*trans*-10-octadecenoic acid, and *cis*-9-octadecenoic acid (oleic acid) were generated from linoleic acid (Fig. 1*B*, *4*). Thus, the combined action of these four enzymes generated saturated products of oleic acid and *trans*-10-octadecenoic acid from linoleic acid (i.e., these four enzymes catalyzed the saturation of an polyunsaturated fatty acid).

In our previous studies, we revealed that linoleic acid could be converted into 10-hydroxy-*cis*-12-octadecenoic acid by CLA-HY (Fig. 1*B*, *2*), as well as into CLA1 and CLA2 along with 10-hydroxy-*cis*-12-octadecenoic acid by CLA-HY, CLA-DH, and CLA-DC (Fig. 1*B*, *3*). We purified the resulting 10-hydroxy-*cis*-12-octadecenoic acid by HPLC and used it as a substrate for reactions containing each enzyme together with the oxidoreduction cofactors (i.e., FAD, NADH, or NADPH) that enhanced CLA synthesis by CLA-HY, CLA-DH, and CLA-DC (14). 10-Hydroxy-*cis*-12-octadecenoic acid was converted into linoleic acid and *trans*-10, *cis*-12-octadecadienoic acid by CLA-HY in the presence of FAD and NADH (*SI Appendix*, Fig. S1). The same substrate was converted into 10-oxo-*cis*-12-octadecenoic acid by CLA-DH in the presence of $NAD^+$ (*SI Appendix*, Fig. S2). As before, we purified the resulting *trans*-10,*cis*-12-octadecadienoic acid and 10-oxo-*cis*-12-octadecenoic acid by HPLC and used them as substrates in subsequent reactions.

None of the enzymes converted *trans*-10,*cis*-12-octadecadienoic acid under any conditions tested. By contrast, 10-oxo-*cis*-12-octadecenoic acid was converted into 10-oxo-*trans*-11-octadecenoic acid by CLA-DC in the absence of cofactors (*SI Appendix*, Fig. S3). HPLC-purified 10-oxo-*trans*-11-octadecenoic acid was converted into 10-oxo-octadecanoic acid by CLA-ER in the presence of FAD/FMN and NADH (*SI Appendix*, Fig. S4) and was converted into 10-hydroxy-*trans*-11-octadecenoic acid by CLA-DH in the presence of NADH (*SI Appendix*, Fig. S5). Purified 10-oxo-octadecanoic acid was converted into 10-hydroxy-octadecanoic acid by CLA-DH in the presence of NADH (*SI Appendix*, Fig. S6), and this product was, in turn, converted into *cis*-9-octadecenoic acid and *trans*-10-octadecenoic acid by CLA-HY in the presence of FAD and NADH (*SI Appendix*, Fig. S7). The 10-hydroxy-*trans*-11-octadecenoic acid could also be converted into *cis*-9,*trans*-11-CLA and *trans*-9,*trans*-11-CLA by CLA-HY in the presence of FAD and NADH (*SI Appendix*, Fig. S8).

These results demonstrate that the linoleic acid-saturation metabolism of *L. plantarum* consists of multiple reactions. The first reaction of linoleic acid metabolism is hydration of the carbon–carbon double bond at the Δ9 position, catalyzed by CLA-HY, to generate 10-hydroxy fatty acid. The second reaction is dehydrogenation of the hydroxy group at C10, catalyzed by CLA-DH, to generate 10-oxo fatty acid. The third reaction is isomerization of the carbon–carbon double bond at Δ12, catalyzed by CLA-DC, to generate the conjugated enone structure, 10-oxo-*trans*-11-fatty acid. The fourth reaction is hydrogenation of the carbon–carbon double bond at Δ11, catalyzed by CLA-ER, to generate the carbon–carbon single bond. The fifth reaction is hydrogenation of the oxo group at C10, catalyzed by CLA-DH, to generate 10-hydroxy fatty acid. The last reaction is dehydration of hydroxy group at C10, catalyzed by CLA-HY, to generate *cis*-9 and *trans*-10 monoenoic fatty acids. Through a branch of the saturation-metabolism pathway, conjugated fatty acids are generated by the combined actions of three of the enzymes, CLA-HY, CLA-DH, and CLA-DC. The branched pathway starts with hydrogenation of the oxo group at C10 in 10-oxo-*trans*-11-fatty acid, catalyzed by CLA-DH, to generate 10-hydroxy-*trans*-11-fatty acid; the final reaction is dehydration of hydroxy group at C10 in 10-hydroxy-*trans*-11-fatty acid, catalyzed by CLA-HY, to generate *cis*-9,*trans*-11– and *trans*-9,*trans*-11–conjugated fatty acids (Fig. 2). As we reported previously, C18 fatty acids with Δ9 and Δ12 diene systems such as α-linolenic acid, γ-linolenic acid, and stearidonic acid undergo the same transformations in *L. plantarum* AKU 1009a (20), indicating that the corresponding intermediates (hydroxy, oxo, conjugated, and partially saturated *trans*-fatty acids) are produced by the combined actions of these enzymes. The revealed fatty acid-saturation metabolism consists of similar reactions in known fatty acid biosynthesis and degradation pathway; however, it is a pathway that uses only free fatty acids as substrates but not CoA- or acyl carrier protein-activated fatty acids.

In the experiments described above, we revealed the pathway of unsaturated fatty acid metabolism in *L. plantarum* and the enzymes involved in this metabolism. These enzymes function as catalysts of hydration/dehydration (CLA-HY), oxidation of hydroxy groups/reduction of oxo groups (CLA-DH), migration of carbon–carbon double bonds (CLA-DC), and saturation of carbon–carbon double bonds (CLA-ER). The genes that encode CLA-DH, CLA-DC, and CLA-ER form a gene cluster in *L. plantarum*. When we searched for gene clusters containing *cla-dh*, *cla-dc*, and *cla-er* in other microorganisms using the Kyoto Encyclopedia of Genes and Genomes database (www.genome.jp/kegg), we found that *Lactobacillus casei* and *Lactobacillus rhamnosus* have the same gene cluster, as well as the CLA-HY gene. Furthermore, many species of lactic acid bacteria have one or more of these four genes. For example, *Lactobacillus salivarius* has *cla-dc*, *cla-er*, and *cla-hy*; and *Lactobacillus amylovorus*, *Lactobacillus johnsonii*, *Lactobacillus helveticus*, and *Lactobacillus crispatus* have *cla-dh*, *cla-er*, and *cla-hy*. Therefore, acting in concert, these species may mediate the polyunsaturated fatty acid-saturation metabolism in gastrointestinal tract. The in vivo distributions of these strains in relation to the fatty acid profiles and the health conditions of host organisms are also of interest.

The reactions and enzymes we identified will be useful for modifying the properties of fatty acids in foods. The apparent isomerization reaction catalyzed by the combined activities of CLA-HY, CLA-DH, and CLA-DC will be useful for production of *cis*-9,*trans*-11– and *trans*-9,*trans*-11–conjugated fatty acids. The other isomerization reaction catalyzed by CLA-HY will be useful for production of *trans*-10,*cis*-12–conjugated fatty acids. Furthermore, the dehydration reaction catalyzed by CLA-HY might determine the ratio of *trans* to *cis*-fatty acids. In other words, enhancing *cis*-dehydration by CLA-HY could be useful for reducing the amounts of *trans*-fatty acid in foods. This might be possible as we reported previously that the CLA1/CLA2 ratio (*cis*/*trans* ratio) can be controlled by optimizing the reaction conditions (14, 16). Furthermore, not only in the food industry but also in the chemical industry, the reactions found in the fatty acid-saturation metabolism are useful (e.g., enzymatic production of hydroxy fatty acids for polymer industry).

