

Veit J. Erpenbeck, Delphine C. Malherbe, Stefanie Sommer, Andreas Schmiedl, Wolfram Steinhilber, Andrew J. Ghio, Norbert Krug, Jo Rae Wright and Jens M. Hohlfeld

Am J Physiol Lung Cell Mol Physiol 288:692-698, 2005. First published Dec 10, 2004;
doi:10.1152/ajplung.00362.2004

You might find this additional information useful...

This article cites 33 articles, 21 of which you can access free at:

<http://ajplung.physiology.org/cgi/content/full/288/4/L692#BIBL>

This article has been cited by 3 other HighWire hosted articles:

Interactions of nanoparticles with pulmonary structures and cellular responses

C. Muhlfeld, B. Rothen-Rutishauser, F. Blank, D. Vanhecke, M. Ochs and P. Gehr

Am J Physiol Lung Cell Mol Physiol, May 1, 2008; 294 (5): L817-L829.

[\[Abstract\]](#) [\[Full Text\]](#) [\[PDF\]](#)

Intratracheal Recombinant Surfactant Protein D Prevents Endotoxin Shock in the Newborn Preterm Lamb

M. Ikegami, K. Carter, K. Bishop, A. Yadav, E. Masterjohn, W. Brondyk, R. K. Scheule and J. A. Whitsett

Am. J. Respir. Crit. Care Med., June 15, 2006; 173 (12): 1342-1347.

[\[Abstract\]](#) [\[Full Text\]](#) [\[PDF\]](#)

Surfactant protein D decreases pollen-induced IgE-dependent mast cell degranulation

D. C. Malherbe, V. J. Erpenbeck, S. N. Abraham, E. C. Crouch, J. M. Hohlfeld and J. R. Wright

Am J Physiol Lung Cell Mol Physiol, November 1, 2005; 289 (5): L856-L866.

[\[Abstract\]](#) [\[Full Text\]](#) [\[PDF\]](#)

Updated information and services including high-resolution figures, can be found at:

<http://ajplung.physiology.org/cgi/content/full/288/4/L692>

Additional material and information about *AJP - Lung Cellular and Molecular Physiology* can be found at:

<http://www.the-aps.org/publications/ajplung>

This information is current as of September 28, 2009 .

Surfactant protein D increases phagocytosis and aggregation of pollen-allergen starch granules

Veit J. Erpenbeck,^{1,2} Delphine C. Malherbe,³ Stefanie Sommer,¹ Andreas Schmiedl,⁴ Wolfram Steinhilber,⁵ Andrew J. Ghio,⁶ Norbert Krug,¹ Jo Rae Wright,³ and Jens M. Hohlfeld^{1,2}

¹Fraunhofer Institute of Toxicology and Experimental Medicine; ²Department of Respiratory Medicine, Hannover Medical School; ⁴Department of Anatomy, Hannover Medical School, Hannover; ⁵Altana Pharma AG, Konstanz, Germany; ³Department of Cell Biology, Duke University Medical Center, Durham; and ⁶Environmental Protection Agency, Chapel Hill, North Carolina

Submitted 27 September 2004; accepted in final form 4 December 2004

Erpenbeck, Veit J., Delphine C. Malherbe, Stefanie Sommer, Andreas Schmiedl, Wolfram Steinhilber, Andrew J. Ghio, Norbert Krug, Jo Rae Wright, and Jens M. Hohlfeld. Surfactant protein D increases phagocytosis and aggregation of pollen-allergen starch granules. *Am J Physiol Lung Cell Mol Physiol* 288: L692–L698, 2005. First published December 10, 2004; doi:10.1152/ajplung.00362.2004.—Recent studies have shown that surfactant components, in particular the collectins surfactant protein (SP)-A and -D, modulate the phagocytosis of various pathogens by alveolar macrophages. This interaction might be important not only for the elimination of pathogens but also for the elimination of inhaled allergens and might explain anti-inflammatory effects of SP-A and SP-D in allergic airway inflammation. We investigated the effect of surfactant components on the phagocytosis of allergen-containing pollen starch granules (PSG) by alveolar macrophages. PSG were isolated from *Dactylis glomerata* or *Phleum pratense*, two common grass pollen allergens, and incubated with either rat or human alveolar macrophages in the presence of recombinant human SP-A, SP-A purified from patients suffering from alveolar proteinosis, a recombinant fragment of human SP-D, dodecameric recombinant rat SP-D, or the commercially available surfactant preparations Curosurf and Alveofact. Dodecameric rat recombinant SP-D enhanced binding and phagocytosis of the PSG by alveolar macrophages, whereas the recombinant fragment of human SP-D, SP-A, or the surfactant lipid preparations had no effect. In addition, recombinant rat SP-D bound to the surface of the PSG and induced aggregation. Binding, aggregation, and enhancement of phagocytosis by recombinant rat SP-D was completely blocked by EDTA and inhibited by D-maltose and to a lesser extent by D-galactose, indicating the involvement of the carbohydrate recognition domain of SP-D in these functions. The modulation of allergen phagocytosis by SP-D might play an important role in allergen clearance from the lung and thereby modulate the allergic inflammation of asthma.

innate immunity; antigen processing; allergy; lung

THE AIRWAYS ARE CONTINUOUSLY exposed to inhaled particles. In the allergic patient, inhalation of allergen particles leads to an inflammatory reaction that is initiated by resident airway cells and perpetuated by invasion of inflammatory cells (12). Grass pollen belongs to the group of allergens that is associated with the highest prevalence of airborne allergies (13). However, due to their size of ~30 μm , grass pollen does not reach the terminal airways. A recent study has shown that grass pollen releases pollen starch granules (PSG) that consist of frag-

mented pollen cytoplasm (30). In contrast to grass pollen, these PSG are smaller in size and respirable and, therefore, enter the airways, where they come into contact with the pulmonary surfactant layer and resident airway cells.

Alveolar macrophages are the predominant cells in the alveolus that serve as the first line of defense against inhaled material (7, 18). They clear inhaled material from the lung by phagocytosis, and they release microbicidal and immunomodulatory mediators. In a previous study, Currie and coworkers (5) have shown that alveolar macrophages bind and phagocytose pollen-derived allergen particles. Allergen uptake was C-type lectin receptor mediated and induced upregulation of inducible nitric oxide synthase mRNA and release of nitric oxide.

