ORIGINAL RESEARCH

# The Role of Secreted Frizzled-related Protein-1 in Allergic Asthma

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## Abstract

Although allergic asthma is a highly prevalent chronic inflammatory condition, the underlying pathogenesis driving T-helper cell type 2 inflammation is not well understood. Wnt/β-catenin signaling has been implicated, but the influence of individual members of the pathway is not clear. We hypothesized that SFRP-1 (secreted frizzled-related protein-1), a Wnt signaling modulator, plays an important role in the development of allergic inflammation in asthma. Using an *in vivo* house dust mite asthma model, *SFRP-1*$^{-/-}$ mice were sensitized, and their BAL fluid was collected to evaluate airway inflammation. *SFRP-1*$^{-/-}$ mice exhibited less inflammation with reduced cellular infiltration and concentration of IL-5 in bronchoalveolar lavage fluid compared with wild-type (WT) mice. Similar findings were observed in WT mice treated with SFRP-1 inhibitor, WAY316606. Alveolar macrophages from sensitized *SFRP-1*$^{-/-}$ mice demonstrated reduced alternative polarization compared with WT, indicating that macrophages could mediate the alteration in inflammation seen in these mice. These findings suggest that SFRP-1 is an important potentiator of asthmatic airway inflammation.

**Keywords:** Wnt pathway; SFRP-1; asthma; macrophage

Asthma is a prevalent chronic inflammatory disease of the airways that causes airway hyperresponsiveness, mucus secretion, and airway remodeling. Recurrent exacerbations contribute to a significant burden on healthcare systems and patients of all age groups (1, 2). The classification of asthma endotypes and phenotypes has evolved to include several distinct mechanistic pathways, and the allergic, or T2-high endotype, is the most common (3, 4). It is characterized by atopy, sensitization to aeroallergens, younger onset, and T-helper cell type 2 (Th2)-skewed cellular and cytokine responses (5). While the underlying cellular and molecular mechanisms promoting this Th2-driven process are not fully understood, the alveolar macrophage and its alternatively activated phenotype are implicated in the pathogenesis of asthma (6–10). A better understanding of the cell mediators and signals that promote the development of allergic asthma may provide insight into effective treatment options for patients.

The Wnt signaling pathway is an evolutionarily conserved cascade of cell signaling proteins vital for processes including embryogenesis, maintenance of homeostasis, and response to injury (11, 12). Aberrant Wnt signaling is involved in a number of chronic pulmonary diseases, including asthma (13–18). Our group demonstrated that SFRP-1 (secreted frizzled-related protein-1), an endogenous Wnt signaling modulator, is important for alveolar formation *in utero*, participates in the pathogenesis of emphysema, and regulates progenitor cell differentiation after injury (19, 20). However, the potential influence of SFRP-1 on Th2 inflammation, macrophage polarization, or the allergic asthma phenotype is not known.

To investigate the role of SFRP-1 in allergic asthma, the murine chronic house dust mite (HDM) exposure model of asthma in wild type (WT) and *SFRP-1*$^{-/-}$ mice was utilized, and airway inflammation, cytokine levels, and airway resistance were compared. In the present study, we demonstrate that inhibition or

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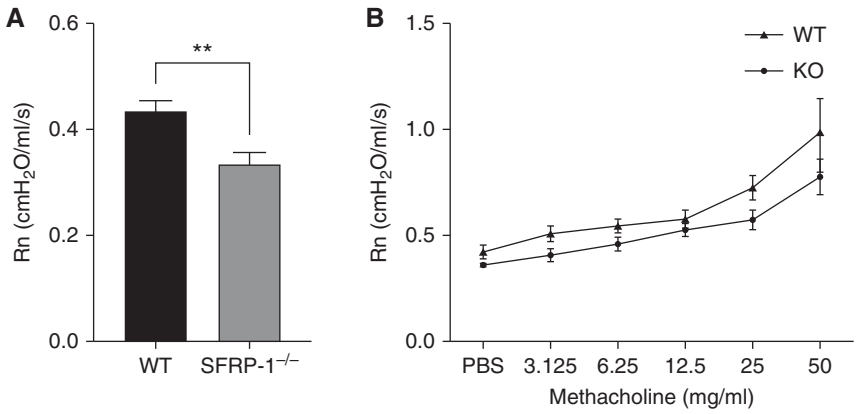


**Figure 1.** Ablation of SFRP-1 (secreted frizzled-related protein-1) reduces baseline airway resistance. (*A*) SFRP-1$^{-/-}$ mice have lower airway Newtonian resistance (Rn) at baseline compared with wild type (WT). (*B*) In response to methacholine challenge, SFRP-1$^{-/-}$ mice have similar airway resistance compared with WT. Each condition included 9–11 mice with 4 measurements at each point during the challenge. $^{**}P < 0.01$ with Student's *t* test. Data are reported as mean ± SEM. KO = knockout.

ablation of SFRP-1 led to reduced eosinophilic airway inflammation, reduced IL-4 and IL-5 levels in bronchoalveolar lavage fluid (BALF), and reduced alternatively activated alveolar macrophage phenotype after HDM sensitization. Some of the results have been previously reported in the form of an abstract (21–23).

