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Membrane vesicles of *Clostridium perfringens* Type A strains induce innate and adaptive immunity

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Abstract

Vesicle shedding from bacteria is a universal process in most Gram-negative bacteria and a few Gram-positive bacteria. In this report, we isolate extracellular membrane vesicles (MVs) from the supernatants of Gram-positive pathogen *Clostridium perfringens* (*C. perfringens*). We demonstrated vesicle production in a variety of virulent and nonvirulent type A strains. MVs did not contain alpha-toxin and NetB toxin demonstrated by negative reaction to specific antibody and absence of specific proteins identified by LC-MS/MS. *C. perfringens* MVs contained DNA components such as 16S ribosomal RNA gene (16S rRNA), alpha-toxin gene (*plc*) and the perfringolysin O gene (*pfoA*) demonstrated by PCR. We also identified a total of 431 proteins in vesicles by 1-D gel separation and LC-MS/MS analysis. In vitro studies demonstrated that vesicles could be internalized into murine macrophage RAW264.7 cells without direct cytotoxicity effects, causing release of inflammation cytokines including granulocyte colony stimulating factor (G-CSF), tumor necrosis factor-alpha (TNF- α) and interleukin-1 (IL-1), which could also be detected in mice injected with MVs through intraperitoneal (i.p.) route. Mice immunized with *C. perfringens* MVs produced high titer IgG, especially IgG1, antibodies against *C. perfringens* membrane proteins. However, this kind of antibody could not provide protection in mice following challenge, though it could slightly postpone the time of death. Our results indicate that release of MVs from *C. perfringens* could provide a previously unknown mechanism to induce release of inflammatory cytokines, especially TNF- α , these findings may contribute to a better understanding of the pathogenesis of *C. perfringens* infection.

Keywords

C. perfringens; membrane vesicles; immunity

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Introduction

Outer membrane vesicles (OMVs) are spherical bilayer structures with average diameter of 20–500 nm which are usually released from Gram-negative bacteria during cell growth. Though the detailed molecular mechanisms of OMV biogenesis is still not clear, it is probably facilitated by 2-heptyl-3-hydroxy-4-quinolone (PQS)-mediated outer leaflet expansion (Schertzer and Whiteley, 2012). OMVs have been found during the growth of various Gram-negative bacteria such as *Escherichia coli* (Wai et al., 1995), *Helicobacter pylori* (Fiocca et al., 1999), *Actinobacillus actinomycetemcomitans* (Kato et al., 2002), *Neisseria meningitidis* (Ferreiros et al., 1991), *Pseudoalteromonas antarctica* (Nevot et al., 2006), *Pseudomonas aeruginosa* (Bomberger et al., 2009), *Shigella flexneri* (Kadurugamuwa and Beveridge, 1998) and *Vibrio cholera* (Chatterjee and Das, 1967; Kondo et al., 1993). Vesicles containing lipopolysaccharide (LPS), lipoproteins, as well as proteins from outer membrane, periplasm, and cytoplasm, have been included in long-distance delivery of multiple bacterial virulence factors (Bomberger et al., 2009), promoting bacterial internalization by human monocytes and modulate their innate immune response (Pollak et al., 2012), mediating short-term protection against outer membrane acting stressors (Manning and Kuehn, 2011) as well as horizontal gene transfer (Velimirov and Hagemann, 2011). OMVs have also been demonstrated to be immunogenic and protective against related pathogens challenge in some studies (Avila-Calderon et al., 2012; Marzoa et al., 2012; McConnell et al., 2011; Muralinath et al., 2011; Park et al., 2011; Roier et al., 2012; Schild et al., 2009).

Different from Gram-negative bacteria, Gram-positive bacteria only contain single, cytoplasmic membrane inside the cell wall, but an outer membrane is absent (Silhavy et al., 2010). However, it has been reported that a few Gram-positive bacteria including *Bacillus cereus*, *Bacillus subtilis*, *Streptococcus sanguis* (Dorward and Garon, 1990), *Staphylococcus aureus* (Dorward and Garon, 1990; Gurung et al., 2011), *Bacillus anthracis* (Rivera et al., 2010) and *Mycobacterium ulcerans* (Prados-Rosales et al., 2011) also produce membrane vesicles (MVs) similar as Gram-negative bacteria. Isolated MVs containing relative toxins important for infection may deliver active toxins to host cell (Rivera et al., 2010) and cause host cell death (Thay et al., 2013). Importantly, MVs containing toxins are immunogenic in BALB/c mice and induce a robust IgM response to toxin components, thus are protective against *B. anthracis* challenge (Rivera et al., 2010).

C. perfringens is a Gram-positive anaerobic spore forming bacterium, able to produce approximately 17 different kinds of protein toxins and other enzymes responsible for associated lesions and symptoms (Cooper and Songer, 2009), including gas gangrene (Bryant et al., 2000), necrotizing enteritis (Miclard et al., 2009) and bacteremia (Atia et al., 2012). *C. perfringens* is also the third most common cause of foodborne illness, mainly through the secreted *Clostridium perfringens* enterotoxin (CPE) (Lahti et al., 2008). *C. perfringens* strains are classified into five toxin types (A, B, C, D and E), based on the production of four major toxins (alpha, beta, epsilon, and iota) (Petit et al., 1999). *C. perfringens* type A is consistently recovered both from the intestinal tracts of animals and from the environment, while others (types B, C, D and E) are less common in the intestinal tracts of animals (Songer, 1996).

C. perfringens type A is known to cause necrotic enteritis in chicken and responsible for two types of infectious diseases in humans including gas gangrene (clostridial myonecrosis) and food poisoning due to the ability of a few strains (~5% of all *C. perfringens* type A isolates) to produce CPE (Ohtani et al., 2013). Among the toxins secreted in type A strains, alpha-toxin has been shown to be a key virulence factor, due to its ability to insert into the plasma membrane of cells, producing gaps in the membrane that disrupt normal cellular function (Sakurai et al., 2004). Another pathogenic critical toxin named NetB was firstly discovered in an Australian *C. perfringens* type A strain (Keyburn et al., 2008). This plasmid encoded, pore-forming toxin (Keyburn et al., 2010a), have been proved to be a critical virulence factor in the pathogenesis of NE in chickens besides alpha toxin (Keyburn et al., 2010b).

