

# Innate immunity of surfactant protein A in experimental otitis media

Osama Abdel-Razek<sup>1</sup> , Lan Ni<sup>1</sup>, Fengyong Yang<sup>1</sup> and Guirong Wang<sup>1,2</sup>

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## Abstract

Surfactant protein A (SP-A) plays an important role in innate immune response and host defense against various microorganisms through opsonization and complement activation. To investigate the role of SP-A in non-typeable *Haemophilus influenzae* (NTHi)-induced acute otitis media, this study used wild type C57BL/6 (WT) and SP-A knockout (KO) mice. We divided mice into an infection group in which the middle ear (ME) was injected with NTHi and a control group that received the same treatment using normal saline. Mice were sacrificed on d 1, 3, and 7 after treatment. Temporal bone samples were fixed for histological, cellular, and molecular analyses. Ear washing fluid (EWF) was collected for culture and analyses of pro-inflammatory cytokines and inflammatory cells. SP-A-mediated bacterial aggregation and killing and phagocytosis by macrophages were studied *in vitro*. SP-A expression was detected in the ME and Eustachian tube mucosa of WT mice but not KO mice. After infection, KO mice showed more severe inflammation evidenced by increased ME mucosal thickness and inflammatory cell infiltration and higher NF- $\kappa$ B activation compared to WT mice. The levels of IL-6 and IL-1 $\beta$  in the EWF of infected KO mice were higher compared to infected WT mice on d 1. Our studies demonstrated that SP-A mediated NTHi aggregation and killing and enhanced bacterial phagocytosis by macrophages *in vitro* and modulated inflammation of the ME in otitis media *in vivo*.

## Keywords

*Haemophilus influenzae*, innate immunity, NF- $\kappa$ B signaling, otitis media, SP-A

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## Introduction

Otitis media (OM) represents an inflammation of the middle ear (ME) cleft mucosa, which includes the mastoid process, tympanic cavity, and the Eustachian tube (ET).<sup>1</sup> OM is the most common disease in early childhood and one of the most frequent reasons to visit the pediatrician.<sup>2,3</sup> It is estimated that during the first year of life, 60–80% of children will have at least one attack of OM.<sup>4</sup> There is a significant global health burden of OM; about 31 million children with 709 million new cases per year are going to develop chronic suppurative OM, resulting in about 21,000 deaths every year from complications such as meningitis.<sup>5</sup> The majority of antibiotic prescription is due to OM. It is also the primary indication for ventilation tube insertion, which is the most commonly performed surgery on children.<sup>6</sup> Viral and bacterial pathogens can cause acute OM (AOM). The leading bacterial pathogens are *Streptococcus pneumoniae*, non-typeable *Haemophilus*

*influenzae* (NTHi), and *Moraxella catarrhalis*. Viral infection is responsible for about 20% of AOM.<sup>7,8</sup>

Innate immunity plays a major role for OM susceptibility in early life before the development of specific adaptive immunity.<sup>4</sup> The effectors of innate immunity include host defense proteins, antimicrobial peptides, and cytokines and chemokines that attract phagocytes to the affected site and enhance their phagocytic and microbicidal capacity.<sup>9</sup> Surfactant protein A (SP-A) is one of the innate immunity molecules, belonging to the

<sup>1</sup>Department of Surgery, SUNY Upstate Medical University, USA

<sup>2</sup>Department of Microbiology and Immunology, SUNY Upstate Medical University, USA

### Corresponding author:

Guirong Wang, Department of Surgery, UH Room 8715, SUNY Upstate Medical University, 750 E Adams St, Syracuse, NY 13210, USA.  
Email: wangg@upstate.edu



family of collagenous C-type lectins. Many types of epithelial cells, including alveolar epithelial type II cells, express and secrete SP-A proteins on the mucous surface.<sup>10</sup> SP-A is expressed not only in the lung but also in other extrapulmonary organs, including the tongue, oral epithelium, and in the ME and ET.<sup>11,12</sup>

SP-A is a hydrophilic and multimeric protein, which contributes to the innate immune response.<sup>13</sup> In the absence of specific antimicrobial Abs, SP-A functions in the first-line defense as a pattern recognition receptor (PRR); it can bind to various microorganisms and increases the phagocytosis of *S. pneumoniae* and *H. influenzae*, the most common pathogens in OM.<sup>4,12,14</sup> SP-A also plays an important role in innate immune responses through opsonization and complement activation.<sup>15–17</sup> The agglutination of target pathogens provides a first line of defense that can then be enhanced by killing and clearance mediated by phagocytic cells that carry receptors for SP-A.<sup>18</sup> SP-A opsonizes Gram-negative bacteria and modifies the conformation of LPS for macrophage binding.<sup>19</sup> SP-A also modulates the expression of the pro-inflammatory cytokines IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , which play an important role in fighting infections. A previous study demonstrated that SP-A is expressed in the normal human and porcine ET.<sup>13</sup> Although the role of SP-A in the defense of the tubotympanum remains to be proven, it is likely that a deficiency of SP-A expression may contribute to the pathogenesis of OM.<sup>20</sup>

A genome-wide linkage analysis found the susceptibility loci of OM within the 17q12 and 10q22.3 regions.<sup>21</sup> The human SP-A locus in chromosome 10q22–q23 consists of two functional genes: *sftpal* (SP-A1) and *sftpa2* (SP-A2). Both gene products are required for fully functional SP-A. Genotyping analysis of OM patients and healthy controls revealed that SP-A haplotype and genotypes were associated with OM.<sup>12</sup> However, it is unclear how SP-A functions in the pathogenesis of OM.

