研究成果の刊行に関する一覧表レイアウト

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S. Sato, S. Kaneto, N. Shibata, Y. Takahashi, H. Okura, **Y. Yuki**, J. Kunisawa, H. Kiyono	Transcription factor Spi-B–dependent and –independentpathways for the development of Peyer's patch M cells	Mucosal Immunol			2012(Dec5)
Fukuyama Y, Tokuhara D, Kataoka K, Gilbert RS, McGhee JR, **Yuki Y**, Kiyono H, Fujihashi K	Novel vaccine development strategies for inducing mucosal immunity	Expert Rev. Vaccines	11	376-79	2012
IG. Kong, A. Sato, **Y. Yuki**, T. Nochi, H. Takahashi, S. Sawada, M. Mejima, S. Kurokawa, K. Okada, S. Sato, D. Briles, J. Kunisawa, Y. Inoue, M. Yamamoto, K. Akiyoshi, and H. Kiyono	Nanogel-based PspA intranasal vaccine prevents invasive disease and nasal colonization by Pneumococcus	Infect. & Immun	81	1625-1634	2013
Y. Fukuyama, D. Tokuhara, S. Sekine, K. Aso, K. Kataoka, J. Davydova, M. Yamamoto, RS Gilbert, Y. Tokuhara, K. Fujihashi, J. Kunisawa, **Y. Yuki**, H. Kiyono, JR McGhee, K. Fijihashi	Potential roles of CCR5+CCR6+ dendric cells induced by nasal ovalbumin plus Flt3 lig and expressing adnovirus for mucosal IgA response	PloS One	8	e60453	2013
van Riet E, Ainai A, Suzuki T, Hasegawa H.	Mucosal IgA responses in influenza virus infections; thoughts for vaccine design	Vaccine	30	5893-5900	2012
Ainai A, Tamura S, Suzuki T, Ito R, Asanuma H, Tanimoto T, Gomi Y, Manabe S, Ishikawa T, Okuno Y, Odagiri T, Tashiro M, Sata T, Kurata T, Hasegawa H	Characterization of neutralizing antibodies in adults after intranasal vaccination with an inactivated influenza vaccine	J Med Virol	84	336-344	2012

Ⅳ. 研究成果の刊行物・別冊

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RNAi suppression of rice endogenous storage proteins enhances the production of rice-based *Botulinum* neutrotoxin type A vaccine

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ABSTRACT

Mucosal vaccines based on rice (MucoRice) offer a highly practical and cost-effective strategy for vaccinating large populations against mucosal infections. However, the limitation of low expression and yield of vaccine antigens with high molecular weight remains to be overcome. Here, we introduced RNAi technology to advance the MucoRice system by co-introducing antisense sequences specific for genes encoding endogenous rice storage proteins to minimize storage protein production and allow more space for the accumulation of vaccine antigen in rice seed. When we used RNAi suppression of a combination of major rice endogenous storage proteins, 13 kDa prolamin and glutelin A in a T-DNA vector, we could highly express a vaccine comprising the 45 kDa C-terminal half of the heavy chain of botulinum type A neurotoxin (BoHc), at an average of 100 μg per seed (MucoRice-BoHc). The MucoRice-Hc was water soluble, and was expressed in the cytoplasm but not in protein body I or II of rice seeds. Thus, our adaptation of the RNAi system improved the yield of a vaccine antigen with a high molecular weight. When the mucosal immunogenicity of the purified MucoRice-BoHc was examined, the vaccine induced protective immunity against a challenge with botulinum type A neurotoxin in mice. These findings demonstrate the efficiency and utility of the advanced MucoRice system as an innovative vaccine production system for generating highly immunogenic mucosal vaccines of high-molecular-weight antigens.

1. Introduction

Although several plants have been shown to be useful for vaccine production [1], there is accumulating evidence that the seed crop rice is one of the most suitable systems for vaccine production, storage, and delivery [2]. We have previously developed rice expressing the B subunit of cholera toxin (CTB) vaccine, MucoRice-CTB, which possesses mucosal immunogenicity and prevents diarrhea in the event of *V. cholerae* and heat-labile enterotoxin-producing enterotoxigenic *Escherichia coli* challenges [3–6]. This rice-based vaccine has proven to be stable at room temperature for three years and thus could be used as a cold-chain-free vaccine [3,6].

In this study, we co-introduced antisense sequences specific for genes encoding endogenous rice storage proteins to block expression of these proteins and allow space for the increased accumulation of vaccine antigen in rice seed [7]. A previous report showed the feasibility of increasing the accumulation of an endogenous seed storage protein, cruciferin, by using an antisense sequence to reduce the production of another authentic seed protein (napin) in *Brassica napus* seeds [8]. In general, there are two types of protein storage organelles, called protein bodies (PB-I and -II), in rice seeds. Alcohol-soluble prolamins are expressed in PB-I (10, 13 16 kDa) and alkali-soluble glutelins (A and B) are expressed in PB-II [9–11]. To examine whether suppression of the production of prolamins or glutelins can effectively increase the expression of a transgene-encoded vaccine antigen, we investigated the effects of RNAi suppression of 13 kDa prolamin and/or glutelin A in a T-DNA vector on expression of a candidate vaccine.

To increase the versatility of the MucoRice system, it is necessary to develop high-yield vaccines for antigens with high molecular

Abbreviations: Ab, antibody; BoHc, a nontoxic subunit fragment of *Clostridium botulinum* type-A neurotoxin; BoNT/A, *C. botulinum* neurotoxin type-A; CT, cholera toxin; CTB, cholera toxin B-subunit; ELISA, Enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; LPS, lipopolysaccharide; mCTA/LTB, A subunit of mutant cholera toxin E112K with the pentameric B subunit of heat-labile enterotoxin from enterotoxigenic *Escherichia coli*; PB, protein body; PBS, phosphate-buffered saline; RNAi, RNA interference; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SIgA, secretory IgA; T-DNA, transfer DNA.

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weight. In the original study, we successfully expressed a low-molecular-weight CTB antigen (monomer, 11 kDa) at 30 μg per seed [3]. Here, we chose a nontoxic 45 kDa fragment of the C-terminal half of the heavy chain of botulinum neurotoxin type A (BoHc) to use as an example of a high-molecular–weight vaccine antigen to evaluate the advanced MucoRice expression system. Although the botulinum neurotoxin is known as oral poisons and is absorbed from the gut to reach peripheral nerve terminals via the blood circulation, the toxin also acts as an inhalant poison, which is absorbed from the airway [12]. The Hc fragment of type A (BoHc) has been successfully used as a nasal vaccine against botulism in mice and nonhuman primates [13].

Here, we could successfully express high yields of high molecular-weight BoHc in a soluble form with the use of an optimized RNAi vector. Because the rice-based BoHc vaccine (or MucoRice-BoHc) was water-soluble and could be purified easily by standard gel filtration, our results demonstrate that advanced MucoRice system can be used for the preparation of purified antigen for nasal immunization and the induction of protective immunity against a neurological toxin.

2. Materials and methods

2.1. DNA construction, transformation of rice plants, and purification of rice-based BoHc

The sequences encoding BoHc were synthesized with optimized codon usage for rice [3] and inserted into a binary T-DNA vector (pZH2B/35SNos) [14] with an overexpressing cassette of BoHc and a combination cassette for RNAi suppression of either 13 kDa prolamin or glutelin A or both storage proteins (Fig. 1A) as described previously [7]. A RNAi casette containing no RNAi trigger sequences for rice endogenous storage proteins was called pZH2BiK. RNAi cassettes containing RNAi trigger sequences for the suppression of the genes encoding 13 kDa prolamin and glutelin A were constructed and called pZH2Bik45 and pZH2BikG1B, respectively. The RNAi trigger sequence for the gene encoding 13 kDa prolamin was a 45 bp fragment of rice 13 kDa prolamin gene comprising coding sequence 1–45. The RNAi trigger sequence for glutelin gene was a 129 bp fragment of the rice glutelin A gene comprising coding sequence 142–270. The *Acs* I–*Mul* I fragment of the BoHc expression cassette was subcloned into pZH2BiK, pZH2Bik45, pZH2BikG1B, and pZH2Bik45-G1B. The expression vectors were used to transform a japonica variety of rice, *Nipponbare*, by using an *Agrobacterium*-mediated method described previously [3] and the recombinant BoHc produced was termed MucoRice-BoHc. The rice expressing BoHc together with a combination cassette for RNAi suppression of both 13 kDa prolamin and glutelin A was polished and extracted by using PBS and then purified by using gel filtration on a Sephadex G-100 column.

