

Surfactant protein A is defective in abrogating inflammation in asthma

Ying Wang,¹ Dennis R. Voelker,² Njira L. Lugogo,¹ Guirong Wang,³ Joanna Floros,^{3,4}
Jennifer L. Ingram,¹ Hong Wei Chu,² Tony D. Church,¹ Pitchaimani Kandasamy,² Daniel Fertel,¹
Jo Rae Wright,¹ and Monica Kraft¹

Departments of ¹Medicine and Cell Biology, Duke University Medical Center, Durham, North Carolina; ²Department of Medicine, National Jewish Health, Denver, Colorado; and ³Center for Host Defense, Inflammation and Lung Disease (CHILD) Research, Department of Pediatrics, and ⁴Obstetrics and Gynecology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania

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Wang Y, Voelker DR, Lugogo NL, Wang G, Floros J, Ingram JL, Chu HW, Church TD, Kandasamy P, Fertel D, Wright JR, Kraft M. Surfactant protein A is defective in abrogating inflammation in asthma. *Am J Physiol Lung Cell Mol Physiol* 301: L598–L606, 2011. First published July 22, 2011; doi:10.1152/ajplung.00381.2010.—Surfactant protein A (SP-A) regulates a variety of immune cell functions. We determined the ability of SP-A derived from normal and asthmatic subjects to modulate the inflammatory response elicited by *Mycoplasma pneumoniae*, a pathogen known to exacerbate asthma. Fourteen asthmatic and 10 normal control subjects underwent bronchoscopy with airway brushing and bronchoalveolar lavage (BAL). Total SP-A was extracted from BAL. The ratio of SP-A1 to total SP-A (SP-A1/SP-A) and the binding of total SP-A to *M. pneumoniae* membranes were determined. Airway epithelial cells from subjects were exposed to either normal or asthmatic SP-A before exposure to *M. pneumoniae*. IL-8 protein and MUC5AC mRNA were measured. Total BAL SP-A concentration did not differ between groups, but the percentage SP-A1 was significantly increased in BAL of asthmatic compared with normal subjects. SP-A1/SP-A significantly correlated with maximum binding of total SP-A to *M. pneumoniae*, but only in asthma. SP-A derived from asthmatic subjects did not significantly attenuate IL-8 and MUC5AC in the setting of *M. pneumoniae* infection compared with SP-A derived from normal subjects. We conclude that SP-A derived from asthmatic subjects does not abrogate inflammation effectively, and this dysfunction may be modulated by SP-A1/SP-A.

epithelial cell; mycoplasma; SP-A1

ASTHMA IS A CHRONIC INFLAMMATORY disease with a pattern of inflammation that includes T helper-2-like cytokines (13, 63). An atypical bacterium, *Mycoplasma pneumoniae*, can exacerbate asthma through increased inflammation and mucus hypersecretion (2, 6, 21, 28, 29). Recent studies show that 50% of patients experiencing their first presentation of asthma have acute *M. pneumoniae* airway infection (7). Thus *M. pneumoniae* is a pathogen that can exacerbate and potentially precipitate asthma.

One important first line of defense against inhaled challenges is the pulmonary innate immune system, which includes surfactant proteins. Multiple studies now show that surfactant proteins A (SP-A) and D (SP-D) are members of a family of innate immune molecules, termed collectins, that facilitate pathogen clearance by acting as opsonins and by regulating a variety of immune cell functions (16, 29, 43, 47, 50, 60, 64). Human SP-A is encoded by two genes, SP-A1 (*SFTPI*) and

SP-A2 (*SFTP2*), and each gene encodes several polymorphic variants (30, 12). The gene-specific protein products are distinguished from one another by four “core” amino acids in their collagen-like regions. These differences appear to affect both structure and function (17, 42–44, 57–59). Core amino acid residue 85 as shown by site-directed mutagenesis is a critical residue for the functional and structural differences between SP-A1 and SP-A2 (58). Tagaram et al. (55) showed that the ratio of SP-A1 to total SP-A content (SP-A1/SP-A) varies as a function of lung disease and age. However, asthma was not evaluated. Given the functional differences between the two human SP-A gene products, the collective evidence indicates that the content of human functional SP-A is best described by the relative levels of the two gene products, rather than the total SP-A.

We have demonstrated that SP-A binds to lipids (47) and proteins (29) on *M. pneumoniae*, attenuating its growth and pathogenicity. SP-A and SP-D modulate macrophage cytokine production in response to infectious agents, and SP-A- and SP-D-null mice exhibit increased susceptibility to infection and inflammation caused by bacteria and viruses (1, 8, 24, 33–36, 41, 50, 64). In addition, SP-A plays a role in regulating the expression of BAL proteins involved in host defense and redox regulation (1, 23). Therefore, surfactant proteins have multiple roles in attenuating the effects of environmental insults.

However, relatively little is known about the role of SP-A or SP-D in asthma and allergic inflammation in humans (20, 22, 46, 56, 61, 65). SP-A and SP-D have been shown to inhibit allergen-induced lymphocyte proliferation and histamine release by immune cells from asthmatic children (60). Haley and coworkers (22) reported that SP-A and SP-D levels are elevated in a murine model of adaptive allergic response, whereas Van de Graaf and colleagues (56) demonstrated that SP-A levels were lower in BAL from asthmatic subjects compared with normal subjects. Pettigrew and colleagues (46) reported an association between wheezing and coughing in newborn infants and proposed that polymorphisms within the SP-A gene may be associated with an increased risk of asthma. Specifically, the 6A/1A haplotype was found to be associated with increased risk of persistent cough or wheeze. In this study, we determined the level of total SP-A and the percentage SP-A1 in BAL fluid in normal and asthmatic subjects, and compared the effects of SP-A isolated from normal and asthmatic subjects on abrogating inflammation in airway epithelial cells infected with *M. pneumoniae*, a pathogen known to exacerbate asthma.

Address for reprint requests and other correspondence: M. Kraft, Dept. of Medicine, Duke Univ. Medical Center, MSRB M275, Research Dr., Durham, NC 27710 (e-mail: monica.kraft@duke.edu).

