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A Combination of Flt3 Ligand cDNA and CpG Oligodeoxynucleotide as Nasal Adjuvant Elicits Protective Secretory-IgA Immunity to *Streptococcus pneumoniae* in Aged Mice

Yoshiko Fukuyama,* Janice D. King,† Kosuke Kataoka,‡ Ryoki Kobayashi,* Rebekah S. Gilbert,‡ Susan K. Hollingshead,‡ David E. Briles,† and Kohtaro Fujihashi*†

Our previous study showed that a combination of a plasmid-expressing Flt3 ligand (pFL) and CpG oligodeoxynucleotides (CpG ODN) as a combined nasal adjuvant elicited mucosal immune responses in aged (2-y-old) mice. In this study, we investigated whether a combination of pFL and CpG ODN as a nasal adjuvant for a pneumococcal surface protein A (PspA) would enhance PspA-specific secretory-IgA Ab responses, which could provide protective mucosal immunity against *Streptococcus pneumoniae* infection in aged mice. Nasal immunization with PspA plus a combination of pFL and CpG ODN elicited elevated levels of PspA-specific secretory-IgA Ab responses in external secretions and plasma in both young adult and aged mice. Significant levels of PspA-specific CD4+ T cell proliferative and PspA-induced Th1- and Th2-type cytokine responses were noted in nasopharyngeal-associated lymphoreticular tissue, cervical lymph nodes, and spleen of aged mice, which were equivalent to those in young adult mice. Additionally, increased numbers of mature-type CD8, CD11b-expressing dendritic cells were detected in mucosal inductive and effector lymphoid tissues of aged mice. Importantly, aged mice given PspA plus a combination of pFL and CpG ODN showed protective immunity against nasal *S. pneumoniae* colonization. These results demonstrate that nasal delivery of a combined DNA adjuvant offers an attractive possibility for protection against *S. pneumoniae* in the elderly.

*Streptococcus pneumoniae* (the pneumococcus) is a major human bacterial pathogen and significant cause of morbidity, resulting in >40,000 deaths in the United States each year (1). Most notably, severe illness and mortality caused by pneumococci have sharply increased in the elderly (2). The effectiveness of the currently licensed pneumococcal polysaccharide vaccine (PPV) for adults consists of capsular polysaccharides derived from 23 serotypes (PPV23). In fact, PPV23 elicited predominant polysaccharide-specific polymeric IgA2 Ab responses in the serum, saliva, and tears (3, 4). This vaccine is effective in young adults; however, its effectiveness becomes reduced at ages >75 y (5, 6). It has been previously reported that older adults have a less effective Ab response to PPV23 than do their younger counterparts (7–9). These observations clearly indicate that the development of a new generation of vaccines is essential to provide effective protection against *S. pneumoniae* in the elderly. Because pneumococcal infection is a major upper respiratory tract (URT) disease, which occurs on mucosal surfaces, one should take into account a mucosal vaccine strategy for developing an effective vaccine that could induce pathogen-specific immunity in the URT. Indeed, it has been shown that nasal immunization with pneumococcal surface protein A (PspA)-based vaccines in mice provided effective protective immunity against pneumococcal colonization and invasive infection (10–13). It has been shown that an age-associated decline occurs in 1-y-old mice for the induction and regulation of Ag-specific mucosal immunity in the gastrointestinal tract, but not in the nasopharyngeal-associated lymphoreticular tissue (NALT)-based immune system (14, 15). These results suggest that NALT-based immunity is less affected by aging. However, nasal immunization failed to induce mucosal immune responses in aged (2-y-old) mice despite the presence of an intact NALT-induced systemic immune response when native cholera toxin was used as nasal adjuvant (14, 15). Furthermore, effectiveness of the current licensed trivalent nasal vaccine FluMist, which consists of type A (H1N1 and H3N2) and type B live attenuated influenza virus strains, has only been demonstrated in people 2–49 y of age (16). In this regard, it is essential to develop a new generation of mucosal adjuvants that are effective in the elderly. To this end, when aged (2-y-old) mice were nasally immunized with OVA plus plasmid-expressing Flt3 ligand (pFL) and CpG oligodeoxynucleotide (CpG ODN) as a combined nasal adjuvant, significant anti-OVA secretory-IgA (S-IgA) Ab responses were noted in external secretions, which were essentially equivalent to those observed in young adult mice (17). Furthermore, a combination of pFL and CpG ODN induced a more balanced Th1- and Th2-type cytokine production by CD4+ T cells, which may be essential to support induction of both humoral and cell-mediated
protective immunity against mucosal viral and bacterial pathogens (17–20).

In this study, we examined the potential of a combination of pFL and CpG ODN as a nasal adjuvant for the induction of specific immune responses against S. pneumoniae infection in the elderly. In this regard, aged mice were nasally immunized with PspA plus a combined nasal adjuvant pFL and CpG ODN for testing the enhancement of PspA-specific IgA Ab responses and protection against bacterial colonization of the nasal mucosa.

