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DIFFERENTIAL SUSCEPTIBILITY OF HUMAN SP-B GENETIC VARIANTS ON LUNG INJURY CAUSED BY BACTERIAL PNEUMONIA AND THE EFFECT OF A CHEMICALLY MODIFIED CURCUMIN

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Abstract

Staphylococcus aureus is a common cause of nosocomial pneumonia frequently resulting in acute respiratory distress syndrome (ARDS). Surfactant protein B (SP-B) gene expresses two proteins involved in lowering surface tension and host defense. Genotyping studies demonstrate a significant association between human SP-B genetic variants and ARDS. Curcumins have been shown to attenuate host inflammation in many sepsis models. Our hypothesis is that functional differences of SP-B variants and treatment with curcumin (CMC2.24) modulate lung injury in bacterial pneumonia. Humanized transgenic mice, expressing either SP-B T or C allele without mouse SP-B gene, were used. Bioluminescent labeled *S. aureus* Xen36 (50 µl) was injected intratracheally to cause pneumonia. Infected mice received daily CMC2.24 (40 mg/kg) or vehicle alone by oral gavage. Dynamic changes of bacteria were monitored using *in vivo* imaging system. Histological, cellular and molecular indices of lung injury were studied in infected mice 48h after infection. *In vivo* imaging analysis revealed total flux (bacterial number) was higher in the lung of infected SP-B-C mice compared to infected SP-B-T mice ($p < 0.05$). Infected SP-B-C mice demonstrated increased mortality, lung injury, apoptosis and NF-κB expression compared to infected SP-B-T mice. Compared to controls, CMC2.24 treatment significantly reduced the following: mortality, total bacterial flux and lung tissue apoptosis, inflammatory cells, NF-κB

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expression ($p<0.05$), and MMPs-2, -9, -12 activities ($p<0.05$). We conclude that mice with SP-B-C allele are more susceptible to *S. aureus* pneumonia than mice with SP-B-T allele; and that CMC2.24 attenuates lung injury thus reducing mortality.

Keywords

Allele; Chemically-modified Curcumin; Pneumonia; Surfactant protein B; *Staphylococcus aureus*

INTRODUCTION

Bacterial pneumonia is one of the major causes of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) (1). ALI and ARDS are life-threatening conditions with an incidence of 79 per 100,000 in the United States (2). *Staphylococcus aureus* is a common gram-positive and opportunistic pathogen, which causes half a million infections a year including pneumonia and approximately 20,000 deaths per year in the United States (2,3).

Surfactant deactivation has been shown to be an important mechanism for mediating lung injury. Alveolar Type II epithelial cells in the lung secrete four surfactant proteins that are distributed on the surface of the alveoli. The hydrophobic surfactant protein B (SP-B) is of particular importance (4). The SP-B gene expresses two protein products, SP-B^M and SP-B^N, involved in lowering surface tension and promoting host defense, respectively (5). The main function of SP-B^M protein is to form the monolayer of phospholipids on the surface of alveoli to reduce the surface tension, preventing the collapse of alveoli and maintaining respiration. SP-B^N functions as a host defense molecule and plays a role in pulmonary bacterial clearance (5). Human SP-B gene has an important single nucleotide polymorphism (SNP rs1130866 i.e. SP-B C/T1580) in the N-terminal sapolin-like domain that produces SP-B^N protein. The SP-B C/T1580 polymorphism forms two common genetic alleles, SP-B C and T alleles, with differing ability to maintain respiratory homeostasis and optimal host defense. Wang et al. has shown in an *in vitro* study that proteins from SP-B C and T alleles contain different post-translational modifications, in particular the SP-B C allele has one additional glycosylation site compared to the T allele. This altered glycosylation may impact SP-B protein processing and function (6).

Humanized transgenic (hTG) mouse models provide a powerful tool for studying the pathophysiological function of human genetic gene/variants (alleles) in clinically important diseases (7,8). The hTG model can elucidate subtle differences in phenotypes caused by human genetic variants and overcome study limitations in infectious diseases *in vivo* (9). We recently generated hTG SP-A mice and showed that the formation of the tubular myelin (TM) *in vivo* requires both SP-A1 and SP-A2 gene products (10). Thus, hTG mice are an ideal *in vivo* system to study functional differences between SP-B C and T alleles in bacterial pneumonia. Additionally, to monitor the changes of bacterial dynamic growth we have used bioluminescent labeled *S. aureus* and an *in vivo* imaging system (11). The advanced hTG mouse model provides us with a unique opportunity to investigate functional

differences of SP-B genetic variants *in vivo* and to monitor dynamic changes in bacterial growth in our pneumonia model.

During infection, increased neutrophil infiltration, lung tissue apoptosis, cytokine synthesis, and degradation of lung matrix result in severe lung injury. Curcumin [1,7-bis-(4-hydroxy-3-methoxy phenyl)-1,6-heptadiene-3,5-dione], a component of turmeric, is extracted from the rhizomes of the plant *curcuma longa*, which possesses several pharmacological properties including anti-inflammatory and anti-oxidant effects. Curcumin also selectively inhibits the activities of inducible matrix metalloproteinases (MMPs), and down-regulates expression of pro-inflammatory cytokines through modulation of NF- κ B and related signaling pathways (12,13). We have found that a novel chemically-modified curcumin, CMC2.24, is more potent than natural curcumins in the inhibition of apoptosis, inflammation, and inducible MMPs, all of which contribute to tissue injury; and CMC2.24 displays enhanced bioactivity and bioavailability with decreased toxicity (14,15). In the present study we investigated the differential effects of human SP-B C and T alleles in the presence or absence CMC2.24 by bacterial pneumonia model in humanized SP-B transgenic mice. We have observed differential susceptibility to bacterial pneumonia between hTG SP-B-C and SP-B-T mice and the protective roles of CMC2.24 in the lung injury of infected mice.

