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Review

Interactions of surfactant protein A with pathogens

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Abstract

The lung is an organ with a large inner surface that is continuously in contact with the environment. Infection of this organ is prevented by several mechanisms. A recently described defence system is collectin-mediated innate immunity of the lung. Collectins are multimeric proteins characterized by carbohydrate recognition domains bound to collagen stalks. Surfactant protein (SP)-A and SP-D are collectins that are present in the epithelial lining fluid of the lung. SP-A interacts with viruses, bacteria and fungi. Furthermore, SP-A binds to various other inhaled glycoconjugates. SP-A receptors on phagocytic cells have been described that are important to ensure rapid pathogen clearance. This innate defence system of the lung may be particularly important during infections in young children when the acquired immune system has not yet become fully established. Also in later life SP-A could be very important to prevent the lungs from infections by pathogens not previously encountered. In addition, SP-A may limit the inflammatory response in the lungs, thus preventing damage to the delicate lung epithelia. Recently, evidence was presented that SP-A may modulate the allergic response to various glycosylated inhaled antigens. The presence of SP-A (and SP-D) in other organs indicates that these collectins may have a general role in mucosal immunity. In this review the interactions of SP-A with a variety of pathogens and its implications are discussed.

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1. Introduction

Gas exchange in the lung takes place in the alveoli. Pulmonary surfactant is a complex mixture of lipids and proteins that forms a thin film at the air-water interface of the alveoli and thus reduces the surface tension. It protects the alveoli against collapse at end-expiration, reduces the work of breathing, and precludes alveolar oedema [1]. Furthermore, surfactant components may have important functions in innate lung defence. Isolated surfactant contains approx. 90% lipids and 10% proteins by weight. The surfactant lipids are mainly phospholipids (95%), of which phosphatidylcholine (PC) represents 70–80%. The major phospholipid component of lung surfactant is dipalmitoylphosphatidylcholine (DPPC), which is responsible for the low surface tension at end-expiration. Surfactant comprises two types of surfactant proteins: SP-A and SP-D (large hydrophilic proteins), and SP-B and SP-C (small hydrophobic proteins). SP-A and SP-D are related and belong to a subgroup of mammalian lectins called 'collectins' (or C-type lectins, group III). These proteins consist of oligomers with carboxy-terminal carbohydrate recognition domains bound via a 'neck' region to amino-terminal collagen-like domains. In lung lavage most SP-A is associated with surfactant lipids, whereas most SP-D is not. For both SP-A and SP-D the main sites of synthesis and secretion are the bronchiolar Clara cells and alveolar type II cells. In Clara cells, SP-A and SP-D are present in secretory granules [2], that could potentially be secreted in an acute phase reaction upon an infectious challenge [3]. The fact that SP-A could be detected in tracheal and bronchial glands, and in the epithelium of conducting airways [4], also suggests the importance of non-surfactant-associated pulmonary functions of SP-A, and contributes to the proposed role of SP-A in the host defence of the lung. Indeed, accruing evidence suggests that the most important functions of SP-A and SP-D are their roles in host defence. Furthermore, SP-A may have a role in the structural organization of surfactant. In this review I will focus on some of the properties of SP-A, its interactions with various glycoconjugates and pathogens, and its putative functions in lung defence. The reader is referred to the chapter of Tino and Wright in this issue for information on the interactions of SP-A with epithelial cells and phagocytes.

SP-A was the first of the surfactant proteins that was purified [5], and analysed for its primary structure [6]. It is an abundant protein in bronchoalveolar lavage and relatively easy to purify. Butanol extraction is a widely used method to purify SP-A, but it has been reported that some of the functional characteristics of SP-A are lost during this extraction procedure [7]. The molecular mass of the monomeric form is 28–36 kDa and human SP-A comprises 248 amino acid residues. The primary structure of SP-A comprises four domains: an amino-terminal domain, a collagenous domain, a neck domain and a carbohydrate recognition domain (see [8] and the chapter by McCormack in this issue for details). The carbohydrate binding domain (CRD) contains a Ca^{2+}-dependent specific carbohydrate binding site. The positions of the four cysteine residues in this region are conserved in all members of the class of calcium-dependent lectins. Disulphide bonds have been described between residues 135 and 226, and residues 204 and 218 [9]. The asparagine at position 187 of the CRD is *N*-glycosylated.

Because SP-A was the first surfactant-associated protein discovered, the properties and putative functions of SP-A were studied more extensively than those of the other surfactant proteins. Obviously, SP-A is not directly responsible for the surface tension lowering properties of pulmonary surfactant, although SP-A has possibly a regulating role [10,11]. It should be kept in mind that studies with knock-out mice have shown that SP-A is not absolutely required for breathing [12]. In a recent study the surface properties, morphology and protein composition of surfactant subtypes were reported [13]. It was found that surface film formation from different

surfactant fractions was dependent on surfactant structure. Interestingly, at low surfactant concentrations extracted surfactants showed a delayed adsorption compared to natural surfactants, which suggests that the SP-A-dependent structural organization is important to ensure efficient surface film formation. In vivo, this structural organization is represented by tubular myelin which is formed from lamellar bodies after they have been secreted into the epithelial lining fluid of the alveolar space. In vitro studies indicated that tubular myelin formation depends on the presence of SP-A (and SP-B) [14]. In tubular myelin SP-A has a preferential localization which suggests that SP-A-SP-A interactions form the scaffold of this structure [15]. Tubular myelin may fulfil a role in vivo as an extracellular surfactant reservoir that is not susceptible to inactivation by inhaled particles, pathogens and transudated serum proteins. Thus, SP-A may protect surfactant function under pathological conditions.

