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# Intranasal vaccination with pneumococcal surface protein A plus poly(I:C) protects against secondary pneumococcal pneumonia in mice

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abstract

Effective pneumococcal vaccines are required for preventing secondary bacterial pneumonia, a life-threatening condition, during epidemics of influenza. We examined whether nasal administration of a low dose of pneumococcal surface protein A (PspA) plus polyinosinic–polycytidylic acid (poly(I:C)) could protect against a fatal secondary pneumococcal pneumonia after influenza A virus infection in mice. PspA-specific IgG but not IgA level was higher in the airways and blood of mice nasally administered a low dose of PspA plus poly(I:C) than in mice nasally administered PspA alone or poly(I:C) alone. Binding of PspA-specific IgG increased C3 deposition on the bacterial surface. The survival rate during secondary infection was higher in mice immunized with PspA plus poly(I:C) than in mice immunized with poly(I:C) alone. The significant reduction in bacterial density in the lung and blood was associated with increased survival of immunized mice with secondary pneumonia. Passive transfer of sera from mice immunized with PspA plus poly(I:C) increased the survival of mice infected with secondary pneumonia. Our data suggest that an intranasal PspA vaccine has promising protective effects against secondary pneumonia after influenza and that PspA-specific IgG plays a critical role in this protection.



## 1. Introduction

Although influenza is a seasonal viral infection associated with significant morbidity and mortality [1], most of the deaths during the 1918 influenza pandemic were caused by secondary bacterial pneumonia, primarily Streptococcus pneumoniae [2,3]. Although recent studies on the 2009 pandemic of H1N1 virus infection showed that the most frequent cause of death was viral pneumonia [4–6], bacterial coinfection by S. pneumoniae was found in fatal cases or cases with severe respiratory failure associated with confirmed pandemic H1N1 virus infection [7,8].

Although pneumococcal conjugate vaccine provides protective immunity against pneumonia and invasive disease in infants [9,10], polysaccharide-based vaccines are not ideal because they must include multiple polysaccharide serotypes and do not protect against strains with nonvaccine serotypes [11]. Previous investigators have examined several pneumococcal proteins as potential vaccine candidates with promising results [12–15]. One of these candidates is pneumococcal surface protein A (PspA), which is a choline-binding protein exposed on the cell surface [16–18]. PspA is present on all pneumococcal strains, and anti-PspA antibody increases bacterial clearance and induces cross-protection against infection from strains with different serotypes [19]. Anti-PspA antibodies neutralize the anticomplementary effect of PspA, increasing C3 deposition on PspA-bearing bacteria [20,21]. Human antibody to PspA could protect mice from fatal pneumococcal infection, suggesting that PspA can be an effective human vaccine [22].

We reported previously on increased bacterial clearance in the lung of mice given intranasal immunization of PspA with a Toll-like receptor (TLR) ligand [23]. Intranasal immunization of each TLR agonist in combination with PspA works as a potent mucosal adjuvant in the induction of PspA-specific antibodies in the airway and blood. In the present study, we developed a murine model of secondary pneumococcal pneumonia and studied the effect of intranasal immunization with PspA with polyinosinic–polycytidylic acid (poly(I:C)), a double-stranded RNA (dsRNA) ligand for both TLR3 and melanoma-associated 5 [24] in this model.

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## 2. Materials and methods

### 2.1. Viral and bacterial strains

Influenza virus (H1N1) A/New Caledonia strain was obtained from The Research Foundation for Microbial Diseases, Osaka University. S. pneumoniae WU2 strain (serotype 3) was grown in Todd-Hewitt Broth (BD, Franklin Lakes, NJ) supplemented with 0.1% yeast extract (THY) to mid-log phase and washed twice with Dulbecco's phosphate-buffered saline (PBS) without $CaCl_2$ and $MgCl_2$ (Sigma–Aldrich, St. Louis, MO). Bacteria were suspended in THY, and the aliquots were snap frozen in liquid nitrogen and stored at −80 °C until use.

### 2.2. Immunogen and adjuvant

The recombinant plasmid pUAB055 [25] containing the 0.9 kb pspA Rx1 gene encoding $PspA_{32-333}$ was transformed into Escherichia coli strain BL21 (DE3) (Invitrogen, Carlsbad, CA) for protein production. The recombinant PspA (rPspA) was purified with Ni-NTA agarose (Qiagen, Valencia, CA). The purified His-tag PspA was purified further with an ion exchange column Mono Q 5/50 GL (GE Healthcare Bio-Sciences, Piscataway, NJ), followed by gel filtration chromatography with Superdex 75 10/300 GL (GE Healthcare Bio-Sciences). We used poly(I:C) (InvivoGen, San Diego, CA), which is a synthetic analog of dsRNA, as the adjuvant for PspA [23].

### 2.3. Immunization of mice

6–8-Week-old C57BL/6 mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). Mice were anesthetized by subcutaneous administration of ketamine (2.46 mg/mouse) and xylazine (0.216 mg/mouse). The anesthetized mice were immunized intranasally with 0.5 μg of PspA together with 10 μg of poly(I:C) or 10 μg of poly(I:C) alone in 12 μl of PBS once a week for 3 weeks. Mice were sacrificed 1 week after the last immunization, and serum and bronchoalveolar lavage (BAL) fluid were sampled for PspA-specific antibody assays. All animal experiments were performed in accordance with institutional guidelines for the Osaka University animal facility.

### 2.4. PspA-specific antibody assays

Microtiter plates (Thermo Fisher Scientific, Waltham, MA) were coated overnight at 4 °C with 100 μl of 1 μg/ml of PspA. The plates were washed with PBS containing 0.05% Tween 20 (PBS-T). Serially diluted serum and BAL fluid were added to the plates, and the plates were incubated for 30 min at 37 °C. The plates were washed three times with PBS-T and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Zymed, San Francisco, CA) for 30 min at 37 °C. After the incubation, the plates were washed three times with PBS-T, 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma–Aldrich) diluted with substrate buffer (1 M diethanolamine, 0.5 mM $MgCl_2$) was added, and the plates were incubated for 30 min at room temperature in the dark. The optical density was read at 405 nm with a microplate reader (Bio-Rad Laboratories, Hercules, CA).

### 2.5. Secondary pneumonia model

Anesthetized mice were infected intranasally with $1 \times 10^3$ plaque-forming units (PFU) of influenza virus (H1N1) A/New Caledonia strain in 30 μl of PBS and infected intranasally with $6 \times 10^3$ colony-forming units (CFU) of S. pneumoniae WU2 strain in 30 μl of PBS 5 days after the viral infection. Two weeks after the last immunization, immunized mice were similarly infected intranasally with influenza virus, followed 5 days later by nasal infection with S. pneumoniae. Mice were sacrificed and lung and blood samples were obtained 2, 6, 16, 24, 48, and 72 h after pneumococcal infection. The lungs were homogenized in 2 ml of PBS, and quantitative culture of the lung homogenates and blood samples were performed on sheep blood agar.

### 2.6. Lung histopathology

Lungs were excised from mice and fixed in 10% formalin. The fixed lungs were embedded in paraffin, and 5 μm-sliced sections were stained with hematoxylin–eosin. Four types of histopathological changes (peribronchiolitis, perivasculitis, interstitial pneumonitis, and alveolitis) were scored independently by a pathologist who was unaware of the animal status and were given histopathological scores using a 0–4 scale as described previously [26,27]. BAL fluid was obtained from the nasally immunized mice after pneumococcal infection at the indicated times. Cells were stained with Diff-Quik (Sysmex, Kobe, Japan), and the cell morphology was determined in cell monolayers prepared using a Cytospin 2 (Shandon Southern Products, UK).

### 2.7. Immunoblotting

S. pneumoniae WU2 strain and rPspA protein were lysed in sample buffer (60 mM Tris–HCl pH 6.8 containing 5% glycerol, 1.6% SDS, 0.1 M DTT, and 0.002% bromophenol blue), and the lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After the electrophoresis, the gel was transferred to a polyvinyl difluoride membrane (Bio-Rad Laboratories), the membrane was blocked with 5% skim milk for 1 h at room temperature and then incubated with sera from PspA-immunized mice at a dilution of 1:5000 for 1 h at room temperature. After the incubation, the membrane was washed three times and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (SouthernBiotech, Birmingham, AL) at a dilution of 1:10,000 for 1 h at room temperature. The separated bands were detected with an ECL kit (GE Healthcare Bio-Sciences).

### 2.8. Antibody binding and C3 deposition assay

S. pneumoniae WU2 strain was grown in THY to mid-log phase. The bacteria were harvested by centrifugation and washed once with PBS. The pellet was suspended with an appropriate amount of PBS to prepare a bacterial suspension at a concentration of $1 \times 10^8$ CFU/ml, and 100 μl of Alexa-Fluor 488-conjugated goat anti-mouse IgG antibody (Invitrogen, Eugene, OR) or 90 μl of the bacterial suspension was incubated with 10 μl of heat-inactivated mouse serum for 30 min at 37 °C. After the incubation, the suspension was washed once with PBS, suspended in 90 μl of gelatin-veronal buffer with $Mg^{2+}$ and $Ca^{2+}$ with 10 μl of naive mouse serum, and then incubated for 30 min at 37 °C. Following the incubation, the bacterial suspension was washed once with PBS, suspended in 100 μl of fluorescein isothiocyanate-conjugated goat anti-mouse C3 antibody (MP Biomedicals, Solon, OH), and incubated for 30 min on ice. After the incubation, the bacterial suspension was washed twice with PBS and suspended in 500 μl of 1% formaldehyde. The samples were kept on ice in the dark until analyzed by flow cytometry using a BD FACSCalibur™ with CELLQuest software.

### 2.9. Passive immunization

Mice were infected intranasally with $1 \times 10^3$ PFU of influenza virus and infected intranasally with $6 \times 10^3$ CFU of S. pneumo-

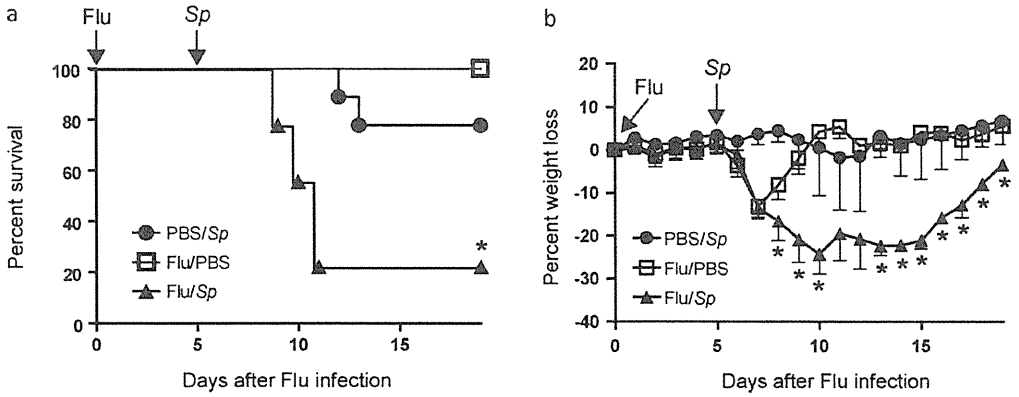


Fig. 1. Survival (a) and change in body weight (b) of mice after influenza virus infection. The data for the mice (n=9) administered PBS nasally, followed 5 days later by administration of S. pneumoniae are shown as PBS/Sp (closed circles). The data for mice (n=7–9) that received influenza virus, followed by administration of PBS or S. pneumoniae are shown as Flu/PBS (open squares) or Flu/Sp (closed triangles). *P<0.05 (versus Flu/PBS and PBS/Sp).

niae WU2 strain 5 days after the viral infection. Immediately after pneumococcal infection, 100 μl of serum obtained from mice immunized intranasally with PBS alone, with 10 μg of poly(I:C) alone, or with 0.5 μg of PspA plus 10 μg of poly(I:C) was administered intraperitoneally to mice with secondary pneumococcal pneumonia. The survival of the infected mice was monitored.

### 2.10. Statistical analysis

All data are expressed as the mean ± SD. Student's t-test, the Mann–Whitney test or one-way ANOVA and Turkeys' multiple comparison methods were used to analyze the data. We used the Kaplan–Meier log-rank test for the survival analysis. P values <0.05 were considered significant.

## 3. Results

### 3.1. Secondary bacterial pneumonia

All mice that received the nasal challenge of influenza virus followed by PBS (Flu/PBS) and 80% of mice that received the nasal challenge of PBS followed by S. pneumoniae (PBS/Sp) survived. By contrast, only 20% of mice that received the nasal challenge of influenza virus followed by S. pneumoniae (Flu/Sp) survived during the observation period (Fig. 1a). Body weight declined transiently by 15% after the PBS challenge in mice that received the Flu/PBS challenge but did not change in mice that received the PBS/Sp challenge (Fig. 1b). By contrast, body weight decreased by 25% during the observation period in surviving mice that received the Flu/Sp challenge.

