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Chemical and structural modifications of pulmonary collectins and their functional consequences

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The lung is continuously exposed to inhaled pathogens (toxic pollutants, micro-organisms, environmental antigens, allergens) from the external environment. In the broncho-alveolar space, the critical balance between a measured protective response against harmful pathogens and an inappropriate inflammatory response to harmless particles is discerned by the innate pulmonary immune system. Among its many components, the surfactant proteins and specifically the pulmonary collectins (surfactant proteins A [SP-A] and D [SP-D]) appear to provide important contributions to the modulation of host defense and inflammation in the lung. Many studies have shown that multimerization of SP-A and SP-D are important for efficient local host defense including neutralization and opsonization of influenza A virus, binding *Pneumocystis murina* and inhibition of LPS-induced inflammatory cell responses. These observations strongly imply that oligomerization of collectins is a critical feature of its function. However, during the inflammatory state, despite normal pool sizes, chemical modification of collectins can result in alteration of their structure and function. Both pulmonary collectins can be altered through proteolytic inactivation, nitration, *S*-nitrosylation, oxidation and/or crosslinking as a consequence of the inflammatory milieu facilitated by cytokines, nitric oxide, proteases, and other chemical mediators released by inflammatory cells. Thus, this review will summarize recent developments in our understanding of the relationship between post-translational assembly of collectins and their modification by inflammation as an important molecular switch for the regulation of local innate host defense.

Keywords: collectins, surfactant proteins, nitric oxide, biomarker, lung

Abbreviations: SP, surfactant protein; LPS, lipopolysaccharide; NO, nitric oxide; MPO, myeloperoxidase; SNO, *S*-nitrosothiol; TLR2/TLR4, Toll-like receptors 2 and 4; BAL, broncho-alveolar lavage; SIRP-1α, signal inhibitory regulatory protein-1α; SHP-1, tyrosine-protein phosphatase-1; NF-κB, nuclear factor kappa B; rSP-D, recombinant SP-D; SIN-1,3-morpholinosydnonimine

Introduction

The pulmonary system and the lung-lining fluid in particular, create a unique set of challenges for the innate immune system; presenting it with a wide range of inhaled particles in the context of local conditions ideally suited to the generation of reactive chemical species, such as oxidants and nitrating species. Critical in regulating innate immune responses in the lung are the collectins, surfactant proteins A (SP-A) and D (SP-D), and nitric oxide (NO). The role of NO in cellular signaling has become one of the most rapidly growing areas in biology over the past three decades, and it appears that the vast diversity of its signaling capabilities

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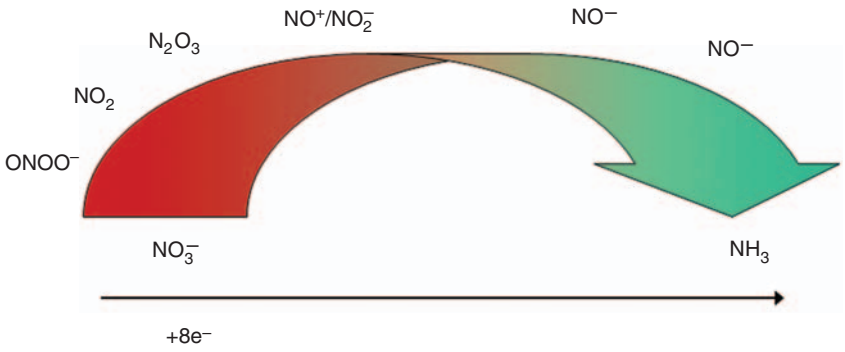

Fig. 1. The redox spectrum of nitrogen. Nitrogen is capable of forming a number of oxides by redox transfer through a variety of oxidation states. Species on the left will generally lose electrons to form nitrate. Gain of electrons will lead to full reduction and the formation of ammonia.

is, at least in part, related to its unique oxidative chemistry. Recent research has highlighted the importance of SP-A and SP-D as targets for NO-mediated signaling events. Within the course of this review, we shall briefly examine the chemistry of NO and relate this to known modifications of SP-A and SP-D.

