Surfactant Protein D Augments Bacterial Association but Attenuates Major Histocompatibility Complex Class II Presentation of Bacterial Antigens

Soren Hansen, Bernice Lo, Kathy Evans, Pavlos Neophytou, Uffe Holmskov, and Jo Rae Wright

Department of Cell Biology, Duke University and Medical Center, Durham, North Carolina; Medical Biotechnology Center, University of Southern Denmark, Odense, Denmark; and Mendel Center for Biomedical Sciences, Nicosia, Cyprus

Surfactant protein D (SP-D) is a secreted pattern recognition molecule associated with lung surfactant and mediates the clearance of pathogens in multiple ways. SP-D is an established part of the innate immune system, but it also modulates the adaptive immune response by interacting with both antigen-presenting cells and T cells. In a previous study, antigen presentation by bone marrow-derived dendritic cells was enhanced by SP-D. As dendritic cell function varies depending on the tissue of origin, we extended these studies to antigen-presenting cells isolated from mouse lung. Flow cytometric studies showed that SP-D binds calcium dependently and specifically to lung CD11c-positive cells. Opsonization of fluorescently labeled *Escherichia coli* by SP-D enhanced uptake by lung dendritic cells. SP-D facilitated the association of *E. coli* and antigen-presenting cells by increasing the frequency of CD11+ cells associated with *E. coli* by up to 10-fold. In contrast to the effect on bone marrow-derived dendritic cells, SP-D decreased the antigen presentation of ovalbumin, expressed in *E. coli*, to ovalbumin-specific major histocompatibility complex class II-specific T-cell hybridomas by 30–50%. The reduction of antigen presentation did not depend on whether the dendritic cells were isolated from the lungs of nonstimulated mice or mice that had been exposed to LPS aerosols. Our results show that SP-D increases the opsonization of pathogens, but decreases the antigen presentation by lung dendritic cells, and thereby, potentially dampens the activation of T cells and an adaptive immune response against bacterial antigens—during both steady-state conditions and inflammation.

Keywords: lung; collectins; SP-D; opsonization; antigen presentation

Dendritic cells are professional, antigen-presenting cells that survey and sample the environment by efficiently capturing antigens via both receptor- and nonreceptor-mediated endocytosis (1, 2). Antigens are processed through lysosomes to the major histocompatibility complex (MHC) peptide-loading compartment of the endoplasmic reticulum (3). Upon maturation induced by microbial products, inflammation, or just signals provided constitutively, dendritic cells migrate to the lymphoid organs, where they present the processed antigen on MHCs to T cells (4). Depending on the origin of dendritic cells, the signals that they received in peripheral tissue, and their subsequent expression of costimulatory molecules and cytokines, they may lead the T-cell response in an immunogenic (T helper cell [Th]

CLINICAL RELEVANCE

This work concerns SP-D's immunoregulatory role, and is important for understanding the disease mechanisms of many inflammatory disorders (e.g., asthma, pneumonia). It contradicts previous findings using *in vitro*-derived antigen-presenting cells.

type 1 and 2), tolerogenic (Treg) direction, or even lead to depletion of autoreactive T-cell populations (5).

The lungs are a continuously exposed interface between the body and the environment, and numerous inhaled pathogens, harmless microorganisms, and allergens challenge the tight regulation between an active immune defense and tolerance. Lung dendritic cells play a pivotal role in this regulation, and are, on the one hand, essential for mounting an adequate immune response against inhaled pathogens like *Mycobacterium tuberculosis*; on the other hand, lung dendritic cells are also decisive in the pathogenesis of asthma (6–8).

The microenvironment in the lung is a critical factor in determining the outcome of dendritic cell maturation and the subsequent fate of T cells. Microbial products (e.g., LPS) elicit a strong proimmunogenic response via Toll-like receptors on the surface of dendritic cells (9). We sought to determine whether lung surfactant protein D (SP-D), as part of the local microenvironment of the lung, scavenges microorganisms, and, hence, modulates the engagement of inflammatory receptors or engages other receptors. If so, SP-D could be essential for the dendritic cell's ability to activate T cells.

SP-D is a member of the collectin family of proteins, defined structurally as containing a collagen-like region (Gly-Xaa-Yaa triplets) and a conserved, calcium-dependent lectin domain (C-type). Via their lectin domains, they recognize and bind to foreign patterns of glycoconjugates found on the surface of microorganisms (10, 11). Collectins subsequently neutralize the microorganisms by increasing the membrane permeability, aggregation, and by inhibition of microbial growth. Binding of collectins to microorganisms may further lead to increased uptake and killing by phagocytes that recognize the microorganism-collectin complex by means of collectin receptors on the surface of the phagocytes (10, 11). SP-D is also an activation ligand for monocytes that respond by increasing surface expression of the endocytic macrophage mannose receptor (12).

SP-D is synthesized by type II cells located in the lower airways, and Clara cells and submucosal glands in the upper airways. In addition, SP-D has also been found in many nonpulmonary tissues, including the trachea, salivary glands, and endothelial cells (13–15). It is synthesized as a polypeptide chain of ~46 kD, which, through interactions in an α -helical coil-coiled neck region, the collagen-like region, and cysteines in the

(Received in original form June 2, 2006 and in final form August 8, 2006)

This work was supported by the Benzon Foundation (S.H.), the Danish Medical Research Council (S.H.), the Carlsberg Foundation (S.H.), and by National Institutes of Health grant HL-68072 (J.R.W.).

Correspondence and requests for reprints should be addressed to Jo Rae Wright, Ph.D., Department of Cell Biology, Box 3709, Duke University Medical Center, Durham, NC 27710. E-mail: j.wright@cellbio.duke.edu

Am J Respir Cell Mol Biol Vol 36, pp 94–102, 2007

Originally Published in Press as DOI: 10.1165/rcmb.2006-0195OC on August 10, 2006

Internet address: www.atsjournals.org

N-termini, oligomerizes to molecules of 12 polypeptide chains (dodecamer). Simultaneously, engagement of multiple SP-D lectin domains of a single SP-D-dodecamer ensures the necessary avidity and specificity for the recognition of foreign microbial patterns. SP-D has been shown to bind to several pulmonary pathogens, including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Haemophilus influenzae*, and influenza A virus, and to increase the phagocytosis of these microorganisms (16–18). It also binds and aggregates *Mycobacterium tuberculosis* and Group B streptococci, but it does not increase the phagocytosis of these microorganisms (16, 19). Mice made genetically deficient in SP-D have a steady-state increase of radical oxygen species, resulting in oxidative stress, and this likely accounts for the observed increase of proinflammatory cytokines (IL-1, IL-6, and TNF- α) upon microbial challenge (20, 21). This may partly explain why, upon bacterial challenge with Group B streptococci or *H. influenzae*, SP-D-deficient mice clear the microorganism as well as do wild-type mice (16). However, upon viral challenge with influenza A virus and respiratory syncytial virus, SP-D-deficient mice are more susceptible than wild-type mice (22, 23).