2.2. Preparation of recombinant proteins

A recombinant BoHc was constructed and produced by use of the *E. coli* expression system as previously described [13]. A nontoxic form of chimeric mucosal adjuvant that combines the A subunit of mutant cholera toxin E112K with the pentameric B subunit of heat-labile enterotoxin from enterotoxigenic *E. coli* (mCTA/LTB) was constructed and produced by use of the *Brevibacillus choshinensis* expression system as previously described [15]. The level of LPS contamination in the purified BoHc and mCTA/LTB (<10 endotoxin units/mg protein) were measured by using a Limulus Test (Wako).

2.3. Protein analyses

Total seed protein was extracted from transgenic rice plant seeds as described previously [3]. Briefly, seeds of rice plants were ground to a fine powder by using a Multibeads shocker (Yasui Kikai, Osaka, Japan) and extracted in the sample buffer (2% [w/v] SDS, 5% [w/v] β-mercaptoethanol, 50 mM Tris–HCl [pH 6.8], and 20% [w/v] glycerol) and the proteins were separated by SDS-PAGE followed by Western blot analysis with rabbit anti-BoHc antibody (Ab), which was established in our laboratory using *E. coli*-derived recombinant BoHc. The level of BoHc accumulated in the rice seeds was determined by densitometry analysis of a Western blot against a standard curve generated with the use of purified *E. coli*-derived BoHc, as previously described [3].

2.4. Immunohistochemical and immune electronmicroscopic analyses

To microscopically evaluate the localization of BoHc in the MucoRice-BoHc seed, a frozen section of the rice seed was reacted with polyclonal rabbit anti-BoHc Ab and visualized with the use of 3,3′-diaminobenzidine. We confirmed that normal rabbit IgG as a control showed no immune-reactivity in MucoRice-BoHc seed. The distribution of BoHc expressed in rice seeds was analyzed by using immunoelectron microscopy with polyclonal rabbit anti-BoHc Ab as described previously [16].

2.5. Immunization, sample preparation and ELISA for detection of BoHc-Ab

To examine the mucosal immunogenicity of MucoRice-BoHc, the purified material of MucoRice-BoHc (100 μg) alone, *E. coli*-derived rBoHc (100 μg) alone, MucoRice-BoHc (25 μg) with or without CT (1 μg, List Biological Laboratories, Campbell, CA) or MucoRice-BoHc (25 μg) with mCTA/LTB (10 μg) dissolved in 20 μl of PBS, or PBS vehicle alone was intranasally immunized in mice (10 μl/nostril, $N=10$) on 3 occasions at 1 wk intervals. The serum and nasal wash were collected prior to immunization, and 1 wk after each immunization. BoHc-specific Ab responses were determined by using BoHc-specific enzyme-linked immunosorbent assay (ELISA) as described previously [17]. Endpoint titers were expressed as the reciprocal log 2 of the last dilution that gave an OD450 of 0.1 greater than that of the negative control.

2.6. Preparation of botulinum neurotoxin A (BoNT/A)

BoNT/A from *C. botulinum* type-A 62 was purified from the culture supernatant as previously described [18]. The toxicity of purified BoNT/A (1.1×10^8 mouse i.p. LD_{50}/mg protein) was assayed by time to death after intravenous injection into mice [19].

2.7. Neutralizing assay

To analyze the protective activity of antigen-specific mucosal Ab immune responses induced by the use of purified MucoRice-BoHc as a nasal vaccine against toxin-induced neurological death, we performed a toxin challenge study, as described previously with some modification [17]. Briefly, the immunized mice were intraperitoneally challenged with 100 ng (1.1×10^4 i.p. LD_{50}) or 500 ng of BoNT/A (5.5×10^4 i.p. LD_{50}) diluted in 100 μl of 0.2% gelatin/PBS, and their survival was observed for 7 days.

2.8. Data analysis

Differences between groups were assessed by Tukey's *t*-test for ELISA data and the log-rank (Mantel–Cox) test for survival analyses. *P* values < 0.05 were considered to indicate statistical significance.

3. Results

3.1. Establishment of rice-expressed botulinum type A neurotoxin vaccine

To increase the levels of high-molecular weight vaccine antigens, such as BoHc, that can be accumulated in rice grains, we constructed T-DNA vectors containing expression cassettes with four combinations of RNAi triggers specific for different storage protein genes together with a vaccine gene cassette (Fig. 1). Several independent transgenic rice lines were generated for each of the four types of MucoRice and the BoHc accumulation levels in seeds were determined by densitometry analysis of Western blots. For each of the four types of MucoRice, the plant line with the highest levels of BoHc antigen accumulated in the seed was selected and advanced to the T3 generation by self-crossing to obtain homozygous lines.

SDS-PAGE showed that the content of 13 kDa prolamin and/or glutelin in MucoRice BoHc lines were approximately half that of the WT (Fig. 2B). Examination of the expression levels of BoHc by densitometry analysis revealed that the expression level of BoHc in rice seeds suppressing both 13 kDa prolamin and glutelin A reached an average of 100 μg per seed, whereas rice seeds carrying the vector with no RNAi trigger obtained an average of 10 μg BoHc per seed. Seeds carrying vectors that suppressed 13 kDa prolamin alone or glutelin A alone obtained an average of 10 μg or 30 μg BoHc per seed, respectively. All further experiments were performed with MucoRice-BoHc derived from rice suppressing both 13 kDa prolamin and glutelin A.

When MucoRice-BoHc was extracted by PBS, densitometry analysis showed that an average 85% of all the MucoRice-BoHc was recovered in PBS (Fig. 2C). Because the MucoRice-BoHc was soluble in PBS, we were able to purify the protein from polished rice without the aleurone layer by using gel filtration (Fig. 2C). The yield of purified BoHc from the total amount of harvested rice seeds was approximately 68% (an average of 68 μg per seed). The level of LPS contamination in purified MucoRice-BoHc was less than 10 endotoxin units/mg protein, which was equivalent to that in highly purified rBoHc from the *E. coli* expression system.

3.2. MucoRice-BoHc accumulated in the cytoplasm between protein bodies in rice seeds

Because expression of BoHc was under the control of the 13 kDa prolamin-specific promoter as a rice-seed-specific promoter (Fig. 1), we examined the location of BoHc expression in MucoRice-BoHc seeds. The results of immunohistochemistry showed that BoHc accumulated in the endosperm cells under