## Methods

### Mice

Male and female *SFRP-1*$^{-/-}$ mice (35–45 weeks of age) and WT age-matched littermates were bred as previously reported (19). Female C57BL/6 mice (seven to nine weeks of age) were purchased from Jackson Laboratory and utilized for experiments involving a pharmacologic inhibitor of SFRP-1. To achieve 80% power to detect a 20% difference in the degree of airway hyperresponsiveness to the 0.01 ml inhaled dose of 50 mg/ml methacholine between control mice and experimental mice (α 0.05 and 1-β 0.2), at least 6 mice were included per group. To achieve 80% power to detect a 20% difference in cell counts between control and experimental mice (α 0.05 and 1-β 0.2), at least 6 mice were included per group. For cytokine measurements, to achieve 80% power to detect a 20% difference in IL-4 and IL-5 concentrations, at least 6 mice were required per group. Mice were maintained under pathogen-free conditions at Columbia University Medical Center. All protocols were approved by the Institutional Animal Care and Use Committee of Columbia University.

### House Dust Mite Sensitization

Mice were first sedated with isoflurane (induction with 5%, maintenance with 2–3%) and then given HDM extract (Dermatophagoides pteronyssinus, lot no. 297835, Greer Laboratories Inc) intranasal (i.n.) in PBS (40 μg/25 μl) or i.n. PBS (25 μl) 5 times per week for 3 weeks (24). Details are described in the data supplement.

### BAL and Cell Counting

Lungs were lavaged with 1 ml and then 1.5 ml of sterile PBS, and the cells were centrifuged and resuspended in PBS. Total cell concentrations were counted using an automated cell counter. Cytospin preparations were stained with Diff Stain kit (IMEB Inc), and differential cell counts were performed using morphological criteria.

### Immunocytochemistry for Alternatively Activated Macrophages and β-Catenin

Cytospin preparations from HDM- and PBS-sensitized mice were evaluated for alternative macrophage polarization. Mac3 (anti-Mac3 diluted 1:50, BD Biosciences cat# 550,292) was utilized as a general macrophage marker. Ym1 was utilized as a marker of alternative polarization, and Ym1 + macrophages were identified by a dual stain containing Mac3 and Ym1 (anti-mouse Ym1/Chitinase 3-like 3 diluted 1:50 [R&D Systems, cat# AF2446]). Slides were counterstained with DAPI and visualized by confocal microscopy. Double-positive cells were counted, and fluorescence intensity of Ym1 was measured on ImageJ software. To assess nuclear translocation of β-catenin, cytospin preparations from vehicle and WAY316606-treated mice were stained for β-catenin after permeabilization with 0.3% Triton X-100. Slides were counterstained with DAPI and visualized by confocal microscopy. Measurement and comparison of nuclear and cytosolic β-catenin signal in macrophages were performed using the "Cyt/Nuc" ImageJ macro (25).

### *In Vivo* Airway Resistance and Hyperresponsiveness (flexiVent)

After sedation with pentobarbital 75 mg/kg, the neck was dissected, and the trachea was cannulated using an 18-gauge beveled tracheal tube. The mouse was then

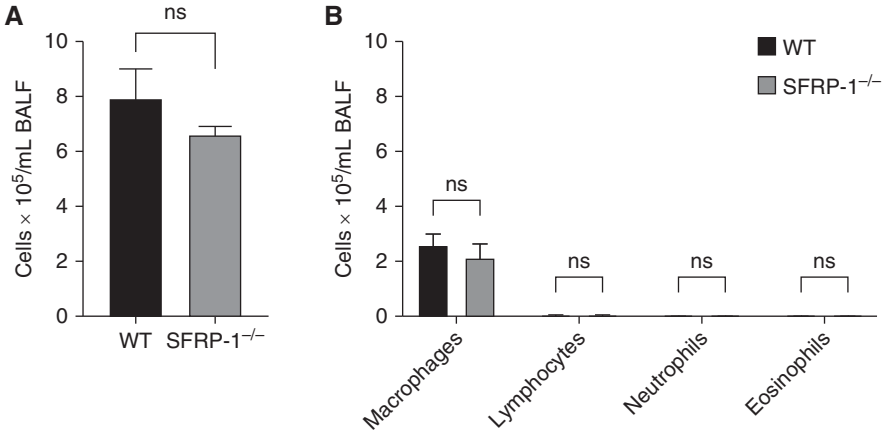


**Figure 2.** Loss of SFRP-1 does not alter the BAL fluid (BALF) cellular profile. Comparative analysis of BALF obtained from WT and SFRP-1$^{-/-}$ mice shows no difference in (*A*) total and (*B*) differential cell counts. Each condition included seven mice. Data are reported as mean ± SEM. ns = not significant.

