



The Canonical but Not the Noncanonical Wnt Pathway Inhibits the Development of Allergic Airway Disease

This information is current as of August 27, 2018.

Hendrik Beckert, Helen Meyer-Martin, Roland Buhl, Christian Taube and Sebastian Reuter

J Immunol published online 22 August 2018
<http://www.jimmunol.org/content/early/2018/08/21/jimmunol.1800554>

Supplementary Material <http://www.jimmunol.org/content/suppl/2018/08/21/jimmunol.1800554.DCSupplemental>

Why *The JI*? [Submit online.](#)

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at:
<http://www.aai.org/About/Publications/JI/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/alerts>



The Canonical but Not the Noncanonical Wnt Pathway Inhibits the Development of Allergic Airway Disease

Hendrik Beckert,* Helen Meyer-Martin,[†] Roland Buhl,[†] Christian Taube,* and Sebastian Reuter*

Asthma is a syndrome with multifactorial causes, resulting in a variety of different phenotypes. Current treatment options are not curative and are sometimes ineffective in certain disease phenotypes. Therefore, novel therapeutic approaches are required. Recent findings have shown that activation of the canonical Wnt signaling pathway suppresses the development of allergic airway disease. In contrast, the effect of the noncanonical Wnt signaling pathway activation on allergic airway disease is not well described. The aim of this study was to validate the therapeutic effectiveness of Wnt-1–driven canonical Wnt signaling compared with Wnt-5a–driven noncanonical signaling in murine models. *In vitro*, both ligands were capable of attenuating allergen-specific T cell activation in a dendritic cell–dependent manner. In addition, the therapeutic effects of Wnt ligands were assessed in two different models of allergic airway disease. Application of Wnt-1 resulted in suppression of airway inflammation as well as airway hyperresponsiveness and mucus production. In contrast, administration of Wnt-5a was less effective in reducing airway inflammation or goblet cell metaplasia. These results suggest an immune modulating function for canonical as well as noncanonical Wnt signaling, but canonical Wnt pathway activation appears to be more effective in suppressing allergic airway disease than noncanonical Wnt activation. *The Journal of Immunology*, 2018, 201: 000–000.

Wnt signaling pathways represent a complex family of glycoproteins, their receptors, coreceptors, and downstream signaling molecules. They are involved in a variety of cellular responses, which can be categorized in β -catenin–dependent canonical signaling and β -catenin–independent noncanonical pathways. The canonical Wnt signaling pathway is well described. Binding of Wnt ligands to their receptors leads to the congregation with coreceptors like LRP5/6, and this induces intracellular signaling via suppression of cytoplasmic degradation of β -catenin by the inhibition of its degradation complex. As a result, β -catenin accumulates in the cytoplasm and translocates into the nucleus and induces gene expression.

The noncanonical signaling pathways are less well described and more variable in their intermediate signaling molecules and biological responses. The best described pathways are the planar cell polarity and the calcium-dependent pathway. Induction of planar cell polarity activates in a commonly LRP5/6-independent manner a signaling cascade, including JNK and the small GTPases Rac-1 and RhoA. Activation of the calcium-dependent pathway promotes NFAT-mediated gene expression (1).

Wnt signaling is well known for its involvement in organ development in embryogenesis (2, 3) and also for the regulation of several mechanisms in later life, such as stem cell self-renewal and cell-migration, -fate, or -polarization (4–7). Recently, it has become apparent that Wnt signaling is also involved in the modulation of immune responses (8). Thus, it is not surprising that dysfunctional Wnt signaling has been associated with the development and progression of several diseases (2, 3). The role of Wnt signaling in the development and exacerbation of different lung diseases, including asthma, chronic obstructive pulmonary disease, and idiopathic pulmonary fibrosis has recently been reviewed (9–11). Depending on disease type, Wnt signaling partners and predominant cell types involved in disease pathophysiology disease promoting and suppressing properties of Wnt have been described. A detailed analysis of the variety of Wnt signaling processes is required to better understand the contribution of Wnt signaling pathways in disease pathophysiology.

Bronchial asthma is a heterogeneous syndrome with many different clinical and immunological phenotypes (12–14). The best described of these is allergic asthma, in which dendritic cell (DC)–driven induction of a Th2 cell response against a harmless Ag is responsible for the pathologic condition (15, 16). This failure in the regulation of adaptive immunity results in eosinophil-dominated chronic inflammatory responses in the lung, which are responsible for remodeling processes and airway hyperresponsiveness (17). Current treatments focus on the suppression of inflammation but fail to cure the disease and are ineffective in some asthma phenotypes (18–22).

Interestingly, it has been demonstrated in different models that activation of the canonical Wnt pathway attenuates inflammatory and allergic processes in the lung (23–26). Induction of Wnt-1 overexpression in murine club cells reduced the migration of allergen-loaded DC from the lung and decreased all hallmarks of the disease in both prophylactic and therapeutic models (26). Thus, the canonical Wnt signaling pathway might be an interesting therapeutic target for the treatment of allergic asthma. Noncanonical Wnt signaling especially mediated by Wnt-5a is associated with structural and airway remodeling processes (27–30).

*Department of Pulmonary Medicine, University Medical Center Essen–Ruhrlandklinik, Essen, North Rhine–Westphalia 45239, Germany; and [†]Department of Pulmonary Medicine, III. Medical Clinic, University Medical Center of the Johannes Gutenberg University, D-55131 Mainz, Germany

ORCID: 0000-0003-0098-3548 (H.B.).

Received for publication April 18, 2018. Accepted for publication July 26, 2018.

Address correspondence and reprint requests to Dr. Sebastian Reuter, Experimentelle Pneumologie, Universitätsmedizin Essen–Ruhrlandklinik, Tüschener Weg 40, 45239 Essen, Germany. E-mail address: sebastian.reuter@rlk.uk-essen.de

The online version of this article contains supplemental material.

Abbreviations used in this article: BAL, bronchoalveolar lavage; BD, Becton Dickinson; BMDC, bone marrow–derived DC; cDC, conventional DC; DC, dendritic cell; HDM, house dust mite; MCh, methacholine; MFI, mean fluorescence intensity; MHCII, MHC class II; PAS, periodic acid–Schiff; tLN, tracheal lymph node; TM, test medium; Treg, regulatory T cell.

Copyright © 2018 by The American Association of Immunologists, Inc. 0022-1767/18/\$35.00

In contrast, the relationship between noncanonical Wnt signaling and immune regulation in asthma is not well described.

The aim of this study was to validate Wnt-1 as a potential therapeutic molecule and to determine immunological function in the Wnt-5a–triggered noncanonical pathway in murine models of bronchial asthma. The immune regulatory functions of both ligands were analyzed in an allergen-specific DC/T cell interaction assay in vitro. The therapeutic effectiveness of Wnt-1 and Wnt-5a was further characterized in two murine in vivo models of allergic airway disease.

Materials and Methods

Animals

C57BL/6J, DO11.10, and BALB/c mice were obtained from the Translational Animal Research Center of the Johannes Gutenberg University Medical Center. All mice were females and used at the age of 8–12 wk. Animal procedures were conducted in accordance with current federal, state, and institutional guidelines, and all experiments were approved by the local regulatory authorities.

Experimental protocols

OVA- and house dust mite (HDM)–specific murine models of allergic airway disease were used to assess the effects of canonical Wnt-1/ β -catenin and noncanonical Wnt-5a signaling in vivo (Fig. 1A). For sensitization toward the model Ags, C57BL/6J animals received an i.p. injection of 20 μ g OVA (Sigma-Aldrich) or HDM (*Dermaphagoides pteronyssinus*, Greer Laboratories) suspended in 2 mg aluminum hydroxide (Imject Alum; Pierce, Rockford, IL) in a total volume of 100 μ l (PBS) on days 0 and 14. Allergic airway disease was induced by challenge via the airways on day 28, 29, and 30. In the OVA model, an OVA solution (1% in PBS) was nebulized daily for 20 min with an ultrasonic nebulizer (NE-U17; Omron, Hoofddorp, the Netherlands). In the HDM model, 1 μ g HDM dissolved in 40 μ l PBS was instilled in the nostrils of anesthetized (isoflurane) mice on day 28, 29, and 30. Readouts were performed 48 h after the last challenge. To analyze the effects of Wnt ligands, anesthetized (isoflurane) animals received Wnt-1 or Wnt-5a 500 ng intranasally in 40 μ l PBS 1 h before each challenge.

Assessment of allergic airway disease and organ preparation

Invasive measurement of lung function was performed on anesthetized, intubated mice by mechanical ventilation in response to increasing doses of methacholine (MCh) (6.25, 12.5, 25, 50, 100 mg/ml) with the Flexi Vent System (SCIREQ, Montreal, QC, Canada) as previously described (31). A forced sinusoidal breathing maneuver (snapshot) was performed to analyze total resistance of the airway system and total compliance of the system. Percentage change to PBS nebulization was calculated to avoid differences in parameter appearing because of different start values.

Blood was taken, sera was prepared, and samples were stored at -20°C until Ig determination via ELISA.

Lungs were lavaged with 1 ml PBS. Total bronchoalveolar lavage (BAL) cell count was determined via trypan blue exclusion. Differential cell counts for macrophages, lymphocytes, neutrophils, and eosinophils were performed on cytocentrifuged preparations stained with Hemacolor Stain Set (Merck, Darmstadt, Germany). After perfusion with PBS, the left lung lobe was ligated at the bronchus, removed, transferred to a tube, submerged with PBS, and stored on ice until single cell preparation for flow cytometric analysis. Right lung lobes were fixed by inflation and immersion in 10% formalin and embedded in paraffin as already described. Tissue sections were stained H&E or periodic acid–Schiff (PAS). To assess airway inflammation, five randomly chosen areas of each H&E-stained slide were scored by two experienced observers who were unaware of the experimental group. Inflammation was scored on a scale from 0 to 4 (32). PAS-positive goblet cells were quantified per millimeter of basal membrane on at least three different representative airways on PAS-stained slides.

Flow cytometric analysis

Flow cytometric analysis was used to assess DC and regulatory T cell (Treg) populations in the lung and draining lymph node ex vivo and DC activation and T cell proliferation in vitro (Fig. 1B).