MATERIALS AND METHODS

1. Mice

hTG SP-B mice (FVB/N strain background) carrying either human SP-B C or T allele without mouse SP-B gene were used in this project. The hTG SP-B-C and SP-B-T mice were recently generated by our laboratory (Generation and characterization of these hTG SP-B mice will be described in a subsequent paper) and maintained in the Department of Laboratory Animal Resources of SUNY Upstate Medical University. The hTG SP-B mice were bred at least 10 generations to stabilize the transgenic SP-B expression. Human SP-B expression of hTG SP-B mice were examined by Western blot analyses and immunohistochemistry. The presence of human SP-B (hSP-B) gene and the absence of mouse SP-B (mSP-B) gene in hTG SP-B-C and SP-B-T mice were confirmed by genotyping PCR analysis with primer pair 1458/189 (sense: GGAGGGCCAGGAACAAACAGG; antisense: CATAACAGATGCCGTTTGAGTC) for hSP-B gene and primer pair 75/76 (Sense: ATCCTCCCTTCTCTGCTCTCC; antisense: TGTGCTTATTGCGTCTGTGG) for mSP-B gene, respectively. To further examine whether there is mouse SP-B mRNA expression in hTG SP-B-C and SP-B-T mice, total RNAs from lung tissue were isolated and reverse transcription PCR (RT-PCR) was performed with primer pair 739/614 (sense TACTCCGTCATCCTGCTCGA and antisense GCTGCTCCACAAATTGCTTG for hSP-B mRNA) and primer pair (sense CCTGCCCCTGGTTATTGACTACTT within mSP-B exon 4 and antisense GGACACAGCCACAGCCAGCACAC within mSP-B exon 7) for mSP-B mRNA. Both male and female mice, 8–12 weeks old, were distributed into 3 experimental groups: the pneumonia group (Pneu, *S. aureus* infection only), pneumonia plus CMC2.24 treatment group (Pneu + CMC2.24, *S. aureus* infection plus CMC2.24), and the control group (sham, treated with sterile vehicle). All protocols related to animal experiments were approved by the institutional animal care and use committee of SUNY Upstate Medical

University. Experiments were performed according to the National Institutes of Health guidelines and ARRIVE guidelines on the use of laboratory animals.

2. Curcumin derivative

Tri-ketonic chemically-modified curcumin (CMC2.24) having enhancing zinc-binding ability (vs. di-ketonic natural curcumin) was obtained from Dr. Francis Johnson's laboratory. CMC2.24 (10 mg) was suspended in 3 ml of 2% carboxymethyl cellulose vehicle, and then each mouse was administrated 0.3 ml of the CMC2.24 solution daily (equal to 40 mg of CMC2.24/kg mouse weight) or the same volume of vehicle alone (control group) by oral gavage (16).

3. *S. aureus*- induced pneumonia model

Pilot experiments were performed to establish the *S. aureus* Xen36 pneumonia model using different doses of bacteria to infect mouse lung. The results indicated that a dose of 5×10^8 CFU/mouse in 50 μ l of bacterial solution was optimal, because mice infected at this level of bacteria produced enough bioluminescent signal in the lung to be detected by the *in vivo* imaging system and had a reasonable survival rate at 48 h after infection. Therefore, direct intratracheal inoculation of bioluminescent *S. aureus* Xen36 at a dose of 5×10^8 CFU/50 μ l/mouse was used to infect mice in all subsequent experiments (17). In brief, hTG SP-B mice were anesthetized using intraperitoneal ketamine/xylazine (90 mg/kg ketamine, 10 mg/kg xylazine) injection. A 0.5-cm mid-line neck incision was made to expose the trachea. In the sham group, 50 μ l of sterile vehicle was injected into the trachea by the same method. After infection, bioluminescence signal was monitored and quantified by an *in vivo* imaging system (Xenogen-200 series, Caliper Life Sciences, Hopkinton, MA). Buprenorphine (0.05 mg/kg body weight) was injected for postoperative analgesia every 8–12 h. Mice were returned to their cages in a temperature-controlled room (22 °C) with 12 h light and dark cycles, and monitored every 4 h. Mice were anesthetized with isoflurane (2%) at several time points after infection (0, 12, 24, 28, 32, and 48 h) (11). At 48 h after *S. aureus* infection, mice were sacrificed under anesthesia. Blood and bronchoalveolar lavage fluid (BALF), lung tissue were harvested or fixed for further study. In the CMC2.24-treated group, the mice were administered a dose of CMC2.24 (40 mg/kg) by oral gavage 30 min before bacterial infection, and then received second dose of CMC2.24 at 24 h after infection. In addition, to identify whether the outcomes of CMC2.24 treatment result from the effect of bacterial infection and induced inflammation, we performed a non-infectious lung injury model in hTG SP-B-C and SP-B-T mice by means of intranasal treatment with 50 μ l/mouse hydrochloric acid (HCl, 0.1 N) in saline, in which the mice were administered a dose of CMC2.24 (40 mg/kg) or vehicle by oral gavage 30 min before HCl treatment (n= 8 mice/group). The mice were then sacrificed 6 h post-treatment for further analysis.