METHODS

Subjects. Subjects were recruited via newspaper and radio advertisements from the general Research Triangle, North Carolina community. The asthmatic subjects fulfilled criteria for asthma (3) exhibiting a provocative concentration of methacholine resulting in a 20% fall in the forced expiratory volume in 1 s (FEV₁) < 8 mg/ml and reversibility, as demonstrated by at least a 12% increase in the FEV₁ or the forced vital capacity with inhaled albuterol. All asthmatic subjects were atopic, as demonstrated by at least one positive skin test to common North Carolina allergens associated with a correlative clinical history. All normal subjects had no clinical history of atopy and were skin test negative. We required that subjects used no controller medications, but did not discontinue any medications. Thus only subjects on as-needed short-acting β_2 -agonists only were recruited. Exclusion criteria included postbronchodilator FEV₁ < 50% predicted; inpatient status; upper or lower respiratory tract infection within 1 mo of study; use of inhaled corticosteroids, leukotriene modifiers, and/or theophylline preparations within 4 wk of study; long-acting β_2 -agonists within 2 wk of study; smoking history greater than 5 pack-yr or any cigarette use within the previous 2 yr; and significant nonasthma pulmonary disease or other medical problems. All subjects provided consent in Duke University Health System Institutional Review Board-approved protocol.

Bronchoscopy. Subjects underwent bronchoscopy with endobronchial protected brushing and bronchoalveolar lavage (BAL) as previously described (32). The brushing of the proximal airways was performed under direct visualization by using a separate protected cytological brush for each pass, with a total of five passes. BAL was performed via instillation of warm, sterile saline in 60-ml aliquots with return via gentle hand suction for a total of 300 ml. Subjects were discharged when their FEV₁ achieved 90% of their prebronchoscopy, postalbuterol value.

Purification of SP-A from BAL of normal and asthmatic subjects. SP-A from normal and asthmatic subjects was extracted from BAL by the calcium chelation method, as previously described (53). The SP-A was recovered in high yield and at high concentrations, up to 200 μ g per BAL sample. The total isolated SP-A was quantified by ELISA using polyclonal antibodies made against SP-A isolated from the lavage of patients with pulmonary alveolar proteinosis (Hu-PAP-SP-A) (55).

Determination of SP-A1/SP-A in the BAL of normal and asthmatic subjects. SP-A1/SP-A in the BAL was measured by ELISA as previously described (55). In brief, human SP-A1 variant (6A²), the most common variant, was expressed in mammalian CHO-K1 cell line and purified by mannose-Sepharose 6B column. In vitro expressed SP-A1 (6A²) and total SP-A, purified from a normal subject BAL of known concentration, were used as standards. SP-A1 and total SP-A were measured via ELISA using an SP-A1-specific antibody (hsp_A1-Ab⁶⁸⁻⁸⁸_Col) and total SP-A antibody (Hu-PAP-SP-A) (55).

Production of SP-A1 and SP-A2 variants for mycoplasma membrane binding assays. The most frequent human allelic variants of SP-A1 and SP-A2, harboring the [50V, 219R] and [91A, 223Q] polymorphisms, were expressed in Freestyle-HEK-293 cells [American Type Culture Collection (ATCC), Manassas, VA] from a PEE14 plasmid (Lonza, Berkshire, UK) under the control of the glutamine synthetase promoter (45). The proteins were purified by affinity chromatography using mannose-Sepharose (45). Binding of the allelic variants to solid-phase mycoplasma membranes was performed as described previously (47).

Binding of SP-A to *M. pneumoniae*. The binding of purified SP-A from BAL to mycoplasma membranes was measured as previously described (47). In brief, 100-ng mycoplasma membranes were adsorbed to microtiter plates in 0.1 mM NaHCO₃, pH 9.6 at 4°C overnight. Human SP-A purified from BAL of normal and asthmatic subjects at concentrations between 0 and 100 ng was added to each

well and incubated with the solid-phase membranes for 2 h at 37°C. The total bound SP-A was detected with 5 μ g/ml horseradish peroxidase-conjugated polyclonal antibody, generated in rabbits and isolated using protein A-Sepharose. Orthophenylenediamine (1 mg/ml) was used as the color development reagent. The SP-A bound to each sample, as well as the area under the binding curve, was used in data analysis.

To determine the maximal binding of recombinant SP-A1 and SP-A2 variants to mycoplasma membranes, 100 ng recombinant proteins expressed in HEK-293 cells, [50V, 219R] SP-A1 and [91A, 223Q] SP-A2, were incubated with the solid-phase mycoplasma membranes. The bound SP-A variants were detected as described above.

Airway epithelial cell culture. Freshly isolated airway epithelial cells from endobronchial brushing were cultured with BEGM (Lonza, Walkersville, MD) supplemented with penicillin and streptomycin. After reaching confluence, cells were trypsinized and seeded onto collagen-coated polyester Transwell insert membranes of 12-mm diameter with the concentration of 4×10^4 per well. After 2 wk at an air-liquid interface, the cells showed signs of differentiation as determined by the presence of cilia and mucus production. To determine the purity of cultured epithelial cells, two cytospin slides were made from the initial epithelial cell suspension, fixed in 4% paraformaldehyde and immunostained by using an anti-human pan cytokeratin antibody (Sigma, St. Louis, MO). Immunostaining demonstrated >98% of cells positive for cytokeratin.

***Mycoplasma pneumoniae* culture.** *M. pneumoniae* (ATCC, strain 15531) was inoculated in SP4 broth (Remel, Lenexa, KS) at 35°C until adherent. The concentration was determined by plating serial dilutions of *M. pneumoniae* in agar plates (Remel). Colony-forming units (cfu) were counted after incubation for 14 days.

Mycoplasma infection of cultured airway epithelial cells. Freshly isolated airway epithelial cells from endobronchial brushing were cultured at an air-liquid interface for 2 wk to allow differentiation with ALI media (Lonza). Cells were infected on the apical surface by *M. pneumoniae* (ATCC) with dose of 50 cfu per cell and incubated for 48 h with and without pretreatment with either normal or asthmatic SP-A (NSP-A and ASP-A, respectively), added 30 min prior to *M. pneumoniae*.

