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Pulmonary Surfactant Protein A Up-Regulates Activity of the Mannose Receptor, a Pattern Recognition Receptor Expressed on Human Macrophages

Alison A. Beharka,* Cecilia D. Gaynor,* Byoung K. Kang,* Dennis R. Voelker,† Francis X. McCormack,‡ and Larry S. Schlesinger2*

Inhaled particulates and microbes are continually cleared by a complex array of lung innate immune determinants, including alveolar macrophages (AMs). AMs are unique cells with an enhanced capacity for phagocytosis that is due, in part, to increased activity of the macrophage mannose receptor (MR), a pattern recognition receptor for various microorganisms. The local factors that “shape” AM function are not well understood. Surfactant protein A (SP-A), a major component of lung surfactant, participates in the innate immune response and can enhance phagocytosis. Here we show that SP-A selectively enhances MR expression on human monocyte-derived macrophages, a process involving both the attached sugars and collagen-like domain of SP-A. The newly expressed MR is functional. Monocyte-derived macrophages on an SP-A substrate demonstrated enhanced pinocytosis of mannose BSA and phagocytosis of Mycobacterium tuberculosis lipoarabinomannan-coated microspheres. The newly expressed MR likely came from intracellular pools because: 1) up-regulation of the MR by SP-A occurred by 1 h, 2) new protein synthesis was not necessary for MR up-regulation, and 3) pinocytosis of mannose BSA via MR recycling was increased. AMs from SP-A−/− mice have reduced MR expression relative to SP-A+/+. SP-A up-regulation of MR activity provides a mechanism for enhanced phagocytosis of microbes by AMs, thereby enhancing lung host defense against extracellular pathogens or, paradoxically, enhancing the potential for intracellular pathogens to enter their intracellular niche. SP-A contributes to the alternative activation state of the AM in the lung. The Journal of Immunology, 2002, 169: 3565–3573.

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Abbreviations used in this paper: AM, alveolar macrophage; PRR, pattern recognition receptor; MR, mannose receptor; MDM, monocyte-derived macrophage; SP-A, surfactant protein A; CRD, carbohydrate recognition domain; APP, alveolar protein; APP-S, APP (human) polypeptides that contain one (human) or two (rat) N-linked oligosaccharide attachment sites, a hydroxyproline-rich collagen-like domain, and carbohydrate recognition domains (CRDs) (8). Subunits of SP-A assemble into trimers and then further associate into 18mers composed of six trimers through interchain disulfide bond formation at the N terminus and noncovalent interactions between the collagen-like sequences. The resulting “bouquet of tulips” configuration, containing CRDs arranged on stalk-like scaffolding of collagen tails (9), provides a high valency of binding sites for microbe and host cell molecules (8). Human SP-A isolated from patients with the lung disease alveolar proteinosis (APP-SP-A) forms even larger aggregates by self-association of multiple “bouquets” (10, 11). Mutational analyses have been performed on recombinant rat SP-A synthesized in insect cells. Wild-type recombinant rat SP-A produced in this manner retains the functional properties of the natural protein despite simplified, mannose-rich glycosylation and incomplete proline hydroxylation and oligomeric assembly (8). Studies using recombinant proteins with site-directed mutations have revealed that the attached carbohydrate of SP-A is important for...
SP-A-mediated phagocytosis of *Mycobacterium tuberculosis* (*M.tb*) (12) and that the collagen-like domain is required for complete oligomeric assembly and binding to the SP-A receptor on alveolar epithelial cells (8).

Recent studies using SP-A−/− mice provide evidence that SP-A plays an important role in innate immunity (13). SP-A−/− mice are susceptible to infection with a variety of extracellular bacteria (13–16). SP-A can interact with both microorganisms and leukocytes in vitro (reviewed in Refs. 7 and 17). Our laboratory has demonstrated that SP-A enhances phagocytosis of the intracellular pathogen *M.tb* by human macrophages and that this response is mediated through a direct interaction between SP-A and the macrophage (12). SP-A-enhanced phagocytosis of *M.tb* by macrophages, including human AMs, occurs rapidly (within 1–2 h). Because phagocytosis of *M.tb* is a receptor-mediated event, one mechanism for the effect of SP-A on macrophages may be to enhance the surface expression and/or function of phagocyte receptors (18, 19). In our prior work, inhibitor studies suggested a role for the macrophage MR in SP-A enhancement of phagocytosis (12). Here we examined the effect of SP-A and its distinct structural components on MR expression and function. Our results demonstrate that SP-A up-regulates surface expression of functional MR, but does not alter complement receptor (CR) expression on human macrophages. Both the collagen region and sugars of SP-A are needed to optimize this effect.

**Materials and Methods**

**Buffers, reagents, and media**

Dulbecco’s PBS with and without Ca²⁺ and Mg²⁺ ions (Life Technologies, Grand Island, NY) and RPMI 1640 medium with 1-glutamine (RPMI) (Life Technologies) were purchased. RPMI medium was used alone or with 20 mM HEPES buffer (pH 7.2; Sigma-Aldrich, St. Louis, MO) and 1 mg/ml human serum albumin (HSA) (Calbiochem, La Jolla, CA). Poly-myxin B sulfate (PMB) was purchased from Sigma-Aldrich. Lipophosphoinositol (LAM) from the Erdman strain of *M. tb* was provided by Dr. P. J. Brennan and colleagues (Colorado State University, Ft. Collins, CO; National Institutes of Health, National Institute of Allergy and Infectious Diseases Contract 25147).

