

ORIGINAL ARTICLE

Impact of a Met(11)Thr single nucleotide polymorphism of surfactant protein D on allergic airway inflammation in a murine asthma model

Carla Winkler,^{1,2,*} Olaf Bahlmann,^{3,*} Janika Viereck,⁴ Lars Knudsen,^{3,5} Dirk Wedekind,⁶ Heinz Gerd Hoymann,¹ Jens Madsen,⁷ Thomas Thum,^{4,8,9} Jens M. Hohlfeld,^{1,2,5} and Matthias Ochs^{3,5,8}

¹Fraunhofer Institute for Toxicology and Experimental Medicine, Hannover, Germany

²Department of Respiratory Medicine, Hannover Medical School, Hannover, Germany

³Institute of Functional and Applied Anatomy, Hannover Medical School, Hannover, Germany

⁴Institute of Molecular and Translational Therapeutic Strategies, IFB-Tx, Hannover Medical School, Hannover, Germany

⁵Biomedical Research in Endstage and Obstructive Lung Disease Hannover (BREATH), Member of the German Center for Lung Research (DZL), Hannover, Germany

⁶Institute for Laboratory Animal Science, Hannover Medical School, Hannover, Germany

⁷Department of Child Health, Sir Henry Wellcome Laboratories, Academic Unit for Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, Southampton, United Kingdom

⁸REBIRTH Cluster of Excellence, Hannover, Germany

⁹National Heart and Lung Institute, Imperial College London, London, United Kingdom

ABSTRACT

The surfactant-associated proteins SP-A and D are pattern recognition molecules with collectin structure. A single nucleotide polymorphism (SNP) exchanging a methionine (Met) for a threonine (Thr) in the amino-terminal SP-D domain influences the oligomeric structure and function of the protein. In this study, we investigated the susceptibility of mice transgenic for the human SP-D Met(11)Thr SNP to allergic airway inflammation and consequences for microRNA (miRNA, miR) expression. Mice expressing either human Met or Thr SP-D were sensitized and challenged with ovalbumin (OVA) in an acute model of allergic asthma. The influence of the SP-D polymorphism on the allergic airway inflammation was evaluated by lung function measurement, pulmonary inflammation parameters, morphological analysis and miRNA expression. Airway hyperresponsiveness, allergic inflammation, and mucus metaplasia were not significantly different between mice expressing one or the other allelic variant of SP-D. OVA sensitization and challenge led to significant airway hyperresponsiveness in wildtype mice and significantly lower eosinophil numbers and interleukin 5 levels in Thr SP-D mice. OVA challenge induced an up-regulation of miR-21 and 155 in Thr SP-D mice and a downregulation of miR-21 in Met SP-D mice. Our results show that murine expression of human polymorphic SP-D variants does not significantly influence the severity of allergic airway inflammation. MiR-21 and 155 are differentially regulated in transgenic mice in response to allergic inflammation. Further studies are required to elucidate the impact of this SNP on inflammatory conditions of the lung.

KEYWORDS allergic asthma, microRNAs, protein multimerization, single nucleotide polymorphism, surfactant protein D

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*Both authors contributed equally to the study.

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Dr. Bahlmann's current address: Institute for Cellular and Molecular Anatomy, Goethe University, Frankfurt, Germany.

Address correspondence to Matthias Ochs, MD, Institute of Functional and Applied Anatomy, Hannover Medical School, Carl-Neuberg-Str. 1, Hannover 30625, Germany. E-mail: ochs.matthias@mh-hannover.de

INTRODUCTION

The surfactant protein D is a hydrophilic protein, which belongs to the family of collagenous lectins, termed collectins. These proteins are composed of a carbohydrate recognition domain (CRD) connected via a neck region with a collagenous domain that ends in an amino-terminal lectin domain. SP-D units form cruciform dodecamers that in turn assemble to a "fuzzy ball"-like multimeric structure (SP-D) [1, 2].

Three single nucleotide polymorphisms (SNP) are described that result in an exchange of amino acids in the protein. A SNP in the SP-D gene switching ATG to ACG causes an exchange of a methionine (Met) for a threonine (Thr) at position 11 in the amino-terminal region of the protein [3]. This SP-D SNP influences assembly, function and concentration of SP-D [4–6]. In addition to trimers and dodecamers Met SP-D forms higher order multimers while Thr SP-D predominantly forms trimers. The oligomeric structure in turn influences the collectin function of the protein. The high molecular weight form of SP-D reveals a higher binding affinity to aggregate gram-positive and gram-negative bacteria, Influenza A virus and mannan [5, 6]. Via binding of their head domains to signal-regulatory protein (SIRP)-1 α SP-D multimers appear to silence pro-inflammatory mediator production while trimers bind with their tail domain to Calreticulin/CD91 and appear to induce mediator production [7, 8]. SP-D concentration decreases with the number of C alleles with highest levels in individuals with the T/T allele and lowest in individuals with the C/C allele; SP-D serum levels are 1035.2 ± 88.6 ng/mL for the Met and 744.1 ± 86.5 ng/mL for the homozygous Thr genotype (concentration average $\pm$ SEM) [5, 9]. The frequency of this SNP is reported for a healthy Danish population. 47.1% of individuals are heterozygous for the Met(11)Thr SNP, 35.4% carry the homozygous Met and 17.5% the Thr SP-D genotype [5].

