By Binding SIRPα or Calreticulin/CD91, Lung Collectins Act as Dual Function Surveillance Molecules to Suppress or Enhance Inflammation

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Summary

Surfactant proteins A and D (SP-A and SP-D) are lung collectins composed of two regions, a globular head domain that binds PAMPs and a collagenous tail domain that initiates phagocytosis. We provide evidence that SP-A and SP-D act in a dual manner, to enhance or suppress inflammatory mediator production depending on binding orientation. SP-A and SP-D bind SIRPα through their globular heads to initiate a signaling pathway that blocks proinflammatory mediator production. In contrast, their collagenous tails stimulate proinflammatory mediator production through binding to calreticulin/CD91. Together a model is implied in which SP-A and SP-D help maintain a non/ anti-inflammatory lung environment by stimulating SIRPα on resident cells through their globular heads. However, interaction of these heads with PAMPs on foreign organisms or damaged cells and presentation of the collagenous tails in an aggregated state to calreticulin/CD91, stimulates phagocytosis and proinflammatory responses.

Introduction

The collectin family of molecules are generally thought to function as components of the innate immune system, acting to protect the host by recognizing pathogen associated molecular patterns (PAMPs) on microorganisms. They have a unique structure with globular heads expressing the recognition domains and conserved collagen-like tails connected through a hinge region. Collectin monomers are highly oligomerized, first into trimers and then further to form complex sertiform or cruciate structures. The group includes mannose binding lectin (MBL), conglutinin, the surfactant proteins SP-A and SP-D, and due to structural similarity, C1q.

By binding to microorganisms through their globular heads, the collectin family can enhance or initiate phagocytosis and inflammatory effects. Subsequent interaction of receptors on phagocytic cells with the collagen-like tails is suggested and a number of potential receptors have been considered (Stuart et al., 1997; Tenner, 1998). However, a C1q (for collagenous tail C1q receptor) described first by Ghebrehiwet et al. (1994) and later identified as cell surface calreticulin (Stuart et al., 1997) seems to be a prime candidate. The collagenous tails of the collectins bind calreticulin (Coppolino and Strayer, 2002). Recently, both CD14 and TLR2 have been shown to bind SP-A or SP-D and due to structural similarity, C1q.

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general suppression of proinflammatory mediator production.

Putting these together leads to an overall model in which SP-A and SP-D help maintain the non- or anti-inflammatory environment in the lung due to their stimulation of the ITIM-containing SIRPα on resident alveolar cells through their globular head, C-lectin domains. However, when these same domains interact with PAMPs on foreign organisms, apoptotic cells, or cell debris, presentation of the collagenous tails in an aggregated state to calreticulin/CD91 on the alveolar cells now can initiate ingestion, along with proinflammatory and proimmunogenic responses.

Results

Surfactant Proteins A and D Inhibit Macrophage Cytokine Production

The potential for lung collectins to inhibit production of proinflammatory cytokines was confirmed initially in vitro with alveolar macrophages lavaged from normal human donors. Pretreatment of the cells with SP-A or SP-D before 18 hr stimulation with LPS led to reduced production of cytokines and chemokines (Figure 1A). A similar effect was seen on the mouse macrophage cell line, RAW-264 (Figure 1B). Importantly, the nonlung collectins, C1q and MBL were unable to inhibit cytokine production, and in fact, enhanced levels above those produced by LPS alone.

Surfactant proteins bind to rough LPS (Greene et al., 1998) and also interact with CD14 (Sano et al., 2000). In the experiments reported here, a smooth (noncollectin binding) LPS was employed. However, to confirm suppression of stimuli other than LPS the enhanced production of cytokines in response to H2O2 was also blocked by SP-A and SP-D, but not C1q (Figure 1C). In the lung, the surfactant proteins are generally found bound to lipids of the pulmonary surfactant through their globular heads (Ogasawara et al., 1994; Sano et al., 1998). However, SP-A and SP-D still suppressed proinflammatory mediator production in the presence of surfactant phospholipids containing hydrophobic surfactant components (SP-B, SP-C) (data not shown).

