IgG fractions showed a neutralization titre of 1:10 (Table IV).

The peak polymeric IgA fraction (about 600 kD) from the nasal wash samples, as measured using an IgA ELISA, contained no IgG antibodies when measured using an IgG ELISA; however, the peak monomeric IgG fractions (about 150 kD) from the nasal wash comprised about 1/4 of IgA (data not shown). By contrast, about 1/10 of the peak dimeric IgA (about 380 kD) from the serum samples comprised IgG antibodies, whereas about 1/10 of the peak monomeric IgG fractions from the serum comprised IgA (data not shown). This suggests that nasal polymeric IgA is responsible for the neutralization activity observed in the peak polymeric IgA fractions (about 600 kD) from the nasal wash samples. Serum monomeric IgG appears to be responsible for the neutralization activity observed in the peak monomeric IgG fractions (about 150 kD) from the serum, because the IgA content of the IgG fractions was very small. In those nasal monomeric IgG fractions that contained a relatively high amount of IgA, both IgG and IgA may be responsible for the neutralization activity. Taken together, these results show that the main neutralizing antibody in the nasal mucus is highly polymeric IgA, while the main neutralizing antibody in the serum is monomeric IgG.

DISCUSSION

In the present study, neutralizing antibody responses and their properties were examined in nasal and serum samples from healthy adults after intranasal administration of a concentrated, inactivated split A/Uruguay (H3N2) vaccine (containing 45 μg HA per dose). The first intranasal administration of a concentrated split vaccine in young adults was conducted by Kuno-Sakai et al. [1994] and showed that both serum HI- and nasal HA-specific IgA antibodies were induced after two aerosol vaccinations, which protected against a challenge infection with a cold-adapted live virus vaccine. In the present trial, neutralizing antibody responses were examined in both serum and nasal wash samples obtained from adults given five doses of vaccine, with an interval of 3 weeks between doses. The nasal wash samples were concentrated to ensure that nasal and serum neutralization titres were assayed at equivalent levels (Table I).

To measure the concentration of IgA and IgG antibodies in the concentrated nasal wash samples, the standardized nasal wash samples were adjusted to 1 mg/ml of total protein, and contained about 1/10 amount of IgA and IgG found in natural nasal mucus [Kurono and Mogi, 1987]. Previous studies show that the total amounts of IgA and IgG increase between pre-vaccination and post-vaccination in BALB/c mice [Tamura et al., 1990, 2010]; however, the results of the present study show that the amount of total IgA (and other antibodies) recovered from the nasal mucus showed small variations at each sampling time, although this was not related to vaccination status (data not shown). Even allowing for small variations in the recovery of total IgA and IgG from the nasal mucus of each subject, the neutralization titres in the standardized nasal wash samples after vaccination appeared to be a reasonable reflection of the absolute antibody titre in the nasal mucus.

A $\geq$4-fold increase in the nasal neutralization titre was observed after the second vaccination in the four younger subjects, whereas a rise in the serum neutralization titre was observed only after the fifth vaccination in the three younger subjects (Table II and Fig. 1). Intranasal administration of a vaccine tends to induce inferior serum antibody responses, but superior nasal IgA responses, compared with intramuscular injection [Atmar et al., 2007]. The present study also showed that neutralization titres correlated well with HI titres, although the HI titres were lower than the corresponding neutralization titres (Table III). This result confirms the work of Okuno et al. [1990], who showed that HI titres are sometimes lower than the corresponding neutralization titres, depending on the strain of influenza A or B virus used in the HI assay.

Healthy adults who had already acquired immunity to influenza viruses due to previous natural infections or vaccinations (seropositive adults) showed both nasal and serum antibody responses induced by the nasal vaccine (Tables II and III, and Fig. 1). Clinical trials show that intranasal administration of inactivated vaccines induces both mucosal and systemic antibody responses in seropositive adults [Kuno-Sakai et al., 1994; Hashigucci et al., 1996; Muszkat et al., 2000; Greenbaum et al., 2002; Durrer et al., 2003; Treanor et al., 2006; Atmar et al., 2007]. The induction of antibody responses in seropositive people by the nasal vaccine can be explained by the notion that the seropositive people have immunological memory for influenza viruses. Previous reports show that administration of an intranasal split vaccine plus adjuvant induces both local and systemic antibody responses in naive mice, and that the adjuvant is not required for a booster dose to induce an enhanced anamnestic immune response 4 weeks later [Tamura et al., 1989, 1992]. Administration of an adjuvant together with the vaccine stimulates innate immunity via several classes of pattern-recognition receptors (such as Toll-like receptors), which leads to the acquisition of specific immune responses, including immunological memory [Tamura et al., 1991, 2005; Tamura and Kurata, 2004].

Analysis of nasal wash and serum samples after passage through Superose 6 columns showed that the major component of nasal mucus antibodies was highly polymeric IgA, while that of serum antibodies was IgG (Fig. 2). In those subjects that received five doses of the intranasal A/Uruguay (H3N2) vaccine, the highly polymeric nasal IgA fractions were responsible for the majority of the neutralizing activity, whereas

the serum IgG fractions were responsible for the majority of the neutralizing activity in the serum (Table IV). These data are in agreement with those obtained in a previous mouse model experiment, in which IgA antibodies with neutralizing activity purified from the respiratory tract of mice immunized intranasally with HA molecules from the A/Puerto Rico/8/34 (H1N1) virus were polymeric, whereas the purified IgG antibodies with neutralizing activity were monomeric [Tamura et al., 1990]. Further study of the detailed structure of IgA, which has higher MW than expected for dimeric IgA [Song et al., 1995] remains to be performed.

Previous studies show that IgA in the respiratory tract is more cross-reactive with variant influenza viruses than IgG [Tamura et al., 1990, 1991]. This cross-reactivity seems to depend on the polymeric nature of IgA [Taylor and Dimmock, 1985; Palladino et al., 1995]. Taken together, these data suggest the potential for intranasally administered inactivated vaccines to induce cross-protection against antigenic variants of viruses in pre-immunized adults.

Both serum and mucosal HA-specific ELISA antibody responses after nasal vaccination need to be examined and compared with the corresponding neutralization and HI titres. In addition, neutralizing antibody responses to other influenza vaccines (from different strains, different subtypes or types of viruses, and from different forms of vaccines such as subvirion and whole virus vaccines) after nasal vaccination remain to be examined to compare the efficacy of nasal vaccines with that of the parenteral vaccine. Some of these studies are ongoing.

In conclusion, intranasal administration of an A/Uruguay split vaccine containing 45 μg HA resulted in induced nasal and serum neutralizing antibody responses in four out of five healthy adult subjects, with a neutralization titre of >1:40 after the second and the fifth administrations, respectively. These neutralizing antibody responses were largely due to the induction of nasal polymeric IgA and serum monomeric IgG.

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Nanogel-Based PspA Intranasal Vaccine Prevents Invasive Disease and Nasal Colonization by *Streptococcus pneumoniae*

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To establish a safer and more effective vaccine against pneumococcal respiratory infections, current knowledge regarding the antigens common among pneumococcal strains and improvements to the system for delivering these antigens across the mucosal barrier must be integrated. We developed a pneumococcal vaccine that combines the advantages of pneumococcal surface protein A (PspA) with a nontoxic intranasal vaccine delivery system based on a nanometer-sized hydrogel (nanogel) consisting of a cationic cholesteryl group-bearing pullulan (cCHP). The efficacy of the nanogel-based PspA nasal vaccine (cCHP-PspA) was tested in murine pneumococcal airway infection models. Intranasal vaccination with cCHP-PspA provided protective immunity against lethal challenge with *Streptococcus pneumoniae* Xen10, reduced colonization and invasion by bacteria in the upper and lower respiratory tracts, and induced systemic and nasal mucosal Th17 responses, high levels of PspA-specific serum immunoglobulin G (IgG), and nasal and bronchial IgA antibody responses. Moreover, there was no sign of PspA delivery by nanogel to either the olfactory bulbs or the central nervous system after intranasal administration. These results demonstrate the effectiveness and safety of the nanogel-based PspA nasal vaccine system as a universal mucosal vaccine against pneumococcal respiratory infection.

The use of polysaccharide-based injectable multivalent pneumococcal conjugate vaccines (PCV7, -10, and -13) has diminished the number of fatal infections due to pneumococci expressing the particular polysaccharides present in the vaccine (1–3). However, *Streptococcus pneumoniae* remains a problematic pathogen (4, 5) because of the large number of different capsular polysaccharides associated with virulent disease in humans. In particular, nonvaccine strains are emerging pathogens that result in morbidity and mortality due to pneumococcal diseases, including pneumonia and meningitis (6–8).

Clinical demand to overcome these problems has prompted the preclinical development of universal serotype-independent pneumococcal vaccines that are based on a surface protein common to all strains. Pneumococcal surface protein A (PspA), a pneumococcal virulence factor (9–13), is genetically variable (14) but highly cross-reactive (9, 10). PspA is commonly expressed by all capsular serotypes of *S. pneumoniae* (15) and is classified into 3 families (family 1, clades 1 and 2; family 2, clades 3 through 5; and family 3, clade 6) according to sequence similarities (14). Given that parenteral immunization with PspA induces cross-reactive neutralizing immune responses in mice (16–18) and humans (19), using PspA as a serotype-independent common antigen for the development of pneumococcal vaccines seems to be an ideal strategy.

Pneumococcal infection is generally preceded by colonization of the upper airway (20, 21). Nasal carriage of pneumococci is the primary source for spread of the infection among humans (22, 23). Therefore, an optimal vaccine strategy to prevent and control the spread of pneumococcal disease would induce protective immunity against both colonization and invasive disease. Several studies have confirmed the efficacy of PspA as a nasal vaccine antigen by coadministering PspA with a mucosal adjuvant such as cholera toxin (CT) or cholera toxin subunit B (CTB) to mice (24–26). The mice subsequently mount antigen-specific immune responses in not only the systemic compartment but also the respiratory mucosal compartment (24, 25, 27), where bacterial colonization occurs (20). PspA-specific secretory immunoglobulin A (sIgA) antibodies induced by intranasal immunization with PspA and an adjuvant (i.e., a plasmid expressing Flt3 ligand cDNA) provide protection against pneumococcal colonization (28). In addition, studies in mice have revealed that this protection is mediated by antigen-specific interleukin 17A (IL-17A)-secret-

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ing $CD4^+$ T cells induced by intranasal immunization with pneumococcal whole-cell antigen (29, 30).

