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Genetic Variation in SP-A2 Leads to Differential Binding to Mycoplasma pneumoniae Membranes and Regulation of Host Responses

Julie G. Ledford,*†‡ Dennis R. Voelker,§ Kenneth J. Addison,*‡ Ying Wang,*† Vinayak S. Nikam,† Simone Degan,¶ Pitachaimani Kandasamy,§ Sasipa Tanyaratsrisakul,§ Bernard M. Fischer,† Monica Kraft,*† and John W. Hollingsworth*‡

Mycoplasma pneumoniae is an extracellular pathogen that colonizes mucosal surfaces of the respiratory tract and is associated with asthma exacerbations. Previous reports demonstrate that surfactant protein-A (SP-A) binds live M. pneumoniae and mycoplasma membrane fractions (MMF) with high affinity. Humans express a repertoire of single-amino acid genetic variants of SP-A that may be associated with lung disease, and our findings demonstrate that allelic differences in SP-A2 (Gln223Lys) affect the binding to MMF. We show that SP-A2−/− mice are more susceptible to MMF exposure and have significant increases in mucin production and neutrophil recruitment. Novel humanized SP-A2–transgenic mice harboring the hSP-A2 223K allele exhibit reduced neutrophil influx and mucin production in the lungs when challenged with MMF compared with SP-A2−/− mice. Conversely, mice expressing hSP-A2 223Q have increased neutrophil influx and mucin production that are similar to SP-A2−/− mice. Using tracheal epithelial cell cultures, we show that enhanced mucin production to MMF occurs in the absence of SP-A and is not dependent upon neutrophil recruitment. Increased phosphorylation of the epidermal growth factor receptor (EGFR) was evident in the lungs of MMF-challenged mice when SP-A was absent. Pharmacologic inhibition of EGFR prior to MMF challenge dramatically reduced mucin production in SP-A−/− mice. These findings suggest a protective role for SP-A in limiting MMF-stimulated mucin production that occurs through interference with EGFR-mediated signaling. SP-A interaction with the EGFR signaling pathway appears to occur in an allele-specific manner that may have important implications for SP-A polymorphisms in human diseases. The Journal of Immunology, 2015, 194: 6123–6132.

Surfactant protein-A (SP-A) is a highly oligomeric protein component of pulmonary surfactant that belongs to the collagén domain containing-C type lectin (collectin) superfamily. Members of the collectin family typically contain an N-terminal collagen-like domain and a C-terminal carbohydrate-recognition domain (CRD). The CRD binds a variety of ligands, including pathogen-derived carbohydrate moieties, in a Ca2+-dependent reaction. For numerous pathogens, SP-A binding to cell surface glycans results in enhanced phagocytosis (1–3).

Human SP-A is encoded by two genes designated SP-A1 and SP-A2 (4–6). Several recent studies identified associations of specific SP-A alleles with infant wheezing (7), tuberculosis (8), respiratory distress syndrome (9, 10), respiratory syncytial virus infections (11), chronic obstructive pulmonary disease (12), and susceptibility to ozone (13). Additionally, we showed that SP-A extracted from asthma subjects has decreased binding affinity for Mycoplasma pneumoniae compared with SP-A extracted from normal controls and that asthmatic SP-A is defective at abrogating M. pneumoniae–induced Muc5AC expression (14).

M. pneumoniae is an extracellular human pathogen that is frequently the causative agent for “walking pneumonia.” M. pneumoniae is classified as a Mollicute and can cause a variety of airway diseases, including bronchiolitis, bronchitis, and bronchiectasis, and it recently was associated with asthma exacerbations (15–18). Mycoplasmas are characterized by their unusually small size (0.15–0.3 μm in diameter) and absence of a cell wall, which renders them resistant to many antibiotics. Mycoplasmas contain a trilayered cell membrane composed of lipoprotein, glycolipid, and lipoglycan components that are antigenic and capable of inducing a host pathogenic response (19, 20).

Although mycoplasmas lack the LPs found in Gram-negative bacteria, they express several cell surface ligands capable of interacting with SP-A. One class of high-affinity ligands for SP-A is composed of disaturated phosphatidylglycerols (21). Kannan et al. (22) also identified a specific membrane protein, MPN372, which also binds SP-A with high affinity. SP-A binding to M. pneumoniae inhibits the growth of the organism (21). Thus, evaluation of mycoplasma membrane fractions (MMF) offers an opportunity to evaluate specific interactions with SP-A.

