

REVIEW

SP-D and regulation of the pulmonary innate immune system in allergic airway changes

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Summary

The airway mucosal surfaces are constantly exposed to inhaled particles that can be potentially toxic, infectious or allergenic and should elicit inflammatory changes. The proximal and distal air spaces, however, are normally infection and inflammation free due to a specialized interplay between cellular and molecular components of the pulmonary innate immune system. Surfactant protein D (SP-D) is an epithelial-cell-derived immune modulator that belongs to the small family of structurally related Ca^{2+} -dependent C-type collagen-like lectins. While collectins can be detected in mucosal surfaces of various organs, SP-A and SP-D (the 'lung collectins') are constitutively expressed in the lung at high concentrations. Both proteins are considered important players of the pulmonary immune responses. Under normal conditions however, SP-A^{-/-} mice display no pathological features in the lung. SP-D^{-/-} mice, on the other hand, show chronic inflammatory alterations indicating a special importance of this molecule in regulating immune homeostasis and the function of the innate immune cells. Recent studies in our laboratory and others implied significant associations between changes in SP-D levels and the presence of airway inflammation both in animal models and patients raising a potential usefulness of this molecule as a disease biomarker. Research on wild-type and mutant recombinant molecules *in vivo* and *in vitro* showed that SP-D binds carbohydrates, lipids and nucleic acids with a broad spectrum specificity and initiates phagocytosis of inhaled pathogens as well as apoptotic cells. Investigations on gene-deficient and conditional over expressor mice in addition, provided evidence that SP-D directly modulates macrophage and dendritic cell function as well as T cell-dependent inflammatory events. Thus, SP-D has a unique, dual functional capacity to induce pathogen elimination on the one hand and control of pro-inflammatory mechanisms on the other, suggesting a potential suitability for therapeutic prevention and treatment of chronic airway inflammation without compromising the host defence function of the airways. This paper will review recent findings on the mechanisms of immune-protective function of SP-D in the lung.

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Introduction

The pulmonary immune system is faced with the dual task of elimination of inhaled pathogens and maintenance of an inflammation-free mucosal environment. Although physical barriers filter out most of the inhaled material, a large amount of small particles (< 10 µm in diameter) still reaches the distal air spaces where they encounter components of the innate immune system: alveolar macrophages, dendritic cells and the lung collectins including surfactant protein D (SP-D). Originally identified as a surfactant-associated protein [1], SP-D is expressed not

only in the alveolar epithelial cells but also in the epithelium of proximal airways. Although it is constantly released into the airways and alveolar spaces, *de novo* SP-D expression markedly increases in response to acute lung injury and inflammation. Previous work from our laboratory and others showed that SP-D increased in a time-dependent fashion, coincident with the resolution of the inflammatory response several days after a single allergen challenge [2, 3] or ozone (O₃) exposure [4] in mice. This increased expression was mediated by pro-inflammatory cytokines (IL-4/IL-13 and IL-6, respectively), and was preceded by mRNA transcription [3, 5]. Although a

number of transcription factors were implicated in the rapid induction of SP-D mRNA, the exact pathways that can lead to an enhanced release of this lung collectin still need to be discovered.

A large body of evidence suggests that SP-D expression in the airways is essential for restoring immune homeostasis following the acute inflammatory response [2, 3, 6–8]. How this molecule works exactly is not well understood but the C-terminal lectin 'head' is implicated in the direct suppression of immune and inflammatory cell activation. On the other hand, the N-terminal collagen tail of SP-D is thought to facilitate the clearance (phagocytosis) of pathogens and apoptotic cells (similar to the triple-helical collagen portion of other molecules with related basic structures such as SP-A, mannose-binding lectin and C1q). Identification of the membrane structures that can mediate these different functions is the current subject of intense investigations by a number of laboratories. The picture is complicated by the fact that SP-D undergoes post-translational modifications in response to oxidative changes in the lung chemical environment, which can profoundly alter the molecule's complex oligomeric structure as well as its function.

Most of our current mechanistic information on the immunomodulatory role of SP-D are derived from studies conducted in mice. These studies, however, have shown not only relevance to human disease but also a potential for therapeutic manipulation of the pulmonary immune system. Models of low or no SP-D expression in different mouse strains and gene-manipulated mice mimic patients who have low SP-D levels in the lung due to genetic reasons or as a consequence of chronic inflammation. For example, SP-D-deficient mice display chronic airway inflammation and a constitutive activation of macrophages and dendritic cells in the proximal and distal air spaces with an increased presence of apoptotic cells. We recently described that current smoker COPD patients had markedly diminished SP-D levels in the lung and lower SP-D was associated with a more severe disease [9]. We also showed that bronchoalveolar macrophages from COPD patients with decreased SP-D had an inability to clear apoptotic cells [10]. Further, changes in serum SP-D levels in healthy volunteers were associated with changes in sputum dendritic cell phenotype in response to O₃ inhalation [11]. Locally administered SP-D has been protective in mouse models of airway inflammation and it is possible that it will work in patients too. SP-D has a high degree of evolutionary conservation and showed biological cross-reactivity among different classes of vertebrates including humans [12–15]. SP-D is not only an anti-inflammatory agent but also an important opsonin that binds a wide range of common respiratory pathogens. Thus, unlike the traditional immunosuppressive therapies, an SP-D-based anti-inflammatory approach may have the advantage of a preserved host defence function.

The surfactant protein D structure and function: general considerations

SP-D was described in 1988 as a surfactant-associated collagenous glycoprotein with primary sequence similarities to SP-A, by the laboratory of E. Crouch [1]. Pulmonary surfactant, a phospholipid material that lines the air-liquid interface in the distal air spaces (discovered by Clements and colleagues in the early 1960s [16, 17]), contains four unique proteins: SP-A, SP-B, SP-C and SP-D. The small, hydrophobic molecules of SP-B and SP-C are essential in the structural organization of surfactant and the maintenance of low surface tension. On the other hand, SP-A and SP-D are large, hydrophilic molecules with host defence and immune regulatory functions. By the mid 1980s, cDNA and genomic sequencing revealed that, unlike SP-B and SP-C, lung collectins contain collagenous sequences [18–21] and belong to a separate class of molecules with similar collagen-like lectin structures.

