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Role of surfactant protein A and D (SP-A and SP-D) in human antiviral host defense

Kevan L. Hartshorn

Boston University School of Medicine, Department of Medicine, Boston MA

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1. ABSTRACT

SP-A and SP-D contribute to host defense against respiratory viral infection. The most extensive body of evidence relates to influenza A viruses (IAV), and evidence from gene-deleted mice also indicate a role for surfactant collectins in defense against respiratory syncytial virus (RSV) and adenovirus. Some important respiratory pathogens including rhinovirus and metapneumovirus have not yet been examined. Viral pathogens that enter the body via the respiratory tract (e.g., Ebola virus), replicate in the lung (e.g., human immunodeficiency virus or HIV) or infect the lung in immuno-compromised hosts (e.g., herpes simplex virus or HSV) are inhibited by collectins. SP-A and SP-D are expressed in other mucosal surfaces (e.g., the eye or genitourinary tract) where they may play roles in antiviral defense. In addition to direct antiviral activities, the SP-A and SP-D modulate innate and adaptive immunity and inflammation associated with infection. The relative importance of antiviral vs anti-inflammatory effects of SP-A and SP-D in viral infections and the potential use of these collectins as therapeutics for viral infections are under investigation.

Table 1. Current data regarding antiviral activities and host defense roles of SP-A and SP-D during viral infections

VIRUS	SP-D	SP-A
IAV	Strongly inhibits infectivity by binding to oligomannose sugars on HA; Viral titers and inflammation increased in SP-D -/- mice; Contributes strongly to antiviral activity of BALF	Antiviral activity mediated by binding of viral HA to sialylated glycan on SP-A; Activity less strong than SP-D; Viral titers and inflammatory responses modestly increased in SP-A -/- mice
RSV	Viral titers increased in SP-D -/- mice; SP-D binds RSV G protein; Thr11 and Ala160 SNPs are associated with reduced risk for severe RSV infection	Viral titers and inflammation increased in SP-A -/- mice; SP-A binds to F protein; Some SP-A haplotypes correlate with risk
Parainfluenza	SP-D inhibits Sendai virus in vitro; SP-D levels rise in parainfluenza virus-infected sheep	SP-A levels rise in parainfluenza virus-infected sheep
Adenovirus		Increased viral titers and inflammation in SP-A -/- mice
SARS coronavirus	Inhibits virus in vitro; Elevated serum levels in SARS	
HSV		HSV binds to sialylated glycan on SP-A CRD reducing infectivity
HIV	Inhibits infectivity in vitro by binding to oligomannose glycans on gp120; Activity weaker than against IAV	Inhibits infection of CD4 cells with HIV; Promotes DC-Sign mediated transfer of infectious virus to T lymphocytes

2. INTRODUCTION

Our understanding of innate immunity to viral infections is rapidly evolving. This review will focus on the innate defense collectins, SP-A and SP-D, in antiviral defense. Important insights have been obtained using purified natural or recombinant proteins or human bronchoalveolar lavage fluids (BALF) in vitro and SP-A or SP-D gene-deleted mice in vivo. Some studies have associated levels of SP-A or SP-D in human BALF or blood, or single nucleotide polymorphisms of SP-A and SP-D, with viral infection. Table 1 lists major respiratory viruses and the level of evidence for antiviral effects of SP-A or SP-D. After a brief discussion of the anatomic locations in which SP-A and SP-D are expressed, we will present the clinical data that is currently available regarding their role in viral infections. It is likely that substantial additions to this clinical data base will be made in the near future. We then discuss in detail, the mechanisms of antiviral activity of the surfactant collectins against different viruses, placing most emphasis on IAV since this virus has been most extensively studied. The contributions of the collectins to direct viral inhibition and to modulation of immune responses through other means will be explored. We then evaluate the potential contributions of SP-A or SP-D during bacterial super-infections (e.g., pneumonia), that frequently complicate respiratory viral infections. Finally, we discuss the implications of these findings for therapy of viral infections using recombinant collectins or methods which increase endogenous production of the collectins.

3. SP-A AND SP-D IN HUMAN ANTIVIRAL HOST DEFENSE

3.1. Preliminary anatomic considerations –

The collectins are capable of recognizing distinctive patterns of carbohydrates present on various microbial organisms, or on infected cells. There are at least three host defense collectins in humans, two originally isolated from pulmonary fluids, SP-D and SP-A, and another from serum, mannose-binding lectin (MBL). Recent findings indicate that SP-D is not only present in the lung but also in a variety of mucosal or other epithelial surfaces in the body (1). SP-A and SP-D can be viewed as part of the mucosal immune system, and SP-D in particular resembles IgA or human beta-defensins (hBDs) in terms of its anatomical distribution. Like hBD2, SP-D expression by mucosal epithelial cells is upregulated during several inflammatory or infectious states (e.g., IAV infection or pulmonary hypersensitivity (2-4). Three additional collectins have been identified in cows, conglutinin, CL43 and CL46, all of which appear to be closely related to SP-D (5-7) but have different saccharide and microbial binding properties.

The bulk of this review will deal with the contributions of SP-A and SP-D to host defense in the respiratory tract. Although the major sites of production of SP-A and SP-D are in the lower airways and lung, they are also expressed in the upper respiratory tract, including the trachea and the Eustachian tube (8). Since several respiratory viruses induce otitis media (9-11), it may be that collectin expression in the Eustachian tube contributes to antiviral defense. There appears to be minimal expression of SP-D in saliva (12, 13). Saliva does have strong inhibitory activity for IAV and HIV but other factors play a greater role in this innate inhibitory activity (14). The relative contribution of tracheal and upper airway expression, vs. lower airway expression, of SP-A or SP-D contribute to defense against respiratory viral infections has not thus far been evaluated.

It should also be noted that expression of SP-D is not restricted to the respiratory tract but that there are low levels in blood and in a variety of mucosal or epithelial surfaces that serve as barriers to viral infections (15). For instance, SP-D is expressed in the prostate and cervical mucosa where it could modulate infection with HIV. In vitro studies suggest such a role in Chlamydia infection (16, 17). SP-D is also expressed in the eye and it contributes to host defense against *Pseudomonas aeruginosa* (18, 19). Several viruses (including IAV or HSV (20)) can use the eye as a portal of infection and hence SP-D could contribute to antiviral defense in the eye.