The complex pulmonary phenotype of unchallenged SP-D-deficient mice that comprise pulmonary oxidative stress, foamy macrophages, abnormalities in phospholipid homeostasis, and emphysema makes it difficult to directly correlate observations to lack of SP-D or indirect effects of the phenotype (24, 25). A potential link between SP-D and adaptive immunity is, however, illustrated by the observation of increased accumulation of activated T cells in peribronchial and perivascular tissue of the SP-D-null mice. The T cells (CD4 and CD8) show an increased expression of CD25 and CD69 (26). This finding is consistent with previous work showing that SP-D *in vitro* dampens the T cell activation induced by anti-CD3 antibodies, plant lectins, and phorbol ester. The effect is partly mediated by inhibition of IL-2 secretion (27). In addition, we observed that SP-D increases antigen presentation of bacterial-derived antigen by bone marrow-derived dendritic cells that subsequently and efficiently activate antigen-restricted T-cell hybridomas (CD4+) to increase secretion of IL-2 (28). Dendritic cells are considered a heterogeneous cell population, and their lineage, origin, and degree of maturation are important determinants of their effect on T cells (29). Studies have also shown differences between bone marrow-derived dendritic cells and dendritic cells isolated from the spleen or lymph nodes (30, 31). Bone marrow-derived dendritic cells (BMDCs) are still considered a paradigm for dendritic cells isolated from various tissues and analyzed *ex vivo*. With the differences and the heterogeneity in mind, we have extended our previous studies on the effect of SP-D on antigen presentation to also include lung antigen-presenting cells. Murine lung dendritic cells are relatively rare, and account for 2–5% of the single cell suspension obtained after digestion of a whole mouse lung. They are, furthermore, hard to distinguish from alveolar macrophages due to mutual CD11c expression (32). Few studies have been conducted using lung dendritic cells, and none have investigated the effect of lung surfactant proteins, as part of the natural local microenvironment, on their ability to endocytose and present antigens to T cells. In the present study, we report that SP-D binds specifically to lung antigen-presenting cells, and facilitates the association between bacteria and antigen-presenting cells. In contrast to its effect on BMDCs, we find that SP-D inhibits the antigen presentation via MHC class II and the subsequent activation of CD4 T cells.

MATERIALS AND METHODS

Materials

Media, balanced salt solutions, antibiotics, and other additives used for cell culture were obtained from Gibco Invitrogen (Carlsbad, CA). FCS

was obtained from HyClone (Logan, UT). Peptone, yeast extract, and agar used for growing *E. coli* were obtained from Difco Laboratories (Detroit, MI). Unless noted, all other reagents were obtained from Sigma Chemical Company (St. Louis, MO).

Antibodies

Rat anti-mouse CD16/32 (Fc block, clone 2.4G2), biotin-labeled rat anti-mouse Gr1 (Ly-6G, clone RB6-8C5), phycoerythrin (PE)-labeled mouse anti-mouse I-A^b (clone AF6-120.1), FITC-labeled hamster anti-mouse CD11c (clone HL-3), and peridinin chlorophyll protein (PerCP)-CY5.5-labeled rat anti-mouse CD11b (M1/70) were purchased from Pharmingen BD (San Diego, CA). Fluorophore-labeled antibodies were used at a concentration of 0.2–1.0 μ g/200,000 cells in a volume of $\sim$ 100–200 μ l, according to the manufacturer's instructions.

Bacteria and Fluorescent Labeling of Bacteria

To allow assessment of the antigen presentation of bacterial-derived antigens, ovalbumin (OVA) was expressed in *E. coli* HB:101 (American Type Culture Collection no. 33694). The pGEX-OVA construct was prepared as previously described (33) and transformed into *E. coli* HB:101. Herein, OVA is expressed as an isopropyl-beta-D-thiogalactopyranoside (IPTG)-inducible fusion protein of glutathione-S-transferase, and is mostly associated with inclusion bodies. For every assessment of antigen presentation, a *E. coli* HB:101 (pGEX-OVA) culture was started $\sim$ 4 h in advance in Luria Bertani (LB)-media and induced with IPTG (0.4 mM) when the optical density (OD) at 600 nm reached 0.6. Bacteria were harvested after 2 h of expression, washed three times in PBS, and the density was estimated by remeasuring the OD at 600 nm. To correlate absorbance at 600 nm with the density of the bacteria, an extinction factor ($OD_{600/1\text{ cm}} = 1 = 7.7 \times 10^7$ bacteria/ml) was previously calculated by correlating absorbance with colony-forming units on LB plates.

E. coli HB:101 were labeled with Texas Red-X (Molecular Probes, Eugene, OR). *E. coli* isolated from colonies grown overnight on LB plates were washed three times in PBS and the density was calculated as described previously here. Before labeling, cells were washed once in buffer (100 mM NaCO₃/NaHCO₂, pH 9.3), and 5×10^9 bacterial cells were dissolved in 1 ml of the same buffer. A total of 50 μ g of Texas Red-X was added to the bacteria, and incubated at room temperature for 3 h with end-over-end rotation. Excess Texas Red-X was reacted and removed by washing with PBS with 50 mM glycine, pH 8.6, and finally by PBS alone. Bacteria were stored at -80°C in 20% glycerol.

Purification and Labeling of SP-D

Recombinant rat SP-D was expressed in serum-free media by Chinese hamster ovary cells, as previously described (34). In brief, SP-D was purified by maltose affinity chromatography and elution using EDTA. The content of endotoxin in SP-D preparations was measured using the Limulus amoebocyte lysate assay (QCL-1000; Biowhittaker, Walkersville, MD). Preparations of SP-D were only used when the content of endotoxin was less than 5 pg endotoxin per μ g of SP-D.

SP-D was labeled with Texas Red-X, according to the manufacturer's instructions. Approximately three molecules of Texas Red-X were conjugated per polypeptide chain of SP-D. Excess Texas Red-X was removed by extensive dialysis.

Generation of Bone Marrow-Derived Dendritic Cells

Isolation of bone marrow stem cells and *in vitro* generation of dendritic cells followed the principles of Inaba and coworkers (35), with some minor modifications. C57BL/6 mice (6–10 wk) were killed by CO₂ inhalation, and bone marrow cells isolated by flushing the tibia, femur, and humerus using Hanks' buffered salt solution (HBSS) with 5% FCS and 2 mM EDTA. Erythrocytes were lysed by 1-min incubation in Gey's salt solution, and cells were seeded (1×10^6 /ml) in 24-well plates in RPMI 1640 with 10% heat-inactivated FCS, 100 U/ml penicillin-streptomycin, 20 μ g/ml gentamycin, and 50 μ M β -mercaptoethanol. Conditioned medium from X63 cells (B-cell myeloma) expressing murine granulocyte-macrophage colony-stimulating factor (provided by Dr. Nicchitta, Duke University, Durham, NC) was added to the bone marrow culture to a final concentration of 5% (vol/vol). Cells were grown for 6 d, and the medium was replaced on Days 2 and 4. On Day 6,

nonadherent and loosely attached immature dendritic cells were isolated. Fresh, conditioned granulocyte-macrophage colony-stimulating factor medium was added at every replacement of the media. The dendritic cells were further enriched by means of magnetic cell sorting and negative selection of cells expressing Gr-1 (28). To validate the content of dendritic cells and their immature status, cells were routinely analyzed by means of flow cytometry and found to contain 85–90% CD11c-positive cells, with low to moderate expression of MHC class II (I-A^b), CD80, and CD86.

Evaluating the Usage of Paramagnetic Anti-CD11c Beads for Isolation of Lung Antigen-Presenting Cells

Several methods were evaluated to optimize the enrichments of lung antigen-presenting cells. Among these, anti-CD11c beads (Miltenyi Biotech, Auburn, CA) were used to enrich antigen-presenting cells from digested lungs (*see below*). The purity, in terms of CD11c expression estimated by flow cytometry, was > 95%. However, functional studies using these cells showed that the beads changed the characteristics of the cells. Fluorophore-labeled SP-D bound to the cells to a greater extent after isolation by means of paramagnetic beads. These observations were verified by homogenous T-cell hybridomas derived from the BW5147 lymphoma and by biotinylated anti-CD4 antibodies (Pharmingen), streptavidin-conjugated paramagnetic beads, and an additional source of beads (Nanomag silica; Micromod Partikeltechnologie, Rostock-Warnemunde, Germany).