To characterize further the model of secondary pneumonia, we next examined the bacterial density in the lung and blood of mice. The bacterial densities of S. pneumoniae were approximately $10^3$ CFU in the lung tissue of mice that received the PBS/Sp challenge 2 h after the pneumococcal challenge and $10^3$ to $10^4$ CFU/lung 16–72 h after the challenge (Fig. 2b). None of these mice was bacteremic between 2 and 72 h after the pneumococcal challenge, except for one of five mice at 48 h after the challenge (Fig. 2a). By contrast, the bacterial density in the lung tissue increased from $10^3$ CFU/lung at 2 h to $10^6$ CFU/lung 16 h after the pneumococcal challenge in mice receiving the Flu/Sp challenge (Fig. 2b). At 16, 24, 48, and 72 h after the pneumococcal challenge, the bacterial density in the lung tissue was significantly higher in mice that received Flu/Sp than in those that received PBS/Sp at the same time points (P<0.05). In these mice that received Flu/Sp, the bacterial density was higher than $10^7$ CFU in the lung tissue, and three of four mice had bacteremia 72 h after the pneumococcal challenge (Fig. 2a).

A histopathology study showed minimal inflammatory changes in the lungs of mice that received PBS/PBS and in mice that received PBS/Sp (Fig. 3a). Marked inflammatory changes were evident in mice that received Flu/PBS or Flu/Sp compared with mice that received PBS/PBS or PBS/Sp. Interstitial pneumonitis was prominent only in mice that received Flu/Sp and not in mice that received Flu/PBS. These data suggest that advanced pulmonary inflammation was caused by secondary pneumonia.

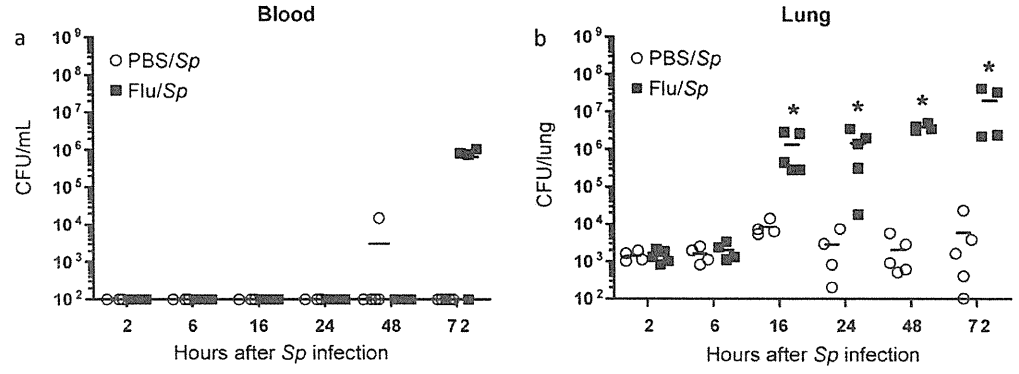


Fig. 2. Bacterial densities in blood (a) and lung (b) of mice given PBS (open circles) or influenza virus (closed squares) nasally followed by administration of S. pneumoniae. Lung tissue and blood were harvested 2, 6, 16, 24, 48, and 72 h after pneumococcal challenge, and bacterial density was counted. Values represent the $\log_{10}$ CFU/mL or CFU/lung (mean ± SD) for 4–5 mice per group. *P<0.05 versus PBS/Sp at the same time points.

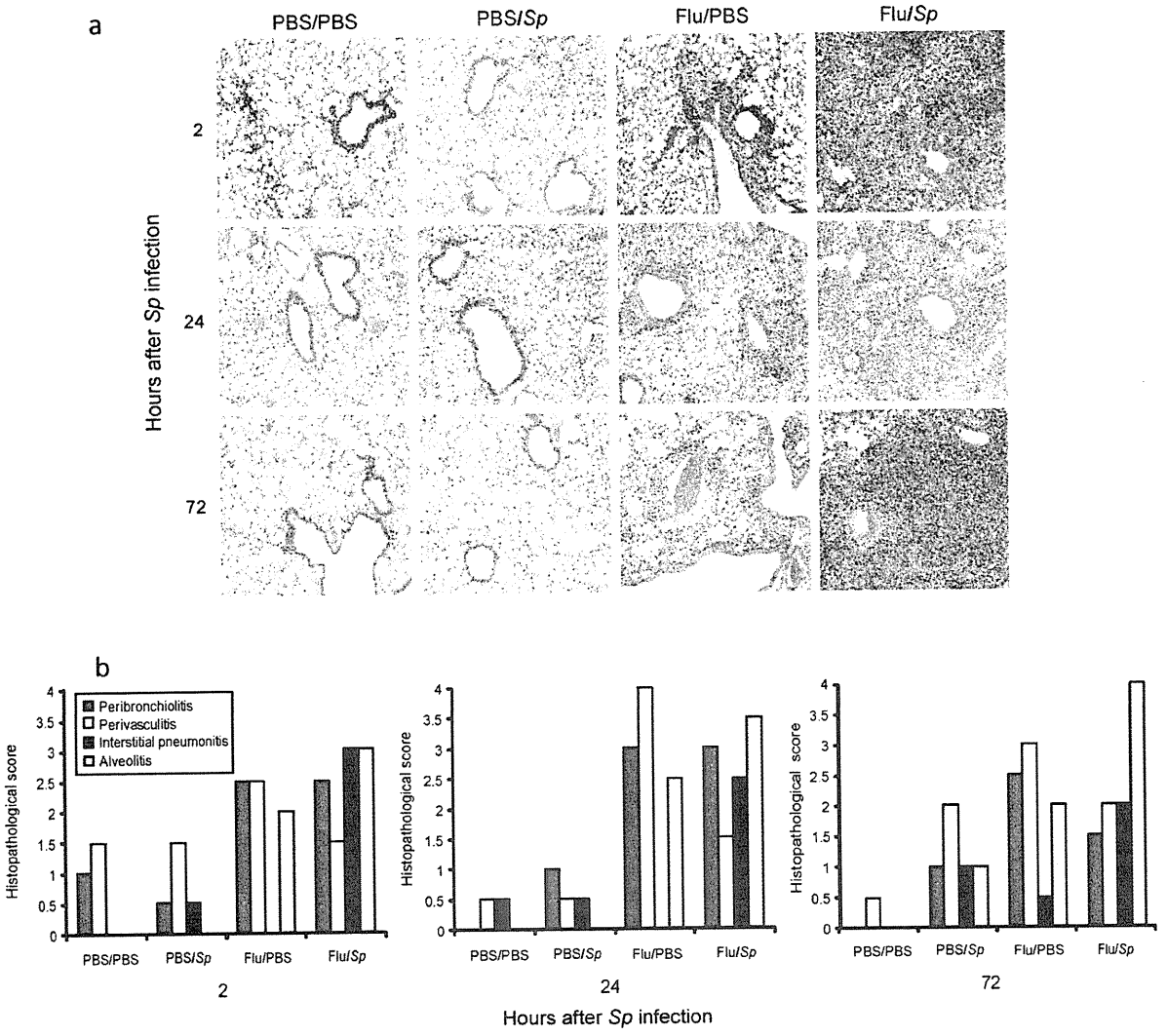


Fig. 3. Histopathological findings in lung tissues from mice with secondary pneumonia (a) and the histopathological scores of lung tissues (b). Mice were given PBS nasally followed by administration of PBS (PBS/PBS), PBS followed by administration of S. pneumoniae (PBS/Sp), influenza virus followed by administration of PBS (Flu/PBS), or influenza virus followed by administration of S. pneumoniae (Flu/Sp). Mice administered PBS or influenza virus nasally were sacrificed at the times indicated after intranasal administration of S. pneumoniae or PBS challenge, and their lungs were excised, fixed, and stained with hematoxylin–eosin. The histopathology was scored from microscopic analysis. Two mice per group were examined.

### 3.2. PspA-specific IgG and its function

A negligible level of PspA-specific IgG, IgG1 or IgG2a was found in serum from mice immunized nasally with poly(I:C) alone (PBS/poly(I:C)) or PspA alone (Fig. 4a and b). A higher level of PspA-specific IgG was found in serum from mice immunized nasally with PspA plus poly(I:C) (PspA/poly(I:C)). PspA-specific IgG in serum comprised both PspA-specific IgG1 and IgG2a, and a low level of PspA-specific IgG was detected in BAL fluid of these mice (Fig. 4b and c). No PspA-specific IgA was detected in BAL fluid in mice immunized nasally with PspA alone or with PspA/poly(I:C) (data not shown).

The serum from mice immunized nasally with PBS/poly(I:C) exhibited no binding to the cell lysates of the WU2 strain or rRx1 PspA protein. Faint binding was detected in the serum from mice immunized nasally with PspA/poly(I:C) irrespective of whether they received the nasal challenge of influenza virus followed by S. pneumoniae (Fig. 4d). Binding of mouse IgG was observed to bacteria treated with serum from mice immunized nasally with PspA/poly(I:C) regardless of whether they had received the nasal challenge of influenza virus followed by S. pneumoniae (Fig. 4e). No binding was observed to bacteria treated with serum from mice immunized nasally with PBS/poly(I:C), suggesting the binding of PspA-specific IgG to the surface of the WU2 strain. Similarly, C3 binding was found on the bacteria treated with serum from mice immunized nasally with PspA/poly(I:C) regardless of whether they received the nasal challenge of influenza virus followed by S. pneumoniae (Fig. 4f). No binding was observed to bacteria treated with serum from mice immunized nasally with PBS/poly(I:C), suggesting that the increased C3 deposition was caused by PspA-specific IgG.

### 3.3. Protective effect of intranasal PspA vaccine

The survival rates did not differ between mice immunized nasally with PspA alone or with PBS alone (Fig. 5a). By contrast, the survival rate was significantly higher in mice immunized nasally with PspA/poly(I:C) than in mice immunized nasally with PBS/poly(I:C) ($P<0.0001$, Fig. 5b). Body weight was significantly