To rule out the presence of an inhibitor in the purified ASP-A, 10 μ g/ml NSP-A and 10 μ g/ml ASP-A were mixed and added to the airway epithelial cells 30 min prior to *M. pneumoniae* infection. Additionally, to determine specificity of SP-A in experiments, NSP-A purified from lavage as described above was selectively depleted from the preparations by using affinity adsorption to mannose-Sepharose. Aliquots containing 25–30 μ g SP-A in a volume of 250 μ l were mixed with mannose-Sepharose beads that had previously been equilibrated with 5 mM Tris-HCl, pH 7.4. The samples were incubated with the mannose-Sepharose in either the presence of 2 mM CaCl₂, or 2 mM EDTA. In the presence of CaCl₂, SP-A will bind to the affinity matrix and be removed from solution, whereas in the presence of EDTA, the protein will fail to bind the matrix and remain in solution. Incubations were conducted at 4°C in microcentrifuge tubes with continuous tumbling to keep the beads in suspension. Following this incubation, the samples were centrifuged at 400 rpm for 3 min at 4°C, and the supernatants were recovered and retained. The resultant pellets were washed once by resuspension in 50 μ l of buffer containing either CaCl₂ or EDTA as appropriate and mixed for 15 min at 4°C. The wash solution was separated from the beads by centrifugation, and the resultant supernatants were pooled with the first elutions from the beads. The preparations of SP-A were reconstituted into tissue culture medium, and concentrations were determined by ELISA and used for treatment of primary cultures of human bronchial epithelial cells.

Detection of MUC5AC mRNA and interleukin-8 protein expression. Airway epithelial cells were placed in TRIzol 48 h after *M. pneumoniae* infection for determination of MUC5AC expression (31) by

Table 1. Subject characteristics

	Asthma (n = 14)	Normal (n = 10)	P Value
Sex	8M:6F	4M:6F	0.02
Age	33 ± 7	26 ± 4	0.44
Medications	albuterol	none	
FEV ₁ , liters	2.9 ± 0.2	3.4 ± 0.2	0.15
FEV ₁ , % predicted	83 ± 4	102 ± 5	0.007
FVC, liters	4.1 ± 0.9	4.6 ± 1.2	0.38
FVC, % predicted	91 ± 5%	108 ± 7%	0.06
FEV ₁ /FVC	71 ± 2	78 ± 2	0.52
Methacholine PC20	0.63 ± 0.3 mg/ml	> 25 mg/ml	0.0001

Values are means ± SE. M, male; F, female; methacholine PC20, provocative concentration of methacholine resulting in a 20% fall in the forced expiratory volume in 1 s (FEV₁); FVC, forced vital capacity.

quantitative real-time PCR (RT-PCR) (11). The housekeeping gene GAPDH was used as an internal standard. The threshold cycle (C_T) was recorded, and the comparative C_T method was used to represent the relative gene expression levels (62). Data are expressed as fold change from uninfected control. Culture supernatant was collected for IL-8 protein measurement by ELISA (R&D, Minneapolis, MN).

Gel electrophoresis under nonreducing and native conditions. Purified SP-A from the BAL of normal and asthmatic subjects was subjected to electrophoresis under nonreducing and native conditions as described by Wang et al. (57). For nonreducing SDS-PAGE, purified SP-A was mixed with a loading buffer without β-mercaptoethanol and heated at 95°C for 10 min. The samples were subjected to electrophoresis on 4–20% acrylamide gradient gels at 90 V for 1 h and 70 V for 5 h. For native PAGE, the purified SP-A was not denatured by β-mercaptoethanol, SDS, and heating. Electrophoresis was performed at 4°C using 4–20% acrylamide gradient gels at 50 V for 1 h and 110 V for 17 h.

Detection of protein oxidation. SP-A protein carbonylation levels were detected by using the OxyBlot Protein Oxidation Detection Kit (Millipore, Billerica, MA). Briefly, carbonyl groups introduced into SP-A protein by oxidative reactions were derivatized with 2,4-dinitrophenyl hydrazine (DNPH), 200 ng of DNPH-derivatized SP-A protein was blotted to nitrocellulose membrane, and immunodetection of oxidized proteins was performed by using rabbit anti-DNPH and goat anti-rabbit IgG (horseradish peroxidase-conjugated) antibodies. To exclude the artifactual increases in carbonyl content raised from tiny amounts of nucleic acid contamination in the purified SP-A (38), the purified SP-A samples were pretreated with 20 μg/ml DNase I (Sigma) and RNase A (Invitrogen, Carlsbad, CA) at 37°C for 1 h, then blotted to nitrocellulose. Blots were exposed to XAR films following enhanced chemiluminescent detection and were quantified by densitometry.

Statistical analysis. Statistical evaluation was performed with JMP statistical software (SAS, Cary, NC). Data are expressed as mean fold changes ± SE, since data were normally distributed. Area under the curve (AUC) analyses of the SP-A/mycoplasma binding curves were performed by using the trapezoidal rule (52). Total SP-A and SP-A1/SP-A was compared between groups by Student's *t*-test. Correlations were reported by using Spearman correlation coefficient. Significance is denoted by *P* < 0.05.

RESULTS

Subject characteristics. Subject characteristics are shown in Table 1. Subjects were considered of mild severity by American Thoracic Society criteria (3).

BAL SP-A1/SP-A is altered in asthmatic subjects. The total BAL SP-A level was determined by ELISA using SP-A specific polyclonal antibody and normalized by total BAL volume. There was no difference between the groups (asthma vs.

