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Flagellin-deficient outer membrane vesicles as adjuvant induce cross-protection of *Salmonella* Typhimurium outer membrane proteins against infection by heterologous *Salmonella* serotypes

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ABSTRACT

Salmonella enteric serovar infections result in high morbidity and mortality worldwide. Cross-protective vaccines are an effective strategy in controlling salmonellosis caused by multiple serotypes. In our previous study, outer membrane vesicles (OMVs) derived from flagellin-deficient *Salmonella* Typhimurium (*S.* Typhimurium) were proven effective in mediating cross-protection against infection by multiple *Salmonella* serotypes; OMVs also exhibit potent adjuvant effects. In this study, we further investigated the adjuvant capacities of flagellin-deficient *S.* Typhimurium OMVs. Our finding showed that outer membrane proteins (OMPs) in combination with flagellin-deficient *S.* Typhimurium OMVs could function as adjuvants and invoke stronger humoral, cellular, mucosal, and cross-protective immune responses compared to conventional aluminum (alum). Furthermore, as an adjuvant, OMVs could induce significantly higher cellular immune responses and display enhanced cross-protection for OMPs against wild-type virulent *Salmonella* Choleraesuis and *Salmonella* Enteritidis challenge. In summary, OMVs function as a potent adjuvant with the capability of conferring greater cross-protection against infection by multiple *Salmonella* serotypes, and may be of great value as an effective vaccine adjuvant in enteric diseases.

1. Introduction

Salmonella is a predominant cause of gastroenteritis, which leads to high morbidity and mortality rates worldwide (Feasey et al., 2012; Heithoff et al., 2015). Most *Salmonella* serovars are considered to be non-typhoidal *Salmonella* (NTS) and cause over 93.8 million cases of gastroenteritis worldwide (Eng et al., 2015). Due to the prevalence of multiple drug-resistant *Salmonella* strains, cross-protection vaccines present an attractive option to control *Salmonella*-associated diseases (Mahan et al., 2012). However, vaccines presently in use, such as subunit vaccines, only induce incomplete Th1-cell response (Zhang et al., 2001). Live attenuated vaccines devoid of functional DNA adenine methylase (Dam) showed partial cross-protection, but the risk of virulence restoration was associated with them due to insufficient attenuation of the pathogen in immune-comprised or elderly individuals (Haesebrouck et al., 2004); both these vaccines exhibit partial immunity or limited cross-protection against challenge by heterologous *Salmonella* serotypes (Heithoff et al., 2015). An ideal vaccine should offer high cross-protection and safety, thus, facilitating the control of heterologous *Salmonella* infection.

Outer membrane vesicles (OMVs) are naturally secreted from the surface of most gram-negative bacteria, and possess excellent intrinsic stimulatory ability due to their integral outer membrane and periplasmic contents (Ellis, 2010; Kulp and Kuehn, 2010). Because of the strong immunoreactivity of OMVs, such that they can induce both strong humoral and cellular immune responses (Sanders and Feavers, 2011), an OMV-based vaccine against *Neisseria meningitidis* has been engineered and licensed for use in humans (Acevedo et al., 2014). Our

Abbreviations: NTS, non-typhoidal *Salmonella*; OMVs, outer membrane vesicles; LPS, lipopolysaccharide; OMPs, outer membrane proteins; Alum, aluminum; ELISA, Enzyme-linked immunosorbent assay; BCA, bicinchoninic acid assay; *S.* Typhimurium, *Salmonella* Typhimurium; S. Choleraesuis, *Salmonella* Choleraesuis; *S.* Enteritidis, *Salmonella* Enteritids; OMV_{ST}, OMVs from Typhimuruim; OMV_{SE}, OMVs from Enteritids; OMV_{SC}, OMVs from Choleraesuis; OMP_{ST}, OMPs from Typhimuruim; OMP_{SE}, OMPs from Enteritids; OMP_{SC}, OMPs from Choleraesuis; OMP_{Mix}, OMPs from mixed serotypes of *Salmonella*; nOMVs, native OMVs; sOMVs, spontaneous OMVs

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previous study using natural OMVs obtained from flagellin-deficient *Salmonella* Typhimurium (*S.* Typhimurium) presented a similar result, confirming that OMVs also possess extensive vaccine potential (Liu et al., 2016a). Additionally, the use of OMVs to mount an immune response is a novel strategy for vaccine formulation and delivery of antigens from other pathogens to induce protective immune responses, reflecting a new direction of current possibilities for vaccine development (Gnopo et al., 2017).

In addition to research on immunogenicity of OMVs, there is increasing interest in OMVs functioning as self-adjuvants for immune-stimulating molecules (Aghasadeghi et al., 2011). This function is mediated through interactions between the pathogen-associated molecular patterns (PAMPs) present on OMVs and the Toll-like receptors (TLRs) on the surface of antigen-presenting cells (APCs), thereby enhancing immune responses (Alaniz et al., 2007; Sanders and Feavers, 2011). Adjuvants as immune stimulators are conventionally required in vaccines to enhance the immune responses (Gnopo et al., 2017; Sanders and Feavers, 2011). Although conventional adjuvants currently used to promote and sustain immune responses, they still have limitations in the type of immune response. For example, alum adjuvants mainly elicit Th2-based responses, and CpG oligodinucleotides mainly increase Th1-based responses (Gnopo et al., 2017; Heeg and Zimmermann, 2000; Sanders and Feavers, 2011). Therefore, we would develop a new approach for applying flagellin-deficient OMVs in a tailored vaccine design strategy; this strategy serves as a directed immune regulation adjuvant system to enhance more comprehensive immune responses (Laughlin et al., 2015).

