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Salmonella Typhimurium derived OMV nanoparticle displaying mixed heterologous O-antigens confers immunogenicity and protection against STEC infections in mice

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Abstract

Shiga toxin-producing *Escherichia coli* (STEC) is one of the major pathogens responsible for severe foodborne infections, and the common serotypes include *E. coli* O157, O26, O45, O103, O111, O121, and O145. Vaccination has the potential to prevent STEC infections, but no licensed vaccines are available to provide protection against multiple STEC infections. In this study, we constructed an engineered *S.* Typhimurium to rapidly produce the outer membrane vesicle (OMV) with low endotoxic activity to deliver the O-antigen of *E. coli*. *S.* Typhimurium OMV (STmOMV), which displays mixed heterologous O-antigens, was systematically investigated in mice for immunogenicity and the ability to prevent wild-type STEC infection. Animal experiments demonstrated that STmOMV displaying both *E. coli* O111 and O157 O-antigens by intraperitoneal injection not only induced robust humoral immunity but also provided effective protection against wild-type *E. coli* O111 and O157 infection in mice, as well as long-lasting immunity. Meanwhile, the O-antigen polysaccharides of *E. coli* O26 and O45, and O145 and O103 were also mixedly exhibited on STmOMV as O-antigens of the O111 and O157 did. Three mixed STmOMVs were inoculated intraperitoneally to mice, and confer effective protection against six *E. coli* infections. The STmOMV developed in this study to display mixed heterologous O-antigens provides an innovative and improved strategy for the prevention of multiple STEC infections.

Keywords OMV, STEC, O-antigen, Genetically engineered *Salmonella*

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Introduction

Escherichia coli (*E. coli*), a facultative anaerobic Gram-negative bacillus, is one of the essential commensal organisms found in the gastrointestinal tract of various animal species, including poultry and humans [1]. Although most *E. coli* strains are non-pathogenic and reside in all mammals, certain strains possess pathogenic properties and have the potential to induce a multitude of clinical illnesses in humans and other animals [2]. Shiga toxin-producing *Escherichia coli* (STEC) strains are among the pathogenic *E. coli* and a significant foodborne pathogen associated with gastrointestinal diseases, inducing diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS), which may lead to renal failure and neurological complications in humans [1]. In addition to serotype O157, the U.S. Food and Drug Administration (FDA) has recognized six additional serotypes among more than 200 STEC serotypes, called the "Big Six" (*E. coli* O26, O45, O103, O111, O121, and O145), as the most frequently isolated serotypes responsible for foodborne illnesses within various nations [3, 4]. Nearly 169,600 individuals are reported to fall ill each year from eating food contaminated with these serotypes in the US [3]. In Argentina, the incidence of HUS associated with STEC is among the highest worldwide, posing a significant public health concern [5]. Furthermore, many cases of severe foodborne illnesses due to STEC infections are also reported annually in China, Australia, Tanzania, and Egypt [3, 6, 7], while the global prevalence of the illness developed by these serogroups is probable to be inaccurately assessed or unseen. Contrary to other types of bacteria, the use of antibiotics worsens STEC illnesses given that medications can stimulate STEC, which leads to an increase in the production of Shiga toxins and the release of more toxins when the bacteria break down [8]. Hence, there remains a critical need for the development of vaccines capable of providing broad protection across multiple STEC serotypes to effectively mitigate the risk of STEC-related infections.

Vaccines are known to confer effective and long-term protective immunity, which is cheaper than diagnosis and treatment after an infection has emerged, and, most importantly, they can prevent or reduce the occurrence of this infection in susceptible populations or animal species, making vaccination an important intervention in reducing the burden and severity of infectious diseases [9]. Two vaccines against *E. coli* O157 infection in cattle have been developed and licensed thus far: Epitopix SRP (Willmar, USA), which uses cell membrane extracts generated from iron-restricted cultures, and Econiche (Econiche Corp, Belleville, Canada), which is based on type III secreted proteins (T3SPs) [9, 10]. Although both vaccines induced antibody responses that were effective in substantially reducing *E. coli* colonization, they could only provide protection against serotype O157, however, lacked cross-protection against non-O157 serotypes in STEC. Several research efforts are reportedly underway to develop new vaccines to address this challenge by identifying candidate alternative vaccine targets such as flagella, proteins associated with T3SS-mediated adhesion, or subunits of Stx [1, 11]. Furthermore, novel vaccine strategies were reported, such as bacterial ghosts, in which the bacteria are modified and the expression of lysis genes are controlled so that the bacteria are lysed to form an empty bacterial cell envelope, which has the same composition as living cells and is not infectious but induces a mucosal immune response, and DNA-based vaccines or adjuvant-boosted vaccines, which have been described to elicit a host immune response and to confer protection against STEC challenge in different animal models [10]. In summary, in addition to selecting the optimal vaccine targets, the immune responses induced by candidate vaccines should also be improved to provide broad protection.

Outer membrane vesicle (OMV) are spherical nanoscale structures naturally released from the outer membrane of gram-negative bacteria during the growth [12]. OMV is mainly composed of bacterial outer membrane components containing the major immunogenic antigens, including lipopolysaccharide (LPS), lipoproteins, and peptidoglycan, which makes OMV attractive as vaccine carriers [13, 14]. In recent years, bacteria have also been engineered for the production of OMV to increase the yield of OMV and to reduce their reactogenicity, while maintaining OMV immunogenicity. Furthermore, OMV can be decorated by heterologous antigens and is effective in promoting the immunogenicity of both homologous and heterologous antigens compared to traditional preparations such as subunit proteins and live attenuated vaccines [15, 16]. O-antigen is a component of the lipopolysaccharide (LPS) layer on the outer membrane of Gram-negative bacteria and represents an attractive target for the development of specific vaccines due to their surface exposure and high variability [17, 18]. Polysaccharide antigens, in most cases, are considered T-cell-independent antigens that do not have the ability to stimulate T cells helping for the promotion of antibody class switching, affinity maturation, or the development of memory B and T cells; nevertheless, when polysaccharides are covalently attached to suitable carriers, they can evoke a T-cell-dependent immune response against polysaccharide [19]. Polysaccharide vaccines have been successful in providing immunity against various bacterial infections, including invasive infections caused by pathogens such as *Salmonella* Typhi and *Neisseria meningitidis* [9, 20, 21]. The specificity and effectiveness of these vaccines depend on the accurate identification and formulation of the polysaccharide antigens, which can

induce an adaptive immune response to produce protective antibodies, thereby preventing bacterial colonization and invasion. The objectives of this work were not only to construct an engineered *S.* Typhimurium to rapidly produce OMV with low endotoxic activity for delivering O-antigen of *E. coli*, but also to evaluate the immunogenicity of OMV, which displays mixed O-antigen, and the ability to fight infection of multiple wild-type STEC in mice, therefore to provide a promising vaccine strategy for vaccine development of STEC (Fig. 1).

