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Overexpression of MicA induces production of OmpC-enriched outer membrane vesicles that protect against *Salmonella* challenge

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ABSTRACT

Outer membrane vesicles (OMVs) derived from bacteria are promising candidates for subunit vaccines. Stresses that modulate the composition of outer membrane proteins (OMPs) are important for OMV synthesis. Small RNAs (sRNAs) expressed in response to stress regulate OMPs, although the mechanism underlying sRNA-mediated OMV biogenesis and its utility for developing vaccine platforms remains to be elucidated. Here, we characterized the role of a sRNA, MicA, which regulates OmpA, a major OMP involved in both production of OMVs and reactive immunity against *Salmonella* challenge. A *Salmonella* strain overexpressing MicA generated more OMVs than a control strain. In addition, OmpC was the major component of MicA-derived OMV proteins. MicA-derived OMVs induced Th1- and Th17-type immune responses *in vitro* and reduced *Salmonella*-mediated lethality in a mouse model. Thus, OmpA-regulatory sRNA-derived OMVs may facilitate production of *Salmonella*-protective vaccines.

1. Introduction

Outer membrane vesicles (OMVs) are produced by the pinching off of the outer membrane during bacterial growth [1,2]. Bacterial OMVs comprise a spherical bilayer membrane containing outer membrane proteins (OMPs), RNAs, lipopolysaccharide (LPS), phospholipids, and some periplasmic constituents; these OMVs measure 20–500 nm in diameter [3–5]. OMVs perform diverse cellular functions, including transport of biological molecules required for communication between bacterial cells and between bacteria; and host cells [5–7] and for induction of pro-inflammatory cytokines in the host [8–10].

Reports suggest that OMVs are highly immunogenic; indeed, mice immunized with bacterial OMVs are protected against challenge with disease-causing bacteria [11,12]. *Salmonella* Typhimurium-derived OMVs provoke pro-inflammatory responses by host cells and induce *Salmonella*-specific adaptive immune responses *in vivo* [9]. Thus, mice immunized with *Salmonella* Typhimurium-derived OMVs are protected against *Salmonella*-borne diseases, suggesting that OMVs could be potential vaccines.

Despite the multiple roles played by bacteria-derived OMVs, their biogenesis is not fully understood. To date, three potential biogenesis mechanisms have been suggested for Gram-negative bacteria [1,2]. Studies also found that environmental and genetic factors affect OMV production [13–15]. Many genes regulate vesiculation [16,17] and play a role in regulating OMV production [1,2,5]. However, identification of such factors is a challenge that must be overcome if we are to understand the biogenesis of OMVs.

Small-noncoding RNAs (sRNAs) play important roles in regulating gene expression in many bacterial species [18]. sRNAs show marked conservation of sequences across species and are expressed in response to multiple stresses. Despite the number of sRNAs identified, their functions remain unclear. Systematic studies aimed to identify the role of sRNAs by utilizing them at saturation levels (via high-copy number plasmids). Indeed, transcription, bacterial motility, biofilm formation, and antibiotic resistance have all been characterized using such a system [19–21].

During production of OMVs, many sRNAs regulate the expression of multiple genes involved in the synthesis of OMPs, which are important for OMV production. OmpA, the most abundant integral OMP of *E. coli* and other enterobacteria, plays a role in the

Abbreviations: OMV, outer membrane vesicle; OMP, outer membrane protein; sRNA, small RNA; LPS, lipopolysaccharide; IP, intraperitoneal; CFU, colony forming units; p.o, per os.

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pathogenesis of various bacterial infections and in maintaining the structure of the outer membrane [22]. In addition, analysis of protein components within OMV fractions derived from many bacterial species shows that OmpA resides within OMVs; indeed, it is a major component of OMVs. However, one report shows that OmpA levels are inversely correlated with the number of OMVs, and that induction of sRNA VrrA (a homolog of *E. coli* MicA in V. *cholera*) increased OMV production in a manner comparable with the loss of OmpA [23].

OMVs show great potential for biotechnological application as carriers for antibiotics or as efficient vaccine particles. In particular, sRNA VrrA could be used for large-scale production of OMVs, but the strain by itself must be modified before use as a vaccine carrier because of the inherent toxicity of *V. cholera*. To overcome these hurdles, components that generate higher amounts of OMVs from bacterial cells that induce normal immunogenic responses need to be identified and exploited as a platform for vaccine design.