Genetic association studies point to a higher susceptibility to allergic rhinitis [10], emphysema [11], chronic obstructive pulmonary disease [12] and pneumonia-associated multiple organ dysfunction syndrome and acute respiratory distress syndrome [9] in individuals with the Thr mono- or biallelic genotype. Interestingly, patients who receive lung allografts survive longer and are less susceptible to lung allograft dysfunction when they are homozygous for the Met SP-D variant [13]. In contrast to reports associating the Thr SP-D variant to lung disease a higher frequency of the Met SP-D variant has been found in infants hospitalized for treatment of severe respiratory syncytial virus bronchiolitis [14]. Finally, there is no association between asthma and SP-

D SNPs in a study on 322 asthmatic children [15] (for review see [16, 17]).

The murine SP-D gene on chromosome 14 shows 76% identity to the human homologue located on chromosome 10 [18, 19]. Mice deficient in SP-D develop an emphysema-like morphology of the lung; they show an accumulation of foamy macrophages in the alveolar lumen and hyperplasia and hypertrophy of alveolar epithelial type II (AE II) cells [20, 21]. Stereological exploration of a Met(11)Thr SNP mouse model revealed that mice expressing human SP-D variants exhibit a partially normalized phenotype compared to SP-D $^{-/-}$ mice [4].

The aim of the present study was to investigate the impact of this human Met(11)Thr SNP on the inflammatory phenotype and on expression of two microRNAs (miRs) involved in allergic inflammation in a murine model of OVA-induced allergic airway inflammation.

MATERIAL AND METHODS

Animals

C57BL/6.Cg-*Sftpd*^{tm1Haw} SP-D deficient knock-out mice, C57BL/6.Cg-*Sftpd*^{tm1HawZDW}Tg(*Rosa26-SFTPD*^{rs721917-T})Haw mice expressing the human Met and C57BL/6.Cg-*Sftpd*^{tm1HawZDW}Tg(*Rosa26-SFTPD*^{rs721917-C})Haw mice expressing the human Thr variant of SP-D (referred to as SP-D $^{-/-}$ and Met resp. Thr SP-D mice) were a kind gift of Jens Madsen, University of Southampton, United Kingdom. The generation of the mouse model is described in a previous paper [4]. C57BL/6 wildtype (wt) mice were purchased from Charles River, Germany. Mice were supplied with food and water ad libitum. Met mice (12 male/20 female) were 12 to 13 weeks, Thr mice (13 male/19 female) 12 to 17 weeks, SP-D $^{-/-}$ (14 male/15 female) 4 to 46 weeks, and wt mice (12 male/20 female) 7 to 9 weeks old.

Induction of an Allergic Airway Inflammation

Wt, SP-D $^{-/-}$, Met SP-D, and Thr SP-D mice were sensitized according to the standard acute OVA model as described before [22]. Briefly, 10 μ g OVA (Sigma-Aldrich, Steinheim, Germany) adsorbed to 1.5 mg aluminum hydroxide (alum) in 200 μ L phosphate-buffered saline per mouse were injected intraperitoneally on days 0, 14, and 21. Control animals were sham-sensitized with 1.5 mg alum adsorbed to saline, accordingly. All mice including controls were challenged with 1% OVA aerosol for 20 minutes on two consecutive days (day 27, 28). All

animal experiments were conducted following approval of the appropriate authority (Bezirksregierung Hannover).

For details about the measurement of pulmonary function see supplemental material and references [22, 23].

Analysis of Pulmonary Inflammation

Mice were euthanized directly after lung function measurement 24 hours after the last OVA aerosol challenge and lungs were lavaged two times with 0.8 mL of 0.9% NaCl containing protease inhibitor (Complete Protease Inhibitor Cocktail Tablets, Roche Diagnostics GmbH, Mannheim, Germany).

Cytospin preparations from bronchoalveolar lavage (BAL) cells were stained according to the Pappenheim standard protocol. Analysis of cytokines in the cell-free supernatant was performed using a Th1/Th2 10-plex from MSD (Meso Scale Discovery, Rockville, USA). Human SP-D in BAL and serum was determined by ELISA measurements (Biovendor, Heidelberg, Germany).

Stereological Analysis

Stereological analysis was performed according to the recommendations published by Hsia *et al.* [24]. First, we applied systematic uniform random sampling (SURS) to sample large to medium sized airways within alcian blue periodic acid Schiff reagent (AB-PAS) stained Paraplast-embedded tissue sections. We estimated epithelial mucus content using the stereology toolbox software newCast (Visiopharm, Version 3.6.5.0, Hørsholm, Denmark). Slides were examined using the 40 $\times$ objective. All stereological measurements were performed by the same, blinded investigator. For further details regarding fixation and removal of the lungs and the stereological investigation see supplemental material.

RNA Isolation and microRNA Reverse Transcription Polymerase Chain Reaction

RNA was isolated from lung parenchyma of wt, SP-D^{-/-}, Met and Thr mice using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Mature miRNAs were detected by reverse transcription polymerase chain reaction in a C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, USA) applying TaqMan MicroRNA Assays specific for miR-21 and miR-155 (Applied Biosystems, Foster City, USA). Each sample was run in du-

plicates. The small nuclear RNA (snoRNA202) was amplified as an internal control.