To analyze cell populations ex vivo, single cell suspensions of lungs and tracheal lymph nodes (tLN) were prepared and stained. Draining lymph nodes were disrupted and transferred over a 70- μ m cell strainer into a 5-ml

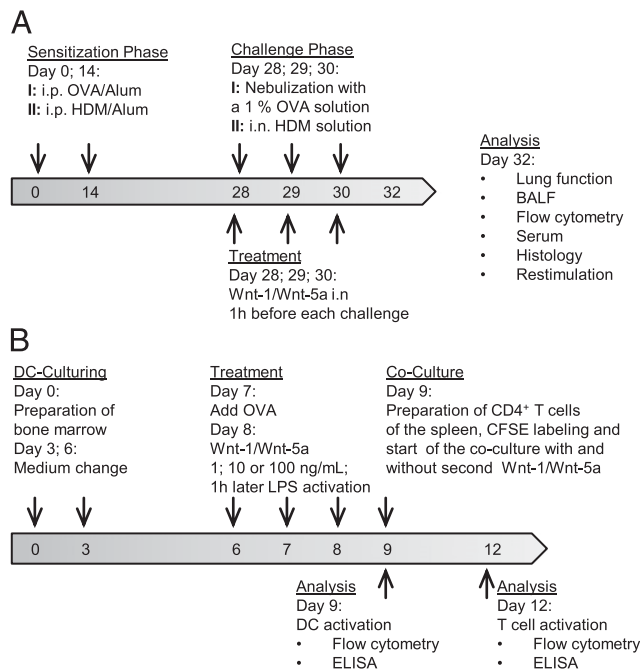


FIGURE 1. Treatment protocols for OVA- and HDM-specific models of allergic airway disease (A) and the murine allergen-specific DC/T cell interaction model (B).

round bottom tube (Becton Dickinson [BD], Heidelberg, Germany). Cells were washed, and cell count was determined. Cell numbers were adjusted to 1×10^7 cells/ml test medium (TM; IMDM plus 10% FCS [PAA]/1% penicillin-streptomycin [Sigma-Aldrich]), and 1×10^6 cells were used for each staining. Lungs were cut and transferred into a 50-ml vial. Collagenase type I (0.5 mg/ml; Sigma-Aldrich) was added. After an incubation time of 45 min at 37°C in a shaking water bath, cells were resuspended at least three times through a 0.9×40 -mm cannula in a 10-ml syringe. Cells were transferred over a cell strainer (70 μ m; BD) into a new vial. To eliminate RBCs, lysis was performed using Gey solution. Cells were washed twice, and cell counts were determined. Cell numbers were adjusted to 1×10^7 cells/ml cell medium (IMDM plus 10% FCS; PAA, 1% penicillin-streptomycin; Sigma-Aldrich), and 1×10^6 cells were used for each staining.

Unspecific binding was blocked with Fc receptor–blocking Abs (α CD16/CD32; BD). To identify DC populations, the cells were stained with FITC-labeled anti-MHC class II (MHCII) (eBioscience, San Diego, CA) and PE-Cy7-labeled anti-mouse CD11c (BD). To further subdivide DC populations, cells were additionally stained with PE-labeled anti-CD103 (BD), PercP-Cy5.5-labeled anti-CD11b (BD), and V450-labeled or APC-Cy7-labeled anti-Ly6c (BD) (gating strategy: Supplemental Fig. 1A). Master mixes with suitable Ab concentrations were prepared and added to the samples. Following incubation, cells were washed and finally resuspended in flow cytometry fixation buffer (4% PFA). The total cell number for different cell types was calculated by multiplying the absolute cell number by the percentage of cells.

To analyze Tregs, adjusted single cell suspensions of either lung or draining lymph nodes were stained with PerCP-Cy5.5-labeled anti-mouse CD3 (BD Biosciences), PE-Cy7-conjugated anti-mouse CD4 (BD Biosciences), and PE-labeled anti-mouse CD25 (BD). Following incubation and washing, intracellular staining against Foxp3 was performed by using the Foxp3 staining buffer set (eBioscience). Following fixation and permeabilization, Foxp3 was stained by incubation with APC-labeled anti-mouse Foxp3 Ab (gating strategy: Supplemental Fig. 1B).

Flow cytometric measurements were performed on a FACSCanto II (BD) using Diva software. Final analysis of flow cytometric data and graphics were achieved using FlowJo software (Tree Star). Flow cytometric analysis of in vitro assays is described in detail in the in vitro section.

Ag stimulation of lung and lymph node single cells in vitro

To assess in vitro Ag-specific cytokine production by lung and lymph node cells, single cell suspensions of lung or lymph nodes were performed under sterile conditions as described above. The 1×10^6 cells were stimulated by incubating for 72 h with or without 250 μ g/ml OVA

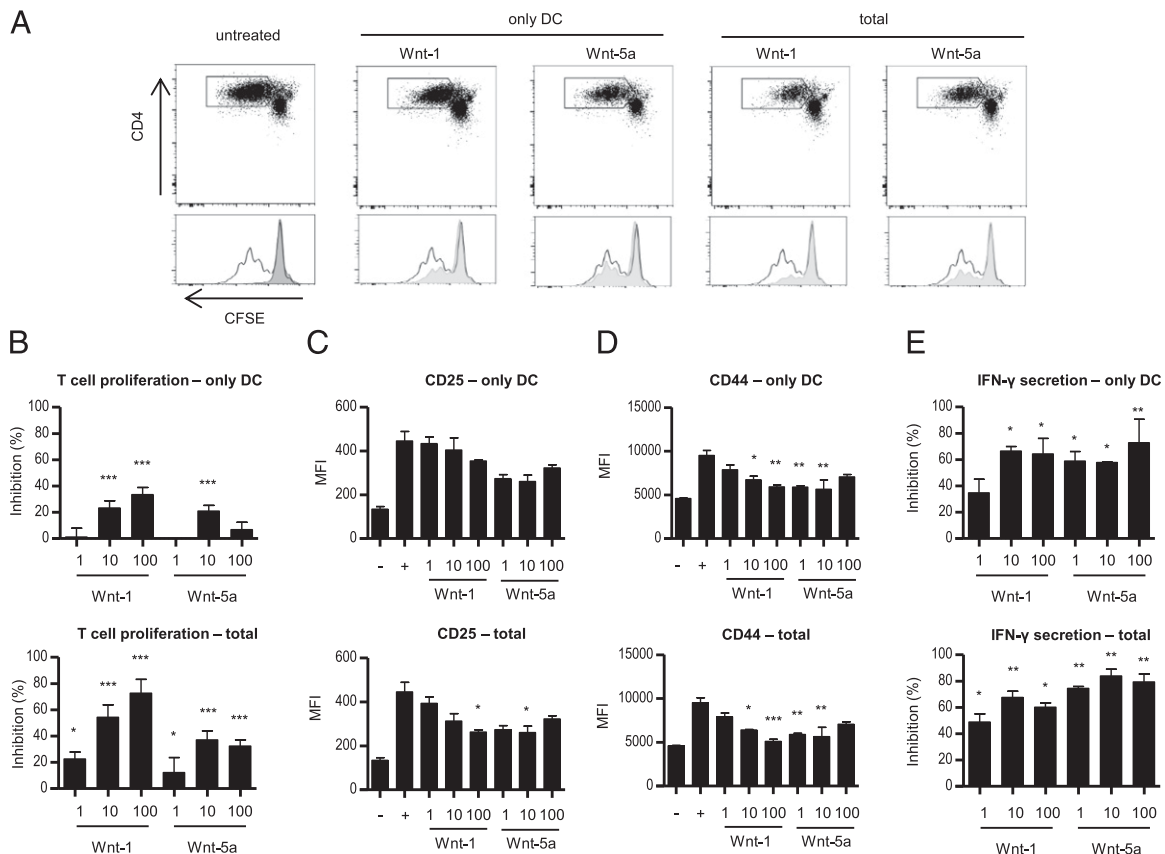


FIGURE 2. Effect of Wnt-1 and Wnt-5a on allergen-specific DC/T cell interactions in vitro. **(A)** Dot plots depict proliferation of CFSE-labeled CD4⁺ T cells cocultured with untreated, activated, OVA-loaded BMDC or BMDC treated with Wnt-1 or Wnt-5a only during activation (only DC) or during the complete coculture (total). Histograms show CFSE expression of T cells cocultured with untreated, activated, OVA-loaded BMDC (black line) against coculture with untreated nonactivated BMDC (dark gray shade) or against coculture with activated BMDC treated with corresponding presented Wnt ligand (light gray shade). **(B)** Inhibition of T cell proliferation after treatment with different doses of Wnt ligands only during BMDC activation (only DC) or during the complete cell culture (total) compared with untreated activated BMDC. **(C)** MFI of surface-expressed CD25 on CFSE-labeled CD4⁺ T cells cocultured with the indicated BMDC groups. **(D)** MFI of surface-expressed CD44 on CFSE-labeled CD4⁺ T cells cocultured with the indicated BMDC groups. **(E)** Inhibition of IFN-γ secretion of T cells cocultured with activated BMDC treated with the indicated Wnt ligand concentration compared with T cells cocultured with untreated activated BMDC. * $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.001$ versus positive control, t test.

(grade V; Sigma-Aldrich) or HDM (Greer Laboratories) at 37°C. After incubation, supernatants were harvested and stored for ELISA analysis at -20°C.

Cytokine ELISA

To determine cytokine concentrations in supernatants of restimulated lymph node and lung cells, ELISA assays were performed according to the manufacturer's procedure: IL-5, IFN-γ, IL-10 (OptEIA; BD), and IL-13 (R&D Systems, Minneapolis, MN) levels were analyzed. To calculate Ag-specific cytokine production, cytokine concentration from supernatants of unstimulated cells was subtracted from the corresponding stimulated samples. Percentage of inhibition of cytokine production was calculated in the in vitro experiments as previously described (33), in short, as follows:

$$\% \text{inhibition} = 100 - \left(\frac{\text{cytokine concentration sample}}{\text{mean cytokine concentration positive control}} \times 100 \right)$$

Ig ELISA

Serum was obtained 48 h after the last challenge. OVA-specific IgG1 and IgG2b titers were determined according to the manufacturer's protocol using ELISA (BD). All Abs were used in concentrations recommended by the manufacturer. OVA-specific IgE titers were analyzed using a previously described protocol (34, 35). HDM-specific IgE, IgG1, and IgG2b (BD) assessments were performed in a similar way. Plates were coated with HDM (25 μg/ml), and detection Abs against IgE, IgG1, and IgG2b were used in 1:250 dilutions. The Ab titer was defined as the