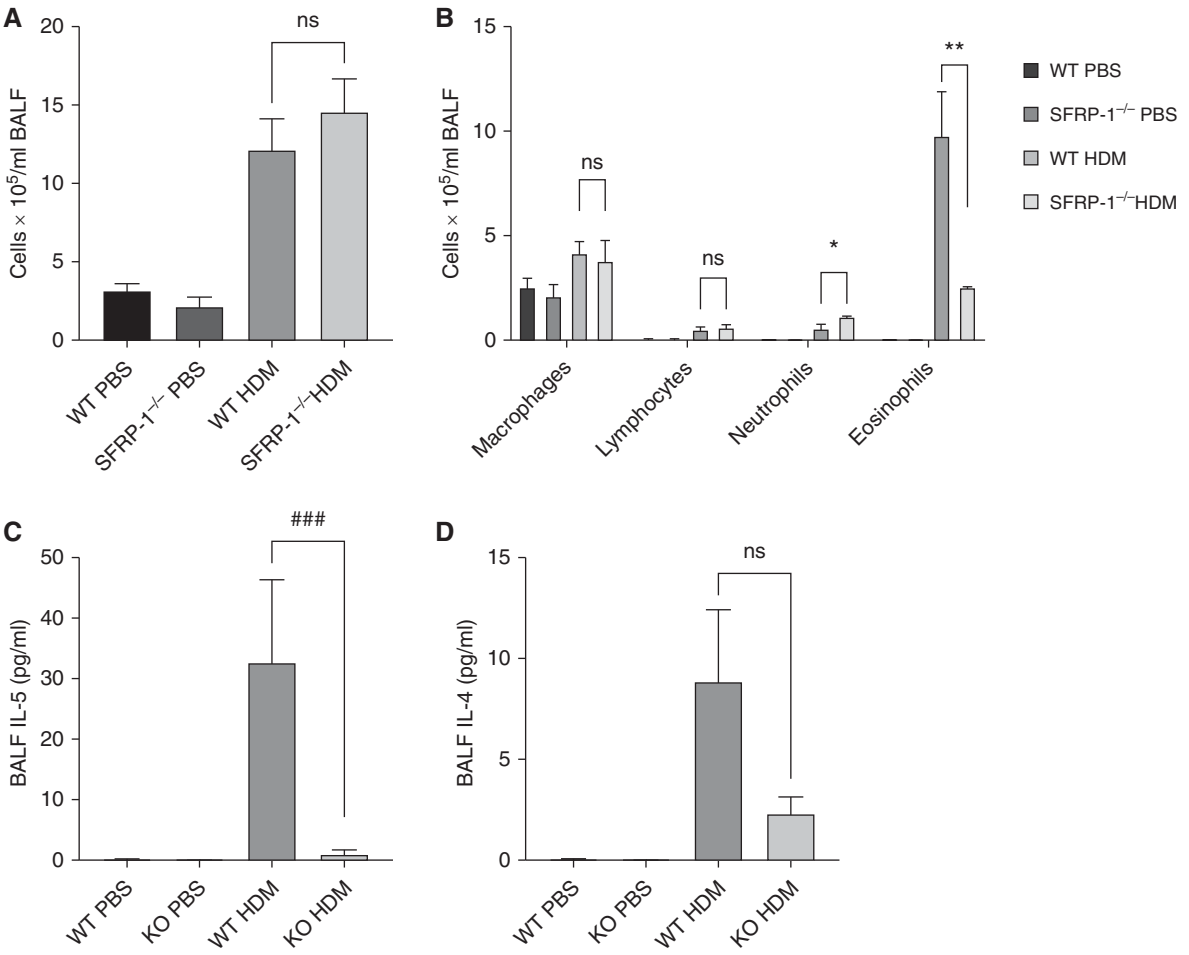


**Figure 3.** Ablation of *SFRP-1* attenuates allergic inflammation after house dust mite (HDM) sensitization. (*A*) BALF total cells. (*B*) BALF cell differential. $^{*}P < 0.05$ using Student's *t* test and $^{**}P < 0.01$ using Student's *t* test. (*C*) BALF IL-5. (*D*) BALF IL-4. Each condition included 6–10 mice, and cytokines were measured in duplicate for each. Normality was determined using the Shapiro-Wilk and Kolmogorov-Smirnov tests. $^{###}P < 0.0001$ using Mann-Whitney test to compare *SFRP-1*$^{-/-}$ with WT HDM. Data are reported as mean ± SEM.

attached to a mouse ventilator (flexiVent; SCIREQ) and ventilated with a tidal volume of 8 ml/kg and frequency of 150 breaths per minute. After three to five minutes of equilibration on the ventilator at 37 °C (Homeothermic Blanket System; Harvard Apparatus), mice were given succinylcholine 0.5 mg every 14 minutes by intraperitoneal injection. A graded methacholine challenge was initiated, and airway resistance was measured using the flexiVent system.

### BAL Cytokine Assay

Using Bio-Plex Pro Mouse Cytokine Th1/Th2 Assay (Bio-Rad Laboratories), the concentrations of cytokines IL-2, IL-4, IL-5, IL-10, IL-12 (p70), GM-CSF, IFN-γ, and TNF-α in the BALF were measured in accordance with the manufacturer's instructions. The lower limit of detection for the cytokines was 3.72, 6.98, 3.57, 2.95, 1.62, 5.1, 1.84, 5.8 pg/ml, respectively. Repeat studies were performed by Eve Technologies using the Mouse High Sensitivity T Cell 18-Plex Discovery Assay Array (MDHSTC18). In this study, Luminex xMAP technology was used for multiplexed quantification of 18 mouse cytokines, chemokines, and growth factors.

The multiplexing analysis was performed using the Luminex 200 system by Eve Technologies Corp. Eighteen markers were simultaneously measured in the samples using Eve Technologies' Mouse High Sensitivity 18-Plex Discovery Assay (MilliporeSigma) according to the manufacturer's protocol. The 18-plex consisted of GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p70), IL-13, IL-17A, KC/CXCL1, LIX, MCP-1, MIP-2, and TNF-α. Assay sensitivities of these markers range from 0.06–9.06 pg/mL for the 18-plex. Individual analyte sensitivity values are available in the MilliporeSigma MILLIPLEX MAP protocol. All samples were assayed in at least duplicate, and prepared standards were included in all of the runs.