The purpose of this study is to investigate the possibility of *C. perfringens* to release MVs in vitro condition and identify the potential pathogenic effects for the bacteria. We report that MVs are produced and released by *C. perfringens* type A strains, triggering innate and adaptive immune responses.

Materials and methods

Bacterial strains and culture conditions

Strains used in these experiments are listed in Table 1. *C. perfringens* strains were routinely cultured at 37°C in trypticase-peptone-glucose (TPG) broth consisting of 5% (w/v) Bacto-Tryptone (Difco Laboratories, Detroit), 0.5% (w/v) proteose peptone (Difco), 0.4% (w/v) glucose and 0.1% (w/v) sodium thioglycolate (Leslie et al., 1989). All experiments were performed in anaerobic conditions provided by BD GasPak EZ Anaerobe Container System.

Vesicle isolation and purification

MVs were isolated as described before with minor modifications (Gurung et al., 2011; Lee et al., 2009; Rivera et al., 2010). Briefly, *C. perfringens* strains were inoculated into 1.5 L of TPG broth and grown until the optical density at 600 nm (OD₆₀₀) reached 1.0 at 37°C anaerobically. Cells were removed by centrifugation at $16,000 \times g$ for 20 min and the supernatant was decanted and passed through a 0.45 µm pore size polyvinylidene difluoride filter (Millipore). Vesicles were pelleted by centrifugation ($40,000 \times g$, 2 h, 4°C) and washed twice with Dulbecco's Phosphate-Buffered Saline (DPBS, Mediatech). The crude membrane vesicles were then suspended in 1 ml 40% OptiPrep (Sigma) in 10 mM HEPES/0.85% NaCl, pH 7.4 and placed at the bottom of a centrifuge tube. Different concentrations of OptiPrep were prepared in 10 mM HEPES/0.85% NaCl solution and layered on top of the 40% solution as follows: 1 ml 30%, 1 ml 20% and 1 ml 10% OptiPrep. MVs were separated by ultracentrifugation at $110,000 \times g$ for 16 h at 4°C. Equal fractions were removed sequentially from the top and analyzed by electron microscopy to identify the presence of MVs. All fractions containing MVs were pooled together and centrifuged ($110,000 \times g$, 2 h, 4°C) and pellets were resuspended in DPBS (Mediatech) and concentration was measured by BCA Protein Assay Kit (Thermo Scientific).

Electron microscopy

Transmission electron microscope (TEM) was used to analyze the morphology and integrity of extracellular vesicles isolated from culture supernatants. Vesicles were placed on carbon coated 400 mesh grids for 1 min, and visualized by negative staining with 0.5% uranyl acetate for 1 min. A Philips CM12 microscope operating at 80 kV was used to analyze the morphology of vesicles. Digital images were recorded with a Gatan model 791 side-mount CCD camera.

DNA analysis in MVs

DNA associated with CP4 MVs was released by GES reagent (5 M guanidinium thiocyanate, 100 mM EDTA, 0.5 % (v/v) Sarkosyl) according to the methods described by Pitcher et al. (Pitcher, 1989) and quantified by spectrophotometry using NanoDrop technology. Briefly, MVs were pre-treated with DNase I (Sigma) at 37 °C for 1 h to digest any DNA possibly bound to the outer surface of MVs, then the MVs were washed with phosphate buffered saline (PBS) and centrifuged again at $110,000 \times g$ for 2 h at 4°C. After that, the MV pellets were resuspended in 500 µl of GES reagent and transferred to a 1.5-ml microcentrifuge tube, standing at room temperature for 5 minutes, then the lysates were cooled on ice and 0.25 ml of cold 7.5 M ammonium acetate was added. After 10 min incubation on ice, 0.5 ml of phenol:chloroform:isoamyl alcohol (25:24:1) was added, mixed, and centrifuged for 15 min ($16,000 \times g$). The supernatant (500 µl) was transferred to a microcentrifuge tube, and nucleic acids were precipitated overnight with 50 µl of 3 M sodium acetate and 1 ml of ice-cold ethanol (96%). After centrifugation, the DNA pellet was washed with 70% ethanol, dried, and resuspended in 50 µl water. The DNA obtained was used for PCR using primers targeting to chromosome encoded 16S rRNA, *plc* and *pfoA* genes as well as the plasmid encoded *netB* gene (Table 2), while CP4 whole cell DNA was used as a positive control.

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) of ingel digested proteins from MVs

Two independent purified CP4 MVs in PBS were mixed together and assayed for protein using a micro BCA assay according to the manufacturer's instructions (Thermo Fisher Scientific, Rockville, IL). Twenty two µg of total protein were subsequently electrophoresed by SDS-PAGE using a 4–15% Criterion gel from BioRad (BioRad, Hercules CA) and stained with BioSafe Coomassie Blue (BioRad, Hercules CA). An entire gel lane of electrophoresed proteins was digested with trypsin following reduction with DTT and alkylation with iodoacetamide, followed by peptide extraction from the gel lane (Shevchenko et al., 1996). LC-MS/MS analysis was carried out using a LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an Advion nanomate ESI source (Advion, Ithaca, NY), following ZipTip C18 sample clean up according to the manufacturer's instructions (Millipore, Billerica, MA). Cleaned-up peptides from above (10% injected, by volume) were eluted from a C18 precolumn (100-µm id $\times$ 2 cm, Thermo Fisher Scientific) onto an analytical column (75-µm ID $\times$ 10 cm, C18, Thermo Fisher Scientific) using a 5% hold of solvent B (acetonitrile, 0.1% formic acid) for 5 min, followed by a 5–7% gradient of solvent B over 5 min, 7–15% gradient of solvent B over 45

