Microbes and Infection 22 (2020) 312–321

Contents lists available at ScienceDirect

Microbes and Infection

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

Original article

Pneumococcal conjugate vaccine modulates macrophage-mediated innate immunity in pneumonia caused by *Streptococcus pneumoniae* following influenza

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ARTICLE INFO

Article history:
Received 5 September 2019
Accepted 10 December 2019
Available online 17 January 2020

Keywords:
Pneumococcal conjugate vaccine
Pneumococcal colonization
Influenza virus infection
Secondary pneumococcal pneumonia
Innate immunity
Monocyte/macrophages

ABSTRACT

Pneumococcal conjugate vaccination (PCV) may prevent influenza-related pneumonia, including *Streptococcus pneumoniae* pneumonia. To investigate PCV efficacy against secondary pneumococcal pneumonia following influenza, PCV was administered intramuscularly 2 and 5 weeks before *S. pneumoniae* serotype-3 colonization of murine nasopharynges followed by intranasal challenge with a sublethal dose of influenza A virus. Bacterial and viral loads, including innate immune responses were compared across conditions. PCV vaccination improved the survival of mice with secondary pneumococcal pneumonia and significantly reduced the pulmonary bacterial burden. Increased monocyte/macrophage influx into the lungs, alleviated loss of alveolar macrophages and decreased neutrophil influx into the lungs occurred in PCV-treated mice irrespective of pneumococcal colonization. Higher monocyte chemoattractant protein 1 levels and lower levels of CXCL1, interferon-γ, interleukin-17A, and IL-10, were detected in PCV-treated mice. Additionally, PCV treatment activated the macrophage intracellular killing of *S. pneumoniae*. Collectively, PCV potentially modulates the host's innate immunity and specific antibodies induction. Macrophage-related innate immunity should be further explored to elucidate the efficacy and mechanisms of PCV versus influenza-related life-threatening diseases.

Humans have experienced several influenza pandemics over the past century [1,2].

Influenza-related mortality is not only due to the viral infection itself but can also result from secondary bacterial pneumonia that is most commonly caused by *Streptococcus pneumoniae* [3–6]. *S. pneumoniae* is a human commensal flora present in the nasopharynx, and an increased nasopharyngeal pneumococcal density is believed to be an important risk factor for secondary bacterial pneumonia during or after a viral upper respiratory tract infection [7–9]. Previous studies have demonstrated that influenza infection induces alterations in a variety of the host's innate immune mechanisms, such as dysregulation of cytokine production and dysfunction of inflammatory cells, which may predispose the individual to secondary bacterial pneumonia [10–15].

Pneumococcal conjugate vaccines (PCVs) are effective in preventing pneumococcal diseases such as community-acquired pneumonia, otitis media, and sepsis [16,17]. Previous studies have also demonstrated that PCVs are not only effective in preventing influenza-related pneumococcal diseases, but also eliminate *S. pneumoniae* from the nasopharynx [18,19]. To evaluate human-related effects of PCVs, it is often necessary to establish *S. pneumoniae* colonization of the nasopharynx followed by the induction of a challenge with the influenza virus. However, most previous studies introduced the influenza virus infection first and then challenged with *S. pneumoniae*, mainly because of difficulties in establishing constant and reproducible nasopharyngeal colonization of *S. pneumoniae* in mice. We previously reported a human-

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https://doi.org/10.1016/j.micinf.2019.12.005
1286-4579/

relevant *S. pneumoniae* colonization model and evaluated a newly developed diagnostic kit for pneumococcal pneumonia [20]. As such, we postulated that a human-relevant secondary *S. pneumoniae* pneumonia model could be utilized to evaluate the efficacy of PCVs and their mechanisms of action, including specific antibody responses, innate host defense responses, and nasopharyngeal colonization.

Abundant evidence indicates that respiratory viruses contribute to secondary bacterial infections through several mechanisms, such as viral destruction of the respiratory epithelium and viral upregulation of bacterial adhesion molecules (e.g., platelet-activating factor receptor [PAF-R]) [21,22]. However, there is limited evidence regarding the effects of pneumococcal infection on viral infections. In addition to their expected anti-pneumococcal activity, PCVs have been reported to prevent virus-associated pneumonia [18,23]. These data suggest that *S. pneumoniae* plays a major role in the development of viral pneumonia, although its mechanisms are poorly understood.

The aforementioned background information prompted us to evaluate the effect of PCVs on host defense systems in pneumococcal and viral pneumonia, and to compare these to secondary pneumococcal pneumonia that manifests after influenza. The data obtained suggest that PCVs might help activate host defense systems, not only through specific antibody induction but also via the modulation of macrophage-related immunity and cytokine/chemokine responses to influenza virus infection.

1. Materials and methods

1.1. Mice

Specific pathogen-free, 6-week-old C57BL/6 mice were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). All mice were maintained at the Laboratory Animal Research Centre of Toho University School of Medicine. All experiments were performed under the guidelines for Proper Conduct of Animal Experiments (Science Council of Japan) and were approved by the Institutional Animal Care and Use Committee (approval numbers 17-55-220 and, 17-55-58).

1.2. Infectious agents

S. pneumoniae 'ATCC 6303' serotype 3 (ST 3) strain was obtained from the American Type Culture Collection (ATCC), while clinical isolates of *S. pneumoniae* ST 15A strain were stocked at Toho University Hospital. *S. pneumoniae* ST 3 strain was used for colonization and for the secondary pneumococcal pneumonia model with the vaccine serotype, while the *S. pneumoniae* ST 15A strain was used for the secondary pneumococcal pneumonia model with the non-vaccine serotype. The bacteria were incubated on Mueller-Hinton agar (Becton, Dickinson [BD] & Co., Sparks, MD, USA) supplemented with 5% defibrinated sheep blood at 37 °C for 14 h. The culture was scraped from the agar and suspended in Todd-Hewitt broth (Difco, Detroit, MI, USA) that was supplemented with 0.5% yeast extract (Bacto™ Yeast Extract, BD) and cultured at 37 °C in 5% CO_2 until attaining log phase. The bacteria in the broth were quantitated by measuring the absorbance at 660 nm and then plotting the optical density on a standard curve generated using known colony-forming unit (CFU) values. The bacterial culture was then diluted to the desired concentration. H1N1 influenza virus strain A/PR/8/34 (PR8) was obtained from the ATCC. A plaque-forming unit (PFU) assay was performed to determine the influenza virus infection inoculum and titers in the lungs of infected mice. Influenza virus RNA was also detected by quantitative real-time PCR analysis as previously described [24]. We used the following PCR primers: Influenza A Virus (M gene), 5′-CGTTCT CTCTATCATCCCGTCAG-3' (forward) and 5′-GGTCTTGTCTTTAGCCAT TCCATG-3' (reverse).

