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Surfactant protein D inhibits early airway response in *Aspergillus fumigatus*-sensitized mice

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Clinical and Experimental Allergy

Summary

Background The surfactant protein SP-D has been reported to reduce bronchial hyper-responsiveness, blood eosinophilia, and T-helper type 2 cytokines in models of allergic asthma. However, little is known about the functional effect of SP-D on the early airway response upon allergen inhalation, which is an important feature of this disease.

Objective We investigated whether SP-D is able to reduce the immediate allergen-induced mediator release and the early bronchial obstruction in addition to its effects on airway inflammation and bronchial hyperresponsiveness in an *Aspergillus fumigatus* mouse asthma model.

Methods *A. fumigatus*-sensitized mice were treated with a recombinant fragment of human SP-D or placebo. Lung functions were measured in orotracheally intubated, spontaneously breathing animals using body plethysmography. In addition, passively sensitized precision-cut lung slices (PCLS) were used to determine the effect of SP-D on allergen-induced histamine release.

Results SP-D inhibited the allergen-induced early airway response and reduced airway hyperresponsiveness compared with placebo. Eosinophilia in bronchoalveolar lavage and lung tissue was reduced after SP-D treatment, possibly by reducing eotaxin levels in the lung. Furthermore, SP-D treatment reduced the allergen-induced histamine release from PCLS.

Conclusions These data suggest that SP-D not only reduces allergen-induced eosinophilic inflammation and airway hyperresponsiveness but also provides protection against early airway obstruction by inhibition of early mediator release.

Keywords allergic inflammation, asthma, collectins, lung function, pulmonary surfactant

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Introduction

It has been suggested that pulmonary surfactant plays a role in asthma [1], both by exerting biophysical effects on airway calibre and by modulating the immune response to inhaled allergens [2]. Among the various surfactant constituents, surfactant protein A (SP-A) and surfactant protein D (SP-D), both of which are members of a family of innate immune molecules called collectins [3], play an important role in innate host defence and immunomodulation [4].

SP-A and SP-D bind to structures expressed by a wide variety of microorganisms and to particles and to aller-

gens [4, 5]. Besides their well-established role in innate immunity, there is accumulating evidence that these collectins also have an important function in regulating adaptive allergen-specific immune responses. For example, Wang et al. [6, 7] showed that these collectins can block the interaction between glycosylated allergens and specific IgE. Furthermore, it was recently reported that SP-A inhibits the maturation of dendritic cells with decreased major histocompatibility complex class II and CD86 expression, which resulted in a reduced T cell allostimulatory ability [8]. SP-A and SP-D have been shown to inhibit lymphocyte proliferation and IL-2

production [9, 10]. Cheng et al. [11] described that SP-A suppressed the production and release of IL-8 by eosinophils. Overall, these collectins appear to decrease the degree of allergic inflammation *in vitro* through interaction with dendritic cells, lymphocytes, eosinophils, mast cells, and the allergens themselves [1, 7, 12].

Besides the well-studied functions of SP-A and SP-D *in vitro*, both collectins (in particular SP-D) have been suggested to reduce the allergen-induced inflammation in mouse asthma models. A well-established mouse model of allergic hypersensitivity is the sensitization and challenge with *Aspergillus fumigatus*, a ubiquitous fungus of clinical importance [13, 14]. It can produce asthma and allergic bronchopulmonary aspergillosis, which is characterized by elevated IgE, eosinophilia, and airway hyper-responsiveness [15, 16]. It has been shown that SP-D, like SP-A, binds carbohydrate components of *A. fumigatus* [17, 18]. Two recent studies in mice that used this allergy model reported a reduction of blood eosinophilia and a shift from T-helper type 2 (Th2) to Th1 cytokine profile in spleen supernatants after treatment with these collectins, in particular, SP-D [19, 20]. A decrease in bronchial hypersensitivity after treatment with SP-D was observed by Strong et al. [20] and Takeda et al. [21]. A human recombinant 60 kDa truncated fragment of SP-D and rat recombinant SP-D, respectively, effectively down-regulated the allergic inflammatory reaction. Interestingly, the observed effects appeared to be mediated through the carbohydrate recognition domain (CRD) of the collectin [21], but the effectiveness of the truncated fragment of SP-D alone suggested that the quaternary structure of a dodecamer is not required [20].

Apart from airway inflammation with eosinophils and bronchial hyperresponsiveness, asthma also typically involves episodic bronchial obstruction. So far, investigations on the effect of SP-D on functional consequences during the early airway response have not been reported. Therefore, we investigated whether a recombinant fragment of human SP-D (rfhSP-D) is effective in reducing the allergen-induced early-phase bronchoconstriction by measuring lung functions in *A. fumigatus*-sensitized BALB/c mice. In the same animals, the developing airway hyperresponsiveness was determined 48 h after allergen inhalation and the pulmonary inflammatory response was characterized by investigation of bronchoalveolar lavage (BAL) differential cell count, inflammatory cell influx into lung tissue, and cytokine expression in BAL and lung tissue.

As airway hyperresponsiveness and allergic inflammation were investigated 48 h after allergen challenge, it was not possible to determine immediate mediator release in these animals. Therefore, precision-cut lung slices (PCLS) from naïve mice were obtained and passively sensitized with serum from *A. fumigatus*-sensitized mice. These PCLS were used to determine the effects of SP-D on allergen-induced immediate histamine release.

Material and methods

Animals

Female BALB/c mice (Charles River, Sulzfeld, Germany) that weighed about 20 g at the time of the first sensitization were kept in a pathogen-free rodent facility (Fraunhofer ITEM, Hannover, Germany). They had free access to food and water. Care and use of experimental animals was strictly performed according to the 'Guide for the Care and Use of Laboratory animals' published by the National Institute of Health (NIH publication 85-23, revised 1985).

Study design

BALB/c mice were randomized to three groups (control, placebo, SP-D, $n = 12-16$). Not all animals were evaluable for the entire set of parameters, but at least 10 animals were analysed for each parameter. Animals were sensitized (day 0), boosted (day 14), treated (days 19 and 20), and challenged (day 21) as follows: all mice were sensitized intraperitoneally with *A. fumigatus* extract (Afu). All animals except those from the control group were boosted with Afu aerosol. The control group received saline instead. The SP-D group was treated intranasally with a recombinant fragment of human SP-D, and the placebo group and control group with saline. On day 21, the mice of the placebo and SP-D groups were challenged with inhaled Afu-extract. The controls inhaled diluent.

Lung function was measured before, during, and after Afu-challenge in intubated spontaneously breathing mice. Forty-eight hours after the challenge, airway responsiveness to inhaled methacholine was tested. Then animals were killed, blood was taken to determine total serum IgE, a lung lavage was performed for cell differentials and cytokine determination, and the lungs were removed for cytokine determination in lung tissue.