In this study, we confirmed SP-A expression in mouse ME and ET mucosa, and found that SP-A plays an important role in the innate immune response in the pathogenesis of OM through modulating inflammation and cytokine expression, enhancing bacterial clearance and regulating NF- $\kappa$ B signaling activation *in vivo* and *in vitro*.

## Materials and Methods

### Mice

Twenty-five male and female (8–10 wk old) wild type (WT) C57BL/6 mice and 70 SP-A knockout (KO) mice with C57BL/6 background were used for this study. KO mice were bred in the animal core facility at

SUNY Upstate Medical University and were maintained under pathogen-free conditions. WT mice were originally purchased from Jackson Laboratories (Bar Harbor, ME). We bred the WT mice in the same animal facility, and the offspring were used in our experiments. All animal experiments and protocols were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee, SUNY Upstate Medical University, those of the National Institutes of Health guidelines on the use of laboratory animals, and “ARRIVE” on the use of laboratory animals. All mice survived throughout the experiments.

### Bacterial strain and culture

Clinical isolate of NTHi 3655 strain was kindly provided by Dr. Allen F. Ryan (University of California, San Diego, CA) and was used for this study. Bacterial culture and preparation of diluted bacterial solution were performed as described previously.<sup>22,23</sup>

### Animal surgery and OM model

The mice were anesthetized by i.p. injection with a combination of ketamine (90 mg/kg) and xylazine (10 mg/kg; i.e., 0.1 ml/100 g animal mass). Otoscopic examination was performed for all mice before treatment to ensure that tympanic membranes were normal and that no ME effusion was found. A 30 G needle was used to inject the bacterial solution into the anterior part of the mouse tympanic membrane. The experimental group received 5  $\mu$ l of the bacterial solution ( $3.5 \times 10^5$  CFU/ear), and this was replaced with 5  $\mu$ l normal saline in the control group. Mice were sacrificed 1, 3, and 7 d after injection of the bacteria or saline. Tissues were fixed or MEs were washed with 200  $\mu$ l normal saline. Ear washing fluid (EWF) from the ME was used for cell counting and cytokine and CFU analysis.

### Histopathological analysis

Temporal bones were removed immediately after animal sacrifice and fixed in 4% paraformaldehyde for 48 h followed by de-calcification in Cal-Ex solution for 6 h and then 10% formaldehyde. After dehydration, specimens were embedded in paraffin, sectioned at a thickness of 4 mm, and stained with hematoxylin and eosin (H&E) for histological examinations, as described previously.<sup>24,25</sup>

### Inflammatory cell analysis

The numbers of neutrophils and macrophages comprising ME cellular infiltrates were assessed by manually counting cell types in five randomly selected clusters of

cellular ME effusions for each ME in a 400 $\times$  high-power field. The numbers were then averaged for statistical analysis.

### Bacterial phagocytosis by macrophages

Bacterial phagocytosis by alveolar macrophages was performed, as described previously.<sup>26,27</sup> The phagocytic index (PI) was calculated as the percent of bacteria-positive macrophages (cells that phagocytized at least one bacterium) multiplied by the average number of bacteria per bacteria-positive macrophage.<sup>26</sup>

### SP-A-mediated bacterial aggregation and killing

SP-A-mediated bacterial NTHi aggregation and killing were performed, as published earlier.<sup>9,28</sup> SP-A protein at concentrations of 0, 10, and 20  $\mu$ g/ml was added into bacteria solution of NTHi in the presence of 2  $\mu$ M of CaCl<sub>2</sub>; the bacteria were incubated for 1 h at 37°C, and then bacterial aggregation was examined under the microscope. For bacterial killing, bacterial solution was monitored for 5 h in the presence of SP-A or BSA, and then CFUs of the bacterial solution were determined by using agar plate culture at 37°C for 16 h.

### Immunofluorescence analysis

Immunofluorescence analysis was used to examine SP-A expression and the NF- $\kappa$ B p65 signaling activation with mouse SP-A Ab and phosphorylated NF- $\kappa$ B (p65) Ab, respectively, in the ME and ET mucosa, as described previously.<sup>29</sup>

### ELISA assay

Concentrations of IL-1 $\beta$  and IL-6 in EWF were measured by ELISA kits (Thermo Fisher Scientific, Pittsburgh, PA), as in our previous work.<sup>30</sup>

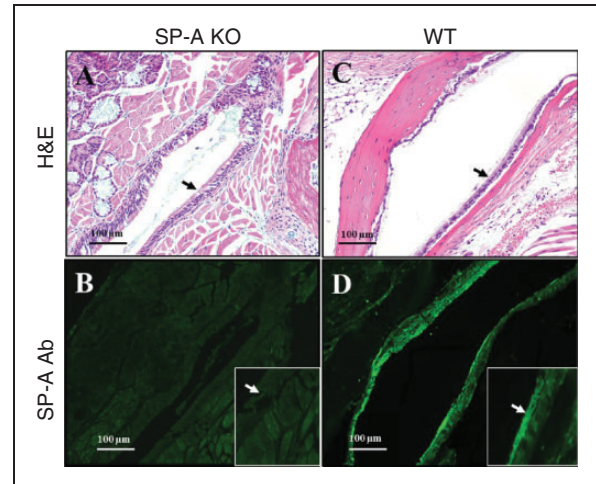
### Statistical analysis

Data were expressed as means $\pm$ SEM, and statistical analyses were performed using SigmaStat v3.5 (Jandel Scientific, San Rafael, CA). Student's *t*-test or ANOVA was performed to assess the statistical significance of differences. A *P*-value of <0.05 was considered statistically significant.