Materials and methods

Growth conditions, plasmids, and bacterial strains

Table 1 shows a detailed list of the bacterial strains and plasmids used in the current research. The wild-type *E. coli* was cultivated using either trypticase soy broth (TSB) (T861601, Macklin, China) or TSB agar (L1015, Solarbio Biotech, China) and *Salmonella* was cultivated either in Luria-Bertani (LB) broth (L1010, Solarbio Biotech, China) or on LB agar [22] at 37℃. If required, the culture media were introduced with 25 μg/ml of chloramphenicol (A600118, Sangon Biotech, China) or streptomycin (S875203, Macklin, China). To promote the growth of the *asd* gene mutant strains, 2,6-diaminopimelic acid (DAP) were added at a final concentration of 50 μg/ml (D1377, Sigma Aldrich, USA) [23]. To culture bacteria for allelic exchange assays, we performed counterselection using the *sacB* gene on LB agar complemented with 5% sucrose (A610498, Sangon Biotech, China).

Construction of mutant strains and polysaccharide synthesis plasmids

The Δ*pagP81*, Δ*pagP81*::P_{lpp}*lpxE*, Δ*tolR*, and Δ*asd* are successively integrated into SW121 (Δ*rfbP* Δ*fliC* Δ*fljB*) via the allelic exchange approach. The suicide plasmid pYA4278 was used for this process, consistent with the methodology outlined in a prior study [24]. Transformation of all plasmids into the corresponding strains was performed via electroporation in this study. To ensure the successful selection of transformants, the LB agar plate cultures were supplemented with the appropriate antibiotics or DAP. Details of the primers used throughout the study can be found in Supplementary Table 1. Suicide plasmid pYA4288, pYA4295, and pSS021 stored in our laboratory were used to generate *pagP81*, Δ*pagP81*::P_{lpp}*lpxE*, and *asd* mutations respectively. The *pagP81* and Δ*pagP81*::P_{lpp}*lpxE* mutations were successively integrated into the SW121 (Δ*rfbP* Δ*fliC* Δ*fljB*) through the allelic exchange using conjugation with the χ7213, which carries the pYA4288 or pYA4295 plasmid,

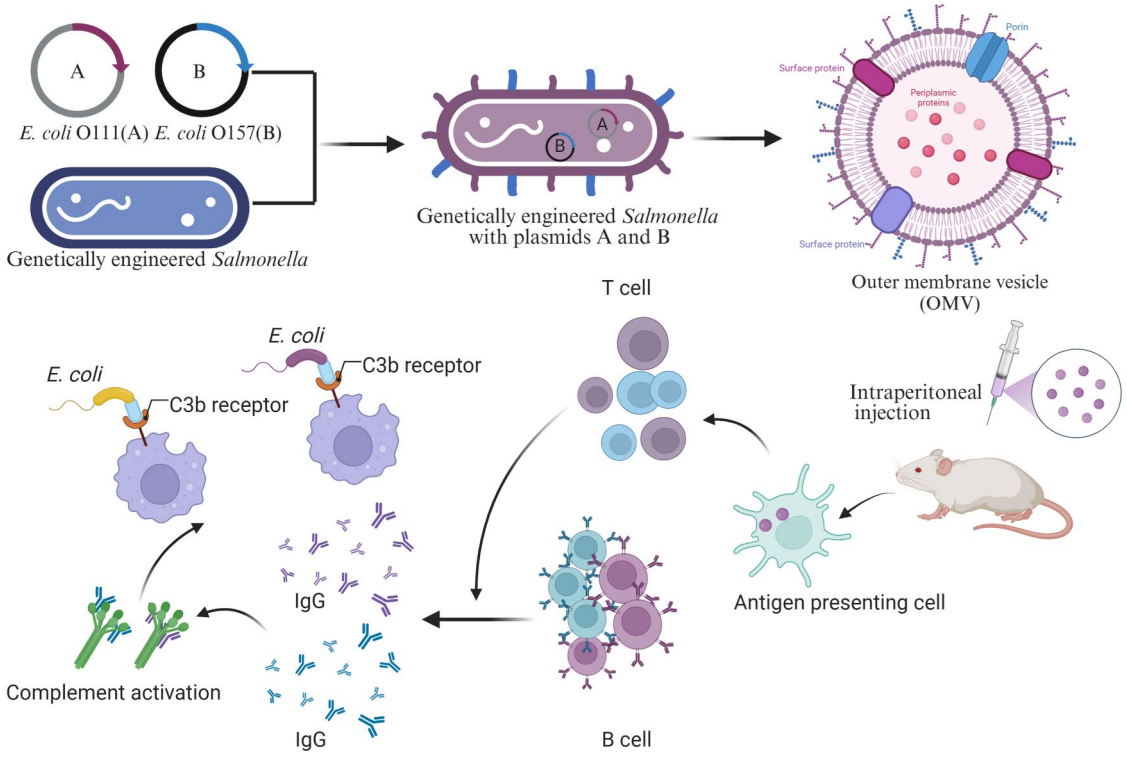

Fig. 1 The schematic diagram of *S.* Typhimurium derived OMV displaying mixed heterologous O-antigens induce an immune response in mice. Two recombinant plasmids synthesizing different *E. coli* O-antigens were introduced into engineered *Salmonella* to isolate OMV. The mice were immunized with OMV displaying mixed *E. coli* O-antigen by intraperitoneal injection. The O-antigen-specific IgG antibodies induced by our OMV possess significant biological functionality to provide protection against the infection of wild-type *E. coli*

