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SP-A Preserves Airway Homeostasis During *Mycoplasma pneumoniae* Infection in Mice[1]

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The lung is constantly challenged during normal breathing by a myriad of environmental irritants and infectious insults. Pulmonary host defense mechanisms maintain homeostasis between inhibition/clearance of pathogens and regulation of inflammatory responses that could injure the airway epithelium. One component of this defense mechanism, surfactant protein-A (SP-A), exerts multifunctional roles in mediating host responses to inflammatory and infectious agents. SP-A has a bacteriostatic effect on *Mycoplasma pneumoniae* (Mp), which occurs by binding surface disaturated phosphatidylglycerols. SP-A can also bind the Mp membrane protein, MPN372. In this study, we investigated the role of SP-A during acute phase pulmonary infection with Mp using mice deficient in SP-A. Biologic responses, inflammation, and cellular infiltration, were much greater in Mp infected SP-A$^{-/-}$ mice than wild-type mice. Likewise, physiologic responses (airway hyperresponsiveness and lung compliance) to Mp infection were more severely affected in SP-A$^{-/-}$ mice. Both Mp-induced biologic and physiologic changes were attenuated by pharmacologic inhibition of TNF-α. Our findings demonstrate that SP-A is vital to preserving lung homeostasis and host defense to this clinically relevant strain of Mp by curtailing inflammatory cell recruitment and limiting an overzealous TNF-α response. ***The Journal of Immunology,*** **2009, 182: 7818–7827.**

Primary pulmonary defense against environmental irritants such as allergens, viruses, and microbes is supplied by the pulmonary innate immune system. This complex system is comprised of cellular components such as macrophages, neutrophils and NK cells, and soluble regulatory factors, which include the surfactant proteins. In addition to their biophysical role in regulating surface tension at the air-liquid interface of the lung (1, 2), the constituents of pulmonary surfactant are now recognized as important regulators of the lung innate immune system. The pulmonary immune functions of surfactant are primarily mediated by surfactant protein (SP)[3]-A and SP-D, which are members of the collectin family of proteins.

The pulmonary collectins consist of an amino terminal collagen-like region that promotes noncovalent oligomerization. The carboxy terminal domains of collectins have C-type lectin structure and activity, which enables SP-A and SP-D to bind numerous carbohydrate ligands expressed on the surface of pathogens. One consequence of SP-A binding is pathogen opsonization, which leads to robust engulfment by phagocytes. Additional immune functions have been attributed to SP-A, such as inhibition of T cell proliferation by its collagen-like domain (3) and regulation of dendritic cell functions in vitro (4). SP-A also modulates the production of inflammatory cytokines (5, 6) by various cell types suggesting a role for SP-A in mediating the conversion from innate to adaptive immunity. Previous studies have found that SP-A reduces TNF-α production by LPS stimulated macrophages in vitro (6) and that mice lacking SP-A and challenged with LPS instilled intratracheally have increases in lung TNF-α that can be reversed by exogenously administering SP-A to the airway during LPS administration (7).

Studies have also provided strong evidence for the protective role of SP-A in mycoplasmal infection. Results from in vitro studies show that SP-A mediates alveolar macrophage killing of *Mycoplasma pulmonis* through a nitric-oxide mechanism (8). Although *M. pulmonis* is a useful pulmonary pathogen for examining immune function in mice, fewer studies have used the clinically relevant strain of *Mycoplasma pneumoniae* (Mp), which is of greater concern for human health. SP-A binds Mp by ligation to the surface protein MPN372 and to disaturated phosphatidylglcerols (9, 10). Although SP-A does have the potential to directly kill a number of bacteria, its effects on Mp are bacteriostatic (9, 10).

Mp is the causative agent of atypical pneumonia and accounts for a large percentage of community-acquired pneumonia in the general population. Not only is Mp responsible for a spectrum of respiratory tract infections such as tracheobronchitis, bronchiolitis, pharyngitis, and croup (11), it is also believed to be associated with other chronic pulmonary diseases such as asthma and chronic obstructive pulmonary disease (12). Previous investigations demonstrated that >50% of chronic stable asthma patients had evidence of airway infection with Mp (13). Because SP-A is expressed in alveolar type II cells, Clara cells, and submucosal glands of the respiratory tract, the potential for SP-A to interact with invading

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[3] Abbreviations used in this paper: SP, surfactant protein; Mp, *Mycoplasma pneumoniae*; BALF, bronchoalveolar lavage fluid; AHR, airway hyperresponsiveness; RT, resistance; BCA, bicinchoninic acid; PAS, Periodic acid-Schiff; WT, wild type; PPLO, pleuropneumonia-like organisms.

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Mp in human lungs is quite high and may aid in limiting Mp colonization during pulmonary infections.

The interactions between microbial pathogens and SP-A are considered an important component of innate pulmonary host defense, and SP-A null mice have been a valuable tool in determining the role of SP-A in this facet of pulmonary immunity. SP-A null mice maintain normal surfactant homeostasis and respiratory function when unchallenged. However, an important phenotype of SP-A null mice is their deficiency in host defense. Diminished phagocytic ability of lung macrophages is observed in the SP-A knockout mice as well as the ability of these cells to produce superoxide and hydrogen peroxide during certain infections. SP-A null mice also have impaired clearance of bacteria and viruses such as group B Streptococcus, *Haemophilus influenza*, Respiratory Syncytial Virus and *Pseudomonas aeruginosa* (14–17). Following infection with *M. pulmonis*, SP-A null mice demonstrate increased CFUs and proinflammatory cytokine levels in bronchoalveolar lavage fluid (BALF) (18).

Previous studies comparing BALB/c mice with C57BL/6 mice have shown that when infected with Mp, C57BL/6 mice do not develop airway obstruction and have less pulmonary inflammation and cytokine production as compared with BALB/c infected mice (19). We therefore sought to determine the role of SP-A in Mp-mediated lung immunity by using mice lacking the SP-A gene that were backcrossed onto the C57BL/6 genetic background. Our findings show that SP-A null mice infected with Mp have a robust inflammatory cell recruitment and significantly elevated airway hyperresponsiveness (AHR) as compared with wild-type (WT) controls. Pharmacologic inhibition of TNF-α expression during the infection attenuated these biologic and physiologic outcomes in the SP-A null mice. These findings reveal a novel protective role of SP-A, which preserves airway and pulmonary homeostasis by limiting the pulmonary proinflammatory sequela and changes in lung function that arise during the acute phase of an Mp infection.

Materials and Methods

Mp culture and quantitation

Mp from American Type Culture Collection (cat. no.: 15531) was grown in SP4 broth (Remel) at 35°C until adherent. Mp concentration was determined by plating serial dilutions of Mp on pleuropneumonia-like organisms (PPLO) agar plates (Remel). Colonies were counted under 10× magnification on plates after incubation for 14 days. For in vivo infection, adherent Mp were washed by centrifuging at 6000 rpm for 5 min and resuspended in sterile saline for infection at a concentration of 1×10^8 Mp/50 μl inoculum. To determine Mp levels in the BALF, 10 μl of diluted samples in SP-4 broth were plated on PPLO plates. Lung tissue was collected after lungs were lavaged and perfused and RNA extracted by phenol-chloroform methods. cDNA was prepared according to standard protocols and Mp was quantified by RT-PCR using primers (forward 5′ CGC CGC AAA GAT GAA TGA C 3′, reverse 5′ TGT CCT TCC CCA TCT AAC AGT TC 3′) specific for the Mp-specific P1-adhesin gene. Relative amounts of Mp P1-ahdesin present in each infected lung were measured based on values obtained from a standard curve for Mp P1-adhesin and were normalized to the mammalian housekeeping gene cyclophilin that was present in the lung tissue. The size of the RT-PCR Mp P1-adhesin product (103 bp) was verified by gel electrophoresis; no expression was detected in saline treated lung samples.

Mice

An inbred strain of SP-A-deficient mice was generated by disrupting the murine gene encoding SP-A by homologous recombination as previously described (20). SP-A null mice were backcrossed for 12 generations onto the C57BL/6 background which were purchased from Charles River Laboratories. WT C57BL/6 mice were purchased from Charles River Laboratories and bred in house to account for environmental conditions. All mice used in experiments were age (8–12 wk) and sex (males) matched and were on protocols approved by the Institutional Animal Use and Care Committee at Duke University.