In previous studies using wild-type (WT) and SP-A−/− mice, we showed that SP-A is protective against live M. pneumoniae in-
fection (23). Because SP-A binds *M. pneumoniae* and acts as an opsonin, we sought to determine whether the phenotypes observed in *M. pneumoniae*-infected SP-A2/− mice were due to the increased pathogen burden or innate recognition of pathogen-derived material. To address this issue, we used a preparation of MMF instilled into WT and SP-A2/− mice to determine the role of SP-A in regulating the inflammatory response to membrane components in the absence of *M. pneumoniae* colonization.

Our findings show that SP-A2/− mice have enhanced mucin production and neutrophil recruitment at 12 h after challenge with MMF, whereas WT mice have negligible mucin production. We also discovered that genetic variation in SP-A2 at position 223, which is associated with multiple lung diseases (7, 24), leads to dramatic differences in binding to MMF. We generated humanized knock-in transgenic mice that lack mouse SP-A, but express (h) SP-A2, with either Gln or Lys at position 223. Mice that express the Gln223 variant, which binds MMF poorly, have an enhanced response to MMF compared with those expressing the Lys223 variant. Using mouse tracheal epithelial cell (MTEC) cultures grown at air–liquid interfaces (ALIs), we were able to determine that the effect of SP-A in the regulation of MMF-induced Muc5AC is independent of neutrophil recruitment. In further studies, we show that MMF challenge in SP-A2/− mice results in enhanced phosphorylation of the epidermal growth factor receptor (EGFR) on airway epithelial cells and that pharmacologic inhibition of EGFR results in attenuated mucin production following MMF stimulation. Taken together, these findings provide novel insight into how genetic alterations in human (h)SP-A2 at position 223 can alter binding affinity for a stimulus and, thereby, mediate the host response by reducing EGFR signaling and mucin production.

### Materials and Methods

#### Generation of SP-A humanized transgenic mice

Site-specific knock-in transgenic mice harboring alleles for hSP-A were generated with assistance of the Duke BAC core and Transgenics Shared Resource at Duke University, which is currently funded by the National Institutes of Health National Cancer Institute Center Core grant P30 CA014236 to the Duke Cancer Institute. Two targeting vectors were created that contained the sequence for hSP-A2, only differing at codons for the Gln223 variant. Using mouse tracheal epithelial cell (MTEC) cultures and vectors were linearized and underwent homologous recombination at the targeting vector, and forward: 5′-GAG CCA AGT TCC TCA CAG CAG AAG-3′; reverse: 5′-AGA CGT CCT TGG GAA GGA CTC CCC-3′, which amplifies the 9.5-kb long arm of the targeting vector, and forward: 5′-GAG CCA AGT TCC TCA CAG CAG AAG-3′; reverse: 5′-AGA CGT CCT TGG GAA GGA CTC CCC-3′, which amplifies the 2.5-kb short arm of the targeting vector.

#### Mouse tracheal cell isolation

Mice were euthanized, and tracheas were removed by dissection and immediately placed in Ham's F-12 medium on ice. Excess connective tissue, muscle, vasculature, and nerves were removed from the trachea, and the mucosal linings were scraped and collected by scraping and centrifugation at 8000 × g for 15 min at 4°C. The tracheas were placed in 10 ml Ham's F-12 medium containing 0.1% protease, and lung tissue was obtained for further analysis. For analysis of mucin production, mice were harvested either at 16 or 24 h post-MMF exposure, as noted in the text for each experiment; for p-EGFR staining, mice were harvested 12 h after MMF exposure. Some mice were given an EGFR inhibitor, AG1478 (Selleckchem), via i.p. injection 30 min prior to receiving oropharyngeal delivery of MMF. AG1478 was solubilized in DMSO and diluted to a concentration of 10 mg/kg mouse weight in sterile saline. The vehicle control was DMSO diluted in sterile saline.