1.3. Vaccination

The 13-valent PCV (PCV13; Prevenar [Pfizer, Tokyo, Japan]) consisted of capsular polysaccharides conjugated to CRM197 carrier protein of pneumococcal serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F. Mice were vaccinated intramuscularly with 100 μL of PCV13 (diluted 1:12 in saline) or saline control, and were boosted 3 weeks later. Two weeks after that, the mice were colonized with *S. pneumoniae* or challenged with influenza virus.

1.4. IgG measurement using whole-cell ELISA

Antibodies specific to *S. pneumoniae* ST 3 and ST 15A antigens were measured by whole-cell ELISA using established methods as previously described [25]. Briefly, *S. pneumoniae* ST 3 or ST 15A was grown to late log phase, washed, and resuspended in phosphate-buffered saline (PBS) to an OD_{580} of 1.0. Ninety-six-well plates were coated with 50 μL of bacterial suspension, refrigerated overnight, and then blocked with PBS +1% bovine serum albumin prior to use. Sera were diluted in PBS +1% bovine serum albumin before its addition to the well and binding to bacterial antigens; and were detected with anti-mouse secondary antibodies conjugated to peroxidase (Jackson ImmunoResearch, West Grove, PA, USA).

1.5. Colonization and secondary pneumococcal pneumonia models

In the secondary pneumococcal pneumonia model with vaccine serotype, C57BL/6 mice were anaesthetized intramuscularly with ketamine at 50 mg/kg of body weight and xylazine at 10 mg/kg, and then inoculated intranasally with *S. pneumoniae* ST 3 in 10 μL of PBS containing 1×10^4 CFUs to develop asymptomatic colonization. Seven days after pneumococcal colonization, anaesthetized mice were challenged intranasally with 40 PFUs of PR8 virus in 30 μL of sterile PBS. In contrast, we could not construct the lethal secondary pneumococcal pneumonia following influenza virus infection model when the mice were inoculated intranasally with 10 μL *S. pneumoniae* ST 15A (Fig. S1). Survival was measured over 15 days (n = 10 mice in each group). At the indicated time points, the bacterial burden in the nasal wash and lungs (n = 5 mice in each group) were measured by plating serial 10-fold dilutions of nasal wash and lung homogenates onto blood agar plates. The plates were incubated at 37 °C in 5% CO_2 overnight, and CFUs were enumerated 24 h later.

1.6. Analysis of lung cells by flow cytometry

The excised lung tissue (without perfusion) was minced and incubated at 37 °C in 5% CO_2 for 50 min in Roswell Park Memorial Institute (RPMI) 1640 medium containing 2% fetal bovine serum, 0.5 mg/mL collagenase D (Roche Diagnostics GmbH, Mannheim, Germany), and 150 μg/mL DNase (Roche Diagnostics). Samples were filtered through a 70 μm cell strainer (BD Falcon). Cells were centrifuged, and the red blood cells were lysed using BD Pharm Lyse (BD Biosciences). Cells were collected from the lungs of each mouse (n = 3 mice in each group); the leukocytes were counted with a hemocytometer while the frequencies of neutrophils, macrophages and dendritic cells were analyzed via flow cytometry. Cell suspensions with stain buffer (PBS plus 2% bovine serum albumin) were incubated with an anti-Fc receptor-blocking antibody (purified anti-mouse CD16/32 antibody, clone 93) from BioLegend (San Diego, CA, USA) for 15 min on ice. Cells were then washed with

stain buffer and their surfaces were stained for 30 min on ice using each experimental design combination of peridinin chlorophyll protein complex (PerCP)/Cy5.5 anti-mouse CD11b antibody (clone M1/70), allophycocyanin (APC)/Cy7 anti-mouse/human CD11c antibody (clone N418), fluorescein isothiocyanate (FITC) anti-mouse Ly6G antibody (clone 1A8), phycoerythrin (PE)/Cy7 anti-mouse F4/80 antibody (clone BM8), and PE anti-mouse CD86 antibody (clone GL-1) from BioLegend; APC anti-mouse MHC class II (I-A/I-E) antibody (clone M5/114.15.2) from Tonbo Biosciences (San Diego, CA, USA). Cells were washed with stain buffer and fixed with 4% paraformaldehyde in PBS for 15 min. Flow cytometry was performed with a BD FACSCanto II (BD Biosciences), and analyzed using the FlowJo software (TreeStar, Ashland, OR, USA). Dead cells that strongly emit autofluorescence were excluded from the analysis due to their size and granularity (Fig. S2).

1.7. ELISA

Cytokines present in lung supernatants (n = 3 mice in each group), including monocyte chemoattractant protein 1 (MCP-1), CXCL1, IFN-γ, interleukin (IL)-4, IL-10, and IL-17A were measured using mouse ELISA kits (R&D Systems, MN, USA) according to the manufacturer's protocols.

1.8. RNA isolation and gene expression analysis

Total RNA was isolated from the lungs using TRIzol Reagent (Invitrogen), according to the manufacturer's instructions (n = 3 mice in each group). For quantitative reverse transcription-PCR analysis, 1 mg of total RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Data analysis, using the SYBR Green real-time RT-PCR technique, was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems). We used the following PCR primers: IFN-α, 5′-TACTCAGCAGACCTTGAACCT-3' (forward) and 5′-CAGTCTTGGC AGCAAGTTGAC-3' (reverse); IFN-γ, 5′-GAACTGGCAAAAGGATGGT GA-3' (forward) and 5′-TGTGGGTTGTTGACCTCAAAC-3' (reverse); IL-4, 5′-CTCATGGAGCTGCAGAGACTCTT-3' (forward) and 5′-CATT-CATGGTGCAGCTTATCGA-3' (reverse); IL-10, 5′-TTTGAATTCCCTGGG TGAGAA-3' (forward) and 5′-GCTCCACTGCCTTGCTCTTATT-3' (reverse); IL-17A, 5′-TTTAACTCCCTTGGCGCAAAA-3' (forward) and 5′-CTTTCCCTCCGCATTGACAC-3' (reverse); PAF-R, 5′-TATACTGGG GGTGGTTGCCAA-3' (forward) and 5′-GCAGGTCAGCCATAGTGAGAT TC-3' (reverse); and β-actin, 5'-AGAGGGAAATCGTGCGTGAC-3' (forward) and 5'-CAATAGTGATGACCTGGCCGT-3' (reverse). Relative fold changes in transcript levels were calculated using the $2^{-\Delta\Delta CT}$ method (where *CT* is the threshold cycle) [26], using the housekeeping gene that encodes β-actin as a reference standard for the amount loaded and the quality of the cDNA.