3.2. Clinical and epidemiological evidence for a role of SP-A and SP-D in respiratory viral infections

Relatively little firm clinical or epidemiological data supporting the role for SP-A or SP-D in respiratory infections have been gathered thus far. One problem

encountered is redundancy in innate defense mechanisms. Furthermore, whereas frank deficiency of MBL led to its identification as an important host defense protein in humans (21), inherited complete deficiencies of SP-A or SP-D have not been observed. Useful clinical data and inferences have, however, been obtained through study of polymorphic variants of the surfactant collectins, and through study of states associated with acquired decreases in SP-A or SP-D.

3.2.1. Genetic epidemiology

One fairly common single nucleotide polymorphism (SNP) of SP-D involves exchange of Threonine for Methionine at amino acid 11 in the N-terminal region of SP-D. Subjects homozygous for threonine 11 have lower serum levels of SP-D and this variant of SP-D tends to form trimers more abundantly than dodecamers or larger multimers in vitro (22). The potential significance of this structural variation will be evaluated in depth below. The Thr11 variant has been associated with reduced risk of atopy in African-American children (23) and reduced risk for severe RSV infection (24). In contrast, the Met11 variant is associated with lower risk of MTB infection (25). A recent study found that the Ala to Thr polymorphism at amino acid 160 in the CRD of SP-D also confers increased risk for RSV infection. It is clear that larger studies involving association of SNPs in SP-D and SP-A with respiratory viral infections will be of interest. This is especially true for IAV since SP-D gene-deleted mice have increased severity of IAV infection (see below) and the Thr11 variant (or other recombinant trimeric forms) of SP-D have reduced ability to inhibit IAV in vitro (26).

There are two SP-A genes (SP-A1 and 2) in humans and multiple haplotypes. Some haplotypes of SP-A have been associated with increased risks for infection (25, 27, 28). There are associations between certain SP-A haplotypes and risk of RSV infection. No differences were found between activity of SP-A1 and 2 against IAV. In vitro activity of SP-A against IAV is determined by the type of sialic acid present on SP-A and preparations lacking sialic acid lack activity against IAV (29).

Although MBL is predominantly found in blood, low levels are detected in the lung during IAV infection in mice (30) and during other pulmonary infection in humans (31). There are polymorphisms of MBL, some of which are associated with markedly reduced levels of the protein in blood and increased risk for infections of various kinds, including respiratory viral infections (32). MBL may play a role in limiting dissemination of viral infections through the blood stream but may also contribute directly to host defense in respiratory viral infection in the lung.

3.2.2. Acquired deficiency of SP-A or SP-D

Some conditions are associated with acquired deficiency of SP-D, including cystic fibrosis, chronic smoking and chronic obstructive lung disease (33-35). These conditions also predispose to, and are worsened by, respiratory viral infection. Diabetes mellitus also predisposes to severe IAV infection. In a mouse model of diabetes increased severity of IAV infection was linked to inhibition of host defense activity of SP-D by elevated glucose (36). The mechanisms of reduced SP-D or SP-A levels in some chronic inflammatory conditions could be degradation by neutrophil or bacterial proteases (37) or complexing with other released neutrophil proteins like defensins (38). SP-A and SP-D also bind to DNA and SP-D mediates clearance of DNA and apoptotic cells from the lung, and this could possibly be a mechanism of reduced levels of SP-D in some chronic inflammatory conditions.

3.3. Important biochemical and structural points for understanding functional attributes of SP-A and SP-D

The collectins all have four basic structural domains: the N-terminal, collagen, neck and carbohydrate recognition domains (CRD). The basic structural unit of all collectins is the trimer. However, the quaternary structures of collectins differ. One class of collectins includes SP-D and conglutinin which have larger collagen domains and can form crosslike structures through attachment of 4 trimers together at the N-terminus. SP-D also can exist in much larger multimeric forms including some that have up to 32 trimers in one molecule. CL43 and CL46 are highly homologous to SP-D and conglutinin. CL43 is unique among collectins in that it naturally exists exclusively as trimers (39, 40). The other family of collectins includes SP-A and MBL. These collectins structurally resemble the complement component C1q. MBL can directly fix complement whereas SP-A inhibits complement activation (41). Binding of collectins to pathogens is usually mediated by attachment of the CRD to carbohydrates on the pathogen surface; however, there are exceptions as noted below for SP-A. The collagen domain is important for maintaining structure, for interacting with specific cellular receptors, and for mediating some of the homeostatic functions of SP-D in the lung; however its role in antiviral activity is not clear. The N-terminus is critical for multimeric assembly and the ability to form multimers is an important determinant of antiviral activity.

3.4. Detailed survey of antiviral activities and mechanisms of inhibition

There are two levels that SP-A or SP-D can impact on antiviral defense: direct antiviral activities and modulation of immune responses. We will consider direct antiviral activities. The most extensive data set available regarding viral neutralization by collectins relates to IAV; hence, we will devote most space to the discussion of IAV, but will also review the data currently available regarding other viruses.

3.4.1. IAV

IAV poses a major public health challenge and because innate immune mechanisms appear to be particularly important in the first line of host defense for this virus. IAV contributes tremendous morbidity and mortality especially in the very young and elderly. Annual mortality from IAV in the USA is ~36,000 (42). Mortality in non-pandemic years is concentrated in the elderly, but IAV also causes work loss in healthy adults (43) and is a major contributor to hospitalization in children (44, 45). A major concern in the case of IAV is a potential recurrent pandemic (46, 47). Although IAV elicits strong adaptive

immune responses, it is prone to rapid genomic variation either through small incremental mutations or major changes resulting from exchange of genome segments with those of other strains (reassortment). A recent example is the H1N1 strain derived from swine. These genomic changes allow IAV to escape immune responses generated against prior strains. There is a window period of 5-7 days prior to arrival of CD8+ T cells in the lung after exposure to a new IAV strain, and it is during this time that innate defense is critical (48).

3.4.1.1. Evidence that collectins inhibit IAV and mechanism of inhibition

The first evidence of the role of collectins in immunity against IAV was the observation that mammalian serum beta-inhibitors of IAV are calcium-dependent lectins (49), which were later demonstrated to be conglutinin and MBL (50-52). SP-D (and to a lesser extent, SP-A) was then shown to inhibit infectivity of IAV and to contribute strongly to the antiviral activity of human bronchoalveolar lavage fluids (BALF) (53-56).