Statistical Analysis

Statistical analysis of functional, morphological, and miRNA data were performed using ANOVA and Newman-Keuls test for posthoc multiple comparisons (GraphPad Prism software, La Jolla, USA). Functional data were log transformed before analysis. Variables were expressed as mean + SD. A probability value of $P < .05$ was accepted as being significant.

RESULTS

Impact of the Human Met(11)Thr SP-D Polymorphism on Allergic Airway Inflammation

Sensitization and challenge of wt animals with OVA led to increased airway resistance in response to methacholine challenge compared to sham-sensitized animals ($P < .05$) (Figure 1A). However, no significant changes were observed comparing the airway hyperreactivity between animals expressing either the human Met SP-D or Thr SP-D polymorphism. Compared to wt control mice OVA-sensitized mice carrying the human Thr SP-D polymorphism revealed significantly lower total cell numbers after induction of the acute allergic airway inflammation ($P < .05$), these mice also possessed a lower percentage of eosinophils ($P < .05$) (Figure 1B) and absolute eosinophil numbers ($P < .05$, data not shown). Comparing the mice harboring the Thr polymorphism to the mice carrying the Met SP-D variant differences regarding the eosinophilic inflammation were not observed.

The alleviated eosinophilic inflammation was also reflected by lower interleukin (IL)-5 levels in the BAL in OVA-sensitized transgenic mice, with lowest levels in Thr SP-D mice compared to wt mice ($p < 0.001$) (Figure 2A). IL-4 and further cytokines reflecting a Th2-biased inflammation like IL-10, IL-2 were not found to be significantly different between OVA-sensitized groups. Human SP-D levels in BAL and serum were evaluated in Met SP-D and Thr SP-D mice, but no differences were observed neither with regard to the inflammation-driven regulation of pulmonary SP-D nor generally between both mice strains (Figure 2B). Interestingly, serum levels in these humanized mouse strains were found to be 300 times higher compared to levels in the pulmonary compartment. Analysis of additional parameters characteristic for the allergic inflammation

