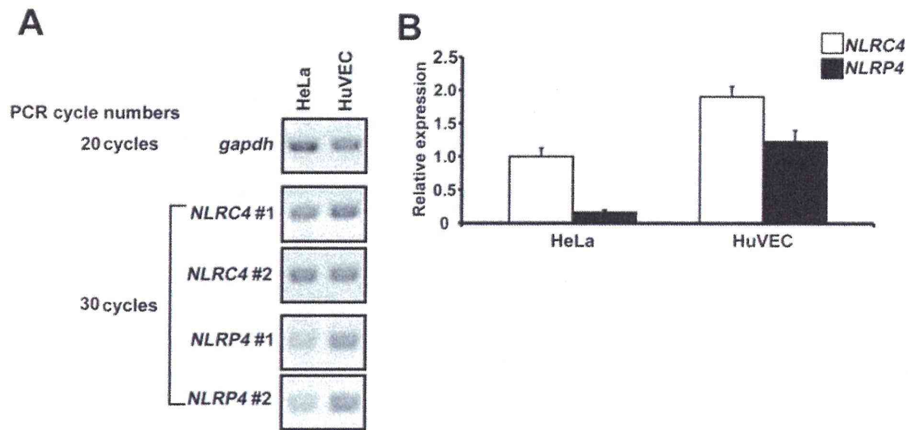
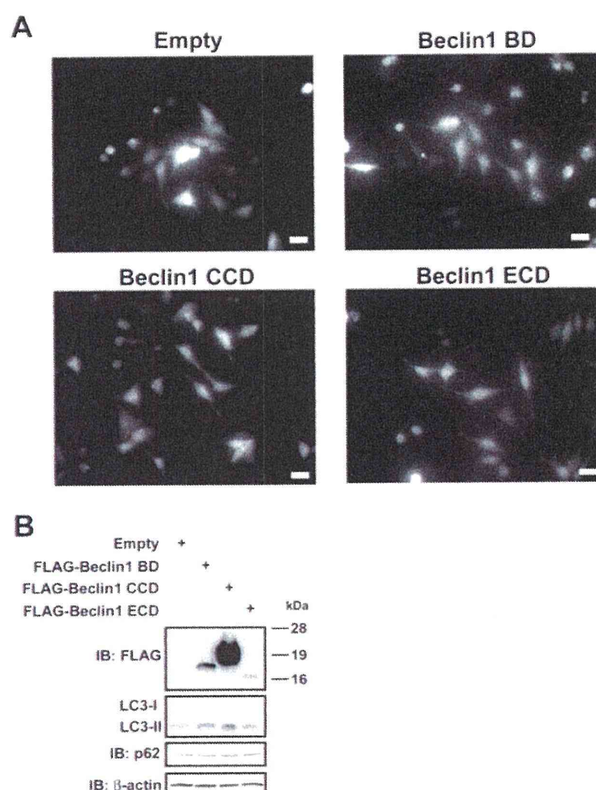


Supplemental Figure 3. The RNA level of *NLRC4* and *NLRP4* in HeLa cells or HuVECs.

A and *B*, The total RNA was extracted from HeLa cells or HuVECs, and then was reverse-transcribed. The cDNA were subjected to PCR (A) or quantitative PCR (B). *B*, Quantitative PCR was performed with NLRC4#1 or NLRP4#1 primer pairs (Table III). The *gapdh* was used as an internal control. The data showed relative expression to the level of *NLRC4* in HeLa cells (*NLRC4* in HeLa cells = 1). The graph shows the mean ± SD.

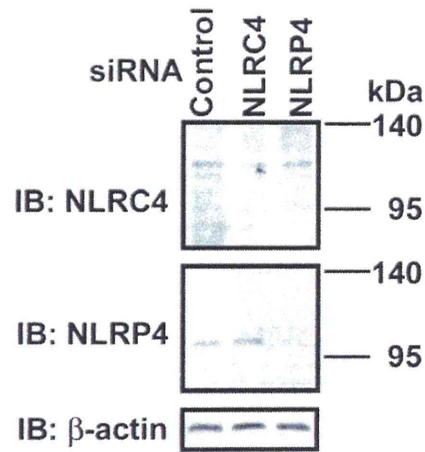
Downloaded from http://jimmunol.org/ by guest on May 24, 2012



Supplemental Figure 4. Beclin1 ECD does not function as a modulator of autophagy in HeLa cells.

A, HeLa cells were co-transfected with GFP-LC3 and truncated mutants of Beclin1, and then fluorescent microscopy analyses were performed at 24 h post-transfection. Scale bar = 20 μm. *B*, Cells pre-transfected with indicated plasmids were cultured for 24 h without protease inhibitors. Cells were lysed, and were subjected to immunoblotting analysis to examine the levels of LC3-II and p62.

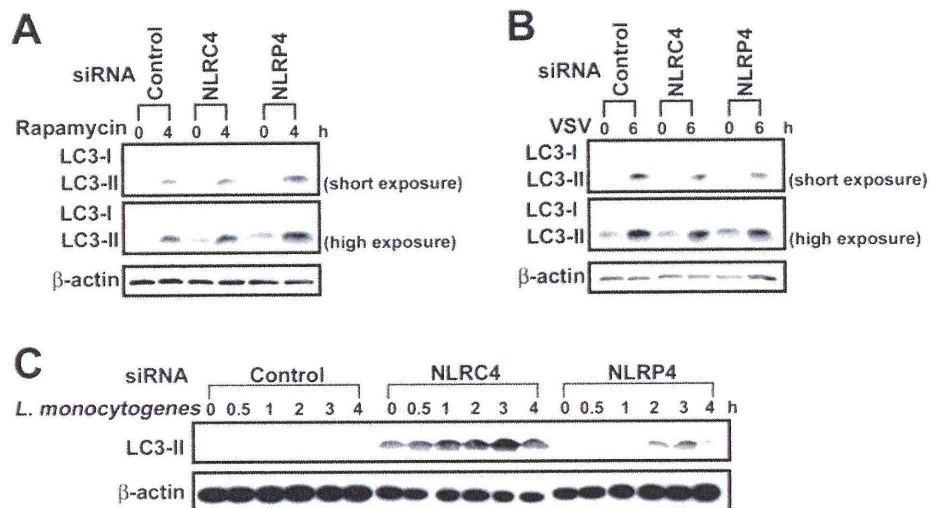
Downloaded from http://jimmunol.org/ by guest on May 24, 2012



Supplemental Figure 5. Reduction of NLRC4 or NLRP4 protein by transfection with NLRC4 or NLRP4 siRNA.