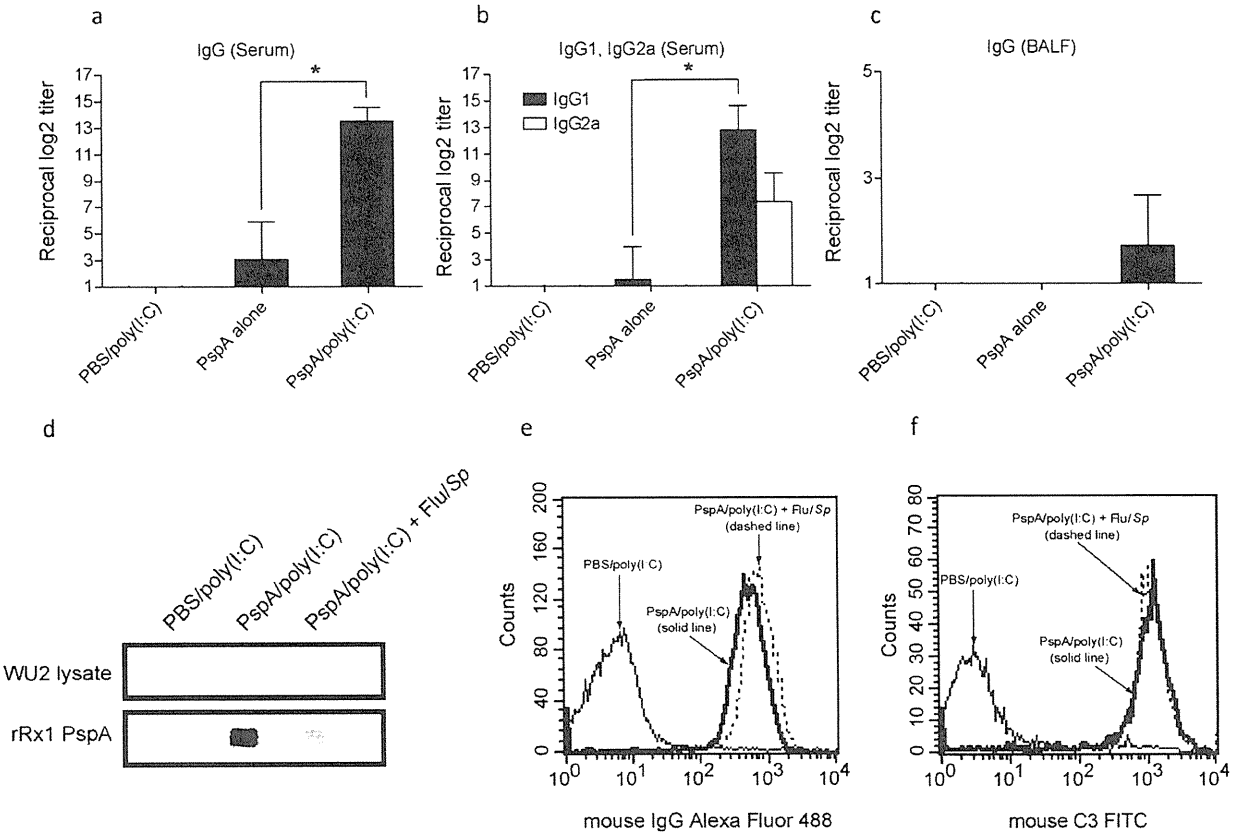


Fig. 4. Induction of PspA-specific IgG levels in serum (a), IgG1 and IgG2a levels in serum (b), and IgG levels in BALfluid (c), immunoblot analysis of lysates of S. pneumoniae WU2 strain and recombinant Rx1 PspA with sera from mice immunized with PBS/poly(I:C) and PspA/poly(I:C) (d), the binding of PspA-specific antibodies (e), and C3 deposition on the bacterial surface (f). Sera from mice immunized with PBS/poly(I:C), PspA alone or PspA/poly(I:C) were used for the determination of PspA-specific antibodies. Sera from mice immunized with PBS/poly(I:C), PspA/poly(I:C), or PspA/poly(I:C) and followed by pneumococcal infection (PspA/poly(I:C) + Flu/Sp) were used for the immunoblot analysis. Sera from mice immunized with PspA/poly(I:C) (solid line) or sera from mice immunized with PspA/poly(I:C) + Flu/Sp (dashed lines) were tested for the ability to bind to the pneumococcal surface and for the ability to bind C3 on the pneumococcal surface by flow cytometry. Sera from mice immunized with PBS/poly(I:C) (grey areas) were used as the control. The results are expressed as mean ± SD for 9–10 mice per group for the measurement of PspA-specific antibodies. *P<0.05 versus PspA alone.

higher in mice immunized nasally with PspA/poly(I:C) than in mice immunized nasally with PBS/poly(I:C) at 2–6 days after the pneumococcal challenge (P<0.005, Fig. 5c).

Bacterial density in the lung was 500-times lower at 24 h, 6000-times lower at 48 h, and 13,000-times lower at 72 h after the pneumococcal challenge in mice immunized nasally with PspA/poly(I:C) than in mice immunized nasally with PBS/poly(I:C) (Fig. 6b). The bacterial density in blood was also lower in mice immunized nasally with PspA/poly(I:C) than in mice immunized nasally with PBS/poly(I:C) at 48 h and 72 h after the pneumococcal challenge (Fig. 6a).

A histopathology study of nasally immunized mice showed similar inflammatory cell infiltration in the lung tissue in mice immunized nasally with PspA/poly(I:C) and in mice immunized with PBS/poly(I:C) (Fig. 7a). The observed alveolitis was slightly milder in mice immunized nasally with PspA/poly(I:C) than in mice immunized nasally with PBS/poly(I:C) at 24 h and 72 h after the pneumococcal challenge. The total number and the differen-

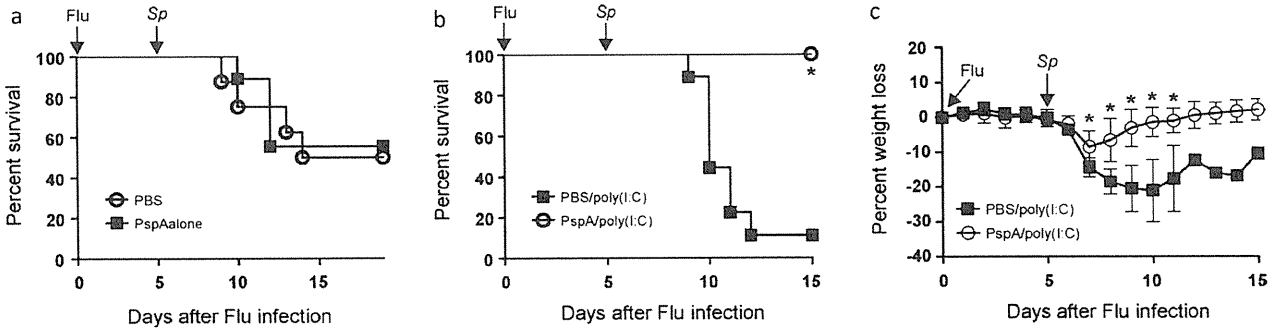


Fig. 5. Survival of C57BL/6 mice immunized nasally with PspA alone or with PBS after secondary pneumonia (a), nasally immunized with PBS/poly(I:C) or with PspA/poly(I:C) after secondary pneumonia (b), and the change in body weight of C57BL/6 mice immunized nasally with PspA/poly(I:C) or PBS/poly(I:C) alone after secondary pneumonia (c). In (a), the groups of mice (n=8–9) immunized nasally with PspA alone or with PBS are shown as PBS (open circles) or PspA alone (closed squares). In (b) and (c), the group of mice (n=9–10) were nasally immunized with PBS/poly(I:C) (closed squares) or with PspA/poly (I:C) (open circles).

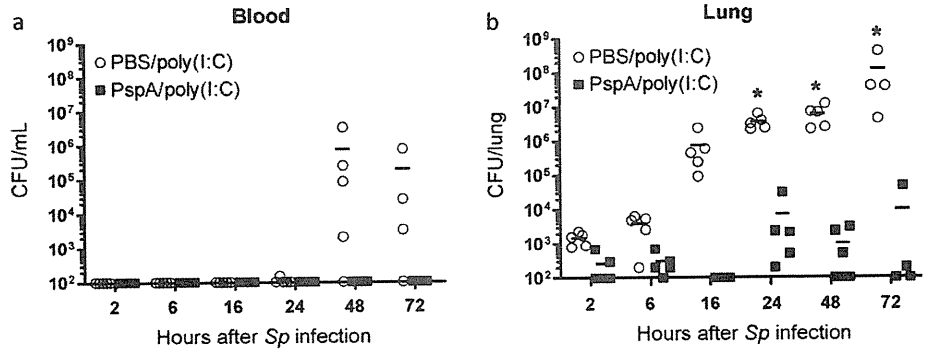


Fig. 6. Bacterial densities in blood (a) and lung (b) of mice immunized nasally with PBS/poly(I:C) (open circles) or PspA/poly(I:C) (closed squares) after secondary pneumonia. Lungs and blood were harvested 2, 6, 16, 24, 48, and 72 h after pneumococcal infection, and bacterial density was counted. Values represent the $\log_{10}$ CFU/mL or CFU/lung (mean ± SD) for 4–6 mice per group. *P<0.05 versus PBS/poly(I:C).

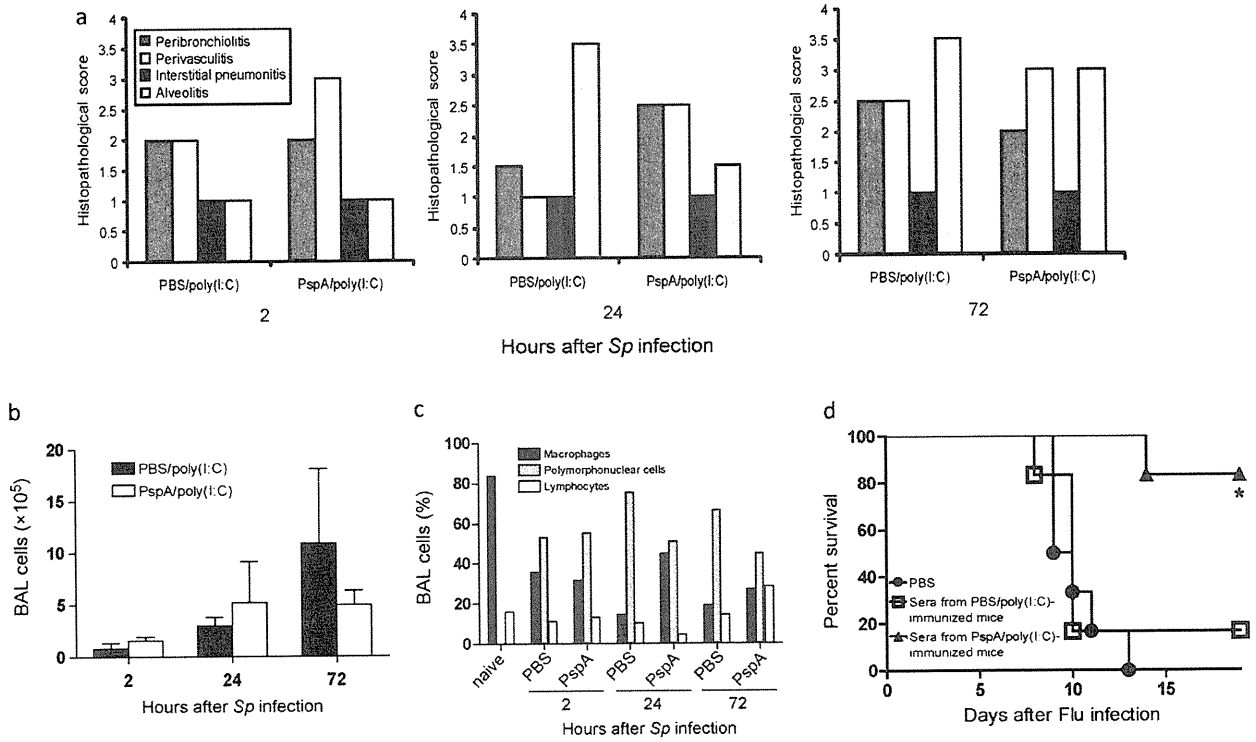


Fig. 7. Histopathological findings of lung tissues (a), the total cell count (b), and differential cell count (c) of BAL cells from nasally immunized mice after secondary pneumonia, and the effect of passive immunization in mice infected with secondary pneumonia (d). Nasally immunized mice were sacrificed at the times indicated after pneumococcal infection, and their lungs were removed, fixed, and stained with hematoxylin–eosin. The histopathology was scored using microscopic analysis. BAL was performed in the nasally immunized mice after pneumococcal infection at the times indicated, and the total number of BAL cells and cell morphology were determined. Three mice per group were examined for analysis of BAL cells. Immediately after pneumococcal infection, 100 μl of PBS (closed circles), 100 μl of serum from mice nasally immunized with PBS/poly(I:C) (open squares) or with PspA/poly(I:C) (closed triangles) was administered intraperitoneally to mice with secondary pneumonia. Six mice per group were examined. *P<0.05 versus PBS/poly(I:C) and PBS.

tial count of BAL cells did not differ significantly between mice nasally immunized with PspA/poly(I:C) and those immunized with PBS/poly(I:C) (Fig. 7b and c). These data suggest that the protective effect of nasal immunization with PspA/poly(I:C) occurred through the suppression of bacterial growth but not through the suppression of pulmonary inflammation.

### 3.4. Protection by passive immunization

The survival rate was higher in mice that received intraperitoneal administration of 100 μl of serum from mice immunized nasally with PspA/poly(I:C) than in mice that received intraperitoneal administration of 100 μl of PBS or serum from mice immunized nasally with PBS/poly(I:C) (P<0.05, Fig. 7d).

## 4. Discussion

In this study, we demonstrated the protective effects of a low dose of PspA plus poly(I:C) against a fatal secondary pneumococcal pneumonia after influenza virus infection in mice. Since nasal challenge with the WU2 strain at $2 \times 10^7$ CFU caused fatal pneumonia in untreated, healthy C57BL/6 mice (Piao et al., unpublished data), the challenge dose of WU2 strain required for a fatal pneumonia in mice nasally infected with $1 \times 10^3$ PFU of influenza virus (H1N1) A/New Caledonia was about 3300-times lower than in untreated

mice. Altered pulmonary defense caused by prior influenza virus infection caused rapid bacterial growth in the lung, and bacteremia developed subsequently in these mice.

Previous reports suggest several possible mechanisms to explain the impaired host defense against pneumococcal pneumonia after influenza virus infection, such as the production of IL-10 [28], interferon-γ (IFN-γ) [29], and type 1 IFNs [30], and desensitization to bacterial Toll-like receptor ligands [31]. Shahangian et al. suggested that the production of type 1 IFNs in the lung triggered by prior influenza virus infection inhibits CXC chemokine production and subsequently decreases the pulmonary influx of polymorphonuclear (PMN) cells [30]. We found a marked influx of PMN cells into BAL fluid from mice immunized nasally with PspA/poly(I:C) or PBS/poly(I:C) after secondary pneumococcal pneumonia. A similar finding of the influx of granulocytes into BAL fluid has been shown in a murine model of secondary pneumococcal pneumonia [28]. In addition, King et al. recently reported a >1800-reduction in the growth of the PspA$^-$ mutant relative to that of the wild-type S. pneumoniae D39 strain in mice with prior influenza virus infection, which was highly significant compared with the growth of PspA$^-$ mutant in mice without prior influenza virus infection [32]. This finding suggests that PspA is a critical pathogenic factor in this murine model of secondary pneumonia.

