) Representative immunohistochemistry image (left) and the quantification (right) of an alpha smooth muscle actin (α-SMA) staining visualised with DAB, obtained from paraffin-embedded lung tissue sections of wild type (WT) and transgenic (Tg) mouse airways exposed to repeated allergen (ovalbumin) challenge vs PBS-treated controls. Group sizes are n = 8, 8, 5, 10 for the respective groups (left to right). (C) WNT-5A and (D) α-SMA immunoblot of whole lung homogenates (treated as in (B)), normalised against β-actin. Group sizes are n = 8, 8, 5, 10 for the respective groups (left to right). (E) Histological staining as in (B) of total collagen deposition, measured with a Sirius Red staining. (F) Collagen 1α1 immunoblot as in (C). (F) Histological staining as in (B) of a periodic acid Schiff staining, indicative of mucus. (H) MUC5A relative gene expression of whole lung homogenates (treated as in (B)) subjected to RT-qPCR. Group sizes are n = 8, 6, 5, 6 for the respective groups (left to right). Data is expressed at the mean ± SEM. * vs WT PBS, $ vs WT OVA.

Figure 4: WNT-5A induces eosinophilic infiltration in OVA-treated animals. (A) Representative immunohistochemistry image (left) and the quantification (right) of eosinophils’ endogenous peroxidase activity, visualised with DAB, obtained from paraffin-embedded lung tissue sections of mouse airways exposed to repeated allergen (ovalbumin) challenge vs PBS-treated controls. (B) Immunohistochemistry staining as in (A) of a CD68 antibody staining, indicative of macrophages. Group sizes are n = 8, 8, 5, 10 for the respective groups (left to right). Data is expressed at the mean ± SEM. * vs WT PBS, $ vs WT OVA.
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**Figure 5:** induced WNT-5A expression increases production of Th2-cytokines. (A/B/C) Relative gene expression (left panel) and protein (right panel) of IL-4 (A), IL-5 (B) and IL-13 (C) obtained from whole lung homogenates of wild type (WT) and transgenic (Tg) mouse airways exposed to repeated allergen (ovalbumin) challenge vs PBS-treated controls, and subjected to RT-qPCR or ELISA. (D) Relative serum IgE levels obtained from blood serum of animals treated as in (A/B/C). Group sizes are n = 8, 8, 5, 10 for the respective groups (left to right). Data is expressed at the mean ± SEM. * vs WT PBS, $ vs WT OVA.
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(a) Whole lung homogenate

(b) Whole lung homogenate

(c) Whole lung homogenate

(d) Serum IgE (absorbance)
Discussion

In this study we sought to characterize the effects of smooth-muscle-derived WNT-5A in the lungs, using transgenic mice expressing a doxycycline-inducible TetO-WNT-5A construct in combination with SM-22-rtTA to drive expression of rtTA under control of the smooth-muscle-specific SM-22 promoter. As we were specifically interested in the effects of induced WNT-5A in the context of allergic asthma, we subjected both WT and transgenic mice to chronic ovalbumin exposure to induce an allergic asthmatic-like state. We found that increased WNT-5A expression from smooth muscle failed to increase bronchoconstriction. WNT-5A also had no effects on airway smooth muscle thickness and deposition of extracellular matrix components. However, we consistently found that smooth-muscle derived WNT-5A increased parameters of Th2 immunity in ovalbumin-treated animals. Mucus secretion, eosinophilic infiltration and serum IgE were all significantly elevated compared to WT animals exposed to ovalbumin. In addition we found that the Th2 cytokines IL-4, IL-5 and IL-13 were increasingly expressed in whole lung tissue of OVA-exposed animals.

We were not able to corroborate previously observed effects of WNT-5A on smooth muscle contraction and matrix remodelling. These studies were done with immortalized human smooth muscle cells and may therefore reflect species-specific differences. However, more likely is the possibility that the high endogenous expression of WNT-5A in smooth muscle may have obscured potential effects. We found that basal expression levels of WNT-5A in airway smooth muscle and elastic arteries were already very high and our previous studies have shown that WNT-5A in freshly isolated ASM is higher than that in cultured ASM cells. Moreover, whereas our previous studies showed effects of WNT-5A knockdown on TGF-β-induced matrix protein and α-SMA expression, recombinant WNT-5A by itself did not have such effects. Collectively, these previous studies and our current findings therefore suggest that WNT-5A is required but not sufficient to promote airway remodelling. Future studies in (cell-specific) WNT-5A knock-out mice are required to test that hypothesis.

