



HUMAN & MOUSE CELL LINES

Engineered to study multiple immune signaling pathways.

Transcription Factor, PRR, Cytokine, Autophagy and COVID-19 Reporter Cells
ADCC, ADCC and Immune Checkpoint Cellular Assays



The Journal of Immunology

RESEARCH ARTICLE | FEBRUARY 15 2001

Surfactant Protein A Enhances Alveolar Macrophage Phagocytosis of Apoptotic Neutrophils¹ ✓

Trista L. Schagat; ... et. al

J Immunol (2001) 166 (4): 2727–2733.

<https://doi.org/10.4049/jimmunol.166.4.2727>

Related Content

Role of Surfactant Proteins A, D, and C1q in the Clearance of Apoptotic Cells In Vivo and In Vitro: Calreticulin and CD91 as a Common Collectin Receptor Complex

J Immunol (October,2002)

Surfactant Protein D Reduces Alveolar Macrophage Apoptosis In Vivo

J Immunol (September,2002)

Pulmonary Surfactant Protein A Inhibits Macrophage Reactive Oxygen Intermediate Production in Response to Stimuli by Reducing NADPH Oxidase Activity

J Immunol (June,2004)

Surfactant Protein A Enhances Alveolar Macrophage Phagocytosis of Apoptotic Neutrophils¹

Trista L. Schagat, Jessica A. Wofford, and Jo Rae Wright²

Surfactant protein A (SP-A) is an innate immune molecule that binds foreign organisms that invade the lungs and targets them for phagocytic clearance by the resident pulmonary phagocyte, the alveolar macrophage (AM). We hypothesized that SP-A binds to and enhances macrophage uptake of other nonself particles, specifically apoptotic polymorphonuclear neutrophils (PMNs). PMNs are recruited into the lungs during inflammation, but as inflammation is resolved, PMNs undergo apoptosis and are phagocytosed by AMs. We determined that SP-A increases AM phagocytosis of apoptotic PMNs $280 \pm 62\%$ above the no protein control value. The increase is dose dependent, and heat-treated SP-A still enhanced uptake, whereas deglycosylated SP-A had significantly diminished ability to enhance phagocytosis. Surfactant protein D also increased phagocytosis of apoptotic PMNs by $\sim 125\%$. However, other proteins that are structurally homologous to SP-A, mannose-binding lectin and complement protein 1q, did not. SP-A enhances phagocytosis via an opsonization-dependent mechanism and binds apoptotic PMNs ~ 4 -fold more than viable PMNs. Also, binding of SP-A to apoptotic PMNs does not appear to involve SP-A's lectin domain. These data suggest that the pulmonary collectins SP-A and SP-D facilitate the resolution of inflammation by accelerating apoptotic PMN clearance. *The Journal of Immunology*, 2001, 166: 2727–2733.

During the resolution of inflammation, polymorphonuclear neutrophils (PMNs)³ undergo programmed cell death, or apoptosis. This results in plasma membrane changes that cause them to be recognized as nonself and subsequently phagocytosed by macrophages (1, 2). These changes include exposure of phosphatidylserine on the outer leaflet of the plasma membrane (3, 4), loss of sialic acid residues on surface Ags (5), and decreased expression of the glycosylphosphatidylinositol-linked protein CD16 (6). If macrophage recognition and phagocytosis of the apoptotic cells are not efficient, PMNs can proceed into the later stages of apoptosis in which their plasma membranes break down and intracellular enzymes leak into the surrounding tissue, resulting in tissue damage and prolonged inflammation (7, 8).

Both soluble factors and phagocyte membrane receptors have been identified that recognize apoptotic cells and mediate their clearance (reviewed in Ref. 9). Soluble mediators that have been identified include β_2 -glycoprotein (10) and complement proteins, which becomes deposited on the apoptotic cell surface (11). Also, thrombospondin acts in concert with the phagocyte receptors $\alpha_v\beta_3$ and CD36 to bridge the apoptotic cell with the phagocyte (12). In addition to $\alpha_v\beta_3$ and CD36, other phagocyte receptors that have been identified are the LPS receptor CD14, class A and class B scavenger receptors, and an ATP-binding cassette transporter,

ABC-1. Inhibition studies suggest that an unidentified phosphatidylserine receptor and lectin(s) are also involved. As discussed in the review by Savill (13), multiple interactions exist between the apoptotic cell and its phagocyte.

Immunoregulatory proteins have been identified in the lungs. Among these proteins are surfactant proteins A and D (SP-A and SP-D), which are in the collectin protein family of innate immune molecules. Collectins are oligomeric proteins characterized by N-terminal collagen-like domains and C-terminal lectin or carbohydrate binding domains (14, 15). All family members are pattern recognition molecules that bind nonself moieties, thereby targeting the foreign material for rapid clearance by immune cells. SP-A in particular can bind to a variety of substrates, including carbohydrates (16), surfactant lipids and lipid vesicles (17, 18), and proteins (i.e., myosin (19)). Binding of SP-A to both bacteria and lipid vesicles enhances their phagocytosis by macrophages (20–24). The macrophages with which SP-A and SP-D interact are the resident immune cells in the lungs, the alveolar macrophages (AM).

Several *in vivo* models of pulmonary injury demonstrate the importance of AM phagocytosis of apoptotic PMNs in the resolution of inflammation (25, 26), and it has been suggested that chronic pulmonary inflammation may be a result of inefficient clearance of apoptotic PMNs (27). We hypothesize that when PMNs undergo apoptosis, the changes that occur at the PMN plasma membrane allow SP-A and SP-D to recognize them as nonself. The proteins would then bind the apoptotic PMNs and target them for rapid phagocytosis by AMs. In this way, SP-A and SP-D would protect the pulmonary tissue by quickly resolving inflammation.

To test this hypothesis, we used a well-established assay for detecting macrophage phagocytosis of apoptotic PMNs (2) and showed that SP-A and, to a lesser extent, SP-D accelerate AM phagocytosis of apoptotic PMNs. We then designed a more sensitive flow cytometric assay and showed that SP-A's effect was time, dose, and opsonization dependent. The effect was not, however, affected by heat treatment of SP-A, but it was partially dependent on SP-A's N-linked carbohydrates. Furthermore, SP-A binds apoptotic PMNs significantly more than viable PMNs, and

Department of Cell Biology, Duke University Medical Center, Durham, NC 27710

Received for publication May 3, 2000. Accepted for publication November 27, 2000.

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

¹ This work was supported by Grant HL51134 from the National Heart, Lung, and Blood Institute, National Institutes of Health.

² Address correspondence and reprint requests to Dr. Jo Rae Wright, Box 3709, Department of Cell Biology, Duke University Medical Center, Durham, NC 27710. E-mail address: j.wright@cellbio.duke.edu

³ Abbreviations used in this paper: PMN, polymorphonuclear neutrophil; SP-A, surfactant protein A; SP-D, surfactant protein D; AM, alveolar macrophage; C1q, complement protein 1q; MBL, mannose-binding lectin; CM-DiI, chloromethylbenzamido derivative of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; CTB, CellTracker Blue; MPO, myeloperoxidase.

this binding does not appear to involve SP-A's carbohydrate recognition domain. Neither the structurally homologous serum collectin mannose-binding lectin (MBL) nor complement protein 1q (C1q) stimulated PMN uptake. Also, SP-A's effect was specific to AMs, as peritoneal macrophages did not show enhanced phagocytosis of apoptotic PMNs in the presence of SP-A.

