C-Terminal *Clostridium perfringens* Enterotoxin-Mediated Antigen Delivery for Nasal Pneumococcal Vaccine

Hidehiko Suzuki¹,², Akihiro Watari², Eri Hashimoto¹,³, Miki Yonemitsu¹, Hiroshi Kiyono³,⁴,⁵, Kiyohito Yagi², Masuo Kondoh²*, Jun Kunisawa¹,³,⁴,⁵,⁶,⁷*

¹ Laboratory of Vaccine Materials, National Institute of Biomedical Innovation, Health and Nutrition, Osaka 567–0085, Japan, ² Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565–0871, Japan, ³ Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Sciences, The University of Tokyo, Tokyo 108–8639, Japan, ⁴ International Research and Development Center for Mucosal Vaccines, The Institute of Medical Science, The University of Tokyo, Tokyo 108–8639, Japan, ⁵ Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, Tokyo, Japan, ⁶ Department of Microbiology and Infectious Diseases, Kobe University Graduate School of Medicine, Kobe 650–0017, Japan, ⁷ Graduate School of Medicine, Graduate School of Pharmaceutical Sciences, and Graduate School of Dentistry, Osaka University, Osaka 565–0871, Japan

* kunisawa@nibiohn.go.jp (JK); masuo@phs.osaka-u.ac.jp (MK)

Abstract

Efficient vaccine delivery to mucosal tissues including mucosa-associated lymphoid tissues is essential for the development of mucosal vaccine. We previously reported that claudin-4 was highly expressed on the epithelium of nasopharynx-associated lymphoid tissue (NALT) and thus claudin-4-targeting using C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) effectively delivered fused antigen to NALT and consequently induced antigen-specific immune responses. In this study, we applied the C-CPE-based vaccine delivery system to develop a nasal pneumococcal vaccine. We fused C-CPE with pneumococcal surface protein A (PspA), an important antigen for the induction of protective immunity against *Streptococcus pneumoniae* infection, (PspA-C-CPE) effectively delivered fused antigen to NALT and consequently induced antigen-specific immune responses. In this study, we applied the C-CPE-based vaccine delivery system to develop a nasal pneumococcal vaccine. We fused C-CPE with pneumococcal surface protein A (PspA), an important antigen for the induction of protective immunity against *Streptococcus pneumoniae* infection, (PspA-C-CPE). PspA-C-CPE binds to claudin-4 and thus efficiently attaches to NALT epithelium, including antigen-sampling M cells. Nasal immunization with PspA-C-CPE induced PspA-specific IgG in the serum and bronchoalveolar lavage fluid (BALF) as well as IgA in the nasal wash and BALF. These immune responses were sufficient to protect against pneumococcal infection. These results suggest that C-CPE is an efficient vaccine delivery system for the development of nasal vaccines against pneumococcal infection.

Introduction

Because various pathogens infect through mucosal tissues, the induction of protective immunity at mucosal tissues is a primary strategy to prevent infectious diseases. In vaccine development, injection-based immunization induces systemic immune responses but not mucosal...
immune responses and so fails to prevent invasion of pathogens at mucosal sites. In contrast, mucosal vaccines (e.g., nasal and oral vaccine) induce both systemic and mucosal immune responses [1]. Therefore, mucosal vaccines have been considered to be ideal for the prevention of and protection from infectious diseases. It is generally accepted that the development of an effective and safe vaccine delivery system is essential for the development of mucosal vaccine against respiratory and intestinal infectious diseases.

Mucosa associated-lymphoid tissues (MALTs) play a pivotal role in the induction of antigen-specific immune responses against mucosally administered antigens, since the tissues have been shown to contain all the necessary immunocompetent cells for the initiation of antigen-specific immune response [2–4]. Therefore, the delivery of antigen to MALT is a promising approach for the development of mucosal vaccine [5,6]. A primary target of vaccine delivery is MALT epithelium, where M cells are located and play an important role in antigen uptake from the lumen and transport into MALTs [3]. Targeting M cells by using specific antibodies [7,8] and bacterial invasion molecules [9] as vaccine delivery vehicle efficiently deposited antigen to MALT and induced immune responses against conjugated antigens. Another target is epithelial cells, which cover the entire mucosal tissues and form tight junctions to seal off the intercellular space. Tight junctions are composed of claudin, occludin, tricellulin, and zonula occludens [10]. There are more than 20 members of the claudin family, whose expression profiles and functions differ among tissues.

We previously found that claudin-4 was highly expressed in nasopharynx-associated lymphoid tissue (NALT) [11] and thus targeting caludin-4 would be a logical delivery candidate for a nasal vaccine. To this end, we used C-terminal fragment of Clostridium perfringens enterotoxin (C-CPE), a non-toxic element of Clostridium perfringens (CPE) that binds to claudin-4 [12,13]. Our previous study showed that intranasal immunization of ovalbumin (OVA) fused C-CPE induced OVA-specific systemic and mucosal immune responses by claudin-4 binding of C-CPE [11,14]. These findings allow us to examine whether claudin-4-targeting vaccines using C-CPE were effective for generating mucosal vaccines against infectious diseases.