SP-A and SP-D Inhibit P38 Activation

Blockade of P38 Map kinase prevents production of inflammatory mediators (Carter and Hunninghake, 2000) and the P38 inhibitor SB-203580 showed a similar pattern of inhibition as the surfactant collectins (Figure 1B). SP-A or SP-D blocked LPS stimulation of P38 phosphorylation in human alveolar macrophages (Figure 2A) and LPS or H2O2-induced P38 phosphorylation in RAW-264 cells (Figures 2B and 2C). In contrast, C1q and MBL induced a modest increase in P38 phosphorylation (Fig-
SP-A or SP-D, but Not C1q, Activates SHP-1 and SIRPα

The preceding experiments indicated that lung collectin suppression is likely an early upstream event involving decreased phosphorylation of src family kinases such as Hck, either from reduced activation and/or increased effects of a tyrosine phosphatase. As shown in Figures 3A and 3B, SP-A and SP-D (but not C1q) were found to increase both phosphorylation and phosphatase activity of SHP-1.

Activation of SHP-1 suggested that the collectins were binding to a surface receptor or complex containing an ITIM domain. SIRPα is a heavily glycosylated, ITIM-containing, membrane receptor present on the surface of a number of cell types (Veillette et al., 1998). Thus, addition of SP-A or SP-D, but not C1q, was found to increased tyrosine phosphorylation of SIRPα (Figure 3C). The lung collectins also initiated rapid association of SHP-1 with SIRPα as shown by coimmunoprecipitation (Figure 3D).

**SP-A and SP-D Bind Directly to SIRPα**

To demonstrate interaction of the collectins with SIRPα, SP-A, SP-D were shown to prevent binding of an anti-SIRPα antibody to RAW-264 cells (Figures 4A and 4B). Binding of a control antibody, F480, was not affected (data not shown). In the other direction, CD47 (as an Fc-Fc fusion product), a known ligand for SIRPα was unaffected and human Fc fragments did not alter SP-A or C1q binding (data not shown).

Furthermore, anti-SIRPα and FITC-labeled SP-A (but not C1q) colocalized on the cell surface (Figure 4D). COS cells transfected with a SIRPα construct and which expressed this protein on their surface, (data not shown) were found to bind FITC-labeled SP-A and SP-D (Figure 4E) but not C1q. Finally, RAW macrophages pretreated with a blocking anti-SIRPα antibody prior to the addition of the surfactant proteins showed restoration of LPS induced cytokine production (Figure 4F).
SP-A and SP-D Inhibition Is Based on Globular Head Binding to SIRPα

The data in Figure 1 demonstrated that SP-A and SP-D inhibited P38 activation and proinflammatory mediator production whereas C1q and MBL enhanced. All four collectins share similar collagenous tails but differ in their globular heads. To confirm that the anti-inflammatory activities of SP-A and SP-D were mediated by their globular head regions, a variety of SP-A and SP-D mutants were examined. Collagen domain deletion mutants (CDM mutants) exhibit a functional head domain but no functional tails (Ogasawara and Voelker, 1995). Both the SP-A and SP-D CDM proteins were able to suppress cytokine production and P38 phosphorylation (Figures 5A and 5B). In addition, FITC-labeled SP-A CDM (but not SP-A tails) bound to COS cells transfected with SIRPα (Figure 5C).

Stimulation of Macrophage Inflammatory Responses by the Collagenous Tail Domains of the Pulmonary Collectins and Calreticulin

In contrast to the effect of SIRPα ligation by SP-A or SP-D globular heads, collectin tail domains may actively enhance or stimulate proinflammatory mediator production (Figures 6A–6C). Thus, the two collectins that lack SIRPα binding and activating properties, C1q and MBL, each enhance P38 activation, NF-κB reporter activity, and production of mediators. Importantly, isolated SP-A collagenous tails or mutants lacking functional head groups (SP-A switch) did the same, enhancing the effects of LPS stimulation but also directly initiating the production of cytokines, P38 phosphorylation, and activation of NF-κB (Figures 6A, 6B, and 6C). Similar effects were seen on cells from C3H-HeJ mice lacking functional TLR-4 and LPS responses (data not shown). To support a role for surface calreticulin in the proinflammatory action of the collectins, an antibody to calreticulin was shown to block induction of inflammatory mediator production by SP-A tails (Figure 6D) or C1q (data not shown).