Materials and Methods

Mice

Young adult (6- to 8-wk-old) C57BL/6 mice were purchased from the Frederick Cancer Research Facility (National Cancer Institute, National Institutes of Health, Frederick, MD). Aged C57BL/6 mice (18 mo) were obtained from the National Institute of Aging and the aging mouse colonies at Charles River Laboratories (Wilmington, MA). Upon arrival, all mice were transferred to microisolators, maintained in horizontal laminar flow cabinets, and provided sterile food and water in a specific pathogen-free animal facility at the University of Alabama at Birmingham Immunobiology Vaccine Center. All mice used in these experiments were free of bacterial and viral pathogens.

PspA and the combined nasal adjuvant

Recombinant PspA was purified by nickel affinity chromatography from Escherichia coli BL21 (DE3) carrying pUAB055 (21), which comprised the first 302 of the 558 aa of PspA/Rx, including all of the α-helical region and some of the proline-rich region (22, 23). The open reading frame plasmid (pORF)-mFlt3l (pFL), comprising the pORF and the full-length mouse FL cDNA gene (InvivoGen, San Diego, CA), was used as a combined nasal adjuvant (17, 19). Plasmid DNA was purified using Qiagen plasmid kits (Qiagen, Valencia, CA). A synthetic ODN containing CpG motif 1826 (CpG ODN) was also used as a nasal adjuvant (17, 18).

Nasal immunization and sample collection

Young adult and aged mice were nasally immunized four times at weekly intervals with 1 μg PspA, 50 μg pFL, and 10 μg CpG ODN. Other groups of aged mice were nasally immunized three times at weekly intervals with 5 μg PspA, 50 μg pFL, and/or 10 μg CpG ODN. Plasma and saliva samples were collected 1 wk after the last nasal immunization. Saliva was obtained from mice following i.p. injection of 100 μg pilocarpine hydrochloride (Sigma-Aldrich, St. Louis, MO). Nasal washes (NWs) were collected 1 wk after the last immunization as well as 5 d after nasal challenge by gently flushing the nasal passage with 1 ml Ringer’s lactate (Abbot Laboratories, North Chicago, IL).

PspA-specific ELISA

The isotypes of PspA-specific Abs in plasma, saliva, and NWs were determined by ELISA as previously described (13, 24). Briefly, 96-well Falcon microtest assay plates (BD Biosciences, San Jose, CA) were coated with 100 ng/ml PspA in PBS. After blocking with 1% BSA (Sigma-Aldrich) in PBS, 2-fold serial dilutions of samples were added and incubated overnight at 4°C. HRP-labeled goat anti-mouse μ, γ, or α chain-specific Abs (SouthernBiotech, Birmingham, AL) were added to individual wells. For IgG Ab subclass analysis, biotinylated mAbs specific for IgG1, IgG2a (and cross-reacting with IgG2c), IgG2b, and IgG3 (BD Biosciences) and peroxidase-conjugated goat anti-biotin Ab (Vector Laboratories, Burlingame, CA) were used for detection. The color reaction was developed for 15 min at room temperature with 100 μl 1.1 mM 2.2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (EMD Biosciences, La Jolla, CA). Endpoint titers were expressed as the reciprocal log2 of the last dilution that gave an OD at 415 nm of 0.1 greater than background.

PspA-specific ELISPOT

Mononuclear cells from the spleen, cervical lymph nodes (CLNs), nasal passages (NPs), and submandibular glands (SMGs) were isolated as described previously (14, 19, 24–26). Cells were subjected to an ELISPOT assay to determine the numbers of PspA-specific Ab-forming cells (AFCs). Briefly, 96-well nitrocellulose plates (Millititer HA; Millipore, Billerica, MA) were coated with 100 ng/ml PspA for analysis of anti–PspA-specific AFCs. The numbers of PspA-specific AFCs were quantified using a CTL ImmunoSpot analyzer (Cellular Technology, Shaker Heights, OH).

Flow cytometric analysis

To characterize the phenotype of dendritic cells (DCs), mononuclear cells (0.2–1 × 10⁶) from NALT, CLNs, and NPs were isolated 1 wk after the last immunization. The cells were stained with FITC-conjugated anti-CD11b, PE-labeled anti-CD11c, allopolycoymin-tagged anti-B220, and biotinylated anti-CD8 mAbs followed by PerCP-Cy5.5-streptavidin (BD Biosciences). In some experiments, mononuclear cells were incubated with FITC-conjugated anti-CD40, PE-labeled anti-CD80, CD86, or I-Ab, and biotinylated anti-CD11c (BD Biosciences) mAbs followed by PerCP-Cy5.5-streptavidin. These samples were subjected to flow cytometry (FACSCalibur; BD Biosciences) for cell subset analysis.

