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Immunomodulatory role of outer membrane vesicles of *Shigella* in mouse model

Soma Mitra, Ritam Sinha, Dhrubajyoti Nag, Hemanta Koley *

Division of Bacteriology, National Institute of Cholera and Enteric Diseases, P-33, C.I.T. Road, Scheme XM, Beliaghata, Kolkata 700010, India

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ABSTRACT

In our previous studies, we discussed the protective efficacy of the two types of vaccine formulation namely SOMVs (single-serotype outer membrane vesicles) and MOMVs (multi-serotype outer membrane vesicles). Here, we compared the immunogenic roles of these two types of formulations and also studied general immunomodulation by *Shigella* OMVs in adult BALB/c mice. The production of various pro-inflammatory (TNF-α, IL-1β, IL-6, IL-12, IL-18, IFN-γ) and anti-inflammatory (IL-4 and IL-10) cytokine profile were assessed by in vivo, ex vivo and in vitro studies. MOMVs treated mice showed significantly enhanced cytokine production compared to SOMVs treated mice. MOMVs treatment has also upregulated iNOS mRNA synthesis in macrophages. Overall the OMVs of *Shigella* were found to show a mixed Th1/Th2 response and maintain the balance between pro-inflammation and anti-inflammation in mice. This will be crucial in the development of the next generation OMVs based vaccine against shigellosis.

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1. Introduction

Vesicles are likely a key factor in effecting an inflammatory response to host. Outer membrane vesicles or OMVs produced by colonizing bacteria encounter and may be taken up by epithelial cells and macrophages to trigger an immediate innate host response. The ability of outer membrane vesicles to trigger inflammatory responses was thoroughly investigated by Alaniz et al. [1]. Their analysis demonstrated that OMVs from *Salmonella enterica* serovar Typhimurium are potent stimulators of pro-inflammatory cytokine secretion and immune cell activation. A pro-inflammatory response to OMVs was also observed for several other pathogens like *Pseudomonas aeruginosa* [2], *Shigella flexneri* [3], *Vibrio cholerae* [4], *Neisseria meningitidis* [5,6], *Helicobacter pylori* [7]. Likewise, OMVs purified from the fish pathogen *Vibrio anguillarum* stimulated the production of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 when inoculated into flounder [8].

The induction of pro-inflammatory and anti-inflammatory cytokines are important in determining whether the immune system is successful in providing protection against specific pathogenic organisms [9,10] like *Shigella* which causes shigellosis, a significant public health burden predominantly in children under the age of 5 years, in developed and developing countries, leading to possibly 1.1 million deaths annually worldwide [11]. In order to develop a novel OMVs based vaccine against shigellosis, the balance between pro-inflammatory and anti-inflammatory cytokines is therefore needs to be understood. Here, we have studied the immunomodulatory role of *Shigella* OMVs, analyzing the pro-inflammatory and anti-inflammatory cytokine level in mouse model and also compared between our newly developed OMVs based vaccine formulations: SOMVs (single-serotype outer membrane vesicles) and MOMVs (multi-serotype outer membrane vesicles).

2. Methodology

2.1. Animals

BALB/c mice, six to seven weeks old, were taken from animal resource department of National Institute of Cholera and Enteric Diseases (NICED) and were caged in a group of five at 25 °C with 75% humidity. They were fed sterile food and water under the care of full time staff and in accordance with the rules of the institutional animal ethics committee (Apro/77/24/11/2010, Reg. No. NICED/ CPCSEA (AW) 215/2009–2015).

2.2. Analysis of pro-inflammatory and anti-inflammatory cytokines in mouse serum

Mice were immunized following the same strategy as explained in our previous publication [12]. Blood was collected on six

* Corresponding author.
E-mail address: hemantakoley@hotmail.com (H. Koley).

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consecutive days till day 28, after the last dose of immunization [12]. TNFα, IL-1β, IFN-γ, IL-6, IL-10 and IL-4 were measured in both immunized and non-immunized mouse serum with specific ELISA kits (Invitrogen, USA) following the instruction manual; Briefly, 50 μl of standards or test samples were added to the appropriate microtiter plate wells (supplied with the kit) followed by the addition of 50 μl biotin conjugate and incubated for 90 min at room temperature. The wells were washed and 100 μl of Streptavidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated again for 30 min at room temperature. After washing, 3,3′,5,5′ tetramethyl-benzidine (TMB) substrate solution was added to each well and incubated for another 30 min at room temperature. The enzyme-substrate reaction was terminated by the addition of 100 μl sulphuric acid solution and the colour change was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of the cytokines in the samples was determined by comparing the optical density of the samples to the respective standard curves.