Surfactant protein A (SP-A) and surfactant protein D (SP-D) belong to the family of C-type lectins that use C-type lectin receptors on alveolar macrophages to bind specifically to the cell surface (15, 22, 25). SP-A and SP-D play an important role in the innate immune defense because they bind to pathogens and modulate their phagocytosis and clearance by alveolar macrophages. For example, SP-D binds to *Pseudomonas aeruginosa* and stimulates phagocytosis by rat alveolar macrophages (26), whereas SP-A enhances the phagocytosis of respiratory syncytial virus, *Haemophilus influenzae*, *Streptococcus pneumoniae*, and group A *Streptococcus* (2, 31). Previous work has demonstrated that SP-A and -D bind to allergens as well (21, 32). In addition, potent anti-inflammatory effects of SP-A and SP-D have been observed in asthma animal models (20, 29). We hypothesized that SP-A and -D play a role in allergen clearance by binding to allergen and enhancing the phagocytosis by alveolar macrophages.

To test this hypothesis, PSG from *Dactylis glomerata* or *Phleum pratense* were isolated and incubated with rat and human alveolar macrophages in the presence of various surfactant components. We investigated the effect of recombinant human SP-A (rhSP-A), SP-A obtained from bronchoalveolar lavage (BAL) of patients with alveolar proteinosis (AP-SP-A), a recombinant fragment of human SP-D (rfhSP-D), recombinant rat SP-D (rrSP-D), as well as the natural surfactant preparations Alveofact and Curosurf, on binding and uptake of PSG by rat and human alveolar macrophages. Furthermore, the binding of SP-A and SP-D to PSG and the aggregation of PSG in the presence of surfactant components were examined.

Address for reprint requests and other correspondence: V. J. Erpenbeck, Fraunhofer Institute of Toxicology and Experimental Medicine, Nikolai-Fuchs-Str. 1a, 30625 Hannover, Germany (E-mail: erpenbeck@item.fraunhofer.de).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

MATERIALS AND METHODS

Reagents. rhSP-A (5.3 mg/ml in 10 mM Na-phosphate buffer, pH 7.5, endotoxin content 44 pg/mg) and rhSP-D [rhSP-D neck/carbohydrate recognition domain (CRD) from *Escherichia coli*, 3.6 mg/ml in 10 mM NaHPO₄ buffer, pH 7.5, endotoxin content 40 pg/mg] were kindly provided by Altana Pharma. Curosurf (80 mg/ml, 74 mg/ml total phospholipids) was kindly provided by Nycomed Pharma (Unterschleissheim, Germany), and Alveofact (65 mg/ml) was kindly provided by Boehringer Ingelheim Pharma (Biberach, Germany). In addition, SP-A was purified from BAL of patients suffering from alveolar proteinosis (AP-SP-A) as previously described (33). A sample of AP-SP-A at a concentration of 2 mg/ml with <200 pg endotoxin/mg SP-A was used. rrSP-D was purified by maltose affinity chromatography from the media supernatant of cultured Chinese hamster ovary cells stably transfected with a full-length rat SP-D cDNA clone as previously described (6). The concentration of the rrSP-D was 0.075–0.122 mg/ml, and the endotoxin level was <460 pg/mg. The rrSP-D is assembled as SP-D dodecamers and a subpopulation of multimers morphologically indistinguishable from those of natural rat-SP-D (3).

Grass pollen (*D. glomerata*) was obtained from Biopol Laboratory (Spokane, WA); *P. pratense* pollen was obtained from Allergon (Ängelholm, Sweden). All other reagents, unless otherwise specified, were purchased from Sigma Chemical (St. Louis, MO).

Cell preparation. Male Sprague-Dawley rats were obtained from Charles River (Raleigh, NC) or Taconic Farms (Germantown, NY). The rats weighed 301–470 g. Alveolar macrophages were obtained by BAL. Rats were killed by pentobarbital sodium injection, and the lungs were removed and lavaged eight times with 10 ml of PBS. The resulting BAL was centrifuged at 250 g for 10 min, and the supernatant was carefully removed. After being resuspended in 2 ml of culture medium (RPMI 1640; Cambrex, Walkersville, MD) supplemented with 10% heat-inactivated FCS, 1% penicillin-streptomycin, and 1% L-glutamine (GIBCO, Carlsbad, CA), the cells were counted in a Neubauer hemocytometer with trypan blue. Some experiments were performed in serum-free medium with or without supplementation of CaCl₂.

We received human BAL cells from five healthy nonsmoking, nonatopic volunteers by performing BAL during fiber-optic bronchoscopy at the Environmental Protection Agency. Patients gave their written informed consent, and the procedure was approved by the institutional review board.

Isolation and fluorescence labeling of PSG. PSG were isolated from whole grass pollen (*D. glomerata* or *P. pratense*) based on a protocol that was previously described by Currie et al. (5). In brief, 200 mg of pollen were added to 20 ml of deionized, autoclaved water containing 0.05% (vol/vol) Tween 20 in a 50-ml tube. The suspension was vortexed for 3 min and rotated for 2 h at 4°C. Whole pollen and pollen fragments were removed by centrifugation at 50 g for 4 min, and the supernatant was passed through a 20- μ m nylon mesh (VWR International, Hannover, Germany). The filtrate was centrifuged at 2,500 g for 10 min, and the pellet was resuspended in 20 ml of sterile deionized water. This volume was passed through a polycarbonate membrane with a pore size of 3 μ m (Osmonics, Minnetonka, MN) using a 20-ml syringe and a filter holder. The filtrate was centrifuged as before, and the resulting pellet was resuspended in 1 ml of sterile PBS and stored at 4°C. To determine the number of PSG, an aliquot was diluted in PBS (1:100) and counted in an improved Neubauer chamber. The isolation procedure yielded 12.0×10^8 – 19.2×10^8 PSG. Analysis of PSG by scanning electron microscopy revealed a monodisperse particle distribution with a maximum diameter of 1.34 ± 0.03 μ m (*D. glomerata*) and 1.50 ± 0.04 μ m (*P. pratense*).

PSG were fluorescently labeled with Alexa Fluor 488 or Alexa Fluor 546 fluorescent dyes (Molecular Probes, Eugene, OR). For the staining procedure 4×10^8 PSG were resuspended in 500 μ l of PBS containing 0.1 M sodium bicarbonate. The suspension was transferred

into the vial of reactive dye, the stir bar was removed, and the vial was rotated for 1 h at room temperature in the dark. After the staining procedure the volume was transferred into a 15-ml tube, diluted to a final volume of ~15 ml, and centrifuged at 2,500 g for 12 min. The supernatant was removed, and the pellet was resuspended in 15 ml of PBS and centrifuged as before. After removing the supernatant, we resuspended the pellet in 1 ml of PBS, counted the PSG again, and added the appropriate amount to the assays.