#### Production of recombinant SP-A2

The human cDNAs encoding for SP-A2 [91A, 223Q] and [91A, 223K] were constructed in a PEE14 vector under control of an hCMV-MIE promoter (21, 26). The plasmids were purified in large scale and used to transfect Freestyle HEK-293 cells using 293-fect, which ensures mammalian oligosaccharide patterns and sufficient hydroxylation of prolines within the collagen-like domain. After cell transfection, the cells were grown in suspension culture in serum-free FreeStyle 293 Expression Medium. At 48 h after transfection, the culture supernatants were harvested by centrifugation and filter sterilized. The supernatants were brought to 85% saturation with ammonium sulfate, and the crude protein pellet was harvested by centrifugation and subsequently dialyzed against 5 mM Tris-Cl (pH 7.4). The dialyzed protein preparation was adjusted to 10 mM CaCl2 and applied to a mannose Sepharose affinity column. The affinity column was washed with 10 volumes of binding buffer, and the protein was eluted with 7 mM EDTA, 5 mM Tris-Cl (pH 7.4). The protein eluate was concentrated, and EDTA was removed using a Centricron Jumbosep apparatus with a 10-kDa cutoff filter. Endotoxin was removed from the protein using a Mustang E adsorption filter. The purity content of the final preparation was determined using Limulus amebocyte lysis. Aliquots of the protein were stored at ~20°C prior to use. This affinity-purification procedure, which requires that the C-type lectin activity of the protein be functional (27), ensures that the isolated SP-A proteins are properly folded and active.

#### MMF preparation and binding assay

Membrane fractions from *M. pneumoniae* were prepared as previously described (26). Briefly, *M. pneumoniae* (strain FH; ATCC 15531) was grown for 5 d at 37°C in polystyrene flasks containing 100 ml SP-4 medium. After 5 d, adherent and nonadherent *M. pneumoniae* cells were collected by scraping and centrifugation at 8000 × g for 15 min at 4°C. The pellet was washed twice with PBS by centrifugation and resuspended in PBS. The mixture was layered on a discontinuous sucrose gradient (60, 52, 48, and 40%) and centrifuged at 10,000 rpm at 4°C for 30 min. Cells were recovered from the 48–52% interface, mixed with PBS, and centrifuged at 8000 × g at 4°C for 15 min. The purified *M. pneumoniae* pellet was resuspended in PBS, mixed with three volumes of distilled water, and incubated on ice for 30 min. The cells were probe sonicated on ice for a total of 2 min, using 30-s sonication and 30-s cooling cycles. Polymyxin B (100 μg/ml) was added to the lysed *M. pneumoniae* cells and centrifuged at 100,000 × g at 4°C for 1 h. The pellet was washed twice with polymyxin B and centrifuged at 100,000 × g at 4°C for 1 h. The final *M. pneumoniae* membrane pellet was resuspended in PBS, homogenized, and used as MMF.

The binding of purified SP-A to MMF was measured as previously described (21). MMFs (100 ng protein/well) were absorbed to microwell plates in 0.1 mM NaHCO3 (pH 9.6) at 4°C overnight. Following adsorption, the wells were blocked with buffer A (20 mM Tris [pH 7.4], 150 mM NaCl, and 5 mM CaCl2) containing 2% BSA for 1 h at 37°C. SP-A at various concentrations was added to each well in buffer A with 2% BSA. The binding reaction was performed for 2 h at 37°C. The bound SP-A was detected by ELISA using 5 μg/ml HRP-conjugated primary Ab. Orthophenylenediamine (1 mg/ml) was used as the color development reagent for detecting the bound HRP-conjugated Ab.