The redox spectrum of nitrogen

Nitric oxide has proven to be a paradoxical molecule within the pulmonary system. Despite its critical roles in the maintenance of airway and blood vessel tone, as well as its contributions to immune defense, it also appears to be involved in a number of pathological processes, particularly those involving inflammation.[1] This contradictory behavior has been highlighted by the successes and failures of NO in the treatment of pulmonary disease.[2] Similar to oxygen, a spectrum of redox states and reactivity exists for nitrogen (Fig. 1), ranging from fully reduced (*i.e.* ammonia) to fully oxidized (*i.e.* nitrate). In combination with its free radical nature, the ability of NO to act as either an oxidant or reductant allows it to participate in a wide variety of both physiological and pathological processes.[3] Its participation in redox chemistry lies at the heart of NO's capacity to modify proteins post-translationally at a number of sites including cysteine and tyrosine residues. Tyrosine nitration[4] and cysteine *S*-nitrosylation[5] each produce structural changes with functional consequences similar to phosphorylation, methylation, and carbonylation.

Protein tyrosine nitration is a covalent protein modification resulting from the addition of a nitro ($-NO_2$) group onto one of the two equivalent *ortho*-carbons of the aromatic ring of tyrosine residues. The reaction can be catalyzed by metalloproteins such as myeloperoxidase (MPO), eosinophil peroxidase (EPO), myoglobin, and the cytochrome P-450s potentially via the generation of nitrogen dioxide.[6] Myeloperoxidase also catalyzes protein nitration by peroxynitrite, the product of the near diffusion-limited reaction of NO with superoxide.[7] An important element of tyrosine nitration is that certain residues are more prone to modification, thus it is a relatively specific chemical reaction.

S-Nitrosylation is a covalent modification of thiol groups by formation of a thionitrite or *S*-nitrosothiol (SNO). The modification of thiol residues by NO to produce a *S*-nitrosothiol was originally proposed as a protein modification by Stamler and colleagues,[8] and a wide variety of proteins have since been found to be susceptible to *S*-nitrosylation.[9–11] These proteins cover a wide range of functions, including kinases, channels, transcription factors, structural proteins, proteases, and respiratory enzymes. *S*-Nitrosylation is in many ways analogous to phosphorylation: post-translational modification targeted at specific amino acid motifs, leading to changes in protein activity, protein–protein interactions, or subcellular localization of target proteins.[12] There are three separate pathways by which SNO can be formed: (i) via the formation of a higher oxide of nitrogen through auto-oxidation of NO: (ii) via direct reaction followed by electron abstraction; or (iii) via catalysis at metal centers.[13] The conditions present within the lung lining would appear to favor nitrosylation, and indeed loss of SNO has been indicated in pulmonary disease.[14] However, like tyrosine nitration, SNO formation occurs at specific residues. Indeed, two different 'SNO motifs' have been identified: (i) a hydrophobic motif in which a cysteine residue is buried within a hydrophobic region; and (ii) a hydrophilic motif flanked by acidic and basic residues.[9,11] The SNO-mediated signaling is involved in the regulation of breathing, ventilation–perfusion matching, vascular tone, and respiratory and peripheral muscle function. Altered SNO signaling is linked to pulmonary hypertension, septic shock, asthma, cystic fibrosis and pneumonia. The biology of SNO signaling, which is of primary relevance to pulmonary physiology in health and disease, nonetheless remains largely unexplored and ripe for discovery.[14]

In addition to nitration and nitrosylation, NO can participate in other protein modifications, particularly

when in concert with other oxidants. However, these changes, such as dityrosine formation and cysteine oxidation, can also occur in the absence of NO. It is important to understand that NO can modify proteins in a number of ways and that the type of modification produced will be dependent on the concentration of potential targets, the flux of NO, the environment, and the presence of co-factors.