## Results

### SP-A expression in the ET and ME mucosa of WT mice

We performed H&E histological and immunofluorescence analysis with SP-A Ab to examine the expression of SP-A protein in the ME and ET mucosa of WT and



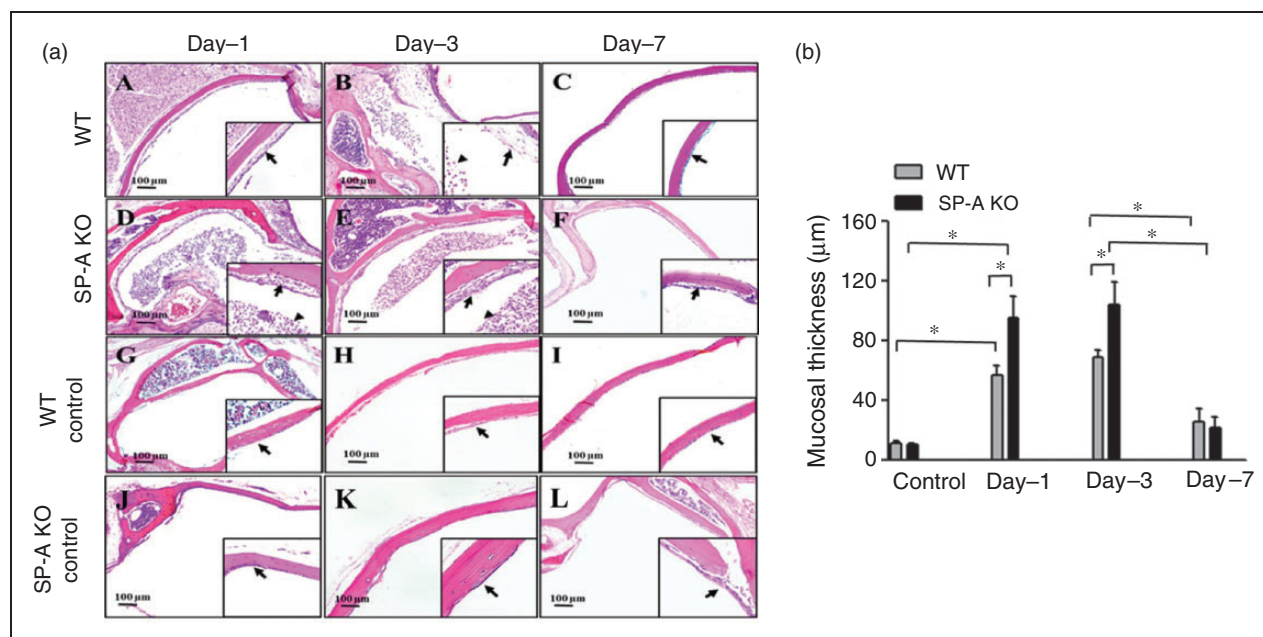
**Figure 1.** Surfactant protein A (SP-A) expression in the Eustachian tube (ET) mucosa of wild type (WT) and SP-A knockout (KO) mice by immunofluorescence assay. (A) Light microscopic histology of SP-A KO mouse ET. (B) Immunofluorescence assay of the same mouse. No SP-A was detected in the ET mucosa. (C) Light microscopic histology of WT mouse ET. (D) Immunofluorescent staining of the same mouse. Green represents SP-A expression. Black arrows point to respiratory type mucosa of the ET; white arrows point to cells expressing SP-A in (D) and absence of expression in (B). *n*=3 mice per each group.

SP-A KO mice. SP-A expression was detected in the ME and ET mucosa of WT mice (Figure 1D), as expected, but not in SP-A KO mice (Figure 1B).

### Histopathological comparison of the ME between WT and SP-A KO mice after infection

The degree of inflammation in the ME was quantified by determination of the mucosal thickness and the percentage of the ME cavity covered by inflammatory cells. The ME mucosa developed characteristic inflammatory changes during bacterial NTHi infection. There was substantial swelling of the epithelial and stromal layers of the mucosa, and a considerable inflammatory exudate in the ME cavity. In WT mice, these changes were most prominent 1–3 d after NTHi inoculation (Figure 2A and B). The ME cavity resembled its baseline appearance by 7 d after infection (Figure 2C). The MEs of SP-A KO mice also showed mucosal thickening and an infiltrate of inflammatory cells on d 1–3 (Figure 2D and E). Further analysis indicated that the inflammatory reaction in SP-A KO mice was much stronger, as we observed significantly increased mucosal thickness (Figure 2b; *P* < 0.05) and cellular recruitment (Figure 2D and E) compared to WT mice. No inflammatory changes were observed in the WT and SP-A KO control (saline) group at any time point (Figure 2J–L).