4. *In vivo* imaging analysis

The mice were monitored for 48 hours after infection. Photographs were captured with a cooled CCD camera (Xenogen-200 series, Caliper Life Sciences, Hopkinton, MA). Pseudo-colored images of photon emissions were covered on gray scale images of the mouse to obtain spatial localization of the bioluminescent signals. For each *in vivo* imaging

experiment: 5 mice were placed in the induction chamber at one time and anesthetized with isoflurane (2% in oxygen), and then placed into the IVIS-200 imaging chamber with continuous anesthesia. Images were performed for an initial exposure (5 min) by an *in vivo* imaging system (18).

5. Inflammatory cell analysis in BALF

After harvest, the BALF was centrifuged at 250×g. The supernatants were saved in −20°C freezer for further analysis. The pellets were re-suspended and wash with 1 ml of sterile saline, and then the cells were mounted on a slide by cytospin centrifuge at 1000 rpm for 3 min. (Hettich ROTOFIX 32A). Slides were stained by using the Hema-3 Stain Kit. Cells were examined by Nikon Eclipse TE2000-U research microscope (Nikon, Melville NY).

6. Histopathological analysis

After sacrifice, the lungs were fixed in 10% neutral formalin for at least 24 hours, and embedded in paraffin. Approximately 5 μm-slides of lung tissues from eight mice for each group were prepared and stained with Hematoxylin and Eosin (H&E). Digital photos were taken with a light research microscope (Nikon, Melville NY) and used for quantitative analysis according to the histological lung injury score system described previously (19). All slides were examined blindly by two experienced investigators. In brief, lung slides were evaluated using a 0–2 scale. The presence of alveolar (A) and interstitial neutrophils (B), alveolar hyaline membranes (C), proteinaceous debris (D) filling the air spaces, and alveolar septal thickening (E) were scored in twenty high power fields for each slide. The resulting scores were calculated by the following formula: $\text{Score} = [(20 \times A) + (14 \times B) + (7 \times C) + (7 \times D) + (2 \times E)] / (20 \times 100)$.

7. Immunohistochemistry (IHC)

To identify human SP-B expression in the lung tissues of hTG SP-B mice, tissue sections on the slides from sham group were examined by the IHC method. In brief, the sections were de-waxed, and blocked endogenous peroxidase activity in 3% H₂O₂ for 10 min. After incubating with 10% goat serum for 40 min at room temperature, the sections were incubated with anti-SP-B antibody (1:400, Hycult biotech, Plymouth Meeting, PA) at 4°C overnight. Subsequently, biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) at 1:2000 dilution was used for hybridization at 37°C for 30 min. DAB was used as a chromogenic substrate to develop color in the section for 3–10 min. Finally, the sections were counterstained for 1–3 min with hematoxylin.

8. Apoptotic cells by TUNEL assay

Lung tissue sections (~5μm) were incubated at 60°C for 20 min, and then de-paraffinized in xylene twice every 10 min, treated with different concentration-grades of alcohol [100%, 90%, 80% and 70% ethanol/ddH₂O], and then rinsed in phosphate buffer saline (PBS, pH7.5). Apoptotic cells were detected by means of a deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. Cell apoptosis was quantified by counting the number of TUNEL-positive cells in 20 random fields at ×400 magnification (20).

9. Western blotting analysis

Frozen lungs were homogenized in RIPA buffer containing a cocktail of protease- and phosphatase-inhibitors (Roche), and the supernatants were used for Western blot analysis (20). Total protein concentrations of samples (lungs and BALF) were determined using the BCA micro assay kit (Thermo Scientific, Rockford, IL). Total protein (40 µg) was resolved by reducing (for NF-κB, Caspase-3, Bcl-2) and non-reducing (for SP-B) 12% SDS-polyacrylamide gel electrophoresis, and then transferred onto PVDF membranes at 4 °C (Bio-Rad, USA). After that, the blot was blocked in 5% non-fat milk of Tris-buffered saline, and detected using a primary antibody against NF-κB (1:400, Santa Cruz Biotech), Caspase-3 (1:400, Santa Cruz Biotech), and Bcl-2 (1:400, Santa Cruz Biotech), as well as a anti-SP-B antibody (1:2000, Hycult biotech, Plymouth Meeting, PA). Thereafter, an anti-rabbit secondary antibody conjugated with horseradish peroxidase was applied (20). To standardize each protein in the blot, the blot was stripped and reprobed with β-Actin antibody (1:400, Santa Cruz Biotech). Immuno-products were detected using Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL) and the blots were exposed to X-film (Pierce Biochemicals, FL). Human BALF and proteins from sham mouse lung tissue were used as controls. The bands on films were quantified by Image J software version 1.48 (Wayne Rasband, NIH, Bethesda, MA).