Lung Collectins Exhibit a Dual Inflammatory Role In Vivo

The effect of surfactant proteins was examined in an inflammatory model in the murine peritoneum, a site lacking endogenous and potentially interfering SP-A and SP-D. Mice injected intraperitoneally with SP-A one hour prior to the initiation of inflammation by LPS showed suppression of proinflammatory mediator production at this site (Figure 7A). As predicted from the model, C1q, which lacks the ability to bind and activate SIRPα, enhanced cytokine production, presumably by acting on calreticulin/CD91 through its collagenous tails. To determine the effect of collectin orientation in the presence of a natural ligand, SP-A or C1q were added to RAW macrophages simultaneously with necrotic cell debris, which express ligands for the globular heads. This would be expected to direct the collagenous tails toward the responding macrophage or tissue cell. Necrotic cell debris did not significantly enhance cytokine production alone, however, in the presence of either SP-A or C1q, proinflammatory mediator production was enhanced (Figure 7B). This suggests that a natural ligand for the globular heads not only promotes engulfment (data not shown), but also the production of proinflammatory mediators. To confirm that this effect was mediated through CD91, cell debris plus collectins was shown unable to elicit a proinflammatory response in CD91-deficient mouse embryonic fibroblasts (MEF) while a proinflammatory response occurred in wild-type MEFs (data not shown).

To examine the effect of orientation in vivo, SP-A or C1q were added to the cell debris (to allow globular head binding and collagenous tail presentation) before injection into the peritoneum. For both molecules, inflammatory mediator generation was enhanced (Figure 7C). To investigate the potential hierarchy between inhibitory or enhancing signals, macrophages were pretreated with SP-A for 30 min prior to addition of necrotic cell debris preincubated with C1q. As predicted, pretreatment of the macrophages with SP-A decreased the
ability of C1q coated debris to enhance cytokine production (Figure 7D).

Discussion

The lung collectins, SP-A and SP-D, like their counterparts MBL and C1q, act as innate immune system molecules that recognize foreign organisms, cell debris, apoptotic cells, and the like to mediate their removal by phagocytosis. As with other innate immune system molecules they may also initiate inflammatory responses linked to host defense and induction of adaptive immunity. It is suggested that they act by recognizing PAMPs through binding sites on their globular heads and by stimulating phagocytic cells via binding of their collagenous tails to calreticulin on the cell surface. This mediates intracellular signaling through CD91 (LRP). In complete contrast, SP-A and SP-D (but, not MBL or C1q) also serve as anti-inflammatory stimuli that, we suggest, help maintain normal tissue homeostasis at sites such as the lung that are constantly exposed to inflammatory stimuli. SP-A appears to be localized only to the lungs, but SP-D is also found at other mucosal surfaces where it may serve a similar protective function. Anti-inflammatory effects appear to be mediated by binding of SP-A and SP-D head groups to the ITIM-containing molecule, SIRPα, leading to suppression of mediator production. These proposed dual effects are modeled in Figure 8. They lead to the suggestion that the lung collectins by themselves act as dual function surveillance molecules, serving normally to maintain a noninflammatory environment, but, upon recognition and binding to foreign materials, reversing both their orientation and their effects to initiate host defense reactions, inflammation, and enhancement of adaptive immunity.

The Host Defense and Proinflammatory Effects of SP-A and SP-D

The effects include binding foreign material, opsonization, enhanced clearance, and inflammation in response to potentially injurious stimuli. In addition, we have suggested that the collectins have been suborned, not only for recognizing foreign or “danger” signals, but also unwanted and damaged cells within the host itself (Ogden et al., 2001; Schagat et al., 2001; Vandivier et al., 2002). Data supporting the importance of SP-A in host defense comes from studies of SP-A deficient mice which exhibit increased susceptibility to airway challenge with group B Streptococci, Pseudomonas aeruginosa, or Staphylococcus aureus. However, despite the decreased resistance, the mice also developed more severe pulmonary inflammation which was corrected by instillation of purified SP-A (LeVine et al., 1998; LeVine et al., 1999). SP-D is also reported to act in pulmonary host defense based on its binding to a variety of microorganisms (Kuan et al., 1992; Restrepo et al., 1999) and rough, but not smooth forms of LPS (Greene et al., 1998). Here again, however, SP-D−/− mice exhibit increased pulmonary inflammatory responses (LeVine et al., 2000).