Mp inoculations and TNF-α inhibition

Male mice 8–12 wk of age were anesthetized by i.p. injection of 10 μl/g body weight of a 12% ketamine (100 mg/ml) and 5% xylazine (20 mg/ml) mix. Mice were infected with either 50 μl of sterile saline or 50 μl of $\sim 1 \times 10^8$ Mp units in sterile saline by intranasal instillation. LMP-420 is an anti-inflammatory boronic acid-containing purine nucleoside analog that transcriptionally inhibits TNF-α production (21). Some mice received i.p. injections of sterile LMP-420 (25 mg/kg) or vehicle (5% sorbitol) 4 h before Mp infection. For i.p. injections, a 10 mg/ml stock solution was prepared in 5% sorbitol (pH 9.0) in sterile water and further diluted in 5% sorbitol as necessary to deliver the desired dose in a volume of 0.2 ml. Those mice receiving LMP-420 or vehicle also received booster doses at 24 and 48 h post Mp infection. This dose, 25 mg/kg, was chosen based on previous (unpublished) studies that have shown that maximal effects of LMP-420 occur at doses over 15 mg/kg. This dose was well-tolerated and resulted in ~90% inhibition of circulating TNF. LMP-420 was synthesized by Scynexis under provisions of a Material Transfer Agreement between LeukoMed and Duke University.

Airway physiology

Direct measurements of respiratory mechanics in response to methacholine were made using the flexivent system (Scireq) and reported as total pulmonary resistance (RT) $cmH_2O/ml/s$ or quasi-static compliance (Cst). Anesthesia was achieved with 60 mg/kg sodium pentobarbital injected i.p. and mice were ventilated with a computer-controlled small animal ventilator with a tidal volume of 7.5 ml/kg and a positive end-expiratory pressure of 3 cm H_2O. The mice were then given a neuromuscular blockade (0.8 ml/kg pancuronium bromide). Measurements of respiratory mechanics were made by the forced oscillation technique. Response to aerosolized methacholine (0, 10, 25, and 100 mg/ml) was determined by resistance measurements every 30 s for 5 min, ensuring the parameters calculated had peaked. Lungs were inflated to estimated total lung capacity after each dose to maintain airway patency. The resistance or compliance measurements were then averaged at each dose per sample group and graphed (RT $cmH_2O/ml/s$) along with the initial baseline measurement premethacholine challenge.

Isolation of BALF and cells from lung tissue

Mice were euthanized with a lethal dose of pentobarbital. To quantify cells in lung of saline and Mp-infected SP-A null and WT mice, animals were subjected to bronchoalveolar lavages with 6 ml of 37°C PBS (0.2 mM EDTA). Cells collected from BALF were pelleted and resuspended in RBC lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) and incubated on ice for 10 min. Cells were then resuspended in buffer (HBSS, -Ca/-Mg, 10 mM EDTA, 5% FCS). After the lungs were lavaged, they were perfused via the right ventricle with PBS until lung lobes were visually free of blood. Lung lobes were carefully removed while avoiding contamination with lymphatic tissue and then minced and incubated with agitation in digestion solution consisting of HBSS (+Ca/Mg) supplemented with Collagenase A and DNase I for 60 min at 37°C. After incubation, the digestion was stopped by adding EDTA and FCS to achieve a final concentration of 20 mM EDTA/5% FCS solution. The digested tissue was then passed through a 40 μM cell strainer (BD Falcon) and washed with HBSS. In 15-ml conical tubes, 2 ml of 4% Optiprep (Axis-Shield) was loaded beneath the cells in buffer and 2 ml of 14.5% Optiprep was loaded directly under the 4% layer. Samples were centrifuged at $600 \times g$ for 20 min at room temperature and allowed to decelerate without braking. The interface layer containing the myeloid and lymphoid cells were harvested and washed in HBSS.

BALF analysis

The first milliliter of BALF obtained from experimental mice was analyzed for total protein by bicinchoninic acid (BCA; ThermoScientific) and for levels of TNF-α by ELISA (BD Biosciences) according to the manufacturer's instructions.

FACS analysis

Cells from BALF or digested lungs were counted and stained with a panel of Abs to distinguish between lymphoid or myeloid cells. The stained cell preparations were analyzed by flow cytometry for cell surface marker expression of the Ab panels. CD8, CD3, MHC II, Ly-6G, and GR-1 Abs were purchased from BD Biosciences. CD11c and CD11b were from eBiosciences and CD4 was purchased from Caltag Laboratories. Flow cytometry was performed in the Duke Human Vaccine Institute Flow Cytometry Core Facility that is supported by the National Institutes of Health award AI-51445. Initially, cells were examined by forward-scatter vs side-scatter

to separate those smaller nongranular lymphoid populations from the larger granular myeloid populations. The percentage of cells within each of these gates was used to calculate the total number of lymphoid cells and myeloid cells examined from the total number of cells obtained from the lung digests as counted on a hemacytometer.

Histological analysis

WT or SP-A null mice were given either saline or Mp by intranasal instillation as described above. Three days after infection, mice were euthanized by a lethal dose of Nembutal followed by exsanguination. The lungs were then perfused with 10 ml of warm PBS and inflated by gravity flotation with 4% paraformaldehyde fixative. Lungs were paraffin embedded, sections were cut at 4-μm thickness and stained with either H&E, Periodic acid-Schiff (PAS), or with an anti-Mp Ab (Affinity Bioreagents). The PAS staining measurements were analyzed quantitatively using a computer-based color deconvolution module of ImageJ version 1.42d (National Institutes of Health). For each slide (representing one mouse), eight representative nonoverlapping images of the large airways were randomly taken at $\times$20 magnification. For each image, the tissue around the large airway was manually erased in Photoshop (Adobe) to allow selective assessment of the airway epithelium. ImageJ was calibrated to obtain the measurements in microns. The positive cells of PAS staining within the airways were recognized by the deconvolution module using a threshold method and their percentages to total epithelial cells were automatically generated.

Statistical analysis

Airway physiology measurements were analyzed by ANOVA for multiple comparisons between groups and were verified by *t* test with PRISM (GraphPad software). All *t* tests were obtained from analysis of data in PRISM. Data sets with significant variance between comparison groups were analyzed by *t* test using Welch's correction in PRISM.

Results

AHR in Mp-infected mice

To determine whether SP-A is critical in controlling Mp pulmonary infection and inflammation, we chose to examine AHR to methacholine challenge 3 days after infection using WT and SP-A null mice on a C57BL/6 background. This time point was chosen based on previously published reports in which AHR was studied following Mp challenge in the Mp-sensitive strain of BALB/c mice, which are more susceptible to Mp infection than C57BL/6 mice (22). In these studies, the greatest levels of AHR as compared with controls was directly associated with the heightened cellular influx into the lung which occurred ~3 days after infection.

Three days after infection, WT and SP-A null mice were analyzed for AHR by methacholine challenge and the average RT value (cmH_20/ml/s) was assessed. Shown in Fig. 1*A*, all animals had minimal AHR to the methacholine challenge when instilled with saline. WT Mp-infected mice responded with significantly elevated airway responsiveness over their saline controls only at the highest tested dose of methacholine provocation (100 mg/ml). In contrast to WT mice, the infected SP-A null mice had significantly elevated AHR, which was evident even at the 25 mg/ml dose of methacholine (Fig. 1*A*).

In addition to increased airway resistance in the Mp-infected SP-A null mice, they also showed significantly reduced dynamic compliance as shown in Fig. 1*B*. Dynamic compliance measures the ease with which the lungs can be extended and a drop in compliance values indicates increased stiffness in the lungs. The compliance measurements in the WT infected mice were comparable to saline-treated mice. However, the compliance of the infected SP-A null mice was significantly lower at the baseline prechallenge measurement as well as throughout the methacholine challenges (Fig. 1*B*).

Histology of Mp-infected lungs

Lung histology provided additional evidence for an important role of SP-A in mediating lung inflammation to Mp infection. In Fig. 2*A*, minimal cellular infiltrate (as indicated by arrows) was observed 3 days after infection near the large airway in WT mice. In comparison, as shown in Fig. 2*D*, large areas of cellular infiltrate consisting of mononuclear cells were observed in the SP-A null mice in perivascular regions and adjacent to the large airways. Additionally, histological analysis demonstrated that cellular inflammation was not limited to the large airway in the Mp-infected mice. As shown in Fig. 2*B*, various inflammatory cells were identified near bronchioles in the perivasculature in WT infected mice. However, in the SP-A null mice, not only were more inflammatory cells detected in the perivasculature surrounding the bronchioles, but cells were observed in the bronchiole lumen as well (Fig. 2*E*). Even greater disparity of inflammation was observed in the alveoli. WT mice had very few cells in the alveoli as shown in Fig. 2*C*. In striking contrast, SP-A null mice had high levels of cellular infiltrate in the alveoli and foci formation suggesting these infected mice also suffered from pulmonary edema (Fig. 2*F*).