Gene-deleted mice have been very useful for study of potential antiviral activities of collectins. SP-D knockout mice (SP-D -/-) show increased viral titers, weight loss and inflammatory responses after IAV infection (57, 58). These effects can be corrected by instillation or over-expression of wild type or mutant forms of SP-D in the lung (2, 59, 60). Sensitivity of various IAV strains depends on the level of glycosylation of the envelope proteins, especially the HA (61). Strains lacking high mannose oligosaccharides on the globular head of the HA are resistant to SP-D, MBL or bovine serum collectins (49, 52, 56, 62, 63). Such strains generally have increased ability to replicate and cause illness in mice and their replication is not altered in SP-D -/- mice compared to controls (64, 65). Of note, the mouse-adapted PR-8 strain that is used in many murine studies of IAV infection has no high mannose oligosaccharides on its envelope proteins and is resistant to SP-D or MBL (52). Other mouse-adapted strains (e.g., WSN) similarly lack glycosylation on the HA. During the process of mouse adaptation loss of HA glycosylation has been demonstrated along with loss of susceptibility to lectin-mediated inhibition (66). Hence, murine studies with most mouse-adapted strains characterize the immune response occurring in the absence of a direct contribution of SP-D.

It is of interest that pandemic strains of IAV including those isolated in 1918, 1968 (H3N2) and 1977 (re-introduction of H1N1) have fewer glycan attachments on the HA globular head and acquire increased glycans as they evolved in the human population (67). In the case of the re-introduced H1N1 strains and H3N2 strains, the initial pandemic strains were resistant to SP-D but later strains that had more glycosylation are highly sensitive (64). Based on knowledge of glycosylation of the 1918 strain it is likely that it too is resistant to SP-D (68). Human isolates of H5N1 also have reduced glycosylation on the HA head compared to recent human H3N2 or H1N1 strains and are resistant to SP-D in vitro (61). Overall these results suggest that lack of HA glycosylation of pandemic strains may be one factor allowing more rapid spread or greater virulence, since they evade inhibition by SP-D or MBL.

Glycosylation of the HA head appears to confer adaptive advantages to IAV strains after introduction into the human population while also rendering the viruses more sensitive to collectin-mediated inhibition. This suggests that there is trade off in which added glycosylation protects the HA from antibody mediated neutralization (69) and possibly has other beneficial effects (e.g., reducing binding affinity of the HA which makes the virus less dependent on NA activity) (70), while possibly attenuating disease severity through increased sensitivity to collectins. Of interest, unlike the H1N1 and H3N2 subtypes, the H2N2 subtype had a relatively brief duration in the human population, possibly due to more limited ability of the H2 HA to accommodate added glycans near its globular tip (71).

The mechanism of direct inhibition of IAV by collectins has not been entirely worked out. SP-D and MBL appear to inhibit IAV during an early phase of the infection cycle (e.g., blocking viral uptake per se and/or HA-induced fusion) (72, 73). SP-D also is a potent viral agglutinin and this may contribute to viral neutralization by reducing effective particle numbers (56).

3.4.1.2. Structural requirements for IAV inhibition by SP-D

Binding of the CRD of SP-D to high mannose oligosaccharides on the viral HA is central to neutralization (55). Of interest, however, truncated trimers composed of only the neck and CRD (NCRD) have greatly reduced antiviral activity compared to full length SP-D. In addition, full length trimers of SP-D (including the N-terminus and collagen domain) have reduced activity compared to higher order multimers. It is likely that loss of the multimeric structure results in substantial loss in binding affinity. We have shown, for instance, that cross-linking NCRDs with certain mAbs directed against SP-D restores antiviral activity (74). Furthermore, a construct containing the N terminus, neck and CRD of rat SP-D (called SP-Dcdm; see Figure 1) forms small dodecamers and has greatly increased antiviral activity compared to trimeric NCRDs (59, 75).

The collagen domain of SP-D has been demonstrated to be critical for restoring normal lung homeostasis to SP-D -/- mice (59, 76). Transgenic expression of constructs lacking the collagen domain (SP-Dcdm) or containing the N-terminus and collagen domain of SP-A fused to the NCRD of SP-D did not rescue the abnormal phenotype of the SP-D -/- mice (i.e., the mice still had abnormal phospholipid accumulation and chronic inflammatory changes). Furthermore, the ability of SP-D to form multimers is critical for homeostatic lung functions of SP-D, since over-expression of the rSP-Dser15,20 mutant (that only forms full length trimers) did not restore normal homeostasis and even antagonized activity of endogenous SP-D in wild type mice (77). Of note, however, the collagen domain and multimerization per se were not required for antiviral activity against IAV since SP-Dcdm

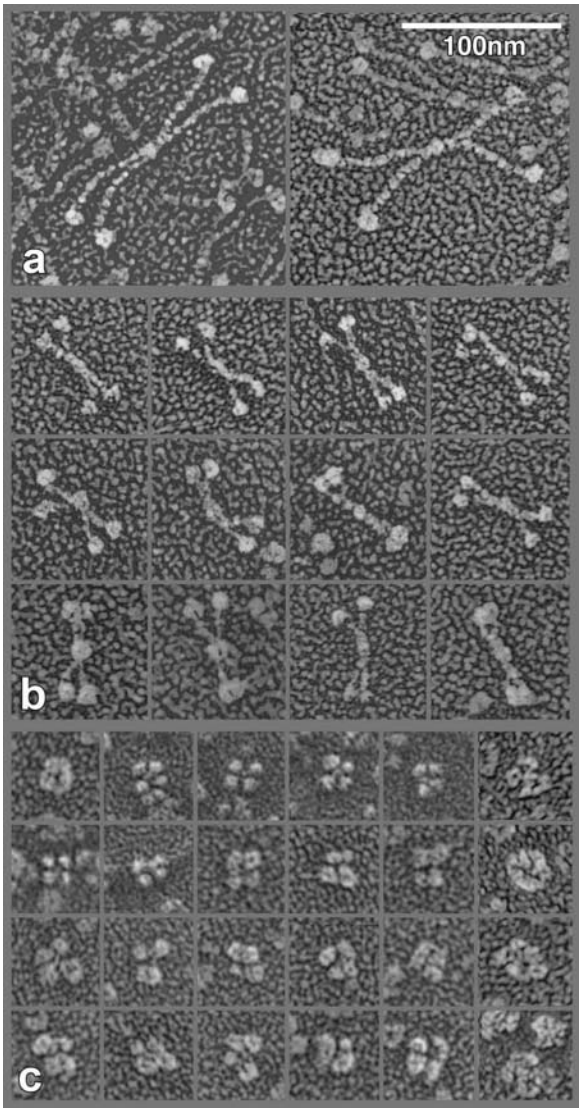

Figure 1. Ultrastructural analysis of collagen deletion mutants as compared to wild-type SP-D**.** *Panel a)* Recombinant rat SP-D dodecamers. Note the central hub, four rigid arms, and terminal globular domains. The arms of SP-D have an overall span of greater than 100nm. Previous proteolytic mapping demonstrated that the arms correspond to the pepsin-resistant, triple helical collagen domains, while the globules correspond to the collagenase-resistant C-terminal lectin domains. *Panel b)* Mini-SP-D consists of dodecamers with shortened arms. *Panel c)* SP-Dcdm consists predominantly of multimers of globular domains, with a large proportion of assemblies containing four globules, consistent with a dodecamer. Note that some assemblies, particularly in the top row, show a central connecting structure, presumed to include the N-peptide. Selected larger assemblies are shown in the column at far right. Figure taken with permission from (75).