The emerging theme from these studies is that the lung collectins recognize a significant number of microorganisms and play a role in their clearance. On the other hand, they also appear to be important in regulating pulmonary inflammatory responses. The degree of inflammation has consistently been related to the levels of SP-A or SP-D in knockout or overexpressing mice with decreased levels associated with increased inflammation and vice versa. It is suggested that the dual function model outlined in Figure 8 explains these apparent dichotomous effects.

Similar conflicting data have been reported in studies of SP-A on macrophages in vitro suggesting either an increase (Blau et al., 1994; Kremlev and Phelps, 1994; Ofek et al., 2001; Widmann et al., 1999) or decrease (Arias-Diaz et al., 2000; Greene et al., 1996; Harrod et al., 1999; Rosseau et al., 1999; Sano et al., 1999) of inflammatory mediator production. Complete explanations for these varied effects are likely complex and may reflect differences in the cells examined, the stimuli used and the preparations of SP-A employed. However, the results are also consistent with the dual effect outlined in Figure 8. Binding to macrophages by the C-lectin domains is suggested to block stimulatory signaling pathways leading to NF-κB- (and AP-1) mediated transcription and/or subsequent translation of the proinflammatory mediators. On the other hand, interaction of the collagenous tail regions of the collectins with calreticulin/CD91 is suggested to induce or enhance production of proinflammatory mediators. This dual effect is hypothesized to represent a unique set of regulatory and discriminatory functions for collectins in the lung (or, for that matter in other mucosal sites known to express SP-D) leading to both protection from inflammation by casual environmental stimuli and the high oxygen tension at this site but also, when invaded or injured, to the opposite effect, namely initiation of protective inflammatory responses.

In this model, binding of SP-A or SP-D to a bacterium, virus, yeast, or damaged cell through recognition by the globular heads would present the collagen tails to the macrophage or other responding cells, likely in an aggregated form optimal for stimulation of this proinflammatory function. The concept would, for example explain the observations of Rosseau et al. (1999) that soluble SP-A was inhibitory to Candida-induced cytokine production, even when preincubated with the macrophages, whereas presentation of the SP-A on the Candida or by precoating the plate on which the macrophages were cultured, was not. These latter two circumstances, we would suggest, led to collagen tail stimulation of membrane calreticulin and CD91. In like manner, Sano et al. (Sano et al., 1999) demonstrated inhibition of TNFα production by SP-A if the macrophages were presented with smooth LPS (with which the SP-A C-lectin domains do not interact) but mild stimulation of this cytokine if SP-A binding, rough forms of LPS were used.

C1q has previously been reported to stimulate production of IL-8, IL-6 and MCP-1 (van den Berg et al., 1999). This work is confirmed herein as C1q (and MBL) not only enhanced an LPS effect, but also induced the mediators on their own or when bound to cellular debris. C1q and MBL do not exhibit anti-inflammatory functions through their globular head groups (do not bind SIRPα) and are proposed to mediate the proinflammatory effect through their collagenous tails. SP-A tails, or SP-A and SP-D constructs lacking head group function also enhanced
Figure 4. SP-A and SP-D Bind Directly to SIRPα

SP-A and SP-D block anti-SIRPα binding to the cell surface. Cells were pretreated with the lung collectins or C1q prior to staining with an anti-SIRPα antibody (A) flow cytometry or (B) immunofluorescence.

(C) CD47 blocks SP-A binding to macrophages. Macrophages were pretreated with CD47 for 20 min prior to addition of FITC labeled SP-A or C1q and analysis by flow cytometry.

(D) SP-A colocalizes with SIRPα on the cell surface. RAW-264 cells were examined after costaining with FITC-labeled SP-A or C1q and anti-SIRPα antibody.

(E) COS cells were transiently transfected with SIRPα or vector control for 24 hr and binding of FITC-labeled SP-A, SP-D, or C1q to the cells detected by flow cytometry.