In this study, we examined the role of MicA from *E. coli* (which is highly conserved among enterobacteria) [24] in the synthesis of OMVs and its effect on immune responses against subsequent *Salmonella* challenge. We observed that MicA was an efficient inducer of OMVs in that its overexpression generated production of more OMVs than a control set. Analysis of other protein components within MicA-derived OMVs revealed that OmpC was the major protein. Further *in vitro* analysis of immunogenicity using MicA-derived OMVs showed that they promoted Th1- and Th17-type immune responses. Moreover, vaccinating mice with MicA-derived OMVs protected them against lethality induced by *Salmonella* infection. Overall, these data suggest that exogenous expression of MicA in *Salmonella* induces production of immunogenic OMVs; therefore, OMVs could be utilized for biotechnological production of large amounts of immunogenic vaccines.

2. Materials and methods

2.1. Ethics statement

The study was approved by the Institutional Animal facility Pohang University of Science and Technology Biotech Center, Pohang, Republic of Korea (approval number 20130005).

2.2. Strains and plasmids

E. coli MG1655 or *Salmonella* Typhimurium 14028S were used as standard strains in this study. Each strain was transformed with plasmids pBRplac and pBRplac-MicA [21] to evaluate the role of MicA.

2.3. Purification of OMVs

E. coli or *Salmonella* strains were cultured to an optical density (OD_{600}) of 3.0 at 37 °C in a tryptic soy broth containing IPTG (0.1 mM) and ampicillin (100 μg/mL). OMVs were purified from bacterial culture medium as described [25]. The concentration of OMVs was measured using a bicinchoninic acid (BCA) assay kit (Thermo Scientific).

2.4. Characterization of OMVs

OMVs were characterized by transmission electron microscopy (TEM), dynamic light scattering (DLS), and nanoparticle tracking analysis (NTA), as described previously [25,26]. TEM, DLS, and NTA were performed using a JEM 1011 electron microscope (JEOL), a Zetasizer (Malvern Instruments Ltd.), and a LM-10HS (Malvern Instruments Ltd.) chamber, respectively.

2.5. Characterization of OMV-derived proteins

Overexpressed MicA or MicA-derived OMVs obtained from *E. coli* MG1655 and *Salmonella* Typhimurium 14028S cells (1×10^9) were analyzed in 12% TGX stain-free gels (Bio-Rad), followed by imaging with ChemiDoc-MP (Bio-Rad). Cultured cells used for OMV preparation were spotted after diluting 1–10^4-fold in LB (Luria-Bertani) broth onto LB/ampicillin plates for the cell viability assays. The plates were then cultivated at 37 °C for 16 h and photographed. Total proteins were prepared from cell lysates or OMVs as described previously [27]. The amounts of OMPs derived from OMVs were analyzed using ImageLab™ Software (v. 5.2.1; Bio-Rad). For Q-TOF/MS analysis, gels were stained with Coomassie blue and protein bands excised and subjected to in-gel trypsin digestion [28]. MS/MS was performed using nano-ESI and a MicroQ-TOF III mass spectrometer (Bruker Daltonics, 255748 Germany), according to the vendor's instructions. The MS/MS results were interpreted by Mascot (Matrix Science) and the peptide homologies searched against the NCBI BLAST database.

2.6. In vitro analyses of cytokine production

RAW 264.7 cells (1×10^5 cells per well) were cultured in Dulbecco's Modified Eagle's Medium (Gibco BRL) supplemented with 10% fetal bovine serum and antibiotics (100 U/mL streptomycin). Cells were then treated with centrifuged and filtered bacterial culture medium, LPS (*E. coli* O55:B5) (Merck Millipore), centrifuged bacterial culture medium, ultracentrifuged supernatant, and OMVs for 12 h. Cytokine (IL-6 and TNF-α) levels in cell culture medium were measured using commercially available enzyme-linked immunosorbent assay kits (ELISA; R&D Systems) and a Versa Max ELISA Microplate Reader (Molecular Devices) [29,30].

2.7. Lethal dose determinations following Salmonella-induced sepsis

To determine the lethal dose of *Salmonella* Typhimurium 14028S used to infect mice, 1×10^6, 1×10^7, and 1×10^8 colony forming units (CFU) in 100 μL of PBS were injected intraperitoneally (i.p.) and survival rates recorded every 12 h for 3 days ($n = 10$ mice/group).