2. Carbohydrate binding specificity of SP-A

The first indications that SP-A may have a role in host defence were obtained 12 years ago. Drickamer and co-workers described a sequence similarity between SP-A and mannose binding proteins, and suggested that SP-A could also have carbohydrate-binding properties [16]. Shortly afterwards, calcium-dependent binding of SP-A to monosaccharides was described and it was proposed that SP-A may play a role in the lung defence [17]. Two reasons were supporting this notion: SP-A is able to bind carbohydrates, and SP-A is structurally similar to C1q [18]. In these early studies it was found that SP-A binds to agarose-bound D-mannose, L-fucose, D-galactose and D-glucose but not very well to *N*-acetyl-D-galactosamine and *N*-acetyl-D-glucosamine [17]. Under the conditions employed, the threshold Ca^{2+} concentration was 0.6 mM and maximal binding was observed at 1 mM Ca^{2+}. Ba^{2+}, Sr^{2+}, and Mn^{2+}, but not Mg^{2+} could substitute for Ca^{2+}. Binding of ^{125}I-labelled SP-A to bovine serum albumin linked monosaccharides showed similar binding characteristics [19].

In another study the carbohydrate-binding characteristics of normal human SP-A and SP-A from alveolar proteinosis patients were compared with those

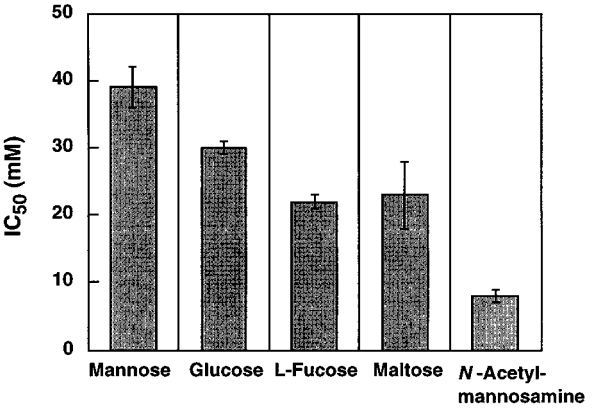

Fig. 1. Carbohydrate binding specificity of SP-A. IC_{50}, concentration of the sugar resulting in a 50% reduction of the binding to mannan-coated microtitre wells. Galactose, D-fucose, mannosamine, glucosamine, galactosamine, *N*-acetylglucosamine, and *N*-acetylgalactosamine were not inhibitory. Data are taken from [20].

of mannan-binding protein (MBP) and conglutinin in an enzyme-linked lectin-binding assay [20]. It was found that several monosaccharides (in D-configuration, except fucose of which both L- and D-configurations were tested) competed for the binding of SP-A to mannan from *Saccharomyces cerevisiae*. The order of inhibiting potency was: *N*-acetylmannosamine > L-fucose = maltose > glucose > mannose (Fig. 1). Galactose, D-fucose, glucosamine, mannosamine, galactosamine, *N*-acetylglucosamine, and *N*-acetylgalactosamine were non-inhibitory. The discrepancy between this study and the earlier studies, especially with respect to the affinity for galactose, may be explained by the different binding assays employed. It must be noted that the affinities for mannose and galactose may be mutually exclusive in C-type lectins. According to the elegant studies of Drickamer [21] with MBP and McCormack and co-workers [8] with rat SP-A, mannose-binding specificity is determined by the motif Glu-Pro-Asn (Glu^{195}-Pro^{196}-Arg^{197} in rat SP-A), whereas galactose-binding specificity is determined by the motif Gln-Pro-Asp. Therefore, the observed binding of SP-A to immobilized galactose may be an artifact, induced by the coupling of the monosaccharide to agarose [17] or to bovine serum albumin [19].

Childs and co-workers observed that human SP-A binds in a Ca^{2+}-dependent fashion to the glycolipids galactosyl ceramide and lactosyl ceramide [19]. The binding of SP-A to glycolipids was dependent on the

assay system (thin layer chromatograms vs. microwells) and it was observed that SP-A binds both saccharide and lipid moieties of glycolipids. Interestingly, SP-A binds with a different affinity to galactosyl ceramides that differ only in having either α-hydroxy or non-hydroxy fatty acids, an observation later confirmed by Hynsjö et al. with lactosyl ceramides [22]. The apparent affinities of SP-A for these respective galactosyl ceramides were also dependent on the assay system. This study showed again that results of binding studies of SP-A to various ligands, including cell surface ligands, should be interpreted with caution. Another remark that can be made here concerns the use of ^{125}I-labelled SP-A in binding studies. Several investigators have shown that radioiodination procedures readily inactivate SP-A [20,23], probably because SP-A is extremely sensitive to oxidative stress [24]. It must be stressed that other workers did not appear to experience problems using radioiodinated SP-A. Nevertheless, one must be aware that the fraction of total SP-A that has been labelled may behave differently from the unlabelled SP-A in tests designed to screen for biological activities.

In a detailed study into the binding of ^{125}I-SP-A to glycolipids Kuroki and co-workers confirmed the binding to galactosyl ceramides and the observation that this binding involves both saccharide and lipid moieties [25]. This finding was corroborated by the fact that SP-A did not bind to galactosylsphingosine. No binding of SP-A was observed to glucosylceramide, sulphatide, Forssman antigen, G_{M1}, G_{M2}, and asialo-G_{M1}. In addition to galactosyl ceramides, SP-A bound to asialo-G_{M2}. The oligosaccharide derived from asialo-G_{M2} competed with the solid-phase asialo-G_{M2} for SP-A binding [25]. Using native (not iodinated) human SP-A Hynsjö and collaborators confirmed the work of Childs and Kuroki [22]. It is not clear whether glycolipid binding of SP-A is involved in defence mechanisms such as binding to enveloped viruses and subsequent enhancement of phagocytosis or prevention of epithelial binding and entry of pathogens.