**Antibodies**

Mouse anti-human mAb against CR1 (anti-CD35; clone J3D3), CR4 (anti-CD11c; clone BU15), and CR3 (anti-CD11b; clone Bear 1) were purchased from Immunotech (Westbrook, ME). Bear 1 recognizes an epitope on the α-chain of CR3 (20), BU15 recognizes the α-chain of CR4 (21), and J3D3 recognizes CR1 (22). Mouse mAb IgG1 from Immunotech was used as the isotypic control for the above mAb. Purified and PE-labeled mouse anti-human MR and its purified and PE-conjugated subtypic control mAb; mouse IgG1κ, were purchased from BD PharMingen (San Diego, CA). Polyclonal rabbit anti-human MRs and rabbit anti-mouse MRs were provided by Dr. P. Stahl (Washington University, St. Louis, MO). A polyclonal rabbit anti-human Ab against SP-A was used (12). Normal rabbit serum (NRS) was used as one control for the experiments involving polyclonal Ab. FITC- or PE-conjugated goat anti-rabbit Ab and FITC- or PE-conjugated goat anti-mouse Ab were used as secondary Abs and were purchased from Cappel (West Chester, PA).

**SP-A proteins**

The SP-A proteins used in this study were developed and purified as previously described (23) (Fig. 1). In brief, bronchoaveolar lavage was used to obtain APF-SP-A from healthy volunteers (native human SP-A) (24). Native rat SP-A was purified from silica-pretreated Sprague Dawley rat lungs (25, 26). Recombinant rat SP-A (which is deficient in hydroxyproline content and hence designated SP-AΔHyp) was produced from SF-9 insect cells after infection with a recombinant baculovirus containing a 1.6-kb cDNA for rat SP-A (23). Recombinant rat SP-A proteins devoid of oligosaccharides at one or both of the consensus sequences for N-linked glycosylation were generated by amino acid substitutions at the Asn (SP-Ahyp,thr1) or Asn and Ser sites (SP-Ahyp,thr1,ser187) (23). Carbohydrate-deficient recombinant SP-A proteins retain structural and bio-logic functions, including oligomerization, aggregation of phospholipid liposomes, binding to immobilized carbohydrate, inhibition of lipid secretion from type II cells, and competition for receptor occupancy on type II cells (23). The synthesis of the mutant recombinant rat SP-A protein containing a nested deletion of the proximal collagen-like region (Gly9–Gly36) (TM2), truncation of the protein at the neck region resulting in a protein lacking the collagen and the NH₂-terminal regions (TM1-2-3), and an amino acid substitution at the Asn187 site (TM1-2-3,ser187) were made as previously described (27, 28). Purity of the SP-A preparation was assessed by SDS-PAGE. Bacterial endotoxin levels were determined using the Limulus amebocyte lysate kit (BioWhittaker, Walkersville, MD). Endotoxin levels in SP-A preparations ranged from undetectable to 300 pg/μg protein, with an average of 15 pg of endotoxin per microgram of protein.

**Human monocytes and macrophages**

Blood was obtained from healthy adult volunteers who were purified protein derivative skin test negative. Mononuclear cells from single donors were isolated from heparinized blood on Ficoll-sodium diatrizoate (Pharmacia Fine Chemical, Piscataway, NJ) and cultured in Teflon wells (Savillex, Minnetonka, MN) for 1 (monocytes) or 5 days (MDMs) in the presence of 20% autologous serum (1.5–2.0 × 10⁶ mononuclear cells/ml) at 37°C (29). On the day of each experiment, PBMCs were removed from Teflon wells and washed extensively, and the monocyte or MDM fraction was purified by adherence.

**FIGURE 1.** Schematic of rat and recombinant rat (hyp) SP-A. Native rat SP-A is composed of two isoforms, which are defined by the presence or absence of an interchain disulphide-forming N-terminal extension composed of amino acids Ile-Lys-Cys (IKC). The long and short isoforms are otherwise identical, containing an N-terminal segment (NTS) denoted domain 1, a collagen-like region divided by a midpoint interruption at Gly9–Gly36 into domains 2 and 3, a neck region, and a carbohydrate recognition domain. Sites of N-linked glycosylation (complex branching structure) are found at Asn1 of the NTS and Asn187 of the CRD. Recombinant rat SP-A synthesized in insect cells is similar to the native protein, except for simplified mannos-rich glycosylation (Y-shaped structure), incomplete proline hydroxylation, and oligomeric assembly as nonamers rather than octadecamers. Mutant recombinant SP-A devoid of attached carbohydrate at the 1 (hyp, thr1) or 1 and 187 (hyp, thr1, ser187) positions were generated by Asn1Thr and Asn187Ser substitutions. Truncated mutant (TM) recombinant SP-A contains deletions of first half of the collagen-like region (TM2), the NTS, and the entire collagen-like region (TM1, 2, 3), or the NTS, the collagen-like region and the carbohydrate attached to Asn187 (TM1, 2, 3, ser187) are shown.
Breeder pairs of gene-targeted SP-A-deficient mice (SP-A−/−) and wild-type (SP-A+/+) controls of the same strain (129J background) were kindly provided by Drs. J. Whitsett and T. Korthaghen (University of Cincinnati, Cincinnati, OH) (30). The mice used in the current study were the progeny of these original breeders and were bred at the University of Iowa (Iowa City, IA). Female and male 8- to 14-wk-old mice were housed under barrier conditions with an environmentally controlled atmosphere. All conditions and handling of the animals were approved by the Animal Care and Use Committee at University of Iowa and followed National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Mice were sacrificed via CO2 asphyxiation. Resident peritoneal macrophages were obtained by peritoneal lavage with ice-cold HBSS supplemented with 10 mM HEPES (Life Technologies). AMs were obtained by bronchoalveolar lavage with 37°C HBSS supplemented with 10 mM HEPES (31).