PspA-specific CD4⁺ T cell responses

CD4⁺ T cells from NALT, CLNs, and spleen were purified 1 wk after the last immunization using an automated magnetic-activated cell sorter (autoMACS) system (Milteny Biotec, Auburn, CA), as described previously (14, 19, 24–27). This purified CD4⁺ T cell fraction (>97% CD4⁺; >99% cell viability) was resuspended in RPMI 1640 (Cellgro Mediatech, Washington, DC) supplemented with HEPES buffer (10 mM), l-glutamine (2 mM), non-essential amino acid solution (10 mM), sodium pyruvate (10 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (80 μg/ml), and 1% FCS complete RPMI 1640 (4 × 10⁶ cells/ml) before being cultured in the presence of 1 μg/ml PspA and T cell-depleted, irradiated (3000 rad) splenic APCs for 2 or 5 d. To assess PspA-specific T cell proliferative responses, an aliquot of 0.5 μCi tritiated [H]thymidine (ThD) (Amersham Biosciences, Arlington Heights, IL) was added during the final 18 h of incubation, and the amount of [H]ThD incorporation was determined by scintillation counting. The supernatants of T cell cultures not incubated with ThD were then subjected to a cytokine-specific ELISA, while T cells were subjected to cytokine-specific quantitative RT-PCR analyses as described below.

Cytokine-specific ELISA

Levels of IFN-γ and IL-4 in culture supernatants of CD4⁺ T cells purified from NALT, CLNs, and spleen were measured by cytokine-specific ELISA as described previously (14, 19, 24–27). Briefly, the immunoplates (Becton Dickinson, Franklin, Pittsburgh, PA) were coated with anti-cytokine capturing mAb. After blocking with 3% BSA in PBS, serial 2-fold diluted samples and standards were added and then incubated overnight at 4°C. The plates were washed and the respective detection mAb was added. After incubation overnight at 4°C, HRP-labeled goat anti-biotin Ab (Vector Laboratories) was added and incubated for 1 h at room temperature. The color reaction was developed for 15 min at room temperature with 100 μl 1.1 mM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (EMD Biosciences). The detection limits for each cytokine were 106.3 pg/ml for IFN-γ and 4.66 pg/ml for IL-4.

Quantitative analysis of cytokine-specific mRNA

The CD4⁺ T cells were harvested after 2 d of incubation for total RNA extraction. Aliquots of extracted RNA (25 μg/ml) were subjected to reverse transcription reaction and were treated with 1 μl 10 μg/ml RNase H (Invitrogen, Carlsbad, CA). The levels of synthesized cDNA were measured using a NanoView RNA/DNA calculator (GE Healthcare, Piscataway, NJ). Sample cDNA and external standards were amplified with cytokine-specific primers and SYBR Green I by using a LightCycler (Roche Applied Science, Indianapolis, IN). The concentration of sample cDNA was determined using linear, diluted external standards obtained by an identical PCR protocol with the LightCycler (19, 24, 27).

Pneumococcal infection

S. pneumoniae strain EF3030 (11) was among the human isolates of capsular group 19 that were examined previously and found to be essentially noninvasive in mice (28). Three weeks after the last immunization, mice were challenged with 1–2 × 10⁶ CFU EF3030 via the nasal route. Five days after challenge, NWs were collected as described above. NPs were removed from the nasal cavity and washed in 1 ml Ringer’s lactate. The numbers of bacterial colonies were determined by plating NWs and NPs on blood agar (BD Biosciences) and incubation at 37°C overnight.

Statistical analysis

The results are presented as the mean ± 1 SEM. PspA-immunized young adult (6- to 8-wk-old) and aged (18 mo) mouse groups were compared with the respective mice immunized with PspA alone using an unpaired Mann–Whitney U test with Statview software (Abacus Concepts, Cary, NC) designed for Macintosh computers. The p values < 0.05 were considered significant.
Results

A combination of pFL and CpG ODN as nasal adjuvant induced mucosal and systemic Ab responses in aged mice

We initially examined whether pFL and CpG ODN as combined nasal adjuvant would enhance PspA-specific immune responses. Young adult mice nasally immunized four times at weekly intervals with 1 μg PspA plus a combination of pFL and CpG ODN exhibited PspA-specific S-IgA Ab responses in NWs, saliva, and plasma (Fig. 1A). In contrast, aged mice nasally immunized four times at weekly intervals with 1 μg PspA plus a combination of pFL and CpG ODN failed to induce PspA-specific S-IgA Ab responses in NWs or saliva. Although PspA-specific IgG Ab responses were detected in plasma of aged mice, the levels were significantly lower than those seen in young adult mice (Fig. 1B).