1.9. Macrophage pneumococcal challenge

Macrophages of the lungs were prepared from vaccinated or unvaccinated mice (n = 10 mice in each group) using a mouse Macrophage Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocols, and seeded with 2×10^4 cells per well in 96-well tissue culture plates (BD Falcon) overnight. Macrophages were infected with *S. pneumoniae* (MOI 1000) for 1 h at 37 °C, followed by washing to remove extracellular non-adherent bacteria and a 1-h treatment with antibiotics (penicillin [20 U/ml]) to eliminate residual or extracellular adherent bacteria. After 1 h and 4 h of incubation, the infected macrophages were lysed for the counting of the viable bacterial number, as previously described [27].

1.10. Statistical analysis

All results are expressed as means ± standard deviation (SD). The data were analyzed using the GraphPad Prism 6 software (GraphPad, Inc., La Jolla, CA, USA). Survival curves were constructed using the Kaplan–Meier method and compared using the log-rank (Mantel–Cox) test. Statistical significance among multiple groups was determined by 2-way analysis of variance (ANOVA), followed by Tukey's multiple comparison post hoc test for comparisons between the groups. A *P*-value <0.05 was considered statistically significant.

2. Results

2.1. PCV improves the survival of mice with secondary pneumococcal pneumonia following influenza virus infection in the setting of S. pneumoniae nasal colonization

Prior colonization with *S. pneumoniae* is reportedly a major predisposing factor to secondary bacterial pneumonia following influenza virus infection [28]. To establish a human-relevant model, mice were colonized with *S. pneumoniae* for 1 week and then challenged with sublethal influenza virus doses. PCV was administered 21 and 42 days before the influenza virus challenge (Fig. 1A).

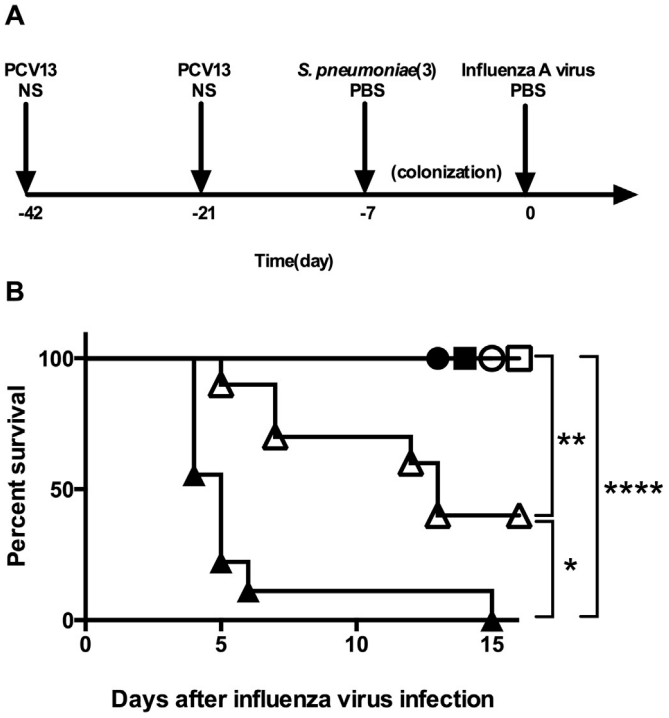

Fig. 1. Effect of pneumococcal conjugate vaccinations (PCVs) on survival during secondary bacterial pneumonia following influenza virus infection of mice with pneumococcal colonization. (A) Overall experimental design. Mice were administered either with PCV or normal saline intramuscularly and boosted three weeks later. Two weeks after that, the mice were inoculated intranasally with either phosphate-buffered saline (PBS) or 10^4 colony-forming units per mouse of *S. pneumoniae* ST 3. Seven days after colonization, the mice were challenged with PBS or 40 plaque-forming units of influenza A virus. (B) Survival was measured over 15 days. The solid circle represents the Naïve-Sp-Mock group, the open circle represents the Vacc-Sp-Mock group, the solid squares represent the Naïve-Sham-IAV group, the open squares represent the Vacc-Sham-IAV group, the solid triangle represents the Naïve-Sp-IAV group, and the open triangle represents the Vacc-Sp-IAV group. Survival curves were constructed using the Kaplan–Meier method and were analyzed using log-rank tests (n = 10 mice in each group). The data are representative of two independent experiments. *P < 0.05, **P < 0.01, ****P < 0.0001. IAV, influenza A virus.

No mortality was observed in mice with either *S. pneumoniae* colonization alone or influenza virus infection alone, regardless of the PCV treatment. In contrast, 100% mortality was observed in mice with *S. pneumoniae* colonization plus influenza virus infection. PCV treatment significantly improved the survival of mice with *S. pneumoniae* colonization plus influenza virus infection (40%, $P < 0.05$) (Fig. 1B).

2.2. Effect of PCVs on the burdens of S. pneumoniae in the nasopharynx/lungs, influenza virus in the lungs, and anti-pneumococcal antibody in the serum

We next asked whether the increased survival rate was due to decreased viral or bacterial load. Hence, we investigated the effects of PCVs on viral loads in the lungs and of pneumococcal densities in the nasopharynx and lungs. In the *S. pneumoniae* colonization groups without influenza virus infection, approximately 10^4 CFUs of *S. pneumoniae* were observed from day 0 to day 5 after PBS inoculation. In addition, increases in pneumococcal burden were observed in the nasopharynx of PCV and non-PCV-administered mice with *S. pneumoniae* colonization following influenza virus infection on days 5. There were no PCV effects on the pneumococcal burden in the nasopharynx during this observation period (Fig. 2A). In contrast, drastic increases in pneumococcal burden were observed in the lungs of non-PCV-administered mice with *S. pneumoniae* colonization following influenza virus infection on days 3 and 5; PCV treatment significantly reduced pneumococcal burdens in the lungs of these mice (Fig. 2B). There were no significant differences in the pneumococcal burdens of mice with *S. pneumoniae* colonization alone (Fig. 2A), and PCV did not alter the lung viral titers regardless of pneumococcal colonization or PCV treatment (Fig. 2C). Higher levels of specific IgG titers, an OD450 of $\geq$2, were found in the serum of mice treated with PCV, and no difference in the antibody responses was observed between the influenza virus-challenged and mock groups (Fig. 2D). There was no correlation between serotype 3 IgG antibody levels and nasopharyngeal colonization. These results suggest that specific antibodies contribute to decreased pneumococcal densities in the lungs, but not in the nasopharynx and the reason for the difference in the vaccine effect on the survival rate is thought to be the difference in the rate of increase in the amount of *S. pneumoniae* during the late stage of influenza infection.