10. MMPs activity by zymography

Aliquots of BALF containing 10 µg total protein were loaded onto a 10% polyacrylamide gel containing 0.1% (wt/vol) gelatin (from denatured type I collagen) under reducing conditions to determine different molecular levels of MMP-2 and MMP-9. After electrophoresis, the gel was washed with renaturing buffer (2.5% Triton X-100) for 30 min, and incubated with 100 ml of developing buffer (40 mM Tris, 200mM NaCl, and 10 mM CaCl₂; pH 7.5) at room temperature for 30 min and then at 37°C for 24 h with gentle agitation. The gel was then stained in 0.05% (wt/vol) Coomassie Brilliant Blue, 30% (vol/vol) methanol, and de-stained in 10% (vol/vol) acetic acid for 1 h and repeatedly for additional 3 h. For MMP-12 analysis, BALF containing 10 µg of total proteins was used on a 12% polyacrylamide gel containing 0.05% (wt/vol) casein following the same above protocol. PageRuler prestained protein ladders (10–170 kDa) were used as molecular weight markers (Thermo Scientific, Rockford, IL). Densitometry determinations were carried out using Image J software version 1.48 (Wayne Rasband, National Institutes of Health, Bethesda, MA).

11. Statistical analysis

All data are presented as means ± SEM. Data were compared using Student t-test or ANOVA by SigmaStat software (version 3.5). Animal survival analysis was performed by the Kaplan-Meier survival method. For all comparisons, p<0.05 was considered statistically significant.

RESULTS

Human SP-B expression in hTG SP-B mice

To examine human SP-B protein expression in the lung tissues of hTG SP-B-C and SP-B-T mice, BALF and lung tissues from Sham SP-B-C and SP-B-T mice (n=5 for each) were analyzed by Western blot and IHC. The results showed that the levels of SP-B protein are similar in the lung of hTG SP-B-C and SP-B-T mice compared to that of human lung (Fig. 1A). The results from IHC analysis indicate human SP-B protein expression in alveolar epithelial type II cells in the lung of hTG SP-B-C and SP-B-T mice (Figs. 1B and C). Genotyping analysis of the DNA from mouse lung showed mouse SP-B gene deficiency in hTG SP-B-C and SP-B-T mice but human SP-B DNA (Fig. 1D). Furthermore, mRNA expression of mouse and human SP-B gene were analyzed by RT-PCR with appropriate controls. The results indicate that no mouse SP-B mRNA but only human SP-B mRNA was expressed in the lung of hTG SP-B-C and SP-B-T mice (Fig. 1E). These data demonstrate that only human SP-B gene but not mouse SP-B was expressed in the lung of hTG SP-B-C and SP-B-T mice.

In vivo measurement to *S. aureus* infection in hTG SP-B-C and SP-B-T mice using bioluminescence analysis

To study functional differences of human SP-B genetic variants in the bacterial pneumonia we measured bacterial dynamic changes in the lungs of hTG SP-B-C and SP-B-T mice after intratracheal infection of bioluminescent labeled *S. aureus* at six time points i.e. 0, 12, 24, 28, 32, 48 h after infection. The results from *in vivo* image analysis showed the level of bioluminescence was significantly higher ($p<0.01$) in the infected SP-B-C mice from 24h to 48h after infection, compared to infected SP-B-T mice (Fig. 2). For infected SP-B-C mice, the levels of bioluminescence increased rapidly from 0 to 24h after infection, remained high between 24 and 32 h, and then decreased (Fig. 2B). In infected SP-B-T mice, the peak time of bioluminescence was at 12 h after infection, then the level decreased slowly (Fig. 2B). In addition, there are different mortality rates between infected SP-B-C and SP-B-T mice (62.8% vs. 33.3%, $p<0.01$) by 48h after infection (Fig. 2C). These results indicate that greater resistance to *S. aureus* Xen36 bacterial infection exists in SP-B-T mice compared to SP-B-C mice.

The effect of CMC2.24 on *S. aureus* resistance in hTG SP-B-C and SP-B-T mice using *in vivo* bioluminescence analysis

To study the effect of CMC2.24 in bacterial pneumonia, infected SP-B-C and SP-B-T mice were administered a daily dose of CMC2.24 (40 mg/kg) or vehicle (control). The results showed significantly decreased bacterial load in the CMC2.24 treated group compared to the control (Fig. 3). For SP-B-C mice, the bioluminescence levels were significantly lower ($p<0.01$) in the CMC2.24 treated group from 24 to 48h after infection, compared to the control (Fig. 3B). Similar effects were observed in SP-B-T mice (Fig. 3C). Furthermore, we observed lower mortality rate in the CMC2.24 treated SP-B-C mice compared to the untreated SP-B-C mice (50% vs. 76%, $p<0.05$) (Fig. 3D), but no difference was observed between CMC2.24 treated and untreated SP-B-T mice (32% vs. 33%).

Lung histology

To assess the effects of human SP-B genetic variants and CMC2.24 on lung injury in pneumonia we examined lung histopathology of the three groups (Sham, Pneu, Pneu + CMC) at 48 h after infection. The results showed obvious pathological changes in lung histology 48 h after infection with or without CMC2.24 treatment (Pneu, Pneu + CMC) but not in Sham mice (Fig. 4A). CMC2.24 treated mice showed decreased lung injury by histology and scores compared with control mice 48 h after infection, including fewer neutrophils in the alveolar space and interstitial membrane, decreased accumulation of proteinaceous debris, and thinner alveolar walls in the lung (Fig. 4A). Furthermore, quantitative analysis indicates the lung injury scores of both CMC2.24 treated SP-B-C and SP-B-T mice (Pneu + CMC) are lower ($p < 0.01$) compared to the Pneu mice, but larger than that of Sham mice (Fig. 4B). The lung injury scores of infected SP-B-C mice with and without CMC2.24 are larger ($p < 0.01$) than those of infected SP-B-T mice with and without CMC2.24, respectively (Fig. 4B).

