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Natural or engineered mutations in Surfactant Protein-D alter allergic asthmatic responses in mice and man

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Abstract

Background—Surfactant protein D (SP-D) has been proposed to be protective in allergic airway responses.

Objective—We aimed to determine the effect of SP-D deficiency on murine and human airway allergy.

Methods—Immunological responses of SP-D gene deficient mice (*Sftpd*^{−/−}) at baseline and following four *Aspergillus fumigatus* exposures were assessed. In addition, the significance of a single nucleotide polymorphism (Met¹¹Thr) in the human SP-D gene (known to decrease SP-D function) on asthma susceptibility was investigated.

Results—Macrophage BALF levels and lung CD-4⁺ T-cells were increased in naive *Sftpd*^{−/−} mice in association with increased lung CCL17 levels. Th2-associated antibody levels (IgG1 and IgE) were significantly lower in 4–6 week old *Sftpd*^{−/−} mice (p<0.05). Accordingly, naive *Sftpd*^{−/−} splenocytes released significantly less IL-4 and IL-13 upon anti-CD3/CD28 stimulation (p<0.01). Following intranasal allergen exposures, a modest decrease in BALF eosinophilia was observed in *Sftpd*^{−/−} mice compared to wild type mice (p<0.01). Translational studies in a pediatric population of Caucasians with asthma revealed that a single nucleotide polymorphism in the *SFTPD* gene, affecting SP-D levels and pathogen binding, was associated with lower asthma susceptibility (p<0.05).

Conclusion—*Sftpd*^{−/−} mice have an impaired systemic Th2 response at baseline and modestly reduced pulmonary eosinophilia following allergen exposure. Translational studies revealed that a mutation in the *SFTPD* gene was associated with lower asthma susceptibility in Caucasians. Taken together, these results support the hypothesis that SP-D-dependent innate immunity influences atopy and asthma.

Clinical implications—SP-D deficiency results in increased pulmonary inflammation and decreased susceptibility to asthma, perhaps related to impaired endotoxin and pathogen clearance.

Keywords

allergy; lung; surfactant protein D; polymorphism; eosinophil; IL-13; *Aspergillus*; endotoxin

INTRODUCTION

Pulmonary surfactant, a complex mixture of lipids and proteins, is essential for lung function. In addition to preventing airway collapse by lowering surface tension, surfactant represents a natural barrier against inhaled pathogens and other harmful particles. While surfactant associated proteins A and D (SP-A and SP-D) have a fundamental role in pulmonary host defense,^{1, 2, 3} their role in allergen-induced lung inflammation is not well defined.^{4, 5} House dust mite (*Dermatophagoides pteronyssinus*),⁶ *Aspergillus fumigatus*,⁷ and pollen starch granules⁸ have been reported to bind to SP-D and SP-A.⁵ Both surfactant proteins are able to inhibit in vitro lymphocyte proliferation, binding of allergen-specific IgE and mast cell histamine release.^{9, 10, 11} Furthermore, a recent study suggests that SP-D binds to pollen starch granules and inhibits mast cell degranulation.¹²

Treatment of mice with either recombinant or purified human SP-A or SP-D significantly decreases allergen-induced airway inflammation.^{9, 13–15} Indeed, in a murine model of allergic bronchopulmonary aspergillosis, Madan et al. reported that treatment with pharmacological doses of recombinant SP-D reduced cellular lung infiltration.¹³ In splenic cell cultures, SP-D treatment shifted Th2 type responses (decreased IL-4 and IL-5) toward Th1 responses (increased IFN γ).¹³ Taken together, these results suggest that SP-A and SP-D might have a protective function by inhibiting allergic responses. However, it is important to note that these experiments are limited by their pharmacological approach and the potential contamination of SP-D with other products such as LPS. An alternative approach involves the examination of natural mutations in *SFTPD* as well as the analysis of the effect of *Sftpd* gene deletion in mice on immunity, inflammation and allergic responses.