### Histology and Immunohistochemical Staining

Slides of fixed mouse lung tissue (5 μm) were deparaffinized according to standard procedures, and hematoxylin and eosin staining were performed. For immunohistochemical staining, sections were labeled with monoclonal mouse anti-major basic protein (Invitrogen) followed by polyclonal rabbit anti-mouse HRP (Vector Laboratories). Antibody binding was detected using 3–3′ diaminobenzidine

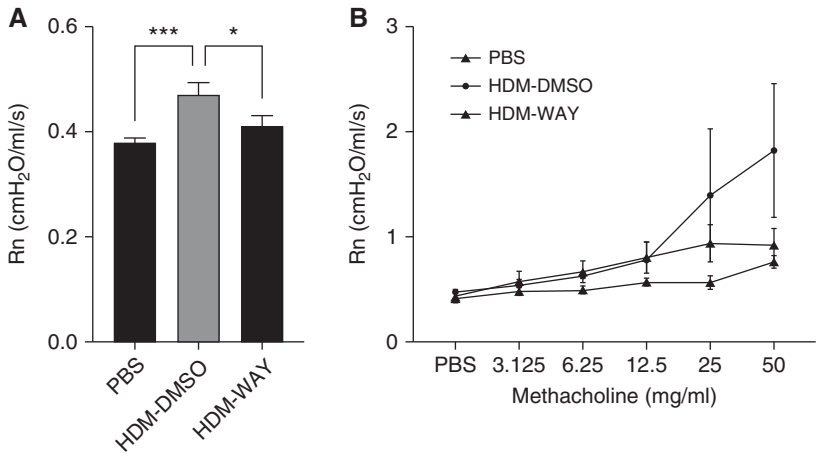


**Figure 4.** Inhibition of SFRP-1 in HDM-sensitized mice decreased airway resistance (Rn). (*A*) The HDM-WAY group had lower baseline Rn compared with HDM-DMSO. (*B*) WAY316066 administration did not significantly affect airway resistance in response to graded methacholine challenge. $*P < 0.05$ and $***P < 0.001$ using one-way ANOVA and Holm-Sidak's test. Each condition included 6–8 mice, with two measurements taken at each point in the challenge. WAY = WAY316066 administration.

(DAB)-hydrochloride (Vector Laboratories) as substrate-chromogen. Slides were counterstained with hematoxylin.

### Statistical Analysis

Statistical analyses were performed using Prism 8, Graphpad software. Power analysis was conducted on Stata/BE 17.0. Details are provided in the data supplement.

## Results

### Ablation of SFRP-1 Leads to Reduced Airway Resistance

To explore the influence of SFRP-1 on airway resistance, lung mechanics were studied in WT and *SFRP-1*$^{-/-}$ mice using the flexiVent apparatus. At baseline, prior to administration of nebulized bronchoconstrictors, *SFRP-1*$^{-/-}$ mice had a lower Newtonian resistance (Rn), which reflects airway resistance (Figure 1A). In response to a graded methacholine challenge, *SFRP-1*$^{-/-}$ mice had similar Rn compared with WT mice (Figure 1B). BALF was collected to analyze baseline cell populations and cytokines. Total cell counts and cell differentials were similar in the *SFRP-1*$^{-/-}$ and WT mice (Figures 2A and 2B).

### Ablation of *SFRP-1* Leads to Reduced Airway Inflammation after Chronic HDM Exposure

Chronic HDM sensitization to investigate the role of SFRP-1 in airway inflammation in allergic asthma was performed. Twenty-four hours after the last dose of HDM or PBS, WT and *SFRP-1*$^{-/-}$ mice were killed, and BALF was collected for cellular and cytokine analysis. Total cell counts did not differ between WT and *SFRP-1*$^{-/-}$ mice who received HDM (Figure 3A). Cell differential counts showed a decrease in eosinophils in the *SFRP-1*$^{-/-}$ mice treated with HDM compared with WT (Figure 3B). There were significantly fewer BALF IL-5 concentrations in the *SFRP-1*$^{-/-}$ mice treated with HDM compared with WT (Figure 3C). There was no significant difference in BALF concentrations of IL-4 (Figure 3D), IL-2, IL-10, IL-12 (p70), GM-CSF, IFN-γ, and TNF-α (Table E1).

### Inhibition of SFRP-1 Leads to Reduced Airway Resistance and Airway Inflammation in the Chronic HDM Model

After observing the effect of *SFRP-1* ablation on airway inflammation, we explored whether pharmacologic inhibition of SFRP-1 could attenuate allergic inflammation in a murine asthma model. Mice underwent chronic HDM sensitization along with intraperitoneal injections of WAY316606. Twenty-four hours after the last HDM dose, airway mechanics were measured using the Flexivent apparatus, and BALF was collected for cellular analysis and cytokine measurement. At baseline, mice that received WAY316606 had a significantly lower Rn compared with those receiving HDM alone (Figure 4A). With a graded methacholine challenge, there was no difference in airway resistance compared with the HDM group (Figure 4B). There was no difference in baseline compliance or elastance between groups. Total cell count from the HDM-WAY group BALF exhibited fewer total cells than the HDM group (Figure 5A). The absolute eosinophil, macrophage, and neutrophil counts were lower in the HDM-WAY group than HDM (Figure 5B). Cytokine analysis revealed a significantly lower IL-4 (Figure 5C) and lower IL-5 (Figure 5D) in the HDM-WAY group than the HDM group. On examination of lung tissue sections stained for major basic protein, a marker of eosinophilic granules, there was reduced peribronchiolar staining in the HDM-WAY group compared with HDM (Figure 6).