Materials and Methods

Materials

All chemicals used were from Sigma (St. Louis, MO) unless otherwise indicated. PBS, RPMI 1640, DMEM, and IMDM were obtained from Life Technologies (Grand Island, NY). Jurkat cells were obtained from American Type Culture Collection (Manassas, VA). The fluorescent labels FITC, Alexa Fluor 488, chloromethylbenzamido derivative of 1,1'-diocetyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (CM-DiI), and CellTracker Blue (CTB) were obtained from Molecular Probes (Eugene OR). Apoptosis detection reagents (PE-labeled annexin and 7-aminoactinomycin D) and anti-CD95 Ab were purchased from PharMingen (San Diego, CA).

Animals

Male Sprague Dawley rats were obtained from Charles River (Raleigh, NC) or Taconic Farms (Germantown, NY). Rats weighed ~200–400 g.

Protein purification

SP-A was purified from the bronchoalveolar lavage fluid of patients with alveolar proteinosis as previously described (28). Briefly, SP-A was extracted from lavage fluid with butanol and sequential solubilization in octylglucoside and 5 mM Tris, pH 7.4. The SP-A was treated with polymyxin B agarose beads to reduce endotoxin contamination. All SP-A preparations had <0.1 pg endotoxin/ μ g SP-A by the *Limulus* amoebocyte lysate assay QCL-1000 (BioWhittaker, Walkersville, MD). SP-A was stored in 5 mM Tris, pH 7.4, at -20°C .

SP-D was purified from rats treated with silica to induce surfactant accumulation as previously described (29). Briefly, 4 wk after rats received an intratracheal administration of silica, their lungs were lavaged, and SP-D was purified from the lavage by ultracentrifugation and affinity and gel filtration chromatography. Purified SP-D was dialyzed into PBS and stored at 4°C .

MBL was purified from rat serum (Pel-Freez, Rogers, AR) by affinity and gel filtration chromatography as previously described (30).

Human serum C1q was purchased from Advanced Research Technologies (San Diego, CA).

Modification of SP-A

The *N*-linked oligosaccharides of SP-A were enzymatically removed by treatment with *N*-glycosidase F (Calbiochem, La Jolla, CA) using a slight modification of a published protocol (31). SP-A deglycosylation was conducted at 37°C for 16 h in 20 mM sodium phosphate (pH 7.4), 6 mM *n*-octyl- β -*D*-glucopyranoside, and 10 mM EDTA with 1 μ g enzyme/25 μ g SP-A. The reaction mixture was buffer exchanged into 10 mM sodium phosphate buffer, pH 7.4, using a Centricon-100 concentrator (Amicon, Beverly, MA). Mock-treated SP-A was prepared exactly as described above, except *N*-glycosidase F was omitted. Gel electrophoresis was used to confirm that the SP-A was completely deglycosylated. Final protein concentrations were determined with the bicinchoninic protein assay kit (Pierce, Rockford, IL).

SP-A was labeled with the amine-reactive probe FITC or Alexa Fluor 488 according to the manufacturer's instructions. Briefly, 500 μ g of SP-A was dialyzed into 10 mM sodium borate, pH 9.5, labeled with 50 μ g of FITC for 1 h or dialyzed into 5 mM HEPES, pH 7.0, then labeled with Alexa Fluor 488 for 1 h. Excess FITC label was removed by dialysis against 5 mM Tris, pH 7.4, and excess Alexa label was removed by dialysis against 5 mM HEPES, pH 7.0.

Cell isolation

AMs were obtained by lung lavage of rats killed by sodium pentobarbital injection. Lungs were lavaged five to seven times with 10 ml of PBS and 0.2 mM EGTA. Cells were collected by centrifugation for 10 min at $330 \times g$ and resuspended in the appropriate buffer. Macrophages were generally >98% pure as determined by hematoxylin differential stain.

Peritoneal macrophages were obtained by peritoneal lavage of rats 4–5 days after i.p. thioglycolate treatment (32). The peritoneum was lavaged five times with 10 ml PBS and 0.2 mM EGTA. Cells were collected by

centrifugation for 10 min at $330 \times g$ and resuspended in the appropriate buffer.

Peripheral PMNs were isolated as previously described with minor modifications (33, 34). Briefly, rats were anesthetized (initial experiments were performed with halothane/metofane, and later experiments yielding similar results were performed with i.m. injection of 0.1 mg of acepromazine, 7 mg of ketamine, and 29 mg of xylazine per 250 g rat), and the jugular was cannulated with a 23-gauge butterfly needle attached to a three-way stop cock. An exchange of 3–4 ml of 6% hetastarch solution with 100 U/ml heparin for 3–4 ml of blood was performed until 40 ml was exchanged per rat. The first 10 ml of blood was collected in a syringe containing 10 ml of the hetastarch solution. After the exchange, blood was collected until the rats died. Blood was allowed to settle 30–45 min at room temperature, and the RBC-depleted fraction was collected and centrifuged at $330 \times g$ for 10 min. Cells were resuspended in 2 ml of PBS and underlaid with a five-step Percoll gradient. Step densities were 1.081, 1.085, 1.089, 1.093, and 1.097. Gradients were centrifuged at $500 \times g$ for 30 min at room temperature, and the fractions at the interfaces of 1.085–1.089 and 1.089–1.093 density layers were pooled, washed, and resuspended in the appropriate buffer. PMN purities were generally >80% as determined by hematoxylin differential stain (EM Science, Gibbstown, NJ).

PMN apoptosis

Freshly isolated PMNs were induced to undergo apoptosis by 24-h culture or by UV radiation (10-min exposure, followed by 3- to 4-h culture) as previously described (35, 36). Apoptotic PMNs were then washed and suspended in the appropriate assay medium. The percent apoptosis was evaluated by PharMingen's apoptosis detection kit using flow cytometry according to the manufacturer's specifications. Freshly isolated PMNs were $27 \pm 5\%$ annexin positive, 24 h-cultured PMNs were $44 \pm 12\%$ annexin positive, and UV-irradiated PMNs were $80 \pm 4\%$ annexin positive.

Jurkat cell apoptosis

Apoptosis of Jurkat cells was induced by incubating them with anti-Fas mAb (37). Briefly, cells were cultured in RPMI 1640 and 10% heat-inactivated FCS and treated with 2 μ g of anti-CD95 Ab/ 5×10^5 cells/ml for 25 h. Cells were then washed, and the percent apoptosis was evaluated by annexin staining. Jurkat cells were 38% annexin positive after Ab treatment.

Fluorescent labeling

Apoptotic PMNs were labeled with either CM-DiI for confocal microscopy or with CTB for flow cytometry. CM-DiI labeling was performed according to the manufacturer's guidelines with slight modifications; PMNs were suspended at 2×10^6 cells/ml PBS, then treated with 0.75 μ g of CM-DiI/ 10^6 cells for 10 min at 37°C followed by 15 min on ice. Cells were then washed and suspended at 2.5×10^7 cells/ml IMDM and 0.1% BSA.

