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Surfactant protein D regulates chemotaxis and degranulation of human eosinophils

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Summary

The collectin surfactant protein D (SP-D) is an important component of the pulmonary innate host defence. Up to now, little is known about the regulation of eosinophil function by SP-D. Various murine models of pulmonary hypersensitivity suggest that SP-D may be a potent anti-allergic protein. We investigated the modulation of eosinophil chemotaxis and degranulation by human SP-D. SP-D markedly inhibited the chemotaxis of eosinophils triggered by eotaxin, a major tissue-derived CC-chemokine, as shown in a modified Boyden chamber assay. In addition, degranulation of ECP in response to Ca^{2+} ionophore, immobilized IgG and serum from allergic patients was inhibited by SP-D. In a fixed-cell enzyme linked immunosorbent assay and in flow cytometry, SP-D bound to eosinophils. This binding was saturable and was inhibited by the addition of maltose and ethylenediaminetetraacetic acid, suggesting the involvement of the carbohydrate recognition domain of SP-D. In addition, flow cytometry showed significant interaction of SP-D with CD32 (FcγII receptor) on eosinophils, which might explain the inhibitory effect of SP-D on the IgG and serum-triggered eosinophil cationic protein degranulation of eosinophils. Our data further support the concept of an anti-inflammatory function of SP-D in the lung of patients with allergic diseases.

Keywords cd32, chemotaxis, degranulation, eosinophil, granulocytes, surfactant protein D

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Introduction

Pulmonary surfactant protein D (SP-D) is a large multi-meric protein, which belongs to the collectin family of carbohydrate-binding proteins and that is secreted by alveolar type-II cells and non-ciliated bronchial epithelial cells into the airways. SP-D is predominantly assembled as a cruciform-like dodecamer, consisting of four trimeric subunits associated at their amino-termini. Each trimeric subunit contains four major domains: a short N-terminal cross-linking domain, a triple helical collagen domain, a neck region and a carboxy-terminal carbohydrate recognition domain (CRD). SP-D is known to play an important role in the innate immunity of mucosal surfaces, especially the lung, protecting against inhaled micro organism and allergen challenge [1–4]. Binding of SP-D to pathogens can cause their agglutination, enhanced killing and clearance by neutrophils and alveolar macrophages *in vitro* [5]. Recently, it has become obvious that SP-D may also be of particular importance in regulating allergic

responses. Besides its ability to bind to various allergens as well to inhibit specific IgE binding to allergens, it has been shown to inhibit lymphocyte proliferation and allergen-induced histamine release from human basophils [6–9]. So far, contradictory data have been reported about the abundance of SP-D in allergic airway inflammation. Whereas severalfold increased SP-D levels have been found in bronchoalveolar lavage (BAL) of patients with bronchial asthma [10], various murine models of allergic pulmonary inflammation showed increased or decreased levels of SP-D in BAL and lung tissue [11–13]. However, in murine models for allergic pulmonary hypersensitivity induced by *Aspergillus fumigatus* (Asp f) or dust mite allergens, inhaled SP-D reduced airway hypersensitivity and Th2 cytokine response as well as blood and tissue eosinophilia [14–16]. In SP-D^{−/−} mice, intrinsic hyper-eosinophilia, increased pulmonary eosinophilia and pulmonary damage as well as severalfold increased levels of IL-5 and IL-13 have been found. Intranasal treatment with SP-D was effective in rescuing these effects. The authors

suggest that SP-D plays a role in regulating eosinophil infiltration and modulation in response to environmental stimuli [17, 18]. In addition, surfactant protein A (SP-A) has been shown to moderately suppress the release of IL-8 of purified human eosinophils stimulated with ionomycin, PMA or sIgA [19, 20]. To date, there are no data on the interaction of SP-D with eosinophil granulocytes. Therefore, we examined the interactions of purified SP-D with isolated human eosinophils.

Eosinophils are considered as important effector cells in inflammatory reactions associated with asthma, allergic – inflammatory diseases and parasitic infections. Whereas IL-5 plays a critical role in the expansion of the eosinophil pool in the bone marrow and blood in response to allergen provocation, the recruitment of eosinophils from the circulation to sites of inflammation is regulated by locally released chemotactic agents, most notably the CC-chemokine eotaxin-1 [21, 22]. In tissue, eosinophils are activated by cytokines and other mediators including sIgA and immobilized IgG, leading to the release of toxic proteins and reactive oxygen species [22]. These toxic proteins i.e. eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDN) and major basic protein (MBP) can lead to serious epithelium damage, when secreted.

Previously, SP-D has been described to stimulate the chemotaxis of neutrophils and alveolar macrophages and to inhibit allergen-induced histamine release from human basophils *in vitro* [5, 6, 23–25]. So far, no data are available on the interaction between SP-D and eosinophils. The goal of this study was to determine the role of SP-D in the chemotaxis and the degranulation of human eosinophils *in vitro*. Our findings substantiate the hypothesis that SP-D may function as an anti – inflammatory protein in the lung of patients with allergic diseases.

Materials and methods

Preparation of human surfactant protein D

Native SP-D was isolated by affinity and gel-chromatography from BAL fluid obtained from patients with idiopathic pulmonary alveolar proteinosis (PAP) undergoing therapeutic lavages as described previously [26]. The concentration of SP-D (426 µg/mL) in the fraction (GF 88–7) used was determined by enzyme linked immunosorbent assay (ELISA), and SP-D was judged to be pure by sodium dodecyl sulphate polyacrylamide gel electrophoresis silver staining and Western blot analysis with specific antibodies to SP-D (Fig. 1a). In addition, gel filtration was performed to characterize the oligomeric state of SP-D according to Griese et al [27]. We showed that more than 95% of the SP-D was organized as 18-mer or higher oligomers (data not shown). All preparations were also examined for endotoxin levels using the Limulus Amebo-