2.3. Effects of PCVs on the influx of neutrophils and monocyte/macrophages into the lungs, the depletion of alveolar macrophages, and the surface CD86 expression of alveolar macrophages during influenza infection

We next examined the effect of PCV on the accumulation of neutrophils ($Ly6G^+$, $CD11b^+$), monocyte/macrophages ($F4/80^+$,

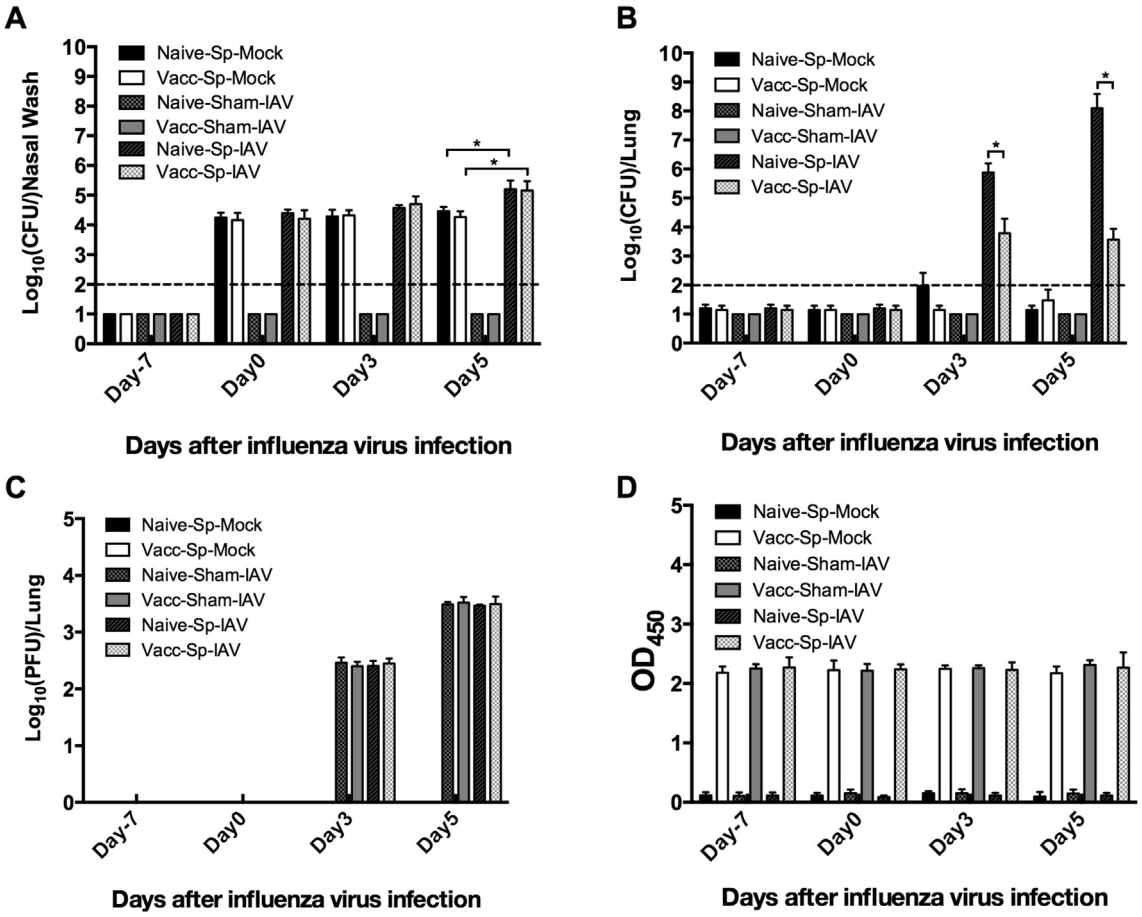

Fig. 2. Effect of pneumococcal conjugate vaccinations on bacterial and viral clearance during secondary bacterial pneumonia following influenza virus infection in mice with pneumococcal colonization. Bacterial burden in the (A) nasopharynx or (B) lung. Seven days after colonization, the mice were challenged with phosphate-buffered saline (PBS) or 40 PFUs of influenza A virus; the mean CFUs ± SDs are represented as log10 (n = 5 mice in each group). (C) Viral burden in the lungs after pneumococcal colonization; the mean PFUs ± SD are represented (n = 5 mice in each group). (D) Anti-serotype 3 strain IgG measured by whole-cell ELISA in serum; the data are representative of two independent experiments (n = 5 mice in each group). *P < 0.05. IAV, influenza A virus.

CD11b$^+$, Ly6G$^-$), alveolar macrophages (F4/80$^+$, CD11c$^+$, CD11b$^-$, Ly6G$^-$), and dendritic cells (CD11c$^+$, F4/80$^-$, Ly6G$^-$) in the lungs (Fig. S2). The number of neutrophils increased in influenza-infected mice 3 and 5 days post-infection as compared to mice with *S. pneumoniae* colonization alone; in the influenza virus infection groups, these numbers were significantly lower in PCV-treated mice (Fig. 3A). Interestingly, in the influenza virus infection groups, higher levels of monocyte/macrophages were observed in vaccinated mice regardless of *S. pneumoniae* colonization (Fig. 3B). In addition, the number of alveolar macrophages decreased in influenza-infected mice 3 and 5 days post-infection; these numbers were significantly higher in PCV-treated mice (Fig. 3C).

In contrast, there were no PCV effects on the number of dendritic cells during this observation period (Fig. 3D). Furthermore, the number of alveolar macrophages expressing CD86 increased in vaccinated mice 3 and 5 days after influenza infection regardless of *S. pneumoniae* colonization (Fig. 4A and B). In addition, the number of monocyte/macrophages expressing CD86 increased in vaccinated mice 5 days after influenza infection regardless of *S. pneumoniae* colonization (Fig. 4C and D). These results suggest that PCV induced migration of monocyte/macrophages which were influenza virus dependent, alleviated the depletion of alveolar macrophages, and activated the macrophages that are responsible for early bacterial clearance in mice challenged with influenza virus, irrespective of pneumococcal colonization.