To evaluate the effects of gastrointestinal bacteria on the profile of fatty acids in host tissues, we monitored endogenous formation of the fatty acid intermediates of polyunsaturated fatty acid-saturation metabolism (i.e., hydroxy and oxo fatty acids) in mice bred in either germ-free or specific pathogen-free (SPF) conditions. In the colon, small intestine, and plasma of both groups of mice, we detected 10-hydroxy-*cis*-12-octadecenoic acid, 10-hydroxyoctadecanoic acid, and 10-oxooctadecanoic acid. By contrast, we did not detect 10-oxo-*cis*-12-octadecenoic acid or 10-oxo-*trans*-11-octadecenoic acid derived from linoleic acid or metabolites derived from α-linolenic acid and γ-linolenic acid. There were significant differences in the levels of hydroxy fatty acids between SPF and germ-free mice: in particular, we observed higher levels of 10-hydroxy fatty acids derived from linoleic acid, oleic acid, or both in SPF mice than in germ-free mice (Fig. 3). We also detected a structurally related hydroxy fatty acid, 13-hydroxy-9-*cis*-octadecenoic acid, which is produced by lactic acid bacteria (27), at higher levels in SPF mice than in germ-free mice (Fig. 3). These differences in the levels of hydroxy fatty acids could be clearly observed in the small intestine, the primary site of fatty acid absorption. These results indicate that gastrointestinal microbes play roles in modifying the fatty acid profiles of their host mice,

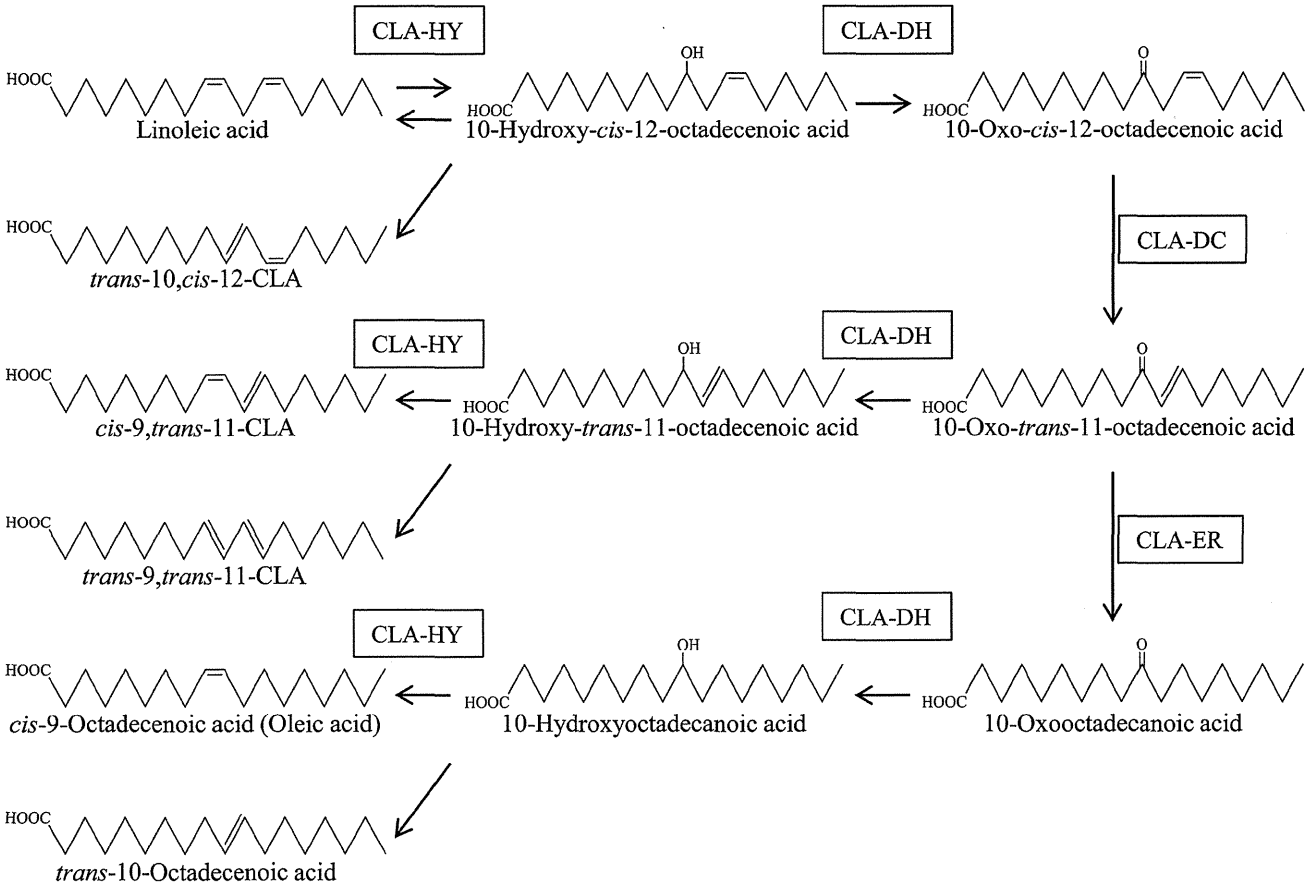


**Fig. 2.** Polyunsaturated fatty acid-metabolism pathway.

in particular, by increasing the levels of hydroxy fatty acids that are initial intermediates of polyunsaturated fatty acid-saturation metabolism. The investigations using monocolonize germ-free mice with *L. plantarum* or a strain deficient in one or more of the fatty acid saturating genes to see whether the host accumulates intermediates are also future interests.

The intermediates found in the polyunsaturated fatty acid-saturation metabolism described here are predicted to exert specific physiological functions. For example, hydroxy and oxo fatty acids act as ligands for PPARγ (28); oxo-fatty acids found in tomato juice are potent PPARα activators and decrease the amount of triacylglycerol in obese diabetic mice (29). Therefore, functional investigations of these fatty acid intermediates of the polyunsaturated fatty acid-saturation metabolism may provide new methods for improving our health by altering lipid metabolism related to the onset of the metabolic syndrome. Exploration of the lipid metabolism of gastrointestinal microorganisms at the enzymatic and genetic levels, and integration of these findings with metagenomic information, might enable us to promote health by controlling intestinal lipid metabolism.

## Materials and Methods

**Chemicals.** Standard samples of *cis*-9,*trans*-11-CLA, *trans*-9,*trans*-11-CLA, and 10-hydroxy-*cis*-12-octadecenoic acid were prepared as described previously (11, 13). Linoleic acid and fatty acid-free (<0.02%) BSA were purchased from Sigma. All other chemicals were of analytical grade and were obtained commercially.

**Cloning and Expression of Recombinant CLA-ER Proteins in *E. coli*.** Primers were designed to amplify the *cla-er* sequence from *L. plantarum* AKU 1009a genomic DNA. The PCR-amplified product was ligated into the pET101/D-TOPO expression vector (Invitrogen), according to the manufacturer's instructions. The resulting plasmid, pCLA-ER, was transformed into *E. coli* Rosetta2 (DE3) (Novagen) to generate *E. coli* Rosetta/pCLA-ER. The integrity of the cloned gene was verified by DNA sequencing using a Beckman-Coulter CEQ8000.

**Expression of Recombinant Proteins in *E. coli*.** Transformants (*E. coli* Rosetta/pCLA-HY, *E. coli* Rosetta/pCLA-DH, *E. coli* Rosetta/pCLA-DC, and *E. coli* Rosetta/pCLA-ER) were cultured in Luria–Bertani medium at 37 °C for 1 h with shaking at 300 rpm, and then isopropyl-β-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM. After addition of IPTG, the transformed cells were cultivated at 20 °C for 6 h with shaking at 300 rpm. The cells were harvested by centrifugation (12,000 × *g*; 10 min), washed twice with 0.85% NaCl, and stored at −20 °C until further use.

**Purification of Enzymes from Transformants.** To determine the approximate concentration of protein eluted in chromatography, effluents were monitored by UV detection at 280 nm. Enzymes were purified using a fast protein liquid chromatography system (Amersham Pharmacia Biotech) equilibrated with binding buffer [20 mM potassium phosphate buffer (KPB), 50 mM imidazole; pH 7.4] or standard buffer (20 mM KPB, 1 mM DTT, 10% (vol/vol) ethylene glycol; pH 6.5). Fractions with enzymatic activity were collected and concentrated using an Amicon Ultra YM-10 (Millipore). All procedures were carried out at 4 °C.