HuVECs were transfected with indicated siRNAs. At 48 h after transfection with NLRC4 or NLRP4 siRNA, the cells were lysed, and then immunoblotting analysis was performed.

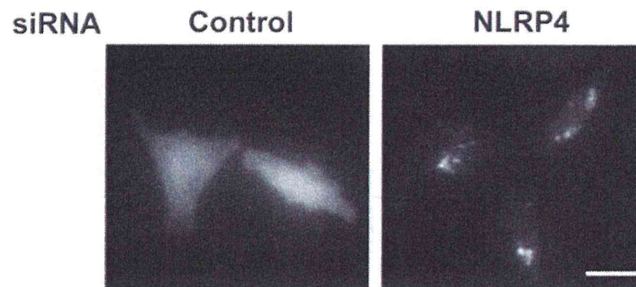
Downloaded from http://jimmunol.org/ by guest on May 24, 2012



Supplemental Figure 6. NLRC4 and NLRP4 negatively regulate the autophagy by invasive bacteria but not by rapamycin or VSV.

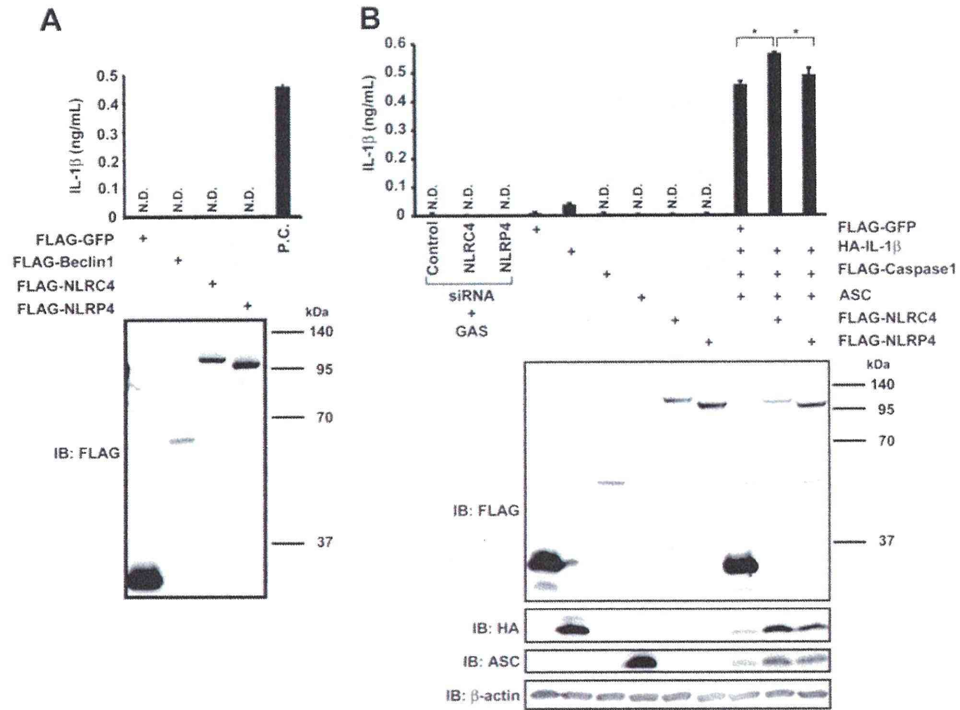
A, *B* and *C*, HeLa cells pre-treated with siRNA were additionally treated with protease inhibitors for 4 h, and then were further treated with 1 μM of rapamycin (*A*) or were infected with VSV at an MOI of 1 (*B*) or *Listeria monocytogenes* at an MOI of 50 (*C*) for the indicated period. The levels of LC3-II in each sample were examined by immunoblotting analysis.

Downloaded from http://jimmunol.org/ by guest on May 24, 2012



Supplemental Figure 7. The knockdown of NLRP4 enhances GFP-LC3 vacuolization. HeLa cells were co-transfected with NLRP4 siRNA and GFP-LC3 expression plasmid, and then fluorescent microscopy analysis was performed. Representative pictures were shown from 10-15 cells examined. Scale bar = 20 μm.

Downloaded from http://jimmunol.org/ by guest on May 24, 2012



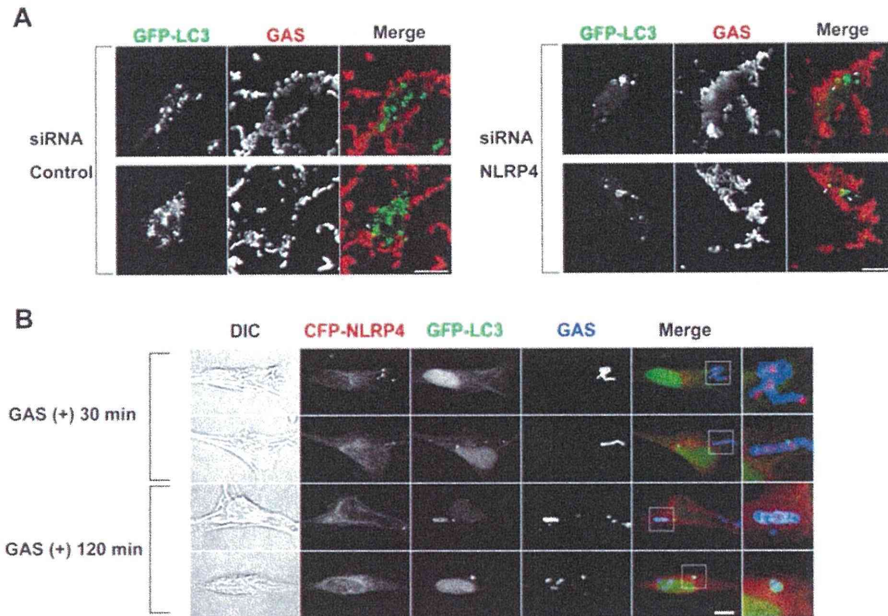
Supplemental Figure 8. NLRP4 does not regulate IL-1β maturation through the inflammasome activation in HeLa cells.

