Induction of TNF-α release from human buffy coat cells by *Pseudomonas aeruginosa* is reduced by lung surfactant protein A

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Abstract Lipopolysaccharide (LPS) induction of TNF-α release is a central event in the pathophysiology of Gram-negative bacterial septicaemia. Lung surfactant protein A (SP-A) mediates pathogen/host cell interactions. Binding of SP-A to *Pseudomonas aeruginosa* LPS and the effects of SP-A with LPS or whole bacteria on buffy coat cells were investigated. SP-A interacts with *P. aeruginosa* LPS in a concentration and calcium dependent manner, either through the lipid A portion of LPS or through another lectin/carbohydrate interaction. SP-A decreased TNF-α secretion induced by bacteria or LPS from buffy coat cells, in a concentration dependent manner.

Key words: Lung surfactant protein A; Collectin; Innate immunity; Tumour necrosis factor-α; Lipopolysaccharide; *Pseudomonas aeruginosa* LPS

1. Introduction

Pseudomonas aeruginosa is an opportunistic lung pathogen which adheres to cilia of damaged or exfoliated human respiratory cells [1]. In cystic fibrosis, staphylococcal airway injury permits colonisation by *P. aeruginosa*. Patients are at first colonised with non-mucoid strains, but later mucoid variants emerge. The extracellular alginate they produce increases their adherence to ciliated epithelium [2]. Despite the high antibody levels present against such strains and intensive antibiotic therapy, they cannot always be completely eradicated. Alginate also interferes with antibody coating and inhibits phagocytosis of *P. aeruginosa* [3]. Bacterial proteases produced by *P. aeruginosa* cause significant damage to the airways. Elastase and alkaline protease are secreted in vivo over prolonged periods in the airways [4]. Elastase increases the permeability of epithelial cells and destroys tight junctions.

Lipopolysaccharide (LPS) is a complex glycolipid and the major component of the outermost membrane of Gram-negative bacteria. LPS provides a potent and pleiotropic stimulus for immune cells, both in vitro and in vivo [5,6], and it has been implicated in the clinical syndrome of Gram-negative septic shock. Although the polysaccharide portion of LPS varies considerably, the lipid A domain is highly conserved between otherwise diverse strains of Gram-negative bacteria. The lipid A portion of the molecule is responsible for most of the biological effects of LPS [5,7].

LPS induction of cytokine release, particularly tumour necrosis factor-α (TNF-α) and interleukin (IL)-1 is probably the central event in the pathophysiology of Gram-negative bacterial septicaemia. LPS stimulation of leukocytes and endothelial cells leads to the release of cytokines and inflammatory mediators that enhance host defences against bacteria [8]. In acute *P. aeruginosa* infections, the organism is covered with a smooth LPS and, during chronic lung infections, an altered rough LPS is present [9].

There are several cell surface receptors for LPS. CD14, which is expressed on the surface of monocytes and neutrophils [10,11], binds LPS with high affinity. Anti-CD14 antibodies block cellular LPS responses [12]. Low or intermediate concentrations of LPS do not induce cellular responses in CD14 knockout mice [13,14]. Conversely transgenic mice that overexpress CD14 show hypersensitivity to LPS [15]. The initial rate and extent of CD14-dependent internalisation of LPS increases with LPS aggregate size [16]. Cellular responses to LPS are increased by the LPS-binding protein (LBP), which binds LPS aggregates and transfers LPS monomers to CD14 [17,18]. CD18, the β2 integrin which is a subunit of complement receptors CR3 and CR4 [19], is a transmembrane signalling receptor for LPS [20]. The macrophage scavenger receptor type A also binds LPS and protects against excessive cytokine release by activated macrophage [21]. At concentrations of LPS in excess of 100 ng/ml, the activation of cells can take place in the absence of LBP and CD14. The low-affinity signalling receptor on neutrophils and other leukocytes is thought to be L-selectin [22]. Whole bacteria are thought to interact with more than one cell surface receptor. Cell wall preparations from group B streptococci type III induce TNF-α release from human monocytes through interaction with CD14 and CD18 [19].