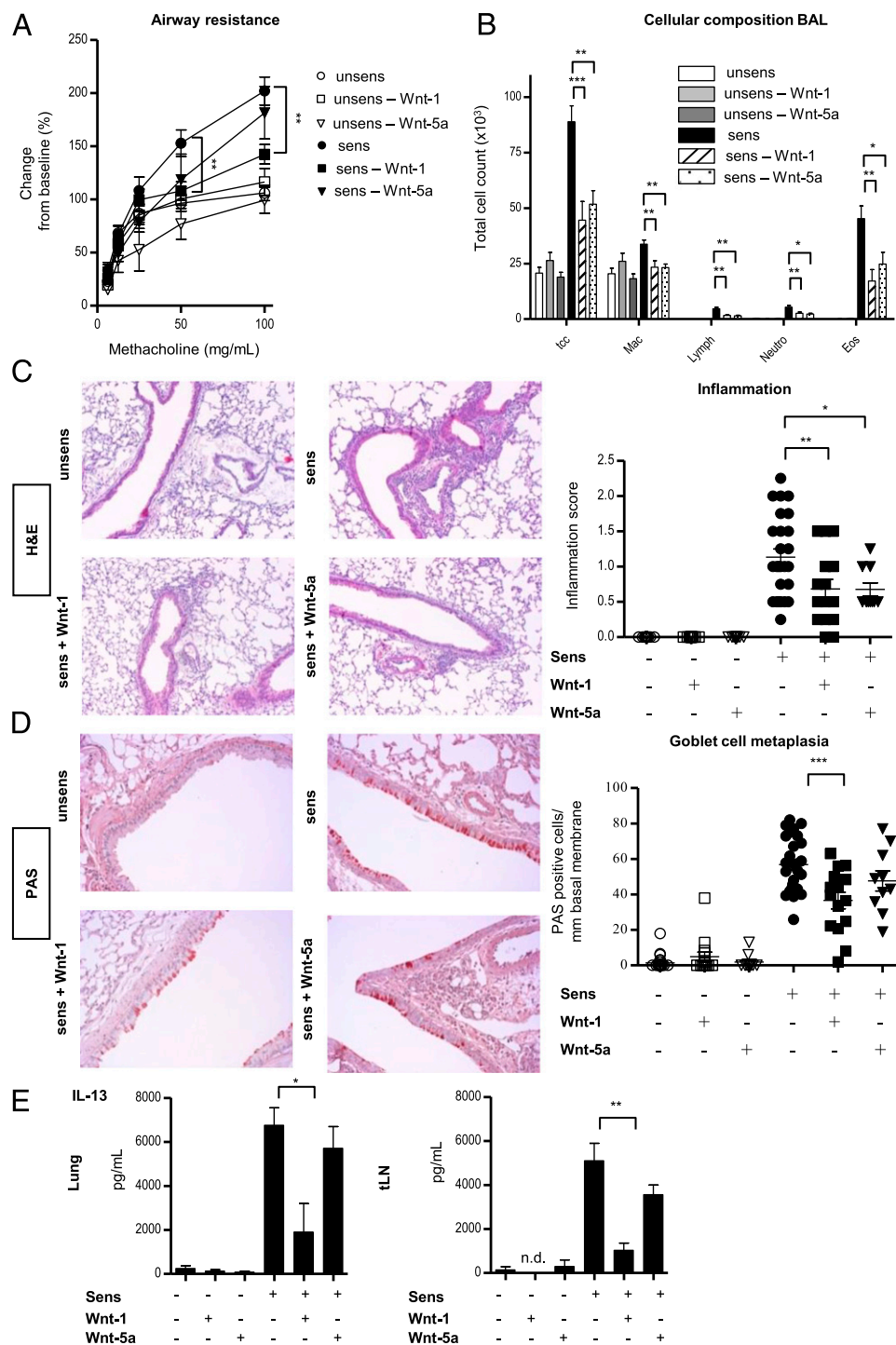
reciprocal serum dilution yielding an absorbance reading of OD = 0.2 after linear regression analysis.

In vitro assays

Bone marrow-derived DCs (BMDC) from BALB/c mice were generated as described previously (36). On day 7, immature BMDC were treated with OVA (5 μg/ml; Merck-Calbiochem), and cells were incubated overnight. After incubation, cells were supplemented with or without increasing doses of recombinant Wnt-1 (1, 10, and 100 ng/ml) (BioVision, Milpitas, CA) or Wnt-5a (1, 10, and 100 ng/ml). To compare the effects of activated versus immature BMDC, a subset of each group was stimulated with LPS (1 μg/ml) (Calbiochem) at 60 min after treatment with the Wnt ligands. After 24 h, DCs were stained for the expression of CD11c and MHCII and the costimulatory molecules PE-conjugated anti-mouse CD80 (BD) or anti-mouse CD86 (eBioscience) and APC-conjugated anti-mouse CD40 (BD). Expression patterns were measured via flow cytometry and analyzed via FlowJo (gating strategy: Supplemental Fig. 1C).

To analyze effects on T cell proliferation, OVA-loaded BMDC were generated, treated with Wnt ligands, and activated with LPS as described above. Twenty-four hours after treatment, DC were cocultured for 72 h with CD4-positive CFSE-labeled OVA transgenic T cells. Splenocytes received from spleens of DO11.10 animals were purified by using MACS Separator-LS Columns (Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-mouse CD4 (clone H129.19; Institute for Immunology, Mainz, Germany) to achieve a CD4-positive cell population. Cells were adjusted to 1×10^7 cells/ml, and CFSE (CFDA-SE; Invitrogen Life Technologies, Darmstadt, Germany) was added to a final concentration of 2.5 μM. Cells were incubated for 4 min at 37°C. Reaction was stopped by adding MEM plus 10% FCS. Following centrifugation, cells were washed twice with

FIGURE 3. Effect of Wnt ligands on the development of allergic airway disease in an OVA model. **(A)** Change of airway resistance in response to rising doses of MCh in untreated animals sensitized and challenged (sens) with OVA or with added 100 ng/ml Wnt-1 (sens-Wnt-1) and Wnt-5a (sens-Wnt-5a) and corresponding unsensitized and challenged (unsens; -Wnt-1; -Wnt-5a) controls. **(B)** Composition of BAL. **(C)** H&E-stained representative lung slides (original magnification $\times 100$) and inflammatory score assessed of untreated unsens and sens mice and sens mice treated with Wnt-1 and Wnt-5a. **(D)** PAS-stained representative lung slides (original magnification $\times 200$) and mucus-positive cells per millimeter of basal membrane of all analyzed groups. **(E)** Allergen-specific-induced concentrations of IL-13 in supernatants of ex vivo-stimulated single cell suspensions of lungs or draining lymph nodes (tLN) from unsens and sens animals and corresponding groups treated with Wnt-1 and Wnt-5a. (A), (B), and (E) show mean $\pm$ SE; (C) and (D) show single animals values, mean $\pm$ SE ($n = 10$ – 25 animals per group from three independent experiments). * $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.001$ versus positive control, ANOVA.



MEM plus 5% FCS and finally adjusted to 5×10^5 cells/ml in TM. Likewise, BMDC were adjusted to 1×10^5 cells/ml, and equal volumes of the cells were plated out together. Cocultures were estimated in TM with and without secondary application of Wnt-1 or Wnt-5a in the same doses used above. Cells were incubated for 72 h and transferred to 1.5-ml reaction tubes and centrifuged. Supernatants were collected in new tubes and stored at -80°C until concentrations of IFN- γ were measured via ELISA. Cells were resuspended in flow cytometry buffer and surface stained with PercP-Cy5.5-labeled anti-mouse CD3 (BD), PE-Cy7-conjugated anti-mouse CD4 (BD), PE-conjugated anti-mouse CD25 (BD), and APC-conjugated CD44 (BD). Flow cytometric measurements and analysis were performed as already described. Mean fluorescence intensity (MFI) of CFSE expression was assessed (gating strategy: Supplemental Fig. 1D). To calculate the inhibition of T cell proliferation, the MFI for the negative

control was determined. The ΔMFI for negative controls versus MFI for positive controls and samples was calculated.

$$\Delta\text{MFI sample or positive control} = \text{mean MFI negative control} - \text{MFI sample or positive control}$$

The mean ΔMFI of the positive control was determined. Finally, to calculate percentage of inhibition in comparison with positive control, the following formula was used:

$$\% \text{inhibition} = 100 - ([\Delta\text{sample} / \text{mean} \Delta\text{positive control}] \times 100)$$

Statistical analysis

ANOVA was used to determine differences between all groups. Before comparisons for all pairs, an Anderson-Darling test was assessed to control

gaussian approximation, and then an appropriate test (unpaired Student *t* test or Mann–Whitney *U* test) was used for analyses. The *p* values for significance were set at 0.05. Values for all measurements are expressed as mean $\pm$ SEM.

Results

Effect of canonical and noncanonical Wnt signaling on allergen-specific DC/T cell interaction in vitro

Neither exposure to Wnt-1 nor Wnt-5a affected the expression of costimulatory molecules CD40, CD80, and CD86 on LPS-activated DC (Supplemental Fig. 2).

In OVA-loaded unstimulated and LPS-stimulated preparations, Wnt ligands were present only during DC activation phase (DC only) or during the complete coculture (total) (Figs. 1B, 2A–E). Application of Wnt-5a before DC activation or during the whole culture was associated with decreased T cell proliferation. This effect was comparable to that seen with Wnt-1, but Wnt-1–induced suppression was more effective at reducing T cell proliferation and showed a clear dose dependency, which was not apparent with Wnt-5a (Fig. 2B).

Contact with activated DCs resulted in increased expression of CD25 (Fig. 2C) and CD44 (Fig. 2D) on T cells. Exposure to Wnt-1 or Wnt-5a only during DC priming did not significantly affect the expression of CD25 (Fig. 2C). When given during the whole culture period, treatment with Wnt-1, and especially Wnt-5a, resulted in significant suppression of CD25 expression (Fig. 2C). In contrast, both ligands prevented the upregulation of CD44 on T cells (Fig. 2D). Interestingly, Wnt-1 demonstrated a clear dose–response effect, whereas Wnt-5a was effective at doses of 1 and 10 ng/ml only. In comparison with T cells cultivated with activated DC, both Wnt-1 and Wnt-5a markedly suppressed the release of IFN- γ already when given at low doses and when supplemented only to DC (Fig. 2E).

In summary, both Wnt-1 ligand and Wnt-5a ligand suppressed allergen-specific DC/T cell interactions in vitro. Although high doses were necessary to suppress proliferation, low doses were capable of inhibiting cytokine secretion.

Administration of Wnt ligands during allergen challenge attenuates the development of allergic disease

Administration of Wnt-1 reduced airway hyperresponsiveness in relative (Fig. 3A, Supplemental Fig. 3) and absolute changes (data not shown) of airway resistance and compliance, eosinophils in BAL fluid (Fig. 3B), tissue inflammation (Fig. 3C), and goblet cell metaplasia (Fig. 3D). In contrast, Wnt-5a had no effect on airway hyperresponsiveness (Fig. 3A, Supplemental Fig. 3). Numbers of eosinophils in BAL fluid and inflammation in lung were decreased by Wnt-5a comparable to Wnt-1 (Fig. 3B, 3C). However, exposure to Wnt-5a did not significantly reduce the number of PAS-positive cells in the bronchial epithelium (Fig. 3D). In addition, Wnt treatment had an effect on the cytokine secretion of ex vivo Ag-stimulated lung and draining lymph node cells.

