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

Delphine C. Malherbe,¹ Veit J. Erpenbeck,² Soman N. Abraham,³
Erika C. Crouch,⁴ Jens M. Hohlfeld,² and Jo Rae Wright¹

Departments of ¹Cell Biology and ³Pathology, Duke University Medical Center, Durham, North Carolina;

²Fraunhofer Institute of Toxicology and Experimental Medicine, Hannover, Germany; and

⁴Department of Pathology and Immunology, Washington University, St. Louis, Missouri

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Malherbe, Delphine C., Veit J. Erpenbeck, Soman N. Abraham, Erika C. Crouch, Jens M. Hohlfeld, and Jo Rae Wright. Surfactant protein D decreases pollen-induced IgE-dependent mast cell degranulation. *Am J Physiol Lung Cell Mol Physiol* 289: L856–L866, 2005. First published June 24, 2005; doi:10.1152/ajplung.00009.2005.—Mast cells play a key role in allergy and asthma. They reside at the host-environment interface and are among the first cells to make contact with inhaled microorganisms and particulate antigens. Pulmonary surfactant proteins A and D (SP-A and SP-D) function in lung host defense by enhancing microbe phagocytosis and mediating other immune cell functions, but little is known about their effects on mast cells. We hypothesized that SP-A and/or SP-D modulate IgE-dependent mast cell functions. Pollen starch granules (PSG) extracted from *Dactylis glomerata* and coated with trinitrophenol (TNP) were used as a model of an inhaled organic particulate allergen. Our data revealed that SP-D inhibited by 50% the release of β -hexosaminidase by peritoneal mast cells sensitized with IgE anti-TNP and stimulated with TNP-PSG. In contrast, SP-A had no effect. Furthermore, SP-D aggregated PSG in a dose-dependent manner, and this aggregation was mediated by SP-D's carbohydrate recognition domain. A single arm SP-D mutant (RrSP-Dser15,20) neither aggregated PSG nor inhibited degranulation, suggesting that multimerization of SP-D is required for maximal PSG aggregation and inhibition of PSG-induced mast cell degranulation. This study is the first to demonstrate that SP-D modulates IgE-mediated mast cell functions, which are important in asthma and allergic inflammation.

collectins; allergen; lung; innate immunity; immunoglobulin E

PULMONARY SURFACTANT IS A mixture of phospholipids and four proteins [surfactant protein (SP)-A, SP-B, SP-C and SP-D] that lines the lung alveoli (49). Surfactant has the following two known functions: a mechanical role in preventing alveolar collapse during breathing and a host defense role in lung innate immunity. Two of the four surfactant proteins have been shown to participate in host defense. SP-A and SP-D bind to bacteria, viruses, and fungi (12, 49) and enhance pathogen uptake by alveolar macrophages (38, 45), neutrophils (21), and dendritic cells (8). In addition, SP-A and SP-D are chemoattractants for alveolar macrophages (50) and neutrophils (13). Cytokine production by alveolar macrophages (36, 39) and dendritic cell maturation (7) are modulated by SP-A, and both SP-A and SP-D inhibit mitogen-stimulated lymphocyte proliferation (4–6). These in vitro findings are corroborated by studies showing that SP-A and SP-D null mice are more susceptible to bacterial and viral infections than are wild-type animals (24–27).

SP-A and SP-D are members of the collectin family of proteins (12, 49). The collectins are composed of four domains, a short amino-terminal domain, a triple helical collagen region, a trimeric coiled-coil domain (neck), and a carboxy-terminal C-type lectin carbohydrate recognition domain (CRD; see Refs. 12 and 49). Binding of the collectins to bacteria is mediated in large part via interaction of the CRD with oligosaccharides on the pathogen (11). Both SP-A and SP-D are oligomeric and consist of three monomers that assemble into a trimer that undergoes further multimerization. Six SP-A trimers assemble in an octadecameric structure resembling a bouquet of tulips, whereas four trimers of SP-D form a dodecamer in the shape of a cruciform (12, 49). Several functions of SP-A and SP-D have been shown to be dependent on their state of oligomerization (10, 20).

Although the effects of SP-A and SP-D on many types of innate and adaptive immune cells have been investigated, relatively little is known about their regulation of mast cell function. Mast cells, which reside at the interface of the host and the environment (skin, small intestine, lung), execute diverse functions that contribute to the immune response (1, 31). For example, mast cells have Fc ϵ RI receptors that, when bound by IgE and cross-linked to an allergen, induce a signaling cascade leading to the release of mast cell granule contents (3, 23). This degranulation process releases mediators, including histamine, which is a vasoactive amine that provokes a variety of responses that occur in allergy and asthma, such as vasodilatation and smooth muscle constriction (3). Until recently, mast cells were considered to be harmful to the host because of their key role in allergy and asthma. However, recent data have revealed that mast cells also contribute to host defense (32–34).

A few recent studies have investigated interactions of surfactant proteins with allergens and regulation of allergen-mediated cellular responses. For example, both SP-A and SP-D bind via their CRDs to dust mite allergens in a calcium-dependent manner (47). This interaction of surfactant with dust mite allergens inhibits the binding of specific IgE to the allergen (47). Similar results were obtained with allergens from *Aspergillus fumigatus* (29). Moreover, SP-A and SP-D inhibit dust mite *Dermatophagoides pteronyssinus* (Der p)-stimulated histamine release from human peripheral blood mononuclear cells (PBMCs) in a dose-dependent fashion. SP-A and SP-D also inhibit allergen-induced lymphocyte proliferation (48).

Address for reprint requests and other correspondence: J. R. Wright, Box 3709, Dept. of Cell Biology, Duke Univ. Medical Center, Durham, NC 27710 (e-mail: j.wright@cellbio.duke.edu).

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Furthermore, SP-A and -D protected mice against allergic bronchopulmonary aspergillosis (30, 42), and SP-D protected mice against allergic responses induced by dust mite allergens (43). In addition, Malhotra and colleagues (35) showed that SP-A binds to a variety of pollen grains and that it facilitates pollen grain binding to the lung epithelial cell line A549.

The goal of the current study was to test the hypothesis that SP-A and -D modulate IgE-dependent mast cell functions using a model system of pollen starch granules (PSG). Taylor et al. (44) recently demonstrated that pollen grains, which are too large to penetrate the lower airways, are composed of allergen-containing starch granules that range in size from 0.5 to 3 μm and can reach the lower airways where they could potentially trigger an asthma attack. We hypothesized that surfactant proteins interact with this type of particulate allergen and thereby inhibit PSG-stimulated mast cell degranulation. In our study, PSG were extracted from *Dactylis glomerata* (orchard grass), a common grass pollen allergen. Given the absence of murine IgE antibodies against PSG, we used an experimental setting that provided specificity to the PSG by sensitizing the mast cells with anti-trinitrophenol (TNP) IgE and by coating the PSG with TNP. Our findings reveal that SP-D increased PSG binding to mast cells. SP-D also aggregated PSG, and this process was mediated by SP-D's lectin domain. Furthermore, SP-D decreased PSG-stimulated mast cell degranulation. Aggregation of PSG and inhibition of mast cell degranulation were dependent on SP-D's oligomerization state. In contrast, SP-A neither modulated PSG-stimulated mast cell degranulation nor aggregated PSG. In summary, this study is the first showing that SP-A and SP-D differentially interact with an allergen and that SP-D inhibits IgE-dependent mast cell functions, thereby revealing a potential important role for SP-D in allergy and asthma.