Table 1 Bacterial strains and plasmids used in this study

Strains or plasmids	Descriptions	Source
***S. enterica*serovar Typhimurium strains**		
χ3761	Wild type *S.* Typhimurium, UK-1	[65]
SW121	Δ*rfbP* Δ*fliC* Δ*fljB*	Lab stock
SW122	Δ*rfbP* Δ*fliC* Δ*fljB* Δ*pagP81*	This study
SW123	Δ*rfbP* Δ*fliC* Δ*fljB* Δ*pagP81*::P_{lpp}*lpxE*	This study
SW124	Δ*rfbP* Δ*fliC* Δ*fljB* Δ*pagP81*::P_{lpp}*lpxE* Δ*tolR*	This study
SW125	Δ*rfbP* Δ*fliC* Δ*fljB* Δ*pagP81*::P_{lpp}*lpxE* Δ*tolR* Δ*asd*	This study
***E. coli*strains**		
χ7232	*endA1 hsdR17* (*rK-*, *mk+*) *glnV44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169* λpir *deoR* (φ*80dlac* Δ(*lacZ*)*M15*)	[66]
χ7213	*thi-1 thr-1 leuB6 glnV44 fhuA21 lacY1 recA1* RP4-2-Tc::Mu[λ pir] Δ*asdA4* Δ(*zhf-2*::Tn*10*)	[66]
G7572	Wild type *E. coli* O111:H8, which was isolated from human faeces and are resistant to streptomycin	Lab collection
G2734	Wild type *E. coli* O157:H7, which was isolated from human faeces and are resistant to streptomycin	Lab collection
G1652	Wild type *E. coli* O26:H46, which was isolated from human faeces	Lab collection
G2511	Wild type *E. coli* O45:K1:H10, which was isolated from human faeces	Lab collection
G1442	Wild type *E. coli* O103, which was isolated from human faeces	Lab collection
G1345	Wild type *E. coli* O145, which was isolated from human faeces	Lab collection
Suicide and cloning vectors		
pYA4278	*sacB mobRP4* R6K *ori* Cm^+, derived from pRE112	[24]
pYA4288	*pagP8* deletion	[17]
pYA4295	*ΔpagP* deletion, and P_{lpp}*lpxE* insertion	[17]
pSS021	for Δ*asd* mutation, suicide plasmid	[30]
pSS264	for Δ*tolR* mutation, suicide plasmid	This study
Recombinant plasmids		
pSW047	Asd^+ vector, pSC101 ori, P_{trc} promoter, the same strain as pG8R184	[29]
pSW048	p15a ori, *cat*, P_{lpp} promoter	Lab collection
pSW049	pSW047 vector carrying genes involved in the biosynthesis of *E. coli* O111 O-antigen (*wbdH-wbdI* fused with *wbdJ-wbdM*)	This study
pSW050	pSW048 vector carrying genes involved in the biosynthesis of *E. coli* O157 O-antigen (*wbdN-wbdQ* fused with *wbdR*, and *gne*)	This study
pSW051	pSW047 vector carrying genes involved in the biosynthesis of *E. coli* O26 O-antigen (*wxz-wbuC*)	This study
pSW052	pSW048 vector carrying genes involved in the biosynthesis of *E. coli* O45 O-antigen (*wbhP-wbhS* fused with *tll-wbhW*)	This study
pSW053	pSW047 vector carrying genes involved in the biosynthesis of *E. coli* O145 O-antigen (*wckD-wbuC*)	This study
pSW054	pSW048 vector carrying genes involved in the biosynthesis of *E. coli* O103 O-antigen (*wbtA-gne*)	This study
Strains for isolating OMV		
OMV1	Isolation from SW125 electroplated with pSW049	
OMV2	Isolation from SW124 electroplated with pSW050	
OMV3	Isolation from SW125 electroplated with pSW049 and pSW050	
OMV4	Isolation from SW125 electroplated with pSW051	
OMV5	Isolation from SW124 electroplated with pSW052	
OMV6	Isolation from SW125 electroplated with pSW051 and pSW052	
OMV7	Isolation from SW125 electroplated with pSW053	
OMV8	Isolation from SW124 electroplated with pSW054	
OMV9	Isolation from SW125 electroplated with pSW053 and pSW054	

to result in strains of SW122 (Δ*rfbP* Δ*fliC* Δ*fljB* Δ*pagP81*) or SW123 (Δ*rfbP* Δ*fliC* Δ*fljB* Δ*pagP81*::P_{lpp}*lpxE*). The pSS264 plasmid was constructed for generating *tolR* mutation in this study. The TolR-1 F/TolR-1R, TolR-2 F/TolR-2R, and Vec-F and Vec-R primers were applied to amplify the left homology arm, right homology arm of the *tolR* gene, and vector DNA fragments, respectively. The DNA segments possess homologous areas allowing recombination in their 5′ flanking regions. The DNA fragments of the *tolR* gene were coupled to pYA4278 through Gibson Assembly Master Mix (E55510, New England Biolabs, USA) to result in pSS264 for deleting the whole open reading frame of the *tolR* gene. The Δ*tolR* was integrated into the SW123 (Δ*rfbP* Δ*fliC* Δ*fljB* Δ*pagP81*::P_{lpp}*lpxE*) through the allelic exchange using conjugation with the χ7213 harboring the pSS264 to yield the strain of the SW124 (Δ*rfbP* Δ*fliC* Δ*fljB* Δ*pagP81*::P_{lpp}*lpxE* Δ*tolR*). The same approach was also utilized for removing the *asd* gene (Table 1).

The cloning vectors used in this study for the synthesis of O-antigen were pSW047, which contained the pSC101 replicon, the *asd* gene, and the P_{trc} promoter, and pSW048, which had the p15a replicon, the *cat* gene, and the P_{lpp} promoter. Taking the construction of the plasmid to clone the O-antigen gene cluster of *E. coli* O111 as an example, we deleted the *manB* and *manC* genes responsible for mannose synthesis in the O-antigen synthesis gene cluster and only cloned the remaining gene clusters (*wbdH-wbdI* fused with *wbdJ-wbdM*) into the pSW047 backbone. In brief, universal primers Vec-1 F/Vec-1R were applied to amplify DNA fragments of the pSW047 vector, and primers O111-1 F/O111-1R were used to amplify an 8077-bp DNA fragment from the *E. coli* O111 genome, and the DNA fragment was ligated to the vector pSW047 through Gibson Assembly Master Mix, to generate the plasmid pSW049 for the biosynthesis of *E. coli* O111 O-antigen polysaccharide (*wbdH-wbdI* fused with *wbdJ-wbdM*). The same method was used for the construction of other plasmids for synthesizing O-antigen polysaccharides in this study.

Production and purification of OMV

OMV derived from engineered *Salmonella* was isolated and purified following a previously published protocol [25]. Briefly, the corresponding engineered *Salmonella* strains were shakily cultivated (180 rpm) in 6 L fresh LB broth with or without appropriate antibiotic at 37°C until the bacterial optical density value at 600 nm (OD_{600}) reached 0.9. The supernatant of bacterial culture was harvested by centrifugation at 6500 rpm at 4℃ and the clear liquid was filtered through a 0.45 μm membrane filter. Then OMV was isolated from the filtered clear supernatant by ultracentrifugation (60,000 × g, 3 h, 4℃). The OMV obtained by ultracentrifugation in the previous step was collected and resuspended in sterile PBS (BL302A, Biosharp, China), followed by purification of the OMV via density gradient centrifugation (150, 000 × g, 3 h, 4℃) in discontinuous OptiPrep™ medium (5-15%-25-40%) (00122, Serumwerk Bernburg, Germany). Finally, the purified OMV was gathered and filtered through a 0.22 μm membrane filter for sterilization.

Characterization of OMV

The structure morphology of the OMV purified in this study was observed by negative-staining EM, which was performed by Hangzhou Yanqu Information Technology Co., Ltd. The total protein concentration of OMV was determined and normalized based on a bicinchoninic acid protein assay (ZJ101, Yazyme, China). The LPS was purified from *Salmonella* based on the described methods [26, 27]. The LPS samples or OMV were separated using a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and subsequently went through silver staining to establish their LPS profile (2 μg of samples in each lane), following the method described by Hitchcock and Brown [28]. The OMV was transferred onto nitrocellulose membranes after separation, and the western blot analysis was performed as previously described using specific rabbit anti-O-antigen antisera, which was previously obtained in the lab [29, 30]. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (BS13278, Bioworld Technology, China) was used as the secondary antibody.

Animal experimental studies

Mice. The 7-week-old female or male BALB/c mice used for the experiment were purchased from Hunan SJA Laboratory Animal Co., Ltd. All animal experiments were adhered to the rules specified in the Guide for the Care and Use of Laboratory Animals. Conscientious efforts were made to avoid animal suffering during the experiments. All arrived animals were given a seven-day acclimatization period before the start of the experiment.