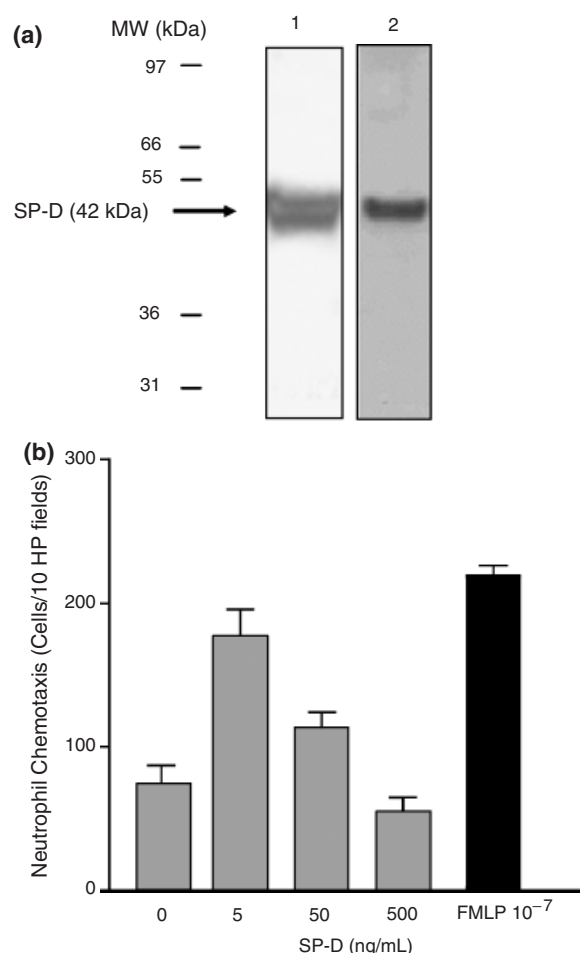


Fig. 1. (a): Characterization of purified human surfactant protein D (SP-D). Native SP-D was isolated by affinity and gel-chromatography from BAL fluid obtained from a patient with idiopathic pulmonary alveolar proteinosis undergoing therapeutic lavage. SP-D was judged to be pure by SDS poly acrylamide gel electrophoresis silver staining (lane 1) and Western blot analysis (lane 2) with specific antibodies to SP-D. Molecular mass markers are shown on the left. (b): Effect of SP-D on the chemotaxis of neutrophils. Cells (5×10^5 /well) were allowed to migrate towards various concentrations of SP-D. In each experiment, cells transversed a 3 µm pore size membrane, adherent cells to the lower surface of the membrane were stained with DiffQuick and counted from 4×10 high-power fields by light microscopy ($\times 400$). Data are expressed as average standard error of the mean of cells/10 high-power fields. Individual experiments were set up in triplicate and reproduced twice with different cell preparations.

cyte Lysate system (Bio Whittaker, Walkersville, MD USA). The assay was linear over a range of 0.1 to 1 EU/mL (10 EU = 1 ng of endotoxin). The amount of endotoxin was 16.6 pg/µg SP-D, similar to the SP-D preparations used in previous *in vitro* studies [5, 6, 14]. The biological activity of the purified SP-D has been proven by Ca^{2+} and carbohydrate-dependent agglutination of a clinical isolate of *Pseudomonas aeruginosa* (Ps 12, non-mucoid, from a patient with cystic fibrosis) as described previously [28]. The chemotactic activity of SP-D for neutrophils was

tested in a modified Boyden chamber assay as described in the following paragraphs. We observed that SP-D was chemotactic at a concentration of 5 ng/mL, decreasing with higher concentrations (Fig. 1b).

Donors

After informed consent, peripheral venous blood was obtained from seven healthy, non-atopic donors (30.1 ± 2.3 years, four female), who were not taking any medications for the last 4 weeks. Their serum IgE was less than 100 U/mL as measured by UniCAP 100 (Pharmacia Diagnostics, Freiburg, Germany) and eosinophil counts were less than 5% in differential blood counts.

For cell stimulation, blood was taken from a 25-year-old female donor, who was allergic to house dust mite (Prick + + +, clinical symptoms of rhinitis when exposed) taking no medication for the last 4 weeks.

Cell purification

Eosinophils were isolated by negative selection using the modified method of Hansel et al. [29]. Briefly, the buffy coat of citrat-anticoagulated venous blood was sedimented with 6% HEAS (Fresenius Kabi GmbH, Bad Homburg, Germany). Granulocytes were then isolated by centrifugation of the leucocyte-rich phase through a 1.077 g/mL Percoll density gradient (Amersham Pharmacia Biotech, Uppsala, Sweden). Contaminating erythrocytes were lysed by hypotonic lysis (1 min, 0 °C) and further purification was achieved by depletion of neutrophils by a magnetic cell separation system using anti-CD16 MikroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the resulting eosinophils was $\geq 98\%$ as determined by microscopic examination of Pappenheim cytospin preparations. Eosinophil viability as tested by exclusion of trypan blue was $> 98\%$. To avoid activation during isolation, cells were kept in Ca^{2+} and Mg^{2+} free medium.

Neutrophils were isolated by eluting the MACS column after the isolation of the eosinophils outside the magnetic field. The purity and viability of the resulting neutrophils was $\geq 97\%$ as determined by microscopic examination as described for eosinophils.

For further experiments, cells were resuspended at 5×10^5 eosinophils/mL or 5×10^6 neutrophils/mL in Tyrode buffer (137 mM NaCl, 2.2 mM KCl, 1 mM Tris, 1.5 mM CaCl_2) supplemented with 0.4% bovine serum albumin (BSA; Paesel & Lorei, Hanau, Germany) and were immediately used for experiments.

Chemotaxis assay

Chemotaxis was performed with a modified Boyden chamber assay using 24-well flat bottom tissue plates and 3 μm polyethyleneterephthylan membrane inserts

(Becton Dickinson Labware Europe, Le Point de Claix, France). Briefly, the lower compartment of each chamber was filled with 500 μL of the stimulus diluted in Tyrode buffer containing 0.4% BSA. Membrane inserts were filled with 100 μL of cell suspension and placed in the pre-filled lower compartments. Negative controls consisting of medium and positive controls consisting of medium containing 500 ng/mL recombinant human eotaxin (PeproTech, Rocky Hills, NJ, USA) for eosinophils or 10^{-7} M f-MLP (Sigma, Deisenhofen, Germany) for neutrophils were included in each experiment. In experiments involving maltose, 10 mM maltose was added to the upper and lower compartment. The chambers were then incubated for 60 min, 37 °C, 5% CO_2 . After incubation, non-migrated cells in the upper wells were removed by scraping and the migrated cells were stained with DiffQuick (Dade Behring Ag, Düringen, Switzerland) on the membrane. Membranes were blinded with numbers by an independent person to reduce bias. Then adherent cells to the lower surface of the membrane were counted from 4 $\times$ 10-high power fields by light microscopy ($\times 400$). Data are presented as cells per high-power field or as chemotactic index. This index accounts the means of counted cells per 10 high-power fields that migrated towards a specific stimulus, divided by the means of counted cells per 10 high-power fields that migrated towards medium only. Basal migration, measured as the number of cells migrating in the presence of buffer alone, averaged 4.6 ± 0.6 cells/10 power field for eosinophils. Individual experiments were set up in triplicate and reproduced at least twice with different cell preparations.