A, HeLa cells were transfected with indicated plasmids, and were cultured for 24 h. The cells were primed with 1 μg/ml of LPS for 16 h, and then were stimulated with 5 mM of ATP for 3 h. The supernatants were recovered, and were subjected to cytokine ELISA. P.C. = positive control by using 0.5 ng/mL IL-1β protein standard solution. N.D. = not detected. The graph shows the mean ± SD. *B*, HeLa cells pretreated with siRNA were

Downloaded from http://jimmunol.org/ by guest on May 24, 2012

infected with GAS at an MOI of 50 for 4 h. The supernatants were recovered, and were subjected to IL-1β ELISA. Furthermore, to confirm the function of NLRP4 in the inflammasome activation, HeLa cells were transfected with the plasmids encoding each inflammasome components. At 24 h post-transfection, culture supernatants were recovered, and were subjected to IL-1β ELISA. N.D. = not detected. The graph shows the mean ± SD where * indicates $p < 0.05$.

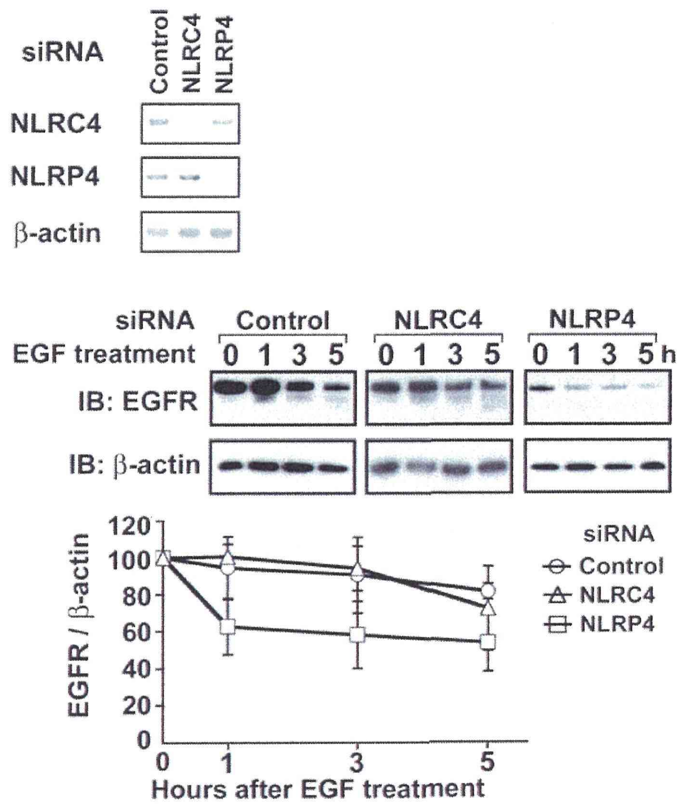
Downloaded from http://jimmunol.org/ by guest on May 24, 2012



Supplemental Figure 9. The dynamics of subcellular localization of NLRP4 and LC3 following GAS infection.

A, Other representative pictures as shown in Fig. 4*A*. Arrowheads in merge images showed GAS-containing LC3 vacuoles. Scale bar = 20 μm. *B*, Other representative pictures as shown in Fig. 4*C*. Scale bar = 20 μm. Three independent experiments gave similar results.

Downloaded from http://jimmunol.org/ by guest on May 24, 2012



Supplemental Figure 10. NLRP4 controls the endocytic degradation process of EGFR.

A549 cells were transfected with control dsRNA or pooled dsRNAs targeting NLRC4 or NLRP4. Forty-eight hours after transfection, the cells were treated with EGF (200 ng/ml) for the indicated period. The levels of EGFR degradation were examined by immunoblotting analysis. Representative immunoblotting images of at least three independent experiments are shown.

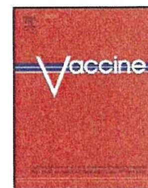
Downloaded from http://jimmunol.org/ by guest on May 24, 2012

Vaccine 29 (2011) 1754–1761

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine



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

Hirokazu Ezoe[a], Yukihiro Akeda[a], Zhenyu Piao[a], Taiki Aoshi[b,c], Shohei Koyama[e], Takeshi Tanimoto[d], Ken J. Ishii[b,c], Kazunori Oishi[a,*]

[a] Laboratory of Clinical Research on Infectious Diseases, International Research Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Japan
[b] Laboratory of Adjuvant Innovation, National Institute of Biomedical Innovation, Japan
[c] Laboratory of Vaccine Science, WPI Immunology Frontier Research Center, Osaka University, Japan
[d] The Research Foundation for Microbial Diseases, Osaka University, Kagawa, Japan
[e] Department of Respiratory Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan

ARTICLE INFO

Article history:
Received 27 September 2010
Received in revised form 7 December 2010
Accepted 23 December 2010
Available online 8 January 2011

Keywords:
Influenza virus
Secondary pneumonia
Streptococcus pneumoniae
PspA
Poly(I:C)
Nasal vaccine

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.
© 2010 Elsevier Ltd. All rights reserved.

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.

* Corresponding author. Tel.: +81 6 6879 4253; fax: +81 6 6879 4255.
E-mail address: oishik@biken.osaka-u.ac.jp (K. Oishi).

0264-410X/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2010.12.117

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 1×10^3 plaque-forming units (PFU) of influenza virus (H1N1) A/New Caledonia strain in 30 μl of PBS and infected intranasally with 6×10^3 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 1×10^8 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 1×10^3 PFU of influenza virus and infected intranasally with 6×10^3 CFU of *S. pneumo-*

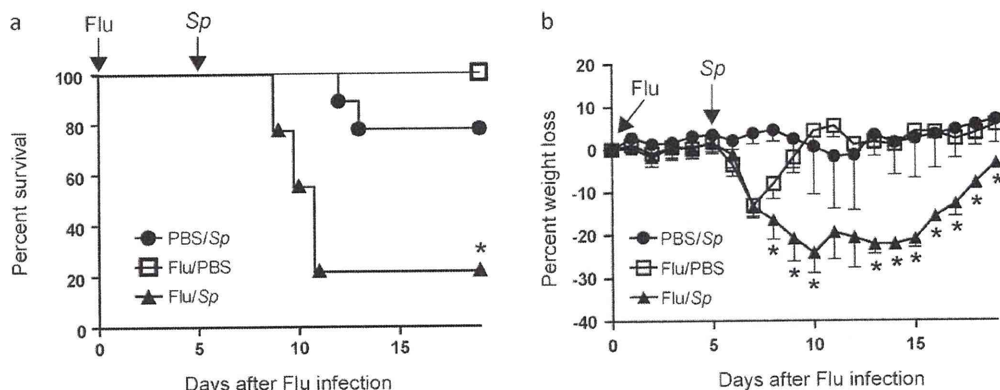


Fig. 1. Survival (a) and change in body weight (b) of mice after influenza virus infection. The data for the mice ($n=9$) administered PBS nasally, followed 5 days later by administration of *S. pneumoniae* are shown as PBS/*Sp* (closed circles). The data for mice ($n=7$–9) that received influenza virus,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