#### Mouse tracheal cell isolation

Mice were euthanized, and tracheas were removed by dissection and immediately placed in Ham’s F-12 medium on ice. Excess connective tissue, muscle, vasculature, and nerves were removed from the trachea, and a longitudinal incision was made exposing the mucosal lining. The trachea was removed, and muscle, vasculature, and nerves were removed from the trachea, and the mucosal linings were scraped and collected by scraping and centrifugation at 8000 × g for 15 min at 4°C. The medium supernatant was removed, and the cell pellet was resuspended in 5 ml Versene (Life Technologies) for 15 min at 37°C. Next, 10% PBS–Ham’s F-12 medium was added to the tube and centrifuged (900 rpm, 5 min, 4°C). The supernatant was removed, and the cell pellet was resuspended in 6 ml 10% FBS–Ham’s F-12 medium.
Culture media and supplements

DMEM–Ham’s F-12 medium was used for both the harvest and culture of MTECs. The culture medium was supplemented with 250 ng/ml amphotericin B solution (HyClone), 20 ng/ml choleter (List Biological), 104 μg/ml bovine pituitary extract (Lonza), 5 μg/ml insulin (Sigma), 5 μg/ml human apo-transferlin (Sigma), 0.1 μM dexamethasone (Sigma), 5 ng/ml mouse epidermal growth factor (Sigma), 0.01 μM retinol (Sigma), 20 U/ml nystatin (Sigma), and 50 μg/ml gentamicin. FBS (Atlanta Biologicals) was used to prepare 10 and 5% serum-rich media used at different steps in the ALI culture protocol.

In vitro culture of MTECs

Costar Transwell (12 mm, 0.4 μm pores) 12-well plates were used to culture the MTECs according to methods previously described (28). The polyester membrane was coated with 300 μg/ml rat tail collagen in 0.02 N glacial acetic acid at room temperature for 1 h. The membranes were washed with PBS and conditioned with Ham’s F-12 medium for 1 h. The medium was removed from the apical and basolateral sides, and 1 ml 10% FBS culture medium was added to the basolateral part of each well. All of the cells were plated evenly between the 12 wells in 500 μl 10% FBS culture media. The plate was incubated at 37 °C in an air–5% CO2 atmosphere for 72 h, without changing the medium. After the initial seeding period of 72 h, the medium on both the apical and basal sides of the membrane was replaced every other day. When the cells reached 80% confluence (6–8 d), the culture medium was changed to 5% FBS and replaced daily, only on the basolateral side, establishing an ALI. When the cells reached full confluence, the medium was changed to serum-free Ham’s F-12 culture medium and replaced daily. The cells were maintained at ALI for 14 d.

Real-time PCR

Mouse tissues and MTECs were collected into 1 ml TRI Reagent (Sigma). RNA was isolated using the standard TRI Reagent/chloroform extraction method. The RNA was quantified using a NanoDrop Spectrophotometer ND-1000. cDNA was synthesized from 1 μg total RNA using a Bio-Rad cDNA Synthesis Kit. Real-time PCR was performed using Bioline 2x SensiFAST SYBR No-ROX mix. The samples were analyzed for expression levels of mouse MUC5AC using forward and reverse primers specific to the gene (forward: 5′-GAG GGC CCA GTG AGC ATC TCC-3′, reverse: 5′-TGG GAC AGC AGT ATT CAG T-3′). The relative levels of expression obtained were normalized to the mammalian housekeeping gene Cyclophilin using primers specific to the gene (forward: 5′-AGC ACT GGA GAG AAA GGA TTT GG-3′, reverse: 5′-TCT TCT TGC TGC TCT TGC CAT T-3′).

Histological analysis

Mice were euthanized by CO2 asphyxiation. Left lung lobes were excised, removed, and fixed in 10% buffered formalin phosphate (Fisher Scientific). After 3–5 d, the lung lobes were transferred from formalin to 70% ethanol. The lobes were embedded in paraffin, cut to 4 μm, mounted on a slide, and stained with periodic acid–Schiff (PAS) to detect mucin production in the secreted product of MTEC. The lobes were embedded in paraffin, cut to 4 μm, mounted on a slide, and stained with periodic acid–Schiff (PAS) to detect mucin production in the secreted product of MTEC. Paraffin-embedded sections were stained according to standard immunohistochemical protocols using either an enhanced ABC (Vector Laboratories), according to the manufacturer’s instructions. For hSP-A staining, sections were blocked for 30 min with 5% donkey serum in TBS-T and incubated with primary Ab at 1:50 dilution overnight at 4 °C. Following secondary Ab (Stain Boost HRP Rabbit; Cell Signaling) for 30 min at room temperature. The isotype control (Rabbit (DA1E) mAb IgG; Cell Signaling) was used at the same concentration, and staining was carried out using the same conditions as for the specific Ab staining. Sections were visualized by Vector Red and hematoxylin staining, according to the manufacturers’ instructions. For hSP-A staining, sections were blocked using the M.O.M. Immunoprotection reagent (Vector Laboratories) according to the manufacturer’s instructions, and the primary Ab hSP-A was added at 1:3000 dilution for 30 min. Ab reactivity was visualized using DAB substrate (Vector Laboratories) and counterstained with hematoxylin.
Because SP-A is known to bind with high affinity to disaturated phosphatidylglycerols and Mpn372 (21, 22), we sought to determine which of these interactions could play a role in MMF regulation of Muc5AC expression. Membrane fractions were prepared from normal M. pneumoniae (WT MMF) and from M. pneumoniae lacking the binding protein (MMF Δ372) and subsequently used to induce Muc5AC expression in the lungs of WT and SP-A−/− mice 24 h postchallenge compared with saline controls (n = 3 experiments). (D) Scale bars, 100 µm. *p < 0.05, **p < 0.01, ***p < 0.001, Student t test.