Assessment of eosinophil cationic protein degranulation

Ninety-six – well tissue plates were coated with PBS / BSA 3% for 2 h at 37 °C and washed three times with PBS to block non-specific sites on the plate. For some experiments, Maxi-Sorb 96-well plates (Nunc, Kamstrup, Denmark) were coated overnight at 4 °C with 100 μL of dilutions of serum from an atopic subject (1 : 10), human IgG (1 mg/mL; Sigma) and sIgA (1 mg/mL; Sigma, Deisenhofen, Germany) made with coating buffer (15 mM Na_2CO_3 , 35 mM NaHCO_3 , pH 9.6). After washing, non-specific sites were blocked with Tyrode/BSA 1% for 2 h at 37 °C, than the plate was washed again. Different concentrations of SP-D and/or Ca^{2+} ionophore (A 23187, Sigma) were added to the wells. Purified eosinophils were suspended at a concentration of 5×10^5 /mL in Tyrode buffer supplemented with 0.4% BSA. Subsequent degranulation experiments were performed on the previously prepared plates with 100 μL each of the cell suspension for 2 h at 37 °C, 5% CO_2 . Tyrode-0.4% BSA served as control medium. After centrifugation for 5 min. at 800 g cell-free supernatants were collected and ECP concentrations were measured with a commercially available

radioimmunoassay kit with a lower limit of detection of 2 ng/mL (UniCap 100, Pharmacia Diagnostics, Uppsala, Sweden).

Binding of pulmonary surfactant protein to eosinophils

A fixed-cell ELISA method was used to detect the SP-D binding to isolated eosinophil granulocytes. After isolation, cells were fixed with 10% (wt/vol) paraformaldehyde in PBS for 10 min and resuspended at 5×10^5 /mL in carbonate-buffer (15 mM Na_2CO_3 , 35 mM NaHCO_3 , pH 9.6). 96-well microtiter plates were coated with 100 μ L/well of the cell suspension overnight at 4 °C. After washing, non-specific sites were blocked with TBS (50 mM Tris-HCl, 150 mM NaCl, 2 mM Ca^{2+} , pH 7.4), 1% BSA for 1 h at 37 °C. Cells were incubated with various concentrations of SP-D for 2 h at 37 °C in TBS. Bound SP-D was cross-linked using 0.125% glutaraldehyde. The plates were blocked, washed again and bound SP-D was detected by a specific polyclonal rabbit anti-human SP-D antibody (1:10 000) and consecutive incubation with horseradish-peroxidase conjugated goat anti-rabbit polyclonal IgG (1:10 000; Sigma) as a second antibody. Colour was generated with ABTS (Boehringer-Mannheim, Mannheim, Germany) and H_2O_2 and read at 405 nm. Control preparations including cells without SP-D but the antibodies served as blanks that were subtracted. All assays were run in triplicate.

Flow cytometry

After isolation, eosinophils were resuspended in TBS and incubated with SP-D under varying conditions for 2 h, 4 °C. To study the binding conditions of SP-D to eosinophils, 5×10^5 cells each were incubated with 5 μ g/mL SP-D in 100 μ L TBS with and without 10 mM maltose or 10 mM EDTA. Non-SP-D-treated cells served as negative control.

To study receptor binding of SP-D to eosinophils, 2.5×10^6 isolated cells were pre-incubated with or without 5 μ g/mL SP-D in 500 μ L TBS for 2 h at 4 °C. The following monoclonal antibodies were added to 5×10^5 pre-incubated eosinophils each: Mouse IgG2b anti-human CD11b-phycoerythrin (PE), mouse IgG1 anti-human CD18-PE, mouse IgG2b anti-human CD32-PE, mouse IgG2a anti-human CCR3-PE (all R&D Systems, Wiesbaden, Germany) and mouse IgG1 anti-human SIRP α -PE (BD Pharmingen, Heidelberg, Germany).

The specific polyclonal rabbit anti-human SP-D antibody (1:5000) and a goat anti-rabbit IgG – fluorescein-isothiocyanate (FITC) antibody (Imgenex, San Diego, CA, USA) were used to analyse the binding of SP-D to eosinophils. Mouse IgG1-PE, mouse IgG2a-PE, mouse IgG2b-PE and mouse IgG2b-PE (all BD Pharmingen,) were used as isotype controls to determine non-specific bind-

ing; these values were subtracted from the respective specific antibody staining.

After the different *in vitro* treatments as mentioned above, eosinophils (1×10^5 /tube) were washed two times and were incubated with the respective monoclonal antibodies for 40 min, washed two times and analysed by flow cytometry. Ten-thousand eosinophils from each sample were analysed. The results were expressed as mean fluorescence intensity (MFI). Calculations were performed with Cell Quest analysis software.

Statistical analysis

Experiments were repeated at least twice. Degranulation and chemotaxis in the presence and absence of SP-D and binding of SP-D to eosinophils were compared by the paired *t*-test or by the ANOVA, followed by the Tukey–Kramer multiple comparison post-test for statistical significance ($P < 0.05$). Data are expressed as means $\pm$ standard error of the mean (SEM).

Results

Surfactant Protein D inhibits the eotaxin-triggered chemotaxis of eosinophils. As mentioned above, SP-D was chemotactic for neutrophils with a maximum at 5 ng/mL decreasing with higher concentrations (Fig. 1b). For eosinophils, we did not observe any significant chemotaxis towards SP-D at physiologic concentrations (5 ng/mL to 5000 ng/mL/).