and rSP-Dser15,20 over-expressing SP-D-/- mice contained viral replication and virus-induced inflammation (59, 60). The NCRD of SP-D also is important for some of its homeostatic functions since SP-D -/- mice over-expressing a chimeric protein containing the N-terminus and collagen domains of SP-D coupled to the NCRD of bovine conglutinin did not normalize the inflammatory phenotype of the mice in the absence of infection (although phospholipid homeostasis was restored) (60). This chimeric molecule has increased IAV neutralizing activity compared to wild type SP-D (78) and viral replication and the resultant inflammation was minimal in the mice over-expressing this protein. Hence, transgenic expression of several mutant forms of SP-D inhibits both viral replication and IAV-induced inflammation in mice lacking wild type SP-D. This suggests that the increased inflammatory response triggered by IAV in SP-D -/- mice is not directly related to the underlying pro-inflammatory phenotype of the mice per se, but to increased viral replication in the absence of SP-D. These studies also show that the collagen domain of SP-D per se is not required for host defense against IAV.

3.4.1.3. The role and mechanism of SP-A in inhibition of IAV

SP-A is present in higher concentrations in respiratory lining fluid than SP-D and contributes to host defense against IAV, RSV, and various bacteria (79, 80). The viral neutralizing activity of SP-A is reduced compared to SP-D or MBL and SP-A contributes less than SP-D to the antiviral activity of BAL fluid against common human strains of IAV (13, 56). SP-A -/- mice do exhibit increased inflammatory responses and variably increased viral titers after infection with IAV (81, 82), although these changes are much less pronounced than in SP-D -/- mice. Furthermore, mice lacking both SP-A and SP-D have a pattern of infection nearly identical to SP-D -/- mice (58). IAV infection results in reactive increases in SP-D, but not SP-A, in the lung (2). Overall these results indicate that SP-D plays a more important role than SP-A in the innate response to IAV infection. The mechanism of anti-IAV activity of SP-A differs from that of the other collectins: instead of binding to glycans on the viral HA and NA, the viral HA binds to a highly sialylated glycan present on the CRD of SP-A (29, 56, 83, 84). SP-A has a similar mechanism of inhibition of herpes simplex virus (HSV) (85). Hence, SP-A inhibits IAV through a mechanism similar to that of mucins, gp-340 or pentraxins (12, 13, 86, 87). The sialic acids on SP-A are partially susceptible to cleavage by the NA since oseltamivir potentiates activity of SP-A in vitro (86). Consistent with its different mechanism of action, SP-A has activity against IAV strains resistant to SP-D (e.g., the PR-8 strain or bovine serum inhibitor resistant strains). In particular the presence of alpha(2,3)-linked sialic acids on SP-A allows it to inhibit IAV strains with avian like binding specificity (56, 88-90). Hence, SP-A may provide a level of protection against avian strains or other pandemic strains lacking glycans on the HA head (90).

3.4.1.4. The unique properties of porcine SP-D

Pigs are an important reservoir and possible "mixing vessel" for IAV strains in that they can be infected with both human and avian strains. The recent swine-derived H1N1 strain that has adapted to humans contains

components of human, swine and avian strains. Porcine SP-D differs from other forms of SP-D and from the serum collectins in that, like SP-A, it has a glycan attachment on the lateral surface of the CRD (89, 91, 92). This enables porcine SP-D to inhibit IAV via a dual mechanism that involves both CRD binding to high mannose glycans on the virus and binding of the viral HA to sialic acids on porcine SP-D. As in the case of mucins and gp340, the ability of SP-A or porcine SP-D to inhibit avian like strains of IAV (e.g., those with an alpha(2,3)-linked sialic acid binding preference) is determined by the density of alpha(2,3)-linked sialic acids on SP-A or porcine SP-D (29, 89). Porcine SP-D also inhibits swine IAV strains that are not inhibited by human SP-D. Of interest deliberate addition of alpha(2,3)-linked sialic acids in vitro on porcine SP-D caused greatly increased inhibition of the PR-8 strain that binds preferentially to this linkage (89).

3.4.1.5. The potential role of MBL in IAV infection

MBL is a strong inhibitor of IAV (30, 52, 63, 93) and has the additional property of being able to fix complement on IAV-infected cells (93). As noted significant levels of MBL are present in the lung during infection so that it may contribute to host defense against IAV. Studies are underway to characterize IAV infection in MBL -/- mice (K. Hartshorn and K. Takahashi, unpublished data).

3.4.2. RSV

RSV is also a major public health problem in infants and the elderly and there is strong evidence of a role for SP-A and SP-D in defense against RSV. For instance, SP-A and SP-D -/- mice show increased inflammation and increased viral titers during infection with RSV (57, 94). As noted above, polymorphic variants of SP-A and SP-D have been associated with increased susceptibility to severe RSV infection (24, 27). Also reduced levels of SP-A and SP-D have been found in children with severe RSV infection (95). RSV infection of perinatal lambs caused reduction in SP-D mRNA and increase SP-A mRNA (96). This appears to be in contrast with IAV infection in mice or parainfluenza virus infection of lambs where increased SP-D levels are seen after infection (97). The reduction in SP-D mRNA was not seen in lambs pre-treated with vascular endothelial growth factor (VEGF) (96). There are some features of the interaction of collectins with RSV that appear to be distinct from their interactions with IAV. For example, wild type SP-D NCRD trimers have been reported to inhibit RSV in vitro and in vivo (98) and SP-A has been found to bind to the envelope proteins of RSV via its calcium-dependent lectin property (99). Whereas SP-D was found to bind to the RSV G protein, SP-A bound the RSV fusion protein. Further studies of the mechanism of action of SP-A and SP-D against RSV would be of great interest and importance.