2.8. Quantification of serum antibodies

Serum concentrations of type-II-collagen-specific IgG_1 antibodies were measured using IgG_1 ELISA Quantitation Kits (Bethyl Laboratories, Montgomery, TX), according to the manufacturer's protocol. Successive measurement of OMV-specific antibodies was performed by coating microplates with prepared OMVs (0.1 μg/mL in PBS), instead of capture antibodies, for 24 h at 4 °C.

2.9. In vivo analysis of cytokine production induced by MicA-derived OMVs

To assess the immune response generated by MicA-derived OMVs *in vivo*, 100 μg of PBS-resuspended OMVs prepared from MicA-overexpressing *Salmonella* strains were intraperitoneally (i.p.) injected into 6-week-old C57BL/6 mice (The Jackson Laboratories, Bar Harbor) ($n = 5$ per group); mice received three injections at intervals of 1 week and were sacrificed on Day 7 post-immunization. Cells (1×10^6) from the spleen were isolated and seeded in a 24-well plate coated with anti-CD3 and anti-CD28 antibodies (0.1 μg/mL; eBioscience). After incubation at 37 °C for 12 h, supernatants were collected and the IFN-γ, IL-17, and IL-4 concentrations measured using commercially available enzyme-

linked immunosorbent assay kits (R&D Systems) and a Versa Max ELISA Microplate Reader (Molecular Devices).

2.10. Effect of immunization with MicA-derived OMVs

To evaluate the effects of MicA-derivedOMVs as vaccine candidates *in vivo*, mice were immunized (both i.p. and orally) with OMVs (5 μg in PBS) from MicA-expressing *Salmonella* strains. Mice ($n = 5$ per group) received three doses over 3 weeks, with a 1 week-interval in between. On Day 24 post-immunization, mice were challenged with *Salmonella* (1×10^7 CFU) and survival monitored every 12 h for 3 days as described previously [25].

2.11. Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc.). A *t*-test was used for single comparisons and one-way analysis of variance for multiple comparisons. Statistical significance was set at $P < 0.05$. The results of survival analyses were statistically compared using the long-rank test method.

3. Results and discussion

3.1. Induced production of OmpC-enriched OMVs by MicA derived from E. coli

OmpA is an OMP that is highly conserved among Gram-negative bacteria [22]. A stress-responsive *E. coli* sRNA, MicA, regulates OmpA expression and has a sequence that is highly conserved among Gram-negative bacteria [24]. Overexpression of VrrA, a homolog of MicA in *V. cholera*, induces production of OMVs [23] but the mechanism underlying their synthesis is not fully understood. Therefore, it is expected that modulation of OmpA production by inhibitory sRNAs or hitherto uncharacterized sRNA-mediated regulatory routes could affect the dynamics of OMV production. To characterize the dynamics of OMV synthesis, we overexpressed MicA in *E. coli* MG1655 cells using a high-copy plasmid. We observed that a protein band (designated band *A*) disappeared upon MicA overexpression (Fig. 1A). This band was identified as OmpA by Q-TOF/MS analysis (Fig. S1A). In addition, we prepared and quantified OMVs from the same number of *E. coli* cells (the viability of which was not affected by MicA overexpression) (Fig. 1B). The concentration of control- and MicA-derived OMVs was 0.1 or 0.8 mg/mL, respectively, suggesting that MicA is involved in overproduction of OMVs. When we further characterized the protein components of purified MicA-derived OMVs (Fig. 1C), we found that only MicA-derived OMVs showed a strong major band (designated band *B*). This band was identified as OmpC by Q-TOF/MS (Fig. S1B). Taken together, the above data suggest that MicA induces overproduction of OMVs, and that the major protein component of OMVs is OmpC.

3.2. Characterization of MicA-derived OMVs from Salmonella

The main purpose of this study was to characterize the immunogenic role of OmpA-regulatory sRNA-derived OMVs and to ask whether these components could be used as a vaccine platform. Due to the weak immunogenicity of *E. coli*, we needed to alter the model system if we were to fully understand the immunogenic role of MicA. One possible model was based on the *V. cholerae* strain [23]; however, this strain is too toxic to be used as a vaccine platform. Therefore, we selected a *Salmonella* strain in which MicA is present and shows high sequence similarity and conserved function with MicA expressed by *E. coli* [24]. Moreover, *Salmonella* strains are widely used to characterize virulence factors both *in vitro* and *in vivo,* and have been used as a vaccine platform [9].