Lung surfactant protein A (SP-A) is a member of the collectin family of mammalian proteins. Collectins contain both globular domains, the calcium-dependent carbohydrate binding domains (C-type lectins), and extended collagen-like regions. Other members of the collectin family are lung surfactant protein D and the plasma lectins, mannose binding lectin, conglutinin and CL-43 [23–25]. SP-A is an abundant protein associated with surfactant that has roles in the structure [26,27], metabolism [28], and function [29,30] of surfactant as well as in host defence [31].

The range of immunologically significant targets which SP-A recognises is not completely known. The current hypothesis is that SP-A is likely to bind to a wide range of inhaled materials and to mediate their interaction with cells (phagocytes or specialised epithelium) as part of a natural defence system [23–25,32,33]. SP-A has been shown to play a role in

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Abbreviations: SP-A, lung surfactant protein A; LPS, lipopolysaccharide; TNF-α, tumour necrosis factor alpha; BC, buffy coat; *P. aeruginosa*, *Pseudomonas aeruginosa*; IL, interleukin; LBP, LPS binding protein

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host defence mechanisms, and is known to bind to influenza virus [34], *Mycobacterium tuberculosis* [35] and *Klebsiella pneumoniae* [36].

SP-A binds specifically and competitively to alveolar macrophages. SP-A positively affects the ability of the alveolar macrophage to perform host defence functions, such as phagocytosis [37] and generation of reactive oxygen species [38]. However, the role of the collectins in cytokine induction is currently unclear; some investigators have observed increased TNF-α induction [39–41] while others have seen the opposite effect [42,43] and there has been one report where SP-A alone increased TNF-α release, but had the opposite effect in the presence of LPS [44]. We have looked at the effect of SP-A on total blood leukocytes which contain a receptor capable of interacting with SP-A [45], rather than limiting the study to one cell type. This is the first investigation of TNF-α release using SP-A and a respiratory pathogen.

2. Methods

2.1. Purification of SP-A

SP-A was purified from human lung lavage fluid from alveolar proteinosis patients by the method of Strong et al. [46]. Briefly, human bronchoalveolar lavage fluid was made 10 mM $CaCl_2$ and incubated at 4°C overnight to maximise SP-A aggregation. The suspension was clarified by centrifugation at $10\,000\times g$ for 1 h at 4°C. The resulting pellet was extracted with 6 M urea, 20 mM Tris-HCl, pH 7.4, 5 mM EDTA (100 ml per gram of pellet). The extract was centrifuged at $10\,000\times g$ for 1 h and the supernatant dialysed against 10 mM Tris-HCl, pH 7.4, 5 mM EDTA. The dialysate was made 10 mM $CaCl_2$ and the pH adjusted to 7.4, before it was loaded to a 15-ml column bed of maltose-agarose (Sigma, Poole, UK), equilibrated with 10 mM Tris-HCl, pH 7.4, 10 mM $CaCl_2$. After loading, the column was washed to background with 10 mM Tris-HCl, pH 7.4, 10 mM $CaCl_2$. Non-specifically-bound proteins were eluted with 10 mM Tris-HCl, pH 7.4, 1 M NaCl, 10 mM $CaCl_2$. SP-D was removed with 100 mM $MnCl_2$, 10 mM Tris-HCl, pH 7.4. The remaining bound proteins were eluted with a gradient of 0–50 mM EDTA in 10 mM Tris-HCl, pH 7.4. Fractions containing SP-A were pooled, concentrated and further purified by gel filtration (Superose 6; Amersham Pharmacia Biotech, St. Albans, UK).

Some preparations of SP-A were found to cause induction of TNF-α release from buffy coat (BC) cells. To ensure a low background of TNF-α production, SP-A was incubated for 30 min at room temperature with polymyxin B agarose (Sigma) to remove LPS and protein G agarose (Sigma) to remove IgG, prior to use.

2.2. Source of bacteria and bacterial products

Pseudomonas aeruginosa was obtained from National Collection of Industrial and Marine Bacteria, Aberdeen, UK, strain reference NCIMB 12050. *P. aeruginosa* was grown in Luria broth at 37°C. LPS was obtained from this strain of *P. aeruginosa* as described below. Lipid A was purchased from Sigma.