CLA-HY and CLA-ER were purified as His-tagged proteins. *E. coli* Rosetta/pCLA-HY and *E. coli* Rosetta/pCLA-ER cells (8 g) in 1.5 L of culture broth were suspended in binding buffer and disrupted with an Insonator 201M ultrasonic oscillator (Kubota). After ultracentrifugation (100,000 × *g*; 60 min) of the cell lysate, the resulting supernatant containing His-tagged CLA-HY or His-tagged CLA-ER was loaded onto a Ni-Sepharose column (His Trap HP; GE Healthcare) preequilibrated with binding buffer. After washing, the bound proteins were eluted with elution buffer (20 mM KPB, 250 mM imidazole; pH 7.4). Active fractions were collected and concentrated with a Centriprep

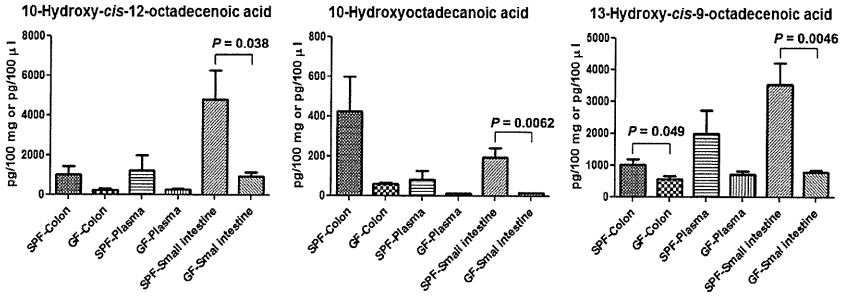


**Fig. 3.** Detection and quantitative analyses of polyunsaturated fatty acid-saturation metabolism intermediates in mice. Lipids extracted from colon (100 mg), intestine (100 mg), or plasma (100 µL) of SPF or germ-free (GF) mice were analyzed by LC-MS/MS-based lipidomics as described in *Materials and Methods*. Data are presented as means ± SEM ($n$ = 8). The statistical significance between mean values was determined by unpaired $t$ test with Welch's correction.

YM-3 (Millipore) and applied to a Hi-load 26/60 Superdex 200 prep-grade column (GE Healthcare) equilibrated with 50 mM KPB (pH 6.5). Active fractions were collected, dialyzed with 50 mM KPB (pH 6.5) including 50% (vol/vol) glycerol, and stored at −20 °C until use.

For purification of CLA-DH, *E. coli* Rosetta/pCLA-DH cells (8 g) in 1.5 L of culture broth were suspended in BugBuster Master Mix (Merck) (30 mL) and incubated for 20 min at room temperature. After ultracentrifugation (100,000 × *g*; 60 min) of the cell lysate, the resulting supernatant was concentrated with a Centriprep YM-3 and applied to a HiLoad 26/60 Superdex 200 prep-grade column that had been equilibrated with standard buffer and eluted. CLA-DH was purified further using a Mono Q 10/100 GL column and a Superdex 200 10/300 GL column (GE Healthcare). The purified CLA-DH was dialyzed with 50 mM KPB (pH 6.5) including 50% (vol/vol) glycerol and stored at −20 °C until use.

For purification of CLA-DC, *E. coli* Rosetta/pCLA-DC cells (8 g) in 1.5 L of culture broth were suspended in standard buffer and disrupted with an Insonator 201M ultrasonic oscillator. After centrifugation (20,000 × *g*; 30 min) of the cell lysate, solid sulfate was added to the resulting supernatant to 50–80% saturation. The precipitate was recovered by centrifugation, dissolved in 10 mL of standard buffer, and then dialyzed three times against 2 L of standard buffer for 8 h. CLA-DC was purified further using a Phenyl Superose HR 10/10 column and a Mono Q 5/50 GL column (GE Healthcare). Purified CLA-DC was dialyzed with 50 mM KPB (pH 6.5) including 50% (vol/vol) glycerol and stored at −20 °C until use.

**Reaction Conditions.** Reactions were performed in test tubes (16.5 × 125 mm) that contained 1 mL of reaction mixture (20 mM KPB; pH 6.5) with 0.1% (wt/vol) fatty acid complexed with BSA [0.02% (wt/vol)] as the substrate and purified enzymes (CLA-HY, CLA-DH, CLA-DC, and CLA-ER) in various combinations. The reactions were performed with 5 mM NADH, 5 mM $NAD^+$, 0.1 mM FMN, or 0.1 mM FAD under microaerobic conditions in a sealed chamber with an $O_2$ absorber (Anaeropack Kenki; Mitsubishi Gas Chemical) and gently shaken (120 strokes per minute) at 37 °C for 12 h. The oxygen concentration under microaerobic conditions was maintained below 0.1% (<1,000 ppm) and monitored with an oxygen indicator (Mitsubishi Gas Chemical). All experiments were performed in triplicate, and the averages of three separate experiments that were reproducible within ±10% are presented in the figures.

**Lipid Analyses.** Before lipid extraction, *n*-heptadecanoic acid was added to the reaction mixture as an internal standard. Lipids were extracted from 1 mL of the reaction mixture with 5 mL of chloroform/methanol/1.5% KCl in $H_2O$ (2:2:1, by volume), according to the procedure of Bligh-Dyer, and concentrated by evaporation under reduced pressure (30). The resulting lipids were dissolved in 2 mL of methanol and 3 mL of benzene and then methylated with 300 µL of 1% trimethylsilyldiazomethane at 28 °C for 30 min. After methylation, the resulting fatty acid methyl esters were concentrated by evaporation under reduced pressure. The resulting fatty acid methyl esters were analyzed by gas–liquid chromatography (GC) using a Shimadzu GC-1700 gas chromatograph equipped with a flame-ionization detector and split-injection system, fitted with a capillary column (SPB-1; 30 m length × 0.25 mm i.d.; Supelco). The initial column temperature was 180 °C for 30 min but was subsequently increased to 210 °C at a rate of 60 °C/min and then maintained at that temperature for 29.5 min. The injector and detector were operated at 250 °C. Helium was used as a carrier gas at a flow rate of 1.4 mL/min. The fatty acid peaks were identified by comparing retention times to known standards.

**Isolation and Identification of Reaction Products.** Reaction products were separated by reverse-phase HPLC using a Shimadzu LC-10A system equipped with a Cosmosil column (5C18-AR; 20 × 250 mm; Nacalai Tesque). The mobile phase was acetonitrile-$H_2O$ (8:2, by volume) at a flow rate of 3.0 mL/min, and the effluent was monitored by UV detection (205 and 233 nm). The methyl esters of purified fatty acids were transformed to the pyrrolidide and trimethylsilyl (TMS) derivatives. Pyrrolidide derivatives were prepared by direct treatment of the purified fatty acid methyl esters with pyrrolidine-acetic acid [10:1 (vol/vol)] in screw-cap tubes for 1 h at 115 °C, followed by extraction with dichloromethane. The organic extract was washed with water and dried over anhydrous $Na_2SO_4$, and then the solvent was removed under vacuum in a rotary evaporator. The TMS derivatives were prepared by direct treatment of the purified fatty acid methyl esters with a mixture of TMS agent (pyridine/hexamethyldisilazane/trimethylchlorosilane; 9:3:1, by volume) in screw-cap tubes for 30 min at 60 °C, followed by extraction with chloroform. The chemical structures of purified fatty acid methyl esters, pyrrolidide derivatives, and TMS derivatives were determined by mass spectroscopy (MS), and the chemical structures of purified free fatty acids were determined by 2D proton NMR ($^1$H-NMR) techniques including $^1$H-$^1$H double-quantum–filtered chemical-shift correlation spectroscopy and 2D nuclear Overhauser effect spectroscopy, as described previously (20).

**Mice.** SPF and germ-free BALB/c mice (9 wk; female) were obtained from CLEA Japan and maintained under SPF and germ-free conditions with a sterile diet (CL-2; CLEA Japan), respectively, at the Experimental Animal Facility, Institute of Medical Science, The University of Tokyo. Isolated tissues were immediately frozen by liquid nitrogen and always kept at −80 °C before fatty acid analysis. All experiments were approved by the Animal Care and Use Committee of the University of Tokyo and conducted in accordance with their guidelines.

**Fatty Acid Analysis in Mice.** Liquid chromatography–tandem MS (LC-MS/MS)-based lipidomics was performed as described (31). Briefly, samples were subjected to solid-phase extraction using a Sep-Pak C18 cartridge (Waters) with a deuterium-labeled internal standard (arachidonic acid-d8, leukotriene $B_4$-d4, 15-hydroxyeicosatetraenoic acid-d8, prostaglandin E2-d4). Lipidomic analyses were performed using an HPLC system (Waters UPLC) with a linear ion-trap quadrupole mass spectrometer (QTRAP 5500; AB SCIEX) equipped with an Acquity UPLC BEH C18 column (1.0 mm × 150 mm × 1.7 µm; Waters). Samples were eluted with a mobile phase consisting of water/acetate [100:0.1 (vol/vol)] and acetonitrile/methanol [4:1 (vol/vol)] (73:27) for 5 min; increased to 30:70 after 15 min, increased to 20:80 after 25 min, and held for 8 min; and then increased to 0:100 after 35 min and held for 10 min with flow rates of 70 µL/min (0–30 min), 80 µL/min (30–33 min), and 100 µL/min (33–45 min). MS/MS analyses were conducted in negative-ion mode, and fatty acid metabolites were identified and quantified by multiple-reaction monitoring. Quantitation was performed using calibration curves constructed for each compound, and recoveries were monitored using added deuterated internal standards.