**Figure 2.** Inflammatory changes in the middle ear (ME) of SP-A KO and WT mice over different time points in panel (a). ME response to non-typeable *Haemophilus influenzae* (NTHi) in WT C57BL/6 mice (A–C) and SP-A KO mice (D–F), as well as WT controls (G–I) and SP-A KO controls (J–L). The ME of WT mice demonstrated inflammation of the ME mucosa and a few cellular infiltrates in the ME cavity on d 1 (A). By d 3 after infection with NTHi, the ME cavities of WT mice were filled with inflammatory cells (B). By d 7, the ME mucosa looks normal (C). In SP-A KO mice, by d 1 after NTHi infection, SP-A KO mice had much more inflammatory cells evident in the ME cavity (D). By d 3 after infection, the MEs of SP-A KO mice showed a larger increase in inflammatory cell infiltrate and a significantly increased mucosal thickness (E) compared to infected WT mice ( $P < 0.05$ ). Black arrows indicate mucosal thickening; arrowheads point to cellular infiltration. WT controls (G–I) and SP-A KO controls (J–L) showed normal histology without inflammation. Statistical analysis of mucosal thickness was shown in panel (b). Original magnifications, 100 $\times$ .  $n = 5$  mice per time point in each group.

### Neutrophils and macrophages in the ME of WT and SP-A KO mice after infection

A large number of neutrophils and macrophages were recruited into the ME of WT and SP-A KO mice at 1 and 3 d after NTHi infection (Figure 3). The number of neutrophils peaked on d 1 and then declined over d 2 and 3 after NTHi inoculation and was almost cleared by d 7. SP-A KO mice showed more neutrophils than WT mice on d 1 (Figure 3E and G). The number of macrophages in the ME increased significantly, with a peak on d 3 after infection. SP-A KO mice showed more macrophages than WT mice on d 3 (Figure 3F and H). On d 7, only a few inflammatory cells (predominantly macrophages) were observed in the model.

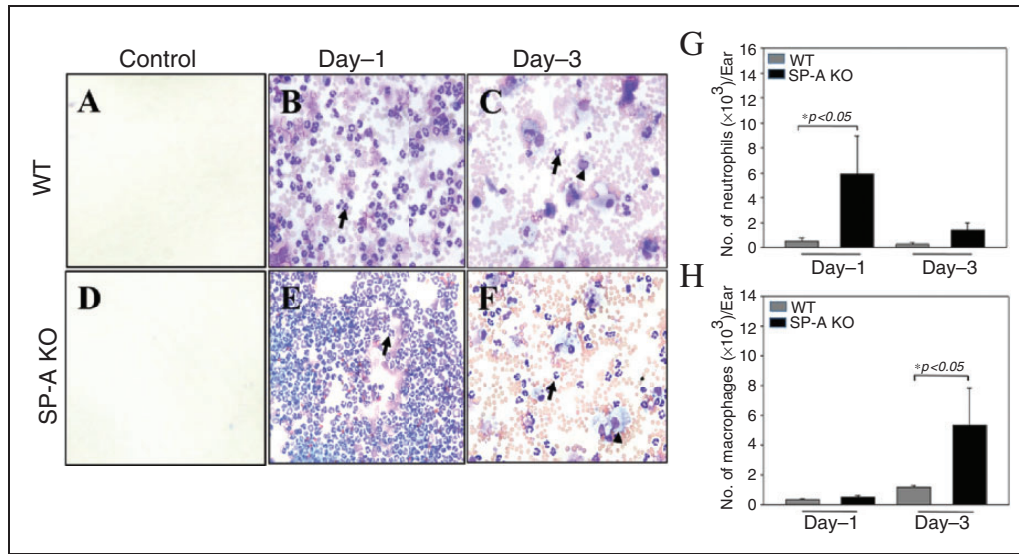
### Pro-inflammatory cytokine analysis and bacterial CFUs in the ME of WT and SP-A KO mice after infection

The levels of pro-inflammatory cytokines IL-1 $\beta$  and IL-6 in MEF were measured by ELISA. The results showed increased IL-1 $\beta$  and IL-6 expression in the

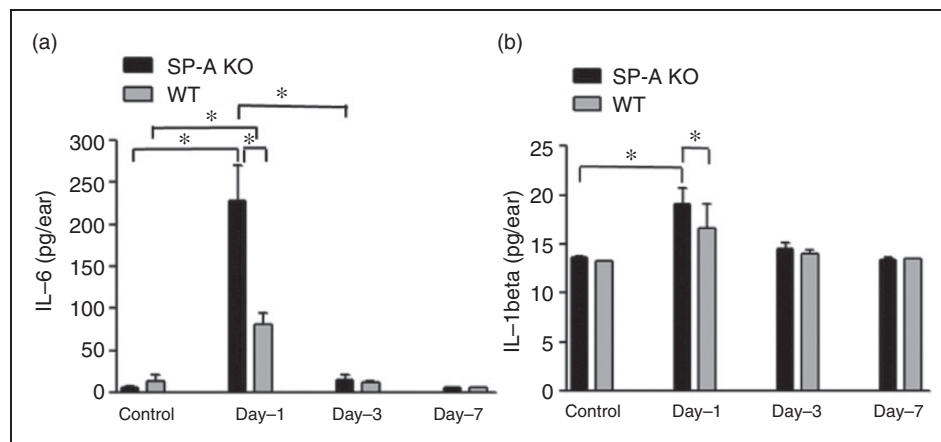
ME after NTHi infection (Figure 4). The level of IL-6 in infected mice increased significantly on d 1 compared to controls. SPA-KO mice showed a significantly higher IL-6 level compared to WT mice on d 1 after NTHi infection (Figure 4a). Similarly, increased IL-1 $\beta$  expression was observed in the ME of infected mice, and SPA-KO mice showed significantly higher IL-1 $\beta$  expression compared to WT mice on d 1 after NTHi infection (Figure 4b). Furthermore, we analyzed the CFU counts in the MEF on d 1, 3, and 7 after infection. We observed that there were remarkable CFU ( $10^6$ – $10^7$  CFU/ear) on d 1 and 3 but few CFU on d 7. The counts of CFU in SP-A KO mice were higher than those in WT mice.