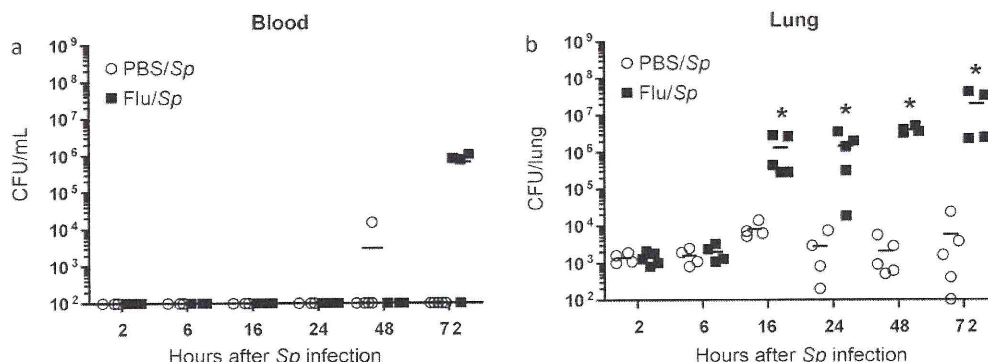


Fig. 2. Bacterial densities in blood (a) and lung (b) of mice given PBS (open circles) or influenza virus (closed squares) nasally followed by administration of *S. pneumoniae*. Lung tissue and blood were harvested 2, 6, 16, 24, 48, and 72 h after pneumococcal challenge, and bacterial density was counted. 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.

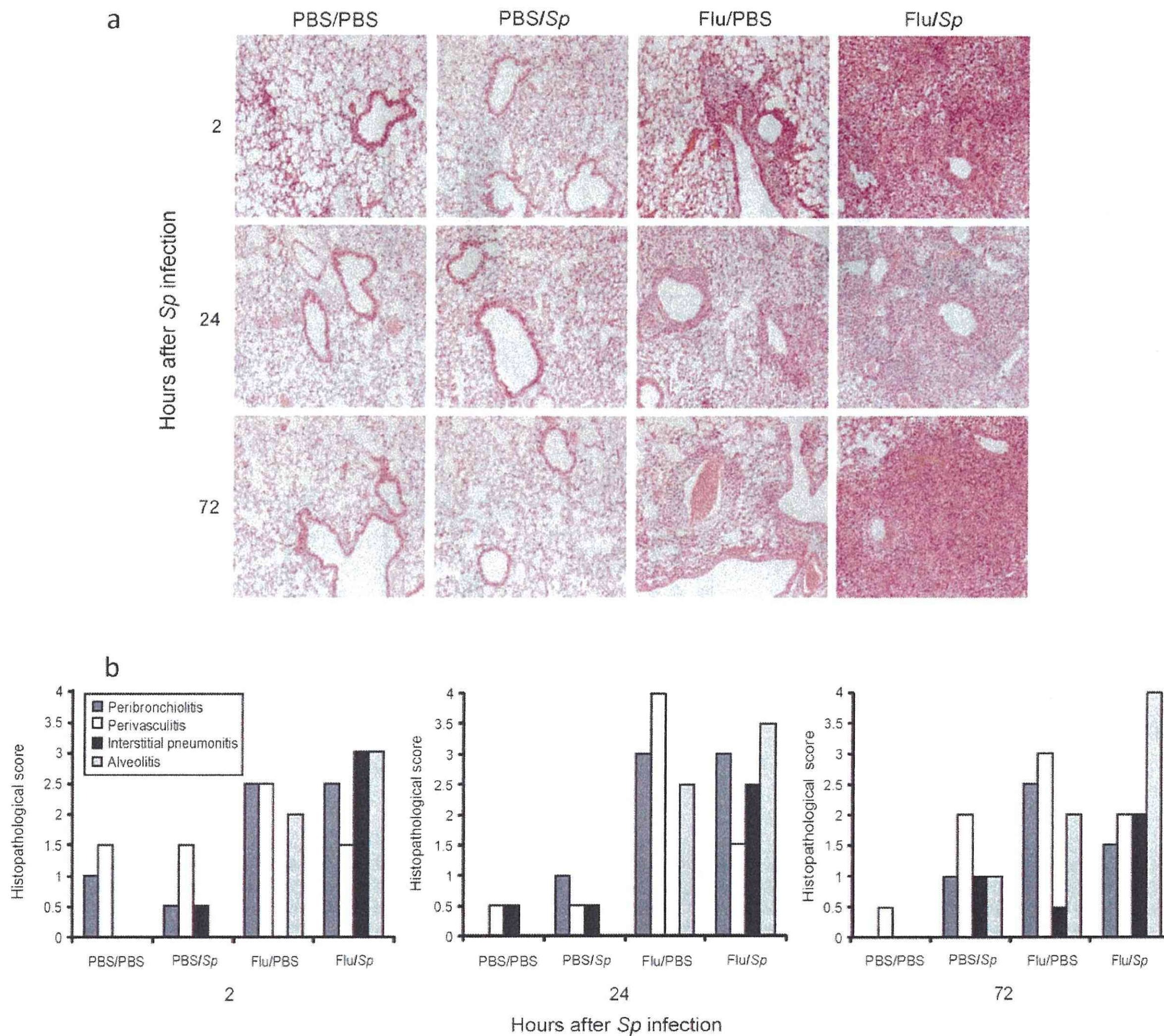


Fig. 3. Histopathological findings in lung tissues from mice with secondary pneumonia (a) and the histopathological scores of lung tissues (b). Mice were given PBS nasally followed by administration of PBS (PBS/PBS), PBS followed by administration of *S. pneumoniae* (PBS/*Sp*), influenza virus followed 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