Immunization of mice. 5–10 mL of ether-soaked (10009318, Sinopharm, China) skimmed cotton wool was spread on the bottom of the glass container, then the mouse was put in, the lid was put on, and the animal entered anesthesia for 20–30 s. Mice were injected intraperitoneally with 100 μl of PBS containing 1 μg of OMV per mouse after anesthetic, and an intraperitoneal injection of 100 μl PBS or OMV derived from *Salmonella* containing empty vector (named as OMV vector) was administered as a negative control. Two weeks after the initial immunization, mice were received a subsequent dose of the identical OMV as a boost. The vaccinated mice were closely monitored for abnormalities, overall changes in physical health, as well as reduced food intake.

In addition, any signs of disheveled fur, diarrhea, illness, and death were documented.

Immunogenicity of OMV in mice. To evaluate the immunogenicity of OMV displaying mixed O-antigens, 64 female or male mice were randomly divided into 8 groups (8 mice per group), including 4 experimental groups (1 OMV1 group, 1 OMV2 group, and 2 OMV3 groups) and 4 control groups (2 PBS control groups, 2 OMV vector control groups). To assess the immunogenicity of physical mixtures of three different OMVs, 144 female mice were randomly divided into 18 groups (8 mice per group), including 6 experimental groups and 12 control groups (6 PBS control groups, 6 OMV vector control groups). These three OMVs were physically mixed in PBS and administered intraperitoneally to six groups of mice on days 0 and 14 with a dose containing 1 μg of each OMV in 100 μl PBS after anesthesia with ether. Two weeks after the primary immunization, animals were administered an additional dose of the same OMV or PBS that was the same as the prior dosage. After mice were anesthetized with ether (10009318, Sinopharm, China), blood samples were collected by mandibular venipuncture at 14, 35, 49, and/or 120 days after the initial immunization. The serum was isolated from the blood cells using centrifugation at a speed of 3500 rpm for a total of 15 min, then aspirated with as much of the supernatant as possible, transferred to a clean 0.5-ml tube, and centrifuged again at 3500 rpm for 5 min. The supernatant was once again gathered into a 0.5-ml tube and then preserved at -20°C if it was needed. To evaluate the IgA in mice triggered by OMV displaying mixed O-antigens, the vaginal canal of each female mouse was rinsed with 100 μl of PBS at designated time intervals, and the vaginal wash was stocked in 1.5 ml tubes. The vaginal wash was kept at low temperatures throughout the entire process and harvested as it is used, avoiding repeated freezing and thawing. In addition, fecal shedding of female and male mice was weighed and homogenized at a concentration of 100 mg/ml in PBS with 0.1 mg/ml soybean trypsin inhibitor (SG2033, Beyotime, China) and then centrifuged at a speed of 3500 rpm for a total of 15 min to collect the supernatant of fecal samples, respectively. The concentration of *E. coli* LPS-specific IgG and/or IgA antibodies in serum, fecal samples, and/or vaginal wash was quantified via enzyme-linked immunosorbent assay (ELISA) in accordance with established protocols [31–33].

Survival assay. To determine the minimum lethal dose of wild-type *E. coli* to mice, wild-type *E. coli* was cultivated in 50 ml of TSB broth to an OD_{600} of approximately 0.85 (~1×10^9 CFU/ml). The strain pellet was collected by centrifugation of the bacterial suspension and resuspended in 1 ml of sterile PBS after being rinsed twice in sterile PBS (~1×10^9 CFU/20 μl). Subsequently, the bacteria were subjected to a 2-fold serial dilution in sterile PBS, and then 100 μl was administered intraperitoneally to the mice, and the survival of the mice was recorded. The exact dose was calculated by making a gradient dilution of the corresponding 100 μl of bacterial solution and dropping it on an LB agar plate for counting. The protection rates of the immunized mice were assessed at week 8 post-initial immunization by the intraperitoneal injection challenge according to the minimum lethal dose of wild-type *E. coli* in this work. Mice that experienced the challenge were observed each day for signs of disheveled fur, agitation, digestive issues, sickness, and death. These observations were documented over a period of 12 days following the challenge. After the experiment, each mouse was euthanized by cervical dislocation after intraperitoneal injection of an overdose of pentobarbital (200 mg/kg).

Bacterial loads in feces. To investigate the level of wild-type *E. coli* O111 and O157 in the fecal shedding of immunized mice following infection with *E. coli* via the oral route [34], 48 female mice were randomly divided into 8 groups (6 mice per group), including 4 experimental groups (1 OMV1 group, 1 OMV2 group, and 2 OMV3 groups) and 4 control groups (2 PBS control groups, 2 OMV vector control groups). Mice were provided unrestricted access to water added with streptomycin (5 g/l) (S875203, Macklin, China) for three days preceding infection to clean the intestinal flora. Furthermore, the fecal shedding was determined to be devoid of streptomycin-resistant *E. coli* at the point of infection. The wild-type *E. coli* O111 and O157, which were isolated from human feces and are resistant to streptomycin, were cultivated in 50 ml of TSB broth to an OD_{600} of approximately 0.85 (~1×10^9 CFU/ml), respectively. The strain pellet was collected by centrifugation of the bacterial suspension and resuspended in 1 ml of sterile PBS (approximately 1×10^9 CFU/20 μl) after being rinsed twice in sterile PBS. Then, mice were orally infected with 20 μl of a bacteria suspension comprising about 1×10^9 CFU of the *E. coli* strain at 5 weeks after the first immunization, which is a non-lethal dose. Following the infection, water with added streptomycin was given back. Subsequently, fecal shedding was collected every three days and documented for the challenged strain. Mice were individually placed in sanitized cages devoid of bedding on days 3, 6, 9, and 12 post-infection, and an amount of 3–5 fecal pellets was obtained in a 1.5-ml tube. Fecal pellets were weighed and homogenized in phosphate-buffered saline (0.1 g/ml). The quantification of bacteria per 1 g of feces was conducted by applying serially diluted or undiluted fecal homogenate over MacConkey agar (M8560, Solarbio Biotech, China) plate containing 25 μg/ml streptomycin. After the experiment, each mouse was euthanized by

cervical dislocation after intraperitoneal injection of an overdose of pentobarbital (200 mg/kg).

Complement deposition assays

Serum samples from the identical group of female mice obtained earlier on day 49 after primary vaccination were blended and inactivated at 56°C for 30 min. The wild-type *E. coli* O111 or O157 strain of 1×10^7 CFU was suspended in 80 µl of PBS with 1% BSA and mixed with 20 µl of heat-inactivated pooled serum from the relevant group. The mixtures were then incubated at 37°C for 30 min. Subsequently, the treated *E. coli* cells were washed twice with PBS and exposed to 100 µl of PBS-1% BSA solution containing 50 µl of baby rabbit complement (CL3441-R, Cedarlane, Canada) and incubated at 37 °C for 30 min. The treated *E. coli* strain in the previous step was washed twice with PBS, and then the *E. coli* strain was stained with FITC-conjugated goat anti-rabbit complement polyclonal antibody (ab182878, Abcam, UK) at a final concentration of 1:100 in PBS with 1% BSA on ice for 25 min. Finally, the strain was resuspended in 500 µl of 2% paraformaldehyde after two washes with PBS, and the samples were examined using flow cytometry (FongCyte, Beijing Challen Biotechnology Co., Ltd). Sera for negative controls were obtained from mice injected with OMV vector. Reaction systems containing only bacteria, bacteria and fluorescent antibodies, or complement and bacteria were also set as negative controls. The experiment was conducted three times, a representative result is displayed.