Our separate studies showed that young adult mice nasally immunized with 5 μg PspA alone resulted in increased levels of PspA-specific IgG Ab responses when compared with those of mice given 1 μg PspA alone, indicating that a higher dose of Ag could enhance Ag-specific immune responses. In this regard, when mice were nasally immunized three times with an increased dose of PspA (5 μg) plus identical doses of pFL and CpG ODN, significant S-IgA Ab responses were noted in NWs, saliva, and plasma, which were essentially equivalent (no significant differences) to the strong responses observed in young adult mice immunized with only 1 μg PspA (Fig. 1A, 1B). Similarly, high levels of PspA-specific plasma IgG, including IgG1, IgG2a, and IgG2b subclass Ab responses, were noted in aged mice given this increased dose of nasally PspA plus a combined adjuvant (Fig. 1B). In contrast, when aged mice were nasally immunized with 5 μg PspA plus pFL or CpG ODN, essentially no PspA-specific S-IgA Ab responses were detected in NWs or saliva (Fig. 2A, 2B). Although PspA-specific IgG responses were induced in plasma of mice given pFL or CpG ODN as a nasal adjuvant, the levels of anti-PspA IgG Abs were significantly lower than those in mice given a combination of pFL and CpG ODN (Fig.
While nasal immunization with 5 μg PspA alone resulted in essentially no PspA-specific Ab responses in external secretions or plasma (data not shown). To further support these findings, increased numbers of PspA-specific IgA and IgG AFCs were detected in NPs, SMGs, spleen, and CLNs of aged mice given nasal PspA plus a combination of pFL and CpG ODN, although the numbers of anti-PspA IgA and IgG AFCs in aged mice were lower than those of young adult mice (Fig. 3). Furthermore, PspA (5 μg) without nasal adjuvant failed to induce PspA-specific IgA AFCs in various mucosal and systemic tissues (<5 AFCs/10^6 cells). These results indicated that administration of a combination of pFL and CpG ODN as nasal adjuvant induced effective mucosal and systemic immunity against S. pneumoniae.

A combination of pFL and CpG ODN as a nasal adjuvant enhances PspA-specific CD4+ T cell responses in aged mice

We next assessed PspA-specific CD4+ T cell responses in aged mice induced by a combination of pFL and CpG ODN as nasal adjuvant. One week after the last immunization, CD4+ T cells from NALT, CLNs, and spleen were cultured with or without PspA in the presence of APCs for 5 d. CD4+ T cells from aged mice given nasal PspA plus a dual adjuvant showed significantly higher levels of proliferative responses, which were essentially identical to those of young adult mice (Fig. 4A). Furthermore, aged mice given nasal PspA plus a combination of pFL and CpG ODN showed significantly higher levels of IFN-γ and IL-4 production by CD4+ T cells than those of aged mice given nasal PspA alone (Fig. 4B). Although the levels of IFN-γ and IL-4 production by NALT CD4+ T cells in aged mice were equal to those seen in young adult mice, these cytokine responses in CLNs and spleen of aged mice were reduced when compared with those in young adult mice (Fig. 4B). Thus, quantitative RT-PCR analysis confirmed that PspA-stimulated CD4+ T cells contained significantly higher levels of IFN-γ and IL-4–specific mRNA in NALT, CLNs, and spleen of both aged and young adult mice nasally immunized with PspA plus a combination of pFL and CpG ODN than in mice given nasal PspA alone (Fig. 4C). These results clearly show that nasal application of a double DNA adjuvant successfully elicits a balanced Ag-specific Th1 (IFN-γ) and Th2 (IL-4) response by CD4+ T cells in mucosal and systemic lymphoid tissues of aged mice.

A combination of pFL and CpG ODN increased DCs in mucosal lymphoid tissues

Because our previous studies showed that a combination of pFL and CpG ODN as nasal adjuvant targets mucosal DCs, we next compared the frequency and phenotype of DCs in various mucosal inductive and effector tissues of young adult and old mice. Higher numbers of CD11c+ DCs were noted in NALT, CLNs, and NPs of both aged and young adult mice given nasal PspA plus a combination of pFL and CpG ODN when compared with age-matched control

![FIGURE 4. PspA-induced CD4+ Th1- and Th2-type cytokine and proliferative responses in aged mice given nasal PspA plus a combination of pFL and CpG ODN. Young adult mice were nasally immunized weekly for 4 consecutive wk with 1 μg PspA, 50 μg pFL, and 10 μg CpG ODN. Aged mice were given nasally 5 μg PspA, 50 μg pFL, and 10 μg CpG ODN three times at weekly intervals. The CD4+ T cells (4 × 10^6 cells/ml) from each mouse group were purified from NALT, CLNs, and spleen 1 wk after the final immunization and cultured with 1 μg/ml PspA in the presence of irradiated splenic feeder cells (8 × 10^6 cells/ml). A. The stimulation index was determined as cpm of wells with PspA/wells without PspA (controls). B. Culture supernatants were harvested after 5 d of incubation and analyzed for the respective cytokine by ELISA. C. Total RNA was extracted from CD4+ T cells after 2 d of incubation and subjected to quantitative RT-PCR analysis. The values shown are the mean ± SEM of 25 mice in each experimental group. *p < 0.05 when compared with mice immunized with PspA alone.](http://www.jimmunol.org/Downloadedfrom)
mice given PspA alone (Table I). To characterize the phenotype of these expanded DCs, we assessed cell surface expression of CD8, CD11b, B220, costimulatory molecules (CD40, CD80 and CD86), and MHC class II (MHC II) by CD11c+ DCs in various mucosal tissues.