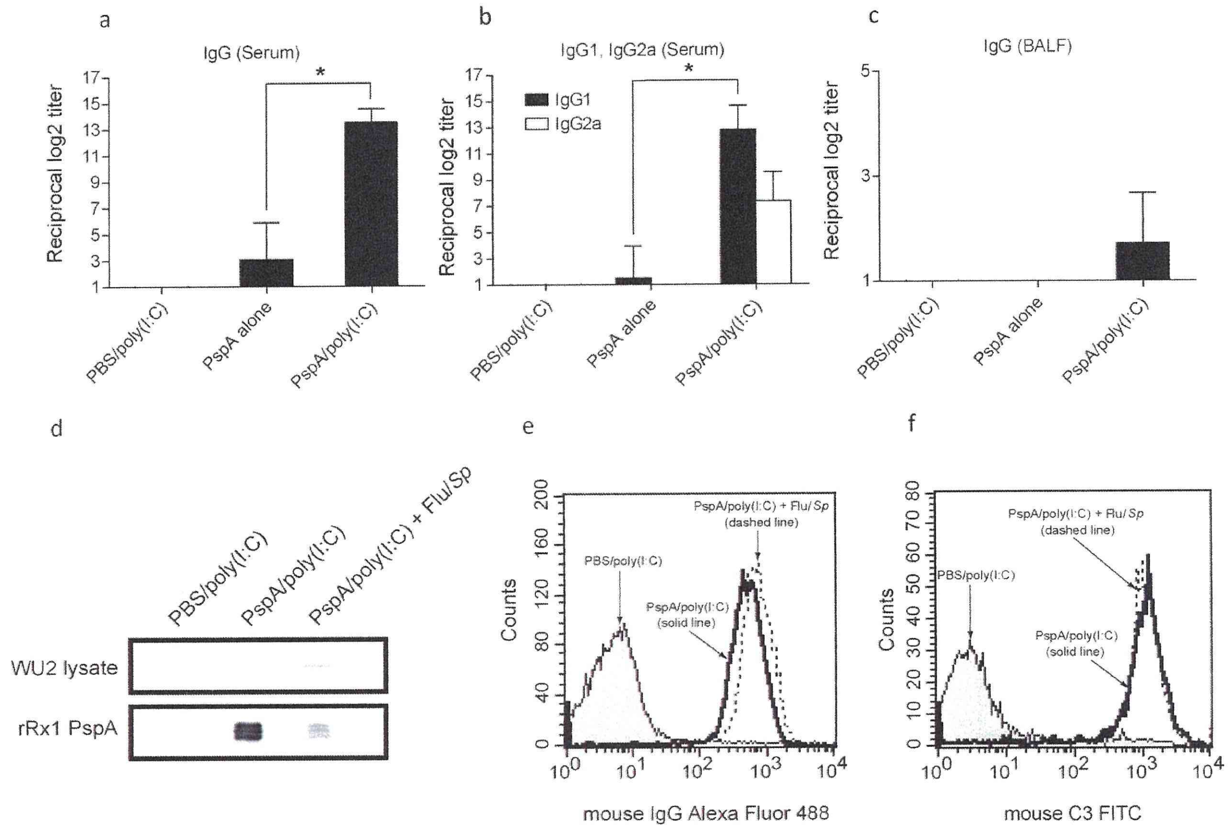


Fig. 4. Induction of PspA-specific IgG levels in serum (a), IgG1 and IgG2a levels in serum (b), and IgG levels in BAL fluid (c), immunoblot analysis of lysates of *S. pneumoniae* WU2 strain and recombinant Rx1 PspA with sera from mice immunized with PBS/poly(I:C) and PspA/poly(I:C) (d), the binding of 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-

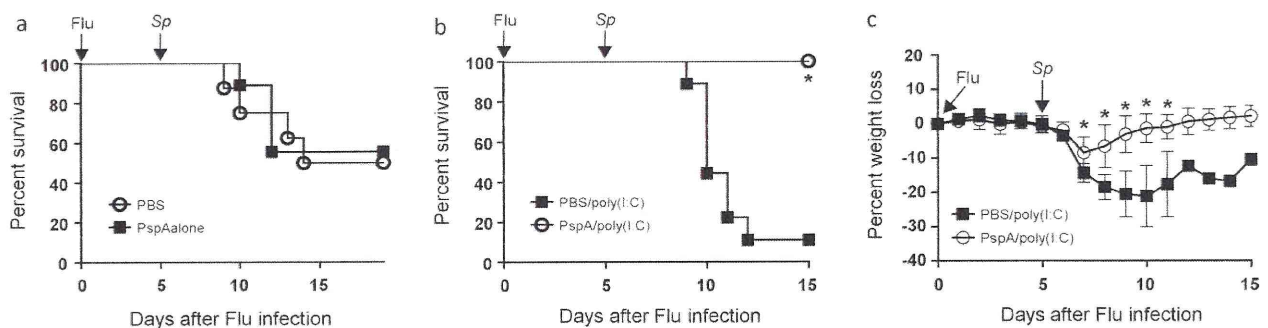


Fig. 5. Survival of C57BL/6 mice immunized nasally with PspA alone or with PBS after secondary pneumonia (a), nasally immunized with PBS/poly(I:C) or with PspA/poly(I:C) after secondary pneumonia (b), and the change in body weight of C57BL/6 mice immunized nasally with PspA/poly(I:C) or PBS/poly(I:C) 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).

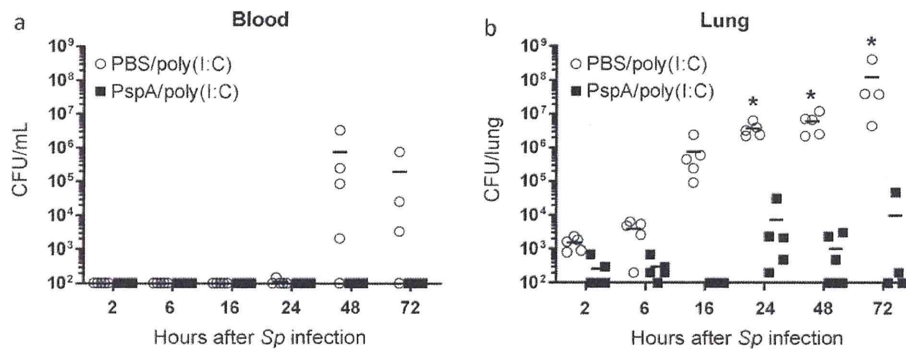


Fig. 6. Bacterial densities in blood (a) and lung (b) of mice immunized nasally with PBS/poly(I:C) (open circles) or PspA/poly(I:C) (closed squares) after secondary pneumonia. Lungs and blood were harvested 2, 6, 16, 24, 48, and 72 h after pneumococcal infection, and bacterial density was counted. Values 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).