Because the lung histology of the Mp-infected SP-A null mice suggested the presence of pulmonary edema associated with alveoli, BALF was assessed by BCA as a quantitative measure of total protein present. Given that BALF was evaluated from several different experiments, data are expressed as fold increase over WT

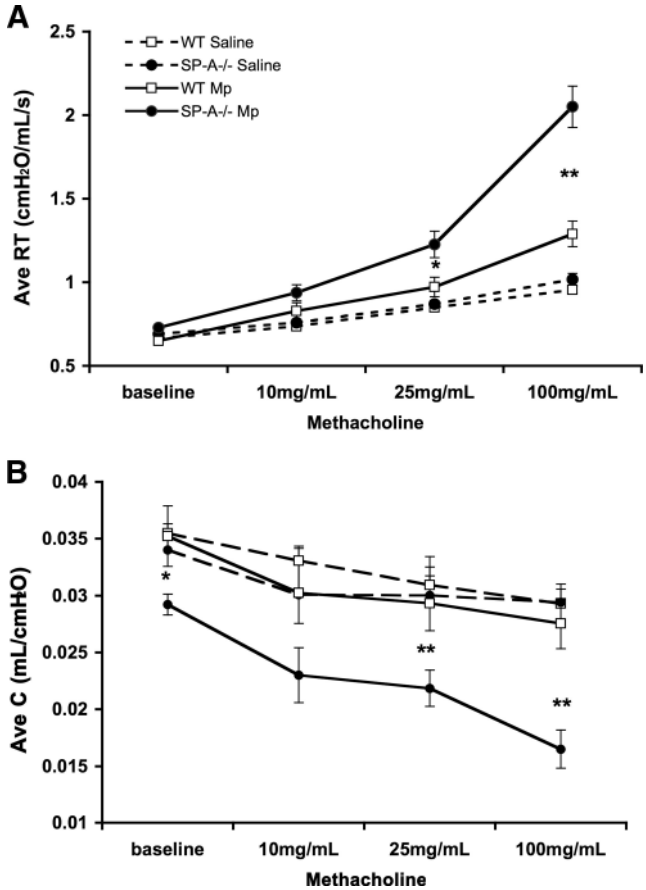

FIGURE 1. Effect of SP-A on Mp -induced airway responsiveness to methacholine challenge. WT and SP-A$^{-/-}$ mice were instilled intranasally with Mp (~1 $\times$ 10^8/mouse) and airway responsiveness to methacholine challenge was analyzed 72 h post infection by Flexivent. *A*, The average resistance was significantly elevated during methacholine challenge in the Mp infected SP-A null mice as compared with WT controls. *B*, The average compliance is significantly lower in the SP-A null Mp-infected mice at baseline and during methacholine provocations. Data are combination of three independent experiments, $n = 8$–12 mice/group. *, $p < 0.05$; **, $p < 0.01$ and is SP-A$^{-/-}$ Mp infected vs all other groups at the respective dose concentrations.

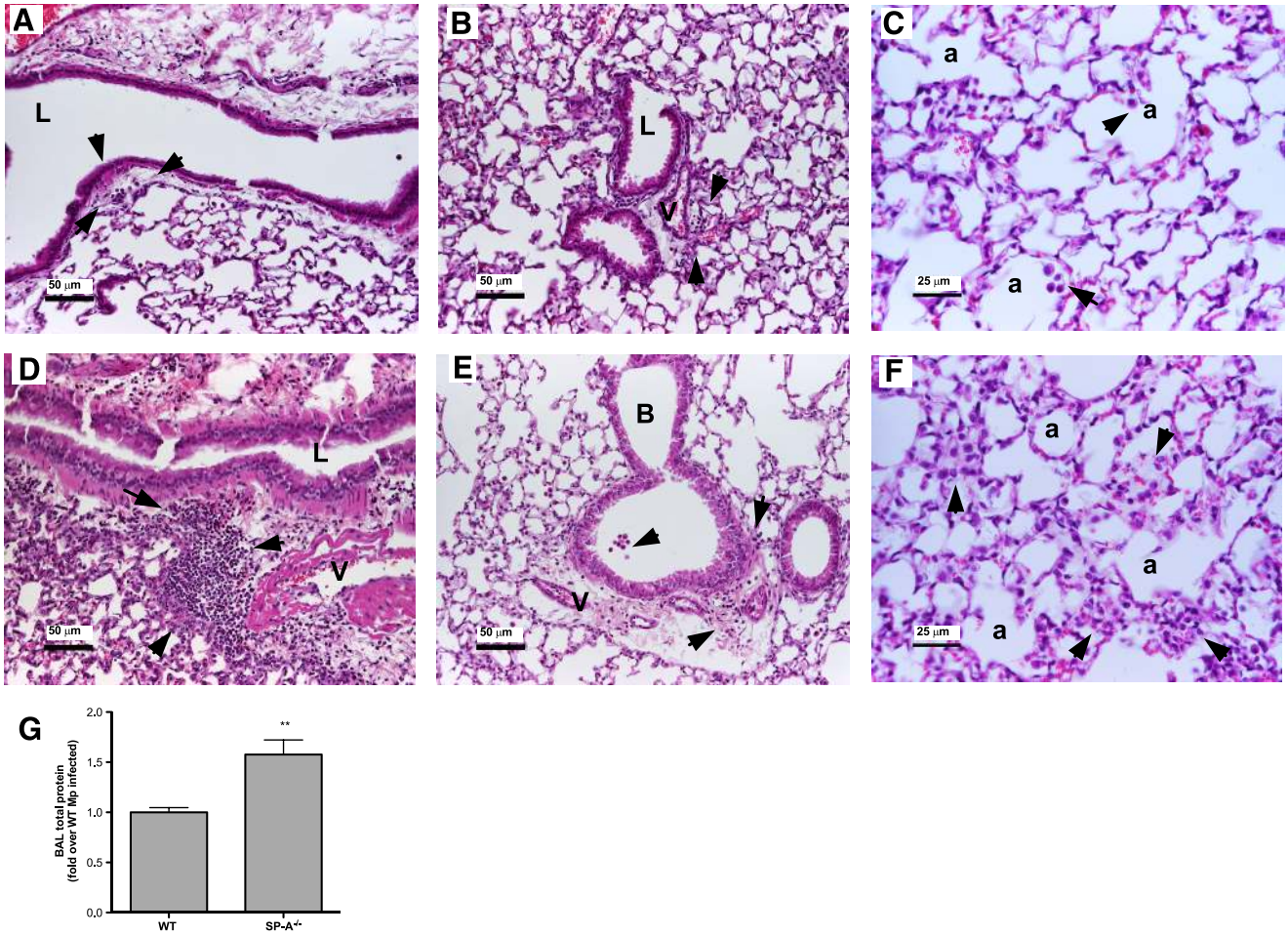

FIGURE 2. Histological analysis of cellular infiltration in Mp-infected mice. Three days (72 h) postinfection, mice were sacrificed and lungs fixed with paraformaldehyde for histological analysis. Lung sections were stained with H&E and visualized by microscopy. L, lumen; V, vessel; a, alveolus are labeled and arrows indicate cellular infiltrate. *A*, Minimal cellular infiltration is observed in and around the large airway of WT infected mice. *B*, Some perivascular infiltrate is detected near bronchioles. *C*, Few cells are seen in the alveoli and consist primarily of macrophages. *D*, Large areas of lymphoid infiltrate are observed in and around the large airway of the SP-A null infected mice. *E*, Cellular infiltrate is detected in the airway lumen and in the perivasculature of the bronchioles. *F*, Many cells are seen in the alveoli and which appear to be comprised of mononuclear cells as well as macrophages. Arrows indicate areas of foci formation indicative of pulmonary edema. For *A*, *B*, *D*, and *E*, scale bars are 50 microns and pictures were taken at ×20 magnification. *C* and *F* scale bars are 25 microns and pictures were taken at ×40 magnification. Each picture is representative of five independent infected mice. *G*, Total protein was measured in BAL by BCA assay. Data shown are combination of three independent experiments. $n = 11$ and 13, respectively and **, $p < 0.01$.

measurements within each experiment. There was no significant difference in total protein measured in BALF between the saline treated WT and SP-A null mice. However, as shown in Fig. 2*G*, the total protein measured in the BALF was significantly elevated in the SP-A null Mp-infected mice over their WT controls. Histological analyses suggest that mice lacking SP-A have more pulmonary edema during Mp infection as compared with WT infected controls. The increased total protein in BAL fluid and pulmonary edema, as suggested by histological analyses, could lead to the significantly reduced compliance as measured in these mice.

