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Immunization with *Escherichia coli* Outer Membrane Vesicles Protects Bacteria-Induced Lethality via Th1 and Th17 Cell Responses

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Outer membrane vesicles (OMVs), secreted from Gram-negative bacteria, are spherical nanometer-sized proteolipids enriched with outer membrane proteins. OMVs, also known as extracellular vesicles, have gained interests for use as nonliving complex vaccines and have been examined for immune-stimulating effects. However, the detailed mechanism on how OMVs elicit the vaccination effect has not been studied extensively. In this study, we investigated the immunological mechanism governing the protective immune response of OMV vaccines. 

Vaccination with *Escherichia coli*-derived OMVs prevented bacteria-induced lethality and OMV-induced systemic inflammatory response syndrome. As verified by adoptive transfer and gene-knockout studies, the protective effect of OMV immunization was found to be primarily by the stimulation of T cell immunity rather than B cell immunity, especially by the OMV-Ag–specific production of IFN-γ and IL-17 from T cells. By testing the bacteria-killing ability of macrophages, we also demonstrated that IFN-γ and IL-17 production is the main factor promoting bacterial clearances. Our findings reveal that *E. coli*-derived OMV immunization effectively protects bacteria-induced lethality and OMV-induced systemic inflammatory response syndrome primarily via Th1 and Th17 cell responses. This study therefore provides a new perspective on the immunological detail regarding OMV vaccination. The Journal of Immunology, 2013, 190: 4092–4102.

*Escherichia coli*, normally regarded as a harmless bacterium, is a representative member of the normal intestinal flora, widely exploited as an experimental workhorse (1). Behind its friendly nature, *E. coli* simultaneously carries a harmful side in which only a slight genetic recombination can bring about highly pathogenic strain that most frequently causes the scourge of bacterial infections worldwide including sepsis, neonatal meningitis, pneumonia, bacteremia, and traveler’s diarrhea (2). This double-sided nature of *E. coli* strongly urges the need for the development of a preventive measure to ameliorate the global burden. Vaccination seems to be the only realistic protective strategy in the foreseeable future in overcoming the *E. coli*-related diseases.

**Abbreviations used in this article:** BAL, bronchoalveolar lavage; DC, dendritic cell; LB, Luria–Bertani; OMV, outer membrane vesicle; SIRS, systemic inflammatory response syndrome.

Vaccination is the administration of antigenic material, a vaccine, to produce immunity to a disease for prevention (3). It is regarded as the most cost-effective method of preventing infectious diseases. Prevention of the disease by vaccination requires the induction of protective immunity in a way in which immune response is triggered only to harm the pathogen shunning normal healthy cells. This is best achieved by the active immunization strategy having the ability to induce specific long-term protective memory, the hallmark of adaptive immunity. Dendritic cells (DCs), as the professional APC, are the first immune cell to evoke adaptive immunity by connecting the innate and adaptive immune responses (4). DCs primarily recognize the pathogenic components and evoke both B cells and T cells for Ab and cytokine production, thereby activating the adaptive immunity in full. Both B and T cells are important in conferring protective immunity to fight Ag-associated diseases. However, prevention of a certain pathogenic disease may require the activation of primarily either the humoral B cell response or cellular T cell response (5, 6). Thus, the key to eradicate the pathogenic infection is the activation of the specific immune response; for example, the specific Ig family in the B cell response or the right Th cell subtypes in the T cell response. However, many of the few licensed vaccines for use in humans are single Ag vaccines lacking the ability to provoke both the B and T cell responses (7, 8). In this sense, the development of new multiple complex vaccines that are both safe and strong activators of protective immunity is necessary.

Extracellular vesicles, otherwise known as membrane vesicles, are spherical bilayered phospholipids of 20–200 nm in diameter produced ubiquitously from all Gram-negative bacteria and some Gram-positive bacteria investigated to date (9–11). Previous biochemical and proteomic studies have revealed that the Gram-negative bacteria-derived extracellular vesicles, referred to as outer membrane vesicle (OMVs), are composed of outer membrane proteins, LPSs, outer membrane lipids, periplasmic proteins, DNA, RNA, and other factors associated with virulence (12–14).
Gram-negative bacteria–derived OMVs, which resemble the outer membrane and periplasm in composition, are proinflammatory and immunologic facsimiles (15, 16). We recently showed that OMVs have the ability to induce systemic inflammatory response syndrome (SIRS), a whole-body inflammatory state characterized by various inflammatory symptoms including hypothermia, tachypnea, and leukopenia, which can lead to sepsis (16). In contrast, recent studies revealed the potential of OMVs derived from Neisseria meningitidis, Acinetobacter baumannii, Porphyromonas gingivalis, Salmonella enterica serovar Typhimurium, Helicobacter pylori, or Vibrio cholerae in inducing protective immunities against bacterial infections in mice (15, 17–24). Most of these studies regarding OMV immunizations are focused on examining the potential of OMVs to evoke innate or adaptive immunity for vaccination. However, the protective vaccination effects involving both B and T cells against bacteria-induced sepsis models and the specific immune mechanisms of how OMV immunization provides such protection have not been elucidated.