2.4. Effects of PCVs on pulmonary chemokine and cytokine production

To examine the mechanisms of PCV efficacy, we measured the levels of chemokines (MCP-1 and CXCL-1) and cytokines (IFN-γ, IL-4, IL-10, and IL-17A) in the lungs. Previous studies have demonstrated that CXCL-1, MCP-1, IFN-γ, IL-10, and IL-17A in particular were reported to be key factors in the immune response to pneumococcal infection after influenza virus infection [14,15,29–33]. Sequential increases in CXCL-1, MCP-1, IFN-γ, IL-10, and IL-17A were observed from day 0 to day 5 after influenza virus infection, but not in IL-4 (Fig. 5A–F). Significantly higher levels of MCP-1 were found in PCV-treated mice on days 3 and 5 regardless of pneumococcal colonization (Fig. 5A). In contrast, lower levels of IFN-γ, CXCL1, IL-10, and IL-17A were found in PCV-treated mice that were infected with influenza virus (Fig. 5B–E). There were no significant differences in CXCL-1, MCP-1, IFN-γ, IL-10, IL-17A, or IL-4 production in mice with *S. pneumoniae* colonization alone (Fig. 5A–E). Furthermore, influenza virus infection also induced high levels of IFN-α, IFN-γ, IL-17A, IL-10, and PAF-R encoding mRNAs in mouse lungs, whereas the expression levels of these genes were reduced

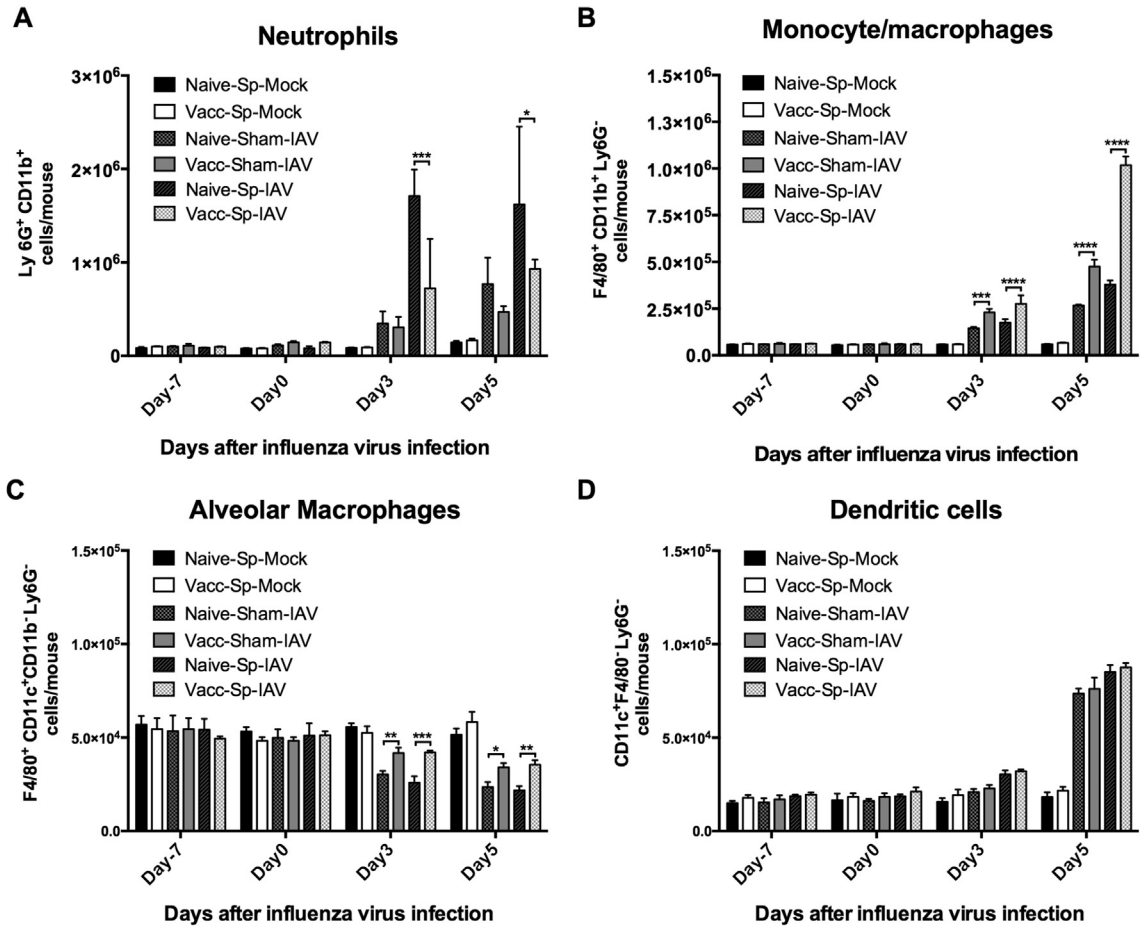

Fig. 3. Pneumococcal conjugate vaccinations induce changes in cell accumulation responses during secondary bacterial pneumonia following influenza virus infection in mice with pneumococcal colonization. The lungs were harvested and analyzed by flow cytometry. (A) Neutrophils, (B) Monocyte/macrophages, (C) Alveolar macrophages, and (D) Dendritic cells from the lungs of pneumococcal conjugated-vaccinated and unvaccinated mice were assessed during coinfection with influenza virus and pneumococcus. The vaccinated and unvaccinated mice were inoculated intranasally with either phosphate-buffered saline (PBS) or 10^4 colony-forming units per mouse of *S. pneumoniae* ST 3. Seven days after colonization, the mice were challenged intranasally with PBS or 40 plaque-forming units of influenza A virus. Bars indicate means ± standard deviation (n = 3 mice in each group). The data are representative of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. IAV, influenza A virus.