Streptococcus pneumoniae (S. pneumoniae) is a key respiratory pathogen and causes pneumonia, meningitis, and otitis media [15,16], which are classifed more than 90 serotype [17,18]. Polysaccharide-based injection-type vaccines are currently used as pneumococcal vaccines and success to reduce the incident of pneumococcal disease [19,20]. However, the effect of these polysaccharide-based injection-type vaccines are only induced serotype specific immune responses. Thus, they do not cover all stains of S. pneumoniae and thus are ineffective for unrelared strains. Therefore, it is necessary to develop a pneumococcal vaccine which is effective for all strains of S. pneumoniae. Pneumococcal surface protein A (PspA) is a choline-binding surface protein of S. pneumoniae and protects S. pneumoniae from killing by apolactoferrin [21]. PspA has high antigenicity, is expressed on all isolates of S. pneumoniae [22]. Additionally, PspA induces cross-reactivity among different strains [23]. Moerover, PspA induces cross active immune responses not only in mice but also in human [23,24]. Thus, PspA is considered to be an ideal vaccine antigen for the development of a pneumococcal nasal vaccine. In this study, we used C-CPE as a nasal delivery vehicle of PspA to create a nasal vaccine against pneumococcal infection.

**Materials and Methods**

**Mice**

Female BALB/c mice (age, 6 to 7 weeks) were purchased from SLC, Inc. (Shizuoka, Japan). In some experiments, we checked murine condition at least once per day. Since mice havindg
30% of body weight loss would lead to death soon, we monitored body weight everyday. We killed mice if they reach to 30% reduction in their body weight or after 14 days after infection. All experiments were approved by the Animal Care and Use Committee of Graduate School of Pharmaceutical Sciences, Osaka University (#22-7-0) and the Animal Care and Use Committee of the National Institute of Biomedical Innovation (approved # DS25-3R4), and conducted in accordance with their guidelines.

Cell culture
A mouse fibroblast cell line (L cells) and mouse claudin-4-expressing L cells were kindly provided by Dr. S. Tsukita (Kyoto University, Kyoto, Japan) [12]. L cells and claudin-4-expressing L cells were cultured in modified Eagle’s medium supplemented with 10% fetal bovine serum in a 5% CO₂ atmosphere at 37°C.

Preparation of PspA-C-CPE fusion protein
PspA cDNA was amplified by polymerase chain reaction (PCR) amplification (forward primer: 5’-agggtacccgaatctccctgatcc-3’, KpnI site is underlined; reverse primer: 5’-gcttaattaattctgggctggagtttc-3’ PacI site is underlined). pET16b-OVA-C-CPE, a G4S linker was inserted between OVA and C-CPE [11], and PspA PCR products were digested by using KpnI and PacI. The PspA fragment was inserted into pET-16b-C-CPE to yield pET16b-PspA-C-CPE. We also prepared PspA fragment for pET16b-PspA by PCR amplification of PspA cDNA using different primers (forward primer: 5’-atgatgctatatagatgcatatggaagaatctcccgtagcc-3’, Ndel site is underlined; reverse primer: 5’-gcttaattaattctgggctggagtttc-3’ BamHI site is underlined). pET16b (Novagen, Darmstadt, Germany) and PspA PCR products were digested by using Ndel and BamHI. The resulting PspA fragment was inserted into pET16b to yield pET16b-PspA.

To obtain recombinant protein, the plasmids were transformed into *Escherichia coli* strain BL21 (DE3). Protein production was induced by using isopropyl-D-thiogalactopyranoside. The culture pellets were sonicated in buffer A (10 mM Tris-HCl [pH8.0], 400 mM NaCl, 5 mM MgCl₂, 0.1 mM PMSF, 1 mM 2-mercaptoethanol, and 10% glycerol). After centrifugation, the supernatants were loaded onto HiTrap HP (GE Healthcare, Pittsburgh, PA, USA) columns. PspA or PspA-C-CPE was eluted with buffer A containing 100~500 mM imidazole. The eluted protein was loaded into a PD-10 column (GE Healthcare) for exchange with phosphate-buffered saline (PBS). The concentration of purified protein was measured by using a BCA protein assay kit (Pierce Chemical, Rockford, IL). The purity of the eluted protein was confirmed by using the NuPAGE electrophoresis system (Life Technologies, Carlsbad, California, USA) followed by staining with Coomassie brilliant blue.

Flow cytometric analysis
Claudin-4-expressing L cells were incubated with PspA or PspA-C-CPE for 1 h at 4°C. The cells were washed with 0.1% bovine serum albumin (BSA) in PBS and incubated with mouse anti-His tag antibody (clone 13/45/31-2, mouse IgG1, Pierce) for 1 h at 4°C. After being washed with 0.1% BSA in PBS, the cells were incubated with fluorescein-labeled goat anti-mouse IgG (H+L) antibody (Rockland, Gilbertsville, PA, USA) for 30 min at 4°C. The cells were washed with 0.1% BSA in PBS and analyzed by flow cytometry (FACSCalibur, Becton Dickinson, New Jersey, USA).