PMNs were labeled with CTB by incubating cells in IMDM, 0.1% BSA, and 25 μ M CTB for 40 min at 37°C in 5% CO_2 followed by a second incubation for 30 min in fresh medium without CTB. Labeled PMNs were then suspended at 2.5×10^7 cells/ml IMDM in 0.1% BSA.

Macrophages were labeled according to manufacturer's specifications in the PKH2 green fluorescent linker kit (Sigma). Briefly, macrophages were suspended at 2×10^7 cells/ml diluent A, then treated with an equal volume of 4×10^{-6} M PKH2 dye for 5 min at room temperature. To stop the labeling, an equal volume of 1% BSA in PBS was added to the mixture for 1 min. Cells were then washed with 1% BSA in PBS, followed by IMDM and 0.1% BSA, and suspended at 2×10^6 /ml.

Myeloperoxidase-based phagocytosis assay

Uptake was evaluated based on methods previously described (1, 38, 39). Freshly isolated AMs were suspended at 1×10^6 cells/ml IMDM and 10% rat serum and adhered to 48-well tissue culture plates for 1 h at 37°C in 5% CO_2 . Wells were then washed twice with IMDM, and cultured PMNs were added to each well at 4×10^6 cells/ml IMDM with additional protein where indicated. Incubations were performed at 37°C in 5% CO_2 for 30 min unless otherwise indicated. Wells were then washed three times with ice-cold PBS, fixed with 2% glutaraldehyde in PBS, and stained for myeloperoxidase (MPO) activity. To stain for MPO activity, the following was added to each well: 0.25 μ l of HBSS, 0.1% BSA, 0.25 μ l of 0.1 M phosphate buffer (pH 6.2), 10 μ l of H_2O_2 , and 10 μ l of 1.25 mg/ml *o*-dianisidine. The reaction was conducted for 15 min at room temperature in the dark, then terminated with addition of 10 μ l of 1% sodium azide. Control studies showed that AMs were 100% MPO negative, and PMNs

were 100% MPO positive. Using light microscopy, cells were scored for the percentage of AMs that had PMNs associated with them. Sample wells were blinded, and a minimum of five fields of view at $\times 100$ objective and 500 macrophages were scored for each sample.

Flow cytometry phagocytosis assay

PKH2-labeled macrophages were incubated 1/3 with CTB-labeled apoptotic PMNs at a final concentration of 1.25×10^6 macrophages/ml IMDM and 0.1% BSA in BSA-precoated tubes in the presence of the indicated proteins. Cells were incubated at 37°C with gentle shaking for 60 min unless otherwise indicated. Cells were then washed with ice-cold PBS and 2 mM EDTA and fixed in 1% formaldehyde and 0.1% BSA in PBS for flow cytometry. Cells were analyzed for both labels, and PKH2-positive cells that were also positive for CTB were considered to have phagocytosed PMNs. Dual-labeled cells compared with total PKH2-positive cells were expressed as the percentage of macrophages that had phagocytosed PMNs.

SP-A binding assay

Cells were labeled using a slight modification of the protocol described in PharMingen's Apoptosis Detection Kit. Briefly, cells were washed twice with ice-cold PBS, then suspended at 1×10^6 cells/ml binding buffer (10 mM HEPES/NaOH (pH 7.4), 0.14 mM NaCl, and 2.5 mM CaCl_2). Aliquots (100 μl) were used for flow cytometry; cells were incubated with 25 $\mu\text{g/ml}$ FITC-SP-A or Alexa-SP-A for 15 min on ice, then with 5 $\mu\text{l/tube}$ PE-annexin for 15 min at room temperature. The binding reaction was stopped by adding 0.4 ml of ice-cold binding buffer/tube. Cells were analyzed by flow cytometry within 1 h or were fixed in 1% formaldehyde and stored at 4°C until analysis.

PMN studies measuring binding of different doses of SP-A and modified SP-A were performed as described, except that before labeling, annexin-positive cells were purified using the MACS apoptotic cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's specifications.

Confocal microscopy

Phagocytosis was conducted as described above, except CM-DiI-labeled PMNs were used. After cells had been fixed, smears were made of 2×10^5 cells. Slides were air-dried, then treated with 10 μl of mounting medium (PBS, 50% glycerol, and 2% 1, 4-diazabicyclo[2.2.2]octane), covered with a coverslip, and sealed with nail polish. Cells were then examined under oil at $\times 630$ magnification. Images were captured using dual-channel excitation. The percent phagocytosis was determined by scoring AMs that had clearly internalized PMNs.

Statistical analysis

All data are expressed as the mean \pm SEM, and statistical analysis was performed using one-tailed Student's *t* test for unpaired samples.

Results

SP-A increases AM phagocytosis of apoptotic PMNs

We examined the ability of SP-A to enhance AM phagocytosis of apoptotic PMNs using a well-established visual assay that detects phagocytosed PMNs by staining for MPO activity (2). At concentrations that stimulated phagocytosis of bacteria (21, 40), SP-A and, to a lesser extent, SP-D increased AM phagocytosis of apoptotic PMNs (280 ± 62 and $158 \pm 16\%$ of control value, respectively; Fig. 1).

The visual assay used in these experiments only allows detection of relatively intact PMNs in AMs. Macrophages break down apoptotic PMNs very rapidly, which results in loss of concentrated MPO activity and an inability to accurately quantitate phagocytosis. Although the percent increase in phagocytosis induced by SP-A was high, the actual percent phagocytosis measured was low ($\leq 6\%$ of AMs contained detectable MPO), and a time course was impossible to detect using the visual assay. We suspected that AMs were rapidly degrading PMNs, and we therefore designed a flow cytometric assay that allowed the detection of phagocytosed PMNs even after they were broken down by AMs. PMNs and AMs were labeled with different fluorescent dyes before phagocytosis. AMs were then analyzed for PMN label. Dual-labeled AMs were considered indicative of phagocytosis. These dyes have been designed

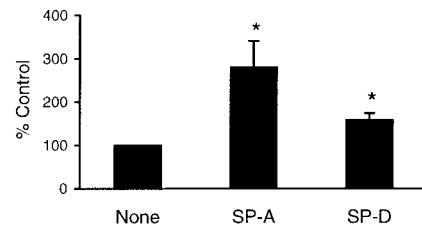


FIGURE 1. SP-A and SP-D increase AM phagocytosis of apoptotic PMNs. Peripheral rat PMNs were made apoptotic by 24-h culture in the absence of serum, then added to adhered AMs at a ratio of 3:1 for 30 min in the absence of protein (none) or in the presence of SP-A (25 $\mu\text{g/ml}$) or SP-D (1 $\mu\text{g/ml}$). Cells were then fixed and stained for MPO activity and visually scored for the proportion of AMs that had phagocytosed PMNs. Data are expressed as the percent increase in PMN-AM association between no protein controls and the addition of protein. AM phagocytosis of apoptotic PMNs was $2.0 \pm 0.3\%$ under control conditions. Apoptosis of PMNs was determined to be $44 \pm 12\%$ by annexin staining. Data shown are the mean \pm SEM ($n = 3-6$). *, $p < 0.05$ vs none.

for in vivo studies, and cell-to-cell transfer is considered negligible during the time course of these assays (41, 42).