FIGURE 1. Inflammatory responses to MMF in WT and SP-A−/− mice. WT and SP-A−/− mice were challenged with 5 µg MMF, and macrophages (A) and neutrophils (B) were quantified by cyto-centrifugation analysis of BALF 24 h after challenge. (C) Sections of lung were scored for PAS staining at 12 and 24 h after MMF challenge. Representative PAS (D) and Muc5AC (E) staining for WT and SP-A−/− MMF-challenged mice 24 h postchallenge compared with saline controls (n = 3 experiments). (D) Scale bars, 100 µm. *p < 0.05, **p < 0.01, ***p < 0.001, Student t test.
and ELISA (~70 pg/ml lavage) (Fig. 3B). The humanized transgenic mice expressed hSP-A at much lower levels than we anticipated, in the range of pg/ml. Murine SP-A is normally expressed in the range of μg/ml. We were unable to detect hSP-A at this low level with Western blot of lavage fluid due to limitations of the Ab. However, we detected hSP-A expression using more sensitive IHC methods. Tissue sections obtained from human lung biopsy were used as a positive control for hSP-A staining (Fig. 3C). No hSP-A was detected in sections from WT (Fig. 3D) or SP-A2−/− mice (Fig. 3E). Mice harboring the hSP-A2 transgenes gave positive reactions to the Ab in the alveolar regions and in alveolar macrophages (which are known to internalize SP-A) for SP-A2 223K−/− (Fig. 3F) and SP-A2 223Q−/− (Fig. 3G) mice.

**Genetic variation in SP-A2 results in differential responses to MMF**

The humanized SP-A mice that express either SP-A2 223K or SP-A2 223Q were challenged with MMF and compared with WT mice to determine the immune response after 16 h. Mice that express SP-A2 223K had little PAS stain, similar to WT challenged mice. PAS stain was significantly more pronounced in SP-A2 223Q−/− mice, similar to SP-A2−/− mice (Fig. 4A, 4B). Similarly, mice that express SP-A2 223Q had enhanced neutrophil recruitment and increased KC in response to MMF challenge (Fig. 4C, 4D). Thus, SP-A2 223Q–transgenic mice essentially phenocopied SP-A2−/− mice. Conversely, SP-A2 223K mice were protected from enhanced neutrophil influx and had lower KC levels compared with the SP-A2 223Q variant and, thus, appeared more like WT mice.

**MMF regulation of Muc5AC expression occurs in the absence of neutrophils**

We next sought to determine whether MMF induction of mucin production and Muc5AC expression in the absence of SP-A binding were due to enhanced neutrophil recruitment. MTECs from WT and SP-A2−/− mice were cultured at an ALI and stimulated with MMF. Real-time PCR analysis of MMF-challenged MTECs revealed that these cells responded much like the whole lung in the

See **FIGURE 2.** SP-A binds to MMF in an isoform-specific manner. (A) Human SP-A isoforms were examined for interaction with solid-phase MMFs. APP denotes the protein purified from alveolar proteinosis patients, which was used as a positive control. High-affinity binding was detected by ELISA. (B) MMF was prepared from a mutant strain of *M. pneumoniae* that lacks the SP-A binding protein (MMF Δ372) and was compared with WT MMF preparations for the ability to upregulate Muc5AC RNA expression in WT and SP-A2−/− mice. *n = 10 mice/group; two experiments combined. *p < 0.05, **p < 0.01, Student t test.