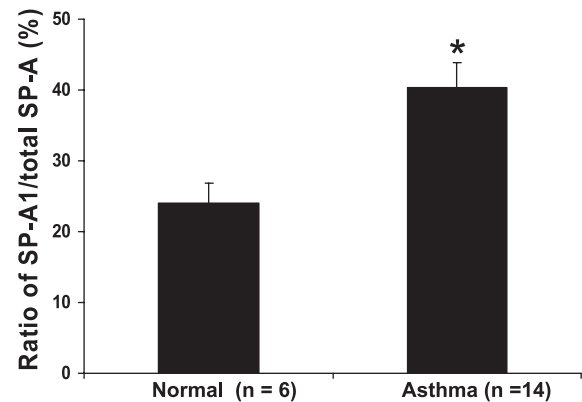


Fig. 1. Ratio of surfactant protein A (SP-A)1 to total SP-A (SP-A1/SP-A) is increased in the bronchoalveolar lavage (BAL) from asthmatic subjects. In vitro-expressed SP-A1 variant 6A² and total SP-A, purified from a normal subject BAL of known concentration, were used as standards. SP-A1 and total SP-A were measured via ELISA using SP-A1 and total SP-A specific antibodies. Data are expressed as means ± SE. **P* < 0.01 between normal and asthma subjects.

normal: 5,190 ± 900 vs. 4,800 ± 828 ng/ml, *P* = 0.78). SP-A1/SP-A was significantly higher in asthmatic compared with normal subjects (40.4 ± 3.5 vs. 24.1 ± 2.8% in asthma vs. normal subjects, *P* = 0.002) (Fig. 1).

Binding of ASP-A to *M. pneumoniae* is decreased compared with NSP-A. The concentration-dependent binding of NSP-A and ASP-A to solid-phase *M. pneumoniae* membranes (SP-A-bound) is shown in Fig. 2. AUC analysis of the SP-A binding curves revealed a significantly lower AUC for ASP-A curves compared with NSP-A curves at 87.2 ± 9.9 vs. 162 ± 31.2 (*P* = 0.03).

Comparison of mycoplasma membrane binding by different SP-A variants. In an additional series of experiments we compared the binding of the two most frequent human SP-A

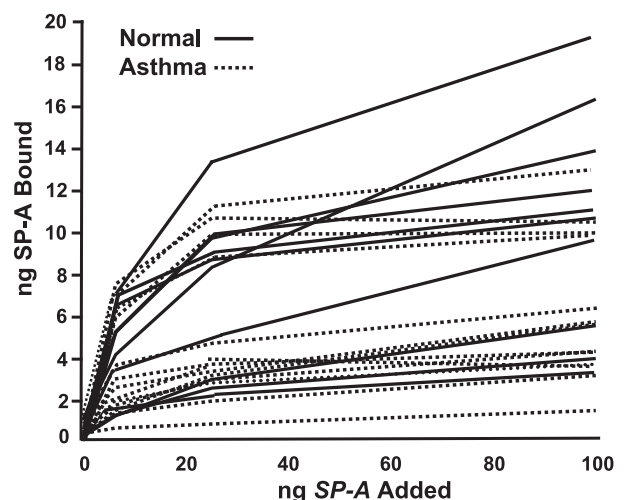


Fig. 2. SP-A binding to solid-phase mycoplasma membranes is markedly reduced in asthmatic subjects. One hundred nanograms of mycoplasma membranes were adsorbed to microtiter wells in 0.1 mM NaHCO₃, pH 9.6. The concentration-dependent binding of normal (NSP-A) and asthmatic SP-A (ASP-A) to solid-phase *Mycoplasma pneumoniae* membranes (SP-A bound) was detected by ELISA. Area under the curve (AUC) analysis of the SP-A binding curves revealed a significantly lower AUC for ASP-A curves compared with NSP-A, *P* = 0.03.

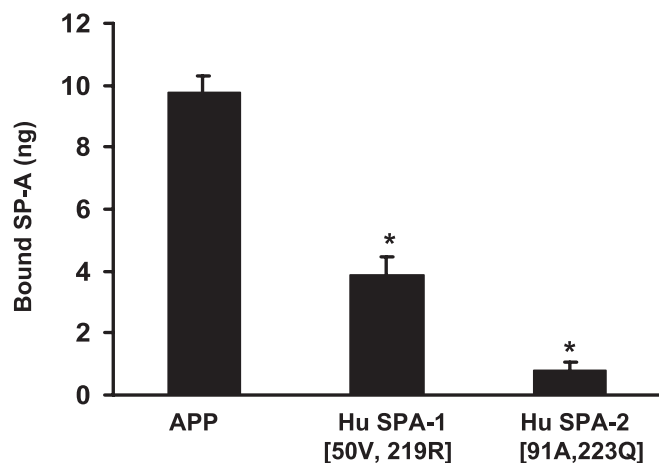


Fig. 3. SP-A variants exhibit different binding properties to mycoplasma membranes. One hundred nanograms of mycoplasma membranes were adsorbed to microtiter wells in 0.1 mM NaHCO₃, pH 9.6. Purified SP-A (100 ng) from the subjects with alveolar proteinosis (APP), or recombinant proteins expressed in HEK-293 cells, [50V, 219R] SP-A1 and [91A, 223Q] SP-A2 (Hu SPA-1 and Hu SPA-2, respectively), were incubated with the solid-phase membranes for 2 h at 37°C. The bound proteins were detected by ELISA with horseradish peroxidase-conjugated polyclonal anti-SP-A antibody. Orthophenylenediamine (1 mg/ml) was used as the color development reagent. Non-specific binding was blocked at each step of the procedure by using a buffered 3% bovine serum albumin. Values shown are means \pm SE. * P < 0.05.

allelic variants, purified recombinant [50V, 219R] SP-A1 and purified recombinant [91A, 223Q] SP-A2 protein. These two proteins bound to mannose-Sepharose affinity columns, demonstrating that they were active and normally folded molecules. All binding values were determined at the concentrations of proteins that approximated maximum binding for a fixed amount of mycoplasma ligand. Our data indicate that the most abundant allelic variant of SP-A2 has negligible binding to mycoplasma (0.8 ± 0.3 ng), compared with the most abundant allelic variant of SP-A1 (4.1 ± 0.6 ng) (Fig. 3).

