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Clostridium perfringens enterotoxin-based protein engineering for the vaccine design and delivery system

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ABSTRACT

Clostridium perfringens is a major cause of food poisoning worldwide, with its enterotoxin (CPE) being the major virulence factor. The C-terminus of CPE (C-CPE) is non-toxic and is the part of the toxin that binds to epithelial cells via the claudins in tight junctions; however, C-CPE has low antigenicity. To address this issue, we have used protein engineering technology to augment the antigenicity of C-CPE and have developed a C-CPE-based vaccine against *C. perfringens*-mediated food poisoning. Moreover, C-CPE has properties that make it potentially useful for the development of vaccines against other bacterial toxins that cause food poisoning. For example, we hypothesized that the ability of C-CPE to bind to claudins could be harnessed to deliver vaccine antigens directly to mucosa-associated lymphoid tissues, and we successfully developed a nasally administered C-CPE-based vaccine delivery system that promotes antigen-specific mucosal and systemic immune responses. In addition, our group has revealed the roles that the nasal mucus plays in lowering the efficacy of C-CPE-based nasal vaccines. Here, we review recent advances in the development of C-CPE-based vaccines against the major bacterial toxins that cause food poisoning and discuss our C-CPE-based nasal vaccine delivery system.

Contents

Abbreviations: Stx, Shiga toxin; Stx2B, Shiga toxin 2B subunit; STEC, Shiga toxin (Stx)-producing *Escherichia coli*; CT, cholera toxin; CTA, cholera toxin A subunit; CTB, cholera toxin B subunit; CPE, *C. perfringens* enterotoxin; C-CPE, C-terminus of *C. perfringens* enterotoxin; IL, interleukin; MALTs, mucosal associated lymphoid tissues; GALT, gut-associated lymphoid tissues; NALT, nasopharynx-associated lymphoid tissue; ECL2, second extracellular loop; PspA, pneumococcal surface protein A; Ttll1, tubulin tyrosine ligase-like family, member 1.

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1. Introduction

Vaccination is an effective means of preventing infectious diseases. Among the various types of vaccines that have been developed, toxoid vaccines have proven effective against toxin-mediated bacterial infections, including diphtheria and tetanus [1]. However, many toxin-mediated bacterial infections lack effective vaccines, such as infectious diarrhea, which caused an estimated 1.3 million deaths worldwide in 2015 [2]. Several bacteria, including *Escherichia coli*, *Vibrio cholerae*, and *Clostridium perfringens*, and their toxins have been identified as major causes of such food- and waterborne diseases.

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) are a common cause of food poisoning in both developing and developed countries. From May 1 through July 4 of 2011, 2971 cases of STEC infection were reported in Germany, including 18 fatal cases [3], and an estimated 265,000 STEC infections occur annually in the United States [4]. Currently, there is no vaccine against STEC in clinical use. STEC produce two major types of virulence factor—Stx1 and Stx2. Compared with Stx1-producing strains, Stx2-producing strains are more frequently associated with serious clinical manifestations such as hemorrhagic colitis, hemolytic uremic syndrome [5,6], and neurological disorders [7]. Furthermore, epidemiological evidence has shown that Stx2-producing *E. coli* serotype O157:H7 is the most common serotype associated with large outbreaks of hemorrhagic colitis and hemolytic uremic syndrome [8,9]. At the molecular level, Stx2 comprises a single A subunit (Stx2A) linked to five identical non-toxic B subunits (Stx2B) [10,11]. Stx2B binds to the cellular receptor globotriaosylceramide on the surface of endothelial cells [12] and disrupts protein synthesis in cells by injuring the eukaryotic ribosome, resulting in cell death [13–15]. Recent studies have shown that Stx2B-specific antibodies block the binding of Stx2 to endothelial cells *in vitro* [16] and that these antibodies neutralize Stx2 to protect mice from lethal infection [16]. Therefore, Stx2B is a potentially useful antigen for the development of vaccines against STEC infection.