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# Vitamin-mediated regulation of intestinal immunity

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The intestine is exposed continuously to complex environments created by numerous injurious and beneficial non-self antigens. The unique mucosal immune system in the intestine maintains the immunologic homeostasis between the host and the external environment. Crosstalk between immunocompetent cells and endogenous (e.g., cytokines and chemokines) as well as exogenous factors (e.g., commensal bacteria and dietary materials) achieves the vast diversity of intestinal immune functions. In addition to their vital roles as nutrients, vitamins now also are known to have immunologically crucial functions, specifically in regulating host immune responses. In this review, we focus on the immunologic functions of vitamins in regulating intestinal immune responses and their roles in moderating the fine balance between physiologic and pathologic conditions of the intestine.

**Keywords: intestinal immunity, vitamin, IgA, regulatory T cells, allergy, inflammation**

## INTRODUCTION

The primary physiologic function of intestine is to serve as the chief site of nutrient absorption into the body. However, intestinal tissues also comprise a unique immune system that can discriminate between pathogens and harmless or beneficial antigens such as commensal microorganisms and dietary constituents (1). To prevent unnecessary inflammatory responses and hypersensitivity to harmless or beneficial materials, the intestinal immune system usually becomes unresponsive to these factors through the induction of oral tolerance (2). At same time, the intestinal immune system acts as the first line of defense against pathogens. For the coordinated operation of this complex network, the intestinal immune system is customized with cooperative immunocompetent cells, including the specialized antigen-sampling M cells; antigen-presenting cells [e.g., dendritic cells (DCs) and macrophages]; IgA-producing plasma cells (PCs); polarized $CD4^+$ T cells such as regulatory T ($T_{reg}$), Th1, Th2, and Th17 cells; mast cells; and innate lymphoid cells (1, 3, 4). Accumulating evidence has demonstrated that the disruption of oral tolerance underlies pathogenic conditions such as intestinal inflammation and food allergy (5).

Coordination of the numerous diverse intestinal immunological functions is achieved through the immunological crosstalk among immunocompetent cells via endogenous molecules (e.g., cytokines and chemokines). In addition to these endogenous factors, components of the gut environment, such as commensal bacteria and dietary materials, influence intestinal immunological functions. Recent advances in genetic identification have revealed that commensal bacteria play an important role in the development and maintenance of not only intestinal or mucosal immunity but also the host immune system [reviewed in Ref. (6)]. Although the underlying molecular and cellular mechanisms are not fully understood, nutritional components derived from the diet, either directly absorbed or metabolized or synthesized *de novo* by commensal bacteria, clearly are essential and influential exogenous factors for the development, maintenance, and regulation of the intestinal immune system (7, 8). This idea is underscored by the fact that nutrient deficiencies often are associated with impaired intestinal immunity (9). For instance, a recent study shows that angiotensin I converting enzyme 2 regulates intestinal amino acid metabolism and consequently affects the ecology of commensal bacteria, which leads to the transmittable colitis (10). Another recent study has demonstrated that commensal bacteria from kwashiorkor, a form of acute malnutrition that occurs by inadequate intake of dietary protein, perturb the metabolism of amino acids and carbohydrates (11).

Vitamins are organic compounds that the host organism cannot synthesize in sufficient quantities and that therefore need to

be supplied exogenously by the diet or commensal bacteria. Some vitamins (e.g., vitamin B family and vitamin C) are water-soluble, whereas others (e.g., vitamins A, D, E, and K) are hydrophobic. Both hydrophilic and hydrophobic vitamins and their metabolites have diverse functions in many biologic events, including immunologic regulation. Indeed, vitamin deficiency results in high susceptibility to infection and immune diseases (12). Previously vitamins were thought to regulate the immune system in an indiscriminant manner, but accumulating evidence has revealed specific functions of individual vitamins and their metabolites in immune responses.

In this review, we discuss recent progress regarding our understanding of the immunologic functions of particular vitamins and their contributions toward maintaining the immunologic balance between physiologic and pathologic conditions of the intestine.

## VITAMIN A REGULATES CELL TRAFFICKING AND DIFFERENTIATION IN THE INTESTINE

Vitamin A, especially its metabolite retinoic acid (RA), has emerged as a critical mediator of mucosal immune responses [reviewed in Ref. (13)]. Vitamin A is a fat-soluble essential micronutrient obtained from diets as all-trans-retinol, retinyl esters, or β-carotene and is metabolized into retinol in tissues (14). Retinol then is converted mainly to the all-trans isoform of RA through oxidation by alcohol dehydrogenases (ADH) and retinaldehyde dehydrogenases (RALDH) (**Figure 1**).

The importance of vitamin A in the regulation of intestinal immunity has long been indicated. Indeed, vitamin A deficiency leads to increased susceptibility to various pathogens and vitamin A supplementation reduces the morbidity and mortality due to infectious diseases (e.g., diarrheal infections and measles) (15). During the past few years, our molecular and cellular understanding of the roles of vitamin A in the regulation of intestinal immunity has increased greatly. A key discovery was that RA regulates cell trafficking by inducing the expression of the gut-homing molecules α4β7 integrin and chemokine receptor CCR9 on lymphocytes and thus determining the gut tropism of these cells (16, 17). Epithelial cells and DCs, especially CD103$^+$ DCs, in the intestine uniquely express RALDH and thus are capable of synthesizing RA; therefore the lymphocytes activated by intestinal DCs and epithelial cells express α4β7 integrin and CCR9, which allow them to return to the intestinal compartment (**Figure 1**). In agreement with this understanding, vitamin-A-deficient mice lack T cells and IgA-PCs in the intestine (16, 17). Several lines of evidence have demonstrated that GM-CSF induces the RALDH expression in DCs and RA itself, IL-4, and MyD88-mediated toll-like receptor pathway enhance the induction of RALDH expression (**Figure 1**) (18, 19).

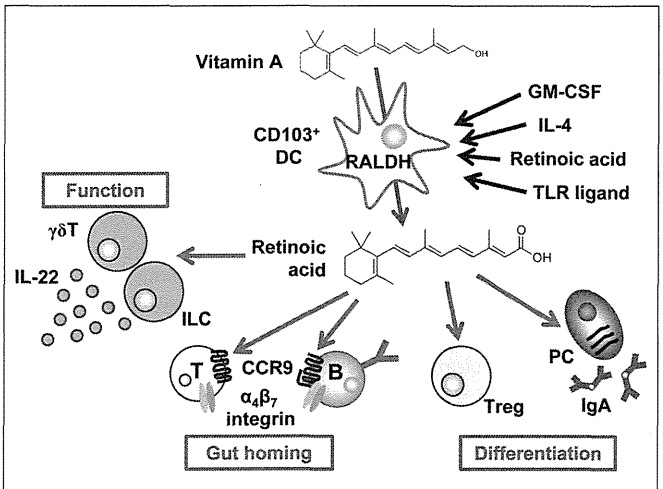


**FIGURE 1 | Regulation of cell trafficking, differentiation, and function by the vitamin A metabolite retinoic acid**. CD103$^+$ dendritic cells (DCs) express retinaldehyde dehydrogenases (RALDH) by GM-CSF, IL-4, TLR ligand, and retinoic acid (RA), which enable them to convert vitamin A into RA. RA then induces CCR9 and α4β7 integrin in T and B cells, causing them to migrate into the intestine. In addition, retinoic acid affects cell differentiation, such as the preferential differentiation of T cells into regulatory T ($T_{reg}$) cells and B cells into IgA-producing plasma cells (PCs). RA also enhances IL-22 production from γδT cells and innate lymphoid cells.

Retinoic acid plays an important role in determining not only the gut tropism of lymphocytes activated in the intestine but also cell differentiation. For example, through the cooperative effects of TGF-β, RA promotes class switching of IgM$^+$ B cells to those expressing IgA (**Figure 1**). Therefore, antagonism of RA results in reduced IgA production (17, 20). Another study demonstrated that Runx proteins mediate effects downstream of RA and TGF-β1 signaling in IgA class switching (21).