MATERIALS AND METHODS

Materials and animals. Cell culture medium, antibiotics, balanced salt solutions, and buffers were purchased from Invitrogen (Carlsbad, CA). Heat-inactivated FBS was from Hyclone (Logan, UT). Recombinant cytokine interleukin (IL)-3 was from R & D Systems (Minneapolis, MN). Unless otherwise noted, all other materials and reagents were purchased from Sigma (St. Louis, MI). C57BL/6 mice were purchased from Charles River (Raleigh, NC). All animal procedures were approved by the Duke University Institutional Animal Care and Use Committee.

Cultured peritoneal mast cells. A peritoneal lavage of four C57BL/6 mice was performed with Hanks' balanced salt solution, and peritoneal mast cells were cultured as previously described (51). Briefly, cells were cultured in DMEM with 10% heat-inactivated FBS and penicillin/streptomycin in the presence of 5 ng/ml recombinant IL-3 and 10 ng/ml recombinant stem cell factor. Cell purity was assessed with a toluidine blue staining or by fluorescence-activated cell sorter (FACS) analysis with a double staining for c-kit and IgE receptors (Pharmingen, San Diego, CA). Routinely, 95% of the cells were positive for c-kit and IgE receptors.

Surfactant proteins. SP-A was purified as previously described by butanol extraction from therapeutic bronchoalveolar lavage of patients suffering from alveolar proteinosis (36). SP-D was isolated as previously described from supernatant of Chinese hamster ovary cells stably transfected with a cDNA clone of full-length rat SP-D (15). Rat SP-D mutant RrSP-Dser15,20 was isolated as previously described (9, 10). SP-D preparations were purified by sequential affinity chromatography on maltosyl-agarose, and dodecamers were isolated by gel filtration chromatography under nondenaturing conditions. The level

of endotoxin in the protein preparations was assessed by Limulus Amebocyte Lysate assay (BioWhittaker, Walkersville, MD). SP-A preparations contained 0.6 pg endotoxin/ μg protein, and wild-type SP-D preparations contained <0.25 pg endotoxin/ μg protein. RrSP-Dser15,20 contained 75 pg endotoxin/ μg protein.

Protein binding assay. Cells (2×10^5) were incubated for 2 h on ice in Dulbecco's PBS containing 1% BSA with increasing concentrations of FITC-labeled SP-A or FITC-labeled SP-D (22). After the 2-h incubation, cells were washed two times by centrifugation (200 g, 4°C) and were fixed in PBS + 1% paraformaldehyde. The samples were analyzed by flow cytometry. For the calcium dependency study, cells were incubated in buffer containing either 0.9 mM CaCl_2 or 1 mM EDTA. The carbohydrate inhibition study was performed by addition of 20 mM sugar (D-maltose, D-galactose, or galactosamine for SP-D and N-acetylmannosamine, D-mannose, or D-galactose for SP-A) at the beginning of the 2-h incubation. For control experiments, surfactant proteins were heat-inactivated by incubation for 10 min at 95°C.

PSG. PSG were extracted as previously described from pollen grains of *D. glomerata* (Biopol, Spokane, WA; see Ref. 14). Briefly, 200 mg pollen grains resuspended in 20 ml deionized water with 0.05% Tween 20 were rotated for 2 h at 4°C. The suspension was centrifuged at 50 g for 5 min and filtered through a 20- μm nylon mesh (VWR International, West Chester, PA). The filtrate was centrifuged for 10 min at 2,000 g. The pellet was resuspended in 20 ml deionized water and was filtered through a 3- μm polycarbonate filter (Osmonics, Minnetonka, MN). The filtrate was centrifuged as before, and the PSG were resuspended in 1 ml PBS, separated into aliquots, and stored at 4°C. There is no murine IgE antibody raised against pollen commercially available, but many commercial antibodies are raised against TNP. We took advantage of this property, and, for all assays, the PSG were coated with TNP and mast cells were sensitized with IgE anti-TNP (Pharmingen). This procedure enabled us to have a system in which mast cells were specifically stimulated by TNP-coated PSG (TNP-PSG). On the day of the experiment, PSG were coated with TNP by incubating the PSG in borate buffer, pH 8.2, with 2,4,6-trinitrobenzenesulfonic acid for 30 min in a water bath at 37°C (J. S. Shin, personal communication). TNP-PSG were washed four times with PBS before use.

PSG binding assay. Cells (1×10^5) were sensitized overnight with IgE anti-TNP at 0.5 $\mu\text{g}/\text{ml}$ (Pharmingen). Cells were resuspended in PBS + 1% BSA and were stimulated with FITC-labeled TNP-coated PSG (100 PSG/1 cell) for 30 min at 37°C. After incubation, cells were washed two times by centrifuging the samples and resuspending them in PBS + 1% BSA. Next, the cells were either fixed in PBS + 1% paraformaldehyde and later stained with the nuclear dye 7-aminoactinomycin (7-AAD; Pharmingen) to be analyzed by flow cytometry or were cytospun (Cytospin 2 by Shandon, 400 rpm, 2 min) on slides and were stained with an Hematoxylin and Eosin Hemacolor kit (Harleco, Gibbstown, NJ) according to the manufacturer's instructions. The slides were analyzed by microscopy (Zeiss Axioskop 2 plus) with $\times 40$ magnification.

β -Hexosaminidase release assay. Cells were seeded in a 96-well plate at a density of 1×10^5 cells/well and were sensitized overnight with IgE anti-TNP at 0.5 $\mu\text{g}/\text{ml}$ (Pharmingen). Cells were resuspended in Tyrode buffer (135 mM NaCl, 5 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 5.6 mM glucose, 1 mg/ml 0.1% BSA, an 20 mM HEPES, pH 7.4) and were stimulated for 30 min at 37°C with either soluble anti-IgE (Pharmingen) at a concentration of 250 ng/ml or with TNP-coated PSG at a ratio of 100 PSG/1 cell in the presence or absence of surfactant proteins at various concentrations. After the 30-min incubation, cells were centrifuged at 250 g for 10 min, and supernatant was collected from each well to perform the colorimetric assay. Total cell content of β -hexosaminidase was obtained by lysing cells that were sensitized with IgE but not stimulated with TNP-PSG using Tyrode buffer containing 0.1% Triton X-100. Supernatants were incubated with p-nitrophenyl N-acetyl- β -D-glucosaminide for 30 min



at 37°C, and the enzymatic reaction was stopped by adding 0.1 M carbonate buffer $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ at pH 10.1. Absorbance at 405 nm was measured with a Bio-Rad plate reader, and the data are expressed as percent release where percent release = absorbance of supernatant/absorbance total cell content, with the total cell content being the sum of the spontaneous β -hexosaminidase release in the medium and the β -hexosaminidase cell content. For some experiments in which cells were preincubated with SP-D, the cells were sensitized overnight with IgE (0.5 $\mu\text{g}/\text{ml}$), the unbound IgE was washed away, and the cells were incubated with 5 $\mu\text{g}/\text{ml}$ SP-D for 2 h at 4°C; the unbound SP-D was washed away before performing the degranulation, as described above. In some assays, the SP-D mutant RrSP-Dser15,20 was used at a concentration of 5 $\mu\text{g}/\text{ml}$.

Aggregation assay. PSG (2×10^7) were coated with TNP and then labeled with FITC (Molecular Probes, Eugene, OR). PSG were incubated in PBS containing 1% BSA in the presence of SP-D or SP-A for 30 min at 37°C. Samples were then centrifuged at 250 g for 10 min and resuspended in PBS containing 1% BSA, and this step was repeated one time. Samples were fixed with PBS with 1% paraformaldehyde and were analyzed by flow cytometry. For some assays, samples were incubated with 5 $\mu\text{g}/\text{ml}$ SP-D and 20 mM D-maltose, or 20 mM D-galactose or 20 mM galactosamine. The SP-D mutant RrSP-Dser15,20 was also used at the concentration of 5 $\mu\text{g}/\text{ml}$ in the aggregation assays.