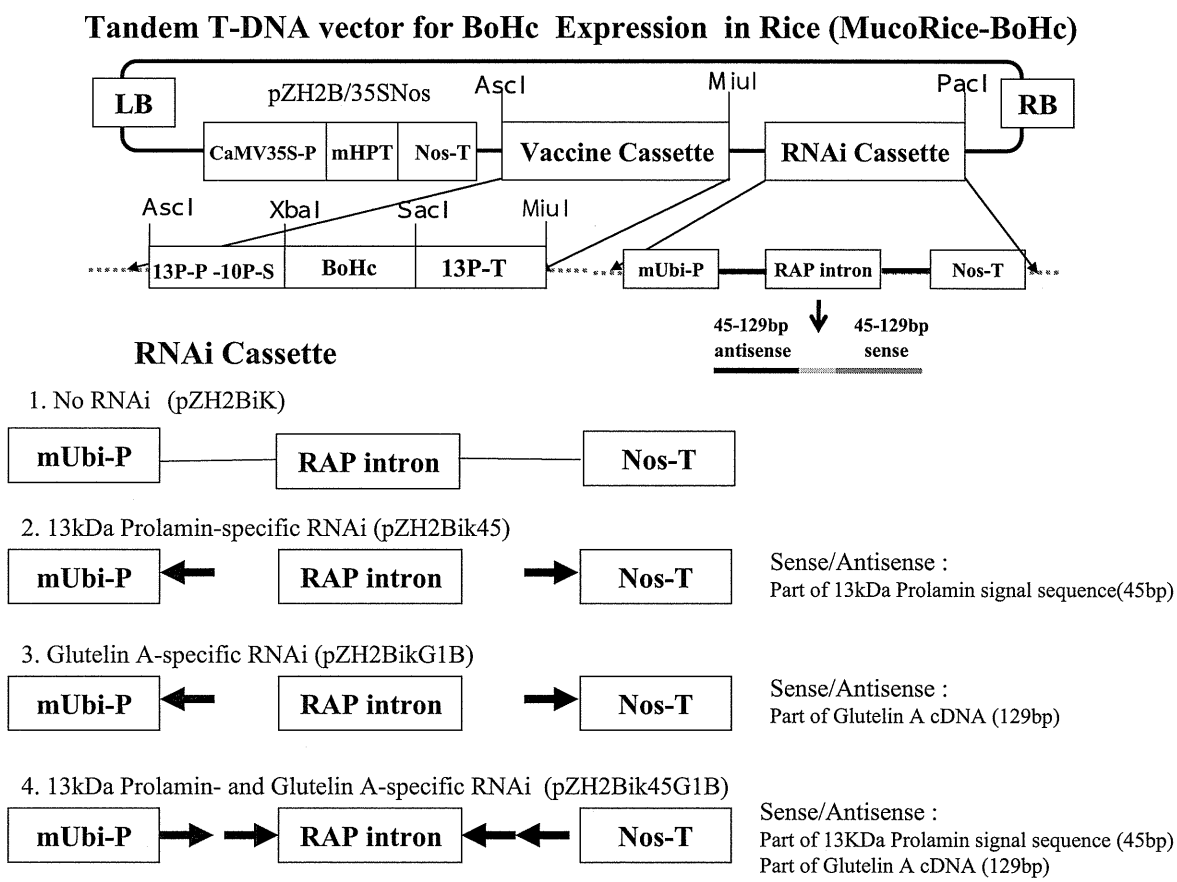

Fig. 1. T-DNA plasmids for RNAi suppression of storage proteins and overexpression of BoHc in rice seed. We constructed a tandem T-DNA plasmid containing a BoHc overexpression cassette with BoHc vaccine antigen sequences controlled by the promoter of rice 13 kDa prolamin, and a combination cassette of RNAi triggers for suppression of major rice endogeneous storage proteins, 13 kDa prolamin and/or glutelin controlled by the ubiquitin promoter. CaMV35S-P, cauliflower mosaic virus 35S promoter; mHPT, mutant hygromycin phosphotransferase; 13P-P, 13 kDa prolamin promoter; 10P-S, signal sequence of 10 kDa prolamin; 13P-T, 13 kDa prolamin terminator; Nos-T, nos terminator; RAP intron, rice aspartic protease intron; Ubi-P, ubiquitin promoter; LB, T-DNA left border; RB, T-DNA right border.

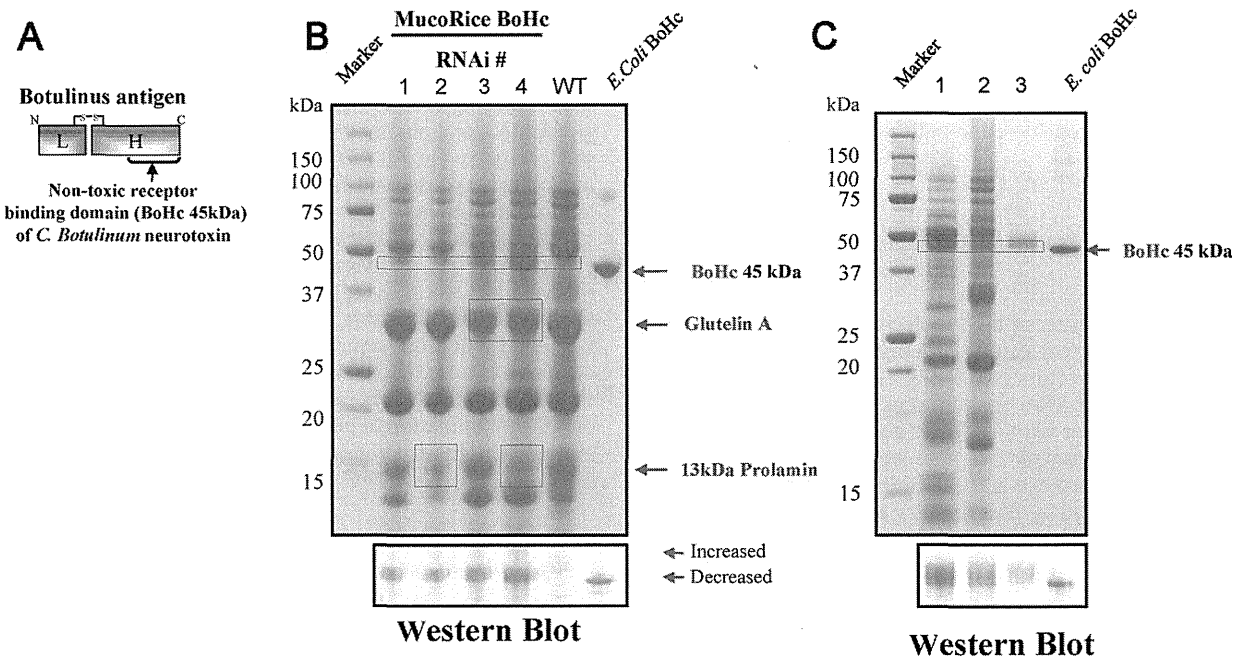

Fig. 2. Expression and purified MucoRice-BoHc. (A) Conceptual scheme of BoHc antigen expressed in MucoRice or *E. coli*. (B) SDS-PAGE analysis comparing protein expression among MucoRice-BoHc lines containing various T DNA plasmids: #1, no RNAi suppression; #2, RNAi suppression of 13 kDa prolamin; #3, RNAi suppression of glutelin A; #4, RNAi suppression of 13 kDa prolamin and glutelin A. WT indicates wild-type rice, and *E. coli* BoHc indicates recombinant BoHc from *E. coli* system. The results showed predominant expression of MucoRice-BoHc with a molecular weight of approximately 48 kDa (red arrowhead, *E. coli* derived BoHc with 45 kDa). Endogenous rice proteins (blue arrows, glutelin A and 13 kDa prolamin) were reduced in MucoRice-BoHc with RNAi suppression of glutelin and/or 13 kDa prolamin compared with levels in non-transformed WT rice. Western blotting revealed that a transgenic protein of 48 kDa specifically reacted with anti-BoHc Ab. (C) The samples were analyzed by SDS-PAGE and Western blot with rabbit anti-BoHc. 1, extract from MucoRice-BoHc transgenic #4 seed (with RNAi suppression of both 13 kDa prolamin and glutelin A) powder produced by using PBS; 2, extract from the MuocRice-BoHc #4 seed powder produced by re-extracting the PBS extract with SDS-sample buffer [2% (w/v) SDS, 5% (w/v) β-mercaptoethanol, 50 mM Tris–HCl (pH 6.8)]; 3, purified MucoRice-BoHc, which was isolated from PBS-extract of the MucoRice-BoHc #4 seed powder by using a gel filtration on Sephadex G-100 column. rBoHc indicates BoHc purified from *E. coli*.

the aleurone layer in MucoRice-BoHc seeds, whereas immune-reactivity was absent in wild-type rice seeds (Fig. 3A).

Furthermore, immune-electron-microscopy analysis of MucoRice-BoHc seeds showed that the vaccine antigens were unexpectedly expressed in cytoplasm at the interspace between PB-I and PB-II (Fig. 3B). It is interesting to note that destruction of PB-II was found in MucoRice-BoHc seeds but not WT rice. Because expression level of rice storage proteins including 13 kDa prolamin and glutelin A in MucoRice-BoHc seeds were suppressed (Fig. 2B), the RNAi knockdown on MucoRice system most likely accounts for the abnormal storage organelle formation.