Cells isolated from sensitized and challenged animals treated with Wnt-1, but not Wnt-5a, showed reduced secretion of IL-13 after restimulation with allergens (Fig. 3E). IL-5, IL-10, and IFN- γ secretion was not, or only minorly, affected by treatment with the Wnt ligands (data not shown).

In summary, treatment with Wnt-1 and Wnt-5a moderated the development of allergic airway disease. Whereas Wnt-1 treatment had a positive effect on all analyzed asthma markers, Wnt-5a mainly reduced inflammation in the lung and BAL fluid. There were no obvious side effects associated with administration of Wnt ligands in unsensitized and challenged animals.

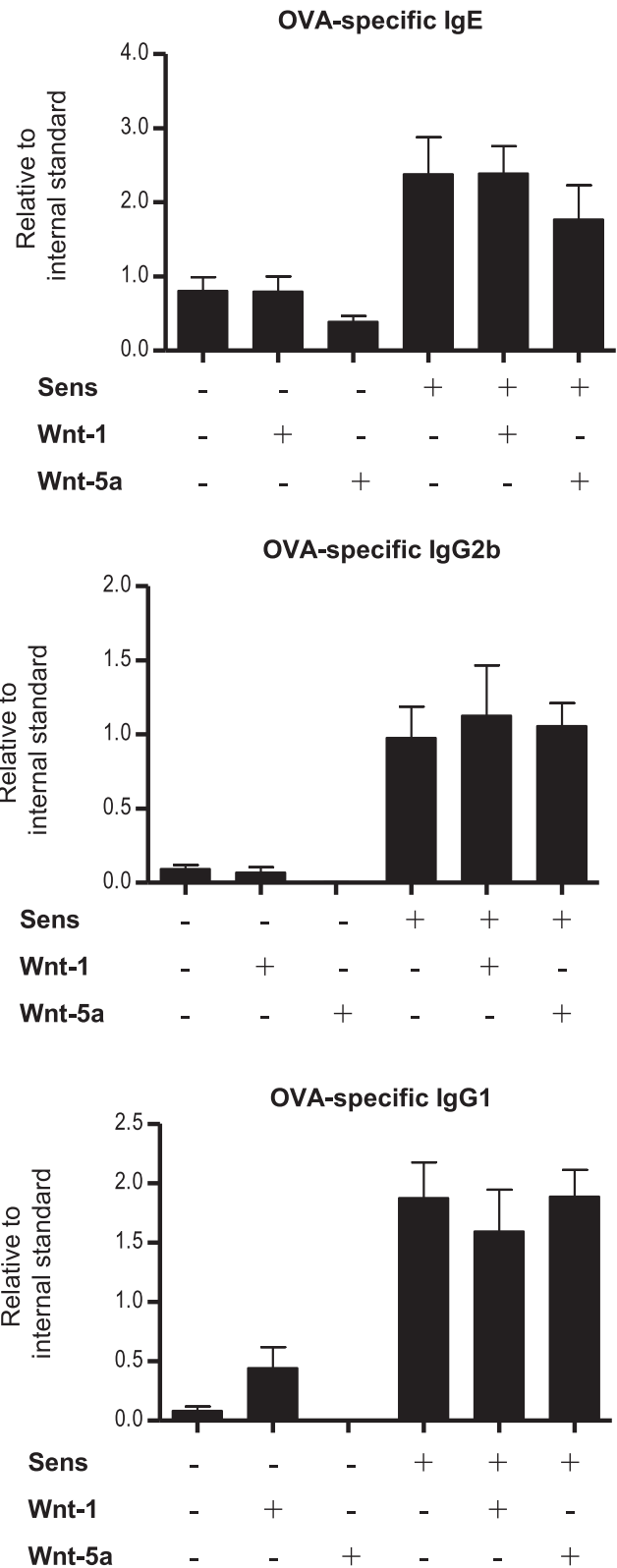


FIGURE 4. Effect on systemic Igs. OVA-specific Ig titers were measured using ELISA and compared with a laboratory internal standard. Graphs show mean $\pm$ SE ($n = 10$ – 25 animals per group from three independent experiments).

Local treatment with Wnt ligands did not affect systemic Ig secretion

Sensitization and challenge led to increased titers of all three measured OVA-specific Igs (IgE, IgG1, and IgG2b) (Fig. 4).

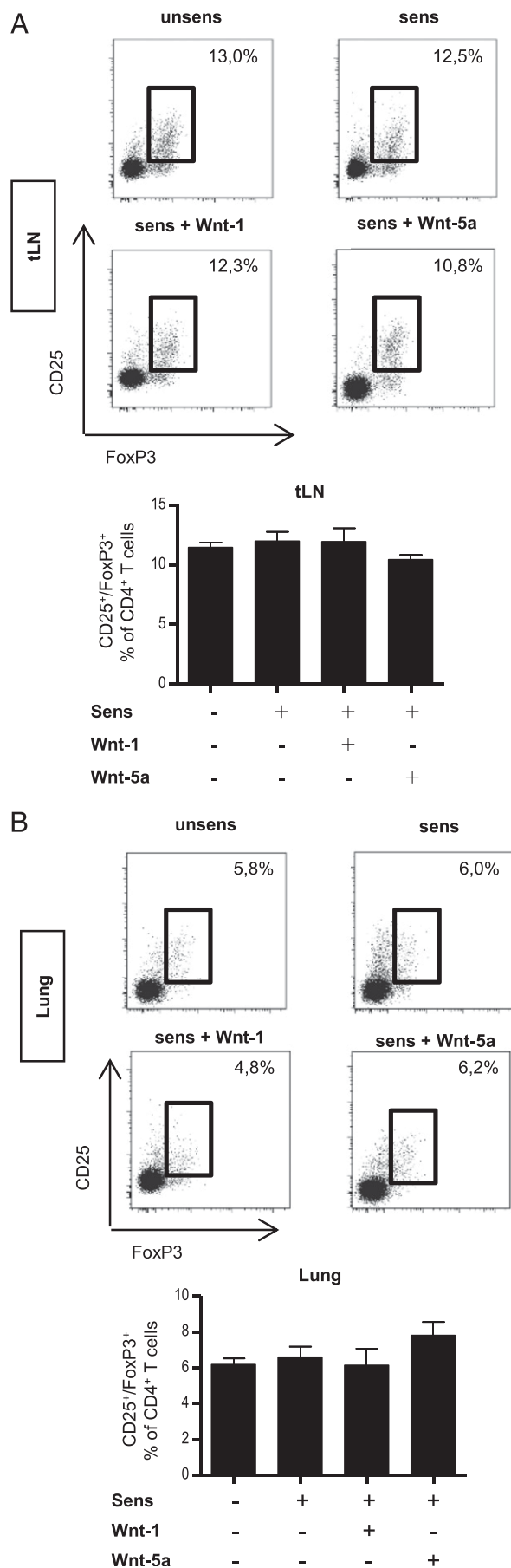


FIGURE 5. Effect of Wnt ligands on the number of Tregs. Representative example dot plots and graphs depict percentage of Foxp3⁺ and

Treatment of sensitized animals with Wnt ligands during challenge did not affect Ig titers.

Immunomodulatory effects of Wnt treatment appear to be independent of Tregs

In both lung and tLN preparations, the proportion of CD25⁺/Foxp3⁺ expressing Tregs among T effector cells was comparable across all investigated groups (Fig. 5).

Wnt-1 affects composition of DC subtypes in the lung

Although there were no significant differences in the proportions of CD11b⁺ and CD103⁺ conventional DC (cDC) subclasses in the draining lymph node (Fig. 6A), treatment with Wnt altered cDC subset composition in the lung (Fig. 6B). Compared with negative controls, sensitized and challenged animals demonstrated a nearly 2-fold increase in the ratio of CD11b⁺ to CD103⁺ cells. Compared with positive controls, animals treated with Wnt-1 showed a CD11b versus CD103 cDC proportion that was in favor of CD103⁺ cells. Animals treated with Wnt-5a showed a composition of CD11b⁺ and CD103⁺ similar to that of the positive control.

In summary, the regulatory properties of Wnt ligands do not appear to be mediated by Tregs. Wnt-1, but not Wnt-5a, affected the composition of cDC subtypes in the lung.

Wnt-1 demonstrates immunosuppressive properties in an HDM-specific model of allergic airway disease

In animals sensitized and challenged with HDM, Wnt ligands did not protect against the development of airway hyperresponsiveness and lung inflammation (Fig. 7A, 7C, Supplemental Fig. 4A). However, administration of Wnt-1 still reduced the number of inflammatory cells, especially eosinophils, in BAL (Fig. 7B) and the number of mucus-positive cells in the lung (Fig. 7D). In addition, allergen-specific secretion of IL-13 in the lung was reduced in sensitized and challenged animals treated with Wnt-1 (Fig. 7E). Wnt-1 had no effect on other analyzed lung cytokines (IL-5, IL-10, or IFN- γ) or cytokine secretion in draining lymph nodes (data not shown). In contrast to the OVA model, Wnt-5a treatment did not attenuate any investigated asthma markers in the HDM model (Fig. 7A–E, Supplemental Fig. 4A). Similar to the OVA model, Wnt treatment had no effect on Ig secretion (Supplemental Fig. 4B).

Discussion

In addition to their well-described function in embryogenesis (37, 38) and structural and repair processes (39), recent data suggest that Wnt signaling pathways have immune regulatory properties (8, 11). Indeed, transgenic lung-specific induction of the canonical Wnt-1 ligand and activation of the β -catenin-dependent pathway with LiCl has been shown to attenuate or prevent the development of allergic airway disease in a therapeutic model (26). The functions of the canonical Wnt pathway in allergic airway disease have been shown to be comparable to those of the GSK3 β inhibitor TDZD-8 (40), genetically reduced expression of the Wnt antagonist Dkk1 (24), and the knockdown of canonical Wnt-10b (23).

The present study analyzed if application of canonical Wnt-1 ligands is sufficient to attenuate the development of allergic

CD25⁺ cells within the CD4⁺/CD3⁺ cell population in (A) draining lymph nodes (tLN) and (B) lung of unsensitized and challenged (unsens) and sensitized and challenged (sens) animals as well as sens animals treated with Wnt-1 and Wnt-5a. Graphs depict mean $\pm$ SE. (n = 10–25 animals per group from three independent experiments).