min, 15–35% gradient of solvent B over 60 min, 35–40% gradient of solvent B over 28 min, 40–85% gradient of solvent B over 5 min, 85% hold of solvent B for 10 min and finally a return to 5% in 1 minute and another 10 minute hold of 5% solvent B. All flow rates were 400 nl/min. Solvent A consisted of water and 0.1% formic acid. Data dependent scanning was performed by the Xcalibur v 2.1.0 software (Andon et al., 2002) using a survey mass scan at 60,000 resolution in the Orbitrap analyzer scanning mass/charge (m/z) 400–1600, followed by collision-induced dissociation (CID) tandem mass spectrometry (MS/MS) of the 14 most intense ions in the linear ion trap analyzer. Precursor ions were selected by the monoisotopic precursor selection (MIPS) setting with selection or rejection of ions held to a ± 10 ppm window. Dynamic exclusion was set to place any selected m/z on an exclusion list for 45 s after a single MS/MS. Tandem mass spectra were searched against *C.*

perfringens proteins downloaded from Uniprot (<http://www.uniprot.org/uniprot/?query=taxonomy:1502>) and appended with common contaminant proteins (e.g., human keratins, trypsin, etc). This search was performed using Thermo Proteome Discoverer 1.3 (Thermo Fisher Scientific, Rockville, IL) considering fully tryptic peptides with up to two missed cleavages. Iodoacetamide derivatives of cysteines and oxidation of methionines were specified as variable modifications. Proteins were identified at 99% confidence with XCorr score cut-offs (Qian et al., 2005) as determined by a reversed database search. The protein and peptide identification results were also visualized with Scaffold v 4.2.0 (Proteome Software Inc., Portland OR) and which uses Bayesian statistics to reliably identify more spectra (Keller et al., 2002). Proteins were accepted that passed a minimum of two peptides identified at 0.1% FDR and a 99.9% protein confidence by the Protein Profit algorithm, within Scaffold.

Uptake of vesicles by macrophages

CP4 MVs were labeled with the 1% (vol/vol) lipophilic fluorophore dialkylcarbocyanine iodide (DiI) (Invitrogen) as described (Oliveira et al., 2010; Parker et al., 2010). Briefly, vesicle suspensions were incubated with 1% (vol/vol) DiI for 30 min at 37°C. The vesicle suspensions were ultracentrifuged at $110,000 \times g$ for 2 h and washed three times with DPBS. Labeled MVs were resuspended in PBS and the protein concentration was determined using BCA Protein Assay Kit (Thermo Scientific) to prepare 3 mg/ml solutions and stored at 4°C. Negative controls included “fake vesicle preparation” made from culture medium incubated with DiI alone and washed three times with DPBS in the same condition (Oliveira et al., 2010).

Murine macrophage RAW264.7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Thermo scientific) supplemented with 10% (vol/vol) fetal bovine serum (FBS) at 37°C with 5% CO₂. Cells were plated onto the wells of a 24-well plate (5×10^5 cells per well) covered with glass coverslips (Biokeystone) and cultured for 24 h. After three washes with PBS, DiI-labeled MVs were inoculated into culture medium at final concentration of 3 $\mu\text{g/ml}$. Cells exposed to the negative control mentioned above were also included. After culturing for 2 h, both cells were washed three times with PBS and then fixed with 4% formaldehyde in PBS for 10 min and blocked with sea block buffer (Fisher) for 1 h at room temperature. Plasma membranes and nuclei were labeled with cholera toxin subunit B (CtxB) (1 $\mu\text{g/ml}$) and 4',6-diamidino-2-phenylindole (DAPI) as described

previously (Oliveira et al., 2010; Vyas et al., 2001). Cells were then visualized with a Leica SP5 confocal microscope (Germany) with a 100 × oil immersion optic.

To further examine the internalization phenomenon, indirect immunofluorescence was employed as an alternative method. After similar fixation and blocking procedure as described above, RAW264.7 cells were incubated for 1 h at room temperature with rabbit anti-CP4 whole cell serum. Cells incubated with regular rabbit serum were used as a negative control. Cells were washed three times with PBS and incubated with goat anti-rabbit IgG FITC-labeled antibody (Santa Cruz) for another 1 h at room temperature. Samples were then visualized with a Leica SP5 confocal microscope (Germany) with a 100 × oil immersion optic. Co-localization was analyzed by Image J software (Wayne Rasband National Institutes of Health, Bethesda, MD, USA) and the results were shown as Manders' coefficients (E.M.M.Manders, 1993).

Cytotoxicity assay

To determine whether the CP4 MVs could induce cytotoxic effects on macrophage cells, RAW264.7 cells in 24-well plates (5×10^5 cells/well) were exposed to several different concentrations of MVs ranging from 2.5 µg/ml to 80 µg/ml. The supernatants from each group were collected after 24 h treatment and detected by Lactate dehydrogenase (LDH) cytotoxicity kit (Biovision) according to the instructions. Briefly, the collected culture supernatants from each well were mixed with LDH reaction mix and incubated for 30 min at room temperature and measured by 450 nm. Supernatants from cells in the absence of MVs or treated with cell lysis solution (Biovision) were used as negative and positive control separately. All experiments were performed in triplicate sets.

Mouse experiments

All mouse studies had been approved by the Arizona State University Animal Care and Use Committee. Female 6 to 7-week-old BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA) and acclimated for 7 days before starting the experiments. Immunization was carried out with 3 doses (days 0, 14, and 28) of 10 µg of CP4 MVs in 100 µl buffered saline with gelatin (BSG) (Curtiss, R., 1965) via the i.p. route. Nonvaccinated control mice (100 µl BSG) were housed in parallel with vaccinated mice for the duration of the experiment. A total of three independent rounds of immunizations with five animals per group were conducted. Serum and vaginal wash samples were collected on days 10, 20 and 35 for antibody determination and cytokine analysis. Two weeks after the last immunization, mice were challenged with *C. perfringens* CP4 strain (1.7×10^8 CFU/mice) by i.p. route. Deaths were recorded intermittently until the end of study.