Flow cytometry. FACS analysis was performed at the Duke University Comprehensive Cancer Flow Cytometry and at the Human Vaccine Institute facilities. Briefly, for the binding of FITC-labeled surfactant proteins to mast cells, the mast cell population was gated using its forward and scatter properties, and the amount of fluorescence obtained in the FITC channel was measured. In a similar way, for the binding of FITC-labeled TNP-PSG to mast cells, the cell population was gated as 7-AAD positive, and the amount of fluorescence obtained in the FITC channel was measured. For the aggregation experiments, the forward and scatter properties of the aggregates were acquired as previously described (19, 41). Samples (10,000 events) were analyzed with FLOWJO software.

Statistics. Statistical analysis was performed with the *t*-test (package included in the Microsoft Excel software). Results were statistically significant at $P < 0.05$.

RESULTS

SP-A and SP-D bind to peritoneal mast cells in a dose- and calcium-dependent manner. Binding of SP-A and SP-D in the presence of calcium or EDTA to mast cells was assessed by flow cytometry using FITC-SP-A in concentrations up to 40 $\mu\text{g}/\text{ml}$ or FITC-SP-D in concentrations up to 10 $\mu\text{g}/\text{ml}$. Binding of SP-D (Fig. 1A) or SP-A (Fig. 1B) to mast cells is dose dependent. In addition, when cells were incubated in the presence of 1 mM EDTA, the amount of cell-associated fluorescence was greatly reduced at all protein concentrations tested, demonstrating that the binding of both SP-A and SP-D is calcium dependent. In addition, SP-A and SP-D binding to mast cells was similar whether the cells were sensitized with IgE or not sensitized (data not shown). Because mast cell degranulation assays are conventionally performed in Tyrode buffer, the binding of SP-D to mast cells in Tyrode buffer was also analyzed. When the binding studies were performed in Tyrode buffer instead of PBS + 1% BSA, SP-A binding to mast cells was not altered, and SP-D binding to mast cells was reduced substantially, probably because of the high glucose content (5.6 mM) in Tyrode buffer. However, SP-D binding in Tyrode buffer was still statistically significant at the dose of 5 $\mu\text{g}/\text{ml}$ (data not shown).

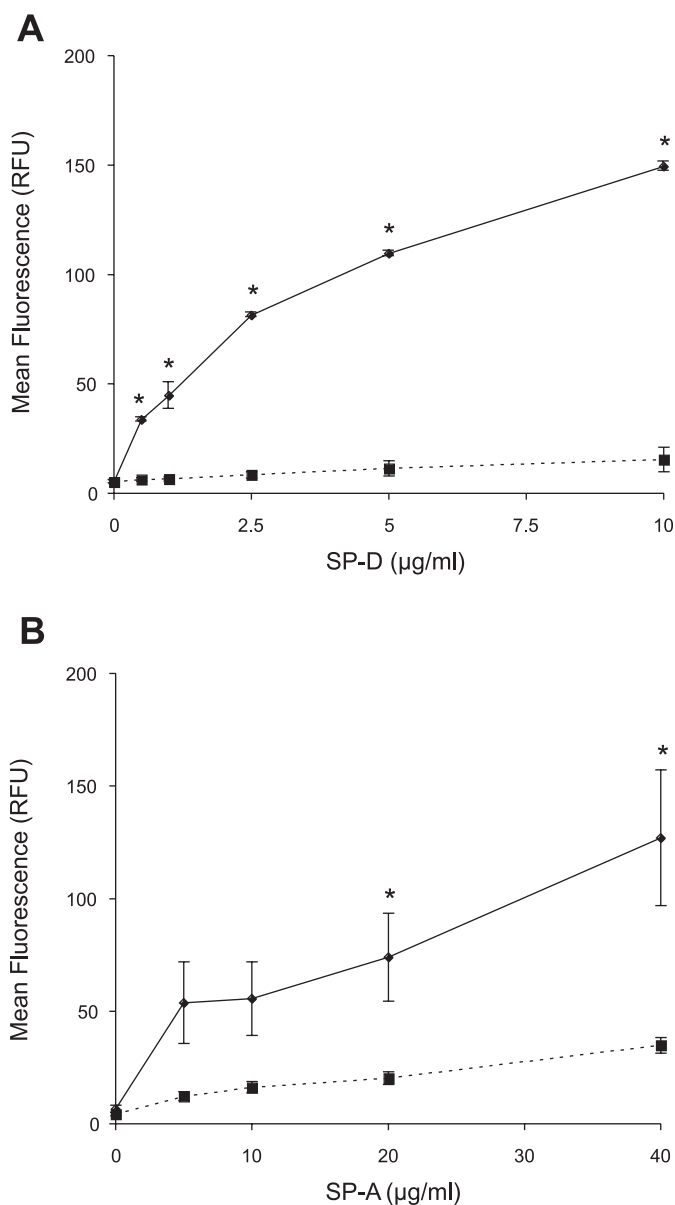


Fig. 1. Surfactant protein (SP) A and SP-D bind to mast cells in a dose-dependent and calcium-dependent manner. Mast cells were incubated for 2 h on ice with increasing concentrations of FITC SP-D (0–10 $\mu\text{g}/\text{ml}$; A) or FITC SP-A (0–40 $\mu\text{g}/\text{ml}$; B) in the presence of 0.9 mM CaCl_2 (◆) or 1 mM EDTA (■). After the incubation, cells were washed two times, fixed, and analyzed by flow cytometry; $n = 5$ experiments and $*P < 0.05$ for SP-A and $n = 3$, $*P < 0.03$ for SP-D compared with EDTA treatment. RFU, relative fluorescence units.

To determine which domain of the proteins was involved in the binding to mast cells, the effects of competing carbohydrates were analyzed. For SP-D, binding to mast cells was inhibited by maltose and galactose but not by galactosamine. Also, SP-D binding was inhibited by heat inactivation (Table 1). These results suggest that SP-D binding to mast cells is mediated by its lectin domain and requires tertiary structure, which is destroyed by heating. For SP-A, binding to mast cells was inhibited by heat inactivation but not by the tested sugars (Table 2). Therefore, binding of SP-A to mast cells does not appear to be mediated via its CRD.

Table 1. *SP-D binds to mast cells through its lectin domain*

Treatment	Binding, %control
SP-D	100
SP-D + maltose	11.1 ± 6.3*
SP-D + galactose	15.6 ± 7.0*
SP-D + galactosamine	103.0 ± 38.2
SP-D + EDTA	7.1 ± 1.4*
Heat-inactivated SP-D	11.9 ± 2.7*

Values are means ± SE; $n = 3$ experiments. SP-D, surfactant protein D. Mast cells were incubated for 2 h on ice with 5 $\mu\text{g/ml}$ FITC SP-D and 20 mM sugar (D-maltose, D-galactose, or galactosamine). Heat inactivation treatment was a 10-min incubation of FITC SP-D at 95°C, and EDTA treatment was the incubation in the presence of 1 mM EDTA. After the incubation, cells were washed two times, fixed, and analyzed by flow cytometry. Results are expressed as %control, where 100% was the amount of cell-associated fluorescence when cells were incubated with SP-D only. * $P < 0.01$ compared with SP-D treatment.