In this study, to investigate the mechanism of how Gram-negative bacteria–derived OMVs elicit a vaccination effect, we used OMVs derived from E. coli as the model strain to study the effect of adaptive immune response. Vaccination with E. coli–derived OMVs potently protected bacteria-induced lethality and effect of adaptive immune response. Using this vaccination model, we revealed derived OMVs potently protected bacteria-induced lethality and effect of adaptive immune response. Vaccination with E. coli–derived OMVs potently protected bacteria-induced lethality and OMV-induced SIRS. Using this vaccination model, we revealed the detailed immunologic mechanisms on how OMV immunization provided protection against bacterially induced sepsis.

Materials and Methods

Mice and cell cultures

Both C57BL/6 and BALB/c background wild-type and BALB/c background IFN-γ−/−, IL-17−/−, and IL-4−/−deficient mice (5-wk-old males) were used. Experimental protocols were approved by the Institutional Animal Care and Use Committee at Pohang University of Science and Technology (Pohang, Republic of Korea) with approval number 2010-01-0007.

Preparations of OMVs derived from E. coli

OMVs were isolated from E. coli as described previously (16). Briefly, bacterial cultures were pelleted twice at 5000 × g for 15 min, and the supernatant fraction was filtered through a 0.45-μm vacuum filter followed by the ultracentrifugation with a QuixStand Benchtop System (Amersham Biosciences) for concentration. OMVs were prepared after another filtration through a 0.22-μm vacuum filter and pelleted by centrifugation in a 45 Ti rotor (Beckman Instruments) at 150,000 × g for 3 h at 4°C. Purified OMVs were resuspended in PBS, aliquoted, and stored at −80°C until further use.

Immunization protocol and induction of bacterial sepsis

For OMV immunization, mice were administered i.p. three times every week with 100 μl PBS containing OMVs derived from E. coli. Sera were obtained by eye bleeding at indicated times to measure the Ab titer. For the bacteria-induced sepsis model, mice were i.p. injected with E. coli. Survival was monitored every 12 h for 5 d. Blood, bronchoalveolar lavage (BAL) fluid, and peritoneal fluids from OMV- and sham-immunized mice were collected at various time points after the bacteria challenge for evaluation.

Protocol for OMV-induced systemic inflammation

Our previous study suggested that OMVs induce SIRS (16). To test the protective effect of OMVs on this model, 5 μg OMVs were applied i.p. three times at 12-h intervals. During the experiment, rectal temperature was evaluated using a digital thermometer (Natume) at indicated times, and systolic blood pressure was measured using a computerized tail-cuff system (Visitek Systems). To count the number of platelets, blood was collected by cardiac puncture in EDTA tubes and diluted to 1:100 in 1% ammonium oxalate. Finally, the number of platelets was counted using a light microscope.

Determination of bacteria CFU

After bacteria injection, bacterial burden in the blood and BAL fluid was examined at various time points. The fluids were serially diluted in sterile PBS, and multiple titrations were plated on Luria–Bertani (LB) agar plates. CFUs were determined after overnight incubation at 37°C.

Measurement of cytokines

The levels of cytokines in fluids and cell culture supernatants were measured by ELISA in accordance with the manufacturer’s instructions (R&D Systems): IL-6 and TNF-α in serum, BAL, and peritoneal fluids, IL-12p70, IL-6, IL-4, IL-1β, and IL-23 in supernatant of DCs, and IFN-γ, IL-17, IL-4, and IL-10 in supernatant of isolated splenic T cells and CD4+ T cells.

Ex vivo studies of isolated DCs and peritoneal macrophages

DCs from whole bone marrow of C57BL/6 mice were prepared using a high concentration of GM-CSF (Promega) (20 ng/ml). Macrophages were obtained from peritoneal exudate cells of OMV- or sham-immunized mice by peritoneal lavage with 9 ml cold PBS and were grown in RPMI 1640 media containing 10% FBS, 2-ME (50 μM), and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). To determine the cellular uptake of OMVs vs E. coli by DCs and macrophages, respectively, OMVs (10 μg/ml) or opsonized E. coli (5.0 × 10⁹ CFU) was applied to the cells grown on 0.1% gelatin-coated coverslips and incubated for 24 h. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and then incubated with laboratory-made rabbit polyclonal anti-OMV or anti–E. coli Ab and/or anti-F4/80 Ab (macrophage marker). After the treatment of Alexa Fluor–conjugated secondary Abs (Invitrogen), the cells were counterstained with Hoechst (Sigma-Aldrich). The FV1000 Olympus confocal microscope (Olympus) was used for analysis. Conditioned medium was collected from OMV-treated cells after 24 h, and cytokines were measured by ELISA.