Asthmatic SP-A is defective in abrogating MUC5AC expression. The baseline MUC5AC mRNA expression, measured by RT-PCR threshold cycle and normalized to GAPDH expression, was not different between the asthma and control epithelial cells prior to *M. pneumoniae* exposure (delta C_T: MUC5ACC_T-GAPDH C_T: 3.89 ± 0.47 vs. 3.89 ± 0.63 in the asthma and control groups, respectively, $P = 0.42$). The relative MUC5AC mRNA expression resulting from exposure of epithelial cells to *M. pneumoniae*, in the presence and absence of NSP-A and ASP-A, compared with negative, uninfected control, respectively, is shown in Fig. 4. *M. pneumoniae* infection had no significant effect on MUC5AC mRNA expression in the normal control group, but it significantly increased MUC5AC mRNA in the asthmatic epithelial cells compared with negative, uninfected control (normal group fold change from negative, uninfected control: 0.91 ± 0.16 , $P = 0.63$; asthmatic group fold change from negative, uninfected control: 2.57 ± 0.50 , $P = 0.04$). The addition of NSP-A significantly attenuated the effects of *M. pneumoniae* on MUC5AC expression in asthmatic airway epithelial cells to the level of the uninfected control (Fig. 4). In contrast, addition of ASP-A did not completely abrogate MUC5AC mRNA expression after *M. pneumoniae* infection in asthmatic epithelial cells, and the fold change was significantly higher than in the setting

of NSP-A (fold changes in MUC5AC expression in asthmatic airway epithelial cells were 0.75 ± 0.25 for the MP50 + NSP-A condition and 1.55 ± 0.22 for the ASP-A + MP50 condition, $P < 0.05$). The combination of NSP-A and ASP-A completely abrogated MUC5AC mRNA expression, similar to NSP-A alone in the asthmatic preparation, ruling out the presence of an SP-A inhibitor in the purified SP-A from asthmatic subjects. The addition of NSP-A and ASP-A alone also had no significant effect on MUC5AC mRNA in the normal control group (data not shown).

Asthmatic SP-A is defective in attenuating interleukin-8 secretion. Changes in IL-8 relative to the corresponding negative, uninfected control are shown in Fig. 5. Baseline IL-8 levels were not significantly different between groups without exposure to *M. pneumoniae* (112.8 ± 40.5 vs. 112.4 ± 63.3 ng/ml in asthmatic and control cells, respectively, $P = 0.99$). Although IL-8 expression increased in both asthmatic and normal airway epithelial cells after exposure to *M. pneumoniae*, the increase was not statistically significant in the normal control group (normal controls: 112.4 ± 63.3 ng/ml at baseline vs. 134.4 ± 73.8 ng/ml after *M. pneumoniae* infection, $P = 0.42$; asthma: 112.8 ± 40.5 ng/ml at baseline vs. 260.4 ± 123.4 ng/ml after *M. pneumoniae* infection, $P = 0.05$). In the asthmatic cells, NSP-A prevented the elevated IL-8 expression, but ASP-A had no significant effect (Fig. 5).

The combination of both NSP-A and ASP-A exhibited a similar effect upon IL-8 levels as NSP-A alone in the asthmatic group, suggesting that an inhibitor is not present in the purified ASP-A (Fig. 5).

Specificity of SP-A effects on decreasing MUC5AC and IL-8 in the presence of M. pneumoniae infection. When NSP-A was removed via adsorption to mannose-Sepharose beads in the

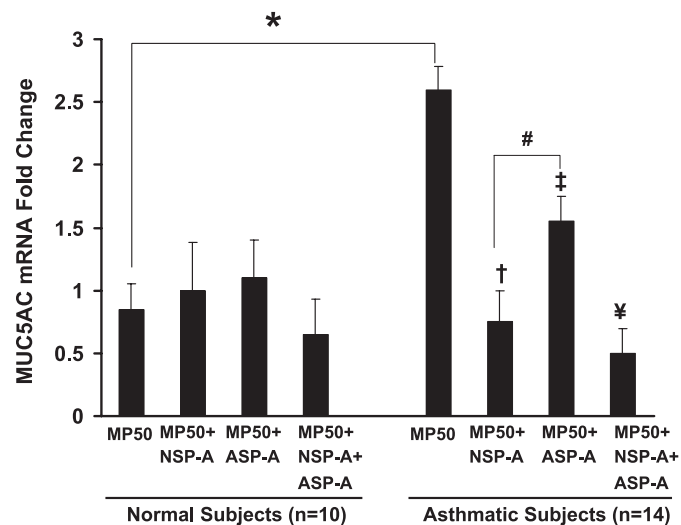


Fig. 4. SP-A from asthmatic subjects is defective in attenuating MUC5AC mRNA expression by asthmatic epithelial cells following *M. pneumoniae* infection. MUC5AC mRNA expression from airway epithelial cells isolated from normal control and asthmatic subjects exposed to *M. pneumoniae* at 50 cfu/cell (MP50) alone and after exposure to NSP-A (MP50 + NSP-A), ASP-A (MP50 + ASP-A), each at 20 μ g/ml, and a combination of both NSP-A and ASP-A (MP50 + NSP-A+ASP-A). The data, representing fold change from negative, uninfected control, are expressed as means \pm SE. * P < 0.01 between MP50 in normal vs. asthmatic subjects; † P < 0.01 between MP50 and MP50 + NSP-A; ‡ P < 0.01 between MP50 and MP50 + ASP-A; # P < 0.01 between MP50 and MP50 + NSP-A+ASP-A; # P < 0.05 between MP50 + NSP-A and MP50 + ASP-A.