Three coding polymorphisms, which alter a single amino acid, have been described in the human *SFTPD* gene: Met¹¹Thr, Ala¹⁶⁰Thr and Ser²⁷⁰Thr. Of these three point mutations only the first one, replacing a methionine (ATG) by a threonine (ACG) at position 11 in the N-terminal region affects SP-D function.¹⁶ Several studies have demonstrated that the Thr form is associated with lower SP-D serum levels.^{16, 17} In individuals with the Thr/Thr genotype, SP-D fails to oligomerize properly, forming mainly trimeric but not the more complex multimeric forms, resulting in impaired pathogen binding.¹⁶ Finally, the Met¹¹Thr polymorphism has been associated with increased susceptibility to tuberculosis in a Mexican population and severe respiratory syncytial virus bronchiolitis in a Finnish pediatric population.^{18, 19}

In the absence of SP-D and detectable pathogens, mice develop emphysema and pulmonary inflammation as they age.^{20, 21} Notably, BALF macrophages are increased in number and show signs of activation.^{20, 21} In the lung of *Sftpd*^{-/-} mice, an accumulation of CD45 cells around blood vessels and airways is observed.²² FACS analysis of *Sftpd*^{-/-} mice

demonstrate an increase in activated CD4 T cells and memory T cells.²² Taken together, these observations suggest increased inflammation in lungs of *Sftpd*^{-/-} mice, which might affect systemic immune responses.

In the present study, we demonstrate that *Sftpd*^{-/-} mice have impaired systemic Th2 immune responses as defined by lower Th2-associated antibody levels and reduced ability of *Sftpd*^{-/-} splenocytes to release Th2 cytokines upon CD3/CD28 stimulation. Accordingly, we demonstrate that *Sftpd*^{-/-} mice exposed to *Aspergillus fumigatus* antigen developed less BALF eosinophilia compared to wild type mice. Furthermore, translational studies revealed that a mutation affecting SP-D levels and function was associated with decreased asthma susceptibility in a pediatric Caucasian population.

MATERIAL AND METHODS

Patients

The inclusion criteria are: (a) a diagnosis of asthma and (b) age >5 years and <18 years upon initiation of the study; the exclusion criteria are: (a) presence of a co-morbid lung condition including cystic fibrosis, congenital anomaly, or bronchopulmonary dysplasia; and (b) dependency on oral steroids or an immunosuppressive agent for a medical condition other than asthma. Asthma was diagnosed in accordance with the American Thoracic Society (ATS) criteria by demonstrating a 12% or greater increase in FEV1 after a bronchodilator or after a 2-week trial of oral corticosteroids. These children subsequently underwent skin prick testing, including positive and negative controls to a panel of 11 relevant environmental antigens indigenous to the Ohio valley (Greer Laboratories, Lenoir, NC).

The patients in the asthma cohort for this study were sequentially recruited from a DNA and clinical data registry of patients recruited from CCHMC allergy clinics. Genomic DNA was isolated from buccal swabs using the ZR Genomic DNA II Kit from Zymo Research (Orange, CA). Of the 1068 subjects in the registry thus far, 426 have asthma and have consented for this study. For the present study, we restricted our asthma cohort to the 226 Caucasian children [age (mean $\pm$ SD): 10.2 $\pm$ 5.1 years; 55% males / 45% females; 81% atopy]

For the control reference group, race- and socioeconomic class-matched children were recruited from non-allergy clinics at CCHMC. An eligibility screening form was used to identify individuals with no history of asthma or allergy symptoms. A total of 292 children were recruited, including 120 Caucasians [age: 8.9 $\pm$ 3.6 years; 45% males / 55% females; 27.5% atopy].

SFTPD genotyping

DNA was eluted with 40 μ l of water and an aliquot was diluted 1:20 for PCR use. We used the LightTyper 96 PCR kit according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN). The primers were selected using the LightCycler Probe Design Software 2.0; the SP-D specific primers were CTAAACCATGTCCATGAAGCATA and GCCACTCTCCACTGAGCTA; the anchor probe was labeled with LCred640 linked to GTGCTTGACCCCTGGTCATGTG; the sensor probe (ACTCCCACAGAACAACGCC)

was labeled with fluorescein and designed to span the T-> C mutation. DNA samples were amplified for 50 cycles and results read using the LightTyper (Roche Diagnostics).

Animals

Sftpd^{-/-} mice were generated by targeted gene inactivation as described.²³ *Sftpd*^{-/-} mice survive and breed normally in the barrier facilities at Cincinnati Children's Hospital Medical Center. Swiss Black *Sftpd*^{+/+} mice were used as controls. All mice were maintained under specific pathogen-free conditions and according to institutional guidelines. Water was available continuously through automatic ports and a commercial mouse diet was provided *ad libitum*.