Fig. 2 shows confocal microscopic images of the individually labeled cells (A and B) and images of AMs that had phagocytosed PMNs (C-F). The percent AM phagocytosis of PMNs was determined by scoring confocal images to confirm that the flow cytometric assay was an accurate measure of phagocytosis and not just a measure of cell association. The flow cytometric assay, like the MPO-based assay, showed that SP-A enhanced AM phagocytosis of apoptotic PMNs, and this was confirmed by confocal microscopy. However, baseline phagocytosis in these three assays was significantly different, presumably due to the sensitivity of each assay and the standard set for determining phagocytosis (MPO assay, $2.0 \pm 0.3\%$ phagocytosis; flow cytometry assay, $6 \pm 1\%$

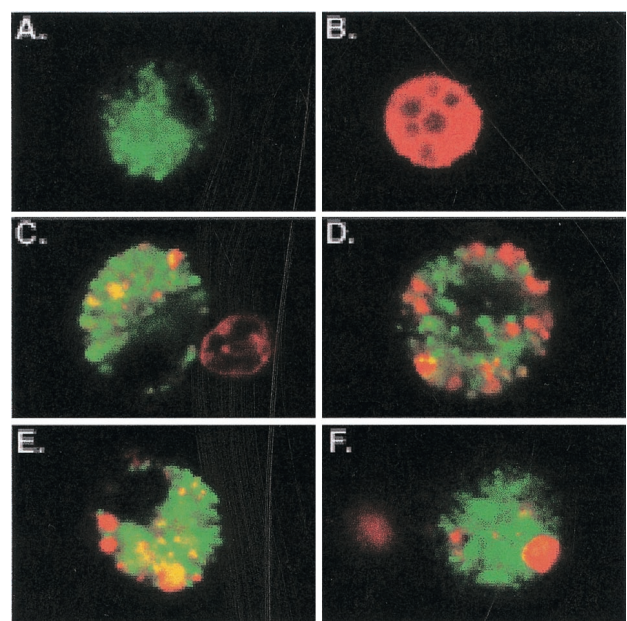


FIGURE 2. Confocal images of AM phagocytosis of apoptotic PMNs. Rat AMs were labeled with PKH2 (A), and apoptotic PMNs were labeled with CM-DiI (B). These cells were coincubated for 60 min, then fixed and examined by confocal microscopy. C-F, AMs that have phagocytosed PMNs. All images were captured under dual channel excitation (488 and 540 nm).

phagocytosis; confocal assay, 47 and 23% phagocytosis). Phagocytosis in the presence of 25 $\mu\text{g/ml}$ SP-A for the three assays was $3.7 \pm 0.2\%$ (MPO assay), $18 \pm 3\%$ (flow cytometry assay), and 61 and 53% (confocal assay).

The effect of SP-A dose on AM phagocytosis was determined (Fig. 3). Fig. 3A shows an example of the flow analysis of PKH2-positive AMs for the CTB-PMN label. Fig. 3B is the dose-response curve of SP-A's enhancement of phagocytosis. Phagocytosis was increased in a dose-dependent manner, with a plateau in the effect at 10–25 $\mu\text{g/ml}$.

The time course of stimulation was also determined (Fig. 4). SP-A's effect peaked at 60 min, then approached control levels.

We also examined the effect of SP-A on the regulation of peritoneal macrophage phagocytosis of apoptotic PMNs. Baseline phagocytosis of peritoneal macrophages was significantly more than phagocytosis by AMs (29 ± 11 and $6 \pm 1\%$, respectively). SP-A did not, however, enhance peritoneal macrophage phagocytosis above the baseline ($28 \pm 7\%$ phagocytosis in the presence of 25 $\mu\text{g/ml}$ SP-A).

SP-A's structural requirements for enhancement of phagocytosis

We examined the effect of modifying several aspects of SP-A's structure on its ability to enhance AM phagocytosis of apoptotic PMNs. After boiling the protein for 10 min, there was no change in its ability to enhance phagocytosis (Fig. 5). However, when SP-A was deglycosylated, its ability to enhance phagocytosis was significantly reduced (Fig. 5). Mock-treated SP-A (SP-A exposed to the deglycosylation reaction but with no *N*-glycosidase F) had an identical effect on phagocytosis as untreated SP-A (data not shown).

Effect of SP-A homologues on AM phagocytosis of apoptotic PMNs

The ability of other collectins to stimulate phagocytosis of apoptotic PMNs was examined. The lung collectin SP-D significantly stimulated phagocytosis, although to a lesser extent than SP-A

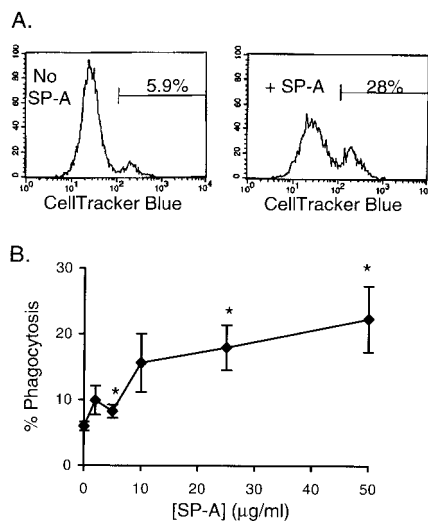


FIGURE 3. SP-A increases AM phagocytosis of apoptotic PMNs as determined by flow cytometry. PMNs were UV irradiated to induce apoptosis (average, $80 \pm 4\%$ by annexin staining), then CTB-labeled and coincubated with PKH2-labeled AMs for 60 min at 37°C in the presence of the indicated concentrations of SP-A. Cells were washed, fixed, and then analyzed by flow cytometry. A, Analysis of PKH2-positive cells for CTB label in the absence and the presence of 25 $\mu\text{g/ml}$ SP-A. B, Dose-response curve for SP-A. Data are expressed as the percentage of AMs that were positive for PMN label. Data are the mean \pm SEM ($n = 4-9$). *, $p < 0.05$ vs no SP-A.

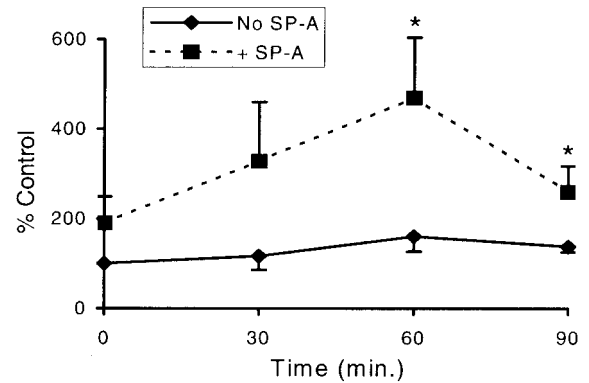


FIGURE 4. Time course for SP-A's increase in AM phagocytosis of apoptotic PMNs. PMNs were UV irradiated to induce apoptosis (average, $80 \pm 4\%$ by annexin staining), then CTB-labeled and coincubated with PKH2-labeled AMs in the presence or the absence of 25 $\mu\text{g/ml}$ SP-A for the indicated times at 37°C . Cells were analyzed by flow cytometry, and data are expressed as the percentage of AMs that were positive for PMN label. Data are the mean \pm SEM ($n = 5-6$). *, $p < 0.05$ vs none.

(Fig. 1 and Table I). The serum collectin MBL did not significantly enhance phagocytosis at either 2 or 10 $\mu\text{g/ml}$ (Table I). We also examined the ability of the structurally homologous C1q to enhance phagocytosis, but no effect was seen (Table I).