The gene for SP-D is clustered together with the SP-A1, SP-A2 and mannose-binding lectin (MBL) genes on the long arm of chromosome 10q22–23 [22] and is located proximally to the centromere at about 80–100 kb from the SP-A2 gene. The collagen-like sequences are encoded by several short exons [19, 23–25]. The overall length of the collagen domain in each specific collectin is determined by the number of tandem exon duplications. The intron-exon organization (one intron is inserted between two exon duplicates) resembles the genes of non-fibrillar collagens [23] suggesting an evolutionary relationship. The neck domain and the carbohydrate recognition domain (CRD) are each encoded by single discrete exons. The similar structure of the collectin genes indicates that they evolved by duplication from a common ancestral gene, which was probably formed by exon shuffling between genes encoding non-fibrillar collagens and a primordial lectin [26].

While the first collagenous lectin, bovine conglutinin was discovered over a hundred years ago, the significance of carbohydrate recognition, a common essential function of all collectins, was only recognized in the 1950s (reviewed by Gupta and Surolia [27]). The first direct demonstration of lectin-mediated binding of SP-D to Gram-negative bacteria and the resultant bacterial aggregation was published in 1992 by Kuan and colleagues, suggesting a possible role for this molecule in pulmonary host defence [28]. The capability to differentiate between various carbohydrate moieties on pathogen surfaces is today considered an important functional feature of collectins.

In 1989, Tenner and colleagues showed that the triple-helical collagen structure of the first component of complement C1q [29] was similar to that of the collectins and that it could mediate phagocytosis through the C1q-receptor (C1qR) by monocytes/macrophages [30]. The C1qR was later shown to be identical to cell surface calreticulin [31–33]. Although binding to calreticulin does

not explain all the biological activities of SP-D, these discoveries prompted a large array of studies on the capability of lung collectins to directly modulate innate immune cell function. Today, we have compelling evidence that SP-D is a potent cellular modulator of immune responses in the lung (reviewed by Wright and colleagues [34–37]). The most recent piece of this evidence comes from studies on genetically modified mice [38–43]. Even though there were discrepancies related to the different background strains or the methods used for genetic manipulations, the collectin gene inactivation generally increased susceptibility to infectious agents, enhanced acute inflammatory response to pathogen exposure and vulnerability to chronic inflammation [26, 38, 40, 42–57]. It is important to note that under normal, non-inflammatory conditions only the SP-D^{-/-} mice but not the SP-A^{-/-} mice display an abnormal immune activation, highlighting the importance of SP-D vs. SP-A in an essential regulation of the pulmonary immune system.

Role of surfactant protein D in allergen and pathogen uptake by phagocytes

The consequence of pathogen binding and aggregation is a more efficient phagocytic elimination. In addition to direct opsonization, collectin-enhanced allergenic particle and pathogen uptake by phagocytes may occur through the regulation of cell-surface pattern recognition receptor expression [34]. Pattern recognition receptors play a major part in host defence and control tissue homeostasis within multicellular hosts [58]. Interactions between collectins and these receptors are significant during both infectious and allergen-induced challenges in the lung. For instance, enhanced mannose receptor expression was shown on human monocyte-derived macrophages involving both the attached sugars and the collagen-like collectin domain. In contrast, alveolar macrophages from collectin-deficient mice had reduced mannose receptor expression when compared with wild-type mice [59]. Such a positive regulation between collectins and pattern recognition receptors was also suggested in another study, using a model of allergic sensitization: Lack of SP-D in gene-deficient mice resulted in a failure to increase TLR4 expression during allergic inflammation in comparison with wild-type mice [60].

Surfactant protein D pattern recognition: carbohydrate and lipid binding

A large body of evidence suggests that CRD-dependent attachment is important between collectins and inhaled particles (reviewed by Hakansson and Reid [61]) and that there is an essential role for the collagen-like domain and the degree of oligomerization for pathogen aggregation and subsequent phagocytosis [62]. SP-D recognizes a wide

variety of pathogen surfaces by a broad specificity that is achieved by a very open binding pocket in the CRD. Pattern recognition by the collectins entails the ability to bind lipopolysaccharide, lipoteichoic acid and mannan over the less suitable terminal monosaccharides and macropatterns of mammalian glycoconjugates. Distinguishing between self and non-self can be also achieved through the recognition of a specific orientation of hydroxyl groups of hexoses. These are predominantly found in mannose, fucose, *N*-acetyl-*D*-glucosamine and glucose [63], which are often located in repeating structures on the surface of microbes as a result of glycosylation enzymes that differ from higher organisms. Further, organization of the individual low-affinity sugar-binding sites into trimeric arrays in the collectin CRD allows multivalent ligands to have a matching ligand topology (bridging a distance of 45–53 Å), which is important to achieve high-affinity binding [26, 33, 35].

In addition to the common carbohydrate-binding activities the collectins have, SP-D is characterized by a relative monosaccharide preference [64]. For instance, while both SP-A and SP-D could recognize the carbohydrate structure of *Aspergillus fumigatus* [65–67], the interaction with SP-A is inhibited with mannose but with SP-D it can be blocked with maltose [68]. The order of carbohydrate preference for human SP-D in solid-phase competition assays using maltosyl-bovine serum albumin as the ligand, is approximately: maltose (inositol) > glucose, mannose, fucose > galactose, lactose, glucosamine > *N*-acetylglucosamine (reviewed by Crouch [69]). Saccharide specificity may be determined by the trimeric neck+CRD domains, given that bacterially expressed trimeric CRDs show the same saccharide-inhibition profile as natural human SP-D [70].