Lung apoptosis

We examined apoptotic cells and apoptosis-related protein (biomarker) expression in the lung tissues of three experimental groups (Sham, Pneu, Pneu + CMC) by TUNEL assay. As shown in the Figure 5A, apoptotic cells exhibit brown nucleus in infected mice but not for Sham mice. Lung tissues from infected SP-B-C mice (Pneu) showed more apoptotic cells compared to infected SP-B-T mice (Pneu) ($p < 0.01$) (Figs. 5A and B). CMC2.24 treated mice showed decreased apoptotic cells ($p < 0.01$) when compared to their respective controls (Pneu).

We also examined the expression of two apoptosis-related proteins in the lung tissues by Western blot analysis. Representative Western blots are shown for each experimental group (Figs. 6A and B). Caspase-3 (Cap-3), as one biomarker of cell apoptosis, correlated positively with apoptosis. The results showed significant increase of Cap-3 expression in the lungs of infected SP-B-C and SP-B-T mice compared to Sham mice (Fig. 6A, $p < 0.01$). CMC2.24 treated SP-B-C and SP-B-T mice showed decreased levels of Cap-3 expression compared to their respective control mice (Fig. 6A, $p < 0.01$). We further examined another biomarker of apoptosis, Bcl-2 as an inhibitor of apoptosis. The expression of Bcl-2 decreased in infected SP-B-C and SP-B-T mice compared to Sham mice (Fig. 6B, $p < 0.01$). CMC2.24 treatment caused increased levels of Bcl-2 expression in the lung tissues from infected SP-B-C and SP-B-T mice compared to SP-B-C ($p < 0.01$) and SP-B-T ($p < 0.05$) controls, respectively (Fig. 6B).

Inflammatory cells in BALF

We assessed number of inflammatory cells in the BALF from three experimental groups: Pneu, Pneu + CMC, as well as Sham mice. As shown in the Fig. 7A, the BALF from Sham mice consisted of 98% alveolar macrophages without neutrophils. On the other hand, a larger increase in inflammatory cells (neutrophils and macrophages/monocytes) was observed in the BALF of Pneu mice, along with decreased neutrophils and macrophages in the BALF of Pneu + CMC treated mice. Quantitative analysis showed the number of neutrophils in the BALF of infected SP-B-C and SP-B-T mice with or without CMC2.24

was larger than that of Sham mice (Fig. 7B, $p<0.01$). The number of neutrophils decreased significantly in the BALF of both SP-B-C ($p<0.01$) and SP-B-T ($p<0.05$) after CMC2.24 treatment compared with their respective untreated groups (Fig. 7B). Similar results were observed for macrophages/monocytes in the BALF from infected SP-B-C and SP-B-T mice (Fig. 7C).

Lung NF- κ B activation

Previous studies have shown that one of two SP-B gene products is involved in host defense and curcumins can regulate host inflammation induced by sepsis through attenuating NF- κ B activation (12,13). We therefore examined the levels of NF- κ B p65 and phosphorylated-I κ B- α (p-I κ B- α) in the lung using Western blotting analysis with antibodies against NF- κ B p65 and p-I κ B- α . The results showed increased levels of NF- κ B p65 and p-I κ B- α in the lung of infected groups (Pneu and Pneu + CMC) compared to Sham mice (Fig. 8, $p<0.01$). Differences in the levels of NF- κ B p65 and p-I κ B- α expression were observed between infected SP-B-C and SP-B-T mice (Fig. 8, $p<0.05$). The levels of NF- κ B p65 and p-I κ B- α in the lungs of infected SP-B-C mice were higher than those observed in CMC2.24 treated mice (Fig. 8, $p<0.01$). The levels of NF- κ B of p-I κ B- α in infected P-B-C were significantly higher than that of infected SP-B-T mice ($p<0.05$).

SP-B levels in BALF

We determined the levels of SP-B protein in the BALF from SP-B-C and SP-B-T mice at 48 h in the Pneu, Pneu + CMC, as well as Sham mice. The level of SP-B protein in BAL fluids from sham mice was higher than those observed in infected mice (Fig. 9A, $p<0.01$). SP-B levels in BALF from CMC2.24 treated SP-B-C and SP-B-T mice were higher than their respective untreated groups (Fig. 9B, $p<0.05$).

MMPs activity in BALF

Previous studies have shown CMC2.24 can inhibit MMP activity (14). Therefore, we examined MMP-2, -9, and -12 activities in the BALF using zymographic analysis. The results demonstrate that the BALF from sham mice has minimal activity of MMP-2, -9, and -12; but infected SP-B-C and SP-B-T mice exhibit increased MMP-2, -9, and -12 activities (Fig. 10, $p<0.01$). CMC2.24 treated SP-B-C and SP-B-T mice had decreased MMP-2, -9, and -12 activities compared to their respective untreated groups (Figs. 10B–D, $p<0.05$).