3. Interactions of SP-A with viruses

SP-A has been reported to act as an opsonin in the phagocytosis of herpes simplex virus type 1 by rat alveolar macrophages [26]. Compared to the opsonic capacity of serum, SP-A was found to be twice as potent. The SP-A-mediated phagocytosis of FITC-labelled herpes simplex virus type 1 was time- and concentration-dependent. Preincubation of the macrophages with SP-A followed by neutralization of cell-bound SP-A with $F(ab')_2$ fragments of anti-SP-A abolished herpes simplex virus type 1 phagocytosis. SP-A binds herpes simplex virus type 1 as was shown indirectly by the increased binding to virus-infected HEp-2 cells expressing viral proteins at the cell surface [27]. Binding of SP-A to infected cells was inhibited by heparin, but not by yeast mannan. Interestingly, deglycosylated SP-A, obtained by digestion with *N*-glycosidase F, did not bind to infected cells. These observations suggest that the carbohydrate moiety of SP-A is involved in recognition of viruses [27]. The carbohydrate moiety of SP-A is not required for stimulation of alveolar macrophages since deglycosylated SP-A still stimulated the production of free radicals by these cells. Furthermore, heparin did not influence SP-A-mediated stimulation of alveolar macrophages, suggesting that the molecular requirements for virus binding differ from those for binding to macrophages.

SP-A binds influenza virus A (H3N2) in a saturable, concentration-dependent fashion in the presence of Ca^{2+} [28]. The binding of SP-A in the presence of Ca^{2+} to immobilized influenza virus could be inhibited partially by mannose suggesting that part of the interaction between the virus and SP-A involves the carbohydrate binding propensity of this collectin. Interestingly, MBP binding to influenza virus is more successfully competed for by mannose and more dependent on Ca^{2+} than SP-A binding. The virus-mediated agglutination of red blood cells was inhibited by both collectins [28]. However, compared to the effects of SP-D on the inhibition of haemagglutination activity of influenza A virus, the effects of SP-A and MBP were relatively small [29]. A recent study by Hartshorn and co-workers confirmed the relatively small effect of SP-A, compared to the other collectins, on influenza A virus-mediated haemagglutination [30]. In line with results by other groups it was observed in this study that SP-A-influenza A virus interactions are not Ca^{2+}-dependent and are not dependent on the lectin activity of SP-A. SP-A (and

MBP) may bind influenza virus A (H3N2) partly via interaction with viral neuraminidase [28]. Benne and co-workers observed concentration- and time-dependent binding of SP-A to influenza A (H3N2) virus-infected HEp-2 cells [31]. Neither the presence of mannan nor the presence of heparin could prevent SP-A binding to the infected cells. Interestingly, removal of the *N*-linked carbohydrate of SP-A or only its sialic acid residues completely prevented binding of SP-A to infected HEp-2 cells. Infection of LLC (MK2D) cells with influenza A (H3N2) virus was prevented by preincubation of the virus with SP-A [31]. Viral infectivity was measured by the appearance of viral proteins on the cell surface. After removal of the carbohydrate moiety of SP-A by enzymatic digestion with *N*-glycosidase F, or after neuraminidase treatment, SP-A no longer prevented viral infection of the cells. It may be concluded that SP-A binds to influenza A virus via its sialic acid residues and thereby neutralises the virus. Neither influenza B virus infection of LLC cells nor mumps virus infection of Vero cells could be prevented by SP-A, although also for these viruses sialic acid residues function as cellular receptors. Semliki Forest virus infection of L cells was not affected by SP-A [31].

In a subsequent study Benne and collaborators showed that SP-A, but not SP-D, is an opsonin for influenza A (H3N2) virus phagocytosis by rat alveolar macrophages [32]. The association and uptake of FITC-labelled virus by alveolar macrophages was studied by flow cytometry. At 60 μg/ml SP-A a 3-fold increased association of virus with cells was observed. Trypan blue quenching studies indicated that about half of the associated virus was internalized. Influenza A virus phagocytosis could not be inhibited in the presence of mannan. In contrast, removal of the sialic acid residues by neuraminidase or *N*-glycosidase F treatment resulted in a complete inhibition of SP-A-induced phagocytosis [32]. Hartshorn et al. showed that preincubation of influenza A virus with SP-A enhanced the ability of this virus to stimulate the respiratory burst of neutrophils. In contrast to the other collectins tested SP-A did not protect neutrophils against virus-induced deactivation [30].

Some viruses, like human immunodeficiency virus-1 (HIV-1), infect alveolar macrophages. The route of entry could include CD4 or non-CD4 receptor-mediated mechanisms, like SP-A-dependent delivery. In this way SP-A would function as a Trojan horse. A recent study by Guay et al. showed that SP-A does not modulate entry of HIV-1 into alveolar macrophages [33]. In contrast, MBP inhibits HIV-1 infection of U937 monocytoid cells by binding to mannose residues on the CD4 binding site of gp120 [34]. Sternberg and co-workers measured SP-A levels in bronchoalveolar lavage from immunosuppressed, HIV-positive patients [35]. It was found that amounts of SP-A were significantly lower in patients than in healthy, uninfected volunteers, suggesting that HIV infection lowers SP-A levels. In contrast, Phelps and Rose found elevated levels of SP-A in bronchoalveolar lavage from HIV-infected patients [36]. However, in this study the fact that these patients had *Pneumocystis carinii* pneumonia was not taken into account. Sternberg et al. showed that in HIV-positive patients with *P. carinii* pneumonia SP-A levels were significantly higher than in HIV-positive patients without pneumonia [35]. Other additional infections of HIV-infected patients did not have such a dramatic effect on SP-A levels. In line with these results are observations done in a rat infection model. Experimental infections of rats with *P. carinii carinii* resulted in dramatically elevated SP-A levels in whole lungs [37]. Moreover, Williams and collaborators observed that isolated *P. carinii* from infected rats contained SP-A [38]. In contrast with the results of Sternberg et al., Downing and coworkers demonstrated that in bronchoalveolar lavage from HIV-infected individuals without evidence of opportunistic pulmonary infection SP-A levels were markedly increased compared with normal control individuals [39].