In contrast to elastic arteries, muscular arteries had much lower amounts of endogenous WNT-5A expression, which may underlie the observed effects on
inflammation. In addition, we were not able to detect differences in WNT-5A expression between wild-type and transgenic mice in elastic arteries, possibly owing to the high endogenous expression. Muscular arteries are located more distally from the heart and distribute blood to the lungs, whereas elastic arteries are located more proximal and receive blood directly from the heart. Muscular arteries also have relatively more smooth muscle compared to elastic arteries. It is possible therefore that the pro-inflammatory effects mediated by WNT-5A are of muscular origin. However, more detailed studies need to be undertaken in order to verify this, as also airway smooth muscle-derived WNT-5A may have contributed to this effect. Another alternative explanation may reside in the differential activity of the SM-22 promoter among the different smooth muscle cell types 28, which would affect expression of the rtTA protein and therefore degree of transgenicity.

The link between WNT-5A and inflammation has been demonstrated in several diseases (see 29 for a review on this topic). Although we did not look into this ourselves, the WNT-5A-inflammation link is most clearly described for innate immunity to combat foreign infections. Microbial products or IFN-γ 30-32 elicit a strong WNT-5A response in monocytes. Also, various tissue-resident cells display increased WNT-5A expression induced by pro-inflammatory cytokines like IL-1β 33, IL-6 34 and TNF-α 35. Conversely, WNT-5A also drives the production of various inflammatory mediators 32,36-38, likely to sustain innate immune responses, and primarily underlies activation of the NF-κB pathway 36,39. In line with this, secreted WNT-5A affects antigen-specific T helper 1 (Th1)-cells. Supernatant of bone marrow stromal cells treated with WNT-5A promotes migration of T-cells 35, presumably through increased production of chemokines 40. In addition, WNT-5A increased IL-12 production and the subsequent generation of IFN-γ-producing T cells 37. It is worth noting that lymphocytes, both in the absence and presence of CD3/CD28 polyclonal stimuli to promote activation and T-cell expansion, do not express WNT-5A, but do express Frizzled-5 (FZD-5) 31, which is further increased upon activation of T-cells 41. This suggests the effects of endogenous WNT-5A on T-cell immunity is mediated by external sources.

Our results hint towards a role for WNT-5A in T helper 2 (Th2)-cell immunity. However, there is surpris-
ingly little direct evidence in the literature that can corroborate this. Peripheral blood mononuclear cells treated with IL-4 or IL-13 have elevated levels of WNT-5A in healthy adults. One study investigated the genetic profile of endobronchial biopsies from mild-to-moderate asthmatics, stratified into ‘Th2-high’ and ‘Th2-low’ subphenotypes on the basis of a signature of three IL-13 inducible genes. They found that WNT-5A was positively correlated with the Th2-high signature. Also FZD-5, a receptor for WNT-5A, was upregulated in Th2-high asthma. Moreover, WNT-5A expression is increasingly expressed in asthmatic PBMCs of Korean subjects. WNT-5A expression in airway epithelial brushings of asthmatics, correlated to fractional exhaled nitric oxide (FeNO) and then stratified into different asthma phenotypes, showed that atopic individuals with an early disease onset and high percentage of bronchoalveolar lavage (BAL) lymphocytes have strongly elevated levels of WNT-5B compared to other phenotypes. WNT-5B and WNT-5A are paralogous genes and share a high degree of sequence similarity. Future efforts should entail more conclusive studies to characterize the relationship between WNT-5A and Th2 driven inflammation in asthma. Nonetheless, the results obtained in these studies provide an exciting groundwork. As we previously shown, WNT-5A also elicits effects on smooth muscle contraction and extracellular matrix remodelling in human airway smooth muscle cells, thus potentially conferring additional clinical benefits over current treatment strategies that primarily target inflammation.