We next examined, whether SP-D could influence the chemotaxis of eosinophils vs. the chemo-attractant eotaxin. Eotaxin triggered eosinophil chemotaxis in a dose-dependent manner (chemotactic index (CI): 100 ng/mL: 2.6 ± 0.5 ; 500 ng/mL: 12.3 ± 1.60 ; 1000 ng/mL: 15.8 ± 6.4). The chemotaxis of eosinophils towards 500 ng/mL eotaxin was inhibited by the addition of SP-D (Fig. 2a). Significant inhibition was observed at SP-D concentrations from 5 to 500 ng/mL (CI: 5 ng/mL SP-D: 4.5 ± 1.1 , $P = 0.01$; 50 ng/mL SP-D: 2.7 ± 0.8 , $P = 0.003$; 500 ng/mL SP-D: 4.2 ± 1.8 , $P = 0.04$), with a maximum at 50 ng/mL (Fig. 2a). At higher concentrations, this effect waned (CI: 5000 ng/mL SP-D: 9.9 ± 2.5 , $P = 0.47$). Denaturation of SP-D at 95 °C for 10 min abolished the inhibitory effect on eosinophil chemotaxis towards eotaxin (CI: 50 ng/mL SP-D: 9.6 ± 1.6).

The addition of 10 mM maltose abolished the observed inhibitory effect of SP-D on eosinophil chemotaxis towards eotaxin (Table 1). As the locomotion and activation of eosinophils are dependent on the presence of sufficient extracellular Ca^{2+} it was not possible to assess the Ca^{2+} dependency of the inhibitory effect of SP-D.

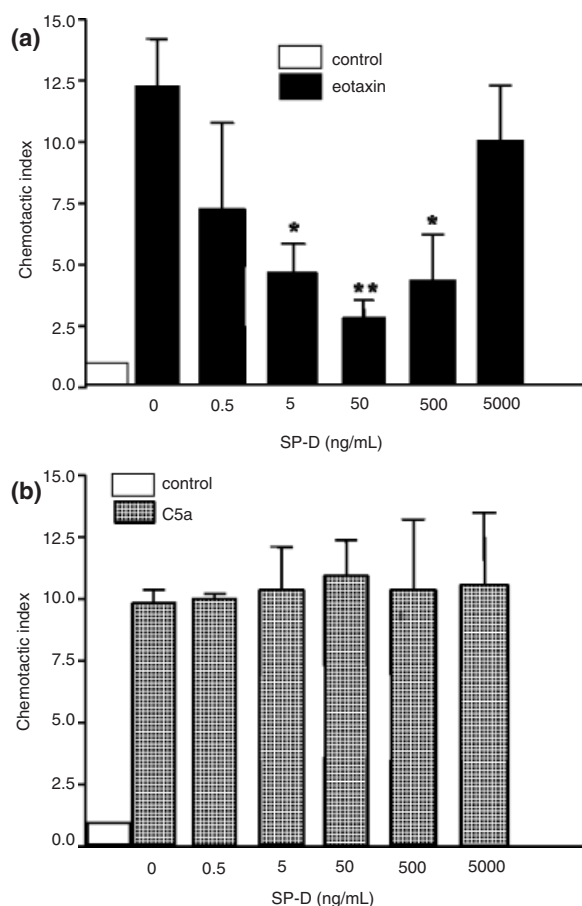


Fig. 2. Effect of surfactant protein D (SP-D) on the chemotaxis of eosinophils stimulated by (a) eotaxin (500 ng/mL) and (b) C5a (100 ng/mL). Cells (5×10^4 /well) were allowed to migrate towards various concentrations of SP-D with/or the stimulus for 1 h at 37% °C, 5% CO₂. In each experiment, cells transversed a 3 µm pore size membrane, adherent cells to the lower surface of the membrane were stained with DiffQuick and counted from 4×10 high-power fields by light microscopy ($\times 400$). Data are presented as a chemotactic index accounting the means of counted cells per 10 high-power fields which migrated towards a specific stimulus divided by means of counted cells per 10 high-power fields that migrated towards medium only. Individual experiments were set up in triplicate and reproduced at least three times with different cell preparations. Basal migration (control), measured as the number of cells migrating in the presence of buffer alone, averaged 4.6 ± 0.6 cells/10 power fields.

Surfactant Protein D does not influence the C5a-triggered chemotaxis of eosinophils. To further examine the inhibitory effect of SP-D on eosinophil chemotaxis, we stimulated the cells with the anaphylatoxin C5a. Stimulation of eosinophils with 100 ng/mL recombinant human C5a provoked a similar chemotactic response of eosinophils as observed for 500 ng/mL eotaxin (CI C5a: 10.1 ± 1.9 vs. CI eotaxin: 12.4 ± 1.6). The addition of SP-D to the experiment did not inhibit the C5a-triggered chemotaxis of eosinophils (Fig. 2b).

Table 1. Carbohydrate dependence of the effect of SP-D on the eotaxin-triggered chemotaxis of eosinophils

Chemoattractant	CI $\pm$ SEM	P*
Maltose 10 mM	1.1 ± 0.8	
Eotaxin 500 ng/ml	17.5 ± 4.4	
Eotaxin 500 ng/ml + maltose 10 mM	18.1 ± 6.5	0.87
Eotaxin 500 ng/ml + SP-D 50 ng/ml	4.4 ± 1.5	0.002
Eotaxin 500 ng/ml + maltose 10 mM + SP-D 50 ng/ml	20.6 ± 10.7	0.45

Eosinophils were allowed to migrate towards different chemoattractants using a modified Boyden chamber. Data are presented as chemotactic index accounting the means of counted cells which migrated towards a specific stimulus divided by means of counted cells which migrated towards medium only.

*The P-value assessed by paired *t*-test is referred to the CI of eotaxin alone.

Surfactant protein D inhibits the eosinophil cationic protein degranulation of stimulated eosinophils. The inhibitory effect of SP-D on the eotaxin-triggered chemotaxis prompted us to determine the effect of SP-D on the ECP release of stimulated human eosinophils. We first showed that increasing concentrations of SP-D (5–5000 ng/mL) did not trigger any ECP degranulation. Next, we stimulated the purified eosinophils with Ca²⁺-ionophore, immobilized human sIgA, human IgG and serum from an atopic patient with subsequent inhibition studies with SP-D. The Ca²⁺-ionophore triggered ECP degranulation was inhibited by SP-D at 5 ng/mL ($P=0.04$), 50 ng/mL ($P=0.06$), 500 ng/mL ($P=0.03$) and 5000 ng/mL ($P=0.02$) (Fig. 3a). In addition, 500 ng/mL SP-D could reduce significantly the ECP-release on serum from an atopic subject ($P=0.007$) and IgG ($P=0.047$). For sIgA, the inhibition did not reach statistical significance ($P=0.09$). The addition of 10 mM maltose to the experiment itself inhibited the degranulation of ECP of eosinophils. Therefore, it was not possible to verify the carbohydrate dependency of SP-D on this inhibitory effect.