3.3. Nasal MucoRice-BoHc induces not only systemic but also mucosal antigen-specific Ab immune responses

To examine whether purified MucoRice-BoHc antigen maintained sufficient immunogenicity to induce a protective Ab response, mice were nasally immunized with PBS (vehicle control) or 100 μg of purified MucoRice-BoHc or rBoHc from an *E. coli*–BoHc expression system. Antigen-specific Ab responses were assessed by using *E. coli*-derived BoHc as a coating antigen for ELISAs. After nasal immunization, the levels of BoHc-specific IgG Ab titers were not significantly different between mice nasally immunized with MucoRice-BoHc and those nasally immunized with *E. coli*-derived rBoHc (Fig. 4A). However, a detectable level of rBoHc-specific secretory IgA (SIgA) Ab titers was found in the nasal washes of mice that were nasally immunized with MucoRice-BoHc (Fig. 4A).

To confirm whether the immunogenicity of MucoRice-BoHc was sufficient for the induction of antigen-specific mucosal immunity, mice were nasally immunized with 25 μg of purified MucoRice-BoHc with or without CT or nontoxic chimera mCTA/LTB as mucosal adjuvants. Both nasal MucoRice-BoHc with CT and nasal MucoRice-BoHc with mCTA/LTB induced brisk rBoHc-specific serum IgG and nasal IgA Ab immune responses (Fig. 4B).

3.4. Nasal MucoRice-BoHc induces protective immunity against botulinum neurotoxin

Next, a challenge test with BoNT/A was performed on all BoHc-vaccinated mice and control mice to examine the quality of BoHc-specific Ab induced. Mice that were vaccinated with 100 μg of purified MucoRice-BoHc or *E. coli*-derived rBoHc without the presence of mucosal adjuvant, but not the control mice, were partially protected against the high lethal dose (100 ng, 1.1×10^4 i.p. LD_{50}) of intraperitoneally injected BoNT/A (Fig. 5A). The mice that were nasally immunized with 25 μg of purified MucoRice-BoHc with CT or mCTA/LTB were completely protected against the extraordinarily high lethal dose (500 ng, 5.5×10^4 i.p. LD_{50}) of intraperitoneally injected BoNT/A, since the mice nasally immunized with 25 μg of MucoRice-BoHc or PBS only failed to protect against the same amount of toxin (Fig. 5B). In the group of mice that were nasally immunized with 25 μg of MucoRice-BoHc with CT or mCTA/LTB, there were no clinical signs of toxin-associated disease over a 1 wk observation period following the BoNT/A challenge test.

4. Discussion

One of our major goals was the adoption of RNAi technology to advance the MucoRice system by co-introduction of antisense sequences specific for endogenous rice storage proteins. We used

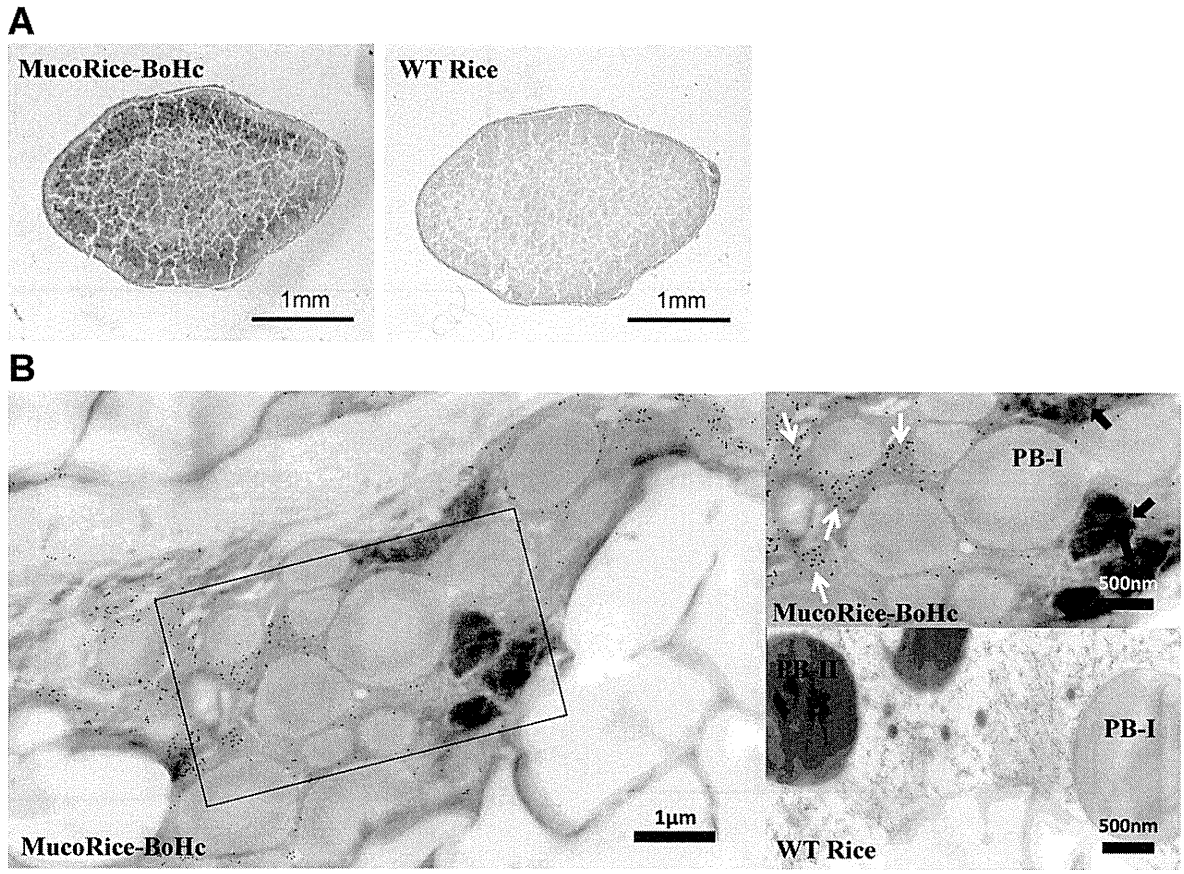

Fig. 3. Immunohistochemical and immune electronmicroscopic analyses of MucoRice-BoHc seed. (A) Immunohistochemical analysis showed that BoHc had accumulated in the whole MucoRice-BoHc seed, whereas it was not detected in a WT-rice seed. (B) Immune electronmicroscopy of MucoRice-BoHc showed that BoHc in the endosperm cells of MucoRice-BoHc was observed as black spots (white arrows). BoHc was predominantly localized in cytoplasm as the interspace between PB-I and PB-II. Destruction of PB-II structure was found in MucoRice-BoHc seed (black arrows) when compared with WT rice seed.

a high-molecular-weight protein vaccine antigen – the 45 kDa C-terminal half (BoHc) of botulinum neurotoxin [13,17] – to showcase the practicality of using this system to improve the capacity for vaccine antigen accumulation in rice seed. To optimize the choice of endogenous storage protein to suppress, we performed RNAi suppression of rice 13 kDa prolamin, glutelin A, or both by altering the sequences included in RNAi cassette in the T-DNA vector and then assessed the level of expression of the antigen MucoRice-BoHc. SDS-PAGE and western blot analysis revealed that suppressing both 13 kDa prolamin and glutelin A greatly improved the production of high-molecular-weight vaccine antigen, BoHc (100 μg/seed) when compared the T-DNA vector without RNAi (10 μg/seed) (Fig. 2B). Thus, introduction of the RNAi technology into the MucoRice vaccine antigen expression system allowed a high-molecular-weight vaccine antigen to be expressed in MucoRice with high yields.

Another unique feature of the BoHc antigen produced by using the advanced MucoRice system was its water-solubility. Because proteins in PBs are not soluble in water [10,11], the unique and advanced property of MucoRice-BoHc comes from its expression in cytoplasm between PB-I and PB-II of endosperm cells in rice seed (Fig. 3B). Because we use prolamin promoter and signal for targeting BoHc to PB-I or PB-II in rice endosperm cells, we cannot explain logically why the vaccine antigen accumulated in cytoplasm and not in PBs. However, BoHc antigen location and level of accumulation might be attributed to abnormal storage organelle formation including destruction of PB-II structure caused by the knock-down of glutelin A in the advanced MucoRice system (Fig. 3B). The MucoRice-BoHc from rice powder of polished rice could be easily dissolved in and extracted from PBS and could be purified by a single-step gel filtration without endotoxin contamination. Thus, the high expression level and PBS-solubility of MucoRice-BoHc confer an economic advantage over vaccine products that are expressed in bacteria or other plant systems.