**Materials and methods**

**Generation of tetracycline inducible TetO-WNT-5A;SM-22-rtTA mice**

TetO-WNT-5A mice were generated as described previously. In brief, a 1.1 kb XbaI fragment was isolated from a TetO-FLAG-WNT-5A vector that was kindly provided by Dr. Roel Nusse and Dr. Renée van Amerongen, Stanford University, USA, and inserted into a pTRE-Tight vector containing an additional 0.68 kb β-globin intron. The 2.4 kb expression cassette was XhoI digested from the plasmid backbone, gel-purified and injected into C57Bl/6J oocytes by standard procedures. TetO-WNT-5A positive founders were identified by PCR using transgene specific primers (see Table 1).

TetO-WNT-5A mice were then crossed with FVB/N-Tg(Tagln-rtTA)E1Jwst/J mice (The Jackson Laboratory,
(Thermo Scientific, #R0611) on a 1% agarose gel (89 mM Tris-HCl, 89 boric acid, 2 mM EDTA) mixed with 0.01% v/v SYBR® Safe DNA Gel Stain (Invitrogen, #S33102) to visualise DNA.

**Animals**

Female mice were subjected to ovalbumin challenge studies, while males were subjected to characterization and contraction studies. Animals were group-housed (2-4 per cage) in climate controlled animal quarters and provided with food and water *ad libitum*. Only siblings were housed together to avoid stress and injury. A 12h/12h light/dark cycle was maintained. Animals were 10-32 weeks of age at the start of the experiment, weighing 21-35 g and were randomly assigned to the different experimental groups. Group sizes were calculated based on airway eosinophil infiltration. Earlier studies with the ovalbumin-challenge model within the University Medical Center Groningen have shown an average of 16 ± 5 eosinophils. Assuming equal variance, alpha of 0.05, 1-beta of 0.8 and an effect size of 40% a group size of 10 can be obtained. Some samples were lost either due to death of the animals or post-processing issues of the animal tissue. These samples were not replaced as the
ovalbumin-challenge for this group of animals yielded a very robust infiltration of airway eosinophils (average of 47 ± 17 for the ovalbumin-treated animals); almost three times the size of earlier studies and therefore maintaining sufficient power.

**Blood and tissue collection**

24 hours following cessation of the last inhalational exposure, animals were killed by exsanguination following a subcutaneous injection of 40 mg/kg ketamine and 0.5 mg/kg dexmedetomidine dissolved in 100 µl solvent. 0.5-1.0 mL blood was collected via cardiac puncture in 1.5 mL Eppendorf tubes and spun down to obtain serum. Lungs were harvested as follows: the post-caval, inferior lobe and left lobe were snap frozen in liquid nitrogen for mRNA and protein analysis. The leftover lungs were then inflated with 600 µL of a saline/Tissue-Tek® mixture (1:1 v/v), of which the superior lobe was subsequently fixed in formalin and later embedded in paraffin for immunohistochemistry. The middle lobe was snap frozen in liquid nitrogen and used for immunohistochemistry.

**Immunohistochemistry**

Paraffin-embedded or frozen lung tissue was cut with a Microm HM 340E microtome (Thermo Scientific). Transverse cross-sections of 5 µm were used for analyses. In short, if necessary tissue sections were deparaffinised in xylene and rehydrated in a serial dilution of ethanol. Heat-induced epitope retrieval was performed where necessary in a Decloaking chamber™ Nxgen pressure cooker (Biocare Medical). Sections were washed in PBS and blocked in normal serum from the species in which the secondary antibody was generated (either goat (Dako, #X0907) or rabbit (Dako, #X0902)). Primary antibody diluted in 1% BSA in PBS was incubated overnight at 4°C, then washed with PBS and incubated with 0.3% H₂O₂ in PBS for 15 min. Subsequently, sections were incubated with secondary antibody for 1 h at room temperature. Finally, sections were washed with PBS and developed with diaminobenzidine (Sigma-Aldrich, #D5637) for 5 min, followed by a hematoxylin counterstain (Sigma-Aldrich, #MHS32). Sections were then rinsed in tap water for 5 min, dehydrated in ethanol-xylene and mounted with KP-mounting medium (Klinipath, #7275).