A higher frequency of CD8+ and CD11b+ DCs was noted in NALT and NPs of both aged and young adult mice given nasal PspA plus a combination of pFL and CpG ODN (Table I). Additionally, CLNs as draining lymph nodes for NALT and NPs showed an increased frequency of the B220+ DC subset (Table I). DCs from CLNs, NALT, and NPs of aged mice given nasal PspA plus a combination of pFL and CpG ODN expressed increased levels CD40, CD80, CD86, and MHC II when compared with aged mice given PspA alone (Table II). Uptregulation of these costimulatory molecules by aged DCs resembles that seen in young adult mice given the nasal double adjuvant. Similarly, increased numbers of CD8+ and CD11b+ DC subsets were noted in SMGs and spleen (data not shown). Taken together, these results indicate that nasal administration of a combination of pFL and CpG ODN preferentially increases mature DC numbers as well as their activation in both mucosal inductive and effector lymphoid tissues.

**Aged mice given nasal PspA plus a combination of pFL and CpG ODN prevented bacterial colonization in the nasal cavity**

Because nasal pFL and CpG ODN as mucosal adjuvant induced PspA-specific mucosal S-IgA Ab responses in the URT mucosa of aged mice, it was important to examine the functional properties of these PspA-specific S-IgA Abs for the prevention of *S. pneumoniae* colonization. Aged mice vaccinated with PspA plus a combination of pFL and CpG ODN were nasally challenged with *S. pneumoniae* 3 wk after the last immunization. Aged mice given PspA-based vaccine showed essentially no pneumococci in either NWs or NPs (Fig. 5). Similarly, vaccinated young adult mice showed complete protection against *S. pneumoniae* colonization in the nasal mucosa. In contrast, both aged and young adult mice given nasal PspA alone failed to provide sufficient protection after nasal challenge (Fig. 5). Thus, high numbers of *S. pneumoniae* CFUs were seen the NWs and NPs of both groups of mice. Furthermore, aged mice given either PspA plus pFL or CpG ODN revealed high numbers of bacterial CFUs in both NWs and NPs (Fig. 5). The numbers of *S. pneumoniae* CFUs were essentially the same as those mice given PspA alone. These results clearly showed that PspA-specific mucosal S-IgA Abs elicited by nasal PspA-based vaccine containing pFL and CpG ODN as combined mucosal adjuvant provide complete protection from bacterial infection in aged mice.

**Discussion**

The present study showed that a nasal vaccine consisting of PspA with a combination of pFL and CpG ODN effectively induced Ag-specific mucosal and systemic immune responses in aged mice. Thus, high levels of PspA-specific S-IgA and IgG Abs were detected in the external secretions and plasma of aged mice. Additionally, pFL and CpG ODN as a combined nasal adjuvant elicited increased numbers of DCs and balanced Th1- and Th2-type cytokine responses by mucosal and systemic CD4+ T cells in aged mice. These results agree with our previous studies that pFL plus CpG ODN as a combined nasal adjuvant targets mucosal DCs for the induction of a balanced Th1- and Th2-type cytokine and Ag-specific Ab responses (17). Thus, although a higher Ag dose was required to achieve the same results as seen in young adult mice, mucosal adjuvanticity of pFL and CpG ODN was effective in aged mice even if a different type of Ag was employed. Of importance, this combined nasal adjuvant system induced functional PspA-specific S-IgA Abs to prevent nasal colonization of *S. pneumoniae*. To our knowledge, the current study is the first to show that mucosal vaccination successfully induces protective S-IgA Ab responses to a respiratory bacterial pathogen in aged mice. Thus, our evidence suggests the potential for using a combined nasal adjuvant for the induction of specific Ab responses to other types of pathogens to protect the immunosenescent from infectious diseases. This should be especially important for adults where an intranasal mucosal vaccine could be easily delivered by drops or by a nasal mist.