Therefore, the intranasal vaccination route is an improved route for preventing colonization of the nasal cavity by pneumococci. A leading obstacle to the practical use of nasal vaccine with a protein-based pneumococcal antigen is the need to coadminister a toxin-based mucosal adjuvant (e.g., CT) for effective induction of antigen-specific immune responses (31, 32). However, the use of such toxin-based adjuvants is undesirable in humans, as it carries the concern that the toxin may reach the central nervous system (CNS) or redirect the vaccine antigen into the CNS through the olfactory nerve in the nasal cavity (33, 34). To bypass these concerns, we recently developed a nasal vaccine delivery system based on a non-toxin-based mucosal antigen carrier, a cationic cholesteryl pullulan (cCHP) nanogel (35).

Here we show the efficacy of a nanogel-based nasal pneumococcal vaccine in which PspA is incorporated into a cCHP nanogel (cCHP-PspA). We also characterized the cCHP-PspA-induced PspA-specific Th17 and antibody responses against *S. pneumoniae*. Mice immunized with nasal cCHP-PspA were protected from lethal challenge with *S. pneumoniae* and had fewer pneumococci on their respiratory mucosae. These results suggest that a nontoxic nasal vaccine comprising nanogel-based PspA offers a practical and effective strategy against pneumococcal infection by preventing both nasal colonization and invasive diseases.

MATERIALS AND METHODS

Mice. Female BALB/c mice (aged 6 to 7 weeks) were purchased from SLC (Shizuoka, Japan). All of the mice were housed with *ad libitum* food and water on a standard 12-h–12-h light-dark cycle. All experiments were performed in accordance with the guidelines provided by the Animal Care and Use committees of the University of Tokyo and were approved by the Animal Committee of the Institute of Medical Science of the University of Tokyo.

Recombinant PspA. Recombinant PspA of *S. pneumoniae* Rx1, which belongs to PspA family 1, clade 2 (14), was prepared as described previously, with slight modifications (26). Briefly, a plasmid encoding PspA/Rx1 (pUAB055; amino acids 1 through 302) (GenBank accession no. M74122) was used to transform *Escherichia coli* BL21(DE3) cells. This construct contains amino acids 1 through 302 of the PspA protein from strain Rx1 plus a 6×His tag at the C terminus (26). The sonicated cell supernatant was loaded onto a DEAE-Sepharose column (BD Healthcare, Piscataway, NJ) and a nickel affinity column (Qiagen, Valencia, CA). This was followed by gel filtration on a Sephadex G-100 column (BD Healthcare).

Preparation of cCHP-recombinant PspA complex for intranasal vaccination. A cCHP nanogel (size, ~40 nm) generated from a cationic cholesteryl group-bearing pullulan was used for all experiments. The cCHP-PspA complex for each immunization was prepared by mixing 7.5 μg PspA with cCHP at a 3:1 molecular ratio (volume, 18 μl per mouse) and incubating the mixture for 1 h at 45°C. Before the complex was used in *in vivo* studies, the fluorescence resonance energy transfer (FRET) of fluorescein isothiocyanate (FITC)-PspA and a tetramethyl rhodamine isothiocyanate (TRITC)-cCHP nanogel was measured with a fluorescence spectrometer (model FP-6500; Jasco, Easton, MD) as described previously (37). FRET analyses confirmed that the cCHP nanogel appropriately formed nanoparticles after the incorporation of PspA (see Fig. S1 in the supplemental material). Dynamic light scattering analysis showed that the cCHP nanogel maintained the same nanoscale size (32.8 ± 0.2 nm) even after the incorporation of PspA. Lipopolysaccharide (LPS) contamination of purified PspA and cCHP (<10 endotoxin units/mg protein) was measured with a *Limulus* test (Wako, Osaka, Japan).

Immunization. Once weekly for 3 consecutive weeks, female BALB/c mice were immunized intranasally with cCHP-PspA, PspA plus CT (1 μg; List Biological Laboratory, Campbell, CA), PspA alone, or phosphate-buffered saline (PBS) only. Some experiments included an irrelevant antigen as a control; in these studies, mice were immunized intranasally with a complex of cCHP nanogel and a recombinant nontoxic receptor-binding fragment of *Clostridium botulinum* type A neurotoxin subunit antigen Hc (cCHP-BoHc/A) (35). Serum, nasal wash fluid (NW), and bronchoalveolar lavage fluid (BALF) samples were harvested 1 week after the last immunization. For NWs, 200 μl sterile PBS was flushed through the posterior choanae (38). BALF was harvested by instilling 1 ml of sterile PBS through a blunt needle placed in the trachea (38).

Bacterial strain. We used the kanamycin-resistant pneumococcal strain *S. pneumoniae* Xen10 (Caliper Life Sciences, MA), derived from the wild-type strain A66.1, which expresses PspA of family 1, clades 1 and 2 (39). *S. pneumoniae* Xen10 carries a stable copy of the modified *Photorhabdus luminescens lux* operon at a single integration site on the bacterial chromosome (40). The virulence of *S. pneumoniae* Xen10 is comparable to that of the parent strain (40, 41). For challenge studies, *S. pneumoniae* 3JYP3670, which expresses PspA of family 2, clade 4, was used (10). All of the *S. pneumoniae* strains were grown in brain heart infusion (BHI) broth at 37°C in 5% CO_2.

Pneumococcal infection model. To evaluate the efficacy of intranasal vaccination with cCHP-PspA, mice were challenged 1 week after the last immunization. The cell densities of exponentially growing *S. pneumoniae* Xen10 cultured at 37°C in BHI broth were estimated from the optical density at 600 nm (OD_{600}); cells were pelleted and then diluted with PBS. Lethal (2×10^5 CFU) and sublethal (2×10^4 CFU) challenge doses diluted in 50 μl sterile PBS were administered intranasally to isoflurane-anesthetized mice. Mice were restrained vertically for 5 min to ensure inhalation of the organisms into the trachea. In addition, mice were inoculated intranasally with a lethal challenge dose (5×10^4 CFU) of strain 3JYP3670 in the same way as that for strain Xen10. Nasal passages and lung tissues were homogenized in 500 μl sterile PBS for 1 min, and the numbers of bacterial colonies were determined by plating samples on LB agar plates containing kanamycin (200 μl/ml).

***In vivo* imaging of immunized and challenged mice.** Bioluminescence of bacteria was monitored for 1 min 24, 48, and 72 h after lethal challenge by using an Ivis charge-coupled device (CCD) camera (Xenogen, Alameda, CA). Total photon emission from the entire thorax of each mouse was quantified by using the LivingImage software package (Xenogen). The results are provided as numbers of photons/s/cm^2/sr.

Antibody titer and subclass analysis. Antibody titers were determined by using enzyme-linked immunosorbent assay (ELISA) as described previously, with slight modifications (25). In brief, samples (2-fold serial dilutions) were loaded into individual wells, and the plate was coated with 1 μg/ml recombinant PspA and incubated. Goat anti-mouse IgA, IgG, IgG1, IgG2a, IgG2b, IgG3, and IgM (dilution factor, 1:4,000) conjugated with horseradish peroxidase were used as secondary antibodies. Reactions were visualized by using the TMB microwell peroxidase substrate system (XPL, Gaithersburg, MD). The endpoint titer is expressed as the reciprocal $\log_2$ of the last dilution that gave an OD_{450} that was 0.1 unit greater than that of the negative control.

PspA-specific $CD4^+$ T cell responses. By using anti-CD4 microbeads (Miltenyi Biotec, Sunnyvale, CA) according to the manufacturer's instructions, $CD4^+$ T cells were isolated from the spleens and cervical lymph nodes (CLNs) of mice intranasally immunized with cCHP-PspA, PspA alone, or PBS only. The purified $CD4^+$ T cells were resuspended at 1×10^6 cells/ml in RPMI 1640 (Cellgro, Mediatech, Washington, DC) supplemented with 10 mM HEPES, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μl/ml streptomycin, and 10% fetal calf serum and then cocultured with irradiated (2,000 rad) splenic antigen-presenting cells (2×10^6 cells/ml) from naïve BALB/c mice for 5 days at 37°C in 5% CO_2 in the presence of 1 μg/ml PspA. Cytokine levels in $CD4^+$ T cell culture supernatants were determined by using cytokine-specific DuoSet ELISA kits

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(R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Radioisotope counting assay. To trace the distribution of PspA after intranasal immunization, PspA was labeled with indium chloride (Nihon Medi-Physics, Tokyo, Japan) anhydride (Dojindo, Kumamoto, Japan) via N-terminal and ε-Lys amino groups, using diethylenetriaminepentaacetic acid as described previously (42). ^{111}In-labeled PspA was administered alone or as a complex with cCHP nanogel. The radioisotope counts in the nasal passage, olfactory bulbs, and brain 10 min and 1, 6, 12, 24, and 48 h after instillation were estimated with a γ-counter (Wizard model 1480; PerkinElmer, Waltham, MA). The results are provided as standardized uptake values (SUVs), calculated as radioisotope counts (cpm) per gram of tissue divided by the ratio of the injected dose (1×10^6 cpm) to body weight (in grams).

Flow cytometric analysis. Mice were immunized intranasally with FITC-PspA in cCHP nanogel, FITC-PspA alone, or PBS only; 6 h later, mononuclear cells were prepared from the nasal passages of each group by mechanical dissociation through 70-μm nylon mesh, as described previously (38, 43). Isolated cells were stained with phycoerythrin (PE)-Cy7-conjugated anti-CD11c (BD Bioscience) and analyzed by flow cytometry. The percentage of PspA$^+$ cells in the CD11c$^+$ fractions was calculated for each experimental group.

Data analysis. Data are expressed as means ± standard deviations (SD). Statistical analysis for most comparisons among groups was performed with Tukey's *t* test; differences were considered statistically significant when the *P* value was <0.05. For survival data, the Fisher exact test was used to compare the numbers of alive versus dead mice in the cCHP-PspA, PspA-CT, and PBS-only groups with those in the PspA-only group.