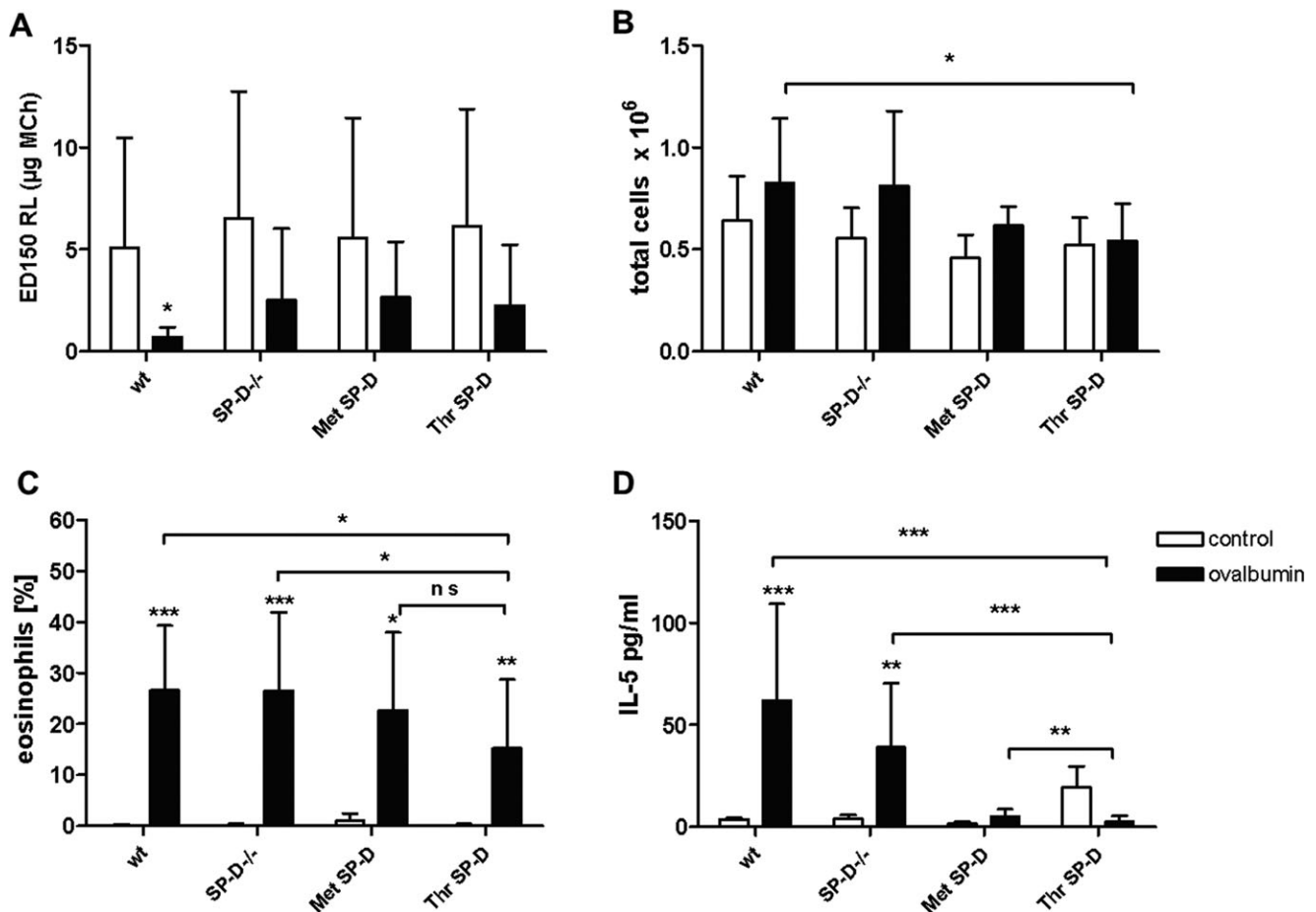


FIGURE 1. Measurement of airway hyperresponsiveness and pulmonary inflammation. (A) Inhaled dose of MCh in μg required to increase pulmonary resistance (R_L) by 150% above baseline ($\text{ED}_{150} R_L$) measured in anesthetized, orotracheally intubated, and spontaneously breathing mice ($n = 16$). Characterization of the cellular inflammation in the BAL fluid assessing (B) total cell counts and (C) eosinophils in% and (D) IL-5 ($n = 10$). Data are presented as mean + SD, * $P < .05$, ** $P < .01$, *** $P < .001$, ns nonsignificant, OVA-sensitized group compared to the respective control group or as otherwise indicated by lines.

like OVA-specific IgE levels in serum, the number of Th2-polarized CD4⁺ lymphocytes in draining lymph nodes and the allergen-specific proliferation of these cells also revealed no differences between OVA-sensitized Met SP-D and Thr SP-D mice (data not shown).

Impact of the Human Met(11)Thr SP-D Polymorphism on Height and Mucus Content of Airway Epithelium

In response to OVA sensitization and challenge mice developed mucus metaplasia in the airway epithelium (Figure 3A). Mucus metaplasia was restricted to large and medium sized airways while small distal airways were devoid of mucus (Figure 3B,C). Furthermore, eosinophils in modest numbers infiltrated the peribronchial and especially the perivascular connective

tissue (Figure 3D). Quantification of epithelial mucus demonstrated a significant increase in wt mice for estimation of volume to surface ratio (V_S) ($P < .05$; Figure 4B) and volume density of mucus (V_V) ($P < .05$; data not shown). Estimation of the ratio of surface area covered by epithelial mucus to total epithelial surface area (S_S) revealed a significant rise in epithelial mucus in all OVA-sensitized groups of mice, compared to the wt control group ($P < .05$; data not shown). A significant difference in epithelial mucus content between groups of mice carrying the SP-D Met(11)Thr SNP was not observed. Because estimation of the total height of epithelium (volume to surface ratio) revealed no significant difference between OVA and sham-sensitized mice (Figure 4A) the rise in epithelial mucus content is apparently not accompanied by a substantial hypertrophy of the respiratory epithelium.

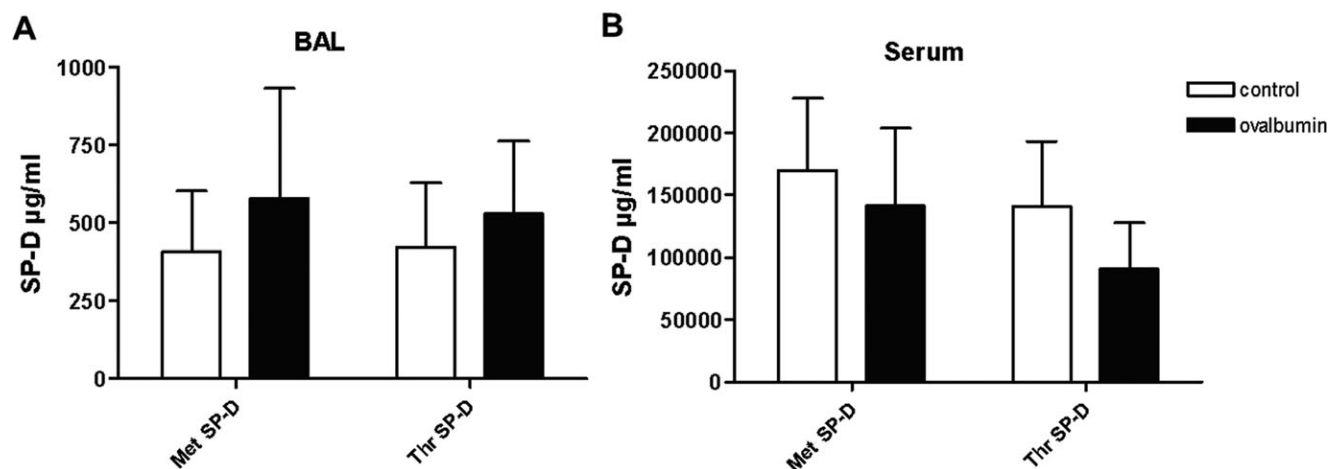


FIGURE 2. Human SP-D in BAL (A) and serum (B), ($n = 10$). Data are presented as mean + SD.