Surfactant proteins and their post-translational modifications

Collectins belong to the superfamily of Ca^{2+}-dependent lectins (C-type lectins). Nine different members have been identified so far in the collectin family: mannose-binding lectin (MBL), conglutinin, SP-A, SP-D, collectin (CL)-43, CL-46, CL-P1 CL-L1 and the very recently identified CL-K1. All collectins form multimers, which increases their affinity to immune cells and pathogens.[15] The basic structural unit of the collectins is the trimer formed via the triple helix collagen tail. Two types of multimerization of triple helices have been observed in collectins – the bouquet and the cruciform. Mannose-binding lectin and SP-A form octadecamers of six trimeric subunits, which resemble a bouquet of flowers,[16] whereas SP-D, conglutinin, and CL-46 are assembled into dodecamers of four or more trimeric subunits and form a cruciform-like structure.[17,18] The amino-acid sequences of mature SP-A and SP-D consist of four structural domains: (i) an N-terminal domain; (ii) a collagen domain; (iii) an α-helical neck region; and (iv) a globular carbohydrate recognition domain (CRD) involved in Ca^{2+}-dependent binding of various groups of ligands.

Surfactant protein A structure and modification

The basic structural unit of SP-A is the trimer formed by the triple helix of the collagen tail and stabilized by N-terminal disulfide bonds.[19] In the case of human SP-A, there are two closely related chains: SP-A1 and SP-A2, and each trimer contains one SP-A1 chain and two SP-A2 chains. In the secreted form of SP-A, trimers are further assembled into hexamers (*i.e.* 18 total chains), arranged in a bouquet structure.[20,21] Allelic variants of SP-A are apparently associated with altered oligomerization, possibly depending on the number of cysteines contained in the primary structure of both the N-terminal and the collagen-like region.[22]

Surfactant protein A is post-translationally modified by the addition of an N-linked oligosaccharide,[23] formation of disulphide bonds, and signal peptide cleavage. These modifications are important for intracellular trafficking of the immature molecule[24] and are essential for SP-A-mediated clearance of influenza A.[25] Surfactant protein A is also a target for modification by reactive species produced during inflammatory disease.[26] Surfactant protein A is readily nitrated by exposure to peroxynitrite or activated alveolar macrophages, and modified SP-A is unable to aggregate lipids or bind *Pneumocystis*.[27,28] It has been shown that MPO can catalyze tyrosine nitration and dityrosine formation in human SP-A and that these changes are associated with a decreased ability to aggregate lipids and bind to mannose.[29] These results indicate that nitration, in addition to oxidation, may cause inhibition of protein function. Interestingly, mouse SP-A does not contain any redox-active cysteines and thus is not susceptible to either cysteine oxidation or the formation of SNO. Oligomerization of SP-A is important for its function, as trimeric fragments of SP-A fail to bind to the TLR4/MD2 receptor complex.[30] Polymorphisms may also lead to alteration of structure and function of the SP-A molecule[20] with specific polymorphisms in SP-A associated with increased susceptibility to pathogens.[31]

Surfactant protein D structure and modification

The SP-D primary translation product is, like SP-A, composed of three polypeptide chains that are held together by disulphide bonds.[32] The oligomerization of the CRDs of SP-D into a trimer is mediated by its neck region, an α-helical coiled-coil with a centrally placed tyrosine ring.[33] A single aromatic side chain in the center of the three helices appears to drive oligomerization towards a trimeric assembly. The secreted form of SP-D is further assembled into a large cruciform dodecamer (12 subunits), and, at over 100 nm in diameter, it is one of the largest and most flexible molecules found in the innate immune system.[32] Critical to this oligomerization are the cysteine residues at positions 15 and 20 in the hydrophobic N-terminus.[15,34] Mutation of these residues results in the secretion of trimers, which represent a single arm of the dodecamer.[15] The positioning of cysteines 15 and 20, in a hydrophobic domain suggests that NO may control oligomerization of SP-D by nitrosylation.[35] In addition, there are five tyrosine residues in native SP-D, making it a potential target for protein nitration. Peroxynitrite causes nitration of tyrosine residues at positions 228, 306 and 314 of SP-D *in vitro* and a significant decrease in SP-D-dependent aggregation of LPS-coated beads.[36]