RESULTS

Intranasal vaccination with cCHP-PspA induces protective immunity against lethal challenge with *S. pneumoniae*. To evaluate whether intranasal cCHP-PspA vaccination induces protective immunity against pneumococcal challenge, we vaccinated mice with cCHP-PspA, PspA-CT, PspA alone, or PBS only. One week after the last immunization, we lethally challenged vaccinated mice with the virulent strain *S. pneumoniae* Xen10 (2×10^5 CFU), which is *S. pneumoniae* A66.1 rendered bioluminescent by the integration of a modified *lux* operon into its chromosome (40). The PspA expression level of strain Xen10 was confirmed to be comparable to that of the parent strain (see Fig. S2 in the supplemental material). We then evaluated survival rates after lethal challenge over a 2-week period. The survival rate of the cCHP-PspA-vaccinated group was 100%, as was that for PspA-CT-vaccinated mice (Fig. 1). In contrast, most of the mice intranasally immunized with PspA alone (survival rate, 0%) or with PBS (20% survival) died within 8 days of challenge with *S. pneumoniae* Xen10 (Fig. 1). The survival rates of the groups immunized with cCHP-PspA or PspA-CT were higher and were statistically significant compared to that of the group immunized with PspA alone ($P < 0.01$). The results from the PspA-only and PBS-only groups did not differ ($P > 0.05$). In addition, immunization with the irrelevant antigen BoHc/A incorporated into cCHP (cCHP-BoHc/A) (35) did not protect mice from challenge with *S. pneumoniae* Xen10 (see Fig. S3). Because PspA family 2 (clades 3 through 5) and family 1 (clades 1 and 2) constitute 94 to 99% of clinical isolates of pneumococci (14, 44–49), we also challenged mice with the strain 3JYP3670, which expresses PspA belonging to clade 4 of family 2 (10). Unlike mice inoculated with cCHP-BoHc/A, PspA alone, or PBS only, mice nasally immunized with cCHP-PspA were protected from lethal challenge with 3JYP3670 (PspA of clade 4) (10), as was the case with Xen10 expressing PspA of clades 1 and 2 (see Fig. S4).

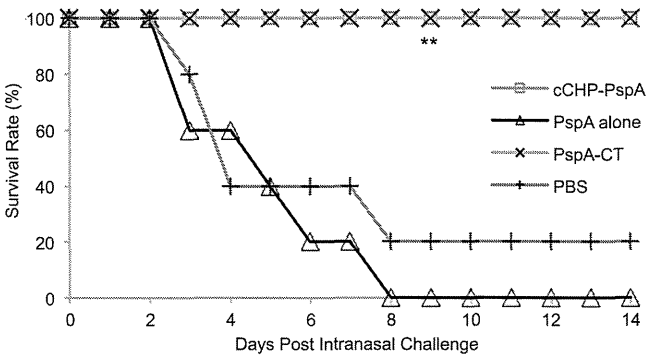

FIG 1 Intranasal vaccination with cCHP-PspA induced protective immunity against pneumococci. One week after the final immunization, mice were challenged with *S. pneumoniae* Xen10 (2×10^5 CFU/mouse), and survival was monitored. Data are representative of three independent experiments, and each group consisted of 5 mice. *P* values were calculated by using the Fisher exact test to compare the numbers of alive versus dead mice in each group with the result obtained for the PspA-only group. **, $P < 0.01$ compared with the group immunized with PspA alone. Abbreviations: cCHP, cationic cholesteryl group-bearing pullulan; CT, cholera toxin; PspA, pneumococcal surface protein A.

Intranasal vaccination with cCHP-PspA enhances bacterial clearance from BALF and the lung. To assess whether intranasal immunization with cCHP-PspA prevented pulmonary infection with pneumococci, we performed *in vivo* bioluminescence imaging of *S. pneumoniae* Xen10 after lethal challenge (2×10^5 CFU) of mice intranasally vaccinated with cCHP-PspA, PspA alone, or PBS. The lungs of mice immunized with PspA alone or with PBS only (control group) showed high-intensity photon signals in a pattern consistent with that of full-blown lung infection (Fig. 2A). In contrast, the lungs of mice immunized with cCHP-PspA lacked bioluminescence, indicating the absence of pulmonary infection. Forty-eight and 72 h after infection, photon counts of the cCHP-PspA-vaccinated group were significantly lower than those of the other two groups (Fig. 2B).

To investigate whether intranasal immunization with cCHP-PspA hastened bacterial clearance from the lung, we counted the bacteria in the BALF and lung tissues of mice intranasally vaccinated with cCHP-PspA, PspA alone, or PBS and sublethally challenged with *S. pneumoniae* Xen10 (2×10^4 CFU). Three hours after challenge, bacterial numbers in BALF (Fig. 2C) and lung tissue (Fig. 2D) did not differ among the three vaccination groups. However, 24 h after challenge, the bacterial counts in the BALF and lung homogenates from the cCHP-PspA-vaccinated groups were significantly lower (about 100-fold) than those for the mice immunized with PspA alone or PBS only (Fig. 2C and D).

Intranasal vaccination with cCHP-PspA reduces bacterial colonization in the nasal cavity. We next examined whether intranasal cCHP-PspA immunization affected nasal carriage of pneumococci in mice challenged with *S. pneumoniae* Xen10. Three days after challenge, bacterial numbers in NWs (Fig. 3A) and nasal passages (Fig. 3B) of mice immunized with the cCHP-PspA nasal vaccine were decreased significantly (approximately 100-fold) compared to those for the two control groups.

Intranasal vaccination with cCHP-PspA induces strong Th17 and Th2 responses. We then examined the type of immune

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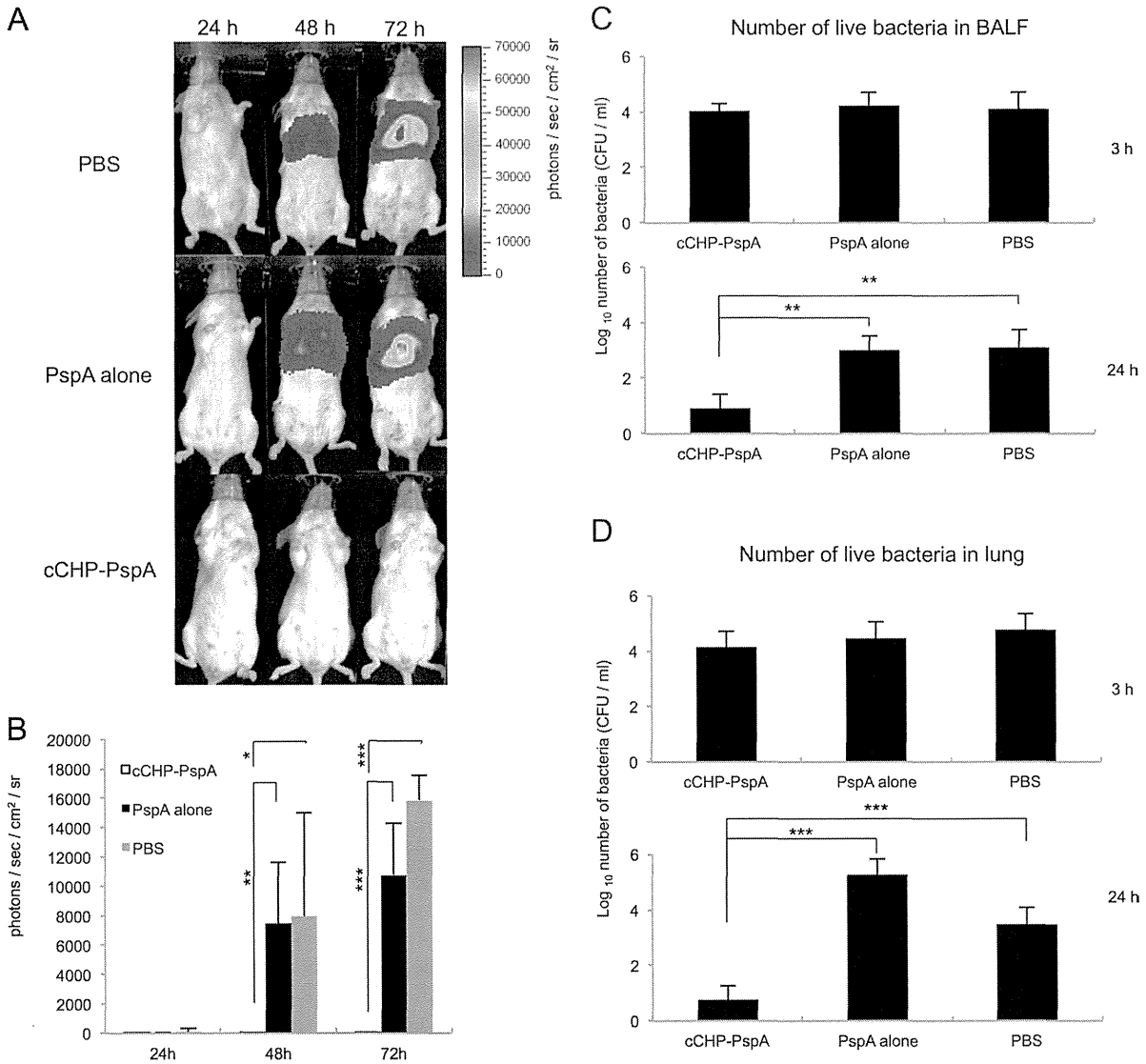

FIG 2 *In vivo* imaging revealed no sign of pneumococcal infection in the lungs of mice immunized intranasally with cCHP-PspA; these mice also showed enhanced bacterial clearance from the BALF and lung. Images (A) and average photon counts (B) show bioluminescence due to *S. pneumoniae* Xen10 in each group of mice infected intranasally with *S. pneumoniae* Xen10 (2×10^5 CFU/mouse) and imaged 24, 48, and 72 h after infection. (C and D) One week after the final immunization, mice were challenged with a sublethal dose (2×10^4 CFU/mouse) of *S. pneumoniae* Xen10. BALF and lung tissues were collected, and the numbers of *S. pneumoniae* Xen10 organisms 3 and 24 h after challenge were determined. Data are representative of three independent experiments, and each group consisted of 5 mice. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Abbreviations: BALF, bronchoalveolar lavage fluid; cCHP, cationic cholesteryl-group-bearing pullulan; PspA, pneumococcal surface protein A.

responses elicited by intranasal cCHP-PspA vaccination. Compared with PspA alone or PBS, cCHP-PspA induced higher levels of IL-17 in $CD4^+$ T cells from the spleen, CLNs, and nasal passages (Fig. 4A). The cCHP-PspA-vaccinated group produced high levels of IL-4 and IL-13, the hallmark cytokines of a Th2-type immune response, but only scant amounts of gamma interferon (Fig. 4B to D). These results show the potential of a cCHP-PspA nasal vaccine as an advanced pneumococcal vaccine that can induce a Th17 response together with a Th2-type immune response.