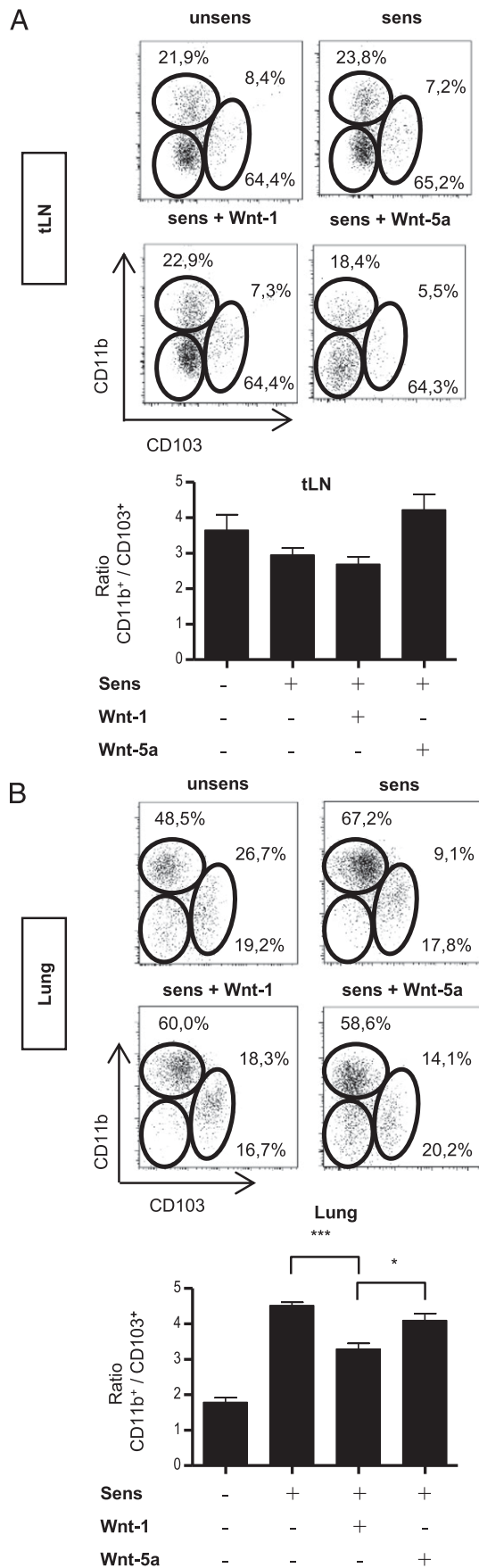


FIGURE 6. Composition of DC subtypes in the lung. Dot plots showing representative examples of CD11b- and CD103-expressing cells within the CD11c⁺/MHCII⁺/Ly6c⁻ cell population of unsensitized and challenged (unsens) and sensitized and challenged (sens) animals and sens animals

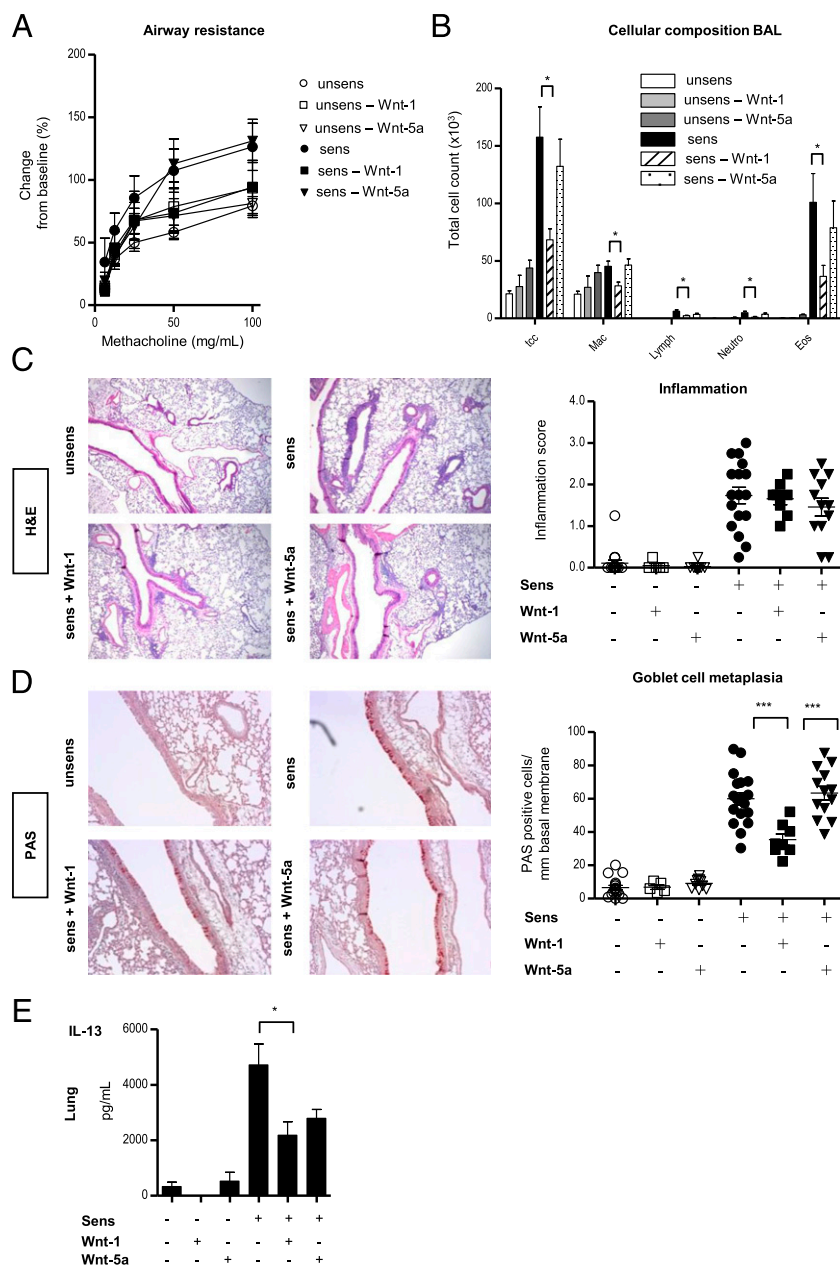
airway disease. Moreover, it compared the immunological effectiveness of the canonical Wnt activation pathway with that of the noncanonical pathway (with Wnt-5a as the ligand for the noncanonical pathway). The obtained data will help to further evaluate a possible therapeutic potential of manipulating Wnt signaling for allergic asthma. Still, further studies in chronic exposure models will be necessary to assess this approach mimicking therapeutic application. It has previously been shown that the canonical ligand Wnt-1 affects the interaction of DC and T cells in vitro (26). This process represents a key event in the interface of innate to adaptive immunity (41). DCs in the lung are essential for the presentation of inhaled Ags in the draining lymph nodes and the induction of corresponding T cell responses (42, 43). Indeed, dysregulated responses, breaking of tolerance and activation of T cells against harmless Ags, are associated with the development of many diseases, including allergic asthma (44, 45). Therefore, preventing DC activation and keeping DCs in a tolerogenic state could be a promising approach to prevent the development and progression of allergic diseases.

Interestingly, application of Wnt-1 during the DC activation phase failed to suppress the expression of classical activation markers, such as CD80, CD86, or CD40. However, Ag-specific activation of T cells was attenuated when T cells were cultivated with activated Wnt-treated DC. In these experimental setups, Wnt-1 dose-dependently suppressed the proliferation and expression of activation markers and cytokine secretion. Application of the noncanonical Wnt ligand Wnt-5a also suppressed DCs but to a lesser extent than Wnt-1. It should be noted that although high doses of the ligands were necessary to suppress proliferation, low doses of both ligands were capable of inhibiting cytokine secretion of allergen-specific activated T cells. These results are consistent with previous studies using a human MLR assay. In these assays, monocytes differentiated to tolerogenic DC in the presence of Wnt-5a, which failed to induce an MLR (46). Furthermore, a human and murine DC/T cell interaction model independent of a specific allergen showed that canonical Wnt-3a and noncanonical Wnt-5a ligands failed to modulate the expression of classical DC activation markers but were capable of suppressing the activation of T cells (46, 47). In these studies, a DC-specific increased expression of tolerogenic molecules like TGF- β and IL-10 were seen after treatment of DC with Wnt-3a and Wnt-5a, respectively (47). Based on these results, it was concluded that Wnt ligands act as immune regulators. Another mechanism by which Wnt-mediated T cell suppression appears seems to be induction of the immunoregulatory enzyme IDO-1 in DC treated with noncanonical Wnt-5a ligands, demonstrating the immunoregulatory capacity of Wnt ligands (48). In the current investigations, in vitro assays indicated that both canonical Wnt signaling mediated by Wnt-1 and noncanonical Wnt signaling induced by Wnt-5a modulated DC activation. This suppression resulted in reduced activation of allergen-specific T cells.

In vivo models of acute allergic airway disease were used to evaluate immunoregulatory properties and suppressive potential of this approach based on our in vitro observations. C57BL/6 mice sensitized and challenged with an allergen were treated with either recombinant Wnt-1 or Wnt-5a during allergen challenge. Application of Wnt-1 attenuated all hallmarks of allergic airway disease.

treated with Wnt-1 and Wnt-5a. Ratio of CD11b versus CD103 in the above groups in (A) draining lymph nodes (tLN) and (B) lung. Graphs show mean $\pm$ SE. ($n = 10$ –25 animals per group from three independent experiments). * $p \leq 0.05$, *** $p \leq 0.001$ versus positive control, ANOVA.

FIGURE 7. Effect of Wnt ligands on the development of allergic airway disease in a HDM model. **(A)** Change in airway resistance in response to increasing doses of MCh in animals untreated, sensitized, and challenged with HDM (sens) or treated with Wnt-1 (sens-Wnt-1) and Wnt-5a (sens-Wnt-5a) and corresponding unsensitized and challenged (unsens; -Wnt-1; -Wnt-5a) controls. **(B)** Composition of BAL. **(C)** H&E-stained representative lung slides (original magnification $\times 100$) and inflammatory score assessed of untreated unsens and sens mice and sens mice treated with Wnt-1 and Wnt-5a. **(D)** PAS-stained representative lung slides (original magnification $\times 200$) and mucus-positive cells per millimeter of basal membrane of all analyzed groups. **(E)** Allergen-specific-induced concentrations of IL-13 in supernatants of ex vivo-stimulated single cell suspensions of lungs from unsens and sens animals and corresponding groups treated with Wnt-1 and Wnt-5a. (A), (B), and (E) depict mean $\pm$ SE; (C) and (D) show single animals values, mean $\pm$ SE. ($n = 8$ –18 animals per group from three independent experiments). $*p \leq 0.05$, $***p \leq 0.001$ versus positive control, ANOVA.



Treatment with Wnt-1 improved airway function, reduced the migration of inflammatory cells into the bronchoalveolar space and lung tissue, and suppressed the development of mucus-producing goblet cells and release of IL-13. These results demonstrate that induction of canonical Wnt signaling can attenuate or prevent the development of disease-driving inflammatory responses in the lung. They confirm the anti-inflammatory capacity of this pathway seen in other organs and disorders, including the intestine/inflammatory bowel disease (49) and the brain/experimental autoimmune encephalomyelitis (50). Taken together, these data suggest that manipulation of the canonical Wnt signal could be a useful therapeutic option to treat diseases resulting from misguided adaptive immune responses like autoimmune or allergic diseases.