The structural variability of SP-D isoforms in health and during inflammatory disease presents an interesting dilemma in terms of its quantitation in BAL or serum. Since native SP-D is a symmetrical dodecamer, its tail domains are inaccessible for antibody generation; in fact, the majority of antibodies to date recognize its

carboxyl domain (EAV, MFB, AJG unpublished observations). During lung injury, the quaternary structure of SP-D can be disrupted resulting in the formation of SNO–SP-D trimers/monomers that may confound the measurement of total SP-D by commercially available ELISA kits. In addition, disruption of the alveolar–capillary barrier during injury or remodeling can permit selected SP-D isoforms into the serum. Therefore, the precise quantitation of SP-D and its reliability as a biomarker requires a re-assessment of antibody epitopes, use of appropriate internal standards, and correlation with quaternary structures and post-translational modifications through the use of gel-filtration, native gels, SDS-PAGE, determination of SNO/nitrotyrosine, and assessment of cross-linked isoforms. In short, there is a need to know what one is measuring by knowing what to measure.

Clinical implications of collectin's multimeric structural modifications

Variation within the SP-D gene may result in alterations in SP-D levels, multimeric structure, and increased susceptibility to infectious diseases. One SP-D polymorphism (rs721917, position 31 from the transcription start site), often termed Met11Thr, which results in either a threonine or a methionine at position 11 in the mature SP-D protein has raised particular interest in relation to alteration of structure, function and association with disease.[37] Previous studies have demonstrated the existence of different structural SP-D isoforms (including trimers, cruciform dodecamers, and higher-order multimers) purified from lung washing of subjects with pulmonary alveolar proteinosis,[18] human serum, or amniotic fluid.[38,39] In such samples, the Met/Met genotype was associated with oligomerization, and subjects with Thr/Thr genotype exhibited reduced serum SP-D levels.[38] In addition, subjects with the Thr/Thr genotype appeared to have only 'low molecular mass' forms of SP-D (such as trimers), while Met/Met subjects had both 'high and low molecular mass' forms (such as multimers and trimers).[37]

Functional differences have been observed between purified oligomeric and trimeric SP-D. The high molecular mass forms of amniotic fluid-derived SP-D demonstrate preferential binding to both Gram-negative and Gram-positive bacteria, fungal mannan and influenza virus, while the low molecular mass form of SP-D preferentially bind to rough LPS, suggesting differential pathogen-related effects of each form.[38] Recombinant trimeric subunits of SP-D have the same saccharide selectivity as higher-order oligomers but appear to show a weak and more restricted range of biological activities.[15,34,40] Taken together, the degree of multimerization of SP-D is a critical determinant of both aggregating activity and potency in enhancing bacterial uptake in neutrophils.[40] The Met11 allele and Met/Met homozygosity in infants are associated with more severe respiratory syncytial virus infection,[41] while adults with the Thr11 allele have increased susceptibility to *Mycobacterium tuberculosis* infection,[42] and SP-D isolated from these individuals appears to have less neutralizing activity against influenza A virus.[43] Griese *et al*.[44] also described differences in multimeric organization of SP-D between BAL of healthy children and those suffering from gastro-esophageal reflux disease (GERD).