### Ablation of SFRP-1 Leads to Altered Polarization of Alveolar Macrophages after Chronic HDM Exposure

As alternatively activated macrophages are associated with allergic asthma and responsible for producing inflammatory signals, we assessed the presence of a marker of alternative polarization, Ym1, on alveolar macrophages from HDM-sensitized *SFRP-1*$^{-/-}$ and WT mice. Macrophages in cytospin preparations from BALF were identified using Mac3 staining, a general macrophage marker. The average fluorescence intensity of Ym1 in macrophages was significantly lower in the *SFRP-1*$^{-/-}$ compared with the WT group (Figure 7), suggesting that after HDM sensitization, there is less alternative polarization in alveolar macrophages of *SFRP-1*$^{-/-}$ mice compared with WT.

### Inhibition of SFRP-1 Leads to Increased Nuclear Translocation of β-Catenin

SFRP-1 is a recognized modulator of the Wnt signaling pathway but may have other downstream targets by which it acts. To assess the effect of SFRP-1 inhibition on β-catenin, the target of the canonical Wnt signaling pathway, we measured nuclear and cytosolic fluorescent signal of β-catenin in macrophages after permeabilization and counterstaining of the nucleus in cytospin preparations from vehicle and WAY316606-treated mice (Figure 8). The nuclear:cytosolic ratio was significantly increased in the WAY316606-treated mice compared with

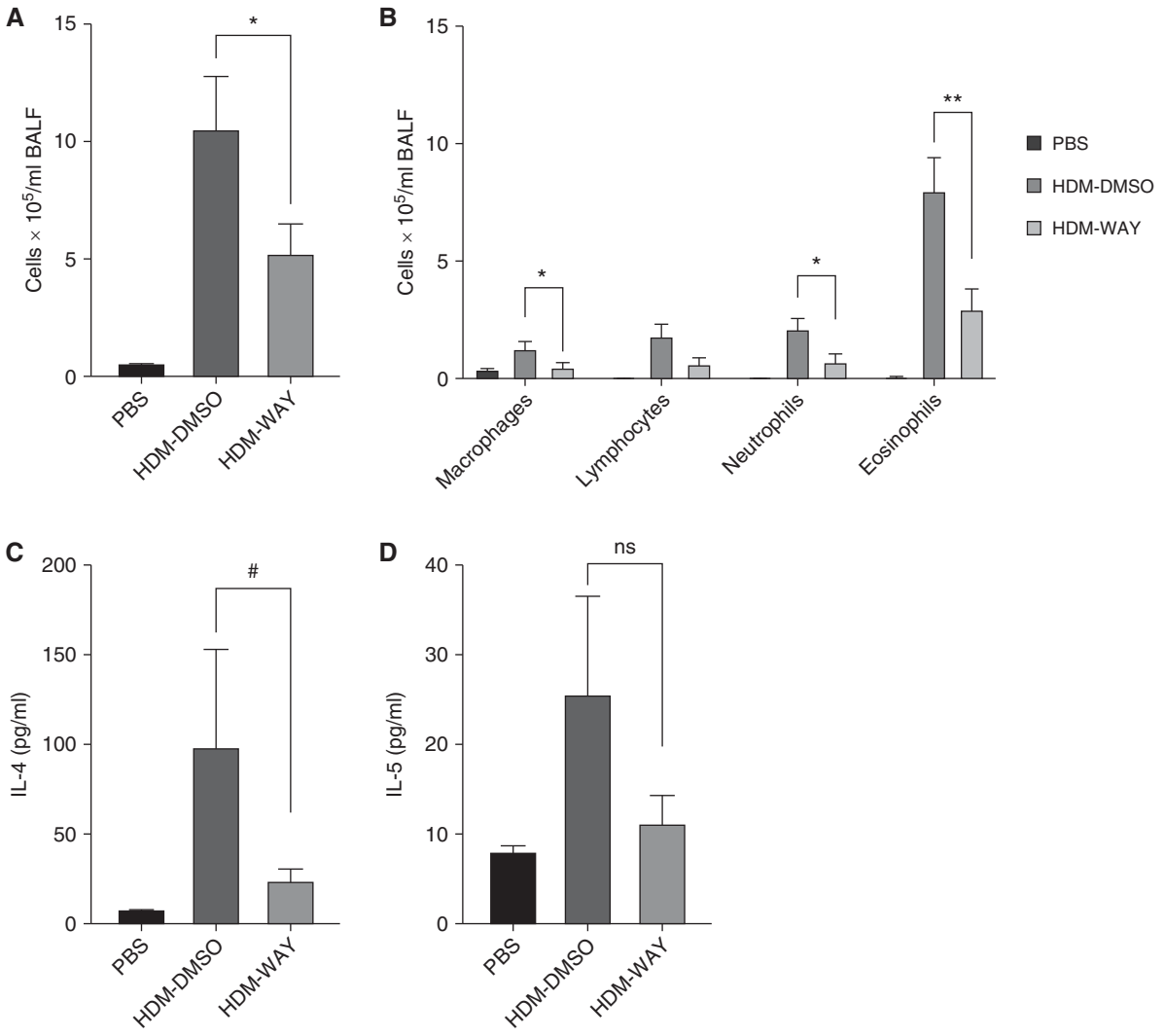


**Figure 5.** SFRP-1 inhibition reduces bronchoalveolar inflammatory infiltrate in mice sensitized with HDM. (*A*) Total cell count obtained from BALF 24 hours after last dose of HDM. (*B*) BALF cell differential from HDM-sensitized mice treated with WAY316606 has significantly less eosinophils, neutrophils, and macrophages. $^{*}P < 0.05$ using Student's *t* test and $^{**}P < 0.01$ using Student's *t* test. (*C*) HDM-WAY group had lower IL-4 and (*D*) IL-5 in BALF. Normality was determined using the Shapiro-Wilk and Kolmogorov-Smirnov tests. $^{\#}P < 0.05$ using Mann-Whitney test to compare *SFRP-1*$^{-/-}$ with WT HDM. Each condition included six to ten mice, and cytokines were measured in duplicate for each. Data are reported as mean ± SEM.

vehicle-treated, indicating that SFRP-1 alters canonical Wnt signaling.