Isolation of Murine Lung Antigen-Presenting Cells

Mice were killed by intraperitoneal injection of pentobarbital (Nembutal, 150 mg/kg body weight) with 200 U of heparin. To remove intravascular cells, the renal arteries were transected, and the vascular system of the lungs was perfused through the pulmonary artery of the heart with 10 ml PBS with 100 U heparin. Only lungs that were well perfused, as judged by their degree of whiteness, were removed and processed further. Lungs were minced with a razor blade and suspended in 5 ml of HBSS (with calcium and magnesium) with 1.0 mg/ml collagenase XI and 0.2 mg/ml DNAase I. The digestion proceeded for 1 h with shaking (200 rpm) at 37°C. To inactivate the enzymes, EDTA and FCS were added to final concentrations of 15 mM and 5%, respectively. A single cell suspension was obtained by passing the digest through a 70- μ m mesh and, if needed, trace amounts of erythrocytes were lysed by 1-min incubation in Gey's lysis solution (0.83% NH₄Cl, 0.1% KHCO₃). After lysis and centrifugation, the cell pellet was suspended in HBSS with 5% FCS, 2 mM EDTA, and 100 U/ml penicillin-streptomycin. This balanced salt solution, with additives, was also used for the subsequent density gradient centrifugation. Suspended cells were layered on top of a 4.0% solution of Optiprep (Axis-Shield PoC AS, Rodeloeke, Norway), placed above a 16% Optiprep solution, and centrifuged at 600 $\times$ g for 20 min at room temperature, without applying the brakes at the end. Low-density cells were isolated from the 4–16% interface. These low-density lung cells were used directly for the analysis of SP-D binding and the SP-D-mediated bacterial association (*see below*). For analysis of antigen presentation, antigen-presenting cells were further enriched by flow cytometric sorting. Low-density cells were incubated with Fc-block and, subsequent, with PE-labeled anti-mouse CD11c. Gates were set with care to avoid selection of CD11c-negative cells with high autofluorescence.

LPS Preparation and Aerosol Exposures of Mice

Exposures were performed as previously described, with only minor modifications (36). Briefly, LPS (*E. coli* 0111:B4) was aerosolized using a nebulizer, and mice were exposed in a Hinner chamber. Lyophilized LPS was dissolved to a concentration of 10 mg/ml HBSS and stored in aliquots at –80°C. Immediately before exposure, an LPS solution of 0.215 mg LPS/ml HBSS was made from the frozen stock and vortexed at slow speed for 10 min. A total of 65 ml of the freshly made LPS solution were loaded into the nebulizer, to which 30 PSI of pressure was applied, resulting in an approximate flow rate of 35 liters/min. After 3 h of exposure, ~ 10 ml of the LPS solution remained inside the nebulizer.

SP-D Binding to Mouse Lung Antigen-Presenting Cells

The binding of SP-D labeled with Texas Red-X to low-density lung cells was studied by means of flow cytometry. Low-density lung cells were resuspended in HBSS (with calcium and magnesium), with 5% heat-inactivated human serum or in HBSS with 2 mM EDTA, pH 7.4, and 5% heat-inactivated human serum. Initial experiments showed that substitution of human serum with FCS inhibited the binding of labeled SP-D to the cells (data not shown). We assume that additional SP-D-like serum collectins (conglutinin and CL-43) within the FCS compete with labeled SP-D for binding sites on the cells. Cells (0.1 ml of 2.0×10^6 cell/ml) were incubated in 96-well, V-bottom plates, or for 2 h on ice with varying amounts of labeled SP-D (0–25 μ g/ml). Before flow cytometric analysis, cells were washed three times in PBS with 1 mM CaCl₂, pH 7.4, and fixed with 1% formaldehyde in PBS. To further characterize and subgroup the cells, Fc block and antibodies specific for mouse MHC class II (MHC-II PE), CD11c (CD11c FITC), and CD11b (PerCP-Cy5.5) were added to the cell/SP-D mixture for the last 30 min of the 2 h incubation on ice. In these studies, a fixed amount of labeled SP-D (8 μ g/ml) was used, and the amplification and compensation of the four channels were initially adjusted to isotype controls and samples labeled with a single fluorophore, respectively.

SP-D-Mediated Association between Bacteria and Lung Antigen-Presenting Cells

Association of Texas Red-X-labeled *E. coli* by low-density lung cells was studied by means of flow cytometry. Low-density lung cells and *E. coli* HB:101, labeled with Texas Red-X, were prepared as described previously here, and resuspended in HBSS (with calcium and magnesium) containing 5% heat-inactivated human serum. Cells (0.1 ml of 2.0×10^6 cell/ml) were incubated in the absence or presence of SP-D (8 μ g/ml) in 96-well plates (round bottomed), with varying amounts of labeled *E. coli* HB:101, for 1.5 h at 37°C. The plate was then incubated on ice for 5 min and subsequently incubated for 30 min with Fc block and antibodies specific for mouse MHC class II (MHC II PE), CD11c (CD11c FITC), and CD11b (PerCP-Cy5.5). Before flow cytometric analysis, cells were washed and fixed as described previously here.

Antigen Presentation Experiments

Antigen presentation experiments were performed as described by Svensson and colleagues, with some modification (37). In brief, antigen-presenting cells were allowed to take up bacteria expressing OVA and present antigens to syngeneic OVA-specific T-cell hybridomas. IL-2 secretion to the supernatant by the T-cell hybridomas was used to estimate T-cell activation and antigen presentation. The T-cell hybridomas recognize the OVA epitope OVA^{258–276} in the context of MHC class II^b, and were generated as described previously (28). They were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS, 55 μ M β -mercaptoethanol, and 100 U/ml penicillin-streptomycin, and grown to a maximum density of 2×10^6 cell/ml.

Mouse lung antigen-presenting cells (CD11c positive) or BMDCs from C57/BL6 mice (MHC haplotype b) were resuspended to a density of 4.0×10^6 cells/ml DMEM with low glucose (1 mg/ml) and 5% heat-inactivated FCS (no antibiotics). A total of 50 μ l of the suspension of antigen-presenting cells (200,000 cells) was added to a well of a 96-well plate (round bottomed) and incubated with 50 μ l of bacteria resuspended in the same media in the presence or absence of SP-D (8 μ g/ml), using various amounts of bacteria. The bacteria (*E. coli* HB:101 OVA) were freshly grown, induced with IPTG to express the OVA fusion protein, and titered by OD 600 nm, as described previously here. Antigen-presenting cells were incubated with bacteria for 3.5 h at 37° with 5% CO₂. Cells were washed within the wells three times with DMEM containing 10% FCS, 55 μ M β -mercaptoethanol, and 100 U/ml penicillin-streptomycin, and 2×10^5 T-cell hybridomas, resuspended in 130 μ l of the same media, were added. T-cell hybridomas were incubated with antigen-presenting cells for 24 h at 37°C in the presence of 5% CO₂. The supernatant was harvested, and its content of IL-2 was measured by means of ELISA (mouse IL-2 mini-kit; Endogen, Woburn, CA).