### NF- $\kappa$ B signaling activation in the ME of WT and SP-A KO mice after infection

Phosphorylated NF- $\kappa$ B p65 (p-NF- $\kappa$ B p65) as a biomarker of NF- $\kappa$ B signaling activation was examined using immunofluorescence analysis with specific Ab against p-NF- $\kappa$ B p65 (Figure 5). The positive epithelial cells with p-NF- $\kappa$ B p65 Ab and total epithelial cells



**Figure 3.** Inflammatory cells recruitment on d 1 and 3 after infection. Cells composing the ME cell infiltrate after NTHi infection. The ME were washed with 200  $\mu\text{l}$  saline, and cytopsin was used to prepare the slides. Inflammatory cell recruitment was noted on d 1 in WT mice and almost exclusively consisted of neutrophils (arrows) (B). The inflammatory exudate observed on d 3 after NTHi infection showed more macrophages (arrowheads), but cells were almost cleared by d 7. In SP-A KO mice, recruitment of inflammatory cells was more pronounced, with significantly larger numbers of neutrophils on d 1 (E) and more macrophages on d 3. The inflammatory exudate exceeded that of WT mice on d 1 and 3. We further assessed the ME infiltrate by quantifying the participation of neutrophils versus macrophages. In WT mice, neutrophils comprised the majority of ME cellular infiltrates on d 1 and 3 after NTHi inoculation. Macrophages were subsequently recruited and were the primary inflammatory cells at later time points. No neutrophils were observed by d 7. Neutrophils (arrows) (G); macrophages (arrowheads) (H). Original magnifications, 400 $\times$ .



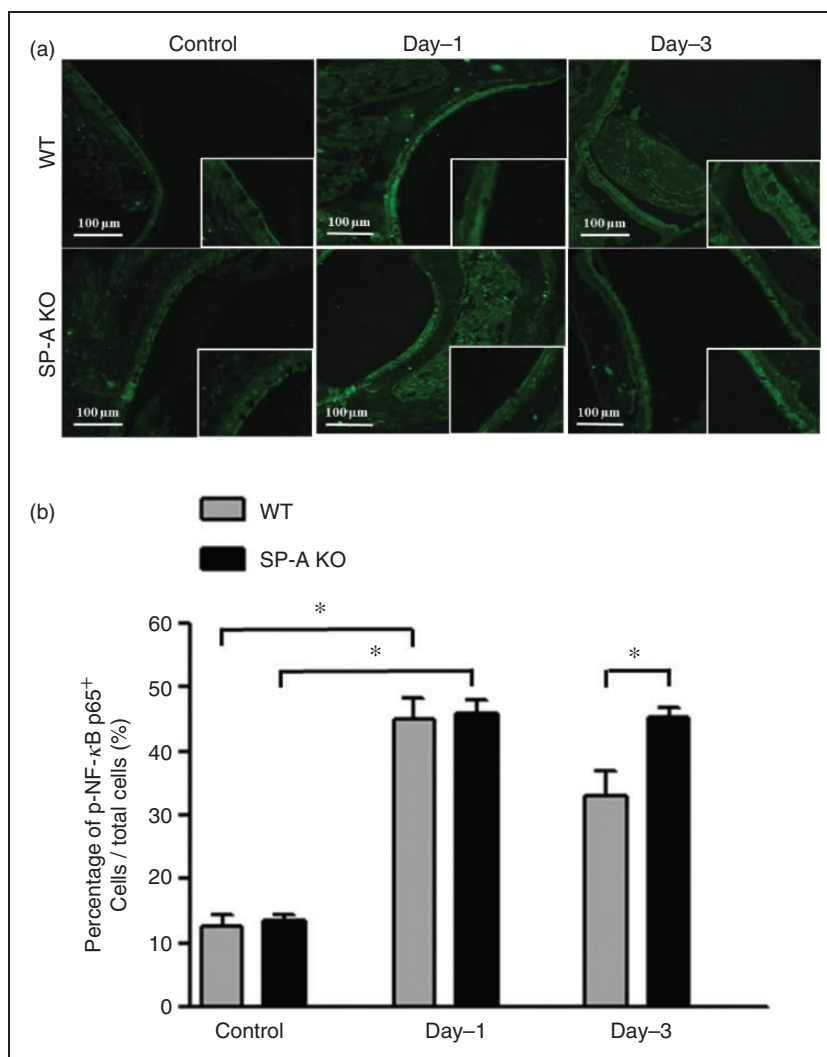
**Figure 4.** Pro-inflammatory cytokines in mouse ear wash. Levels of pro-inflammatory cytokines in ME wash during acute otitis media (OM) and control mice. (a) IL-6 and (b) IL-1 $\beta$  were determined by ELISA. Data are represented as the mean  $\pm$  SEM.  $n=4$  mice per group.  $*P < 0.05$ .

were analyzed at 200 $\times$  using a fluorescence Eclipse TE2000-U microscope (Nikon, Tokyo, Japan). The results showed an increased number of p-NF- $\kappa$ B p650-positive cells in the ME epithelia cells on d 1 and 3 in infected mice compared to controls (Figure 5a and b), suggesting increased NF- $\kappa$ B signaling activation in the ME after NTHi infection. Further analysis indicated that SP-A KO mice had higher levels of

NF- $\kappa$ B signaling activation on d 3 compared to WT mice (Figure 5b).