Another well-known bacterium that causes toxin-mediated disease is *Vibrio cholera*. *Vibrio cholera* enters the body via contaminated water, colonizes the intestinal tract, and induces cholera [17], which is a potentially fatal gastrointestinal infection with symptoms that include watery diarrhea and vomiting and that can lead to acidosis, drowsiness, and unconsciousness [18]. Overcrowded areas in underdeveloped and developing countries with poor sanitation and unsafe drinking water are at particularly high risk of experiencing cholera outbreaks. In 2010, a large outbreak in Haiti caused 697,256 cases of cholera and 8534 deaths [19]. Among the 200 serotypes of *V. cholerae* identified so far, only two pathogenic strains have been found that secrete cholera toxin (CT), the major virulence factor involved in the development of cholera [20,21]. At the molecular level, CT is structurally similar to Stx in that it comprises a single ADP-ribosyltransferase–active A subunit (CTA) linked to five identical B subunits (CTB) [22]. CTB binds with high affinity to the ganglioside GM1, which is found in the lipid rafts of most mammalian cells [23,24]. After receptor-binding, CTA is internalized by host cells, where it activates the G protein that catalyzes ADP-ribosylation of adenylate cyclase, thus resulting in massive fluid and electrolyte loss and leading to the development of serious clinical symptoms including watery diarrhea [25]. Similar to Stx2, the blocking of CTB receptor binding is one potential way of preventing CT-mediated pathogenicity [26,27]. None of the cholera vaccines in current use are based on CT, because its toxicity has limited its development as a mucosal vaccine. However, CT-specific secretory IgA plays an important role in protecting against CT-induced diarrhea [28,29], and several studies in mice have demonstrated that CTB-specific antibody alone has sufficient neutralizing ability to prevent CT-induced or live *V. cholerae*-induced diarrhea [30,31].

Clostridium perfringens is another major food poisoning bacterium that produces diarrhea-inducing toxins. The pathogenicity of *C. perfringens* is mediated by more than 16 different toxins, and the variability in the toxin armamentarium provides a classification system in which *C. perfringens* isolates can be divided into five types (A–E) [32]. For example, *C. perfringens* are the second most common cause of food poisoning in the United States, where they cause about 1,000,000 cases annually, and most of them are caused by type A strains [33,34]. Type B strains cause fatal hemorrhagic dysentery in sheep [35], type C strains cause fulminant disease in both humans and animals [36], and type D strains cause enterotoxemia in sheep and goats [37]; the role of type E strains in disease remains unclear.

C. perfringens enterotoxin (CPE), which is predominantly produced by type A strains, is the main virulence factor responsible for causing *C. perfringens*–mediated gastrointestinal disease [33]. The most persuasive evidence for this etiology was obtained from a rabbit ileal loop model [38], in which *C. perfringens* harboring an inactivated version of the gene encoding CPE was found to be avirulent and did not induce fluid accumulation in the intestine. CPE induces fluid secretion by acting as a pore-forming toxin. It binds to the surface of epithelial cells via claudins and forms a hexameric oligomer on the cell surface [39,40]. The oligomer inserts itself into the cell membrane and forms a pore [41] through which calcium floods into the cell and causes cell death [42–44]. In the intestine, epithelial cell death due to CPE induces fluid accumulation and electrolyte loss, leading to diarrhea [45]. CPE also binds to internal organs, especially the liver, and can cause a potentially lethal release of potassium into the blood [46].

At the molecular level, CPE is a 319–amino acid, single-chain polypeptide comprising a toxic N-terminal domain and a non-toxic C-terminal receptor-binding domain. The N-terminus of CPE is critical for its cytotoxicity because it contains the amino acid residues necessary for oligomerization and pore formation [40,47]. In contrast, the C-terminus of CPE (C-CPE) is the receptor-binding fragment, and it is reported that recruitment of anti-C-CPE monoclonal antibodies to the active site of claudin binding is necessary to neutralize CPE and protect against CPE-mediated pathogenicity [48–50]. Among several C-terminal fragments that have been produced, the fragment comprising amino acids 194–319 has high solubility and affinity for claudin and is capable of enhancing mucosal absorption of drugs [51,52]. However, this fragment has low antigenicity [53] and, when administered alone, does not induce sufficient immune responses to protect against CPE. To address this issue, we succeeded in augmenting the antigenicity of C-CPE by using protein engineering technology.