Inflammatory cell recruitment into the lung parenchyma and airspace during Mp infection

Three days after Mp challenge, BALF and lung tissue digests were collected and used to evaluate inflammatory cell recruitment. No difference was found between the WT and SP-A$^{-/-}$ saline treated mice in the total number of cells harvested from BALF (Fig. 3*A*). Likewise, the numbers of cells obtained from the lung digests were not significantly different in the saline-treated WT and SP-A$^{-/-}$ mice (Fig. 3*B*). However, BALF and lung tissue from mice lacking SP-A contained significantly more inflammatory cells 72 h after Mp infection compared with WT mice (Fig. 3, *A* and *B*).

Cells from the BALF were analyzed by staining and differential counting as well as by flow cytometry methods. By differential staining, cells obtained from BALF of saline-treated mice in both groups contained >90% alveolar macrophages (data not shown). For flow cytometry analysis, cells considered alveolar macrophages were autofluorescent and stained positive for the cell surface marker CD11c while they had low to moderate expression of MHC class II (CD11c^{+}, MHC II^{low}) (23). In agreement with cytospin analysis, > 90% of cells recovered from BALF of saline treated mice were alveolar macrophages by flow cytometry (data not shown). Although there were elevated numbers of dendritic cells, inflammatory monocytes, neutrophils, T cells, and B cells, as detected by flow cytometry in the BALF of SP-A null mice after Mp infection, no single cell population analyzed was significantly greater than in the WT infected mice (data not shown).

To examine those cells not yet infiltrating the airspace and therefore not collected in the lavage process, we enzymatically digested the lung to obtain cells from the lung tissue from which myeloid

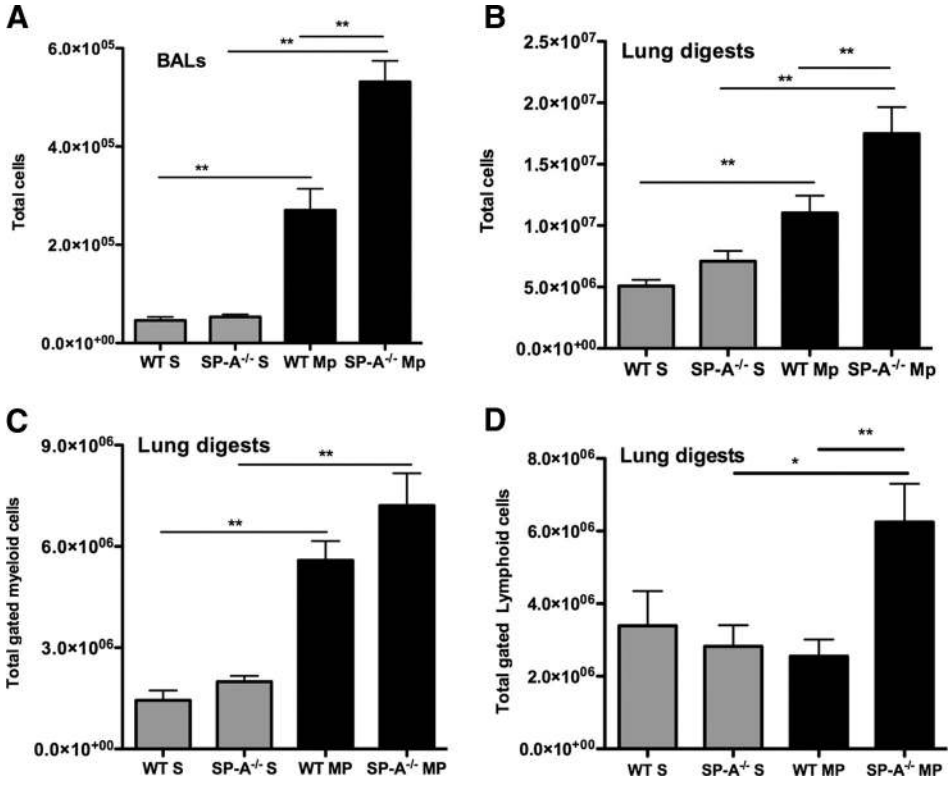

FIGURE 3. Inflammatory cell recruitment into the lung tissue post Mp infection. *A*, BALF was collected 72 h post Mp infection and total cells were counted. *B*, After the vasculature was perfused and airways were lavaged, whole lungs were enzymatically digested and single cells isolated by gradient centrifugation. Isolated cells from the lung digests were analyzed by flow cytometry with a panel of Abs against specific cell surface markers to distinguish myeloid (*C*) or lymphoid (*D*) cells. The results shown are the mean ± SEM from three independent experiments, $n = 12$/group and **, $p < 0.01$.

and lymphoid cells were enriched by gradient centrifugation (24). As shown in Fig. 3, *C* and *D*, the number of myeloid or lymphoid cells from the lung digests of SP-A$^{-/-}$ saline or control saline treated mice were not significantly different. Although the total number of gated myeloid cells, Fig. 3*C*, trended toward increased values in the Mp infected SP-A$^{-/-}$ mice compared with Mp infected WT mice, this did not achieve statistical significance. Interestingly, the number of gated lymphoid cells was significantly higher in the mice lacking SP-A as compared with WT controls when infected only 3 days with Mp (Fig. 3*D*). Those cells in the lymphoid gate were further analyzed to determine the presence of significantly more T lymphocytes in the infected SP-A null mice as compared with WT infected controls (data not shown).

Quantitation of Mp infection in bronchoalveolar lavages and lung tissue

To determine whether the heightened cellular inflammation and AHR in the SP-A null mice were associated with an increase in Mp burden, we first examined Mp counts in BALF 72 h after infection. It has been reported that C57BL/6 mice are more resistant to Mp infection than other strains of mice. Therefore, it is not surprising that from an inoculum of 10^8 Mp, both WT and SP-A null mice had only 10^4 live Mp present in BALF after 3 days of infection. The amount of Mp measured in the BALF was not significantly different between the WT and SP-A null mice (Fig. 4*A*).

Most mycoplasma species infecting mammalian cells adhere tenaciously to the epithelial linings of the respiratory tract but rarely invade the tissue. This adhesion of mycoplasma to host cells is a requirement for colonization and infection (25). Because adherence is vital to the infectivity of mycoplasma, Mp units are therefore typically measured in infected animals from the lung tissue as well as from the lung lavages. The most defined adhesin protein on Mp is adhesin-P1 and is known to be the major structure responsible for interaction with host cells (26). Measurement of adhesin-P1 expression by PCR is often used to estimate the number of Mp (27, 28). For this analysis, lungs were lavaged to remove cells in the airspaces, perfused to remove cells from pulmonary circulation, and the tissue associated adhesin-P1 expression was quantified. As shown in Fig. 4*B*, WT mice had very low levels of Mp adhesin-P1 after 3 days of infection. In contrast, the amount of the surface adhesion marker in the SP-A null mice was elevated ~25-fold over WT levels. Mp binding to airway epithelial cells was verified by immunohistochemistry using a *Mycoplasma pneumoniae* specific Ab. As shown in Fig. 4*C*, more Mp is detected in the infected large airways of SP-A null mice as compared with WT controls.

TNF-α production during Mp infection

Mp is known to be a potent activator of cellular TNF-α production. SP-A has been shown to inhibit TNF-α release when induced by LPS, or intact bacteria, both in vitro and in vivo (6, 29, 30). TNF-α is a potent cytokine that not only triggers, but also maintains the inflammatory response, and is vital in cell recruitment and activation. Twelve hours following Mp infection, levels of TNF-α present in the BAL were analyzed by ELISA. Both groups of saline-treated mice had minimal detectable levels of TNF-α (data not shown). Not surprisingly, TNF-α levels were dramatically increased in WT mice 12 h after Mp infection. Strikingly, the elimination of SP-A from the lung resulted in a 5-fold increase in TNF-α production in response to Mp infection (Fig. 4*D*).