Flow cytometry
PBMCs (29) were incubated with SP-A proteins (10 μg/ml) or HSA (control) in Teflon wells for various time periods. After washing, cells were incubated with rabbit anti-MR, NRS, or isotypic control Ab, C. Anti-SP-A, polyclonal anti-MR, monoclonal anti-MR, NRS, or isotypic control Ab was added to appropriate wells with the appropriate PE-conjugated subtype control mAb served as negative controls. Additionally, in the initial experiments, after washing, the cells were incubated with rabbit anti-human MR Ab or NRS followed by PE-conjugated anti-rabbit secondary Ab. Subsequent experiments used PE-conjugated mAb against human MR or the appropriate PE-conjugated subtype control mAb. As a positive control for up-regulation of the MR, MDMs in monolayer culture on glass coverslips were incubated with IL-4 (Genzyme, Cambridge, MA) for 20 h before staining (5). To determine whether new protein synthesis was involved in MR up-regulation, 10 μg/ml cycloheximide (CHX) was added to select wells for 60 min before addition of SP-A. To determine whether LPS was contributing to the response, LPS at double the highest level found in the SP-A preparations (15 ng/ml) was added to select wells. Additionally, in some experiments, the LPS neutralizer PMB (5 μg/ml) (32) was added to select wells 30 min before or simultaneous with SP-A. Because the results were the same with both approaches, experiments using PMB were combined.

In experiments using mouse peritoneal or alveolar macrophages, 5 × 10^5 cells were incubated with rabbit anti-mouse MR (1/200) or NRS (control). After washing, cells were incubated with FITC-conjugated goat anti-rabbit Ig as the secondary Ab.

Cells were fixed in paraformaldehyde and were analyzed for mean fluorescence intensity (MFI) and percentage of positive cells (95/5% cutoff) by PE-conjugated mAb against human MR or the appropriate PE-conjugated subtype control mAb. As a positive control for up-regulation of the MR, MDMs in monolayer culture on glass coverslips were incubated with IL-4 (Genzyme, Cambridge, MA) for 20 h before staining (5). To determine whether new protein synthesis was involved in MR up-regulation, 10 μg/ml cycloheximide (CHX) was added to select wells for 60 min before addition of SP-A. To determine whether LPS was contributing to the response, LPS at double the highest level found in the SP-A preparations (15 ng/ml) was added to select wells. Additionally, in some experiments, the LPS neutralizer PMB (5 μg/ml) (32) was added to select wells 30 min before or simultaneous with SP-A. Because the results were the same with both approaches, experiments using PMB were combined.

ELISA to determine possible cross-reactivity between SP-A and Abs against MR
APP-SP-A from 0 to 500 ng/well was adhered to a Costar medium binding ELISA plate (Cambridge, MA) using 0.05 M carbonate/bicarbonate buffer (pH 9.6). After washing, nonspecific binding sites were blocked with 5% BSA in PBS at 60 min at 4°C. Anti-SP-A, polyclonal anti-MR, monoclonal anti-MR, NRS, or isotypic control Ab was added to appropriate wells overnight at 4°C. After washing, the appropriate HRP secondary Ab (Bio-Rad Laboratories, Richmond, CA) was incubated with the monolayers for 2 h at room temperature. The developing reaction (Bio-Rad Laboratories) was stopped with 5% oxalic acid (J. T. Baker Chemical Company, Phillipsburg, NJ). The plate was read at 405 nm on a microplate reader (Bio-Tek Instruments, Winooski, VT). The mean ± SD of the absorbance for triplicate wells of each type was calculated. The OD of control wells devoid of Ag or primary Ab (typically ≤0.10) was subtracted out from each test group.

Immunofluorescence microscopy
MDMs in monolayer culture on glass coverslips were incubated with APP-SP-A (10 μg/ml) or HSA for 1 h at 37°C and then were washed, incubated for an additional 10 min at 37°C, and then cooled to 4°C. The cells were next fixed with paraformaldehyde for 5 min, washed, and incubated with monoclonal anti-IR (1/500) in 0.2% BSA in PBS without Ca2+ and Mg2+ ions for 1 h, followed by FITC-conjugated goat anti-mouse Ab (1/100; Cappel) for 1 h. In certain experiments, lysosome-associated membrane protein-1 Ab, which stains intracellular late endosomal and lysosomal compartments (33), served as a control to confirm a nonsurface-associated cell membrane. To determine the possible effect of LPS contamination of the SP-A preparation, certain MDM monolayers were incubated with either 5 μg/ml PMB before SP-A or 15 ng/ml LPS.

The coverslips were mounted on glass slides and examined by confocal, scanning laser microscope (Zeiss, Thornwood, NY). Fluorescence intensity of each cell section was quantitated using the line intensity option of the Laser Sharp Reference Program (Bio-Rad Laboratories). A line was placed over a minimum of five fields of view at 3, 6, and 9 o’clock on the cell, and MFI of that area of the cell was calculated using the software program. The values presented represent specific MFIs in which the MFI of the subtypic control slides has been subtracted out. MFI was determined for 20 cells per coverslip in each test group, three coverslips per donor, and a minimum of two donors were used for each treatment.

Iodination of mannose-BSA
Mannose-BSA was iodinated by the lactoperoxidase method in the core radioisotope facility at the Veterans Affairs Medical Center (Iowa City, IA). Specific activity was typically 2–4 mCi/μg mannose-BSA protein, and 95% of total counts being TCA precipitable. The preparation was used within 3 wk of iodination (29).