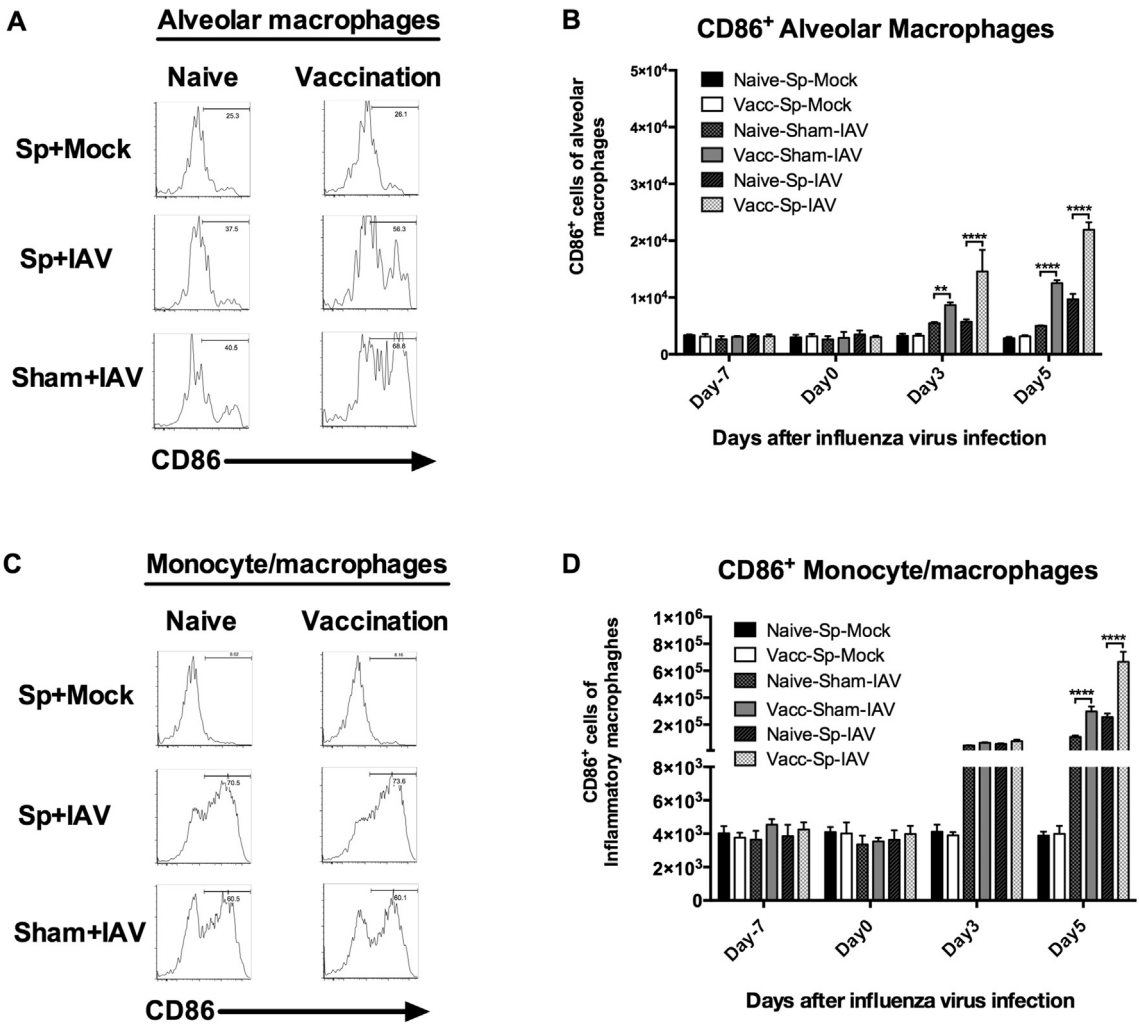

Fig. 4. Pneumococcal conjugate vaccinations activated the alveolar and monocyte/macrophages during secondary bacterial pneumonia following influenza virus infection in mice with pneumococcal colonization. The number of alveolar macrophages (A, B) and monocyte/macrophages (C, D) expressing CD86 were analyzed by flow cytometry. Bars indicate means ± standard deviation (n = 3 mice in each group). The data are representative of three independent experiments. **P < 0.01, ****P < 0.0001. IAV, influenza A virus.

in PCV-administered mice (Fig. S3). There were no significant differences in gene expression levels in the lungs of mice with *S. pneumoniae* colonization alone (Fig. S3). These results indicate that PCV administration induces an increase in MCP-1 in mice challenged with influenza virus irrespective of pneumococcal colonization and appears to decrease IFN-γ, CXCL1, IL-10, and IL-17A production that follows influenza virus infection in the lung.

2.5. *Effects of PCVs on macrophage phagocytic function and intracellular killing in* S. pneumoniae *infections*

Macrophages are critical effectors in the early innate response to bacteria in tissues. Phagocytosis and the killing of bacteria are interrelated functions essential for bacterial clearance, but whether PCVs activate the macrophages, which become challenged with *S. pneumoniae,* is unknown. To examine the effects of PCVs on macrophage phagocytosis and intracellular killing in *S. pneumoniae* infections, we investigated whether PCVs altered the ability of macrophages to inhibit the growth of *S. pneumoniae* in vitro. Macrophages of the lungs were prepared from C57BL/6 mice and infected with *S. pneumoniae*. As shown in Fig. 6, there were no significant differences in the intracellular pneumococcal burdens of macrophages 1 h post-infection (Fig. 6A). In contrast, PCV treatment significantly reduced intracellular pneumococcal burdens of macrophages 4 h post-infection (Fig. 6B). These results suggest that PCV administration activate the macrophage intracellular killing of *S. pneumoniae*.

3. Discussion

We compared the effects of PCVs on mice with influenza infection and those with secondary pneumococcal pneumonia following influenza in a human-relevant pneumococcal colonization model. Our data on the effects of PCVs on influenza infection alone demonstrated that PCVs might potentially impact the host innate immune system through a mechanism other than the induction of specific antibody against vaccine-covered serotypes of *S. pneumoniae*. Notably, PCVs induced significant increases in the accumulation of MCP-1-related macrophages and alleviated the depletion of alveolar macrophages in the lungs of influenza-infected mice.

We utilize a different model than most other studies evaluating post-influenza bacterial pneumonia. Other studies typically treat with influenza and then challenge with bacteria 6–7 days later. Certainly, in the lethal secondary pneumococcal pneumonia model following influenza virus infection without *S. pneumoniae*

