International Immunology, Vol. 31, No. 2, pp. 91–100
doi:10.1093/intimm/dxy071
Advance Access publication 16 October 2018

Development of a bivalent food poisoning vaccine: augmented antigenicity of the C-terminus of *Clostridium perfringens* enterotoxin by fusion with the B subunit of *Escherichia coli* Shiga toxin 2

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Received 15 August 2018, *editorial decision* 8 October 2018; *accepted* 11 October 2018

Abstract

Food poisonings caused by *Clostridium perfringens* and Shiga toxin (Stx)-producing *Escherichia coli* (STEC) occur frequently worldwide; however, no vaccine is currently available. Therefore, we aimed to develop a bivalent vaccine against *C. perfringens* and STEC infections. Although it has been considered that the C-terminal region of *C. perfringens* enterotoxin (C-CPE) could be a good vaccine antigen to block the binding to its receptor, it was insufficient for induction of a protective immune response because of the low antigenicity. However, the fusion of C-CPE with Stx2 B subunit (Stx2B) augmented the antigenicity of C-CPE without affecting the antigenicity of Stx2B. Indeed, high levels of C-CPE-specific neutralizing IgG were found in the serum of mice immunized with the fusion protein Stx2B–C-CPE. Additionally, comparable and substantial levels of Stx2B-specific neutralizing IgG were induced in mice receiving Stx2B–C-CPE or Stx2B alone. These antibody responses against C-CPE and Stx2B lasted for at least 48 weeks, which were sufficient for protective immunity *in vitro* and *in vivo*, indicating that Stx2B–C-CPE could induce long-term protective immunity. As an underlying mechanism, *ex vivo* stimulation with Stx2B, but not with C-CPE, induced cytokine production from splenic T cells collected from mice immunized with Stx2B–C-CPE, suggesting that Stx2B-specific, but not C-CPE-specific, T cells were induced by the immunization with Stx2B–C-CPE and plausibly promoted immunoglobulin class switching of both Stx2B- and C-CPE-specific B cells from IgM to IgG. These findings collectively indicate that Stx2B–C-CPE is a T-cell-antigen-supplement-type bivalent vaccine, which could be an efficient against *C. perfringens* and STEC infections.

Keywords: bacterial infection, diarrhea, hyperkalemia, hemolytic uremic syndrome (HUS), T cell response

Introduction

Diarrheal diseases caused an estimated 1.3 million deaths worldwide in 2015 (1). Especially among children younger than 5 years in low-income countries, diarrheal diseases are a major cause of illness and death (2). In the USA, 19507 cases of infection with specific bacterial or parasitic pathogens and 75 deaths were identified in 2014 by the Foodborne Diseases Active Surveillance Network coordinated by the Centers for Disease Control and Prevention (3). Among these cases, two types of infectious bacteria, *Clostridium perfringens* and Shiga toxin (Stx)-producing *Escherichia coli* [STEC, also known as

enterohemorrhagic *E. coli* (EHEC)] were a common cause of food poisonings in both developing countries and developed countries such as the USA, the UK and Japan (3, 4).

Clostridium perfringens causes food poisoning through the production of *C. perfringens* enterotoxin (CPE) (5). Symptoms include diarrhea and systemic complications such as hyperkalemia (6). *Clostridium perfringens* produces CPE in the small intestine after consumption of food containing at least 10^5–10^7 bacteria. CPE is produced as a single chain polypeptide of 319 amino acids and acts as a pore-forming toxin. In the intestine, the C-terminal region of CPE (C-CPE, residues 194–319) binds to claudin 4 (CLDN4) expressed on epithelial cells as a tight junction protein (7, 8). Upon binding, the N-terminal region of CPE forms physiological pores by oligomerization and disrupts the plasma membrane, resulting in aberrant ion influx and induction of cell death (6). Following the destruction of the intestinal epithelial barrier, CPE enters blood circulation and induces cell death in the liver and kidney by the CLDN4-dependent manner, resulting in hyperkalemia and death by cardiac arrest.

Similarly, STEC infection exerts severe clinical manifestations such as hemorrhagic colitis, hemolytic uremic syndrome (HUS) and neurological disorders accompanied with acute encephalopathy by producing Stx (also known as Vero toxin) in one or more of two forms, Stx1 and Stx2 (9). Epidemiologically, compared with strains producing Stx1 alone, Stx2-producing *E. coli* strains are more frequently associated with HUS (4). Indeed, the Stx2-producing *E. coli* serotype O157:H7 is the most prevalent serotype associated with large outbreaks and incidences of hemorrhagic colitis and HUS (4, 10). Stx2-producing *E. coli* infects via food with 50~100 CFU of bacteria, adheres to the intestinal epithelium by forming the attaching and effacing lesion, grows and then produces Stx2. Stx2 belongs to the AB toxin family, and as such comprises subunits A and B. The B subunit mediates the binding to glycosphingolipid globotriaosylceramide (Gb3) on target cells (11, 12). Then, the A subunit enters the cytoplasm, reaches the endoplasmic reticulum by retrograde transport and releases adenine at position 4324 of 28S rRNA in the 60S ribosome by its RNA *N*-glycosidase activity. Then, protein synthesis is inhibited, resulting in cell death locally and systemically (11–13). Collectively, these findings suggest that inhibition of binding of CPE and Stx2 to target cells can prevent their serious pathogenicity.

Here, we aimed to develop a bivalent vaccine for CPE and Stx2 to prevent systemic intoxication induced by *C. perfringens* or STEC infections because these toxins cause severe systemic symptoms and death. Because both C-CPE and Stx2 B subunit (Stx2B) are non-toxic and associated with receptor binding (8, 14, 15), they are potential vaccine antigens. The immunoprophylactic potential of Stx2B has been reported previously (14, 16, 17); however, we found in the current study that C-CPE has low immunogenicity and hence it is difficult to induce sufficient CPE-specific immune responses. We previously reported a nasal vaccine with C-CPE–mediated antigen delivery; in this vaccine, the receptor binding activity of the C-CPE portion of the antigen–C-CPE fusion protein facilitated the binding of fusion protein to the mucosal surface leading to strong antigen-specific antibody responses (15). Historically, carrier protein-mediated conjugation with a small peptide antigen is known to augment the antigenicity of peptide antigen (18). These findings prompted us to prepare a Stx2B–C-CPE fusion protein to induce immune responses against both C-CPE and Stx2B and thus act as a bivalent vaccine antigen against *C. perfringens* and STEC infections.