2.3. Purification of LPS

LPS was purified from *P. aeruginosa* by the method of Darveau and Hancock [47] with slight modifications. Bacterial cells were harvested in the mid-logarithmic phase and suspended at 10 ml/g (wet weight) in 10 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$. The cells were lysed in a bead beater (Stratech Scientific, UK) for 3 min. The cell lysate was sonicated for 2×30 s at a probe intensity of 75% and the suspension was incubated at 37°C for 2 h. After incubation, the solution was made 0.1 M tetrasodium EDTA, 10 mM Tris-HCl and 2% SDS at a pH of ~9.5. The sample was then subjected to centrifugation at $50\,000\times g$ for 30 min at 20°C to remove peptidoglycan. The supernatant was decanted and 200 μg/ml pronase (Sigma) was added. The sample was incubated overnight at 37°C with constant shaking. Two volumes of 0.375 M $MgCl_2$ in 95% ethanol were added, mixed and cooled to 0°C. The sample was then centrifuged at $12\,000\times g$ for 15 min at 2°C. The pellet obtained was suspended in 25 ml of 2% SDS-0.1 M tetrasodium EDTA, dissolved in 10 mM Tris-HCl (pH 8), and sonicated as described above. Incubation at 85°C for 30 min ensured denaturation of SDS-resistant proteins. Pronase was added to 25 μg/ml, and the sample was incubated overnight at 37°C with constant agitation. After incubation, LPS was precipitated with two volumes of 0.375 M $MgCl_2$ in 95% ethanol at 0°C as described above, followed by centrifugation at $12\,000\times g$ for 15 min at 2°C.

The pellet was resuspended in 15 ml of 10 mM Tris-HCl (pH 8), sonicated as described above, and centrifuged at 1000 rpm to remove insoluble Mg^{2+}-EDTA crystals. The supernatant was then centrifuged at $200\,000\times g$ for 2 h at 15°C in the presence of 25 mM $MgCl_2$. The pellet of LPS was resuspended in distilled water.

2.4. Solid phase binding assays

Microtitre plate wells (Polysorp; Nunc, Kamstrup, Denmark), were coated with LPS from *P. aeruginosa* (100 μl per well; 1 μg per well) in 100 mM Na_2CO_3 coating buffer (pH 9.6) for 2 h at room temperature. The plates were then washed four times with 250 μl/well of PBS-0.05% (w/v) Tween-20 (PBS-Tween) before the non-specific sites were blocked with BSA (5%, w/v) in PBS-Tween (300 μl per well). The bound LPS was incubated with serial dilutions of SP-A (100 μl per well; maximum 10 μg) in PBS, containing $CaCl_2$ (0.5 mM) with or without EDTA (maximum 5 mM) or mannan (maximum 50 μg/ml) or Lipid A (maximum 100 μg/ml) for 2 h at room temperature. After further washing, polyclonal rabbit anti-human SP-A in PBS-Tween-0.5 mM $CaCl_2$ was added to each well (total 100 μl). Incubations with primary antibody were carried out for 2 h at room temperature. The plates were washed four times in PBS-Tween-$CaCl_2$ and alkaline-phosphatase-conjugated goat antibodies to rabbit IgG (Sigma) added and left for 2 h at room temperature. After four further washes with 200 μl/well PBS-Tween, 100 μl/well of *p*-nitrophenyl phosphate (*p*NPP; 1 mg/ml; Sigma) in 25 mM Tris-HCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, pH 7.4, was added and incubated for 30 min or until sufficient colour developed. The plates were read at 405 nm.

2.5. Preparation of buffy coat cells

Buffy coat (BC) cells were prepared from EDTA treated fresh human blood (50 ml). The blood was centrifuged (10 min, $800\times g$). The interface between the plasma and red blood cells, which contains the leukocytes, was removed for further centrifugation (6 min, $800\times g$). The white cell pellet was washed twice in PBS and pelleted by centrifugation (6 min, $450\times g$). The pellet was resuspended in equal volumes of plasma and RPMI 1640 media containing 2 mM glutamine (Gibco-BRL, Paisley, UK). Each test required approximately 1×10^7 cells in 500 μl.