Our previous study of a sublethal pneumonia model using the WU2 strain showed that intranasal immunization of mice with a high dose (2.5 μg) of PspA and 10 μg of a TLR agonist (Pam3CSK4, poly(I:C), lipopolysaccharide, or CpG1826) increased the level of PspA-specific IgG in blood and PspA-specific IgA in the airways [23]. Among these TLR agonists, poly(I:C) is a synthetic analog of dsRNA. Since Poly I:PolyC12 U (Ampligen®), a synthetic dsRNA compound, which can act as a mucosal adjuvant for influenza virus [33], is applicable for humans [34], we used poly(I:C) as a mucosal adjuvant for PspA in this study. Although a previous study employing a PspA DNA vaccine suggested that a balanced IgG1/IgG2a immune response to PspA might increase the complement deposition, and the protection against pneumococcal infections [35], our previous study demonstrated the effects of nasal immunization with PspA plus different TLR agonists on bacterial clearance from the airways were equivalent between the different TLR agonists despite the balance of IgG1/IgG2a immune responses to PspA [23].

An intranasal administration of a low dose of PspA and poly(I:C) induced a higher level of PspA-specific IgG in blood and a low level of PspA-specific IgG, but not IgA, in BAL fluid of mice. PspA-specific IgG induced in serum bound to the surface of the WU2 strain and caused the strong deposition of C3 on the WU2 strain. This nasal vaccine with a low dose of PspA caused a marked reduction of the bacterial growth in the lung (500–13,000-times lower), and inhibited bacteremia completely, and subsequently improved the survival rate of mice with secondary pneumonia. Furthermore, the productions of tumor necrosis factor (TNF)-α and macrophage inflammatory protein (MIP)-2α in serum of mice nasally immunized with PspA/poly(I:C) were completely suppressed at 72 h after secondary pneumonia, compared with those in sera of mice nasally immunized with PBS/poly(I:C) alone (data not shown). These data suggest a complete suppression of bacteremia subsequently inhibited the systemic inflammatory responses in mice nasally immunized with PspA/poly(I:C).

Although our recent study confirmed the role of PspA-specific IgA in bacterial clearance of the serotype 19F strain of EF3030, which was relatively avirulent and caused bacterial colonization in the airway [36], PspA-specific IgA seems to not be essential in a model of bacteremic pneumonia using a virulent serotype 3 strain of WU2 in mice. By contrast, the successful protection of mice from death by passive transfer of anti-PspA serum shown in this study suggests that PspA-specific IgG plays a pivotal role through binding to the bacterial surfaces and increasing C3 deposition on the bacterial surfaces in a fatal model of secondary pneumonia using the WU2 strain.

A recent study also reported that nasal immunization with 1.0 μg of PspA plus 4 μg of cholera toxin B subunit reduced the bacterial load of serotypes 2, 3, and 4 pneumococci in the lungs of mice with secondary pneumonia after influenza virus infection measured 24 h after pneumococcal challenge compared with control mice [32]. However, the magnitude of the reduction in the bacterial load in the lung by this intranasal PspA vaccine was only less than 100-fold, and no induction of PspA-specific antibodies or increased survival of mice with secondary pneumonia was observed.

In conclusion, intranasal vaccination with a low dose of PspA plus poly(I:C) induced a high level of PspA-specific IgG in serum but a low level of PspA-specific IgG in BAL fluid. PspA-specific IgG bound to invasive pneumococci and led to the deposition of C3 on this bacteria. Intranasal vaccination had a marked antibacterial effect in a fatal model of pneumococcal pneumonia after influenza virus infection. Our data suggest that intranasal vaccination with a low dose of PspA plus poly(I:C) is highly protective against secondary pneumococcal pneumonia, which is seen frequently in children and adults during pandemic influenza and epidemics of seasonal influenza.

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Research Article Open Access

# Comparative Effects of Toll-Like Receptor Agonists on a Low Dose PspA Intranasal Vaccine against Fatal Pneumococcal Pneumonia in Mice

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## Abstract

To develop a cost-effective pneumococcal vaccine, we compared the effects of a panel of Toll-like receptor (TLR) agonists on a low dose pneumococcal surface protein A (PspA) nasal vaccine in a fatal pneumococcal pneumonia model using a serotype 3 strain. The mice were nasally immunized with 10 μg of the TLR agonist (TLR 2, 3, 4 and 9) and 0.1μg of PspA once per week for three weeks. A high level of PspA-specific immunoglobulin G (IgG) was detected in sera of mice that were nasally administered a low dose of PspA plus each TLR agonist, while no PspA-specific IgG were detected in sera of mice that had been nasally administered a low dose of PspA alone. A relatively low level of PspA-specific IgG was also detected in the airway of mice that had been nasally administered a low dose of PspA plus each TLR agonist. The binding of PspA-specific IgG increased the deposition of C3 on the bacterial surface. Bacterial density in the lung and blood was significantly decreased in mice that had been administered a low dose of PspA plus each TLR agonist, compared with mice that received a low dose of PspA alone 24 h after a bacterial challenge. Furthermore, significant increases in survival rate were found in a murine model of fatal pneumonia that had been nasally administered a low dose of PspA plus each TLR agonist, compared with mice that received a low dose of PspA alone. The rank order of TLR agonists on the effect of increasing survival rate was LPS > Pam3CSK4 > Poly(I:C) and CpG 1826. These data suggest a potentially new strategy for the development of a cost-effective intranasal vaccine with a low dose PspA plus TLR agonist that would be effective against life-threatening bacteremic pneumococcal pneumonia.

Keywords: PspA; TLR agonist; Intranasal vaccine; Streptococcus pneumoniae; Pneumonia

Abbreviations: PspA: Pneumococcal surface protein A; TLR: Toll-Like Receptor; LPS: Lipopolysaccharide; Poly(I:C): Polyinosine-polycytidylic acid; CpG ODN 1826: CpG-Containing Oligodeoxynucleotide 1826; BALF: Bronchoalveolar lavage fluid; NW: Nasal wash

## Introduction

S. pneumoniae is a leading human pathogen that causes a wide variety of diseases, ranging from otitis media to pneumonia, bacteremia, and meningitis in both children and adults. Pneumococcal infections can occur at any age but are more frequent in infants, the elderly and immunocompromised patients. Despite the development of effective treatments, the pneumococcus has remained a significant cause of morbidity and mortality worldwide [1,2]. Because of this, a clear need for an effective vaccine for the prevention of disease exists. Currently licensed polysaccharide-based pneumococcal vaccines only elicit protective antibodies against the infection of serotypes that are included in the vaccine. In addition, invasive diseases attributable to non-vaccine serotypes of S. pneumoniae have increased greatly [3,4]. Therefore, the search for new vaccine candidates that elicit protection against a broader range of pneumococcal strains is an important goal. To broaden the protection, the use of pneumococcal proteins represents a feasible and preferable alternative. Several pneumococcal proteins are currently under investigation as potential candidates for such a vaccine [5,6]. One of these proteins, PspA has recently undergone phase one clinical trials in humans and has been found to be safe and highly immunogenic [7,8]. PspA is a surface protein of S. pneumoniae that is found on all pneumococci and is broadly expressed among different serotypes of pneumococci [8]. Antibodies to PspA generated in mice [9,10] or humans [7,8] are capable of passively protecting mice against infections with different serotypes. PspA is, therefore, an attractive candidate for use as future protein-based pneumococcal vaccines.

Since S. pneumoniae enters the host primarily through the respiratory mucosa, vaccination strategies designed to target the airways are of great interest. An appropriate mucosal adjuvant is required to elicit an antigen-specific immune response in both the mucosal and systemic compartments [11]. We previously reported that each of the TLR agonists used in this study is an effective nasal adjuvant for the PspA antigen at a high dose (2.5 μg per mouse), and that it elicited the production of PspA-specific IgA in the airways and PspA-specific IgG in plasma. Because of this, it was capable of reducing the bacterial load in both the nasopharynx and lungs after a challenge with pneumococci with different serotypes [12]. Nasal immunization with a high dose of PspA alone could induce a certain level of PspA-specific IgG in the plasma and increased mouse survival, compared with mice that were nasally immunized with PBS alone, in a fatal pneumonia model in this study. These data suggest that nasal immunization with the reduced

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dose of PspA in combination with a TLR agonist was able to prevent the development of fatal pneumonia in this model. In the present study, we therefore examined the issue of whether nasal immunization using different TLR agonists in conjunction with a low dose (0.1μg per mouse) of PspA could confer protection against fatal pneumococcal pneumonia in healthy mice.

## Materials and Methods

### Mice and bacterial strains

Female C57BL/6 mice (6- to 8-wk-old) were purchased from Charles River Japan, Kanagawa, Japan. Mice were transferred to microisolators and maintained in horizontal laminar flow cabinets. They were provided sterile food and water in a specific pathogen-free facility. All mice used in these experiments were free of bacterial and viral pathogens. All animal experiments described in this study (protocol number; 08008) were performed in accordance with institutional guidelines for the Osaka University animal facility. S. pneumoniae WU2 strain with serotype 3, expressing PspA belonging to family 1, clade 2, was grown in Todd-Hewitt Broth (BD, Franklin Lakes, NJ) supplemented with 0.1% yeast extract (THY) to the mid-log phase and washed twice with phosphate-buffered saline (PBS) without $CaCl_2$ and $MgCl_2$. Bacteria were suspended in THY, and aliquots were snap frozen in liquid nitrogen and stored at -80°C until used.

### Recombinant PspA and adjuvants

Recombinant PspA/Rx1 (amino acids 1 to 302) was prepared as previously described [8]. To extensively remove LPS from the PspA preparations, we used an LPS removal column, ProteoSpin$^R$, (Norgen, Thorold, Canada). Four TLR agonists, including Escherichia coli K12 LPS (TLR4 agonist), Pam3CSK4 (TLR1/2 agonist), Poly(I:C) (TLR3 agonist) or CpG ODN 1826 (TLR9 agonist) were selected to use as adjuvants. The LPS, Pam3CSK4 and Poly(I:C) were purchased from InvivoGen (san Diego, CA). CpG ODN 1826 was purchased from Hokkaido System Science (Sapporo, Japan). Each of these adjuvants was used in a dose of 10 μg for nasal immunization.

### Nasal immunization

Mice were immunized intranasally three times at weekly intervals with 12μl of PBS containing 10μg of each TLR agonist and 0.1μg of PspA, 0.1μg of PspA alone or 12 μl of PBS on days 0, days 7 and days 14. The mice were euthanized on day 21 to obtain serum, bronchoalveolar lavage fluid (BALF) and a nasal wash (NW). The time points of nasal immunization and sampling for the determination of antibody levels were determined according to our previous study [13]. After removing the mandible, the nasal cavity was gently flushed with 1 ml of PBS from the posterior opening of the nose. The NW obtained from the anterior openings of the nose was collected. BALF was obtained by irrigation with 1 ml of PBS using a blunted needle inserted into the trachea after a tracheotomy [13].

### PspA-specific antibody assays

PspA-specific antibody titers of IgG or IgA in Serum, BALF and NW were determined by ELISA as previously described [12]. PspA was used as the coating antigen (1 μg/ml). A 100μl sample was added to each well, followed by incubation at 37°C for 30 min. The plate was washed, and then reacted with 100μl of alkaline phosphatase-conjugated goat anti-mouse IgA, IgG, IgG1 or IgG2a (Zymed, San Francisco, CA) and the OD at 405 nm was then measured. End-point titers were expressed as the reciprocal $\log_2$ of the last dilution giving an $OD_{405}$ of 0.1 OD unit above the $OD_{405}$ of negative control samples obtained from non-immunized mice.

### IgG binding and C3 deposition assays

Antibody binding was analyzed by whole cell ELISA. Frozen stock of S. pneumoniae WU2 (family 1 and clade 2) were plated onto blood agar, incubated overnight and then grown in THY to an $OD_{600}$ of 0.4~0.5 and harvested by centrifugation. The bacterial cells were washed, resuspended in PBS, and fixed with 80% ethanol at room temperature for 1 h. The ethanol-killed bacteria were washed twice with PBS, and the pellet resuspended in PBS to an $OD_{600}$ of about 0.2. 50μl of the ethanol-killed bacteria were coated in ELISA overnight at 4°C. The following day, the wells were washed 3 times with 0.05% of Tween-20 in PBS (PBST). The plates were then blocked with 3 % of skim milk in PBST at room temperature for 1 h. After 3 washes with PBST, 50μl of diluted serum in 1 % of skim milk in PBST were added to the plates, which were then incubated at 37°C for 2 h. The plates were washed 3 times with PBST, and then reacted with 100μl of alkaline phosphatase-conjugated goat anti-mouse IgG (Zymed, San Francisco, CA). The OD at 405 nm was then measured. The end-point titers were expressed as the reciprocal $\log_2$ of the last dilution giving an $OD_{405}$ of 0.1 OD unit above the $OD_{405}$ of negative control samples obtained from non-immunized mice.