SDS-PAGE analysis indicated that the molecular weight of MucoRice-BoHc (48 kDa) was slightly higher than that of rBoHc from *E. coli* (45 kDa) (Fig. 2B and C). Because there are nine glycosylation-sequon (N-X-T/S) positions in the sequence of BoHc [20], we considered that several sugar chains attach to MucoRice-BoHc at these positions but not to rBoHc from *E. coli*. SDS-PAGE, and Western blot analysis detected a least 3–4 bands corresponding to MucoRice-BoHc, whereas *E. coli*-derived rBoHc was detected as a single band only (Fig. 2B and C). These results suggest that MucoRice-BoHc is a mixture of fully and partially glycosylated protein.

We next examined whether MucoRice-BoHc was highly immunogenic when compared with *E. coli*-derived rBoHc after nasal administration in mice. When mice were nasally immunized with 100 μg of purified MucoRice-BoHc or *E. coli*-derived rBoHc, there are no statistical differences in BoHc-specific IgG immune responses and protective immunity against high lethal dose of a neurotoxin challenge test between mice immunized with MucoRice-BoHc and *E. coli*–derived rBoHc. Taken together, these results demonstrate that the quality of the toxin neutralization

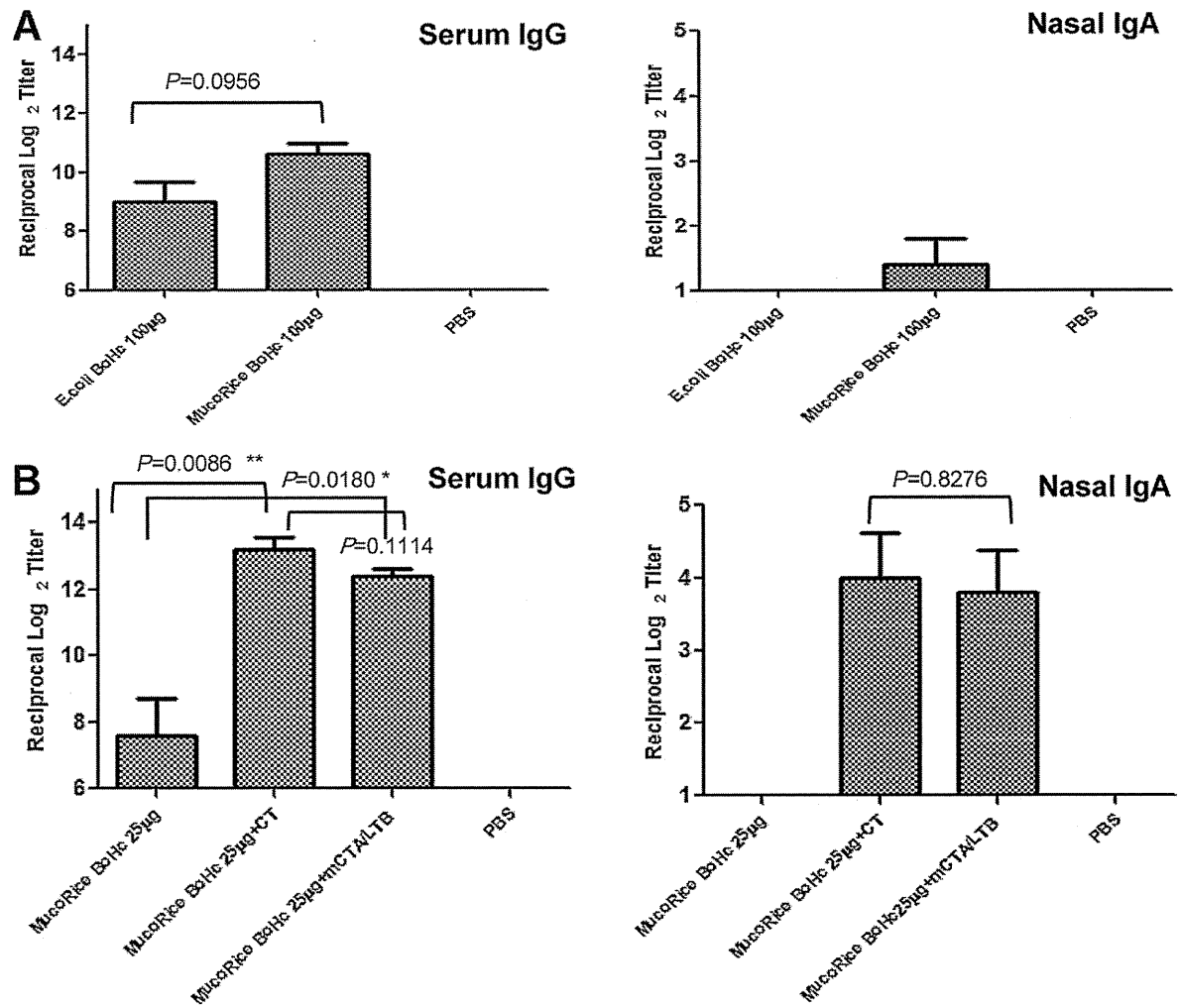

Fig. 4. Immunogenicity of purified MucoRice-BoHc produced by using advanced MucoRice system. (A) Mice were nasally immunized with 100 μg of purified MucoRice-BoHc alone on 3 occasions at 1-week intervals and compared with mice that were nasally immunized with same dose of recombinant BoHc prepared by means of a standard *E. coli* system (*E. coli*-BoHc). (B) In a separate study, mice were immunized with nasal vaccine containing 25 μg purified MucoRice-BoHc with or without mucosal adjuvant cholera toxin (1 μg) or nontoxic chimera adjuvant (mCTA/LTB, 10 μg) on 3 occasions at 1-week intervals. Data are expressed as means ± standard deviation ($N = 10$). The experiments were conducted three times. $^{*}P < 0.05$; $^{**}P < 0.01$.

Ab activity induced by MucoRice-BoHc was comparable to that induced by *E. coli*-derived rBoHc and that the immunogenicity of the MucoRice-BoHc and *E. coli*-derived rBoHc mucosal vaccines was equivalent.

To examine whether MucoRice-BoHc could induce high levels of mucosal immune response when nasally administered together with mucosal adjuvant, mice were immunized with nasal vaccine composed of 25 μg of MucoRice-BoHc and mucosal adjuvant CT or mCTA/LTB. As one might expect, antigen-specific serum IgG and nasal IgA Ab responses were rapidly induced in mice immunized with nasal MucoRice-BoHc plus adjuvant. Further, there was no difference between the antigen-specific immune responses induced by nasal MucoRice-BoHc administered with CT or mCTA/LTB. Native CT is a potent enterotoxin and induces high total and vaccine-specific immunoglobulin E responses; however, use of the mCTA/LTB chimera adjuvant did not have the same effect in our previous study [15]. To confirm the quality of BoHc-specific antibodies induced by MucoRice-BoHc plus CT or mCTA/LTB, we performed a challenge test on vaccinated mice by using an extraordinarily high lethal dose (5.5×10^4 mouse i.p. LD_{50}) of intraperitoneally injected BoNT/A. Regardless of the adjuvant used, the immunized mice survived after the challenge, indicating that they had gained full protection from the neurotoxin. In contrast, the non-immunized mice died within 12 h. These results suggest that MucoRice-BoHc with nontoxic chimera adjuvant CTA/LTB has the potential to be used as a promising nasal vaccine against botulism.