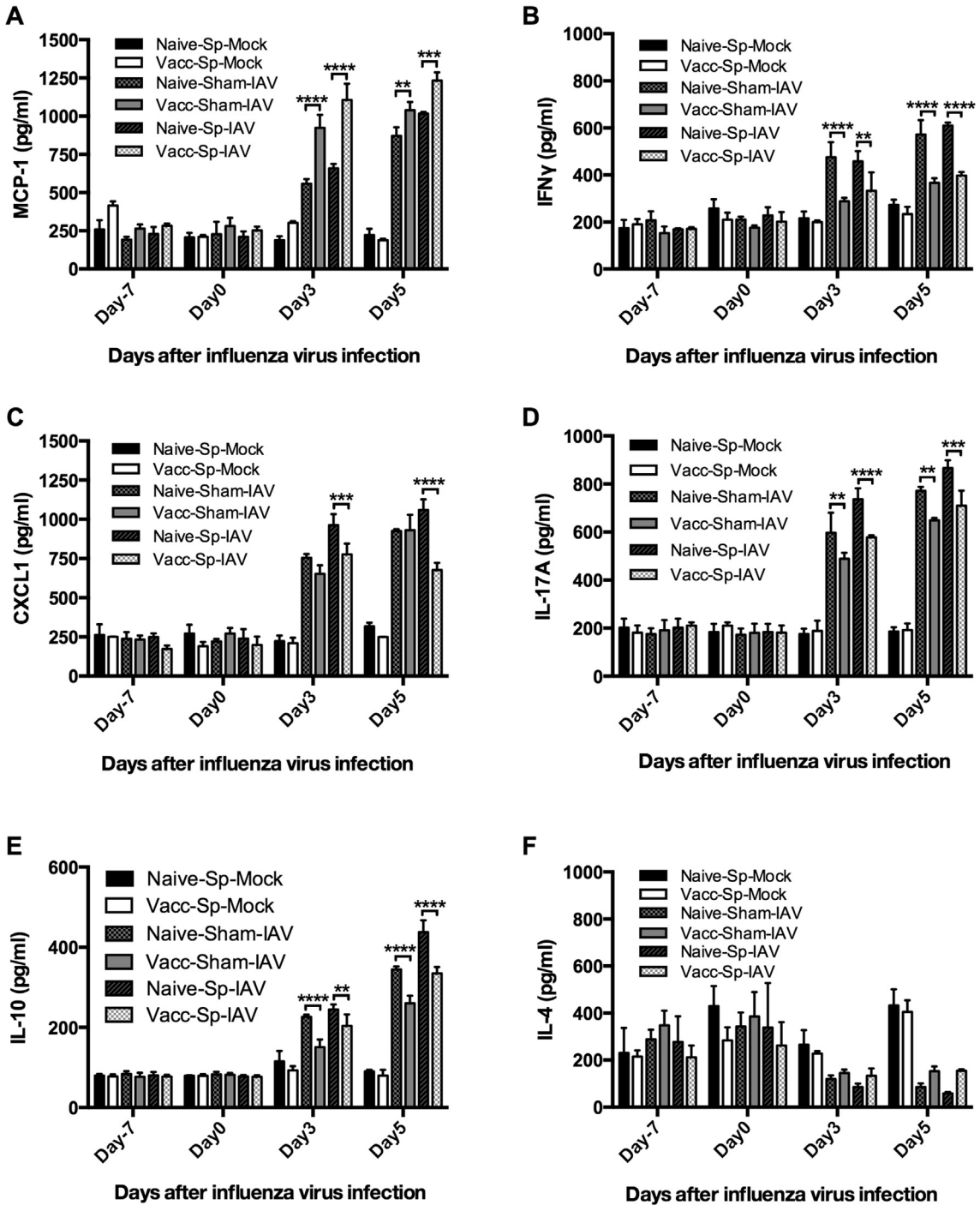

Fig. 5. Pneumococcal conjugate vaccinations promote monocyte chemoattractant protein 1 (MCP-1) production during coinfection with influenza virus and pneumococcus. Protein levels of (A) MCP-1, (B) interferon (IFN)-γ, (C) CXCL-1, (D) interleukin (IL)-17A, (E) IL-10, and (F) IL-4 in the lung were determined using ELISA. The vaccinated and unvaccinated mice were colonized with phosphate-buffered saline (PBS) or 10^4 colony-forming units per mouse of *S. pneumoniae* ST 3. Seven days after colonization, the mice were challenged intranasally with PBS or 40 plaque-forming units of influenza A virus. Bars indicate means ± standard deviation (n = 3 mice in each group). The data are representative of three independent experiments. **P < 0.01, ***P < 0.001, ****P < 0.0001. IAV, influenza A virus.

colonization, PCV administration significantly improved survival of mice with *S. pneumoniae* plus influenza virus infection. Consistent with the survival curves, significantly lower bacterial burdens of the lungs were observed in vaccinated mice than in unvaccinated mice (Fig. S4). However, we treated the mice nasal passages with bacteria and then gave influenza at a later time point. We were surprised to find that a human-relevant secondary *S. pneumoniae* pneumonia model was useful for evaluating the efficacy of PCVs and their mechanisms of action, including nasopharyngeal colonization, cytokine responses, and innate host defense responses.

Our data showed that influenza infection induced excess pneumococcal carriage in the nasopharynx in the acute phase. Furthermore, we did not observe a reduction in *S. pneumoniae* quantities in the nasopharynx after PCV vaccination in our

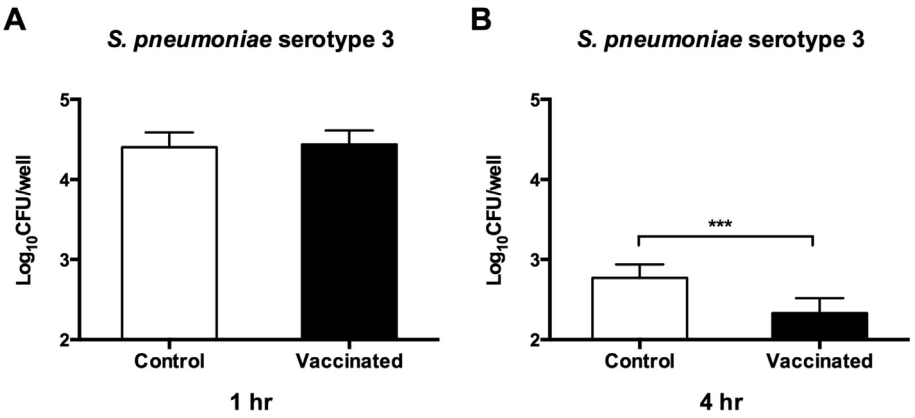

Fig. 6. Effects of pneumococcal conjugate vaccine on *S. pneumoniae* infection in macrophages. (A, B) Macrophages from the lungs of pneumococcal conjugated-vaccinated and unvaccinated mice were infected with *S. pneumoniae* ST 3 for 1 h at an MOI of 1000. After 1 h and 4 h of incubation, the infected macrophages were lysed and the mean CFUs were ascertained. CFUs ± SDs are represented as log10 (n = 10 mice in each group). The data are representative of two independent experiments. ***P < 0.001.

colonization model. Notably, in the *S. pneumoniae* ST 3 colonization model, approximately 10^4 CFUs of *S. pneumoniae* were observed from day 0 to day 35 after *S. pneumoniae* inoculation. There were no PCV effects on the pneumococcal burden in the nasopharynx during this observation period (Fig. S5). Mina and colleagues previously reported that pneumococcal growth in the upper respiratory tract following influenza virus infection was significantly reduced by a live attenuated influenza vaccine, whereas pneumococcal conjugate vaccination provided no benefit [34]. In contrast, Khan and colleagues demonstrated that vaccination with PCVs leads to a greater reduction in *S. pneumoniae* (vaccine serotype 6A strain) nasopharyngeal density in mice during influenza virus coinfection [35]. The efficacy of PCVs on pneumococcal colonization of the nasopharynx may be influenced by several factors such as *S. pneumoniae* strains/capsular types, colonization models, and the conditions of influenza infection.