Inhibition of TNF-α attenuates AHR

In an allergic airway model, blockage of TNF-α has been shown to inhibit late phase AHR in mice and further studies show decreased AHR in TNF-α null mice (31). To determine whether the increased AHR observed in Mp-infected SP-A null mice may be due to overproduction of TNF-α, and therefore a myriad of other effects due to TNF signaling, we inhibited TNF-α transcription with the pharmacologic inhibitor LMP-420 (32). LMP-420 is an anti-inflammatory nucleoside analog that is a potent and highly

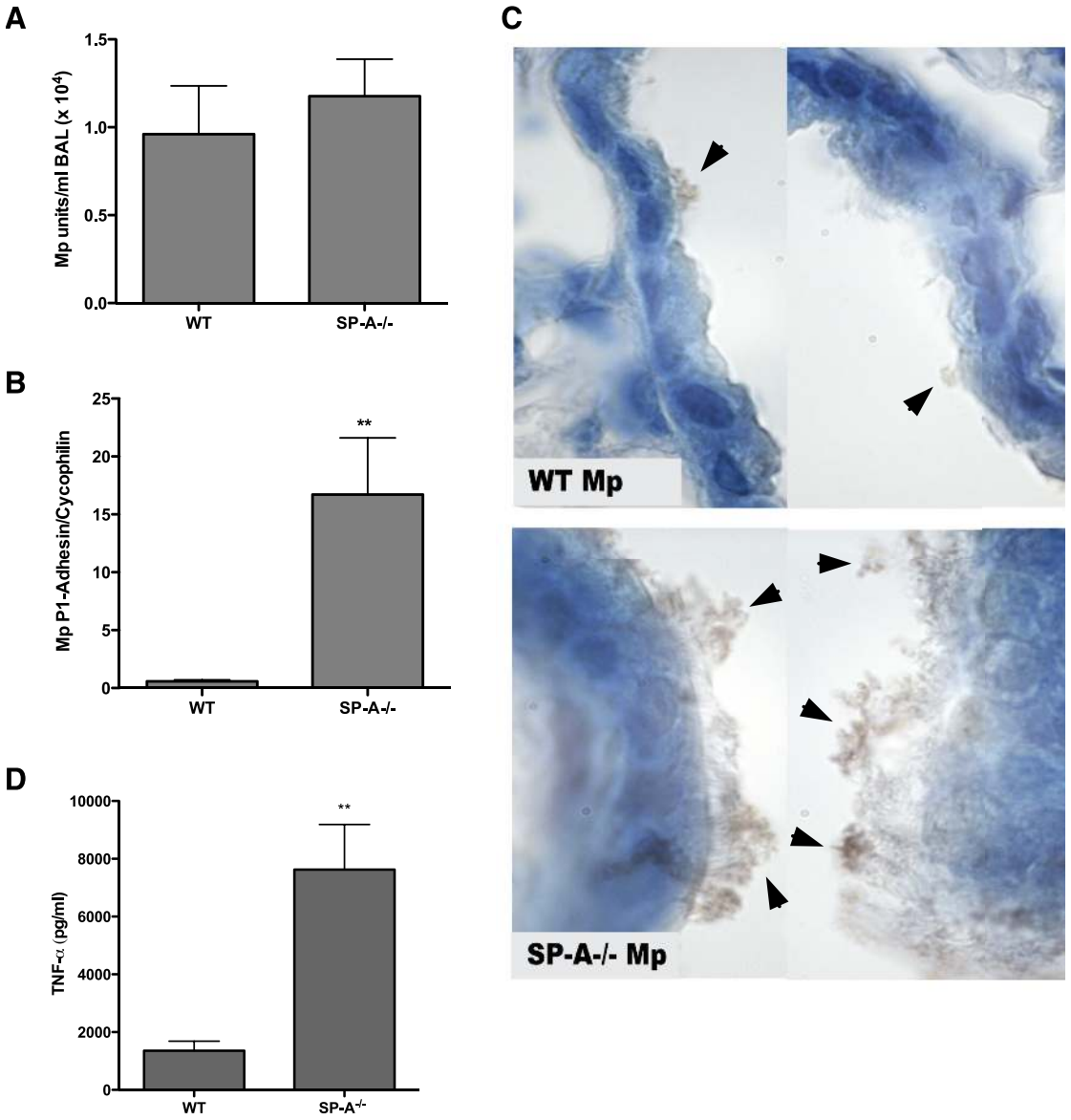

FIGURE 4. Mp quantitation from BALs and lung tissue. WT and SP-A null mice were sacrified and the vasculature perfused before *A*, BALs and *B*, lung tissue collection 72 h after infection. Dilutions of BALF were plated on PPLO agar plates and Mp counts were determined with the aid of a microscope at ×4 magnification following 2 wk of incubation. Relative Mp units (assessed by RT-PCR) of Mp specific P1-adhesin gene were standardized to the housekeeper gene cyclophilin. Results are combination of three experiments with $n = 12$ and 12 and **, $p < 0.01$. *C*, Infected lungs were fixed and sections analyzed (×100 oil-immersion) by immunohistochemistry with an anti-Mp HRP Ab. Arrows indicate areas of staining associated with ciliated airway epithelial cells. *D*, TNF-α was analyzed from BALF collected 12 h post Mp infection by ELISA. Data are mean ± SEM from three independent experiments. $n = 12$/group and **, $p < 0.01$.

selective inhibitor for TNF-α (21). Mice were injected 4 h before Mp infection i.p. with LMP-420 followed by booster injections 24 and 48 h post infection. The amount of TNF-α present in BALF 12 h after Mp infection was assessed by ELISA to verify attenuation, which was ~90%, in the presence of LMP-420 and levels were undetectable on day 3 (data not shown). Indices of inflammation and Mp content were then assessed 3 days after infection to compare null mice injected with vehicle before infection with Mp, in which they continued to have significantly higher AHR compared with WT infected mice. However, this heightened AHR was significantly attenuated if the SP-A null mice were pretreated with the TNF inhibitor, LMP-420, before infection (Fig. 5*A*). This suggests that SP-A can modulate factors related to physiologic airway function during the acute phase of an infection, and in the absence of SP-A, Mp enhances TNF signaling and secretion, leading to an enhancement in AHR.

As discussed above, dynamic compliance was also significantly elevated in Mp infected SP-A null mice before methacholine challenge. However, compliance was attenuated at baseline as well as with methacholine challenge in the Mp infected SP-A null mice in which TNF-α activity had been inhibited with LMP-420 treatment (Fig. 5*B*). This latter result suggests LMP-420 also reduced lung edema. Although LMP-420 administration did decrease total BAL protein in the infected SP-A null mice as measured by BCA by ~25%, this decrease did not achieve statistical significance (data not shown).

The inflammatory cell response in the lung is decreased by TNF-α inhibition

AHR to methacholine was attenuated in the SP-A null mice if TNF-α was inhibited during the infection period. This could be due to numerous consequences of the elevated TNF-α. We initially