SP-D increases TNP-PSG binding to mast cells. Malhotra and colleagues (35) showed that SP-A increased the binding of pollen grains to lung epithelial cells. This prompted us to test whether surfactant proteins enhance PSG binding to mast cells. Mast cells were incubated for 30 min at 37°C in the presence of FITC-labeled TNP-PSG at a ratio of 100 PSG/cell with increasing concentrations of SP-A or SP-D. Quantitative analysis was performed by flow cytometry, and cells were also observed by microscopy. Microscopic observations showed that SP-A did not aggregate TNP-PSG, and the amount of TNP-PSG bound to the mast cell surface was similar in the presence or absence of SP-A (Fig. 2). In contrast, when cells were incubated with TNP-PSG and SP-D, large TNP-PSG aggregates were observed, and the number of TNP-PSG interacting with mast cells was increased (Fig. 2). To discriminate between TNP-PSG associated with cells and the large TNP-PSG aggregates, the samples that were analyzed by FACS were also labeled with the nuclear dye 7-AAD (Fig. 3A). The flow cytometry data revealed that SP-D dose dependently increased TNP-PSG binding to mast cells up to 300% at the highest SP-D concentration of 10 $\mu\text{g/ml}$ (Fig. 3B). This effect was not saturable at the SP-D doses that were tested. In contrast, SP-A did not alter TNP-PSG binding to mast cells (Fig. 3C).

SP-D aggregates TNP-PSG. The finding that incubation of TNP-PSG with SP-D resulted in formation of large PSG aggregates lead us to hypothesize that SP-D interacted directly with TNP-PSG and induced their aggregation. TNP-PSG were incubated for 30 min at 37°C with increasing concentrations of SP-A or SP-D, and aggregate size was measured by flow cytometry (Fig. 4A). SP-D induced a dose-dependent size increase of TNP-PSG aggregates when PSG were incubated with SP-D concentrations ranging from 0 to 2.5 $\mu\text{g/ml}$ (Fig. 4B). The effects of SP-D on aggregation reached a plateau at concentrations >5 $\mu\text{g/ml}$ SP-D (Fig. 4B), which induced a fivefold increase in PSG aggregation. In addition, SP-D also dose dependently aggregated TNP-PSG extracted from *Lolium perenne* and from *Phleum pratense* (data not shown). The SP-D-induced aggregation of TNP-PSG was observed in both PBS + 1% BSA and in Tyrode buffer (data not shown). In addition, SP-D aggregated both uncoated and TNP-coated PSG (data not shown), consistent with our previous results (17). Therefore, PSG aggregation was not the result of TNP labeling

of the PSG. In contrast, SP-A had no effect on TNP-PSG aggregation (Fig. 4C).

Through its CRD, SP-D binds carbohydrate structures on pathogen surfaces. Because PSG are carbohydrate rich, we investigated whether SP-D interacted with TNP-PSG through its lectin domain. The ability of carbohydrates, which bind to SP-D's CRD, to inhibit aggregation was also analyzed by FACS. D-Maltose was the most potent inhibitor of SP-D-induced TNP-PSG aggregation, reducing it by 70% compared with the aggregation obtained when TNP-PSG were incubated with SP-D only (Table 3). D-Galactose was less efficient than maltose at inhibiting SP-D-mediated TNP-PSG aggregation, inducing only a 50% reduction. Galactosamine, a sugar with very low affinity toward the CRD of SP-D, did not inhibit PSG aggregation. In addition, EDTA inhibited TNP-PSG aggregation by SP-D (Table 3). These results suggest that SP-D interacts with TNP-PSG through its CRD.

SP-D decreases TNP- and PSG-induced mast cell degranulation. Because SP-A and SP-D have been shown to reduce the release of histamine from basophils of allergic patients stimulated with dust mite allergens (48), we hypothesized that SP-A and SP-D would also alter IgE-mediated degranulation of mast cells. Peritoneal mast cells were stimulated for 30 min at 37°C with PSG at a ratio of 100 PSG/cell and increasing concentrations of SP-A or SP-D (Fig. 5). As a measure of mast cell degranulation, the release of β -hexosaminidase was evaluated. PSG were coated with TNP, and mast cells were sensitized with IgE anti-TNP. This procedure creates a system in which mast cells were specifically stimulated by TNP-PSG. Indeed, when mast cells were sensitized and then stimulated with noncoated PSG, there was no degranulation ($0.34 \pm 0.05\%$, $n = 3$); in contrast, stimulation with TNP-PSG resulted in the release of ~ 15 – 30% of the cell content of β -hexosaminidase. By themselves, surfactant proteins did not induce mast cell degranulation (data not shown). Although SP-A had no effect on TNP-PSG-induced mast cell degranulation even at the highest concentration tested (40 $\mu\text{g/ml}$) as shown in Fig. 5B, SP-D was able to reduce mast cell degranulation by 50% at the two highest doses tested (5 and 10 $\mu\text{g/ml}$; Fig. 5A). When the degranulation assay was performed in PBS + 1% BSA instead of Tyrode buffer, SP-D reduced TNP- and PSG-induced mast cell degranulation by 50%, comparable to the results obtained

Table 2. *SP-A does not bind to mast cells through its lectin domain*

Treatment	Binding, %control
SP-A	100
SP-A + <i>N</i> -acetylmannosamine	92.7 ± 3.7
SP-A + mannose	92.1 ± 5.7
SP-A + galactose	89.1 ± 4.4
SP-A + EDTA	21.9 ± 6.4*
Heat-inactivated SP-A	24.7 ± 3.9*

Values are means ± SE; $n = 5$ experiments. SP-A, surfactant protein A. Mast cells were incubated for 2 h on ice with 20 $\mu\text{g/ml}$ FITC SP-A and 20 mM sugar (D-mannose, D-galactose, or *N*-acetylmannosamine). Heat inactivation treatment was a 10-min incubation of FITC SP-A at 95°C, and EDTA treatment was the incubation in the presence of 1 mM EDTA. After the incubation, cells were washed two times, fixed, and analyzed by flow cytometry. Results are expressed as %control, where 100% was the amount of cell-associated fluorescence when cells were incubated with SP-A only. * $P < 0.01$ compared with SP-A treatment.

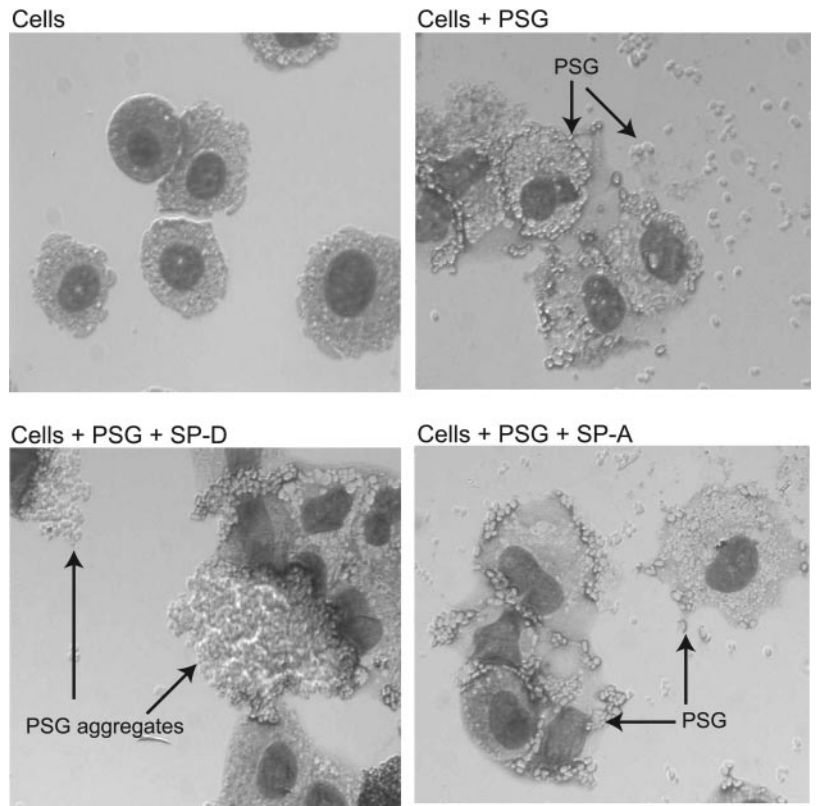


Fig. 2. Representative experiment of SP-D's increase of pollen starch granule (PSG) binding to mast cells. Mast cells were sensitized overnight with IgE (0.5 μ g/ml) and were stimulated for 30 min at 37°C with FITC-labeled trinitrophenol (TNP)-coated PSG in presence of 5 μ g/ml SP-D or 40 μ g/ml SP-A. Cells were cytospun on glass slides, stained with a Hematoxylin and Eosin Hemacolor kit, and analyzed by microscopy (magnification $\times 40$).