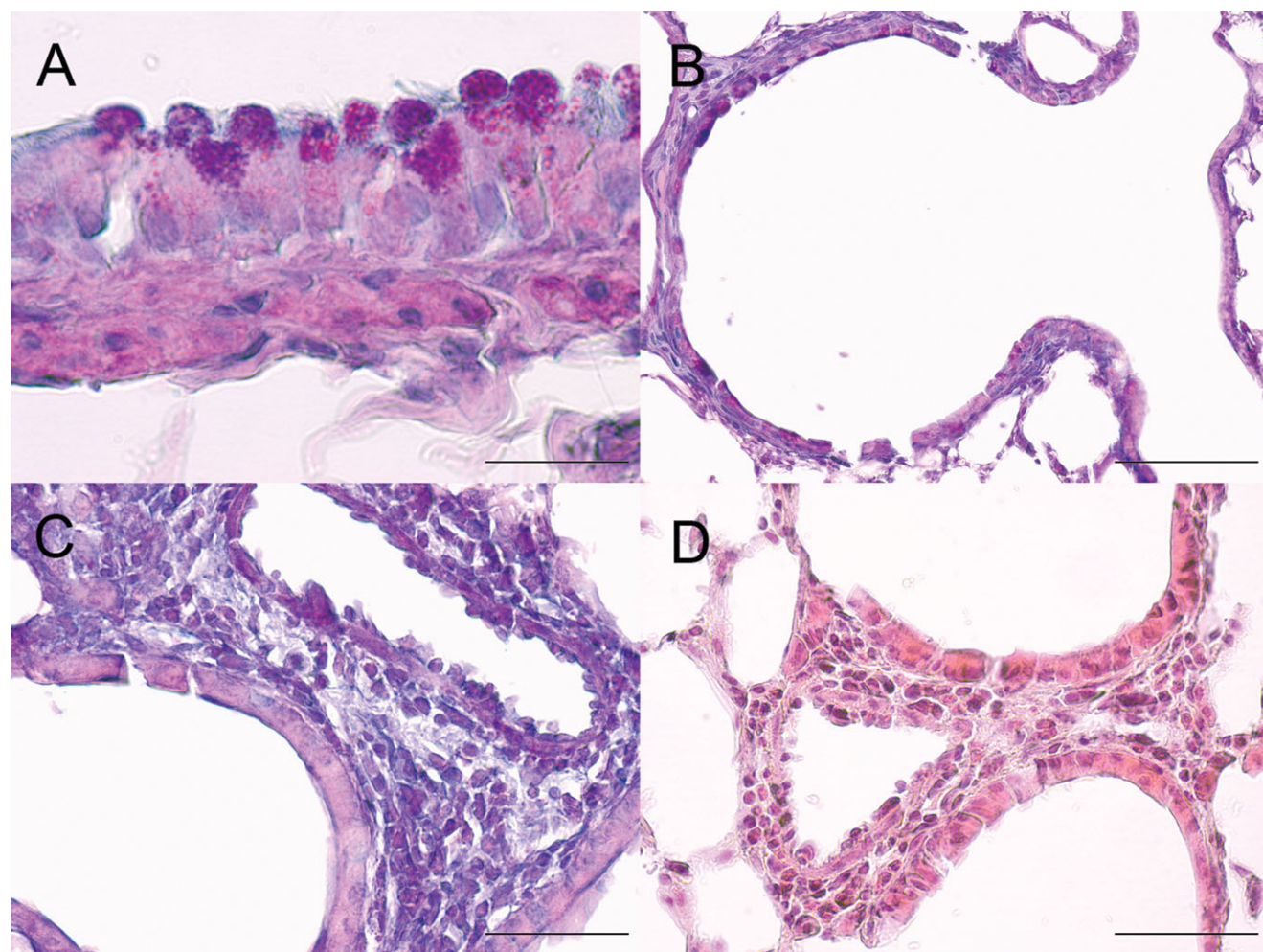


FIGURE 3. Histology of lung tissue. AB-PAS (A-C) and HE (D) staining in wt (A, C, D) and Thr SP-D (B) mice. (A) proximal airway with apical AB-PAS staining in nonciliated cells (scale bar 20 μm), (B) midlevel airway with AB-PAS positive cells branching into small distal airways that are AB-PAS negative (scale bar 100 μm). (C) and (D): distal airway and adjacent small blood vessel with eosinophilic infiltration of perivascular and peribronchial tissue (scale bar 50 μm).