Protocols

Mice were subjected to repeated intranasal *Aspergillus fumigatus* or saline exposures (100 µg in 50 µl) two days in a row on two consecutive weeks. Twenty hours after the last instillation, all mice were sacrificed and parameters analyzed.

Bronchoalveolar lavage (BALF)

Mice were euthanized with carbon dioxide inhalation 20 hours after the last intranasal challenge and lungs were washed with two separate 1 ml doses of 2% fetal calf serum (FCS) in phosphate buffered saline (PBS). After cytocentrifugation, cells were stained by a modified Diff-Quik (HEMA3; Fisher Scientific, Kalamazoo, MI). All samples were evaluated in a blinded manner.

Lung lysates

Lungs were collected in 350 µl of sterile PBS, snap frozen and stored at -80°C. Upon thawing, tissue samples were homogenized for 15–20 seconds. After addition of 350 µl of 0.1% Triton X, lung homogenates were vortexed and kept on ice for 10 minutes. Following centrifugation at 12000 rpm for 10 minutes, supernatants were collected and stored at -80°C until needed.

Flow cytometry

Lungs were enzymatically digested with Liberase CI enzyme blend (0.5 mg/ml; Roche, Basel, Switzerland) and DNase (0.5 mg/ml; Sigma, St. Louis, MO) at 37°C for 30 min. Cells were counted and about 5.10⁶ cells were stained with the following antibody combinations: anti-CD3-FITC/anti-CD19-PE/anti-CD45-APC or anti-CD4-FITC/anti-CD69-PE/anti-CD45-APC (BD PharMingen, San Diego, CA). After two washes, labeled cells were resuspended in vital dye 7-AAD and subjected to flow-cytometry on FACS calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Splenocyte culture

Splenocytes were isolated from *Sftpd*^{-/-} and wild type mice and plated in triplicate at a density of 10⁵ cells per well in 96-well plates. Cells were cultured for 48h at 37°C in the presence of anti-CD3 (5 µg/ml) and anti-CD28 (2 µg/ml) in 24 well plates. After 48h, cultures were pulsed with 1 µCi per well [³H]thymidine for 6h. Isolated splenocytes were

plated at 1×10^6 cells/ml and stimulated with anti-CD3 and anti-CD28 for 48h at 37C. Supernatants were then assayed for cytokines.

ELISA Measurements

IgE, eotaxin-1, eotaxin-2, TARC, JE, MIP2, IFN γ , TNF α , IL-4 and IL-13 levels were measured according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Total antibody levels (IgM, IgA, IgG1) were measured according to the manufacturer's instructions (SouthernBiotech, Birmingham, AL). Allergen-specific IgG1, IgG2a and IgE levels were measured after coating 96-well Immulon plates with 100 μ l of *Aspergillus* (100 μ g/ml) in carbonate buffer and overnight incubation. Blocking was done with 10% FCS in PBS, and all washes were performed with 0.1% Tween-20 in PBS. Plasma samples were diluted 1:5000 for IgG1 (1:5 for IgG2a and IgE), before serial dilutions of 1:4. After 2h incubation, plates were washed and Horseradish Peroxidase (HRP)-conjugated rabbit anti-mouse IgG1 (1:1000) (X56;BD PharMingen) was added. The plates were developed with TMB A and B solutions and quenched with 2M HCL. Optical density was read at 450 nm on an Ultra Microplate Reader (Bio-tek Instruments, Winooski, VT).

Statistical analysis

Murine data are expressed as mean $\pm$ SEM. Statistical significance comparing different sets of mice was determined by Student's unpaired t-test or Mann Whitney's U-test when appropriate. Hardy-Weinberg equilibrium (HWE) as well as allele and genotype frequencies were analyzed with the FINETTI program (<http://ihg.gsf.de/cgi-bin/hw/hwa1.pl>). Finally, we confirmed our results by performing the Armitage's trend test, which takes into account the genotypes rather than the alleles.

RESULTS

Increased leukocytes in the lung SP-D deficient mice

Before assessing the impact of SP-D deficiency on allergic responses, we investigated baseline immune responses in these mice. In order to uncover early events associated with the development of pulmonary inflammation, we focused our study on 4–6 week old *Sftpd*^{-/-} mice. A significant increase in cells recovered from the BALF of *Sftpd*^{-/-} mice was already observed at 4 weeks of age (Figure 1A). Over 98% of these BALF cells were macrophages. In addition, FACS analysis of lung cell suspensions revealed a significant increase in T cells, especially CD4⁺ cells (Figure 1B). Of note, activated T-cells (CD4⁺/CD69⁺ cells) were present in the lungs at baseline and a significant increase was observed in *Sftpd*^{-/-} mice (Figure 1C). Taken together, these findings demonstrate an accumulation of macrophages and activated T-cells in the BALF and lungs, respectively, of naïve 4–6 week old *Sftpd*^{-/-} mice.