It is good to realize that determination of the amounts of SP-A and other surfactant components in bronchoalveolar lavage as markers for (infectious) disease can lead to wrong conclusions. In general, SP-A levels are determined in cell-free lavage fluid and are expressed as amounts per ml lavage fluid. Most investigators do not measure amounts of SP-A in the cellular pellet. However, it is easy to see that, during infections, secreted SP-A may distribute differently between the extracellular compartments. SP-A, bound to some pathogens, may deliver these pathogens to alveolar macrophages resulting in

the presence of more SP-A in the cellular pellet. In addition, aggregates of SP-A and SP-D with pathogens may pellet at low gravitational forces, also leading to lower levels of these surfactant proteins in cell-free lavage fluid. The fact that during *P. carinii* pneumonia SP-A levels were significantly higher than in HIV-positive patients with other additional infections [35] could be related to the observation that part of the *P. carinii* clusters (and SP-A, bound to these clusters) remained in the supernatant. It is clear that slightly different lavage and centrifugation techniques could lead to altered recoveries of collectins in bronchoalveolar lavage fluid and this could also lead to discrepancies as found between the study of Sternberg et al. [35] and that of Phelps and Rose [36]. Furthermore, altered binding of surfactant proteins to the epithelial surface may also lead to different levels of these proteins in retrieved lavage fluid. Since part of the interactions of SP-A and SP-D with pathogens and cells is mediated by Ca^{2+}-dependent carbohydrate binding, lavage in the presence of Ca^{2+}-chelators may increase the yield of these collectins in cell-free lavage fluid. However, this will not release all bound collectins and certainly not internalized SP-A and SP-D. Finally, it is important to realize that, measurement of the altered dynamics of the collectin-dependent defence system during an infectious challenge is probably more informative than the measurement of collectin levels. For diagnostic purposes the levels of surfactant components, if measured both in cell-free lavage and in the (cell) pellet, will continue to be very valuable.

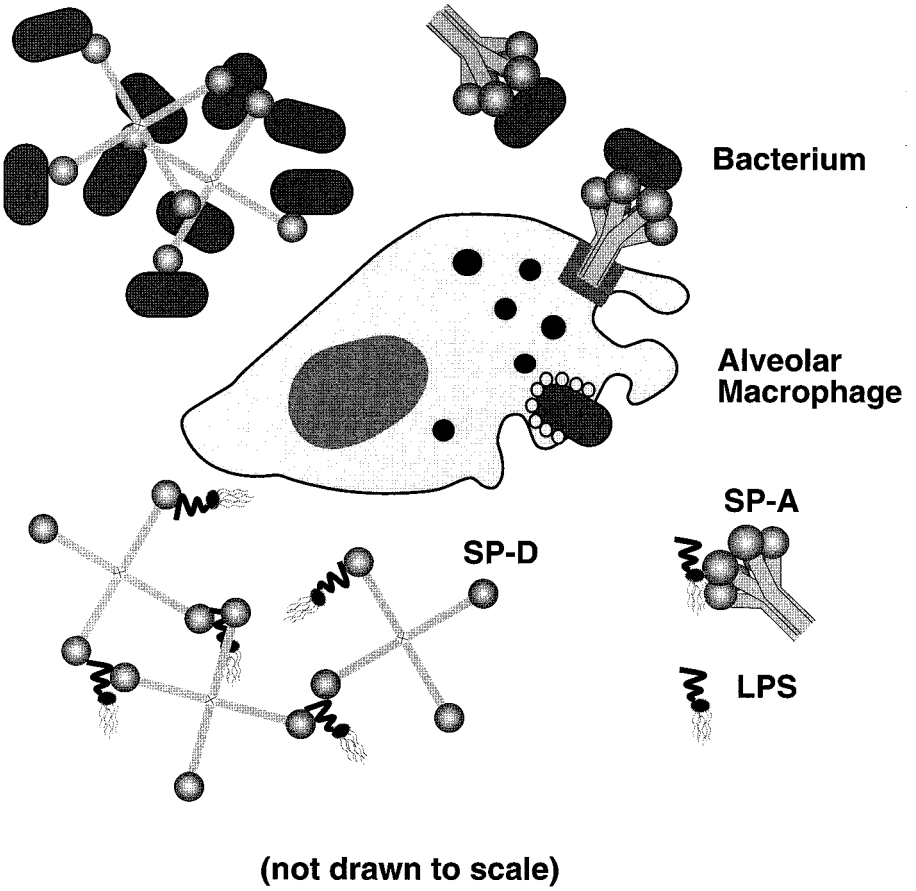

Fig. 2. Interactions of SP-A and SP-D with bacteria and LPS. SP-A acts as an opsonin and mediates uptake of bacteria by alveolar macrophages. SP-A binds lipopolysaccharide (LPS), possibly via its lipid A moiety. In contrast, SP-D binds, and possibly aggregates, LPS via its core polysaccharides and/or O-specific antigens. SP-D agglutinates bacteria.

4. Interactions of SP-A with bacteria

SP-A interacts with a variety of bacteria and enhances uptake of some of these bacteria by phagocytic cells (Fig. 2). In an early study Van Iwaarden and co-workers demonstrated that SP-A from human alveolar proteinosis patients stimulates phagocytosis of radiolabelled, serum-opsonized, *Staphylococcus aureus* by rat alveolar macrophages in a surface phagocytosis assay [40]. Rat surfactant also stimulates phagocytosis in the same assay system and SP-A is the component responsible for this activity. SP-A alone had no effect on the phagocytosis of *S. aureus* by alveolar macrophages, indicating that SP-A may stimulate complement and immunoglobulin mediated phagocytosis. No direct binding of SP-A to *S. aureus* was reported. Later studies indicated that SP-A binds to *S. aureus* [41,42] and may act as an opsonin. In line with the observations of Van Iwaarden et al. it was reported that phagocytosis of complement and IgG coated erythrocytes is enhanced if macrophages are cultured on SP-A coated plates [43]. These early studies indicated that SP-A may facilitate complement and immunoglobulin-mediated phagocytosis by alveolar macrophages.