Phagocytosis assay. Alveolar macrophages (2×10^5) in 1 ml of culture medium were placed into polypropylene tubes (Becton Dickinson, Franklin Lakes, NY). The surfactant preparations (Curosurf, Alveofact, rhSP-A, AP-SP-A, rhSP-D, rrSP-D) were added immediately, and the cells were incubated for 40 min at 37°C in 5% CO₂. One sample without surfactant was placed at 4°C as a negative control. After 40 min, Alexa Fluor 488-labeled PSG were added to the cells at ratios of 10:1 or 20:1. Rat BAL cells were then incubated for 8 h at 37°C (4°C for the negative control). Due to differences in basal phagocytic activity compared with rat cells, human cells were incubated only for 4 h. After the incubation, the cells were centrifuged at 250 g for 5 min, and the supernatant was removed. The cells were fixed with 2 ml of a fixative solution containing 2% formaldehyde and 12 mM NaN₃ for 20 min at room temperature. The fixed cells were centrifuged again as before and resuspended in 350 μ l of PBS for analysis by flow cytometry or confocal microscopy. A nuclear stain was performed with 7-amino-actinomycin D (Beckmann-Coulter, Krefeld, Germany).

Determination of intracellular fate of phagocytosed PSG. To examine the intracellular fate of phagocytosed PSG, we preincubated 2×10^5 rat alveolar macrophages with Alexa Fluor 488-labeled PSG in culture medium at a ratio of 1:20 for 8 h in the presence or absence of rrSP-D (1 μ g/ml). After this preincubation period, cells were spun through a 5%/68% Percoll step gradient to remove free PSG, thereby preventing further phagocytosis of PSG. The Percoll separation reduced the ratio of cells to extracellular PSG from 1:20 to $1:0.5 \pm 0.2$. After two washes with PBS, cells were resuspended in 0.5 ml of culture medium and incubated for another 0 (immediately fixed), 24, 48, or 96 h. After each incubation, vitality of cells was determined by trypan blue staining, and cells were immediately fixed with 2 ml of fixative solution, stained with TO-PRO3 (Molecular Probes), and measured by flow cytometry. Cytospin preparations were used for evaluation with the confocal laser scanning microscope.

Aggregation and binding assay. For the aggregation assay, PSG (2×10^6) were incubated with the surfactant proteins (rhSP-A at 30 μ g/ml, AP-SP-A at 10 μ g/ml, rhSP-D at 30 μ g/ml, and rrSP-D at 1 μ g/ml) in culture medium at 37°C (or 4°C) for 8 h. The PSG were then centrifuged and fixed as described for the cells and subsequently analyzed by flow cytometry and confocal microscopy. For the evaluation by flow cytometry, the size of the granule aggregates was measured in the forward scatter (FSC) channel. A similar method was previously described for cells (28) or bacteria (10). For the calculation, the intensity in the FSC of the PSG incubated at 37°C in the absence of surfactant protein was set to 100%.

For the binding assay, the surfactant proteins (AP-SP-A and rrSP-D) were fluorescently labeled with FITC ($n = 2$) or Alexa Fluor 488 ($n = 1$) as described previously (27). The FITC-labeled surfactant proteins (AP-SP-A at 10 μ g/ml, rrSP-D at 1 μ g/ml) were then added to 2×10^6 Alexa Fluor 546-stained PSG in 1 ml of culture medium, incubated for 8 h, and prepared as in the previous experiments.

Flow cytometry, confocal microscopy. Cells were analyzed by flow cytometry with a FACSCalibur (Becton Dickinson) equipped with a 488-nm argon-ion laser and a 635-nm red diode laser. A minimum of 5,000 cells (gated on positive nuclear stain) was counted from each tube. The percentage of cells that bound/phagocytosed PSG was calculated as the number of positive cells in relation to the total number of cells analyzed. For the analysis of aggregation of the granules, the mean FSC intensities of all counted granules of each sample were compared. The mean FSC of the control at 37°C was set

to 100%, and the change of size/aggregation of the PSG was expressed as a percentage of this control.

Confocal microscopy was performed on a Zeiss LSM 510 META run by LSM 510 software. To determine whether the PSG were adherent to the surface or located inside the cells, three-dimensional images were taken from fixed cells in solution (PBS) in a chamber slide after staining with Evans blue. A minimum of 100 cells was counted from representative samples.

Statistical analysis. The unpaired, two-tailed *t*-test was used for statistical analysis of the data. A Bonferroni correction was used throughout. *P* values <0.05 were considered to be significant. All values are given as means $\pm$ SE.

RESULTS

rrSP-D enhances the binding/uptake of PSG by rat macrophages. Rat alveolar lavage cells, which were >95% alveolar macrophages, were incubated with the surfactant preparations and PSG from *D. glomerata*. Three control samples, one at 4°C, another at 37°C without surfactant preparations, and one sample at 37°C without surfactant and PSG, were included. Analysis of the cells by flow cytometry showed that $14.3 \pm 1.9\%$ of the cells at 37°C were positive compared with $7.1 \pm 1.1\%$ of the cells at 4°C (Fig. 1). Addition of the rrSP-D at a concentration of 1 $\mu\text{g}/\text{ml}$ significantly increased the number of PSG-positive cells ($44.6 \pm 4.6\%$).

In contrast, the addition of rfhSP-D (30 $\mu\text{g}/\text{ml}$), as well as the addition of AP-SP-A (1 $\mu\text{g}/\text{ml}$), rhSP-A (30 $\mu\text{g}/\text{ml}$), and the commercially available surfactant preparations Curosurf and Alveofact (both 250 $\mu\text{g}/\text{ml}$), showed no effect on the PSG uptake/binding by alveolar macrophages (Fig. 1).

The uptake/binding of PSG by macrophages was enhanced dose dependently by rrSP-D. At concentrations of 0.25, 0.5, 1, 2.5, and 5 $\mu\text{g}/\text{ml}$, the percentage of positive cells was increased to 25.9 ± 4.3 , 33.7 ± 4.5 , 40.7 ± 6.3 , 54.7 ± 8.9 , and $65.3 \pm 10.9\%$, respectively. Increasing concentrations of AP-SP-A from 1 to 50 $\mu\text{g}/\text{ml}$ showed no effect (data not shown).