2.6. Cytokine release assay

To investigate the effect of SP-A on the release of TNF-α from BC cells, purified SP-A (100 μl; final concentration of 5.7, 2.9, 1.4 and 0.7 μg/ml) was incubated with BC cells (500 μl) in the presence of *P. aeruginosa* (100 μl of 1:5000 log phase culture ($OD^{600}=0.5$)) or *P. aeruginosa* LPS (10 μg/ml) for 4 h at 37°C. BC cells were incubated with medium or SP-A (1.4 μg/ml) as negative controls. Positive controls contained *P. aeruginosa* or *P. aeruginosa* LPS and BC cells. After 4 h, the cells were removed by brief centrifugation and the supernatants were assayed for TNF-α by ELISA (R&D Systems, Oxon, UK).

3. Results

3.1. Interaction of SP-A with P. aeruginosa LPS

SP-A has previously been shown to interact with LPS from various organisms [42,44,48] in a calcium dependent manner. The interaction of purified *P. aeruginosa* LPS and purified human SP-A was investigated by coating microtitre wells with LPS and probing with SP-A. Under these conditions, SP-A bound to *P. aeruginosa* LPS in a concentration dependent manner (Fig. 1A). The maximum concentration of SP-A available was insufficient to saturate, but an approach to saturation was observed.

In order to investigate how SP-A interacts with *P. aeruginosa* LPS, EDTA was added to chelate calcium ions, or mannan was added to compete for the sugar binding sites of SP-A,