In addition, SP-A-enhanced phagocytosis of non-opsonized bacteria by alveolar macrophages was also reported [44]. Human SP-A (recombinant and from alveolar proteinosis patients) was found to stimulate serum-independent phagocytosis of *Escherichia coli*, *Pseudomonas aeruginosa* and *S. aureus* by rat alveolar macrophages depending on the growth phase of the bacteria. Further support indicating that SP-A itself can act as an opsonin came from the studies of Pikaar and co-workers. These investigators incubated isolated rat alveolar macrophages with *E. coli* in the presence or absence of SP-A. Flow cytometric studies showed that SP-A enhanced the binding of *E. coli* J5 (containing rough LPS) but not of *E. coli* O111 (containing smooth LPS) to macrophages, suggesting that SP-A binding to Gram-negative bacteria is dependent on LPS structure [45]. Electron microscopical studies showed that in the presence of SP-A the number of ingested J5 bacteria increased nearly 6-fold and the attachment even 20-fold over the control incubations without SP-A. Preincubation of alveolar macrophages with SP-A did not enhance the binding or uptake of bacteria. Immuno-electron microscopical studies showed that the attached and ingested bacteria were heavily coated with SP-A on their surface [45]. These observations strongly suggested that SP-A acts as an opsonin in the phagocytosis of bacteria.

SP-A also binds the Gram-positive bacterium *S. aureus* [41,42]. McNeeley and Coonrod showed increased attachment of SP-A-coated *S. aureus* to macrophages without ingestion. SP-A also bound *Streptococcus pneumoniae* (type 25) in a Ca^{2+}-dependent manner but SP-A-coated pneumococci did not associate with macrophages [41]. In contrast to these results, Tino and Wright recently showed that SP-A enhances uptake of *St. pneumoniae* by alveolar macrophages [46]. Furthermore, SP-A was reported to opsonize *S. aureus* and to facilitate phagocytosis of these bacteria by monocytes by binding to the C1q receptor of these cells [42]. It was found that SP-A neither binds to, aggregates, nor stimulates the phagocytosis of *Ps. aeruginosa* [46]. This result was not in line with the results from an earlier report [44]. This discrepancy may be explained by the different phagocytosis assays employed and by the observation done in the earlier study [44] that the SP-A-stimulated phagocytosis varied with the growth phase of the bacteria. In addition to binding to *St. pneumoniae*, Tino and Wright also found that SP-A binds to *Haemophilus influenzae* and group A *Streptococcus*. Only *H. influenzae* was aggregated by SP-A [46]. This indicates that binding, but not aggregation, is a prerequisite for SP-A-stimulated phagocytosis. In an earlier report McNeeley and Coonrod already showed that SP-A binds and aggregates *H. influenzae* type a and that SP-A promotes attachment and ingestion of this organism by macrophages [47]. Interestingly, SP-A bound much less to *H. influenzae* type b and SP-A did neither aggregate this organism nor stimulate uptake by macrophages.

The respiratory pathogen *Mycobacterium tuberculosis* is inhaled into the alveoli of the lung where it is phagocytosed by and multiplied within mononuclear phagocytes. Since SP-A stimulates phagocytosis two studies were devoted to investigate whether *M. tuberculosis* takes advantage of this property of SP-A to gain access to macrophages. Downing and collaborators observed that bronchoalveolar lavage of HIV-infected individuals contains a factor which significantly enhanced the attachment of *M. tuberculosis* to

murine alveolar macrophages by a factor of 3 relative to control individuals [39]. This factor turned out to be SP-A, because the enhancement of *M. tuberculosis* attachment by bronchoalveolar lavage of HIV-infected individuals could be prevented by immunoprecipitation of SP-A from the lavage fluid. Addition of purified SP-A to immunoprecipitated lavage fluid restored the attachment of *M. tuberculosis* to alveolar macrophages. Furthermore, it was observed that SP-A levels in lavage correlated with *M. tuberculosis* attachment, again indicating that *M. tuberculosis* uses SP-A as a Trojan horse. Interestingly, peripheral CD4 lymphocyte counts of HIV-infected individuals were inversely proportional to SP-A levels [39]. Gaynor and co-workers obtained evidence that SP-A enhances phagocytosis of *M. tuberculosis* by a direct interaction with human monocyte-derived macrophages (MDMs) and human alveolar macrophages [48]. Removal of SP-A from monolayers of MDMs by washing before adding bacteria or formation of MDM monolayers on SP-A coated glass coverslips did not diminish the enhanced adherence of *M. tuberculosis*. These experiments suggested that SP-A may have a direct effect on MDMs as an activation ligand. Interestingly, deglycosylated human alveolar proteinosis SP-A and non-glycosylated recombinant rat SP-A failed to enhance *M. tuberculosis* adherence, suggesting that the carbohydrate moieties of SP-A may be important in mediating its interactions with MDMs leading to enhanced phagocytosis of *M. tuberculosis*. In contrast, boiled human alveolar proteinosis SP-A did not loose its stimulating activity. It was suggested that SP-A mediates the reported effect on phagocytosis of *M. tuberculosis* via upregulation of the macrophage mannose receptor activity [48].

Recently, it was reported that SP-A also binds to another mycobacterial species, namely *Bacillus Calmette-Guérin* [49]. SP-A binds this organism in a Ca^{2+}-dependent manner and binding could be inhibited by the addition of carbohydrates, suggesting that the CRD is involved in the binding. Complexes of SP-A with *B. Calmette-Guérin* were taken up by several phagocytic cells via the specific 210 kDa SP-A receptor [49]. It may be concluded that for this mycobacterial species SP-A exerts its effect for a large part as an opsonin rather than by modulating macrophage function.