Measurement of Ab titer against OMV and E. coli proteins

For evaluating the OMV or E. coli protein–specific IgG, serum was harvested from OMV- and sham-immunized mice. The 96-well plates were coated with OMVs (200 ng/well in PBS) or E. coli whole-cell lysate (100 ng/well in PBS), blocked with 1% BSA/PBS, and loaded with serum samples (1:500 dilutions in 1% BSA/PBS). OMV- or E. coli protein–specific IgG was detected after incubation with secondary peroxidase-conjugated anti-mouse IgG Ab followed by the addition of a chemiluminescent substrate.

Adaptive transfer

Serum and spleen from OMV- and sham-immunized mice were obtained 1 wk after the last immunization. For serum transfer, each naive mouse was injected i.p. with 300 μl serum from either OMV- or sham-immunized mice. For splenocyte transfer, spleens were dissociated using a cell strainer and a sterile plunger from a 5-ml syringe (26). After the RBCs were lysed by incubation with ammonium chloride solution, splenocytes were resuspended in RPMI 1640 containing 10% FBS, 2-ME (50 μM), and antibiotics. To determine the distribution of lymphocyte subsets in spleen, isolated splenocytes were stained with CD19-E, CD3e-allophycocyanin, CD4-FITC, and CD8a–PE-Cy5 (BD Biosciences) for 30 min at room temperature and analyzed on a FACScalibur flow cytometer (BD Biosciences) using CellQuest software. T cells were isolated from the spleen cells by positive selection using anti-CD3 magnetic bead (Miltenyi Biotech). CD4+ T cells and CD8+ T cells were separated by positive selection with anti-CD4 and anti-CD8a microbeads, respectively. Cell purity was >90% as assessed by flow cytometric analysis (data not shown). For cytokine secretion experiments of CD4+ T cells, each well of a 96-well plate was seeded with 2.0 × 10⁴ T cells (2.0 × 10⁴/0.1 ml RPMI 1640). Opsonized E. coli (5.0 × 10⁹ CFU) with or without mitomycin C–treated splenocytes (2.0 × 10⁴) isolated from control mice as feeder cells in 10% FBS/RPMI 1640 containing 2-ME (50 μM). After OMV treatment, purified CD4+ T cells for 72 h, IFN-γ, IL-17, and IL-4 levels from cell supernatant were determined by ELISA assay.

Bacteria killing by peritoneal macrophages

Peritoneal macrophages were harvested from OMV- and sham-immunized mice and were plated on 48-well plates at 2.5 × 10⁶ cells in 10% FBS/RPMI 1640 and incubated for 2 h. Cells were then washed with PBS and added with 300 μl RPMI 1640. Opsonized E. coli (5.0 × 10⁹ CFU) in PBS was added to the plate and incubated for 30 min to allow phagocytosis to occur. The wells were washed twice with 500 μl of PBS to remove unengulfed bacteria. One set of plates (T-0) was added to with 100 μl LB broth with 0.1% Tween-20 and was incubated for 1 min at room temperature. The other set of plates (T-90) was further incubated with 0.1% Tween-20 and incubated for 1 min at room temperature for cell lysis. Lysed cells were then plated on LB agar plates overnight, and bacterial colony was counted. The percentage of bacteria killing was determined by calculating the proportions of killed bacteria in the T-90 plate compared with the T-0 plate.
Statistical analysis

Survival curves were compared by log-rank test. All values are expressed as mean ± SEM, and the Student t test was used to calculate p values based on comparisons with the appropriate control.

Results

The immunization effect of OMVs on the protection against bacteria-induced sepsis and OMV-induced systemic inflammation

In order to verify the ability of E. coli–derived OMVs as an effective vaccine candidate, we examined the effect of OMVs immunization on the progression of E. coli–induced sepsis. Immunization of OMVs followed the protocol as shown in Fig. 1A. Mice were i.p. injected with OMVs every week for 3 wk, and 7 d after the final immunization, immunized mice were challenged with lethal dose of E. coli (1.0 × 10⁹ CFU) and monitored every 12 h for 5 d for survival. Only 20% of the sham mice survived at 120 h after the bacteria challenge. In contrast, survival rates of mice injected with sublethal doses of OMVs were significantly improved, with 80 and 100% survival for mice immunized with 0.5 and 1 μg of OMVs, respectively (Fig. 1B). Increase in OMV amount (2 μg) had no further effect on improving vaccine efficacy. Therefore, a final dose of 1 μg was chosen for the vaccine dosage. To confirm whether 1 μg of OMVs cause any inflammatory adverse effect on mice, mice