To further estimate the therapeutic effectiveness of Wnt signaling, a model of allergic airway disease using HDM extract was used. HDM allergens represent the most important indoor allergens for humans (51). In contrast to OVA, HDM proteins have a strong immunogenic potential and can induce robust allergic airway diseases when applied directly into the airways (52). Again, application

of Wnt-1 prior to allergen challenge resulted in reduced airway inflammation and goblet cell metaplasia. In addition, airway reactivity was attenuated, although this did not reach statistical significance. This less dramatic effect could be due to the direct delivery of the HDM allergen into the lung, triggering a stronger response compared with OVA exposure. In addition, HDM has been shown to activate Dickkopf-1 (DKK-1) in platelets, which acts as a Wnt antagonist and therefore might attenuate the effect of the Wnt treatment (24). Nevertheless, these studies further support the notion that the canonical Wnt pathway suppresses immune reactions in different models of allergic airway disease.

Although there are many immunological in vitro and in vivo studies performed with canonical Wnt ligands, there is a lack of data regarding the therapeutic importance of noncanonical Wnt signaling, and pro- as well as anti-inflammatory effects have been described (30). After demonstrating comparable immunosuppressive potential in DC/T cell interaction assays in vitro, we analyzed the immune regulatory impact of Wnt-5a in two in vivo models of allergic airway disease. Although administration of Wnt-5a resulted

in a slight reduction of airway inflammation in BAL and lung tissue, it had no detectable effect on lung function, goblet cell metaplasia, and cytokine secretion in the OVA model, and a lack of effectiveness was confirmed in the HDM-induced model of allergic airway disease. In contrast to Wnt-1, treatment with Wnt-5a did not affect any feature of the disease phenotype in this model.

To further characterize the immune regulatory mechanisms of Wnt signaling in vivo, T cell, B cell, and DC responses were analyzed. Canonical and noncanonical Wnt ligands have been reported to induce Tregs in vitro (47) and in vivo (48). However, another study showed that at least canonical Wnt signaling had a negative effect on Treg function (53). The positive association of membrane-bound DKK-1, a Wnt antagonist, and the suppressive function of Tregs confirmed this observation (54). Analyzing the proportion of CD25⁺/Foxp3⁺ Tregs among the CD4⁺ effector T cells, we did not detect any effect of either Wnt-1 or Wnt-5a in the lung or draining lymph node, consistent with our transgenic assay data. Therefore, we conclude that manipulation of Tregs has only a minor role, if any, in the suppressive mechanism induced by Wnt molecules. Serum levels of allergen-specific Igs were analyzed to determine the effect on B cells. None of the analyzed allergen-specific Igs (IgE, IgG1, and IgG2b) were affected by Wnt-1 or Wnt-5a treatment, meaning that a systemic effect on plasma cells can be excluded. These results are in line with those from Yu et al. (55) showing that mature B cells were unresponsiveness to canonical Wnt signaling.

Previous in vitro and in vivo studies (26, 46, 47, 49) suggested that the immune regulatory effects of Wnt were mediated by modulation of DC responses. In the current study, we analyzed the composition of DC in the draining lymph nodes and lung. Interestingly, Wnt-1-treated animals demonstrated changes in the composition of DC subsets in the lung. Whereas untreated and Wnt-5a-treated animals showed cDC subsets that were dominated by CD11b⁺ cDC, Wnt-1-treated animals had a cDC composition comparable to the negative control (i.e., a high proportion of CD103⁺ DC). Because CD11b⁺ cDC are thought to be responsible for allergen sensitization (43, 56) and CD103⁺ DC are thought to be responsible for tolerance (57, 58) induction, the modulation of their ratio could be one mechanism by which the in vivo immune suppressive activities of Wnt-1 are mediated. However, this conclusion has to be viewed with caution as the precise function of CD103⁺ DC is still under discussion. Indeed, immunogenic (59) as well as tolerogenic properties (57, 58) have been associated with CD103⁺ DC. Therefore, the precise impact of Wnt ligand-induced change in DC subclass ratio on allergic airway disease needs to be further evaluated.

Taken together, data from our in vitro and in vivo studies clearly demonstrate an immune suppressive capacity of the canonical Wnt ligand Wnt-1 in the context of allergic airway disease. Manipulation of the canonical Wnt signaling pathway could be an interesting approach to complement or replace current therapies for allergic asthma and other DC-driven diseases. Conversely, our data suggest that noncanonical signaling mediated by Wnt-5a is not a suitable treatment target for allergic airway diseases. However, its therapeutic effectiveness in treatment of other disorders needs to be evaluated.

In conclusion, Wnt signaling appears to be a potential therapeutic target in allergic airway disease. Context-dependent investigations are needed to determine the potential effectiveness and tolerability of this approach.

Acknowledgments

We thank Christina Belz and Anke Heinz (Department of Pulmonary Medicine, III. Medical Clinic, University Medical Center of the Johannes Gutenberg University Mainz) for excellent technical assistance. English language editing assistance was provided by Nicola Ryan, independent medical writer.

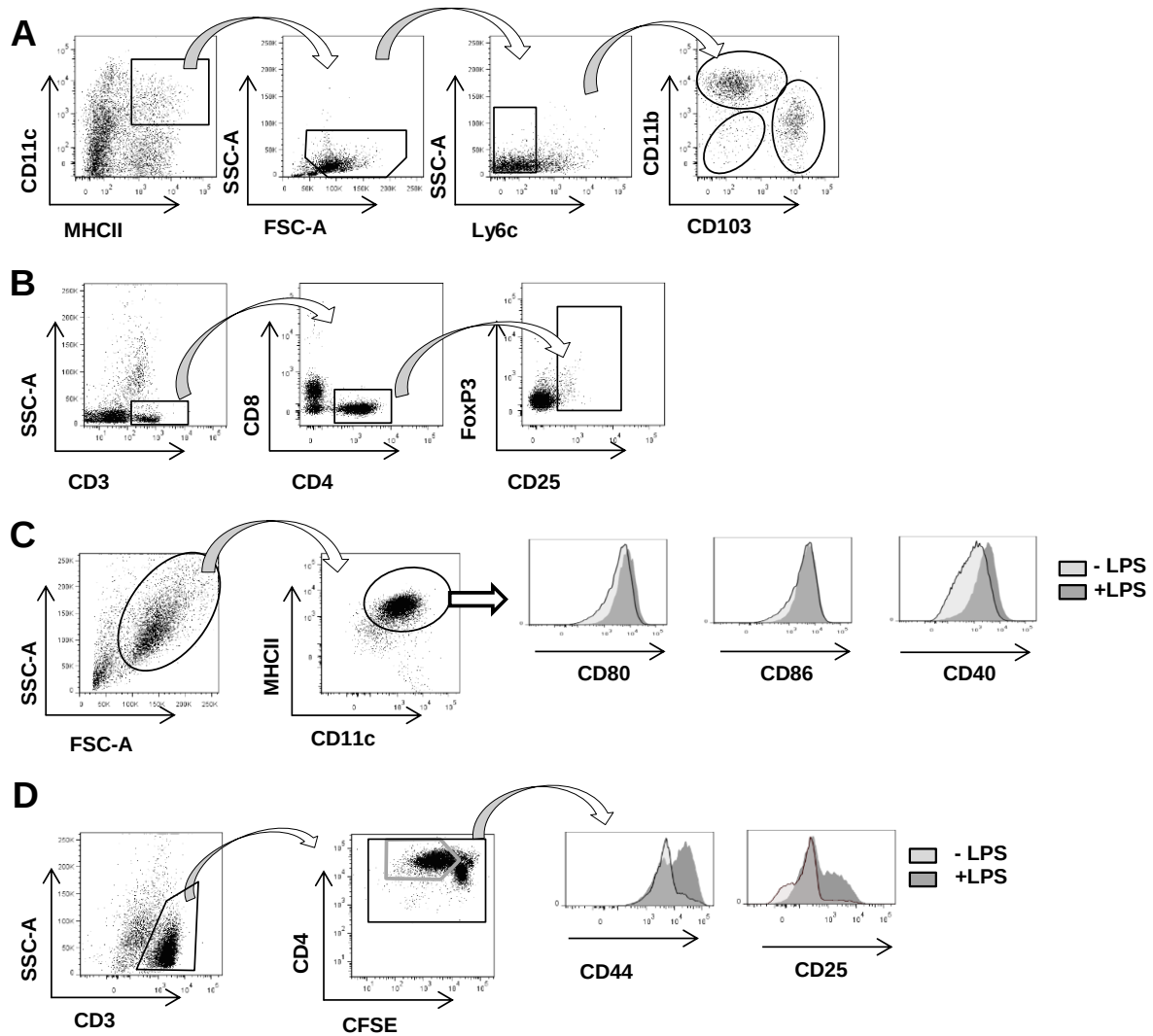
Disclosures

The authors have no financial conflicts of interest.