SP-A increases apoptotic PMN phagocytosis by AMs via an opsonization-dependent, but not activation ligand-dependent, mechanism

It has previously been shown that SP-A can stimulate AM phagocytosis of bacteria by both coating the bacteria, thereby targeting them for phagocytosis (opsonization-dependent phagocytosis) (22, 23, 43), and directly stimulating AMs, thereby causing them to be more phagocytically competent (activation ligand-dependent phagocytosis) (21, 44). To determine the mechanism by which SP-A enhances AM phagocytosis of apoptotic PMNs, these two models were examined using an adherent visual assay. The activation ligand-dependent model was tested by coating wells with SP-A before AM adherence. Adhering AMs to SP-A-coated wells was not sufficient to significantly increase AM phagocytosis of apoptotic PMNs above the control value (Fig. 6). However, when

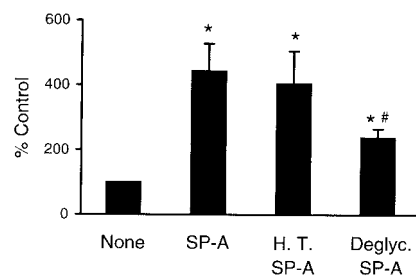


FIGURE 5. SP-A's effect on AM phagocytosis of apoptotic PMNs is not inactivated by heat treatment, but is partially dependent on SP-A's *N*-linked carbohydrates. PMNs were UV irradiated to induce apoptosis (average, $80 \pm 4\%$ by annexin staining), then CTB-labeled and coincubated with PKH2-labeled AMs for 60 min at 37°C in the presence of no protein (none) or 25 $\mu\text{g/ml}$ SP-A (SP-A), heat-treated SP-A (H. T. SP-A), or deglycosylated SP-A (Deglyc. SP-A). Cells were analyzed by flow cytometry, and data are expressed as the percentage of AMs that were positive for PMN label. Data are the mean \pm SEM ($n = 3-4$). *, $p < 0.05$ vs none; #, $p < 0.05$ vs SP-A.

Table I. The effect of SP-A homologues on AM phagocytosis of apoptotic PMNs^a

Treatment	% Control
None	100%
SP-A 25 μ g/ml	213 \pm 33%*
SP-D 0.5 μ g/ml	132 \pm 16%
2 μ g/ml	125 \pm 7%*
MBL 2 μ g/ml	119 \pm 12%
10 μ g/ml	118 \pm 13%
C1q 25 μ g/ml	110 \pm 19%

^a PMNs were UV irradiated to induce apoptosis (average 80 \pm 4% by annexin staining), then CTB-labeled and coincubated with PKH2-labeled AMs for 60 min at 37°C in the presence of the indicated proteins. Cells were then washed, fixed, and analyzed by flow cytometry. Values are reported as % no protein control. AM phagocytosis of apoptotic PMNs was 5.8% under control conditions. Mean \pm SEM. $n = 3-7$.

*, $p < 0.05$ vs none.

apoptotic PMNs were pretreated with SP-A for 15 min, and unbound SP-A was removed by washing the PMNs, AMs phagocytosed opsonized PMNs to the same extent as when SP-A was added directly to the assay (Fig. 6).

SP-A binds apoptotic PMNs

Macrophage phagocytosis of PMNs coincides with PMN apoptosis (2, 39). Because SP-A seems to stimulate phagocytosis via an opsonization-dependent mechanism (Fig. 6), and stimulation increases as PMN apoptosis increases (data not shown), we hypothesized that SP-A binding to PMNs increases as PMNs undergo apoptosis. To test this, we compared SP-A binding to apoptotic vs viable PMNs. PMNs made apoptotic by culturing were dual-labeled with FITC-SP-A and PE-annexin and analyzed by flow cytometry. The intensity of SP-A binding to annexin-positive (apoptotic) vs annexin-negative (viable) PMNs was compared. Fig. 7 is a representative dot plot of these experiments. SP-A bound to annexin-positive PMNs 4 \pm 1-fold more than to annexin-negative PMNs (average \pm SEM; $n = 3$). Annexin alone bound 84% of the PMNs (Fig. 7A), and SP-A alone bound 89% (Fig. 7B). Dual labeling of the PMNs showed that annexin bound 69% and SP-A bound 86% of the cells (Fig. 7C). This binding is dependent on SP-A dose, but does not appear to be saturable at concentrations up to 25 μ g/ml (Fig. 7D). An increase in SP-A binding to apoptotic vs viable PMNs was also seen with the PMNs made apoptotic by UV irradiation; SP-A bound annexin-positive PMNs \sim 2-fold more than annexin-negative PMNs. These data support the opsonization-

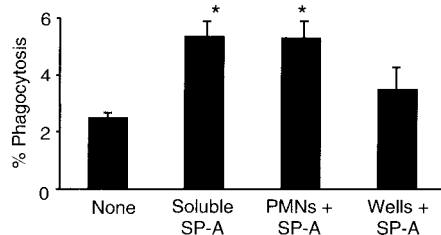


FIGURE 6. SP-A increases AM phagocytosis of apoptotic PMNs via an opsonization-dependent mechanism. PMNs were UV irradiated to induce apoptosis (average, 80 \pm 4% by annexin staining), then incubated with AMs that were adhered to tissue culture plates. The percentage of AMs that had phagocytosed apoptotic PMNs after 60 min was determined under the following conditions: control (no addition of protein), soluble SP-A (25 μ g/ml SP-A was added to the assay), PMNs + SP-A (PMNs were treated with 25 μ g/ml SP-A for 30 min before the assay), wells + SP-A (wells were coated with 25 μ g/ml SP-A before AM plating). Data are the mean \pm SEM ($n = 3-6$). *, $p < 0.01$ vs none.

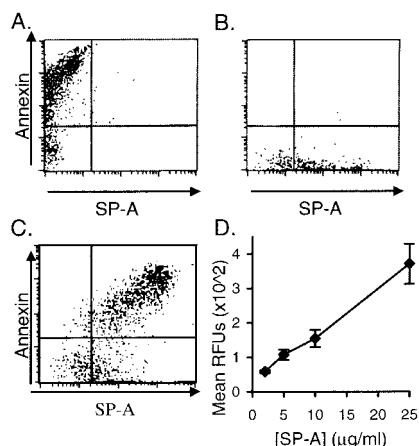


FIGURE 7. SP-A binds annexin-positive PMNs 4 \pm 1-fold more than annexin-negative PMNs. Peripheral PMNs were isolated and made apoptotic by culturing for 24 h in the absence of serum. Apoptotic PMNs were then washed and incubated for 15 min on ice with or without 25 μ g/ml FITC-labeled SP-A, followed by an additional 15-min incubation with or without PE-labeled annexin. Cells were then diluted 5-fold in cold binding buffer and analyzed by flow cytometry within 1 h. The histogram is representative of three experiments. A, PMNs labeled with PE-annexin (84% positive); B, PMNs labeled with FITC-SP-A (89% positive); C, PMNs dual-labeled with PE-annexin and FITC-SP-A (69% positive for both labels); D, graph of the mean relative fluorescence units (RFUs) of Alexa-SP-A bound to annexin-positive PMNs in the presence of increasing concentrations of SP-A. Apoptosis of PMNs was, on the average, 80 \pm 4% by annexin staining. Data are the mean \pm SEM ($n = 3$).

dependent model of SP-A-mediated AM phagocytosis of apoptotic PMNs. Binding of modified SP-A to PMNs and AMs was compared to determine whether the mechanism of binding to the two cells differed (Table II). Neither heat treatment nor deglycosylation of SP-A significantly reduced SP-A binding to apoptotic PMNs. However, both heat treatment and deglycosylation reduced SP-A binding to AMs. The AM binding data are consistent with published results by Oosting et al. (45).