using Tyrode buffer (data not shown). Likewise, SP-A had no effect on degranulation in either buffer (data not shown). In addition, SP-D also reduced mast cell degranulation when mast cells were stimulated with TNP-coated PSG extracted from *L. perenne* (rye grass) and from *P. pratense* (timothy grass; data not shown). In contrast, SP-D did not inhibit mast cell degranulation stimulated by soluble anti-IgE when SP-D and anti-IgE were added simultaneously (Fig. 6) or when mast cells were preincubated with SP-D for 30 min before the addition of soluble anti-IgE or calcium ionophore (data not shown). To determine if SP-D has a direct effect on the mast cells, cells were sensitized with IgE and then incubated with 5 μ g/ml SP-D before stimulation with TNP-PSG or soluble anti-IgE. Preincubation with SP-D did not inhibit mast cell degranulation (Fig. 7). However, addition of 5 μ g/ml SP-D at the same time as the TNP-PSG induced a decrease in mast cell degranulation. These results demonstrate that SP-D does not exert a global inhibitory effect on mast cell degranulation. In addition, they further confirm our hypothesis that SP-D directly interacts with the TNP-PSG by aggregating them and sequestering them away from the cells.

SP-D multimerization is important for PSG aggregation and for inhibition of mast cell degranulation. Several of SP-D's immune functions are mediated by its state of multimerization. For example, oligomerized SP-D is a more effective pathogen agglutinin than is monomeric SP-D (10). We investigated whether a SP-D mutant molecule composed of a trimeric arm only (RrSP-Dser15,20) would be less efficient at mediating TNP-PSG aggregation and degranulation than wild-type SP-D. The RrSP-Dser15,20 arm mutant did not significantly enhance TNP-PSG aggregation (Fig. 8A), thereby demonstrating that SP-D dodecameric structure is essential for maximal PSG

aggregation. Furthermore, the RrSP-Dser15,20 mutant did not inhibit TNP-PSG-induced mast cell degranulation in a statistically significant manner (Fig. 8B). These results suggest that SP-D multimerization is important for both the aggregation of TNP-PSG and the inhibition of mast cell degranulation.

DISCUSSION

Our findings reveal that SP-D modulates IgE-mediated mast cell functions. SP-D, but not SP-A, inhibited TNP- and PSG-induced mast cell degranulation by $\sim 50\%$, and this inhibition required SP-D multimerization. Interestingly, SP-D did not inhibit degranulation induced by either soluble anti-IgE or calcium ionophore, suggesting that the interaction with a particulate allergen was required for inhibition. Furthermore, pretreatment of sensitized mast cells with SP-D did not alter mast cell degranulation capabilities. Both SP-A and SP-D bind to mast cells in a dose-dependent and calcium-dependent manner. In addition, carbohydrates inhibited binding of SP-D to mast cells but not binding of SP-A, suggesting that SP-D binding to mast cells occurs via its lectin domain. Although SP-A had no effect on TNP-PSG aggregation or binding to mast cells, SP-D dose-dependently increased TNP-PSG aggregation and binding to mast cells. The aggregation of TNP-PSG was also inhibited by sugars and is therefore likely mediated by SP-D's lectin domain. Wild-type SP-D was a much more effective agglutinin of TNP-PSG than was RrSP-Dser15,20, a single-arm SP-D mutant, suggesting that SP-D dodecameric structure was necessary for maximal aggregation of TNP-PSG by SP-D. In addition, SP-D multimerization was important for SP-D's inhibition of mast cell degranulation. Collectively, these results