In vitro and vivo inflammation cytokines induced by MVs

To determine whether CP4 MVs could induce inflammation reactions in vitro, RAW264.7 cells were seeded in 24-well cell culture plates to 80% confluence. After washing once with DPBS, cells were exposed to 1 µg/ml, 5 µg/ml or 10 µg/ml MVs in fresh medium for 24 h at 37°C. Supernatants were collected then and the inflammation cytokines of both experimental and control (medium alone) were determined by Bio-Plex Multiplex Cytokine

Assay (Bio-Rad). The serum samples from mice were used to determine the cytokine production in vivo.

***C. perfringens* membrane protein extraction**

The membrane proteins of *C. perfringens* CP4 were isolated according to the methods described previously (Mujahid et al., 2007). CP4 was incubated in 500 ml TPG broth at 37°C overnight and the bacteria were collected by centrifugation at $16,000 \times g$ for 10 min and washed twice with PBS. Then cell pellets were resuspended in 40 ml of osmotic digestion buffer containing 20% sucrose in 20 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, protease inhibitor cocktail (Sigma) and 10 U of mutanolysin (Sigma). The enzymatic digestion was incubated for 2 h at 37°C with shaking. The majority of intact protoplasts were removed by centrifugation at $3000 \times g$ for 1 h followed by centrifugation at $16,000 \times g$ for 30 min to remove cell debris and any remaining protoplasts. The supernatant containing membrane proteins was concentrated by Amicon Ultra-15 Centrifugal Filter Units (10,000 NMWL, Millipore) and the protein concentrations were measured using BCA Protein Assay Kit (Thermo Scientific) and stocked at $-20^\circ C$ until use.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot

CP4 whole cells or MVs were sonicated on ice for 2 min and the protein concentrations were measured using BCA Protein Assay Kit (Thermo Scientific). One ml culture supernatants after harvesting of MVs were concentrated by 10% TCA and resuspended in 100 μ l SDS-PAGE loading buffer (100 mM Tris-HCl, pH6.8; 4% SDS; 0.2% Bromophenol Blue; 20% Glycerol; 200 mM β -Mercaptoethanol). Then 20 μ g whole-cell lysates, MVs samples as well as 10 μ l resuspended culture supernatant samples were separated by 12% SDS-PAGE gels. Coomassie blue staining was performed using PageBlue Protein Staining Solution (Thermo Scientific) according to the instructions. Proteins were electroblotted onto nitrocellulose membranes (Amersham International), and the membranes were probed with rabbit anti-PlcC (Zekarias et al., 2008), anti-NetB and anti-CP whole cell polyclonal antiserum (lab stock) as primary antibodies, separately, followed by a secondary anti-rabbit antibody conjugated with alkaline phosphatase (AP) and developed by 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT solution, VWR). To make anti-NetB antibody, the *netB* gene was cloned into pGEX-4T-2 (GE, Life Science) and the NetB protein was purified as a GST fusion. Rabbit serum was collected after two times injection of purified proteins with Freund's adjuvant on day 0 and day 21, respectively. The anti-CP whole cell serum was collected from a rabbit immunized with formalin inactivated *C. perfringens* cells, which were prepared as described before (Zekarias et al., 2008).

The extracted membrane proteins of CP4 as mentioned above were also separated by 12% SDS-PAGE gels and probed with pooled serum from MVs immunized or mock-vaccinated mice. Then a secondary AP-conjugated anti-mouse antibody and BCIP/NBT solution (VWR) were used to develop the results.

Indirect immunofluorescence assay (IFA)

For IFA experiments, CP4 bacteria were collected from 1 ml overnight TPG culture by centrifugation at $16,000 \times g$ for 10 min and washed twice with PBS. The pellets were

resuspended with 1 ml PBS and incubated with pooled sera from MV immunized mice at the dilution of 1:400, whereas sera from BSG control mice were used as the negative control. After incubation at room temperature for 1 h, the bacteria were washed twice with PBS and then incubated with PBS containing goat anti-mouse IgG-FITC (Southern Biotech) at the dilution of 1:1000 at room temperature for 1 h. After additional twice PBS washes, the pellets were resuspended using 100 μ l PBS and 10 μ l samples were used for microscope analysis using a Leica SP5 confocal microscope (Germany) with a 100 $\times$ oil immersion optic.

Enzyme-linked immunosorbent assay (ELISA)

To measure IgG, IgG1 and IgG2a responses against CP4 in mouse sera and IgA in vaginal washes, purified CP4 membrane proteins resuspended in sodium carbonate-bicarbonate buffer (pH 9.6) were used to coat polystyrene 96-well flat-bottom microtiter plates (Dynatech Laboratories Inc., Chantilly, VA) with 100 ng/well. After incubation at 4°C overnight, the plates were blocked with sea block blocking buffer (Fisher). The serum and vaginal washes were serially diluted and 100 μ l of diluted sample was added to duplicate wells and incubated for 1 h at 37°C. After three times PBS-0.05% Tween-20 washing, the plates were incubated with biotinylated goat anti-mouse IgG, IgG1, IgG2a, or IgA (Southern Biotechnology Inc., Birmingham, AL) antibodies diluted 1:10,000 for 1 h at 37°C. Wells were developed with streptavidin horseradish peroxidase conjugate (Invitrogen, Carlsbad, CA) at the ratio of 1:6,000, followed by 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma). Color development (absorbance) was recorded at 405 nm using a SpectraMax M2 Multi-Mode Microplate Reader (Molecular Devices, LLC). Titers were recorded as the last dilution that resulted in 0.1 greater than background and expressed as the reciprocal log₂ values.

Statistical analysis

All statistics were carried out using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA). Cytokine and antibody titers were expressed as means $\pm$ standard errors and evaluated with *t*-test. Differences were considered significant at *P* < 0.05.