The SP-A enhanced phagocytosis of *Klebsiella pneumoniae* by alveolar macrophages may occur via both mechanisms, the opsonizing activity of SP-A, and the activation of macrophages via an increased activity of the mannose receptor [50]. Binding of SP-A to *K. pneumoniae* was dependent on the strain. SP-A binds to the capsule of strain K21a (containing Manα1Man sequences) as was shown by binding of SP-A-coated particles to the bacterial surface and by SP-A-induced agglutination of the bacteria. In contrast, almost no binding of SP-A to the K2 strain was observed. In addition, SP-A enhances phagocytosis of *K. pneumoniae* K21a but not of K2 serotypes by alveolar macrophages. Pretreatment of macrophages with SP-A resulted in the increased binding and killing of *K. pneumoniae* K21a [50].

Mycoplasma pulmonis accounts for a large percentage of pneumonias and exacerbates other respiratory conditions such as asthma and chronic obstructive pulmonary disease. SP-A binds to *My. pulmonis* in a concentration and Ca^{2+}-dependent manner [51]. Since the binding was not totally dependent on Ca^{2+}, and mannosyl-BSA (up to 2 mM mannose) was not able to inhibit binding of SP-A to mycoplasmas, the authors concluded that the collagen-like domain of SP-A was involved in binding. However, additional experiments are required to rule out the possibility that SP-A binds *My. pulmonis* via its CRD. Preincubation of interferon-γ activated murine alveolar macrophages with SP-A resulted in phagocytosis and mycoplasmal killing. SP-A may mediate killing through generation of NO and/or its toxic metabolites [51].

SP-A may bind Gram-negative bacteria via interaction with its lipopolysaccharides (LPS, endotoxin). SP-A binds to the J5 mutant of *E. coli* but not to its O111 mutant [52]. This suggests that SP-A binds only to rough LPS, a finding confirmed by the binding of SP-A to LPS-coated beads. LPS binding was Ca^{2+}-dependent. SP-A also bound lipid A-coated beads. These studies by Van Iwaarden et al. led to the conclusion that SP-A associates with LPS via the lipid A moiety of rough LPS and in this way protects the lung from infection by Gram-negative bacteria with rough LPS [52]. In contrast, Kalina and coworkers observed SP-A binding to LPS that was Ca^{2+}-independent [53]. Moreover, these investigators noticed that SP-A is capable of binding to LPS from

four different strains of bacteria and to lipid A. SP-D was shown to bind to core polysaccharides and/or O-specific antigens of LPS in a calcium dependent fashion [54]. In vivo, SP-A and SP-D may act in concert with other factors to form a complex that binds Gram-negative bacteria and enhances phagocytosis.

The respiratory tract is continuously exposed to LPS. Therefore, SP-A and SP-D may be involved in the neutralization and clearance of inhaled free endotoxin without eliciting an inflammatory response. Indeed, SP-D was shown to act as an endotoxin scavenger in a rat model of acute respiratory distress syndrome (ARDS) [55]. Subsequently, SP-D-LPS complexes may be taken up by alveolar macrophages without macrophage activation. Like bactericidal/permeability increasing protein (BPI) in the bloodstream SP-D may act to dampen the biological effects of LPS. However, SP-D may also act like LPS-binding protein (LBP) which facilitates transfer of LPS to CD14 on phagocytes and thus enhances macrophage activation. The role of SP-A in neutralizing inhaled endotoxin is not clear either. SP-A levels in lung lavage immediately increase in an acute phase reaction upon inhalation of LPS [3]. However, it is not known whether newly secreted SP-A binds directly to inhaled LPS. Experiments in vitro indicated that the LPS induced secretion of colony-stimulating factors (CSF) by alveolar macrophages and alveolar type II cells could be blocked by SP-A, although SP-A alone, like LPS, had a stimulating effect [53]. It is clear that more work is required to determine the role of SP-A and SP-D in protecting the lungs from inhaled endotoxin.

Until recently, most experiments designed to investigate the role of the collectins SP-A and SP-D in innate lung defence were carried out in vitro. LeVine and co-workers reported the first of a series of studies in murine infection models using mice lacking SP-A [56]. SP-A-deficient mice were produced by targeting the SP-A locus by homologous recombination in embryonic stem cells [12]. These mice survive normally under laboratory conditions indicating that SP-A is not crucial for normal lung function and suggesting that its role in host defence may be of importance (see chapter of Korfhagen et al. in this issue). The studies of LeVine et al. showed, for the first time, that indeed SP-A-deficient mice are more susceptible to bacterial infections. Intratracheal instillation of group B streptococci resulted in a decreased bacterial clearance in SP-A −/− mice [56]. The quantification of bacteria in lung homogenates indicated that, particularly at 6 h after infection, bacterial counts were much higher in the SP-A −/− mice than in the control mice, indicating that SP-A is important in immediate, first-line pulmonary defence. After 24 and 48 h systemic dissemination of bacteria was much higher in SP-A −/− mice as indicated by a relatively high colony count in the spleen, whereas almost no bacteria were found in the spleen of control mice. In line with previous in vitro studies, LeVine and co-workers obtained evidence that SP-A is involved in the stimulation of uptake of bacteria by alveolar macrophages. It was observed that even 48 h after infection fewer bacteria were associated with alveolar macrophages from SP-A −/− mice than with macrophages from control mice. Western blot analysis of lung homogenates revealed that SP-D levels apparently did not increase in response to instillation with group B streptococci [56].

Using another approach, Herting and collaborators also studied the possibility that SP-A protects the lung from infection with group B streptococci [57]. These investigators instilled a rat monoclonal antibody to rabbit SP-A in the lungs of newborn, ventilated, near term rabbits, infected the animals 30 min later and determined bacterial proliferation in these and control animals 5 h after the onset of the experiment. The control animals received physiological saline or nonspecific rat IgG. No significant differences were observed between the bacterial counts in anti-SP-A-treated rabbits and control animals. The authors concluded that SP-A is only of limited importance in host defence against severe neonatal infections with encapsulated group B streptococci [57]. However, this conclusion seems to be a little bit premature since the authors did not show that the monoclonal antibody to rabbit SP-A indeed binds to a domain of SP-A that is involved in host defence. In addition, it was not investigated whether inoculation with less bacteria resulted in infections that did not overwhelm SP-A-mediated lung defence.