After incubation with PSG from *P. pratense* pollen, $25.9 \pm 6.2\%$ of the cells were positive after incubation at 37°C, and rrSP-D increased the percentage of positive cells up to $50.3 \pm 6.6\%$.

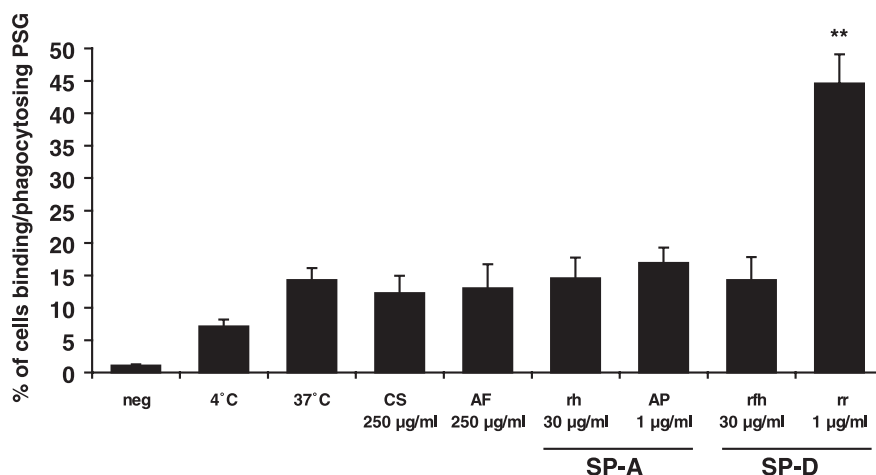
To determine whether rrSP-D increased only binding of PSG to the surface of macrophages or only uptake of PSG by macrophages or both binding and uptake, we performed additional experiments at 4°C, a temperature that disables macro-

phages to phagocytose PSG. At 4°C rrSP-D (1 $\mu\text{g}/\text{ml}$) enhanced binding of PSG to the cells from $7.1 \pm 1.1\%$ to $14.4 \pm 3.3\%$ ($P < 0.01$, Fig. 2), but the percentage of PSG-positive cells was much more increased at 37°C ($40.7 \pm 6.3\%$, $P < 0.01$), indicating that rrSP-D enhances binding as well as uptake at this temperature. These observations were confirmed by evaluation of 100 cells in three-dimensional images obtained by confocal microscopy from one representative experiment. After incubation at 37°C without rrSP-D, PSG were found on the surface of $11 \pm 1.0\%$ of the cells and inside of $13 \pm 1.9\%$ of the cells (cells that showed PSG inside and on the surface were only counted as inside). The rrSP-D increased the percentage of cells with PSG on the surface to $14 \pm 1.7\%$ and cells with ingested PSG to $28 \pm 2.2\%$ (Fig. 3). These data suggest that rrSP-D enhanced both binding and phagocytosis of PSG at 37°C.

Inhibition of rrSP-D-mediated PSG uptake/binding. To identify the mechanism of increased uptake/binding of PSG in the presence of rrSP-D, we added different known inhibitors of SP-D-mediated effects to the samples before addition of the PSG. At 37°C, EDTA (10 mM) completely inhibited basal and SP-D-stimulated uptake/binding of PSG, and the competitive inhibitors D-maltose (100 mM) and D-galactose (100 mM) decreased the effect of rrSP-D (Fig. 2). At 4°C, EDTA, D-maltose, and D-galactose abolished rrSP-D-mediated binding of PSG to macrophages (Fig. 2). These results suggest an involvement of the CRD of rrSP-D and a calcium dependency of the enhanced uptake/binding of PSG in the presence of rrSP-D.

Enhanced uptake/binding of PSG by human alveolar macrophages in the presence of rrSP-D but not rfhSP-D. Human BAL cells ($n = 5$) were used to test whether the effect of rrSP-D might enhance the uptake of PSG by human alveolar macrophages as well. Because of the increased capability of human macrophages to phagocytose/bind the PSG compared with rat macrophages, an incubation time of 4 h was used for all experiments with human macrophages. The incubation of human macrophages together with PSG at a ratio of 1:10 at 37°C showed that $38.0\% (\pm 4.7\%)$ of the cells bound/phagocytosed PSG after 4 h, which was significantly enhanced by rrSP-D ($69.5 \pm 9.7\%$, $P < 0.05$). In contrast, there was no significant difference in the percentage of cells that bound/phagocytosed PSG in the presence of 250 $\mu\text{g}/\text{ml}$ Curosurf ($37.8 \pm 7.9\%$), 250 $\mu\text{g}/\text{ml}$ Alveofact ($39.1 \pm 6.2\%$), 30 $\mu\text{g}/\text{ml}$

Fig. 1. Percentage of rat alveolar macrophages that bound/phagocytosed pollen starch granules (PSG) from *Dactylis glomerata* after 8-h incubation at 4°C, at 37°C, and at 37°C in the presence of Curosurf (CS), Alveofact (AF), recombinant human (rh) surfactant protein A (SP-A), SP-A purified from patients suffering from alveolar proteinosis (AP), recombinant fragment of human (rfh) SP-D, and recombinant rat (rr) SP-D; $n = 5$ –10, ** $P < 0.01$.



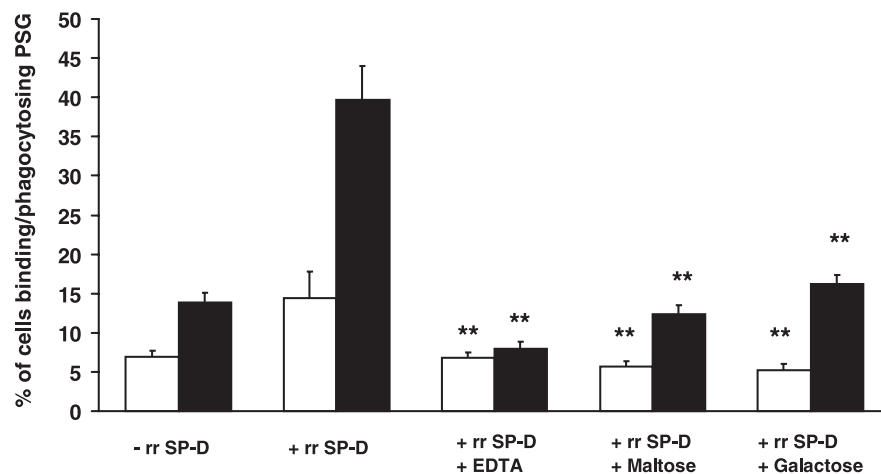


Fig. 2. Incubation of PSG and alveolar macrophages without and with rrSP-D at 4°C (open bars) and at 37°C (closed bars) and inhibition of rr-SP-D-enhanced binding/phagocytosis in the presence of EDTA (10 mM), D-maltose (100 mM), or D-galactose (100 mM); $n = 5-12$, $**P < 0.01$ compared with rrSP-D without inhibitors at corresponding temperatures.