Methods

Mice

Female BALB/c mice (age 6–7 weeks) were purchased from CLEA Japan, Inc. (Tokyo, Japan) and used for experiments after pre-breeding for 1 week. All experiments were approved by the Animal Care and Use Committee of the National Institutes of Biomedical Innovation, Health and Nutrition (Approval No. DS27-48R10) and conducted in accordance with their guidelines.

Preparation of plasmids

C-CPE fragment (GenBank accession no. M98037.1) was cloned into pET16b (Novagen, Darmstadt, Germany) to prepare pET16b-C-CPE, as described previously (8). G4S linker fused C-CPE fragment was prepared from pET16b-PspA-C-CPE as described previously (15) by using *Pac*I and *Sal*I. Cellular DNA was extracted from EHEC serotype O157 strain Sakai as described previously (19). Stx2B DNA (GenBank accession no. NP_050540.1) was amplified by polymerase chain reaction (PCR) using specific primers: forward primer: 5′-GG*GGTACC*ATGAAGAAGATGTTTATGGCGG-3′ with a *Kpn*I site (italicized); reverse primer: 5′-GGGGG*GAATTC*TCAGTCATTATTAAACTGCACTTC-3′ with an *Eco*RI site (italicized) or 5′-GG*TTAATTAA*GTCATTATTAAACTGCACTTCAG-3′ with a *Pac*I site (italicized). The Stx2B PCR products were digested with *Kpn*I [New England Biolabs Japan Inc. (NEB), Tokyo, Japan] and *Eco*RI (NEB) or *Pac*I (NEB). The Stx2B and C-CPE fragments were inserted into pCold I (Takara Bio Inc., Shiga, Japan) by using T4 DNA ligase (NEB) to produce pCold I-Stx2B and pCold I-Stx2B–C-CPE.

Preparation of recombinant proteins

We prepared recombinant protein as described previously (15). To obtain recombinant His-tagged proteins, the plasmids were transformed into *E. coli* strain BL21 (DE3) (Toyobo, Osaka, Japan) or BL21 containing pG-Tf2 (Takara). Protein production was induced by using isopropyl-D-thiogalactopyranoside (Nacalai Tesque, Kyoto, Japan). The culture pellets were sonicated in buffer A [10 mM Tris-HCl (pH 8.0), 400 mM NaCl, 5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM 2-mercaptoethanol and 10% (v/v) glycerol]. After centrifugation at 17 800 × *g* for 15 min at 4°C, the supernatants were loaded onto HiTrap HP columns (GE Healthcare, Pittsburgh, PA, USA). C-CPE, Stx2B and Stx2B–C-CPE were each eluted with buffer A containing 100–500 mM imidazole. Each 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 (Life Technologies, Carlsbad, CA, USA). The purity of the eluted protein was confirmed by using the NuPAGE electrophoresis system (Life Technologies) followed by staining with Coomassie brilliant blue.

Flow cytometric assay

Mouse CLDN4-expressing mouse fibroblast cells (L cells) were kindly provided by Dr Tsukita (Kyoto University, Kyoto, Japan) (7). Human CLDN4-expressing human sarcoma cells (HT1080 cells) were prepared as described previously (20). The CLDN4-expressing L cells and parent L cells were incubated with C-CPE, Stx2B or Stx2B–C-CPE for 1 h at 4°C. The cells were then subjected to the following sequential steps, each of which was preceded by a wash with 2% (v/v) newborn calf serum in PBS: (i) incubation with mouse anti-His tag antibody (clone J099B12, BioLegend, San Diego, CA, USA) for 1 h at 4°C; (ii) incubation with fluorescein-labeled rat anti-mouse IgG (H+L) antibody (clone RMG1-1, BioLegend) for 30 min at 4°C; and (iii) analysis with a flow cytometer (MACSQuant; Miltenyi Biotec, Auburn, CA, USA).

Gb3-binding enzyme-linked immune sorbent assay

Globotriaosylceramide (Gb3)–binding enzyme-linked immune sorbent assay (ELISA) was performed as described previously (21). Gb3 (Nacalai Tesque) was dissolved in chloroform–methanol (2:1). Ninety-six-well immunoplates (Thermo Fisher Scientific, Waltham, MA, USA) were coated with Gb3 (1 μg per well) diluted with carbonate buffer (Sigma Aldrich, St Louis, MO, USA) at 4°C overnight. The plates were then subjected to a series of six steps, each of which was preceded by a wash with 0.05% (v/v) Tween in PBS: (i) the plates were treated with 1% (w/v) bovine serum albumin (BSA) in PBS for 2 h at room temperature; (ii) samples were added to the wells, and the plates were incubated at 37°C for 2 h; (iii) mouse anti-His tag antibody (BioLegend) was added to the wells and incubated at 37°C for 2 h; (iv) goat anti-mouse IgG conjugated with horseradish peroxidase (Southern Biotech, Birmingham, AL, USA) was added to the wells, and the plates were incubated at 37°C for 1 h; (v) tetramethylbenzidine peroxidase substrate (Sera Care Life Sciences Inc., Milford, MA, USA) was added to the wells and the plates were incubated for 2 min at room temperature; and (vi) 0.5 M HCl (Nacalai Tesque) was added to the samples, and the absorbance was measured at 450 nm.

Immunization

Mice were subcutaneously immunized with PBS (mock), 50 μg of Stx2B, 67 μg of C-CPE or 111 μg of Stx2B–C-CPE with or without alum adjuvant (Sigma) once a week for 2 consecutive weeks. One week after the last immunization, serum was collected.