Sensitization and collectin treatment

All mice were sensitized on day 0 by an intraperitoneal and subcutaneous injection of soluble *A. fumigatus* antigens (5 µg each, Greer Laboratories Inc., Lenoir, NC, USA) dissolved in 0.1 mL incomplete Freund's adjuvant. On day 14, the mice were non-invasively boosted in a closed chamber by exposure for 12 min to an aerosol generated from 1% Afu dissolved in saline (SteriPharm, Berlin, Germany) using a Pari LC Star (2.8 µm mass median diameter, Pari Werke, Starnberg, Germany). On 2 consecutive days before Afu challenge (38 and 14 h before challenge), rfhSP-D (0.15 mg/kg; 3 µg in 50 µL 0.9% NaCl per mouse) was applied intranasally to anaesthetized mice (1.35 mg ketamin and 0.027 mg xylazin per mouse in 0.15 mL saline intraperitoneally). The control group and

the placebo group were treated in the same way with 0.9% saline solution.

The rfhSP-D representing residues 179–355 of the mature protein sequence is composed of the trimeric α -helical coiled-coil neck region and three C-type lectin domains of human SP-D and was expressed in *Escherichia coli* and purified from inclusion bodies using a slightly modified protocol described by Strong *et al.* [20]. Transformed *E. coli* cells were kindly provided by Ken B. Reid (Oxford, UK). Endotoxin levels were measured by the Limulus Amebocyte Lysate Assay (BioWhittaker, Walkersville, MD, USA). The rfhSP-D used for the experiments contained 1 pg endotoxin per microgram rfhSP-D. Additional experiments demonstrated that neither the amount of rfhSP-D that was administered to the animals nor the amount of endotoxin that was contained in this rfhSP-D preparation were able to induce a change in total BAL cell count or BAL cell differential when administered in a similar way to naïve mice (data not shown). The amount of rfhSP-D that was administered was chosen according to a previous study by Takeda *et al.* [21].

Challenge and lung function measurements

Allergen-induced early airway response. The technique for lung function measurement has been described recently [22]. On day 21, mice were anaesthetized with intraperitoneal injections of metomidate (38–60 mg/kg) and fentanyl (0.02–0.04 mg/kg) with minimal supplementations as required. Mice were orotracheally intubated under visual control with a standard 20 G $\times$ 32 mm (11/4") Abbocath[®]-T cannula (Abbott, Sligo, Ireland). The intubated, spontaneously breathing animal was then placed in a supine position in a thermostat-controlled body plethysmograph (type 871, designed by Fraunhofer ITEM in cooperation with HSE-Harvard Apparatus, March-Hugstetten, Germany). The orotracheal tube was directly attached to a pneumotachograph (PTM T16375) connected to a Validyne DP 45–14 (both HSE-Harvard) to determine tidal flow. To measure transpulmonary pressure (P_{TP}), a water-filled PE-60 tube was inserted into the oesophagus to the level of the midthorax and coupled to a pressure transducer (model P75, HSE-Harvard). Lung resistance (R_L) was calculated from the integrated signals of P_{TP} and tidal flow [22]. The resistance of the orotracheal tube (0.63 cm H₂O/mL/s) was subtracted. R_L , together with other basic respiratory parameters was continuously recorded using commercial software (HEM 3.4, Notocord, Croissy, France). R_L values were averaged in 10 s segments and maximum R_L values were taken and expressed as percent changes from corresponding baseline values, which were taken as 0%. After recording baseline values, mice were challenged for 10 min with either Afu-aerosol (2% in Water G Chromasolv[®], Product no. 34877, Sigma-

Aldrich, Deisenhofen, Germany; placebo and collectin-treated group) or vehicle (Water G. Chromasolv[®]; control group). Aerosols were generated by a computer-controlled, jet-driven aerosol generator system (Bronchy type III, particle size 2.98 μ m mass median aerodynamic diameter, Fraunhofer ITEM, licensed by Buxco, NY, USA) as previously described for rats [22] and mice [23]. The total inhaled dose (8 μ g Afu) administered via the orotracheal tube was calculated and controlled by a computerized dose-control system based on the continuously measured respiratory volume per minute and aerosol concentration. To prevent hypoxia, the animals were provided with a stream of 30% oxygen during measurements. After the measurements, mice were removed from the chamber and extubated after recovery from anaesthesia.

Airway hyperresponsiveness. Lung function was performed as described above. Forty-eight hours after Afu challenge and after recording baseline values, airway responsiveness to aerosolized methacholine chloride (calculated inhaled doses of 0.10, 0.15, 0.22, 0.33, 0.5, 0.75, 1.13, 1.69, and 2.53 μ g; MCh; Sigma) was assessed [22, 23]. Aerosols were generated from dissolved MCh (5% in saline) as described for the Afu challenge, and lung function variables were registered continuously. Three to 5 min were allowed between doses to permit a return to near-baseline values. Increasing doses of MCh were applied until resistance reached at least 200% of the baseline value. The effective dose of MCh that elicited an increase of R_L above baseline of 200% (ED₂₀₀) was calculated from the individual dose-response curve.

Blood sampling and bronchoalveolar lavage

After mice were killed using an overdose of pentobarbital, blood was taken via the vena cava caudalis, serum isolated, aliquoted, and stored at –20 °C for IgE-antibody determination. Next, BAL fluid was recovered from the distal trachea according to standard procedures using 2 $\times$ 0.8 mL aliquots of saline [23]. The lavage was centrifuged and the aliquoted supernatant was frozen in liquid nitrogen and stored at –70 °C for cytokine determination. The cell pellets were pooled and resuspended in phosphate-buffered saline (PBS). Total and differential cell counts were determined.

Preparation of lung tissue

Following BAL, the right lungs were dissected, carefully removed, and pulverized in liquid nitrogen. The lysing solution contained 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, protease inhibitor (Roche Diagnostics, Mannheim, Germany), and 0.2% SDS, 1% Triton X-100, and 1% Igepal (Sigma). Ultrasonication was performed with 100 mg powder resuspended in 2 mL lysing solution. The

lysate was centrifuged at 2000 *g* for 30 min, the supernatant aliquoted, and stored at -80°C until further analysis. Total protein content of the lysates was measured using a Bio-Rad protein assay (Bio-Rad Laboratories, München, Germany).

The left lungs were filled with Tissue Tek[®] O.C.T. compound (Electron Microscopy Sciences, Washington, PA, USA) 1 : 10 diluted in PBS, frozen in liquid nitrogen, and stored at -80°C for immunohistochemistry.

Histologic and immunohistochemistry studies

Lung sections (7 μm) were cut, dried overnight, and fixed with acetone. A Giemsa stain was performed for identification of inflammatory cell influx.