3.4.3. Parainfluenza viruses

Parainfluenza virus is another common respiratory pathogen related to RSV that can cause severe infection in immunocompromised individuals. Relatively little is known about the interaction of SP-A and SP-D with parainfluenza viruses; however, we have found that SP-D can inhibit hemagglutination activity of Sendai virus (a murine parainfluenza virus) (Hartshorn et al, unpublished data). In neonatal lambs parainfluenza virus type 3 causes viral pneumonia accompanied by increased SP-D, SP-A and beta defensin mRNA, although SP-A protein levels were not increased (97). There was temporal association between elevation of these mRNA levels and decline in viral antigens in lung tissue suggesting that they may have contributed to host defense against the virus. Note that infection of neonatal lambs with RSV, or with a bacterial strain (*Mannheimia haemolytica*) that causes a more long lasting bronchopneumonia (up to 45 days), SP-D expression or protein levels were depressed (100).

3.4.4. Adenovirus

One study has shown decreased virus clearance and increased inflammation in SP-A -/- mice infected with adenovirus (101).

3.4.5. SARS

SP-D and MBL can bind to SARS coronavirus and some studies have shown an association of genotypes that are associated with MBL deficiency and SARS infection (102-105). Serum levels of SP-D are elevated in patients with SARS related pneumonia and have been suggested as a marker of alveolar damage in this condition (106).

3.4.6. Ebola

Ebola virus can be transmitted through a respiratory route and its envelope proteins contain high mannose glycan attachments which serve as attachment sites for MBL (107). Pre-incubation with MBL can inhibit binding of Ebola and Marburg viruses to DC-SIGN and mediate complement attack of pseudotyped viral particles (107). Hence, MBL and by extension the surfactant collectins may contribute to host defense against these highly lethal pathogens.

3.4.7. Herpesviruses

SP-A has been shown to bind to herpes simplex virus and promote its uptake by macrophages (108, 109). Of interest, the mechanism of binding of SP-A to HSV is similar to its binding to IAV (i.e., it involves charge dependent association of the virus with the sialylated carbohydrate on the SP-A CRD). Low levels of MBL in serum have been associated with symptomatic HSV infection (110). Clearly additional studies of the interactions of collectins with HSV would be valuable.

3.4.8. HIV

Patients with AIDS have been found to have elevated levels of SP-A in BAL fluid (111). Although the respiratory route is not an apparent mode of transmission of HIV, HIV replication does occur in the lung and this is likely an important reservoir of virus in advanced HIV disease (112). Hence, SP-D and SP-A probably do have important interactions with HIV in vivo. Since SP-D is expressed in the genito-urinary tract where it interacts with sexually transmitted pathogens like *Chlamydia* (17), it may affect transmission of HIV as well. MBL has neutralizing activity against HIV in vitro although this effect is

attenuated compared to its activity against IAV (113). Several studies have shown an association of lower levels of MBL with increased risk of HIV transmission or progression to AIDS but other studies have not confirmed this (114). In fact, a positive correlation between plasma levels of MBL and progression to AIDS was found in one study (115). We found that SP-D is more potent than MBL at inhibiting HIV but as with MBL the inhibitory activity of SP-D for HIV is much less than its activity vs. IAV (116). SP-A has been found to bind to HIV as well and inhibit infection of CD4+ T cells with the virus (117). Since binding of HIV to DC-SIGN is a mechanism through which HIV can be delivered to CD4 cells, the ability of collectins to inhibit binding of HIV to DC-SIGN on macrophages or dendritic cells is an interesting area for study. Paradoxically, SP-A was found to promote transfer of HIV from dendritic cells to T cells, suggesting that SP-A may promote viral transfer to T cells at mucosal sites (117). Of interest, other innate defense proteins have been found to paradoxically promote HIV infectivity including the defensins HD5 and HD6 (118) and gp340 (119); hence, HIV has adapted to subvert both innate and adaptive immune mechanisms.

3.5 Modulation of innate and adaptive immune responses to viral infection

SP-A and SP-D also modulate viral pathogenesis by altering other aspects of the immune response. The current data suggests that these collectins principally function to reduce the intensity of inflammatory responses in the lung, although the extent to which this results from simple inhibition of viral replication vs. other anti-inflammatory actions is unclear and may differ for different infections. Based on studies of SP-D -/- mice it seems likely that the major mechanism of action of SP-D with respect to IAV is primary reduction of viral load which then leads to reduction in inflammation. SP-A, in contrast, is less potent at inhibiting IAV and in some studies using SP-A -/- mice an increase in IAV related lung inflammation is seen without changes in viral titers (58). Increased inflammation is also seen in SP-A and SP-D -/- mice infected with RSV and various bacteria.

It is conceivable that SP-A or SP-D could also promote inflammatory responses in certain viral infections, although this is speculative at present. Nitrosylation of SP-D has recently been shown to occur in some settings of intense inflammation (e.g., bleomycin lung injury) and this modified form of SP-D has pro-inflammatory effects (120, 121). Severe IAV infection in mice is associated with deleterious activation of reactive nitrogen and oxygen species (48); hence, similar modification of SP-D could occur during viral infection. We will briefly review the interactions of SP-A and SP-D with various components of innate and adaptive immune responses as they may relate to viral infection.

3.5.1. Innate immunity

SP-D binds to or modulates the activity of several soluble innate mediators, including some defensins and gp340. In addition, SP-A and SP-D interact with innate immune cells like neutrophils and macrophages and cellular receptors involved in innate immunity (e.g., Toll like receptors)

3.5.1.1. Gp-340

Binding of SP-D to salivary gp340 was associated with loss of antiviral activity under some conditions (12-14, 86). Lung gp340 bound less avidly to SP-D than salivary gp340 and had cooperative antiviral effects when combined with SP-D in vitro. As noted gp340 is expressed in vaginal fluids as well and on the surface of macrophages and in this location has been found to promote HIV infection (119, 122). Interactions of gp340 with SP-D in vaginal fluids have not been studied.

3.5.1.2. Complement

MBL can directly fix complement on IAV infected cells in vitro (123). SP-D has no known interactions with complement; however, SP-A can inhibit complement activation in the lung through interacting with C1q (124). Further studies will need to be undertaken to determine if the collectins modulate complement activation in vivo during IAV infection.