Uptake of 125I-labeled mannose-BSA by monocyte-derived macrophages
PBMCs in Teflon wells (cells suspension) were incubated with 10 μg/ml APP-SP-A or HSA for 60 min at 37°C. After washing, cells were incubated in PBS containing Ca2+ and Mg2+ with or without 2.5 mg/ml mannan for 20 min at 37°C. One microgram of 125I-labeled mannose-BSA was then added for 10 min at 37°C. The cells were cooled to 4°C and washed six times, and the final pellet was collected and counted using a Beckman gamma counter (Beckman Coulter, Fullerton, CA). In other experiments, 5-day-old MDMs were placed in monolayer culture on glass coverslips. The monolayers were incubated with 10–20 μg/ml SP-A or HSA for 60 min at 37°C. After washing, separate wells were incubated with PBS or 4 mg/ml mannan in PBS for 10 min at 37°C. Then, 1 μg of 125I-labeled mannose-BSA was incubated with the monolayers for 10 min at 37°C. The monolayers were cooled to 4°C and the glass coverslips were transferred to a new 24-well tissue culture plate and were washed eight times. The cells were lysed with 1% SDS and the lysates were counted in a Beckman gamma counter. MR functional activity was defined as mannann-inhibitable uptake of 125I-labeled mannose-BSA. As a positive control, MDMs in Teflon wells were incubated with IL-4 (Genzyme) for 20 h before analysis.

Preparation of LAM-coated microspheres
LAM-coated microspheres were prepared as described (34). Briefly, polybead polystyrene microspheres (Polysciences, Warrington, PA) (2.0 × 10^8) that were 1 μm in diameter were washed two times in 0.05 M carbonate-bicarbonate buffer (pH 9.6) in presiliconized polypropylene tubes (National Scientific Supply, San Rafael, CA) and then incubated with 50 μg of Erdman M.tb strain LAM or buffer (control) for 1 h at 37°C on an Adams Nutator. The microspheres were then washed twice and incubated in 5% HSA in PBS (2 h at 37°C) to block nonspecific binding sites. Lastly, the microspheres were washed, resuspended in 0.5% HSA, and used in the adherence assay described below.

Phagocytosis of LAM- and HSA-coated microspheres by SP-A-treated macrophages
MDMs (2 × 10^5) were plated on a substrate of SP-A or HSA (control) for 90 min (12). After washing, monolayers were incubated with LAM-coated or HSA-coated microspheres (2.0 × 10^5) in RPMI + HEPES + HSA at 37°C for 60 min. After 60 min, the MDMs were washed to remove nonadherent microspheres and were fixed in 10% formalin. In certain wells, through the cell membrane of each cell at four points, quartz to responding to 12. LPS, inhibits MR activity (29). The mean number (±SD) of microspheres per MDM on each of duplicate or triplicate coverslips was determined by counting a minimum of 200 consecutive MDMs per coverslip by phase contrast microscopy.

Statistics
A two-tailed Student’s t test was used for analyzing differences between specific test groups and control groups in each experiment.
Results

**SP-A up-regulates expression of the MR on human macrophages**

SP-A up-regulates the phagocytosis of *M. tb* rapidly (within 60 min) via a direct interaction with the macrophage, indicating that SP-A influences macrophage receptor activity (12). We used flow cytometry to determine whether SP-A up-regulates the expression of the MR on MDMs. The results of a typical experiment are displayed as histograms (Fig. 2) and cumulative data are presented in Table I. The MR was constitutively expressed on MDMs incubated with HSA (control) (Fig. 2A). After exposure to SP-A for 60 min, expression of the MR (based on both percent change in MFI and percent positive cells) was enhanced (*p* < 0.05) when compared with the control (Fig. 2A and Table I). IL-4 up-regulated MR expression as a positive control (5) (Fig. 2C and Table I). SP-A effects can be influenced by LPS contamination (35, 36). Incubation of macrophages with PMB and then SP-A did not affect the SP-A-induced up-regulation of MR expression (Fig. 2B and Table I), and PMB alone had no effect on MR expression (*p* > 0.05) (data not shown). Additionally, incubation with LPS alone did not increase MR expression (*p* > 0.1) (Table I).

To determine whether SP-A selectively up-regulated MR expression or was capable of up-regulating other receptors involved in phagocytosis, we assessed the surface expression of CRs after SP-A incubation by flow cytometry. CR4 was constitutively expressed on MDMs (Fig. 2B). Incubation with SP-A did not significantly alter expression of CR4 (Fig. 2B and Table I) or CR1 and CR3 (Table I).

We next assessed the location of the MR on control or APP-SP-A-treated monocytes or MDMs by confocal microscopy (Fig. 3A). As reported (3), monocytes did not express MR (Fig. 3A), and exposure to SP-A for 1 h did not induce MR expression on monocytes (*p* > 0.1) (Fig. 3B). In contrast, MDMs expressed a low level of MR constitutively (Fig. 3C and Table II), and SP-A markedly increased the expression of MR on MDMs (Fig. 3D and Table II). Similar to the flow cytometry data, incubation of monolayers with LPS or with PMB before SP-A had no effect on MR expression in control-treated or SP-A-treated cells, respectively (Table II). At any given time, 20–25% of total MR protein exists on the cell surface, with the majority residing in intracellular pools (37). For confocal microscopy experiments, MDMs were fixed with paraformaldehyde and thus were nonpermeabilized. Consistent with this, fixed MDMs incubated with Ab to lysosome-associated membrane protein-1, an intracellular late endosomal and lysosomal marker (33), revealed no significant staining (data not shown). Therefore, the MR expression being measured in these experiments represented cell surface protein only.

We next determined whether the increased MR expression was due to outward movement of the intracellular pools or to new protein synthesis by incubating MDMs with CHX before SP-A to prevent protein synthesis. Pretreatment with CHX had only a small effect on the magnitude of SP-A-induced MR up-regulation (Table I), supporting the notion that MR up-regulation was dependent primarily upon recycling of preformed MR rather than on newly synthesized MR.

To rule out cross-reactivity of anti-MR for SP-A as previously reported (38), an SP-A ELISA was performed. Polyclonal anti-human Ab against SP-A recognized APP-SP-A (500 ng/well) with an OD < 0.1 (data not shown). Neither the polyclonal anti-human MR Ab nor the monoclonal anti-human MR recognized APP-SP-A (OD < 0.05) (data not shown). Additionally, APP-SP-A was examined by Western blotting using the Abs mentioned above. Again, the polyclonal anti-human SP-A Ab recognized SP-A and the anti-MR Ab did not (data not shown). To rule out the possibility that SP-A remained on the surface of the MDMs after incubation and washing in our assays and was being recognized by the anti-MR Ab, we performed confocal microscopy experiments as above using a polyclonal Ab to SP-A as the primary Ab instead of the anti-MR Ab. No specific detection of SP-A on the cell surface was found (data not shown). These data are consistent with the results in our earlier study (12) that showed that SP-A is internalized rapidly by the MDMs.