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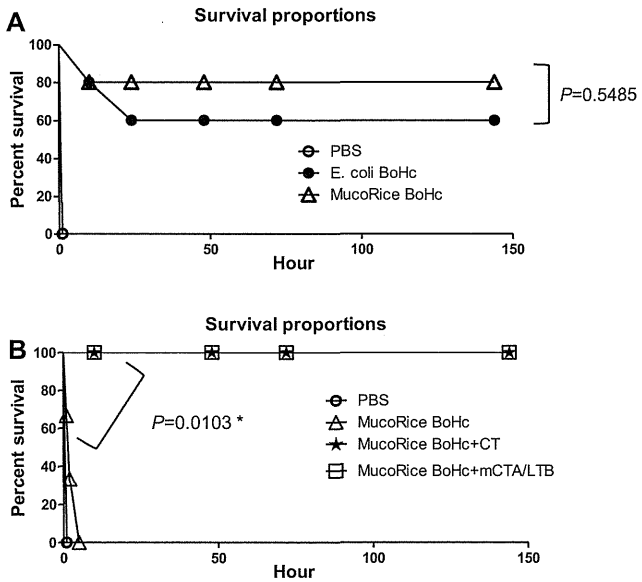

Fig. 5. Induction of protective immunity the against neurotoxin in mice immunized with nasal vaccine containing MucoRice-BoHc and CT or nontoxic mCTA/LTB. (A) Mice immunized with 100 μg of purified MucoRice-BoHc or *E. coli*-derived rBoHc were challenged with a high lethal dose (100 ng, 1.1×10^4 mouse i.p. LD_{50}) of intraperitoneal injection of BoNT/A. (B) Mice immunized with 25 μg of purified MucoRice-BoHc together with CT or mCTA/LTB were challenged with an extraordinarily high lethal dose (500 ng, 5.5×10^4 mouse i.p. LD_{50}) of intraperitoneal injection of BoNT/A. Immunization conditions were described in Fig. 4A and B. The experiments were conducted three times. $^*P < 0.05$; $^{**}P < 0.01$.

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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

(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).

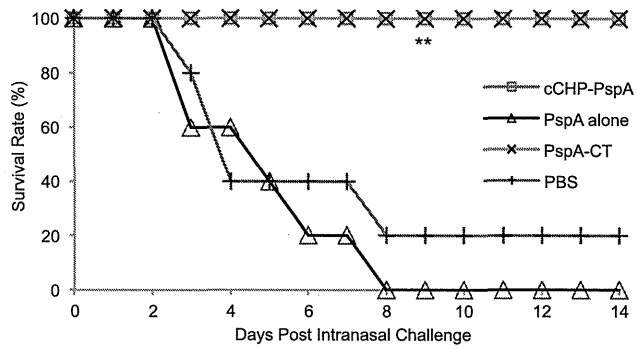

FIG 1 Intranasal vaccination with cCHP-PspA induced protective immunity against pneumococci. One week after the final immunization, mice were challenged with *S. pneumoniae* Xen10 (2×10^5 CFU/mouse), and survival was monitored. Data are representative of three independent experiments, and each group 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

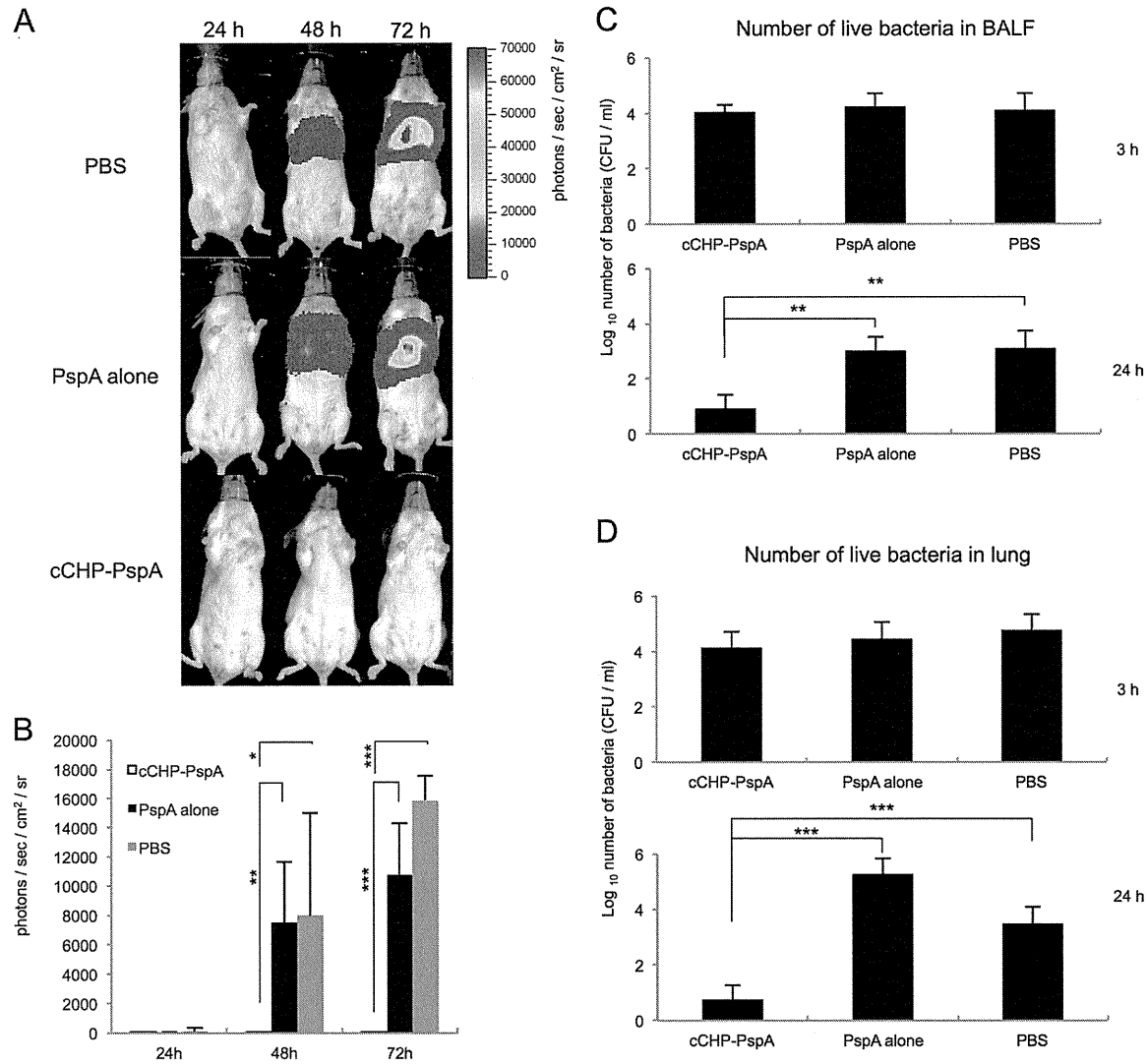

FIG 2 *In vivo* imaging revealed no sign of pneumococcal infection in the lungs of mice immunized intranasally with cCHP-PspA; these mice also showed enhanced bacterial clearance from the BALF and lung. Images (A) and average photon counts (B) show bioluminescence due to *S. pneumoniae* Xen10 in each 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

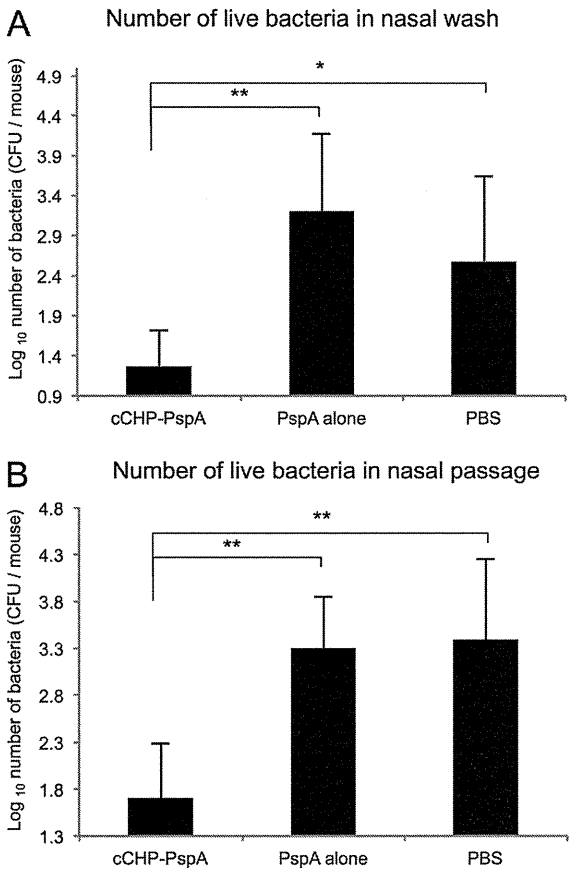

FIG 3 Intranasal vaccination with cCHP-PspA reduced bacterial colonization of the nasal cavity. One week after the final immunization, mice were challenged with a sublethal dose (2×10^4 CFU/mouse) of *Streptococcus pneumoniae* Xen10. Nasal washes and tissues were collected, and the numbers of *S. pneumoniae* 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.