E. Coli-specific serum bactericidal antibody (SBA) assay

Serum samples from the identical group of female mice obtained earlier on day 49 after primary vaccination were blended and inactivated at 56°C for 30 min. Then, heat-inactivated serum samples were added to 96-well U-bottom plates in triplicate and 2-fold serial dilution in TSB broth. 10 µl containing 1000 CFU of wild-type *E. coli* O111 or O157 and 25 µl of baby rabbit complement (CL3441-R, Cedarlane, Canada) were added to the wells. The total reaction volume was 100 µl and the initial serum dilution was 1:2 in this study. The plates were placed in a shaker (180 rpm) and incubated at 37℃ for 1 h. The quantification of viable CFU was performed by titrating 10 µl of the reaction mixture onto TSB agar plates, followed by colony counting after an overnight incubation at 37°C. As negative controls, wells with bacteria and baby rabbit complement were added. The proportion of killed bacteria (per well) was calculated using the formula [1 - (number of CFU in the test well/number of CFU in the negative control well)] × 100.

Adherence inhibition assay

INT407 cells were routinely cultured in DMEM medium (C3113, Vivacell, China) added with 10% fetal bovine serum (FBS) (C04001, Vivacell, China) and 1% penicillin-streptomycin (C3420, Vivacell, China) at 37°C with 5% CO_2. When starting the experiment, the INT407 cells were suspended in DMEM medium added with 10% FBS (without penicillin-streptomycin) and plated in 24-well plates (~5×10^5 cells/well). The wild-type *E. coli* O111 and O157 were cultivated in 20 ml of TSB broth to an OD_{600} of approximately 0.85 (~1×10^9 CFU/ml), respectively. The strain pellet was collected by centrifugation of the bacterial suspension and resuspended in 20 ml of DMEM culture medium after being rinsed twice in the DMEM culture medium. Serum samples from the identical group of female mice obtained earlier on day 49 after primary vaccination were blended and inactivated at 56°C for 30 min. The wild-type *E. coli* O111 or O157 strain of 1×10^7 CFU was suspended in 1 ml DMEM medium or 800 µl of DMEM medium supplemented with 200 µl of inactivated pooled serum (1:5) from the relevant group. The mixtures were then incubated at 37°C for 30 min. After that, the treated bacteria of 1×10^6 CFU/100 µl were used to infect INT407 cells for 2 h at 37°C with 5% CO_2. Three washes with PBS eliminated non-adherent bacteria, while adherent bacteria were retrieved using lysis with 0.1% SDS, serially diluted in PBS, and subsequently plated on MacConkey agar for quantification. The results were calculated as the percentage of adhering bacteria relative to the initial quantity of introduced bacteria, normalized to the proportion of bacterial adhesion when incubated in DMEM medium (without serum).

Statistical analysis

Graph-Pad Prism 8.0 software was employed for statistical analysis. Unless stated otherwise, data were expressed as means±SEM. Parametric tests were performed on data that passed the Gaussian distribution and the equal variance test, if not non-parametric tests were utilized. To evaluate variations in antibody levels and adherence inhibition assay, we performed one-way ANOVA, followed by Bonferroni's multiple comparison tests. Statistical differences of the SBA assay and bacterial loads in feces were assessed by a two-way ANOVA test followed by Tukey's multiple comparison test. Mouse survival differences were analyzed using the log-rank test, with the Kaplan-Meier survival curve used for monitoring. The least significant difference test was utilized for mean comparisons. A p-value of less than 0.05 was deemed statistically significant.

Results

Engineered *S.* Typhimurium OMV can display mixed O-antigens

To obtain a strain capable of producing OMV with low endotoxic activity for delivery of *E. coli* O-antigen, we successively introduced the Δ*pagP81*, Δ*pagP81*::P_{lpp}*lpxE*, Δ*tolR*, and Δ*asd* mutations in the genome of SW121 (Δ*rfbP* Δ*fliC* Δ*fljB*) by allelic exchange via conjugation with the χ7213, harboring the plasmid of pYA4288, pYA4295, pSS264, or pSS021, to generate SW125 (Δ*rfbP* Δ*fliC* Δ*fljB* Δ*pagP81*::P_{lpp}*lpxE* Δ*tolR* Δ*asd*) (Table 1) [17]. Figure 2 showed the O-antigen gene clusters generally located between the *galF* and *gnd* genes on the *S.* Typhimurium and *E. coli* genome and their chemical structures, revealing the differences in glycosyltransferase genes, glycan compositions, and linkages within the O-unit. To rationally design and construct plasmids capable of synthesizing *E. coli* O111 and O157 O-antigen in strain SW125 (Δ*rfbP* Δ*fliC* Δ*fljB* Δ*pagP81*::P_{lpp}*lpxE* Δ*tolR* Δ*asd*), DNA fragment of O-antigen operons involved in the biosynthesis of the *E. coli* O-antigen (Fig. 2C) were integrated with the pSW047 or pSW048 DNA fragment to generate the corresponding plasmids under the control of P_{trc} promoter or P_{lpp} promoter, respectively (Table 1). Subsequently, these plasmids were introduced into the strains SW125 (Δ*rfbP* Δ*fliC* Δ*fljB* Δ*pagP81*::P_{lpp}*lpxE* Δ*tolR* Δ*asd*) or SW124 (Δ*rfbP* Δ*fliC* Δ*fljB* Δ*pagP81*::P_{lpp}*lpxE* Δ*tolR*) to obtain the corresponding engineered *S.* Typhimurium strains, which were used to isolate and purify OMV (Table 1).

OMV isolated from the strain SW125 (Δ*rfbP* Δ*fliC* Δ*fljB* Δ*pagP81*::P_{lpp}*lpxE* Δ*tolR* Δ*asd*) harboring the plasmid pSW049 (named as OMV1) were morphologically characterized by negative-staining EM. The EM image showed that OMV were spherical nanoparticles with most diameters ranging from 50 to 250 nm, indicating successful isolation and purification of OMV from engineered *S.* Typhimurium culture media by a combination of ultrafiltration, ultracentrifugation, and density gradient ultracentrifugation (Fig. 3A). The O-antigen on the surface of OMV was visualized by silver staining and confirmed by western blotting (Fig. 3B, C and D). The OMVs isolated from the strains SW125 harboring pSW049 (O111) and SW124 harboring pSW050 (O157) (named as OMV2) exhibited a typical LPS banding pattern similar to the wild-type strain, compared with the strain SW124 (Δ*rfbP* Δ*fliC* Δ*fljB* Δ*pagP81*::P_{lpp}*lpxE* Δ*tolR*), which failed to show the whole LPS banding pattern due to a lack the glycosyltransferase RfbP, which transfers and links Galactose-1-phosphate (Gal-1-P) to undecaprenyl-phosphate (Und-P) (Fig. 3B) [35, 36]. To investigate whether mixed-*E. coli* O-antigen can be synthesized in engineered *S.*