FIGURE 1. Immunization with OMVs protects against E. coli–induced lethality. (A) Study protocol for the immunization and bacterial challenge. (B) Survival rates of OMV- and sham-immunized mice challenged with E. coli (n = 10, each group). E. coli–derived OMVs (0.5, 1, and 2 μg) and sham (PBS) were i.p. injected at weekly intervals for 3 wk, and then E. coli (1.0 × 10⁹ CFU) was i.p. challenged 1 wk after the last immunization. Survival was monitored every 12 h for 5 d. Results are representative of four independent experiments performed (n = 40 total). (C) Survival rates of OMV- and sham-immunized mice challenged with E. coli after 42 d of immunization (n = 10, each group). Mice immunized with 1 μg of OMVs were challenged with E. coli (5.0 × 10⁹ CFU) 6 wk after the last immunization. Survival was monitored every 12 h for 5 d. For (D) and (E), mice immunized with 1 μg of OMVs were challenged with E. coli (1.0 × 10⁹ CFU) 1 wk after the last immunization. (D) E. coli count in blood and BAL fluid from OMV- and sham-immunized mice collected at various time points after the E. coli challenge (n = 4, each group). All sham-immunized mice died after 12 h of bacterial challenge. (E) The levels of TNF-α and IL-6 in serum, BAL fluid, and peritoneal fluid from OMV- and sham-immunized mice 6 h after the E. coli challenge (n = 5, each group). **p < 0.01, ***p < 0.001.
injected with 1 µg of OMVs following the preset immunization schedule were monitored for adverse effects (Supplemental Fig. 1). No mice injected with the immunization dose of OMVs developed any form of adverse effect, indicating the safety of low-dose OMVs. We next examined the long-term protective effect of OMV immunization. All of the mice immunized with 1 µg of E. coli OMVs survived the lethal-dose E. coli challenge (5.0 × 10^9 CFU) 6 wk after the last immunization, implying that the protective effect of OMVs is maintained (Fig. 1C). Furthermore, OMV immunization provided pathogen-specific protection, as E. coli OMV-immunized mice did not show any protective effect when challenged with S. enteritidis and Staphylococcus aureus (Supplemental Fig. 2).

To evaluate the protective effect of OMV immunization on the bacteria-induced sepsis, OMV- and sham-immunized mice were sacrificed after the E. coli challenge. Blood, BAL fluids, and peritoneal fluids were obtained from the OMV- and sham-immunized mice for further investigation (Fig. 1A). Immunization with OMVs (1 µg) improved bacterial clearance in the blood and BAL fluid, with complete bacteria clearance 24 h after the challenge (Fig. 1D). However, the sham-immunized mice had a significant amount of bacterial CFU in blood and BAL fluid and eventually died after 12 h of bacteria challenge. In addition, to confirm complete bacteria clearance in OMV-immunized mice, we examined bacteria loads in the four major organs, the lung, liver, kidney, and spleen, after 72 h of bacteria challenge. No bacteria colony was observed in all of the organs. Additionally, as an indicator of systemic cytokine shock during sepsis, we measured the level of major proinflammatory cytokines IL-6 and TNF-α in the serum, BAL fluid, and peritoneal fluids of OMV-immunized and sham mice. No increase in the levels of both IL-6 and TNF-α in the serum and BAL fluid was detected in OMV-immunized mice after the E. coli challenge, whereas a significant increase of these cytokines was observed in sham mice (Fig. 1E). However, IL-6 and TNF-α levels in the peritoneal fluid, the site of E. coli injection, were increased even in OMV-immunized mice, suggesting that bacteria challenge still causes local inflammation. Together, these results demonstrate that the immunization with OMVs prevents E. coli–induced lethality via enhancement of bacteria clearance and prevention of systemic inflammation.

Recently, multiple injections of OMVs derived from intestinal E. coli were found to induce severe sepsis in murine model through the induction of SIRS (16). When OMV- and sham-immunized mice were challenged by multiple injections of OMVs as described in Fig. 2A, only OMV-immunized mice did not show the SIRS symptoms such as fall in body temperature (hypothermia) and drop in blood pressure (hypotension), whereas sham-immunized mice did (Fig. 2B, 2C). OMV immunization also prevented the decrease in the platelet level of the peripheral blood, another aspect of OMV-mediated SIRS (Fig. 2D). Moreover, IL-6 and TNF-α induced by multiple injections of OMVs decreased to the basal level in the serum, BAL fluid, and peritoneal fluids of OMV-immunized mice, with an exception of TNF-α level in the peritoneal fluids (Fig. 2E). Furthermore, mice immunized with OMVs had no adverse physical symptoms such as eye exudates formation, seen in sham-immunized mice after the OMV challenge (Supplemental Fig. 3). Together, these results clearly indicate that the pre-exposure of sublethal-dose OMVs prevents the multiple phenomena of OMV-induced SIRS.