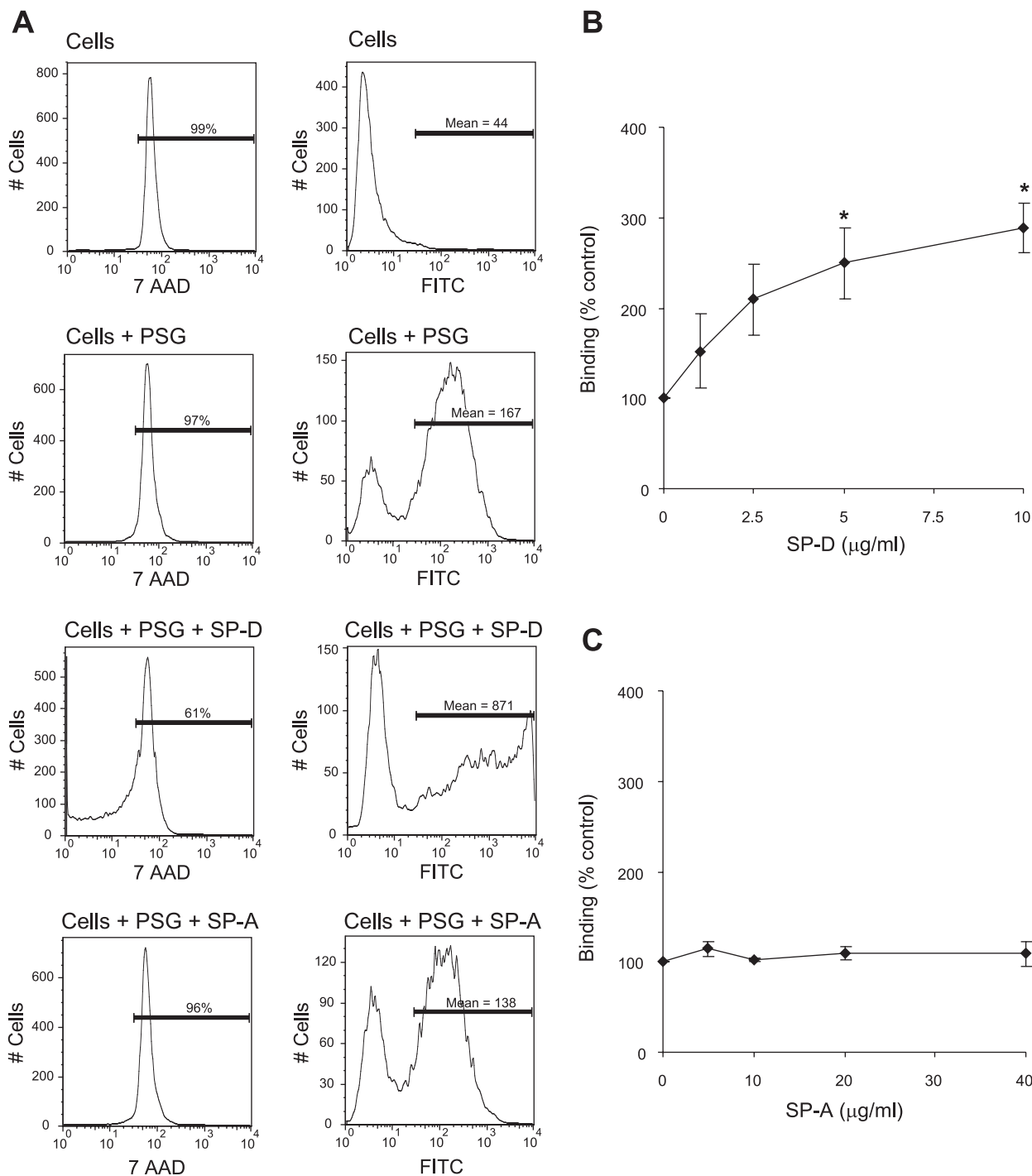


Fig. 3. SP-D increases PSG binding to mast cells. Mast cells were sensitized overnight with IgE (0.5 $\mu\text{g/ml}$) and were stimulated for 30 min at 37°C with FITC labeled TNP-coated PSG in the presence of increasing concentrations of SP-D or SP-A. Cells were then labeled with 7-aminoactinomycin (7-AAD) and were analyzed by flow cytometry. A is a representative fluorescence-activated cell sorter (FACS) plot of the following 4 experimental groups: cells, cells + PSG, cells + PSG + 5 $\mu\text{g/ml}$ SP-D, and cells + PSG + 40 $\mu\text{g/ml}$ SP-A. Panels on left are the 7-AAD-positive cells, and panels on right are the fluorescence measured in the FITC channel. B and C represent the binding of TNP-PSG to mast cells in the presence of increasing concentrations of SP-D (B) and SP-A (C). For B and C, results are expressed as %control, where 100% is the amount of PSG binding in the absence of surfactant protein; $n = 4$, $*P < 0.05$ compared with no protein treatment.

show that SP-D but not SP-A mediates mast cell responsiveness to pollen granules.

In this study, both SP-A and SP-D were shown to bind to mast cells in a dose-dependent and calcium-dependent manner. Binding of SP-A and SP-D to macrophages (37), type II

pneumocytes (22), and dendritic cells (8) has also been observed to be both calcium and dose dependent. Binding of SP-D to mast cells was not saturable at the concentrations tested. Previous studies with type II cells (22) reported similar nonsaturable binding, and it was suggested that protein self-

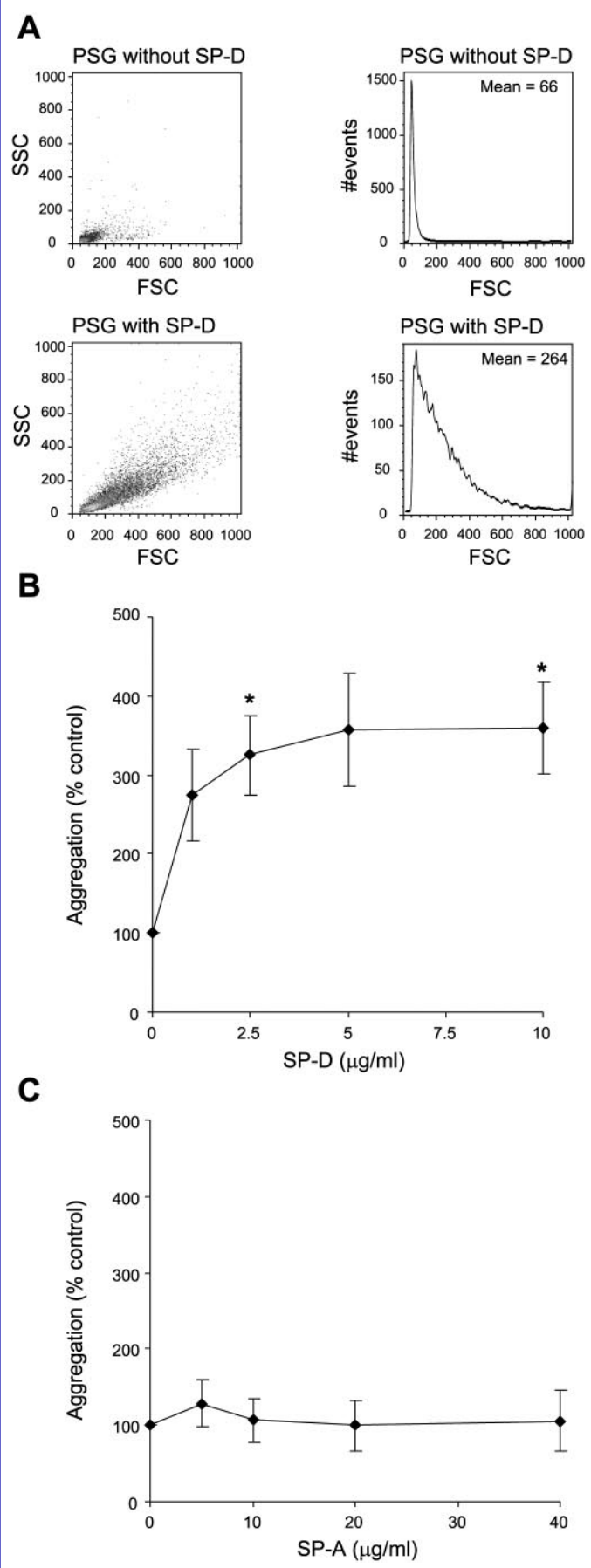


Table 3. SP-D aggregates PSG through its lectin domain

Treatment	Aggregation, %control
No SP-D	100*
SP-D	464 $\pm$ 25
SP-D + D-maltose	136 $\pm$ 4*
SP-D + D-galactose	250 $\pm$ 5*
SP-D + galactosamine	503 $\pm$ 19
SP-D + EDTA	88 $\pm$ 2*

Values are means $\pm$ SE; $n = 5$ experiments. PSG, pollen starch granules. FITC-labeled TNP-coated PSG were incubated for 30 min at 37°C with 5 $\mu\text{g/ml}$ SP-D in the presence of 20 mM sugars. Samples were analyzed by flow cytometry. Results are expressed as %control, where 100% is the amount of aggregation obtained with SP-D. * $P < 0.01$ compared with SP-D treatment.

aggregation, which is concentration dependent (40), is responsible for this effect. In addition, as previously described with other cell types, SP-D binding to mast cells is likely via its lectin domain. In contrast to SP-D, SP-A's lectin domain did not seem to mediate the binding to mast cells. Several receptors for SP-A and SP-D have been described over the years (notably SPR-210 for SP-A and gp-340 for SP-D; see Ref. 49) and signal inhibitory regulatory protein- α (SIRP α) and calreticulin/CD91 were recently identified as SP-A and SP-D receptors on macrophages. Binding of SP-A and SP-D to SIRP α through their lectin domain inhibits macrophage activation and maintains the lung in a resting state, whereas, when surfactant proteins bind pathogens through their lectin domain, the collectin collagen domains trigger macrophage activation via the CD91/calreticulin complex and induce release of proinflammatory mediators (18). The receptors for SP-A and SP-D on mast cells have not been identified.

A previous study showed that SP-A bound to pollen grains and enhanced pollen grain adhesion to A549 cells (35). In addition, Madan and colleagues (28) demonstrated that SP-A and SP-D increased the binding of conidia of *A. fumigatus* to both neutrophils and alveolar macrophages. We found that SP-D dose dependently increased TNP-PSG binding to mast cells. SP-D induced binding of large TNP-PSG aggregates to mast cells instead of single TNP-PSG, and this may account for the increase in fluorescence measured by flow cytometry. In contrast, SP-A had no effect on TNP-PSG binding. Although we do not have an explanation for why SP-A enhances pollen grain binding to A549 cells but not to mast cells, it is possible that the effect may be cell or allergen specific or dependent on the method of isolation of the SP-A. For example, in this study, we extracted PSG from orchard grass pollen grains, whereas Malhotra and colleagues (35) used whole pollen grains from different grass species. Furthermore, previous studies proposed that SP-A isolated by different methods had different functions (46). Malhotra and colleagues used SP-A isolated by ion-

Fig. 4. SP-D aggregates PSG. FITC-labeled TNP-coated PSG were incubated with increasing concentrations of SP-D or SP-A for 30 min at 37°C. Samples were analyzed by flow cytometry. Representative FACS plots of PSG or PSG + 5 $\mu\text{g/ml}$ SP-D are presented in A, with the panels on the left being the forward/scatter plots and the panels on the right being the fluorescence measured in the FSC channel. B and C show the effect of increasing concentrations of SP-D (B) and SP-A (C) on TNP-PSG aggregation. For B and C, results are expressed as %control, where 100% is the amount of PSG aggregation in the absence of surfactant protein; $n = 3$, * $P < 0.05$ compared with no protein treatment. SSC, side scatter; FSC, forward scatter.