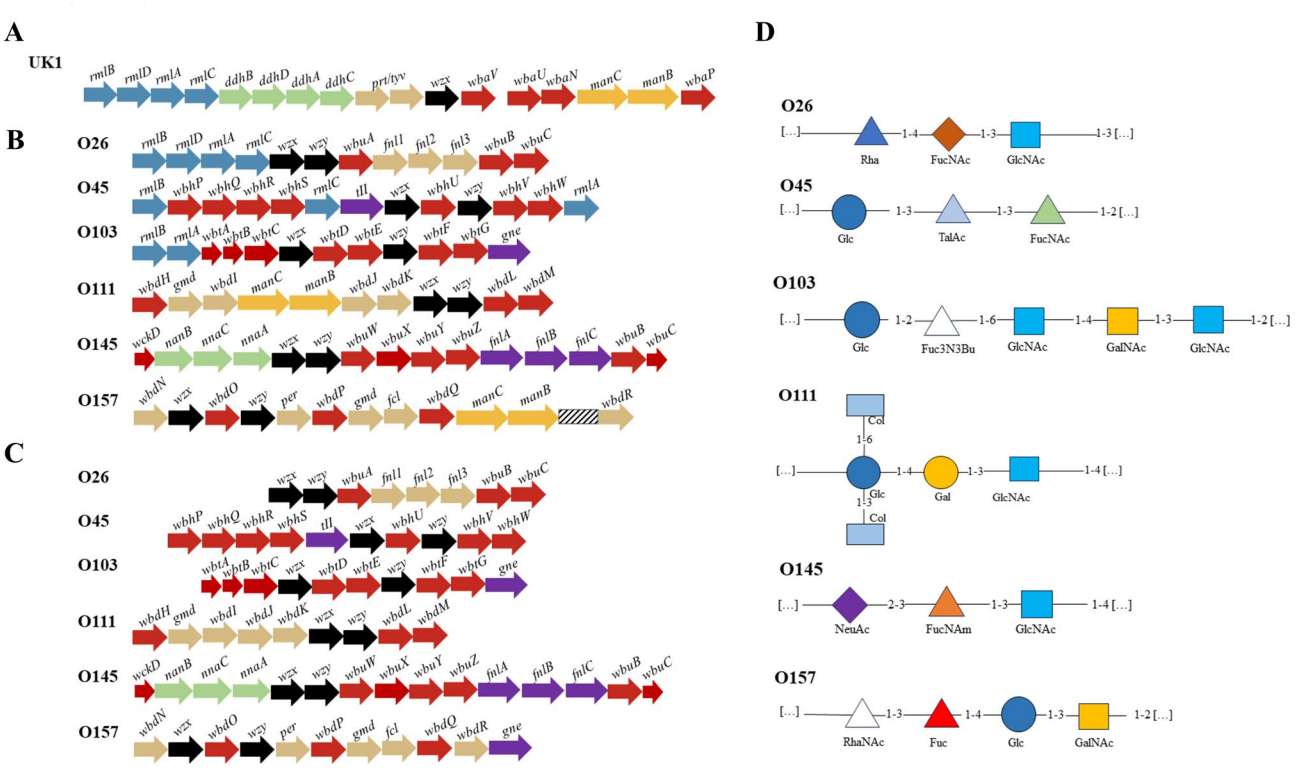

Fig. 2 The schematic diagram of the O-antigen operons of *S.* Typhimurium and *E. coli* and chemical structures of *E. coli* O-antigen. (**A**) The whole O-antigen operon of *S.* Typhimurium. (**B**) The whole O-antigen gene cluster of *E. coli* O26, O45, O103, O111, O145, and O157. (**C**) The truncated O-antigen operon of *E. coli* O26, O45, O103, O111, O145, and O157. (**D**) The chemical structures of *E. coli* O26, O45, O103, O111, O145, and O157

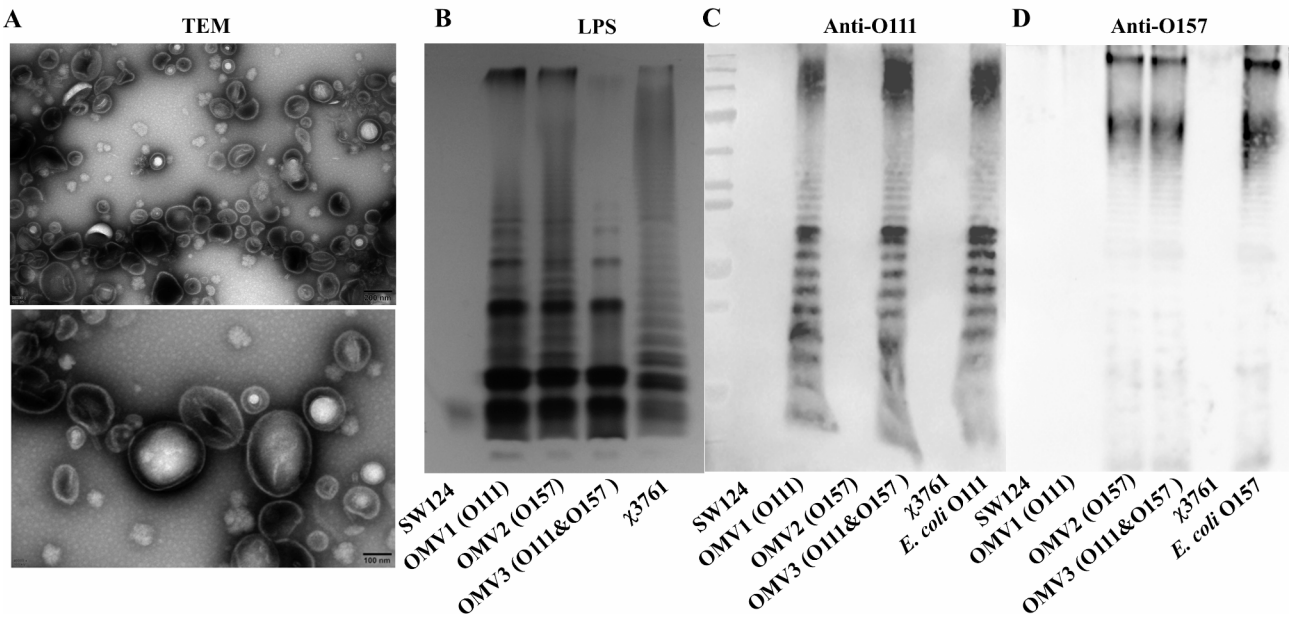

Fig. 3 The characteristics of OMV derived from engineered *S.* Typhimurium for delivering *E. coli* O-antigen. (**A**) OMV derived from engineered *S.* Typhimurium SW125 electroplated with pSW049, which serotype converted to *E. coli* O111, was visualized by negative-staining EM. (**B**) Identification of OMV delivering *E. coli* O111, O157, or mixed O-antigen by their LPS profile and silver staining. (**C**) Determination of OMV delivering *E. coli* O111 and mixed O-antigen by western blotting. The primary antibody used for detection was a rabbit polyclonal antibody specific for *E. coli* O111 O-antigen. (**D**) Detection of OMV delivering *E. coli* O157 and mixed O-antigen by western blotting. The primary antibody used for detection was a rabbit polyclonal antibody specific for *E. coli* O157 O-antigen

Typhimurium and displayed on the surface of its OMV, the strain SW125 with plasmids pSW049 and pSW050, which carry the fragments of the O-antigen gene cluster of *E. coli* O111 and *E. coli* O157, respectively, was used to isolate OMV (named as OMV3). The results of the silver-stained profiles indicated that OMV3 also showed a typical ladder LPS banding pattern (Fig. 3B). The western blotting results revealed that OMV1 (O111) or OMV2 (O157) displayed LPS recognized by sera against the corresponding *E. coli* O111 or O157 O-antigen (Fig. 3C and D), and the O-antigen exhibited by OMV3 (O111&O157) reacted not only with anti-O111 sera but also with anti-O157 sera (Fig. 3C and D). These results indicated that we successfully cloned the O-antigen operons of *E. coli* and engineered *S.* Typhimurium to synthesize and display mixed *E. coli* O-antigen on OMV surface.