ELISA for measurement of antigen-specific serum antibody

Ninety-six-well immunoplates (Thermo Fisher Scientific) were coated with Stx2B or C-CPE (0.5 μg per well) diluted with PBS at 4°C overnight. After the solutions were removed, the plates were treated with 1% (w/v) BSA in PBS for 2 h at room temperature. The plates were then subjected to the following four steps, each of which was preceded by a wash with 0.05% (v/v) Tween in PBS: (i) serial dilutions of samples were added to the wells and the plates were incubated for 2 h at room temperature; (ii) goat anti-mouse IgG, IgM, IgA, IgG1, IgG2a, IgG2b or IgG3 conjugated with horseradish peroxidase (Southern Biotech) was added and the plates were incubated for 1 h at room temperature; (iii) tetramethylbenzidine peroxidase substrate was added and the plates were incubated for 2 min at room temperature; and (iv) 0.5 M HCl (Nacalai Tesque) was added to the samples, and the absorbance was measured at 450 nm.

CPE cytotoxicity assay

African Green Monkey kidney normal cells (Vero cells, JCRB Cell Bank, Osaka, Japan, RRID CVCL_0059) (5×10^4 cells per well) were seeded into 96-well plates and incubated at 37°C overnight in a 5% CO_2 incubator. CPE (0.1 μg) was mixed with serum (40 μl) collected from immunized mice and incubated at 37°C for 1 h; this solution was then added to the Vero cells and incubated at 37°C for 1 h. The viable cell number was measured by using Cell count reagent SF (Nacalai Tesque) and reading absorbance at 450 nm. The survival rate (%) was calculated by the following formula: survival rate (%) = viable cell number after CPE treatment with or without serum/viable cell number of untreated Vero cells.

CPE-induced hyperkalemia model and measurement of serum potassium

Mice were injected intravenously with CPE (100 μg kg^{-1} body weight). Thirty minutes after the injection, a video was taken of each mouse. Blood samples were collected from the heart under anesthesia at 4 h after the injection and the concentration of serum potassium was measured by using a Dri-Chem 7000 system (FUJIFILM, Tokyo, Japan).

Stx2-induced HUS model and measurement of blood urea nitrogen

Mice were injected intravenously with Stx2 (1 ng per mouse) and observed for 1 week. Blood samples were collected at 3 days after the injection and the concentration of blood urea nitrogen (BUN) in serum was measured by using the Dri-Chem 7000 system (FUJIFILM).

T-cell assay

Spleens were collected from mice immunized with Stx2B–C-CPE or mock (PBS) and splenic $CD4^+$ T cells were isolated by using anti-mouse CD4 magnetic beads and a magnetic cell separation system (Miltenyi Biotec) according to the manufacturer's protocols. Antigen-presenting cells (APCs) were prepared from splenic cells by 30 Gy irradiation. Endotoxin was removed from the preparations of Stx2B–C-CPE, Stx2B and C-CPE by using a proteus NoEndoS kit (Protein Ark, Sheffield, UK). $CD4^+$ T cells (2×10^5 cells) were cultured with APCs (1×10^4 cells) and endotoxin-free protein preparations (5 μM) for 4 days. The culture supernatants were collected for measurement of cytokines including IFN-γ, IL-4 and IL-17A by using a BD cytometric bead array mouse $T_h1/T_h2/T_h17$ cytokine kit (BD Biosciences, San Jose, CA, USA).

Statistics

Data are presented as mean ± SD or box and whisker plot. Statistical analyses were performed by using the Student's *t*-test (GraphPad Software, La Jolla, CA, USA).

Results

Preparation of a fusion protein of C-CPE and Stx2B

To design a bivalent vaccine against food poisoning caused by *C. perfringens* and STEC, we prepared a fusion protein with Stx2B at the N-terminus and C-CPE at the C-terminus joined by a G4S linker (Stx2B–C-CPE; Supplementary Figure 1). Stx2B–C-CPE was purified in the same manner as used for Stx2B and C-CPE recombinant proteins; purity was confirmed using a NuPAGE electrophoresis system (Fig. 1A). We then examined the binding activities of C-CPE, Stx2B and Stx2B–C-CPE to CLDN4 and Globotriaosylceramide (Gb3), which are receptors of CPE and Stx2, respectively. Stx2B–C-CPE bound to mouse CLDN4-expressing L cells with efficacy similar to that of C-CPE, but it did not bind to the parent L cells (Fig. 1B), which do not express CLDN4. In contrast, Stx2B did not bind to either CLDN4-expressing or parent L cells (Fig. 1B). Stx2B–C-CPE also bound to human CLDN4-expressing cells (Supplementary Figure 2). Additionally, Stx2B–C-CPE and Stx2B, but not C-CPE, bound to Gb3 (Fig. 1C). These findings collectively indicate that Stx2B–C-CPE exerts dual binding activity to both CLDN4 and Gb3.

Induction of protective immune responses against CPE by Stx2B–C-CPE vaccination

To examine whether Stx2B–C-CPE could induce C-CPE-specific immune responses, mice were immunized subcutaneously with Stx2B–C-CPE, C-CPE alone or C-CPE plus alum once a week for 2 weeks. One week after the last immunization, serum was collected to measure C-CPE-specific antibody responses by ELISA. Mice immunized with Stx2B–C-CPE showed high levels of C-CPE-specific serum IgG (Fig. 2). In contrast, little or no C-CPE-specific IgG responses were induced in mice immunized with C-CPE alone or C-CPE with alum (Fig. 2). Consistent with these results, mice immunized with Stx2B–C-CPE showed high levels of C-CPE-specific serum IgG1, IgG2a, IgG2b and IgG3 compared with mice immunized with C-CPE alone or C-CPE plus alum (Supplementary Figure 3). C-CPE-specific serum IgM antibody production was induced by immunization with C-CPE alone or C-CPE plus alum although the levels were lower than that induced by Stx2B–C-CPE (Fig. 2). C-CPE-specific serum IgA was not detected in any group (Supplementary Figure 4). These results collectively indicate that fusion of C-CPE with Stx2B augmented the antigenicity of C-CPE, and thus Stx2B–C-CPE induced high levels of C-CPE-specific IgG as well as IgM antibody production.