Increased chemokine levels in the lung SP-D deficient mice

Lung levels of the macrophage chemokine JE (CCL2) were significantly increased in 6–7 week-old *Sftpd*^{-/-} mice but not in 4 week old mice (Figure 2A; $p < 0.01$). Consistent with increased pulmonary accumulation of CD4⁺ T-cells, lung levels of TARC (CCL17) were elevated in *Sftpd*^{-/-} mice at both 4 and 6–7 week of age (Figure 2B). In contrast, levels of

Th1 (IFN γ and IL-12p70) and Th2 (IL-4 and IL-13) cytokine levels were similar between wild type and *Sftpd*^{-/-} mice (Figure 2E–H).

Impaired Th2 systemic immune responses in SP-D deficient mice

We were next interested in assessing if the local inflammatory events occurring in the lungs of naïve *Sftpd*^{-/-} mice were associated with systemic changes. Interestingly, the Th2 antibodies IgE (Figure 3A) and IgG1 (Figure 3B) were both significantly lower in *Sftpd*^{-/-} mice compared to wild type mice. In contrast, IgA levels were significantly elevated in the plasma of *Sftpd*^{-/-} mice (Figure 3C). To determine if the lower plasma Th2 antibody levels were associated with impaired systemic Th2 cytokine production, we isolated spleen cells from *Sftpd*^{-/-} mice and wild type controls and stimulated them with a combination of anti-CD3 and anti-CD28. IL-4 and IL-13 levels were significantly lower in culture supernatants of splenocytes isolated from *Sftpd*^{-/-} mice (respectively Figure 3D and E), while IFN γ production was not significantly altered (Figure 3F). As a control, splenocyte proliferation was similar between wild type and *Sftpd*^{-/-} mice. Taken together these results suggest that *Sftpd*^{-/-} mice have an impaired systemic Th2 immune response at baseline.

Impaired allergen-induced BALF eosinophilia in *Sftpd*^{-/-} mice

In order to understand the impact of the impaired Th2 immune phenotype observed in *Sftpd*^{-/-} mice on allergen-induced pulmonary immune responses, we used a murine model of experimental allergic airway inflammation that does not require systemic priming with an adjuvant (Figure 4A). First, we assessed the impact of SP-D deficiency on allergen-induced BALF inflammation. Interestingly, decreased total BALF cell levels were observed in *Sftpd*^{-/-} mice compared to wild type mice following allergen exposure (Figure 5B). Furthermore, the percentage of infiltrating BALF eosinophils was $67.8 \pm 2.5\%$ in wild type mice and only $53.9 \pm 3.2\%$ in *Sftpd*^{-/-} mice, resulting in significantly less eosinophils in the BALF of *Sftpd*^{-/-} mice (Figure 5C). This impaired eosinophil recruitment was not due to decreased eotaxin-2 secretion in the BALF of *Sftpd*^{-/-} mice (Figure 5D). Of note, following saline exposure a significant increase in neutrophil levels was observed in the BALF of *Sftpd*^{-/-} mice compared to wild type mice (respectively 3832 ± 537 and 881 ± 109 total BALF neutrophils; $p < 0.001$) while eosinophils were barely detectable (data not shown).

Altered allergen-induced Th2 responses in *Sftpd*^{-/-} mice

We aimed to assess the impact of allergen exposure on pulmonary Th2 cytokine expression in wild type and *Sftpd*^{-/-} mice. Following four intranasal *Aspergillus* exposures, lung IL-13 levels were increased in wild type and *Sftpd*^{-/-} mice compared to saline challenged controls (data not shown). Compared to wild type mice, IL-13 lung levels were not significantly elevated in *Sftpd*^{-/-} mice following allergen exposure (Figure 4E). Furthermore, SP-D deficiency was not associated with either increased IL-4 lung levels following *Aspergillus* exposure (89 ± 12 vs 88 ± 12 pg/mg of protein) or decreased IFN γ levels. Indeed, IFN γ lung levels even appeared higher in *Sftpd*^{-/-} mice (Figure 4F). Of note, a modest increase in TNF α lung levels was also detected in *Sftpd*^{-/-} mice after *Aspergillus* exposure (Figure 4G).