Intranasal vaccination with cCHP-PspA induces high levels of systemic antibodies. To address whether intranasal administration of cCHP-PspA induced PspA-specific antibody responses, we examined the serum titers of PspA-specific antibodies. PspA-specific IgG responses in the systemic compartment were significantly higher in mice immunized with intranasal cCHP-PspA than in those given PspA only (Fig. 5A). Unlike the predominant IgG response, IgM and IgA titers in the serum samples were very low (Fig. 5A).

Intranasal immunization with cCHP-PspA induced primarily IgG1 antibodies, followed by IgG2b antibodies (Fig. 5B). This pattern indicated skewing toward a Th2-type response and was consistent with the cytokine profiles of the culture supernatants from antigen-stimulated $CD4^+$ T cells prepared from the same mice (Fig. 4B and C).

Intranasal vaccination with cCHP-PspA induces high levels of mucosal antigen-specific sIgA antibodies. We next examined whether vaccinated mice also produced mucosal antigen-specific Ig responses. Intranasal vaccination with cCHP-PspA induced

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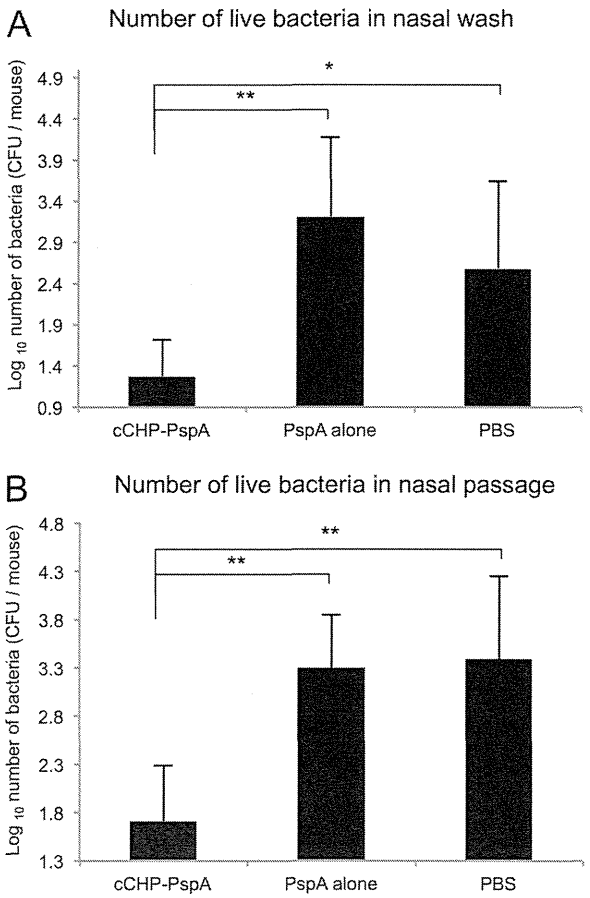

FIG 3 Intranasal vaccination with cCHP-PspA reduced bacterial colonization of the nasal cavity. One week after the final immunization, mice were challenged with a sublethal dose (2×10^4 CFU/mouse) of *Streptococcus pneumoniae* Xen10. Nasal washes and tissues were collected, and the numbers of *S. pneumoniae* Xen10 3 days after infection were determined. Data are representative of three independent experiments, and each group consisted of 5 mice. *, $P < 0.05$; **, $P < 0.01$. Abbreviations: cCHP, cationic cholesteryl group-bearing pullulan; PspA, pneumococcal surface protein A.

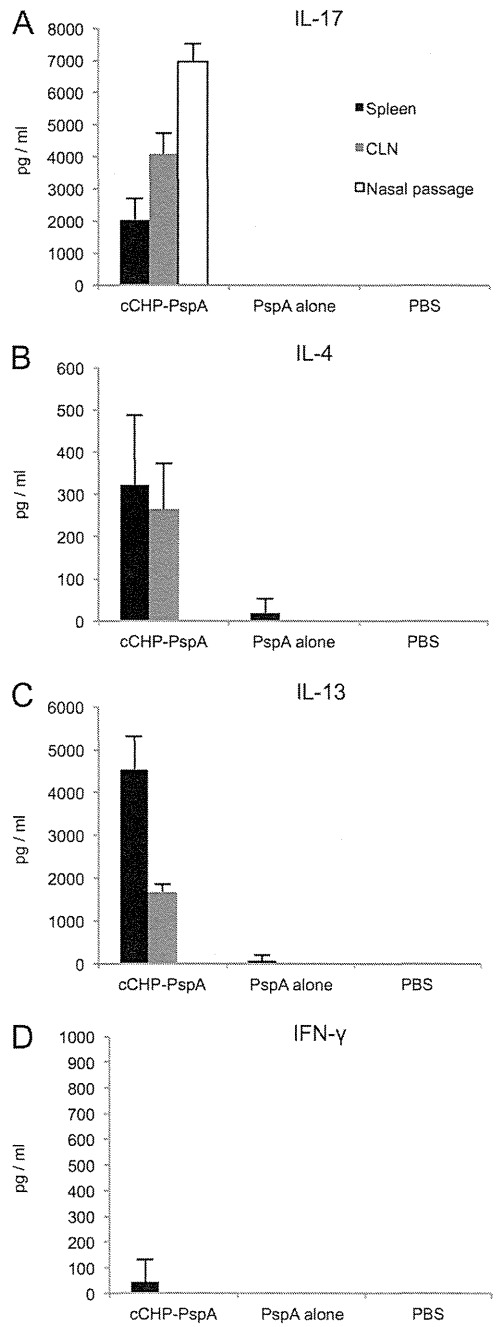

FIG 4 $CD4^+$ T cells from cCHP-PspA-immunized mice produce Th17- and Th2-type immune responses. Cytokines produced by $CD4^+$ T cells isolated from the spleens, cervical lymph nodes, and nasal passages of mice immunized with cCHP-PspA, PspA alone, or PBS only were analyzed. Data are representative of five independent experiments, and each group consisted of 5 mice. Abbreviations: cCHP, cationic cholesteryl-group-bearing pullulan; CLN, cervical lymph node; IFN-γ, gamma interferon; IL, interleukin; PspA, pneumococcal surface protein A.

PspA-specific mucosal IgA antibodies in the nasal secretions (Fig. 6A). In addition, BALF samples from mice intranasally vaccinated with cCHP-PspA contained PspA-specific IgA antibodies (Fig. 6B), and PspA-specific IgG antibodies were detected at high titers in both the NWs and BALF of mice intranasally immunized with cCHP-PspA (Fig. 6C and D). The nasal and BALF antigen-specific IgGs induced by intranasal immunization with cCHP-PspA were primarily of the IgG1 and IgG2b subclasses (Fig. 6E and F), similar to the Ig responses in the systemic compartment (Fig. 5B). Taken together, these results further support the benefit of cCHP-based nanogel as an effective nasal vaccine delivery vehicle for the induction of PspA-specific systemic and mucosal antibody responses against *S. pneumoniae*.

cCHP delivers PspA to dendritic cells (DCs) without CNS accumulation of PspA. The potential for antigen deposition and accumulation in the CNS through the olfactory fossa is one of the great concerns surrounding the use of nasal vaccines (33, 34, 50). To address this important concern, we instilled ^{111}In-labeled PspA alone or in complex with cCHP into the nasal cavities of mice. Beginning 6 h after administration, the nasal passages of mice treated with ^{111}In-labeled cCHP-PspA had higher SUVs than did those of mice treated with ^{111}In-labeled PspA alone, but there was no accumulation of ^{111}In-labeled PspA in the olfactory bulbs or brain throughout the 48-h observation period (Fig. 7A).

The cCHP vaccine delivery system enabled prolonged antigen exposure at the nasal epithelium, allowing continuous antigen

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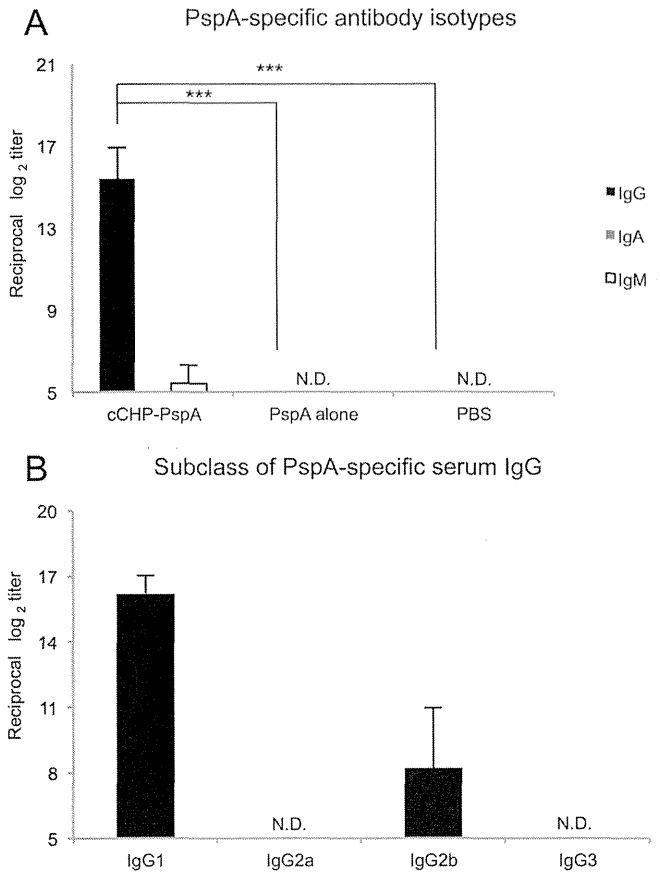

FIG 5 Intranasal vaccination with cCHP-PspA induced high levels of systemic antibodies. The data show the PspA-specific serum IgG level (A) and subclass analysis for IgG1, IgG2a, IgG2b, and IgG3 (B) for each immunized group (cCHP-PspA, PspA alone, or PBS only). Titers of PspA-specific IgG in sera were measured on day 7 after final immunization. Data are representative of three independent experiments, and each group consisted of 5 mice. N.D., not detected by ELISA with samples diluted 1:32. ***, $P < 0.001$. Abbreviations: cCHP, cationic cholesteryl group-bearing pullulan; Ig, immunoglobulin; PspA, pneumococcal surface protein A.

uptake by nasal DCs located in the epithelial layer and lamina propria of the nasal passages for the initiation of antigen-specific immune responses. Whereas 17.8% of the DCs located in the nasal passages had taken up PspA in the mice intranasally immunized with cCHP-PspA, only 0.7% of nasal DCs contained PspA antigen in mice that had been immunized intranasally with PspA alone (Fig. 7B). These results further support the concept that the cCHP-PspA vaccine formulation is an attractive inhalant delivery vehicle that effectively delivers and sustains antigen at the nasal epithelium for continuous antigen uptake by DCs without antigen deposition in the CNS.