(F) Pretreatment of RAW cells with anti-SIRPα reverses the inhibitory effect of SP-A. Cytokines were measured after overnight culture from cells pretreated with anti-SIRPα or an isotype control followed by a 30 min incubation with SP-A and then stimulation with LPS.

and directly induced, production of proinflammatory mediators as well as activation of P38 Map kinase and induction of NFκB and AP-1 reporter constructs. Initial interaction of SP-A with cell debris to orient the molecule for collagenous tail presentation before injection into the peritoneum led to a proinflammatory response.

Collectins can bind via their collagenous tails to cell surface calreticulin. While it is not yet clear how this endoplasmic reticulum, calcium binding, and chaperone gains access to this site, most cell types examined do express calreticulin on their surface (Arosa et al., 1999; Goicoechea et al., 2000). Recent studies have suggested that calreticulin interacts with, and signals through, CD91 (Basu et al., 2001; Orr et al., 2002). Data presented herein support this route for collagenous tail stimulation. While not directly investigated, we suspect that significantly aggregating the collectin tails is required for optimal stimulation of the calreticulin/CD91 system (Ogden et al., 2001; van den Berg et al., 1998) suggesting either low affinity interactions and/or multimerization as a mode of activation of this receptor complex.

In addition to the direct stimulation of proinflammatory mediator through calreticulin and CD91, interaction of collagenous tails with cell surface receptors might also be expected to bring organisms, cells, or molecules captured by the globular head groups into close proximity to the cell. Thus, capture of LPS by SP-A or SP-D could enhance interaction of the LPS with Toll-like receptors on a target cell to which the complex had bound through its tail, in addition to any stimulatory effect the tail binding may have by itself.

The Anti-Inflammatory Effects of SP-A and SP-D

In the normal environment of the resting lung, we suggest that SP-A and SP-D engage the ITIM-containing inhibitory receptor SIRPα expressed on mononuclear phagocytes but also by pulmonary epithelial cells (data not shown). This would maintain a general suppression of mild activation by casual stimuli such as inhaled particles, LPS, lysed cells, or the stray effects of oxidants resulting from the high oxygen tension. An alternative role for lung collectins in protection against oxidant effects may involve direct effects on lipid peroxidation (Bridges et al., 2000). Oxidants initiate inflammatory reactions and many defense mechanisms have been described, particularly those that remove or divert the oxidants themselves. Here we are suggesting two different modes of antioxidant effect via SIRPα blockade of both the active generation of toxic oxidants and suppression of the proinflammatory consequences of oxidant action. These mechanisms would add to the enormously redundant protective processes required for survival in our
highly toxic, oxygen-rich environment. Effects of the lung collectins to bind and divert LPS or to block its interaction with CD14 or TLRs (Sano et al., 2000) would serve only to enhance the overall anti-inflammatory effect.

SP-A and SP-D, but not C1q or MBL, inhibited the production of representative proinflammatory cytokines and chemokines from LPS-stimulated cells both in vitro and in vivo. Importantly, suppression was not seen with lung collectin constructs lacking globular head function but was unaltered in the absence of collagenous tails. Inhibition also extended to a variety of proinflammatory stimuli including H2O2 or cell debris.

The inhibitory effect was seen on NFκB and AP-1 reporter constructs and was very similar in profile and extent to the blockade seen with P38 Map kinase inhibitors. The lung collectins inhibited P38 activation as well as known upstream candidate molecules Vav and the src-family kinase member Hck. Hck is of interest, as a recent report indicates that its overexpression leads to enhanced pulmonary inflammation (Ernst et al., 2002).

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did not appear involved as determined with CD45 null mice. However, SIRPα (also known as SHPS-1) which activates the tyrosine phosphatase, SHP-1 was implicated. Data are presented to show SIRPα phosphorylation and SHP-1 activation and phosphorylation during stimulation with SP-A or SP-D. Once again, the control collectin family members, C1q and MBL were inactive. It is noteworthy that SHP-1 deficient mice also develop spontaneous pulmonary inflammation (Ward, 1978).