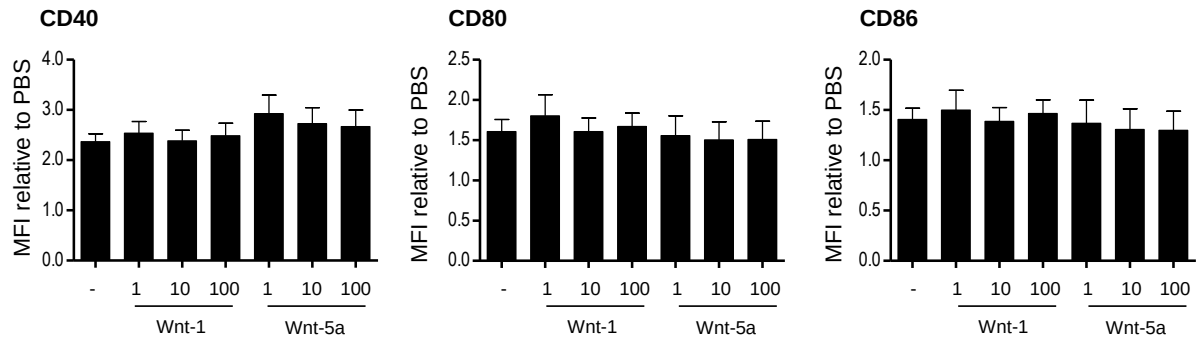
References

- Niehrs, C. 2012. The complex world of WNT receptor signalling. *Nat. Rev. Mol. Cell Biol.* 13: 767–779.
- Clevers, H., and R. Nusse. 2012. Wnt/ β -catenin signaling and disease. *Cell* 149: 1192–1205.
- MacDonald, B. T., K. Tamai, and X. He. 2009. Wnt/ β -catenin signaling: components, mechanisms, and diseases. *Dev. Cell* 17: 9–26.
- Clevers, H., K. M. Loh, and R. Nusse. 2014. Stem cell signaling. An integral program for tissue renewal and regeneration: Wnt signaling and stem cell control. *Science* 346: 1248012.
- Van Camp, J. K., S. Beckers, D. Zegers, and W. Van Hul. 2014. Wnt signaling and the control of human stem cell fate. *Stem Cell Rev.* 10: 207–229.
- Nance, J. 2014. Getting to know your neighbor: cell polarization in early embryos. *J. Cell Biol.* 206: 823–832.
- Nusse, R., and H. Clevers. 2017. Wnt/ β -Catenin signaling, disease, and emerging therapeutic modalities. *Cell* 169: 985–999.
- Staal, F. J. T., T. C. Luis, and M. M. Tiemessen. 2008. WNT signalling in the immune system: WNT is spreading its wings. *Nat. Rev. Immunol.* 8: 581–593.
- Skronska-Wasek, W., R. Gosens, M. Königshoff, and H. A. Baarsma. 2018. WNT receptor signalling in lung physiology and pathology. *Pharmacol. Ther.* 187: 150–166.
- Baarsma, H. A., and M. Königshoff. 2017. “WNT-er is coming”: WNT signalling in chronic lung diseases. *Thorax* 72: 746–759.
- Reuter, S., H. Beckert, and C. Taube. 2016. Take the Wnt out of the inflammatory sails: modulatory effects of Wnt in airway diseases. *Lab. Invest.* 96: 177–185.
- Wenzel, S. E. 2013. Complex phenotypes in asthma: current definitions. *Pulm. Pharmacol. Ther.* 26: 710–715.
- Bhakta, N. R., and P. G. Woodruff. 2011. Human asthma phenotypes: from the clinic, to cytokines, and back again. *Immunol. Rev.* 242: 220–232.
- Anderson, G. P. 2008. Endotyping asthma: new insights into key pathogenic mechanisms in a complex, heterogeneous disease. *Lancet* 372: 1107–1119.
- Kim, H. Y., R. H. DeKruyff, and D. T. Umetsu. 2010. The many paths to asthma: phenotype shaped by innate and adaptive immunity. *Nat. Immunol.* 11: 577–584.
- Locksley, R. M. 2010. Asthma and allergic inflammation. *Cell* 140: 777–783.
- Fahy, J. V. 2015. Type 2 inflammation in asthma—present in most, absent in many. *Nat. Rev. Immunol.* 15: 57–65.
- Martin, R. J., S. J. Szeffer, T. S. King, M. Kraft, H. A. Boushey, V. M. Chinchilli, T. J. Craig, E. A. Dimango, A. Deykin, J. V. Fahy, et al; National Heart, Lung, and Blood Institute’s Asthma Clinical Research Center. 2007. The predicting response to inhaled corticosteroid efficacy (PRICE) trial. *J. Allergy Clin. Immunol.* 119: 73–80.
- Szeffer, S. J., R. J. Martin, T. S. King, H. A. Boushey, R. M. Cherniack, V. M. Chinchilli, T. J. Craig, M. Dolovich, J. M. Drazen, J. K. Fagan, et al; Asthma Clinical Research Network of the National Heart Lung, and Blood Institute. 2002. Significant variability in response to inhaled corticosteroids for persistent asthma. *J. Allergy Clin. Immunol.* 109: 410–418.
- Berry, M., A. Morgan, D. E. Shaw, D. Parker, R. Green, C. Brightling, P. Bradding, A. J. Wardlaw, and I. D. Pavord. 2007. Pathological features and inhaled corticosteroid response of eosinophilic and non-eosinophilic asthma. *Thorax* 62: 1043–1049.
- Green, R. H., C. E. Brightling, G. Woltmann, D. Parker, A. J. Wardlaw, and I. D. Pavord. 2002. Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. *Thorax* 57: 875–879.
- Wenzel, S. E., L. B. Schwartz, E. L. Langmack, J. L. Halliday, J. B. Trudeau, R. L. Gibbs, and H. W. Chu. 1999. Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. *Am. J. Respir. Crit. Care Med.* 160: 1001–1008.
- Trischler, J., T. Shiomi, D. L. Turner, P. L. Sklepkiwicz, M. P. Goldklang, K. F. Tanaka, M. Xu, D. L. Farber, and J. M. D’Armiento. 2016. Immune modulation of the T cell response in asthma through Wnt10b. *Am. J. Respir. Cell Mol. Biol.* 54: 584–593.
- Chae, W. J., A. K. Ehrlich, P. Y. Chan, A. M. Teixeira, O. Henegariu, L. Hao, J. H. Shin, J. H. Park, W. H. Tang, S. T. Kim, et al. 2016. The Wnt antagonist Dickkopf-1 promotes pathological type 2 cell-mediated inflammation. *Immunity* 44: 246–258.
- Guo, Y., A. Mishra, E. Howland, C. Zhao, D. Shukla, T. Weng, and L. Liu. 2015. Platelet-derived Wnt antagonist Dickkopf-1 is implicated in ICAM-1/VCAM-1-mediated neutrophilic acute lung inflammation. *Blood* 126: 2220–2229.
- Reuter, S., H. Martin, H. Beckert, M. Bros, E. Montermann, C. Belz, A. Heinz, S. Ohngemach, U. Sahin, M. Stassen, et al. 2014. The Wnt/ β -catenin pathway attenuates experimental allergic airway disease. *J. Immunol.* 193: 485–495.
- Kwak, H. J., D. W. Park, J.-Y. Seo, J.-Y. Moon, T. H. Kim, J. W. Sohn, D. H. Shin, H. J. Yoon, S. S. Park, and S.-H. Kim. 2015. The Wnt/ β -catenin signaling pathway regulates the development of airway remodeling in patients with asthma. *Exp. Mol. Med.* 47: e198.
- Baarsma, H. A., W. Skronska-Wasek, K. Mutze, F. Ciolek, D. E. Wagner, G. John-Schuster, K. Heinzelmann, A. Günther, K. R. Bracke, M. Daguassat, et al. 2017. Noncanonical WNT-5A signaling impairs endogenous lung repair in COPD. [Published erratum appears in 2017 *J. Exp. Med.* 214: 565.] *J. Exp. Med.* 214: 143–163.