Furthermore, to determine whether the effects of CMC2.24 and the differences between the two SP-B genetic variants results from an effect on bacterial infection or altered inflammation itself, a non-infectious lung injury model was employed by means of intranasal treatment of 50 μ l of hydrochloric acid (HCl, 0.1 N) per mouse, with and without CMC2.24 in hTG SP-B-C and SP-B-T mice. The results showed that the scores of lung injury decreased in the CMC2.24-treated mice compared to vehicle-treated mice 6 h after HCl treatment (CMC2.24 treated vs. vehicle treated, SP-B-C: 0.22 ± 0.03 vs. 0.38 ± 0.03 , $p<0.05$, $n=10$; SP-B-T: 0.15 ± 0.02 vs. 0.31 ± 0.03 , respectively, $p<0.05$ $n=10$). The injury scores of CMC2.24 treated and vehicle treated SP-B-T mice were lower than that of CMC2.24 treated and vehicle treated SP-B-C mice ($p<0.05$), respectively. Analysis of inflammatory cells in the BALF indicated decreased inflammatory cells in the CMC2.24-

treated mice compared to vehicle-treated mice 6 h after HCl treatment (CMC2.24 treated vs. vehicle treated, SP-B-C: $1.8 \pm 0.4 \times 10^7$ vs. $5.2 \pm 0.6 \times 10^7$, $p < 0.05$, $n = 10$; SP-B-T: $1.1 \pm 0.3 \times 10^7$ vs. $4.1 \pm 0.5 \times 10^7$, respectively, $p < 0.05$, $n = 10$). The number of inflammatory cells in BALF of CMC2.24 treated and vehicle treated SP-B-T mice were lower than that of CMC2.24 treated and vehicle treated SP-B-C mice ($p < 0.05$), respectively. These results suggest that CMC2.24 attenuates lung injury in a non-infectious model and SP-B-C mice are more susceptible to non-infectious injury than SP-B-T mice.

Discussion

Pneumonia is the leading cause of infectious morbidity and mortality in the United States. It is a major cause of ALI and ARDS which have very high associated mortality (40–60%). It is currently unclear why some individuals are more susceptible to bacterial pneumonia compared to others, however genetic variations of SP-B with subsequent loss of surfactant activity appear to be critical in ARDS progression (21–23) and may explain clinically observed differences in morbidity and mortality in patients with pneumonia-induced ARDS. In the present study, we investigated the functional differences of hTG SP-B-C and SP-B-T mice in response to *S. aureus* infection with or without CMC 2.24 treatment. We found significantly different resistance profiles of hTG SP-B-C and SP-B-T mice to bacteria using our *in vivo* imaging method, as well as differing lung injury patterns as evidenced by histopathology, cell, and molecular analyses. We also found that CMC2.24 attenuates lung injury after bacterial infection by diminishing lung inflammation, apoptosis, and MMP activation.

SP-B, a key component of pulmonary surfactant, is essential for normal lung function (24). In fact, an acute reduction in SP-B by 75–80% causes lethal respiratory failure in animals (25). Likewise, SP-B levels are decreased by up to 60% in patients with acute lung injury and ARDS due to enhanced SP-B turnover and degradation, contributing to their prolonged respiratory failure (22). The SP-B gene expresses two protein products, SP-B^M and SP-B^N, which are involved in lowering surface tension and innate host defense respectively (5). Although a number of hSP-B polymorphisms and mutations have been identified, the SNP rs1130866 i.e. SP-BC/T1580 is functionally one of the most important (26,27). This SP-BC/T1580 polymorphism is not only associated with pneumonia and pneumonia-induced ARDS (21,28,29), but also with neonatal respiratory distress syndrome (RDS) (30), and interstitial lung disease (ILD) (31). The detailed mechanisms for the increased susceptibility of SP-B C allele to these pulmonary diseases are unknown (6,27,32). The results of this study indicate SP-B-C mice are more susceptible to bacterial infection with increased lung injury severity and lung inflammation compared with SP-B-T mice. Because the only difference between SP-B-C and SP-B-T mice is the SP-B gene, the products of SP-B C and T alleles cause the differential response to bacterial pneumonia in these two mouse lines. We also observed the SP-B level in the BAL fluid of infected SP-B-C mice decreased more than that of infected SP-B-T mice despite similar SP-B levels between normal SP-B-C and SP-B-T mice, suggesting that the key difference is in SP-B processing and/or degradation during *S. aureus* pneumonia.

Our results demonstrate that CMC2.24 has a protective effect on lung injury in this model of bacterial pneumonia, including better survival rate from 28h to 48h after infection in CMC-treated mice. Previous studies demonstrate that curcumins are involved in the modulation of inflammatory signaling pathways and mediators, including reduction in NF- κ B activation and lipid derived inflammatory mediators, inhibition of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (33), and increased expression of histone deacetylase (HDAC) (34). The protective mechanisms of CMC2.24 demonstrated in the current study are its ability to reduce inflammatory cell infiltration at the site of lung infection and to prevent lung tissue apoptosis, as well as decreased activity of the pro-inflammatory signaling pathways NF- κ B p65 and p-I κ B in lung tissue after CMC2.24 treatment. These results are consistent with the previous observations regarding curcumin's effects in the regulation of inflammation (12,13,16,33).

The data from our non-infectious model demonstrated that hTG SP-B-C mice were more susceptible to non-infectious injury compared to SP-B-T mice, and CMC2.24 decreased lung injury and inflammatory cell infiltration in the lung of non-infectious model. By comparisons of the data from bacterial infection and non-infectious models in this study, one possible mechanism of CMC2.24 effects may alter the host inflammatory response, but do not for host-bacterial interaction.

MMPs, a group of complex zinc-containing neutral proteolytic enzymes, are essential for the degradation and turnover of the extracellular matrix (ECM). In particular, inducible MMPs are activated by pulmonary infection, accelerating connective tissue degradation and exacerbating lung (35). MMP-2 is secreted from neutrophils and macrophages as a 72-kDa pro-form that is cleaved into a 64-kDa active form; the corresponding pro- and active forms of MMP-9 have masses of 92 kDa and 83 kDa, respectively. In the present study, the activity of MMP-2, -9, and -12 was induced in the BALF of infected mice and attenuated by CMC2.24 treatment. Collectively, these results indicate CMC2.24 may have therapeutic potential in bacterial pneumonia.