Several studies have suggested that multimeric SP-D is anti-inflammatory, down-regulating the inflammatory responses of effector cells (Fig. 2). Starosta *et al*.[45] reported that loss of biological activity of oxidized SP-D is caused by the alteration and disruption of its quaternary structure. Yamazoe *et al*.[46] also demonstrated that oligomeric SP-D inhibit LPS-induced inflammatory cell responses and that the oligomeric state of SP-D is a critical feature of its function. Multimerization of SP-D is important for efficient influenza A virus neutralization and opsonization.[47] Loss of this anti-inflammatory property may be one mechanism by which post-translational modification of SP-D affects asthma pathogenesis. The multimeric form of SP-D also inhibits eotaxin-mediated eosinophil migration, while trimeric SNO–SP-D blocks this inhibitory activity.[48] These observations strongly imply that oligomerization of SP-D is critical to its control of the inflammatory response of the lung.

Functional consequences of collectin multimeric structure

The role of pulmonary collectins in inflammation is controversial – both pro-inflammatory and anti-inflammatory roles are reported. In 2003, Gardai *et al*.[49] proposed a distinct mechanism of collectin-mediated modulation of inflammation. Using SP-A as the prototype collectin molecule, they suggested that collectins either suppress or enhance lung inflammation, depending on the orientation of their binding to alveolar macrophages via a CRD-dependent or CRD-independent mechanism. According to the 'head or tail hypothesis', a collectin, binding to SIRP-1α on residential alveolar macrophages via its CRD, prevents inflammation through p38 inhibition. In contrast, when interacting with pathogens via its globular head, the collectin molecule presents its collagenous tail to the calreticulin/CD91 receptor complex, activating a pro-inflammatory response via up-regulation of p38-mediated NF-κB activity.[49] However, because SP-D forms a symmetrical cruciform dodecamer, its