**The source of the SP-A affects the degree of MR expression**

To test whether SP-A proteins from different sources were equally capable of up-regulating MR expression, we incubated MDMs with APP, native human or rat SP-A, as well as recombinant SP-A (SP-A*<sup>hr</sup>) and measured MR expression compared with control MDMs using flow cytometry (Table I). All four sources of SP-A examined significantly increased MR expression compared with control cells. Incubation with APP-SP-A consistently induced numerically higher levels of MR expression than did the other proteins, but the APP-SP-A-induced increase was only significantly higher (*p* < 0.05) than the level induced by SP-A*<sup>hr</sup>.

**The carbohydrate- and collagen-like domains of SP-A are both involved in SP-A-induced MR expression**

We next explored the structural components of SP-A that mediate the enhanced MR response. To study the potential influence of the carbohydrate moieties of SP-A, we used recombinant SP-A proteins devoid of oligosaccharides at one (Asn<sup>187</sup>) site (SP-A*<sup>bpy,serv187</sup>) or both (Asn<sup>1</sup> and Asn<sup>187</sup>) sites (SP-A*<sup>bpy,serv1</sup> and SP-A*<sup>bpy,serv1,serv187</sup>) of the consensus sequence sites for glycosylation. The absence of
carbohydrates on the variant proteins used in these studies has been demonstrated previously (23). MDMs were incubated with variant SP-A proteins and MR expression was measured using flow cytometry (Table I). Although not statistically significant, addition of the SP-A carbohydrate variant proteins resulted in a 60% increase in MFI and a 45–61% increase in positive cells relative to control MDMs. Incubation of MDMs with SP-A protein devoid of N-linked carbohydrates at the Asn187 site (SP-Ahyp,ser187) resulted in MR expression similar to that seen with SP-A devoid of carbohydrate (SP-Ahyp,thr1,ser187) and was particularly evident for the percent change in positive cells. These results provide evidence that glycosylation is important for optimal up-regulation of MR expression.

In parallel experiments, we investigated the importance of the collagen-like region of SP-A for up-regulation of MR expression (Table I). SP-A-truncated proteins lacking the first half of the collagen region but still possessing both carbohydrate attachment sites (TM2), lacking the collagen region and the N-terminal segment but still possessing the carbohydrate attachment site at Asn187 (TM1-2-3), or lacking the collagen region, the N terminus, and both sugar attachment sites (TM1-2-3ser187) were added to MDMs. The truncated proteins were capable of increasing MR expression to a small extent, but less than that of APP-SP-A or SP-Ahyp,ser187. The level of MR expression achieved was similar among truncated proteins. Taken together, these studies demonstrate a critical role for both the carbohydrates and collagen region of SP-A in mediating its effect.

### Table I. Effect of SP-A on phagocytic receptor expression on human MDMs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MDM Stained with Ab Against</th>
<th>% Change in MFI</th>
<th>% Change in Positive Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td>MR</td>
<td>165 ± 43d</td>
<td>290 ± 31d</td>
</tr>
<tr>
<td>APP-SP-A</td>
<td>MR</td>
<td>125 ± 20d</td>
<td>283 ± 66d</td>
</tr>
<tr>
<td>APP-SP-A</td>
<td>CR1</td>
<td>−12 ± 17</td>
<td>2 ± 11</td>
</tr>
<tr>
<td>APP-SP-A</td>
<td>CR3</td>
<td>−16 ± 5</td>
<td>−5 ± 4</td>
</tr>
<tr>
<td>APP-SP-A</td>
<td>CR4</td>
<td>−11 ± 7</td>
<td>2 ± 6</td>
</tr>
<tr>
<td>CHX/APP-SP-A</td>
<td>MR</td>
<td>88 ± 18d</td>
<td>236 ± 73d</td>
</tr>
<tr>
<td>LPS</td>
<td>MR</td>
<td>25 ± 13</td>
<td>32 ± 16</td>
</tr>
<tr>
<td>PMB/APP-SP-A</td>
<td>MR</td>
<td>119 ± 24d</td>
<td>277 ± 108d</td>
</tr>
<tr>
<td>Native human SP-A</td>
<td>MR</td>
<td>100 ± 13d</td>
<td>291 ± 81d</td>
</tr>
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<td>Native rat SP-A</td>
<td>MR</td>
<td>105 ± 12d</td>
<td>281 ± 100d</td>
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<tr>
<td>SP-Ahyp,ser187</td>
<td>MR</td>
<td>85 ± 16d</td>
<td>126 ± 45d</td>
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<td>SP-Ahyp,thr1,ser187</td>
<td>MR</td>
<td>67 ± 19</td>
<td>61 ± 23</td>
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<tr>
<td>TM2</td>
<td>MR</td>
<td>52 ± 31</td>
<td>45 ± 20</td>
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<td>TM1-2-3</td>
<td>MR</td>
<td>58 ± 20</td>
<td>70 ± 31</td>
</tr>
<tr>
<td>TM1-2-3ser187</td>
<td>MR</td>
<td>58 ± 29</td>
<td>60 ± 21</td>
</tr>
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</table>

**a** MR or CR 1, 2, or 3 MFI measured using flow cytometry.
**b** MDMs were incubated with SP-A proteins (10 μg/ml) or LPS (15 ng/ml) for 2 h. IL-4 added for 20 h was used as the positive control. CHX or PMB (5 μg/ml) was added prior to APP-SP-A.
**c** Values shown represent the percent change in MFI = ([treatment MFI – control MFI] / control MFI) × 100. Values are corrected for nonspecific binding by subtracting out the MFI from the appropriate nonspecific control (NRS or the appropriate subtypic IgG). Mean ± SEM; n = 6 for IL-4 and APP-SP-A, n = 3 for the others.
**d** Significant relative to MR expression on untreated MDMs at p < 0.05 by Student’s t test.