**FIGURE 3.** Generation of humanized SP-A–transgenic mice. (A) Site-targeted knock-in mice that express either SP-A2 223K or SP-A2 223Q were created by BAC recombineering into the endogenous mouse locus of C57BL/6 ES cells. (B) Murine SP-A (mSP-A) and hSP-A2 expression was determined in targeted mice by real-time PCR of lung tissue and by ELISA of BALF. Real-time PCR was standardized to the housekeeping gene Cyclophilin. Staining by IHC for hSP-A in human lung tissue (positive control) (C), WT murine distal airway (negative control) (D), SP-A2−/− murine distal airway (negative control) (E), SP-A2 223K–targeted mice alveolar macrophages and distal airway (F), and SP-A2 223Q–targeted mice alveolar macrophages and distal airway (G). Original magnification ×40.
production of Muc5AC. Similar to what we observed in MMF-challenged WT mice (Fig. 2B), MMF challenge of WT MTECs also resulted in decreased Muc5AC transcript compared with vehicle-treated controls (Fig. 5A). Likewise, in MMF-challenged MTECs from SP-A2/2 mice, Muc5AC transcript was elevated over vehicle controls (Fig. 5A), similar to our in vivo results (Fig. 2B). Additionally, MTECs derived from SP-A2/2 mice had significantly greater Muc5AC transcript after MMF challenge compared with MTECs derived from WT mice. Interestingly, MTECs from WT mice produced an SP-A transcript, as detected by real-time PCR, and secreted a band that reacted with an anti-SP-A Ab by Western blot analysis (Fig. 5B, 5C). The reactive band, which appears to be present as a nonglycosylated dimer ~50 kDa in size (30), is not detected in apical supernatants from SP-A2/2 MTEC cultures. KC levels were significantly elevated in the basolateral supernatant compartment after MMF stimulation, which is shown as a fold increase over vehicle-stimulated controls. Additionally, significantly more KC was secreted from the SP-A2/2-derived MTECS compared with those grown from WT mice (Fig. 5D). The increased KC produced in the cultures derived from SP-A2/2 mice is a likely mechanism driving the increased neutrophil recruitment in SP-A–deficient mice observed in Fig. 4C. The above findings obtained with ALI cultures demonstrate that the enhanced mucin production observed in SP-A2/2 mice is independent of the recruitment of neutrophils.

EGFR has enhanced phosphorylation in SP-A2/2 mice when challenged with MMF

EGFR is activated by a variety of ligands and undergoes a transformation from an inactive monomer to an active homodimer. This dimerization leads to autophosphorylation and stimulation of tyrosine kinase pathways that initiate several signaling pathways, including those involved in mucin production (31, 32) and IL-8 (or murine KC) production (33). Sections from saline-treated WT (Fig. 6A) and SP-A2/2 (Fig. 6B) mice and MMF-challenged WT (Fig. 6D) and SP-A2/2 (Fig. 6E) mice were stained for p-EGFR. Little staining was evident in the isotype control (Fig. 6C) or in the
WT saline or MMF-challenged mice sections. However, MMF-exposed lung sections from mice lacking SP-A had increased staining for p-EGFR compared with SP-A-sufficient (WT) MMF-challenged mice (Fig. 6E, 6F).

Inhibition of EGF in SP-A\(^{-/-}\) mice results in decreased mucin production

We next sought to determine whether EGF signaling is necessary for MMF-induced mucin production when SP-A is absent. SP-A\(^{-/-}\) mice were treated with the EGF inhibitor AG1478 prior to instillation of MMF and compared with vehicle-treated and MMF-challenged SP-A\(^{-/-}\) mice. SP-A\(^{-/-}\) mice challenged with MMF and treated with vehicle had comparable levels of PAS and p-EGFR staining as previously detected in SP-A\(^{-/-}\) mice challenged with MMF (Fig. 7A, 7C). However, the MMF-challenged SP-A\(^{-/-}\) mice that received treatment with the EGFR inhibitor (AG1478) prior to challenge had significantly decreased PAS staining compared with the vehicle controls (Fig. 7B, 7E). Additionally, SP-A\(^{-/-}\) mice treated with the EGFR inhibitor had decreased Muc5AC RNA compared with vehicle-treated SP-A\(^{-/-}\) mice (Fig. 7F), supporting the conclusion that inhibition of EGF protects from MMF-induced Muc5AC upregulation and mucin production.