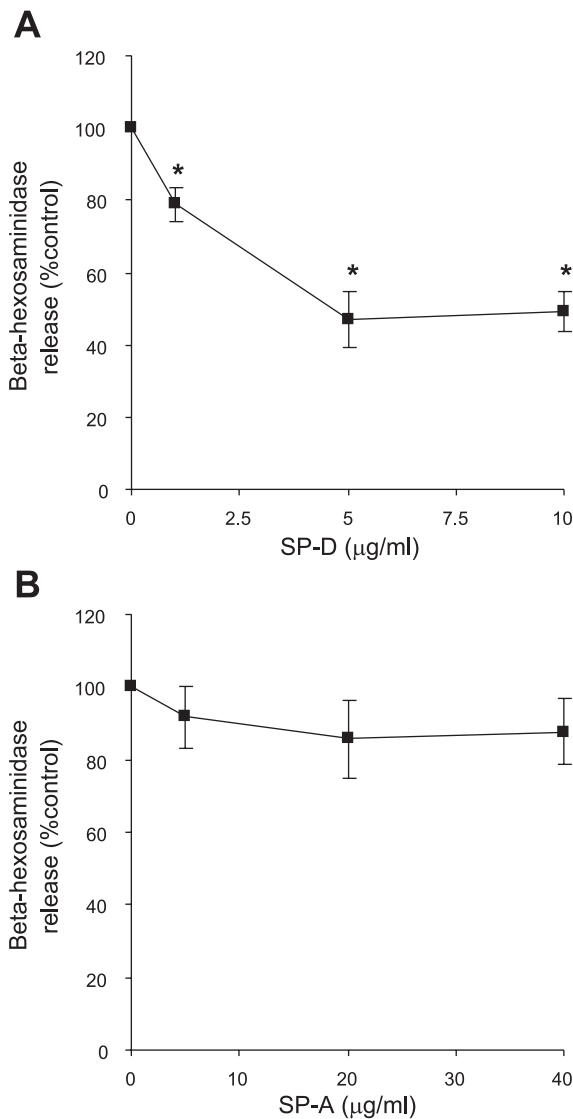


Fig. 5. SP-D inhibits mast cell degranulation. Mast cells were sensitized overnight with IgE (0.5 μg/ml) and were stimulated for 30 min at 37°C with TNP-coated PSG in presence of increasing concentrations of SP-D (A) or SP-A (B). Results are expressed as %control, where 100% is the amount of β-hexosaminidase released in the absence of surfactant protein; $n = 3$, $*P < 0.05$ compared with no protein treatment.

exchange chromatography from human bronchoalveolar lavage, whereas we used butanol-extracted SP-A from bronchoalveolar lavage from alveolar proteinosis patients. Therefore, the discrepancy of the results may be because of the SP-A isolation method.

Previous studies demonstrated that SP-A and SP-D bind via their lectin domains to specific allergens, such as those contained in dust mite or in conidia of *A. fumigatus* (2, 47). It has also been shown that SP-A binds to several types of pollen (35, 47). In line with our previous study showing an increased aggregation of native (non-TNP-coated) PSG by SP-D (17), we were able to demonstrate that SP-D dose dependently aggregated TNP-PSG, whereas SP-A had no effect. The aggregation of TNP-PSG by SP-D was mediated by its CRD. We also performed aggregation experiments with RrSP-Dser15,20, which is a SP-D mutant secreted as a single trimeric molecule.

We found that RrSP-Dser15,20 did not enhance TNP-PSG aggregation compared with the TNP-PSG aggregation obtained with wild-type SP-D. These data are consistent with previous studies showing that multimers of SP-D were more effective at viral aggregation than were SP-D trimers (10) and suggest that SP-D multimerization is required for maximal aggregation of TNP-PSG.

The finding that SP-D inhibits mast cell degranulation correlates well with the observed decrease of histamine release from basophils of allergic patients stimulated with *A. fumigatus* or dust mites in presence of SP-D (29, 48). Indeed Madan and colleagues (29) showed that 10 μg/ml SP-D inhibited the

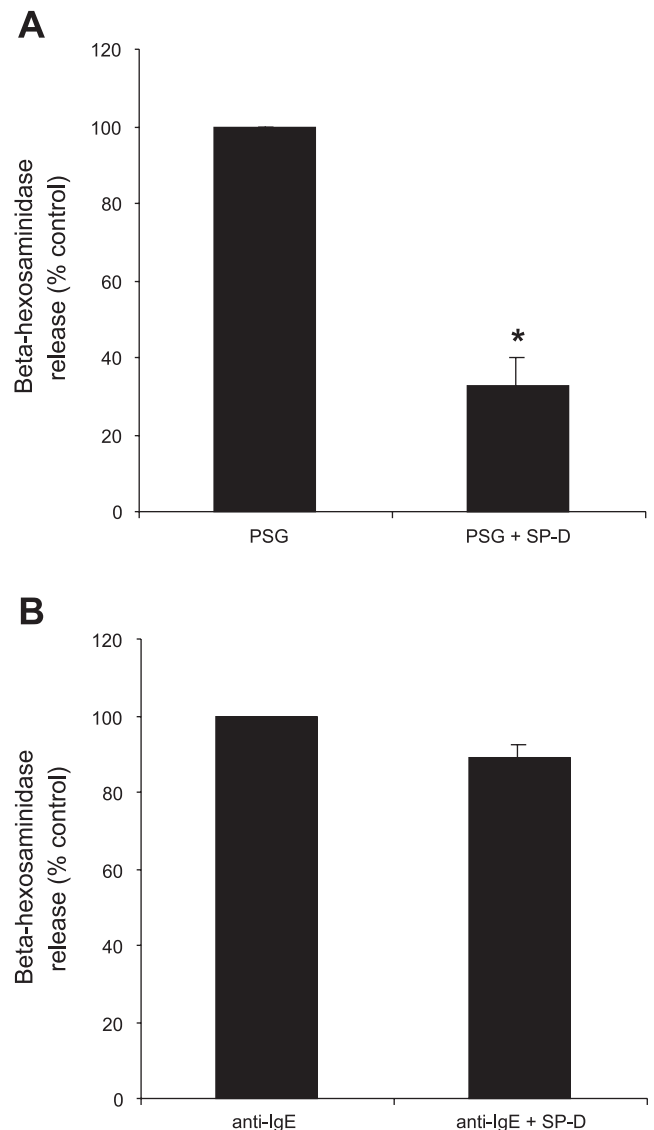


Fig. 6. SP-D inhibits mast cell degranulation when cells are stimulated with particulate allergens. Mast cells were sensitized overnight with IgE (0.5 μg/ml) and were stimulated for 30 min at 37°C with TNP-coated PSG (100 PSG/1 cell; A) or with soluble anti-IgE at 250 ng/ml (B) in the presence or absence of 5 μg/ml SP-D. Results are expressed as %control, where 100% is the amount of β-hexosaminidase released in the absence of surfactant protein, where PSG induced the release of $13 \pm 3\%$ β-hexosaminidase and anti-IgE induced the release of $31 \pm 3\%$ β-hexosaminidase; $n = 3$, $*P < 0.02$ compared with no protein treatment.