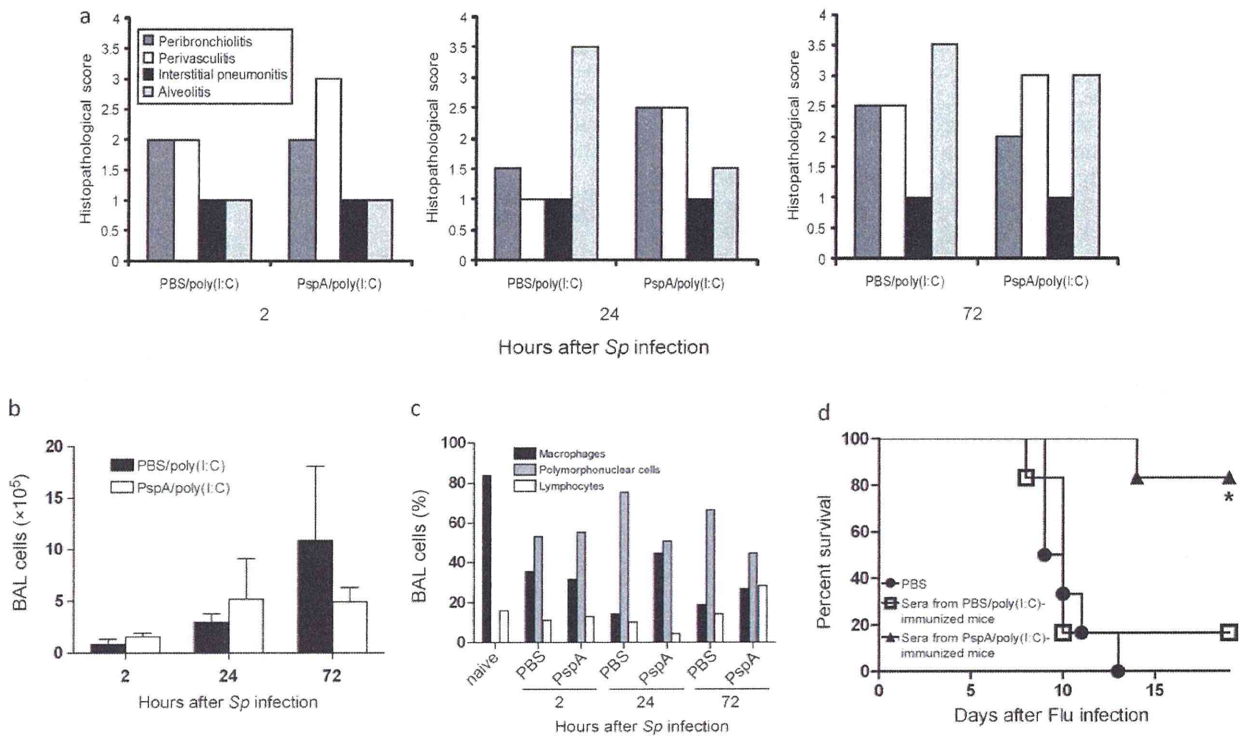


Fig. 7. Histopathological findings of lung tissues (a), the total cell count (b), and differential cell count (c) of BAL cells from nasally immunized mice after secondary pneumonia, and the effect of passive immunization in mice infected with secondary pneumonia (d). Nasally immunized mice were sacrificed 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 2×10^7 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 1×10^3 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.

Acknowledgments

We thank D.E. Briles, University of Alabama at Birmingham, for providing the pneumococcal strain WU2 and the recombinant plasmid pUAB055. We are also grateful to K. Ichida, M. Takahashi, and M. Tsujimoto, Laboratory Diagnostic Service Division, The Research Foundation of Microbial Diseases, Osaka University, for their technical assistance and comments on the histological material.

This work was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), and by the Biomedical Cluster Kansai project, which is promoted by the Regional Innovation Cluster Program and subsidized by the Japanese Government, and by Grants-in-Aid from the Ministry of Health, Labor and Welfare of Japan for "Mechanisms, epidemiology, prevention and control of acute respiratory infection".

References

[1] Rothberg MB, Haessler SD, Brown RB. Complications of viral influenza. Am J Med 2008;121:258–64.
[2] Morens DM, Taubenberger JK, Fauci AS. Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. J Infect Dis 2008;198:962–70.
[3] Chien YW, Klugman KP, Morens DM. Bacterial pathogens and death during the 1918 influenza pandemic. N Engl J Med 2009;361:2582–3.
[4] Louie JK, Acosta M, Winter K, Jean C, Gavali S, Schechter R, et al. Factors associated with death or hospitalization due to pandemic 2009 influenza A(H1N1) infection in California. JAMA 2009;302:1896–902.
[5] Perez-Padilla R, de la Rosa-Zamboni D, Ponce de Leon S, Hernandez M, Quinones-Falconi F, Bautista E, et al. Pneumonia and respiratory failure from swine-origin influenza A (H1N1) in Mexico. N Engl J Med 2009;361:680–9.
[6] Champunot R, Tanjatham S, Kerdsin A, Puangpatra P, Wangsai S, Treebuphachatsakul P, et al. Impact of pandemic influenza (H1N1) virus-associated community-acquired pneumonia among adults in a tertiary hospital in Thailand. Jpn J Infect Dis 2010;63:251–6.
[7] Estenssoro E, Rios FG, Apezteguia C, Reina R, Neira J, Ceraso DH, et al. Pandemic 2009 influenza A in Argentina: a study of 337 patients on mechanical ventilation. Am J Respir Crit Care Med 2010;182:41–8.
[8] 2009 Pandemic influenza A (H1N1) virus infections—Chicago, Illinois, April–July 2009. MMWR Morb Mortal Wkly Rep 2009;58:913–8.
[9] Klugman KP, Madhi SA, Huebner RE, Kohberger R, Mbelle N, Pierce N. A trial of a 9-valent pneumococcal conjugate vaccine in children with and those without HIV infection. N Engl J Med 2003;349:1341–8.
[10] Cutts FT, Zaman SM, Enwere G, Jaffar S, Levine OS, Okoko JB, et al. Efficacy of nine-valent pneumococcal conjugate vaccine against pneumonia and invasive pneumococcal disease in The Gambia: randomised, double-blind, placebo-controlled trial. Lancet 2005;365:1139–46.