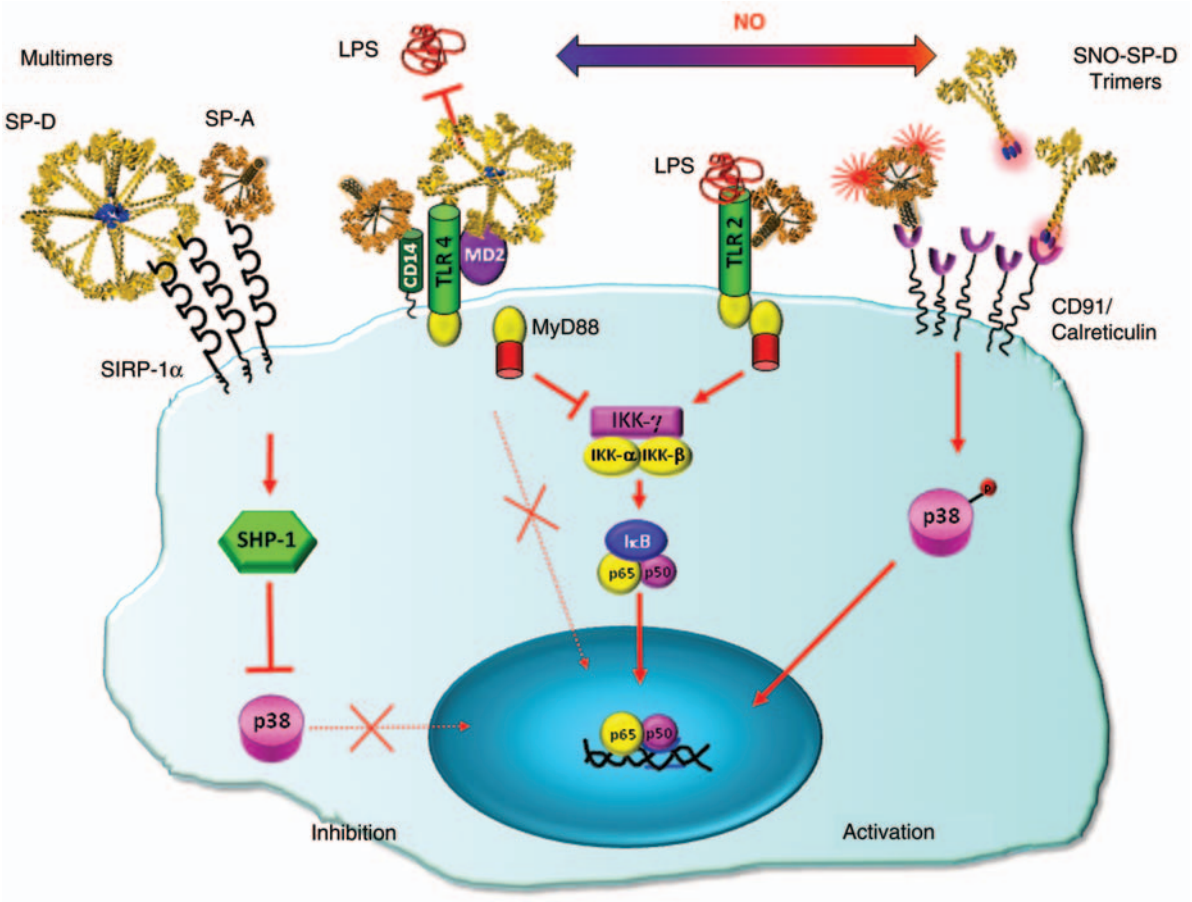

Fig. 2. A model of the pro- and anti-inflammatory functions of pulmonary collectins in macrophages. Under non-inflammatory conditions, binding of the head domains of SP-A or of SP-D (remains in multimeric form in which the tail domains remain buried) to SIRP-1α induces the activation of tyrosine phosphatase SHP-1, resulting in blockage of the downstream signaling through p38 MAP kinase, potentially resulting in the blockages of NF-κB action and the inhibition of inflammatory function. Under inflammatory conditions, the production of NO leads to the formation of SNO–SP-D and the disruption of the multimeric structure. The tail domains now become exposed and bind to calreticulin/CD91. This results in p38 phosphorylation via calreticulin/CD91, potentially leading to NF-κB activation and the production of pro-inflammatory mediators. Binding of the collagenous tail of SP-A (globular head interacts with pathogens) to calreticulin/CD91 receptor complex stimulates cytokine production. Multimeric forms of SP-D, but not its trimers, bind a complex of TLR4/MD-2 and inhibit TLR4-mediated inflammatory responses caused by both smooth and rough serotypes of LPS, indicating that the inhibitory effect of SP-D is dependent upon the formation of its cruciform structure. Surfactant protein A inhibits inflammatory responses stimulated with smooth LPS by direct interactions with TLR2 and TLR4. Surfactant protein A binds to rough LPS and enhances TLR2/TLR4 inflammatory response through interaction with the adaptor molecule MyD88 (myeloid differentiation primary-response protein 88). The MyD88-dependent signaling pathway leads to the activation of NF-κB.

tail domains would seem to be inaccessible, rendering this model incomplete.

Our group has demonstrated that, while the multimeric structure of native SP-D is anti-inflammatory (consistent with its observed effects *in vitro*), disruption of the multimeric structure exposing tail domains renders SP-D pro-inflammatory.[35] We demonstrated that, in its native form, SP-D (like SP-A) binds to SIRP-1α via its CRD domain and inhibits pro-inflammatory responses via SHP-1-mediated inhibition of p38 phosphorylation and NF-κB activation. However, during inflammation, SP-D can become a direct target for post-translational modification by NO. *S*-Nitrosylation of cysteine residues in the hydrophobic tail domain of SP-D *in vitro* by *S*-nitrosocysteine (a donor of NO) or *in vivo* (during bleomycin injury) result in breakdown of multimeric SP-D structure, exposing its *S*-nitrosylated tail domains. In addition, the formation of SNO–SP-D trimers initiates a pro-inflammatory response (Fig. 2) enhancing macrophage migration and lung chemokine production through calreticulin/CD91 binding and p38 activation that can be blocked by CD91 or SP-D antibodies.[35] Importantly, SNO–SP-D, but not trimeric SP-D, is chemo-attractive for macrophages and induces p38 MAPK phosphorylation. In another *in vivo* murine model of lung inflammation, we have demonstrated that $CD4^+$ T-cell immune-reconstituted mice infected with *P. murina*