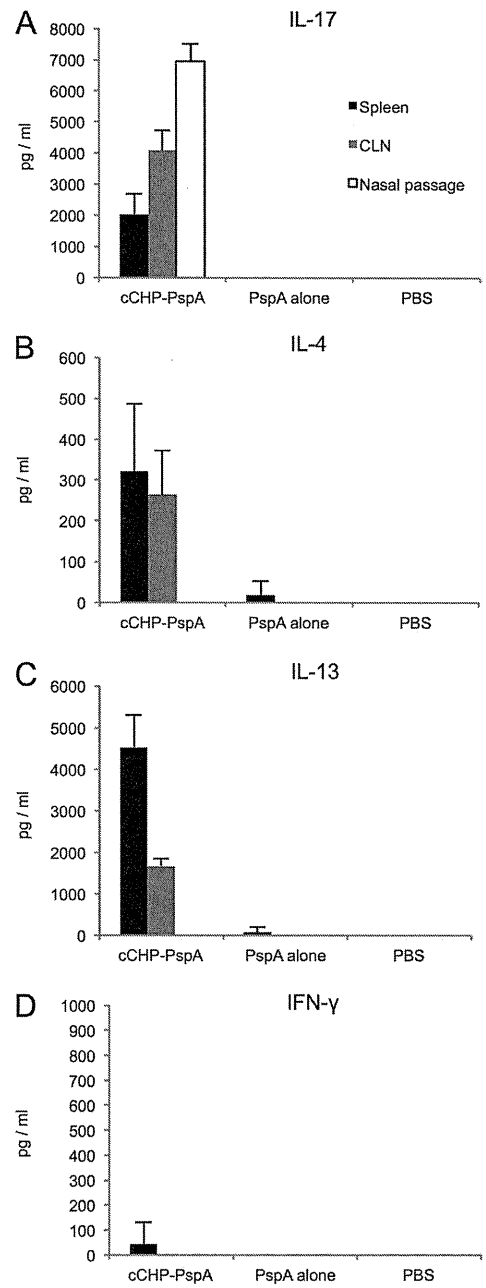

FIG 4 $CD4^+$ T cells from cCHP-PspA-immunized mice produce Th17- and Th2-type immune responses. Cytokines produced by $CD4^+$ T cells isolated from the spleens, cervical lymph nodes, and nasal passages of mice immunized with cCHP-PspA, PspA alone, or PBS only were analyzed. Data are representative of 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

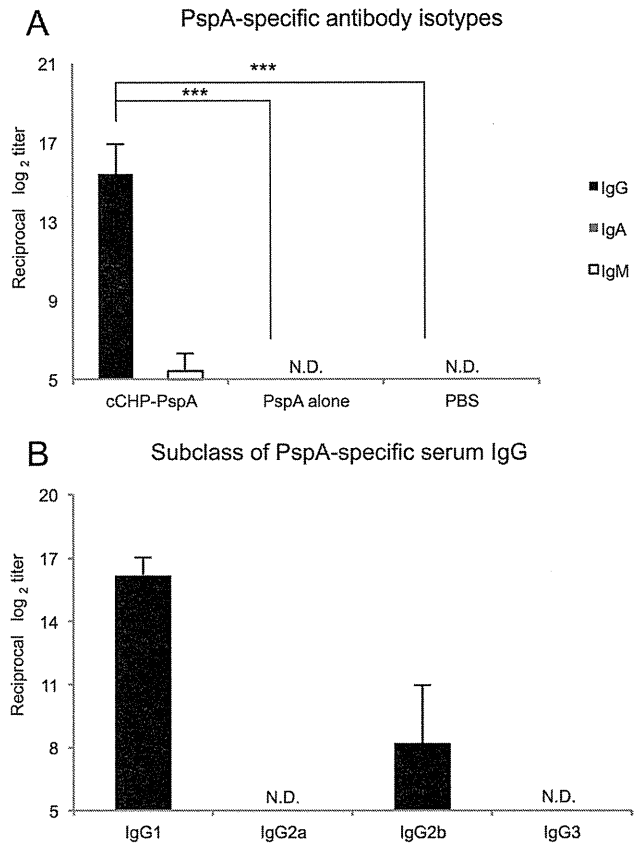

FIG 5 Intranasal vaccination with cCHP-PspA induced high levels of systemic antibodies. The data show the PspA-specific serum IgG level (A) and subclass analysis for IgG1, IgG2a, IgG2b, and IgG3 (B) for each immunized group (cCHP-PspA, PspA alone, or PBS only). Titers of PspA-specific IgG in sera were 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

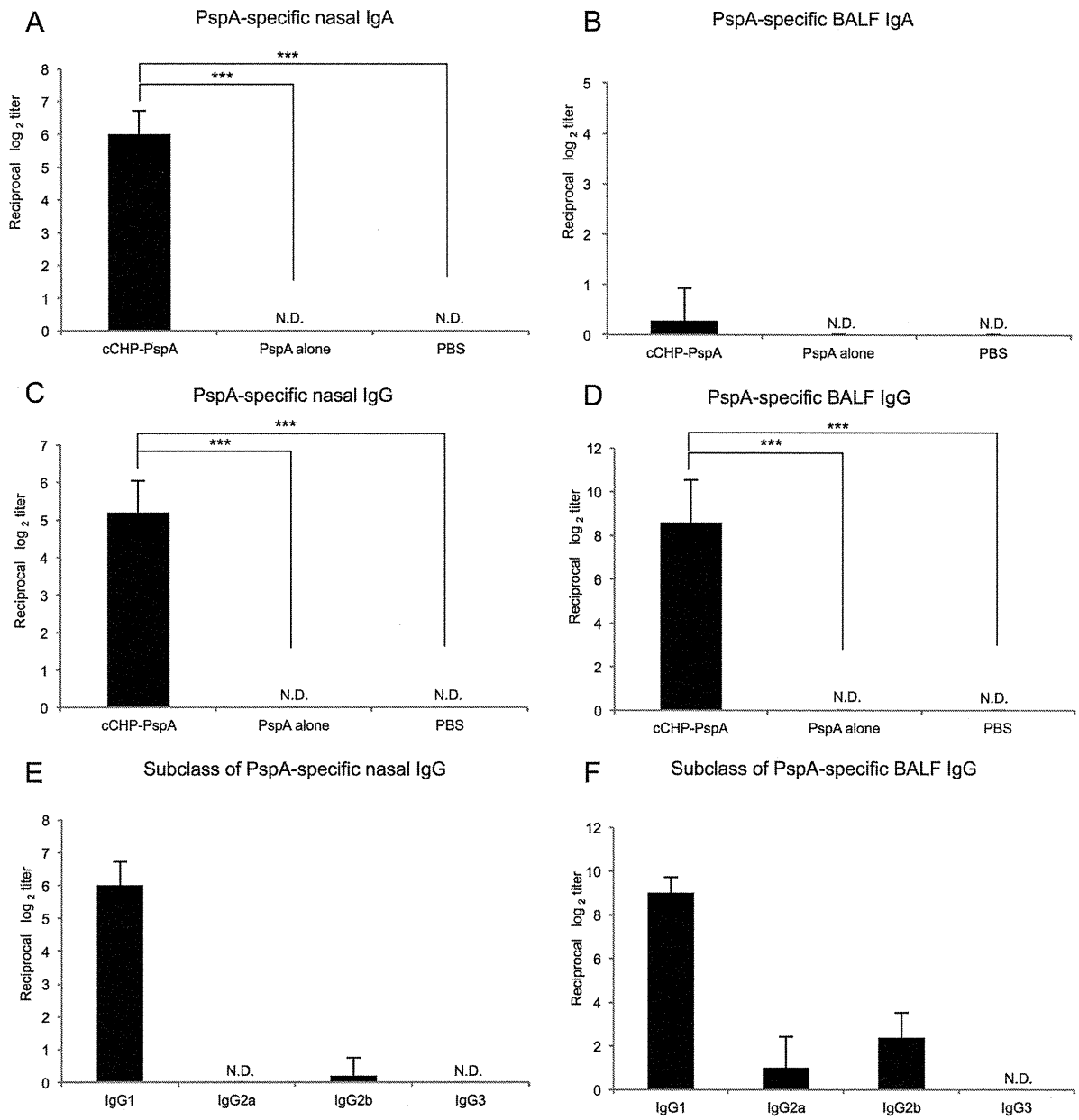

FIG 6 Intranasal vaccination with cCHP-PspA induced strong PspA-specific secretory IgA and IgG responses. Titers of nasal (A and C) and bronchial (B and D) IgA and IgG induced by intranasal immunization with PspA alone or PspA mixed with cCHP are shown. Titers of PspA-specific IgA and IgG in nasal washes 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-