### SP-A-mediated bacterial aggregation

To investigate the potential mechanism of SP-A innate immunity in AOM, we studied SP-A-mediated bacterial aggregation *in vitro*. As shown in Figure 6, SP-A



**Figure 5.** Phosphorylated NF- $\kappa$ B expression in ME mucosa. Anti-phosphorylated-NF- $\kappa$ B p65 Ab was used for the detection of activated and translocated NF- $\kappa$ B level. Based on the IF analysis there is a significant increase in p-NF- $\kappa$ B p65 in ME mucosa on d 1 compared to controls in both SP-A KO and WT mice. The level of p-NF- $\kappa$ B p65 decreased significantly on d 3 in infected WT mice compared to infected SP-A KO mice.  $n=4$  mice per group. \* $P < 0.05$ .

protein (10 and 20  $\mu$ g/ml) in the presence of 2  $\mu$ M  $\text{Ca}^{2+}$  remarkably induced NTHi bacterial aggregation. We examined both human SP-A and mouse SP-A for SP-A-mediated NTHi bacterial aggregation, and the results indicated that both human and mouse SP-A were able to induce NTHi bacterial aggregation (Figure 6).

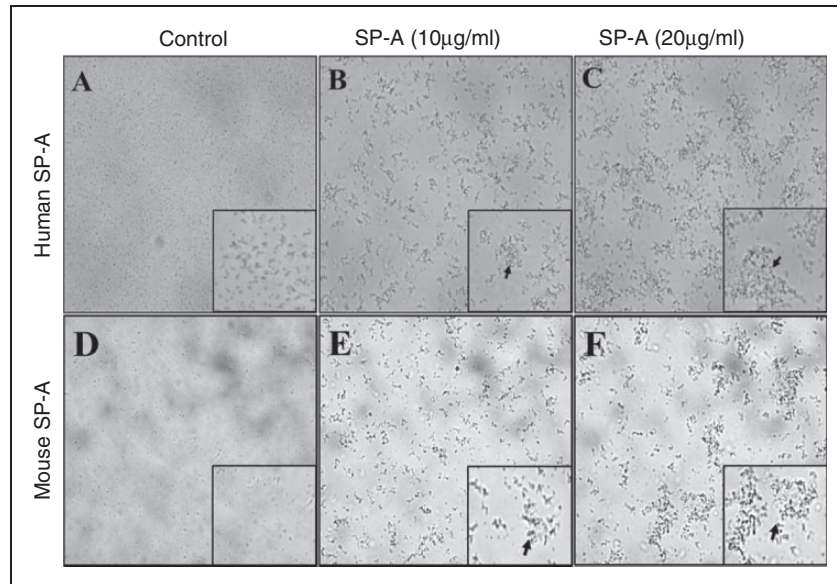
#### SP-A-mediated NTHi killing in vitro

We examined the effect of SP-A on bacterial NTHi killing in normal saline containing 2.5 mM  $\text{Ca}^{2+}$  in the presence or absence of SP-A. The results indicated that in the presence of SP-A (40  $\mu$ g/ml), the bacterial OD value markedly decreased from 2 to 5 h ( $P < 0.05$ ; Figure 7a). To examine the effects of SP-A on bacterial

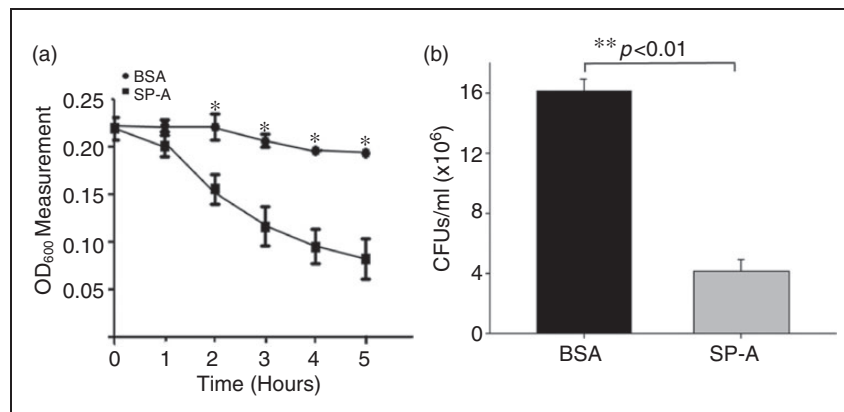
viability further, the CFU of the culture were determined by agar plate culture assay. The results demonstrated that NTHi treated with SP-A (40  $\mu$ g/ml) resulted in significantly lower CFU ( $P < 0.01$ ) compared to the BSA controls (Figure 7b), suggesting that SP-A can be bactericidal under such experimental conditions.