C3 deposition was analyzed by flow cytometry. 10μl or 20μl of heat-inactivated serum was incubated with washed S. pneumoniae WU2 cells in 100μl of a reaction mixture achieving at a final concentration of $10^8$ cfu/ml at 37°C for 30 min. The live bacteria were washed once with PBS and then incubated with 10% fresh normal mouse serum as the source of complement in gelatin veronal buffer with $Ca^{2+}$ and $Mg^{2+}$ (Sigma, St. Louis, MO) at 37°C for 30 min. After washing, live bacteria were incubated with 100μl of fluorescein isothiocyanate-conjugated anti-mouse C3 IgG (MP Biomedicals, Solon, OH) in PBS for 30 min on ice. The samples were fixed with 2% formaldehyde after two washing steps and stored at 4°C. Flow cytometry analysis was conducted using a FACSCalibur apparatus (Becton Dickinson), and 10,000 gated events were recorded.

### Fatal pneumococcal pneumonia model

To determine the protective effects of nasal immunization with PspA plus each TLR agonist, S. pneumoniae WU2 strain at a dose of 2.0 × $10^7$ cfu (3 x $LD_{50}$) suspended in 30μl of sterile saline was intranasally administered to both the immunized and untreated mice 2 weeks after the last immunization. The 2-week interval between the last immunization and the bacterial challenge was maintained to avoid the influence of each TLR agonist on pulmonary defense, since some TLRs are involved in the innate immune response to S. pneumoniae [14-16]. The blood or lungs were aseptically removed from mice that had been anesthetized or euthanized with pentobarbital at 24 h post-bacterial challenge. The lung tissue was homogenized in 2 ml of sterile saline per whole lung tissue prior to culturing. To prevent coagulation, the blood was collected in tubes containing heparin. Quantitative bacterial cultures of blood or lung tissues were performed on horse blood agar. Mortality was monitored for 16 days following the pneumococcal challenge.

### Statistics

Statistical analyses were performed using one-way ANOVA and Tukey's multiple comparison method for antibody titers and bacterial clearance [17], and a log rank (Mantel-Cox) test for analysis of the survival curve with Graphpad Prism (GraphPad Software, Sandiego,

CA). Data were considered to be statistically significant if the p-values were less than 0.05.

## Results

### Immune responses to PspA in mice after nasal immunization with a low dose of PspA plus TLR agonists

While no PspA-specific IgG was detected in sera of mice that had been immunized nasally with PspA alone, increased high levels of PspA-specific IgG were detected in sera of mice that had been immunized nasally with PspA plus and either LPS, Pam3CSK4, Poly(I:C) or CpG 1826 (Figure 1A). The levels of PspA-specific IgG in the sera of mice immunized PspA plus LPS were significantly higher than those of mice that had been immunized with PspA plus and either Poly(I:C) ($p < 0.01$) or CpG 1826 ($p < 0.01$). No significant difference was found among the levels of PspA-specific IgG among mice that had been immunized nasally PspA plus Pam3CSK4, Poly(I:C) or CpG 1826.

The levels of PspA-specific IgG in the BAL fluids and NWs of mice that had been nasally immunized with PspA plus the LPS were 5.17 ± 0.98 and 1.67 ± 1.03, respectively. In contrast, negligible levels of PspA-specific IgG were induced in the BAL fluids and NWs from mice that had been nasally immunized PspA plus Pam3CSK4, Poly(I:C) and CpG 1826. PspA-specific IgA was not detected in the BAL fluid or NWs from these mice that had been immunized nasally with PspA plus the TLR agonist.

No PspA-specific IgG1 or IgG2a was detected in sera of mice that had been immunized nasally with PspA alone (Figure 1B). In contrast, increased levels of PspA-specific IgG1 or IgG2a were found in mice that had been immunized nasally with PspA plus the TLR agonist. The levels of PspA-specific IgG1 in sera of mice that had been immunized nasally with PspA plus LPS were significantly higher than those of mice that were immunized nasally with PspA plus Pam3CSK4 ($p < 0.05$), PspA plus Poly(I:C) ($p < 0.01$) or PspA plus CpG 1826 ($p < 0.01$). No

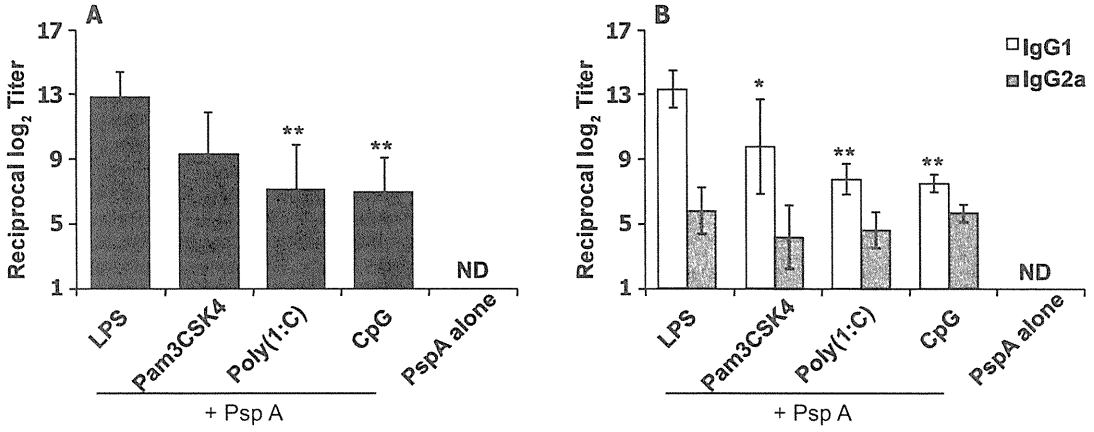


**Figure 1:** Induction of PspA-specific IgG (closed bars)(A), PspA-specific IgG1 (open bars) and IgG2a (gray bars) (B) in serum by intranasal immunization with either PspA plus each TLR agonist or PspA alone. Mice were nasally immunized three times at weekly intervals with 10 μg of TLR agonist and 0.1 μg of PspA. One week after the final immunization, the mice were euthanized to obtain serum, and PspA-specific antibody titers were determined using ELISA. The results are expressed as the mean ± S.D. for six mice per group. *$p < 0.05$, **$p < 0.01$, when compared with mice that were nasally immunized PspA plus LPS. ND, not detected.

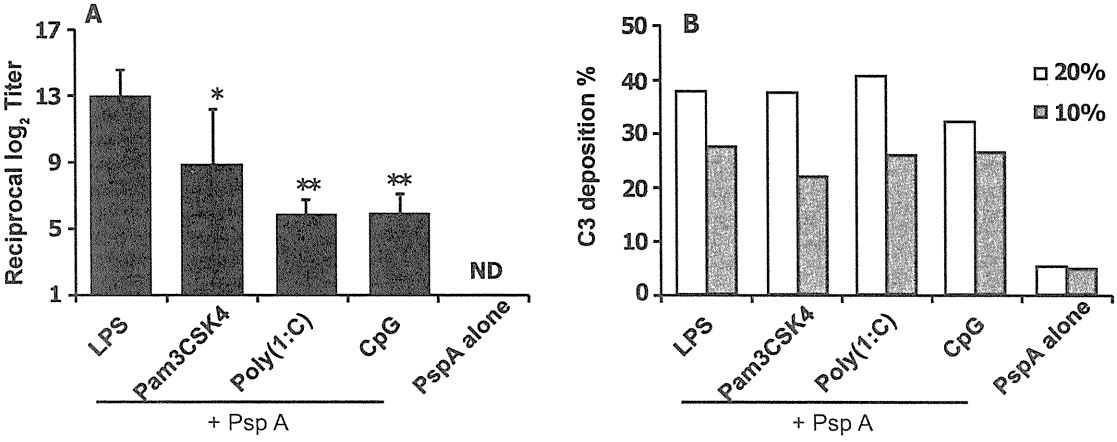


**Figure 2:** Binding of IgG antibodies (A) and C3 deposition (B) on the *S. pneumoniae* WU2 cell surface in the presence of sera from immunized and control mice. Binding The levels of IgG were analyzed by whole cell ELISA, and the C3 deposition were analyzed by flow cytometry using *S. pneumoniae* WU2 cells which were incubated with 20% (open bars) or 10% (gray bars) of pooled sera from mice that were immunized by intranasal immunization with either PspA plus each TLR agonist or PspA alone. The percentage of fluorescent bacteria (greater than 10 fluorescence intensity units) is shown as C3 deposition for each sample. *$p < 0.05$, **$p < 0.01$, when compared with mice that were nasally immunized PspA plus LPS. The results are expressed as the mean ± S.D. for six mice per group. ND, not detected.

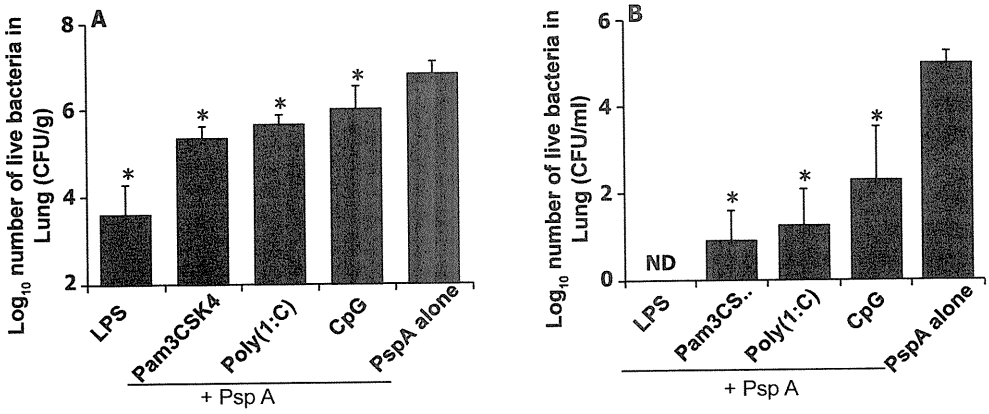


**Figure 3:** The effect of intranasal immunization with PspA plus each TLR against on the bacterial densities in Lung tissue (A) and blood (B) at 24 h post-challenge with the *S. pneumoniae* WU2 strain. An invasive dose of $2 \times 10^7$ cfu / mouse was nasally administered to mice that had been previously immunized with either PspA plus each TLR agaonist or PspA alone. Mice were euthanized to obtain lung tissues or blood from infected mice at the indicated time-point after bacterial challenge, and quantitative bacterial cultures of lung tissue or blood were performed. Values represent the $Log_{10}$ cfu / g or $Log_{10}$ cfu / ml (mean ± S.D.) for six mice per group. *$p < 0.0001$, when compared with mice that were nasally immunized with PspA alone.

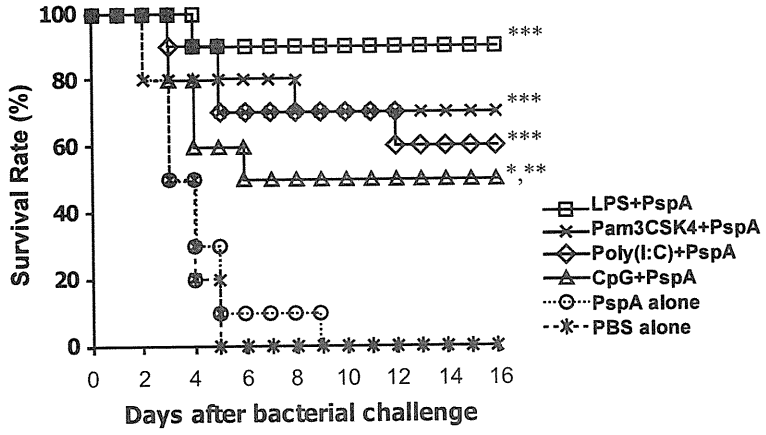


**Figure 4:** Survival of mice that were immunized nasally with a low dose of PspA plus each TLR agonist, a low dose of PspA alone and PBS alone after pneumococcal pneumonia. Immunized mice were intranasally challenged with $2 \times 10^7$ cfu of pneumococcal strain WU2, and the survival of the mice was monitored for 16 days. Results were examined by a Kaplan-Meier survival curve analysis for ten mice per group. *$p < 0.05$, when compared with mice that were nasally immunized PspA alone. **$p < 0.01$, when compared with mice that were nasally immunized PBS alone. ***$p < 0.001$, when compared with mice that were nasally immunized PspA alone or PBS alone.

significant difference was found in PspA-specific IgG1 levels among the sera of mice that were nasally immunized with PspA plus Pam3CSK4, Poly(I:C) or CpG 1826. No significant difference was found in PspA-specific IgG2a among sera of mice that were immunized nasally with PspA plus the TLR agonist. The mean ratios of PspA-specific IgG1 titers to PspA-specific IgG2a titers were 2.26 for LPS, 2.33 for Pam3CSK4, 1.68 for Poly(I:C), and 1.32 for CpG 1826, respectively.