In summary, functional differences of human SP-B genetic variants, i.e. the SP-B C and T alleles were found in the bacterial pneumonia, SP-B-C mice showed increased susceptibility to *S. aureus* infection compared to SP-B-T mice. Differing dynamic loads of bacteria between SP-B-C and SP-B-T mice were also observed by *in vivo* imaging method. CMC2.24 improves mortality and attenuates lung injury in this model of *S. aureus* pneumonia. But the limitation of the study is to focus on the comparison of two human SP-B genetic variants without the comparison to wild type animals, which will warrant to be studied in the future.

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Fig. 1. Expression of human SP-B protein in the lung of hTG SP-B-C and SP-B-T mice
BALF Samples (n=5 for each of SP-B-C and SP-B-T mice) were obtained from Sham hTG SP-B-C and SP-B-T mice. Total proteins in the BALF samples were determined using the BCA micro-assay kit. Five micrograms of each BALF sample were used for Western blotting analysis as described in the method. Panel A shows one representative blot of human SP-B expression and the quantitative results. The results indicate that the SP-B levels in the BALF from SP-B-C and SP-B-T mice are similar compared to human BALF. Panels B and C display human SP-B expression in the lung of SP-B-C and SP-B-T mice (n=5 for each type of mice) determined by immunohistochemical analysis. The results indicate alveolar epithelial type II cells (arrows) with positive SP-B staining in the lung of SP-B-C and SP-B-T mice. Panel D shows representative gel images from genomic DNA genotyping analysis of hTG SP-B-C and SP-B-T mice and controls (+: Positive control, -: Negative control), indicating only the presence of human SP-B (hSP-B) gene but absence of mouse SP-B (mSP-B) gene in hTG SP-B-C and SP-B-T mice. Panel E is representative images of SP-B mRNA expression examined by RT-PCR of hTG SP-B-C and SP-B-T mice and controls (+: Positive control, -: RT-minus control (last second lane) and Negative PCR control (last lane)). The results indicate no mouse SP-B mRNA expression but only human SP-B mRNA expression in the lung of hTG SP-B-C and SP-B-T mice.

Fig. 2. The levels of bioluminescence in infected SP-B-C and SP-B-T mice

The levels of bioluminescent signal, which represents bacterial number in the lung of infected mice, were measured at several time points from 0 to 48 hours by *in vivo* imaging system. The results show that infected SP-B-C mice exhibit higher level of bioluminescence than infected SP-B-T mice from 24h to 48h after infection. (A) The representative image of bioluminescence at each time point in infected SP-B-C and SP-B-T mice; (B) Comparisons of the bioluminescent level at each time point between infected SP-B-C and SP-B-T mice; (C) The survival curves of infected SP-B-C and SP-B-T mice. * $p < 0.05$, ** $p < 0.01$ (n= 15 mice/group)

Fig. 3. The levels of bioluminescence in the mice with pneumonia vs. pneumonia with CMC2.24 treatment

Infected SP-B-C and SP-B-T mice were administered a daily dose of CMC2.24 (50 mg/kg) or vehicle by gavage. The levels of bioluminescence were measured for 48 h after infection by an *in vivo* image system. The levels in the CMC2.24-treated group (Pneu + CMC) were lower compared to the control group (Pneu) for both infected SP-B-C and SP-B-T mice starting at 24h and 28h after infection, respectively. (A) The representative image of bioluminescence at 28h in infected SP-B-C and SP-B-T mice. Comparisons of the bioluminescent level at each time point in infected SP-B-C (B) and SP-B-T (C) mice with and without CMC2.24 treatment; (D) The survival curves of infected SP-B-C mice with and without CMC2.24 treatment. * $p < 0.05$, ** $p < 0.01$ (n=15 mice/group)

Fig. 4. Histology of the lung in infected SP-B-C and SP-B-T mice with and without CMC2.24 treatment

The lung tissue histopathology was analyzed in three groups, i.e. Sham, pneumonia (Pneu), and pneumonia plus CMC2.24 treatment (pneu+CMC) of SP-B-C and SP-B-T mice.

Histological sections from three groups were stained with H/E (A) and the histopathological score of lung injury was assessed (B). Lung histology shows inflammatory cells in alveoli and interstitial membrane, proteinaceous debris, and wider alveolar wall in the lung tissues of infected mice but not in Sham mice. Compared to infected SP-B-T mice with or without CMC2.24, the lung injury score is higher in infected SP-B-C mice with or without CMC2.24, respectively. The score of lung injury is lower in the CMC2.24-treated SP-B-C and SP-B-T mice compared to pneumonia SP-B-C and SP-B-T mice, respectively. Naïve

control = Sham, pneumonia = Pneu, pneumonia with CMC2.24 = Pneu+CMC; Bar = 50 μ m;
 Graphs represent the mean $\pm$ SEM. *p<0.05, ** p<0.01 (n=8 mice/group).

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and SP-B-T mice. naïve control = Sham, pneumonia = Pneu, pneumonia plus CMC2.24 treatment = Pneu+CMC; Bar=50µm; Graphs represent the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ (n=8 mice/group).