rhSP-A ($41.0 \pm 10.5\%$), 10 $\mu\text{g/ml}$ AP-SP-A ($44.0 \pm 7.1\%$), or 30 $\mu\text{g/ml}$ rhSP-D ($43.1 \pm 13.0\%$).

Further experiments with rrSP-D at concentrations of 0.25, 1, and 5 $\mu\text{g/ml}$ showed a dose dependency of this effect (data not shown).

Phagocytosed PSG remain inside alveolar macrophages. To investigate the fate of phagocytosed PSG, macrophages were separated from extracellular PSG by Percoll gradient centrifugation after 8 h of preincubation and taken back into culture for

up to 96 h. Without rrSP-D, the percentage of alveolar macrophages that bound/phagocytosed PSG was stable at 10% for 96 h (Fig. 4). In the presence of rrSP-D, >60% of the cells were PSG positive. After an initial decrease of about 13% during the first 24 h, the percentage remained stable at ~45% up to 96 h (Fig. 4). Similar results were obtained by evaluation of mean fluorescence values of PSG-positive cells, indicating that the majority of PSG remained inside the cells (data not shown).

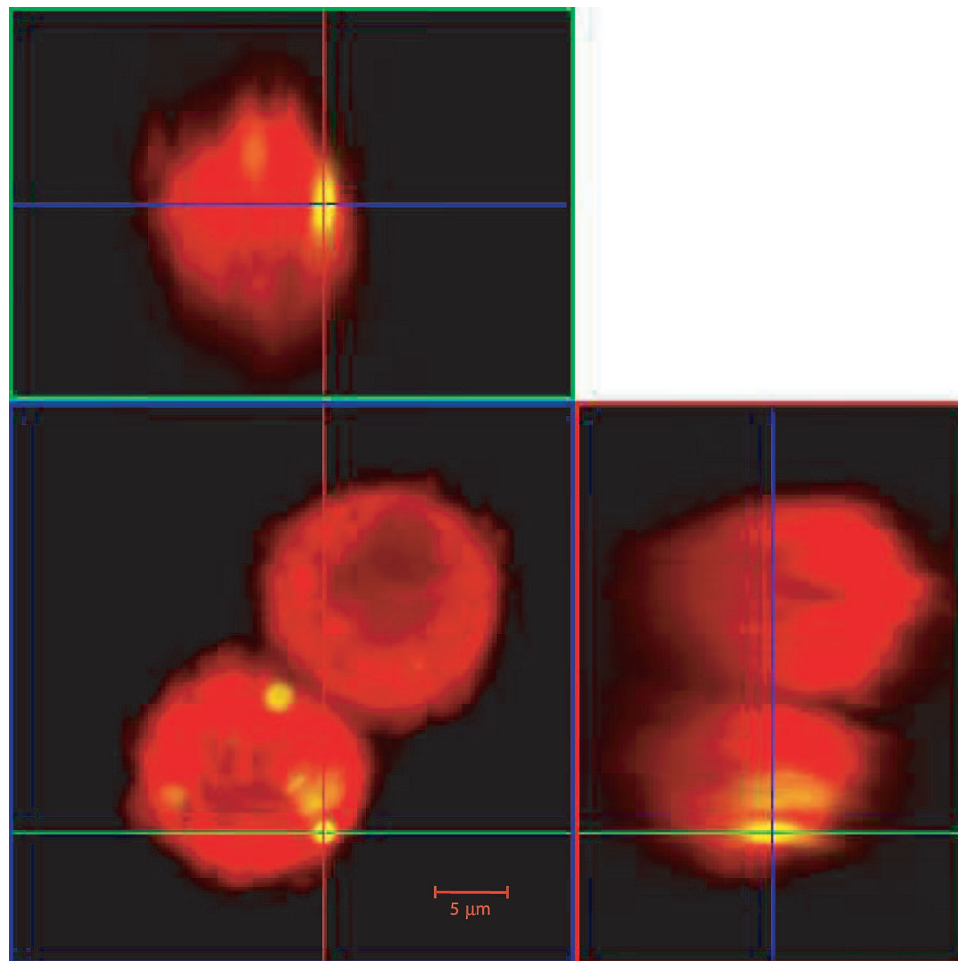


Fig. 3. Representative 3-dimensional image obtained by confocal microscopy. Cells were incubated with Alexa Fluor 488-labeled PSG from *D. glomerata* for 8 h at 37°C and stained with Evans blue to determine intra- or extracellular localization of the PSG. Size bar = 5 μm .

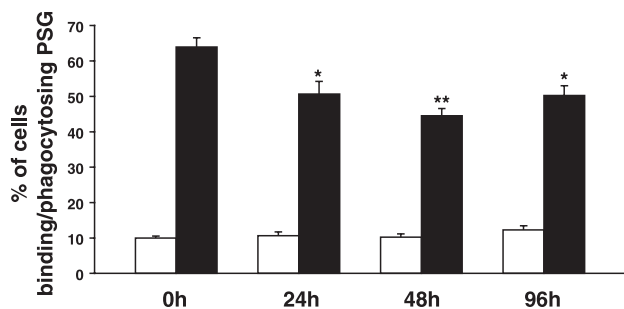


Fig. 4. Preincubation of rat alveolar macrophages with PSG from *D. glomerata* with (closed bars) and without (open bars) rrSP-D (1 $\mu\text{g/ml}$) followed by Percoll gradient centrifugation. Percentage of cells binding/phagocytosing PSG was determined by flow cytometry immediately after the separation (0 h) or after further incubation for 24, 48, and 96 h; $n = 4$, $*P < 0.05$ and $**P < 0.01$ respectively compared with 0 h.