Binding of surfactant protein D to eosinophils. SP-D bound to purified human eosinophils in a dose-dependent manner (Fig. 4) using a fixed-cell ELISA. Significant and reproducible binding of SP-D was observed with concentrations as low as 50 ng/mL SP-D. The binding of SP-D to eosinophils approached saturation at concentrations of 2500–5000 ng/mL SP-D. Higher concentrations were not assayed as they likely lie beyond the physiological range. Heat-denatured SP-D (95 °C, 30 min) failed to bind to eosinophils [500 ng/mL SP-D: 0.37 ± 0.07 vs. 0.04 ± 0.01 OD₄₀₅–OD_{blank} $\pm$ SEM, $P=0.004$].

To test carbohydrate and Ca²⁺ dependency of the binding of SP-D to eosinophils, competition assays were performed in the presence or absence of maltose or EDTA

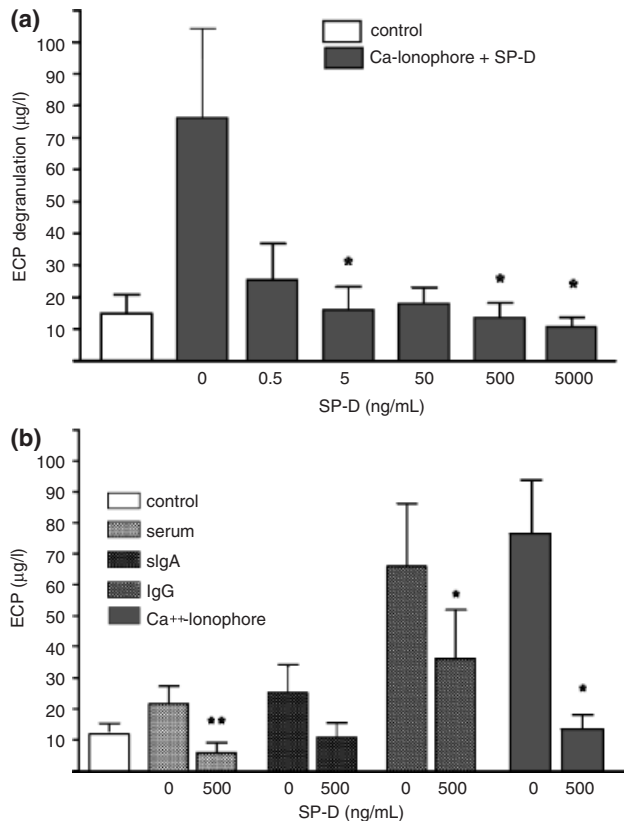


Fig. 3. Effect of surfactant protein D (SP-D) on the eosinophil cationic protein (ECP) degranulation of eosinophils stimulated by Ca^{2+} ionophore (a) or immobilized sIgA, IgG and serum from an atopic patient (b). Purified eosinophils ($5 \times 10^5/\text{well}$) were incubated with SP-D in the presence or absence of the stimulus on previously prepared plates for 2 h at 37°C , 5% CO_2 . Tyrode-0.4% bovine serum albumin (BSA) served as control medium. Cell-free supernatants were collected and ECP concentrations were measured with a commercially available radioimmunoassay kit. Individual experiments were set up in triplicate and reproduced at least three times with different cell preparations.

using flow cytometry. The addition of 10 mM maltose as well as 10 mM EDTA significantly inhibited the binding of SP-D to eosinophils (Fig. 5).

Receptor interaction of surfactant protein D. To examine possible surface receptors for SP-D on eosinophils, we performed flow cytometry for various receptors that have previously been reported to be involved in eosinophil chemotaxis or degranulation.

For the low-affinity IgG Fc receptor, $\text{Fc}\gamma\text{RII}$ (CD32), we showed significant inhibition ($85 \pm 3\%$, $P < 0.01$) of mouse IgG2b anti-human CD32-PE binding to eosinophils when cells were pre-incubated with $5 \mu\text{g}/\text{mL}$ SP-D, suggesting a relevant SP-D-receptor interaction for CD32 on eosinophils (Fig. 6). In addition, we showed that $\text{SIRP}\alpha$ was present on eosinophils, but similar to CCR3, SP-D only weakly interfered with the specific binding of the

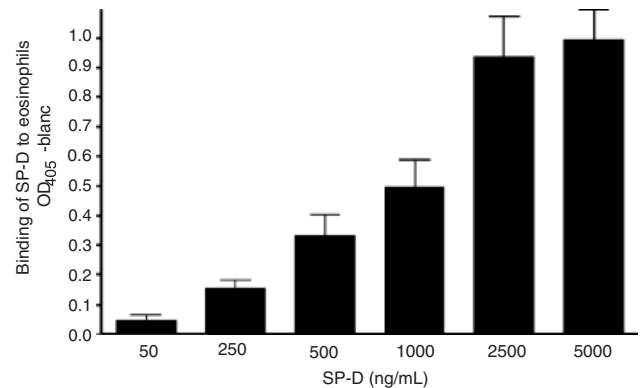


Fig. 4. Binding of surfactant protein D (SP-D) to eosinophils. Eosinophils ($5 \times 10^4/\text{well}$) were fixed in 10% paraformaldehyde, resuspended in carbonate-buffer pH 9.6 and coated to 96-well microtitre plates. Cells were incubated for 1 h at 37°C with various concentrations of SP-D. Binding was detected by a specific polyclonal rabbit anti-human SP-D antibody followed by incubation with horseradish-peroxidase-conjugated goat anti-rabbit polyclonal IgG. Final detection was assessed after the addition of ABTS and H_2O_2 and read at 405 nm. Control preparations including cells with no SP-D but the antibodies served as blanks, which were subtracted from each optic density (OD) measured. Individual experiments were set up in triplicate and reproduced at least three times with different cell preparations.

antibody to this receptor. These experiments suggested no or a minor binding of SP-D to $\text{SIRP}\alpha$ and CCR3. No binding of SP-D to CD11b/CD18 was found.