To characterize the SP-A binding site on apoptotic PMNs, we examined the ability of various sugars to affect SP-A binding. Neither mannose nor mannan, which bind SP-A's lectin domains, affected SP-A binding to the apoptotic PMNs (98 \pm 13 and 99 \pm 15% of the SP-A control, respectively). Galactose also had no effect (97 \pm 9% of the SP-A control). SP-A binding was not calcium dependent (79 \pm 9% of the SP-A control), nor was it inhibited by excess unlabeled SP-A (155 \pm 25% of the SP-A control).

To determine whether SP-A has the capacity to bind other apoptotic cells, we induced apoptosis in Jurkat cells by cross-linking Fas on the surface of the cells (37) and compared SP-A binding to

Table II. SP-A binding to AMs vs apoptotic PMNs: the effect of SP-A modification^a

Treatment	Mean Fluorescence (RFUs)	
	AMs	Apoptotic PMNs
SP-A	58 \pm 3	158 \pm 24
Heat-treated SP-A	21 \pm 2*	137 \pm 43
Deglycosylated SP-A	34 \pm 1*	104 \pm 8

^a AMs were isolated from bronchoalveolar lavage of normal rats. Apoptotic PMNs were isolated from UV-irradiated rat peripheral PMNs by annexin-coupled magnetic beads. Cells were incubated with 10 μ g/ml of the indicated Alexa Fluor 488-labeled proteins for 15 min on ice followed by 15 min at room temperature, then fixed and analyzed by flow cytometry. RFUs, Relative fluorescence units. Mean \pm SEM. $n = 3$.

*, $p < 0.05$ vs SP-A.

the apoptotic vs the viable cells. Apoptosis was detectable in 38% of the cells 25 h after induction, and SP-A bound apoptotic Jurkat cells 3-fold more than viable cells.

Discussion

This study demonstrates that the pulmonary collectin, SP-A, can accelerate macrophage phagocytosis of apoptotic PMNs. This occurs via an opsonization-dependent mechanism, and the effect is partially mediated by the *N*-linked carbohydrates on SP-A. The other pulmonary collectin, SP-D, also enhances phagocytosis of PMNs, although to a lesser extent than SP-A. The serum collectin, MBL, did not significantly enhance phagocytosis. Because PMN apoptosis and subsequent phagocytosis by AMs are necessary for inflammation to be resolved (25–27), our data suggest that the pulmonary collectins may protect pulmonary tissue by facilitating the resolution of inflammation.

Collectins all bind to nonself molecular patterns. Before the current study the reported consequence of this binding was enhanced uptake of bacteria and viruses by phagocytes. The current study suggests that SP-A and SP-D can recognize apoptotic PMNs and enhance their phagocytosis by AMs. This effect was not shared by the serum collectin, MBL, or the structurally homologous complement component, C1q. Previous studies had demonstrated that C1q bound to apoptotic cells, but only in the presence of serum (46). As our experiments were performed without serum, it is not surprising that C1q was unable to enhance phagocytosis. It is possible that MBL may also require serum or other cofactors to enhance phagocytosis of apoptotic cells.

The ability of SP-A to enhance macrophage phagocytosis of apoptotic PMNs was not common to all phagocytes, as peritoneal macrophages did not show increased phagocytosis of apoptotic PMNs in the presence of SP-A. The peritoneal macrophages are, however, primed, because they have migrated into the peritoneum in response to an inflammatory stimulus. This priming may affect the macrophages' ability to respond to SP-A, and it remains to be determined how the activation state of AMs may affect SP-A's regulation of their phagocytosis of apoptotic PMNs.

SP-A enhanced AM phagocytosis in a time-dependent manner, with a peak in enhancement observed at 60 min. It was interesting that SP-A's effect seemed to decrease after 60 min, although this is not statistically significant. This may be due to a decrease in the effective concentration of SP-A in the medium over the course of the assay, perhaps by AM uptake and degradation (47). Alternatively, an apparent reduction in phagocytosis may be due to a breakdown or dissipation of the PMN label after phagocytosis by the AMs, because fluorescence of the PMN label is dependent on its local concentration.

Multiple changes occur on the surface of apoptotic cells, revealing cryptic patterns that SP-A may then recognize as nonself. For example, changes in carbohydrates occur on the plasma membranes of apoptotic cells (5). Although the details of these changes remain to be determined, they appear to involve, at least partially, the loss of sialic acid residues on surface structures. Because mono- and disaccharides have been shown to inhibit phagocytosis of apoptotic cells (48–50), it has been speculated that a lectin receptor is involved in phagocytosis, and several lectin receptors on nonprofessional phagocytes have been identified (48, 50, 51).

It does not appear, however, that SP-A is interacting with the apoptotic PMNs via its lectin domain. Mannan and mannose, which bind to SP-A's lectin domain, did not inhibit SP-A binding to the apoptotic PMNs. Also, the tertiary structure of this domain is what imparts the protein's lectin ability; heat treatment of SP-A eliminates binding to immobilized D-mannose (16), attenuates enhancement of AM phagocytosis of bacteria (21), and reduces SP-A

binding to AMs, which is consistent with our data (45). Interestingly, SP-A's ability to mediate AM phagocytosis of apoptotic PMNs is not eliminated by heat treatment. This observation suggests that some domain other than the lectin domain is responsible for the interaction of the protein with apoptotic PMNs.

Although heat treatment of SP-A did not reduce phagocytosis or SP-A binding to apoptotic PMNs, it did significantly reduce SP-A binding to AMs. Studies attempting to characterize the SP-A receptor on AMs (reviewed by Tino and Wright (52)) suggest that SP-A has multiple interactions with AMs. Our data suggest that heat treatment of SP-A inhibits some of these interactions, but not those interactions required for mediation of AM phagocytosis of apoptotic PMNs.

An SP-A-mediated function that is not dependent on the higher structure of the protein is its ability to interact with lipid vesicles (17, 18). SP-A binds to a variety of lipids, including dipalmitoylphosphatidylcholine. Heat treatment of SP-A does not inhibit its ability to bind lipids, but it does decrease its ability to aggregate them. The only lipid change on the surface of apoptotic cells that has been identified is exposure of phosphatidylserine (3, 4). This phospholipid is actively partitioned to the inner leaflet of the plasma membrane in viable cells, but when cells become apoptotic, this asymmetry is lost, and phosphatidylserine is exposed on the surface of the cell. It seems unlikely that SP-A is binding to phosphatidylserine on apoptotic cells, because it has been previously shown that SP-A does not bind phosphatidylserine (18). It is possible that other unidentified changes in the phospholipid composition of the outer plasma membrane are responsible for SP-A binding or that SP-A may bind phosphatidylserine in the context of the plasma membrane.