SIRPα is a heavily glycosylated, ITIM-containing, transmembrane protein. Ligation of its extracellular domain by natural ligands such as CD47 or by crosslinking antibody leads to tyrosine phosphorylation by unknown kinases (Kharitonenkov et al., 1997). Recruitment, phosphorylation (by receptor tyrosine kinases), and activation of SHP-1 follows. In the studies reported here, binding of anti-SIRPα antibodies to macrophages was reduced in the presence of SP-A or SP-D. Transfection of SIRPα into COS cells rendered them able to bind the lung collectins. Data were also presented showing colocalization of the surfactant proteins with SIRPα. We do not know the domains on SIRPα recognized by the globular heads of SP-A and SP-D, nor whether these are the same or different for each collectin. However, SIRPα’s glycosyl groups are reasonable candidates.

A potential mechanism is proposed in which lung collectins bind by their globular heads to SIRPα under normal conditions. SIRPα activation would suppress the production of proinflammatory mediators potentially triggered by daily insults to the lungs thereby main-
taining an immunologically quiet and noninflamed environment. However, when these same head domains interact with PAMPs on foreign organisms, apoptotic cells, or cell debris, presentation of the collagenous tails in an aggregated state to calreticulin/CD91 on the cells can initiate phagocytosis and/or proinflammatory and proimmunogenic responses. In addition, the collectins would also aid in the resolution of inflammation by contributing to the clearance of apoptotic cells. Thus, a unique dual inflammatory role for these important components of the innate immune system is proposed.

Experimental Procedures

Antibodies and Reagents
Antiphosphotyrosine 4G10, anti-SIRPα, and anti-VAV were from Upstate (Lake Placid, NY) and anti-SHP-1 from Santa Cruz (San Diego, CA). Anti-CRT antibody was from Affinity BioReagents (Golden, CO), antiphospho-P38 and phospho-MekK2 from Cell Signaling Technology (Beverly, MA) and anti-SIRPα. P84 was a gift from Dr. A. Ullrich, Max-Planck-Institut fur Biochemie, Martinsried, Germany. SP203580 was obtained from Calbiochem (San Diego, CA) and smooth LPS (E. coli 0111:B4) from ListBiological Laboratories (Campbell, CA). Mice used were 6-week-old female C57Bl/6k from Harland-Sprague-Dawley (Frederick, MD).

Cell Culture
Murine RAW 264.7 and J774 macrophages (ATCC) were cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 mg/ml streptomycin, and 100 U/ml penicillin under a humidified 5% CO2 atmosphere at 37°C. Alveolar macrophages were isolated by bronchoalveolar lavage from normal human volunteers and cultured as described previously (Vandivier et al., 2002).

Isolation and Purification of Collectins
SP-A was isolated from whole lung lavage fluid taken from patients with pulmonary alveolar proteinosis, as previously described (Allen et al., 2001). SP-D was expressed in CHO cells and stripped of LPS after purification as described previously (Vandivier et al., 2002). MBL was purified from acute-phase human plasma as reported previously (Ogden et al., 2001). Purified human C1q came from Quidel Corp (San Diego, CA). SP-A collagenous tails were purified as described previously (Ogden et al., 2001; Reid, 1976). Briefly, SP-A was dialyzed overnight against 0.15 M NaCl, 0.1 M Na acetate, [pH 4.5], and incubated for 4 hr at 37°C with 0.3 mg/ml pepsin. After centrifugation for 10 min at 10,000 g, the supernatant was fractionated on an ACA34 column and tails eluted with Tris-buffered saline with 5 mM Ca2+.

Stimulation of Cytokines
Macrophages were cultured at 1 x 10^6 cell/ml overnight and then pretreated with 10 μg of the collectins or 25 μM SB203580 for 20 min prior to 18 hr LPS stimulation. Supernatants were centrifuged to remove cellular debris and stored at −70°C until analyzed by ELISA. Alternative stimuli included 500 μM H2O2, or necrotic cell debris. This latter comprised broken cell membranes and cytosol in the 15,000 x g supernatant of five cycles of freeze-thaw treatment of Jurkat T cells. Mice were injected intraperitoneally with 50 μg of SP-A or C1q or 1 ml of sterile PBS and 1 hr later with 50 μg of LPS, 200 μg of cell debris, or sterile PBS as a control. The peritoneal cavity was lavaged after 90 min. Cytokines (TGF-β1, TNFα, MCP-1, and either MIP-2 for mouse or IL-8 for human cells) were measured by ELISA using antibodies from R&D Systems (Minneapolis, MN) as described (Fadok et al., 2001).