Results

Purification of *C. perfringens* membrane vesicles and detection by TEM

Six *C. perfringens* type A strains from different sources and backgrounds including CP273, CP404, CP888, CP971, CP4 and JGS4143 were used to isolate membrane vesicles (Table 1). TEM analysis revealed that all six selected strains could produce vesicles ranging from approximately 20 nm to 400 nm diameter (Fig. 1). Then we selected CP4 strain for further analysis, the whole-cell lysates, concentrated culture supernatants and MVs samples were subjected to SDS-PAGE (Fig. 2A) and western blot analysis using anti-CP4 whole cell serum (Fig. 2B), anti-PlcC antibody (Fig. 2C) and anti-NetB antibody (Fig. 2D) as primary antibodies. We were interested to see whether *C. perfringens* MVs had cross reactivity to *C. perfringens* whole cells since Gram-negative bacterial OMVs have been shown to be immunogenic and protective against the pathogen from which they were derived (Bishop et al., 2012; McConnell et al., 2011). Western blot assay using the anti-CP4 whole cell serum

demonstrated that CP4 MVs showed strong cross reactivity (Fig. 2B), which indicated that the MVs possibly contain a variety of *C. perfringens* surface exposed proteins. Alpha-toxin, which is the most important toxin for *C. perfringens* type A strains, as well as a newly identified important toxin NetB, were measured by western blot analysis. The results indicated that alpha-toxin and NetB toxin were secreted into the supernatant during in vitro culture conditions, but with no detectable levels in CP4 MVs in our analyses (Fig. 2C and D).

Presence of DNA in membrane vesicles

It has been demonstrated that OMVs from some Gram-negative bacteria contain DNA components, including not only plasmid DNA but also chromosomal DNA (Klieve et al., 2005; Kolling and Matthews, 1999; Renelli et al., 2004). In order to determine whether *C. perfringens* MVs also contained DNA components, we performed a DNA extraction from CP4 MVs and amplified the 16S rRNA, *plc*, *pfoA* and *netB* genes. Our analysis included MVs that were pretreated with DNase I to eliminate any extra-vesicular DNA contamination. The results demonstrated that CP4 MVs with or without DNase I treatment, we were able to obtain PCR products corresponding to the chromosome encoded 16S rRNA, *plc* and *pfoA* genes but not plasmid encoded *netB* gene (Fig. 3). These results indicated that the CP4 MVs contained at least partial CP4 chromosomal DNA even if not the complete chromosome.

Identification and subcellular distribution of CP4 MVs proteins

In order to determine the protein composition of CP4 MVs, one dimensional (1-D) electrophoresis separation followed by LC-MS/MS was performed as described before and a total of 431 proteins were identified listed in Supporting Information (S-Table 1, 2 and 3). All proteins detected were classified according to the cellular component (Fig. 4A and B) based on the databases provided by Scaffold software. In summary, most identified proteins were classified as membrane (24%) or cytoplasm (18%) category. Intracellular organelle (9%), ribosome (8%), as well as plasma membrane (10%) proteins were more abundant (Fig. 4A and B). Our results correlate well with other examples of extracellular vesicles containing a variety of proteins, such as membrane proteins, cytoplasmic and intracellular proteins (Ayalew et al., 2013; Lee et al., 2007; Lee et al., 2009; Pierson et al., 2011). In contrast to previous study which indicated that vesicles secreted from another Gram-positive bacteria *Staphylococcus aureus* were especially enriched with extracellular proteins (Lee et al., 2009), our data demonstrated that only a small part of “extracellular region” (0.4%) proteins were identified in CP4 MVs (Fig. 4B). The results also confirmed that *C. perfringens* MVs contain a lot of bacterial surface exposed proteins as shown before (Fig. 2B).

Functional classification of identified proteins

The biological functions of identified proteins in CP4 MVs were categorized and the results indicated that the proteins are involved in various activities, including metabolic processes (47%), establishment of localization (20%), biological regulation (6%), responses to stimulus (2%) and immune system processes (0.2%) (Fig. 4C and D). Consistent with a

previous study (Lee et al., 2009), CP4 MVs contained a lot of ribosomal proteins, DNA polymerases and several metabolic enzymes, suggesting the vesicles might facilitate the transfer of functional enzymes. Since we were more interested in proteins possibly associated with virulence of *C. perfringens*, we noticed that one kind of toxin-Beta2 toxin was identified in the MVs sample (S-Table 3). Beta2 toxin, encoded by a large plasmid of *C. perfringens* (Gibert et al., 1997), has been demonstrated to be present in both type A and type C strains and the Beta2 toxin positive isolates are highly associated with enteric diseases in domestic animals, particularly with enteritis in piglets (Fisher et al., 2005; Harrison et al., 2005; Thiede et al., 2001; Waters et al., 2003). The LC-MS/MS results also confirmed the absence of alpha-toxin and NetB toxin in CP4 MVs shown in western blot previously (Fig. 2C and D).

***C. perfringens* MVs could enter into macrophages without obvious direct cytotoxic effects**

It has been shown that extracellular vesicles could be internalized into different cell lines (Oliveira et al., 2010; Parker et al., 2010; Sharpe et al., 2011). In this study, RAW264.7 cells were incubated with DiI-labeled (red) CP4 MVs and both direct fluorescent or indirect immunofluorescence (IFF) were performed. The microscopy analysis indicated that the MVs (red) could be internalized by macrophage cells (green) (Fig. 5A), while no visible fluorescence was observed in mock infected cells (Fig. 5B). The results of IFF showed at least partial co-localization of CP4 MVs (red) with antigenic vesicular components (green) (Fig. 5C, D, E and F).

Lactate dehydrogenase (LDH) activities were assayed to determine whether the co-incubation of *C. perfringens* MVs with RAW 264.7 cells could result in cytotoxic effects. LDH is a cytoplasmic enzyme retained by viable cells with intact plasma membranes. Membrane damage results in LDH release to culture supernatants. After exposure to the vesicles for 24 h, RAW 264.7 cells did not show obvious cell lysis even after incubation with 80 µg/ml dose of MVs (Fig. 5G). This result indicated that the *C. perfringens* MVs did not cause acute toxicity to RAW 264.7 cells under our experimental conditions.