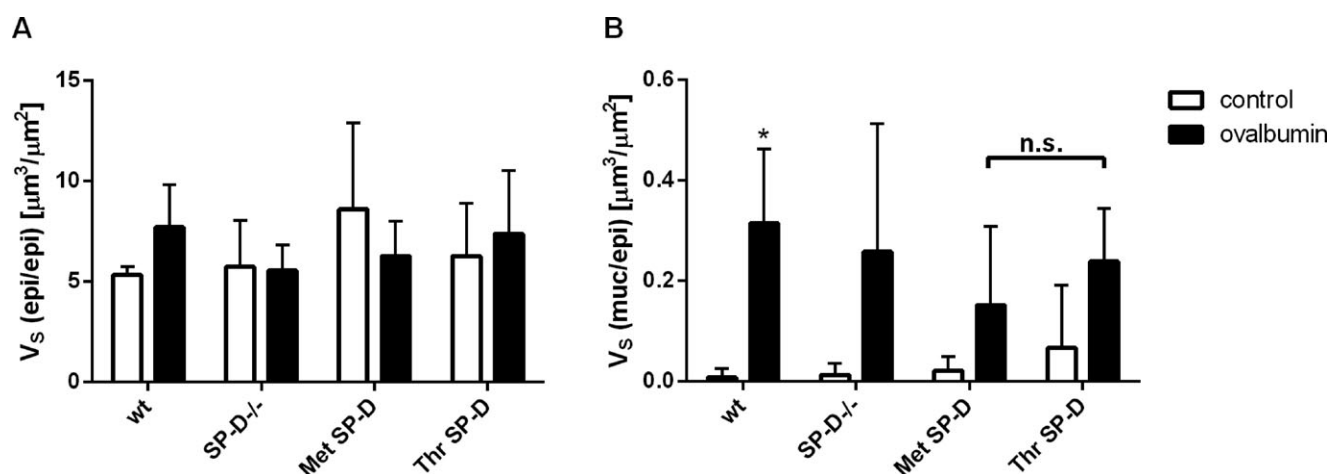


FIGURE 4. Quantitative microscopy. (A) Epithelial volume per surface area of airway epithelium (representing mean epithelial layer thickness), (B) Mucus volume per surface area of airway epithelium (wt $n = 5$, SP-D-/- $n = 3$, Met SP-D $n = 6$, Thr SP-D $n = 6$). Data are presented as mean + SD, ** $P < .05$, n.s. nonsignificant, OVA-sensitized group compared to the respective control group or as otherwise indicated by lines.

Impact of the Human Met(11)Thr SP-D Polymorphism on miR-21 and miR-155 Expression

SP-D-/- animals had higher miR-21 levels and miR-21 was decreased by OVA challenge. In contrast, OVA challenge of Thr SP-D mice led to a significant increase in miR-21 and miR-155 expression, whereas in Met SP-D mice OVA challenge resulted in a decreased expression of miR-21. In OVA-sensitized mice harboring, the Thr SNP levels of both miRs were elevated compared to the wt, knock-out, and animals with the Met variant (for individual P -values see Figure 5A and B).

DISCUSSION

In this study, we investigated the impact of the human SP-D Met(11)Thr SNP on the inflammatory phenotype in OVA-induced allergic airway inflammation and miRNA-related effects. We hypothesized that mice expressing allelic variants of the SP-D show different degrees of allergic inflammation because allelic SP-D variants differ in their microbial binding affinity, they distinctively modulate expression of inflammatory mediators and seem to increase the susceptibility to certain airway diseases like allergic rhinitis [5–8, 10].

Assessing bronchial hyperreactivity, we found that wt OVA-sensitized mice developed significant airway hyperresponsiveness in response to challenge. OVA-sensitized SP-D-/- and transgenic mice responded to methacholine in a comparable way to wt mice but the

differences compared to groups of sham-sensitized mice did not reach significance.

Investigation of cells in the BAL revealed that Thr SP-D mice exhibited lower total cell numbers and a lower percentage of eosinophils in the BAL compared to wt mice. We did not observe a significant difference between mice expressing one or the other allelic variant of SP-D. Comparing SP-D-/- and wt mice some authors report a higher susceptibility of SP-D-/- mice to allergic inflammation [25, 26] while others describe a lower number of eosinophils and airway hyperreactivity in SP-D-/- mice in response to *Aspergillus fumigatus* challenge [27]. We observed an inflammatory phenotype not significantly different in SP-D-/- compared to wt mice.

With regard to cytokine levels, transgenic mice showed significantly lower IL-5 levels in response to OVA challenge, with lowest levels in Thr mice compared to wt mice. Maturation and differentiation of eosinophils is controlled by IL-5 [28]. Thus, lower IL-5 levels parallel lower eosinophil numbers in Thr SP-D mice. Furthermore, IL-5 levels in these mice did not change substantially to allergen challenge; on the contrary, they resembled levels seen in wt sham-sensitized mice. Surprisingly, sham-sensitized Thr SP-D mice had significantly higher IL-5 levels than sham-sensitized wt mice; we have no explanation for this finding yet. Unlike IL-5, IL-4 levels rose significantly in response to allergen challenge in Thr SP-D mice, suggesting that there was no general Th2 cytokine dysregulation. Further investigations will be required to elucidate IL-5 regulation in mice transgenic for the SP-D Met(11)Thr SNP.