#### SP-A-mediated bacterial phagocytosis by macrophages

Alveolar macrophages were obtained from SP-A KO mice by bronchoalveolar lavage. Bacterial phagocytosis by macrophages in the presence or absence of SP-A was examined in this study. The results indicated that



**Figure 6.** SP-A-mediated NTHi bacterial aggregation. The large bacterial aggregation indicated by arrows in (B) and (C) were observed in the presence of SP-A (10 and 20 µg/ml). The lower panels (D), (E), and (F) showed a similar response to mouse SP-A.



**Figure 7.** Effect of SP-A on the NTHi killing *in vitro*. For time-course experiments, NTHi (strain 3655) bacterial solutions in normal saline were added with 40 µg/ml of SP-A or BSA (control) and then incubated for 5 h. Bacterial concentration was monitored by measuring OD at 600 nm. OD<sub>600</sub> value was used to represent bacterial concentration. CFU of the bacterial solution were determined at the time point at 5 h after incubation. The results showed that bacterial CFU decreased significantly by 40 µg/ml of SP-A (three independent experiments). \*\* $P < 0.001$ .

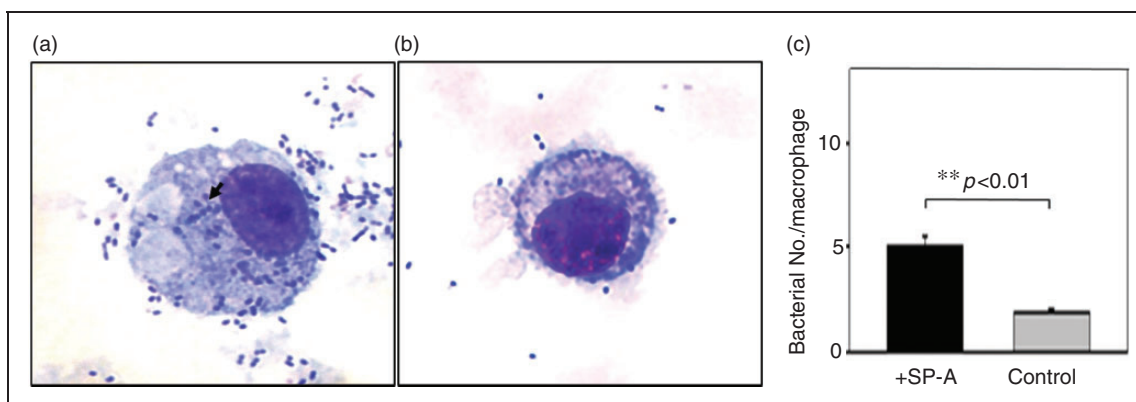
SP-A significantly enhanced bacterial NTHi phagocytosis by alveolar macrophages (Figure 8).

## Discussion

OM is the most common childhood infection, affecting about 80% of children by the age of 3 yr. Although most acute attacks end up in resolution, about 20% of OM sufferers have recurrent episodes. Genetic defects or variants most probably in the innate immune response could be the reason for recurrent attacks of

ear infections. The innate immune system recognizes the presence of microbial infection by using PRRs to identify PAMPs, which represent the molecular signature of pathogens.<sup>31</sup> Because SP-A works as a pattern recognition molecule, it functions as one part of the first lines of defense before the development of specific antimicrobial Abs. SP-A binds to and increases the phagocytosis of *S. pneumoniae* and *H. influenzae*, the most common otopathogens.<sup>4,12</sup> To understand the mechanisms underlying the role of SPA in OM better, SP-A KO and age-matched WT mice were studied in this murine AOM model. Our study reveals that





**Figure 8.** SP-A-mediated enhanced bacterial phagocytosis by macrophages was evaluated on macrophages using an *in vitro* assay in which alveolar macrophages from SP-A KO mice were used. We added SP-A at concentrations of 5  $\mu\text{g/ml}$  or 20  $\mu\text{g/ml}$  to the macrophages, which were then mixed with viable NTHi bacteria in the presence of 2  $\mu\text{M}$  of  $\text{CaCl}_2$ , and the mixtures were incubated for 30 min at room temperature to encourage phagocytosis. Extracellular bacteria were then removed by rinsing with saline. After centrifugation and re-suspension of the pellets, we used the cytospin to create the slides. The representative figures showed a significantly increased ( $P < 0.001$ ) number of bacteria phagocytized (a); when we added SP-A to the macrophage solution in comparison to that of the macrophages without SP-A (b); and SP-A enhanced bacterial phagocytic index by macrophages (c). Original magnifications, 1000 $\times$ .

SP-A is expressed in the ME and ET mucosa of WT mice and that the absence of SP-A is associated with a stronger inflammatory response to NTHi infection of the ME, which manifested as increased mucosal thickness and inflammatory cells compared to WT mice. Further *in vitro* studies demonstrated that the presence of SP-A resulted in increased phagocytic capacity of NTHi by alveolar macrophages, increased bacterial aggregation, and killing of NTHi.