exhibit impaired pulmonary function with substantial increases in the BAL of an *S*-nitrosylated form of SP-D and enhanced macrophage chemotaxis.[50] Both SNO–SP-D formation and enhancement of chemotaxis were abolished by ascorbate treatment *in vitro*. These studies provided generalizable mechanistic evidence that effector cell recruitment is induced by SNO–SP-D.

In addition to *S*-nitrosylation, a number of post-translational modifications of SP-D with functional consequences can occur. Surfactant protein D in both its monomeric and cross-linked forms is nitrated after challenge with allergen or a combination of allergen with LPS.[48] Furthermore, treatment of BAL or rat rSP-D with SIN-1, a generator of peroxynitrite, results in nitration and cross-linking of SP-D *in vitro*. Similar to our findings, Matalon *et al*.[36] recently demonstrated that peroxynitrite causes nitration and cross-linking of native SP-D, and that these modifications of SP-D were accompanied by a significant decrease in SP-D-dependent aggregating activity in the lavage of mice acutely exposed to nitrogen dioxide. Experiments with tyrosine mutants have demonstrated that most of the peroxynitrite dependent cross-linking of the CRD is dependent on the presence of Tyr228.[36] Starosta *et al*.[45] also showed that the quaternary structure of SP-D was altered under oxidative conditions *in vivo* and *in vitro* and that this alteration was associated with a loss of its anti-inflammatory properties. Interestingly, the authors reported that GERD was associated with the formation of non-reducible, covalently linked forms of SP-D with molecular masses of 85 kDa, 120 kDa, 160 kDa and 200 kDa.[44]

In humans with acute allergic inflammation, we have demonstrated alterations in SP-D structure and/or function, which may play a role in pathogenesis.[48] Segmental challenge with allergen or a combination of allergen and LPS caused covalent cross-linking of SP-D and altered its quaternary structure. The formation of covalently cross-linked SP-D in asthmatic patients correlated positively with both eosinophil number and nitrate levels, suggesting a strong relationship between cross-linked SP-D and severity of allergic inflammation. Furthermore, patients from the cross-linked group showed a significant increase in the level of SNO–SP-D and exhibited markedly enhanced levels of Th2 cytokines after challenge with allergen or a combination of allergen and LPS.[48] The appearance of cross-linked SP-D and disruption of its multimeric structure after allergen challenge was not antigen specific and, furthermore, non-asthmatic patients did not develop cross-linked SP-D isoforms.

It remains to be determined whether the presence of oxidatively cross-linked SP-D is a mediator of allergen-induced inflammation or merely a biomarker. In this regard, it is interesting to note that polymorphic variation in the N-terminal domain of the SP-D molecule influences oligomerization, function and the concentration of the molecule in serum.[38] Thus, polymorphic variations, such as the Thr/Thr11,[43] may lead to a differential susceptibility to post-translational modification and inflammatory signaling capacity. From our prior work, we would suppose that variations that alter oligomeric organization of SP-D may lead to increased inflammatory signaling, but larger, population-based studies are necessary to address this question.

Conclusions

Surfactant protein A and SP-D, the so-called pulmonary collectins, play a critical role in regulating innate immune responses within the lung. These proteins possess complex oligomeric structures that are critical to their function both in binding to potential pathogens, but also in regulating immune cell response. Nitric oxide is capable of modifying these proteins via a number of different mechanisms and with varying effects on the structural organization of these critical regulators. Understanding the nature of these modifications and their functional consequences is critical to improving our knowledge of how NO, and other oxidants, play a role in regulating innate immune function.

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