Total collagen abundance was visualised with a Sirius red staining. In short, tissue sections were deparaffinised in xylene and rehydrated in a serial dilution of ethanol. Sections were
incubated in Sirius red solution (Sirius red F33: Klinipath, #80115, picric acid: Sigma-Aldrich, #P6744) for 60 min at room temperature, followed by a 2 min wash with 0.01 N HCl. Sections were counterstained with hematoxylin (Sigma-Aldrich, #MHS32) for 5 min, rinsed with tap water for 5 min, quickly dehydrated in ethanol-xylene and mounted with KP-mounting medium (Klinipath, #7275).

Mucous was visualised with a Periodic Acid Schiff (PAS) staining. Tissue sections were deparaffinised and hydrated to deionized water. Sections were immersed in Periodic Acid solution (Sigma-Aldrich, #3951) for 15 min at room temperature, rinsed in water and immersed in Schiff's Reagent (Sigma Aldrich, #3952016) in the dark for 30 min at room temperature. Sections were rinsed in water, counterstained with hematoxylin (Sigma-Aldrich, #MHS32) for 5 min, rinsed with tap water for 5 min, quickly dehydrated in ethanol-xylene and mounted with KP-mounting medium (Klinipath, #7275).

Digital images were quantified using ImageJ (NIH). Analyses were performed in a blinded fashion. Expression intensity was expressed as staining positive area relative to the length of the basement membrane squared (mm$^2$ / mm$^2$) for airways or to the length of the smooth muscle layer squared for arteries.

**Precision cut lung slices**

Male mice were terminated seven days after doxycycline administration by exsanguination following a subcutaneous injection of ketamine and dexmedetomidine as described above. After euthanization the trachea was cannulated, and lungs were filled through the cannula with a low melting-point 1.5% agarose solution (Gerbu, #1744) dissolved in medium containing CaCl$_2$ (0.9 mM), MgSO$_4$ (0.4 mM), KCl (2.7 mM), NaCl (58.2 mM), NaH$_2$PO$_4$ (0.6 mM), glucose (8.4 mM), NaHCO$_3$ (13 mM) and HEPES (12.6 mM, GIBCO, #15630), supplemented with sodium pyruvate (0.5 mM, GIBCO, #11360), MEM Amino acids (1:50, GIBCO, #11130), MEM Vitamin solution (1:100, GIBCO, #11120), L-Glutamine (1 mM, GIBCO, #25030) and 100 units/mL antibiotics (Penicillin-Streptomycin, GIBCO, #15070), pH = 7.2. Agarose was solidified by placing the animals on ice at 4 °C for 15 min. Lungs were harvested and individual lobes were sliced at a thickness of 250 µm in ice-cold medium, but without supplements (see above), using a tissue slicer (Comprestatome™ VF-300 microtome,
Precisionary Instruments, San Jose, CA, USA). Thereafter, slices were kept at 37 °C under humidified conditions in a CO₂ incubator, and washed every 30 min for four times to remove residual agarose and cell debris in medium containing CaCl₂ (1.8 mM), MgSO₄ (0.8 mM), KCl (5.4 mM), NaCl (116.4 mM), NaH₂PO₄ (1.2 mM), glucose (16.7 mM), NaHCO₃ (26.1 mM), HEPES (25.2 mM), supplemented with sodium pyruvate (1 mM), MEM Amino acids (1:50), MEM Vitamin solution (1:100), L-Glutamine (2 mM), 100 units/mL antibiotics, and streptomycin (100 µg/mL), pH = 7.2.

Lung slice responses were recorded the next day, with the contractile agonist Acetyl-β-methylcholine chloride (MCh, 10⁻¹⁰⁻¹⁰⁻⁴ M, Sigma Aldrich, #A2251). Baseline airway luminal area was determined 120 sec prior to addition of MCh, after which a ten-fold higher dose was administered every 10 min interval. A metal washer was placed on top of the slices to flatten and maintain them in place. A nylon mesh was placed between the washer and lung slice to avoid direct contact of the metal. Bright field images of the lung slices were captured in time-lapse (1 frame per 2 s) with a resolution of 1280 × 960 pxl (1.15 m/pxl) using an inverted microscope (Nikon, Eclipse TS100).