To assess systemic Th2 responses, allergen specific Th2 antibody levels were measured following *Aspergillus* exposures. Allergen-specific IgE was undetectable. Albeit lower, *Aspergillus*-specific IgG1 levels were not significantly different in *Sftpd*^{-/-} mice compared to wild type mice (data not shown).

A single polymorphism (SNP) in the human *SFTPD* gene influences asthma susceptibility

Because the Met¹¹Thr SNP results in a mutation affecting SP-D assembly, function and serum levels,¹⁶ we hypothesized that the Thr/Thr genotype would alter asthma susceptibility. To avoid potential problems caused by genetic differences among races,²⁴ we restricted our cohort to Caucasian children with or without asthma (see Method section). No effect of the Met¹¹Thr SNP was observed on atopy (data not shown) allowing us to group 226 asthmatics and 120 controls for analysis. Using two separate statistical tests, the Met haplotype was associated with increased risk for development of asthma (Table 1; $p < 0.05$). This was confirmed at the genotype level, first by comparing the CC genotype to the TT genotype (Table 1; $p < 0.05$), then by performing the Armitage's trend test, which yielded a common odd ratio of 1.354 ($p < 0.05$). Taken together, these observations suggest that the Thr/Thr genotype is associated with reduced asthma susceptibility.

DISCUSSION

In the present study, a human SP-D mutation that inhibits SP-D binding to pathogens¹⁶ was associated with decreased asthma susceptibility in a Caucasian population. A recent German study concludes that none of the three known amino acid variants in SP-D are associated with bronchial asthma.²⁵ However, while there were only slight differences between the German and American Caucasian populations suffering from asthma,²⁴ the German control population was randomly selected and did not exclude, as we did, all controls with a history of asthma.²⁵ Interestingly, while serum SP-D levels are significantly lower in subjects with the Thr/Thr genotype,^{16, 17} increased BALF and serum SP-D levels have been reported in patients with asthma,^{26, 27} possibly indicating increased lung levels and increased bi-directional permeability of the respiratory epithelium. Since we found an association between the SP-D and asthma, these findings suggest that a defective or absent SP-D might be protective.

However, based mainly on the ability of SP-D to bind some allergens in vitro and to partially protect against the development of allergic airway inflammation, it has been hypothesized that the absence of SP-D would lead to a more severe asthma phenotype following allergen challenge.⁵ Demonstrating this hypothesis has been elusive as only two studies have observed increased Th2 cytokine levels.^{28, 29} A complex cytokine response was described in the study of Madan et al, but the fact that the cytokines measured in lung suspensions were all higher in PBS treated mice than in the *Aspergillus* exposed mice makes any interpretation problematic.²⁸ Another study observed an increase in IL-13 BALF levels accompanied by a modest increase in BALF eosinophilia following allergen exposure (nebulized OVA) in *Sftpd*^{-/-} mice compared to wild type controls.²⁹ However, in a similar protocol with OVA/alum sensitization and two intranasal OVA exposures three days apart, we failed to observe an increase in IL-13 BALF levels in *Sftpd*^{-/-} mice and found no

significant differences in BALF eosinophilia between wild type and *Sftpd*^{-/-} mice (data not shown). Furthermore, in *Aspergillus* treated *Sftpd*^{-/-} mice, we and others found no increase in BALF eosinophilia in *Sftpd*^{-/-} mice.³⁰ Remarkably, we demonstrated a significant reduction in BALF eosinophil levels in 5 week-old *Sftpd*^{-/-} mice compared to wild type mice following multiple intranasal allergen exposures. Albeit modest, the observed decrease might be of importance, since eosinophils have recently been shown to be essential for several experimental asthma features.³¹ Taken together with unchanged IL-4, IL-13 and IFN γ lung levels as well as increased TNF α lung levels, we suggest that rather than a Th2 biased lung environment, *Sftpd*^{-/-} mice develop a pro-inflammatory pulmonary phenotype when allergen exposures occur exclusively through the upper airways (no systemic sensitization). Indeed, we have shown that numerous *Aspergillus*-induced genes were increased in a STAT6 independent way following intranasal *Aspergillus* exposure.³²