It has been shown that CpG ODN is a potent mucosal immunomodulator for the induction of Ag-specific cell-mediated immunity and humoral immune responses (18, 29–32). Indeed, CpG ODN has been shown to enhance Ab responses against *S. pneumoniae* polysaccharide types 19F and 6B (33). Our previous study showed that mice given nasal recombinant protective Ag of anthrax toxin plus CpG ODN exhibited high levels of protective Ag-

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Table I. Comparison of the subpopulation of CD11c+ DCs in mucosal and peripheral lymphoid tissues of mice given nasal PspA plus a combination of pFL and CpG or PspA alone

<table>
<thead>
<tr>
<th>Tissue Source</th>
<th>Mice</th>
<th>Nasal Adjuvant</th>
<th>% of Total Lymphocytes: CD11c&lt;sup&gt;+&lt;/sup&gt;</th>
<th>% of CD11c&lt;sup&gt;+&lt;/sup&gt; DCs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NALT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Young</td>
<td>None</td>
<td>1.7 (±1.2)</td>
<td>12.1 (±1.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pFL + CpG</td>
<td>7.3 (±1.2)*</td>
<td>24.4 (±0.7)*</td>
</tr>
<tr>
<td></td>
<td>Aged</td>
<td>None</td>
<td>2.5 (±0.8)</td>
<td>9.3 (±0.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pFL + CpG</td>
<td>10.4 (±1.5)*</td>
<td>20.6 (±3.5)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pFL + CpG</td>
<td>0.7 (±0.2)</td>
<td>21.1 (±3.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pFL + CpG</td>
<td>4.0 (±0.6)*</td>
<td>27.2 (±5.5)*</td>
</tr>
<tr>
<td></td>
<td>Aged</td>
<td>None</td>
<td>0.5 (±0.2)</td>
<td>20.3 (±2.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pFL + CpG</td>
<td>12.7 (±1.9)*</td>
<td>25.2 (±3.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pFL + CpG</td>
<td>5.3 (±0.9)</td>
<td>14.8 (±3.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pFL + CpG</td>
<td>14.7 (±3.5)*</td>
<td>29.7 (±2.8)*</td>
</tr>
<tr>
<td></td>
<td>Aged</td>
<td>None</td>
<td>4.8 (±0.8)</td>
<td>12.2 (±2.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pFL + CpG</td>
<td>16.3 (±0.8)*</td>
<td>28.0 (±3.1)*</td>
</tr>
</tbody>
</table>

Mice were nasally immunized weekly for four consecutive weeks with 1 µg PspA, 50 µg pFL, and 10 µg CpG ODN and for 3 consecutive wk with 5 µg PspA, 50 µg pFL, and 10 µg CpG ODN in young adult and aged mice, respectively. One week after the final immunization, mononuclear cells from NALT, CLNs, and NPs were stained with a combination of the respective mAbs and subjected to flow cytometry analysis by FACSCalibur.

<sup>a</sup>Mono- and multi- nuclear cells were stained with PE-labeled anti-CD11c mAb.

<sup>b</sup>The values shown are the mean ± SEM of five independent experiments. Each group consisted of five mice.

<sup>c</sup>Mononuclear cells were stained with FITC-conjugated anti-CD11b, PE-labeled anti-CD11c, allophycocyanin-tagged anti-B220 and biotinylated anti-CD8 mAbs followed by PerCP-Cy5.5-streptavidin.

<sup>d</sup>*p < 0.05 when compared with immunized mice with PspA alone.
specific IgG2a and IgA Ab responses in both plasma and external secretions (18). Importantly, these protective Ag-specific Abs neutralized anthrax toxin in vitro (18). Additionally, CpG ODN has been found to successfully enhance Ag-specific immune responses in aged mice (34–36). A recent study showed that addition of CpG ODN to a conjugate vaccine (PPS14-PspA) led to an adjuvant effect via TLR9 activation, which induced Th1-mediated anti-PPS14 Ab responses in aged mice (37). Taken together, these prior studies have shown that CpG ODN was indeed useful as a mucosal adjuvant to induce Ag-specific immune responses in aged mice (38). When aged mice were orally immunized with OVA plus CpG ODN, high levels of OVA-specific mucosal S-IgA and systemic IgG Ab responses were elicited (38). Thus, Peyer’s patches of these aged mice contained significant Th1- but not Th2-type cytokine responses (38). These findings demonstrate that although CpG ODN acts as a mucosal adjuvant and enhances Ag-specific immunity in aged mice, in many cases CpG ODN as an adjuvant most likely elicits polarized Th1-type responses, which may lead to potential problems for the development of safe vaccines due to the induction of significant inflammatory responses. To this end, our recent study demonstrated that nasal immunization with OVA plus a combination of pFL and CpG ODN as combined mucosal adjuvant enhanced OVA-specific S-IgA Ab responses with balanced Th1- and Th2-type responses in 2-y-old mice (17). The present study further confirms the concept of a combined adjuvant by eliciting protective pathogen-specific immunity with both IFN-γ- and IL-4–producing CD4+ T cells. These findings suggest that use of pFL as a Th2-type cytokine inducer with CpG ODN is an essential element for successful mucosal vaccine development in the elderly.