Furthermore, cells containing major basic protein (MBP) in lung sections were identified by immunohistochemistry. After rehydration in PBS, the sections were stained overnight with a rat anti-mouse MBP IgG (kindly provided by Drs N. and J. J. Lee, Mayo Clinic, Scottsdale, AZ, USA) diluted 1 : 500 in 4% donkey serum or rat IgG-purified Ig (Sigma) for negative control. This was followed by two washes and staining with Alexa Fluor[®] 594-labelled donkey anti-rat IgG (1 : 400) for 1 h at room temperature. The slides were examined with a Zeiss Axioskop (Carl Zeiss, Göttingen, Germany) equipped with a filter system for fluorescence detection.

Immunoglobulin E and cytokine determination

Mouse serum was tested for IgE antibodies using a commercially available ELISA (Becton Dickinson, Heidelberg, Germany) according to the manufacturer's instruction.

BAL supernatants and lung lysates were tested for IL-5 and eotaxin using commercially available ELISAs (R & D Systems, Wiesbaden, Germany). The tests were performed according to the manufacturer's instructions. Cytokine concentrations in lung tissue were normalized per milligram total protein.

Detection of exogenous recombinant human and endogenous murine SP-D in bronchoalveolar lavage fluid

BAL supernatants were analysed for exogenous human SP-D using a commercially available ELISA system specific for human SP-D (BioVendor, Heidelberg, Germany). The tests were performed according to the manufacturer's instructions by using a standard curve ranging from 1.56 to 100 ng/mL. Specificity for human SP-D was confirmed as no SP-D was detected in undiluted BAL samples from control and placebo treated animals. Spike experiments with rfhSP-D in BAL samples from control mice revealed a recovery of $95.4 \pm 5.0\%$ ($n = 3$).

Using a second, custom-made sandwich ELISA system, total SP-D (endogenous murine SP-D and exogenous rfhSP-D) was determined. For this assay, a 96-well plate was coated overnight with a monoclonal anti-SP-D antibody (VIF11, BMA, Augst, Switzerland) at a concentration of 2 $\mu\text{g/mL}$ diluted in carbonate buffer (100 mM Na_2CO_3 ; 100 mM NaHCO_3 , pH 9.5). After blocking with 1% bovine serum albumin, 5% sucrose in PBS with 0.05% NaN_3 for 1 h, diluted samples or standards (recombinant mouse SP-D (mSP-D), kindly provided by Dr Dennis Voelker, National Jewish Medical and Research Center, Denver, CO, USA) ranging from 3.125 to 400 ng/mL were added for 1 h. Bound SP-D was detected with a biotinylated monoclonal anti-SP-D antibody (IIE11, BMA) at a concentration of 2 $\mu\text{g/mL}$, followed by incubation with streptavidin-conjugated horseradish-peroxidase (R&D Systems). Finally, substrate solution (R&D Systems, Abingdon, UK) was added and the reaction was stopped by adding 2 N H_2SO_4 . Measurement was immediately performed on a microplate reader at 450 nm. The antibodies were cross-reactive for rat, human, and mouse SP-D. Using mSP-D as a standard, spike experiments with a defined amount of rfhSP-D yielded values that were 11 times higher than the actual amount of rfhSP-D that was contained. Therefore, the sensitivity of this system was 11 times higher for rfhSP-D compared with mSP-D.

Histamine release from precision-cut lung slices

Mouse PCLS were prepared as described previously [24, 25]. Shortly, after injection of pentobarbital (Narcoren, Pharmazeutische Handelsgesellschaft mbH, Garbsen, Germany), the trachea was cannulated and the animals exsanguinated via the *vena cava inferior*. Through the cannula the lung was filled with a low-melting point agarose solution (0.75%, final concentration). In order to solidify the agarose and harden them for cutting, the lungs were placed on ice for 10 min. The lobes were separated and tissue cores were prepared with a rotating sharpened metal tube (diameter 10 mm). These cores were cut into 220 μm thick slices with a Krumdieck tissue slicer (Alabama Research and Development, AL, USA).

Fresh mouse PCLS were incubated in 300 μL medium and treated overnight with 1% serum from mice that had been actively sensitized with *A. fumigatus* by standard protocols [26]. Serum from mice treated with saline solution was used as control. The rfhSP-D (10 $\mu\text{g/mL}$) was pre-incubated for 1 min with the serum, before incubating with the PCLS.

Slices were challenged with *A. fumigatus* extract (1 mg/mL) for 10 min. In some experiments, the rfhSP-D was only added together with the allergen. After allergen challenge, the supernatant was collected, snap-frozen in liquid nitrogen, and stored at -80°C until determination of histamine. Histamine was detected by ELISA (IBL,

Hamburg, Germany) according to the manufacturer's instructions. The slices were dried and weighed to standardize the amount of histamine to the weight of the respective PCLS.

Statistics

Values are reported as mean $\pm$ SEM. Significant differences between groups were compared using a non-parametric Mann-Whitney *U*-test. Correlations were assessed by the Spearman's rank test. Values of $P < 0.05$ were considered significant. Statistics were calculated using GraphPad Prism Version 3.02 (GraphPad Software Inc., San Diego, CA, USA).

Results

Allergen-induced early airway response was weaker in SP-D-treated mice

In placebo-treated animals, challenge with Afu led to an R_L of 339% above baseline (0%). SP-D-treated mice demonstrated a significantly lower R_L in comparison (Fig. 1). Animals in the SP-D group had a resistance of only 96% above baseline. As expected, challenge with vehicle did not increase resistance in the control group, which was 17% above baseline.

Airway hyperresponsiveness was reduced in SP-D-treated mice and correlated with lung resistance

Hyperresponsiveness of Afu-sensitized mice 48 h after challenge was tested using methacholine chloride by inhalation. Placebo-treated mice were hyperresponsive to MCh with a fourfold lower ED_{200} compared with

vehicle-challenged control mice (0.52 ± 0.14 vs. 2.17 ± 0.47 μ g MCh, $P = 0.0017$, Fig. 2). Mice treated with SP-D were less sensitive to MCh as they tolerated a significantly higher dose of MCh (0.92 ± 0.18 μ g MCh) than placebo-treated animals (Fig. 2).

The early airway response measured as an increase in R_L correlated well with airway hyperresponsiveness measured 48 after allergen challenge ($r = -0.66$, $P < 0.0001$, Fig. 3)

Fewer eosinophils in bronchoalveolar lavage and lung tissue following SP-D treatment

Table 1 shows the leucocyte composition in BAL fluid for treatment and control groups. A total cell count that was significantly lower than placebo, but comparable to control animals, was observed for the SP-D-treated group (Table 1). In this group, a significant reduction of eosinophil cell number was observed (Table 1). In the control group, the number of eosinophils was zero while in the placebo group the number of eosinophils was 0.19×10^6 (Table 1). There was a significant correlation between the number of eosinophils and the increase of R_L ($r = 0.72$, $P < 0.0001$) and an inverse correlation between eosinophil number and ED_{200} ($r = -0.63$, $P < 0.0001$).