Therefore, we introduced MicA of *E. coli* into *Salmonella* strain 14028S and examined its effect on production of OMVs and modulation of protein components. First, we analyzed proteins in total cell extracts from control or MicA-overexpressing cells (Fig. 2A). We found that band A (and identified as OmpA by Q-TOF/MS; Fig. S2) disappeared upon overexpression of MicA. Using the same number of cells (Fig. 2B), we prepared OMVs and identified major protein band *B* as OmpC by Q-TOF/MS. The protein band patterns in *Salmonella* cells and OMVs were similar to those in *E. coli* (see Fig. 1). Further quantification of control- or MicA-derived OMVs from *Salmonella* revealed concentrations of 0.5 and 3.3 mg/mL, respectively. Thus, overexpression of MicA generated a ~6.6-fold increase in OMV production. Next, we examined the physiological

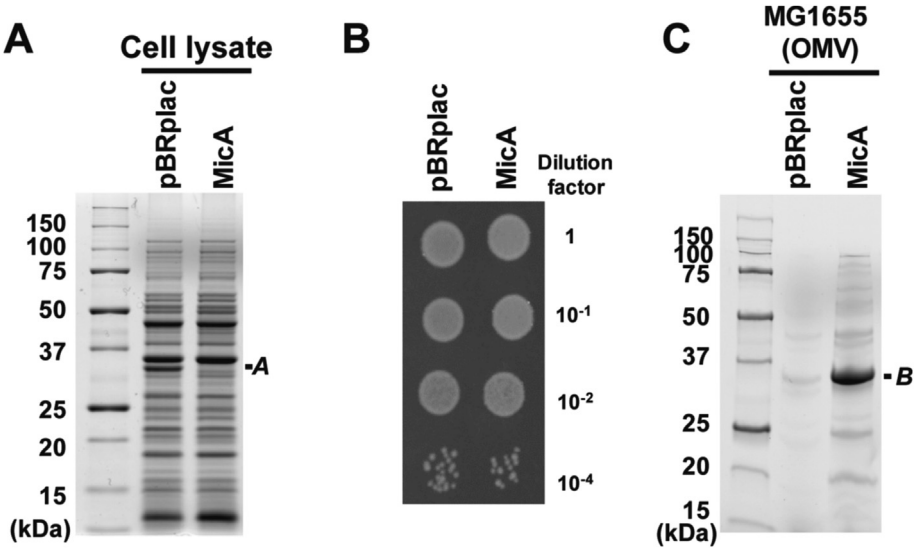

Fig. 1. Role of MicA in outer membrane vesicle (OMV) production by *E. coli*. (A) Analysis of proteins in control or MicA-overexpressing MG1655 *E. coli* cell lysates. Total cellular proteins were resolved on 12% TGX stain-free gels and imaged. (B) Cell viability assays. The cells used to prepare OMVs were serially diluted, spotted onto LB/ampicillin plates, incubated for 16 h, and imaged. (C) Analysis of proteins from control or MicA-derived OMVs. The same volume of OMV-derived proteins was loaded on 12% TGX stain-free gels and imaged.