2. Development of a novel bivalent vaccine against *C. perfringens* and STEC infection

Previously, considering that Stx2B and C-CPE are both non-toxic domains that strongly bind to their respective receptor, we designed a bivalent vaccine (Stx2B–C-CPE) with activity against both Stx2 and CPE (see Fig. 1) [54]. Indeed, in mice administration of C-CPE alone induces a C-CPE-specific IgM, but not IgG, response due to its low antigenicity. In contrast, administered Stx2B–C-CPE, sufficient IgG immune responses with neutralizing activity against CPE were induced [54]. We also confirmed that fusion of Stx2B with C-CPE did not affect the antigenicity of Stx2B, such that Stx2B–C-CPE also induced a strong Stx2B-specific neutralizing IgG response in mice that was comparable with that induced by

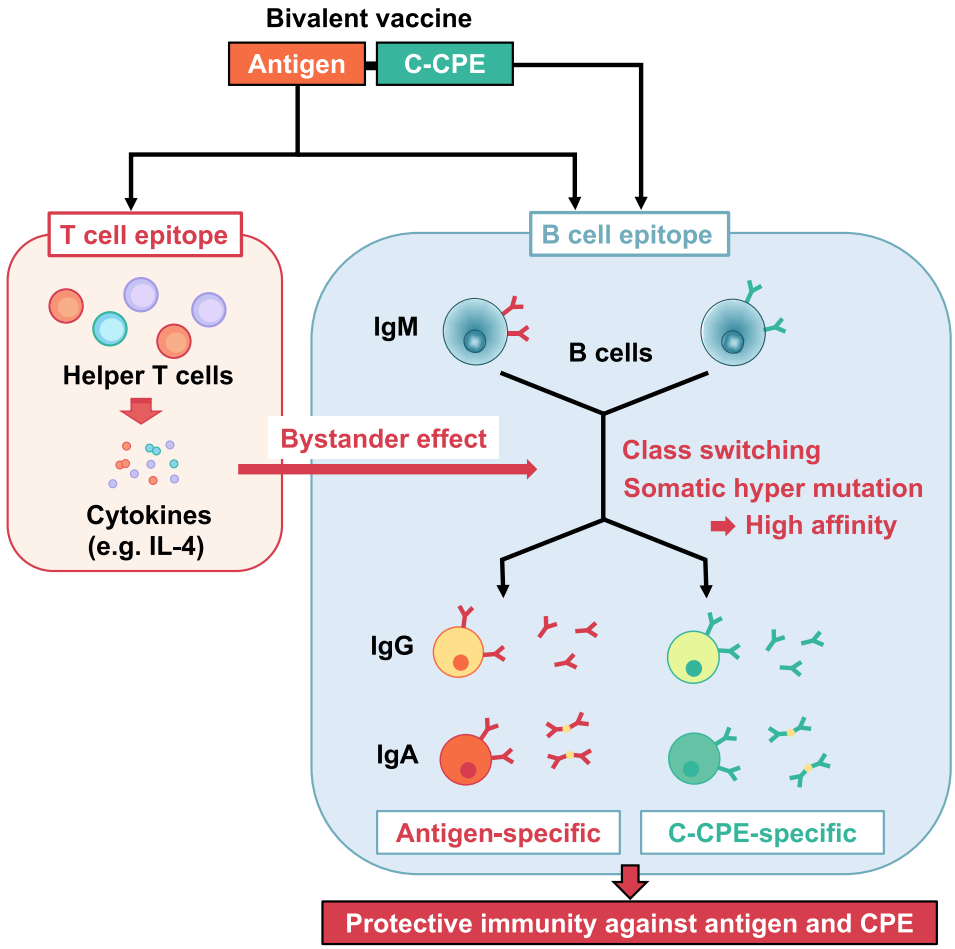

Fig. 1. Antigenicity of C-CPE is augmented. The epitopes derived from C-CPE could only be recognized by B cells, whereas the epitopes derived from fused vaccine antigen are recognized by both T cells and B cells. Antigen-specific T cells support the class switching of antigen-specific as well as C-CPE-specific B cells from IgM to IgG and IgA by producing cytokines such as IL-4. Because class switching is accompanied by somatic hypermutation, the antibodies induced by antigen–C-CPE are considered to have high affinity and provide protective immunity against the antigen-mediated and CPE-mediated pathogenicity.

administration of Stx2B alone. Subcutaneous injection of Stx2B–C-CPE to mice induced protective immune responses against Stx2 and CPE that lasted for at least 48 weeks.