Treatment with SP-D reduced the number of macrophages in BAL compared with placebo (Table 1). Neither neutrophil nor lymphocyte numbers differed in SP-D-treated compared with placebo-treated animals.

Histologic examination of lung sections from sensitized mice after allergen challenge showed a massive influx of inflammatory cells including eosinophils in the peribronchial and perivascular tissue (Figs 4a and d). SP-D treatment markedly reduced the cell influx and the number of eosinophils (Figs 4b and e). In sections from sensitized

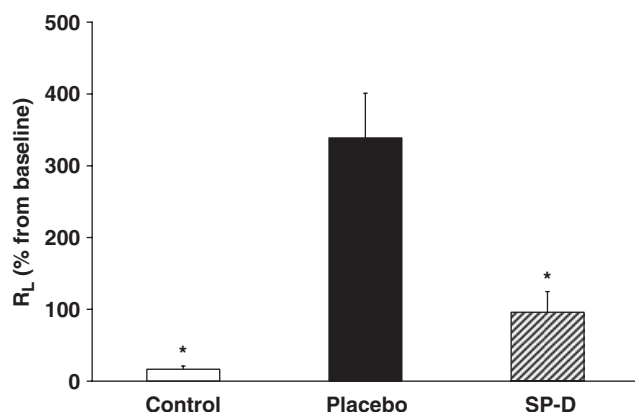


Fig. 1. Comparison of lung resistance (R_L) of placebo- and SP-D-treated mice. R_L was determined directly before, during, and after *Aspergillus* challenge. Values are given as the mean percentage $\pm$ SEM that R_L increased compared with baseline (0%). The asterisks represent significant differences ($P < 0.05$) compared with placebo.

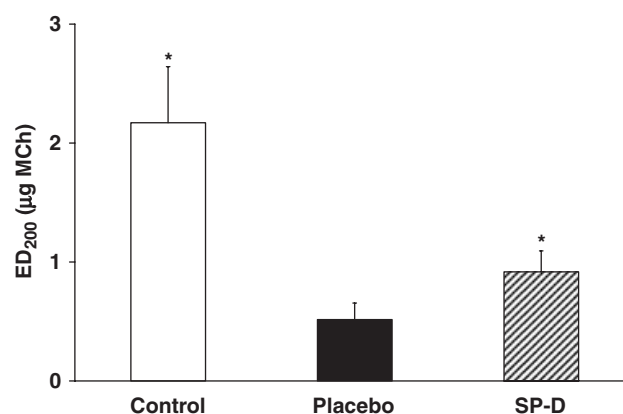


Fig. 2. Comparison of hyperresponsiveness to methacholine chloride (MCh) of placebo- and SP-D-treated mice. The MCh inhalation dose at which the increase in lung resistance reached 200% above baseline was determined. Values are given as mean dose $\pm$ SEM of MCh. The asterisks represent significant differences ($P < 0.05$) compared with placebo.

mice challenged with vehicle, very few inflammatory cells were observed and eosinophils were rarely detected (Figs 4c and f).

Serum immunoglobulin E level was comparable in all groups

Total IgE was determined in the serum of collectin-treated, placebo-treated, and control mice using ELISA. The serum level of the SP-D-treated and control groups was comparable with placebo (placebo $10\,401 \pm 1589$ IU/mL; SP-D 8496 ± 1536 IU/mL; control 6494 ± 1450 IU/mL).

Cytokines in bronchoalveolar lavage and lung tissue after SP-D treatment

IL-5 and eotaxin were analysed in BAL fluid and lung tissue (lysate). All groups showed comparable IL-5 levels

in BAL and lung tissue (Figs 5a and b). In BAL, the eotaxin level was not different between SP-D-treated and placebo-treated mice (Fig. 5c). In lung tissue, however, the eotaxin level was significantly reduced in SP-D-treated mice compared with placebo treated animals (Fig. 5d). Eotaxin levels in BAL ($r = 0.741$, $P < 0.0001$) and lung tissue (0.573 , $P = 0.0006$) correlated with the number of eosinophils.

Levels of exogenous and endogenous SP-D in bronchoalveolar lavage fluid

RfhSP-D was detectable at low levels (83.5 ± 21.6 ng/mL) in animals 62 h after they received their second dose of rfhSP-D. BAL fluids of control mice and placebo-treated mice were negative for rfhSP-D (Fig. 6a). Measurement of total SP-D (endogenous mSP-D plus exogenous rfhSP-D) in all samples demonstrated increased levels of SP-D in placebo-treated mice compared with control mice ($P < 0.01$). Interestingly, SP-D treatment significantly reduced levels of total SP-D compared with placebo-treated mice ($P < 0.05$, Fig. 6b). Importantly, endogenous murine SP-D levels were even lower in the rfhSP-D-treated group, because one has to consider the contribution of exogenous human SP-D in these samples, which was estimated to account for about 10% of the total SP-D signal.

Levels of total SP-D from all BAL samples correlated with BAL eosinophil numbers ($r = 0.7316$, $P < 0.0001$), percentage of eosinophils ($r = 0.7447$, $P < 0.0001$), early airway response ($r = 0.6499$, $P < 0.0001$), and correlated inversely with airway hyperreactivity ($r = -0.5685$, $P = 0.0004$).

SP-D reduced histamine release from precision-cut lung slices

The allergen-induced histamine release from PCLS that were passively sensitized with serum from *A. fumigatus*-sensitized mice was higher than that from unsensitized PCLS ($P < 0.05$, Fig. 7). Treatment with SP-D during sensitization reduced the histamine release from sensitized PCLS ($P < 0.05$). No change in histamine release was detected when rfhSP-D was given together with the allergen (data not shown).

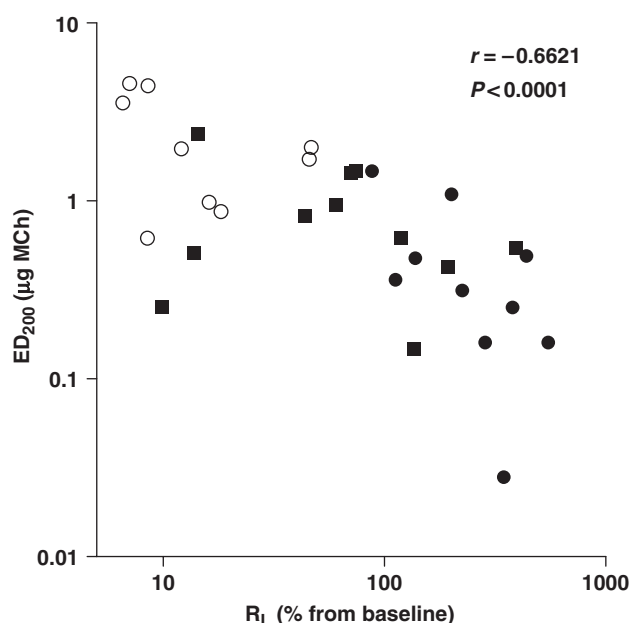


Fig. 3. Correlation of early airway response with airway hyperresponsiveness to methacholine chloride. Spearman R is shown for data obtained from the control group ($\circ$), placebo-treated group ($\bullet$), and the SP-D-treated group ($\blacksquare$).