Binding of SP-D to lipid moieties may also have important functional consequences. Interactions with surfactant lipids is required to optimally orient the CRDs in relation to the air/lipid or lipid/hypophase (the aqueous lining of the pulmonary epithelium) interface to facilitate the uptake of foreign materials embedded in the surfactant layer of the proximal and distal air spaces. Exposed hydrophobic sites could also mediate interactions with hydrophobic domains displayed on various microorganisms, and perhaps stabilize relatively weak lectin-dependent interactions [69]. The major surfactant-associated ligand of SP-D is phosphatidylinositol (PI) but it also binds to glucosylceramide. The physiological significance of this binding is supported by a compelling demonstration that SP-D^{-/-} mice have striking pathological alterations in the lung including increased surfactant DPPC pool size and abnormalities in the morphology and function of type II alveolar epithelial cells and alveolar macrophages and consequentially in lung structure [26, 38]. Because it is unlikely that SP-D has a primary role in normal surfactant metabolism, these changes could be secondary to an enhanced inflammatory state of the SP-D^{-/-} lung.

Binding to nucleic acids and removal of apoptotic cells

SP-D binds nucleic acids from a variety of origins, including components of disrupted cells (cell debris), liberated and cell surface DNA, thereby contributing to general clearance activities. SP-D effectively binds and aggregates alveolar macrophage DNA, and it enhances the uptake of DNA by human monocytic cells. Binding to cell-surface DNA might be one mechanism by which collectins mediate enhanced phagocytosis of apoptotic cells. Indeed, SP-D is capable of recognizing and binding to apoptotic cells and facilitates their removal by macrophages [71–75]. Collectins may recognize damaged cells via either their CRD [76], collagen region [72] or independently from those [77].

Several receptors were proposed as candidates to mediate apoptotic cell removal by collectins. Among these, currently the calreticulin/CD91 complex appears to be the most accepted [74]. The role of the collagen domain-mediated calreticulin/CD91 binding, however, was contradicted by an *in vivo* study in which the treatment of SP-D^{-/-} mice with a C-terminal mutant SP-D molecule (that does not contain the collagen domain) restored the macrophage defect and reduced the number of apoptotic cells in the lung [55].

Aggregation and phagocytosis of inhaled pathogens and allergens

Critical evidence for the significance of collectin-mediated phagocytosis of inhaled pathogens were provided by studies on bacterial infection. Collectin gene-targeted mice [47] are highly susceptible to group B *Streptococcus* [78, 79], *Haemophilus influenzae* [44] and *Pseudomonas aeruginosa* infections [80]. Collectin-enhanced phagocytosis and aggregation before uptake was also important for *Escherichia coli* [28], *Streptococcus pneumoniae*, *Staphylococcus aureus* [81] and *Cryptococcus neoformans* [82]. Phagocytosis through direct SP-D binding was shown for *P. aeruginosa* [83], *H. influenzae* [44] and the unencapsulated variants of *Klebsiella pneumoniae* [84]. It is important that SP-D is also involved in neutralization and clearance of inhaled endotoxin [85]. SP-D prefers rough LPS to the smooth LPS [86] and unlike SP-A, SP-D does not recognize lipid A as a ligand [87]. In addition, the LPS component of the pathogens has been identified as a major ligand for SP-D on *E. coli* [28], *K. pneumoniae* and *P. aeruginosa* [70]. The binding occurs via the CRD, and accordingly is Ca dependent.

Fungal pathogens and allergens frequently gain access to the host via the respiratory tract. Under normal conditions, fungal infections of the lung are very rare, although fungi like *C. neoformans* and *A. fumigatus* are commonly found in the environment and are continuously inhaled. This suggests that efficient defence mechanisms must exist to protect against these pathogens. The acidic

capsule of the fungi is the most important in their pathogenesis as it inhibits phagocytosis and is poorly immunogenic, and thus it enables the infectious agents to persist for extended periods in the host [88, 89]. Both rat and human SP-D can bind to *Pneumocystis carinii* via its CRD [90] and mediate its aggregation *in vitro* [91]. Unlike SP-A, SP-D enhances the agglutination of unencapsulated *C. neoformans* [82]. The clinical importance of these observations is illustrated by the fact that a heightened susceptibility of children with cystic fibrosis to a range of infectious and allergenic pathogens is linked with extremely low levels of SP-A and SP-D in the BAL fluid [92].

Depending on the host's immunological and genetic status, different types of *A. fumigatus*-associated respiratory diseases have been recognized, the most common of which is allergic bronchopulmonary aspergillosis (ABPA). When the lung of an immunocompromised subject is exposed to *A. fumigatus*, a systemic infection, invasive aspergillosis ensues. In an immunocompetent subject, however, it often results in IgE-mediated asthma. A number of studies using murine models of ABPA or allergic airway sensitization have emphasized the importance of lung collectins in protection against *A. fumigatus*-induced pathologies. Treatment with recombinant collectins not only protected against mortality but also inhibited the Ig, eosinophil and Th2 cytokine responses in murine models [3, 93–97].

In addition to fungi, both lung collectins were shown to bind inhaled allergenic particles of a wide range of sources. For example, pollen starch granules extracted from *Dactylis glomerata* (Cocksfoot grass) were aggregated by the CRD of SP-D [98, 99] and this interaction inhibited the release of β -hexosaminidase by peritoneal mast cells. SP-A also readily bound water-extractable particles of various pollen grains including pollen from *Populus nigra italica* (Lombardy poplar), *Poa pratensis* (Kentucky blue grass), *Secale cereale* (Cultivated rye) and *Ambrosia elatior* (Short ragweed) [100]. SP-D and SP-A were also shown to bind to whole mite extracts (*Dermatophagoides pteronyssinus*) and the purified allergen Der p 1 in a carbohydrate-specific and calcium-dependent manner [101, 102]. Lung collectins, in fact, inhibited mite-extract-specific IgE binding and histamine release *in vitro* [103] indicating a protective importance of calcium and sugar-dependent allergen binding by collectins [104].