For the intracellular cytokine analysis, mononuclear cells were isolated from the lung and nasal passages as previously reported [25, 26]. The isolated cells were incubated in RPMI1640 medium containing 10% fetal calf serum with 50 ng/mL of phorbol 12-myristate 13-succinate (Sigma-Aldrich, St Louis, MO), 750 ng/mL of ionomycin (Sigma-Aldrich), and 5 μg/ml of...
brefeldin A (BioLegend, San Diego, CA) for 4 h at 37°C. After washing with PBS, cells were stained with zombie (BioLegend) for 15 min at room temperature and subsequently treated with anti-mouse CD16/32 (clone 93, BioLegend) for 15 min at room temperature. After washing with PBS containing 2% newborn calf serum, the cells were stained with fluorescein isothiocyanate-rat anti-mouse CD4 (clone RM4-5, BioLegend) for 30 min at 4°C, fixed with 4% paraformaldehyde for 15 min at 4°C, permeabilized with Perm/Wash (BD Biosciences, San Diego, CA), and then stained with phycoerythrin-rat anti-mouse IL-17 (clone TC11-18H10, BD Biosciences) for 30 min at 4°C as previously reported. The cells were analyzed by flow cytometry (Miltenyi Biotec, Auburn, CA).

Immunohistologic analysis

NALT from mice was embedded in Tissue-Tek OCT compound (Sakura Finetek Japan, Tokyo, Japan) and cut into 6-μm sections. Sections were fixed with 100% acetone for 1 min at 4°C, and nonspecific binding was blocked by treating with 2% fetal calf serum in PBS for 30 min at room temperature. Sections were washed with 0.05% Tween in PBS and incubated with PspA or PspA-C-CPE (both were biotinylated by using a biotinylation kit from Thermo Fisher Scientific (Massachusetts, USA)) and fluorescein-conjugated *Ulex europaeus* agglutinin 1 (UEA-1) at 4°C overnight. After being washed with 0.05% Tween in PBS, sections were stained with Alexa Fluor 546-conjugated streptavidin for 30 min at room temperature. Sections were washed with 0.05% Tween in PBS and stained with 4,6-diamidino-2-phenylindole (DAPI). Finally, sections were washed with 0.05% Tween in PBS, mounted in Fluoromount (Diagnostic BioSystems, California, USA), and observed by fluorescence microscopy (BZ-9000, Keyence, Osaka, Japan).

Immunization

Mice were nasally immunized with vehicle (PBS), 5 μg of PspA alone, 2 μg of C-CPE alone or PspA-C-CPE once weekly for 3 consecutive weeks. One week after the last immunization, serum, nasal wash fluid, and bronchoalveolar lavage fluid (BALF) were collected. Nasal wash fluid was obtained by using 200 μL PBS. BALF were collected by using 1 mL PBS.

Measurement of PspA-specific antibody production by enzyme-linked immune sorbent assay (ELISA)

PspA-specific antibody production was determined by ELISA. Accordingly, 96-well immunoplates were coated with PspA (0.05 μg/well) at 4°C overnight. The immunoplates were treated with 1% BSA in PBS for 2 h at room temperature to prevent nonspecific binding. After the plates were washed with 0.05% Tween in PBS, 2-fold serial dilutions of samples were added to wells, and the plates were incubated at 4°C overnight. After the plates were washed with 0.05% Tween in PBS, goat anti-mouse IgG or IgA conjugated with horseradish peroxidase (Southern-Biotech, Birmingham, AL) was added to the immunoplates and incubated for 1 h at room temperature. After the plates were washed with 0.05% Tween in PBS, PspA-specific antibodies were detected by using TMB peroxidase substrate and reading the absorbance at 450 nm.

*S. pneumoniae* culture and infection

*S. pneumoniae* Xen10 (parental strain, A66.1 serotype 3; Caliper Life Sciences) were growth in brain–heart infusion broth at 37°C in a 5% CO₂ atmosphere, with no aeration. *S. pneumoniae* Xen10 cells were washed and diluted with D-PBS. One week after the last immunization, mice
were nasally challenged with $5.0 \times 10^6$ CFU of \textit{S. pneumoniae} Xen10. The survival of mice was monitored for 14 days.

**Data analysis**

Data were expressed as the mean ± SEM. Statistical analysis was performed by using the non-parametric Mann–Whitney’s U test. (GraphPad Software, California)

**Results**

**Preparation of PspA-fused C-CPE protein**

To investigate whether a C-CPE based claudin-4-targeting vaccine delivery system can be used as a nasal pneumococcal vaccine, we genetically fused PspA with C-CPE (PspA-C-CPE). We previously found that C-terminus of C-CPE is an active portion to interact with claudin-4 [27]. Thus, we fused PspA with N-terminus of C-CPE to maintain the claudin-4-binding activity of C-CPE (Fig 1A). Purification of PspA and PspA-C-CPE proteins was confirmed by Coomassie brilliant blue staining (Fig 1B).