## IgG binding and C3 deposition on the bacterial surface

While no binding of mouse IgG was observed in the case of bacteria treated with sera from mice that were immunized nasally with PspA alone, the levels of mouse IgG found on bacteria that were treated with sera from mice immunized nasally with the PspA plus LPS, Pam3CSK4, Poly(I:C) or CpG 1826 (Figure 2A) was increased. The levels of mouse IgG were significantly higher in sera from mice that were immunized nasally PspA plus LPS compared to mice that were nasally immunized with PspA plus Pam3CSK4 ($p < 0.05$), PspA plus poly (I:C) or PspA plus CpG 1826 ($p < 0.01$). These levels of mouse IgG in serum from mice that were immunized nasally with PspA plus the TLR agonist closely corresponded with the levels of PspA-specific IgG induced in serum. The frequencies of C3 depositions were substantially increased in bacteria that had been pretreated with 10% and 20% sera from mice that were immunized nasally with PspA plus each of the TLR agonists, compared with those of mice that were immunized nasally with PspA alone (Figure 2B). No differences were found in the frequencies of C3 deposition on bacteria that were pretreated with sera from mice nasally immunized with PspA plus each TLR agonist.

## Bacterial clearance from the lungs and the blood

The bacterial densities (mean ± S.D. for $Log_{10}$ cfu / g) reached 6.83 ± 0.26 in the lung and 4.89 ± 0.3 in blood from mice that were immunized nasally with PspA alone at 24 h post-challenge (Figure 3A and 3B). Significant decreases were found in bacterial density in the lungs of mice that were immunized nasally with PspA plus either

LPS, Pam3CSK4, Poly(I:C) or CpG 1826 compared with mice that were immunized nasally with the PspA alone ($p < 0.0001$). No significant differences were found in the bacterial densities in the lung among mice immunized nasally with PspA plus LPS, Pam3CSK4, Poly(I:C) or CpG 1826 (Figure 3A). Significant decreases were also found in blood from mice that were immunized nasally with PspA plus either Pam3CSK4, Poly(I:C) or CpG 1826 compared with mice that were immunized nasally with the PspA alone ($p < 0.0001$). No bacteria were detected in blood samples from any of the mice that were immunized nasally with PspA plus LPS agonist. No significant differences were found among mice immunized nasally with PspA plus LPS, Pam3CSK4, Poly(I:C) or CpG 1826 (Figure 3B).

## Protection by PspA plus each TLR vaccine against fatal pneumococcal pneumonia

As shown in Figure 4, the Kaplan-Meier analysis demonstrated significant protection as evidenced by the mean survival rate for mice that were immunized nasally with PspA plus each TLR agonist compared with mice that were immunized nasally with PspA alone or PBS alone. The survival rate was 90% for mice immunized nasally with the PspA plus LPS agonist ($p < 0.0001$ for PspA alone or PBS alone), 70% for mice immunized nasally with PspA plus Pam3CSK4 agonist ($p = 0.0008$ for PspA alone or $p = 0.0006$ for PBS alone), 60% for mice immunized nasally with PspA plus Poly(I:C) agonist ($p = 0.0005$ for PspA alone or $p = 0.0003$ for PBS alone), 50% for mice immunized nasally with PspA plus CpG 1826 agonist ($p = 0.0127$ for PspA alone or $p = 0.0062$ for PBS alone), respectively. No significant differences were found among mice that were immunized nasally with PspA plus LPS, Pam3CSK4, Poly(I:C) or CpG 1826.

## Discussion

The findings reported herein provide a demonstration of the protective effects of the nasal vaccination of a low dose of PspA plus each TLR agonist against a fatal model of pneumococcal pneumonia with serotype 3 S. pneumoniae WU2. Nasal vaccination of a low dose of PspA plus each TLR agonist induced a high level of PspA-specific IgG in the serum and a low level of PspA-specific IgG in the airways of mice. The binding of PspA-specific IgG in sera resulted in an increase in C3 deposition on the bacterial surfaces. Subsequently, the bacterial densities in the lung tissues and blood were significantly decreased in mice that were immunized nasally with PspA plus TLR agonist, compared with the values for mice immunized nasally with a low dose of PspA alone. The reduction in bacterial densities in lung tissues could be explained by the sufficient extravasation of PspA-specific IgG into the alveolar space of mice that were immunized nasally with a low dose of PspA plus each TLR agonist [18]. Bacterial invasion into the blood circulation was readily suppressed by PspA-specific IgG in sera of mice immunized nasally with a low does of PspA plus each of the TLR agonists. The survival of infected mice that were immunized nasally with a low dose of PspA plus TLR agonist was significantly increased compared with those of mice that were immunized nasally with a low dose of PspA alone. These findings are in contrast with findings reported in our previous study, showing no significant difference in the survival of infected mice between nasal immunization with a high dose (2.5μg) of PspA plus TLR agonist and a high dose of PspA alone in a fatal pneumonia model using the WU2 strain [12]. The findings reported herein on the effect of a low dose of PspA plus TLR agonist also suggest possibilities for the development of a cost-effective PspA intranasal vaccine with the goal of preventing a fatal pneumonia.

We recently reported on the pivotal role of PspA-specific IgA on the bacterial clearance of a less virulent serotype 19F strain in the upper airway in a mouse model of bacterial colonization [19]. Since PspA-specific IgG was shown to be sufficient for protecting mice against a fatal bacteremic pneumonia caused by a virulent serotype 3 strain in this study, PspA-specific IgA may not be essential for invasive pneumococcal infections, such as bacteremic pneumonia.

While the rank orders of PspA-specific IgG induced in sera were LPS > Pam3CSK4 > Poly(I:C) and CpG 1826, no difference was found in the frequency of C3 deposition on bacterial surfaces in immune sera induced by a low dose of PspA plus each TLR agonist in this study. This discrepancy between the levels of PspA-specific IgG and the frequencies of C3 deposition may be explained by the similar ratios of PspA-specific IgG1 titers to IgG2a titers (1.32 to 2.33) among mice that were immunized nasally with a low dose of PspA plus each TLR agonist in this study, because the C3 binding activity of the IgG2a isotype is superior to those of other IgG isotypes [20]. Although antibacterial effects in the lung and blood were the highest in mice that were immunized nasally with a low dose of PspA plus LPS than those in mice immunized nasally with a low dose of PspA plus the other TLR agonists at 24 h post-infection, no significant difference was found in the survival rate of mice immunized nasally with a low dose of PspA plus each TLR agonist. This discrepancy may be explained by subsequent bacterial growth in the lungs and blood in mice that had been immunized nasally with a low dose of PspA plus LPS as well as mice immunized nasally with a low dose of PspA plus the other TLR agonists later than 24 h post-infection.

Despite similar C3 binding activities of immune sera, the rank orders for the survival rates of the immunized mice were LPS > Pam3CSK4 > Poly(I:C) and CpG 1826, and were in agreement with those of plasma levels of PspA-specific IgG reported in this study. Although LPS or Pam3CSK4 demonstrated a superior adjuvant effect among the TLR agonists tested in this study, these bacterial products are highly toxic to humans. In contrast, a synthetic analogue of a dsRNA compound, such as Poly (I:C) or Poly I:PolyC12 U (Ampligen®), or CpG ODN would be expected to be applicable as a safe mucosal adjuvant in humans [21,22].

In conclusion, the data presented here provide evidence to indicate that intranasal immunization with a vaccine containing a low dose of PspA plus each TLR agonist elicited a high level of systemic PspA-specific IgG, and was capable of preventing the development of fatal pneumococcal pneumonia in mice. An intranasal administration of each TLR agonist in combination with a low dose PspA significantly increased the survival rates of the infected mice in the following order: LPS > Pam3CSK4 > Poly(I:C) and CpG ODN 1826. This study confers an important insight regarding strategies for a cost-effective PspA protein-based vaccine against invasive pneumococcal infections.

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# The Nasal Dendritic Cell-Targeting Flt3 Ligand as a Safe Adjuvant Elicits Effective Protection against Fatal Pneumococcal Pneumonia▽

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We have previously shown that a pneumococcal surface protein A (PspA)-based vaccine containing DNA plasmid encoding the Flt3 ligand (FL) gene (pFL) as a nasal adjuvant prevented nasal carriage of Streptococcus pneumoniae. In this study, we further investigated the safety and efficacy of this nasal vaccine for the induction of PspA-specific antibody (Ab) responses against lung infection with S. pneumoniae. C57BL/6 mice were nasally immunized with recombinant PspA/Rx1 (rPspA) plus pFL three times at weekly intervals. When dynamic translocation of pFL was initially examined, nasal pFL was taken up by nasal dendritic cells (DCs) and epithelial cells (nECs) but not in the central nervous systems, including olfactory nerve and epithelium. Of importance, nasal pFL induced FL protein synthesis with minimum levels of inflammatory cytokines in the nasal washes (NWs) and bronchoalveolar lavage fluid (BALF). NWs and BALF as well as plasma of mice given nasal rPspA plus pFL contained increased levels of rPspA-specific secretory IgA and IgG Ab responses that were correlated with elevated numbers of $CD8^+$ and $CD11b^+$ DCs and interleukin 2 (IL-2)- and IL-4-producing $CD4^+$ T cells in the nasal mucosa-associated lymphoid tissues (NALT) and cervical lymph nodes (CLNs). The in vivo protection by rPspA-specific Abs was evident in markedly reduced numbers of CFU in the lungs, airway secretions, and blood when mice were nasally challenged with Streptococcus pneumoniae WU2. Our findings show that nasal pFL is a safe and effective mucosal adjuvant for the enhancement of bacterial antigen (Ag) (rPspA)-specific protective immunity through DC-induced Th2-type and IL-2 cytokine responses.

Streptococcus pneumoniae is a leading human pathogen causing diseases ranging from otitis media to pneumonia, bacteremia, and meningitis. This bacterium, commonly termed the pneumococcus, can result in an estimated 1.6 million deaths per year worldwide, more than half of which are young children in developing countries (2). Although pneumococcal capsular polysaccharide and pneumococcal protein-capsular conjugate vaccines can provide protective immunity against pneumonia and invasive diseases in adults and infants, a strong need still exists for a new generation of effective vaccines for the prevention of all potential S. pneumoniae infections. In this regard, the multivalent polysaccharide vaccines do not provide protection against strains with nonvaccine serotypes (28, 41). Of importance, pneumococcal surface protein A (PspA) has been extensively investigated as a candidate vaccine antigen (Ag) to prevent pneumococcal infection (5, 37). For instance, PspA-specific antibody (Ab) enhances bacterial clearance and induces cross-protection against infection with strains of different serotypes (4, 31). Further, previous studies have demonstrated that PspA-specific Abs overcome the anticomplementary effect of PspA, allowing increased complement activation and C3 deposition on PspA-bearing bacteria (27, 30).

Nasal immunization has been shown to preferentially induce Ag-specific Ab responses in the respiratory tract (20) and other mucosal lymphoid tissues (10, 25, 26). To induce maximal levels of Ag-specific immune responses in both mucosal and systemic lymphoid tissue compartments, it is often necessary to use a mucosal adjuvant (16, 22, 39). Although native cholera toxin and related Escherichia coli enterotoxin are potent mucosal adjuvant for enhancement of Ag-specific immune responses, their application for human use is not warranted since they can cause diarrhea or Bell's palsy (6, 23, 29). Moreover, these toxins are known to migrate into and accumulate in the olfactory tissues when given nasally (40). In this regard, our previous studies demonstrated that nasal application of a DNA plasmid (pFL) containing the gene of the Flt3 ligand (FL), which is a kind of cytokine, preferentially expanded $CD8^+$ dendritic cells (DCs) and subsequently induced Ag-specific mucosal immune responses mediated by interleukin 4 (IL-4)-producing $CD4^+$ T cells when mice were nasally administrated ovalbumin with pFL as the mucosal adjuvant (19). Further, a combination of nasal pFL and CpG oligonucleotides as a

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double DNA adjuvant enhanced mucosal and systemic immune responses via induction of plasmacytoid DCs as well as $CD8^+$ DCs in mucosal compartments (11, 17). Nasal administration of an adenovirus vector encoding FL cDNA also showed enhancement and maintenance of long-term immunity (17, 32).