DISCUSSION

We showed that cCHP-PspA-vaccinated mice survived a lethal challenge with *S. pneumoniae* (Fig. 1; see Fig. S4 in the supplemental material), whereas mice vaccinated with cCHP complexed with an irrelevant antigen (BoHc/A) did not (see Fig. S3 and S4). Importantly, compared with those of mice inoculated with control constructs, the respiratory tracts of mice immunized with intranasal cCHP-PspA had less colonization and invasion by pneumococcal organisms (Fig. 2 and 3). Intranasal administration of cCHP-PspA resulted in enhanced PspA-specific Th17 responses (Fig. 4A) and mucosal IgA and systemic IgG antibody responses (Fig. 5 and 6), all of which are involved in establishing protective immunity against pneumococci (10, 28–30). To our knowledge, the current study is the first to show the efficacy of a nasal vaccine not only for inducing protective immune responses but also for preventing nasal colonization by use of a single protein antigen (PspA) without adding any biologically active adjuvant.

The precise mechanisms underlying the efficacy of cCHP-PspA as a nasal vaccine against *S. pneumoniae* lung infection remain to be elucidated. However, we speculate that serum and BALF IgGs, the main isotype of antibody induced by the cCHP-PspA nasal vaccine in the lower respiratory compartment (Fig. 5A and 6D), play key roles in survival against lethal challenge with *S. pneumoniae*, given that antibody titers of PspA-specific IgA in the BALF were low (Fig. 6B) and therefore might contribute only minimally to protection against invasive diseases. This hypothesis is supported by the results of a previous study (28) in which $IgA^{-/-}$ mice immunized with intranasal PspA-adjuvant (i.e., a plasmid expressing Flt3 ligand cDNA) mounted a protective immune response against lethal challenge with *S. pneumoniae*. Our current study shows that the cCHP-PspA nasal vaccine effectively induced antigen-specific sIgA antibodies in the upper airways (Fig. 6A). Immunization of $IgA^{-/-}$ mice with intranasal PspA-adjuvant did not prevent pneumococcal colonization of the nasal cavity (28). In light of the findings of the previous study (28) and our current one, serum antigen-specific IgG antibodies are crucial to preventing invasive disease associated with clinical signs, whereas antigen-specific sIgA antibodies are essential for preventing colonization of the upper respiratory tract by *S. pneumoniae*.

In addition to the essential role of sIgA in protection from nasopharyngeal colonization by pneumococci, IL-17A-producing $CD4^+$ T cells play an important role in preventing pneumococcal nasal colonization in mice immunized with intranasal pneumococcal whole-cell antigen (29, 30). Recent studies have found that IL-17 promotes multiple aspects of humoral immunity by enhancing B cell proliferation and isotype switching (51), B cell recruitment to the respiratory mucosa, and expression of the polymeric immunoglobulin receptor on the airway epithelium (52). In the current study, we found that intranasal immunization with cCHP-PspA generated Th17 cells in the nasal passages, draining lymph nodes, and systemic compartment (Fig. 4A). Therefore, our findings suggest that intranasal immunization with cCHP-PspA induces both humoral and cellular immune responses, which are required for protective immunity against pneumococcal colonization and invasive disease. In addition to their essential role in antipneumococcal immunity (29, 30), Th17 responses are a hallmark of autoimmunity (53). Therefore, future studies should carefully examine whether the Th17 responses induced by intranasal immunization with cCHP-PspA are associated with any adverse effects.

As one might expect, the protective immunity induced by nasal cCHP-PspA was not observed when an irrelevant antigen, BoHc/A, was incorporated into cCHP (cCHP-BoHc/A) (35) and used as a nasal vaccine (see Fig. S3 and S4 in the supplemental material). Moreover, mice immunized intranasally with cCHP-PspA (PspA of clades 1 and 2) were protected against challenge with pneumococcal strain 3JYP3670, which expresses PspA of clade 4 (10), whereas mice immunized with cCHP-BoHc/A, PspA

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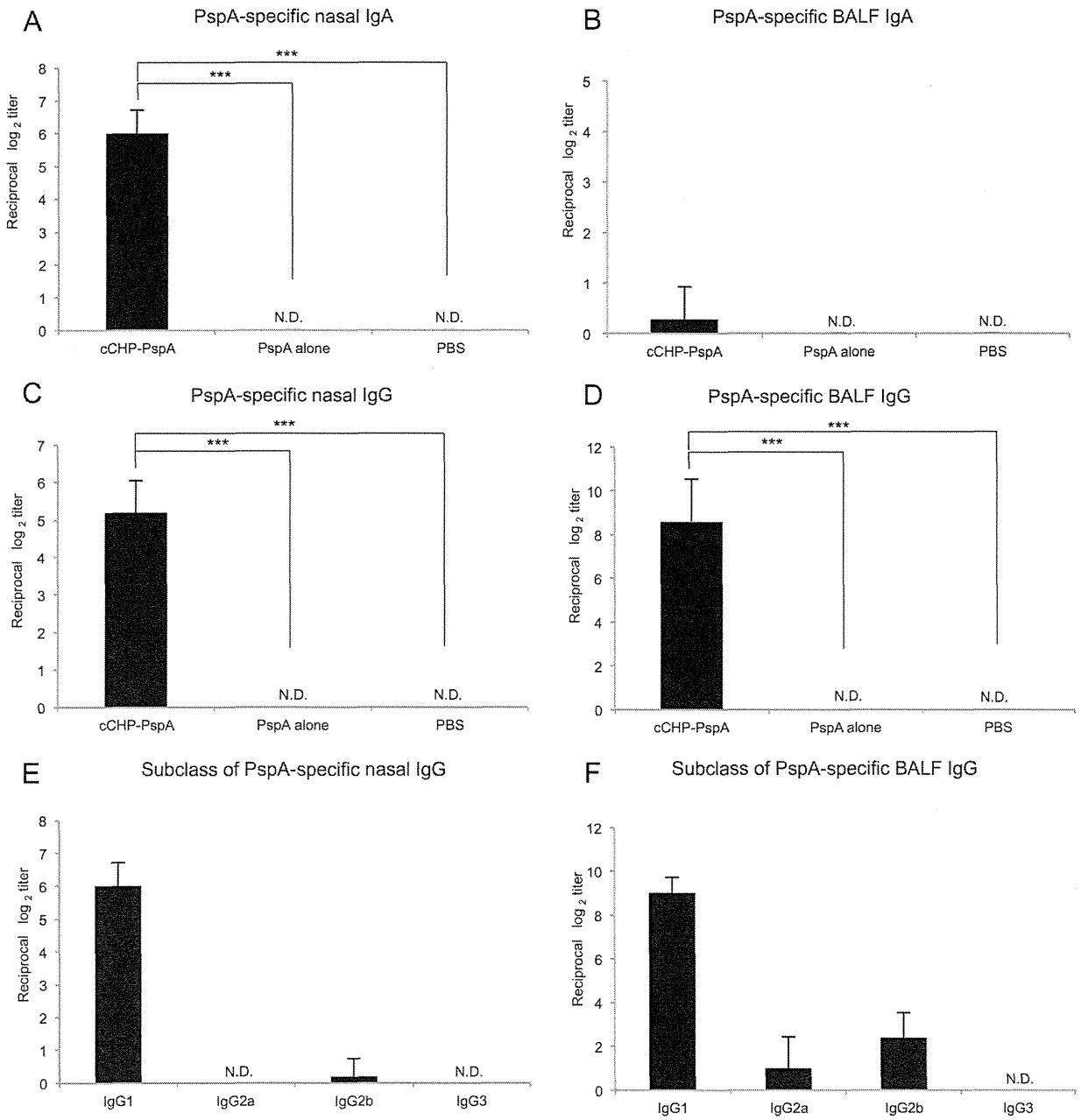

FIG 6 Intranasal vaccination with cCHP-PspA induced strong PspA-specific secretory IgA and IgG responses. Titers of nasal (A and C) and bronchial (B and D) IgA and IgG induced by intranasal immunization with PspA alone or PspA mixed with cCHP are shown. Titers of PspA-specific IgA and IgG in nasal washes and BALFs were measured on day 7 after final immunization. Intranasal cCHP-PspA vaccination induced high levels of IgG1 and IgG2b in mucosal secretions of the upper (E) and lower (F) airways. Data are representative of five independent experiments, and each group consisted of 5 mice. N.D., not detected in undiluted samples. ***, $P < 0.001$. Abbreviations: BALF, bronchoalveolar lavage fluid; cCHP, cationic cholesteryl-group-bearing pullulan; Ig, immunoglobulin; PspA, pneumococcal surface protein A.

alone, or PBS were not (see Fig. S4). These findings highlight the potential advantage of nasal vaccination of cCHP-PspA in inducing antigen-specific protective immunity with subtype cross-reactivity.