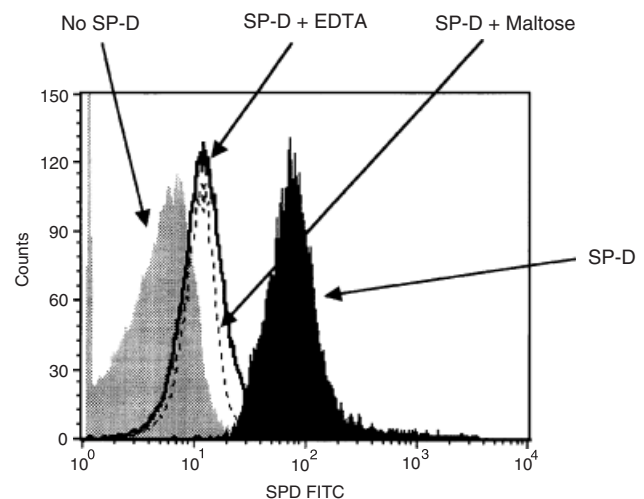


Fig. 5. Carbohydrate and Ca^{2+} dependency of surfactant protein D (SP-D) binding to eosinophils. 5×10^5 eosinophils were incubated with $5 \mu\text{g}/\text{mL}$ SP-D in $100 \mu\text{L}$ tris buffered saline with and without 10 mM maltose or 10 mM ethylenediaminetetraacetic acid. Non-SP-D-treated cells served as negative control. The specific polyclonal rabbit anti-human SP-D antibody (1 : 5000) and a goat anti-rabbit IgG-fluorescein isothiocyanate (FITC) antibody were used to analyse the binding of SP-D to eosinophils. In the following flow cytometry, 10^3 eosinophils from each sample were analysed. The results were expressed as mean fluorescence intensity. Calculations were performed with Cell Quest analysis software.

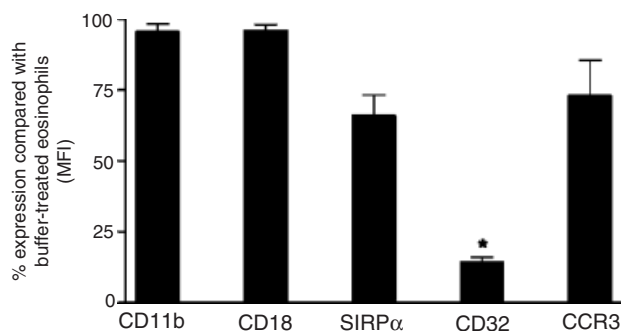


Fig. 6. Interaction of surfactant protein D (SP-D) with eosinophil surface receptors. 2.5×10^6 isolated cells were preincubated with or without $5 \mu\text{g}/\text{mL}$ SP-D in $500 \mu\text{L}$ tris buffered saline for 2 h, 4°C . Monoclonal antibodies were then added to 5×10^5 pre-incubated cells each. The values of isotype controls to determine non-specific binding were subtracted from the respective specific antibody staining. Using flow cytometry, 10^3 eosinophils from each sample were analysed. The results are presented as % expression of each receptor after SP-D treatment compared with buffer-treated eosinophils. MFI, mean fluorescence intensity; * $P < 0.01$: SP-D-treated vs buffer-treated eosinophils.

Discussion

This study demonstrated for the first time the direct effects of SP-D on eosinophils *in vitro*. SP-D inhibited eotaxin-triggered chemotaxis and ECP-degranulation of eosinophils stimulated by immobilized IgG and serum of allergic patients. Furthermore, SP-D bound to eosinophils in a saturable, Ca^{2+} - and carbohydrate-dependent manner, suggesting the involvement of the CRD. Flow cytometry showed significant interaction of SP-D with Fcγ II (CD32) on eosinophils which might explain the inhibitory effect of SP-D on the IgG and serum-triggered ECP degranulation of eosinophils. Taken together, these data provide new evidence for the role of SP-D in eosinophil-mediated inflammatory reactions as critical components in allergic disease.

Previously, numerous experiments have suggested that SP-D may protect against allergen challenge at various levels. These protective mechanisms seem to involve direct binding to allergens [6, 8], inhibition of IgE cross-linking [6, 8], suppression of the activation of sensitized basophils [6], suppression of B and T cell proliferation [7, 9] and modulation of dendritic cells, macrophages and T cell proliferation [4]. In murine models for allergic lung disease, SP-D might inhibit eosinophil trafficking and migration to the lungs. Madan et al. [14] showed in a murine model of ABPA that intranasal administration of SP-D significantly decreased peripheral and pulmonary eosinophilia. Levels of IL-2, IL-4 and IL-5 were decreased in the SP-D-treated groups, indicating a marked shift from T-helper type 2 (Th2) to Th1 immune response. Similar protective effects have been reported in murine models of lung allergy induced by Der p [15, 16]. In SP-D^{-/-} mice,

intrinsic hyper-eosinophilia, increased pulmonary eosinophilia and pulmonary damage as well as increased levels of IL-5 and IL-13 have been found. Intranasal treatment with SP-D or with a recombinant fragment containing the CRD was effective in rescuing these effects. The authors suggest that SP-D has a role in regulating eosinophil infiltration and modulation in the lung in response to environmental stimuli [17, 18].

From our results, a direct inhibitory effect of SP-D on eosinophil chemotaxis and degranulation may also contribute to the above-observed *in vivo* effects. Because of its Ca^{2+} and carbohydrate-dependent binding, it is likely that this inhibitory effect is mediated by the CRD of SP-D.

For eosinophil chemotaxis, we observed a biphasic effect with a maximum of inhibition at $50 \text{ ng}/\text{mL}$ SP-D. For neutrophils, SP-D was chemotactic at similar low concentrations decreasing with higher concentrations consistent with previous findings [5, 24, 25]. Biphasic dose-response curves are not unusual for chemotaxis and similar to that observed for other well-characterized chemo-attractants [30]. However, the underlying mechanism for the biphasic effect remains to be determined.