Cytokines induced by MV stimulation

In the presence of CP4 MVs, RAW264.7 cells secreted a series of inflammatory cytokines such as G-CSF (Fig. 6A), TNF-α (Fig. 6B), and IL-6 (Fig. 6C). Induction of TNF-α was dominant among these cytokines, incubation with 10 µg/ml of CP4 MVs caused release of 28545 ± 2800 (n=2) pg/ml TNF-α ($P<0.01$), 19473 ± 1322 pg/ml G-CSF ($P<0.01$), and 1232 ± 121.6 pg/ml IL-6 ($P<0.01$). The same trend in cytokine production was observed in mice immunized with MVs at the dose of 10 µg/mice, though a much lower concentration compared to macrophage cells. After 35 days post-infection, G-CSF in sera achieved 92.94 ± 3.945 pg/ml ($P<0.01$) (Fig. 6D), whereas 1098 ± 63.41 pg/ml ($P<0.01$) for TNF-α (Fig. 6E), and 60.79 ± 1.28 pg/ml for IL-6 ($P<0.001$) (Fig. 6F), significantly increased compared to serum from mock-vaccinated mice at days 35, respectively.

Serum from MV immunized mice react with *C. perfringens* membrane proteins and bind to the CP4 cell surface

Purified *C. perfringens* membrane proteins were subjected to SDS-PAGE (Fig. 7A) and probed with pooled serum from mice. Consistent with previous results of western blot (Fig. 2B) and LC-MS/MS assays (Fig. 4, S-Table 1), the western blot results clearly demonstrated that the pooled serum from CP4 MV immunized mice reacted to at least six protein bands from membrane proteins (Fig. 7B), whereas the serum from mock-vaccinated mice showed no reaction (Fig. 7C). To further confirm the interaction between collected serum and *C. perfringens* bacteria, immunofluorescence was performed and the results showed that obvious fluorescence was observed when CP4 bacteria were incubated with the serum from mice immunized by CP4 MVs (Fig. 7D), while no visible fluorescence could be detected in mock serum incubation control (Fig. 7E). Taken together, the results indicated that the serum from mice immunized with CP4 MVs could bind to the surface membrane proteins of CP4 bacteria, also confirmed the presence of membrane proteins in MVs.

Immune responses in mice

The immune response to CP4 MVs was studied in BALB/c mice. A significant rise of IgG titers against CP4 membrane proteins was observed in mice at 10, 20 and 35 days in a time dependent manner, while no detectable antibodies were observed in BSG control mice (Fig. 8A). To further understand the nature of the immune response, isotyping was carried out. The results indicated that levels of all antibody isotypes (IgG1, IgG2a) were significantly higher in animals immunized with MVs than in control animals. However, IgG1 titers predominated over IgG2a titers at all time points, suggesting a bias toward a Th2 type immune response (Fig. 8B). Compared to the systemic immune response, the mucosal immune antibody IgA levels were obviously much lower (Fig. 8C), indicating that the i.p. injection of MVs induced mainly systemic immune reactions other than mucosal immunity.

To determine whether the antibodies induced by CP4 MVs could provide protective effects in mice, *C. perfringens* CP4 challenge was conducted. The results showed that 40% of mice died in 20 h post challenge in MV immunized mice compared to 80% death in non-immunized mice. However, the high antibody titers induced by MVs could not protect mice from challenge in our experiments, since all mice died in 28 h though a little bit longer than non-immunized mice which all died in 24 h (Fig. 8D).

Discussion

Extracellular vesicles have been demonstrated to be a universal product during bacterial growth in vitro and in vivo, especially for Gram-negative bacteria. In this study, *C. perfringens*, an important Gram-positive pathogen, produced membrane vesicles during growth under in vitro culture conditions. All six tested type A strains from different sources could release membrane vesicles into supernatants, no matter whether it was a necrotic enteritis outbreak high virulence strain (CP4) (Thompson et al., 2006) or a moderate virulent strain (CP404). The molecular mechanisms of membrane vesicles formation have not been fully understood. A number of models have been proposed in Gram-negative bacteria, including loss of lipoprotein connections between the bacterial outer membrane and the

underlying peptidoglycan layer, accumulation of misfolded proteins in the periplasmic space or a PQS mediated bilayer-couple model (Schertzer and Whiteley, 2012). Compared to Gram-negative bacteria, only a few of Gram-positive bacteria have been demonstrated to secrete membrane vesicles and much less is known about the formation mechanisms.

It has been observed that bacterial toxins components could be associated with either OMVs in Gram-negative bacteria (Chatterjee and Chaudhuri, 2011; Donato et al., 2012; Kim et al., 2010; Rompikuntal et al., 2012) or MVs in Gram-positive bacteria (Rivera et al., 2010; Thay et al., 2013). As an important potential biological weapon, *Bacillus anthracis* produces membrane vesicles containing biologically active toxins such as lethal factor (LF) and edema factor (EF) toxins, and serve as an important vesicular transport system for anthrax toxins and some other virulence factors (Rivera et al., 2010). Western blot analysis using CP4 MV preparations showed that the two important toxins for *C. perfringens* type A isolates (alpha-toxin and NetB) were not included in MVs, which was also confirmed by LC-MS/MS analysis. On the other hand, the presence of Beta2 toxin in CP4 MVs demonstrated by LC-MS/MS assay still need to be confirmed further. The proteins identified in CP4 MVs mainly consisted of a lot of membrane proteins, cytoplasmic proteins, as well as many other subcellular fraction components (S-Table 1, 2 and 3), but only a few of extracellular proteins. Consistent with other bacterial membrane vesicles (Parker et al., 2010; Sharpe et al., 2011), CP4 MVs could be internalized by murine macrophage RAW264.7 cells. Vesicles from other bacteria have been shown to facilitate the transfer of virulent proteins between bacterial cells, and trafficking of cell-cell signals (Mashburn-Warren and Whiteley, 2006), therefore, the complex protein components identified in CP4 MVs therefore could possibly provide an unexplored approach for *C. perfringens* to communicate with host cells.