OMV displaying mixed O-antigens elicited robust O-antigen-specific humoral immune responses

In order to investigate the immunogenicity of OMV displaying mixed *E. coli* O-antigen, 64 female mice were randomly divided into 8 groups of 8 mice each, including 4 experimental groups (1 OMV1 group, 1 OMV2 group, and 2 OMV3 groups) and 4 control groups (2 PBS control groups, 2 OMV vector control groups), and immunized with 100 μl containing 1 μg of OMV1 (O111), OMV2 (O157), or OMV3 (O111&O157) by the intraperitoneal route on day 0 and boosted with the same dose on day 14, respectively, and 100 μl of PBS and OMV vector were served for control groups. Serum IgG and IgG isotype subclasses IgG1 and IgG2a responses to *E. coli* O111 and/or O157 LPS were measured by ELISA 2 weeks, 5 weeks, and/or 7 weeks after primary immunization. Both OMV1 (O111) and OMV3 (O111&O157) triggered a significant increase in the level of *E. coli* O111 LPS-specific IgG on day 14 after initial immunization compared to the PBS and OMV vector control group (Fig. 4A). In addition, *E. coli* O111 LPS-specific IgG levels increased over time after inoculation, and there was no significant difference in *E. coli* O111 LPS-specific IgG levels between sera from group OMV1-immunized mice and sera from group OMV3-immunized mice (Fig. 4B and C). Although OMV2 (O157) or OMV3 (O111&O157) elicited less specific IgG against *E. coli* O157 LPS in mice at 2 weeks after the initial immunization (Fig. 4A), they triggered similarly significant *E. coli* O157 LPS-specific IgG levels in mice at 5 weeks and 7 weeks after the initial immunization compared to the control group (Fig. 4B and C). The OMV3 (O111&O157) induced significantly higher levels of O111-specific antibodies than O157-specific antibodies in mice (Fig. 4B and C). Further analysis showed that IgG2a was the predominant subtype of *E. coli* O157 LPS-specific IgG provoked by the OMV2 (O157) (Fig. 4D, E and F). On the contrary, the mice vaccinated with OMV1 (O111) mounted a similar level of anti-*E. coli* O111 LPS IgG1 and IgG2a immune response (Fig. 4D,

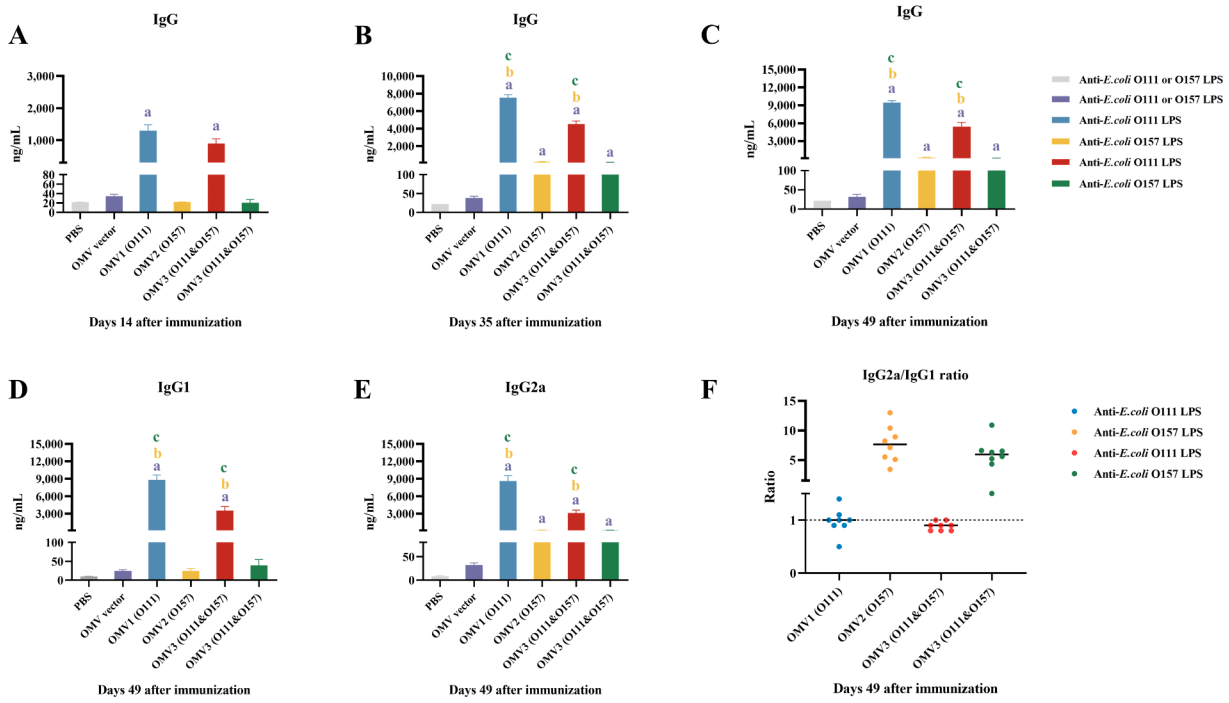

Fig. 4 Humoral immune response to LPS from *E. coli* O111 or O157 in female mice induced by the OMV for delivering *E. coli* O-antigen. Serums of immunized female mice were gathered at days 14, 35, and 49 post-initial vaccination. Quantitative ELISA was applied to analyze specific IgG at days 14 (**A**), 35 (**B**), and 49 (**C**) post-initial vaccination against LPS from *E. coli* O111 and/or O157, as well as the subtypes IgG1 (**D**) and IgG2a (**E**). (**F**) The ratio of IgG2a/IgG1. The results displayed the precise levels of antibodies, as measured by a standard curve, in female mice intraperitoneally inoculated with the OMV delivering *E. coli* O-antigen at the scheduled weeks. The standard differences between the mice in each group were shown by the error bars. Data are presented as the means ± SEM ($n = 8$). Statistical differences were assessed by a one-way ANOVA test followed by Bonferroni's multiple comparison test; superscript letters a, b, and c indicate $P < 0.05$ for comparison with *E. coli* LPS-specific IgG antibody levels in the OMV vector group, *E. coli* O157 LPS-specific IgG antibody levels in the OMV2 group, and *E. coli* O157 LPS-specific IgG antibody levels in the OMV3 group, respectively

E and F). Interestingly, although OMV3 (O111&O157) displayed both *E. coli* O111 and O157 O-antigen, it induced similar levels of *E. coli* O111-LPS-specific IgG1 and IgG2a subtypes in mice, and *E. coli* O157-LPS-specific levels of IgG1 and IgG2a subtypes remained IgG2a-dominant (Fig. 4D, E and F). These results imply a shift towards a Th1-biased immune response against *E. coli* O157 O-antigen elicited by OMV2 (O157) and OMV3 (O111&O157), whereas a balance between the Th1 and Th2 responses against *E. coli* O111 O-antigen stimulated by OMV1 (O111) and OMV3 (O111&O157).