### Table II. Effect of SP-A on macrophage mannose surface receptor expression

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MFI/cell&lt;sup&gt;c&lt;/sup&gt; (mean ± SEM)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA</td>
<td>13 ± 2.1</td>
<td>4</td>
</tr>
<tr>
<td>APP-SP-A</td>
<td>68 ± 2.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td>PMB/APP-SP-A</td>
<td>66 ± 2.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td>LPS</td>
<td>13 ± 7.0</td>
<td>2</td>
</tr>
</tbody>
</table>

**a** MR expression was visualized using a mAb against MR and confocal microscopy.
**b** MDMs adhered to glass coverslips in 24-well tissue culture plates were incubated with HSA, APP-SP-A (10 μg/ml), or LPS (15 ng/ml) for 1 h. PMB (5 μg/ml) was added prior to APP-SP-A to neutralize LPS (PMB/APP-SP-A).
**c** MFI was measured for 20 cells per coverslip (see Materials and Methods). Triplicate coverslips were used in each experiment. Values shown are corrected for nonspecific binding by subtracting out the MFI from cells stained with the IgG1κ subtypic control mAb.
**d** Significantly different from HSA control; p < 0.05 by Student’s t test.

**FIGURE 3.** MDMs treated with APP-SP-A demonstrate enhanced expression of the MR. One-day-old monocytes (A and B) or 5-day-old MDMs (C and D) in monolayer culture were incubated with HSA (A and C) or APP-SP-A (10 μg/ml) (B and D) for 1 h. After washing, cells were fixed in paraformaldehyde and sequentially stained with mouse anti-human MR mAb or subtypic control mAb (data not shown) followed by a secondary FITC-anti-mouse Ab. Glass coverslips were mounted and visualized by confocal microscopy. A representative experiment is presented (n = 2–6).
SP-A enhances the uptake of 125I-labeled mannose-BSA by MDMs

To assess whether the MR newly induced by SP-A was functional, we studied mannan-inhibitable pinocytosis of 125I-labeled mannose-BSA, an assay of MR recycling (39). When APP-SP-A (10 μg/ml) was added to MDMs in suspension, the uptake of mannose-BSA was increased from 0.55 ± 0.13 to 1.64 ± 0.61 ng (198 ± 35% increase, n = 4, p < 0.05) and was comparable to the increase seen with IL-4 (0.55 ± 0.13 to 1.16 ± 0.37 ng for control vs IL-4, respectively; 110% increase, n = 6, p < 0.05). The addition of APP-SP-A (10 μg/ml) to MDM monolayers also enhanced the uptake of 125I-labeled mannose-BSA compared with control cells. However, the percent increase was lower (37 ± 15% increase in mannose-BSA uptake, mean ± SEM, n = 4) than that recorded for MDMs in suspension, suggesting that the state of the cell (adherent vs nonadherent) may be a factor in dictating the magnitude of the response to SP-A.

MDMs in monolayer culture on a substrate of SP-A demonstrate enhanced phagocytosis of LAM-coated microspheres

We have determined that a major capsular lipoglycan, LAM, from S. pneumoniae serves as a ligand for the MR during phagocytosis of bacteria (34). Microspheres coated with LAM serve as model phagocytic particles for studies of MR-mediated phagocytosis (34). To determine whether SP-A enhances phagocytosis of LAM microspheres, we studied mannan-inhibitable phagocytosis of LAM microspheres and control microspheres by MDMs in monolayer culture (40) on an SP-A or HSA substrate (Fig. 4). SP-A significantly enhanced the phagocytosis of LAM microspheres in a mannan-inhibitable fashion (168 ± 63% enhancement, mean ± SEM, p < 0.05, n = 8) compared with the HSA substrate (Fig. 4).

AMs from SP-A−/− mice express less MR than AMs from SP-A+/+ mice

To determine whether SP-A up-regulates MR expression on AMs in vivo, we studied the expression of the MR on alveolar and peritoneal macrophages isolated from SP-A−/− and SP-A+/+ mice using flow cytometry. Alveolar and peritoneal macrophages from both mouse types constitutively expressed MR (Table III). MR expression on peritoneal macrophages did not differ between mouse types. In contrast, the MR expression (reflected by MFI) on AMs from SP-A−/− mice was only approximately one-half of the MR expression on AMs from SP-A+/+ mice (Table III), approaching the level of expression seen on peritoneal macrophages. Thus, this result provides evidence that macrophages exposed to SP-A in the alveolus exhibit up-regulated MR expression and is consistent with our findings using human MDMs in vitro culture.

Discussion

In the healthy host, the majority of immune cells in the lower respiratory tract at the lung/environment interface are AMs, which constitute the first line of defense against organisms, particles, or allergens that reach the distal airspaces. Macrophages are optimally positioned to clear inhaled particles and microbes by phagocytosis. To avoid compromising gas exchange, the host tightly regulates the inflammatory process accompanying AM phagocytosis. To avoid compromising gas exchange, the host tightly regulates the inflammatory process accompanying AM phagocytosis. Therefore, it is not surprising that AMs possess a unique phenotype compared with macrophages in other tissue compartments. This phenotype, which has been termed an “alternative state of activation” (6), includes a greater phagocytic capacity for both nonopsonized and opsonized particles, a reduced oxidative burst, and decreased expression of costimulatory molecules (41). As part of their distinctive repertoire, AMs exhibit enhanced expression of the receptors of innate immunity, such as the MR, which acts as a PRR recognizing an array of mannoproteins and mannosyl-coated microbes (4). AMs originate from circulating monocytes that immigrate into the pulmonary environment, where they encounter SP-A, an interaction that may contribute to the unique biological properties of AMs. SP-A is capable of a direct interaction with AMs through binding to cell surface receptor(s), resulting in modulation of macrophage functions such as enhanced chemotaxis, modified respiratory burst, and increased phagocytosis of microbes and apoptotic cells (7, 42). Here we demonstrate that the interaction between SP-A and human macrophages results in increased expression of the MR.