## Discussion

In this study, we demonstrate that ablation or inhibition of SFRP-1, a modulator of Wnt protein binding, leads to reduced airway inflammation in the chronic HDM asthma model. Studies on alveolar macrophages from these animals demonstrate lower expression of Ym1, a marker of alternative polarization, and increased nuclear translocation of β-catenin, indicating that the attenuated inflammation may be secondary to altered macrophage phenotype and are occurring through changes in canonical Wnt signaling. Overall, these results indicate that SFRP-1 plays an important role in potentiating allergic inflammation in asthma.

Prior studies investigating the role of SFRPs in lung disease demonstrate that SFRP-1 contributes to the pathogenesis of emphysema by upregulating proteins that damage lung tissue after injury (19). SFRP-2 was recently reported to promote airway inflammation in the presence of cigarette smoke and levels correlated with forced expiratory volume in 1 second/forced vital capacity ($FEV_1$/FVC) in individuals with COPD (26). While our report is the first to investigate the role of SFRP-1 in asthma, our results are consistent with the former studies, indicating that SFRP-1 is a driver of airway inflammation in chronic pulmonary disease.

The link between Wnt signaling and the pathogenesis of asthma is not clear, but evidence from human and animal studies suggests a promising connection. Genome-wide association studies have identified polymorphisms in WNT proteins such as

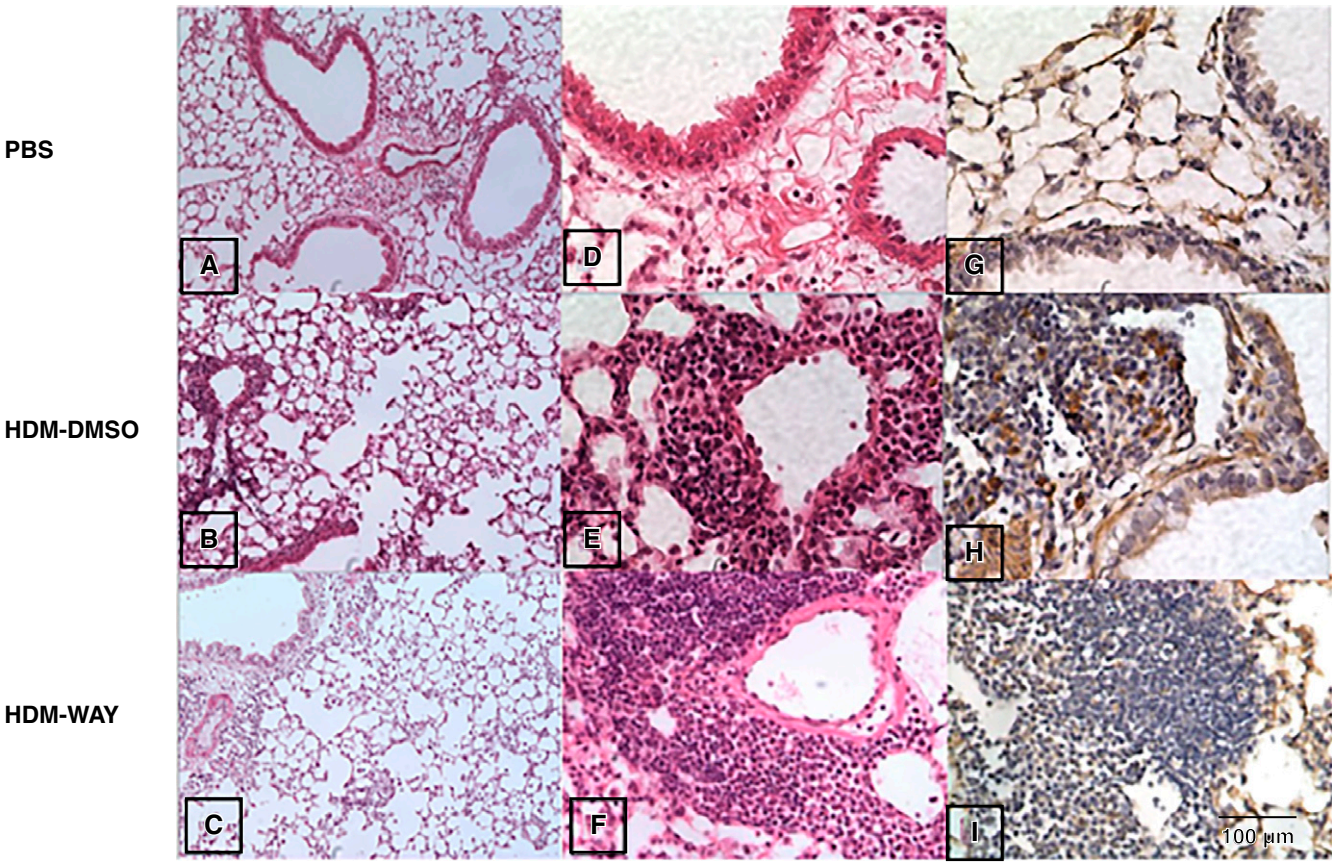


**Figure 6.** SFRP-1 inhibition reduced peribronchiolar eosinophilic infiltration in HDM-sensitized mice. Immunohistochemistry staining for eosinophils (brown color) in lung tissue sections shows reduced staining in HDM-WAY group compared with HDM-DMSO ($n = 4$ per condition). Figures (*A–C*) H&E staining. (*D–F*) H&E staining. (*G–I*) Major basic protein staining. Scale bar, 100 μm. H&E = hematoxylin and eosin.