We then checked the binding activity of PspA-C-CPE to claudin-4. We previously reported that OVA-fused with C-CPE bound to claudin-4 [11]. Likewise, PspA-C-CPE efficiently bound to claudin-4-expressing L cells but not to parent L cells (Fig 2A). We also confirmed that PspA alone did not bind to L cells regardless of whether they expressed claudin-4 (Fig 2A).

We next investigated whether PspA-C-CPE bound to NALT epithelium. As seen for the binding of PspA-C-CPE to claudin-4-expressing L cells, PspA alone did not bind to NALT epithelium, whereas the PspA-C-CPE construct efficiently bound to epithelium (Fig 2B). Additionally, PspA-C-CPE also bound to UEA-1+ M cells, a finding consistent with a previous report that M cells also expressed claudin-4 [28]. These data indicate that PspA-C-CPE maintained binding activity to claudin-4, allowing the efficient binding of PspA-C-CPE to NALT epithelium, including M cells.

**Nasal immunization with PspA-C-CPE efficiently induces PspA-specific antibody responses in both the respiratory and systemic compartments**

Based on the efficient delivery of vaccine antigen to nasal epithelium including NALT by the use of PspA-C-CPE, our next experiment was aimed to investigate the PspA-specific immune responses induced by nasal immunization with PspA-C-CPE. In this study, mice were nasally immunized with mock, PspA alone, or PspA-C-CPE once a week for 3 weeks. One week after the last immunization, serum and respiratory samples (nasal wash and BALF) were collected for ELISA analysis to measure the production of PspA-specific antibodies. Mice nasally immunized with PspA-C-CPE showed higher levels of PspA-specific serum IgG (Fig 3A). In addition to systemic immune compartment, PspA-specific IgA responses were induced in the nasal washes (Fig 3B). Furthermore, mice nasally immunized with PspA-C-CPE showed higher levels of PspA-specific IgA and IgG responses in the BALF (Fig 3C and 3D). These findings indicate that the efficient delivery of PspA by nasal immunization using C-CPE was coincident with the induction of strong PspA-specific antibody responses in the respiratory tract and systemic immune compartments.

**Nasal immunization with PspA-C-CPE induces protective immunity against \textit{S. pneumoniae} infection**

Finally, we evaluated whether the PspA-specific immune response induced by nasal immunization with PspA-C-CPE was sufficient to protect against pneumococcal infection. One week
after the last immunization, mice underwent respiratory challenge with *S. pneumoniae* (5 × 10^6 CFU/mouse). We monitored the survival rate until 14 days after infection. Few mice could survive after pneumococcal infection when they were nasally immunized with mock (less than 15%) or PspA alone (less than 60%) (Fig 4). In contrast, more than 80% of the mice survived in the same condition when mice received nasal immunization with PspA-C-CPE (Fig 4).

IL-17 is known to play important roles in the clearance of pneumococcal infection [29]. Thus, it is possible that IL-17-type innate immunity induced by C-CPE provides protective immunity against pneumococcal infection. However, we found that C-CPE alone did not induce IL-17-producing cells in the nasal passage and lung (S1 and S2 Figs). Consequently, mice receiving nasal administration of C-CPE alone could not survive after pneumococcal infection (S3 Fig). Thus, C-CPE alone did not induce IL-17-type innate immunity, which was
insufficient to protect against pneumococcal infection. These findings indicate that nasal immunization with PspA-C-CPE induced PspA-specific acquired immunity, which was required for the protection against pneumococcal infection.

**Discussion**

In this study, we showed that claudin-4-targeting using C-CPE elicited PspA-specific systemic and respiratory antibody responses that were sufficient to induce protection against pneumococcal infection. Various approaches for a vaccine delivery system have been reported. M cells have been shown to be potential target cells for vaccine delivery to MALTs. Some pathogens (e.g., *Salmonella*, reovirus, *Yersinia*, *E. coli*) use M cells as an invasion site, and the underlying molecular mechanisms including the ligand and receptor have been revealed [9, 30–32]. For instance, σ1, a surface protein of reovirus, binds to α2,3-sialic acid on M cells to invade its host [33]. FimH expressed on enterobacteria acts as a ligand for glycoprotein 2 for the organism’s invasion through M cells [32]. CPE binds claudin-4 which is expressed in intestinal mucosa [34] to show their pathogenesis. It was reported that claudin-4 is also expressed on antigen-sampling M cells in the NALT and GALT [28]. Thus, claudin-4 is a potent target to deliver vaccine to M cells. Our current study extend microbe-based vaccine delivery to MALT by showing the ability of C-CPE to bind to respiratory epithelial cells, including NALT M cells.