Immunoblotting Analysis
Immunoblotting was carried out as described previously with minor modifications (Xiao et al., 2002). RAW 264.7 cells (1 x 10^6 cells/ml) were cultured overnight, preincubated with collectins, and stimulated with LPS for 10 min. Cells were lysed in lysis buffer (20 mM HEPES, 1 mM NaCl, 1 mM DTT, 0.5% Triton X-100, and 1 x Protease Inhibitor Cocktail Set I (Calbiochem, San Diego CA)), resolved on 10% SDS-PAGE, and blotted to a nitrocellulose membrane. The membranes were probed with a phospespecific antibody to P38, or Mkk6. To confirm equal loading, the membranes were stripped and reprobed for P38, or Mkk6 protein.

Transient Cell Transfection and Reporter Gene Assays
pNF-kB-Luc (kb4, 6x, Clontech, Palo Alto CA) and pAP-1-Luc (AP-1, 7x, Stratagene, La Jolla CA) luciferase reporter gene constructs were kindly provided by Dr. Annemie Van Linden, Denver, Colorado, and the SIRPα constructs were provided by Dr. A. Ullrich, Max-Planck-Institut fur Biochemie, Martinsried, Germany. RAW 264.7 cells (3.0 x 10^6/ml) were cultured overnight and transfected with Lipofectamine Plus according to the manufacturer’s instructions. The cells were pretreated with SP-A, SP-D, or C1q as above and luciferase activity, normalized to β-gal, was measured 4 hr after LPS stimulation.

Inhibition of VAV Phosphorylation
RAW 264 macrophages were pretreated as above for 20 min prior to stimulation with 100 ng/ml of LPS. After 5, 10, and 20 min, samples were lysed in lysis buffer (20 mM Tris, 140 mM NaCl, 2.6 mM CaCl2, 1 mM MgCl2, 1% NP-40, and 10% glycerol) containing protease inhibitors and 10 μg/ml Na3PO4. Samples were immunoprecipitated for 2 hr and lysates run on a 10% SDS-PAGE gel. Immunoblots were probed with antiphosphotyrosine 4G10 antibody and equal loading confirmed by stripping and reprobing with an anti-Vav antibody.

SHP-1 Activation Assay
RAW 264 macrophages were incubated overnight, SP-A or SP-D (10 μg/ml) added for various times and samples lysed. Supernatants were collected and immunoprecipitated with 1 μg of anti-SHP-1 or an isotype control. Samples were resuspended in 50 μl of assay buffer (Upstate Phosphatase assay kit) and 25 μl aliquots examined on a 10% SDS-PAGE gel. Immunoblots were probed with antiphosphotyrosine 4G10 antibody and equal loading confirmed by stripping and reprobing with an anti-Vav antibody.

SIRPα and SHP-1 Immunoprecipitation and Western Blots
Cells were incubated for 10 min with the indicated stimuli then lysed in 500 μl of lysis buffer. Lysates were immunoprecipitated with 1 μg anti-SIRPα or anti-SHP-1 antibody and transferred, and probed with antiphosphotyrosine 4G10 overnight. Immunoblots were stripped and reprobed with either anti-SIRPα or anti-SHP-1 to confirm equal protein levels.

SIRPα and Collectin Localization
RAW 264 macrophages were stained on ice with 2 μg of anti-SIRPα P84 antibody in PBS ± 1% HSA and 2% serum for 45 min. Samples were washed twice prior to addition of a Cy3 anti-rat antibody (Jackson, West Grove PA). Staining was analyzed by flow cytometry or fluorescence microscopy. For colocalization studies, FITC-labeled SP-A or C1q or 1 ml of sterile PBS and 1 hr later with 50 μg/ml Hoechst #33258 (Calbiochem). Images were collected with Slidebook on a Leica DMRXA microscope at 62× magnification.

Data are presented as mean ± SEM of at least three independent experiments and the gels shown are representative of three or four separate experiments.

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