Flow Cytometry

Flow cytometric analysis and sorting was performed at the Duke Comprehensive Cancer Center Flow Cytometry Facility (Durham, North Carolina). For analysis, at least 10,000 events per sample were collected.

Statistical Analysis

Data were compared by ANOVA with the Student's *t* test where appropriate. Values were considered significant at $P < 0.05$.

RESULTS

Paramagnetic Beads Enhance SP-D Binding to Isolated Cells

In the course of establishing a procedure for enrichment of lung antigen-presenting cells, we found that SP-D bound to a greater extent (2.5-fold) to lung antigen-presenting cells and BMDCs isolated by magnetic cell sorting (positive selection of CD11c-positive cells) than to cells isolated by alternative methods (data not shown). We verified this effect successfully using homogeneous T-cell hybridomas that only weakly bind SP-D under normal circumstances (*see* MATERIALS AND METHODS). In terms of mean fluorescence, binding of SP-D to T-cell hybridomas isolated using paramagnetic beads was up to 10-fold greater than to cells not exposed to beads. Consequently, we chose to isolate lung antigen-presenting cells by alternative methods.

Isolation of Lung Cells Expressing CD11c, MHC Class II, and CD11b

Low-density cells obtained from perfused lungs digested with collagenase and DNase were isolated by density gradient centrifugation. Depending on the individual mouse and whether mice had been exposed to LPS aerosols, the frequency of CD11c-positive cells within the fraction of low-density cells varied from 5 to 15% (Figures 1C and 1D, Populations I + IV + V). Approximately 53% of the low-density cells expressed MHC class II on the surface (Figure 1A) and, within the CD11c-positive population, 82% expressed MHC class II (Figure 1D, Populations IV + V). No or only very low levels of the costimulatory molecules, CD80 and CD86, were expressed on these cells (data not shown). The mixture of low-density cells was only used to study the interaction with SP-D and opsonization mediated by SP-D. In both setups, discrimination of antigen-presenting cells was done by means of fluorophore-labeled antibodies for MHC class II, CD11b, and CD11c. The subpopulations that express CD11c

are mixed populations of macrophages and dendritic cells at different stages of maturation and activation.

For use in antigen presentation experiments, antigen-presenting cells were further enriched by flow cytometric sorting using PE-conjugated anti-CD11c antibody. The yield was $\sim 50,000$ – $200,000$ CD11c-positive cells per nonstimulated mouse. When mice had been exposed to LPS aerosols, the yield increased to $600,000$ – $800,000$ CD11c-positive cells per mouse. Routine re-analysis of the sorted cells showed that more than 92% were CD11c positive.

SP-D Binds to Lung Cells Expressing MHC Class II, CD11c, and CD11b

To study the interactions between SP-D and antigen-presenting cells of the lung, SP-D was conjugated with Texas Red-X, and the binding was analyzed by flow cytometry. The binding to low-density cells isolated from perfused mouse lungs was calcium dependent and saturable at a concentration of $8.0 \mu\text{g}$ SP-D/ml. At this concentration, SP-D bound in the presence of calcium to $41.6\% (\pm 0.3\%)$ of all the cells, and only $5.4\% (\pm 0.2\%)$ of the cells in the absence of calcium (Figure 1A). Further subgrouping based on expression of MHC class II and CD11b showed that SP-D bound to 97–100% of the cells in populations expressing intermediate to high levels of CD11c (Figures 1C and 1D, Population I, IV and V; Table 1). Both the frequency of positive cells and the amount of SP-D bound to each positive cell, assessed by fluorescence intensity, were influenced by the presence of calcium (Figures 1E and 1F and Table 1). Within these populations (I, IV, and V), the presence of calcium increased the mean fluorescence intensities of from 19–30 arbitrary fluorescence units (FUs) to 896 – $1,660$ FU, and the frequencies from 25–36% to 97–100% (Table 1). Populations devoid of CD11c expression (II, III, and VI) showed some degree of SP-D binding, with frequencies of positive cells ranging from 22 to 74%, but the intensity of fluorescence within these populations was considerably less (13 – 131 FU) than the intensity of populations expressing CD11c. In summary, SP-D binding correlated with expression

Figure 1. SP-D binding to low-density lung cells (A) Calcium-dependent binding of SP-D to low-density lung cells. Low-density lung cells were incubated with varying concentrations of Texas-Red-labeled SP-D in the presence of either calcium (*open squares*) or EDTA (*open circles*). (B–D) Characterization of MHC class II, CD11b, and CD11c expression. Low-density lung cells were incubated with $8 \mu\text{g}$ Texas-Red-labeled SP-D/ml and fluorophore-conjugated monoclonal antibodies raised against MHC class II, CD11b, and CD11c. Cells were gated initially on expression of MHC class II (B). Gates were based on similar analysis using fluorophore-labeled isotype antibody controls and are illustrated as a function of the relative fluorescence intensity of MHC class II expression. Cells with low and high MHC class II expression were subsequently analyzed, respectively, in (C) and (D) for expression of CD11b and CD11c. The defined subpopulations (I–VI) were analyzed separately for binding of Texas-Red-labeled SP-D. (E) SP-D binding to the phenotype: MHC II^{low}, CD11b^{int}, and CD11c^{high} (defined as Population I in [C]). (F) SP-D binding to the phenotype: MHC II^{high},

CD11b^{high}, and CD11c^{high} (defined as Population IV in [D]). Ca²⁺, EDTA, or O refers to incubation in the presence of calcium, EDTA, or without SP-D, respectively. The relative frequencies of cells associated with SP-D are illustrated as a function of the relative fluorescence intensity of Texas-Red-labeled SP-D. The analyses of the remaining populations are summarized in Table 1.

TABLE 1. SUMMARY OF SURFACTANT PROTEIN D BINDING TO DIFFERENT POPULATIONS OF LOW-DENSITY LUNG CELLS

Population	Ca ²⁺ /EDTA	
	Frequency of Cells Associated with SP-D (%)	Fluorescence Intensity (Geometric Mean)
I MHCII ^{low} CD11c ^{high} CD11b ^{int}	99 (1)/36 (2)	937 (4)/30 (1)
II MHCII ^{low} CD11c ^{int} CD11b ^{high}	74 (1)/3.6 (1)	131 (3)/6 (0.1)
III MHCII ^{low} CD11c ^{low} CD11b ^{low}	56 (2)/5.4 (1)	41 (2)/5.4 (0.1)
IV MHCII ^{high} CD11c ^{high} CD11b ^{high}	100 (1)/33 (1)	1660 (4)/27 (1)
V MHCII ^{high} CD11c ^{int} CD11b ^{high}	97 (1)/25 (2)	896 (10)/19 (1)
VI MHCII ^{high} CD11c ^{low} CD11b ^{low}	22 (1)/3.1 (0.2)	13 (1)/6.0 (0.1)

Definition of abbreviations: MHC, major histocompatibility complex; SP-D, surfactant protein D.

Frequencies of cells associated with SP-D are relative frequencies of the individual population. Data are the means ($\pm$ SE) of a single representative experiment, out of a total number of three, performed in duplicate. The populations (I–VI) are defined in Figures 1C and 1D, as is the parental frequency of each population. The expression levels of the individual markers MHCII, CD11b, and CD11c are given in superscript and refer to a high, intermediate (int), or low level of expression defined in Figures 1B–1D.

of CD11c, and was further increased upon coexpression of MHC class II and CD11b.