We next examined whether C-CPE-specific immune responses induced by Stx2B–C-CPE could be sufficient to protect against CPE. CPE was lethal to Vero cells in *in vitro* culture (Fig. 3A). Consistent with low C-CPE-specific antibody production (Fig. 2; Supplementary Figure 3), serum collected from mice immunized with C-CPE alone or mock did not show any protective activities against CPE *in vitro* (Fig. 3A). In contrast, serum collected from mice immunized with Stx2B–C-CPE completely neutralized lethal doses of CPE in Vero cells (Fig. 3A).

These *in vitro* neutralizing activities of Stx2B–C-CPE led us to analyze *in vivo* neutralizing activity. Intravenous injection of mice with CPE led to high serum potassium levels (i.e. hyperkalemia; Fig. 3B) and to paralysis of limbs and general weakness (Supplementary Figure 5). These symptoms were not ameliorated by immunization with C-CPE alone or mock. In contrast, mice immunized with Stx2B–C-CPE showed normal levels of serum potassium and consistently showed no symptoms associated with hyperkalemia (Fig. 3B; Supplementary

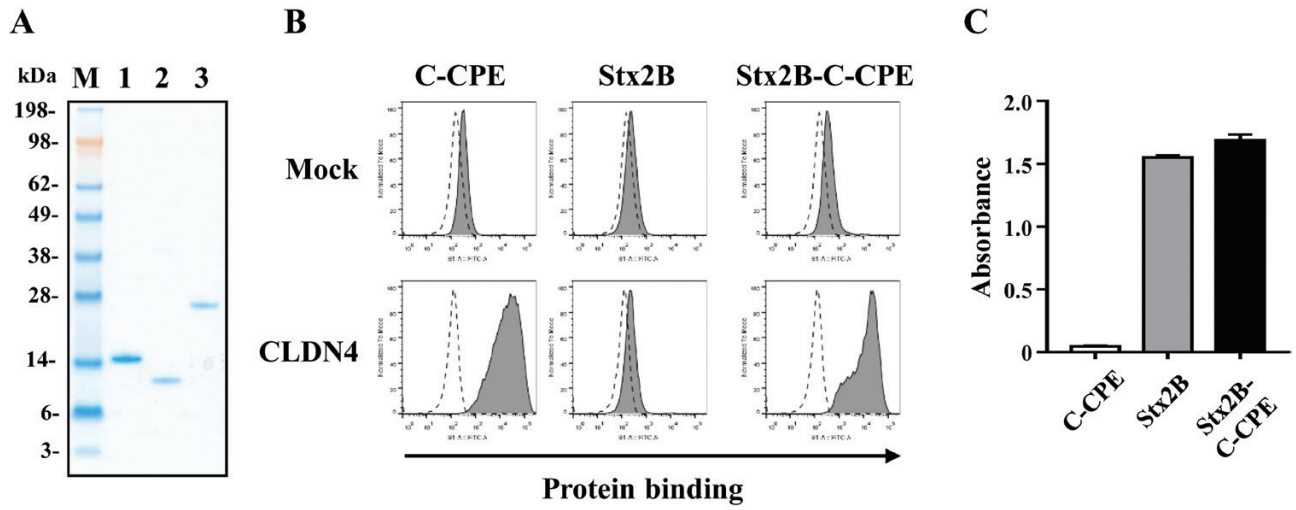

Fig. 1. Preparation of the fusion protein of Stx2B and C-CPE (Stx2B–C-CPE). (A) SDS–PAGE electrophoresis of recombinant proteins. Lane M, size marker; lane 1, C-CPE; lane 2, Stx2B; lane 3, Stx2B–C-CPE. (B) Binding of Stx2B–C-CPE to murine claudin 4-expressing L cells. Gray filled histograms are murine claudin 4-expressing L cells (lower panels) or parent L cells (upper panels) treated with C-CPE, Stx2B or Stx2B–C-CPE. Dotted line histograms are control (non-treated cells). (C) Binding of Stx2B–C-CPE to Gb3. Binding of C-CPE, Stx2B or Stx2B–C-CPE to Gb3 was evaluated by Gb3-ELISA. Data are means ± SD (n = 4). The results shown are representative of two independent experiments.

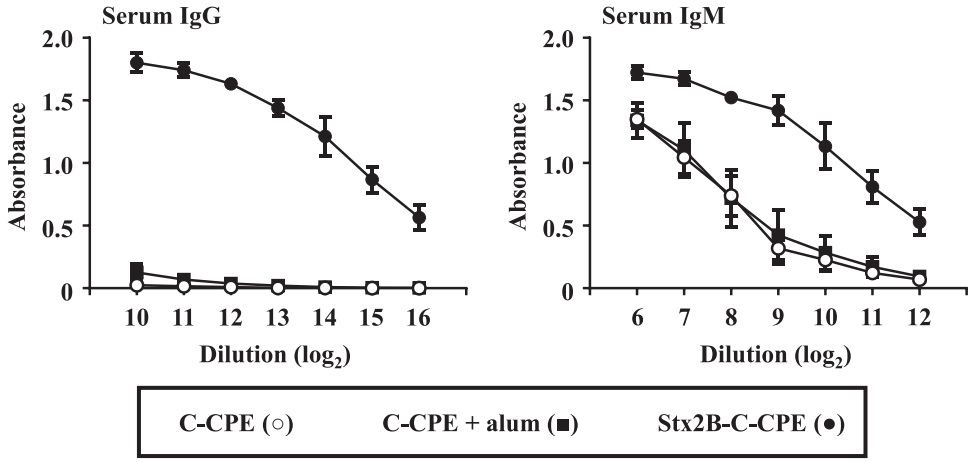

Fig. 2. Induction of C-CPE-specific immune responses by immunization with Stx2B–C-CPE. Mice were subcutaneously immunized with C-CPE alone, C-CPE with alum or Stx2B–C-CPE, once a week for 2 consecutive weeks. One week after the last immunization, serum was collected and C-CPE-specific serum IgG and IgM antibodies were detected by ELISA. Data are means ± SD (n = 4). The results shown are representative of two independent experiments.