Note that cCHP lacks any biologically active adjuvant effect because it cannot activate immune cells by itself (35). The nanogel formulation had no effect on the expression of costimulatory molecules on nasal DCs (see Fig. S5 in the supplemental material), which are supposed to already express high steady-state levels of costimulatory molecules in the mucosal environment in response to numerous inhaled antigens. Our current and previous studies have shown that antigens are released from the nanogel and are taken up efficiently by DCs in the nasal mucosa (Fig. 7B) (35). These studies suggest that cCHP nanogel is an effective carrier that has strong chaperone-like activity, enabling the delivery of PspA across the nasal mucosal epithelial cell layer for subsequent uptake by DCs and initiation of antigen-specific immune responses.

In summary, this study introduced a promising nanometer-

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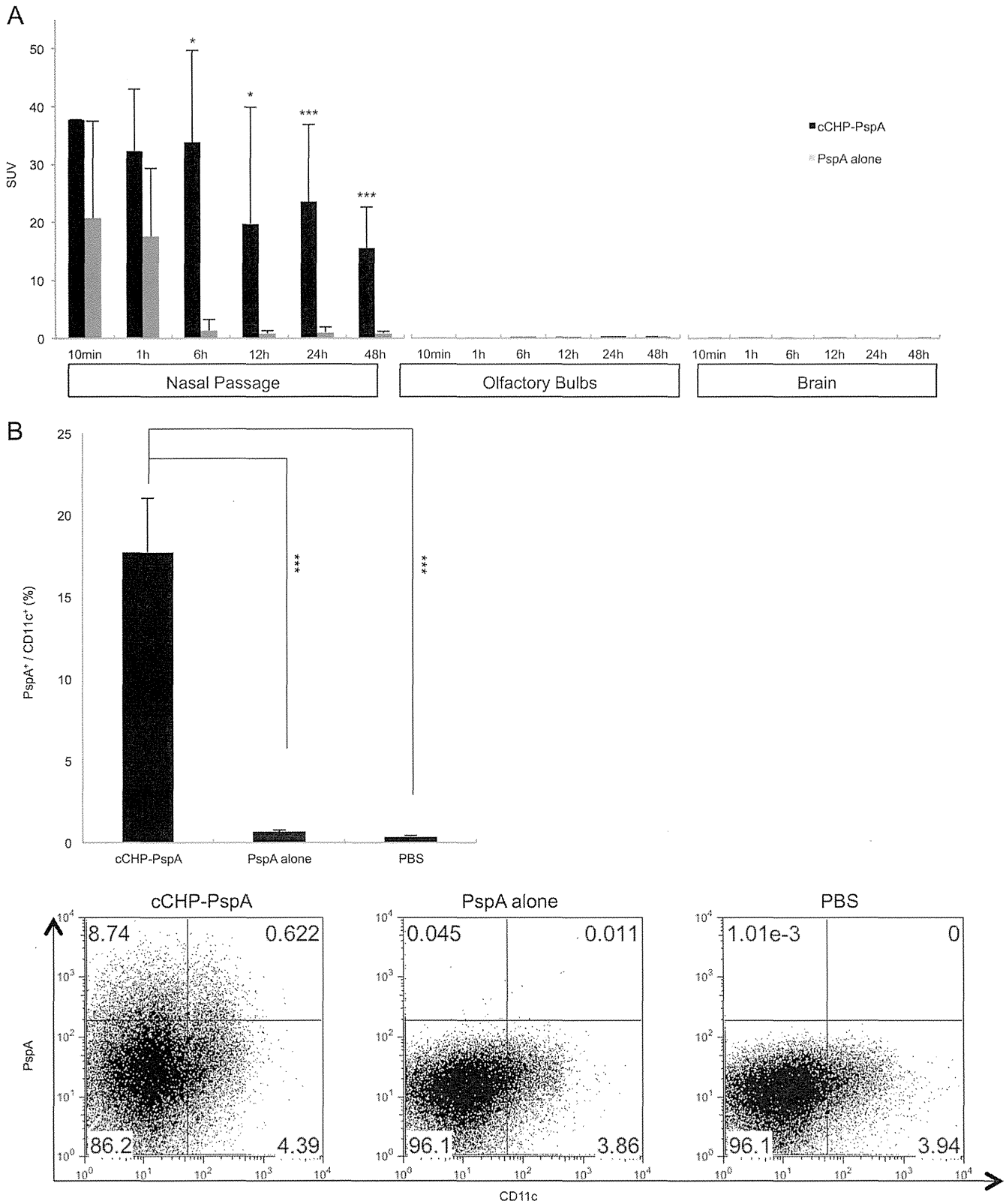

FIG 7 Intranasal vaccination with cCHP-PspA induced no accumulation of PspA in the central nervous system (A) but enhanced the efficiency of uptake of PspA by dendritic cells in the nasal passages (B). (A) ^{111}In-labeled PspA was administered intranasally with or without cCHP nanogel, and the radioisotope counts (SUVs) in the nasal passages, olfactory bulbs, and brain were estimated 10 min and 1, 6, 12, 24, and 48 h after instillation. (B) Dendritic cells in the nasal passages of mice immunized intranasally with cCHP-PspA, PspA alone, or PBS were analyzed by flow cytometry 6 h after immunization. Data are representative of three independent experiments, and each group consisted of 5 mice. *, $P < 0.05$; ***, $P < 0.001$. Abbreviations: cCHP, cationic cholesteryl group-bearing pullulan; PspA, pneumococcal surface protein A.

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sized carrier-based pneumococcal nasal vaccine that incorporates cCHP nanogel and the pneumococcal serotype-independent protein antigen PspA. The antigen-specific immune responses induced by this vaccine effectively protected mice against the respiratory pathogen *S. pneumoniae*. Our results confirmed that cCHP nanogel is a promising candidate carrier of a protein antigen for a mucosal vaccine that induces humoral and cellular immune responses against PspA to combat colonization and invasion of the airways by respiratory pathogens.

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We declare that we have no conflicts of interest.

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Nanogel-based antigen-delivery system for nasal vaccines

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Nasal vaccination is considered a potent and practical immunization route for the induction of effective immunity to infectious diseases. Successful nasal vaccines require efficient delivery to, and retention of antigens within, nasal mucosa, including both the inductive (e.g., nasopharynx-associated lymphoid tissues) and effector (e.g., turbinate covered with single-layer epithelium) tissues, where antigen-specific immune responses are initiated and executed, respectively. We developed an approach towards successful nasal vaccination by using self-assembled nano-sized hydrogel particles, known as nanogels, which are composed of a cationic type of cholesteryl group-bearing pullulan. Here, we review the merging of nanotechnological and immunological concepts leading to the development of next-generation nasal vaccines, and demonstrate the applicability of novel nanogel-based vaccine for the prevention of infectious diseases.

Keywords: nanogel; nasal vaccine; chaperone; nanotechnology; antigen delivery

Introduction

Mucosal administration of vaccines can effectively induce both systemic and mucosal antigen antigen-specific immune responses if the appropriate antigen-delivery vehicle and/or mucosal adjuvant is employed (Yuki & Kiyono, 2003). Mucosal vaccination therefore has the potential to improve the efficacy of current parenterally delivered vaccines and provide a basis for preventing various infectious diseases by protective mucosal and systemic immunity (Holmgren & Czerkinsky, 2005). However, most inactivated or subunit vaccines are poor immunogens for the induction of antigen-specific responses by both systemic and mucosal immune systems when given mucosally. Co-administration with biologically active mucosal adjuvant, for example cholera toxin (CT) or heat-labile enterotoxin (LT), can overcome this disadvantage of mucosal antigen exposure (Yuki & Kiyono, 2003). However, most potent mucosal adjuvants are toxin-based and thus not suitable for humans. Intranasal immunization with vaccine antigens together with CT or LT is effective in inducing protective immunity (Ryan et al., 1999; S. Yamamoto et al., 1997), but the redirection of adjuvants or antigens to the central nervous system (CNS) have raised concerns about their safety, even when toxin activity is removed (van Ginkel, Jackson, Yuki, & McGhee, 2000).

The development of an adjuvant-free and safe nasal-vaccine-delivery system has the potential to address the disadvantages and safety concerns associated at present with

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mucosal vaccines. To accomplish this goal, we developed an effective chaperone-type vaccine-delivery system with self-assembled nano-sized cationic hydrogel particles, known as nanogels, composed of an amphiphilic polysaccharide, ethylenediamine group functionalization of cholesteryl group-bearing pullulan (Ayame, Morimoto, & Akiyoshi, 2008). The nanogel forms complex nanoparticles with various proteins. When holding a protein molecule in a segregated nanomatrix, the nanogel acts as an artificial chaperone to protect it from aggregation and denaturation and can assist in its refolding after release (Nomura, Ikeda, Yamaguchi, Aoyama, & Akiyoshi, 2003). In addition, cancer-specific recombinant antigens, for example human epidermal growth factor receptor 2 complexed with nanogel, act as an injection-type cancer vaccine to induce both anti-tumor cytotoxic T cells and antibody production enhancing T helper (Th) cells by effective antigen delivery to, and subsequent incorporation by, antigen processing and presenting cells, including macrophage and dendritic cells in peripheral lymph nodes (Ikuta et al., 2002). A Phase I clinical trial demonstrated the effect of the nanogel system, especially for the treatment of esophageal cancer (Tsuji et al., 2008). In our recent study, we demonstrated the advantages of cationic nanogel as a novel adjuvant-free and safe carrier for mucosal vaccines. For example, nasally administered nanogel holding a subunit type of *Clostridium botulinum* antigen (BoHc-nanogel) or pneumococcal surface protein A (PspA-nanogel) induced notable levels of antigen-specific systemic and mucosal immune responses, even in the absence of biologically active mucosal adjuvants (e.g., CT). Further, mice nasally immunized with BoHc-nanogel or PspA-nanogel were protected against a systemic challenge with botulinum toxin and an intranasal challenge with *Streptococcus pneumoniae*. In addition, nasal administration with nanogel holding radioisotope-labeled BoHc showed no evidence for the redirection of antigen to the CNS, including the olfactory bulb and brain (Nochi et al., 2010).

In this review, we introduce the unique characteristics of nanotechnology and the mucosal immune system that have allowed the merging of material engineering and mucosal immunology and the development of nanogel-based nasal vaccines as the next generation of mucosal vaccines for the prevention of infectious diseases.