Table 1. Cellular composition of bronchoalveolar lavage fluid^a

Group	Total cells	Eosinophils		Macrophages		Neutrophils		Lymphocytes	
	(10^6)	(10^6)	(%)	(10^6)	(%)	(10^6)	(%)	(10^6)	(%)
Control $n = 10$	0.24 ± 0.04	$0.00 \pm 0.00^*$	$2.0 \pm 1.0^*$	0.21 ± 0.04	$88.6 \pm 1.6^*$	$0.02 \pm 0.01^*$	$6.5 \pm 1.4^*$	$0.01 \pm 0.00^*$	2.7 ± 0.6
Placebo $n = 16$	0.39 ± 0.06	0.19 ± 0.04	39.9 ± 5.6	0.17 ± 0.02	53.1 ± 5.5	0.00 ± 0.00	1.1 ± 0.4	0.02 ± 0.00	4.5 ± 0.8
SP-D $n = 15$	$0.21 \pm 0.04^*$	$0.07 \pm 0.02^*$	27.1 ± 4.2	$0.12 \pm 0.02^*$	63.6 ± 4.3	0.01 ± 0.00	3.9 ± 1.3	0.01 ± 0.00	5.4 ± 1.0

^aValues are given as mean $\pm$ SEM.

*Significant differences compared with placebo group.

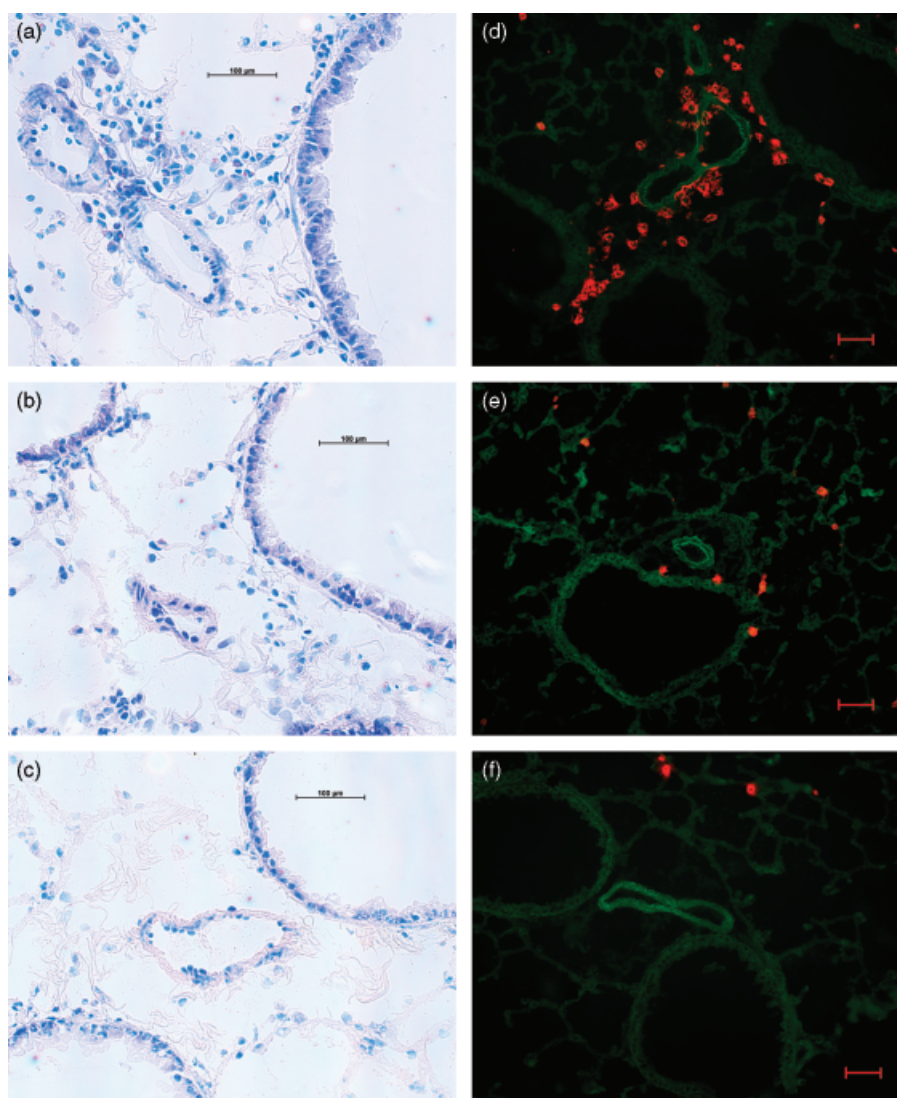


Fig. 4. Effect of recombinant fragment of human SP-D treatment on influx of inflammatory cells was investigated by histologic examination of Giemsa-stained lung sections (a–c). Infiltration of perivascular and peribronchial tissue by eosinophils was demonstrated after staining with a rat anti-mouse major basic protein antibody and Alexa Fluor[®] 594-labelled donkey anti-rat IgG in the placebo-treated group (a, d) SP-D-treated group (b, e), and in the control group (c, f). Bars = 100 µm.

Discussion

The surfactant protein SP-D has been reported to reduce bronchial hyperresponsiveness, blood eosinophilia, and Th2 cytokines in models of allergic asthma [19–21, 27, 28]. The studies performed so far have focused on the inflammatory response and on airway responsiveness to methacholine. The early airway response starting immediately after allergen exposure is an important pathophysiologic feature of allergic asthma that can be effectively treated by fast-acting substances like β_2 -agonists. This is the first study that demonstrates the beneficial effects of SP-D on allergen-induced bronchoconstriction during the early airway response.

One possible explanation for the inhibition of the allergen-induced bronchoconstriction is that the release

of bronchoconstrictive mediators from airway cells was inhibited. Both SP-D and SP-A have been found to inhibit binding of allergen-specific IgE to *A. fumigatus* allergen extracts [17] and to mite allergen extracts by either steric hindrance or competitive binding [6]. In line with this, it was demonstrated that both SP-D and SP-A can inhibit mediator release from human basophils and whole blood cells *in vitro* [7, 17]. The decreased histamine release from sensitized PCLS challenged with *A. fumigatus* allergen is in line with these observations. We conclude that mast cells as the major source for histamine release are less responsive to allergen-induced degranulation and mediator release after treatment with SP-D. This might explain the positive *in vivo* effects of SP-D on the early-phase bronchoconstriction.