SP-D Enhances Bacterial Association to Lung Cells Expressing MHC Class II and CD11c

The ability of SP-D to enhance phagocytosis of *E. coli* HB:101 by mouse lung antigen-presenting cells was analyzed using flow cytometry and Texas Red-X-labeled bacteria. Low-density lung cells were incubated for 1.5 h with varying amounts of bacteria, and in the absence or presence of SP-D (8 μ g/ml). Before analysis, fluorophore-labeled antibodies specific for CD11b, CD11c, and MHC class II were included to allow further characterization of the low-density cells (Figure 2). Subgrouping was based on expression of MHC class II (Figure 2A), and further subgrouping hereof was based on expression of CD11b and CD11c (Figure 2B). To account for the high autofluorescence of cells derived from the alveolar space and the low level of MHC class II expression on macrophages and immature dendritic cells, the gate was intentionally set diagonally and at a low level of expression (Figure 2A). SP-D enhanced the association of *E. coli* with CD11c-positive cells (Figures 2C and 2E). The enhancement was detectable at a bacteria:cell ratio of 1:4, and was still significant at a ratio of 25:1. A 3- and 10-fold increase in frequency was observed for the two populations (IV and III), characterized by MHCII⁺, CD11c⁺, CD11b⁺, and MHCII⁺, CD11c⁺, and CD11b⁺, respectively. SP-D also enhanced bacterial uptake by 2.7-fold in cell Population II, characterized by MHCII⁺, CD11c⁺,

and CD11b⁺, but only at the single ratio of 0.25 bacteria per cell (Figure 2F). There was no effect of SP-D on Population I, characterized by expression of MHCII⁺, CD11c⁺, and CD11b⁺, which otherwise constitutes the largest population (61%) (Figure 2D).

SP-D Decreases Antigen Presentation of Bacterial Antigens by Lung Antigen-Presenting Cells, but Increases Presentation by BMDCs

Antigen presentation was assessed by means of IL-2 secretion by OVA-specific T-cell hybridomas that were stimulated by cells presenting bacterial-derived OVA. The antigen-presenting cells were allowed to phagocytose bacteria in the absence or presence of SP-D, and were not fixed before coincubation with the T-cell hybridomas. Although lung antigen-presenting cells were just as efficient at presenting antigens as BMDC, there was, in terms of responsiveness to the amount of bacteria present, a great difference between the two cell types (Figure 3). Presentation by lung antigen-presenting cells was efficient, peaked even at the two lowest tested ratios of bacteria:cell (1.0 and 3.5), and was diminished at ratios of 12 or more bacteria per cell (Figure 3A). In contrast, presentation by BMDC reached its maximum at a ratio 43 bacteria per cell. Presentation by lung antigen-presenting cells isolated from mice exposed to LPS aerosols was more similar to presentation by BMDCs, and peaked at bacteria:cell ratios of 3.5 and 12 (Figure 3B). It was inhibited, but not abolished, at a ratio of 43. Both lung antigen-presenting cells and BMDCs were inhibited at ratios above 43 bacteria per cell. The presence of SP-D during phagocytosis of the bacteria led to an increase of antigen presentation by BMDCs. Although antigen presentation, in terms of levels of IL-2 secretion, varied among identical experiments performed at different occasions (discussed subsequently here), the increased secretion of IL-2 mediated by SP-D was never less than 2.4-fold (140%) of the secretion in the absence of SP-D (Figure 3C). SP-D had the opposite effect on mouse lung antigen-presenting cells, regardless of whether they were isolated from nonstimulated mice or mice exposed to LPS aerosols (Figures 3A–3C). When SP-D was present during phagocytosis of the bacteria, subsequent antigen presentation by lung antigen-presenting cells was decreased by as much as 25–40%. At certain bacteria:cell ratios, the effect mediated by SP-D was only minimal or even absent; however, in contrast to the enhancing effect of SP-D on antigen presentation by BMDCs, SP-D always attenuated antigen presentation by lung antigen-presenting cells.

DISCUSSION

This study demonstrates that SP-D interacts with lung antigen-presenting cells to attenuate antigen presentation of bacterial-derived antigen, although it facilitates association between the same cells and bacteria. We show that SP-D binds to calcium—and dose dependently to CD11c-positive lung cells. Unlike previous data and our own observations using BMDC, the enhanced binding of SP-D correlates with decreased antigen presentation via MHC class II. Our observation emphasizes the importance of using naturally occurring antigen-presenting cells when addressing the functionality of SP-D and its modulation of the adaptive immune response.

Antigen-presenting cells in the mouse lung are relatively rare, and constitute only 5–10% of the total number of lung cells obtained after vascular perfusion and physical and enzymatic disruption. Nearly all protocols on isolation of antigen-presenting cells from mouse lung have applied magnetic microbeads to isolate sufficient numbers of CD11c-positive cells.

Figure 2. SP-D enhancement of *E. coli* HB101 association with low-density lung cells. Immature low-density cells were incubated with varying numbers of Texas-Red-labeled *E. coli* HB:101 in the presence or absence of SP-D (8 μ g/ml) for 30 min at 37°C. Lung cells were collected and incubated with fluorophore-conjugated antibodies against MHC class II, CD11b, and CD11c, washed, and fixed for analysis by FACS. (A) Dot plot of MHC class II expression versus side scatter. Gates were based on similar analysis using PE-labeled isotype antibody control. (B) Dot plot of CD11b versus CD11c expression of MHC class II-positive cells defined in (A). (C–F) Relative frequency of low-density lung cells positive for Texas-Red-labeled *E. coli* HB:101 as a function of the number of labeled *E. coli*, within the four populations of MHC class II-positive cells defined in (B). Filled squares, incubation in the presence of SP-D; open squares, incubation in the absence of SP-D. In the absence of Texas-Red-labeled *E. coli*, SP-D changed neither the autofluorescence (< 0.5% positive cells) of the cells nor the distribution of the populations (data not shown).

In this study, we found that this approach is not suitable when addressing the binding and function of SP-D. Preliminary experiments using BMDCs showed that SP-D binds more avidly to these cells when they were enriched by positive selection using anti-CD11c beads than cells enriched by alternative methods. This observation was subsequently confirmed by our findings that SP-D binds only minimally to unselected T-cell hybridomas, but binds to a much greater extent (10-fold enhancement) to T-cell hybridomas selected using anti-CD4 magnetic beads. Because the two type of beads tested were made of either a glucose polymer or silica, we speculate that SP-D interacts through its lectin domain with the glucose moiety of the glucose polymer; however, we have no explanation for the binding to cells isolated using silica-based magnetic beads.

To study the effect of SP-D on lung antigen-presenting cells, we used gradient centrifugation to obtain low-density cells from enzymatically digested lungs that had been previously perfused to eliminate contamination with blood-derived antigen-presenting cells. To avoid the effect of contaminating CD11c-negative cells on antigen presentation, the lung cells selected by means of density alone were used only to study binding of SP-D and SP-D-mediated opsonization. Subsequent flow cytometric analysis, using various fluorophore-labeled antibodies in combination with fluorophore-labeled SP-D or *E. coli*, allowed us to characterize the SP-D binding and SP-D-mediated opsonization by CD11c-positive cells within the low-density fraction, and at the same time characterize the phenotype of these cells in terms of expression of CD11b and MHC class II. Binding of SP-D was dose dependent, saturable, and correlated with expression of CD11c.