In addition to the effects of RA on DCs and B cells, RA affects T cell differentiation. Indeed, preferential differentiation of T cells into $T_{reg}$ cells is mediated by CD103$^+$ DCs that are capable of producing RA and activating latent TGF-β (22–24). Reciprocally, RA failed to enhance differentiation of naïve T cells into Th17 cells in the absence of DCs (25). In this regard, DCs in the intestinal lamina propria of vitamin-A-deficient mice reportedly show impaired production of IL-6, a cytokine that is essential in the differentiation of Th17 cells (26) although there are controversial reports on the production of IL-6 by MLN-DCs from vitamin-A-deficient mice (27). On the other hand, RA–RA receptor α signal in T cells requires T cell effector responses regardless T cell subsets (26), which is in line with a previous report that Th17 cells require a low concentration of RA (20). In agreement with these functions of RA, vitamin-A-deficient mice have decreased numbers of both $T_{reg}$ and Th17 cells in the intestine mainly due to the defect of T cell trafficking into the small intestine (25, 26, 28). In addition, segmented filamentous bacteria, Th17-inducing commensal bacteria, is decreased in vitamin A-deficient condition by high levels of mucin by goblet cells, which also leads to the impaired Th17 cell differentiation (29). Taken together, intrinsic and extrinsic factors for T cell differentiation are affected by the RA.

In addition to conventional αβ T cells, a recent study has demonstrated that RA enhanced IL-22 production by γδT cells and innate lymphoid cells, which are involved in the attenuation of intestinal inflammation (30). RA also affects non-lymphoid cells in the lymph node initiation. Indeed, RA produced by neurons

adjacent to the lymph node anlagen induced CXC13 expression in stromal organizer cells and consequently led to the initial clustering of lymphoid tissue inducer cells (31). Therefore, RA has diverse functions in the regulation of versatile immunological events including cell trafficking, differentiation, cytokine production, and lymphoid organogenesis.

The various roles of RA in the mucosal immune system, especially regulating cell trafficking into the intestine, enable us to consider clinical applications of this metabolite. In general, parenteral immunization fails to achieve efficient antigen-specific immune responses in the intestine because it does not induce the necessary gut-homing molecules for the migration of antigen-sensitized immune cells into the intestine. A recent study demonstrated that the addition of RA at the time of subcutaneous vaccination increased the accumulation of antigen-specific T cells and IgA-producing PCs in the intestine and concurrently induced protective immunity against intestinal pathogens (e.g., *Salmonella*) (32). These findings suggest that exogenous RA treatment might be used to stimulate the production of gut-migrating $T_{reg}$ cells for the control of intestinal inflammation and allergy. Additional investigation into the immune functions of RA is warranted to advance potential clinical applications of this vitamin A metabolite.

## MEMBERS OF THE VITAMIN B FAMILY CONTROL CELL METABOLISM AND ACTS AS LIGANDS IN THE REGULATION OF INTESTINAL IMMUNITY

Initially thought to be a single vitamin, vitamin B currently is recognized as a family comprising eight different members. All B vitamins are water-soluble, and they are involved in various pathways of cell metabolism. Among the B vitamins, vitamin B6 is essential for metabolism of nucleic acids, amino acids, and lipids and thus influences cell growth. Consequently, vitamin B6 deficiency leads to various impairments of immunity, such as lymphoid atrophy and reduced numbers of lymphocytes (33); conversely, vitamin B6 supplementation bolsters these weakened immune responses (34). A previous study suggested the involvement of the lipid mediator sphingosine 1-phosphate (S1P) in vitamin-B6-mediated immune regulation. S1P has been shown to regulate cell trafficking, especially cell egress from organized lymphoid tissues in both systemic (e.g., thymus, bone marrow, and lymph nodes) and mucosal (e.g., intestine) compartments [reviewed in Refs. (35, 36)]. The cell trafficking is determined by the S1P gradient that is achieved through the coordinated production of S1P and its degradation, which is mediated by S1P lyase and S1P phosphohydrolase (35). S1P lyase requires vitamin B6 as a co-factor for the degradation of S1P (37), and the administration of a vitamin B6 antagonist impair S1P lyase activity and thus create an inappropriate S1P gradient. These defects lead to impaired trafficking of lymphocytes from lymphoid tissues and consequently reduced numbers of lymphocytes in the periphery (38, 39).

Like vitamin B6, vitamin B9 (that is, folate or folic acid) is essential for nucleic acid and protein synthesis (40), and inadequate levels of vitamin B9 dramatically alter the immune response. Previous studies suggested that vitamin B9 deficiency inhibits the activity of $CD8^+$ T cells and NK cells; in turn, this inhibition is associated with decreased resistance to infections (41).

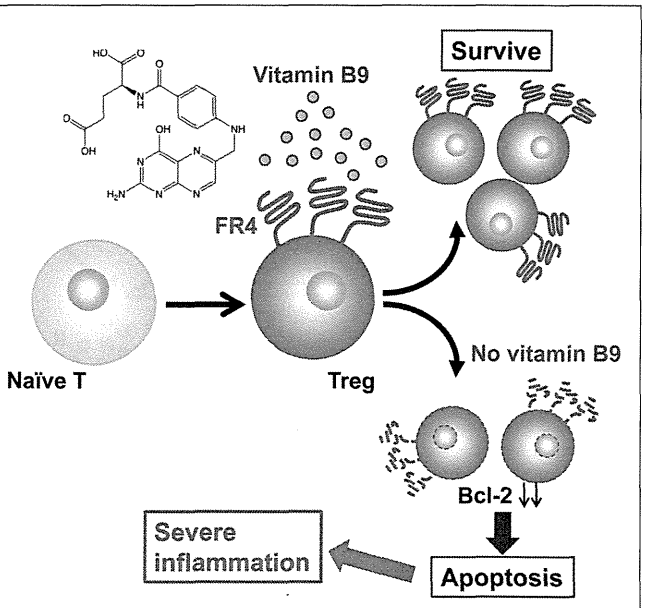


**FIGURE 2 | Vitamin B9 is required for the survival of regulatory T cells and subsequent maintenance of immunologic homeostasis in the intestine**. Once naïve T cells differentiate into regulatory T ($T_{reg}$) cells, they express folate receptor 4 (FR4), and require vitamin B9 for their survival. The absence of sufficient amounts of vitamin B9 induces the apoptosis of $T_{reg}$ cells, with decreased expression of Bcl-2 and subsequent increased intestinal inflammation.

Folate receptor 4, a vitamin B9 receptor, is highly expressed on the surfaces of $T_{reg}$ cells (42), implying a specific function of this vitamin in these cells. In particular, our recent study revealed that vitamin B9 is crucial in the maintenance of $T_{reg}$ cells (43). In the absence of vitamin B9, naïve T cells can differentiate into $T_{reg}$ cells, but differentiated $T_{reg}$ cells fail to survive owing to the decreased expression of anti-apoptotic molecules (e.g., Bcl-2) (**Figure 2**). As a result, mice maintained on a vitamin-B9-deficient diet have decreased numbers of intestinal $T_{reg}$ cells (43). As a result, the impaired survival of $T_{reg}$ cells in these mice leads to their increased susceptibility to intestinal inflammation (**Figure 2**) (44).