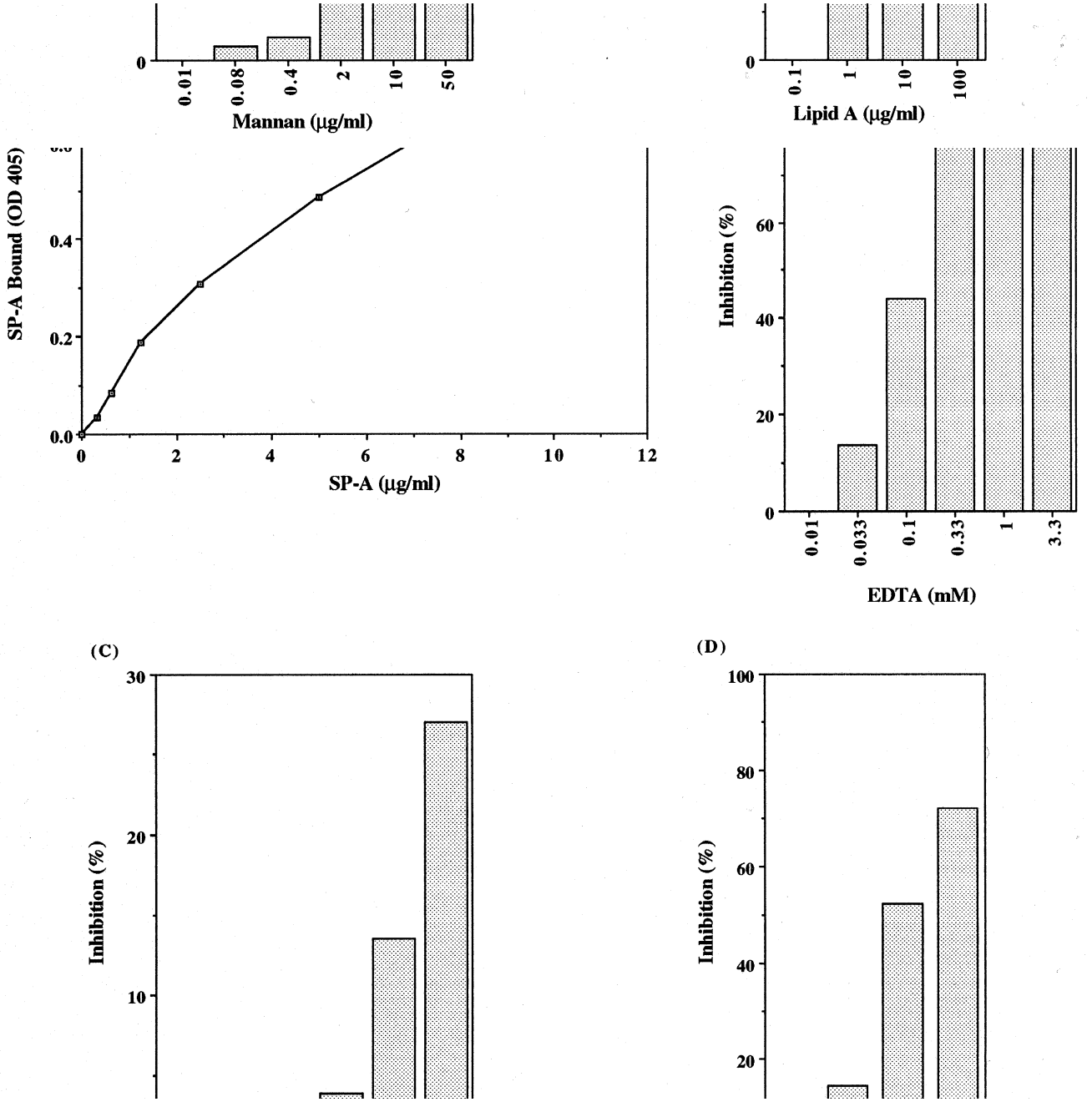

Fig. 1. Binding of SP-A to *P. aeruginosa* LPS. Each well of a microtitre plate was coated with 1 μg of LPS and blocked. A: SP-A was incubated with LPS in the presence of 0.5 mM Ca^{2+} at the concentrations shown. Polyclonal anti-SP-A antibodies were followed by goat anti-rabbit IgG-alkaline phosphatase. The assay was developed with pNPP and the optical density was read at 405 nm (OD_{405}). The amount of SP-A bound was taken as a direct reading from the optical density (arbitrary units). Inhibition of SP-A binding was determined at 5 μg/ml SP-A with (B) EDTA, (C) mannan and (D) Lipid A (all at concentrations shown). Inhibition (%) was calculated from the OD_{405} of the 0.5 mM Ca^{2+} value and the OD_{405} values obtained with inhibitors.

or lipid A was added to compete for lipid A binding. Removal of calcium ions with EDTA almost completely inhibited the binding of SP-A to *P. aeruginosa* LPS (Fig. 1B). Competition for SP-A binding sites between the fixed concentration of *P. aeruginosa* LPS and variable concentration of mannan resulted in a significant but low level of inhibition of SP-A binding (Fig. 1C). Competition for SP-A carbohydrate binding sites between the fixed concentration of *P. aeruginosa* LPS and variable concentration of lipid A resulted in a significant level of inhibition of SP-A binding (Fig. 1D). The major in-

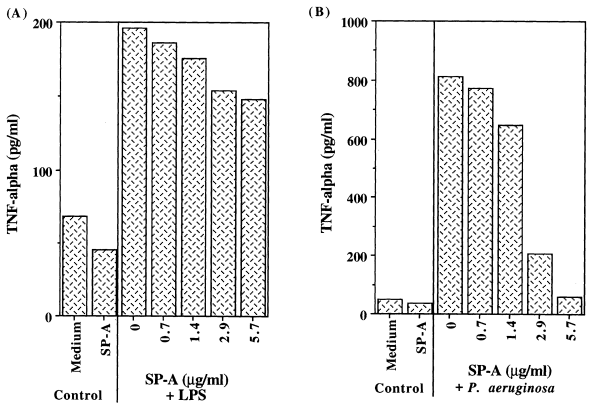

Fig. 2. Effect of SP-A on (A) *P. aeruginosa* LPS or (B) whole bacteria induced TNF-α release by buffy coat cells. Buffy coat (BC) cells were isolated from whole blood by centrifugation. The concentrations of purified SP-A shown were incubated with BC cells in the presence of *P. aeruginosa* LPS or whole bacteria for 4 h at 37°C. BC cells were incubated with medium or SP-A (1.4 μg/ml) to give two negative controls. The positive control contained only *P. aeruginosa* LPS or whole bacteria and BC cells. All incubations were 4 h. The supernatants were assayed for TNF-α by ELISA.

teraction between SP-A and LPS is through the lipid A portion of LPS, which can be inhibited by 70% by competition with free Lipid A. However, carbohydrate binding via the CRD of SP-A appears to make a contribution to the interaction; addition of mannan inhibits SP-A binding by 30%.