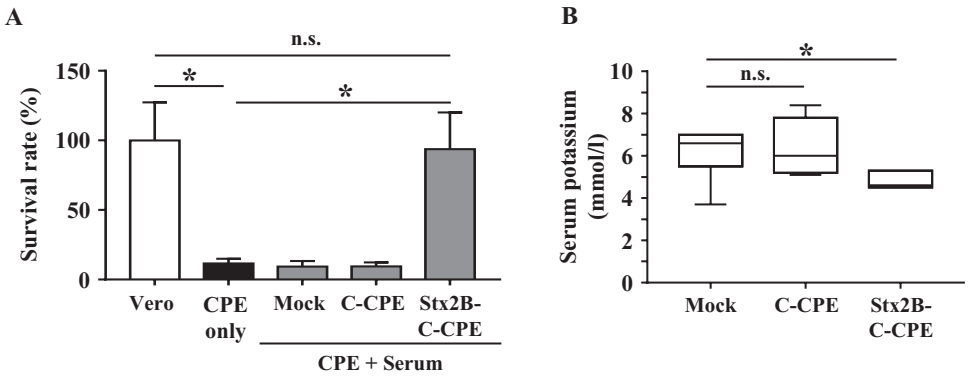

Fig. 3. Induction of protective immune responses against CPE by immunization with Stx2B–C-CPE. (A) Neutralization of CPE lethality in Vero cells. Serum was collected from mice immunized with PBS (Mock), C-CPE or Stx2B–C-CPE. CPE pre-incubated with or without one of these sera was then added to Vero cells. The survival rate (%) was calculated based on viable cell number at 1 h after incubation. Data are means ± SD (n = 4). The results shown are representative of two independent experiments. (B) Protective immunity against CPE injection in mice. Mice were subcutaneously immunized with Stx2B–C-CPE, C-CPE or Mock once a week for 2 consecutive weeks. Ten days after the last immunization, mice were intravenously injected with CPE (100 μg kg^{-1} body weight). Four hours after CPE injection, serum was collected and the potassium concentration was measured. The normal range of serum potassium concentrations is from 3.8 to 5.0 mmol l^{-1}. Data are combined from two independent experiments (n = 7). An asterisk means a significant difference as calculated by using the Student's t-test (*P < 0.05). 'n.s.' means no significant difference.

Figure 5). These results collectively indicate that immunization with Stx2B–C-CPE could induce protective immune responses *in vitro* and *in vivo* against CPE-mediated pathogenesis of *C. perfringens*.

Induction of protective immune responses against Stx2 by Stx2B–C-CPE vaccination

We next examined Stx2B-specific immune responses following immunization with Stx2B–C-CPE. Unlike C-CPE, Stx2B was highly immunogenic. Therefore, immunization with Stx2B alone was sufficient to induce Stx2B-specific serum IgG (Fig. 4) and IgM (Supplementary Figure 6). Similar levels of Stx2B-specific serum IgG and IgM were detected in mice immunized with Stx2B–C-CPE (Fig. 4; Supplementary Figure 6), suggesting that fusion of Stx2B with C-CPE did not affect the immunogenicity of Stx2B. Comparable levels of Stx2B-specific serum IgG1, IgG2a, IgG2b and IgG3 and no detectable levels of Stx2B-specific serum IgA were detected in mice immunized with Stx2B–C-CPE or Stx2B (Supplementary Figures 6 and 7), which was similar to the immune responses against C-CPE.

We next examined whether Stx2B-specific immune responses induced by immunization with Stx2B–C-CPE could protect against Stx2-mediated pathogenicity. Stx2 causes HUS accompanied with an increased level of serum BUN and acute encephalopathy resulting in death (22). As expected, intra-peritoneal injection with Stx2 resulted in high levels of serum BUN in mock-immunized mice (Fig. 5A). Consistent with high levels of Stx2B-specific antibody production, mice immunized with Stx2B–C-CPE or Stx2B showed significantly lower levels of serum BUN even after Stx2 injection (Fig. 5A). Consistently, all mock-immunized mice died within

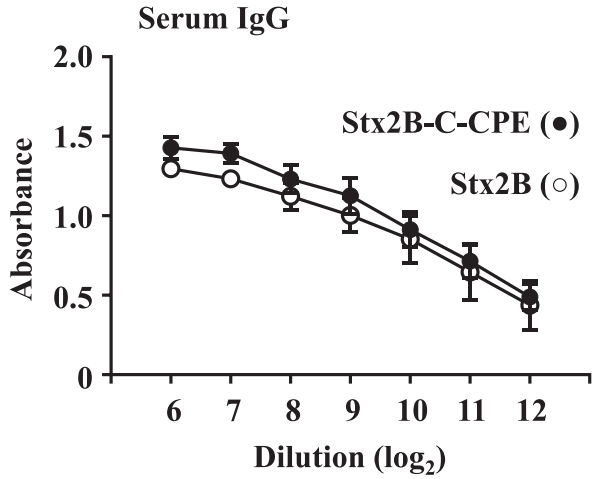

Fig. 4. Induction of Stx2B-specific immune responses by immunization with Stx2B–C-CPE. Mice were subcutaneously immunized with Stx2B–C-CPE or Stx2B once a week for 2 consecutive weeks. One week after the last immunization, serum was collected and Stx2B-specific serum IgG antibody was detected by ELISA. Data are means ± SD (n = 4). The results shown are representative of two independent experiments.