We also found that T cells responses against the fusion protein, which explained a mechanism underlying the class switching from IgM to IgG against CPE. Cytokines from helper T cells are crucial for the induction of somatic hypermutation and immunoglobulin class switching of B cells from IgM to IgG [55–57]. In an *ex vivo* study in which we collected T cells from mice immunized with Stx2B–C-CPE, we found that stimulation with Stx2B–C-CPE or Stx2B alone, but not C-CPE alone, induced the production of the cytokines interferon gamma, interleukin (IL) 4, and IL-17. This meant that these T cells recognized epitopes derived from Stx2B but not C-CPE, suggesting that Stx2B-specific T cells support the class switching of C-CPE-specific B cells from IgM to IgG (see Fig. 1). In addition to the Stx2B-specific IgG antibody immune response, C-CPE-specific IgG was also induced by Stx2B–C-CPE. Because class switching is accompanied by somatic hypermutation [58], the antibodies induced by Stx2B–C-CPE were considered to have high affinity and to be capable of neutralizing their respective toxins (see Fig. 1).

Generally, IgG antibodies neutralize toxins by binding to them and inducing complement activation and opsonization via Fcγ receptors expressed on phagocytes such as macrophages and neutrophils [59]. Mouse IgG antibodies have several subclasses, including IgG1, IgG2a, IgG2b, and IgG3 [55,59]; subclasses IgG2a and IgG2b show preferential binding to Fcγ receptors [59]. T_h1 cytokines such as interferon gamma promote isotype switching of IgM to IgG2a, whereas T_h2 cytokines, such as IL-4, promote isotype switching to IgG1, IgG2b, or IgG3 [55–57]. Therefore, our finding that Stx2B–C-CPE induced the production of interferon gamma, IL-4, and IL-17 by T cells indicates the induction of T_h1, T_h2, and T_h17 responses, respectively. Thus, in addition to directly neutralizing Stx2 and CPE, Stx2B–C-CPE may facilitate the degradation of these toxins through Fcγ receptors and promote complement-mediated phagocytosis of IgG–toxin complexes.

3. Development of a novel oral vaccine against *C. perfringens* infection and cholera

Another example of augmenting the antigenicity of C-CPE is a fusion protein comprising C-CPE and the non-toxic B subunit of CT (CTB), which is responsible for the binding of CT to target cells [60]. Nasal administration of uncoupled antigens together with CTB induces higher antigen-specific immune responses than administration of antigen alone [61]. Oral administration of an uncoupled antigen with CTB is also reported to induce protective systemic and mucosal immune responses against *Naegleria fowleri* infection [62]. Furthermore, because CTB possesses at least four antigenic domains, it has high antigenicity such that CTB alone induces CT-specific immune responses [63,64]. Therefore, CTB could be useful as either an antigen or an antigen carrier for oral immunization against CT.

Because blocking the binding of CT and CPE to their target receptors would protect against toxin-induced disease, we designed a bivalent oral vaccine by genetically fusing the binding fragments of these two toxins (CTB–C-CPE) [60]. Both C-CPE- and CT-specific intestinal IgA antibody immune responses, as well as serum IgG antibody immune responses, were induced in mice orally administered CTB–C-CPE. In a murine intestinal loop model, CTB–C-CPE vaccination inhibited CPE-mediated diarrhea and intestinal villous disruption. Furthermore, mice immunized with CTB-C-CPE showed decreased incidence of hyperkalemia and increased the survival rate when mice were intravenously challenged with CPE. Moreover, the CT-specific antibody responses that were induced in the systemic and intestinal compartments in the CTB–C-CPE immunized mice were comparable with those induced by CTB alone, and serum from mice immunized with CTB–C-CPE or CTB alone showed comparable neutralizing activities to block the binding of CT to GM1. As a result, the development of diarrhea upon oral challenge with CT was prevented by vaccination with CTB–C-CPE or CTB. The high levels of serum IgG and its neutralizing activities against CPE and CT were maintained in the CTB–C-CPE immunized mice for at least 48 weeks after the final immunization.

Similar to Stx2B–C-CPE, oral administration of CTB–C-CPE also induced T-cell responses [60], suggesting that CTB-specific T-cell responses likely contributed to the induction of class switching and therefore augmented the antigenicity of C-CPE. Indeed, C-CPE-specific IgG1, IgG2b, and IgG3 were found to be induced by oral administration of CTB–C-CPE (see Fig. 1).