SP-A was originally identified as a surfactant-associated protein, dominantly expressed in lung alveolar epithelial type II cells. SP-A functions are important in both maintaining lung homeostasis and protecting it from infection. SP-A plays important roles in innate immune responses to a wide range of respiratory pathogens such as viruses, fungi, and bacteria such as *Mycobacterium*, *Pseudomonas aeruginosa*, and *H. influenzae*.<sup>32</sup> Recognition and binding of this diverse variety of incoming pathogens by SP-A trigger various immune responses, including opsonization, leading to enhanced phagocytosis and killing by recruited macrophages and neutrophils via oxidative mechanisms, aggregation of pathogens thereby hindering their entry into host cells, and direct microbicidal activities by increasing cellular membrane permeability.<sup>28</sup> Yu et al. found that SP-A was up-regulated in rat ME after induction of OM by NTHi and that expression of SP-A was also identified in the ME effusion of humans.<sup>33</sup>

SP-A also assists in the clearance of apoptotic cells and in modulating local inflammation. SP-A protein is, however, widely expressed throughout the body,

including the female reproductive tract, urinary tract, gastrointestinal tract, the eye, ear, nasal compartment, central nervous system, and the skin.<sup>10</sup> The functions of SP-A at these extrapulmonary sites are relatively under-investigated, but it is emerging that SP-A contributes significantly to the regulation of inflammation and protection from infection at these sites.<sup>32</sup> As stated before, SP-A is expressed in the ET epithelial cells. The ET links the ME and nasopharynx and can potentially be infected by pathogens from the upper respiratory system, which may lead to OM. In a recent study, OM was induced in mice by injection of LPS derived from *Klebsiella pneumoniae* directly to the ME, and it was noted that these mice had significantly increased SP-A expression in the ME, indicating that SP-A may be up-regulated due to LPS stimuli.<sup>13</sup>

Genome-wide linkage studies identify regions of the genome that harbor disease susceptibility loci by typing microsatellite markers or single nucleotide polymorphisms (SNPs) spaced across the genome in sets of affected relatives.<sup>34</sup> In an evaluation of 588 patients undergoing tympanostomy tube insertion for chronic or recurrent OM with DNA analysis, three important chromosomal regions were identified as important influencers: 10q, 19q, and 3p.<sup>35</sup> Casselbrant et al. used the genetic relationships across implicated *loci* tool to identify possible candidate genes within their linkage regions, which identified a cluster of chemokine genes on 17q12 and several surfactant protein genes near 10q22.3. Possible candidate genes at these sites includes pulmonary surfactant-associated protein gene *SFTPA2* in the 10q22.3 region.<sup>21</sup> The human



SP-A locus in chromosome 10q22–q23 consists of two very similar genes: *SFTPA1* (Protein SP-A1) and *SFTPA2* (Protein SP-A2). Both gene products are required for fully functional SP-A protein. Several alleles that differ by a single amino acid have been identified for each gene. The frequency of specific SP-A haplotypes and genotypes differs between children with recurrent OM compared to a control population.<sup>12</sup> Using a candidate gene approach, Ramet et al. reported an over-representation of the 6A<sup>4</sup>-1A<sup>5</sup> haplotype in children with recurrent OM and in children diagnosed with their first episode of AOM before the age of 6 mo; there was also an under-representation of the 6A<sup>2</sup>-1A<sup>0</sup> haplotype in the latter subgroup.<sup>12,36</sup>

Using an immunofluorescence staining method, we have shown the presence of SP-A in the ET mucosa of WT mice. Paananen et al. demonstrated the presence of SP-A and SP-D in porcine ET mucosa using immunostaining with human mAb against SP-A and a porcine polyclonal Ab against SP-D, which suggested that the function of these proteins may not be essential for surface activity but may have an immune defense purpose against ME infection.<sup>37</sup> Our results showed that pro-inflammatory IL-6 and IL-1 $\beta$  peak after 24 h, with a significantly lower level in WT compared to SP-A KO mice, indicating that SP-A may modulate these pro-inflammatory cytokines. This is in accordance with a previous study that concluded that SP-A can modulate lung inflammation in humans by modulating LPS-induced production of pro-inflammatory cytokines by alveolar macrophage and increase the antibacterial and antiviral functions of macrophages.<sup>38</sup> We have also shown that the presence of SP-A significantly increased the phagocytic capacity of alveolar macrophages. SP-A can enhance phagocytosis through opsonization and also directly stimulate phagocytosis by the up-regulation of cell surface phagocytic receptors in macrophages.<sup>39</sup> We have shown a significant increase in phosphorylated NF- $\kappa$ B (p65) in ME mucosa of infected mice on d 1 and 3 compared to controls, which decreased significantly in infected WT on d 3 compared to infected SP-A KO mice. Previous studies found that SP-A and SP-D can interact with the various cell receptors, resulting in increased phosphorylation of p38 MAPK and the modulation of the NF- $\kappa$ B signaling pathway with enhanced expression of inflammatory factors in infection and sepsis.<sup>9,40</sup> It is necessary to investigate the cellular and molecular mechanisms of the role of SP-A as well as of human SP-A genetic variants that are associated with altered susceptibility in AOM in the future.

In summary, the results from this study demonstrate that SP-A contributes to the innate immunity of the ME through enhancing bacterial phagocytosis and

killing and modulates inflammation of the ME mucosa possibly through regulation of inflammation and NF- $\kappa$ B signaling activation.

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### Declaration of conflicting interests

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### ORCID iDs

Osama Abdel-Razek  <https://orcid.org/0000-0002-8450-4067>

Guirong Wang  <https://orcid.org/0000-0002-3288-8572>

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