Airway luminal area was quantified in a blinded fashion using ImageJ (NIH).

**Allergen exposure**

To induce a chronic allergic asthmatic response, female mice were sensitized to chicken-derived ovalbumin (OVA) (Sigma-Aldrich, #A5378), using Aluminum Hydroxide (AlOH₃) (Inject™, Thermo Scientific, #77161) as an adjuvant to promote an IgE and Th2 skewed response, as previously described 21 (Figure 3). In brief, animals were sensitized on days 1, 14 and 21 by means of an intraperitoneal injection of 10 µg OVA together with 1.5 mg of AlOH₃ dissolved in 200 µl saline. Subsequently, animals were exposed to aerosolized OVA (1% w/v in saline) or saline for 20 min/day on two consecutive days/week for four weeks. Exposures were carried out in a custom-built Perspex chamber (9 L) with a De Vilbiss Model 646 nebulizer. Air flow was set at a rate of 40 L/min, providing aerosol with an output of 0.33 mL/min.

**Western blot analysis**

Lung homogenates were first pulverised with a pestle and mortar in liquid nitrogen and subsequently sonicated in RIPA lysis buffer (65 mM Tris, 155 mM NaCl, 1% Igepal CA-630, 0.25% sodium deoxycholate, 1 mM EDTA, pH 7.4, and
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A mixture of protease inhibitors: 1 mM Na₃VO₄, 1 mM NaF, 10 µg ml⁻¹ leupeptin, 10 µg ml⁻¹ Pepstatin A, 10 µg ml⁻¹ Aprotinin) and kept on ice for 15 min. Lysates were vortexed vigorously and finally centrifuged for 10 min at 10,000 g. Protein content of the supernatant fractions was determined with a BCA protein assay kit (Thermo Scientific, #23225) and subsequently subjected to SDS-PAGE, using 6% and 10% running gels (depending on protein size). Separated proteins were transferred to PVDF membranes (Carl Roth, 0.45 µm, #T830.1), which were then blocked with ROTI®-Block blocking solution (Carl Roth, #A151.2) for 2 hours at room temperature. Membranes were incubated with primary antibodies overnight at 4 °C in TBST (50 mM Tris-HCl, 150 mM NaCl, 0.05% (w/v) Tween-20, pH 7.4). The next day, after washing in TBST, membranes were incubated with HRP-conjugated secondary antibody for 2 hours at room temperature. Finally, blots were developed using enhanced chemiluminescence substrate (Perkin Elmer, #NEL105001EA). Digital images were quantified by densitometry using LI-COR Image Studio Lite software.

**RT-qPCR**

Lung homogenates were first pulverised with a pestle and mortar in liquid nitrogen prior to addition of lysis buffer. Isolation of animal tissue mRNA was isolated with TRIzol reagent. Equal amounts of cDNA were synthesized using AMV reverse transcriptase (Promega, #A3500) and diluted 15 times with RNase-free ddH₂O. Quantitative real-time PCR was performed on an Illumina Eco Real-Time PCR system using SYBR green as the DNA binding dye (Roche, #04913914001). PCR cycling was performed with denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec and extension at 72 °C for 30 sec for 45 cycles. Analysis of RT-qPCR data was done using LinRegPCR analysis software 22,23. 18S ribosomal RNA, Beta-2-Microglobulin (B2M) and Ribosomal Protein L13A (RPL13A) were used as reference loci for accurate normalization of the RT-qPCR data. Primer sequences are listed in Table 1. Total RNA yield was determined with a NanoDrop ND-1000 spectrophotometer and samples were normalized accordingly.
### Table 1. Primer sequences used

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<th>Species</th>
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**Serum IgE**