When we examined the binding of CTB–C-CPE to the intestinal epithelium, we found that CTB–C-CPE bound to mouse claudin-4-expressing cells and to GM1, but not to parent cells without claudin-4 expression. Since both claudin-4 and GM1 are expressed by intestinal epithelial cells, CTB–C-CPE was found to bind to epithelial cells in mouse intestine, including to the surface of Peyer's patches, which are induction sites of mucosal immune responses in the intestinal tract.

4. C-CPE for claudin-targeted vaccine delivery to mucosal associated lymphoid tissues

Mucosal associated lymphoid tissues (MALTs) are important sites for the induction of antigen-specific secretory IgA antibodies [65]. Examples of MALTs include gut-associated lymphoid tissue (GALT) in the intestinal tract and nasopharynx-associated lymphoid tissue (NALT) in the respiratory tract. Antigens that contact the epithelial surface of GALT and NALT are taken up by M cells located in areas called the follicle-associated epithelium [65–67]. After uptake, the antigens are delivered to antigen-presenting cells such as dendritic cells [68]. The antigen-presenting cells then process the antigens into peptides and transport them to naïve helper T cells, which primes the helper T cells [69]. The antigen-primed helper T cells support the induction of somatic hypermutation by B cells and immunoglobulin class switching in germinal centers [70]. Therefore, MALTs are considered good target for mucosal vaccine antigens to induce antigen-specific immune responses.

The surface of mucosal tissues such as MALTs is covered with a sheet of epithelial cells that controls the diffusion of lipids, proteins, water, ions, and other small molecules across the tissue. The epithelial cells in these sheets are linked and communicate via tight junctions, adherens junctions, desmosomes, and gap junctions [71]. Tight junctions are seals between epithelial cells that bind them together to form a barrier that is virtually impermeable to fluids [72]. Adherens junctions and desmosomes link adjacent epithelial cells and provide strength to the membrane [73]. Gap junctions are transmembrane channels that allow the passage of small molecules and ions between epithelial cells [74]. Of these four structures, tight junctions are located closest to the apical side of the epithelium, and they contain two main transmembrane proteins: claudins and occludin [75,76]. Within tight junctions, claudins are exposed at the apical side, where they control the charge and molecular-size selectivity of the barrier [77]. At least 27 claudin isotypes with tissue-specific expression have been identified in the human genome. Unique profiles of claudin-2, -3, and -4 expression were found in the tight junctions of the follicle-associated epithelium of Peyer's patches, which are inductive sites in GALT, and, specifically, claudin-4 is preferentially expressed in the apex region of follicle-associated epithelium [78]. It has also been reported that claudin-4 is also expressed in NALT [79]. Therefore, claudin-4 targeting is considered a potentially useful strategy for delivering vaccine antigens to the surface of MALTs and enhance mucosal vaccine efficacy.

Many studies have shown that C-CPE binds selectively to claudin-4 and increases paracellular permeability [80,81]. It has also been shown that the second extracellular loop of claudin-4 interacts with the final 17 C-terminal amino acids of CPE [82,83]. Moreover, C-CPE showed no cytotoxicity to cells expressing claudin-4 *in vitro* [84], and in mice nasal administration of C-CPE caused no mucosal injury in the nasal cavity or nasal passages [85]. Therefore, C-CPE has potential as a claudin-4-targeting antigen carrier.

5. Mucosal pneumococcal vaccination using C-CPE-mediated delivery

Streptococcus pneumoniae is a key respiratory pathogen that causes infectious diseases such as pneumonia, otitis media, and meningitis [86,87]. *Streptococcus pneumoniae* has more than 90 serotypes [88,89], as classified according to their polysaccharide capsule, so it has been challenging to develop an effective vaccine antigen against this bacterium. The polysaccharide-based vaccine most often used in current clinical practice is administered by injection; therefore, only serotype-specific systemic immune responses are induced despite its high antigenicity [90]. It was shown that pneumococcal surface protein A (PspA) is a major virulence factor that is produced by all *S. pneumoniae* serotypes [91]. PspA inhibits the formation of, or accelerates the dissociation of, the alternative pathway C3 convertase and also prevents the deposition of C3b onto pneumococci to disrupt complement receptor-mediated pathways of clearance [92]. It has been reported that intraperitoneally injection of single PspA fragments induces cross-protective immune responses against different pneumococcal strains in mice [93], suggesting that PspA is a strong candidate for development into a pneumococcal vaccine.