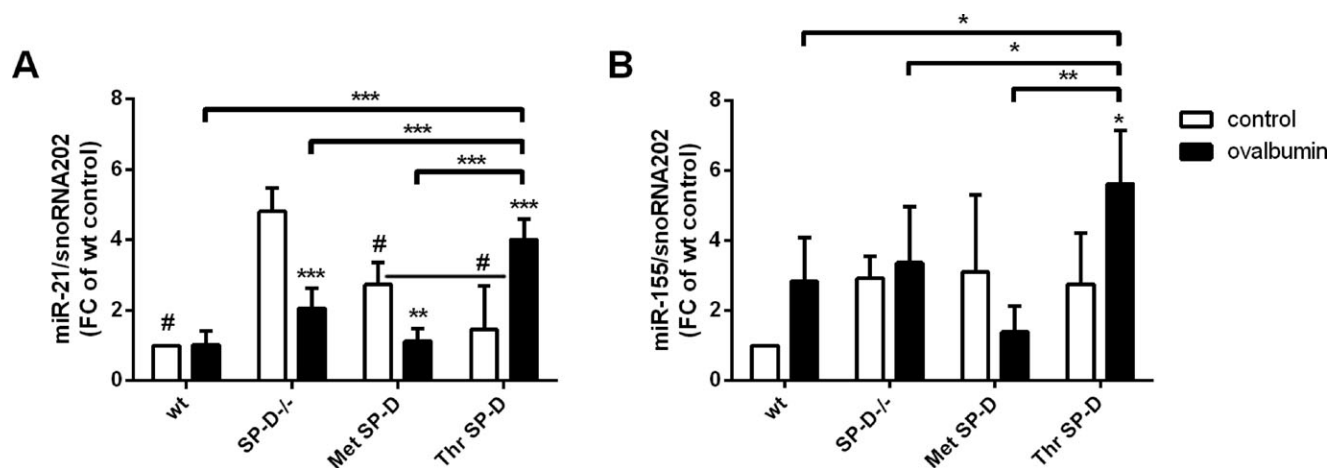


FIGURE 5. Expression patterns of miR-21 (A) and miR-155 (B). Relative miRNA levels in lung tissue ($n = 4$). MiRNA expression is normalized to snoRNA202. FC: fold change. Data are presented as mean $\pm$ SD, * $P < .05$; ** $P < .01$; *** $P < .001$, compared to the respective control groups or as otherwise indicated by lines; # $P < .001$ compared to SP-D-/- control group.

Quantifying epithelial mucus content we did not observe a significant difference between groups of transgenic mice in response to allergen challenge. Compared to wt mice SP-D deficiency had no significant influence on epithelial mucus content, either. It has been shown that intratracheal administration of rat SP-D in mice significantly reduces the number of PAS-positive cells in the airway epithelium [29]. In an *Aspergillus fumigatus* challenge model, epithelial mucus content is not significantly lower in SP-D-/- compared to wt mice [27]. It is possible that mice deficient in SP-D are not entirely deficient in innate immunity. SP-A with its multiple CRDs arranged in a bouquet-like structure may provide a kind of immunological rescue for SP-D-/- mice, helping to clear allergen particles and to lower pro-inflammatory mediators by binding to SIRP-1 α .

With regard to SP-D levels, healthy humans homozygous for Met SP-D possess almost 50% more SP-D in the serum than individuals homozygous for Thr SP-D [5]. Presumably due to ROSA26-driven SP-D expression SP-D serum concentrations in this study were higher than those previously published [5]. The Met/Thr SP-D ratio seen in murine serum samples was similar to the ratio observed in humans. Data on Met and Thr SP-D concentrations in the BAL have not yet been reported; in this study, mean SP-D BAL levels in both groups of transgenic sham-sensitized mice were approximately the same. As far as we can judge from SP-D serum levels, ROSA26-promoted SP-D expression in this mouse model successfully reproduces constitutional differences in human SP-D levels.

In this SP-D Met(11)Thr SNP mouse model, Met SP-D did not alleviate the allergic inflammation com-

pared to Thr SP-D. Results from studies investigating the composition of SP-D under challenging conditions might provide an explanation for this result. Allergen challenge models demonstrate that allergic inflammation (and smoking) can disrupt SP-D multimers and impair protein function [30–33]. Thus, in our asthma model in the course of airway inflammation, large multimers of Met SP-D might have been degraded to SP-D trimers, thereby partly neutralizing its superior microbial binding affinity and silencing of pro-inflammatory mediator production. The oligomerization of SP-D in this SP-D Met(11)Thr SNP mouse model has been documented in unchallenged mice [4]. We did not investigate the oligomerization status in response to allergen challenge and therefore it is possible that postinflammatory modification of SP-D occurred, affecting our results.

MiRs are short single-stranded noncoding RNAs that silence expression of coding genes. MiR-21 is linked to various disease conditions. In myocardial disease, for example, miR-21 appears to promote cardiac fibrosis and hypertrophy and miR-21 silencing by antagomirs has therapeutic effects [34]. Furthermore, miR-21 is upregulated in allergic diseases like allergic asthma, eosinophilic esophagitis and atopic dermatitis [35, 36]. Thus, we hypothesized that allergic inflammation might modulate miRNA expression levels in a SP-D Met(11)Thr SNP mouse model. Our results show that OVA sensitization and challenge is followed by an upregulation of miR-21 and 155 in Thr SP-D mice. In contrast, in Met SP-D mice miR-21 is significantly downregulated implicating different effects of SP-D SNPs on miRNA regulation. Surprisingly, miR-21 levels did not differ between OVA-sensitized and sham-sensitized wt mice

in response to challenge. Our results demonstrate that miR-21 and 155 are differentially regulated in mice transgenic for a human SP-D Met(11)Thr SNP in response to allergen challenge.