A recent study demonstrated an additional function of the vitamin B family in the control of immune responses via mucosa-associated invariant T (MAIT) cells. MAIT cells are unconventional T cells that express a semi-invariant αβ T cell receptor that is restricted by the MHC class I-related molecule MR1; these cells are mostly found in the intestine, liver, and lung (45). Because MAIT cells can react rapidly to bacterial infections (e.g., *Escherichia coli, Klebsiella pneumoniae,* and *Mycoplasma tuberculosis*), it was supposed that the antigen presented to MR1 was bacteria-derived molecules. However, a recent study clarified that, in fact, bacterially produced metabolites of vitamin B9 and vitamin B2 bound to MR1 are presented as antigen to MAIT cells (46). Furthermore, like vitamin B2 derivatives, the vitamin B9 metabolite 6-formyl

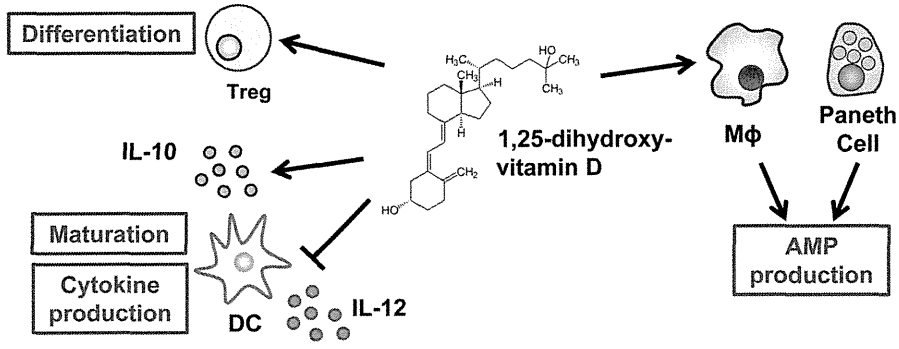


**FIGURE 3 | Vitamin D mediates innate and acquired immunity**. The active form of vitamin D, it metabolite 1,25-dihydroxyvitamin D, inhibits the maturation of dendritic cells (DCs) and their production of IL-12 but simultaneously promotes their production of IL-10. In addition, T cells respond directly to 1,25-dihydroxyvitamin D for their preferential differentiation into $T_{reg}$ cells. As a component of innate immunity, 1,25-dihydroxyvitamin D promotes the production of anti-microbial peptides (AMP) by macrophages and Paneth cells.

pterin binds to MR1 but, unlike vitamin B2 derivatives, fails to activate MAIT cells (46). These findings suggest that, depending on their metabolism by commensal bacteria and presentation by MR1, members of the vitamin B family can act either as positive or negative regulatory ligands for MAIT cells.

## VITAMIN D IS AN INHIBITOR OF IMMUNE RESPONSES

In its typical role of maintaining optimal concentrations of serum calcium, vitamin D is essential to a healthy mineralized skeleton (47). In addition to its effects on calcium and bone metabolism, vitamin D – especially its metabolite 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] – is an important regulator of the immune system, and its deficiency is linked to aberrant immune responses, including intestinal inflammation (48). Regarding a possible mechanism linking vitamin D and intestinal inflammation, $1,25(OH)_2D$ may be important in the creation of an immunologic regulatory or suppressor environment. For example, $1,25(OH)_2D$ inhibits the maturation of DCs and the production of their effector cytokine, IL-12, and simultaneously promotes the production of their inhibitory cytokine, IL-10, thus regulating T cell function and development (**Figure 3**) (49). In addition, T cells directly respond to $1,25(OH)_2D$, with preferential differentiation into $T_{reg}$ cells (**Figure 3**) (50).

Furthermore, vitamin D enhances innate immunity (**Figure 3**). More than 25 years have passed since the anti-microbial function of $1,25(OH)_2D$ against *Mycobacterium tuberculosis* in human monocytes was reported (51). Subsequent studies have revealed the molecular and cellular mechanisms underlying this anti-microbial activity. Once they are activated through Toll-like receptors, macrophages–monocytes express CYP27B1, a key enzyme in the synthesis of $1,25(OH)_2D$ (52), and the vitamin D receptor (VDR) (53). These changes lead to intracrine synthesis of $1,25(OH)_2D$, which enhances the gene expression mediated by vitamin D and the VDR axis. VDR-mediated genes include the anti-microbial molecules cathelicidin (LL-37) and β-defensin 2 (54). Similar $1,25(OH)_2D$-induced production of these anti-microbial molecules occurs in epithelial cells (55) and Paneth cells (56). In addition, $1,25(OH)_2D$ stabilizes tight-junction structures between epithelial cells in the intestinal tract (57). Together, these diverse functions of vitamin D contribute to the creation of the first line of defense against pathogens without the induction of aberrant inflammatory responses.

## CONCLUSION

Clinical evidence has long indicated that inadequate vitamin intake disrupts host immunity, thus predisposing humans to infectious and inflammatory diseases. Accumulating evidence has revealed the molecular and cellular mechanisms underlying myriad functions of vitamins in innate and acquired immune responses. These findings clarify the beneficial roles of vitamins in the maintenance of immunologic homeostasis and inform the design of vitamin analogs as pharmacologic agents for the generation and maintenance of a healthy immune condition. The complex functions of vitamins in the regulation of the immune system merit continued investigation, and these research efforts likely will enable scientists to refine our understanding of the mechanisms underlying the immunologic roles of various vitamins and to advance the development of vitamin-dependent therapeutic agents for the control of infectious and immune diseases.

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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 ($2 \times 10^5$ CFU) and sublethal ($2 \times 10^4$ 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 ($5 \times 10^4$ 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 $1 \times 10^6$ 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 ($2 \times 10^6$ 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 ($1 \times 10^6$ 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 ($2 \times 10^5$ 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).

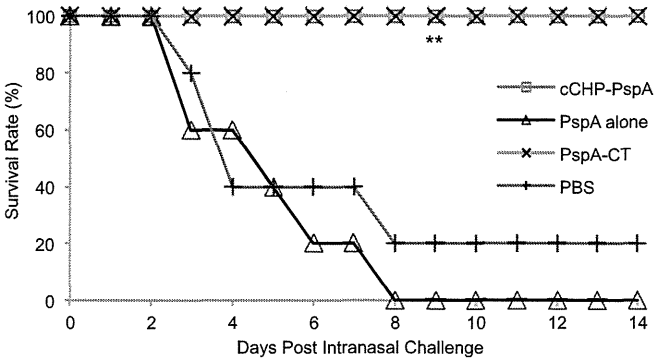


FIG 1 Intranasal vaccination with cCHP-PspA induced protective immunity against pneumococci. One week after the final immunization, mice were challenged with *S. pneumoniae* Xen10 ($2 \times 10^5$ CFU/mouse), and survival was monitored. Data are representative of three independent experiments, and each 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 ($2 \times 10^5$ 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 ($2 \times 10^4$ 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