OMV immunization effect on the activation of innate and adaptive immune responses

Host defense relies on the concerted action of both Ag-nonspecific innate immunity and Ag-specific adaptive immunity. DCs work as the bridge connecting the two immune responses, providing the first line of defense against infection (27). We thus examined how DCs recognize and respond to OMVs derived from E. coli in eliciting the protective effects on acute systemic infection. When bone marrow–derived DCs were treated with E. coli–derived OMVs for 24 h, signals against OMVs were observed in the cytoplasm of DCs, indicating OMVs uptake by DCs (Fig. 3A). Moreover, such OMV-treated DCs efficiently induced immune-modulating cytokines IL-12p70 and IL-6 (Th1- and Th17-polarizing cytokines, respectively), whereas the level of IL-4, a representative Th2-polarizing cytokine, remained unchanged (Fig. 3B). We also observed that OMV treatment to DCs induced an increase in the production of other Th17 cytokines, including IL-1β and IL-23 (Fig. 3C). These results suggest that OMV immunization effectively activates the innate immune responses by DCs and that activated DCs produce Th1- and Th17-polarizing cytokines. Next, we assessed the effect of OMV immunization on the induction of Ag-specific B and T cell responses. During the immunization processes, sera were obtained from OMV- and sham-immunized mice at indicated times, and OMV protein–reactive IgG titer was evaluated (Fig. 4A). A rise in OMV protein-reactive IgG was detected after the immunization with 1 µg of OMVs; Ab titer initially increased at 7 d after the first OMV injection and kept rising throughout the immunization procedure. However, OMV protein-reactive IgG was not detected in the sera of sham-immunized mice. In addition, E. coli protein–reactive IgG was also detected from the sera of OMV-immunized mice 7 d after the last immunization (Fig. 4B). Next, to evaluate the OMV-specific T cell responses, splenic T cells were isolated from OMV- and sham-immunized mice 1 wk after the last immunization and restimulated with OMVs (100 ng/ml) for 24 h. OMV-specific production of IFN-γ and IL-17, the key cytokines produced by Th1 and Th17 cells, respectively, was higher in OMV-immunized mice compared with the control (Fig. 4C). However, OMV-Ag–specific production of IL-4, the key cytokine of Th2 cells, was similar in the two groups. Moreover, an increase in IL-10 from splenic T cells isolated from OMV-immunized mice was also observed (Fig. 4C). These results suggest that the immune-inflammatory response in OMV-immunized mice is being balanced by pro- and anti-inflammatory cytokines (28). Furthermore, this OMV-specific Th1 and Th17 memory T cell response was preserved even 45 d after the final immunization (Fig. 4D). However, no detected levels of IFN-γ and IL-17 were measured in the serum of both sham- and OMV-immunized mice 6 and 12 h after the E. coli challenge. Together, these findings demonstrate that the immunization with E. coli–derived OMVs induces an effective Ab response as well as strong Th1 and Th17 cell responses against OMV Ags.

The effect of OMV immunization on bacteria phagocytosis activity and bacteria-killing ability of peritoneal macrophage

To assess whether OMV immunization directly affects the ability of immune cells to fight the bacterial infection, we compared the phagocytosis and bacteria-killing activity of macrophages isolated from the peritoneum of OMV- and sham-immunized mice. The macrophages were isolated 1 wk after the final immunization. Peritoneal macrophages isolated from OMV-immunized mice had a change in their cellular morphology from a round shape to a large, multipolar, spindle-like shape after the immunization with OMVs, indicating macrophage maturation (Fig. 5A). These activated macrophages have gained a stronger ability to uptake bacteria compared with sham macrophages control. Accordingly, this result suggests that macrophages have changed their cellular morphology by OMV immunization, indicating that macrophages remain...
in the active state even 1 wk after the OMV immunization. Furthermore, peritoneal macrophages isolated from OMV-immunized mice were more effective in killing bacteria compared with sham control (Fig. 5B). These results indicate that OMV immunization enhances bacterial uptake and clearance by macrophages, possibly via induction of Ab and Th1 and Th17 cell responses.

Adoptive transfer of serum and splenocytes for the protection against E. coli–induced lethality
Adoptive transfer is one alternative means to induce active immunization. To find out whether B cell humoral immunity or T cell cellular immunity is important in conferring OMV-induced vaccination, we compared the vaccine effectiveness of serum and splenocyte adoptive transferred mice (Fig. 6A). Naive mice that received splenocytes from OMV-immunized mice conferred potent bacterial clearance effect, having 80% survival after lethal-dose E. coli challenge. In contrast, naive mice that received serum from OMV-immunized mice had no enhanced ability to survive after E. coli challenge compared with control naive mice that received serum or splenocytes from sham-immunized mice. To further investigate which portion of the immunized splenocytes conferred protective immunity on naive recipient mice, spleens were extracted from OMV- and sham-immunized mice for lymphocyte subsets examination. The proportions of lymphocyte subsets in spleen were determined by analyzing the splenocytes stained with CD19-PE, CD3e-allophycocyanin, CD4-FITC, and CD8a–PE-Cy on an FACSCalibur flow cytometer (BD Biosciences) (Fig. 6B, top panel). The results are illustrated as percentages in the table (Fig. 6B, bottom panel). The proportions (percentages) of B cells and T cells were similar in the two groups, indicating the preservation of B cell/CD4+ T cell/CD8+ T cell ratio. These results suggest that T cell–mediated cellular immunity confers a major protective effect of OMV immunization compared with B cell humoral immunity.