Confocal microscopy revealed that the fluorescence of labeled PSG was found inside the cells at all time points regardless of preincubation with rrSP-D. The vitality of the cells after these incubation periods did not show a difference ($96.9 \pm 0.9\%$ viable cells).

PSG are aggregating in the presence of rrSP-D and to a lesser extent in the presence of AP-SP-A. Confocal microscopy reveals that the PSG were aggregating in the presence of rrSP-D. We further investigated this phenomenon by incubating the PSG with the different surfactant preparations without macrophages.

The incubation of PSG with rrSP-D at a concentration of 1 $\mu\text{g/ml}$ induced an aggregation of the PSG up to 2,500% compared with the control at 37°C (set to 100%), as determined by the mean FSC intensity (Fig. 5A). Detectable, but significantly less, aggregation was also found in the presence of AP-SP-A at a concentration of 10 $\mu\text{g/ml}$, whereas no aggregation was found in the presence of rhSP-A or rfhSP-D. Aggregation of the PSG in the presence of rrSP-D was completely blocked by addition of EDTA (10 mM). A significant reduction of PSG aggregation was achieved by adding maltose at a concentration of 10 mM, but the aggregation was not significantly reduced by 10 mM galactose (Fig. 5A). Addition of EDTA to the PSG in the presence of AP-SP-A reduced the aggregation, which did not achieve statistical significance (data not shown). Flow cytometric evaluation of PSG aggregation in binding/uptake experiments with rrSP-D reveals that there was no correlation between the degree of aggregation of the PSG and the percentage of PSG-positive cells (Spearman $r = 0.4818$, $P = 0.13$).

rrSP-D binds to PSG. To demonstrate whether surfactant proteins bind to the PSG, we incubated Alexa Fluor 546-labeled PSG with FITC or Alexa Fluor 488-labeled surfactant proteins (rrSP-D and AP-SP-A). Fluorescently labeled rrSP-D (1 $\mu\text{g/ml}$) induced aggregation of PSG, and labeled rrSP-D coated the surface of large PSG aggregates (Fig. 5B). Binding of rrSP-D was blocked by addition of EDTA at a concentration of 10 mM (Fig. 5C). In contrast, no binding of FITC-labeled

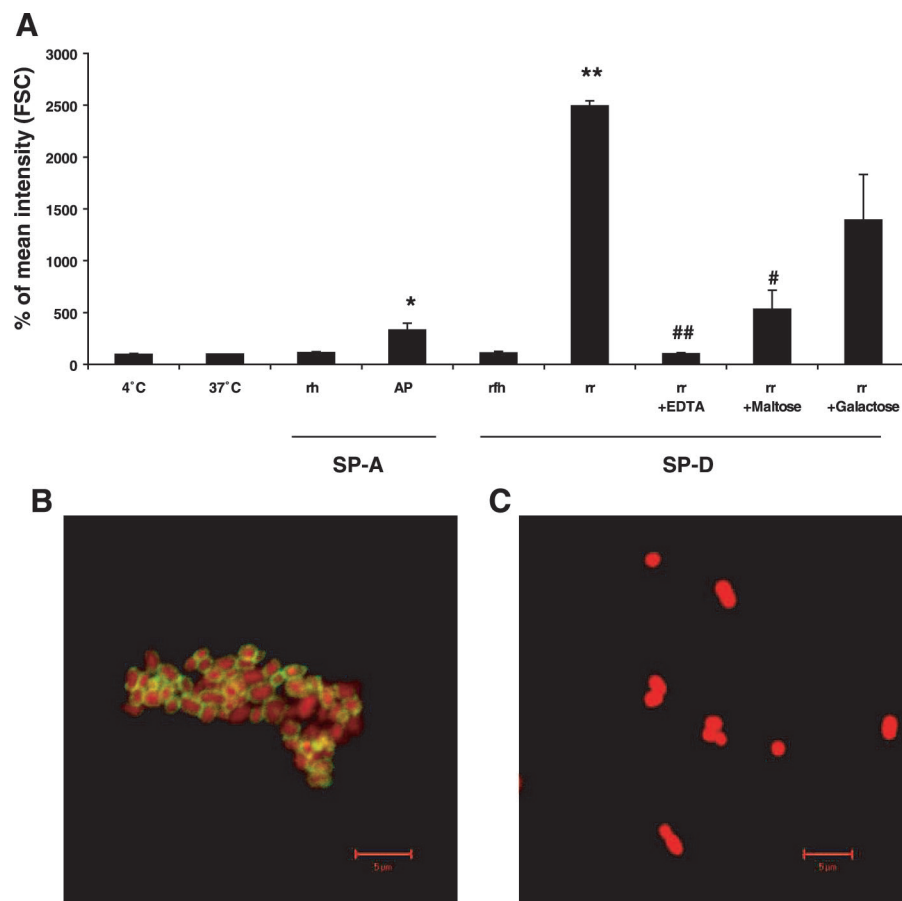


Fig. 5. Aggregation of the PSG from *D. glomerata* expressed as change (%) of the mean forward scatter (FSC) intensity in the presence of rhSP-A (30 $\mu\text{g/ml}$), AP-SP-A (10 $\mu\text{g/ml}$), rfhSP-D (30 $\mu\text{g/ml}$), and rrSP-D (1 $\mu\text{g/ml}$). Inhibition of rrSP-D-induced PSG aggregation was achieved by EDTA (10 mM), D-maltose (10 mM), and D-galactose (10 mM); $n = 4-6$, $*P < 0.05$ and $**P < 0.01$ compared with the control at 37°C , $\#P < 0.05$ and $\##P < 0.01$ compared with rrSP-D without inhibitors (A). PSG aggregation and binding of FITC-labeled rrSP-D (1 $\mu\text{g/ml}$) (B) was completely blocked by EDTA (10 mM) (C). Size bars = 5 μm .

AP-SP-A, even at a higher concentration (10 $\mu\text{g/ml}$), was detectable after the incubation (data not shown).

DISCUSSION

The aim of this study was to investigate the role of SP-A and -D in phagocytosis and clearance of respirable allergen by alveolar macrophages. We demonstrate that full-length dodecameric SP-D significantly increases the binding and phagocytosis of PSG by rat and human alveolar macrophages. In contrast, the rfhSP-D, which consisted only of the trimeric coiled neck region and the globular carboxy-terminal CRD, did not induce binding or phagocytosis of the PSG. Therefore, we conclude that the activity of SP-D on binding and phagocytosis of allergen is dependent on its integrity and molecular assembly.