Discussion

Previous studies demonstrated that SP-A binds to a variety of pathogens and aids in their phagocytosis by alveolar macrophages. Our studies provide insight into a novel role for SP-A by binding to noninfectious membranes derived from pathogenic \textit{M. pneumoniae} and protecting from enhanced mucin production by limiting EGF signaling. Additionally, we show that a specific genetic allelic variant in SP-A2 (position Gln223Lys), which is commonly found in the population, can alter the ability of SP-A to bind to membrane components derived from infectious \textit{M. pneumoniae} and, as a consequence, reduces the protective affect of SP-A in mounting a host defense response. In further studies, we determined that MMF-stimulated mucin production occurred in the absence of neutrophil recruitment and that the protective affect of SP-A was likely occurring at the level of the epithelial cell. Using an EGFR inhibitor, we were able to ablate the enhanced mucin production observed in MMF-challenged SP-A\(^{-/-}\) mice. Taken together, these finding suggest that when SP-A is absent (as in SP-A\(^{-/-}\) mice) or has a lower binding affinity for MMF (as in SP-A223Q/\(-\) mice), MMF will cause increased p-EGFR signaling that results in enhanced mucin production.

\textit{M. pneumoniae} is a well-known human pathogen that is a causative agent of community-acquired “walking” pneumonia, acute bronchitis, and asthma exacerbations. In many of these conditions, mucin production is thought to be an important factor in the pathogenesis of disease. Although management of \textit{M. pneumoniae} infections is typically achieved by the use of macrolides, tetracyclines, or fluoroquinolones, many studies into the mechanisms of \textit{M. pneumoniae} pathogenicity demonstrated that immune-stimulatory factors are inherent in the cell membrane and can persist as secreted factors, both of which can stimulate immune responses independent of \textit{M. pneumoniae} viability. In fact, secreted community-acquired respiratory distress syndrome (CARDS) toxin from \textit{M. pneumoniae} can lead to ciliostasis, increased tissue permeability, and cell death (34, 35). Interestingly, CARDS toxin also was shown to demonstrate high-affinity binding to SP-A (22). Typically, \textit{M. pneumoniae} only expresses CARDS toxin during infection of a live host and expresses poorly in vitro culture systems (36). Therefore, the MMF stimuli used for our studies is unlikely to contain CARDS toxin, and the differences in the response observed between SP-A2 223Q and SP-A2 223K is likely due to the ability to interact with and bind the lipid components in the membrane preparation. Additionally, WT mice challenged with MMF derived from a CARDS-deficient strain of \textit{M. pneumoniae} (lacks Mpn372) also had reduced Muc5AC expression at similar levels to MMF-challenged WT mice (Fig. 2B). These findings suggest that the ability of SP-A to bind to \textit{M. pneumoniae} membrane lipid components, rather than the CARDS protein, is responsible for the protective mechanisms against mucin production.