In this study, we examined the safety and effectiveness of nasal pFL as a mucosal adjuvant for the induction of functional bacterial Ag (recombinant PspA [rPspA])-specific Ab responses for protection against *S. pneumoniae* infection in the lower respiratory tract. Our findings show that nasal rPspA plus pFL adjuvant successfully elicits protective immunity in both the upper and lower respiratory tracts by enhancing mucosal DC-mediated Th2-type and IL-2 cytokine responses without detectable cytokine-mediated inflammation.

## MATERIALS AND METHODS

Mice. Specific-pathogen-free female C57BL/6 mice (6 to 8 weeks old) were purchased from Charles River Japan (Kanagawa, Japan) and used in this study. Upon arrival, these mice were transferred to microisolators, maintained in horizontal laminar flow cabinets, and provided sterile food and water as part of a specific-pathogen-free facility at Osaka University (Suita, Japan), and all experiments were conducted in accordance with the guidelines provided by Osaka University. All of the mice used in these assays were free of bacterial and viral pathogens.

rPspA and adjuvants. Endotoxin-free rPspA was purified by chromatography on a chelating Sepharose 4B column preloaded with $Ni^+$ (GE Healthcare, Piscataway, NJ) from Escherichia coli BL21(DE3) carrying pUAB055, which comprised the first 302 of the 588 amino acids of PspA/Rx1, including all of the α-helical region and some of the proline-rich region (3). The plasmid pORF9-mFlt3L (pFL) consists of the pORF9-mcs vector (pORF) plus the full-length murine FL cDNA gene (InvivoGen, San Diego, CA). These plasmids were purified using the Gene Elute endotoxin-free plasmid kit (Sigma-Aldrich, St. Louis, MO) (19). The Limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD) resulted in <0.1 endotoxin unit of lipopolysaccharide (LPS) per 1 μg of plasmids or rPspA.

Nasal immunization and sample collection. Mice were immunized three times at weekly intervals nasally with 6 μl/nostril phosphate-buffered saline (PBS) containing 5 μg of rPspA and 50 μg of pFL as a mucosal adjuvant. As controls, mice were immunized nasally with 50 μg of pORF (empty plasmid) and 5 μg of rPspA under anesthesia. In some experiments, mice were administered pFL (50 μg), pORF (50 μg), rPspA (1 μg or 5 μg), native cholera toxin (nCT) (1 μg), or PBS alone under anesthesia. Plasma, nasal washes (NWs), and bronchoalveolar lavage fluid (BALF) were obtained as described previously (36).

Dynamic translocation of pFL. On 12 h or 7 days after mice were nasally given pFL (50 μg) alone, mononuclear cells were isolated from nasal mucosa-associated lymphoreticular tissues (NALT) and nasal passages (NPs) as described previously (14, 19), and NALT and NP dendritic cells (DCs) were purified by the AutoMACS cell sorter (Miltenyi Biotec, Auburn, CA) using anti-CD11c monoclonal Ab (MAb) microbeads (19). Further, nasal epithelial cells (nECs) and olfactory nerves and epithelium (ON/E) were isolated from nasal passages and olfactory bulbs, respectively (14, 40). In brief, cells from the nasal mucosa and olfactory bulb were prepared by gentle teasing through stainless screens and were subjected to discontinuous gradient centrifugation using 40% and 55% Percoll. Cells on the surface of the 40% layer were used as nECs and ON/E. To further confirm the presence of nECs and ON/E, the size and granularity of cells were determined by using flow cytometry. DNA was then extracted from NALT, NP-DCs, nECs, and ON/E, and the ampicillin resistance gene (858 bp) contained in the pFL plasmid was detected by a primer-specific PCR method. The sense primer was 5′-**CCA** ATG CTT AAT CAG TGA GGC-3′, and the anti-sense primer was 5′-**ATG** AGT ATT CAA CAT TTC CGT GTC G-3′. The PCR products were separated by electrophoresis in 2% agarose gels and visualized by UV light illumination following ethidium bromide (0.5 mg/ml) staining (19).

Analysis of FL protein synthesis. Twelve hours after nasal administration of pFL (50 μg), empty plasmid (50 μg), rPspA alone, or PBS, DCs from NALT and NPs and nECs and ON/E were purified aseptically as described above and were then cultured for 48 h ($2 \times 10^6$ cells/ml) in complete medium. The concentrations of FL protein secreted into the medium were determined by FL-specific enzyme-linked immunosorbent assay (ELISA) (Quantikine M mouse Flt3 ligand ELISA kit; R & D Systems, Minneapolis, MN). Mice were next nasally immunized weekly for three consecutive weeks with rPspA (5 μg) plus pFL (50 μg) or pORF (50 μg), rPspA alone (5 μg), or PBS, and 1 week after the last immunization, the FL protein in nasal washes (NWs) and bronchoalveolar lavage fluid (BALF) was determined by FL-specific ELISA (R & D Systems).

Detection of inflammatory cytokines in mucosal secretion. In order to determine inflammatory cytokines by nasal application of pFL, NWs and BALF were collected 5 days after the nasal administration of pFL (50 μg), pORF (50 μg), rPspA (1 μg or 5 μg), or native cholera toxin (1 μg). Next, the mucosal secretion samples were subjected to ELISA specific to IL-1β, IL-6 (R & D Systems), and tumor necrosis factor alpha (TNF-α) according to the manufacturer's instructions (eBioscience, San Diego, CA).

rPspA-specific Ab assays. In order to examine mucosal and systemic immune responses to Ag, rPspA-specific IgA and IgG antibody (Ab) levels in plasma, NWs, and BALF were determined by ELISA on day 7 after the last immunization, as described previously (18, 19, 32). Briefly, 96-well Falcon microtest assay plates (BD Biosciences, Oxnard, CA) were coated with 1 μg/ml of rPspA in PBS. After incubating serial dilutions of samples, horseradish peroxidase-conjugated goat anti-mouse IgM, IgG, IgG1, IgG2a, IgG2b, IgG3, or IgA (Southern Biotechnology Associates Inc., Birmingham, AL) was added to wells. The color reaction was developed for 15 min at room temperature. Endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution that gave an optical density at 415 nm ($OD_{415}$) of 0.1 greater than the background level. Further, mononuclear cells obtained from spleen, NALT, cervical lymph nodes (CLNs), mediastinal lymph nodes (MeLNs), NPs, and lungs were subjected to an enzyme-linked immunospot (ELISPOT) assay in order to determine the numbers of Ag-specific Ab-forming cells (AFCs) (18, 19). In brief, mononuclear cells in the spleen, NALT, CLNs, and MeLNs were isolated aseptically by a mechanical dissociation method using gentle teasing through stainless steel screens as described previously (14). For isolation of mononuclear cells from NPs, a modified dissociation method was used based upon a previously described protocol (18). Mononuclear cells from lungs were isolated by a combination of an enzymatic dissociation procedure with collagenase type IV (0.5 mg/ml; Sigma-Aldrich) followed by discontinuous Percoll (Amersham Biosciences, Arlington Heights, IL) gradient centrifugation.

Flow cytometric analysis. To characterize the phenotype of DCs, aliquots of mononuclear cells ($0.2 \times 10^6$ to $1.0 \times 10^6$ cells) were isolated from various lymphoid compartments 1 week after the last immunization with rPspA plus pFL or pORF. The cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD11b, CD8, or B220 MAbs (BD Biosciences). In some experiments, mononuclear cells were incubated with phycoerythrin (PE)-labeled anti-mouse $I\text{-}A^b$, CD11c, CD40, CD80, or CD86 MAbs (BD Biosciences) and biotinylated anti-mouse CD11c MAbs (BD Biosciences), followed by CyChrome-streptavidin. These samples were then subjected to flow cytometry analysis (FACSCalibur; BD Biosciences) for cell subset analysis (19).

rPspA-specific $CD4^+$ T cell responses and cytokine-specific ELISA. $CD4^+$ T cells from lungs, CLNs, and spleen were purified using an automatic cell sorter (AutoMACS) system (Miltenyi Biotec) as described previously (18, 19). The purified $CD4^+$ T cell fraction (>97% $CD4^+$ and >99% viable) was resuspended in RPMI 1640 (Sigma-Aldrich) supplemented with HEPES buffer (10 mM), L-glutamine (2 mM), nonessential amino acid solution (10 μl/ml), sodium pyruvate (10 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (80 μg/ml), and 10% fetal calf serum (FCS) (complete medium; $4 \times 10^6$ cells/ml) and cultured in the presence of T cell-depleted, complement- and mitomycin-treated splenic Ag-presenting cells taken from nonimmunized, normal mice with or without 2 μg/ml rPspA. To assess rPspA-specific T cell proliferative responses, an aliquot of 0.5 μCi of tritiated [$^3$H]TdR (PerkinElmer Japan Co., Ltd., Japan) was added during the final 18 h of incubation, and the amount of [$^3$H]TdR incorporation was determined by scintillation counting (19). The culture supernatants were collected on day five and analyzed using gamma interferon (IFN-γ)-, IL-2-, IL-4-, IL-5-, IL-6-, and IL-10-specific ELISA kits (eBioscience). The detection limit for each cytokine was as follows: 15 pg/ml for IFN-γ, 2 pg/ml for IL-2, 4 pg/ml for IL-4, IL-5, and IL-6, and 30 pg/ml for IL-10.

Pneumococcal infection. Mice were nasally challenged with a serotype 3 *S. pneumoniae* strain (WU2) with a mucoid phenotype at a dose of $1.8 \times 10^7$

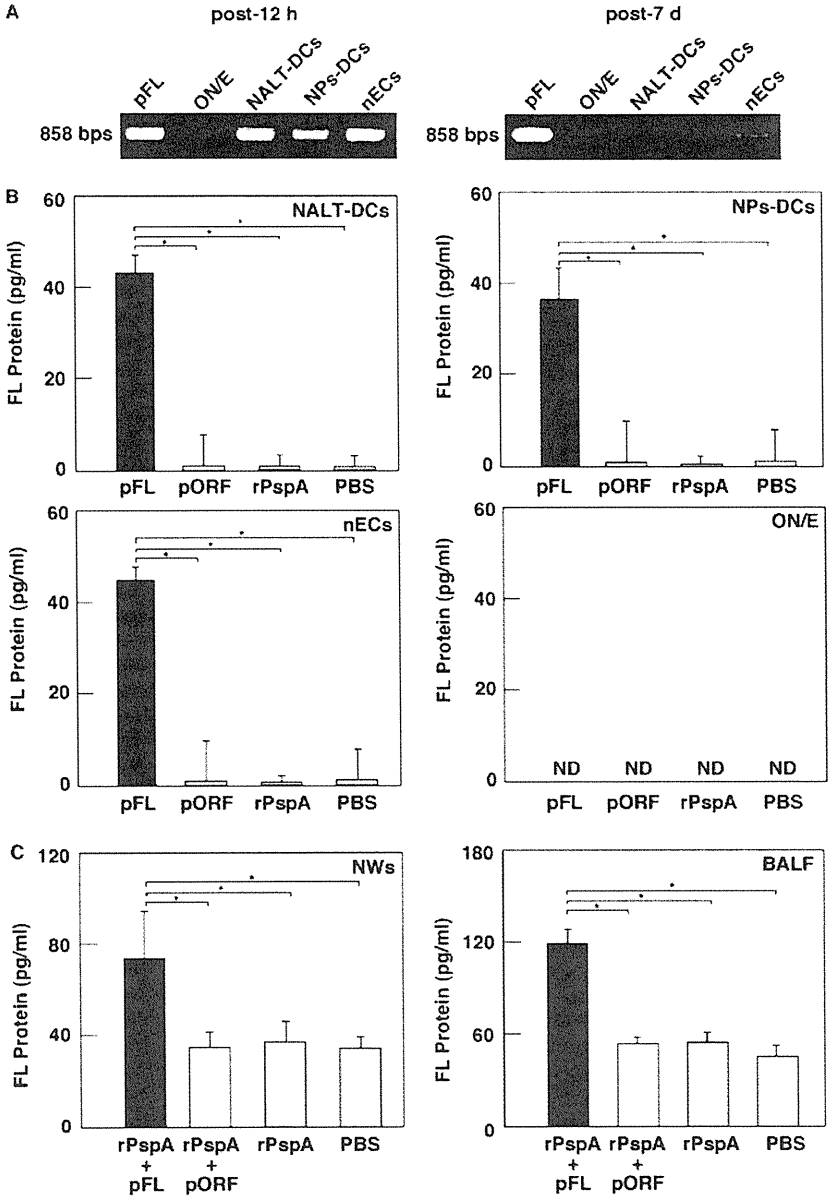


FIG. 1. (A to C) Translocation of FL plasmid after nasal administration of pFL (A), FL protein production by nasal DCs and epithelial cells (B), and expression of the FL protein in mucosal secretions (C). (A) Twelve hours (left) or 7 days (right) after nasal application of pFL (50 μg), the DNA samples were extracted from $1.0 \times 10^5$ (each) cells of the olfactory nerve and epithelium (ON/E; lane 2), NALT-DCs (lane 3), NPs-DCs (lane 4), and nasal epithelial cells (nECs; lane 5). In order to show the presence of plasmid in these cell populations, the ampicillin resistance gene (858 bp) contained in pFL was detected by PCR using specific primers. pFL (0.1 μg) was employed as a positive control (lane 1). (B) Mice were nasally administered pFL (50 μg; black column), pORF (50 μg; white column), rPspA (5 μg; shaded column), or PBS (hatched column). Twelve hours later, NALT-DCs, NPs-DCs, nECs, and ON/E were isolated and cultured ($2 \times 10^6$ cells/ml, respectively) for 48 h in complete medium. The concentration of FL protein secreted in medium was measured by FL-specific ELISA. The values shown are the means ± SEM for 30 mice for each group and a total of three experiments. *, $P < 0.05$ compared with results for the mouse group given pORF, rPspA, or PBS. (C) Mice were nasally immunized weekly for three consecutive weeks with rPspA (5 μg) plus pFL (50 μg; black column) or pORF (50 μg; white column), rPspA alone (5 μg; shaded column), or PBS (hatched column). One week after the last immunization, NWs and BALF (100 μl, respectively) were collected and subjected to FL-specific ELISA. The values shown are the means ± SEM of data for 30 mice for each group and a total of three experiments. *, $P < 0.05$ compared with results for mouse group given pORF, rPspA, or PBS.