The roles of Th1, Th17, and Th2 cytokines on the protection against bacterial sepsis–induced lethality by OMV immunization
One of the key functions of CD4+ T cells during bacterial infection is the production of cytokines that activate inflammatory cells. We observed that OMV-specific production of TNF-α, IFN-γ, and IL-17 was higher in splenic CD4+ T cells isolated from OMV-immunized mice compared with the sham mice (Fig. 7A). IFN-γ– and IL-17–induced Th1 and Th17 cellular adaptive immunity are known to play essential roles in conferring vaccination effects
on several bacterial infections both in humans and mice (6, 26, 29, 30), indicating that there is a correlation between OMV efficacy and IFN-\(\gamma\) and IL-17 production.

Next, we carried out the survival experiments on IFN-\(\gamma\), IL-17, and IL-4 knockout mice (IFN-\(\gamma^{2/2}\), IL-17\(^{2/2}\), and IL-4\(^{2/2}\), respectively). Pre-exposure of OMVs in IFN-\(\gamma^{2/2}\) and IL-17\(^{2/2}\) mice did not exhibit the protective effect of wild-type mice, but showed similar survival with sham mice, but in a delayed manner (Fig. 7B). This delayed lethality in IFN-\(\gamma^{2/2}\) mice was similarly observed in the bacteria CFU titers of blood and BAL fluids in OMV-immunized IFN-\(\gamma^{2/2}\) mice (Supplemental Fig. 4A). However, all of the gene-knockout OMV-immunized mice developed OMV-protein–specific IgG during the immunization periods and showed a similar Ab titer to that of wild-type OMV-immunized mice (Fig. 7C), indicating that the B cell ability of Ab production is preserved in gene-knockout mice. In addition, when we examined the IgG subtype produced after OMV immunization on wild-type, IFN-\(\gamma^{2/2}\), and IL-4\(^{2/2}\) mice, we found that both IgG1 and IgG2a Abs are produced by the OMVs immunization (Supplemental Fig. 4B).

To further explore the mechanism of how such IFN-\(\gamma\)– and IL-17–dependent Th1 and Th17 cell responses elicit functional roles on enhancing vaccine efficacy, we evaluated the bacteria-killing ability of peritoneal macrophages isolated from wild-type and IFN-\(\gamma^{2/2}\), IL-17\(^{2/2}\), and IL-4\(^{2/2}\) mice. In line with our previous survival data, macrophages from IFN-\(\gamma^{2/2}\) and IL-17\(^{2/2}\) mice had lost the ability to kill bacteria compared with wild-type mice, whereas macrophages from IL-4\(^{2/2}\) mice maintained the same bacteria-killing ability (Fig. 7D). Together, these results provide support that survival during bacterial infection by OMV vaccination is associated with the macrophage bacteria-killing ability and that such bacteria clearance is dependent on IFN-\(\gamma\) and IL-17.

**Discussion**

Despite the first discovery of OMVs from Gram-negative bacteria 40 y ago, only the last 10 y have brought better understanding of the roles and compositions of OMVs (13, 31). Thus, many of the fields regarding OMV applications are still in their infancy. Yet the effective immune-stimulating roles of OMVs have boosted the development for their use as the next-generation acellular vaccine,
already having clinically licensed OMVs for use against serogroup B meningococcal disease (32–34). However, the precise vaccination mechanism of how OMVs prevent infectious diseases remains unraveled. Broader knowledge on how and by what means the OMVs confer such protective immunity will increase the likelihood of developing a more effective and safe vaccine. In this