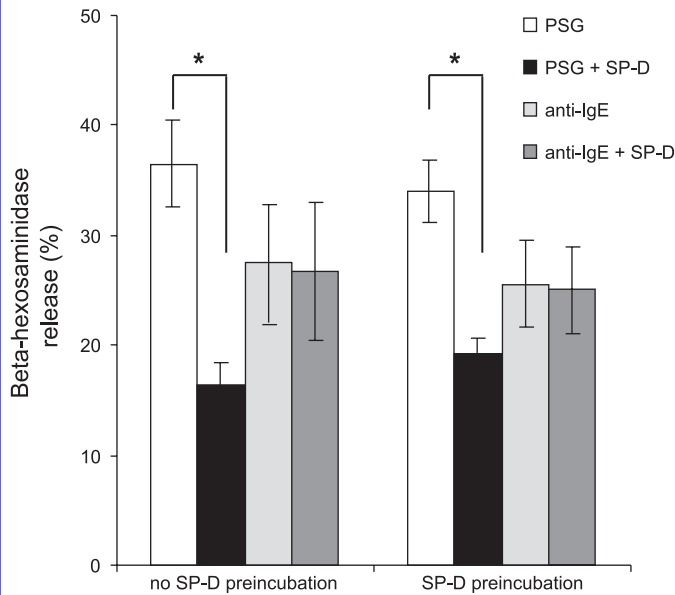


Fig. 7. SP-D preincubation does not affect mast cell degranulation. Mast cells were sensitized overnight with IgE (0.5 μ g/ml) and were incubated with 5 μ g/ml SP-D for 2 h at 4°C before being stimulated for 30 min at 37°C with TNP-coated PSG (100 PSG/1 cell) or with soluble anti-IgE at 250 ng/ml in the presence or absence of 5 μ g/ml SP-D. Results are expressed as %release of β -hexosaminidase; $n = 3$, * $P < 0.03$ compared with PSG treatment.

release of histamine by 50% from basophils stimulated with dust mites.

Our finding that SP-A did not inhibit TNP- and PSG-induced mast cell degranulation is in contrast to previous work by Wang and colleagues (48), who showed that SP-A also inhibited Der p-stimulated histamine release from human PBMCs in a dose-dependent fashion. This discrepancy may be because of the different allergens used in the two studies, or the effect may be cell specific. In addition, the method of purifying the SP-A may have an impact on the results, as described above. The SP-A used in this study was obtained by butanol extraction of lavage of alveolar proteinosis patients, whereas Wang et al. (48) used non-butanol-extracted SP-A. Interestingly, Erpenbeck and colleagues (16, 17) also demonstrated that SP-D, but not SP-A, which was also isolated by butanol extraction, increased PSG uptake by alveolar macrophages.

We found that, to mediate its effect on mast cell degranulation, SP-D had to interact with a particulate allergen, such as the TNP-PSG. In addition, SP-D by itself did not alter mast cell degranulation. The TNP coating of the PSG was a required modification step to induce a specific response to the PSG, since there are no commercially available antibodies against pollen granules in the murine system. This model system has not been tested in vivo, so its biological relevance remains unknown. Importantly, we observed that the SP-D-induced aggregation of the PSG was not affected by the TNP coating. Wang and colleagues (48) showed that pretreatment of PBMCs with surfactant proteins did not inhibit Der p-induced histamine release, thereby suggesting that surfactant proteins may suppress histamine release by binding to the allergens and preventing them from stimulating the basophils. A similar mechanism may be involved in SP-D's inhibition of PSG-induced mast cell degranulation, since preincubation of sensitized mast cells with SP-D did not inhibit mast cell degranulation.

We hypothesize that PSG aggregation by SP-D may prevent the granules from activating mast cells by sequestering the PSG away from mast cells by inducing the formation of large PSG aggregates. In addition, maximal inhibition of mast cell degranulation is also dependent on SP-D multimerization. Indeed, the single-arm mutant RrSP-Dser15,20 did not significantly inhibit TNP- and PSG-induced mast cell degranulation. Previous studies showed that both SP-D and a truncated SP-D molecule were able to reduce the allergy symptoms induced in mice by *A. fumigatus* or dust mite allergens (42, 43). These in vivo studies did not directly measure the release of mast cell mediators, so it is difficult to extrapolate our in vitro findings with mast cells to the in vivo studies.

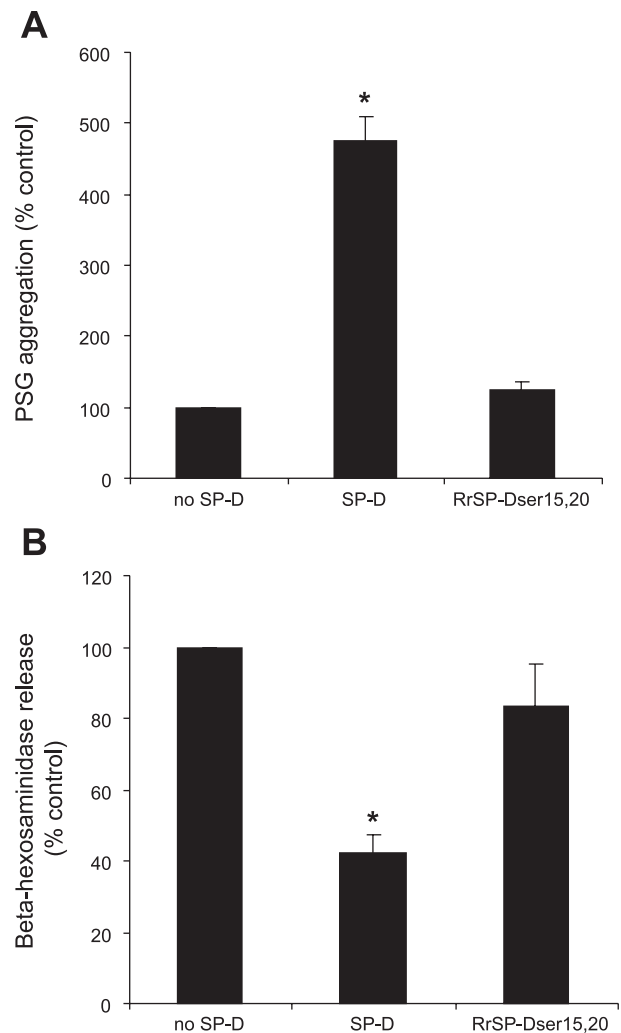


Fig. 8. SP-D multimerization is necessary for PSG aggregation and for inhibition of PSG-induced mast cell degranulation. A: FITC-labeled TNP-coated PSG were incubated for 30 min at 37°C with 5 μ g/ml wild-type SP-D or SP-D mutant RrSP-Dser15,20. Samples were analyzed by flow cytometry. Results are expressed as %control, where 100% is the amount of aggregation obtained with wild-type SP-D; $n = 5$, * $P < 0.01$ compared with no protein treatment. B: mast cells were sensitized overnight with IgE anti-TNP (0.5 μ g/ml) and were stimulated for 30 min at 37°C with TNP-coated PSG in presence of 5 μ g/ml wild-type SP-D or RrSP-Dser15,20. Results are expressed as %control, where 100% is the amount of β -hexosaminidase released in the absence of surfactant protein; $n = 9$, * $P < 0.05$ compared with no protein treatment.



In summary, in contrast to previous studies showing that SP-A and SP-D behaved similarly and could inhibit allergen-induced histamine release from basophils of allergic patients, we found that only SP-D interacted with PSG, and this modulated mast cell IgE-dependent functions. Both of these processes required SP-D multimerization. This study is the first to show that SP-D modulates IgE-mediated mast cell functions, thereby suggesting an important role for SP-D in allergy and asthma.

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