Professional antigen-sampling cells, M cells are covered by short villi and less dense mucus, and thus physically and chemically accessible to mucosally-administered antigen from the lumen of respiratory and intestinal tracts [3]. Basement of M cells form a pocket structure where dendritic cells are located [3], allowing efficient antigen transport to the dendritic cells. Then, dendritic cells present antigen to underlining T cells and B cells for the initiation of antigen-specific immune responses [4]. Our current study demonstrated that PspA-C-CPE bound to M cells, which resulted in the internalization of antigen into NALT for the induction of PspA-specific antibody responses. In addition, we found that PspA-C-CPE bound to not only M cells but also NALT epithelium (Fig 2B), which might further enhance the antigen deposition.

---

**Fig 2. Binding of PspA-C-CPE to claudin-4-expressing cells.** (A) Claudin-4-expressing L cells and parent L cells were treated with PspA or PspA-C-CPE. Their bindings were detected by using an anti-His tag antibody followed by staining with fluorescein-labeled secondary antibody. Violet histograms are PspA or PspA-C-CPE; the green line histogram is control. (B) Binding of PspA-C-CPE to NALT epithelium. NALT sections were fixed with acetone and stained with biotinylated-PspA or biotinylated-PspA-C-CPE followed by staining with Alexa Fluor 546-conjugated streptavidin. M cells were detected by staining with fluorescein-conjugated UEA-1. Yellow arrows indicate PspA-C-CPE bound to M cells. Red, biotinylated-PspA or biotinylated-PspA-C-CPE; green, UEA-1; blue, DAPI. Scale bar is 100 μm (left) or 50 μm (right).

\[\text{doi:10.1371/journal.pone.0126352.g002}\]
into NALT. To this end, our previous study showed that C-CPE reversibly opens tight junctions and enhances mucosal absorption [35]. Therefore, C-CPE likely opens the tight junctions, allowing the fused antigen uptake through the epithelial cell layer. Moreover, claudin-4 contains a clathrin-sorting signal sequence in its C-terminal intracellular region [36, 37], leading to the possibility that PspA-C-CPE is taken up by epithelial cells via clathrin-dependent endocytosis.

Regarding the safety of a PspA-C-CPE-based vaccine, the parent CPE protein is known to cause food poisoning by binding to claudin-expressing intestinal epithelium [34]. CPE has two domains: the N-terminal domain contains the toxic function, whereas the C-terminal domain has the claudin-binding function. In the food poisoning mechanism of CPE, claudin-4-CPE complexes on intestinal cells oligomerize through their N-terminal domains, subsequently leading to alternated membranes permeability and cell death [34]. Because C-CPE lacks the toxic N-terminal domain of CPE [38, 39], C-CPE likely is non-toxic. We found normal levels of aspartate transaminase, alanine aminotransferase, and urea nitrogen in the blood of mice nasally immunized with C-CPE [40]. Therefore, a nasal vaccine delivery system using C-CPE is a safe and effective tool for the development of mucosal vaccines.

Currently, polysaccharide-based vaccine is approved for use as a pneumococcal vaccine in humans. However, this vaccine only induces serotype-specific immune responses. In contrast,
we used PspA as a vaccine antigen in this study because PspA is highly antigenic and induces cross-activity among various pneumococcal strains [23, 41, 42]. PspA-specific IgA responses purportedly prevent colonization or the initial step of invasion of \textit{S. pneumoniae} [43]. In addition, PspA-specific serum IgG responses are also important for the elimination of invasing \textit{S. pneumoniae} [26]. PspA has an ability to interact with and fix complement component C3 and inhibit its deposition. Indeed, PspA-negative \textit{S. pneumoniae} is immediately cleared from blood because of their inability to inhibit complement function [44]. Therefore, it is plausible that PspA-specific serum IgG prevents the PspA-mediated inhibition of complement function and facilitates bacterial elimination in a complement-dependent manner. Although the exact immunologic function of PspA-specific IgA in the respiratory tract remains unknown, surface choline-binding proteins, including PspA, are required for colonization of the nasal cavity [45]. Our current study demonstrated that nasal immunization with PspA-C-CPE induces PspA-specific serum IgG responses and respiratory IgA responses (Fig 3A–3D). Consequently, PspA-C-CPE likely prevents initial invasion by \textit{S. pneumoniae} in the respiratory tract and lethal pathogenesis in the systemic compartments. Although PspA was wildly cross-reactive to various pneumococcal isolate, the distinct degrees of cross-reactivity were reported [41, 46]. In this issue, one possible strategy for the improvement is to use PspA derived from selective clades (e.g., PspA4 and PspA5) for the high degree of cross reactivity [42]. Further, additional pneumococcal antigens such as pneumococcal surface adhesion A and pneumolysin can be combined for the strong protection against pneumococcal infection [47, 48].