Several collectin receptors have been described, and the subject was recently reviewed by Kishore and colleagues (38). Among the receptors, SP-D has only been shown to interact directly with the 340-kDa glycoprotein (gp340) and CD14, but strong evidence suggests that SP-D also interacts with signal-regulatory protein (SIRP)- α (CD172a) and the calreticulin-CD91 complex (39). All four receptors have been found associated with lung macrophages, and the interactions with SP-D are mediated via the lectin domain of SP-D or require the presence of calcium (39–41). The calcium dependency observed in our study is consistent with the binding characteristics of these four collectin receptors. Because CD14, as well as MHC class II, are upregulated upon LPS-induced maturation, we speculate that the observed increase in SP-D binding to CD11c-positive cells expressing MHC class II, compared with CD11c-positive cells

without MHC class expression (Table 1), reflects the increased CD14 expression (42).

We found that SP-D opsonized *E. coli* by facilitating the association between the microorganisms and certain subpopulations of low-density lung cells. As antigen-presenting cells in their immature state only express low levels of MHC class II, we included these cells in the MHC class II-positive population and the subsequent analysis (Figure 2A). SP-D increased by up to 10-fold the frequency of cells interacting with bacteria. The greatest effect of SP-D was seen on the subpopulation characterized by expression of MHC class II and CD11c. We find it very striking that SP-D mediates opsonization by the exact same subpopulations of cells to which it binds in the absence of microorganism. This finding is consistent with the possibility that there are specific SP-D receptors expressed on the subpopulations of CD11c-positive cells. The effects of SP-D on enhancing bacterial uptake did not require the bacteria to be preincubated (not shown); thus, it appears that the receptors recognized SP-D both in the absence and presence of bacteria, and that cells already “coated” with SP-D are able to bind bacteria via the coated SP-D.

The ability of SP-D to mediate opsonization, and even the mechanisms behind SP-D-mediated opsonization, varies among microorganisms and the phagocytic cells tested (43, 44). In general, it appears that, when SP-D aggregates bacteria, it leads to an increase in the number of bacteria taken up by the phagocytic cells, whereas opsonization of nonaggregated microorganisms leads to an increase in frequency of cells that phagocytose (17, 44).

SP-D has also been reported to decrease opsonization of bacteria, and it is plausible that the effect of SP-D depends on the actual microbial pattern and coengagement of multiple pattern-recognition receptors (19).

In our study, SP-D increased the number (frequency) of cells interacting with the bacteria, and not the amount of bacteria interacting with a single CD11c-positive cell (mean fluorescence). Thus, within the heterogeneous low-density cells, SP-D enhanced the interaction of bacteria with the subpopulations characterized by expression of MHC class II, CD11c, and low but detectable levels of CD11b. This observation is in contrast with our previous findings using BMDCs (28). SP-D enhances bacterial uptake by BMDCs by increasing the number of bacteria taken up, and it requires a relative high bacteria:BMDC ratio (25:1) to be efficient (28). The two mechanisms likely reflect the

Figure 3. SP-D modulation of antigen presentation of *E. coli* HB101-OVA by lung antigen-presenting cells and BMDCs. (A) Low-density CD11c-positive lung cells (squares) or BMDCs (circles) were incubated with *E. coli* expressing OVA in the presence (filled symbols) or absence (open symbols) of 8 μ g SP-D/ml for 3.5 h at 37°C. The cells were washed and incubated with OVA²⁵⁸⁻²⁷⁶-specific T-cell hybridomas for 24 h, and the amount of IL-2 in the supernatant was measured by ELISA. (B) Low-density CD11c-positive lung cells from mice exposed to LPS 36 h in advance or nonstimulated BMDCs incubated with *E. coli* HB101-OVA and T-cell hybridomas, as described in (A). Data are means ($\pm$ SE) of duplicate or triplicate analyses, and reflect a representative experiment from a set of at least three independent experiments. * Significant difference in the absence or presence of SP-D with $P < 0.05$, estimated using Student's *t*-test. (C) Summary of the effect of SP-D on the antigen presentation by mouse lung antigen-presenting cells (LAPCs), low-density CD11c cells from mice exposed to LPS 36 h in advance (LAPCs + LPS), and BMDCs. Ratio in parenthesis are the number of antigen-presenting cells versus the number of *E. coli* HB101-OVA (the ratio at which maximal IL-2 signal was observed in the absence SP-D).

differences in expression of SP-D receptors between BMDCs and lung antigen-presenting cells, but this hypothesis was not tested further in this study. The observed magnitude of SP-D-mediated opsonization within our studies (3- to 10-fold increase) exceeds the previously reported effect of SP-D for the opsonization of *P. aeruginosa* by macrophages (2.5- to 3.5-fold) (17).

To assess antigen presentation, antigen-presenting cells within the low-density fraction were further enriched into CD11c-positive cells by flow cytometric sorting. The yield of CD11c-positive cells from nonstimulated mice was $\sim 125,000$ ($\pm 75,000$) per mouse, and, as the antigen presentation assay required $2 \times 200,000$ for duplicate measurement of a single data point, up to 20 mice were processed for isolation of CD11c-positive lung cells in a single setup. This yield is much less than the 2.4×10^6 obtained from a single mouse in a recent study using anti-CD11c magnetic beads for isolation (32). We have no explanations for this difference. Our yield corresponded to 19–50% of the initial number of CD11c-positive cells in the enzymatically digested single suspension from perfused lungs. We observed that just the slightest signs of an incomplete vascular perfusion of the lung lead easily to a threefold increase in the yield of CD11c-positive cells (data not shown). These cells were discarded and not used for experiments. When mice were stimulated with LPS and the CD11c-positive cells isolated 36 h after exposure, the yield increased by ~ 5.6 -fold. This time point was determined experimentally as the time point at which the number of CD11c-positive cells peaked and the number of Gr-1-positive cells declined (data not shown).

The precise phenotype of mouse lung dendritic cells is generally unclear; furthermore, they appear to be relatively heterogeneous (32, 45, 46). Both lung dendritic cells and macrophages express constitutively the otherwise dendritic cell characteristic marker, CD11c. Hence, discrimination between the two types of cells may take advantage of either a high autofluorescence of alveolar macrophages or variation in the level of MHC class II expression (32, 45). We have chosen not to discriminate between the two cell types due to the combination of the limited number of CD11c-positive cells within the lungs and, as we hypothesized, that both cell types present antigen (express MHC class II) and may interact with each other to modulate the effects of SP-D. Looking at them in a mixture with each other mimics the situation *in vivo*.

Thus, the lung CD11c-positive cells used in the present studies represent a mixture of dendritic cells and macrophages originating from both the alveolar space and the lung tissue. In the absence of SP-D, these cells are able to present antigen to OVA-specific T cells even more efficiently than BMDCs (Figure 3A). Similar to BMDCs, their response depends on the amount bacteria (bacterial antigen) introduced. This kinetic varies within identical experiments performed on different days (data not shown), probably owing to minor variation in their degree of activation and maturation during the isolation. The tendency was, however, always the same, and, in comparison with BMDCs, nonstimulated lung antigen-presenting CD11c-positive cells required only 1/10–1/70 the amount of bacteria to give the optimal antigen presentation. The maximum level of IL-2 secretion by the T-cell hybridomas also varied within identical experiments performed on different days. This likely reflects the actual growth status of T-cell hybridomas when they were harvested and coincubated with antigen-presenting cells. BMDCs were always included in the experiment as a positive control for these reasons, and to evaluate the relative level of antigen presentation by lung antigen-presenting cells. We cannot explain why the antigen presentation reaches an optimum and then declines at higher bacteria:cell ratios and with more available antigen. Within the 3.5 h of coincubation with bacteria and antigen-presenting cells,

there were no visible signs of bacterial overgrowth or cell death, visualized by trypan blue staining (data not shown). Only occasionally did we observe nutrient depletion at the end of an experiment, when T-cell hybridomas had grown for 24 h, and only at the absolute highest bacteria:cell ratio used (100). We cannot rule out the possibility that lung antigen-presenting cells may respond to increasing amounts of bacterial toxins that inhibit their ability to present antigens.