Nanogel protein delivery with chaperone function

Therapeutic proteins such as cytokines, antigens and antibodies have attracted increasing interest because of their application in vaccines and immunotherapy for cancers, allergies and infectious diseases (Leader, Baca, & Golan, 2008). Since these proteins are easily denatured and can therefore lose their bioactivity before they reach their target tissue or immune induction site after delivery to patients, interest has focused on the development of a delivery system that can maintain efficient therapeutic effects of specific biologically active proteins at minimum dosages. Recently, nanogels, which are nano-sized hydrogel nanoparticles with a diameter > 100 nm, have attracted a great deal of attention as a nanocarrier in drug-delivery systems (DDS; Kabanov & Vinogradov, 2009). Nanogels can trap bioactive compounds such as drugs, proteins and DNA within their nano-scale polymer networks. We previously reported that nanogel consisting of self-assembled cholesteryl group-bearing pullulan (CHP) spontaneously forms a complex with different proteins by hydrophobic interactions and protects the entrapped proteins from aggregation and the subsequent loss of bioactivity (Figure 1a, b; Sasaki & Akiyoshi, 2010b).

The protective effect of CHP-based nanogels on proteins is similar to the function of existing molecular chaperones in living systems (Hartl, 1996). Native proteins

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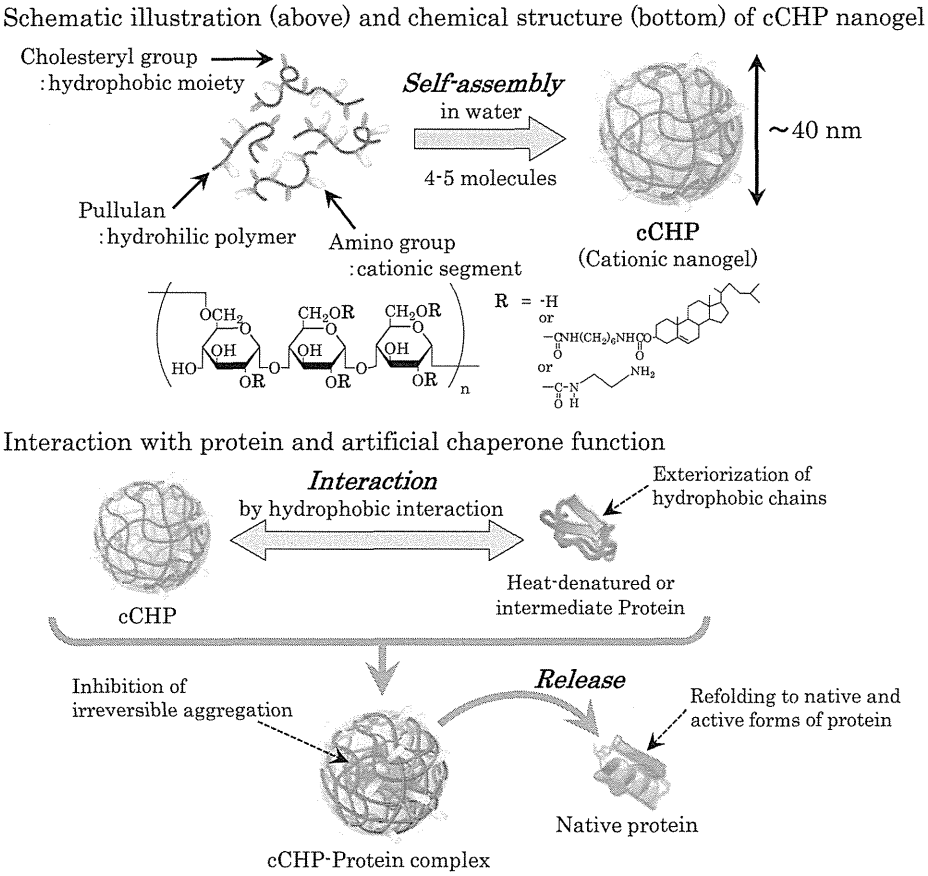

Figure 1. Cationic cholesteryl group-bearing pullulan (cCHP) forms chaperone nanogel.
Note: (a) Schematic illustration (top) and chemical structure (bottom) of cCHP nanogel. (b) Interaction with protein and artificial chaperone function.

partially unfold in response to stresses such as heat or chemical reagents when interior hydrophobic groups are exposed to the exterior surface. Hydrophobic interactions between exposed side-chains usually result in irreversible aggregation. Molecular chaperones selectively trap the folding intermediate proteins or heat-denatured proteins via hydrophobic interactions to prevent irreversible aggregation. Then, with the aid of adenosine triphosphate (ATP) and another co-chaperone, the chaperone releases the folded (refolded) proteins in a native and active form (Taguchi, Ueno, Tadakuma, Yoshida, & Funatsu, 2001). Therefore, the function of molecular chaperones is an important concept that is expected to lead to breakthroughs in DDS, especially in the delivery of peptides and unstable proteins.

CHP-based nanogels have potential to provide an effective protein-delivery system with artificial molecular chaperone function. They can capture proteins within the nanogel network in a denatured form above denaturation temperatures or in the presence of reagents (e.g., urea) that chemically denature proteins (Figure 1b). Because the size of the nanogel network is comparable to that of proteins, the nanogel can hold a protein molecule within the segregated nanomatrix. This feature of nanogels enhances protein stabilization enabling the isolation of trapped protein molecules for inhibition of pro-

tein–protein interactions and aggregation (Nomura et al., 2003; Sasaki and Akiyoshi, 2010a). Additionally, CHP nanogels can form a colloidally stable complex with protein with an overall complex size of about 50 nm, which is suitable for effective intracellular uptake. Cationic-type CHP (cCHP) nanogels, which have a modified cationic ethylenediamine group, can deliver cargo proteins efficiently to different types of mammalian cells (Ayame et al., 2008). We recently reported that cCHP nanogels can deliver proteins into the myeloma cell line J558L and primary $CD4^+$ T cells more efficiently than other carriers such as cationic liposomes and protein transduction domain-mediated protein-delivery systems (Watanabe et al., 2011). Furthermore, the anti-apoptotic protein Bcl-xL delivered by cCHP nanogels efficiently blocked apoptosis of these cells, indicating that the artificial chaperone function of nanogels is a useful tool to deliver proteins and maintain their major bioactivities.

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Mucosal immune system

To develop a mucosal vaccine for nasal delivery, it is necessary to appreciate the presence of a functionally unique mucosal immune system. Although the human body is constantly exposed to a large number of a variety of microbial antigens that enter the body via inhalation, ingestion and sexual contact, the mucosal immune system not only provides a front line of defense against invading pathogens by its induction of active immune responses, including the production of neutralizing secretory IgA (sIgA) antibodies, but also induces immunological unresponsiveness as a quiescent immune response to beneficial antigens, and then creates a homeostatic situation between the host's mucosal surfaces and the external environment (Czerkinsky et al., 1999).

The mucosal immune system consists of both inductive and effector sites and plays a key role in dynamic immune responses including the T-helper cell type 2 (Th2)-mediated secretary immunoglobulin A (sIgA) response, the mucosal cytotoxic T cell response, and the T helper 17 cell response, as well as in the harmonious regulation of these responses by regulatory T cells representing the active and quiescent phases of antigen-specific immune responses. When foreign antigens and pathogens are encountered as a result of ingestion or inhalation, these antigens are taken up by the inductive tissues associated with the digestive and respiratory tracts (Holmgren and Czerkinsky, 2005). Hosts have evolved a family of organized lymphoid tissues known as mucosa-associated lymphoid tissues (MALT) in the regions associated with the aerodigestive tract. Peyer's patches, an example of gut-associated lymphoid tissue, and nasopharynx-associated lymphoid tissue (NALT), are well-characterized members of the MALT family located in the intestinal and respiratory tracts, respectively (Neutra & Kozlowski, 2006). NALT are located at both basal sides of the nasal cavity in rodents but is not found at the same anatomic location in humans. Instead, oropharyngeal lymphoid tissues in the upper respiratory tract, including the adenoids and palatine tonsils in humans, are thought to act as organized mucosa-associated lymphoid structures (Kiyono & Fukuyama, 2004). NALT possess dedicated antigen-sampling cells, known as M cells, in the follicle-associated epithelium (FAE) region, which have the ability to take up antigens from the lumen of the upper respiratory tract. Dendritic cells (DC) immediately underneath the FAE capture and process antigens taken up by the M cells for the initiation of an antigen-specific immune response (Iwasaki, 2007). Classical M cells located in NALT are considered an important gateway to the environment and play an important role in the initiation of antigen-specific mucosal immunity against nasally encountered antigens (Neutra, Frey, & Kraehenbuhl, 1996). Mucosally administered

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antigens can also be taken up from the mucosal surfaces, which are covered by a layer of columnar epithelial cells such as small intestinal or nasal epithelia, by mucosal DC extending into the lumen and migrating to mesenteric or cervical lymph nodes for antigen presentation to naive lymphocytes (Rescigno et al., 2001). We recently demonstrated that respiratory M cells in the turbinates covered with a single layer of nasal epithelium have the potential to act as an alternative antigen sampling site for the initiation of antigen-specific immune responses (Kim et al., 2011).

Antigen-stimulated B and T cells in NALT express α4β1 integrin and the chemokine receptor CCR10, allowing them to be selectively trafficked to the effector sites of the nose, trachea and bronchus, where their ligands, vascular cell adhesion molecule 1 and chemokine ligand CCL28 are expressed (Campbell et al., 2001). The same mucosal migration molecules are involved in the trafficking of activated lymphocytes to the genitourinary tract, which can explain the effective induction of antigen-specific immune responses in the genital tract after intranasal immunization (Kunisawa, Fukuyama, & Kiyono, 2005). Recent studies have demonstrated the important biological role of mucosal DC in the imprinting of gut antigen-primed B and T cells, but not those from nasal or other mucosa, via the production of retinoic acids in gut-associated lymphoid tissue (Iwata et al., 2004; Mora et al., 2006). However, it remains unknown how the respiratory and genital imprinting molecules induced in the NALT originated antigen-specific T and B cells.