WISP-1, WIF-1, Fzd3, and Fzd6 as predictors of asthma susceptibility (16, 17). Wnt3A, Wnt5A, Wnt6, Wnt10A, and Wnt10b are implicated in Th2 type of allergic inflammation in asthma (18, 27), and β-catenin is an important regulator of airway remodeling in asthma (28). Polymorphisms in SFRPs and their association with asthma have not yet been studied. Based on our findings in this study, it would be of interest to explore SFRP polymorphisms in asthma in the future.

In this study, the ablation of SFRP-1 in the murine model of chronic allergic asthma attenuated airway inflammation and airflow limitation. The Rn, which is a measure of resistance affected minimally by chest wall resistance and thus represents mostly airway resistance (29), was decreased in *SFRP-1*$^{-/-}$ mice. This difference was seen at baseline but not with methacholine challenge, which suggests that the role of SFRP-1 may be more related to structural differences rather than reversible smooth muscle contraction. This finding is likely explained by the developmental defect noted in *SFRP-1*$^{-/-}$ mice, characterized by congenital dilation of the alveolar ducts (19).

Regarding airway inflammation, a decrease in total inflammatory cell count, absolute eosinophil count, and IL-4 and IL-5 concentrations was noted in BALF of mice with absent or inhibited SFRP-1. This suggests that SFRP-1 has an important role in promoting Th2-type inflammation in asthma, which causes an eosinophil predominant cellular infiltration of the airways. Prior studies have shown that the downstream effector of Wnt signaling, T-cell factor, is important for early Th2 development, and inhibition of Wnt signaling with Dickkopf 1 reduces the production of signature Th2 cytokines, indicating that this pathway is a mediator of Th2 inflammation (30, 31). Similarly, animal studies demonstrated that overexpression of Wnt-1 during allergen challenge attenuated the development of allergic airway disease (32) and that this pattern is seen only during canonical, not noncanonical, Wnt signaling activation (15). However, the exact mechanism by which the Wnt pathway influences Th2 inflammation has not been elucidated, and our data suggest that SFRP-1 may be one of the critical Wnt signaling components involved in the process.

To investigate a possible cellular mediator that may be contributing to altered inflammation in the *SFRP-1*$^{-/-}$ mouse, we considered the role of macrophages in this process. Alveolar macrophages are among the first cells to respond to inhaled antigens and allergens and are a critical part of the innate immune system. They clear the air spaces, release numerous cytokines and chemokines, and recruit other cells in response to inflammatory stimuli (33). Once stimulated by inflammatory mediators, macrophages are broadly classified into two subgroups, M1, or classically activated, and M2, or alternatively activated macrophages, although it is known that they demonstrate