Of interest is the differential effect of SP-D on the migration of eosinophils and neutrophils or alveolar macrophages *in vitro*. Consistent with previous findings, we observed that SP-D was chemotactic on neutrophils [5, 24, 25]. Similar to our findings, for eosinophils, Crouch et al. [24] determined the CRD as the critical part for the interaction of SP-D with neutrophils as the addition of maltose inhibited the observed chemotactic effect. This conclusion was later confirmed by Cai et al. showing neutrophil chemotaxis towards a recombinant fragment of the SP-D CRD [23].

For alveolar macrophages, but not peripheral blood mononuclear cells, Tino et al. [25] showed that SP-D stimulated the chemotaxis and actin polymerization at concentrations ranging from 10 to $25 \mu\text{g}/\text{mL}$. Up to now, no specific receptor or pathway has been identified to explain the observed effects of SP-D on the chemotaxis of the above-mentioned cells [4].

Previously, some proteins have been suggested to act as SP-D binding receptors. LPS binds to the cellular (CD14) and the soluble form (sCD14) of the LPS-receptor CD14. CD14 has been shown to bind directly to SP-A and SP-D, thereby modulating the LPS-elicited cellular response [31]. As eosinophils do not express CD14, this receptor is not considered to play a role in SP-D binding to eosinophils. Gardai et al. [32] recently showed for macrophages that SP-D can act as an enhancer or suppressor of inflammatory mediator production depending on the receptors and SP-D-binding sites involved. When SP-D bound via the CRD to SIRPα, pro-inflammatory mediator production was blocked through src-family kinases and the p38 map kinase (MAPK) [32]. As SIRPα is known to be expressed by granulocytes, this was considered as a

possible receptor involved in the inhibitory effect of SP-D to eosinophils' actions.

Glycoprotein 340 (Gp 340) is described as a rather soluble SP-A and SP-D-binding protein than a cell surface receptor and was therefore not considered for our experiments [4, 33].

CCR3, CD32 and/or CD18/CD11b have been suggested to be involved in eosinophil activation, degranulation and/or migration [21, 22]. To elucidate the interaction of SP-D with eosinophils, we analysed whether SP-D could bind to the above-mentioned eosinophil surface receptors. Here, we found for the first time a significant interaction of SP-D with FcγII (CD32), the major IgG Fc receptor expressed by human eosinophils [21, 22]. FcγII is a low-affinity receptor for IgG that binds IgG immune complexes and is expressed in a diverse range of cells such as monocytes, macrophages, neutrophils, eosinophils, platelets and B cells [34]. FcγII is involved in a number of immune responses including antibody-dependent cell-mediated cytotoxicity, clearance of immune complexes, release of inflammatory mediators and regulation of antibody production [34]. Previously, Kaneko et al. [35] showed that immobilized IgG1 and IgG3 acting via FcγII (CD32) are responsible for eosinophil degranulation of cytotoxic granule proteins to sera from allergic patients with asthma. These data, taken together with our findings, may explain the inhibitory effect of SP-D on eosinophil ECP degranulation to immobilized sera of allergic patients as well as to IgG.

The reason why SP-D-inhibited eotaxin-triggered eosinophil chemotaxis but not C5a-triggered chemotaxis remains unclear. Eotaxin-induced chemotaxis has been shown to be mediated via CCR3, the major CC chemokine receptor on eosinophils [21, 22]. Kampen et al. [36] found that extracellular signal-regulated kinase (ERK)-1/2 and p38 MAPK were involved in eotaxin-induced chemotaxis and degranulation in eosinophils. However, the upstream signal of p38 MAPK remains unknown [37]. We observed low binding of SP-D to CCR3, not yet sufficient to explain the observed inhibitory effects of SP-D on the eotaxin-triggered chemotaxis. A similar conclusion can be drawn for the SP-D-binding potential of SIRPα, which we clearly demonstrated to be present on human eosinophils.

C5a acts via the G protein-coupled receptor CD88 on granulocytes. There are few data focusing on the C5a-mediated signalling pathway in eosinophils [38]. For neutrophils, it has been demonstrated that, after activation of CD88, the p38 MAPK pathway plays a crucial role in chemotaxis [39], too.

The influence of SP-D on the activation of upstream signals of e.g. p38 MAPK in eosinophils might therefore be crucial for the inhibitory effect on eosinophil chemotaxis and degranulation. Future experiments will investigate these mechanisms in further detail.

In conclusion, the present data suggest that SP-D regulates chemotaxis and degranulation of eosinophils potentially mediated via binding to CD32. As eosinophils are central inflammatory cells for the development of allergic airway diseases like asthma, our results are compatible with the hypothesis of a protective role of SP-D in the pathogenesis and maintenance of allergic asthma.

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References

- 1 Crouch E, Wright JR. Surfactant proteins A and D and pulmonary host defence. *Annu Rev Physiol* 2001; **63**:521–54.
- 2 Hickling TP, Clark H, Malhotra R, Sim RB. Collectins and their role in lung immunity. *J Leukoc Biol* 2004; **75**:27–33.
- 3 Reid KBM. Interactions of surfactant protein D with pathogens, allergens and phagocytes. *Biochemica et Biophysica acta* 1998; **1408**:300–5.
- 4 Kishore U, Greenhough TJ, Waters P et al. Surfactant proteins SP-A and SP-D: structure, function and receptors. *Mol Immunol* 2006; **43**:1303–15.
- 5 Madan T, Eggleton P, Kishore U et al. Binding of pulmonary surfactant protein A and D to *Aspergillus fumigatus* conidia enhance phagocytosis and killing by human neutrophils and alveolar macrophages. *Infect Immun* 1997; **65**:3171–9.
- 6 Madan T, Kishore U, Shah A et al. Lung surfactant proteins A and D can inhibit specific ige binding to allergens of *Aspergillus fumigatus* and block allergen induced histamine release from human basophils. *Clin Exp Immunol* 1997; **110**:241–9.
- 7 Wang JY, Shieh CC, You PF, Lei HY, Reid KBM. Inhibitory effect of pulmonary surfactant protein 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.
- 8 Wang JY, Kishore U, Lim BL, Strong P, Reid KB. Interaction of human lung surfactant proteins A and D with mite (*Dermatophagoides pteronyssinus*) allergens. *Clin Exp Immunol* 1996; **106**:367–73.
- 9 Borron PJ, Mostaghel EA, Doyle C, Walsh ES, McHeyzer-Williams MG, Wright JR. Pulmonary surfactant proteins A and D directly suppress CD3(+)/CD4(+) cell function: evidence for two shared mechanisms. *J Immunol* 2002; **169**:5844–50.