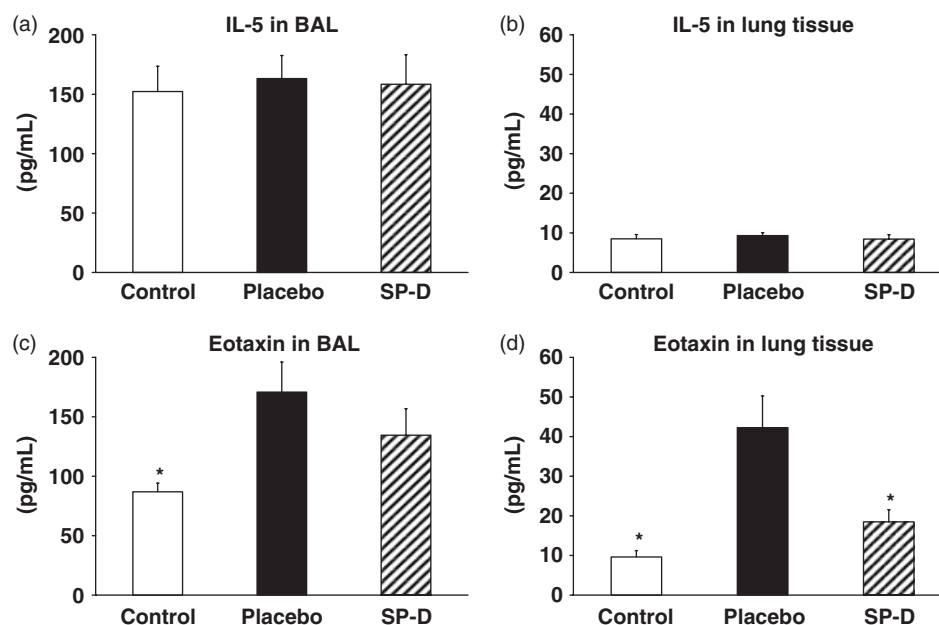


Fig. 5. Comparison of cytokine production in bronchoalveolar lavage (BAL) and lung tissue. Forty-eight hours after allergen challenge, IL-5 (a) and eotaxin (c) were determined in BAL fluid of the different treatment groups. Values are given as mean pg/mL $\pm$ SEM. IL-5 (b) and eotaxin (d) were also determined in lung tissue (lysate) and are given as mean pg/mg total protein $\pm$ SEM. The asterisks represent significant differences ($P < 0.05$) compared with placebo.

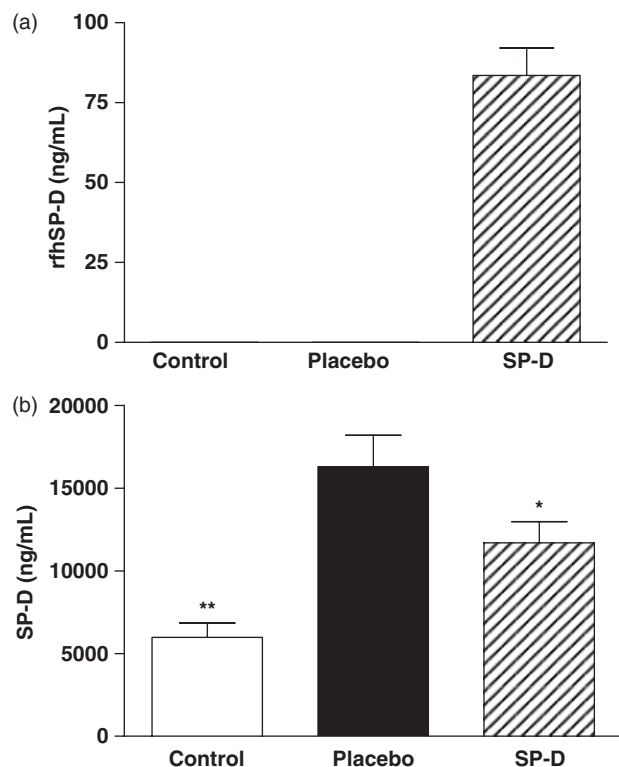


Fig. 6. Exogenous human SP-D (rhSP-D) in bronchoalveolar lavage (BAL) fluid detected by human-specific ELISA system in rhSP-D-treated mice (a). Endogenous murine SP-D and exogenous rhSP-D were determined together by a non-species specific SP-D ELISA in BAL fluid with murine SP-D as a standard (b). The asterisks represent significant differences ($*P < 0.05$; $**P < 0.01$) compared with placebo.

The observed effects of SP-D on mast cells might either depend on the direct interaction of SP-D with these cells or on the interaction of SP-D with other factors involved, namely allergens or Ig. It has been demonstrated that glycosylated allergens such as *A. fumigatus* allergen extracts or *A. fumigatus* conidia were unspecifically bound by CRDs of the collectins [17, 18]. The fact that rhSP-D was only capable of inhibiting histamine release if present during the sensitization period but not if present during the allergen challenge argues for a direct interaction of rhSP-D with allergen-specific Ig. Accordingly,

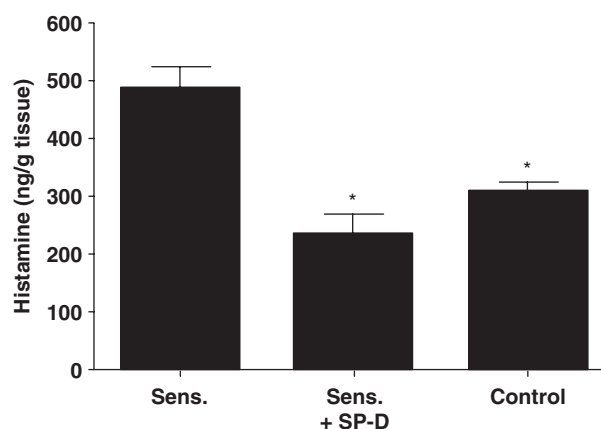


Fig. 7. Histamine release from precision-cut lung slices (PCLS). PCLS were not sensitized (Control) or passively sensitized with serum from *Aspergillus fumigatus*-sensitized mice (Sens.) and subsequently stimulated with *A. fumigatus* allergen. The presence of rhSP-D significantly reduced the histamine release from passively sensitized PCLS ($P < 0.05$).

Nadesalingam et al. [29] recently demonstrated SP-D binding to various types of antibodies including IgE and IgG. Therefore, the beneficial effects of exogenous SP-D on early bronchoconstriction *in vivo* and on histamine release *in vitro* are most likely explained by a direct interaction of SP-D with these Igs.