Each monomer of SP-A is post-translationally modified by *N*-linked glycosylations. These glycosylations are not required for SP-A's lectin activity or for binding to phospholipids (53), but they are involved in viral recognition (31, 54) and aggregation of phospholipid vesicles (17). Interestingly, SP-A's *N*-linked carbohydrates are involved in AM phagocytosis of *Mycobacterium tuberculosis* (20). Also, we and others (45) have found that deglycosylation of SP-A significantly decreases SP-A binding to macrophages. The reduced ability of deglycosylated SP-A to enhance AM phagocytosis of apoptotic PMNs appears to be due to its reduced ability to bind AMs and not to a disrupted interaction with the PMNs.

SP-A not only bound apoptotic PMNs significantly more than viable PMNs, but it also bound apoptotic Jurkat cells significantly more than viable Jurkat cells. This suggests that SP-A is recognizing a motif on the apoptotic PMNs that is common to apoptotic cells regardless of cell type or mode by which apoptosis is induced. Identification of this binding site will not only contribute to a better understanding of how SP-A may regulate apoptotic cell clearance, but will contribute to our understanding of the changes that occur at the cell's plasma membrane during apoptosis.

SP-A is not the first example of an innate immune molecule that plays a role in clearance of apoptotic cells. Both CD14, the primary LPS receptor, as well as complement have been found to mediate phagocytosis of apoptotic cells (11, 55). It seems possible that the clearance of bacteria and the clearance of apoptotic cells have evolved to encompass similar mechanisms. The goal of both systems is the same: to rid the body of unwanted cells or particles. Understanding how these proteins function will lead to a better understanding of how inflammation may be modulated to minimize potentially harmful proinflammatory responses while maximizing the host response against infection.

Acknowledgments

We are grateful to Dr. J. Michael Cook and the staff of the Duke University Flow Cytometry Facility for their excellent technical assistance and help with experimental design and interpretation. We are also thankful to Dr. Rick Fehon's laboratory for training and use of the confocal facility.