[11] Shapiro ED, Berg AT, Austrian R, Schroeder D, Parcells V, Margolis A, et al. The protective efficacy of polyvalent pneumococcal polysaccharide vaccine. N Engl J Med 1991;325:1453–60.
[12] Talkington DF, Brown BG, Tharpe JA, Koenig A, Russell H. Protection of mice against fatal pneumococcal challenge by immunization with pneumococcal surface adhesin A (PsaA). Microb Pathog 1996;21:17–22.
[13] Ogunniyi AD, Woodrow MC, Poolman JT, Paton JC. Protection against *Streptococcus pneumoniae* elicited by immunization with pneumolysin and CbpA. Infect Immun 2001;69:5997–6003.
[14] Arulanandam BP, Lynch JM, Briles DE, Hollingshead S, Metzger DW. Intranasal vaccination with pneumococcal surface protein A and interleukin-12 augments antibody-mediated opsonization and protective immunity against *Streptococcus pneumoniae* infection. Infect Immun 2001;69:6718–24.
[15] Ferreira DM, Darrieux M, Silva DA, Leite LC, Ferreira Jr JM, Ho PL, et al. Characterization of protective mucosal and systemic immune responses elicited by pneumococcal surface protein PspA and PspC nasal vaccines against a respiratory pneumococcal challenge in mice. Clin Vaccine Immunol 2009;16:636–45.
[16] Briles DE, Tart RC, Swiatlo E, Dillard JP, Smith P, Benton KA, et al. Pneumococcal diversity: considerations for new vaccine strategies with emphasis on pneumococcal surface protein A (PspA). Clin Microbiol Rev 1998;11:645–57.
[17] Jedrzejas MJ. Unveiling molecular mechanisms of pneumococcal surface protein A interactions with antibodies and lactoferrin. Clin Chim Acta 2006;367:1–10.
[18] Jedrzejas MJ, Lamani E, Becker RS. Characterization of selected strains of pneumococcal surface protein A. J Biol Chem 2001;276:33121–8.
[19] Nabors GS, Braun PA, Herrmann DJ, Heise ML, Pyle DJ, Gravenstein S, et al. Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies to heterologous PspA molecules. Vaccine 2000;18:1743–54.
[20] Ren B, Szalai AJ, Hollingshead SK, Briles DE. Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface. Infect Immun 2004;72:114–22.
[21] Ochs MM, Bartlett W, Briles DE, Hicks B, Jurkuvenas A, Lau P, et al. Vaccine-induced human antibodies to PspA augment complement C3 deposition on *Streptococcus pneumoniae*. Microb Pathog 2008;44:204–14.
[22] Briles DE, Hollingshead SK, King J, Swift A, Braun PA, Park MK, et al. Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. J Infect Dis 2000;182: 1694–701.
[23] Oma K, Zhao J, Ezoe H, Akeda Y, Koyama S, Ishii KJ, et al. Intranasal immunization with a mixture of PspA and a Toll-like receptor agonist induces specific antibodies and enhances bacterial clearance in the airways of mice. Vaccine 2009;27:3181–8.
[24] Kumar H, Koyama S, Ishii KJ, Kawai T, Akira S. Cutting edge: cooperation of IPS-1- and TRIF-dependent pathways in poly IC-enhanced antibody production and cytotoxic T cell responses. J Immunol 2008;180:683–7.
[25] Briles DE, Ades E, Paton JC, Sampson JS, Carlone GM, Huebner RC, et al. Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of *Streptococcus pneumoniae*. Infect Immun 2000;68:796–800.
[26] Hamelin ME, Yim K, Kuhn KH, Cragin RP, Boukhvalova M, Blanco JC, et al. Pathogenesis of human metapneumovirus lung infection in BALB/c mice and cotton rats. J Virol 2005;79:8894–903.
[27] Prince GA, Prieels JP, Slaoui M, Porter DD. Pulmonary lesions in primary respiratory syncytial virus infection, reinfection, and vaccine-enhanced disease in the cotton rat (*Sigmodon hispidus*). Lab Invest 1999;79:1385–92.
[28] van der Sluijs KF, van Elden LJ, Nijhuis M, Schuurman R, Pater JM, Florquin S, et al. IL-10 is an important mediator of the enhanced susceptibility to pneumococcal pneumonia after influenza infection. J Immunol 2004;172:7603–9.
[29] Sun K, Metzger DW. Inhibition of pulmonary antibacterial defense by interferon-gamma during recovery from influenza infection. Nat Med 2008;14:558–64.
[30] Shahangian A, Chow EK, Tian X, Kang JR, Ghaffari A, Liu SY, et al. Type I IFNs mediate development of postinfluenza bacterial pneumonia in mice. J Clin Invest 2009;119:1910–20.
[31] Didierlaurent A, Goulding J, Patel S, Snelgrove R, Low L, Bebien M, et al. Sustained desensitization to bacterial Toll-like receptor ligands after resolution of respiratory influenza infection. J Exp Med 2008;205:323–9.
[32] King QO, Lei B, Harmsen AG. Pneumococcal surface protein A contributes to secondary *Streptococcus pneumoniae* infection after influenza virus infection. J Infect Dis 2009;200:537–45.
[33] Ichinohe T, Tamura S, Kawaguchi A, Ninomiya A, Imai M, Itamura S, et al. Cross-protection against H5N1 influenza virus infection is afforded by intranasal inoculation with seasonal trivalent inactivated influenza vaccine. J Infect Dis 2007;196:1313–20.
[34] Thompson KA, Strayer DR, Salvato PD, Thompson CE, Klimas N, Molavi A, et al. Results of a double-blind placebo-controlled study of the double-stranded RNA drug polyI:polyC12U in the treatment of HIV infection. Eur J Clin Microbiol Infect Dis 1996;15:580–7.
[35] Ferreira DM, Darrieux M, Oliveira MLS, Leite LCC, Miyaji EN. Optimized immune response elicited by a DNA vaccine expressing pneumococcal surface protein A is characterized by a balanced immunoglobulin G1 (IgG1)/IgG2a ration and proinflammatory cytokine production. Clin Vaccine Immunol 2008;15:499–505.
[36] Fukuyama Y, King JD, Kataoka K, Kobayashi R, Gilbert RS, Oishi K, et al. Secretory-IgA antibodies play an important role in the immunity to *Streptococcus pneumoniae*. J Immunol 2010;185:1755–62.