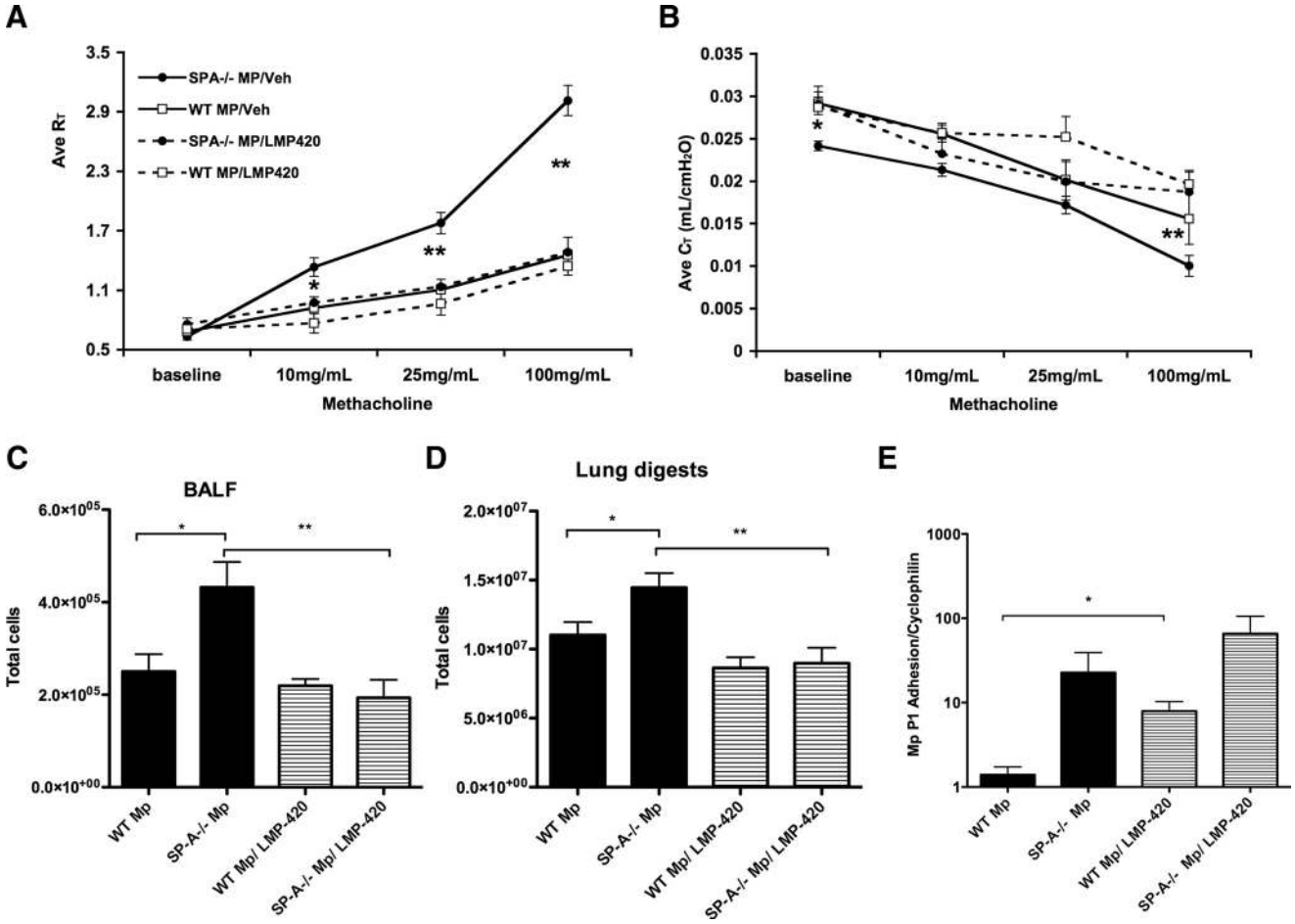

FIGURE 5. Treatment with LMP-420 attenuates AHR and cellular infiltration in SP-A null Mp-infected mice. Before Mp instillation, WT and SP-A$^{-/-}$ mice were treated with the TNF-α inhibitor, LMP-420. Two subsequent i.p. injections of LMP-420 were given 24 and 48 h post Mp infection to maintain inhibition. *A*, Airway responsiveness and *B*, airway compliance to methacholine challenge were analyzed 72 h post infection by Flexivent technology. n = 8–10/group and *, $p < 0.05$; **, $p < 0.01$ and is SP-A$^{-/-}$ Mp infected/vehicle vs all other groups. Total cells from *C*, BALs or *D*, lungs digests of the vehicle or LMP-420 infected mice were analyzed to determine levels of cellular infiltration. Data is the mean ± SEM, n = 8–10/group. *, $p < 0.05$; **, $p < 0.01$. *E*, RT-PCR for Mp P1-adhesin from cDNA extracted from Mp-infected mice in the presence or absence of LMP-420 as standardized to the housekeeper cyclophilin.

sought to determine whether inflammatory cell recruitment could be a factor in the decreased AHR measured in these mice since there were significantly more cells in samples collected from the SP-A null mice when infected with Mp for 3 days (Fig. 1). Total cell counts from BALF as well as lung digests revealed that significantly fewer inflammatory cells were present in the infected lungs in SP-A null mice given the TNF-α inhibitor as compared with those receiving vehicle (Fig. 5, *C* and *D*). Further cellular analysis from lung tissue by flow cytometry revealed that T lymphocytes populations from the lung digests, which were significantly increased as discussed previously, were significantly reduced back to saline control levels in the Mp infected SP-A null mice when TNF-α was inhibited (data not shown).

Interestingly, the amount of Mp present as detected by RT-PCR in both WT and SP-A null mice treated with the TNF-α inhibitor was actually greater than in those mice given vehicle (Fig. 5*E*). Paradoxically, there was a reduction in AHR and in the amount of CD3$^+$ T cells with LMP-420 treatment despite a greater Mp burden. Together these data suggest that the increase in T cells detected in the lungs of the Mp infected SP-A null mice was not due directly to Mp-induced proliferation since the increased Mp levels did not correlate with the decreased numbers of T lymphocytes in the mice treated with LMP-420.

Goblet cell hyperplasia is attenuated by inhibition of TNF-α in airways of Mp infected mice

Mucus hypersecretion has been shown to be an important factor in the pathogenesis of many asthma phenotypes and has been associated with disease fatality due to mucus plugging of airway lumens (33, 34). Several inflammatory cytokines including TNF-α have been shown to induce mucus gene expression and mucus cell hypersecretion in vivo (35–38). We therefore sought to determine whether blocking TNF-α during Mp infection would result in decreased levels of goblet cell hyperplasia. WT and SP-A null mice were treated with vehicle or LMP-420 before infection with Mp as previously described. WT infected mice have very low levels of PAS positive cells identified by histology 3 days after infection. However, lung sections taken from infected mice deficient in SP-A display many PAS positive cells in the large airways (Fig. 6*A*, *left*). When mice are treated with the TNF-α inhibitor during infection, few if any PAS positive cells are detected by histological examination in either the WT or SP-A null mice (Fig. 6*A*, *right*).

To quantify the amount of PAS-positive cells in the Mp infected lungs, pictures were taken randomly of each large airway for a given section. The analysis of the volume percentage of positive cells to PAS stain was calculated by the ratio of the value of the

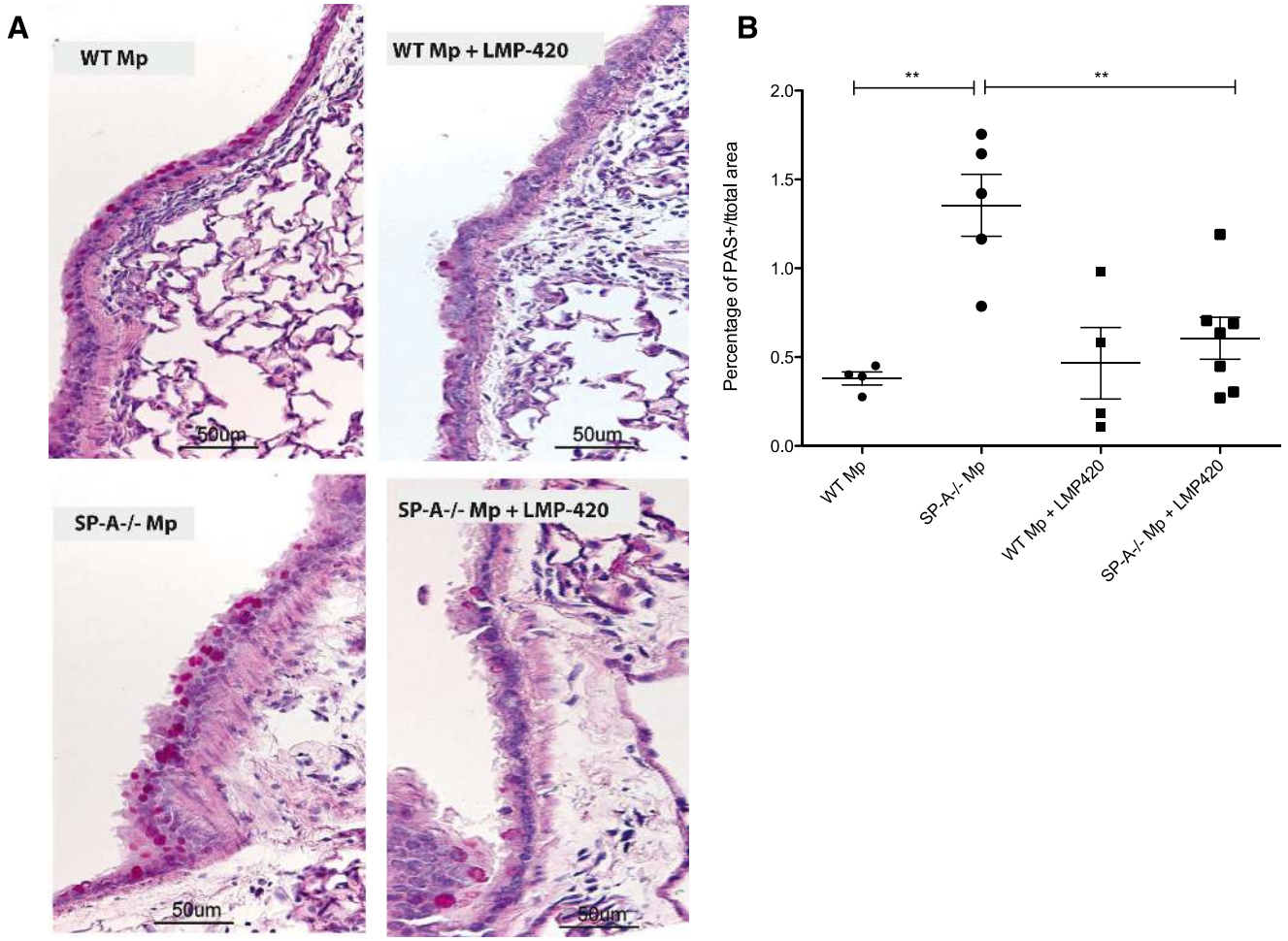

FIGURE 6. Histological analysis of airway mucin production in Mp infected mice. WT and SP-A null mice treated with vehicle (*left*) or the TNF-α blocker, LMP-420 (*right*), before infection with Mp. *A*, Paraffin sections were stained with PAS to detect mucin production (bright pink stain). Pictures were taken at $\times$20 magnification and the bar represents 50 μm. *B*, Slides from two independent experiments were further analyzed by blinded histological analysis. Representative pictures from nonoverlapping large airways were taken at $\times$20 magnification and the percentage of PAS positive stain was determined per area of large airway. Approximately eight pictures were taken from each slide and averaged per specimen. $n = 4$–7/group and **, $p < 0.01$.