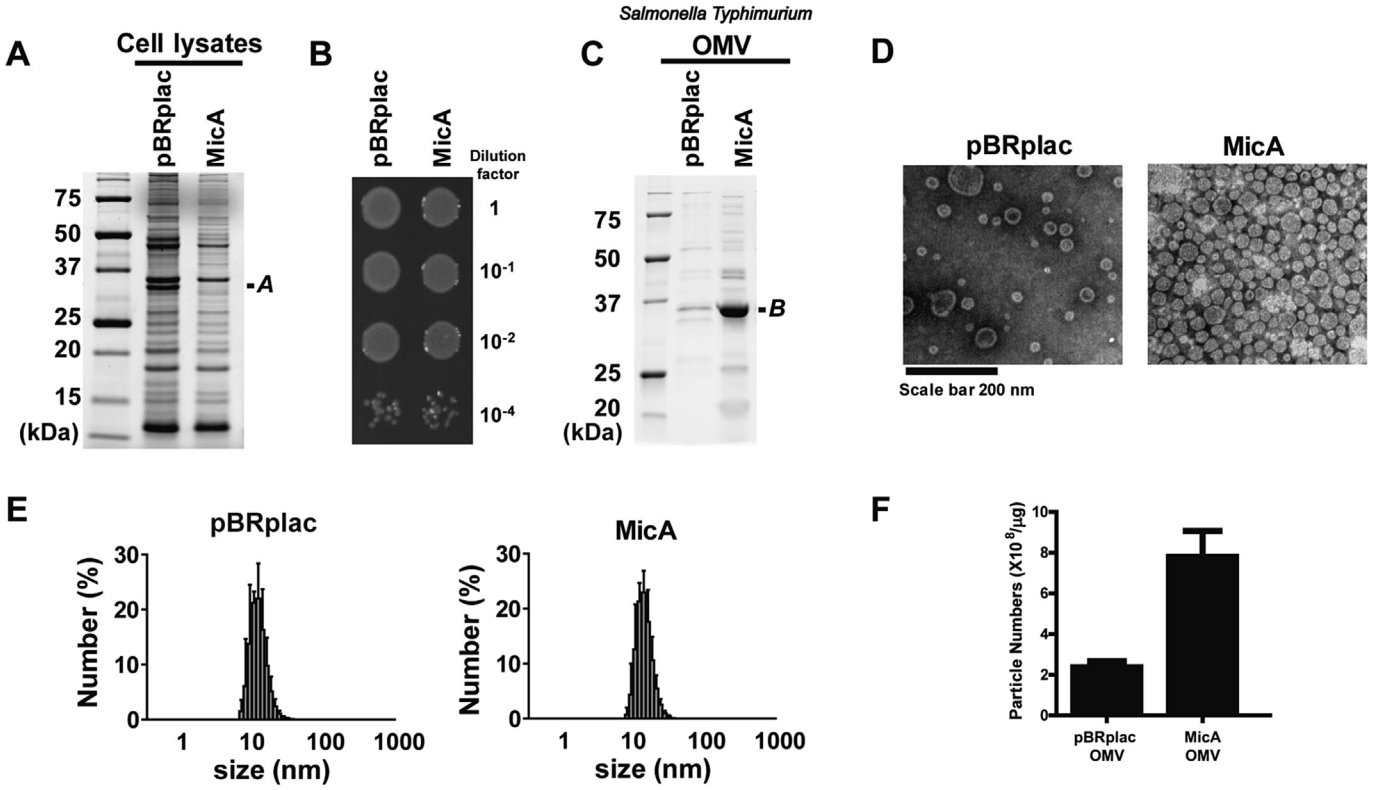

Fig. 2. Effect of MicA on OMV production by *Salmonella*. (A) Analysis of proteins in control or MicA-overexpressing *Salmonella* cell lysates. Total cellular proteins were run on 12% TGX stain-free gels and imaged. (B) Cell viability assays. Assays were performed as described in Fig. 1B. (C) Analysis of proteins in control or MicA-derived OMVs. The same volume of OMVs was separated on 12% TGX stain-free gels and imaged. (D) Transmission electron microscopy (TEM) images of OMVs. (E) OMV sizes determined by dynamic light scattering (DLS). (F) OMV particle numbers determined by nanoparticle tracking analysis (NTA). Asterisk (*) indicates samples showing significant differences from pBRplac-derived OMVs ($P < 0.05$).

characteristics of OMVs from *Salmonella* to see whether they harbored any defects. To this end, we first visualized OMVs by TEM and found that pBRplac- and MicA-derived OMVs were spherical in shape (Fig. 2D). Additional analysis by DLS revealed that the diameters of control- or MicA-derived OMVs ranged from 10 to 50 nm, with no observable variation in shape and size between control- and MicA-derived OMVs (Fig. 2E). NTA showed that the number of MicA-derived OMV particles was 3.3-fold higher than that of control-derived particles when equal amounts of protein were analyzed (Fig. 2F). These data indicate that MicA induces production of more OMVs without affecting their general characteristics; thus, MicA is a promising inducer of OMVs for vaccine development.