Epidemiological studies have demonstrated the potential of PCVs to prevent not only vaccine-covered pneumococcal infections but also pneumonia caused by other respiratory pathogens, such as respiratory syncytial virus, parainfluenza virus, and *Mycoplasma pneumonia* [18,23]. In a double-blind, randomized, placebo-controlled trial of 37,107 fully immunized infants, Madhi and colleagues found that a 9-valent PCV reduced pneumonias associated with 7 respiratory viruses by 31% [18]. These data suggest that *S. pneumoniae* plays a major role in the development of viral pneumonia, although its specific mechanisms are not well understood. Conversely, it is well known that respiratory virus infections, especially influenza virus, sensitize the hosts to secondary bacterial infections likely via the destruction of epithelial barriers and induction of bacterial adhesion molecules [21,22]. It was considered that PCVs may modulate host defense systems, such as inflammatory cellular responses and cytokine/chemokine production, via their activation of several types of immune cells [36]. To our knowledge, there are no data on the effects of PCVs on the host innate responses to influenza infection.

Previous reports have demonstrated that influenza virus infection specifically depletes the airway-resident alveolar macrophages that are responsible for early bacterial clearance and increases the levels of CXCL1 that leads to an increased massive influx of neutrophils into the lungs, leading to a deficit in early bacterial killing, exacerbated inflammatory responses, and worse disease outcome [12,29]. Our data showed that PCVs significantly increased the influx of monocyte/macrophages into the lungs, alleviated the decrease in alveolar macrophages, and decreased the influx of neutrophils post-influenza challenge regardless of *S. pneumoniae* colonization status. These excessive macrophage accumulations were well associated with the overproduction of the macrophage-trafficking chemokine MCP-1. In contrast, CXCL1, IFN-γ, IL-10, IL-17A, and PAF-R were suppressed following PCV treatment; also, irrespective of *S. pneumoniae* colonization. Monocyte accumulation at the site of infections and relief of the depletion in alveolar macrophages during influenza infection may be an important host defense response against a variety of infectious diseases. Additionally, excess production of IFN-γ was reported to cause deterioration in mice with secondary *S. pneumoniae* pneumonia after influenza virus infection. Sun and colleagues reported that excessive IFN-γ targeting alveolar macrophages decreased phagocytic function during the influenza virus infection [15]. In contrast, the reduction of IFN-γ-producing influenza-specific $CD8^+$ T cells in the lungs during coinfection contributes to post-influenza superinfection [37]. Lastly, in a model of influenza-*Staphylococcus aureus* infection, Kudva and colleagues showed that IFN-γ has no role in bacterial outgrowth in superinfected animals [33]. Therefore, the role of IFN-γ in influenza and bacterial infections might depend on the model used. Thus, we investigated whether PCV modulates macrophages expressing CD86, as well as the phagocytic function and intracellular killing of macrophages in *S. pneumoniae* infections. We showed that PCVs increased the number of macrophages expressing CD86 and activated macrophage intracellular killing when exposed to *S. pneumoniae*.

As a result of PCV selection pressure, non-PCV serotypes may predominate in the future [38–41]. However, it has also been reported that the incidence rate of invasive pneumococcal disease caused by the non-vaccine serotype is low in young children, even after the introduction of PCV. There is an ongoing debate over whether PCV protects against pneumococcal diseases caused by the non-vaccine serotypes [42,43]. We investigated the efficacy of PCVs against secondary pneumococcal pneumonia (non-PCV serotype 15A strain) following influenza virus infection. PCV administration slightly prolonged the survival period; additionally, a significant suppression of the lung bacterial burden was demonstrated despite no detectable specific serum IgG against *S. pneumonia* ST 15A (Fig. S6). Certainly, it is necessary to be cautious not to overstate the observed results because there are some limitations in the current study. First, the changes in early influenza-induced responses depended on adaptive immunity was not determined and thus, we need to examine the efficacy of PCV against influenza virus infection in B cell and T cell-deficient (Rag-1-deficient) mice or B cell-deficient (mu-MT) mice without antibodies. Secondly, we did not examine the sources of MCP-1 production. Much of the MCP-1 in

the lung is produced by alveolar macrophages [44]. Thus, cell deletion studies or studies using bone-marrow chimeras might be necessary in future to confirm which cells are the most important for PCV-induced MCP-1 production. Thirdly, we did not analyze macrophages isolated from influenza virus-infected mice to assess influenza virus-dependent effects on intracellular killing conferred by PCV-vaccination. Finally, we did not investigate whether the increased protection conferred by prior vaccination was still present in CCR2 knockout mice or whether this was based on vaccination with the diphtheria-derived carrier protein CRM_{197} alone. PCVs are composed of 13-types of pneumococcal capsular polysaccharides combined with the CRM_{197} as an adjuvant. Protein-conjugated vaccines such as PCVs possess immune-stimulating and memory-stimulating activities through the induction of B and T cells [36]. Rabian and colleagues reported that diphtheria-derived carrier protein stimulates lymphocyte proliferation responses, increases IFN-γ, and produces a higher frequency of helper $CD4^+CXCR5^+$ T cells in PCV (7-valent)-immunized patients [45]. However, there are no studies examining the effects of diphtheria-derived carrier protein on macrophage function and number. The nature, magnitude, and timing of non-specific immunological effects of vaccines and adjuvants remain under investigation [46].

In conclusion, our data shed light on the unexplored potentials of PCVs against pneumococcal and influenza infections. It is likely that the mechanism of this involves macrophage-mediated immune modulation involving several cytokines/chemokines. To overcome the problems of the host's innate immune defects during influenza virus infection or serotype replacement as a result of PCV selection pressure, it is important not only to develop universal vaccines, which induce antipneumococcal adaptive immunity, but also to investigate the enhancement of innate immunity by PCV administration. Since a growing number of individuals are affected by *S. pneumoniae* and influenza infection that causes health deterioration, especially in the context of sequential infection or coinfection, it may be crucial to use pneumococcal and influenza vaccines to lower such risks. A better understanding of the direct and indirect mechanisms of action may be required for the effective and appropriate applications of PCVs.

Declaration of Competing Interest

The authors declare no competing interests.

Acknowledgments

This work was supported by the Japan Society for the Promotion of Science KAKENHI Grant Number 17K10033 to SK, The Project Research Grant No. 28-13 of Toho University School of Medicine to KM, and a Grant−in Aid for Private University Research Branding Project from the MEXT.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2019.12.005.

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