When lung antigen-presenting CD11c-positive cells were obtained from LPS-exposed mice, the optimal bacteria:cell ratio shifted upward (Figure 3B). Again, the kinetic and optimal bacteria:cell ratios varied, but the tendency was persistent. To obtain maximum antigen presentation, lung antigen-presenting cells obtained from LPS-exposed mice required only 10–50% of the amount of bacteria that resulted in maximum antigen presentation by BMDCs. Thus, both steady-state and inflammatory antigen-presenting cells are more sensitive than BMDCs, and SP-D acts similarly on both types of lung antigen-presenting cells.

In many setups, including that described in our previous report, a formalin fixation of the antigen-presenting cells—before coinocubation with the OVA-specific T-cell hybridomas—is included (28). This was not the case in our studies, as we found that the ability of lung antigen-presenting CD11c-positive cells, unlike, for example, BMDCs, absolutely required a nonfixed nature to present antigen and, thereby, activate the OVA-specific T-cell hybridomas. We do not have an explanation for why lung dendritic cells, unlike BMDCs, do not present antigen well when they are fixed. However, this observation may provide at least a partial explanation for the previous findings showing that lung dendritic cells are poor antigen-presenting cells (47).

Regardless of whether lung antigen-presenting cells were harvested from LPS-exposed mice or unexposed mice, SP-D attenuated the antigen presentation. SP-D had the greatest effect on lung antigen-presenting cells from LPS-exposed mice, and lead to a ~ 50% decrease (Figure 3C). This is in sharp contrast to our previous finding on the effect on BMDCs (28). These findings were also verified by the present studies, and SP-D increased antigen presentation by BMDCs up to 170% (Figure 3C).

Thus, although SP-D opsonizes bacteria by both lung antigen-presenting cells and BMDCs, SP-D exerts different effects on the ability of the two cell types to present bacterial antigens. We hypothesize that variation in expression of collectin receptors contributes to this phenomenon. It is possible that SP-D directs phagocytosis of bacteria by lung antigen-presenting cells to receptors that are either less endocytic or less capable of targeting bacterial antigen to the MHC class II lysosomal pathway. We find it striking that the inhibitory effect of SP-D on antigen presentation by lung antigen-presenting cells occurred at the same relatively low ratios of bacteria:cell at which we also observed that SP-D facilitated association between bacteria and the same cells (Figures 2 and 3). The opsonic effect of SP-D is probably mediated via SP-D receptors (discussed previously here). This finding, therefore, supports the notion that SP-D targets bacteria to collectin receptors that do not target the MHC class II lysosomal pathway. Alternatively, and as observed by others in the rat, our findings may be explained by a complex interplay between alveolar macrophages and dendritic cells that exists within our mixture of CD11c-positive lung cells (48).

Our findings on the effect of bacterial antigen presentation are consistent with previous observations showing that SP-D-deficient mice have increased numbers of activated T cells in the perivascular and peribronchial tissue (26). A deficiency of SP-D may lead to increased antigen presentation by antigen-presenting cells in the lung, and subsequent local activation of T cells and exaggeration of the adaptive immune response. Our

findings also concur with the recently reported inhibitory effect of SP-D on the Th2 response in the lung, where it forms a negative feedback with the Th2 cytokines IL-4 and IL-3 (49). In summary, our findings support the concept that SP-D is, on the one hand, an effective component of the first line of defense, where it facilitates bacterial clearance, and, on the other hand, a controller or downregulator of the adaptive immune response via suppression of antigen presentation or the inhibition of T cells (27).

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

1. Sallusto F, Cella M, Danieli C, Lanzavecchia A. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J Exp Med* 1995; 182:389–400.
2. Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* 1991;9:271–296.
3. Trombetta ES, Mellman I. Cell biology of antigen processing *in vitro* and *in vivo*. *Annu Rev Immunol* 2005;23:975–1028.
4. Sallusto F, Lanzavecchia A. Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression. *Immunol Rev* 2000;177:134–140.
5. Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. *Annu Rev Immunol* 2003;21:685–711.
6. Tian T, Woodworth J, Skold M, Behar SM. In vivo depletion of cd11c⁺ cells delays the cd4⁺ T cell response to *Mycobacterium tuberculosis* and exacerbates the outcome of infection. *J Immunol* 2005;175:3268–3272.
7. Holt PG, Upham JW. The role of dendritic cells in asthma. *Curr Opin Allergy Clin Immunol* 2004;4:39–44.
8. van Rijt LS, Jung S, Kleinjan A, Vos N, Willart M, Duez C, Hoogsteden HC, Lambrecht BN. In vivo depletion of lung cd11c⁺ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *J Exp Med* 2005;201:981–991.
9. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 2004;5:987–995.
10. Holmskov U, Thiel S, Jensenius JC. Collections and ficolins: humoral lectins of the innate immune defense. *Annu Rev Immunol* 2003;21:547–578.
11. Wright JR. Immunoregulatory functions of surfactant proteins. *Nat Rev Immunol* 2005;5:58–68.
12. Kudo K, Sano H, Takahashi H, Kuronuma K, Yokota S, Fujii N, Shimada K, Yano I, Kumazawa Y, Voelker DR, *et al.* Pulmonary collectins enhance phagocytosis of *Mycobacterium avium* through increased activity of mannose receptor. *J Immunol* 2004;172:7592–7602.
13. Wong CJ, Akiyama J, Allen L, Hawgood S. Localization and developmental expression of surfactant proteins D and A in the respiratory tract of the mouse. *Pediatr Res* 1996;39:930–937.
14. Madsen J, Kliem A, Tornøe I, Skjoldt K, Koch C, Holmskov U. Localization of lung surfactant protein D on mucosal surfaces in human tissues. *J Immunol* 2000;164:5866–5870.
15. Sorensen GL, Madsen J, Kejlving K, Tornøe I, Nielsen O, Townsend P, Poulain F, Nielsen CH, Reid KB, Hawgood S, *et al.* Surfactant protein D is proatherogenic in mice. *Am J Physiol Heart Circ Physiol* 2006;290: H2286–H2294.
16. LeVine AM, Whitsett JA, Gwozdz JA, Richardson TR, Fisher JH, Burhans MS, Korfhagen TR. Distinct effects of surfactant protein A or D deficiency during bacterial infection on the lung. *J Immunol* 2000; 165:3934–3940.
17. Restrepo CI, Dong Q, Savov J, Mariencheck WI, Wright JR. Surfactant protein D stimulates phagocytosis of *Pseudomonas aeruginosa* by alveolar macrophages. *Am J Respir Cell Mol Biol* 1999;21:576–585.
18. Ofek I, Mesika A, Kalina M, Keisari Y, Podschun R, Sahly H, Chang D, McGregor D, Crouch E. Surfactant protein D enhances phagocytosis and killing of unencapsulated phase variants of *Klebsiella pneumoniae*. *Infect Immun* 2001;69:24–33.
19. Ferguson JS, Voelker DR, Unfar JA, Dawson AJ, Schlesinger LS. Surfactant protein D inhibition of human macrophage uptake of *Mycobacterium tuberculosis* is independent of bacterial agglutination. *J Immunol* 2002;168:1309–1314.
20. Wert SE, Yoshida M, LeVine AM, Ikegami M, Jones T, Ross GF, Fisher JH, Korfhagen TR, Whitsett JA. Increased metalloproteinase activity,