Binding to cell surface receptors: the importance of a dual biological activity

Binding through the collagen tail

The first indications of collectin binding to immune cells came from studies in 1989 investigating the C1qR function on alveolar macrophage [29]. The details of cell function modulation by the lung collectins are still not

well understood. Because these proteins are 'sticky' and strongly bind carbohydrate, lipid and protein ligands, promiscuous binding partners and several putative receptors have been identified. The amino-terminal collagen tail of SP-D may serve as a ligand for the collectin receptor [100, 105]. This receptor, originally termed cC1qR, was later shown to be identical to calreticulin. As the endoplasmic reticulum (ER) membrane fuses with the plasma membrane during phagocytosis, calreticulin (which is a cytoplasmic molecule with an ER retention sequence) is expressed on the cell surface during this process. Calreticulin does not have a transmembrane domain but appears to mediate signalling through the LDL-receptor-related protein, CD91 [32] which is found on macrophages [35, 106, 107]. As calreticulin, CD91, SP-A and SP-D can all bind multiple ligands in a promiscuous manner, the importance and specificity of this receptor-ligand mechanism need further investigations. Although the calreticulin/CD91 complex explains some of the biological actions mediated by SP-D, it is still unclear whether ER recruitment and calreticulin binding are necessary and/or sufficient for phagocytosis [32, 74, 105, 108, 109]. Through collagen receptor binding, collectins were shown to up-regulate pattern recognition receptor expression [59], stimulate cytokine [110–115] and reactive molecular species production [116, 117], induce chemotaxis [118–120] and influence intracellular signalling pathways [109, 119, 121] in innate immune cells.

The collagenous tail may also act as a ligand for receptors other than calreticulin. A 340-kDa glycoprotein (gp-340) was originally identified as a pulmonary SP-D-binding protein [122] but later it was shown to also bind SP-A [123] in the presence of calcium but independent of the CRD region. This protein has both a soluble and a membrane-associated form and functions as an opsonin receptor for various pathogens [124–126]. Gp-340, however, has a questionable significance as its presence on the membrane surface was not necessary to mediate collectin function (chemotaxis) on macrophages [123].

Binding through the carbohydrate recognition domain

CRD-specific interactions with lung cells may be mediated through a number of receptors. Apart from pathogen binding, the CRD region has been involved in modulating functions of various cell types of the immune system. The ability of SP-D to exert chemotactic activity on neutrophils and monocytes for instance was suggested to depend on its lectin activity [127]. In fact, the functionally active part of the lectin head of the lung collectins in immunosuppression appeared to be the CRD [52, 55, 81, 128], which was specifically inhibited by treatment with sugars (glucose) and calcium chelation [103].

Through the CRD, SP-D can also recognize, bind and modulate function of CD14 [129, 130], TLR2 [131, 132]

and TLR4 [121, 132]. SP-D bound both TLR2 and TLR4 in a concentration- and calcium-dependent manner. Epitope mapping with recombinant proteins consisting of the CRD or the neck domain plus CRD, indicated that human SP-D binds the extracellular domains of TLR2 and TLR4 through its CRD by a mechanism different from its binding to PI and LPS [132].

Dual collectin activity on innate immune cells

Early indications of dual collectin activity on macrophages came from studies on SP-A and mycobacterial infections: SP-A enhanced phagocytosis of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG by alveolar macrophages [133, 134] and augmented the production of IL-6 in the presence of infection. In the absence of infection however, SP-A inhibited IL-6 [135]. These studies suggested that the nature of collectin-mediated immune functions may depend on whether an immune intervention is needed following infection or allergen exposure or whether an immunosuppressive/anti-inflammatory action would be necessary to prevent chronic tissue damage during inflammatory changes. SP-A (but not C1q and type IV collagen) was also shown to inhibit *Candida albicans*-induced cytokine production by macrophages [136] independent of *Candida*-SP-A binding or C1qR occupancy. These data confirmed that collectin inhibition of innate immune cells occurs by a direct binding to the cell through the lectin head and it is not related to the collagen receptor binding.

Gardai and colleagues provided *in vitro* as well as *in vivo* evidence that SP-A and SP-D (but not MBL or C1q) binding to signal inhibitory regulatory protein (SIRP- α) on macrophages through their lectin head inhibited LPS- and H₂O₂-stimulated cytokine production [109] as well as phagocytosis [137]. SIRP- α is a type I transmembrane receptor of the Ig superfamily expressed on dendritic cells as well as other leucocytes of the myelomonocytic lineage (monocytes, macrophages and neutrophils). The SIRP- α intracellular domain contains two ITIM as well as two immunoreceptor tyrosine-based switch-like motifs, which recruit Src homology region 2 domain-containing phosphatase (SHP)-1 and -2, thus inhibiting intracellular signalling. SIRP- α is important in regulating a number of dendritic cell functions and is particularly involved in dendritic cell maturation and migration [138].

According to the 'head or tail hypothesis', (Fig. 1) SP-A and SP-D interacting with pathogens via their lectin head, and presenting their collagenous tail on phagocytes to calreticulin/CD91 induce phagocytosis and a pro-inflammatory response [109]. In subsequent studies, the Gardai group demonstrated that SP-A or SP-D binding to SIRP- α on residential alveolar macrophages via the CRD suppressed phagocytic function of these cells through SHP-1 signalling [137]. Thus, SP-D is capable of eliciting