Clark et al. [30] showed that after intranasal administration of a 10 µg dose of rfhSP-D, only 35–40% of the administered dose was recovered by BAL after 1 h and that it was virtually undetectable after 21 h. In another study, only 60% of exogenous SP-D (2–5 µg) and 80% of exogenous SP-A were recovered by BAL 1 h after instillation into rat lungs. Recovery of SP-D was further decreased to 35% at 1 h during LPS-induced inflammation while SP-A recovery was unaffected by LPS [31]. By using a human-specific SP-D ELISA, we detected approximately 2% of the exogenous rfhSP-D in BAL fluid 62 h after the last dose was given. These results demonstrate that most of the exogenous collectin is either cleared from the airway lumen or bound in a way that prevents detection in BAL fluid at the time when the allergen is administered.

Looking at endogenous murine SP-D, we observed that allergen challenge in sensitized animals leads to increased levels of SP-D. This is in line with previous data in asthma mouse models [20, 21], and also fits to data from our recent study with asthma patients [32]. In the present study, total SP-D in BAL fluid, which mostly reflects endogenous murine SP-D, correlated with eosinophil numbers, early airway response, and airway hyperresponsiveness, demonstrating a strong relation to the disease severity.

Interestingly, we found that treatment with exogenous rfhSP-D significantly reduced the increased endogenous SP-D levels. This was rather unexpected and might be explained by either an interaction of exogenous SP-D with endogenous SP-D synthesis and secretion or an inhibition of the allergic inflammation, which in turn leads to decreased endogenous SP-D. The fact that high levels of endogenous SP-D were associated with increased inflammation whereas the administration of exogenous SP-D resulted in a decrease of the allergic inflammation might further indicate that the endogenous SP-D, although present at high levels, was functionally inactivated. Previous studies have shown proteolytic effects of *A. fumigatus* allergens [33] and there is a strong influx of inflammatory proteins during the allergic inflammation [34], both of which could attenuate SP-D function, either by cleavage of the protein or by binding to inflammatory proteins. Further studies have to determine whether the SP-D that is present at the site of allergic inflammation is functionally inactivated and therefore, needs to be replaced by exogenous SP-D.

In addition to the effect of rfhSP-D on the early airway response, airway hyperresponsiveness 48 h after allergen inhalation was also analysed. Mice treated with rfhSP-D

demonstrated a reduced airway hyperresponsiveness. The reduced airway hyperresponsiveness is in line with results recently reported by Strong et al. [20], who determined enhanced pause (P_{enh}) as a measure of airway function in response to MCh in surfactant protein-treated mice. The rfhSP-D molecule was the same as the one used in this study. However, challenge, treatment regimen, and measurement technique were not identical. Strong et al. treated mice after challenge (rescue effect), while we showed a prophylactic effect. Takeda et al. [21] also showed a protective effect of SP-D on the development of airway hyperresponsiveness in ovalbumin-sensitized BALB/c mice treated 24 and 5 h before challenge. The surfactant protein used for treatment was a rat recombinant SP-D molecule instead of a human recombinant SP-D fragment as in our study. Interestingly, the observed effect of SP-D molecules clearly depends on the functional integrity of the CRD, because Takeda et al. also used mutant SP-D with an altered CRD, which had no effect. In contrast, association of SP-D trimers into the dodecameric quarternary structure is not necessary for the observed effects. Our data and the results of others [19, 20] provide evidence that a recombinant fragment of SP-D with an intact CRD but lacking the collagen domain except for a short region of the collagen stalk is sufficient to inhibit both the early- and late-phase allergic reaction in models of allergic asthma.

Treatment with rfhSP-D inhibited the allergic inflammatory response. Leucocyte numbers and eosinophil and macrophage cell counts were significantly lower in the BAL of mice treated with rfhSP-D. The reduced number of eosinophils correlated with improved bronchoconstriction in the collectin-treated animals. A recent study also reported reduction of eosinophil cell number in BAL compared with controls following prophylactic SP-D treatment of ovalbumin-sensitized mice [21]. Furthermore, in previous studies, a lower number of eosinophils was found in peripheral blood of collectin-treated mice sensitized to *A. fumigatus* or house dust mite allergen [19, 20, 27, 28].

To further assess the influence of rfhSP-D on the eosinophilic lung inflammation, we investigated expression of relevant cytokines (IL-5 and eotaxin) in BAL and lung tissue of treated and untreated mice. We found no influence of rfhSP-D on IL-5 levels in either BAL or lung tissue (lysate). Takeda et al. [21] also reported that IL-5 levels in BAL were unaffected by treatment with rat recombinant SP-D. Both studies investigated BAL cytokine levels 48 h after allergen challenge. In contrast, Madan et al. [19] found significantly lower IL-5 levels in collectin-treated compared with placebo-treated mice. However, they analysed IL-5 levels in spleen cells that were cultured for 72 h *in vitro*, while our group and Takeda's determined IL-5 in BAL directly after killing the animals. In contrast to IL-5, eotaxin levels were reduced in

lung tissue of rfhSP-D-treated mice compared with placebo-treated animals. The reduced eotaxin levels strongly correlated with the lower eosinophil cell count in BAL of collectin-treated animals. Eotaxin is synthesized and secreted by a variety of cells. It is believed that macrophages, epithelial cells, and fibroblasts are major sources of eotaxin release [35, 36]. Therefore, treatment with rfhSP-D might down-regulate eotaxin production and release from these cells. Interestingly, the number of macrophages in BAL of mice treated with rfhSP-D was reduced, which might account for lower eotaxin levels. So far, this is the first study to report on the influence of collectins on eotaxin levels. Future studies need to investigate the production site of eotaxin and the potential influence of rfhSP-D on eotaxin expression in this allergen model.

In conclusion, the present findings demonstrate that rfhSP-D is capable of reducing the early airway response upon allergen challenge in an *A. fumigatus* model of allergic asthma. The SP-D-mediated effects on the early airway response most likely result from an inhibition of cellular mediator release. In addition, rfhSP-D decreases airway hyperresponsiveness and eosinophilic inflammation possibly by reducing eotaxin release. We suggest that rfhSP-D may have therapeutic potential in the treatment of both the early airway response and the subsequent inflammatory phase in allergic asthma.