reactive tissue to PAS and the total volume of the large airway. As shown in Fig. 6*B*, SP-A null infected mice have significantly more PAS^+ area per total area in the large airway as compared with WT-infected mice. This parameter is significantly decreased when mice are treated with the TNF-α inhibitor, LMP-420, during infection (Fig. 6*B*).

Discussion

These studies demonstrate novel findings in a mouse model of bacterial infection, that expand upon the protective contribution of SP-A to innate immunity and host defense and elucidate a new role for the protein in mediating airway hyperreactivity. In mice deficient in SP-A, changes in the biologic response to Mp-infection were exacerbated, as demonstrated by enhanced levels of BALF protein, mucus overproduction, and a robust lymphocyte-rich inflammation after 3 days of infection. Coincident with up-regulation of the biologic response, physiologic changes inclusive of AHR and decreased lung compliance were also more severe in the absence of SP-A. Additionally, as detected by immunohistochemistry, there was greater Mp colonization in the large airways of SP-A null mice. Not unexpectedly, and corresponding with the increased burden of Mp to the airway in SP-A deficient mice, BALF concentrations of the proinflammatory cytokine TNF-α were found to be significantly elevated during the acute phase of infection. Inhibition of TNF-α production during Mp infection by systemic intervention resulted in striking reductions in AHR, cellular inflammation, and mucus hypersecretion. We sought to determine possible mechanisms by which TNF-α was contributing to the phenotypes observed, as it not only triggers but also maintains inflammatory responses and is vital in cell recruitment and activation.

SP-A has been shown to bind Mp by recognition of disaturated phosphatidylglycerol surface lipids and the protein, MPN372 (9, 10). Human SP-A binds MPN372 protein in a calcium concentration-dependent, and saturable manner (9). Additionally, by binding the mycoplasma surface lipids, SP-A directly inhibits growth of Mp (10). Although SP-A binding to Mp has been shown to have a bacteriostatic effect in vitro, no such experiments have been published in vivo. Therefore, we sought to determine whether mice lacking SP-A had a greater Mp burden which could lead to the production of greater amounts of TNF-α. Mp colonies derived from BALF were equivalent in both the mice containing SP-A and those lacking SP-A. However, Mp establishes pulmonary infection by binding to ciliated airway epithelial cells where it causes robust cytokine production by lung macrophages and in the process damages airway cilia. The amount of Mp present in the lung tissue was detected by RT-PCR of whole lung tissue for the Mp specific P1-adhesin gene. This analysis over multiple experiments consistently revealed that Mp are present in the lung tissue from infected mice

lacking SP-A at ~25-fold greater numbers than in WT mice containing SP-A. Because the Mp used for infection is adherent when collected from cultures, for which expression of P1-adhesin is necessary, it is unlikely that SP-A acts in any way to alter transcriptional adaptation of the organism. It is more probable that SP-A binds to Mp through MPN372 and/or through surface lipids thereby preventing Mp from binding airway cells. The increase in Mp burden in the lung tissue could prolong the persistence of an overly robust biologic response and further exacerbate AHR in SP-A null mice.

A hallmark of mycoplasma infection is its ability to stimulate macrophages and monocytes to secrete substantial amounts of proinflammatory cytokines, which lead to local and systemic inflammatory responses. One prototypal cytokine, TNF-α, is a principal mediator of inflammation, fever, and septic shock. Because TNF-α is an acute phase cytokine, it was not surprising that at the time of airway function changes, 3 days after Mp-infection, detectable levels of TNF-α in BALF were minimal. In contrast, TNF-α levels measured in BALF peaked 12 h postinfection in both WT and SP-A null mice. Interestingly, TNF-α levels in BALF from those mice lacking SP-A were significantly greater by almost 5-fold as compared with WT infected controls. Previous reports demonstrate that SP-A can inhibit LPS induced TNF-α production in vivo (7). Additionally, SP-A also inhibits peptidoglycan-induced TNF-α secretion by alveolar macrophages (39). Findings in this study suggest SP-A plays an important role in limiting MP-induced TNF-α production in the airway. The heightened increase in TNF-α activity in those mice lacking SP-A could account for the increase in AHR observed in the SP-A null mice by several mechanisms.

Treatment of the infected mice with the TNF-α inhibitor LMP-420 not only significantly decreased AHR in SP-A null mice but also significantly reduced T lymphocyte numbers. Interestingly, SP-A is known to inhibit T cell proliferation in vitro (3) and therefore lack of SP-A in the lung environment would likely result in more proliferation especially in this infection setting considering many mycoplasmas are mitogenic. The data presented in this study show that the T lymphocyte numbers were repressed during Mp infection upon inhibition of TNF-α production independent of whether SP-A was present or absent in the lung environment. This repression suggests that the mycoplasmas are not acting as direct mitogens for lymphocytes and that SP-A is not necessarily acting to repress T cell proliferation during this infection. The findings support the concept that the increase in T lymphocytes was a direct consequence of excess TNF-α secretion. Thus, the decrease in T lymphocytes via reduction in TNF-α production may partially explain the apparent decrease in AHR of the infected SP-A null mice.

Upon examination of histological sections of SP-A null infected lungs, we found that in the absence of SP-A, cellular foci were present adjacent to large airways to a much greater extent than in WT controls and these foci consisted almost exclusively of small mononuclear cells. Recent publications demonstrate that the presence of T-lymphocytes can act directly on airways to induce AHR and act independently of other inflammatory cell recruitment and mucus overproduction (40). We therefore sought to determine which inflammatory cells were present in BALF and lung tissue of the Mp infected mice by flow cytometry. Although there were significantly more cells present in the BALF of SP-A null mice, there was no difference in the number of lymphoid cells. However, upon examination of cells isolated from the lung tissue by flow cytometry we found significantly more lymphoid cells ($CD3^+$ $CD4^+$ T cells and $CD3^+$ $CD8^+$ T cells) in the infected SP-A null mice.

Alternately, events downstream of overzealous TNF-α secretion may also have contributed to augmentation of AHR. For example, mucus hypersecretion has been known for some time to play an important part in the pathogenesis and severity of inflammatory airway disease and asthma. Recent findings show that chronic TNF-α exposure enhances airway mucus gene expression in mice (35). Histological examination and quantitative analysis of infected lungs indicated that mucus production was greater in the mice lacking SP-A. Again, this measurement was significantly decreased by inhibiting TNF-α before and during the infection.

SP-A null Mp infected mice displayed another notable feature observed by histological analysis. In the lung parenchyma, not only were more cellular infiltrates associated with the alveoli but swelling of the alveoli interstitium was also detectable consistent with pulmonary edema (see Fig. 2*F*). Total protein levels were elevated in the BALF from infected mice consistent with the histological analyses. Mice lacking SP-A and infected with Mp had significantly more total protein in BALF than WT mice (see Fig. 2*G*). This finding was particularly interesting since decreases in compliance indicate increased stiffness in the lungs, which is often clinically correlated with increased pulmonary edema (41, 42). Taken together these findings are consistent with the significantly lower lung compliance observed in the infected SP-A null Mp mice and assessed before methacholine provocations. Compliance measures in infected mice lacking SP-A which had been treated with the TNF-α inhibitor, however, had compliance measurements similar to those observed for the control saline treated mice.