MR, a member of the C-type lectin super family (4), binds with high affinity to glycoconjugates containing mannose, fucose, or N-acetyl glucosamine. MR is not present on monocytes, but is constitutively present on macrophages including MDMs and dendritic cells (2, 3). AMs express particularly high levels of functional MR. In the current study, SP-A increased MDM expression of MR, as demonstrated by both flow cytometry and confocal microscopy. The newly expressed MR was functional, as determined by mannan-inhibitable pinocytosis of the ligand mannose-BSA.

Table III. MR expression on primary mouse macrophages from SP-A−/− and SP-A+/+ mice

<table>
<thead>
<tr>
<th>Mouse Type</th>
<th>Cell Type</th>
<th>% Positive</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-A−/−</td>
<td>Alveolar</td>
<td>76 ± 6</td>
<td>293 ± 51</td>
</tr>
<tr>
<td>SP-A+/−</td>
<td>Alveolar</td>
<td>62 ± 11</td>
<td>155 ± 55</td>
</tr>
<tr>
<td>SP-A−/−</td>
<td>Peritoneal</td>
<td>69 ± 19</td>
<td>204 ± 128</td>
</tr>
<tr>
<td>SP-A+/−</td>
<td>Peritoneal</td>
<td>65 ± 12</td>
<td>184 ± 46</td>
</tr>
</tbody>
</table>

*a* Resident peritoneal or alveolar macrophages were isolated from SP-A−/− (wild-type) or SPA+/− mice. Cells were incubated with rabbit anti-mouse MR Ab or NRS (nonspecific control). FITC anti-rabbit Ig Ab was used as a secondary. Fluorescence was measured by flow cytometry. The percentage of positive cells and MFI for the nonspecific control were 4 ± 3 and 27 ± 9, respectively.

*b* Significantly different from wild-type mouse alveolar macrophages, p < 0.05 by Student’s t test, n = 4.

FIGURE 4. MDMs on a substrate of APP-SP-A demonstrate enhanced phagocytosis of Erdman LAM microspheres. MDMs were plated on a substrate of either HSA or APP-SP-A. After washing, monolayers were incubated with either HSA (control) or Erdman LAM microspheres (2.0 × 10⁵) in the presence or absence (control) of soluble mannan. After washing, MDMs were fixed in paraformaldehyde, and microscopic uptake was enumerated by phase microscopy. Data are the mean ± SD of triplicate cover slips per test group. Within a given substrate, an asterisk indicates values significantly different from HSA microspheres, p < 0.05 by Student’s t test. Representative experiment of n = 8.
and phagocytosis of *M. tb* LAM-coated particles after SP-A treatment. It is unlikely that the increased uptake of ligands was due to an SP-A-induced change in affinity of the receptor for the ligand because there are no reports of affinity modification of MR as described for CR3 (43).

The effect of SP-A on MR expression was selective for cell type and receptor type. In contrast to its effect on macrophages, short-term (60-min) incubation with SP-A did not induce MR expression on monocytes. MR expression becomes apparent upon monocyte maturation over 3–4 days in culture (3). Thus, expression is differentiation dependent. The mechanisms for regulation of MR expression have not been clearly elucidated, but potentially include transcriptional regulation via the PU.1 site in the MR gene promoter (44–46). The effect of SP-A is receptor specific under the conditions of our assays. SP-A did not affect expression of CRs on macrophages in our present study or FcγR function in a previous study (12). In contrast with our results, Tenner et al. (18) reported that SP-A increased phagocytosis of both E-IgG by FcR on monocytes and complement-coated erythrocytes by CR1 on macrophages. SP-A up-regulation of cell surface expression of FcγR or CR1 was not addressed in their study (18). Additionally, Kremlev et al. (19) reported that SP-A treatment of the human monocyte cell line THP-1 significantly increased the levels of expression of CD14, ICAM-1, and CD11b as measured by flow cytometry. Possible explanations for these apparent discordant data include the assay conditions such as the ligand density on the phagocytic particles or, alternatively, the cell types used. Another difference between the current study and that by Kremlev et al. (19) is the method used to purify SP-A. Kremlev et al. (19) used an isoelectric focusing method after solubilization of APP-SP-A.

MR is synthesized as a 154-kDa precursor, which is processed to its mature form of 162 kDa in ~90 min. (47, 48). Permeabilization studies indicate that in differentiated macrophages, 80% of the MR is localized intracellularly in vesicles (49, 50). The intracellular pool may include newly synthesized receptor en route to the endosomal apparatus and receptors moving from one pool to another. Newly synthesized MR has a half-life of 33 h as determined by pulse-chase studies. This indicates that on the average each molecule recycles between the cell surface and endosomes hundreds of time before degradation (47).