3.3. Changes in OMP composition induced by MicA lead to increased production of OMVs

Although we showed that overexpression of MicA induces production of OMVs rich in OmpC (Figs. 1 and 2), the factors that induce production of such OMVs have not been characterized. We expected that MicA-induced production of large amounts of OMVs might be driven by the composition of cellular OMPs, including the OmpA/OmpC ratio. To examine this hypothesis, we resolved proteins from the same amounts of control- or MicA-derived OMVs prepared from *Salmonella* (Fig. 3) and quantified the amounts of OmpA and OmpC (Fig. S3). The ratio of OmpC to OmpA protein in a control set was about to 1.5:1 (Fig. S3). However, the ratio in MicA-derived OMVs was about 9:1 (Fig. S3B). Thus, we suggest that a plausible basis for the MicA-mediated production of large amounts

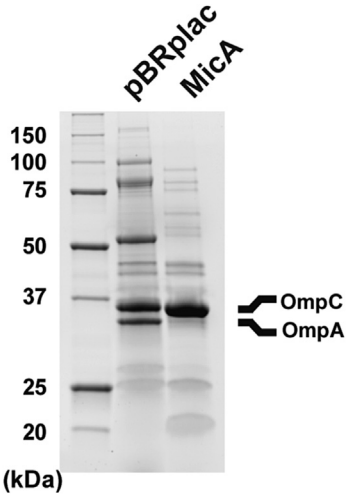

Fig. 3. Composition of outer membrane proteins (OMPs) within MicA-derived OMVs. Protein composition of MicA-derived OMVs from *Salmonella* Typhimurium 14028S cells. The same amounts of protein from control- or MicA-derived OMVs (Fig. 2C) were resolved on 12% TGX stain-free gels and imaged.

of OMVs might be alterations in the composition of OMPs (e.g., the OmpA/OmpC ratio).

3.4. In vitro characterization of MicA-overexpressing derived OMVs and their effects on cytokine production

Induced secretion of cytokines by macrophages exposed to

inflammatory stimuli is indicative of an immune response [31]. To examine involvement of MicA-overexpressing cells in such a response, we examined changes in IL-6 and TNF-α production by murine macrophages (RAW264.7 cells) exposed to culture medium from control or sRNA-overexpressing bacterial cells. We found that medium from a strain overexpressing MicA reproducibly induced expression of IL-6 and TNF-α (Fig. S4), which suggests that MicA induces an inflammatory response. We hypothesized that such a response by cells would be induced by MicA-derived OMVs pinched off into the culture medium. To verify this, we purified OMVs from the culture medium of control or MicA-overexpressing cells and measured cytokine levels. We found that OMVs from MicA-overexpressing cells produced higher levels of cytokines than those from a control set (Fig. S5). Taken together, these data show that MicA-derived OMVs induce an inflammatory response.

3.5. Effect of MicA-derived OMVs on Salmonella Typhimurium-induced sepsis

Finally, to explore the immune responses induced by MicA-derived OMVs *in vivo*, we immunized groups of five mice with MicA *Salmonella*-derived OMVs (control groups received saline). After 3 weeks, all animals remained in good health and exhibited no abnormal behavior (Fig. 4A). OMV-specific IgG_1 was measured to determine whether OMVs induced specific antibodies. Levels of OMV-specific IgG_1 induced by OMVs were 6-fold higher than those measured in the control group (Fig. 4B). Next, we detected increased expression of pro-inflammatory cytokines IFN-γ and IL-17, which are indicative of Th1- and Th17-type immune responses, by cultured spleen cells isolated from mice immunized with MicA OMVs (Fig. 4C). MicA induced a significant increase in the levels of both cytokines when compared with the control group. Th1- and Th17-type immune responses are important for protection against infectious disease because they increase recruitment of both neutrophils and phagocytes [32,33]. MicA-derived OMVs did not stimulate production of IL-4, an indicator of a Th2-type immune response, suggesting that this OmpA-regulatory sRNA mainly induces Th1- and Th17-type immune responses. We next challenged mice that had been vaccinated (i.p. and orally) with OMVs (Fig. 4D) with *Salmonella* Typhimurium (1×10^7 CFU) after determining the lethal dose of bacteria (Fig. S6) and found that all orally and non-immunized mice died 36 h after bacterial challenge; however, I.P.-vaccinated mice survived for 72 h (Fig. 4E), indicating that the MicA-derived OMVs elicit a protective response against *Salmonella* infection. Although the effects induced by oral administration were