3.5.1.3. Defensins

We have shown that SP-D binds to human neutrophil peptides 1 and 2 (HNP-1 and HNP-2) and this interaction actually reduces viral neutralizing activity of SP-D against the Phil82 viral strain (which is normally highly sensitive to inhibition by SP-D) (125). In contrast, binding of HNPs to the PR-8 strain of IAV (normally resistant to SP-D) provides a bridge through which SP-D can bind to this strain. Treatment of human BAL fluid with activated neutrophils or with purified HNPs results in substantial loss of SP-D activity and reduction of IAV inhibitory activity (38, 125). This effect is due in part to proteases released by neutrophils which have known ability to degrade SP-D (37, 126, 127); however, other components released by primary granules of neutrophils (e.g., HNPs) also contribute (38). It is likely that HNPs and SP-D interact in vivo during IAV infection since there is probably release of HNPs from neutrophil granules in the airway during the neutrophilic phase of infection. Some conditions characterized by chronic neutrophilic inflammation (e.g., COPD and cystic fibrosis) are also associated with lower levels of SP-D and SP-A (34, 35). It is possible that neutrophil proteases and HNPs (elevated in the lung in these conditions) contribute to reductions of surfactant collectins and increased susceptibility to viral infection (38). Further studies need to be done to evaluate this hypothesis. Note that instillation of HNPs in mice causes inflammatory effects, although the mechanism of this pro-inflammatory effect has not been determined (128).

In recent studies we have found that human beta-defensins (hBDs) do not bind to SP-D and that combinations of hBDs and SP-D allow for additive viral neutralization of IAV (129). Expression of hBDs and SP-D by respiratory mucosal epithelial cells is upregulated in vitro and in vivo during respiratory viral infections (2, 97, 130-133); hence, additive antiviral activity of these innate proteins could contribute during the first phase of respiratory viral infection. Retrocyclins are cyclic defensins

found in primates. Retrocyclins have strong IAV neutralizing activity and, like HNPs bind to SP-D; however, the retrocyclins have additive antiviral activity when combined with SP-D (129). Retrocyclins are generally not expressed in humans due to the presence of a stop codon in the human gene encoding them; however, a recent study found that retrocyclins can be produced by human cervical epithelial cells after treatment with an aminoglycoside (134). It is possible, therefore, that retrocyclins may interact with SP-D under some circumstances in mucosal sites.

Other soluble mediators - Several other innate mediators including lactoferrin, lysozyme, and thrombospondin were not found to interfere with antiviral activity of SP-D (14). Mucins had cooperative antiviral effects when combined with SP-D or SP-A (14, 86). Combinations of SP-A and SP-D also had additive antiviral effects in vitro (86).

3.5.1.4. Phagocytes

Both SP-D and SP-A promote viral uptake by phagocytes including neutrophils and monocyte/macrophages (90, 135, 136). The mechanism for opsonizing activity of SP-D and SP-A may differ. SP-A has a number of direct effects on macrophages which can lead to increased uptake of various pathogens. For instance, SP-A increases macrophage expression of scavenger, macrophage mannose, Fc and complement receptors (137, 138), which can then mediate increased ingestion of pathogens. SP-A also has a distinct receptor on macrophages that can mediate uptake of mycobacteria (139). The macrophage mannose receptor has been shown to mediate infection of these cells by glycosylated forms of IAV (140), although the impact of SP-A on this response is not known. The ability of SP-D to promote uptake of IAV by neutrophils and to increase IAV-induced neutrophil respiratory burst responses appears to be mediated at least in part by viral aggregation (90, 141). An important current theory regarding interaction of SP-A and SP-D for macrophages is that these collectins bind via their CRD to SIRP-alpha in the uninflamed lung and this leads to down-regulation of macrophage activation (142, 143). In contrast, when SP-A or D are bound to ligands such as LPS or apoptotic cell debris they bind through their collagen domains to distinct receptors (e.g., calreticulin/CD91) with resultant enhancement of activation. It is not clear if a similar model operates with respect to neutrophils, although neutrophils do carry these receptors. Receptors for the collagen domain of SP-D are not required for promotion of IAV uptake or of IAV-induced respiratory burst responses in vitro as we have recently shown with forms of SP-D lacking the collagen domain and or N-terminus (74, 75). It is possible, therefore, receptors for the collectin collagen domain are not as involved in response to viral infection as they are in responses to other infections or insults in the lung. As noted above, the situation may differ for severe viral infection if nitrosylated SP-D is produced. Of interest, pre-incubation of neutrophils with SP-D promotes viral uptake by the cells without increasing respiratory burst responses, whereas uptake and respiratory burst responses are both increased if IAV is first incubated with SP-D followed by addition to neutrophils (144).

3.5.1.5. Toll like receptors (TLRs)

SP-A and SP-D also have many important interactions with TLRs (145, 146). Both collectins bind to TLR2 and TLR4 as well as to CD14 (147, 148), although the mechanisms of binding differ (137). In general SP-A down-regulates activation mediated by these receptors in response to such ligands as LPS, PGN or zymosan (137). Even though SP-A increases TLR2 expression on macrophages it reduces signaling through TLR2 and 4 (149). The most important TLRs involved in pathogenesis of IAV are TLR3 and TLR7 although the possible interaction of these receptors with collectins has not been studied. TLR2 and 4 are, however, involved in RSV infection (150). Furthermore, severe acute lung injury from H5N1 IAV is mediated by TLR4 (151). Hence, the ability of SP-A or SP-D to down-regulate activation through TLR4 could be beneficial in some viral infection settings.

3.5.2. Adaptive immunity

Although SP-A and SP-D have been reported to have a variety of effects on the adaptive immune response very little is known about how they modulate such responses during viral infection. Dendritic cells are critical to mounting an immune response to respiratory viral infections and there is some limited data regarding ability of collectins to modulate dendritic cell functions (152). CD8 cells are critical in clearance of IAV infection but can also cause immune mediated injury. It is possible that the ability of SP-A or SP-D to dampen CD8 cell activation (153-155) could be protective in severe IAV infection. There is some evidence that absence of SP-A modifies the nature of the adaptive response to IAV (2). Antibody responses are important in preventing re-infection with a similar strain of IAV. SP-A and SP-D bind to immunoglobulins and could modulate their activity, but again the role of this phenomenon in viral infection has not been studied (156). One problem with respect to IAV infection is that most studies of the adaptive immune response to IAV in mice have been carried out with mouse adapted strains like WSN or PR-8 that are not inhibited by SP-D (perhaps contributing to their increased pathogenesis in mice). RSV infection is associated with airway hyperreactivity and asthma (157). Polymorphisms of SP-D have been linked to asthma as well (23). It is possible, therefore, that the enhanced severity of RSV infection associated with polymorphisms of SP-D (or RSV infection in SP-D -/- mice) relate to the role of SP-D in reducing allergic airway inflammation and the associated Th2-mediated responses (153).