- 10 Cheng G, Ueda T, Numao T *et al*. Increased levels of surfactant protein A and D in bronchoalveolar lavage fluids in patients with bronchial asthma. *Eur Respir J* 2000; **16**:831–35.
- 11 Wang JY, Shieh CC, Yu CK, Lei HY. Allergen-induced bronchial inflammation is associated with decreased levels of surfactant proteins A and D in a murine model of asthma. *Clin Exp Allergy* 2001; **31**:652–62.
- 12 Kasper M, Sims G, Koslowski R *et al*. Increased surfactant protein D in rat airway goblet and Clara cells during ovalbumin-induced allergic airway inflammation. *Clin Exp Allergy* 2002; **32**: 1251–58.
- 13 Haley KJ, Ciota A, Contreras JP, Boothby MR, Perkins DL, Finn PW. Alterations in lung collectins in an adaptive allergic immune response. *Am J Physiol Lung Cell Mol Physiol* 2002; **282**: L573–84.
- 14 Madan T, Kishore U, Singh M *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.
- 15 Strong P, Reid KB, 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.
- 16 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**:309–7.
- 17 Madan T, Reid KB, Singh M, Sarma PU, Kishore U. Susceptibility of mice genetically deficient in the surfactant protein (SP)-A or SP-D gene to pulmonary hypersensitivity induced by antigens and allergens of *Aspergillus fumigatus*. *J Immunol* 2005; **174**:6943–54.
- 18 Schaub B, Westlake RM, He H *et al*. Surfactant protein D deficiency influences allergic immune responses. *Clin Exp Allergy* 2004; **34**:1819–26.
- 19 Cheng G, Ueda T, Nakajima H *et al*. Surfactant Protein A exhibits inhibitory effect on eosinophils IL-8 production. *Biochem Biophys Res Commun* 2000; **270**:831–5.
- 20 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**:59–62.
- 21 Foster PS, Mould AW, Yang M *et al*. Elemental signals regulating eosinophil accumulation in the lung. *Immunol Rev* 2001; **179**:173–81.
- 22 Rothenberg ME, Hogan PH. The eosinophil. *Annu Rev Immunol* 2006; **24**:147–74.
- 23 Cai GZ, Griffin GL, Senior RM, Longmore WJ, Moxley MA. Recombinant SP-D carbohydrate recognition domain is a chemoattractant for human neutrophils. *Am J Physiol* 1999; **276**: L131–6.
- 24 Crouch EC, Persson A, Griffin GL, Chang D, Senior RM. Interactions of pulmonary surfactant protein D (SP-D) with human blood leukocytes. *Am J Respir Cell Mol Biol* 1995; **12**:410–5.
- 25 Tino MJ, Wright JR. Surfactant proteins A and D specifically stimulate directed actin-based responses in alveolar macrophages. *Am J Physiol* 1999; **276**:L164–74.
- 26 Strong P, Kishore U, Morgan C, Lopez BA, Singh M, Reid KB. A novel method of purifying lung surfactant proteins A and D from the lung lavage of alveolar proteinosis patients and from pooled amniotic fluid. *J Immunol Methods* 1998; **220**: 139–49.
- 27 Griesse M, Maderlechner N, Ahrens P, Kitz R. Surfactant proteins A and D in children with pulmonary disease due to gastroesophageal reflux. *Am J Respir Crit Care Med* 2002; **165**: 1546–50.
- 28 Griesse M, Wiesener A, Lottspeich F, von Bredow C. Limited proteolysis of surfactant protein D causes a loss of its calcium-dependent lectin functions. *Biochim Biophys Acta* 2003; **14**: 1638:157–63.
- 29 Hansel TT, DeVries IJ, Iff T *et al*. An improved immunomagnetic procedure for the isolation of highly purified human blood eosinophils. *J Immunol Methods* 1991; **145**:105–10.
- 30 Senior RM, Skogen WF, Griffin GL, Wilner: effects of fibrinogen derivatives upon the inflammatory response. Studies with human fibrinopeptide. *B J Clin Invest* 1986; **77**:1014–9.
- 31 Senft AP, Korfhagen TR, Whitsett JA, Shapiro SD, LeVine AM. Surfactant protein-D regulates soluble CD14 through matrix metalloproteinase-12. *J Immunol* 2005; **15**: **174**: 4953–9.
- 32 Gardai SJ, Xiao YQ, Dickinson M *et al*. By binding SIRP alpha or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation. *Cell* 2003; **115**:13–23.
- 33 Ligtenberg TJ, Bikker FJ, Groenink J *et al*. Human salivary agglutinin binds to lung surfactant protein-D and is identical with scavenger receptor protein gp-340. *Biochem J* 2001; **359**: 243–8.
- 34 Gessner JE, Heiken H, Tamm A, Schmidt RE. The igg Fc receptor family. *Ann Hematol* 1998; **76**:231–48.
- 35 Kaneko M, Swanson MC, Gleich GJ, Kita H. Allergen-specific IgG1 and IgG3 through Fc gamma RII induce eosinophil degranulation. *J Clin Invest* 1995; **95**:2813–21.
- 36 Kampen GT, Stafford S, Adachi T *et al*. Eotaxin induces degranulation and chemotaxis of eosinophils through the activation of ERK2 and p38 mitogen-activated protein kinases. *Blood* 2000; **95**:1911–7.
- 37 Adachi T, Vita R, Sannohe S *et al*. The functional role of rho and rho-associated coiled-coil forming protein kinase in eotaxin signaling of eosinophils. *J Immunol* 2001; **167**:4609–15.
- 38 Elsner J, Oppermann M, Kapp A. Detection of C5a receptors on human eosinophils and inhibition of eosinophil effector functions by anti-C5a receptor (CD88) antibodies. *Eur J Immunol* 1996; **26**:1560–4.
- 39 Heit B, Tavener S, Raharjo E, Kubes P. An intracellular signaling hierarchy determines direction of migration in opposing chemotactic gradients. *J Cell Biol* 2002; **14**: 159:91–102.