Viewed as a whole, our data favor the idea that short-term incubation of SP-A increases trafficking of preformed MR to the cell surface. First, increased surface expression of MR in response to SP-A can be seen as early as 1 h. Second, uptake of 125I-labeled mannose-BSA at 37°C, a temperature at which receptor recycling occurs, increased in the presence of SP-A. Third, the addition of CHX before SP-A did not significantly reduce the up-regulation of MR, indicating that new protein synthesis was not necessary for the SP-A induction of MR. Phagocytosis of LAM microspheres is rapid and MR specific. Both surface-expressed MR and preformed intracellular MRs that are recycled to the surface are involved in phagocytosis of LAM microspheres. Thus, the fact that CHX had only a small effect on both MR expression and the phagocytosis of microspheres (40) supports the notion that SP-A primarily affects recycling of preformed MR in macrophages rather than induction of newly synthesized MR. They do not, however, rule out that SP-A could also affect transcription and/or posttranscriptional modification of the MR during longer periods of incubation. MR expression and function is highly regulated (4).

The magnitude of the SP-A-induced MR expression was dependent on the type of SP-A used: APP-SP-A consistently induced a larger increase in expression than did native human or native rat SP-A and a significantly larger increase in expression was induced than did recombinant rat SP-A. This is consistent with our previous finding that APP-SP-A was more effective than native human or recombinant rat SP-A in enhancing phagocytosis of *M. tb* by MDMs (12). These differences may reflect the degree of oligomerization and/or self-association of SP-A, or the preferential interactions of human cells with human SP-A species. Thus, both the form of SP-A and the nature of the host cell appear to influence the magnitude of MR response to SP-A.

The present study, combined with our previous work (12), shows that the magnitude of MR expression is also dependent on whether SP-A is presented in solution or on a matrix. We speculate that macrophages will encounter SP-A in different contexts within the alveoli, i.e., immigrating as a monocyte from the microvasculature into the alveoli and as a resident macrophage in the alveolus. These functional differences may give clues as to how blood monocytes uniquely differentiate to the AM.

Our data using macrophages from SPA−/− mice support our hypothesis that continuous interactions between alveolar surface lining constituents, particularly SP-A, and AMs affect the phenotype and function of these cells. AMs from the SP-A−/− mice express less MR than do those from control mice, and they phenotypically resemble peritoneal macrophages. In contrast, no differences in MR expression were found in peritoneal macrophages between SP-A−/− and SP-A+/+ mice. The observed differences in MR expression can be attributed to differences in SP-A levels because surfactant isolated from SP-A-deficient mice appears to function normally, and lungs of these mice appear normal histologically and ultrastructurally, except for the absence of tubular myelin (30).

The primary structure of an SP-A subunit is composed of several discrete domains including the following: 1) a short N-terminal segment, 2) a collagen-like sequence of Gly-X-Y repeats, where X is any amino acid and Y is often proline or hydroxyproline, 3) a hydrophobic neck domain, and 4) a CRD. This monomer trimersizes by the folding of the collagen-like domains into triple helices (8, 51). Six SP-A trimers form an octadecamer through covalent and noncovalent interactions between the N-terminal segment and the first half of the collagen-like domain (8, 51). Although the structures of APP and native and recombinant SP-A are very similar, subtle differences have been noted that affect the level of protein multimerization. Differences in multimerization may potentially affect receptor binding, signaling, and consequently up-regulation of MR expression. APP-SP-A octadecamers can self-associate to form multimolecular complexes not found in SP-A obtained from healthy volunteers (10, 11). In contrast, recombinant SP-A synthesized in invertebrate cells is deficient in hydroxyproline and does not oligomerize to the same extent as native SP-A. Mutants lacking the collagen region can only form trimers and hexamers, whereas mutants lacking both the N-terminal and collagen regions can form only trimers (8). Neither of these variants was capable of stimulating an optimal increase in MR expression, but the experiments do not discriminate between the direct effects of deletional mutations on SP-A/SP-A receptor interactions (8, 52, 53) and indirect effects due to altered oligomerization (52, 53).

Our studies demonstrate that both the sugars and collagen-like domain of the protein play a role in SP-A induction of MR. SP-A contains two sugars at Asn187 in the N terminus region and Asn in the CRD region (8). The carbohydrate moieties of SP-A have been reported to be important for binding to macrophages (12) and herpes simplex virus type 1-infected cells (54), but not to type II epithelial cells (8). Our results indicate that the carbohydrates play a role in SP-A up-regulation of MR expression (12). Although it is true that the insect expression system modifies proteins in a simple, mannose-rich manner, which is different from native SP-A (23), this knowledge does not alter our interpretation of the data. TM2,
which contains high mannose oligosaccharides, remains inactive. Collectively, our data argue most strongly for the three-dimensional oligomeric context of the oligosaccharides being important for full SP-A function.

The precise domains of SP-A involved in receptor binding are not well characterized. This may be due to the existence of more than one SP-A receptor, that a receptor complex is involved in binding, and/or that receptor expression varies with cell type (55). Thus, different cell types may vary in their host cell response to SP-A. Several proteins have been identified as putative receptors for SP-A, some of which are present on macrophages (24, 38, 56–58). Our studies suggest that SP-A binds to its receptor(s) on macrophages and induces the activation of a signal transduction pathway(s) leading to increased MR trafficking to the plasma membrane. The signal transduction pathways involved in this process are the focus of our current studies.

In conclusion, SP-A increases the surface expression of functional MR on macrophages. Up-regulation of the MR by SP-A provides a mechanism for enhanced phagocytosis of invading infectious organisms. Our data indicate that the majority of newly expressed MR comes from the preformed intracellular receptor pool and is induced to traffic to the cell surface by SP-A. The source of SP-A influences the magnitude of MR expressed, and an intact glycoprotein is necessary for optimal SP-A induction of MR expression. In most scenarios, up-regulation of MR expression on a professional phagocyte such as a macrophage would be beneficial for the host by initiating phagocytosis and killing of nonopsonized microbes, thereby participating in first-line host defense. However, intracellular pathogens such as \( M. tb \) may use increased levels of MR to reach their intracellular niche, the AM. The current study supports the idea that the alveolar lung constituent, SP-A, contributes to the outcome of lung invasion by inhaled particles and bacteria by modifying the biological properties of AM.

Acknowledgments

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References