When IgA^+ B cells from the inductive sites migrate to distant effector sites, such as the nasal passage, the cells enter the final stage of differentiation in the presence of IgA-enhancing cytokines, such as interleukin-5 and interleukin-6 produced by Th2 cells, to become IgA-producing plasma cells for the subsequent production of dimeric or polymeric IgA (McGhee, Fujihashi, Beagley, & Kiyono, 1991). The dimeric or

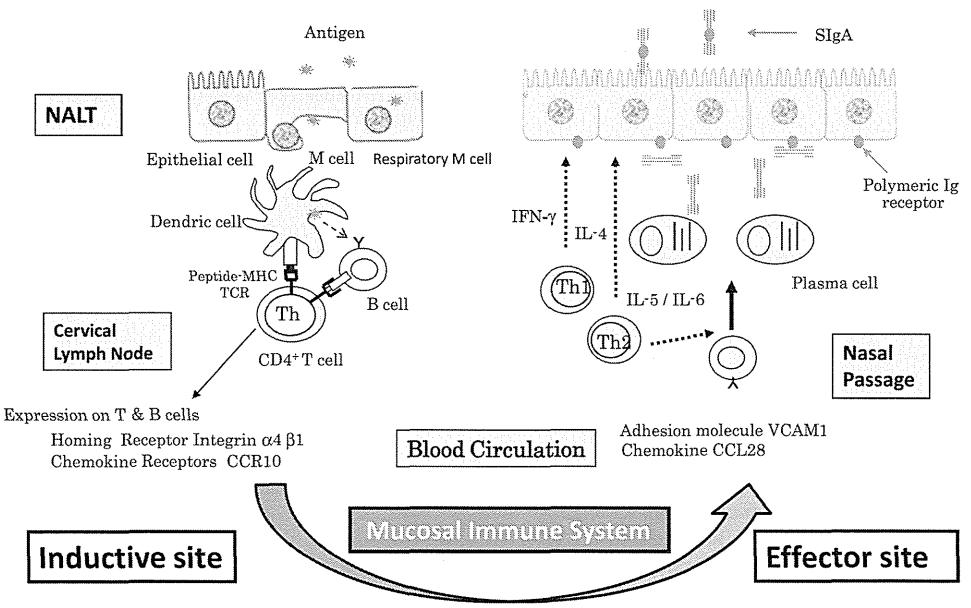

Figure 2. Nasal immune system.
Note: Schematic illustration of the nasal immunization system for the induction of antigen-specific secretory IgA.

polymeric IgA then binds to poly-immunoglobulin receptors (pIgR) expressed on the basolateral surface of epithelial cells for the formation and transport of sIgA. During transport to the apical surface of complexes of dimeric or polymeric forms of IgA and pIgR, the extracellular fragments of pIgR are secreted for the formation of sIgA (Kaetzel, 2005). In conclusion, interconnections between the inductive and effector sites of the mucosal immune system support the development of an intranasal vaccine (Figure 2).

Efficacy of nanogel-based nasal vaccines without adjuvants

An ideal intranasal vaccine will be delivered safely and effectively. To investigate whether a cCHP nanogel could be used as a carrier for an intranasal vaccine, we tested two types of vaccine candidate antigens including recombinant nontoxic receptor-binding fragment (heavy-chain C terminus; BoHc) of *C. botulinum* type A neurotoxin (BoNT/A; Park & Simpson, 2003) and pneumococcal surface protein A (PspA; Briles et al., 2003). We used cCHP nanogel containing 15 amino groups per 100 glucose units to enhance the delivery and adherence efficacy of vaccine antigen for nasal epithelium (Ayame et al., 2008). Fluorescence resonance energy transfer (FRET) is a useful tool to investigate the interaction between the cCHP nanogel and antigens. After fluorescein isocyanate-labeled antigen protein was incubated with tetramethylrhodamine isothiocyanate-labeled cCHP for 5 h at 45°C, FRET signals resulting from complexation were observed (Figure 3a; Nochi et al., 2010). Size analysis of the cCHP and cCHP-antigen complex by dynamic light scattering showed that the cCHP-antigen complexes cCHP/BoHc (32.9 ± 0.3 nm) and cCHP/PspA (32.8 ± 0.2 nm) maintained a size similar to that of the cCHP nanogel (34.8 ± 0.9 nm) and had a polydispersity index of < 0.15, indicating a narrow size distribution. In addition, the complex was positively charged

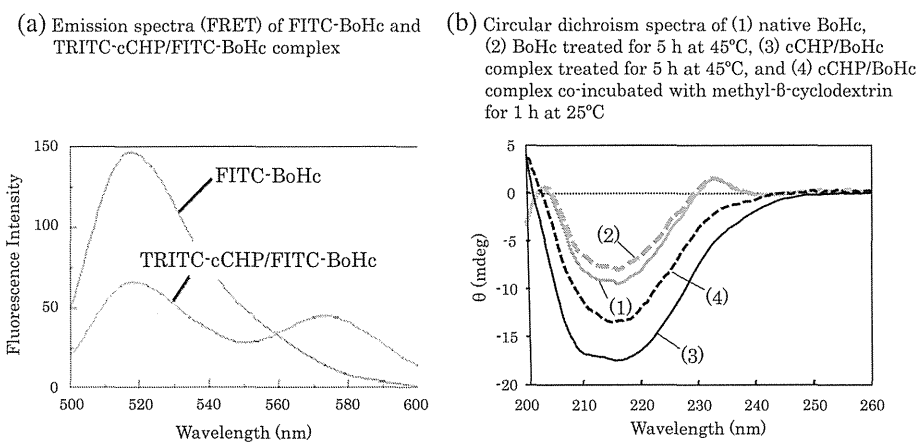

Figure 3. cCHP nanogel interacts with antigen BoHc and is released as a native form of BoHc. Note: (a) Fluorescence response energy transfer was detected from tetramethylrhodamine isothiocyanate (TRITC)-conjugated cCHP carrying fluorescein isocyanate (FITC)-conjugated BoHc, but not from FITC-conjugated naked-BoHc. (b) Circular dichroism analysis showed that the ellipticity (θ) value of BoHc, which was decreased to 15.2 mdeg after BoHc/A was incorporated into the cCHP nanogel, recovered to 9.4 mdeg after the release of BoHc/A from the cCHP nanogel by treatment with methyl-β-cyclodextrin. (1) native BoHc, (2) BoHc treated for 5 h at 45°C, (3) cCHP/BoHc complex treated for 5 h at 45°C, and (4) cCHP/BoHc complex co-incubated with methyl-β-cyclodextrin for 1 h at 25°C.

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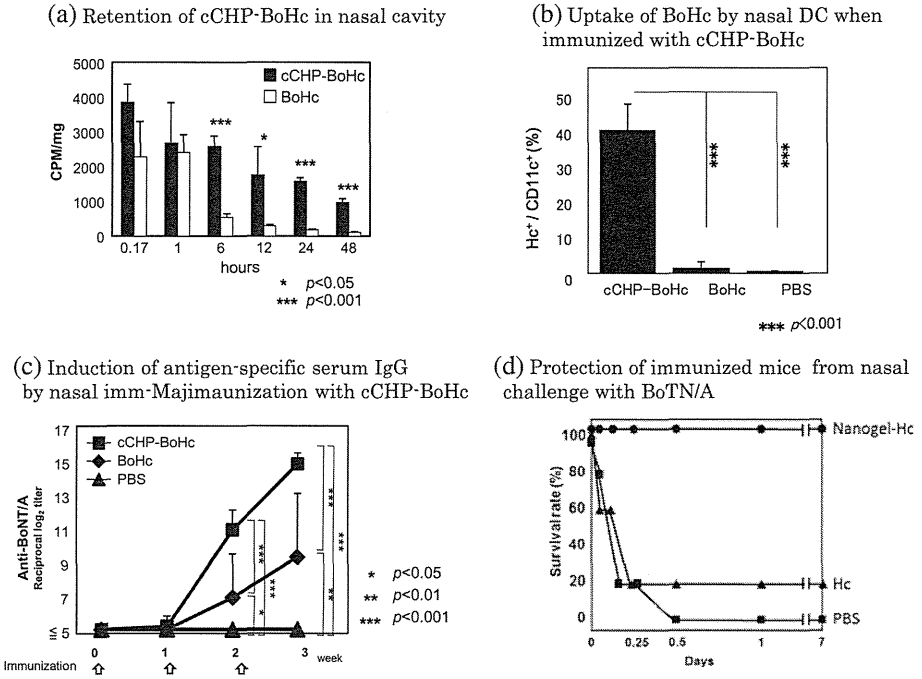

Figure 4. cCHP-BoHc is effectively delivered to the nasal cavity and induces protective immunity.

Note: (a) Direct quantitative study with [^{111}In]-labeled BoHc demonstrated that BoHc/A was retained in the nasal tissues for more than 2 days after intranasal immunization with cCHP nanogel. By contrast, most naked BoHc disappeared from the nasal cavity within 6 h of administration. (b) Flow cytometric analyses showed that BoHc/A released from cCHP nanogel was effectively taken up by CD11cC dendritic cells located in the epithelial layer and lamina propria of the nasal cavity, but naked BoHc/A was not. (c) Strong BoHc-specific serum IgG antibody responses were induced by intranasal immunization with cCHP–BoHc/A, but not by naked BoHc/A or control PBS. (d) Mice intranasally vaccinated with cCHP–BoHc/A were completely protected from intranasal challenge with BoNT/A, but not with naked BoHc/A or control PBS.

(ζ-potential = +7.0 ± 0.5 mV) from modification of the cationic segment to assist contact between the negatively charged mucosal surface and cell membrane. Circular dichroism measurements revealed that the secondary structure of the antigen had changed after internalization within the cCHP nanogel, but recovered on release, indicating that the antigen maintained specific antigenicity (Figure 3b; Nochi et al., 2010). The direct counting assay using radioisotope [^{111}In] (indium 111), which has a half-life of 2 days, clearly shows that the cCHP nanogel holding the [^{111}In]-labeled BoHc was effectively delivered to, and continuously retained in, the nasal mucosa for more than 2 days when administered nasally; most of the [^{111}In]-labeled BoHc administered without the cCHP nanogel had dispersed within 6 h (Figure 4a; Nochi et al., 2010). These results show that the cCHP nanogel is an effective and useful nasal vaccine delivery vehicle, which can continuously deposit antigens to the nasal cavity including both inductive and effector sites.

To address directly whether vaccine antigens (e.g., BoHc) delivered by cCHP nanogel can be taken up from nasal epithelium to initiate antigen-specific immune responses by the airway mucosal immune system *in vivo*, we next performed histochemical stud-