We propose that altered baseline innate immune events influence allergen-induced pulmonary responses. Indeed, at four weeks of age, *Sftpd*^{-/-} mice spontaneously develop emphysema in the absence of detectable viral or bacterial infection.²⁰ As previously described, we demonstrated that 4–6 week old *Sftpd*^{-/-} mice have increased BALF macrophages.²¹ Elevated JE/MCP-1 lung levels are consistent with data from a recent study focusing on hyperoxia in *Sftpd*^{-/-} mice.³³ Furthermore, we found a significant increase in the number of activated CD4/CD69 double positive cells in 5 week old *Sftpd*^{-/-} mice, albeit significantly less than the 30% of CD69 positive CD4 T-cells previously reported in the lungs of adult *Sftpd*^{-/-} mice.²² The increased accumulation of CD4 T-cells observed in the lungs of *Sftpd*^{-/-} mice could be the result of increased TARC levels present in the lungs of these mice.³⁴ While TARC is generally considered a CD4⁺ Th2 cell chemoattractant, it is important to note that LPS has been shown to induce TARC in bone marrow derived dendritic cells.³⁴

While our mice are kept in a pathogen-free barrier facility, *Sftpd*^{-/-} mice are nevertheless chronically exposed to endotoxin and other Toll activated ligands, not only in our allergen preparations,³⁵ but also directly in the vivarium. Indeed, when exposed to intranasal saline instillations, a significant increase in BALF neutrophil numbers is observed in *Sftpd*^{-/-} mice, despite undetectable endotoxin levels in our saline solution. Furthermore, a recent study observed a modest neutrophilic infiltrate in the BALF of naïve 7–8 week old *Sftpd*^{-/-} mice.³³ While non-pathogenic viral infections cannot be excluded, we favor endotoxin exposure as an explanation, in part, because SP-D deficiency has been associated with increased soluble LPS receptor (CD-14) BALF levels and decreased macrophage CD-14 surface expression.³⁶ While activated “foamy” macrophages have been observed in *Sftpd*^{-/-} mice,^{20, 21} the reported downregulation of CD14 surface expression results in an impaired ability of these macrophages to respond to further LPS stimulation in vitro. Indeed, purified BALF macrophages from *Sftpd*^{-/-} mice produced decreased TNF α and IL-12p70 in response to LPS and IFN γ (data not shown). This suggests that compensatory mechanisms designed to maintain homeostasis exist in the lungs of *Sftpd*^{-/-} mice. This may explain why despite signs of inflammation no imbalance in Th1/Th2 cytokines was observed. Nevertheless, SP-D deficient mice do not appear to efficiently adapt to normal endotoxin exposure, as emphysema develops over time. Interestingly, a recent study just showed that

mice lacking Toll-like receptor-4 (which complexes with CD14 to drive LPS cellular responses), also develop emphysema.³⁷

Remarkably, naïve *Sftpd*^{-/-} mice had lower systemic Th2 antibody and cytokine levels compared to wild type control mice. A recent study showing increased baseline levels of IgG1 and IgE in mice lacking Toll-like (TLR) receptor signaling suggested the existence of TLR mediated suppression of IL-4 in naïve mice under pathogen-free conditions.³⁸ We therefore speculate that in *Sftpd*^{-/-} mice, increased endotoxin exposure would signal through TLR receptors and result in impaired IL-4 expression. In the same study, the authors demonstrate that following i.p. allergen sensitization in the presence of alum no differences in antigen-specific titers were observed.³⁸ These findings suggest that experimental asthma models relying on i.p. sensitization with an adjuvant are able to overcome impaired baseline Th2 response. Indeed, two groups using OVA and Aspergillus as allergens demonstrated an increase in serum total IgE levels in *Sftpd*^{-/-} mice.^{29, 30} Haczku et al. also demonstrated a modest increase in Aspergillus-specific IgE and IgG1 levels following two i.p. sensitizations. In our model, a single week after intranasal sensitization, Aspergillus-specific IgG1 levels were similar between wild type and *Sftpd*^{-/-} mice.