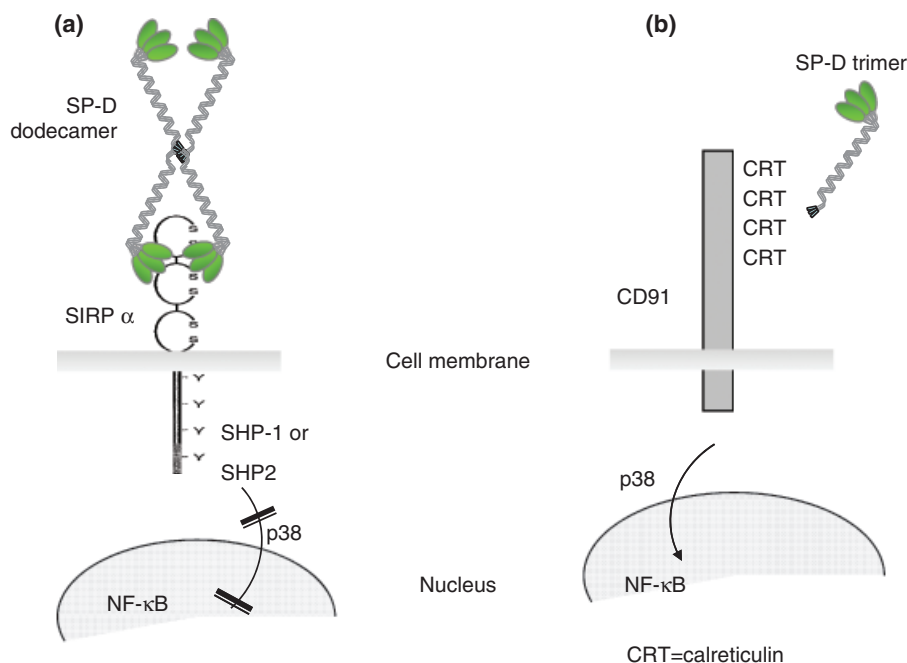


Fig. 1. The 'Head or Tail Hypothesis' (adapted from Gardai et al. [109]): SP-D is capable of differential binding through either the CRD or the collagen domain to cell membrane receptors and eliciting respective anti- or pro-inflammatory, signalling pathways. (A) SP-D is assembled as a lectin head containing the carbohydrate recognition domain (CRD), a neck region and a collagenous tail. SP-D (a 43 kDa monomer) under normal baseline conditions forms a higher order quaternary structure (usually a dodecamer) assembled from four homotrimers with the N-terminal collagen tail of the molecule hidden in the centre of the oligomeric structures bound together by cysteine residues. The C-terminal CRD binding to signal inhibitory regulatory protein (SIRP- α) inhibits cytokine production [109]. SIRP- α is expressed on dendritic cells and macrophages. It contains two ITIM as well as two immunoreceptor tyrosine-based switch-like motifs, which recruit Src homology region 2 domain-containing phosphatase (SHP)-1 and -2, thus inhibiting intracellular signalling [138]. (B) When the collagen domain of SP-D is exposed in lower oligomers (trimers), it can initiate binding to the collagen-receptor calreticulin/CD91. Ligation of this receptor complex induces pro-inflammatory cellular functions mediated by the activation of p38 and NF- κ B signalling molecules. Oxidative changes under pro-inflammatory circumstances can alter the structure of the native SP-D [119, 120, 143] leading to an impaired inhibitory function of the CRD [143] or de-oligomerization of the SP-D molecule from dodecameric to trimeric forms exposing the otherwise hidden N-terminal collagen domain [119].

differential and indeed opposing functions depending on the binding orientation [109]; CRD binding by the native molecule mediates inhibitory signals while collagen binding induces cell activation. While this hypothesis is very elegant and could explain some of the confusions that surround the role of lung collectins in innate immune regulation, a number of questions remain unanswered. For example, in case of SP-D, given the cruciform dodecameric structure, it is still unclear, how the collagen tail may bind calreticulin when pathogens are present. It is also difficult to understand why there are striking differences between the pulmonary phenotype of the SP-A $^{-/-}$ and SP-D $^{-/-}$ mice if SP-A and SP-D operated the same way. And finally, what is the relevance of all the *in vitro* and animal findings to how the human pulmonary immune system functions?

Susceptibility to oxidative damage

One possible way to make the SP-D structure available for the reversible 'head or tail' orientation is by its suscep-

ibility to oxidative post-translational modifications. The SP-D monomer forms a higher order quaternary structure (the normal, native SP-D is a dodecamer assembled from four identical homotrimers) [139]. Because of its long collagen tail, SP-D is capable of forming combined structures of these multimers, the so-called 'fuzzy balls' with a total mass of several million Da and a size of about 100 nm [140]. Under baseline conditions, the N-terminal collagen tail of the molecule is well hidden in the centre of the oligomeric structures bound together by cysteine residues. Unmasking the collagen domain of SP-D in lower oligomers (trimers) may initiate or amplify pro-inflammatory cellular changes that can play a significant modulatory role during airway inflammation. When exposed, the collagen tail of SP-D can serve as a ligand for the collagen-receptor calreticulin/CD91 [32, 74, 141, 142]. Ligation of this receptor complex induces pro-inflammatory cellular functions mediated by the activation of p38 and NF- κ B signalling molecules [109, 119]. Through the collagen receptor, collectins up-regulate pattern recognition receptor expression, stimulate cytokine and reactive

molecular species production, induce chemotaxis and influence intracellular signalling pathways in innate immune cells [109, 119, 120].

Under pro-inflammatory circumstances, oxidative changes can alter the structure of the native SP-D [119, 120, 143]. Nitration of the molecule, for example, resulted in an impaired CRD function [143]. Nitrosylation, on the other hand, induced de-oligomerization of the SP-D molecule from dodecameric to trimeric forms exposing the otherwise hidden N-terminal collagen domain [119]. We have recently showed that O₃ inhalation induced trimeric SP-D formation in the airways of mice [120, 144]. In two recent publications [119, 120], bleomycin-induced acute lung injury resulted in oxidative damage to the SP-D molecular structure with a loss of the dodecameric form. Investigations on the structural integrity of SP-D in the BAL fluid using native gel electrophoresis showed the appearance of SP-D trimers, in addition to dodecamers in the O₃-exposed mice [120]. Based on these results, we propose that inflammation induces conformational changes in SP-D and the appearance of trimers. The trimeric form may function differently as it has an exposed collagen tail (as well as a C-terminal head). Because the collagen tail can serve as a ligand for collectin receptors such as the calreticulin/CD91 [74] and engagement of this receptor may induce *pro*-inflammatory signals in dendritic cells or macrophages, it is possible that there are domain-specific changes in the SP-D molecule induced by oxidative damage. Therefore, loss of SP-D immunoprotection and activation of innate immune cells during allergic airway inflammation, could be due to impaired CRD function and/or exposure of the collagen domain with a calreticulin/CD91-binding ability. Indeed, in a model of O₃-induced exacerbation of allergic airway changes, we recently found that the appearance of abnormal, lower molecular weight forms of SP-D was associated with a heightened activation of alveolar macrophages and dendritic cells in the airways [144]. The pathogenic importance of oxidative modifications in the SP-D molecule is however still hypothetical and will require further investigations.