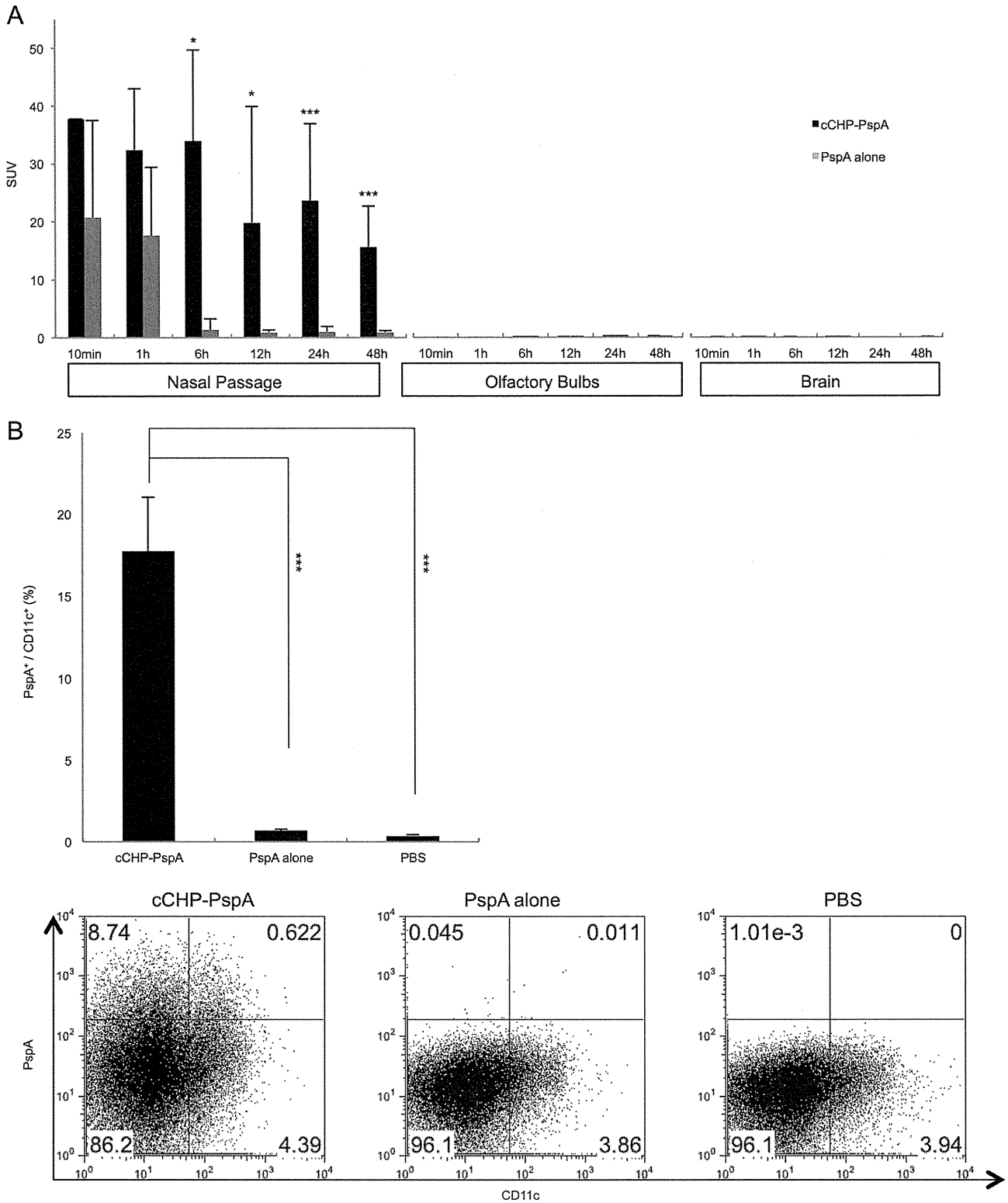

FIG 7 Intranasal vaccination with cCHP-PspA induced no accumulation of PspA in the central nervous system (A) but enhanced the efficiency of uptake of PspA by dendritic cells in the nasal passages (B). (A) ^{111}In-labeled PspA was administered intranasally with or without cCHP nanogel, and the radioisotope 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.

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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Characterization of Neutralizing Antibodies in Adults After Intranasal Vaccination With an Inactivated Influenza Vaccine

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The levels and properties of neutralizing antibodies in nasal wash and serum collected from five healthy adults were examined after intranasal administration of an A/Uruguay/716/2007 (H3N2) split vaccine (45 μg hemagglutinin (HA) per dose; five doses, with an interval of 3 weeks between each dose). Prior to the assays, nasal wash samples were concentrated so that the total amount of antibodies was equivalent to about 1/10 of that found in the natural nasal mucus. Vaccination induced virus-specific neutralizing antibody responses, which increased with the number of vaccine doses given. Neutralizing antibodies were produced more efficiently in the nasal passages than in the serum: A ≥4-fold increase in nasal neutralization titres was observed after the second vaccination in four out of five subjects, whereas a rise in serum neutralization titres was observed only after the fifth vaccination. Nasal and serum neutralizing antibodies were mainly found in the polymeric IgA and monomeric IgG fractions, respectively, after gel filtration. Taken together, these results suggest that intranasal administration of an inactivated split vaccine induces high levels of nasal neutralizing antibodies (primarily polymeric IgA) and low levels of serum neutralizing antibodies (primarily monomeric IgG). ***J. Med. Virol. 84:336–344, 2012.*** © 2011 Wiley Periodicals, Inc.

KEY WORDS: influenza; vaccine; neutralizing antibody

INTRODUCTION

To prevent influenza, protective immunity must be induced in advance by administration of a vaccine. Currently available inactivated vaccines, detergent disrupted split-viruses, or purified glycoproteins (surface antigen vaccines) are given via parenteral injection [Murphy and Webster, 1996]. Parenteral vaccination, that is, vaccination via the non-mucosal route, induces serum IgG antibodies, which are highly protective against homologous virus infection, but less effective against heterologous virus infection. Thus, intramuscular vaccination of seasonal influenza vaccine would be less effective in protecting against a heterologous virus epidemic.

A large number of studies show that the protective immunity induced by influenza virus infection is mainly mediated by secretory IgA (S-IgA) and IgG antibodies within the respiratory tract. S-IgA is carried to the mucus by transepithelial transport, while serum IgG is transported from the serum to the mucus by diffusion [Murphy and Clements, 1989; Brandtzag et al., 1994; Murphy, 1994; Asahi et al., 2002; Asahi-Ozaki et al., 2004]. S-IgA in the upper respiratory tract prevents viral infection, while IgG supports S-IgA-mediated protection by neutralizing newly-generated viruses [Ito et al., 2003; Renegar et al., 2004]. IgG is the main antibody involved in anti-viral protection in the lungs [Ramphal et al., 1979; Palladino et al., 1995; Renegar et al., 1998; Ito et al., 2003]. Also, polymeric S-IgA neutralizes viruses more effectively than monomeric IgA or IgG [Taylor

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