As discussed in the previous section, high expression of claudin-4 has been identified in NALT, and C-CPE specifically binds to claudin-4. Therefore, we developed a nasal vaccine against *S. pneumoniae* by fusing PspA with C-CPE (PspA–C-CPE) [94]. *In vivo* results in mice showed that PspA alone did not bind to NALT epithelium, whereas PspA–C-CPE did, including to M cells. The basement membrane of M cells forms a pocket-like structure that allows the antigens taken up by M cells effectively transport to dendritic cells [95]. On the other hand, C-CPE opens the tight junctions between epithelial cells, thus possibly allowing uptake of PspA–C-CPE through the epithelial cell layer of NALT (see Fig. 2).

We also found higher levels of PspA-specific IgG and nasal IgA in the serum and bronchoalveolar lavage fluid of PspA–C-CPE–immunized mice than in PspA alone immunized mice [94]. PspA plays an important role in the pathogenesis of *S. pneumoniae* infection, and PspA-specific serum IgG may prevent PspA-mediated inhibition of complement function. However, PspA-specific nasal IgA also

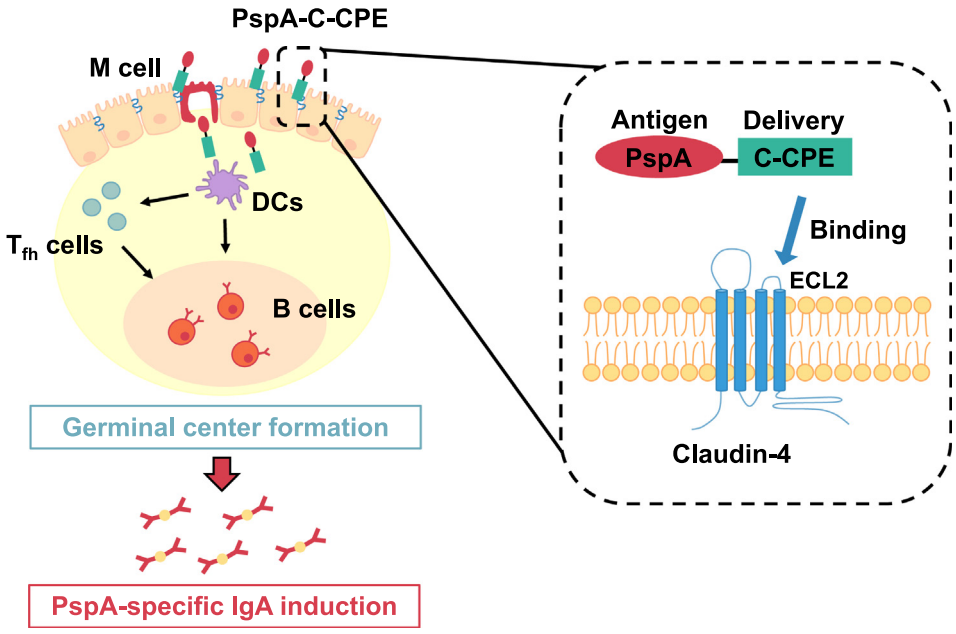

Fig. 2. C-CPE-mediated delivery of vaccine antigen to NALT. Claudin-4 is expressed in NALT. C-CPE delivered pneumococcal surface protein A (PspA) to NALT epithelium including M cells by binding to the second extracellular loop (ECL2) of claudin-4. Dendritic cells (DCs) processed the PspA into peptides and transport them to T cells and B cells. The antigen-primed helper T (Tfh) cells supported class switching of B cells in germinal center, inducing PspA-specific nasal IgA production.

appears to interfere with colonization of the nasal cavity by *S. pneumoniae* [96]. We confirmed that the PspA-specific acquired immunity induced by nasal immunization with PspA–C-CPE provided protection against lethal challenge with *S. pneumoniae* [94]. Together, these results demonstrate the potential of using C-CPE for the targeted delivery of vaccine antigen to NALT for the induction of antigen-specific immune responses (see Fig. 2).

6. Effects of mucus on C-CPE-mediated antigen delivery to MALTs

The mucosal surface is covered with a mucus layer that prevents the binding of foreign antigens, including those in vaccines, to the epithelial cells. Mucus is a water-based gel that contains various proteins, inorganic salts, and lipids [97]. Mucins are the major protein components in mucus, and they form long, linear oligomers via covalent cross-linking of cysteine-rich domains, which contributes to the adhesive and space-occupying properties of mucus [98].