Functional defects in CD4+ T cells are the hallmark of age-associated immune alterations in aged mice (39). Indeed, CD4+ T cells from Peyer’s patches of aging (1-y-old) and aged (2-y-old) mice failed to produce Ag-specific Th2-type cytokines when orally immunized with OVA plus cholera toxin (CT) as mucosal adjuvant (15). In contrast, nasal immunization with OVA plus CT maintained intact CD4+ T cell function in aged mice (14). Despite significant Ag-stimulated Th2-type cytokine production by CD4+ T cells, CT as nasal adjuvant failed to elicit Ag-specific S-IgA Ab responses in the oral-nasopharyngeal mucosa (14).

In contrast, the current study showed that Ag-specific S-IgA Ab responses along with a balanced Th1- and Th2-type cytokine response by CD4+ T cells could be induced in aged mice. The different outcomes between these two studies are most likely due to the distinct differences in adjuvant properties between CT and pFL plus CpG ODN. Because pFL plus CpG ODN as nasal adjuvant specifically targets NALT DCs, an increased number of mature-type DCs in aged mice effectively induces higher amounts of effector CD4+ T cell differentiation than is seen when CT is used as nasal adjuvant. It is possible that intrinsic in vivo cytokine production by CD4+ T cells may be totally different when

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**FIGURE 5.** Effects of the PspA plus pFL and CpG ODN nasal vaccine on nasal colonization of *S. pneumoniae* in aged mice. Three weeks after the last immunization, mice were challenged with 1–2 × 10^6 CFU EF3030. Five days after challenge, NWs (A) and NPs (B) were collected and the numbers of bacterial colonies were determined. Each line represents the median log10 CFU/mouse.

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**Table II.** Comparison of costimulatory molecules and MHC II expression by CD11c+ DCs in mucosal and peripheral lymphoid tissues of mice given nasal PspA plus a combination of pFL and CpG or PspA alone

<table>
<thead>
<tr>
<th>Tissue Source</th>
<th>Mice</th>
<th>Nasal Adjuvant</th>
<th>CD40b</th>
<th>CD80b</th>
<th>CD68b</th>
<th>MHC IIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>NALT</td>
<td>Young</td>
<td>None</td>
<td>0.4 (±0.5)</td>
<td>5.9 (±1.6)</td>
<td>8.9 (±2.6)</td>
<td>45.9 (±1.9)</td>
</tr>
<tr>
<td></td>
<td>Aged</td>
<td>pFL + CpG</td>
<td>4.3 (±0.2)*</td>
<td>10.6 (±2.1)*</td>
<td>16.4 (±2.4)*</td>
<td>75.9 (±3.2)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.4 (±0.2)</td>
<td>4.8 (±1.1)</td>
<td>7.9 (±2.2)</td>
<td>44.9 (±1.8)</td>
</tr>
<tr>
<td>CLNs</td>
<td>Young</td>
<td>None</td>
<td>0.7 (±0.9)</td>
<td>13.9 (±1.1)*</td>
<td>24.9 (±4.1)*</td>
<td>71.7 (±8.9)*</td>
</tr>
<tr>
<td></td>
<td>Aged</td>
<td>pFL + CpG</td>
<td>3.7 (±0.1)*</td>
<td>10.6 (±1.3)</td>
<td>25.8 (±8.5)</td>
<td>85.3 (±0.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.7 (±0.5)*</td>
<td>14.4 (±1.4)*</td>
<td>40.7 (±8.4)*</td>
<td>90.6 (±1.8)*</td>
</tr>
<tr>
<td>NPs</td>
<td>Young</td>
<td>None</td>
<td>0.5 (±0.6)</td>
<td>10.9 (±0.9)</td>
<td>21.2 (±5.2)</td>
<td>82.2 (±1.1)</td>
</tr>
<tr>
<td></td>
<td>Aged</td>
<td>pFL + CpG</td>
<td>2.1 (±0.1)*</td>
<td>26.4 (±1.6)*</td>
<td>37.7 (±5.7)*</td>
<td>92.9 (±0.6)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.6 (±0.7)</td>
<td>17.8 (±0.1)</td>
<td>25.9 (±6.2)</td>
<td>33.3 (±1.9)</td>
</tr>
</tbody>
</table>

Mice were nasally immunized weekly for 4 consecutive wk with 1 μg PspA, 50 μg pFL, and 10 μg CpG ODN and for 3 consecutive wk with 5 μg PspA, 50 μg pFL, and 10 μg CpG ODN in young adult and aged mice, respectively. One week after the final immunization, mononuclear cells from NALT, CLNs, and NPs were stained with a combination of the respective mAbs and subjected to flow cytometry analysis by FACS caliber.