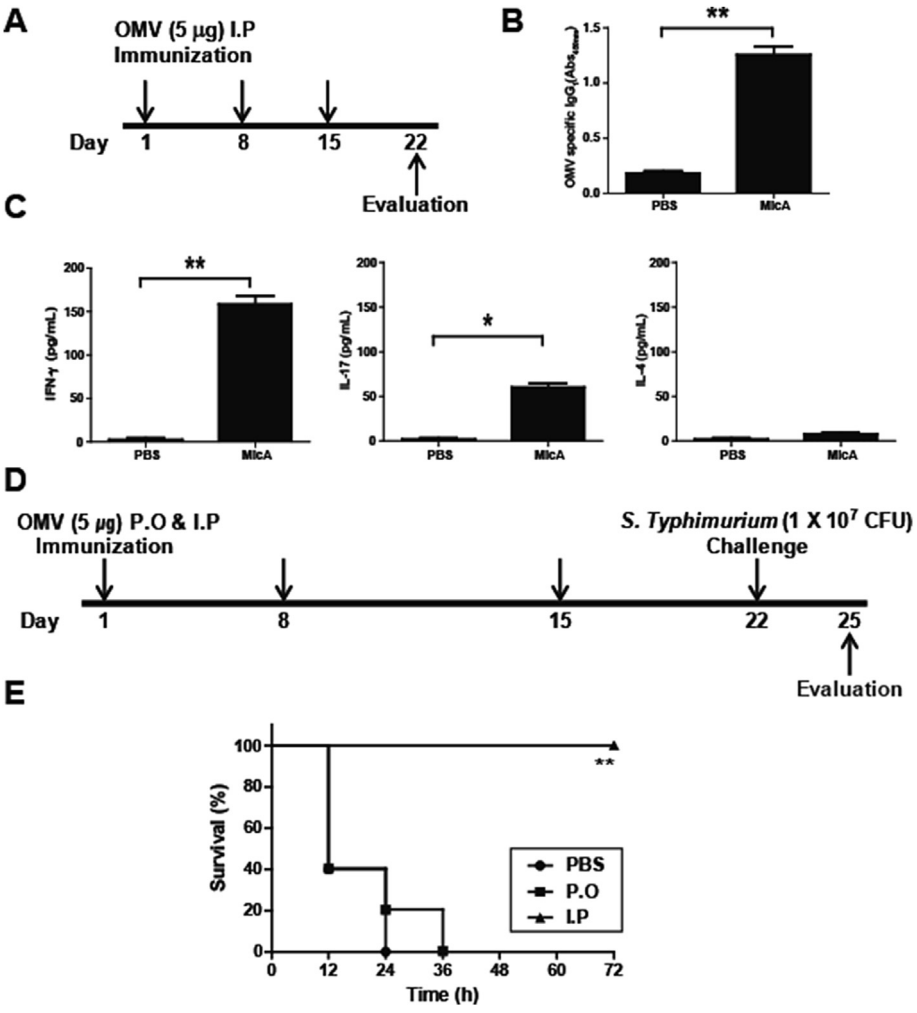

Fig. 4. Effect of MicA-derived OMVs on *Salmonella*-induced sepsis. (A) Protocol for intraperitoneal administration of OMVs to C57BL/6 mice. (B) Serum levels of OMV-specific IgG_1. (C) Expression of Th1- and Th17-type cytokines (IFN-γ and IL-17) and IL-4 in culture supernatants of spleen cells stimulated with anti-CD3 and -CD28 antibodies. (D) Protocol for OMV immunization against *Salmonella* infection. (E) Survival of OMV-vaccinated and control/non-immunized vaccinated mice challenged with *Salmonella* (1×10^7 CFU) (*$P < 0.05$ and **$P < 0.01$ indicate a significant difference *versus* mice vaccinated with phosphate buffered saline).

not as strong as those induced by i.p. administration, the data suggest that MicA-derived OMVs are a potential platform for antigen delivery and for development of improved vaccines against *Salmonella* and other diseases.

In summary, we found that MicA overexpression by *Salmonella* induced production of OMVs very efficiently, along with appropriate immunogenic and protective responses. Thus, OMVs could be utilized as an efficient vaccine platform. One consideration when using MicA-derived OMVs as a vaccine platform is the reduction of any potential toxicity. For example, bacterial protoplast-derived OMVs from which the toxic outer membrane components have been depleted still induce effective immune responses against bacterial infections without systemic toxicity [34]; therefore, we need to develop strategies that avoid the toxicity of bacterial OMVs. One such strategy is to produce OMVs from non-pathogenic bacteria. Another example might be the use of polymyxin B to decrease toxicity as reported previously [35].

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgements

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Transparency document

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Appendix A. Supplementary data

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