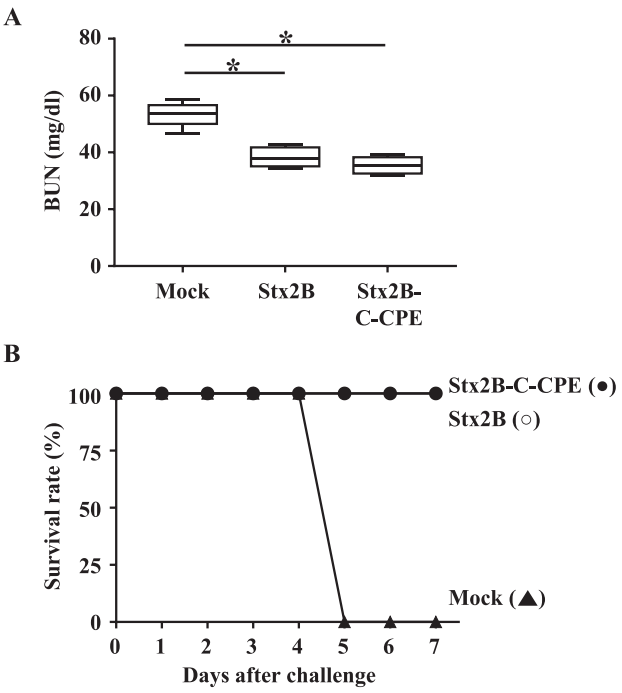

Fig. 5. Induction of protective immune responses against Stx2 by immunization with Stx2B–C-CPE. (A) Serum BUN concentration in mice. Mice were subcutaneously immunized with PBS (Mock), Stx2B or Stx2B–C-CPE once a week for 2 consecutive weeks. Ten days after the last immunization, mice were intra-peritoneally injected with Stx2 (1 ng per mouse). Three days after Stx2 injection, serum was collected and BUN concentration was measured. The normal range of serum BUN concentration is from 8 to 33 mg dl^{-1}. An asterisk means significant difference as calculated by using the Student's t-test (*P < 0.05). 'n.s.' means no significant difference. (B) Survival rate after Stx2 injection. Mice were observed for 7 days after Stx2 injection. The results shown are representative of two independent experiments (n = 4).

5 days of Stx2 injection, whereas all mice survived under the same conditions when they were immunized with Stx2B–C-CPE or Stx2B (Fig. 5B). These results indicate that Stx2B–C-CPE maintained the high immunogenicity of Stx2B, and that this was sufficient to induce protective immune responses against Stx2.

T-cell immune responses induced by Stx2B–C-CPE

To investigate T-cell responses induced by immunization with Stx2–C-CPE, we isolated splenic $CD4^+$ T cells from mice immunized with Stx2B–C-CPE and measured their cytokine production upon stimulation with antigen in the presence of APCs. In agreement with the IgG subclass data (Supplementary Figures 3 and 7), production of IFN-γ (a T_h1-type cytokine) and IL-4 (a T_h2-type cytokine) in culture supernatant was increased by stimulation with Stx2B–C-CPE (Fig. 6). In addition to the T_h1- and T_h2-type cytokines, the stimulated cells also produced IL-17 (Fig. 6), suggesting that Stx2B–C-CPE could induce T_h1, T_h2 and T_h17. We next examined whether the T cells recognized antigen derived from Stx2B, C-CPE, or both. Intriguingly, stimulation with Stx2B produced IFN-γ, IL-4 and IL-17 levels comparable to those produced by stimulation with Stx2B–C-CPE, whereas stimulation with C-CPE did not induce any cytokine (Fig. 6). These results collectively indicate that immunization with Stx2B–C-CPE could induce antigen-specific effector T cells including T_h1, T_h2 and T_h17 cells, which recognized antigen derived from Stx2B.

Long-term antibody production by Stx2B–C-CPE vaccination

To examine whether antibody responses were sustained, we separately monitored antibody responses against C-CPE and Stx2B. Mice immunized with Stx2B–C-CPE showed high levels of C-CPE- and Stx2B-specific IgG in serum for at least 48 weeks after the last immunization (Fig. 7). These results indicate that immunization with Stx2B–C-CPE could induce long-term immune responses against both C-CPE and Stx2B in mice.

Discussion

In this study, we demonstrated the efficacy of Stx2B–C-CPE as a bivalent vaccine against systemic toxicity of CPE and Stx2 produced by *C. perfringens* and STEC, respectively. Both *C. perfringens* and STEC are pathogens causing toxin-type food poisoning; therefore, neutralizing toxin activity is critical to prevent their pathogenicity. A severe symptom of *C. perfringens* is hyperkalemia, which is caused by binding of CPE to CLDN4 in liver and kidney and induces death by cardiac arrest (23–25). STEC, particularly Stx2 producers, are also responsible for large outbreaks, with the binding of Stx2 to Gb3 expressed on vascular endothelial cells of kidney, platelets and red blood cells resulting in severe illnesses (e.g. HUS, acute encephalopathy) and death (4, 9). Here, we demonstrated that Stx2B–C-CPE induces protective and sustained immunity against both CPE and Stx2 in mice. Since there is no murine infectious model currently, we did not check whether Stx2B–C-CPE vaccination effectively prevents