*The values shown are the mean ± SEM of five independent experiments. Each group consisted of five mice.

*p < 0.05 when compared with immunized mice with PspA plus pORF.
a combination of adjuvants is used. Indeed, the ex vivo cytokine assay employed the same number of CD4⁺ T cells in each culture, which may not reproduce the actual in vivo situation. Nevertheless, a combination of pFL and CpG ODN as combined nasal adjuvant elicited pathogen-specific mucosal immunity in aged mice, which was supported by balanced IFN-γ and IL-4 synthesis by CD4⁺ T cells.

Upregulation of DC function in aged mice is presumably one of the key elements needed to overcome T cell immunosenescence. Indeed, others showed that DCs activated in vitro by IL-15 effectively restored T cell function against an intracellular pathogen in aged mice (40). Our previous study showed that an increased frequency of DCs within the T cell and the subepithelial regions of NALT played a key role in the induction of Ag-specific T cell and Ab responses (17, 19, 27). Thus, nasal pFL and CpG ODN as mucosal adjuvant induced Ag-loading subepithelial dome DCs to migrate into the T cell area, which contributed to increased numbers of mature-type DCs, which subsequently stimulated naive CD4⁺ T cells in aged mice (17). Because it has been shown that CD4⁺ T cell help is essential in the induction of Ab-dependent immunity against pneumococcal infection (41–43), it was reasonable to employ pFL and CpG ODN as nasal adjuvant for the induction of Ag-specific immunity to S. pneumoniae.

Indeed, our present study clearly revealed that using nasal pFL and CpG ODN in a PsP-based vaccine significantly activated mature-type DCs for the induction of functional CD4⁺ T cells and pathogen-specific protective immunity in the upper respiratory tract of aged mice. Thus, increased numbers of CD11b⁺, CD8⁺, and B220⁺ DC subsets that coexpressed MHC II, CD40, CD80, and CD86 molecules were noted in NALT, NPs, and CLNs of aged mice given PsP plus a combination of pFL and CpG ODN. The expansion of CD8⁺ and B220⁺ DCs in various mucosal tissues agrees with our previous findings (17, 19). Thus, nasal pFL-induced CD8⁺ DCs in NALT and NPs play a key role in regulation of Th2-type cytokine-mediated Ag-specific Ab responses (17, 19). Because CpG ODN motifs exhibit mucosal adjuvant activity through direct activation of TLR9-expressing plasmacytoid DCs that mediated B cell activation (44, 45), the increased frequency of B220⁺ DCs in CLNs indicate TLR9-mediated activation of this DC subset for the induction of PsP-specific immunity. In contrast, it was an unexpected result that an increased frequency of CD11b⁺ DCs was noted in mice given the PsP-based vaccine with nasal pFL and CpG ODN as mucosal adjuvant. These different results may be due to the antigenicity of the coadministered Ag. Our previous study employed OVA, whereas the present study used PsP as Ag. Because PsP is a bacterial surface protein Ag and induces cross-protective Ab responses, PsP may facilitate expansion of the mature-type CD11b⁺ DC subset. Indeed, it was reported that influenza virus infection induced accumulation of CD11b⁺ DCs in the lungs (46). Furthermore, others showed that bacterial protein Ags plus pFL as mucosal adjuvant resulted in expansion of CD11b⁺ DCs with increased levels of costimulatory molecules (47). Furthermore, our previous studies showed that nasal administration of FL-expressing adenovirus preferentially expanded CD11b⁺ DCs, whereas pFL as nasal adjuvant resulted in increased numbers of the CD8⁺ DC subset (17, 19, 27). A recent report showed that colonic DCs are mainly CD11b⁺ and specifically respond to CpG ODN due to the microbial exposure of the colonic mucosa (48). It seems that the formulation and combination of Ags and adjuvants likely influences the subsets of DCs induced in different lymphoid tissues. Taken together, the activation of these three subsets of DCs is most likely an essential factor for the induction of PsP-specific immunity, since pFL or CpG ODN alone as a nasal adjuvant, which only activates limited DC subsets, failed to elicit protective anti-PsP S-IgA Ab responses in the external secretions of aged mice.

In summary, the current study showed that use of a combined adjuvant was an effective vaccination strategy to correct age-associated immune alterations. This DC-targeting nasal adjuvant not only elicits bacterial Ag-specific S-IgA Ab responses but also generates a more balanced IFN-γ-inducing Th1- and IL-4-producing Th2-type CD4⁺ T cell response. Of importance, the induced PsP-specific S-IgA Abs were protected and controlled against pneumococcal infection in the nasal cavity of aged mice. These results demonstrated that nasal delivery of a combination of pFL and CpG ODN offers an attractive mucosal adjuvant strategy for the development of effective and safe vaccines in the elderly.

Disclosures

The authors have no financial conflicts of interest.

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