In summary, we genetically prepared C-CPE fused with PspA and confirmed that PspA-C-CPE efficiently bound to NALT epithelium, including M cells. These functions led to the induction of PspA-specific antibody responses in both the systemic compartment and respiratory tract; these responses were sufficient to convey protection against pneumococcal infection. These findings suggest that C-CPE is an effective nasal vaccine delivery system for protection against pneumococcal infection.
Supporting Information

S1 Fig. C-CPE alone did not induce IL-17-producing cells at the nasal passage. Mice were nasally immunized with vehicle or C-CPE alone (2 μg) once weekly for 3 weeks. One week after the last immunization, nasal passage were collected for measurement of IL-17-producing cells. Bar is median. Data are representative of two independent experiments (n = 5 for each experiment).

(TIF)

S2 Fig. C-CPE alone did not induce IL-17-producing cells at the lung. Mice were nasally immunized with vehicle or C-CPE alone (2 μg) once weekly for 3 weeks. One week after the last immunization, lung were collected for measurement of IL-17-producing cells. Bar is median. Data are representative of two independent experiments (n = 5 for each experiment).

(TIF)

S3 Fig. C-CPE alone could not provide the protective immunity against pneumococcal infection. Mice were nasally immunized with vehicle or C-CPE alone (2 μg) once weekly for 3 weeks. One week after the last immunization, mice were inhaled challenged with S. pneumoniae (5.0 × 10^6 CFU/mouse), and their survival was monitored for 14 days. Data are representative of two independent experiments (n = 10 for each experiment).

(TIF)

Acknowledgments

We thank Drs. Y. Horiguchi and S. Tsukita for providing C-CPE cDNA and claudin-expressing L cells, respectively. We also thank all members of our laboratory for useful comments and discussions.

Author Contributions

Conceived and designed the experiments: HS HK KY MK JK. Performed the experiments: HS AW EH MY. Analyzed the data: HS AW MK JK. Wrote the paper: HS MK JK.

References

1. Fujikuyama Y, Tokuhara D, Kataoka K, Gilbert RS, McGhee JR, Yuki Y, et al. Novel vaccine development strategies for inducing mucosal immunity. Expert Review of Vaccines. 2012; 11(3):367–79. doi: 10.1586/erv.11.196 PMID: 22380827
2. Dlugonska H, Grzybowski M. Mucosal vaccination—an old but still vital strategy. Annals of Parasitology. 2012; 58(1):1–9. PMID: 23094329
3. Mabbott NA, Donaldson DS, Ohno H, Williams IR, Mahajan A. Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium. Mucosal Immunol. 2013; 6(4):666–77. doi: 10.1038/mi.2013.30 PMID: 23695511
4. Brandtzaeg P. Induction of secretory immunity and memory at mucosal surfaces. Vaccine. 2007; 25(30):5467–84. PMID: 17227687
5. Kunisawa J, Kurashima Y, Kiyono H. Gut-associated lymphoid tissues for the development of oral vaccines. Advanced Drug Delivery Reviews. 2012; 64(6):523–30. doi: 10.1016/j.addr.2011.07.003 PMID: 21827802
6. Kunisawa J, Nochi T, Kiyono H. Immunological commonalities and distinctions between airway and digestive immunity. Trends Immunol. 2008; 29(11):505–13. doi: 10.1016/j.it.2008.07.008 PMID: 18935748
7. Nochi T, Yuki Y, Matsumura A, Mejima M, Terahara K, Kim DY, et al. A novel M cell-specific carbohydrate-targeted mucosal vaccine effectively induces antigen-specific immune responses. J Exp Med. 2007; 204(12):2789–96. PMID: 17984304
8. Shima H, Watanabe T, Fukuda S, Fukuoka SI, Ohara O, Ohno H. A novel mucosal vaccine targeting Peyer's patch M cells induces protective antigen-specific IgA responses. Int Immunol. 2014; 26 (11):619−25. doi: 10.1093/intimm/dxu061 PMID: 24908678

9. Wang X, Hone DM, Haddad A, Shata MT, Pascual DW. M cell DNA vaccination for CTL immunity to HIV. J Immunol. 2003; 171(9):4717−25. PMID: 14568947

10. Furuse M. Molecular basis of the core structure of tight junctions. Cold Spring Harb Perspect Biol. 2010; 2(1):a002907. doi: 10.1101/cshperspect.a002907 PMID: 20182608

11. Kakutani H, Kondoh M, Fukasaka M, Suzuki H, Hamakubo T, Yagi K. Mucosal vaccination using claudin-4 targeting. Biomaterials. 2010; 31(20):5463−71. doi: 10.1016/j.biomaterials.2010.03.047 PMID: 20398936

12. Sonoda N, Furuse M, Sasaki H, Yonemura S, Katahira J, Horiguchi Y, et al. Clostridium perfringens enterotoxin fragment removes specific claudins from tight junction strands: Evidence for direct involvement of claudins in tight junction barrier. J Cell Biol. 1999; 147(1):195−204. PMID: 10508866

13. Van Itallie CM, Betts L, Smedley JB, McClane BA, Anderson JM. Structure of the claudin-binding domain of Clostridium perfringens enterotoxin. J Biol Chem. 2008; 283(1):268−74. PMID: 17977833