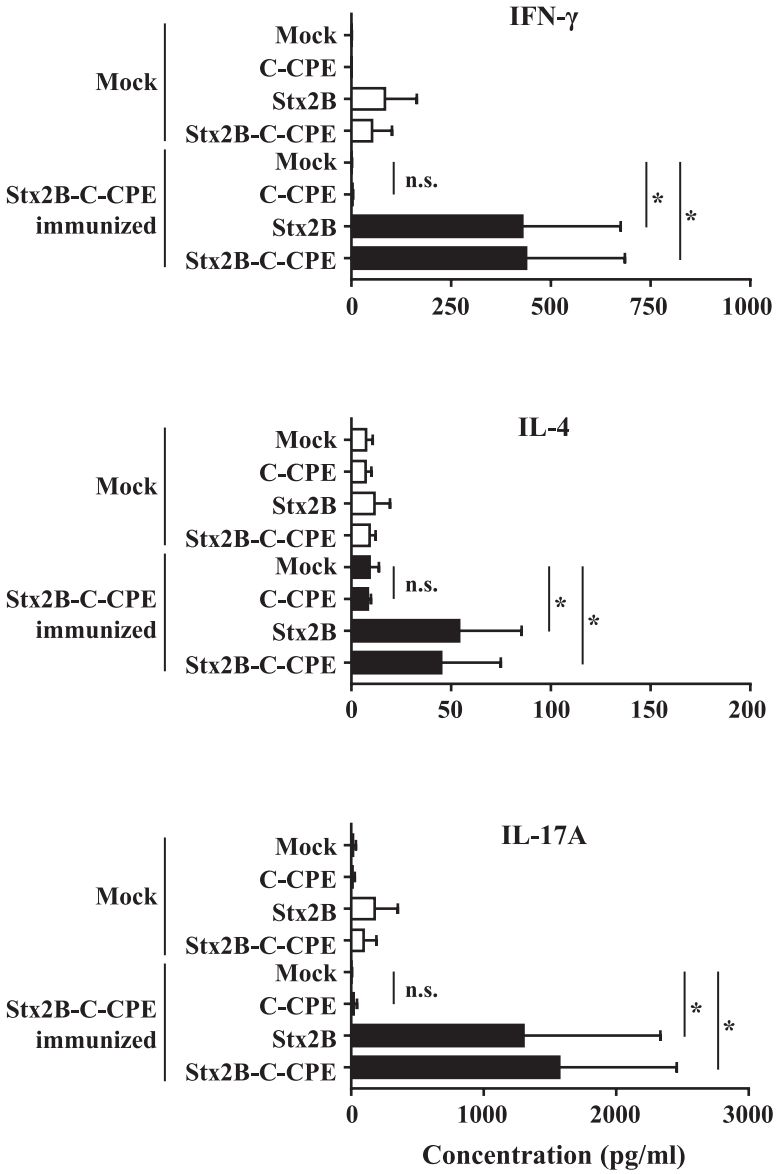

Fig. 6. T-cell activation by immunization with StxB–C-CPE. Splenic CD4⁺ T cells isolated from mice immunized with Stx2B–C-CPE were incubated with APCs in the presence of antigens (C-CPE, Stx2B or Stx2B–C-CPE) or PBS (Mock). Concentrations of cytokines were measured in the culture supernatants by using a BD cytometric bead array mouse $T_h1/T_h2/T_h17$ cytokine kit. An asterisk means significant difference as calculated by using the Student's *t*-test (*$P < 0.05$). 'n.s.' means no statistically significant difference. Data are means ± SD ($n = 4–5$). The results shown are combined from two independent experiments.

systemic intoxication induced by bacterial infection of *C. perfringens* and STEC. Several lines of evidence obtained from clinical and experimental studies showed that the representative systemic symptoms (i.e. hyperkalemia and HUS) are induced by toxins but not by bacteremia (9, 23, 24), suggesting that the neutralizing antibodies induced by Stx2B–C-CPE could suppress the deterioration of systemic pathogenesis. Therefore, we propose that a similar vaccine in humans could potentially decrease the numbers of patients with severe systemic symptoms such as hyperkalemia and HUS.

In addition to these systemic symptoms, both CPE and Stx2 show pathogenicity in the intestine including diarrhea. *Clostridium perfringens* lacking the *cpe* gene does not cause adverse effects in a rabbit ileal loop model and Caco-2 cells derived from human colon (26, 27) and deletion of the *stx2* genes abrogates pathogenicity including cecal fluid accumulation following STEC infection in mice (28), suggesting that CPE and Stx2 play an important role in the development of diarrhea. In the intestine and other organs, CPE and Stx2 bind to CLDN4 and Gb3 expressed on epithelial cells, respectively (24, 29). Therefore, Stx2B–C-CPE could possibly be used as a mucosal vaccine to prevent diarrhea caused by CPE and Stx2. Generally, toxin neutralization in the intestinal lumen is mediated by secretory IgA antibodies, which requires mucosal immunization (30). Thus, application of Stx2B–C-CPE as a mucosal vaccine is a subject of our ongoing study to prevent

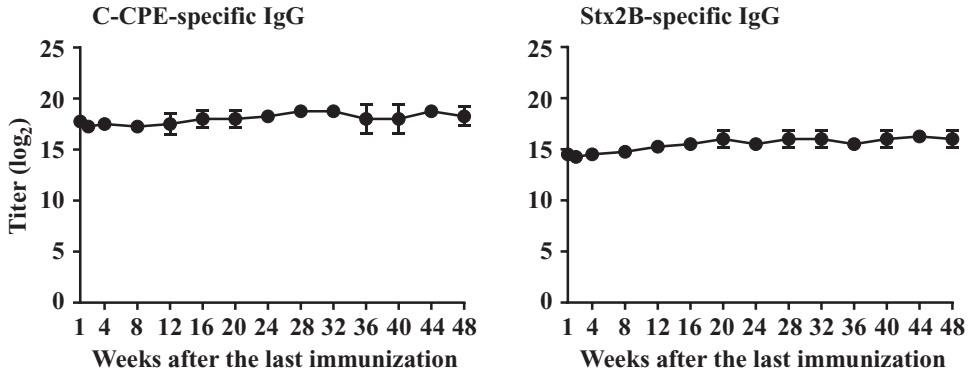

Fig. 7. Long-term antibody production by Stx2B–C-CPE vaccination. Mice were subcutaneously immunized with Stx2B–C-CPE once a week for 2 consecutive weeks. For 48 weeks after the last immunization, serum was collected and C-CPE- and Stx2B-specific serum IgG antibodies were detected by ELISA. Data are means ± SD (n = 4). The results shown are representative of two independent experiments.

the onset of intestinal diseases caused by C. *perfringens* and STEC.

One of the interesting findings in this study is that fusion of C-CPE with Stx2B augmented the antigenicity of C-CPE. The low immunogenicity of C-CPE was predicted in previous studies (31), but the reason was not known. The current study indicates that C-CPE alone could induce specific IgM, but not IgG responses. Generally, B-cell responses, especially immunoglobulin class switching, require cytokines from T_h cells (32). Here, *ex vivo* stimulation with Stx2B–C-CPE or Stx2B, but not C-CPE, induced cytokine production from T cells collected from mice immunized with Stx2B–C-CPE, suggesting that the T cells recognized epitopes derived from Stx2B, but not C-CPE. Therefore, it is plausible that Stx2B-specific T cells help the class switching of C-CPE-specific B cells from IgM to IgG. Since immunoglobulin class switching is generally associated with somatic hypermutation (33), antibody responses induced by Stx2B–C-CPE could show high affinity, which would provide additional neutralizing toxin activity.