- oxidant production, and emphysema in surfactant protein D gene-inactivated mice. *Proc Natl Acad Sci USA* 2000;97:5972–5977.
21. Yoshida M, Korfhagen TR, Whitsett JA. Surfactant protein D regulates NF- κ B and matrix metalloproteinase production in alveolar macrophages via oxidant-sensitive pathways. *J Immunol* 2001;166:7514–7519.
 22. LeVine AM, Elliott J, Whitsett JA, Srikiatkachorn A, Crouch E, DeSilva N, Korfhagen T. Surfactant protein-D enhances phagocytosis and pulmonary clearance of respiratory syncytial virus. *Am J Respir Cell Mol Biol* 2004;31:193–199.
 23. LeVine AM, Bruno MD, Huelsman KM, Ross GF, Whitsett JA, Korfhagen TR. Surfactant protein A-deficient mice are susceptible to group B streptococcal infection. *J Immunol* 1997;158:4336–4340.
 24. Botas C, Poulain F, Akiyama J, Brown C, Allen L, Goerke J, Clements J, Carlson E, Gillespie AM, Epstein C, et al. Altered surfactant homeostasis and alveolar type II cell morphology in mice lacking surfactant protein D. *Proc Natl Acad Sci USA* 1998;95:11869–11874.
 25. Korfhagen TR, Sheftelyevich V, Burhans MS, Bruno MD, Ross GF, Wert SE, Stahlman MT, Jobe AH, Ikegami M, Whitsett JA, et al. Surfactant protein-D regulates surfactant phospholipid homeostasis *in vivo*. *J Biol Chem* 1998;273:28438–28443.
 26. Fisher JH, Larson J, Cool C, Dow SW. Lymphocyte activation in the lungs of SP-D null mice. *Am J Respir Cell Mol Biol* 2002;27:24–33.
 27. Borron PJ, Crouch EC, Lewis JF, Wright JR, Possmayer F, Fraher LJ. Recombinant rat surfactant-associated protein D inhibits human T lymphocyte proliferation and IL-2 production. *J Immunol* 1998;161:4599–4603.
 28. Brinker KG, Martin E, Borron P, Mostaghel E, Doyle C, Harding CV, Wright JR. Surfactant protein D enhances bacterial antigen presentation by bone marrow-derived dendritic cells. *Am J Physiol Lung Cell Mol Physiol* 2001;281:L1453–L1463.
 29. Wilson HL, O'Neill HC. Murine dendritic cell development: difficulties associated with subset analysis. *Immunol Cell Biol* 2003;81:239–246.
 30. Powell TJ, Jenkins CD, Hattori R, MacPherson GG. Rat bone marrow-derived dendritic cells, but not *ex vivo* dendritic cells, secrete nitric oxide and can inhibit T-cell proliferation. *Immunology* 2003;109:197–208.
 31. Garrigan K, Moroni-Rawson P, McMurray C, Hermans I, Abernethy N, Watson J, Ronchese F. Functional comparison of spleen dendritic cells and dendritic cells cultured *in vitro* from bone marrow precursors. *Blood* 1996;88:3508–3512.
 32. Vermaelen K, Pauwels R. Accurate and simple discrimination of mouse pulmonary dendritic cell and macrophage populations by flow cytometry: methodology and new insights. *Cytometry A* 2004;61:170–177.
 33. Neophytou PI, Ozegbe P, Healey D, Quartey-Papafio R, Cooke A, Hutton JC. Development of a procedure for the direct cloning of T-cell epitopes using bacterial expression systems. *J Immunol Methods* 1996;196:63–72.
 34. Dong Q, Wright JR. Degradation of surfactant protein D by alveolar macrophages. *Am J Physiol* 1998;274:L97–105.
 35. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, Muramatsu S, Steinman RM. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 1992;176:1693–1702.
 36. Hollingsworth JW II, Cook DN, Brass DM, Walker JK, Morgan DL, Foster WM, Schwartz DA. The role of Toll-like receptor 4 in environmental airway injury in mice. *Am J Respir Crit Care Med* 2004;170:126–132.
 37. Svensson M, Stockinger B, Wick MJ. Bone marrow-derived dendritic cells can process bacteria for MHC-I and MHC-II presentation to T cells. *J Immunol* 1997;158:4229–4236.
 38. Kishore U, Greenhough TJ, Waters P, Shrive AK, Ghai R, Kamran MF, Bernal AL, Reid KB, Madan T, Chakraborty T. Surfactant proteins SP-A and SP-D: structure, function and receptors. *Mol Immunol* 2006;43:1293–12315.
 39. Gardai SJ, Xiao YQ, Dickinson M, Nick JA, Voelker DR, Greene KE, Henson PM. By binding SIRP α or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation. *Cell* 2003;115:13–23.
 40. Sano H, Chiba H, Iwaki D, Sohma H, Voelker DR, Kuroki Y. Surfactant proteins A and D bind CD14 by different mechanisms. *J Biol Chem* 2000;275:22442–22451.
 41. Holmskov U, Lawson P, Teisner B, Tornoe I, Willis AC, Morgan C, Koch C, Reid KB. Isolation and characterization of a new member of the scavenger receptor superfamily, glycoprotein-340 (gp-340), as a lung surfactant protein-D binding molecule. *J Biol Chem* 1997;272:13743–13749.
 42. Mahnke K, Becher E, Ricciardi-Castagnoli P, Luger TA, Schwarz T, Grabbe S. CD14 is expressed by subsets of murine dendritic cells and upregulated by lipopolysaccharide. *Adv Exp Med Biol* 1997;417:145–159.
 43. Crouch E, Hartshorn K, Ofek I. Collectins and pulmonary innate immunity. *Immunol Rev* 2000;173:52–65.
 44. Hartshorn KL, White MR, Crouch EC. Contributions of the N- and C-terminal domains of surfactant protein D to the binding, aggregation, and phagocytic uptake of bacteria. *Infect Immun* 2002;70:6129–6139.
 45. Kirby AC, Raynes JG, Kaye PM. CD11b regulates recruitment of alveolar macrophages but not pulmonary dendritic cells after pneumococcal challenge. *J Infect Dis* 2006;193:205–213.
 46. Webb TJ, Sumpter TL, Thiele AT, Swanson KA, Wilkes DS. The phenotype and function of lung dendritic cells. *Crit Rev Immunol* 2005;25:465–491.
 47. Nicod LP, Cochand L, Dreher D. Antigen presentation in the lung: dendritic cells and macrophages. *Sarcoidosis Vasc Diffuse Lung Dis* 2000;17:246–255.
 48. Holt PG, Oliver J, Bilyk N, McMenamin C, McMenamin PG, Kraal G, Thepen T. Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells *in vivo* by resident alveolar macrophages. *J Exp Med* 1993;177:397–407.
 49. Haczku A, Cao Y, Vass G, Kierstein S, Nath P, Atochina-Vasserman EN, Scanlon ST, Li L, Griswold DE, Chung KF, et al. IL-4 and IL-13 form a negative feedback circuit with surfactant protein-D in the allergic airway response. *J Immunol* 2006;176:3557–3565.