In this study, we purified OMVs from flagellin-deficient *S.* Typhimurium and combined them with outer membrane proteins (OMPs) from different *Salmonella* serotypes. We further performed animal experiments and analyzed the antibody response and cross-protection capacity in mice through screening for the optimal vaccine composition that conferred the most effective immune protection. The flagellin-deficient OMVs not only presented an efficient vaccine candidate, but also served as a novel vaccine adjuvant, which may be formulated with other vaccine components to control infection by heterologous bacterial serotypes.

2. Materials and methods

2.1. Preparation of antigen

All *Salmonella* serotypes were cultured in Luria-Bertani (LB) broth (Difco, Detroit, MI, USA) at 37 °C. The OMVs were isolated from flagellin-deficient *Salmonella* Typhimurium (*S.* Typhimurium) strain K083, which was constructed in our previous study. In this strain, the genes *fliC* and *fljB* were deleted to completely remove flagellin in OMVs (Liu et al., 2016b). The method of purification used was as previously described (Alaniz et al., 2007; Liu et al., 2016b). OMPs, which were used for vaccination and blocking the antigen, were isolated from wild-type *S.* Choleraesuis S340 (OMP_{SC}), wild-type *S.* Enteritidis S246 (OMP_{SE}) and wild-type *S.* Typhimurium S100 (OMP_{ST}), as previously described (Carlone et al., 1986; Liu et al., 2016c). The protein concentrations of the OMVs and OMPs were measured using a the bicinchoninic acid (BCA) assay kit (Thermos Pierce, Rockford, IL, USA). OMV and OMP antigens (~2 mg/ml) were diluted in saline (total volume 10 μl) or formulated with aluminum (2 mg/ml, Alhydrogel, Brenntag Biosector, Borupvang, Denmark) in saline (total volume 10 μl). OMP_{Mix} was mixed with the same amount (2 μg) of OMPs from the three serotypes, as described in Table 1. All experimental protocols were approved by Nanchang University.

2.2. Ethic statements

All experiments involving animals were conducted in compliance with the guidelines of the Animal Welfare Act and related regulations of Nanchang University for animal experiments (Nanchang, China, Approval No. NCDXYD-2017019). All animal work protocols were approved by the animal welfare committee of Nanchang University. The principles stated in the Guide for the Care and Use of Laboratory Animals were followed. All efforts were made to minimize animal suffering during the experiments.

Table 1
Vaccine formulation strategy for immunization using OMPs or OMVs.[a].

Group of 12 mice	Immunogen and Dose (μg/ one mouse)
1	OMP_{SE} (6) + OMV_{ST} (6)
2	OMP_{SE} + Alum (12)
3	OMP_{SE} (12)
4	OMP_{SC} (6) + OMV_{ST} (6)
5	OMP_{SC} + Alum (12)
6	OMP_{SC} (12)
7	OMP_{ST} (6) + OMV_{ST} (6)
8	OMP_{ST} + Alum (12)
9	OMP_{ST} (12)
10	OMV_{ST} (6)
11	OMV_{ST} (12)
12	OMP_{Mix}(6) + OMV_{ST} (6)[b]
13	PBS control

[a] 6 weeks female BABL/c mice as animal model was selected in this study and immunized with intranasal routes.
[b] 6 μg OMP_{Mix} contained 2 μg OMP_{SE}, 2 μg OMP_{SC} and 2 μg OMP_{ST}.

2.3. Immunization and challenge schedule for animal experiments

Female BALB/c mice (6 weeks old, 16–22 g) were purchased from the Laboratory Animal Science Center of Nanchang University. Mice were divided into groups of 13 and then immunized via the intranasal route with suitable amounts of OMVs or OMPs suspended in 10 μl of PBS buffer (detail in Table 1). PBS alone (10 μl) was administrated intranasally, and these mice served as the negative control. Booster immunizations were then performed 4 weeks after the first immunization. Blood samples were collected by orbital sinus puncture, and vaginal secretions were collected by repeatedly flushing the animals 5 times with 0.1 ml PBS. Following centrifugation, soluble fractions of sera and secretion samples were harvested and stored at −80 °C for further experiments. Five weeks after a booster immunization, the mice were challenged orally with 10^9 colony-forming units (CFU) of *S.* Typhimurium S100 (~10,000-fold LD_{50}) or 10^8 CFU of *S.* Choleraesuis S340 (~1000-fold LD_{50}) or 10^8 CFU of *S.* Enteritidis S246 (~1000-fold LD_{50}) in 20 μl PBS containing 0.01% gelatin (BSG buffer). These three *Salmonella* serotypes were isolated from commonly infected animals, and were able to establish systemic infections in mice after oral administration (Liu et al., 2016c). The challenged mice were monitored daily for 30 days. Immunogenicity studies were conducted, as described in Fig. 1 and Table 1. Animal experiments were performed twice, and the data were combined for analysis.

2.4. Enzyme-linked immunosorbent assay (ELISA)

For analysis of the antibody response by quantitative ELISA, 96-well plates were coated with 1 μg of OMPs or OMVs isolated from the corresponding *Salmonella* strains suspended in 100 μl sodium carbonate-bicarbonate coating buffer (pH 9.6) per well and incubated overnight at 4 °C. To construct standard curves for quantification of each antibody isotype, plates were coated in triplicate with two-fold dilutions of the appropriate purified mouse Ig isotype standard (IgG, IgG1, IgG2a and IgA; BD Biosciences), beginning with 0.5 μg/μl. Plates were washed 3 times with PBST (PBS containing 0.1% Tween 20) and then blocked with 2% BSA solution for 2 h at room temperature. A 100-μl volume of suitable-fold diluted sample was added to the respective wells in triplicate, and the plates were incubated for 1 h at room temperature.