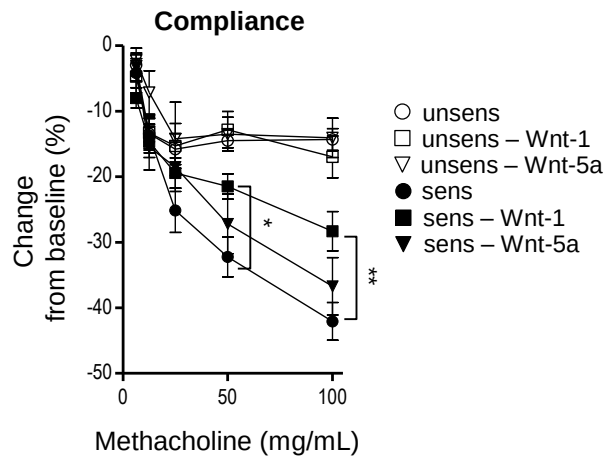
29. Kumawat, K., M. H. Menzen, I. S. T. Bos, H. A. Baarsma, P. Borger, M. Roth, M. Tamm, A. J. Halayko, M. Simoons, A. Prins, et al. 2013. Noncanonical WNT-5A signaling regulates TGF- β -induced extracellular matrix production by airway smooth muscle cells. *FASEB J.* 27: 1631–1643.
30. Kumawat, K., and R. Gosens. 2016. WNT-5A: signaling and functions in health and disease. *Cell. Mol. Life Sci.* 73: 567–587.
31. Martin, H., S. Reuter, N. Dehzad, A. Heinz, I. Bellinghausen, J. Saloga, I. Haasler, S. Korn, H. Jonuleit, R. Buhl, et al. 2012. CD4-mediated regulatory T-cell activation inhibits the development of disease in a humanized mouse model of allergic airway disease. *J. Allergy Clin. Immunol.* 129: 521–528, 528e1–e7.
32. Bopp, T., N. Dehzad, S. Reuter, M. Klein, N. Ullrich, M. Stassen, H. Schild, R. Buhl, E. Schmitt, and C. Taube. 2009. Inhibition of cAMP degradation improves regulatory T cell-mediated suppression. *J. Immunol.* 182: 4017–4024.
33. Dehzad, N., T. Bopp, S. Reuter, M. Klein, H. Martin, A. Ulges, M. Stassen, H. Schild, R. Buhl, E. Schmitt, and C. Taube. 2011. Regulatory T cells more effectively suppress Th1-induced airway inflammation compared with Th2. *J. Immunol.* 186: 2238–2244.
34. Spengel, J. M., E. Mizoguchi, J. P. Brewer, T. R. Martin, A. K. Bhan, and R. S. Geha. 1998. Epicutaneous sensitization with protein antigen induces localized allergic dermatitis and hyperresponsiveness to methacholine after single exposure to aerosolized antigen in mice. *J. Clin. Invest.* 101: 1614–1622.
35. Cruz, E. A., S. Reuter, H. Martin, N. Dehzad, M. F. Muzitano, S. S. Costa, B. Rossi-Bergmann, R. Buhl, M. Stassen, and C. Taube. 2012. *Kalanchoe pinnata* inhibits mast cell activation and prevents allergic airway disease. *Phyto-medicine* 19: 115–121.
36. Bros, M., N. Wiechmann, V. Besche, T. Castor, S. Sudowe, S. Grabbe, and A. B. Reske-Kunz. 2009. A novel plasmid DNA electroporation method allows transfection of murine DC. *J. Immunol. Methods* 343: 13–20.
37. Wang, J., and A. Wynshaw-Boris. 2004. The canonical Wnt pathway in early mammalian embryogenesis and stem cell maintenance/differentiation. *Curr. Opin. Genet. Dev.* 14: 533–539.
38. Tanaka, S. S., Y. Kojima, Y. L. Yamaguchi, R. Nishinakamura, and P. P. L. Tam. 2011. Impact of WNT signaling on tissue lineage differentiation in the early mouse embryo. *Dev. Growth Differ.* 53: 843–856.
39. Whyte, J. L., A. A. Smith, and J. A. Helms. 2012. Wnt signaling and injury repair. *Cold Spring Harb. Perspect. Biol.* 4: a008078.
40. Bao, Z., S. Lim, W. Liao, Y. Lin, C. Thiemeermann, B. P. Leung, and W. S. F. Wong. 2007. Glycogen synthase kinase-3 β inhibition attenuates asthma in mice. *Am. J. Respir. Crit. Care Med.* 176: 431–438.
41. Steinman, R. M. 2012. Decisions about dendritic cells: past, present, and future. *Annu. Rev. Immunol.* 30: 1–22.
42. Lambrecht, B. N. 2001. Allergen uptake and presentation by dendritic cells. *Curr. Opin. Allergy Clin. Immunol.* 1: 51–59.
43. Plantinga, M., M. Guilleams, M. Vanheerswyngheles, K. Deswarte, F. Branco-Madeira, W. Toussaint, L. Vanhoutte, K. Neyt, N. Killeen, B. Malissen, et al. 2013. Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity* 38: 322–335.
44. van Helden, M. J., and B. N. Lambrecht. 2013. Dendritic cells in asthma. *Curr. Opin. Immunol.* 25: 745–754.
45. Lambrecht, B. N., and H. Hammad. 2009. Biology of lung dendritic cells at the origin of asthma. *Immunity* 31: 412–424.
46. Valencia, J., C. Hernández-López, V. G. Martínez, L. Hidalgo, A. G. Zapata, Á. Vicente, A. Varas, and R. Sacedón. 2011. Wnt5a skews dendritic cell differentiation to an unconventional phenotype with tolerogenic features. *J. Immunol.* 187: 4129–4139.
47. Oderup, C., M. Lalevic, and E. C. Butcher. 2013. Canonical and noncanonical Wnt proteins program dendritic cell responses for tolerance. *J. Immunol.* 190: 6126–6134.
48. Holtzhausen, A., F. Zhao, K. S. Evans, M. Tsutsui, C. Orabona, D. S. Tyler, and B. A. Hanks. 2015. Melanoma-derived Wnt5a promotes local dendritic-cell expression of IDO and immunotolerance: opportunities for pharmacologic enhancement of immunotherapy. *Cancer Immunol. Res.* 3: 1082–1095.
49. Manicassamy, S., B. Reizis, R. Ravindran, H. Nakaya, R. M. Salazar-Gonzalez, Y. C. Wang, and B. Pulendran. 2010. Activation of beta-catenin in dendritic cells regulates immunity versus tolerance in the intestine. *Science* 329: 849–853.
50. Suryawanshi, A., I. Manoharan, Y. Hong, D. Swafford, T. Majumdar, M. M. Taketo, B. Manicassamy, P. A. Koni, M. Thangaraju, Z. Sun, et al. 2015. Canonical wnt signaling in dendritic cells regulates Th1/Th17 responses and suppresses autoimmune neuroinflammation. *J. Immunol.* 194: 3295–3304.
51. An, S., L. Chen, C. Long, X. Liu, X. Xu, X. Lu, M. Rong, Z. Liu, and R. Lai. 2013. *Dermatophagoides farinae* allergens diversity identification by proteomics. *Mol. Cell. Proteomics* 12: 1818–1828.
52. Buday, T., and J. Plevkova. 2014. House dust mite allergy models — reliability for research of airway defensive mechanisms. *Open J. Mol. Integr. Physiol.* 4: 27–35.
53. van Loosdregt, J., V. Fleskens, M. M. Tiemessen, M. Mokry, R. van Bortel, J. Meerdink, C. E. G. M. Pals, D. Kurek, M. R. M. Baert, E. M. Delemarre, et al. 2013. Canonical Wnt signaling negatively modulates regulatory T cell function. *Immunity* 39: 298–310.
54. Chae, W. J., J. H. Park, O. Henegariu, S. Yilmaz, L. Hao, and A. L. M. Bothwell. 2017. Membrane-bound Dickkopf-1 in Foxp3⁺ regulatory T cells suppresses T-cell-mediated autoimmune colitis. *Immunology* 152: 265–275.
55. Yu, Q., W. J. Quinn, III, T. Salay, J. E. Crowley, M. P. Cancro, and J. M. Sen. 2008. Role of beta-catenin in B cell development and function. *J. Immunol.* 181: 3777–3783.
56. Mesnil, C., C. M. Sabatel, T. Marichal, M. Toussaint, D. Cataldo, P.-V. Drion, P. Lekeux, F. Bureau, and C. J. Desmet. 2012. Resident CD11b(+)Ly6C(−) lung dendritic cells are responsible for allergic airway sensitization to house dust mite in mice. *PLoS One* 7: e53242.
57. Semmrich, M., M. Plantinga, M. Svensson-Frej, H. Uronen-Hansson, T. Gustafsson, A. M. Mowat, U. Yrlid, B. N. Lambrecht, and W. W. Agace. 2012. Directed antigen targeting in vivo identifies a role for CD103+ dendritic cells in both tolerogenic and immunogenic T-cell responses. *Mucosal Immunol.* 5: 150–160.
58. Khare, A., N. Krishnamoorthy, T. B. Oriss, M. Fei, P. Ray, and A. Ray. 2013. Cutting edge: inhaled antigen upregulates retinaldehyde dehydrogenase in lung CD103+ but not plasmacytoid dendritic cells to induce Foxp3 de novo in CD4+ T cells and promote airway tolerance. *J. Immunol.* 191: 25–29.
59. Nakano, H., M. E. Free, G. S. Whitehead, S. Maruoka, R. H. Wilson, K. Nakano, and D. N. Cook. 2012. Pulmonary CD103(+) dendritic cells prime Th2 responses to inhaled allergens. *Mucosal Immunol.* 5: 53–65.



Supplemental Figure 1. After exclusion of doublets by analysing cell properties investigating FSC-H vs FSC-A and exclusion of autofluorescent cells following gating strategies were used: Gating strategy for: **(A)** murine cDC subsets (DC in lung and draining lymph nodes were characterized upon their expression of CD11c and MHCII. Within this population FSC-A intermediate and high cells were regarded as cDC and inflammatory DC. By excluding Ly6c^{high} expressing inflammatory DC, cDC were detailed investigated. By analyzing CD103 and CD11b cDC subpopulations were specified); **(B)** Murine regulatory T cells (Tregs in lung and tLN were characterized as CD3⁺, CD4⁺ cells expressing CD25 and FoxP3); **(C)** murine DC activation (huge and granular cells were selected based on their properties analysing FSC-A vs. SSC-A. DC were characterized as CD11c⁺/MHCII⁺ cells. Within this population mean fluorescence intensity (MFI) of CD80, CD86 and CD40 was analysed); **(D)** Murine T cell proliferation (T cells were determined upon their expression of CD3. Within this population CD4⁺/CFSE⁺ cells were gated. MFI of CFSE, CD44 and CD25 was analysed).

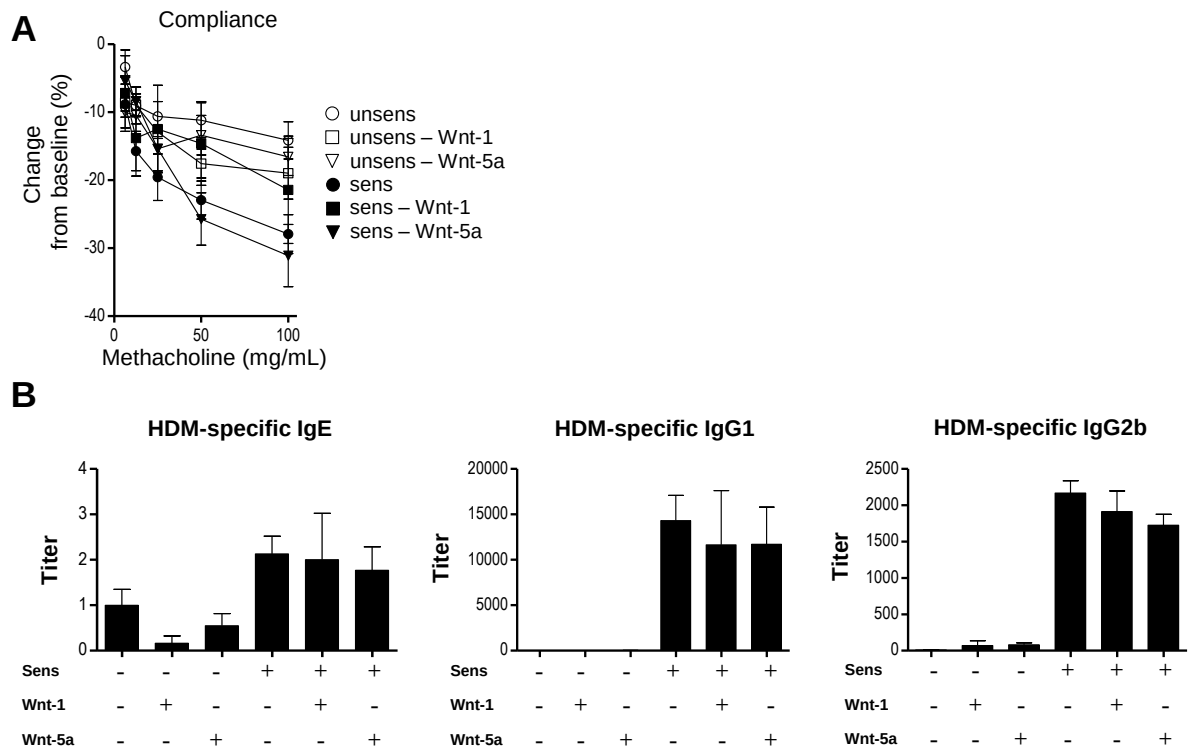


Supplemental Figure 2. Effects of Wnt ligands on expression of co-stimulatory molecules on activated BMDC. Graph bars depict mean relative increase fluorescence intensity compared with non-activated BMDC of activated DC treated with or without different doses of Wnt-1 and Wnt-5a (1, 10 or 100 ng/mL). Two experiments were performed with a duplicate approach each (n=2).



Supplemental Figure 3. Change from baseline in airway compliance in response to increasing doses of methacholine in untreated animals sensitized and challenged (sens) with OVA or treated additionally with Wnt-1 and Wnt-5a, and unsensitised and challenged (unsens) controls. Values are mean $\pm$ standard error. (n=10-25 animals/group from three independent experiments).

* $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.001$ versus positive control, ANOVA.



Supplemental Figure 4. Development of allergic airway disease in a house dust mite (HDM) model.

(A) Change of airway compliance in response to increasing doses of methacholine in untreated animals sensitized and challenged (sens) with HDM or treated additionally with Wnt-1 and Wnt-5a, and corresponding unsensitized and challenged (unsens) controls. (B) HDM-specific IgE, IgG2b and IgG1 titers measured in serum samples of untreated animals sensitized and challenged (sens) with HDM or treated additionally with Wnt-1 and Wnt-5a, and respective unsensitized and challenged (unsens) controls. Values are mean $\pm$ standard error. (n=8-18 animals/group from three independent experiments).

* $p \leq 0.05$, ** $p \leq 0.001$, *** $p \leq 0.001$ versus positive control, ANOVA.