3.2. Cytokine release assay

Interaction between leukocytes and LPS is known to lead to the secretion of TNF-α [8]. Incubation of LPS with BC cells induced TNF-α release (Fig. 2A). Addition of SP-A alone to these cells had little effect on the basal level of TNF-α produced. In the presence of LPS, addition of SP-A led to a decrease in TNF-α secretion and the decrease was proportional to the concentration of SP-A added (Fig. 2A).

Incubation of whole bacteria with BC cells induced a substantial release of TNF-α (greater than observed with LPS alone). In the presence of bacteria, addition of SP-A greatly reduced the release of TNF-α, such that at a final SP-A concentration of 2.9 μg/ml, the BC cells released only 20% of the TNF-α of the positive control and this decreased to 1% in the presence of 5.7 μg/ml (Fig. 2B).

4. Discussion

The goals of our investigation were to determine whether SP-A interacts with the respiratory pathogen, *Pseudomonas aeruginosa*, and to provide more data on the effects of SP-A induction of TNF-α. Previous, contradictory studies on the effect of SP-A in induction of TNF-α used slightly different cell types [40,42]. We have used total blood leukocytes which contain a receptor capable of interacting with SP-A [45].

Our results show that SP-A can interact with *P. aeruginosa* LPS in a concentration and calcium dependent manner, consistent with the findings of other investigators for LPS from other organisms [42,44,48]. However, SP-A binding to *P. aeruginosa* LPS is partially inhibited by mannan, unlike the interaction with *Salmonella minnesota* Re-LPS or *Escherichia coli* J5-LPS [48]. The mode of interaction of SP-A with lipid A is uncertain. Interaction may be with the charged saccharides, or with the fatty acids. SP-A is also likely to interact with other sugars in LPS; this interaction will be dependent on the carbohydrate composition. Our results highlight the difference in polysaccharide components of LPS from different organisms [5,7].

The cytokine release assay suggests that LPS or bacteria interact with BC cells in a different manner when SP-A is present. This could be due to adhesion of the LPS/SP-A or bacterium/SP-A complex via an SP-A receptor rather than via one of the known receptors: CD14, CD18 or L-selectin. Alternatively, SP-A may prevent the interaction of cell surface receptors with LPS or bacteria by competing for binding sites. SP-A binding to the lipid A portion of LPS may break up LPS micelles, an event which would produce smaller aggregates of LPS. Smaller aggregates are known to be less efficient at inducing a TNF-α response [16], probably because they are unable to crosslink receptors.

The SP-A concentration dependence of TNF-α release could indicate that when there is sufficient SP-A present to deal with an infection, then the cells do not signal a requirement for help. However, as soon as there is more LPS or bacteria than can be dealt with by this part of the innate immune system then the endotoxin or pathogen can interact with LBP and CD14. This would then lead to TNF-α secretion to stimulate other arms of the immune system which can dispose of LPS, such as macrophage scavenger receptors [21], or to recruit inflammatory cells. This concept is supported by the observation that SP-A concentration inversely correlated with the number of inflammatory cells in bronchoalveolar lavage fluid from cystic fibrosis patients (A. Mander, personal communication). SP-A mediated removal of LPS represents an immunologically similar mechanism to the scavenger receptor [21]; each reduces the amount of proinflammatory cytokine released.

We conclude that SP-A binds to *P. aeruginosa*, at least partially through LPS, and prevents TNF-α release probably through sterically hindering interaction of the microorganism with cell surface receptors other than SP-A receptors.

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