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References

- Hohlfeld JM, Erpenbeck VJ, Krug N. Surfactant proteins SP-A and SP-D as modulators of the allergic inflammation in asthma. *Pathobiology* 2002; **70**:287–92.
- Hohlfeld JM. The role of surfactant in asthma. *Respir Res* 2002; **3**:4.
- Hoppe HJ, Reid KB. Collectins – soluble proteins containing collagenous regions and lectin domains – and their roles in innate immunity. *Protein Sci* 1994; **3**:1143–58.
- Crouch E, Wright JR. Surfactant proteins A and D and pulmonary host defense. *Annu Rev Physiol* 2001; **63**:521–54.
- Crouch E. Surfactant protein-D and pulmonary host defense. *Respir Res* 2000; **1**:93–108.
- Wang JY, Kishore U, Lim BL, Strong P, Reid KBM. Interaction of human lung surfactant proteins A and D with mite (*Dermatophagoides pteronyssinus*) allergens. *Clin Exp Immunol* 1996; **106**:367–73.
- Wang JY, Shieh C-C, You P-F, Lei H-Y, Reid KBM. Inhibitory effect of pulmonary surfactant proteins A and D on allergen-induced lymphocyte proliferation and histamine release in children with asthma. *Am J Respir Crit Care Med* 1998; **158**:510–8.
- Brinker KG, Garner H, Wright JR. Surfactant protein A modulates the differentiation of murine bone marrow-derived dendritic cells. *Am J Physiol Lung Cell Mol Physiol* 2003; **284**:L232–41.
- Borron P, Veldhuizen RA, Lewis JF *et al.* Surfactant associated protein-A inhibits human lymphocyte proliferation and IL-2 production. *Am J Respir Cell Mol Biol* 1996; **15**:115–21.
- Borron PJ, Crouch EC, Lewis JF, Wright JR, Possmayer F, Fraher LJ. Recombinant rat surfactant-associated protein D inhibits human T lymphocyte proliferation and IL-2 production. *J Immunol* 1998; **161**:4599–603.
- Cheng G, Ueda T, Nakajima H *et al.* Suppressive effects of SP-A on ionomycin-induced IL-8 production and release by eosinophils. *Int Arch Allergy Immunol* 1998; **117** (Suppl. 1):59–62.
- Hohlfeld J, Fabel H, Hamm H. The role of pulmonary surfactant in obstructive airways disease. *Eur Respir J* 1997; **10**:482–91.
- Kauffman HF, Tomee JF, van der Werf TS, de Monchy JG, Koeter GK. Review of fungus-induced asthmatic reactions. *Am J Respir Crit Care Med* 1995; **151**:2109–15.
- Kurup VP, Mauze S, Choi H, Seymour BW, Coffman RL. A murine model of allergic bronchopulmonary aspergillosis with elevated eosinophils and IgE. *J Immunol* 1992; **148**:3783–8.
- Elstad MR. Aspergillosis and lung defenses. *Semin Respir Infect* 1991; **6**:27–36.
- Murali PS, Dai G, Kumar A, Fink JN, Kurup VP. Aspergillus antigen-induced eosinophil differentiation in a murine model. *Infect Immun* 1992; **60**:1952–6.
- Madan T, Kishore U, Shah A *et al.* Lung surfactant protein A and D can inhibit specific IgE binding to the allergens of *Aspergillus fumigatus* and block allergen-induced histamine release from human basophils. *Clin Exp Immunol* 1997; **110**:241–9.
- Allen MJ, Harbeck R, Smith B, Voelker DR, Mason RJ. Binding of rat and human surfactant proteins A and D to *Aspergillus fumigatus* conidia. *Infect Immun* 1999; **67**:4563–9.
- Madan T, Kishore U, Singh MB *et al.* Surfactant proteins A and D protect mice against pulmonary hypersensitivity induced by *Aspergillus fumigatus* antigens and allergens. *J Clin Invest* 2001; **107**:467–75.
- Strong P, Reid KBM, Clark H. Intranasal delivery of a truncated recombinant human SP-D is effective at down-regulating allergic hypersensitivity in mice sensitized to allergens of *Aspergillus fumigatus*. *Clin Exp Immunol* 2002; **130**:19–24.
- Takeda K, Miyyahara N, Rah YH *et al.* SP-D regulates airway function and allergic inflammation through regulation of macrophage function. *Am J Respir Crit Care Med* 2003; **168**:783–9.
- Glaab T, Hoymann HG, Hohlfeld JM *et al.* Noninvasive measurement of midexpiratory flow indicates bronchoconstriction in allergic rats. *J Appl Physiol* 2002; **93**:1208–14.
- Glaab T, Daser A, Braun A *et al.* Tidal midexpiratory flow as a measure of airway hyperresponsiveness in allergic mice. *Am J Physiol Lung Cell Mol Physiol* 2001; **280**:L565–73.

- 24 Held HD, Martin C, Uhlig S. Characterization of airway and vascular responses in murine lungs. *Br J Pharmacol* 1999; **126**:1191–9.
- 25 Martin C, Uhlig S, Ullrich V. Videomicroscopy of methacholine-induced contraction of individual airways in precision-cut lung slices. *Eur Respir J* 1996; **9**:2479–87.
- 26 Wohlsen A, Uhlig S, Martin C. Immediate allergic response in small airways. *Am J Respir Crit Care Med* 2001; **163**:1462–9.
- 27 Singh M, Madan T, Waters P, Parida SK, Sarma PU, Kishore U. Protective effects of a recombinant fragment of human surfactant protein D in a murine model of pulmonary hypersensitivity induced by dust mite allergens. *Immunol Lett* 2003; **86**: 299–307.
- 28 Strong P, Townsend P, Mackay R, Reid KB, Clark HW. A recombinant fragment of human SP-D reduces allergic responses in mice sensitized to house dust mite allergens. *Clin Exp Immunol* 2003; **134**:181–7.
- 29 Nadesalingam J, Reid KB, Palaniyar N. Collectin surfactant protein D binds antibodies and interlinks innate and adaptive immune systems. *FEBS Lett* 2005; **579**:4449–53.
- 30 Clark H, Palaniyar N, Strong P, Edmondson J, Hawgood S, Reid KB. Surfactant protein D reduces alveolar macrophage apoptosis in vivo. *J Immunol* 2002; **169**:2892–9.
- 31 Herbein JF, Wright JR. Enhanced clearance of surfactant protein D during LPS-induced acute inflammation in rat lung. *Am J Physiol Lung Cell Mol Physiol* 2001; **281**:L268–77.
- 32 Erpenbeck VJ, Schmidt R, Günther A, Krug N, Hohlfeld JM. Surfactant protein levels in bronchoalveolar lavage after segmental allergen challenge in patients with asthma. *Allergy* 2006; **61**:598–604.
- 33 Kauffman HF, Tomee JF, van de Riet MA, Timmerman AJ, Borger P. Protease-dependent activation of epithelial cells by fungal allergens leads to morphologic changes and cytokine production. *J Allergy Clin Immunol* 2000; **105**:1185–93.
- 34 Hohlfeld JM, Schmiedl A, Erpenbeck VJ, Venge P, Krug N. Eosinophil cationic protein alters pulmonary surfactant structure and function in asthma. *J Allergy Clin Immunol* 2004; **113**: 496–502.
- 35 Ponath PD, Qin S, Ringler DJ *et al*. Cloning of the human eosinophil chemoattractant, eotaxin. Expression, receptor binding, and functional properties suggest a mechanism for the selective recruitment of eosinophils. *J Clin Invest* 1996; **97**:604–12.
- 36 Gonzalo JA, Lloyd CM, Kremer L *et al*. Eosinophil recruitment to the lung in a murine model of allergic inflammation. The role of T cells, chemokines, and adhesion receptors. *J Clin Invest* 1996; **98**:2332–45.