**FIGURE 4.** *E. coli*-derived OMVs provoke the activation of adaptive immune response and induce the production of both Th1- and Th17-polarizing cytokines. (A) Serum levels of OMV-reactive IgG. Sera were obtained from OMV- and sham-immunized mice during the immunization processes, and then OMV-reactive IgG titer was measured by ELISA (*n* = 4, each group). (B) Sera were obtained from OMV- and sham-immunized mice 7 d after the last immunization, and then serum levels of *E. coli*-reactive IgG were measured by ELISA (*n* = 4, each group). (C) OMV-specific production of IFN-γ, IL-17, IL-4, and IL-10 from splenic T cells. Splenic T cells were isolated from OMV- and sham-immunized mice 1 wk after the final immunization and then stimulated with OMVs (100 ng/ml) for 24 h. The levels of IFN-γ, IL-17, IL-4, and IL-10 in the cell supernatants were measured by ELISA assay (*n* = 3, each group). (D) OMV-specific production of IFN-γ, IL-17, and IL-4 from splenic T cells isolated from OMV- and sham-immunized mice 45 d after the final immunization. The splenic T cells were stimulated with OMVs (100 ng/ml) for 24 h. The levels of IFN-γ, IL-17, and IL-4 in the cell supernatants were measured by ELISA assay (*n* = 3, each group). **p < 0.01, ***p < 0.001.

**FIGURE 5.** Immunization with *E. coli* OMVs enhances macrophage uptake of bacteria, leading to bacteria clearance. (A) Uptake of *E. coli* by peritoneal macrophages after the OMVs immunization. Macrophages were isolated from peritoneal fluids of OMV- and sham-immunized mice 1 wk after the last immunization. After the incubation with opsonized *E. coli* (1.0 × 10⁶ CFU) for 30 min, macrophages were stained with F4/80 (red), the macrophage marker, anti-*E. coli* polyclonal Ab (green), and Hoechst (blue). Scale bars, 5 μm. (B) Bacteria-killing ability of peritoneal macrophages isolated in OMV- and sham-immunized mice 1 wk after the last injection. **p < 0.01. DIC, Differential interference contrast.
sense, in the current study, we investigated the vaccination mechanisms of OMVs using OMVs derived from *E. coli*. We demonstrated that the active immunization with the sublethal dose of *E. coli* OMVs prevents not only *E. coli*-induced lethality, but also OMV-induced SIRS via enhancing bacterial clearance and inhibiting the onset of systemic inflammation. These protective effects were accompanied by the involvement of innate and adaptive immune responses induced by OMVs, eliciting INF-γ and IL-17–dependent Th1 and Th17 cell responses, respectively.

Vaccination has been considered to be the most cost-effective method to prevent infectious diseases. To develop a new vaccine with maximum efficacy, several experimental factors should be considered, including the route of injection, dosage schedule, and the use of the adjuvant (3). Given that *E. coli* is a common causative agent of sepsis, we focused on the systemic immunization via the i.p. route. Vaccine adjuvants affect the quality and magnitude of immunological responses (35). Various adjuvants, including TLR3, -4, -7, and -9 agonists, are used in combination with Ags to enhance the Ag-specific immune responses. OMVs, in contrast, harbor several pathogen-associated molecular patterns including LPS, immune-modulating proteins, and bacterial DNA (13, 16, 36). These OMV components act as TLR ligands, thus rendering OMV a good adjuvant itself.

Previously, studies revealing the proinflammatory characteristics of OMVs as an agent that can stimulate both innate and adaptive immunity in vitro and in vivo have been published (15, 17–20, 23, 24). In particular, using detergent-treated OMVs as a vaccine agent against *N. meningitidis* has been proposed and is being developed for clinical trial (32–34). Detergent-treated OMVs have the advantage in terms of increase in OMV yield, as treatment of detergent to bacteria increases vesicle release (34, 37, 38). However, studies comparing the protein composition of detergent-treated OMVs and native OMVs have shown a difference in the proteomic profiles (39). Among the identified proteins, 64% of proteins from detergent-treated OMVs were cytoplasmic and inner

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**FIGURE 6.** Adaptive T cell immune responses induced by OMVs are essential for the protective immunity to bacterial infection. (A) Mice were injected with either splenocytes (1 × 10⁸) or serum (300 μl) isolated from OMV- and sham-immunized mice 1 d after the challenge (n = 5, each group). Results are representative of two independent experiments performed (n = 10 total). (B) The proportions of various immune cells in spleen obtained from OMV- and sham-immunized mice (n = 5, each group). Splenocytes were stained with CD19-PE, CD3e-allophycocyanin, CD4-FITC, and CD8a–PE-Cy and evaluated on an FACSCalibur flow cytometer (BD Biosciences) using CellQuest software, and the results are illustrated as a table (bottom panel). **p < 0.01.
membrane proteins, whereas most of the proteins from native OMVs were outer membrane proteins (39). This difference in the protein profiles, therefore, might lessen the vaccination efficacy of detergent-treated OMVs.