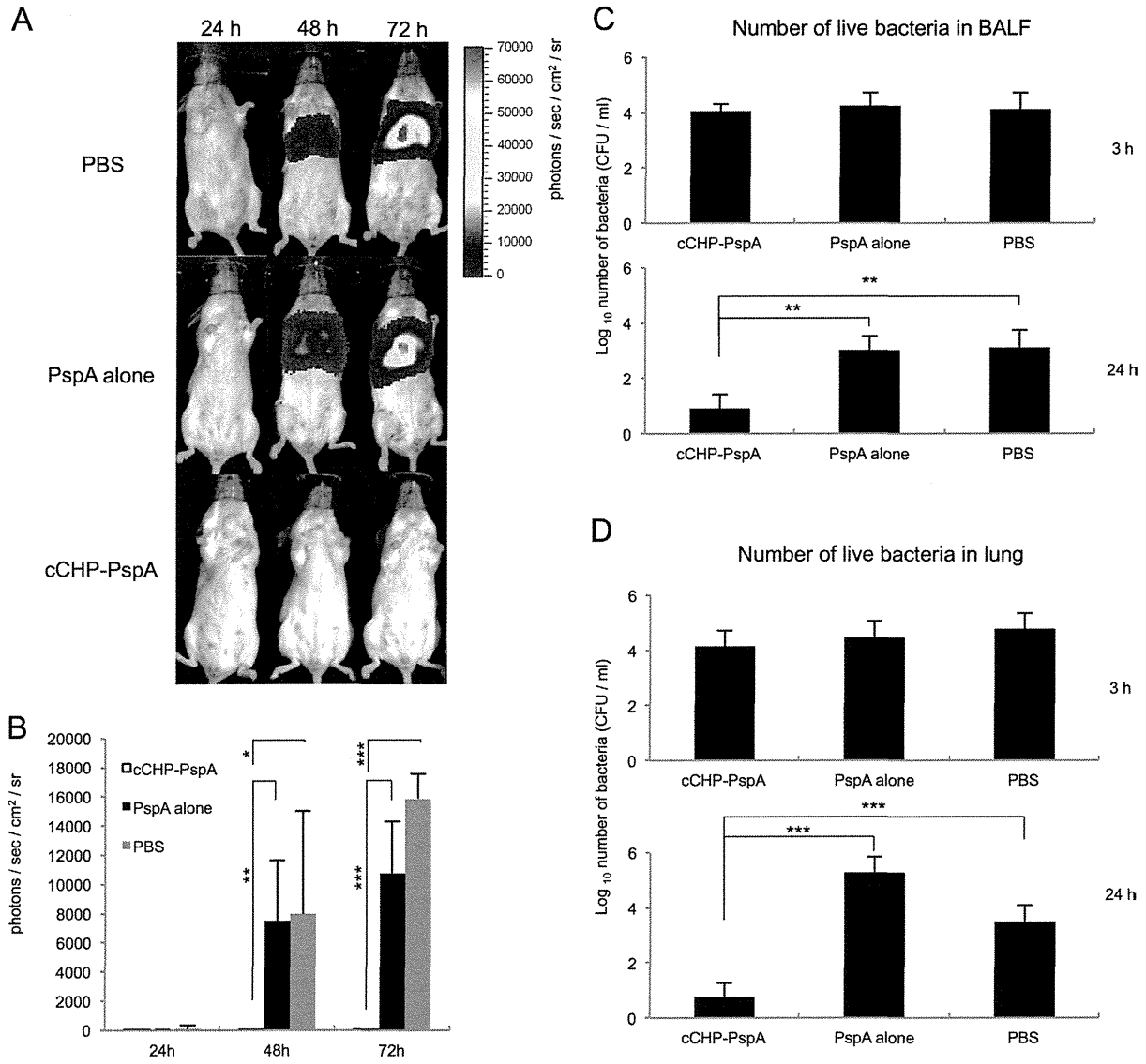


FIG 2 *In vivo* imaging revealed no sign of pneumococcal infection in the lungs of mice immunized intranasally with cCHP-PspA; these mice also showed enhanced bacterial clearance from the BALF and lung. Images (A) and average photon counts (B) show bioluminescence due to *S. pneumoniae* Xen10 in each group of mice infected intranasally with *S. pneumoniae* Xen10 ($2 \times 10^5$ 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 ($2 \times 10^4$ 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

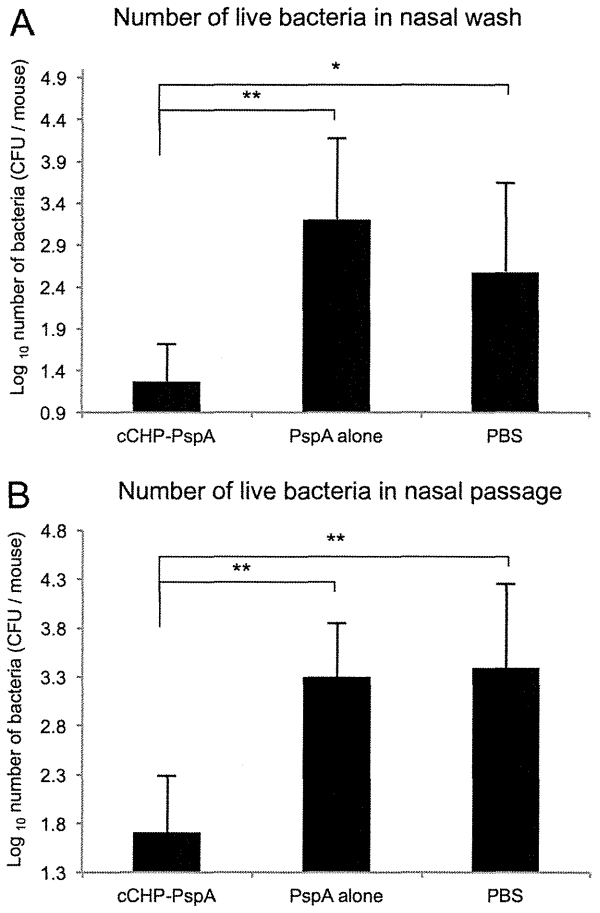


FIG 3 Intranasal vaccination with cCHP-PspA reduced bacterial colonization of the nasal cavity. One week after the final immunization, mice were challenged with a sublethal dose ($2 \times 10^4$ CFU/mouse) of *Streptococcus pneumoniae* Xen10. Nasal washes and tissues were collected, and the numbers of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

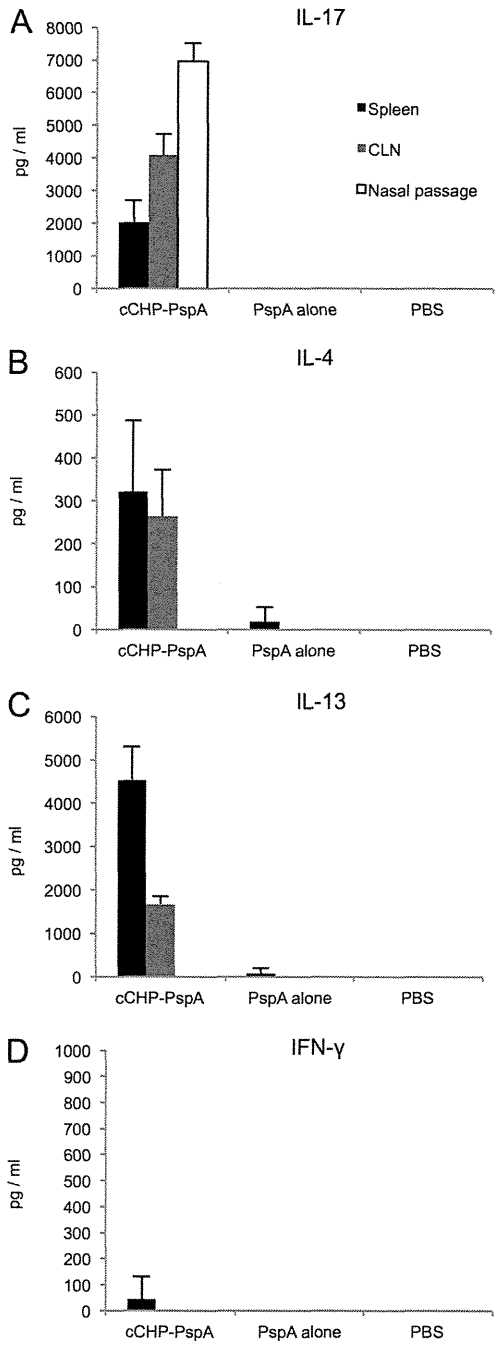


FIG 4 $CD4^+$ T cells from cCHP-PspA-immunized mice produce Th17- and Th2-type immune responses. Cytokines produced by $CD4^+$ T cells isolated from the spleens, cervical lymph nodes, and nasal passages of mice immunized with cCHP-PspA, PspA alone, or PBS only were analyzed. Data are representative of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

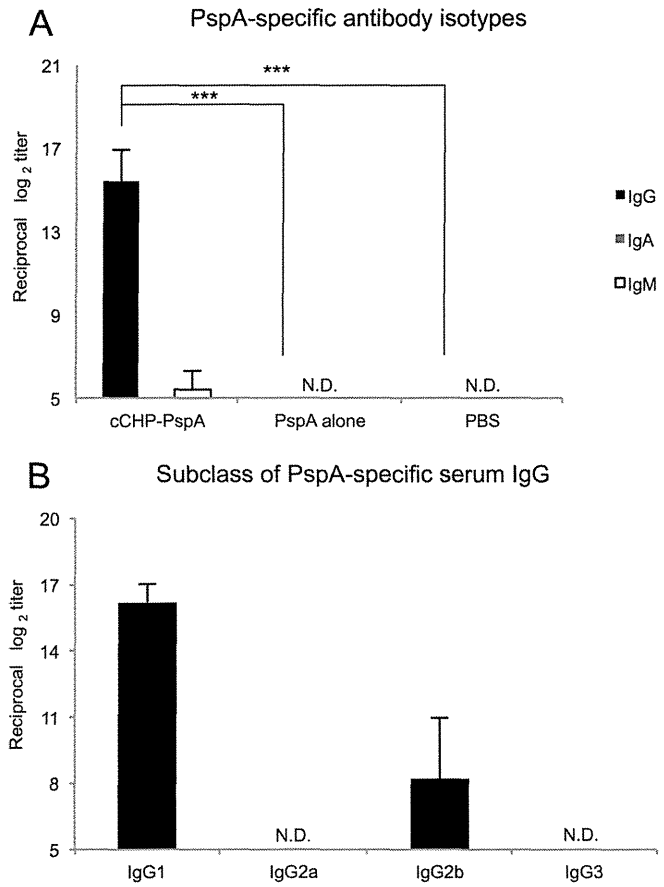


FIG 5 Intranasal vaccination with cCHP-PspA induced high levels of systemic antibodies. The data show the PspA-specific serum IgG level (A) and subclass analysis for IgG1, IgG2a, IgG2b, and IgG3 (B) for each immunized group (cCHP-PspA, PspA alone, or PBS only). Titers of PspA-specific IgG in sera were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