Levels of OVA-specific IgE antibodies in serum were measured by ELISA. OVA-specific IgE antibody was detected using plates coated with 100 µL anti-mouse IgE antibody (1:250, BD Biosciences, #553413) dissolved in 100 mM NaHCO₃ and 34 mM Na₂CO₃, pH 9.5. Next, plates were washed in PBS + 0.05% Tween-20 (v/v), pH 7 and blocked for 2 h at RT in 200 µL blocking buffer (PBS + 1% BSA, pH 7). Afterwards, plates were washed thoroughly and mouse serum was added for 2 h at 37 °C. Plates were then washed extensively and labelled with 100 µL biotinylated OVA (1:200) dissolved in PBS + 10% FBS for 1 h at RT. Plates were washed again thoroughly and incubated with 100 µL streptavidin-horseradish peroxidase (1:3000) dissolved in 50 mM Tris, 137 mM NaCl, 2 mM EDTA, 0.05% Tween-20 (v/v) and 0.5% BSA (w/v) for 30 min at RT. Finally, plates were washed extensively and peroxidase activity was started by adding OPD Peroxidase Substrate tablets (Sigma Aldrich, #P9187) dissolved in PBS. Reaction was stopped after 50 min with 100 µL H₂SO₄ at absorbance was measured with a microplate reader at 490 nm.

**Luminex screening assay**

Cytokine levels of IL-4, IL-5 and IL-13 in lung homogenates were determined
with a Luminex® screening assay (R&D Systems, #LXSAMS) according to the manufacturer’s instructions. Total protein content was normalized with a BCA protein assay kit (Thermo Scientific, #23225) prior to start of the assay. In brief, filter-bottom microplates were pre-wet with 100 µL washing buffer. Liquid was removed through the filter with a vacuum manifold and 50 µL of the diluted microparticle cocktail was added to the plate in addition to 50 µL of either standard or sample (1:2), for 2 h at RT on a horizontal orbital shaker. Liquid was removed while maintaining microparticles and plates were washed thoroughly with washing buffer. Next, 50 µL of diluted Biotin antibody cocktail was added to all wells for 1 h at RT. Plates were washed again and incubated with 50 µL streptavidin-PE for every well for 30 min at RT. Finally, plates were washed, liquid was removed and microparticles were resuspended in washing buffer. Absorbance was measured with the Luminex 100 system using Starstation software (Applied Cytometry Systems).

**Statistical analysis**

All data represents the mean ± SEM. At least seven animals were analysed per treatment group. Data with an n below 4 were not statistically analysed. A Shapiro-Wilk’s test (p>0.05) as well as visual inspection of the respective histograms, normal Q-Q plots and box plots was used to test whether samples were normally distributed (approximately), using IBM SPSS Statistics version 23. Comparisons between two groups were made using an unpaired Student’s t-test for normally distributed data or a Mann-Whitney U test as the non-parametric equivalent. Comparisons between three or more groups were performed using a one-way ANOVA followed by Tukey’s post hoc test for normally distributed data, or with a Kruskal-Wallis H test for non-normally distributed data. A value of p < 0.05 was considered statistically significant. Analyses were performed with GraphPad Prism (GraphPad Software, Inc.).

**Antibodies and chemicals**

The following antibodies were used: WNT-5A (immunohistochemistry 1:300, Abcam, #ab72583) α-smooth muscle actin (immunohistochemistry 1:100, western blot 1:1,000, Abcam, #ab5694), β-actin (western blot 1:2,000, Sigma Aldrich, #A5441), Collagen 1α1 (western blot 1:1,000, Southern Biotech, #1310-01), CD68 (immunohistochemistry 1:100, Bio-Rad, # MCA1957T), peroxidase-conjugated anti-mouse IgG (western
blot 1:3,000, Sigma-Aldrich, #A9044), peroxidase-conjugated anti-rabbit IgG (western blot 1:3,000, Sigma-Aldrich, #A0545), peroxidase-conjugated anti-goat IgG (western blot 1:8,000, Sigma-Aldrich, #A5420). All other chemicals were of analytical grade.

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**Author contributions**

TK conceived the study and designed the experiments, carried out the vast majority of the work, analysed the data and drafted the manuscript. MM carried out part of the *in vivo* work. RG conceived the study and designed the experiments. RS provided the TetO-WNT-5A founder lines. All authors critically revised the manuscript for important intellectual content and approved its final version.

**Competing financial interests**

The authors declare no competing financial interests.
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