It has been observed that either chromosome or plasmid DNA could be packaged into OMVs during their formation (Klieve et al., 2005; Renelli et al., 2004; Velimirov and Hagemann, 2011). Once DNA is encapsulated into OMVs/MVs, it would be protected from exonucleases which was demonstrated in our study, since specific PCR products targeting *C. perfringens* chromosome encoded 16S rRNA, *plc* and *pfoA* genes were observed after DNase I treatment. Plasmid encoded *netB* gene was not found in MVs according to PCR results. In contrast to a previous report which demonstrated that Gram-positive bacteria MVs did not package DNA (Dorward and Garon, 1990), we showed that *C. perfringens* MVs contained at least partial nuclease-resistant chromosome DNA inside vesicles. The export of DNAs with nuclease-resistant membrane vesicles have been shown to occur in a variety of Gram-negative bacteria and provide an approach to facilitate horizontal gene transfer between different strains of Gram-negative bacteria and even across species (Dorward and Garon, 1990; Kadurugamuwa and Beveridge, 1995; Kulp and Kuehn, 2010; Renelli et al., 2004). We did not determine further the gene transfer due to CP4 MV. However, based on the fact that MVs could enter into host cells, it could be possible for *C. perfringens* to transfer DNA components, especially toxin genes, through MVs to host cells where the bacteria could not survive.

The strong cross reaction between CP4 MVs and CP4 whole cell immune sera led us to explore whether CP4 MVs could provide a new approach for *C. perfringens* vaccine design.

In fact, mice immunized with CP4 MVs by i.p. injection could induce high antibody titers against *C. perfringens* membrane proteins shown by ELISA. Although these antibodies may bind to the *C. perfringens* bacterial surface and postpone death time slightly in MV immunized mice, the protective effects were disappointing compared to other bacterial membrane vesicle immunity results (Bishop et al., 2010; Bishop et al., 2012; McConnell et al., 2011). The slightly delayed death time in immunized mice, was caused probably by complement activation based on the binding between MV induced antibodies and bacteria, though we did not measure this directly. Toxins secreted from *C. perfringens* especially alpha-toxin and NetB have been considered to be the key factors to cause disease. The MVs of *C. perfringens* were free of toxin components, at least alpha-toxin and NetB, as shown previously (Fig. 2C and D). Therefore no antibodies against these toxins could be induced. That may explain why the MVs could not provide efficient protection against challenge in mice.

Gram-positive bacteria cell walls consist of as many as 40–100 sheets of peptidoglycan, comprising up to 50% of cell wall material (Siqueira and Rocas, 2007). Therefore it is reasonable that MVs secreted from Gram-positive bacteria include peptidoglycan components such as *N*-acetylglucosamine, which is also confirmed by protein identification (S-Table 1 and 3). Peptidoglycan plays an important role in pathogenesis, including the activation of macrophages/monocytes with consequent release of pro-inflammatory cytokines, such as IL-1 β , IL-6, TNF- α , granulocyte-macrophage colony-stimulating factor (GM-CSF), and G-CSF (Boneca, 2005). Peptidoglycan-containing OMVs from Gram-negative bacteria have been demonstrated to upregulate cytokines related to inflammatory responses in vitro (Kaparakis et al., 2010). Our results demonstrated that CP4 MVs could induce G-CSF, TNF- α and IL-6 secretion in vitro and in vivo conditions, which probably were caused by the peptidoglycan content.

C. perfringens alpha-toxin has been shown to be a key virulence factor for the development of gas gangrene with inflammatory myopathies. Injection of alpha-toxin induced the release of large amount of pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α in BALB/c mice (Oda et al., 2008). TNF- α plays an more important role in the lethality and systemic hemolysis compared to IL-1 β and IL-6 in the study due to the fact that the lethal effect of alpha-toxin is closely related to the release of TNF- α into the bloodstream, while as the administration of anti-TNF- α antibody inhibited the death of mice induced by alpha-toxin. In our study, we showed that injection of *C. perfringens* MVs stimulated dramatic production of inflammatory cytokines, especially TNF- α , in mice. Therefore, the release of MVs may contribute indirectly to shock by stimulating production of endogenous mediators such as TNF- α (Bryant and Stevens, 1996; Bunting et al., 1997), which will benefit *C. perfringens* pathogenicity and infection in addition of secreted toxins.

In conclusion, *C. perfringens* type A strains released MVs containing DNA and various protein components, which could be internalized by macrophage cells RAW264.7 and induced dramatic proinflammatory cytokines in vitro and in vivo, especially TNF- α , which possibly provides an unknown mechanism for *C. perfringens* to cause inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Membrane vesicles (MVs) production in *C. perfringens* Type A strains. Vesicles were visualized by transmission electron microscope (TEM). A to F represented strains CP273, CP404, CP888, CP971, CP4 and JGS4143, respectively. Scale bars: 100 nm.

Fig. 2.

Absence of alpha-toxin and NetB toxin in *C. perfringens* CP4 membrane vesicles (MVs) shown by western blot. (A) *C. perfringens* whole-cell lysates (20 μ g), MVs (20 μ g) and concentrated supernatants (10 μ l) were subjected to SDS-PAGE followed by staining with Coomassie blue. (B) Western blot using anti-CP4 whole cells antibodies. (C) Western blot using anti-PlcC antibodies. (D) Western blot using anti-NetB antibodies. Lane 1, protein marker; lane 2, CP4 whole-cell lysates; lane 3, concentrated *C. perfringens* supernatants;

lane 4, CP4 MVs. Lines to the left indicate the molecular masses of the protein standards in kDa.