14. Suzuki H, Kakutani H, Kondoh M, Watari A, Yagi K. The safety of a mucosal vaccine using the C-terminal fragment of Clostridium perfringens enterotoxin. Pharmazie. 2010; 65(10):766−9. PMID: 21105580

15. Grevers G. Challenges in reducing the burden of otitis media disease: an ENT perspective on improving management and prospects for prevention. Int J Pediatr Otorhinolaryngol. 2010; 74(6):572−7. doi: 10.1016/j.ijporl.2010.03.049 PMID: 20409595

16. Miyaji EN, Oliveira ML, Carvalho E, Ho PL. Serotype-independent pneumococcal vaccines. Cell Mol Life Sci. 2013; 70(18):3303−26. doi: 10.1007/s00018-012-1234-8 PMID: 23269437

17. Park IH, Pritchard DG, Cartee R, Brandao A, Brandileone MC, Nahm MH. Discovery of a new capsular serotype (6C) within serogroup 6 of Streptococcus pneumoniae. J Clin Microbiol. 2007; 45(4):1225−33. PMID: 17267625

18. Calix JJ, Nahm MH. A new pneumococcal serotype, 11E, has a variably inactivated wcjE gene. J Infect Dis. 2010; 202(1):29−38. doi: 10.1086/653123 PMID: 20507232

19. Zhao AS, Boyle S, Butrymowicz A, Engle RD, Roberts JM, Mouzakes J. Impact of 13-valent pneumococcal conjugate vaccine on otitis media bacteriology. Int J Pediatr Otorhinolaryngol. 2014; 78(3):499−503. doi: 10.1016/j.ijporl.2013.12.028 PMID: 2461461

20. Harboe ZB, Dalby T, Weinberger DM, Benfield T, Molbak K, Slotved HC, et al. Impact of 13-Valent Pneumococcal Conjugate Vaccination in Invasive Pneumococcal Disease Incidence and Mortality. Clin Infect Dis. 2014.

21. Shaper M, Hollingshead SK, Benjamin WH Jr., Briles DE. PspA protects Streptococcus pneumoniae from killing by apolactoferrin, and antibody to PspA enhances killing of pneumococci by apolactoferrin. Infect Immun. 2004; 72(9):5031−40. PMID: 15321996

22. CRAIN MJ, Wallyman WD 2nd, Turner JS, Yother J, Taikington DF, McDaniel LS, et al. Pneumococcal surface protein A (PspA) is serologically highly variable and is expressed by all clinically important capsular serotypes of Streptococcus pneumoniae. Infect Immun. 1990; 58(10):3293−9. PMID: 1698178

23. Nguyen CT, Kim SY, Kim MS, Lee SE, Rhee JH. Intranasal immunization with recombinant PspA fused with a flagellin enhances cross-protective immunity against Streptococcus pneumoniae infection in mice. Vaccine. 2011; 29(34):5731−9. doi: 10.1016/j.vaccine.2011.05.095 PMID: 21696689

24. Nabors GS, Braun PA, Hermann DJ, Heise ML, Pyle DJ, Gravenstein S, et al. Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies to heterologous PspA molecules. Vaccine. 2000; 18(17):1743−54. PMID: 10699322

25. Kurono Y, Yamamoto M, Fujihashi K, Kodama S, Suzuki M, Mogi G, et al. Nasal immunization induces Haemophilus influenzae-specific Th1 and Th2 responses with mucosal IgA and systemic IgG antibodies for protective immunity. J Infect Dis. 1999; 180(1):122−32. PMID: 10353870

26. Kong IG, Sato A, Yuki Y, Nochi T, Takahashi H, Sawada S, et al. Nanogel-based PspA intranasal vaccine prevents invasive disease and nasal colonization by Streptococcus pneumoniae. Infect Immun. 2013; 81(5):1625−34. doi: 10.1128/IAI.00240-13 PMID: 23460513

27. Takahashi A, Kondoh M, Masuyama A, Fuji R, Mizuguchi H, Horiguchi Y, et al. Role of C-terminal regions of the C-terminal fragment of Clostridium perfringens enterotoxin in its interaction with claudin-4. J Control Release. 2005; 108(1):56−62. PMID: 16091298

28. Rajapaksa TE, Stover-Hamer M, Fernandez X, Eckelhoefer HA, Lo DD. Claudin 4-targeted protein incorporated into PLGA nanoparticles can mediate M cell targeted delivery. J Control Release. 2010; 142 (2):196−205. doi: 10.1016/j.jconrel.2009.10.033 PMID: 19896996
29. Zhang Z, Clarke TB, Weiser JN. Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. J Clin Invest. 2009; 119(7):1899–909. doi: 10.1172/JCI36731 PMID: 19509469

30. Jensen VB, Harty JT, Jones BD. Interactions of the invasive pathogens *Salmonella typhimurium*, *Listeria monocytogenes*, and *Shigella flexneri* with M cells and murine Peyer's patches. Infect Immun. 1998; 66(8):3758–66. PMID: 9673259