3.6 Role of SP-A or SP-D in bacterial superinfection

Bacterial pneumonia is a major cause of morbidity and mortality during or shortly after IAV epidemics. Recent studies of the 1918 epidemic demonstrate that bacterial superinfection accounted for a significant part of the mortality of that epidemic (158, 159). The most common bacterial agent is *Streptococcus pneumoniae* (160-162); however, an associated of IAV infection with staphylococcal pneumonia has been repeated reported. Most alarming has been the recent observation of methicillin resistant staphylococcal infections resulting in mortality after influenza infection in young subjects (163,

164). IAV also predisposes to other infections that are introduced through the respiratory tract bacterial meningitis and otitis (9, 165-169). These findings suggest that IAV infection causes alterations in respiratory immune responses during and after infection. The ability of IAV to predispose to bacterial superinfection has been confirmed in animal models and there is mounting evidence that IAV alters innate immunity both early and late (e.g., up to 6 months) after infection (161, 162, 170-172). It should be noted that bacterial superinfection and suppression of innate immunity also occur after RSV infection (173) and metapneumovirus infection (42, 174-176).

IAV alters neutrophil recruitment to the lung and impairs neutrophil antibacterial functions in a broad based manner and these alterations correlate with increased susceptibility to bacterial pneumonia or otitis (171, 173, 177). In vitro human neutrophil deactivation by IAV results from binding of the viral HA to sialylated cell surface receptors (178). Pre-incubation of the virus with anti-HA antibodies or collectins protects neutrophils against this deactivation (179). The degree of neutrophil dysfunction is also greater in SP-D -/- mice infected with IAV (180). Hence, neutrophil dysfunction may account in part for concurrent bacterial and IAV infection. The recently discovered PB1-F2 protein that is produced through translation from an alternative reading frame of one of the viral polymerase proteins has been shown to induce apoptosis in immune cells. The PB1-F2 protein has now been shown to be linked to bacterial superinfection, presumably by inducing apoptosis in alveolar macrophages (181, 182). IAV also causes lytic infection of the airway which temporarily damages clearance of bacteria and promotes bacterial adhesion in part through action of the viral neuraminidase (162). SP-D inhibits IAV neuraminidase activity (72) which could be protective against this effect. A staphylococcal protease has been shown to promote IAV infection through facilitating HA cleavage (183). Direct antibacterial effects of SP-A or SP-D could be important in protecting against bacterial superinfections and deficiencies of these proteins could predispose both to IAV and bacterial infection.

It has long been observed that pneumonia may ensue at some time after recovery from IAV infection. Exciting recent findings have shed new light on the pathophysiology underlying this observation. Didierlaurent et al recently demonstrated that IAV infection of mice results in a sustained reduction in TLR2, TLR4, and TLR5 signaling in alveolar macrophages that results in reduced cytokine release and reduced neutrophil recruitment (173). These effects correlate with increased susceptibility to bacterial infection up to six weeks after IAV infection. Excessive production of IL-10 weeks after IAV infection has also been linked to delayed bacterial superinfection (172). Alternatively production of IFN-gamma in response to IAV infection has been linked to bacterial superinfection (184). Note that IFN-gamma production is elevated in SP-D and SP-A -/- mice infected to IAV as compared to infected control mice (2, 81). IAV also leads to generation of myeloid suppressor cells which could contribute to sustained impairment of bacterial defense (185). Natural killer T (NKT) cells were found to counteract the generation of myeloid suppressor cells. It is unknown if SP-A or SP-D interact with, or modify activity of myeloid suppressor or NKT cells.

3.7 Important areas for future research

There are many aspects of response to viral infections that could be modulated by SP-A or SP-D. For instance, type 1 IFNs play a key role in host response to viral infections and many viruses have evolved mechanisms to counteract their activity. Similarly viral sensors including TLRs 3 and 7 or cytoplasmic sensors like RIG, are critical in initiation of innate responses to viral infection in mucosal sites. Little is known about how SP-A or SP-D interact with the type 1 IFN system or with these viral sensors. NK cells are also an important component of innate response to viral infections and interactions of NK cells with collectins have not been explored. Reactive oxygen and nitrogen species are generated during severe viral infections (e.g., severe IAV) and in some cases may have deleterious effects. SP-A and SP-D down-regulate oxidant responses in some models; however, whether anti-oxidant properties of the SP-A or SP-D are important during viral infections requires study.

Further studies of the interaction of SP-A and SP-D with highly pathogenic viruses (e.g., highly pathogenic IAV strains, Ebola, SARS) need to be undertaken. More definitive epidemiological studies associating polymorphic forms of SP-A or SP-D with risk for important viral infections also are needed.

3.8 Therapeutic implications

There are at least two ways to envision translating knowledge of collectin biology into useful antiviral therapeutics. The first could involve use of recombinant forms of collectins optimally designed for viral inhibition (48). The second could be use of other agents known to increase production of SP-D or SP-A in vivo.

3.8.1. Recombinant SP-D as therapy

Isolated trimers composed of the neck and CRD of SP-D (NCRDs) are being explored as therapeutics because they are relatively easy to produce in bacteria and have beneficial activity in various in vitro or murine model systems (186-190). For instance, NCRD can restore some functions of SP-D -/- mice and can ameliorate RSV or aspergillus infections or pulmonary hypersensitivity reactions. It should be noted that many of these studies employed an NCRD construct that also contains a short N-terminal collagen domain fragment which has impact on its activity (191). We have explored the activities of NCRD with regard to IAV, using NCRD without any collagen sequence but with S protein binding site and His tag in the N-terminus (192). As noted above NCRD trimers of wild type human or rat SP-D have minimal activity. We have explored the effect of mutating key residues on the SP-D NCRD on activity of NCRD trimers that have no collagen domain sequence. We have based our approach on the findings that the NCRDs of bovine serum collectins have increased intrinsic antiviral activity for IAV as compared to