5. Interactions of SP-A with fungi and yeasts

Under normal conditions, fungal infections of the lungs are rare. Yet, fungi like *Cryptococcus neoformans* and *Aspergillus fumigatus* are commonly found in the environment and are continuously inhaled. This suggests that efficient defence mechanisms must exist to protect against these fungi. Cryptococcosis, caused by *C. neoformans*, occurs mostly in the immunocompromised host, especially those with defects of cell mediated immunity. It is the leading mycological cause of death among AIDS patients. *A. fumigatus* is one of the major airborne fungi and causes, depending on the host's immune status, many different diseases, ranging from allergic to invasive clinical conditions. Schelenz and co-workers provided the first evidence that SP-A and SP-D might be involved in the immediate protection against fungal lung infections [58]. It was found that SP-A and SP-D bind to the cell wall of acapsular *C. neoformans*. Two serum collectins, MBP and CL-43, but not conglutinin, bound to the cell wall of *C. neoformans*. The CRD is involved in the interaction of SP-A and SP-D with this fungus. Binding was inhibited in the absence of Ca^{2+} and in the presence of sugars. The interaction of human SP-A with *C. neoformans* could be effectively inhibited by *N*-acetylmannosamine and *N*-acetylglucosamine [58]. In contrast, no binding of the collectins was observed with the encapsulated form of the yeast. SP-D, but not SP-A causes agglutination of acapsular *C. neoformans*. In a subsequent study Schelenz presented evidence that SP-A and SP-D bind *Aspergillus* conidia of various species [59].

Madan and co-workers conducted a study to find out whether SP-A and SP-D enhanced binding, phagocytosis, activation, and killing of *A. fumigatus* conidia by human alveolar macrophages and circulating neutrophils [60]. It was found that both SP-A and SP-D bound *A. fumigatus* conidia in a Ca^{2+}-dependent fashion. Mannose and maltose inhibited binding, mannose being more competitive than maltose for SP-A binding and maltose being more competitive than mannose for SP-D. Interestingly, both SP-D and SP-A agglutinated conidia and enhanced binding of conidia to alveolar macrophages and neutrophils. In addition, both collectins acted as opsonins for the uptake of conidia by neutrophils and increased oxidative burst and killing of *A. fumigatus* conidia. These observations strongly suggested that both SP-A and SP-D have important roles in host defence against fungal infections of the lung by enhancing uptake of the conidia, increasing activation of the cellular respiratory burst, and ultimately killing the infectious pathogens. In a subsequent study, Madan and co-workers also provided evidence that SP-A and SP-D play a role in the modulation of allergic sensitization and/or development of allergic reactions to the allergens of *A. fumigatus* (see below). SP-A did not act as an opsonin for phagocytosis of *Candida albicans* by alveolar macrophages [61]. Unexpectedly, SP-A inhibited the up-regulated phagocytosis of serum-opsonized yeast by alveolar macrophages in suspension. C1q and concanavalin A had a similar effect [61].

P. carinii is also a common cause of life-threatening pneumonia in immunodeficient patients. Zimmerman and collaborators were the first to show that the mannose-rich surface membrane glycoprotein gp120 of *P. carinii* is a ligand for rat SP-A [62]. Binding of SP-A to *P. carinii* is time- and Ca^{2+}-dependent and could be inhibited by mannosyl albumin, indicating that SP-A binds with its CRD to *P. carinii*. Further investigations revealed that rat SP-A binds to the carbohydrate domains of the major surface glycoprotein of *P. carinii* [63]. Mutant recombinant SP-A molecules with substitutions for the predicted calcium- and carbohydrate-coordinating residues of the CRD failed to bind to the major surface glycoprotein, indicating CRD mediated binding to *P. carinii*. The oligosaccharide moiety of SP-A is not required for the binding of SP-A to the major surface glycoprotein [63]. In another study it was found that SP-A enhanced attachment of *P. carinii* to rat alveolar macrophage monolayers [38]. SP-A mediated attachment was significantly reduced in the presence of EGTA and mannose, but also by type V collagen. Table 1 gives an overview of the interactions of SP-A with pathogens.

6. Interactions of SP-A with inhaled glycoconjugates

Since SP-A is a calcium-dependent lectin it can potentially bind to any inhaled glycoconjugate bearing sugars that can be recognized by SP-A. It is

conceivable that binding by SP-A (or SP-D) may affect the fate of the inhaled substance. The first study that dealt with SP-A binding to relevant carbohydrate containing particles other than pathogens was the study of Malhotra and co-workers [64]. These investigators showed that SP-A binds to a variety of pollen grains, including pollen from *Populus nigra italica* (Lombardy poplar), *Poa pratensis* (Kentucky blue grass), *Secale cerale* (cultivated rye) and *Ambrosia elatior* (short ragweed) [64]. Interaction of SP-A with *Pop. nigra italica* pollen may involve interaction with 57 kDa and 7 kDa glycoproteins since SP-A binds in a calcium-dependent fashion to aqueous extracts of pollen grains containing these proteins. The addition of 50 mM mannose to the microtitre plate wells prevented this interaction. Moreover, calcium-dependent binding to the pollen grains was lost after aqueous extraction at 4°C for 48 h. Binding of SP-A to pollen grains was found to mediate adhesion of pollen grains to A549 cells [64]. Unfortunately, this study is the only study sofar dealing with collectin binding to plant-derived materials that are able to elicit an allergic response.