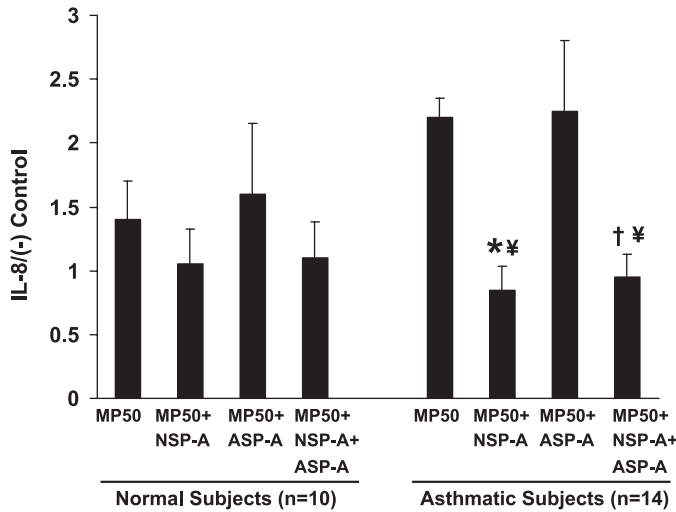


Fig. 5. SP-A from asthmatic subjects is defective in abrogating IL-8 levels secreted by asthmatic epithelial cells following *M. pneumoniae* infection. IL-8 expression from airway epithelial cells from normal control subjects and subjects with asthma exposed to *M. pneumoniae* at 50 cfu/cell (MP50) alone, and after exposure to NSP-A (MP50 + NSP-A), ASP-A (MP50 + ASP-A), each at 20 μg/ml, and a combination of both NSP-A and ASP-A (MP50 + NSP-A+ASP-A). The data, representing the ratio to negative, uninfected control are expressed as means ± SE. **P* < 0.01 between MP50 and MP50 + NSP-A; †*P* < 0.01 between MP50 and MP50 + NSP-A + ASP-A; #*P* < 0.05 between MP50 + ASP-A and MP50 + NSP-A and between MP50 + ASP-A and MP50 + NSP-A+ASP-A.

presence of 2 mM CaCl₂, the resulting solution did not significantly reduce MUC5AC and IL-8 in the setting of mycoplasma infection compared with untreated SP-A (Fig. 6). When 2 mM EDTA is added for the chelation of CaCl₂ in the presence of mannose-Sepharose beads, the resulting solution contains active SP-A, which decreased MUC5AC and IL-8 in a similar fashion to untreated SP-A (Fig. 6). In addition, Western blot analysis showed that there was no endogenous SP-A in apical supernatant of cultured airway epithelial cells from normal and asthmatic subjects before and after *M. pneumoniae* infection (data not shown), which suggests the inhibitory effects on MUC5AC and IL-8 production raised specifically from exogenous added SP-A.

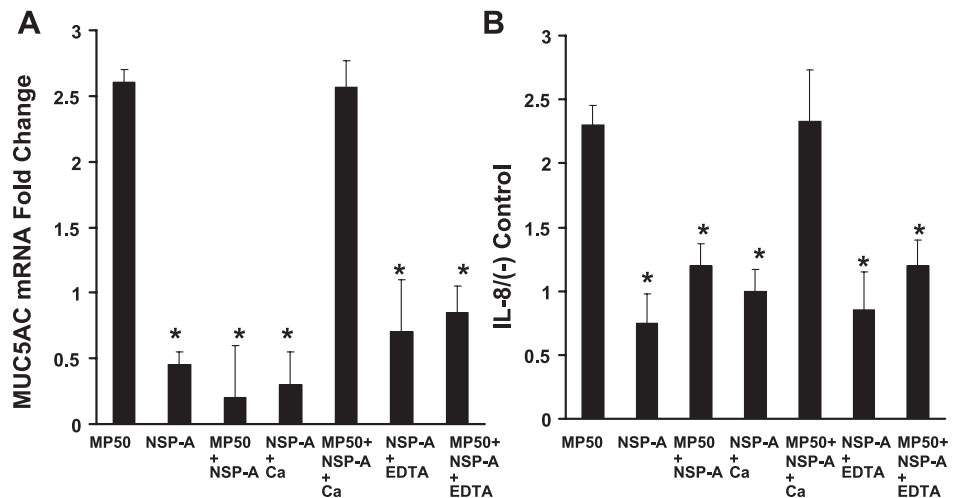


Fig. 6. Reduction of MUC5AC and IL-8 by NSP-A in the setting of mycoplasma infection is specific to SP-A. Purified NSP-A mixed with mannose-Sepharose beads in the presence of 2 mM CaCl₂ (NSP-A + Ca) and SP-A mixed with mannose-Sepharose beads in the presence of 2 mM EDTA (NSP-A + EDTA) was added separately to airway epithelial cells from 4 asthmatic subjects alone or in the presence of *M. pneumoniae* at 50 cfu/cells (MP50). A: fold change in MUC5AC mRNA relative to negative, uninfected control. B: ratio of IL-8 protein to negative, uninfected control. The data, representing the ratio to negative, uninfected control, are expressed as means ± SE. **P* < 0.01 compared with MP50.

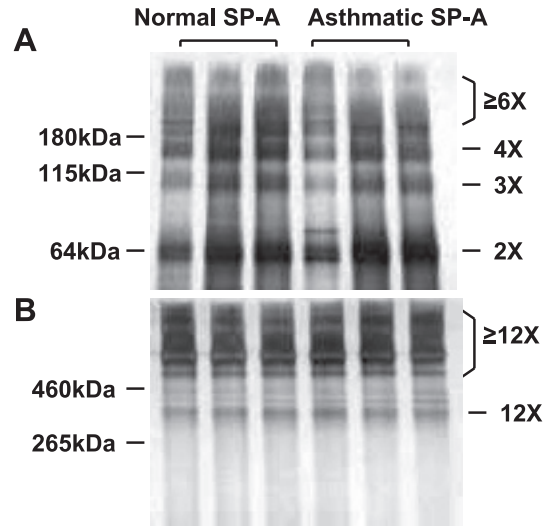


Fig. 7. Oligomerization status of purified SP-A. Three NSP-A samples and 3 ASP-A samples were purified and subjected to 4–20% PAGE under nonreducing conditions (A) or under native conditions (B) followed by silver staining. Numbers on left indicate the molecular mass. Marks on right represent oligomers.

Relationship of SP-A binding to M. pneumoniae and its anti-inflammatory effects. The maximum binding of SP-A to solid-phase *M. pneumoniae* membranes inversely correlated with the IL-8 production where the Spearman correlation coefficient was $r = -0.74$, $P = 0.006$. Thus higher maximum binding of SP-A to *M. pneumoniae* results in lower IL-8 expression by airway epithelial cells pretreated with SP-A after *M. pneumoniae* infection. In addition, SP-A1/SP-A positively correlated with maximal binding, but only in asthmatic subjects, where the Spearman correlation coefficient was $r = 0.71$, $P = 0.003$. In normal subjects, this correlation was not significant (Spearman correlation coefficient: $r = 0.25$, $P = 0.54$).