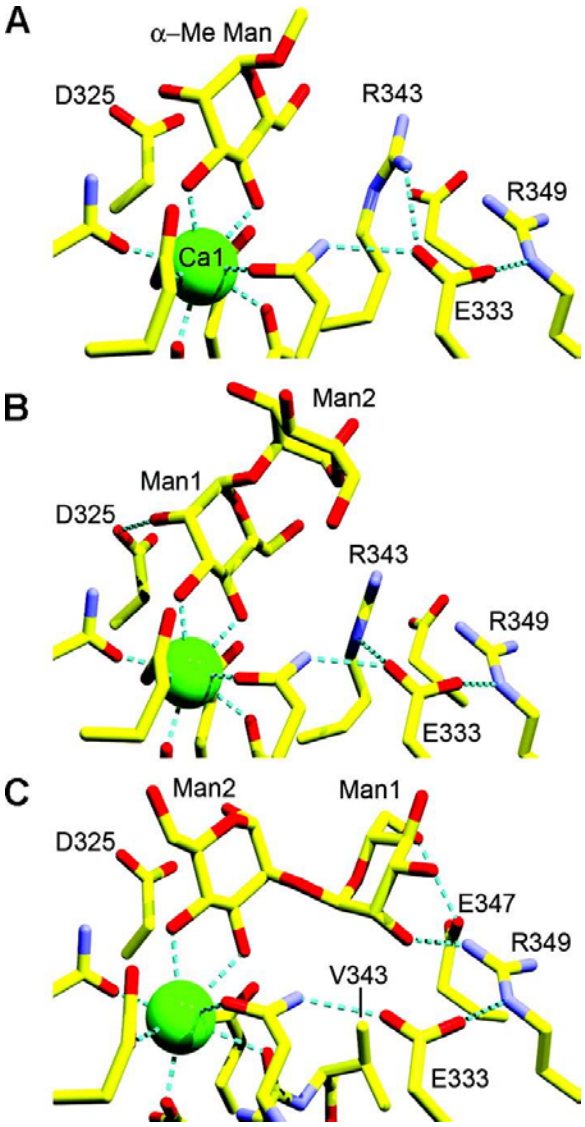

Figure 2. Crystallographic studies of binding of wild type SP-D NCRD and the R343V mutant NCRD to saccharides. Alignment of α-methyl mannoside (α-Me-Man) (panel A) or α(1,2) dimannose (panels B and C) in the lectin binding site of NCRDs are shown. Panels A and B show the wild type NCRD and panel C shows the R343V mutant. Taken with permission from (193).

wild type SP-D NCRDs. Initial crystallographic studies demonstrated that there are two ridges on either side of the saccharide binding pocket of SP-D. These ridges derive from the presence of the charged residues D325 (in human SP-D) and R343. It is now clear that these residues play important roles in modulating the binding properties of NCRDs for specific glycans or pathogens. In an initial study we showed that addition of an RAK (or AAA) insert in human SP-D NCRD conferred a modest increase in mannan binding and antiviral activity compared to wild type SP-D NCRD. The RAK insert is found in CL43 at a position near residue 325 and we believe that it may alter the ability of SP-D to bind to secondary residues on extended mannan chains. Recently we reported that a much greater increase in antiviral activity results from the simple change of R343 for a hydrophobic residue (193). The most effective substitution is valine, although isoleucine or alanine also confer substantial increases in activity. As shown in Figure 2, this substitution alters binding orientation of α(1,2) dimannose to the NCRD. The R343V mutant have greater mannan binding affinity and specifically increased affinity for branched oligomannose chains such as those expressed on viral envelope proteins, including IAV and HIV. R343V has substantially greater antiviral activity than any wild type collectin NCRD we have tested including those of SP-D, conglutinin, CL43 or MBL.

Other mechanisms through which activity of collectins may be increased include modification of the collagen domain or crosslinking of NCRDs through other means. We have found that certain mAbs (or Fab2 fragments thereof) strongly increase antiviral activity of NCRD trimers (74). In addition, an SP-D preparation in which the collagen domain is reduced in size by approximately 50% actually had some increased antiviral activities (75). We have called this preparation mini-SP-D and it is depicted in Figure 1. Another approach involves coupling of NCRDs to Fab1 fragments of mAbs to create bispecific proteins. Tacken et al showed that a bispecific protein containing the human SP-D NCRD coupled to an Fab1 fragment directed against the Fc receptor for IgA resulted in greatly increased opsonizing activity for IAV, bacteria and *Candida* as compared to NCRD alone (194).

3.8.2. Upregulation of surfactant collectin expression

Another promising approach for therapy is use of treatments known to increase endogenous production of SP-A or SP-D. During IAV infection (2) or bacterial pneumonia (195) SP-D levels rise in the lung in response to unknown stimuli. Treatments known to increase SP-A or SP-D generation in vivo include corticosteroids (35), keratinocyte growth factor (KGF) (196), and VEGF (197). This effect of corticosteroids may relate to their beneficial properties in chronic obstructive lung disease or infections like pneumocystis pneumonia; however, corticosteroids have other adverse effects on immunity and more specific means of increasing SP-A and or SP-D production would be valuable. KGF is an approved agent for treatment of mucositis resulting from high dose chemotherapy and has a favorable side effect profile and this agent promotes generation of SP-A, SP-D and other innate defense mediators in the lung. Treatment of mice with KGF markedly increases neutralizing activity of BAL fluid for IAV (McCormack F and Hartshorn K, unpublished data) and this agent deserves study in models of viral infection. VEGF can also modulate surfactant protein metabolism.

4. SUMMARY AND PERSPECTIVE

SP-A and SP-D have important roles in early host defense against viral infections. This has been most clearly demonstrated for respiratory infections and IAV in particular. Further studies of other common respiratory pathogens will hopefully be forthcoming in the near future.

SP-D in particular is widely distributed at mucosal locations and could modify viral infections at other locations like the eye, the genitourinary or gastrointestinal tract; however, relatively little is known in this regard apart from findings of inhibitory activity of collectins against HIV and HSV. More epidemiological studies are needed to establish relationship of SP-A or SP-D polymorphisms with risk for viral infection. SP-A and SP-D clearly modulate inflammatory responses to viral infection and there are many important areas for research into this aspect of their biology. Therapeutic approaches using recombinant forms of SP-D or agents known to increase SP-A and SP-D production offer promise for future therapy of severe viral infections.

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Send correspondence to: Kevan L. Hartshorn, Boston University School of Medicine, EBRC 414, 650 Albany Street, Boston, MA 02118, Tel: 617-638-5638, Fax : 617-638-7530, E-mail: khartsho@bu.edu

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