In another study aimed to determine whether SP-A and SP-D may play a role in the modulation of the allergic response it was investigated whether these collectins interact with mite allergens [65]. It was found that SP-A and SP-D bind to whole mite extracts (*Dermatophagoides pteronyssinus*) and the purified allergen *Der p* I in a carbohydrate-specific and calcium-dependent manner. *Der p* I is one of the three major allergens in the faecal pellets of the house dust mite. SP-A and SP-D did neither bind to deglycosylated *Der p* I nor to recombinant forms of the allergen polypeptides which were expressed in *E. coli* in non-glycosylated forms [65]. Finally, it was demonstrated that SP-A and SP-D inhibit allergen-specific IgE binding to the mite extract. The precise nature of this inhibition was not clear. It could be that the surfactant proteins and IgE share the same binding site or exert a steric hindrance effect, thus preventing binding of IgE. The authors concluded that it is possible that SP-A and SP-D are involved in the modulation of allergen sensitization and/or the development of allergic reactions. Recently, investigators from the same group and others provided evidence that allergen binding by SP-A and SP-D may be a general phenomenon [66]. It was found that these collectins bind to the purified allergens gp55 and gp45 of the opportunistic fungal pathogen *A. fumigatus* in a calcium-dependent fashion. Mannose and maltose could partially block binding of SP-A, respectively SP-D, indicating that the carbohydrate binding domain of these collectins was involved. Deglycosylated allergens were not bound. In line with what was found with mite extracts, SP-A and SP-D could inhibit the ability of allergen-specific IgE from Aspergillosis patients to bind to the allergens. Moreover, both SP-A and SP-D were able to inhibit *A. fumigatus* allergen-induced histamine release from sensitized basophils [66]. Thus, both SP-A and SP-D may modulate the allergic response to a wide variety of glycosylated inhaled antigens.

Table 1
Pathogens bound by SP-A

Herpes simplex virus type 1	[26,27]
Influenza A virus (H3N2)	[28,31]
S. aureus	[41,42]
E. coli J5	[45]
St. pneumoniae	[41]
Group A *Streptococcus*	[46]
Group B *Streptococcus*	[56]
H. influenzae (type a)	[46,47]
M. tuberculosis	[39]
B. Calmette-Guérin	[49]
K. pneumoniae	[50]
My. pulmonis	[51]
C. neoformans	[58]
A. fumigatus	[59,60]
P. carinii	[62,63]

7. SP-A: an acute phase protein?

Intratracheally delivered LPS increased tissue levels of SP-A in rats within 6 h and SP-A levels in lavage 72 h after administration [67]. Furthermore, SP-A mRNA levels increased significantly at 24 h. It was concluded that SP-A reacted in a manner analogous to that of mannose-binding protein, a serum-acute phase reactant. Although these results were in line with what would be expected, the increase of SP-A could also be a response to lung injury. Sugahara and co-workers showed that on the long term intratracheal administration of LPS resulted in a marked proliferation of alveolar epithelial cells with a con-

comitant increase of SP-A mRNA [68]. In another study it was investigated whether SP-A is involved in the immediate reaction to intratracheally aerosolized LPS [3]. In this study SP-A levels in lavage were monitored at short time intervals after LPS administration. SP-A levels rose sharply within the first hour after LPS aerosolization and peaked 10 h after LPS, indicating that SP-A is involved in the acute phase reaction to intratracheally aerosolized LPS. The rapid increase suggested that enhanced secretion rather than synthesis caused this 'acute-phase' behaviour [69]. It was not investigated whether type II cells or Clara cells are responsible for the enhanced secretion. These studies suggest that SP-A (and SP-D) are involved in the immediate response to infectious challenge. The first event may be enhanced secretion of these collectins, possibly by Clara cells that contain granules filled with SP-A and SP-D [2]. The second event may be enhanced transcription, resulting in a higher production of SP-A and SP-D.

8. Future developments

It may be concluded that SP-A interacts with a wide variety of pathogens that potentially could infect the lungs. The experiments using mice lacking SP-A [56] showed, for the first time, that SP-A may have an important role preventing lung infections. Now it is clear that SP-A (and SP-D) are involved in innate host defence, it is worthwhile to delineate the mechanisms that regulate collectin-mediated lung defence upon infectious challenge. It will particularly be important to determine the relative roles of type II cells and Clara cells in this defence system. Studies in vitro and in vivo are required that are aimed to resolve the signals (pathogen-derived mediators, cytokines, chemokines, growth factors) involved in the pro-inflammatory network that lead to enhanced secretion and production of SP-A and SP-D by epithelial cells. In this respect it is of interest to take into account that these surfactant proteins may be involved in the intercellular communication between alveolar macrophages and epithelial cells. Therefore, it will be necessary to find out whether these collectins act as pro-inflammatory mediators. An important issue that has not been addressed is the possibility that, in situ, SP-A (and SP-D) may bind molecules such as C-reactive protein, immunoglobulins, SP-B, proteases, etc., that could exert a variety of effects. Processes like complement activation, pathways that link innate defence with the adaptive immune response, recognition of pathogens, regulation of cell proliferation, etc., could be regulated by SP-A and SP-D in concert with other proteins.

More support for the role of SP-A in innate lung defence may come from the genetic screening of patients with recurrent lung infections, particularly during early childhood. This will not be an easy task, since SP-A is coded by two genes and several allelic variants have been described. The identification of mutations that are correlated with susceptibility to recurrent infections, and perhaps, the allergic response, will help to understand the pathogenesis of lung disease and the design of adequate ways to treat patients. In the near future it is of upmost importance to investigate whether SP-A, SP-D, or hybrid molecules based on domains of these collectins, are able to prevent or to treat lung infection in experimental animals. These studies may result in the development of drugs based on collectins. Finally, the expression of SP-A at sites of the body other than the lung suggests that SP-A-mediated innate defence may be important at a variety of mucosal surfaces [70–73]. It will be exciting to establish the role of surfactant components in tissues other than the lung.

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