Collectin regulation of macrophage function

Pathogen recognition and binding by SP-D lead to aggregation, phagocytosis and a regulated release of inflammatory mediators. The importance of this mechanism is supported by evidence obtained using gene-targeted mice in models of infection and allergic sensitization as these animals show an impaired clearance of a number of bacteria, virus and fungal pathogens. A large body of studies also strongly suggests that direct binding of both collectins to phagocytes in the presence and absence of pathogens may result in different outcomes. However, under normal conditions, the lung epithelium must remain infection and inflammation free. That the lung

collectins serve an anti-inflammatory function has been clearly demonstrated more than a decade ago. Both SP-D and SP-A inhibited TNF- α and other pro-inflammatory cytokines and chemokines by LPS-stimulated human alveolar macrophages *in vitro* [136, 145]. The immune regulatory effects of the lung collectins were later extensively studied in SP-A^{-/-}, SP-D^{-/-} and double (SP-A/SP-D) knockout mice. Interestingly, while the SP-A^{-/-} mice appear normal under baseline conditions, SP-D^{-/-} and double knockout mice show serious signs of constitutive activation of the immune system indicating that SP-D has an essential immunoprotective role. That this immunoprotection parallels pathogen elimination is demonstrated by the fact that in spite of an exaggerated inflammatory response, upon injury or infection mice lacking SP-D have an impaired host defence capability [38, 43, 44, 49, 146, 147]. Cells of both the innate and adaptive immune system in SP-D^{-/-} mice display multiple abnormalities including altered morphology, constitutive activation with a heightened release of cytoplasmic enzymes, cytokines, chemokines and oxidants. The ensuing disease conditions of SP-D^{-/-} mice include an accelerated development of emphysema, susceptibility to various inflammatory stimuli and allergic sensitization and infections.

Indeed, a hallmark of the SP-D^{-/-} lung is the presence of enlarged, foamy macrophages with an increased pro-inflammatory mediator expression and high levels of MMP-9 and MMP-12 [43]. That SP-D protects against pro-inflammatory mediator release including the ones that favour the development of a Th2-type inflammation was more recently confirmed in a model of allergic sensitization. Culture of alveolar macrophages from ovalbumin-sensitized C57BL/6 mice together with SP-D and allergen resulted in an increased production of the immunosuppressive IL-10 as well as IL-12, and IFN- γ cytokines unfavourable for development of Th2-type changes [148]. SP-D also directly inhibited Th2 cytokine release in allergen-stimulated splenic mononuclear cells *in vitro*, derived from Balb/c mice sensitized with *A. fumigatus* [3].

In studies performed in the early 90s, SP-D and SP-A were shown to enhance the amount of oxygen radicals produced by alveolar macrophages and neutrophils [117] and two independent studies showed that SP-A stimulated the release of reactive oxygen metabolites [116, 149]. These reported effects, however, were not confirmed in later studies using SP-D knock-out mice, which had increased oxidant production [44, 147]. In support of the protective function of SP-D against free radical release, a recent report on allergic sensitization in mice showed that recombinant SP-D significantly inhibited allergen and LPS-induced NO production by isolated alveolar macrophages [150]. Interestingly, lung collectins had additional protective effects as they appeared to be potent endogenous inhibitors of lipid peroxidation and oxidative cellular injury [151].

Alveolar macrophages need to home to the lung in order to perform their immune surveillance function without the presence of inflammation. Lung collectins have significant chemotactic activities on alveolar macrophages. Both SP-A- and SP-D-stimulated chemotaxis in a concentration-dependent manner [152] and induced directional actin polymerization in alveolar macrophages *in vitro* [123]. In this latter study, it was also demonstrated that this effect is cell and collectin specific, a SP-A did not stimulate the chemotaxis of monocytes [118], while in another study SP-D attracted these cells [127]. Binding of collectins to macrophages probably occurred independently from the C1qR, because C1q did not show the same chemotactic effect on macrophages.

Taken together, these works indicate that SP-D recognizes and binds pathogens that leads to aggregation, phagocytosis and the regulated release of inflammatory mediators. The dual role of SP-D in immunoprotection and pathogen elimination is demonstrated in gene-deficient mice. In spite of an exaggerated inflammatory response, these animals show an impaired clearance of a number of bacteria, virus and fungal pathogens.

Effects of surfactant protein D on dendritic cells

Dendritic cell maturation

While the role of collectins in regulating alveolar macrophages has been extensively studied, the literature on how these innate immune molecules affect the function of dendritic cells is scant. Studies describing the effects of lung collectins on dendritic cell function indicated a regulatory effect in antigen presentation and T cell stimulation [153–155] but there is virtually no information on the effects of SP-A or SP-D on differentiation or migration of the pulmonary dendritic cell subpopulations. Our studies suggested that lack of SP-D in the lung of SP-D^{-/-} mice resulted in a dendritic cell population with a pro-inflammatory, myeloid phenotype and constitutive TNF- α expression. In contrast, the administration of SP-D in bone marrow dendritic cell cultures suppressed the activation marker and TNF- α expression [156] indicating that native SP-D inhibits the maturation and activation of the pro-inflammatory dendritic cells.

An important implication of both phagocytosis and the direct binding of collectins to antigen-presenting cells is the ensuing regulation of adaptive immune response. Studies on this function showed that SP-A inhibited while SP-D augmented antigen presentation by bone marrow-derived (myeloid) dendritic cells [153, 154]. SP-D, however, inhibited antigen presentation when dendritic cells were isolated from the lung [155]. Under baseline conditions, dendritic cells reside in the lung tissue in an immature state. These resting cells are scattered throughout in the bronchial and alveolar wall capturing inhaled

pathogens but unable to present them to T cells. In response to 'danger' signals such as pathogens, pro-inflammatory cytokines, or necrotic cells, dendritic cells start to mature and switch from an antigen-capturing to an antigen-presenting and a T cell-stimulating, pro-inflammatory state [157]. Although all mature dendritic cells share a common ability to process and present antigen to naïve T cells for the initiation of an immune response, they differ in origin, migratory patterns, localization and cytokine production [158]. Plasmacytoid dendritic cells make up the majority in the lung under baseline conditions [159] and these cells were shown to be tolerogenic in allergen-induced inflammation. On the other hand, myeloid dendritic cells migrate rapidly to the lung during a Th2-type inflammation and exert potent Th2 cell activation [160–164].