CFU (20 μl). Forty-eight hours after the bacterial challenge, the lungs were removed aseptically and homogenized in 9 ml of sterile saline per gram of lung tissues. NWs and blood were collected as described above. Bacterial colonies were counted by plating lungs, NWs, and blood (50 μl, respectively) on horse blood agar (BD Biosciences), followed by incubation at 37°C overnight. The detection limit of bacterial culture was $10^2$ CFU/g. The 50% lethal dose was calculated to be $2.5 \times 10^6$ CFU.

Statistical analysis. Each result is expressed as the mean ± 1 standard error of the mean (SEM). All mouse groups were compared with control mice using an unpaired Mann-Whitney U test by using the Statview software program

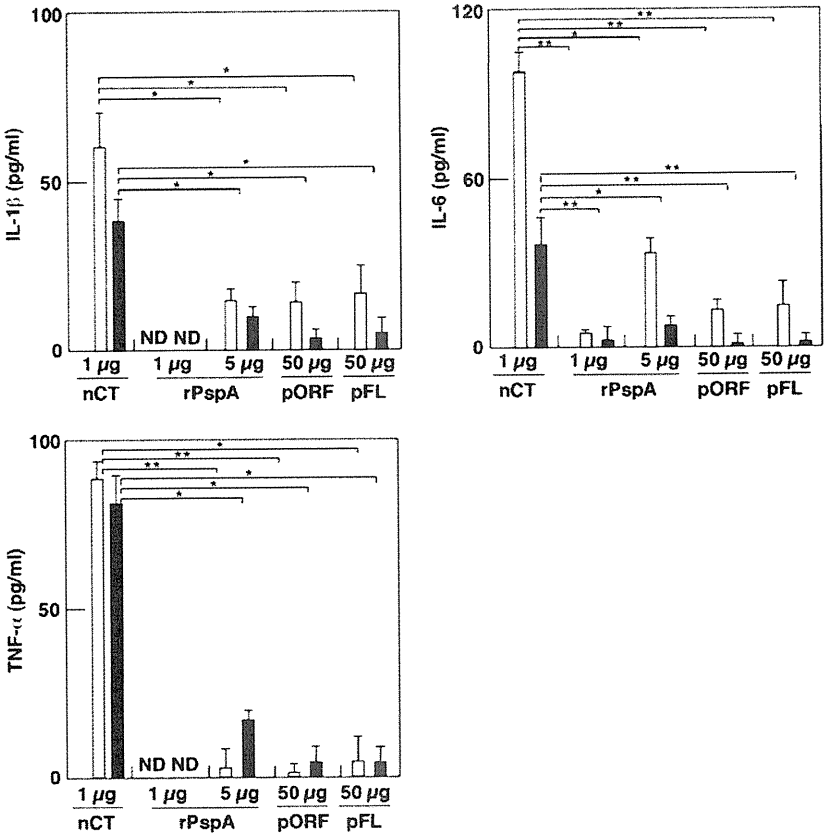


FIG. 2. Inflammatory cytokine production in NWs (white column) and BALF (black column). Mice were nasally administered native cholera toxin (nCT) (1 μg), rPspA (1 or 5 μg), pORF (50 μg), or pFL (50 μg). Five days later, NWs and BALF were collected and subjected to IL-1β-, IL-6-, and TNF-α-specific ELISA. The values shown are the means ± SEM of data for 30 mice for each group and total of three experiments. *, $P < 0.05$; **, $P < 0.01$ (compared with results for mouse group given nCT).

(Abacus Concepts, Cary, NC), designed for Macintosh computers, with Bonferroni's correction. P values of <0.05 or <0.01 were considered significant.

## RESULTS

Tracking plasmid expression and FL protein synthesis. In order to examine safety of pFL for nasal application, we initially traced plasmid-specific ampicillin resistance gene expression by nasal DCs, nECs, and the ON/E. DCs from NALT and NPs, as well as nECs, possessed the ampicillin resistance gene 12 h after nasal administration of pFL (Fig. 1A, left). Of interest, on 7 days after nasal pFL application, the ampicillin resistance gene was detected only in nECs (Fig. 1A, right). Further, NALT-DCs, NP-DCs, and nECs of mice given nasal pFL produced significantly elevated levels of the FL protein compared with those of mice given nasal pORF (empty plasmid), rPspA alone, or PBS (Fig. 1B). In addition, nasal application of the combination of rPspA and pFL resulted in FL protein production comparable to that with nasal application of pFL alone (data not shown). However, FL protein synthesis in mice given nasal rPspA plus pORF was at essentially the same level as that seen in mice given pORF or rPspA alone (data not shown). Thus, NWs and BALF from mice given nasal pFL plus rPspA contained significantly higher levels of FL than those from mice given nasal pORF plus rPspA, rPspA alone, or PBS only (Fig. 1C). On the other hand, of importance, no plasmid-specific genes were essentially detected in the ON/E of mice given nasal pFL (Fig. 1A). Thus, the culture supernatants of ON/E did not contain detectable levels of the FL protein (Fig. 1B). These results show that pFL is largely present in nasal DCs and nECs but not in the ON/E and suggest that pFL on nECs may maintain production of the FL protein.

Nasal pFL induces lower levels of inflammatory cytokines than nCT. Although pFL was not taken up by the central nervous system, it is important to show that FL produced in the nasal cavity does not induce inflammatory responses. In this regard, the levels of IL-1β, IL-6, and TNF-α production in NWs and BALF were examined 5 days after nasal administration with rPspA, pORF, native cholera toxin (nCT), or pFL. The levels of inflammatory cytokine synthesis in NWs and BALF of mice given nasal pFL were essentially the same as or lower than that of mice given nasal rPspA or pORF alone (Fig. 2). Similarly, nasal application of rPspA plus pFL resulted in low levels of inflammatory cytokine production which were similar to those seen in pFL alone (data not shown). Conversely, nasal nCT induced markedly high levels of these inflammatory cytokines (Fig. 2). These results show that nasal pFL application does not elicit unnecessary inflammatory responses in the nasal mucosa.

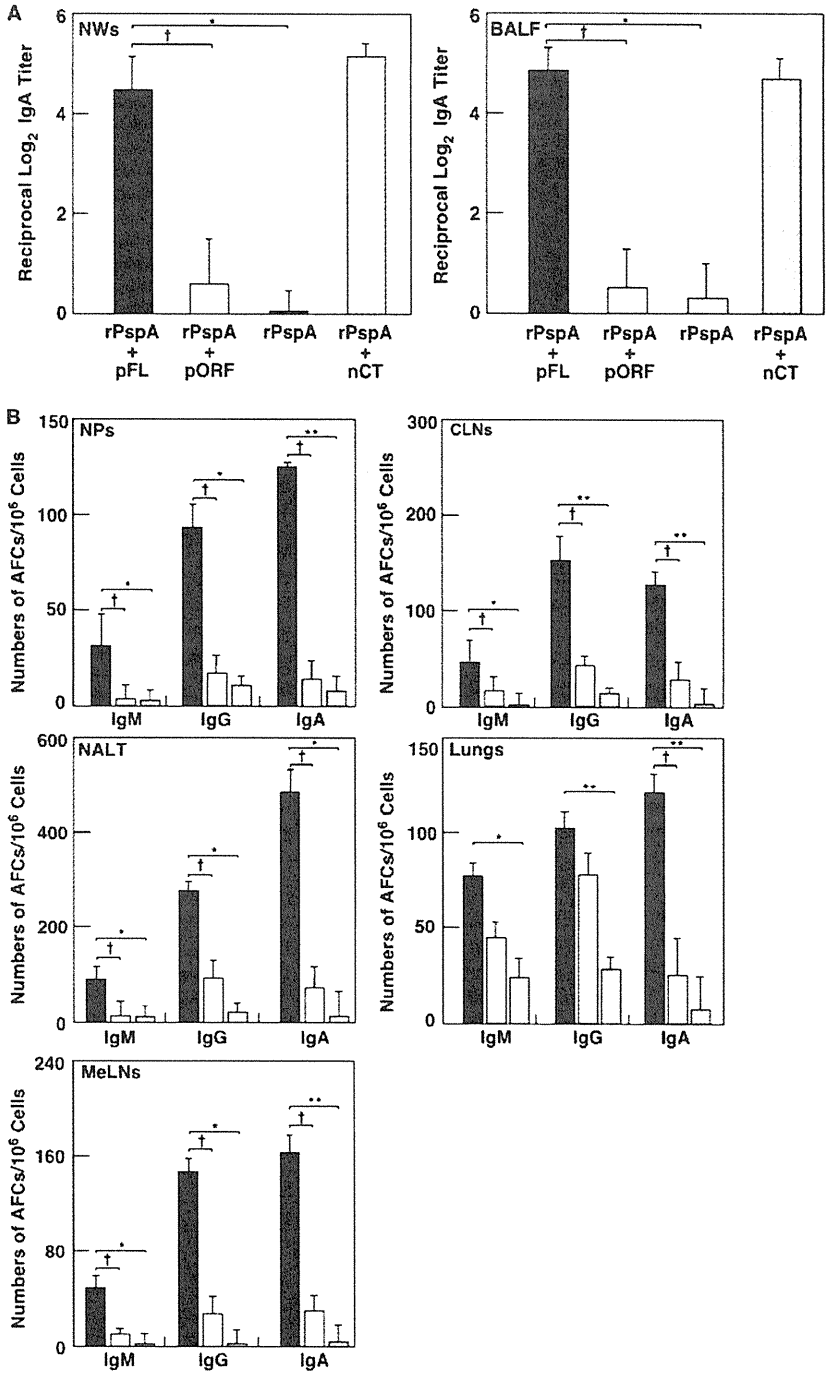


FIG. 3. Mucosal immune responses to rPspA in external secretions and mucosal lymphoid tissues. C57BL/6 mice were nasally immunized three times at weekly intervals with rPspA (5 μg) plus pFL (50 μg; black column) or pORF (50 μg; white column), rPspA alone (shaded column), or rPspA (5 μg) plus nCT (1 μg; hatched column). (A) Seven days after the last immunization, the levels of rPspA-specific IgA Abs in NWs and BALF were determined by rPspA-specific ELISA. (B) Seven days after the last immunization, mononuclear cells isolated from NPs, CLNs, NALT, lungs, and MeLNs were subjected to ELISPOT assay to determine the numbers of Ag-specific IgM, IgG, and IgA Ab-forming cells (AFCs). The values shown are the means ± SEM (n = 20). *, $P < 0.05$; **, $P < 0.01$ (compared with mouse group given rPspA alone). †, $P < 0.05$ (compared with mouse group given rPspA plus pORF).

Induction of rPspA-specific Ab responses in mucosal and systemic tissues of mice given rPspA plus pFL. We next examined whether nasal administration of pFL as a mucosal adjuvant would enhance rPspA-specific Ab responses. Giving mice nasal rPspA plus pFL resulted in significantly increased levels of rPspA-specific IgA Ab responses in NWs and BALF compared with results for mice given nasal rPspA plus pORF or rPspA Ag alone (Fig. 3A). The levels of rPspA-specific IgA