CONCLUSION

This study evaluates for the first time the effect of the human SP-D Met(11)Thr SNP in a mouse model of allergic airway inflammation. We could not find a significant difference in the inflammatory phenotype between mice carrying one or the other polymorphic SP-D variant while miRNA expression was differentially regulated. Future investigations of this mouse model under different challenging conditions will be required to further elucidate the impact of this SNP on airway and lung diseases.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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SUPPLEMENTARY MATERIAL

Fixation, Removal, and Sampling of Mice Lungs

Briefly, mice were sacrificed, the abdomen was opened and the inferior vena cava exposed. We

introduced an intravenous canula and immediately started a preflush perfusion with 5 to 10 mL of a heparinized 0.9% NaCl solution at a hydrostatic pressure of 30 cm H₂O. At the time most of the blood was removed from the circulation the preflush perfusion was stopped. The trachea was exposed and via a small incision a canula was introduced and fixed with a ligature. The lung was then inflated with a hydrostatic pressure of 13 cm H₂O. In the beginning, the pressure was increased slowly to 25 cm H₂O, lowered to 10 cm H₂O, again increased to 25 cm H₂O and finally set to 13 cm H₂O. Then, the perfusion was carried on with 5 mL of a mixture of 1.5% paraformaldehyde (PFA, Merck, Germany) and 1.5% glutaraldehyde (GA, Agar Scientific, United Kingdom) in 0.15 M 2-[4-(2 Hydroxyethyl)-1-piperazinyl]-ethanysulfonic acid sodium salt (HEPES, Merck, Germany) buffer. The lung was removed and finally immersed in fixative for several days.

Subsequently, the lung was embedded in agar (Agar-Agar, Kobe I, Roth, Germany) and prepared for IUR sectioning. The block was placed onto an equally spaced clock and cut at a random angle. With the cut plane at the bottom the block was placed onto a cosine-weighted clock and again cut at a random angle. The cut plane that results is isotropic. Slabs of equal thickness cut with a random start parallel to this plane are isotropic uniform random (IUR). 1.5 mm thick IUR slabs were cut using a tissue slicer and every third slab randomly allocated to paraffin embedding (Surgiplast, Leica, Germany). Paraffin blocks were cut at 3 μ m and stained with AB-PAS and haematoxylin eosin (HE).

Stereological Assessment

Stereology was performed according to the recommendations by Hsia [24]. Mucus content is expressed by volume density of mucus, that is, volume of mucus per volume of epithelium [$\mu\text{m}^3/\mu\text{m}^3$] estimated by point counting and calculated as follows

$$V_V(\text{muc}, \text{epi}) = \frac{\sum P(\text{muc})}{\sum P(\text{epi})}$$

where $P(\text{muc})$ and $P(\text{epi})$ are points of a stereological test point grid hitting AB-PAS positive mucus resp. epithelium.

Volume density of mucus normalized to the surface area the epithelium covers, that is, volume of mucus per surface area of epithelium [$\mu\text{m}^3/\mu\text{m}^2$] is calculated as follows

$$V_S(\text{muc}, \text{epi}) = \frac{\sum P(\text{muc}) \cdot l/p}{2 \cdot \sum I(\text{epi})}$$

where $P(\text{muc})$ are points hitting mucus, $I(\text{epi})$ are intersections of test lines with the epithelium and l/P is the length per point of a combined test line and point grid. $V_S(\text{epi}, \text{epi})$ represents mean epithelial layer thickness.

The ratio of total surface area to surface area covered by epithelial mucus was estimated by intersection counting and calculated as follows

$$S(\text{muc}/\text{epi}) = \frac{I(\text{muc})}{I(\text{epi})}$$

where $I(\text{epi})$ and $I(\text{muc})$ are intersections of a line grid with epithelial mucus resp. with the epithelium.

Measurement of Pulmonary Function

A 24 hours after the final OVA aerosol challenge, pulmonary function was measured invasively to assess AHR as described previously [22, 23]. Briefly, mice were intubated orotracheally and placed in supine position in temperature-controlled body plethysmographs (type 871, HSE-Harvard Apparatus, March-

Hugstetten, Germany). Pulmonary resistance (RL) was calculated from the measured differences in transpulmonary pressure and tidal respiratory flow over each entire breath cycle under isoflurane anesthesia (by HEM 4.2 software, Notocord Systems, Croissy, France). After recording baseline values for 2 min, AHR to aerosolized methacholine chloride (MCh, from 5% solution; Sigma, Deisenhofen, Germany) was determined using a Bronchy type III generator (Fraunhofer ITEM) under continuation of lung function measurement. The total dose inhaled via the orotracheal tube (in μg) was calculated and controlled by a computerized feedback dose control system (Fraunhofer ITEM; [23] based on the continuously measured respiratory minute volume and photometrical aerosol concentration. The mice were exposed to increasing MCh dose steps of 0.063, 0.125, 0.3, 0.5, 1, 2, 4, 8, and 16 μg . From individual dose effect plots (delta% RL vs. MCh dose) the effective inhalation dose in μg MCh required to produce a 150% increase in RL was evaluated for each animal (ED_{150}RL).