31. Clark MA, Hirst BH, Jeffson MA. M-cell surfaceβ1 integrin expression and invasin-mediated targeting of *Yersinia pseudotuberculosis* to mouse Peyer's patch M cells. Infect Immun. 1998; 66(3):1237–43. PMID: 9488419

32. Hase K, Kawanho K, Nochi T, Pontes GS, Fukuda S, Ebisawa M, et al. Uptake through glycoprotein 2 of FimH-bacteria by M cells initiates mucosal immune response. Nature. 2009; 462(7270):226–30. doi:10.1038/nature08529 PMID: 19907495

33. Helander A, Silvey KJ, Mantis NJ, Hutchings AB, Chandran K, Lucas WT, et al. The viral receptor-binding region of poliovirus type 1 is involved in binding to type 1 reovirus to M cell apical surfaces. J Virol. 2003; 77(14):7964–77. PMID: 12829836

34. Smedley JG 3rd, Uzal FA, McClane BA. Identification of a prepore large-complex stage in the mechanism of action of *Clostridium perfringens* enterotoxin. Infect Immun. 2007; 75(5):2381–90. PMID: 17307943

35. Kondoh M, Masuyama A, Takahashi A, Asano N, Mizuguchi H, Koizumi N, et al. A novel strategy for the enhancement of drug absorption using a claudin modulator. Mol Pharmacol. 2005; 67(3):749–56. PMID: 15150207

36. Bonifacino JS, Traub LM. Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem. 2003; 72:395–447. PMID: 12651740

37. Ivanov AI, Nusrat A, Parkos CA. Endocytosis of epithelial apical junctional proteins by a clathrin-mediated pathway into a unique storage compartment. Mol Biol Cell. 2004; 15(1):176–88. PMID: 14528017

38. Hanna PC, Mietzner TA, Schoolnik GK, McClane BA. Localization of the receptor-binding region of *Clostridium perfringens* enterotoxin utilizing cloned toxin fragments and synthetic peptides. The 30 C-terminal amino acids define a functional binding region. J Biol Chem. 1991; 266(17):11037–43. PMID: 16457211

39. Kokai-Kun JF, McClane BA. Deletion analysis of the *Clostridium perfringens* enterotoxin. Infect Immun. 1997; 65(3):1014–22. PMID: 9038311

40. Suzuki H, Kondoh M, Li X, Takahashi A, Matsushita K, Matsushita K, et al. A toxicological evaluation of a claudin modulator, the C-terminal fragment of *Clostridium perfringens* enterotoxin. Mol Immunol. 2011; 48(7):1237–46. PMID: 21812332

41. Bries DE, Hollingshead SK, King J, Swift A, Braun PA, Park MK, et al. Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. J Infect Dis. 2000; 182(6):1694–701. PMID: 11069242

42. Moreno AT, Oliveira ML, Ferreira DM, Ho PL, Darieux M, Leite LC, et al. Immunization of mice with single PspA fragments induces antibodies capable of mediating complement deposition on different pneumococcal strains and cross-protection. Clin Vaccine Immunol. 2010; 17(3):439–46. doi:10.1128/CVI.00430-09 PMID: 20089795

43. Fukuyama Y, King JD, Kataoka K, Kobayashi R, Gilbert RS, Oishi K, et al. Secretory-IgA antibodies play an important role in the immunity to *Streptococcus pneumoniae*. J Immunol. 2010; 185(3):1755–62. doi:10.4049/jimmunol.20090831 PMID: 20585031

44. Tu AH, Fulgham RL, McCrory MA, Bries DE, Szalai AJ. Pneumococcal surface protein A inhibits complement activation by *Streptococcus pneumoniae*. Infect Immun. 1999; 67(9):4720–4. PMID: 10456924

45. Oggunniyi AD, LeMessurier KS, Graham RM, Watt JM, Bries DE, Stroeher UH, et al. Contributions of pneumolysin, pneumococcal surface protein A (PspA), and PspC to pathogenicity of *Streptococcus pneumoniae* D39 in a mouse model. Infect Immun. 2007; 75(4):1843–51. PMID: 17261599

46. Goulart C, Darieux M, Rodriguez D, Pimenta FC, Brandleone MC, de Andrade AL, et al. Selection of family 1 PspA molecules capable of inducing broad-ranging cross-reactivity by complement deposition and opsonophagocytosis by murine peritoneal cells. Vaccine. 2011; 29(8):1634–42. doi:10.1016/j.vaccine.2010.12.074 PMID: 21211592

47. Goulart C, da Silva TR, Rodriguez D, Politano WR, Leite LC, Darieux M. Characterization of protective immune responses induced by pneumococcal surface protein A in fusion with pneumolysin derivatives. PLoS One. 2013; 8(3):e59605. doi:10.1371/journal.pone.0059605 PMID: 23533636
48. Briles DE, Ades E, Paton JC, Sampson JS, Carlone GM, Huebner RC, et al. Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of *Streptococcus pneumoniae*. Infect Immun. 2000; 68(2):796–800. PMID: 10639448