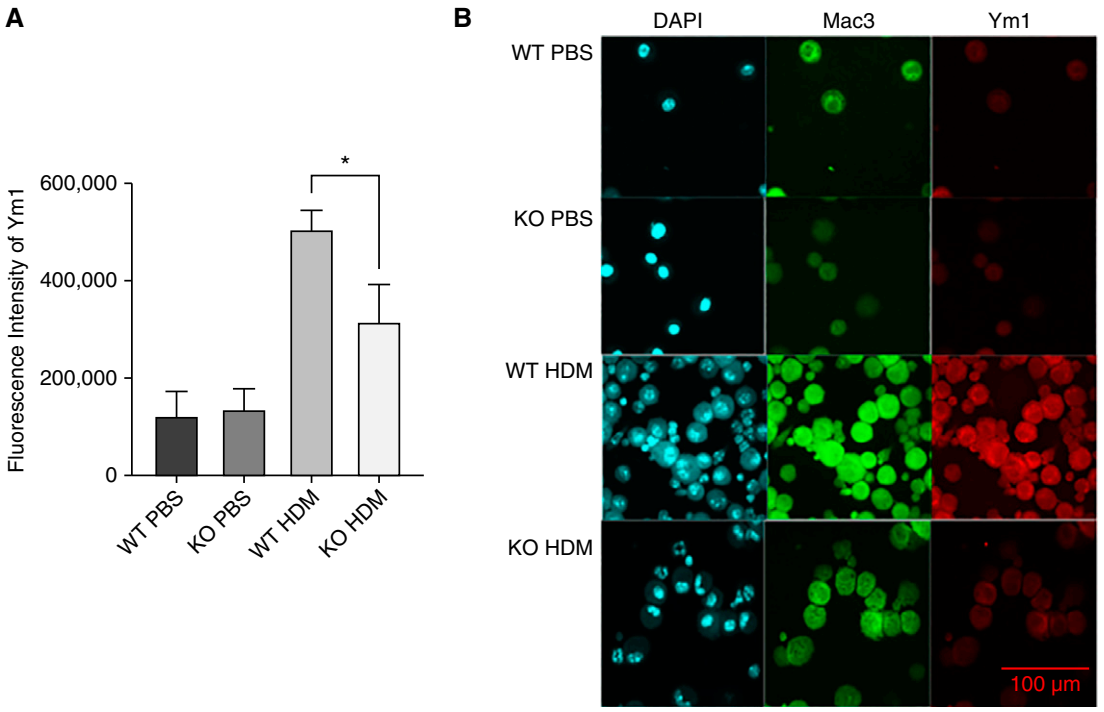


**Figure 7.** Ablation of SFRP-1 leads to reduced alternative polarization of alveolar macrophages after chronic HDM exposure. (*A*) Fluorescence intensity of Ym1 in Mac3 + cells in BALF is lower in *SFRP-1*$^{-/-}$ mice compared with WT ($n = 3$–5 per condition). *$P < 0.05$ using Student's *t* test to compare WT HDM and KO HDM. (*B*) Representative images of Ym1 + Mac3 + cells. Counterstained with DAPI. Scale bar, 100 μm.

plasticity and can depolarize or repolarize into the other subtype (34). In humans and mouse models of allergic asthma, there are greater numbers of M2 macrophages and their products in the BALF, and it is believed that these cells contribute to allergic airway inflammation (8–10, 35). Possible mechanisms by which macrophages are thought to contribute to asthma pathology include altered phagocytosis, clearance of apoptotic cells, and cytokine production (7, 36). Ford and colleagues demonstrated

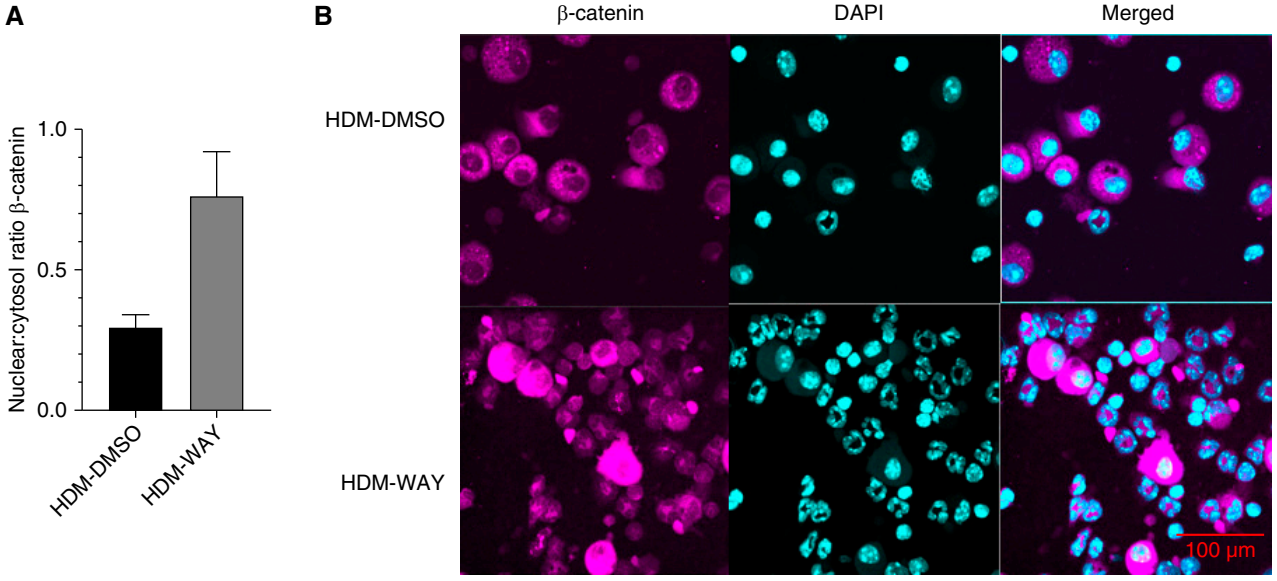


**Figure 8.** Inhibition of SFRP-1 leads to increased nuclear translocation of β-catenin in alveolar macrophages from mice sensitized with HDM. (*A*) Nuclear:cytosol ratio of β-catenin is higher in mice treated with the SFRP-1 inhibitor WAY316606. ($n = 2$–4 per condition). (*B*) Representative images of β-catenin staining in alveolar macrophages. Scale bar, 100 μm.

that adoptive transfer of alternatively activated macrophages enhances Th2-driven inflammation and eosinophil recruitment in the mouse lung (37). Based on these published studies, M2 macrophages were shown to be an important component of allergic airway inflammation, but the mediators controlling M2 macrophages are not defined. In this study, we demonstrate that alveolar macrophages from HDM-sensitized *SFRP-1*$^{-/-}$ have reduced M2 phenotype compared with WT. A role for SFRP-1 in differentiation is consistent with our previous studies that demonstrated SFRP-1 is essential for mediating differentiation of bronchioalveolar stem cells (20) and suggests that SFRP-1 has a broad role in the differentiation of multiple cell types.

Taken together, our results suggest that SFRP-1 potentiates eosinophilic airway inflammation in chronic allergic asthma. Further studies to define the role of individual Wnt proteins in eosinophil biology (38) can add therapeutic targets and increase our understanding of the heterogeneity seen in clinical asthma. Future studies aimed at exploring polymorphisms in *SFRP-1* in asthma would be informative in establishing the strength of the relationship between this signal and clinical severity. Additionally, experiments employing lung or cell-specific *SFRP-1* knock-out would help clarify the mechanisms by which SFRP-1 promotes allergic inflammation. ■

**Author disclosures** are available with the text of this article at www.atsjournals.org.

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