IgG antibodies bind antigen and induce complement activation and opsonization through Fcγ receptors (FcγRs) expressed on phagocytes such as macrophages and neutrophils (34). Mouse IgG antibodies are divided into IgG1, IgG2a, IgG2b and IgG3 subclasses (34, 35). Although IgG1 and IgG3 show low activities for FcγR binding and complement activation, IgG2a and IgG2b preferentially bind FcγRs and activate complement through the classical pathway (34). Taking this into account, we consider it likely that Stx2B–C-CPE vaccination can accelerate degradation of Stx2 and CPE through FcγRs and complement-activating phagocytosis of IgG–toxin complex in addition to the direct neutralization of toxin by antigen binding.

T_h1 cytokines such as IFN-γ promote a switch towards IgG2a, whereas T_h2 cytokines such as IL-4 induce isotype switching to IgG1, IgG2b and IgG3 (35–37). Here, Stx2B–C-CPE induced production of IFN-γ and IL-4 from T cells, indicating induction of T_h1 and T_h2 responses, respectively. Since BALB/c mice are genetically regarded as a T_h2 dominant strain, many existing vaccines could induce the T_h2 response but be weak stimulators of the T_h1 response (38, 39). In addition to the genetic background, the environmental cytokine conditions also influence differentiation of naive T cells to T_h1 or T_h2 cells, which is mostly under the control of APCs such as dendritic cells (DCs) (38, 40). In this respect, induction of T_h1 polarization by Stx2B appears dependent on Gb3-expressing DCs (41, 42). Immunization with Stx2B coupled with the model antigen ovalbumin (Stx2B–OVA) elicits antigen-specific cytotoxic T lymphocytes, humoral immune responses and T_h1 polarization (41). IL-2 production from DCs stimulated with Stx2B–OVA is suppressed by inhibition of Gb3 synthesis (41). Although the mechanisms are not well understood, our results suggest that Stx2B mediates T_h1 polarization following Stx2B–C-CPE vaccination.

In the current study, Stx2B was cloned from STEC serotype O157 strain Sakai (Stx2a) because this is the most common serotype to cause outbreaks and HUS in the USA, Europe and Japan (3, 4). Stx2 belongs to the Stx family, which includes various subtypes such as Stx1a, Stx1c, Stx1d, Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f and Stx2g (43). Of note, the Gb3-binding sites are conserved in all members of the Stx family (44, 45). Indeed, it was previously reported that antibody induced by the B subunit of Stx2a showed neutralizing capacity against not only Stx2a but also other variants (Stx2c and Stx2d) and Stx1 (45). Therefore, it is possible that Stx2B-specific IgG neutralizing antibody induced by immunization with Stx2B–C-CPE could exert cross-reactivity against the other members of the Stx family.

The goal in producing a clinically successful vaccine is to induce long-term protective immunity (46). The maintenance of long-term protective immunity is provided by both immunological memory and long-term production of antibody. The maintenance of long-term production of antibodies is provided by long-lived antibody-secreting cells derived from B cells in a T-cell-dependent manner. Here, long-term Stx2B- and C-CPE-specific antibody production appeared to be induced through activation of $CD4^+$ T cells by Stx2B–C-CPE vaccination.

Another important factor in the production of a clinically successful vaccine is cost (47). Currently, approved multiple vaccines are formed by mixing different antigens after the separate production of each antigen. Thus, these vaccines require several production lines and regulations. In contrast, Stx2B–C-CPE is a single protein that acts as a bivalent

vaccine and therefore the cost to produce Stx2B–C-CPE is the same as a single vaccine.

In summary, the current study provides a promising strategy to develop a novel bivalent vaccine to protect against food poisonings caused by CPE-producing *C. perfringens* and STEC infections. The underlying mechanisms include the augmented antigenicity of C-CPE by fusion with Stx2B, which has high antigenicity to T cells.

Funding

This work was supported by the Research Fellowship for Young Scientists (K.H. is Research Fellow of Japan Society for the Promotion of Science); the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Japan Society for the Promotion of Science under grant numbers JP16H01373 (J.K.), JP15H05790 (J.K.), JP17K08301 (H.S.), JP17H04134 (J.K.), JP26670241 (J.K.), JP26293111 (J.K.), JP18K17997 (K.H.), JP18J00556 (K.H.) and JP18K19400 (M.K.); the Japan Agency for Medical Research and Development (AMED) under grant numbers 17fk0108223h0002 (J.K. and S.Y.), 17ek0410032s0102 (J.K.), 17fk0108207h0002 (J.K.), 17ek0210078h0002 (J.K.), 17ak0101068h0001 (J.K.) and 17gm1010006s0101 (J.K.); the Ministry of Health, Labour and Welfare of Japan (J.K.); the Science and Technology Research Promotion Program for Agriculture, Forestry, Fisheries and Food Industry (J.K.); the Terumo Foundation for Life Sciences and Arts (J.K.); the ONO Medical Research Foundation (J.K.); and the Canon Foundation, the BIKEN Foundation (J.K.). None of these funding sources had a role in study design; in the collection, analysis and interpretation of data; or in the writing of the report.

Acknowledgements

We thank Dr S. Tsukita (Kyoto University) for providing CLDN4-expressing L cells. We also thank BIKEN group and the laboratory members for their helpful discussions. K.H. designed the study, performed experiments, analyzed data and wrote the paper. A.H., H.S., T. Nishino and Y.T. performed experiments and analyzed data. J.K. designed the study and wrote the paper. T. Nagatake, A.M., S.-i.H., M.K. and S.Y. provided helpful discussion. All authors approved the final version of the manuscript.

Conflicts of interest statement: The authors declared no conflicts of interest.

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