Fig. 6. The levels of apoptotic and anti-apoptotic biomarkers in the lung of infected SP-B-C and SP-B-T mice

The levels of apoptosis (Caspase-3) (Panel A) and anti-apoptosis (Bcl-2) (Panel B) biomarkers in the lung tissues were analyzed by Western blot analysis, and quantified by densitometry. The data were normalized by the level of β-actin. The level of caspase-3 increased significantly in the infected mice (Pneu, Pneu + CMC) compared to Sham ($p<0.01$). The levels of caspase-3 in infected SP-B-C mice (Pneu and Pneu + CMC) are higher ($p<0.01$) than those of infected SP-B-T mice (Pneu and Pneu + CMC), respectively. Treatment with CMC2.24 decreased the levels of caspase-3 in both infected SP-B-C and SP-B-T mice. For anti-apoptosis biomarker (Bcl-2), the level of Bcl-2 is lower in the infected mice (Pneu, Pneu + CMC) compared to Sham ($p<0.01$). Treatment with CMC2.24 increased the level of Bcl-2 significantly in both infected SP-B-C and SP-B-T mice. Naïve control = Sham, pneumonia = Pneu, pneumonia plus CMC2.24 treatment = Pneu+CMC; Bar = 50μm; Graphs represent the mean ± SEM. * $p<0.05$, ** $p<0.01$ (n=8 mice/group).

Fig. 7. Inflammatory cells in BALF from infected SP-B-C and SP-B-T mice with and without CMC2.24 treatment

Samples of BALF were prepared from three groups (Sham, Pneu, and Pneu + CMC) of SP-B-C and SP-B-T mice. The cells in each BALF samples were mounted on a slide by the cytopspin centrifuge method. The slides from three groups were stained using the Hema-3 Stain Kit (A). By means of light microscopy, neutrophils (PMN) and macrophages/monocytes per slide were analyzed and quantified at $\times 400$ magnification. The number of neutrophils and macrophages/monocytes were compared among Sham, Pneu, Pneu + CMC groups. In the BALF of the Sham group more than 98% of cells are macrophages, but there are no neutrophils. The number of neutrophils increased significantly in the BALF from infected SP-B-C and SP-B-T mice compared to Sham mice. The numbers of neutrophils and macrophages are larger in the BALF from infected SP-B-C mice compared to infected SP-B-T mice. On treatment with CMC2.24, the number of neutrophils and macrophages in the BALF from both SP-B-C and SP-B-T decreased significantly. Naïve control = Sham, pneumonia = Pneu, pneumonia plus CMC2.24 treatment = Pneu+CMC; Bar = 50μm; Graphs represent the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ ($n = 8$ mice/group).

Fig. 8. Expression of NF-κB p65/p-IκB in the lung of infected SP-B-C and SP-B-T mice
 The levels of inflammatory NF-κB p65 protein in the lung tissues of infected mice and Sham were examined by Western blotting analysis and then quantified by densitometry. The data were normalized by the levels of β-actin. Panels A and B show the Western blotting and the quantitative results, respectively. Compared to the Sham group, infected SP-B-C and SP-B-T mice (Pneu and Pneu + CMC) have higher levels of NF-κB p65 expression. CMC2.24 treatment decreased significantly the levels of NF-κB p65 expression in the lung tissues of infected mice. Naïve control = Sham, pneumonia = Pneu, pneumonia plus CMC2.24 treatment = Pneu+CMC; Graphs represent the mean ± SEM. * $p < 0.05$, ** $p < 0.01$ ($n = 8$ mice/group).

Fig. 9. The levels of secreted SP-B in the BALF of infected SP-B-C and SP-B-T mice
 Samples of BALF were obtained from three mouse groups (Sham, Pneu, and Pneu + CMC) of SP-B-C and SP-B-T mice. The levels of total protein in the BALF of the three groups were determined using the BCA micro-assay kit. Five micrograms of each BALF sample were used for analysis of SP-B by Western blotting analysis as described in the method. Panels A and B show the Western blotting and the quantitative results, respectively. The results showed that the levels of SP-B in the BALF from infected mice (Pneu and Pneu + CMC) decreased significantly compared to Sham mice. The order of the levels of SP-B in the BAL is as Sham > Pneu + CMC > Pneu. Naïve control = Sham, pneumonia = Pneu, pneumonia plus CMC2.24 treatment = Pneu+CMC; Graphs represent the mean $\pm$ SEM. * p <0.05, ** p <0.01 (n=8 mice/group).

Fig. 10. MMPs activity in the BALF of infected SP-B-C and SP-B-T mice

The activities of MMP-2, -9, -12 were examined in the BALF of three groups (Sham, Pneu, and Pneu + CMC) of SP-B-C and SP-B-T mice by gel zymography as described in the method. Panels A and B show the zymographic gels and quantitative results of MMP-2, -9, -12 activities, respectively. No detectable levels of MMP-2, -9, -12 activities were observed in Sham mice. Significant activities of MMP-2, -9, -12 were determined in the BALF of infected mice in which infected SP-B-C mice displayed higher than infected SP-B-T mice. On treatment with CMC2.24, the activity levels of MMP-2, -9, -12 decreased significantly ($p<0.05$) in both infected SP-B-C and SP-B-T mice. Naïve control = Sham, pneumonia = Pneu, pneumonia plus CMC2.24 treatment = Pneu+CMC; Graphs represent the mean $\pm$ SEM. * $p<0.05$, ** $p<0.01$ ($n=8$ mice/group).