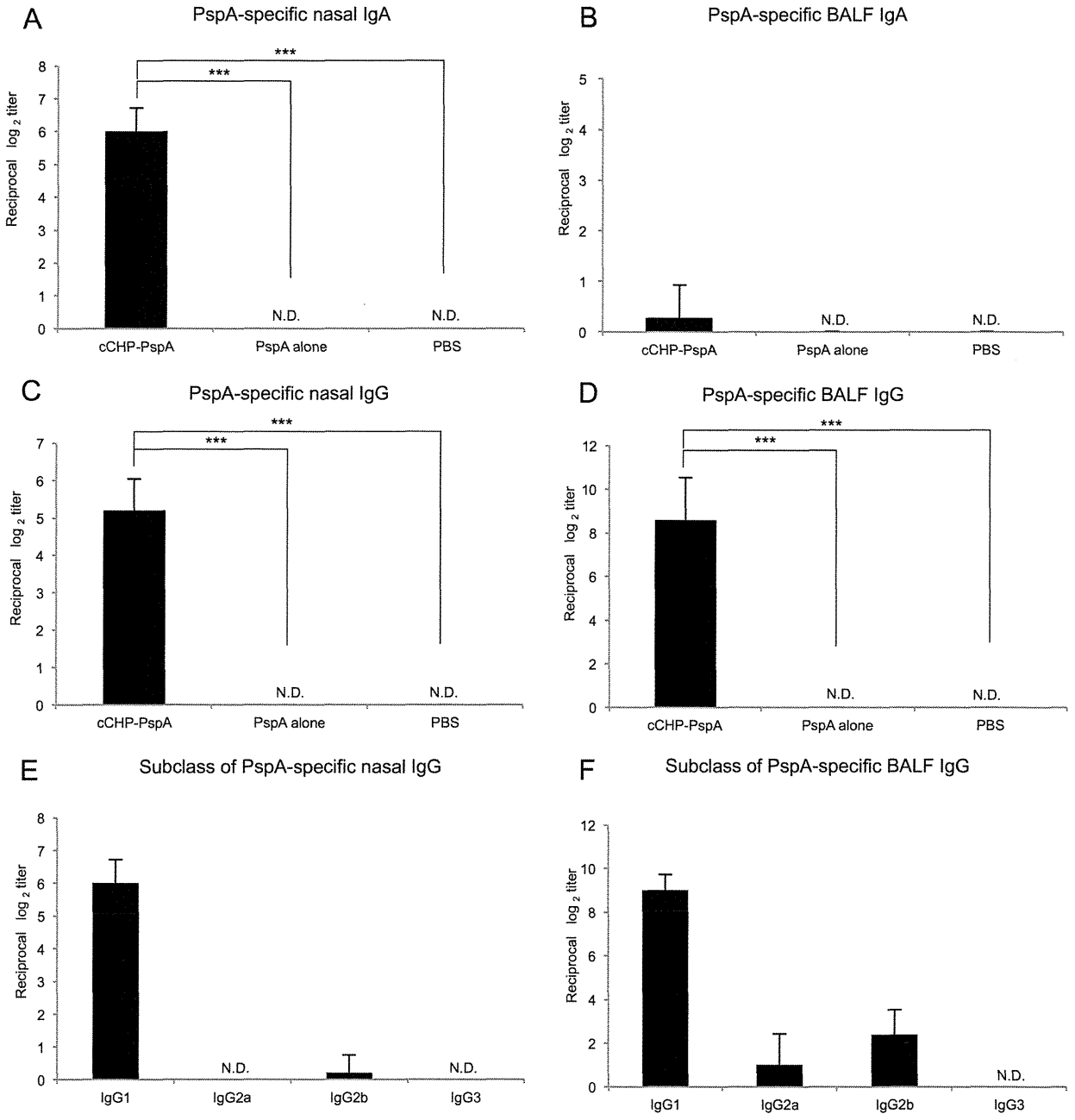


FIG 6 Intranasal vaccination with cCHP-PspA induced strong PspA-specific secretory IgA and IgG responses. Titers of nasal (A and C) and bronchial (B and D) IgA and IgG induced by intranasal immunization with PspA alone or PspA mixed with cCHP are shown. Titers of PspA-specific IgA and IgG in nasal washes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

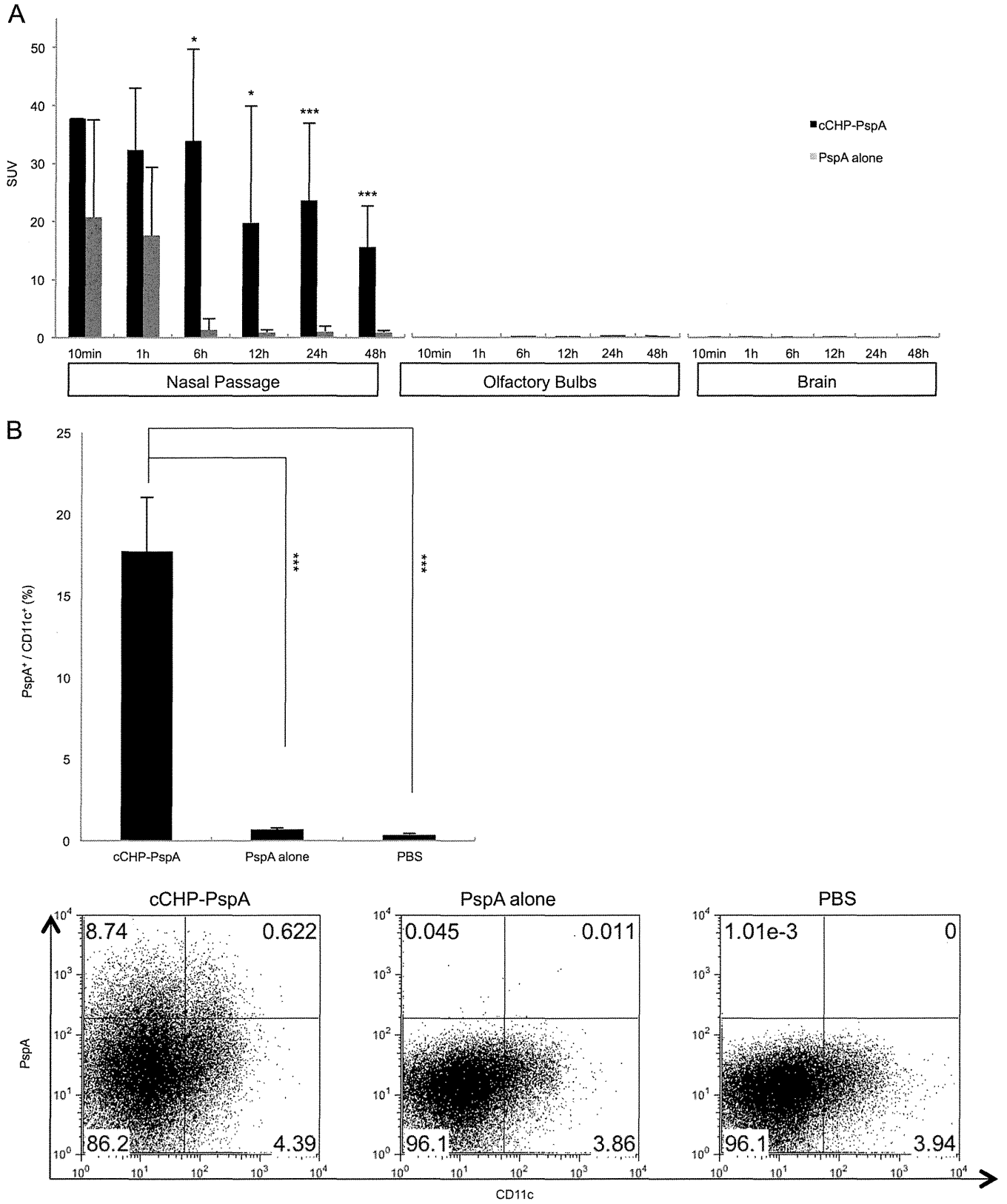


FIG 7 Intranasal vaccination with cCHP-PspA induced no accumulation of PspA in the central nervous system (A) but enhanced the efficiency of uptake of PspA by dendritic cells in the nasal passages (B). (A) $^{111}$In-labeled PspA was administered intranasally with or without cCHP nanogel, and the radioisotope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