Dendritic cells in the BAL of SP-D^{-/-} mice had a myeloid (bone marrow derived) phenotype and constitutive TNF- α expression. In bone marrow-derived dendritic cell cultures, an administration of SP-D suppressed both the activation marker and autocrine TNF- α expression. Thus, SP-D can alter the differentiation and inhibit activation of immature dendritic cells through the inhibition of TNF- α [156]. Interestingly, dendritic cells in the lung of SP-D^{-/-} mice were unable to migrate to the regional lymph nodes and accumulated instead in the airway submucosal tissue (S. Kierstein and A. Haczku, unpublished observation), possibly due to a markedly increased expression of thymus and activation-regulated chemokine (TARC) [3].

Dendritic cell migration

Dendritic cell migration is essential for both the onset and resolution of the inflammatory airway changes. There are a number of potential chemokine receptors that can mediate epithelium-directed or lymph-node-directed migration. For example, Ccr2, Ccr4 and Ccr5 could mediate pro-inflammatory migration of immune cells into the airway epithelium. Lymph node homing of dendritic cells and lymphocytes can be facilitated by Cxcr4 and Ccr7. Nevertheless, in our model of *A. fumigatus*-induced allergic airway inflammation, the Ccl17-Ccr4 and Ccl19/21-Ccr7 ligand-receptor pairs were significantly up-regulated at the mRNA level in a time-dependent manner indicating the importance of these genes in immune cell migration in response to allergen inhalation. The expression of the Ccr4 ligand Ccl17 was selectively induced while the expression of Ccr7 was selectively abolished *in vivo* in SP-D^{-/-} mice [165, 166]. The significance of this effect of SP-D on the Ccr7 expression is that this receptor was shown to be specific not only for lymph node homing but also for immune tolerance [167]. Ccr4 is considered to be specific for Th2 cells and activated dendritic cells;

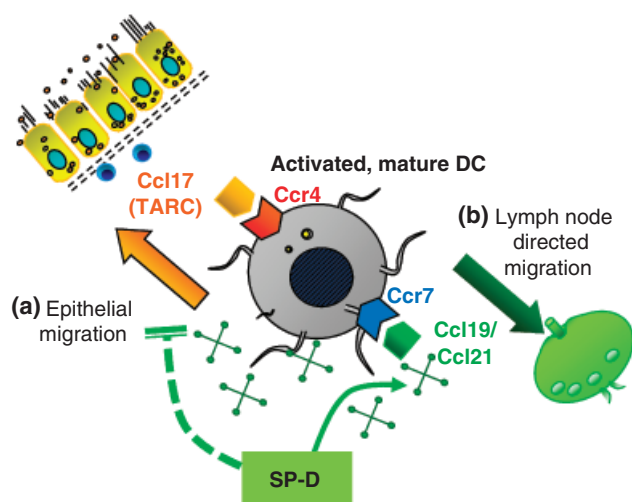


Fig. 2. Hypothetic role of SP-D in dendritic cell migration. In *Aspergillus fumigatus*-induced allergic airway inflammation, the Ccl17-Ccr4 and Ccl19/21-Ccr7 ligand-receptor pairs were significantly up-regulated at the mRNA level in a time-dependent manner in mice [4, 165, 166]. (a) SP-D prevents the epithelial migration of myeloid dendritic cells by inhibiting autocrine Ccl17 release. Ccl17 (thymus and activation-regulated chemokine, TARC) is responsible for attracting Th2 lymphocytes and activated myeloid dendritic cells to the airway submucosal tissue through its receptor, Ccr4 which is expressed specifically on Th2 cells and activated dendritic cells. (b) SP-D promotes the uptake of inhaled pathogens and the migration towards the mediastinal lymph nodes. In addition to the initiation of naïve T cells, lymph-node-directed migration serves the purpose of confining the immune response to the lymphoid area leaving the airways pathogen and inflammation free. This process is driven by Ccl19 or Ccl21, both of which act on the chemokine receptor Ccr7.

therefore, this receptor and its ligand Ccl17 (TARC) are very relevant to studies on the allergic airway response.

Ccl17 is produced by epithelial cells, macrophages and dendritic cells and is responsible for attracting Th2 lymphocytes and activated myeloid dendritic cells to the airway submucosal tissue through its receptor, Ccr4, which is expressed specifically on Th2 cells and activated dendritic cells [164]. Our studies showed that myeloid dendritic cells accumulated in the airways of SP-D^{-/-} mice [156] paralleled by the elevated expression of TARC [6] indicating that the presence of SP-D maybe important in suppressing the release of this pro-inflammatory mediator and a consequent epithelial migration of dendritic cells. Activated myeloid dendritic cells themselves release large amounts of TARC, creating a 'vicious' Th2-type inflammatory circle, which explains the susceptibility of the SP-D^{-/-} mice to allergic sensitization [6]. Because TNF- α is a potent activator of TARC release, it is possible that native SP-D or the CRD prevents epithelial migration of myeloid dendritic cells by inhibiting the TNF- α /TARC pro-inflammatory axis.

Following the uptake of inhaled pathogens, immature dendritic cells mature and start migrating towards the

mediastinal lymph nodes where they exert their antigen-presenting and T cell-stimulating function. Lymph-node-directed migration also serves the purpose of confining the immune response to the lymphoid area leaving the airways pathogen and inflammation free. This process is driven by Ccl19 or Ccl21, both of which act on the chemokine receptor Ccr7. The Ccr7 expression on dendritic cells was shown to be important not only in lymph node homing but also in protecting from autoimmune pathologies, atherosclerosis and allergic airway sensitization. We have found that the Ccr7 gene was highly expressed in the lung of wild-type mice 48 h after allergen inhalation, coincident with the inflammatory resolution [166]. Expression of Ccl19 and Ccr7 in wild-type mice paralleled the appearance of labelled dendritic cells in the thoracic lymph nodes. SP-D^{-/-} mice, however, were not able to induce Ccr7 expression and had virtually no CFSE-labelled cells in the thoracic lymph nodes after allergen challenge [166]. These intriguing data suggested that the presence of SP-D is necessary to suppress TNF- α /TARC-mediated epithelial migration and to promote lymph node-directed migration (Fig. 2). These speculations, however, will need to be substantiated and the significance of SP-D in dendritic cell migrations should be clarified.