In the nasal cavity, mucus is continuously secreted by goblet cells and cleared by the asymmetric beating of cilia [99]. Tubulin glutamylation adds several glutamic acids to the C-terminus tail domain of tubulin to produce the correct structure for ciliary function, especially for asymmetric beating [100,101]. Tubulin tyrosine ligase-like family, member 1 (Ttll1) is a glutamylation-performing enzyme expressed in cilia, and deficiency in this protein leads to loss of asymmetric beating; therefore, Ttll1-knockout (Ttll1-KO) mice show marked mucus accumulation in the nasal cavity [100].

We examined the effects of nasal mucus accumulation on the efficacy of nasally administered PspA–C-CPE in Ttll1-KO mice [102]. We found that PspA–C-CPE failed to induce an effective IgA response because the binding of PspA–C-CPE to the epithelial cells in the NALT was prevented by the dense accumulation of nasal mucus (see Fig. 3). Germinal centers (GCs) are specialized sites where antigens are presented to T and B cells after processing by dendritic cells, and the IgA class switching of B cells occurs with the support of follicular helper T cells [103]. Flow cytometric analysis revealed that nasal immunization with PspA–C-CPE induced

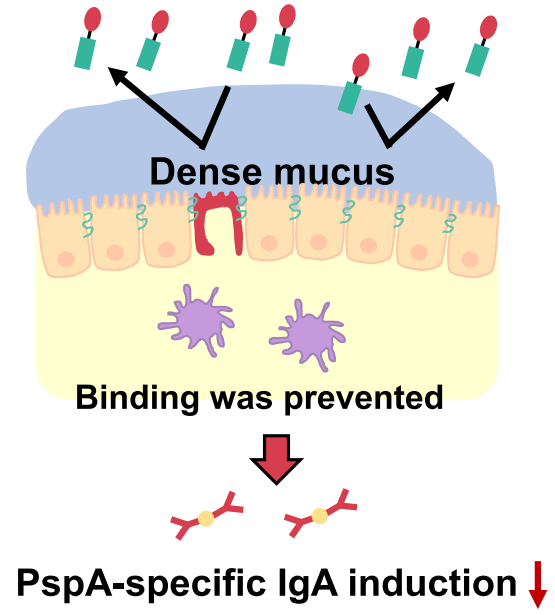

Fig. 3. Binding of PspA-C-CPE to NALT epithelium was prevented. Mucus accumulated in nasal cavity of mice lacking tubulin tyrosine ligase-like family, member 1 (Ttll1-KO). Binding of PspA-C-CPE to NALT epithelium was prevented by the dense accumulation of nasal mucus in Ttll1-KO mice, resulting in a failure of PspA-specific nasal IgA induction.

GC formation as well as $GL7^{high}B220^{+}$ GC B cell proliferation in the NALT of Ttll1-hetero mice. However, smaller size of GCs and fewer $GL7^{high}B220^{+}$ GC B cells were found in Ttll1-KO mice after PspA–C-CPE administration, and the number of follicular helper T cells was also significantly lower in Ttll1-KO mice compared with in Ttll1-hetero mice. Given that the binding of PspA–C-CPE to NALT epithelium was prevented by the dense mucus, we removed the mucus by using *N*-acetylcysteine, a clinical expectorant that cleaves the disulfide bonds in mucin cross-links. Removal of the mucus rescued the PspA-specific nasal IgA antibody response as

well as GC formation, indicating that the mucus not only prevented the delivery of PspA–C-CPE to the epithelium but also affected its efficacy. Therefore, it is necessary to consider the effects of physical barriers, particularly the mucus layer, when developing C-CPE-based mucosal vaccines.

7. Conclusions

Although the bacteria and toxins responsible for causing food poisoning have been identified, food poisoning remains a serious disease that has major impacts on human health worldwide. The World Health Organization estimates that 600 million people contract food poisoning, and 420,000 people die from food poisoning-associated causes each year; therefore, next-generation vaccines and antigen delivery systems are urgently needed. Continued elucidation of the properties and actions of C-CPE will support the development of novel vaccines against the major bacteria and their toxins responsible for food poisoning, as well as the development of new generation of mucosal vaccine.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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