SP-D, like SP-A, belongs to the group III C-type lectin family. SP-D is composed of 43-kDa monomers, each consisting of four major domains: a short cysteine-containing amino-terminal region, a triple helical collagenous domain, a trimeric coiled neck region, and a globular carboxy-terminal CRD (4). These monomers are assembled into trimers, and four of these trimeric subunits undergo disulfide cross-linking within their amino-terminal domains to form a cruciform dodecameric structure. The binding of allergen by the CRD on one side of the molecule and the binding to the cell by the CRD on the other side of the molecule might promote phagocytosis. This might be a possible explanation of our findings, since previous studies have shown that surfactant proteins bind allergen (21, 32) as well as alveolar macrophages in a Ca^{2+} -dependent manner through their CRDs (15). The effect of SP-D appears to be species independent since rrSP-D enhanced phagocytosis by both rat and human alveolar macrophages.

In addition to interacting with macrophages by its CRD, SP-D binding has also been shown via its collagen-like region (9). Thus SP-D might bind to PSG via its CRD and to alveolar macrophages via its collagen-like region. This could also explain why the effect is exclusively seen in the presence of full-length dodecameric rrSP-D but not rfhSP-D, which lacks the collagen-like domain and cysteine-rich region. In addition, SP-A did not enhance phagocytosis, but it increased aggregation of PSG although to a lesser extent compared with SP-D, which might indicate that SP-D-specific binding to alveolar macrophages is causing the increase of phagocytosis.

A phenomenon that was observed during the incubation of PSG with rrSP-D was the aggregation of the PSG. Interestingly, there was no aggregation of the PSG in the presence of rfhSP-D, which suggests that the aggregation of the PSG by SP-D is also dependent on the quaternary structure of the molecule. Previous studies have shown that SP-D aggregates various pathogens (8, 11, 16, 34). These studies also demonstrate that only dodecameric SP-D but not trimeric variants leads to agglutination of the bacteria and that the NH_2 -terminal and collagen domains of SP-D seem to be necessary for bacterial aggregation. Our results are consistent with these findings, and we conclude that the aggregation of PSG and bacteria by full-length dodecameric SP-D occurs via similar mechanisms.

AP-SP-A but not rhSP-A was also able to aggregate the PSG, however, to a much lesser extent compared with rrSP-D. Although no binding of AP-SP-A was visible in our confocal images, our data suggest that AP-SP-A has a low but signifi-

cant potency to aggregate the PSG. The observed differences between SP-A and SP-D in their ability to enhance uptake and aggregation might be due to different binding affinities to the carbohydrate structures on the surface of PSG. Previous studies have shown that binding of SP-A and SP-D to pathogens via the CRD is dependent on the type of carbohydrate ligand present on the surface of the pathogens (1, 19).

Inhibition experiments demonstrated that CRD-mediated functions of SP-A and SP-D can be completely blocked by EDTA and competitively inhibited by various sugars with varying potency. Maltose, for example, is one of the most potent inhibitors for CRD-mediated functions of SP-D, whereas galactose inhibits these functions to a much lesser degree (24). In our experiments, EDTA completely blocked the uptake and aggregation of the PSG, indicating a calcium ion dependency of the process. Moreover, rrSP-D enhanced binding and uptake of PSG in experiments with serum-free medium only after supplementation of calcium. In addition, maltose and, to a lesser extent, galactose competitively inhibited the enhanced uptake and aggregation of the PSG in the presence of rrSP-D. Together, these data suggest that the described processes are dependent on the affinity of the surfactant proteins for specific carbohydrate structures on the surface of the PSG.

Previous studies have shown that aggregation of pathogens does not necessarily lead to enhanced uptake by alveolar macrophages (1) or that aggregation is a phenomenon that can occur independently of phagocytosis (8). Moreover, for some pathogens, aggregation by surfactant proteins might even impair their phagocytosis (34). Although we observed large aggregates of PSG after incubation with rrSP-D, the vast majority of the granules that were found intracellularly were solitary. In addition, there was no correlation between the degree of PSG aggregation and the percentage of PSG-positive cells in experiments performed with rrSP-D. Therefore, we assume that SP-D mediates both aggregation and phagocytosis but that aggregation might not be necessary to enhance phagocytosis.

To determine the fate of phagocytosed PSG, alveolar macrophages were preincubated with PSG and followed up to 96 h after separation of extracellular PSG. The percentage of cells that had ingested PSG in the presence of rrSP-D initially decreased 24 h after Percoll separation but remained stable from 24 to 96 h. Therefore, we assume that the majority of PSG that are phagocytosed by alveolar macrophages remain inside the cells where they undergo either degradation (14) or macrophage-mediated clearance via the bronchotracheal route (17, 23).

In conclusion, our results demonstrate that dodecameric SP-D enhances phagocytosis and aggregation of pollen allergen, which might contribute to an increased allergen elimination from the lung. Thereby, SP-D might reduce allergen load and reduce allergic inflammation in patients with allergic asthma.

ACKNOWLEDGMENTS

We gratefully acknowledge technical assistance of Michael Cook, Lynn Martinek, Tim Oliver, Kathy Evans, Brunhild Volkmann, and Bianca Lavae-Mokhtari.

GRANTS

This work was supported by Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 587/B8 (J. M. Hohlfeld), Deutscher Akademischer Aus-

tauschdienst postdoctoral grant (V. J. Erpenbeck), and National Heart, Lung, and Blood Institute Grant HL-68072 (J. R. Wright).