Characteristics of the oligomerization of SP-A from normal and asthmatic subjects. Purified SP-A from three normal and three asthmatic subjects was analyzed under nonreducing conditions (Fig. 7A) or native conditions (Fig. 7B) by silver staining of the PAGE. The intensity of each band of oligomers was quantified by densitometry (Tables 2 and 3). Under nonre-

Table 2. Quantification of oligomers of SP-A from normal and asthmatic subjects separated by nonreducing gel electrophoresis

Human SP-A	Percentage of Oligomers			
	2×	3×	4×	≥6×
Normal subjects (n = 3)	28.1 ± 0.03	14.4 ± 0.01	14.9 ± 0.01	42.4 ± 0.03
Asthmatic subjects (n = 3)	34.4 ± 0.03	13.7 ± 0.01	14.1 ± 0.01	36.5 ± 0.04

Values are means ± SE. SP-A, surfactant protein A; 2×, 3×, etc., oligomers consisting of 2, 3, etc., monomers.

ducing conditions, where inter- and intramolecular disulfide bonds remain intact, different sets of oligomers exist for both NSP-A and ASP-A that may represent dimers (2×), trimers (3×), tetramers (4×), hexamers, and higher order multimers (≥6×). The relative amount of each oligomer was not significantly different between NSP-A and ASP-A, as demonstrated in Table 2. In addition, we analyzed the SP-A under native conditions, under which SP-A was not treated with SDS, DTT, β-mercaptoethanol, or heating. Higher order multimers (>12×) and very small amount of dodecamers (12×) were observed, and there was no significant difference of the relative amounts between NSP-A and ASP-A (Fig. 7B; Table 3).

Oxidation levels of NSP-A and ASP-A. To determine whether differences in SP-A-mediated suppression of inflammation and mucin production arise from different protein oxidation levels, we measured the oxidized amino acids levels of purified SP-A from seven normal and seven asthmatic subjects. To exclude the artifactual increases in carbonyl content raised from tiny amounts of nucleic acid contamination in the purified SP-A, the purified SP-A samples were pretreated with DNase I and RNase A and showed a ratio of absorbance at 260/280 nm of 0.6, which indicates full removal of nucleic acids (5). No significant difference in oxidized amino acids was detected between NSP-A and ASP-A with or without DNase I and RNase A treatment (Fig. 8).

DISCUSSION

The results of this investigation demonstrate for the first time, in humans, that SP-A isolated directly from asthmatic subjects is deficient in its ability to decrease inflammation, as measured by IL-8 and MUC5AC expression associated with infection of airway epithelial cells *ex vivo*. The maximum amount of SP-A bound to solid-phase *M. pneumoniae* membranes inversely correlated with IL-8 production by airway epithelial cells, and this function is abnormal in asthma. SP-A1/SP-A positively correlated with maximum binding of SP-A to mycoplasma, but only within the asthmatic group. In addition, SP-A1 and SP-A2 variants display different binding to mycoplasma. We hypothesize that pathogen binding by SP-A in asthma may be modulated differently from that in the normal airway. Functions such as phagocytosis stimulated by SP-A1 and SP-A2 proteins have previously been shown to be differentially affected by oxidative stress, as reported by Mikerov et al. (42). We have previously demonstrated that gene and protein expression of the major mucin protein in human airways, MUC5AC, is increased in airway epithelial cells isolated directly from asthmatic subjects after exposure to *M. pneu-*

moniae, compared with airway epithelial cells isolated directly from normal control subjects (31). Therefore, this *ex vivo* model provides a method to test elements of the innate immune response. The lack of a significant anti-inflammatory effect by ASP-A does not appear to be due to the presence of a competitive inhibitor, since mixing experiments with NSP-A and ASP-A resulted in abrogation of inflammation, similar to NSP-A alone. The effect is also specific for SP-A, since decreased MUC5AC and IL-8 were demonstrated when purified SP-A is employed, and the anti-inflammatory effect was absent when total SP-A was removed by mannose-Sepharose.

Surfactant is a lipoprotein complex that reduces surface tension at the air-liquid interface of the lung and also participates in host defense. Surfactant proteins, SP-B and SP-C, are hydrophobic and interact with surfactant lipids and participate in reduction of surface tension. SP-A and SP-D are relatively hydrophilic and are members of the collectin family of proteins that participate in pulmonary immunity. Both SP-A and SP-D are synthesized and secreted by the alveolar type II cell, the airway Clara cell (4), submucosal cells (18), as well as non-pulmonary tissues (49, 37, 39, 40). SP-A and SP-D bind to pathogens largely via interactions of their C-type lectin domains with carbohydrates on bacterial membranes and enhance pathogen phagocytosis (48). SP-A also binds to Toll-like receptor-2 (TLR2) and -4 (TLR4) (51). *M. pneumoniae* also binds to TLR2 to produce MUC5AC and IL-8, and we previously demonstrated that inhibition of TLR2 results in no significant effect of *M. pneumoniae* upon airway epithelial cells (31). Reduced maximum binding of ASP-A to *M. pneumoniae* in our study may permit more *M. pneumoniae* to bind to TLR2 and initiate infection. Alternatively, it is also possible that ASP-A does not bind effectively to TLR2 to attenuate infection independently of interactions with *M. pneumoniae* (51). Additional experiments evaluating binding of SP-A to TLR2 in this model are required to test this latter hypothesis. In either case, there may be at least two mechanisms by which reduced binding interaction between SP-A and *M. pneumoniae* results in a reduced anti-inflammatory effect.