Aluminum hydroxide, which is among the very few licensed vaccine adjuvants used these days, is known to only elicit a humoral B cell response without activating the cellular T cell response (8). However, orchestration of both B and T cells eventually modulates the effective adaptive immunity to fight the disease carrying the immunized Ag. Likewise, both B and T cells are known to participate in the process of protective immunity against infectious diseases, but the exact function and the importance of humoral and cellular immunity are still unraveled and disputed (40). Especially in bacterial infection, it has been thought that protective immunity strictly relies on the function of B cell response, the Ab secretion (41). In contrast, growing evidence provides new insight into the mechanism by which our body reacts against bacterial infection and how Th1 and Th17 cell responses play critical roles in activating such protective immunity (6, 42). By adoptive transfer and gene-knockout mice studies, we demonstrated that protective immunity toward E. coli–derived OMVs strongly depends on the stimulation of T cell immunity, especially IFN-γ- and IL-17–dependent T cell responses, rather than B cell humoral immunity (Figs. 6, 7). However, we could not exclude the possibility that cytokines, antimicrobial peptides, B cells, or non–T cells including NK cells, macrophages, and DCs, may aid the protective effect of OMV immunization. In addition, OMV-immunized mice 7 d after the last immunization may include cytokines or antimicrobial peptides induced by immunization that might influence the outcome of the serum-transfer experiments (43). Therefore, to investigate the importance of specific T cell subsets in relation to other factors and other immune cells, adoptive transfer experiment of CD4+ T cells or CD8+ T cells and a loss of function approach on specific T cells should be carried out.

Moreover, our results in Fig. 7 clearly suggest that IFN-γ and IL-17, but not IL-4, are critical for protection against lethal sepsis in OMV-immunized mice. As other cell types like NK cells and γ-δ T cells can also produce IFN-γ and IL-17, and as protection primarily occurred within the first 48 h after the challenge, it would be important to investigate the roles of other innate immune cells on the protective effect of OMV immunization, especially in relation to the protective effect of IFN-γ– and IL-17–producing Th1 and Th17 CD4+ T cells, respectively. In addition, although IL-17 is mostly known for playing an important role in recruiting...
neutrophils to the site of bacterial infection (44), it is also known for promoting macrophage-mediated bacteria killing (45, 46). Together, although further study is required, our bacteria-killing assay of macrophages isolated from the knockout mouse provides a new view, that both IFN-γ and IL-17 are important in conferring bacteria clearance that finally leads to the survival after bacterial infection (Fig. 7D).

Having been derived from bacteria, OMVs offer many advantages as vaccines compared with other conventional vaccines used clinically these days. First, they are cost effective in that they are less expensive and easily obtainable. Second, they include all of the multiple putative virulence factors while they are still nonliving (13, 16, 36). This is especially advantageous in that it could overcome the limitation of vaccine efficacy in current single Ag vaccines (7).

Finally, because of the vesicular structure, OMVs have the potential to work as both vaccine adjuvant and Ag carrier (36). Therefore, OMVs can enhance the vaccination effect without the aid of adjuvant by loading other Ags inside. However, the fact that OMVs enclose various pattern recognition molecules gives rise to a toxicity issue, as OMVs injected in high doses could induce SIRS (16). Therefore, extra care should be taken when defining the therapeutical window for OMV administration on humans in the future.

Because of the thick cell wall of Gram-positive bacteria, extracellular vesicle secretion was overlooked in these bacteria. Our recent study reported for the first time, to our knowledge, that the active immunization process also taking place in archaea, Gram-negative bacteria, Gram-positive bacteria, and eukaryotic cells (10, 31). Proteomic analysis revealed that extracellular vesicles derived from S. aureus harbor several pathogenic components (10). Furthermore, S. aureus extracellular vesicles induce atopic dermatitis–like skin inflammation in mice (47). These observations provide hints on the possible roles of Gram-positive secreted vesicles as vaccine candidates (10). In fact, recently a study on the immune-activating role of Gram-positive bacteria–derived extracellular vesicles has been published (48). However, the survival of mice immunized with Gram-positive bacteria–derived extracellular vesicles was not as effective compared with our results. Because Gram-negative bacteria OMVs have potent vaccine adjuvant activities, we can speculate that combination of Gram-positive and Gram-negative bacteria extracellular vesicles may be developed as a universal vaccine against pan-bacteria–mediated infectious diseases without the aid of conventional adjuvant.

Collectively, our results illustrate that the active immunization with the sublethal dose of E. coli–derived OMVs efficiently blocks not only E. coli–induced lethality, but also OMV-induced SIRS via inhibiting the onset of systemic inflammation. OMV immunization produces both Th1 and Th17 cell responses, which confer the protection against bacterial sepsis–induced lethality by enhancing bacterial clearance ability of inflammatory cells including macrophages. When combined with other studies, our findings offer sufficient information to redirect the current understanding of bacteria-derived extracellular vesicle vaccination and provides a new direction for vaccine development against multibacteria infections as well as bacteria-derived extracellular vesicle–induced inflammatory diseases

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Disclosures

The authors have no financial conflicts of interest.

References

ROLES OF Th1 AND Th17 RESPONSES IN OMVs VACCINE