In conclusion, the present findings suggest that in the absence of SP-D, a spontaneous inflammation and a skewing of the immune response away from Th2 develops; in a pathogen-free environment, this occurs potentially because of impaired endotoxin clearance. Childhood endotoxin exposure has been proposed to be protective in the context of the hygiene hypothesis.^{39, 40} However, while the Thr¹¹ SP-D variant does not appear to impact LPS binding in vitro, it results in a protein with impaired ability to oligomerize properly and bind numerous pathogens.¹⁶ We speculate that increased mucosal inflammation in individuals with the Thr¹¹ SP-D variant renders them less susceptible to the development of asthma. Additionally, as illustrated by the opposing effects of the CD14-159C>T SNP on asthma susceptibility, gene-environment interactions play a central role in this process.³⁹ It will be therefore of great interest to explore how childhood infections and exposure to bacterial products can affect allergic diseases in individuals with the Thr¹¹ variant especially when polymorphisms in Toll-like receptors are taken into account.

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Abbreviations used

Alum	aluminum potassium sulfate
BALF	bronchoalveolar lavage fluid

i.n.	intranasal
i.p.	intra peritoneal
LPS	Lipopolysaccharide
OVA	ovalbumin
SNP	single nucleotide polymorphism
SP-A/D	surfactant protein A or D
<i>Sftpd</i>^{-/-}	surfactant protein D gene deficient mice

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Figure 1. Increased cellular infiltrate in the lungs of *sftpd*^{-/-} mice

(A) BALF cells were collected in 4–6 week old wild type (WT) and *Sftpd*^{-/-} mice (KO) and total cells counted. Lungs cells were isolated as described in the method section and stained for FACS analysis. (B) After excluding dead cells and CD-45 negative cells, the percentage of CD-4 positive cells was determined. (C) Representative density plots of CD-69 positive lung cells from wild type and *SFTPD*^{-/-} mice are shown.

Figure 2. Chemokine and cytokine lung levels in wild type and *sftpd*^{-/-} mice

Lungs were collected and homogenized as described in the method section. Lung supernatants were used to measure the chemokines (A) CCL2/JE/MCP1, (B) CCL17/TARC and the Th2/Th1 cytokines (C) IL-4, (D) IL-13, (E) IFN γ and (F) IL-12p70 in 4 (open circle) and 6–7 week old (black circle) *Sftpd*^{-/-} mice and their wild type controls. Statistical significance was determined by Student's T-test.

Figure 3. Impaired systemic Th2 profile in *Sftpd*^{-/-} mice at baseline

Total plasma (A) IgA, (B) IgE and (C) IgG1 levels were measured in 4–5 week old mice. Spleen cells from 4–5 week old wild type and *Sftpd*^{-/-} mice were stimulated with anti-CD3 and anti-CD28 as described in the method section. (D) IL-4, (E) IL-13 and (F) IFN γ levels were measured in culture supernatants at 48h. Statistical significance was determined by Mann-Whitney or Student's T-test when appropriate.

Figure 4. Decreases BALF eosinophilia despite increased lung cytokine levels in *Sftpd*^{-/-} mice following repeated *Aspergillus* exposures

(A) Mice were subjected to two consecutive intranasal *Aspergillus fumigatus* exposures followed one week later by two subsequent exposures. BALF were collected 20h following the last intranasal challenge. Total BALF cells (B), eosinophils (C) and (D) eotaxin-2 BALF levels were assessed in 5–6 week old *Sftpd*^{-/-} mice and their controls (17–18 mice total; 5 separate experiments). Lungs were homogenized 20h after the last challenge and the supernatant used to measure (E) IL-13 (F) IFN γ and (G) TNF α levels in *Sftpd*^{-/-} mice and their WT controls (8 mice/group; 2 separate experiments). Statistical significance was determined by Mann-Whitney or Student's T-test when appropriate.

Table 1

Genotype and allele frequency of the T-> C single nucleotide polymorphism affecting the surfactant protein D gene of asthmatic patients and pediatric Caucasian controls.

SNP	Asthmatics (n=226)	Controls (n=120)	Odd ratio (95% c.i.)	p value
<i>Allele frequency</i>				
ATG (Met)	271 (60.0%)	124 (51.7%)	1.401 (1.022–1.920)	0.0360
ACG (Thr)	181 (40.0%)	116 (48.3%)		
<i>Genotype frequency</i>				
TT	87 (38.5%)	36 (30.0%)	1.841 (1.009–2.694)	0.0457
TC	97 (42.9%)	52 (43.3%)	1.421 (0.804–2.513)	> 0.1
CC	42 (18.6%)	32 (26.7%)	1.000	
TT+TC	139 (61.5%)	84 (70.0%)	1.593 (0.942–2.694)	0.0809

Odd ratios, confidence intervals (c.i.) and p values were determined by the FINETTI program as described in the method section