2.3. Analysis of pro-inflammatory and anti-inflammatory cytokines in mouse peritoneal macrophage cells

Peritoneal macrophages from BALB/c mice were isolated and cultured following the protocol explained previously [13]. Briefly, cells were seeded per well at a concentration of 1×10^6 cells/ml in 12 well tissue culture plates (Nunc) and were cultured at 37 °C in a humidified 5% CO_2 atmosphere. The integrity of the monolayer was monitored by inverted Phase contrast microscopy. The day before the experiment, cells were grown in serum free RPMI. Next day, cells were washed three times and the medium was replaced with antibiotic-free, serum-free RPMI, just before adding the OMVs. One control experiment, without any antigen, was set along with the test. Cells were incubated with OMVs for overnight. IL-12, IL-18, IL-6 and IL-10 secretion in the supernatant was measured following the instruction manual of respective ELISA Kits (Invitrogen, USA). A curve of absorbance versus concentration of each cytokine studied here, were plotted. The concentrations of different cytokines in the samples were determined by comparing the optical density of the samples to the standard curve.

2.4. Quantification of iNOS mRNA by real time PCR

The inducible nitric oxide synthase (iNOS) mRNA in SOMVs, MOMVs and untreated macrophages was quantified. iNOS is involved in immune response, produces nitric oxide (NO), as an immune defence mechanism. At first the total mRNA was collected from the macrophages using the mRNA isolation kit from Invitrogen. cDNA was synthesised from these mRNA using Superscript II Reverse Trancriptase kit (Invitrogen) and quantified by Real-time PCR using Syber Green master mix. Fold changes were calculated by $2^{\Delta\Delta Ct}$ method.

2.5. Peritoneal macrophage and CD4+ T cells co-culture

CD4+ T cells were isolated from spleens of BALB/c mice. Spleens were strained over 70 μm nylon cell strainers. The resulting single cell suspension of splenocytes containing CD4+ T cells was then processed following the instruction manual of the CD4+ T cell isolation kit by Invitrogen. The purity of the cell preparations was determined by FACS analysis with phycoerythrin-conjugated anti-CD4+ antibody (BD PharMingen). Routinely, the purity of the cell preparations was >95%. The cells were then washed in RPMI 1640 containing 2% FCS, 5 U/ml penicillin G, 5 μg/ml streptomycin, and 0.1% gentamycin. Prior to the experimentation the media was replaced with serum free RPMI 1640.

CD4+ T cells (2×10^5) were cultured with 10^5 peritoneal macrophages in a final volume of 200 μl in round-bottomed 12-well plates in the presence or absence of antigen for 4 days in serum free RPMI 1640. Prior to the co-culture, the macrophages were stimulated in vitro with the OMVs for 6 h. After 4 days, IFN-γ and IL-4 secretion in the supernatant were measured following the instruction manual of respective ELISA Kits (Invitrogen, USA). The concentrations of different cytokines in the samples were determined by comparing the optical density of the samples to the respective standard curve.

2.6. Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Each experiment was repeated at least three times. Statistical significance was measured by analysis of variance (ANOVA) and two-tailed type 3 Student's *t* test. *P* values $\leqslant$ 0.05 and 0.005 were considered statistically significant and highly significant respectively.

3. Results and discussion

OMVs of *Shigella* have an immunomodulatory role in eliciting protective immune response in a mouse model [12,14]. The compositions of OMVs make significant activators of host innate and acquired immune response pathways. In addition to the potent immunomodulatory molecule lipopolysaccharides, vesicles contain outer membrane proteins and other important innate immune-activating proteins. Altogether, vesicle components appear to act synergistically to modulate the host immune response in ways that can either stimulate the clearance of the pathogen, enhance the virulence of the infection, or both. In addition, the immunogenic properties of OMVs lead to protective mucosal and systemic bactericidal antibody responses that have already been studied in our previous research [12,14].

In this study, mice, immunized with either SOMVs or MOMVs, have elicited significant up-regulation of IFN-γ, TNF-α, IL-6 and IL-10 but no IL-1β and IL-4 were observed in vivo (Fig. 1). IFN-γ and TNF-α were observed maximum on day 25th against both SOMVs and MOMVs followed by a gradual decrease. Whilst IL-6 and IL-10 were seen maximum on the 27th day after fourth oral immunization and then a sharp decrease. IFN-γ (main pro-inflammatory cytokine secreted by Th1 cells) level was significantly higher in immunized mice sera. Whilst IL-10 (main anti-inflammatory cytokine secreted by both Th1 and Th2 cells) too was found in significant concentration in mice sera. These data were found to be statistically significant upon comparing non-vaccinated control to any of the OMVs-type immunized. IL-6, apart from being a pro-inflammatory cytokine, has a role in anti-inflammation and Th2 cell stimulation. It also enhances the secretion of IL-10 [15–17]. IFN-γ and IL-6, both were being secreted in immunized mice sera establishing a mixed Th1/Th2 response in vivo; although the ratio is shifted more towards Th1 response as observed by significantly higher concentration of IFN-γ. These observations explained clearly a balance between pro-inflammatory and anti-inflammatory response in mouse, immunized with OMVs of *Shigella*. However, MOMVs were seen to be more powerful in eliciting cytokine response than SOMVs, with high statistical significance.

During ex vivo studies with mouse peritoneal macrophages, we observed that IL-12 and IL-18 were secreted predominantly by peritoneal macrophages stimulated with either SOMVs or MOMVs for overnight. IL-6 and IL-10 were also found in the macrophage culture supernatant though in lesser amount than IL-12 and IL-18. IL-12 and IL-18 production were much higher

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