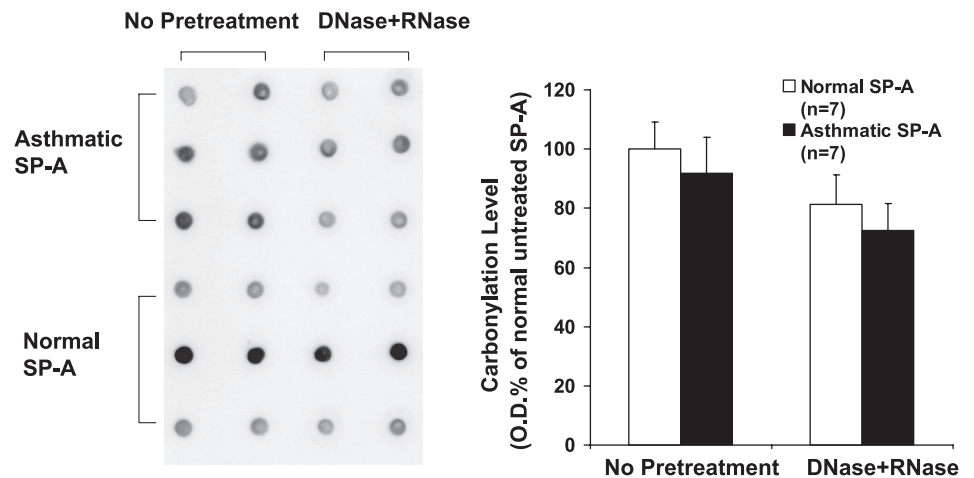
In asthma, the dysfunction of SP-A may contribute to loss of regulation of innate immunity. Human SP-A is encoded by two genes, SP-A1 and SP-A2, and each has been identified with several variants that differ with regard to the functions of oligomerization (17, 58) and phagocytosis (43, 44, 58). SP-A2 protein variants enhanced phagocytosis of *Pseudomonas aeruginosa* more effectively than SP-A1 protein variants (44). However, SP-A2 was more susceptible to loss of this function compared with SP-A1 after exposure to ozone (42). Moreover, the ratio of SP-A1 to total SP-A has been shown to vary as a function of age and lung disease such as cystic fibrosis (55). Because we found the percentage of SP-A1 to be increased in

Table 3. Quantification of oligomers of SP-A from Normal and Asthmatic Subjects Separated by Native Gel Electrophoresis

Human SP-A	Percentage of Oligomers	
	12 ×	≥12 ×
Normal subjects (n = 3)	9.2 ± 0.01	90.7 ± 0.03
Asthmatic subjects (n = 3)	9.8 ± 0.02	90.2 ± 0.04

Values are means ± SE. 12×, Dodecamers; ≥12×, higher order multimers.

Fig. 8. Oxidation levels of purified SP-A from normal and asthmatic subjects. Two hundred nanograms of purified SP-A from normal and asthmatic subjects, with or without DNase and RNase pretreatment, were blotted onto nitrocellulose membrane and oxidized proteins were detected by the OxyBlot method. Blots were exposed to XAR films following enhanced chemiluminescent detection and quantified by densitometry. The data representing the percentage of optical density of the NSP-A without treatment with DNase and RNase are expressed as means \pm SE.



asthma, we hypothesize that the alteration in this ratio may modulate binding to *M. pneumoniae*, as illustrated by the different binding to mycoplasma between SP-A1 and SP-A2 variants and the significant correlation between the maximal binding of SP-A to mycoplasma and SP-A1/SP-A in asthma. The elevated ratio of SP-A1/SP-A in asthma may reflect upregulation of SP-A1 gene expression, or reduced turnover of SP-A1, or both. Despite the relative increases in SP-A1 in asthmatic subjects, the activity of the ASP-A in suppressing IL-8 protein and MUC5AC mRNA is significantly diminished. One possible interpretation of these data could be that mycoplasma sequesters SP-A1 and prevents the protein from negatively regulating Toll-like receptors, thereby enhancing inflammatory responses. Since the SP-A population within the lung consists of heterooligomers of different alleles and different genes, the increase in SP-A1 content in asthmatic subjects may cause more heterooligomers to be sequestered. In addition, the polymorphic variation of both SP-A1 and SP-A2 may also contribute to altered protein function, as suggested by several association studies of SP-A genetic variants and disease susceptibility and/or severity (14–16, 19). SP-A1 and SP-A2 variants demonstrated differences in oligomerization and phagocytosis when exposed to ozone (27, 42), which may explain, at least in part, the variability of SP-A to modulate an insult in an oxidative environment. Moreover, association between human SP-A oligomer size and disease has been previously observed between normal subjects and subjects with birch pollen allergy (25). Our present study did not show significant differences of the oligomerization patterns of NSP-A and ASP-A under nonreducing or native conditions. However, we cannot exclude the existence of certain new oligomeric molecules in ASP-A, which may migrate at many different positions in the gel that cannot be detected either because their band intensity is too low and they migrate along with existing oligomers, or they are too large to enter the gel. In addition, the possibility exists that there are differences in the level of oxidation of specific amino acids, such as cysteine and proline, resulting in the differences in SP-A structure and/or conformation; this needs to be further investigated.

The presence and significance of SP-A1 and SP-A2 and their corresponding variants have not been studied in asthma, but data exist to suggest surfactant protein and phospholipid deficiency or dysfunction (56, 61, 65), although this is controver-

sial (9). Pettigrew and colleagues (46) reported an association between wheezing and coughing in newborn infants and concluded that polymorphisms within the SP-A gene may be associated with an increased risk of asthma. Our present data demonstrated the differences in maximal binding of SP-A1 and SP-A2 variants to mycoplasma. Future studies to further determine additional mechanisms driving our results should include comparing the inhibitory effects of the SP-A1 and SP-A2 variants on IL-8 and MUC5AC production, genotyping of our subjects for SP-A polymorphisms, and evaluating other infectious organisms, such as rhinovirus, to determine whether the abNSP-A function noted in asthma is unique to mycoplasma or is present with other pathogens relevant to asthma. We also realize that our ex vivo model involves only airway epithelial cells and SP-A interacts with several cell types including macrophages, neutrophils, T cells, and dendritic cells (54). This work could be expanded to include the function of ASP-A in these cell types in future studies.

In conclusion, the present study demonstrates that native SP-A derived from BAL of asthmatic subjects is defective in abrogating inflammation, which may be associated with the altered SP-A1/SP-A in asthmatic subjects. How the balance of SP-A1 and SP-A2 gene products affects innate and adaptive immune responses in asthma, and how these functions are regulated by structure/genotype, as well as the effects of oxidation, are possible subjects of future investigation. Abnormalities in SP-A isolated from asthmatic subjects may justify consideration of surfactant protein replacement as a therapeutic option for asthma.

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Present address of G. Wang: Department of Surgery, SUNY Upstate Medical University, Syracuse, NY 13210.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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