アジュバント開発研究の新展開

Advanced Technologies for Adjuvant Research and Development

監修：石井　健，山西弘一
Supervisor : Ken J. Ishii, Koichi Yamanishi

シーエムシー出版

はじめに

ワクチンは現存する医療技術の中でもその起源が最も古く，且つ，有効なもののひとつである。ワクチン開発は，Jenner以来，永らく観察と経験に頼ってきた。しかし近年の免疫学の発展により，アジュバント成分を特異的に認識するTLRなどの宿主細胞受容体を介した自然免疫反応がその後の獲得免疫反応を厳密に制御することが判明し，また，ワクチン抗原探索技術の進歩によって論理的なワクチン設計が可能となってきた。これからのワクチン開発研究では，慢性感染症，癌，アレルギーなどワクチンの対象となる疾患に関する病態研究から，免疫学，疫学，治験製剤の生産，さらには効果判定を科学的かつ効率的に行う臨床研究が必須であり，常に実用化を念頭においたGoal orientedな戦略が必要である。

このような状況の中で，本書はワクチン開発研究になくてはならなくなってきた，アジュバントに関する，基礎研究，開発，審査行政にわたる網羅的な内容を提供する意欲的なものである。国内はもとより，グローバルな視点でユニークかつトップレベルの仕事をされている方々に執筆をお願いした。

まずアジュバントの総論に関してその現状，歴史，そして未来を展望させていただいた。また，安全性と規制の考え方にも言及した。この内容は著者の考え方によるものであり，日本や外国の規制当局の意向を必ずしも反映しないことを留意していただきたい。

そして，アジュバントの免疫の基礎研究内容を自然免疫の立場から大阪大学の石井健，審良静男両氏に，アジュバントのシグナル伝達に関して佐賀医科大学の吉田裕樹氏，Th2アジュバントの作用機序とその臨床応用に関して兵庫医科大学の中西憲司氏，メモリー細胞に直接作用するアジュバント研究の新展開を大阪大学の竹内理氏にお願いした。また，免疫系におけるアジュバントの機能〜記憶T細胞の活性化機構に関して，エモリー大学の荒木幸一氏に執筆をお願いした。アジュバントのメカニズムが自然免疫から獲得免疫のメモリー機能まで多岐にわたっていることがご理解いただけたら幸いである。

次にアジュバントの要素として，微生物由来，合成物質由来のものなど多くが知られている。そのなかで，もっとも古くから認可され，広く汎用されているアジュバントとしてアルミニウム塩（総称：アラム）が知られているが，これを含む粒子状アジュバントの新規免疫学的メカニズムを産業医科大学の黒田悦史氏，ウイルスワクチン，特にインフルエンザウイルスの内因性アジュバントに関する知見をハーバード大学（東北大学）の小山正平氏，医薬基盤研究所の青枝大貴氏にお願いした。最近由来のアジュバント成分に関する研究も盛んであるが，アジュバントとしてのLPSの改良と精製に関して大阪大学の藤本ゆかり氏に，コレラトキシンを利用した新規粘膜アジュバント開発研究の新展開に関して琉球大学の荒川武氏，古くから抗がん免疫療法にて用いられているBCG-CWSのアジュバントとしての開発研究の新展開を㈱MBRの柳義和氏にお

願いした。結核菌に関して新たに理解が進んだCタイプレクチンを介する結核菌由来のアジュバント成分の免疫学的作用機序を九州大学の山崎晶氏に，乳酸菌を利用した経口ワクチンアジュバントに関して東北大学の北澤春樹氏，マラリア原虫由来成分によるアジュバント効果とヘム代謝産物のヘモゾインによるアジュバント作用機序に関して大阪大学のCevayir Coban氏，さらには真菌由来のβグルカンを利用した次世代アジュバント開発研究の内容を北九州市立大学の櫻井和朗氏にお願いした。これらのほかにも多くの微生物由来の成分がアジュバントとして作用することが知られており，今後ますます多くのアジュバントが微生物成分から発見され臨床応用されていくことであろう。

また，合成の物質からもアジュバントが開発されているが，その内容は微生物由来のものに勝るとも劣らないほど多種多様である。すべてを網羅できないことをお詫びするが，今回はオイルエマルジョン由来のアジュバント，とくにMF59というヨーロッパで認可されているアジュバントを中心に㈶日本生物化学研究所の岩田晃氏に，核酸アジュバントのCpGDNAに関してBCG研究所の山本三郎氏，核酸アジュバントのメカニズムとくに樹状細胞活性化に関する知見を大阪大学の改正恒康氏，さらにはY形DNAのアジュバント効果の詳細を京都大学の高倉善信氏にお願いした。

上記のように脂質や核酸がアジュバントとして作用し，その開発研究が非常に盛んであるが，リコンビナントサイトカインそのものもアジュバントとなりえる。その内容を大阪大学の堤康央氏，医薬基盤研究所の角田慎一氏に，そしてIL-15による免疫記憶の活性化によるアジュバントしての可能性を九州大学の吉開泰信氏にお願いした。さらにタンパクのみならず，いろいろな生物活性を持つポリペプチドもアジュバントとなりうるという内容を医薬基盤研究所の小檜山康司氏にお願いした。また，納豆菌由来のポリペプチドからなるナノパーティクルアジュバントに関して大阪大学の明石満氏にお願いした。

このように大変多くのアジュバントがいろいろな経緯，由来で発見され，開発研究に進んでいる現状がお分かりいただけると思う。しかし，アジュバントは非常に強い免疫活性物質であることが多く，使い方を間違うといわゆる「毒」にもなりえる。しかし，それを防ぐ，もしくは最小限にするための技術開発にむけた努力も続いている。特にデリバリーシステムの開発はワクチン，アジュバント開発研究にはなくてはならない必須項目になりつつある。そのなかでもリポソームに関する知見を国立感染症研究所の内田哲也氏にお願いした。また，そのデリバリーのターゲットとして有望視される経鼻粘膜を利用したインフルエンザワクチンの開発状況を国立感染症研究所の長谷川秀樹氏に，また経口ワクチンのターゲット組織である小腸における自然免疫活性化機構とそのアジュバント開発に関して大阪大学の植松智氏に執筆をお願いした。

最後に，開発研究を経て実際の臨床現場で使われているアジュバントに関して肺炎球菌ワクチン（プレベナー）に関する知見を㈱ファイザーの佐々木津氏に，GSK社のASシリーズアジュバントに関してGSK社のGarcon氏にお願いした。

最後に，この本ではあまり触れなかったが，アジュバント開発研究は感染症のみならずガンワ