Effects of surfactant protein D on the adaptive immune response

Pulmonary lymphocytes are hyporesponsive to a variety of antigenic stimuli when compared with peripheral blood leucocytes. Early studies provided evidence that the alveolar lining fluid contributes to the induction and maintenance of such cellular hyporesponsiveness *in vivo* and *in vitro* [168–170]. These observations were more recently confirmed by studies on gene-deficient mice. While no significant alterations have been observed in SP-A-deficient mice, in SP-D^{-/-} animals an increased percentage of both CD4⁺ and CD8⁺ T cells were found together with an increased expression of the activation markers CD69 and CD25 in the BAL [3, 41]. That the presence of SP-D can directly suppress T cell function was demonstrated *in vitro* on isolated lymphocytes in a number of studies [3, 15, 171, 172]. What are the possible mechanisms of T cell SP-D interactions? Binding and sequestering of the antigens may be one possibility to interfere with T cell activation. However, as SP-D was also capable of inhibiting mitogen-induced T cells [3, 14], blocking of antigen-T cell receptor interaction may not be the major pathway for the inhibition of lymphocyte function. Interestingly, SP-A was shown to inhibit T cell proliferation and decrease IL-2 production [173] via its collagen-like domain through surfactant protein receptor 210 (SPR-210; named after its 210 kDa size) [174]. However, no such receptor has been identified for SP-D. Further, SP-D was implicated in T cell inhibition in an

IL-2-independent manner but cell viability was not affected by this collectin [175]. The SP-D effects on mitogen- and Der p-stimulated lymphocytes were attenuated when cells were derived from asthmatic children with acute attacks [103]. These results indicated that T cells that are already activated may be resistant to the inhibitory effects of SP-D.

The *in vivo* consequences of SP-D interaction with T cell-dependent inflammatory events were demonstrated in various models of allergic airway sensitization. Treatment of allergen-sensitized mice with recombinant or purified SP-D was effective in reducing the eosinophilic inflammation and specific IgE production [93–96, 150, 176]. Furthermore, airway hyperresponsiveness was also inhibited and there was a shift from a Th2 cytokine pattern towards a Th1 response *ex vivo* [93, 94] and *in vitro* [3, 148]. SP-A- and SP-D-deficient mice exhibited intrinsic hypereosinophilia [57], increased IL-13 [3, 57, 60] and TARC [3, 177] levels in the BAL indicating a diminished control of inflammation in these animals. The currently studied SP-D^{-/-} mice are either of a mixed background or have a C57BL/6 genotype. An important question would be whether the lack of SP-D would result in more prominent effects in Balb/c mice, which are intrinsically more susceptible to allergic sensitization than C57BL/6 mice. The relevance of this question to disease is that individuals who are genetically predisposed to allergic sensitization of the airways may be protected by sufficient levels of SP-D. However, if susceptibility to allergy is paralleled with low SP-D production (due to genetic or acquired factors), a more severe disease outcome may ensue.

Significance

During the inflammatory airway response, SP-D plays immune-protective roles at multiple levels. The mechanistic significance from the onset to resolution of the inflammatory airway response is highlighted by the fact that SP-D is capable of the differential (pro- or anti-inflammatory) regulation of innate immune cell function. The outcome of SP-D-cell interactions may be dictated by the binding orientation (either via the CRD or through the collagen region [33, 109]), receptor signalling and the cytokine/inflammatory mediator milieu of the local environment [178]. The ability to achieve differential effects on the innate immune response by the same molecules carries obvious benefits. Based upon recent human studies on genetic polymorphisms and chronic inflammatory lung disease, it appears that a deficiency in expression and/or function of SP-D is associated with an enhanced susceptibility to infections and inflammation, particularly in severe conditions.

Four polymorphisms have been identified within the SP-D gene, including the non-synonymous Met11Thr,

Ala160Thr, Ser270Thr, and a synonymous Ala286Ala mutation. The Met11Thr and Ala160Thr polymorphisms in the amino terminal and collagen domain have a frequency exceeding 20%, whereas the Ser270Thr and Ala286Ala polymorphisms are relatively rare. To date, only polymorphism in amino acid 11 has been associated with disease [179, 180]. Importantly, individuals with the Met11Thr-encoding genotype had significantly lower SP-D serum levels than individuals with the Met11Met genotype [181]. The Met11Thr allele is linked with protection from severe RSV infection in infants, whereas the Met11Met allele is associated with RSV bronchiolitis, and the Thr11-coding allele is associated with a susceptibility to *M. tuberculosis* infection [180, 182]. Studies have suggested that the Met11Met allele produces SP-D of both low- and high-molecular-weight forms (monomers, trimers and dodecamers) whereas the Thr11 produces mainly low-molecular-weight structures (monomers) [181]. It was also shown that the high-molecular-weight form of SP-D has an increased binding affinity to complex microorganisms while the low-molecular weight SP-D preferentially bound LPS. Further, in a recent study, significantly more atopic black children had the Met allele compared with non-atopic black children, indicating a differential regulation of allergic sensitization [177]. It appears, therefore, that the different alleles of codon 11 can influence SP-D oligomerization, which can result in markedly different SP-D serum levels and function [181].

Nucleotide polymorphisms that do not result in amino acid changes were also identified in the lung collectin genes. These are called 'silent' or, if exons are involved, 'synonymous' mutations and generally thought to be evolutionarily neutral as these polymorphisms do not alter protein function. However, due to possible codon usage biases, translational stability, splicing, or transcriptional control of expression of the lung collectins may be significantly affected. Indeed, COPD susceptibility was associated with such polymorphisms in the SP-A and SP-D genes [183, 184]. Further studies are needed, however, to understand better the clinical significance of allelic variations in SP-D expression and function and to aid future clinical investigations into the use of collectin preparations for therapeutic manipulations of the pulmonary immune system.

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