Mp respiratory tract infections are thought to account for 20–40% of all cases of community-acquired pneumonia and have been shown in recent years to be associated with wheezing syndromes and asthma exacerbations (12, 43, 44). Additionally, both cellular inflammation and mucus hypersecretion are known causative agents of AHR and both are often present in the airways of asthmatics and are mediated by a TNF-α response. Alternately, recent evidence has also shown that increased amounts of TNF-α can modify airway smooth muscle tone directly and attenuate airway relaxation (45). Although discriminating between these factors cannot clearly define one as the causative agent of enhanced AHR in the infected mice lacking SP-A, these multiple factors should all be taken into consideration as contributors to the heightened response in these mice. Collectively, these findings strongly emphasize the important protective role that SP-A exerts in preserving normal lung homeostasis, airway function, and host defense in response to Mp infections.

Acknowledgments

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Disclosures

The authors have no financial conflict of interest.

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Corrections

Ledford, J. G., H. Goto, E. N. Potts, S. Degan, H. W. Chu, D. R. Voelker, M. E. Sunday, G. J. Cianciolo, W. M. Foster, M. Kraft, and J. R. Wright. 2009. SP-A preserves airway homeostasis during *Mycoplasma pneumoniae* infection in mice. *J. Immunol.* 182: 7818–7827.

We have recently become aware of potential discrepancies between the machine-generated raw data and the data provided by our collaborating pulmonary function laboratory that were used to calculate the average resistance and compliance results on the Flexivent. We therefore repeated the original experiments, and in contrast to our published work, we now find that SP-A KO mice do not have greater airway hyperresponsiveness as compared with wild type (WT) mice. We verified that WT mice have significantly greater airway resistance when infected with *Mycoplasma pneumonia* (Mp) as compared with WT saline controls. These newly generated data are provided here as a replacement for our original Fig. 1.

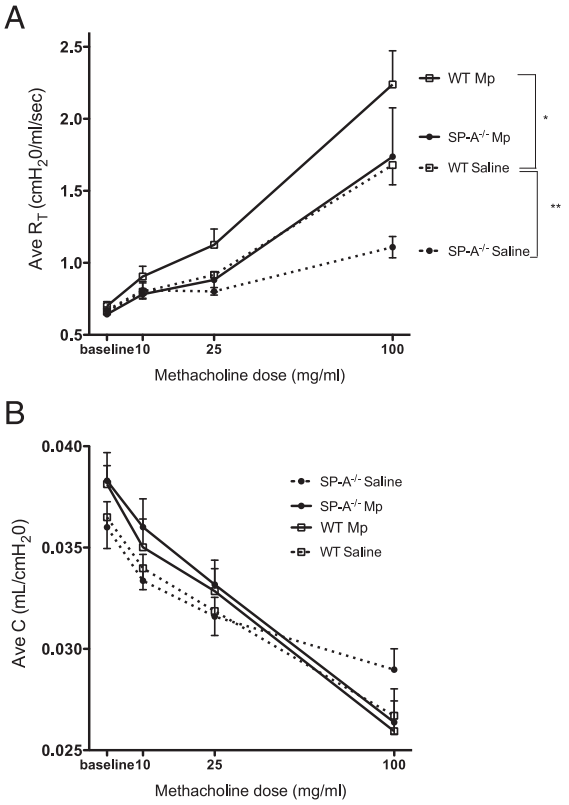

FIGURE 1. Airway physiology measurements after Mp infection. WT and SP-$A^{-/-}$ mice were instilled intranasally with Mp ($\sim 1 \times 10^8$/mouse) or saline as a control and airway responsiveness to methacholine challenge was analyzed 72 h post infection by Flexivent. *A*, The average resistance was significantly increased to methacholine challenge (100 mg/ml) in the Mp infected WT mice ($n = 8$) as compared with saline treated WT mice ($n = 6$), *, $p < 0.05$. WT saline treated mice ($n = 6$) had significantly higher average resistance to methacholine challenge (100 mg/ml) as compared with SP-$A^{-/-}$ saline treated mice ($n = 5$), **, $p < 0.01$. *B*, The average compliance decreased over the course of methacholine challenge in all groups of mice examined.

In addition, we would like to retract Fig. 5*A* and 5*B* from the published article, as they build upon the data in Fig. 1, which we have been unable to verify. In light of the changes to Fig. 1 and the retraction of Figs. 5*A* and 5*B*, the following corrections are needed to the text of the published article.

In the *Abstract*, the sentence "Likewise, physiologic responses (airway hyperresponsiveness and lung compliance) to Mp infection were more severely affected in SP-$A^{-/-}$ mice," is no longer valid and needs to be omitted.

In the last paragraph of the *Introduction*, the phrase ". . .and significantly elevated airway hyperresponsiveness (AHR). . ." is no longer valid and needs to be omitted.

In the *Results* section, in the second paragraph under the subheading *AHR in Mp-infected mice*, the sentence "Shown in Fig. 1*A*, all animals had minimal AHR to the methacholine challenge when instilled with saline," and the sentence "In contrast to WT mice, the infected SP-A null

mice had significantly elevated AHR, which was evident even at the 25 mg/ml dose of methacholine (Fig. 1*A*)," are no longer valid and need to be omitted. The entire third paragraph, "In addition to increased airway resistance in the Mp-infected SP-A null mice, they also showed significantly reduced dynamic compliance as shown in Fig. 1*B*. Dynamic compliance measures the ease with which the lungs can be extended and a drop in compliance values indicates increased stiffness in the lungs. The compliance measurements in the WT infected mice were comparable to saline-treated mice. However, the compliance of the infected SP-A null mice was significantly lower at the baseline pre-challenge measurements well as throughout the methacholine challenges (Fig. 1*B*)," is no longer valid and needs to be omitted.

In the *Results* section, in the first paragraph under the subheading *Inhibition of TNF-α attenuates AHR*, the text "To determine whether the increased AHR observed in Mp-infected SP-A null mice may be due to overproduction of TNF-α, and therefore a myriad of other effects due to TNF signaling. . ." needs to be omitted, as does the following text at the end of that paragraph: ". . .in which they continued to have significantly higher AHR compared to WT infected mice. However, this heightened AHR was significantly attenuated if the SP-A null mice were pretreated with the TNF inhibitor, LMP-420, before infection (Fig. 5*A*). This suggests that SP-A can modulate factors related to physiologic airway function during the acute phase of an infection, and in the absence of SP-A, Mp enhances TNF signaling and secretion, leading to an enhancement in AHR." In the second paragraph, the text "As discussed above, dynamic compliance was also significantly elevated in Mp infected SP-A null mice before methacholine challenge. However, compliance was attenuated at baseline as well as with methacholine challenge in the Mp infected SP-A null mice in which TNF-α activity had been inhibited with LMP-420 treatment (Fig. 5*B*)," is no longer valid and needs to be omitted.

The data shown in Fig. 5A and 5B and part of the text in the figure legend, "AHR" and "*A*, Airway responsiveness and *B*, airway compliance to methacholine challenge were analyzed 72 h post infection by Flexivent technology. n=8–10/group and *, $p < 0.05$; **, $p < 0.01$ and is SP-A$^{-/-}$ Mp infected/vehicle vs all other groups," are no longer valid.

In the first paragraph of the *Discussion,* the following text needs to be omitted: ". . .and elucidate a new role for the protein in mediating airway hyperreactivity. . ." and "Coincident with up-regulation of the biologic response, physiologic changes inclusive of AHR and decreased lung compliance were also more severe in the absence of SP-A." In addition, "AHR" in the sentence "Inhibition of TNF-α production during Mp infection by systemic intervention resulted in striking reductions in AHR, cellular inflammation, and mucus hypersecretion," needs to be omitted. In the last sentence of the second paragraph of the *Discussion*, the text ". . .and further exacerbate AHR. . ." should be disregarded. In addition, in the last sentence of the third paragraph of the *Discussion*, the last sentence "The heightened increase in TNF-α activity in those mice lacking SP-A could account for the increase in AHR observed in the SP-A null mice by several mechanisms," needs to be omitted.

In the first sentence of the fourth paragraph of the *Discussion*, the text ". . .not only significantly decreased AHR in SP-A null mice but also. . ." and the last sentence "Thus, the decrease in T lymphocytes via reduction in TNF-α production may partially explain the apparent decrease in AHR of the infected SP-A null mice," are no longer valid and need to be omitted. Finally, in the last paragraph of the *Discussion*, the sentence "Although discriminating between these factors cannot clearly define one as the causative agent of enhanced AHR in the infected mice lacking SP-A, these multiple factors should all be taken into consideration as contributors to the heightened response in these mice," is no longer valid and needs to be omitted.

We are confident that the other data we reported are valid, as they were collected and analyzed independently of the pulmonary mechanics data. All authors agree to this *Correction* and to the retraction of Figs. 5*A* and 5*B*. We apologize to our colleagues and the scientific community for any inconvenience this might have caused.

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