REFERENCES

- Allen MJ, Harbeck R, Smith B, Voelker DR, and Mason RJ. Binding of rat and human surfactant proteins A and D to *Aspergillus fumigatus* conidia. *Infect Immun* 67: 4563–4569, 1999.
- Barr FE, Pedigo H, Johnson TR, and Shepherd VL. Surfactant protein-A enhances uptake of respiratory syncytial virus by monocytes and U937 macrophages. *Am J Respir Cell Mol Biol* 23: 586–592, 2000.
- Crouch E, Chang D, Rust K, Persson A, and Heuser J. Recombinant pulmonary surfactant protein D. Post-translational modification and molecular assembly. *J Biol Chem* 269: 15808–15813, 1994.
- Crouch E, Persson A, Chang D, and Heuser J. Molecular structure of pulmonary surfactant protein D (SP-D). *J Biol Chem* 269: 17311–17319, 1994.
- Currie AJ, Stewart GA, and McWilliam AS. Alveolar macrophages bind and phagocytose allergen-containing pollen starch granules via C-type lectin and integrin receptors: implications for airway inflammatory disease. *J Immunol* 164: 3878–3886, 2000.
- Dong Q and Wright JR. Degradation of surfactant protein D by alveolar macrophages. *Am J Physiol Lung Cell Mol Physiol* 274: L97–L105, 1998.
- Fels AO and Cohn ZA. The alveolar macrophage. *J Appl Physiol* 60: 353–369, 1986.
- Ferguson JS, Voelker DR, Ufnar JA, Dawson AJ, and Schlesinger LS. Surfactant protein D inhibition of human macrophage uptake of *Mycobacterium tuberculosis* is independent of bacterial agglutination. *J Immunol* 168: 1309–1314, 2002.
- Gardai SJ, Xiao YQ, Dickinson M, Nick JA, Voelker DR, Greene KE, and Henson PM. By binding SIRPalpha or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation. *Cell* 115: 13–23, 2003.
- Gottfredsson M, Erlendsdottir H, Sigfusson A, and Gudmundsson S. Characteristics and dynamics of bacterial populations during postantibiotic effect determined by flow cytometry. *Antimicrob Agents Chemother* 42: 1005–1011, 1998.
- Hartshorn KL, Crouch E, White MR, Colamussi ML, Kakkanatt A, Tauber B, Shepherd V, and Sastry KN. Pulmonary surfactant proteins A and D enhance neutrophil uptake of bacteria. *Am J Physiol Lung Cell Mol Physiol* 274: L958–L969, 1998.
- Holt PG, Macaubas C, Stumbles PA, and Sly PD. The role of allergy in the development of asthma. *Nature* 402: B12–B17, 1999.
- Jaen A, Sunyer J, Basagana X, Chinn S, Zock JP, Anto JM, and Burney P. Specific sensitization to common allergens and pulmonary function in the European Community Respiratory Health Survey. *Clin Exp Allergy* 32: 1713–1719, 2002.
- Knapp PE and Swanson JA. Plasticity of the tubular lysosomal compartment in macrophages. *J Cell Sci* 95: 433–439, 1990.
- Kuan SF, Persson A, Parghi D, and Crouch E. Lectin-mediated interactions of surfactant protein D with alveolar macrophages. *Am J Respir Cell Mol Biol* 10: 430–436, 1994.
- Kuan SF, Rust K, and Crouch E. Interactions of surfactant protein D with bacterial lipopolysaccharides. Surfactant protein D is an *Escherichia coli*-binding protein in bronchoalveolar lavage. *J Clin Invest* 90: 97–106, 1992.
- Lehnert BE. Pulmonary and thoracic macrophage subpopulations and clearance of particles from the lung. *Environ Health Perspect* 97: 17–46, 1992.
- Lohmann-Matthes ML, Steinmuller C, and Franke-Ullmann G. Pulmonary macrophages. *Eur Respir J* 7: 1678–1689, 1994.
- Madan T, Kishore U, Shah A, Eggleton P, Strong P, Wang JY, Aggrawal SS, Sarma PU, and Reid KB. Lung surfactant proteins 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* 110: 241–249, 1997.
- Madan T, Kishore U, Singh M, Strong P, Clark H, Hussain EM, Reid KB, and Sarma PU. Surfactant proteins A and D protect mice against pulmonary hypersensitivity induced by *Aspergillus fumigatus* antigens and allergens. *J Clin Invest* 107: 467–475, 2001.
- Malhotra R, Haurum J, Thiel S, Jensenius JC, and Sim RB. Pollen grains bind to lung alveolar type II cells (A549) via lung surfactant protein A (SP-A). *Biosci Rep* 13: 79–90, 1993.
- Miyamura K, Leigh LE, Lu J, Hopkin J, Lopez BA, and Reid KB. Surfactant protein D binding to alveolar macrophages. *Biochem J* 300: 237–242, 1994.
- Oh YK and Swanson JA. Different fates of phagocytosed particles after delivery into macrophage lysosomes. *J Cell Biol* 132: 585–593, 1996.
- Persson A, Chang D, and Crouch E. Surfactant protein D is a divalent cation-dependent carbohydrate-binding protein. *J Biol Chem* 265: 5755–5760, 1990.
- Pison U, Wright JR, and Hawgood S. Specific binding of surfactant apoprotein SP-A to rat alveolar macrophages. *Am J Physiol Lung Cell Mol Physiol* 262: L412–L417, 1992.
- Restrepo CI, Dong Q, Savov J, Mariencheck WI, and Wright JR. Surfactant protein D stimulates phagocytosis of *Pseudomonas aeruginosa* by alveolar macrophages. *Am J Respir Cell Mol Biol* 21: 576–585, 1999.
- Schagat TL, Wofford JA, and Wright JR. Surfactant protein A enhances alveolar macrophage phagocytosis of apoptotic neutrophils. *J Immunol* 166: 2727–2733, 2001.
- Scherer JM, Stillwell W, and Jenki LJ. Anomalous changes in forward scatter of lymphocytes with loosely packed membranes. *Cytometry* 37: 184–190, 1999.
- Strong P, Reid KB, and 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* 130: 19–24, 2002.
- Taylor PE, Flagan RC, Valenta R, and Glovsky MM. Release of allergens as respirable aerosols: a link between grass pollen and asthma. *J Allergy Clin Immunol* 109: 51–56, 2002.
- Tino MJ and Wright JR. Surfactant protein A stimulates phagocytosis of specific pulmonary pathogens by alveolar macrophages. *Am J Physiol Lung Cell Mol Physiol* 270: L677–L688, 1996.
- Wang JY, Kishore U, Lim BL, Strong P, and Reid KB. Interaction of human lung surfactant proteins A and D with mite (*Dermatophagoides pteronyssinus*) allergens. *Clin Exp Immunol* 106: 367–373, 1996.
- Wright JR, Wager RE, Hawgood S, Dobbs L, and Clements JA. Surfactant apoprotein Mr = 26,000–36,000 enhances uptake of liposomes by type II cells. *J Biol Chem* 262: 2888–2894, 1987.
- Yong SJ, Vuk-Pavlovic Z, Standing JE, Crouch EC, and Limper AH. Surfactant protein D-mediated aggregation of *Pneumocystis carinii* impairs phagocytosis by alveolar macrophages. *Infect Immun* 71: 1662–1671, 2003.