References

- Newman, S. L., J. E. Henson, and P. M. Henson. 1982. Phagocytosis of senescent neutrophils by human monocyte-derived macrophages and rabbit inflammatory macrophages. *J. Exp. Med.* 156:430.
- Savill, J. S., A. H. Wyllie, J. E. Henson, M. J. Walport, P. M. Henson, and C. Haslett. 1989. Macrophage phagocytosis of aging neutrophils in inflammation: programmed cell death in the neutrophil leads to its recognition by macrophages. *J. Clin. Invest.* 83:865.
- Fadok, V. A., D. R. Voelker, P. A. Campbell, J. J. Cohen, D. L. Bratton, and P. M. Henson. 1992. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol.* 148:2207.
- Martin, S. J., C. P. Reutelingsperger, A. J. McGahon, J. A. Rader, R. C. van Schie, D. M. LaFace, and D. R. Green. 1995. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J. Exp. Med.* 182:1545.
- Morris, R. G., A. D. Hargreaves, E. Duvall, and A. H. Wyllie. 1984. Hormone-induced cell death. II. Surface changes in thymocytes undergoing apoptosis. *Am. J. Pathol.* 115:426.
- Dransfield, I., A. M. Buckle, J. S. Savill, A. McDowall, C. Haslett, and N. Hogg. 1994. Neutrophil apoptosis is associated with a reduction in CD16 (Fc γ RIII) expression. *J. Immunol.* 153:1254.
- Savill, J., and C. Haslett. 1995. Granulocyte clearance by apoptosis in the resolution of inflammation. *Semin. Cell Biol.* 6:385.
- Savill, J. 1998. Apoptosis: phagocytic docking without shocking. *Nature* 392:442.
- Platt, N., R. P. da Silva, and S. Gordon. 1998. Recognizing death: the phagocytosis of apoptotic cells. *Trends Cell Biol.* 8:365.
- Balasubramanian, K., J. Chandra, and A. J. Schroitt. 1997. Immune clearance of phosphatidylserine-expressing cells by phagocytes: the role of β 2-glycoprotein I in macrophage recognition. *J. Biol. Chem.* 272:31113.
- Mevorach, D., J. O. Mascarenhas, D. Gershov, and K. B. Elkon. 1998. Complement-dependent clearance of apoptotic cells by human macrophages. *J. Exp. Med.* 188:2313.
- Savill, J., N. Hogg, Y. Ren, and C. Haslett. 1992. Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis. *J. Clin. Invest.* 90:1513.
- Savill, J. 1997. Apoptosis in resolution of inflammation. *J. Leukocyte Biol.* 61:375.
- Holmskov, U., R. Malhotra, R. B. Sim, and J. C. Jensenius. 1994. Collectins: collagenous C-type lectins of the innate immune defense system. *Immunol. Today* 15:67.
- Epstein, J., Q. Eichbaum, S. Sheriff, and R. A. B. Ezekowitz. 1996. The collectins in innate immunity. *Curr. Opin. Immunol.* 8:29.
- Haagsman, H. P., S. Hawgood, T. Sargeant, D. Buckley, R. T. White, K. Drickamer, and B. J. Benson. 1987. The major lung surfactant protein, SP 28-36, is a calcium-dependent, carbohydrate-binding protein. *J. Biol. Chem.* 262:13877.
- Haagsman, H. P., R. H. Elfring, B. L. M. van Buel, and W. F. Voorhout. 1991. The lung lectin surfactant protein A aggregates phospholipid vesicles via a novel mechanism. *Biochem. J.* 275:273.
- Kuroki, Y., and T. Akino. 1991. Pulmonary surfactant protein A (SP-A) specifically binds dipalmitoylphosphatidylcholine. *J. Biol. Chem.* 266:3068.
- Michelis, D., M. Z. Kounnas, W. S. Argraves, E. D. Sanford, J. D. Borchelt, and J. R. Wright. 1994. Interaction of surfactant protein A with cellular myosin. *Am. J. Respir. Cell Mol. Biol.* 11:692.
- Gaynor, C. D., F. X. McCormack, D. R. Voelker, S. E. McGowan, and L. S. Schlesinger. 1995. Pulmonary surfactant protein A mediates enhanced phagocytosis of *Mycobacterium tuberculosis* by a direct interaction with human macrophages. *J. Immunol.* 155:5343.
- Tino, M. J., and J. R. Wright. 1996. Surfactant protein A stimulates phagocytosis of specific pulmonary pathogens by alveolar macrophages. *Am. J. Physiol.* 14:L677.
- Marienchek, W. I., J. Savov, Q. Dong, M. J. Tino, and J. R. Wright. 1999. Surfactant protein A enhances alveolar macrophage phagocytosis of a live, mucoid strain of *P. aeruginosa*. *Am. J. Physiol.* 277:L777.
- Kabha, K., J. Schmiegner, Y. Keisari, H. Parolis, J. Schleppehaefer, and I. Ofek. 1997. SP-A enhances phagocytosis of *Klebsiella* by interaction with capsular polysaccharides and alveolar macrophages. *Am. J. Physiol.* 16:L344.
- Wright, J. R., and D. C. Youmans. 1995. Degradation of surfactant lipids and surfactant protein A by alveolar macrophages in vitro. *Am. J. Physiol.* 268:L772.
- Hussain, N., F. Wu, L. Zhu, R. S. Thrall, and M. J. Kresch. 1998. Neutrophil apoptosis during the development and resolution of oleic acid-induced acute lung injury in the rat. *Am. J. Respir. Cell Mol. Biol.* 19:867.
- Cox, G., J. Crossley, and Z. Xing. 1995. Macrophage engulfment of apoptotic neutrophils contributes to the resolution of acute pulmonary inflammation in vivo. *Am. J. Respir. Cell Mol. Biol.* 12:232.
- Haslett, C. 1999. Granulocyte apoptosis and its role in the resolution and control of lung inflammation. *Am. J. Respir. Crit. Care Med.* 160:S5.
- McIntosh, J. C., S. Mervin-Blake, E. Conner, and J. R. Wright. 1996. Surfactant protein A protects growing cells and reduces TNF- α activity from LPS-stimulated macrophages. *Am. J. Physiol.* 15:L310.
- Wright, J. R., D. F. Zlogar, J. C. Taylor, T. M. Zlogar, and C. I. Restrepo. 1999. Effects of endotoxin on surfactant protein A and D stimulation of NO production by alveolar macrophages. *Am. J. Physiol.* 276:L650.
- Tino, M. J., and J. R. Wright. 1999. Surfactant proteins A and D specifically stimulate directed actin-based responses in alveolar macrophages. *Am. J. Physiol.* 276:L164.
- van Iwaarden, J. F., J. A. G. van Strijp, H. Visser, H. P. Haagsman, J. Verhoef, and L. M. G. van Golde. 1992. Binding of surfactant protein A (SP-A) to herpes simplex virus type 1-infected cells is mediated by the carbohydrate moiety of SP-A. *J. Biol. Chem.* 267:25039.
- Li, Y. M., G. Baviello, H. Vlassara, and T. Mitsuhashi. 1997. Glycation products in aged thioglycollate medium enhance the elicitation of peritoneal macrophages. *J. Immunol. Methods* 201:183.
- Williams, J. H., Jr., K. M. Moser, T. Ulich, and M. S. Cairo. 1987. Harvesting the noncirculating pool of polymorphonuclear leukocytes in rats by hetastarch exchange transfusion (HET): yield and functional assessment. *J. Leukocyte Biol.* 42:455.
- Sugawara, T., M. Miyamoto, S. Takayama, and M. Kato. 1995. Separation of neutrophils from blood in human and laboratory animals and comparison of the chemotaxis. *J. Pharm. Toxicol. Met.* 33:91.
- Fadok, V. A., D. L. Bratton, A. Konowal, P. W. Freed, J. Y. Westcott, and P. M. Henson. 1998. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF- β , PGE $_2$, and PAF. *J. Clin. Invest.* 101:890.
- Afford, S. C., J. Pongracz, R. A. Stockley, J. Crocker, and D. Burnett. 1992. The induction by human interleukin-6 of apoptosis in the promonocytic cell line U937 and human neutrophils. *J. Biol. Chem.* 267:21612.
- Cifone, M. G., R. De Maria, P. Roncaioli, M. R. Rippon, M. Azuma, L. L. Lanier, A. Santoni, and R. Testi. 1994. Apoptotic signaling through CD95 (Fas/Apo-1) activates an acidic sphingomyelinase. *J. Exp. Med.* 180:1547.
- Henson, P. M., B. Zanolari, N. A. Schwartzman, and S. R. Hong. 1978. Intracellular control of human neutrophil secretion. I. C5a-induced stimulus-specific desensitization and the effects of cytochalasin B. *J. Immunol.* 121:851.
- Savill, J. S., P. M. Henson, and C. Haslett. 1989. Phagocytosis of aged human neutrophils by macrophages is mediated by a novel "charge-sensitive" recognition mechanism. *J. Clin. Invest.* 84:1518.
- Manz-Keinke, H., H. Plattner, and J. Schlepper-Schafer. 1992. Lung surfactant protein A (SP-A) enhances serum-independent phagocytosis of bacteria by alveolar macrophages. *Eur. J. Cell Biol.* 57:95.
- Cumberledge, S., and M. A. Krasnow. 1993. Intercellular signalling in *Drosophila* segment formation reconstructed in vitro. *Nature* 363:549.
- Horan, P. K., and S. E. Slezak. 1989. Stable cell membrane labelling. *Nature* 340:167.
- Pikaar, J. C., W. F. Voorhout, L. M. G. VanGolde, J. Verhoef, J. A. G. VanStrijp, and J. F. VanIwaarden. 1995. Opsonic activities of surfactant proteins A and D in phagocytosis of Gram-negative bacteria by alveolar macrophages. *J. Infect. Dis.* 172:481.
- Tenner, A. J., S. L. Robinson, J. Borchelt, and J. R. Wright. 1989. Human pulmonary surfactant protein (SP-A), a protein structurally homologous to C1q, can enhance FcR- and CR1-mediated phagocytosis. *J. Biol. Chem.* 264:13923.
- Oosting, R. S., and J. R. Wright. 1994. Characterization of the surfactant protein A receptor: cell and ligand specificity. *Am. J. Physiol.* 267:L165.
- Korb, L. C., and J. M. Ahearn. 1997. C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. *J. Immunol.* 158:4525.
- Bates, S. R., and A. B. Fisher. 1996. Surfactant protein A is degraded by alveolar macrophages. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 15:L258.
- Hall, S. E., J. S. Savill, P. M. Henson, and C. Haslett. 1994. Apoptotic neutrophils are phagocytosed by fibroblasts with participation of the fibroblast vitronectin receptor and involvement of a mannose/fucose-specific lectin. *J. Immunol.* 153:3218.
- Duvall, E., A. H. Wyllie, and R. G. Morris. 1985. Macrophage recognition of cells undergoing programmed cell death (apoptosis). *Immunology* 56:351.
- Dini, L., F. Autuori, A. Lentini, S. Oliverio, and M. Piacentini. 1992. The clearance of apoptotic cells in the liver is mediated by the asialoglycoprotein receptor. *FEBS Lett.* 296:174.
- Oka, K., T. Sawamura, K. Kikuta, S. Itokawa, N. Kume, T. Kita, and T. Masaki. 1998. Lectin-like oxidized low-density lipoprotein receptor I mediates phagocytosis of aged/apoptotic cells in endothelial cells. *Proc. Natl. Acad. Sci. USA* 95:9535.
- Tino, M. J., and J. R. Wright. 1998. Interactions of surfactant protein A with epithelial cells and phagocytes. *Biochim Biophys Acta* 1408:241.
- McCormack, F. X., H. M. Calvert, P. A. Watson, D. L. Smith, R. J. Mason, and D. R. Voelker. 1994. Structure and function of surfactant protein A: hydroxyproline- and carbohydrate-deficient mutant proteins. *J. Biol. Chem.* 269:5833.
- Benne, C. A., C. A. Kraaijeveld, J. A. van Strijp, E. Brouwer, M. Harmsen, J. Verhoef, L. M. van Golde, and J. F. van Iwaarden. 1995. Interactions of surfactant protein A with influenza A viruses: binding and neutralization. *J. Infect. Dis.* 171:335.
- Devitt, A., O. D. Moffatt, C. Raykundalia, J. D. Capra, D. L. Simmons, and C. D. Gregory. 1998. Human CD14 mediates recognition and phagocytosis of apoptotic cells. *Nature* 392:505.