MMF, which is composed of nonlive \textit{M. pneumoniae} membranes that include lipoproteins and glycolipids, was demonstrated by Chmura et al. (19) to stimulate IL-8 secretion from human bronchial epithelial cells. IL-8 in humans, the equivalent of murine KC, is a potent recruiter of neutrophils. SP-A\(^{-/-}\) and SP-A223Q/\(-\) mice had significantly more neutrophil recruitment and KC levels in BALF in response to MMF challenge compared with WT and SP-A2 223K/\(-\) mice. Because SP-A\(^{-/-}\) and SP-A2 223Q/\(-\) mice also demonstrated enhanced mucin production during MMF challenge, and neutrophils are known to contribute to mucin production via release of elastase (37), we sought to determine, using MTEC cultures, whether the enhanced mucin production was dependent on the presence of neutrophils. We
suspected that if mucin production was tied to neutrophil recruitment, then in the absence of neutrophil recruitment in the MTEC system, MMF would fail to increase Muc5AC upregulation compared with the saline controls. We observed that, although the MTECs derived from SP-A^{−/−} mice had significantly increased Muc5AC expression in MMF-challenged cells compared with vehicle-treated cells, MTECs from WT MMF-challenged mice had significantly repressed Muc5AC expression compared with vehicle controls. This unexpected finding led us to investigate SP-A expression in the WT MTECs. We were able to detect SP-A, as a band ∼50 kDa, from the apical supernatants of MTEC cultures that were derived from WT mice. No SP-A was detected from the SP-A^{−/−} apical supernatants. Although we were surprised to discover that SP-A was synthesized and secreted from MTECs during the month-long growth and differentiation process, the 50 kDa SP-A has been detected previously and is suspected to be a nonglycosylated dimer (30).

The finding that Muc5AC is repressed during MMF challenge of WT MTECs suggests the possibility that SP-A is inducing a negative regulatory pathway that leads to the repression of Muc5AC. Additionally, the MTECs derived from SP-A^{−/−} mice suggest that, when SP-A is absent, MMF is capable of causing Muc5AC upregulation in the absence of neutrophils. Interestingly, previous studies suggested that live *M. pneumoniae* signals almost exclusively through TLR-2 for induction of Muc5AC (38). Although previous work showed that MMF stimulates IL-8 production in a manner independent of TLR-2, the major receptor involved in the inflammatory response to MMF was not defined. Our own experiments using TLR-2–deficient mice challenged with MMF supported the notion that MMF was likely signaling independently of TLR-2 (data not shown).

Nonlive mycoplasma-derived components are composed of a variety of proteins and lipids and were shown to possess Ag activity (39). Because many of the MMF components could signal through non-TLRs, we chose to focus on those non-TLR receptors that are already known to regulate mucin production. Staining by IHC for p-EGFR revealed that MMF challenge upregulates this signal in the large airways when SP-A is absent. EGFR has long been established as an important receptor that regulates mucin production through signaling of various ligands, both endogenous and exogenous. Lipoteichoic acid, although absent from mycoplasmas, is found in the cell walls of many pathogens and was shown to induce mucus via EGFR. Our findings of MMF-EGFR signaling are supported by recent work by Hao et al. (40), demonstrating that live *M. pneumoniae* enhances mucin production by activating the EGFR signaling pathway. Although they also report increased IL-6, a ligand for EGFR, after *M. pneumoniae* challenge, our

![FIGURE 6.](http://www.jimmunol.org/content/186/13/6129/F6.expansion.jpg) **FIGURE 6.** p-EGFR expression is enhanced post-MMF challenge. IHC was performed for detection of p-EGFR 12 h post-MMF or saline challenge. (A) WT saline challenged. (B) SP-A^{−/−} saline challenged. (C) Isotype control. (D) WT MMF challenged. (E) SP-A^{−/−} MMF challenged. (F) Computer-generated quantitative analysis of the percentage of the large airway positive for p-EGFR staining. n = 3–4/group. **p < 0.01.

![FIGURE 7.](http://www.jimmunol.org/content/186/13/6129/F7.expansion.jpg) **FIGURE 7.** Inhibition of EGFR attenuates mucus in MMF-challenged mice. SP-A^{−/−} mice were given AG1478 or vehicle prior to challenge with MMF. Representative PAS-stained lung histology of vehicle-treated (A) versus AG1478-treated (B) mice that were challenged with MMF 16 h postchallenge. p-EGFR staining of vehicle-treated (C) versus AG1478-treated (D) mice that were challenged with MMF. (E) Blinded scoring of PAS-stained lung sections (n = 2 experiments with 11 vehicle and 10 AG1478 mice). (F) Muc5AC expression by real-time PCR from lung tissue of MMF-challenged mice in the presence of absence of EGFR inhibition (n = 2 experiments). **p < 0.01.
disoequilibrium test (TDT) and case-control association studies reveal surfactant protein A (SP-A) susceptibility alleles for respiratory distress syndrome (RDS) and possible race differences. Clin. Genet. 60: 178–187.