Fig. 3. PCR amplification to determine the presence of DNA associated with *C. perfringens* CP4 MVs. (A) 16S rRNA (100bp). (B) *plc* (1197bp). (C) *pfoA* (1503bp). (D) *netB* (1350 bp). Lane 1, 1kb DNA ladder; lane 2, CP4 genomic DNA (control); lane 3, MVs without DNase I treatment; lane 4, MVs with DNase I treatment; lane 5, negative control (no template). Lines to the left indicate the molecular masses of the DNA standards in base pair.

Fig. 4. Subcellular distribution and functional classification of identified proteins in *C. perfringens* CP4 MVs revealed by one dimensional (1-D) gel separation and LC-MS/MS. (A) Identified proteins were classified according to their subcellular distribution. The numbers indicated the amount of proteins in each fraction. (B) Percentage of displayed proteins in each subcellular location (%). (C) Identified proteins were classified according to their functional classification. The numbers indicated the amount of proteins in each fraction. (D) Percentage of displayed proteins in each functional division (%).

Fig. 5.

Internalization and cytotoxicity effect of CP4 membrane vesicles. DiI-stained vesicles (red) were incubated with RAW264.7 macrophage cells, followed by staining of nucleus with DAPI (blue). Results were recorded under Leica SP5 confocal microscope (100 ×). (A) In direct fluorescence assay, cell membrane was stained with FITC-labeled cholera toxin subunit B (green), arrows indicated the internalization of MVs in cells. (B) Negative control using “fake vesicles preparation” as mentioned in Materials and Methods. (C, D and E) In indirect immunofluorescence assay, cells were incubated with rabbit anti-CP4 serum

followed by secondary goat anti-rabbit FITC labeled antibody (green). The merged image demonstrates that DiI-stained compartments co-localize, at least partially, with antigenic vesicular components (arrow). (F) Quantitative analysis of co-localization (yellow fluorescence) from three independent experiments using Image J software. (G) Cytotoxicity effects on cells caused by incubation with CP4 MVs. RAW264.7 cells were incubated with CP4 MVs as the dose indicated, LDH activity in supernatants were measured. Cell lysis solution and culture medium alone were used as positive and negative control respectively.

Fig. 6.

Cytokine responses induced by *C. perfringens* CP4 MVs in RAW264.7 cells (A, B and C) and mice (D, E and F). RAW264.7 cells were incubated with CP4 MVs at a dose of 1 μ g/ml, 5 μ g/ml and 10 μ g/ml, cells without MVs incubation were used as negative control. After 24 h incubation, the cell culture supernatant was collected and cytokines were measured using a Bio-Plex kit (Bio-Rad). (A) G-CSF. (B) TNF- α . (C) IL-6. Mice were inoculated with CP4 MVs at 10 μ g/mice on day 1, day 14 and day 28. Blood samples were collected on day 10, day 20 and day 35 from both immunized mice and mock-vaccinated mice and cytokines were measured. (D) G-CSF. (E) TNF- α . (F) IL-6. Data are presented as mean $\pm$ SEM (n = 2). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. control.

Fig. 7.

Specific reaction between serum from MVs immunized mice and CP4 bacteria. (A) SDS-PAGE analysis of CP4 membrane proteins. (B) Western blot analysis of CP4 membrane proteins using serum from MVs immunized mice as 1st antibody. (C) Western blot analysis of CP4 membrane proteins using serum from mock- vaccinated mice as 1st antibody. For all SDS-PAGE and western assay, lane 1, protein marker (kDa); lane2, CP4 membrane protein. (D) Laser scanning confocal microscopy imaging of the interaction of CP4 bacterial surface with antibodies against MVs. Right panel, colocalization of bacteria (visualized by

differential interference contrast (DIC)) with immunofluorescence (FITC) was seen as green dots (arrow). Scale bar: 1 μ m. (E) Negative control using serum from mock-vaccinated mice.

Fig. 8.

ELISA measurements of serum IgG and mucosal IgA responses in immunized and control mice (n=15/group). (A) Total serum IgG specific for *C. perfringens* CP4 strain membrane proteins (MP). No immune responses were detected to MP in control mice (reciprocal titer, 1:100). (B) Ratio of anti-MP IgG1/IgG2a titers. (C) Mucosal anti-MP IgA. No anti-MP IgA was detected in control mice (reciprocal titer, 1:20). The data represent antibody titers (mean ± SEM, n=3) in pooled sera or vaginal washes from mice immunized with CP4 MVs or BSG and boosted 2 wk and 4 wk later. (D) Survival curve after WT *C. perfringens* challenge in BSG control group and MVs immunized group (10 µg/mice).

Strains used in this study

Table 1

Strains ^a	Isolate source	Necrotic enteritis outbreak related	Toxin PCR ^b (Alpha)	Toxin PCR ^b (NetB)	Source	Reference
CP273	Darkling beetle	yes	Pos	Neg	G. Siragusa Damisco USA Inc	Lab stock
CP404	Ground beef	no	Pos	Neg	G. Siragusa Damisco USA Inc	Lab stock
CP888	Broiler ceca	yes	Pos	Pos	G. Siragusa Damisco USA Inc	Lab stock
CP971	Broiler midgut	yes	Pos	Pos	G. Siragusa Damisco USA Inc	Lab stock
CP4	Field isolate	yes	Pos	Pos	J.F. Prescott Guelph, Can	(Thompson et al., 2006)
JGS 4143	Necrotic enteritis isolate	yes	Pos	Pos	G. Songer Iowa state University	(Barbara et al., 2008)

^a all strains used in this study belong to Type A *C. perfringens*

^b Pos, positive; Neg, negative

Table 2

Primers used in this study

Primers	Sequence (5'-3')
16S rRNA-F	CGCATAACGTTGAAAGATGG
16S rRNA-R	CCTTGGTAGGCCGTTACCC
CPA-F	ATGAAAAGAAAGATTTGTAA
CPA-R	TTATTTTATATTATAAGTTG
Pfo-F	ATGATAAGATTTAAGAAAAC
Pfo-R	TTAATTGTAAGTAATACTAG
NetB-F	ATCATTTTACAAGTTAACTT
NetB-R	ATTGTCCAATTTTAATATTAA