We also assess the levels of IgA antibodies specific to *E. coli* O111 and/or O157 O-antigen in serum, vaginal wash, and fecal shedding triggered by OMV1 (O111), OMV2 (O157), and OMV3 (O111&O157) at 5 weeks following primary immunization by ELISA. The results showed that no significant specific IgA antibody was detected in all experimental groups relative to the PBS and OMV vector control group, suggesting that not only OMV3 (O111&O157) but also OMV1 (O111) and OMV2 (O157) failed to provoke mucosal immunity in this study (Supplementary Fig. 1). The levels of IgG and IgA antibodies specific to *E. coli* O111 and/or O157 O-antigen induced by OMV1 (O111), OMV2 (O157), and OMV3 (O111&O157) in male mice were consistent with the results in female mice (Supplementary Fig. 2).

Sera from mice immunized with OMV displaying *E. Coli* O-antigen mediated high levels of C3 complement deposition on wild-type *E. Coli*

Serum antibody-mediated complement deposition is an essential process through which the host immune system protects against pathogens and modulates the immune response, and it is regarded as a potential in vitro marker of protection against many pathogens [37]. The serum antibody-mediated complement deposition was carried out to investigate the functionality of serum antibodies generated by engineered *S.* Typhimurium OMV displaying single or mixed *E. coli* O-antigens. The pooled serum from immunized female mice in all experimental groups mediated similar levels of C3 deposition on the corresponding *E. coli* strains (Fig. 5A and B). No complement C3 deposition was observed on bacteria when exposed to serum from the OMV vector group (Fig. 5A and B). Reaction systems containing only bacteria, bacteria and fluorescent antibodies, or complement and bacteria were

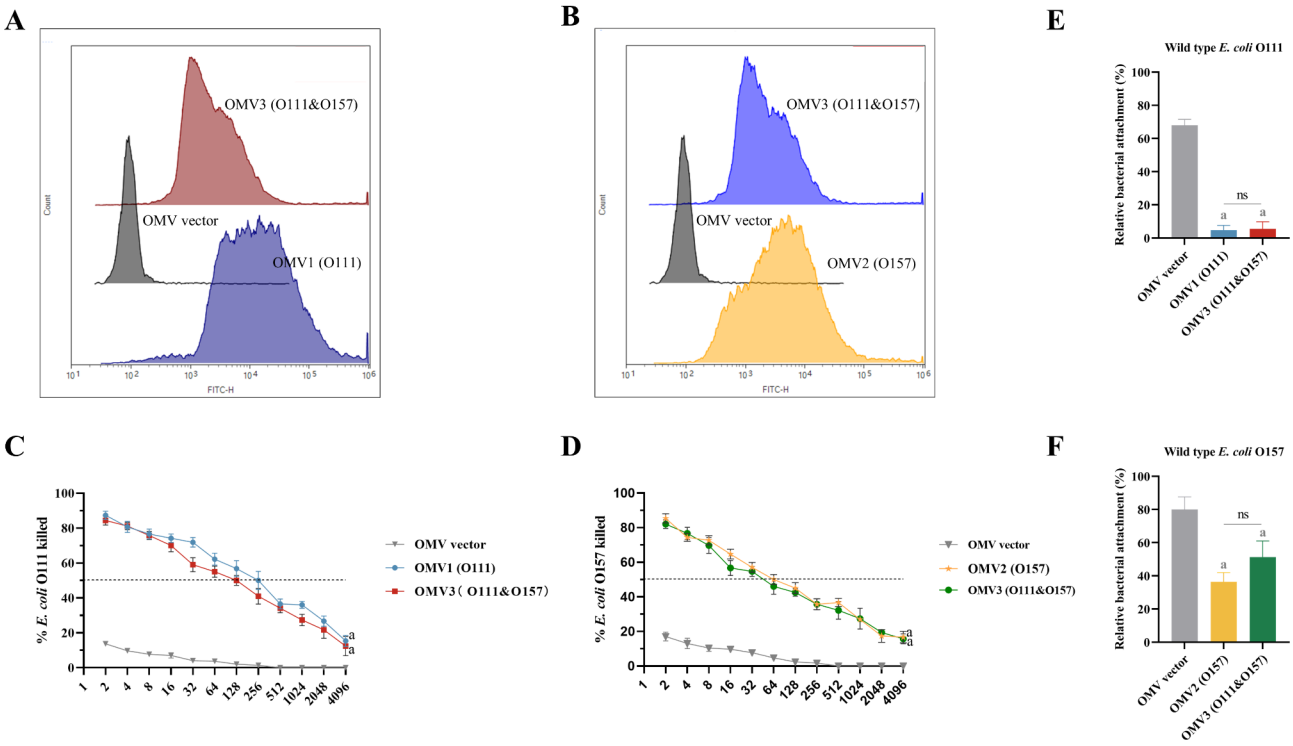

Fig. 5 Detection of serum antibody biological functionality in vitro. (**A**, **B**) Serum antibody-mediated complement deposition. Serum samples from the identical group of female mice obtained earlier on day 49 after primary vaccination were blended and inactivated at 56°C for 30 min. Wild-type *E. coli* O111 (**A**) or *E. coli* O157 (**B**) strain of 1×10^7 CFU was resuspended in 80 µl of PBS with 1% BSA and mixed with 20 µl of heat-inactivated pooled serum from the relevant group. The mixtures were incubated at 37°C for 30 min. The treated wild-type *E. coli* O111 or *E. coli* O157 cells were washed with PBS and exposed to 100 µl of PBS-1% BSA solution containing rabbit complement and incubated at 37°C for 30 min. Then, the *E. coli* strains were stained with FITC-conjugated goat anti-rabbit complement polyclonal antibody and examined using flow cytometry. Sera for negative controls were obtained from mice injected with OMV vector. The experiment was conducted three times, a representative result is displayed. (**C**, **D**) Serum bactericidal antibody assay. Serum samples from the identical group of female mice obtained earlier were added to 96-well U-bottom plates in triplicate and serial 2-fold ratio dilutions in TSB broth. 1000 CFU/10 µl of wild-type *E. coli* O111 (**C**) or O157 (**D**) and 25 µl of baby rabbit complement were added to the wells. The total reaction volume was 100 µl and the initial serum dilution was 1:2 in this study. The plates were placed in a shaker (180 rpm) and incubated at 37°C for 1 h. The quantification of viable CFU was performed by titrating 10 µl of the reaction mixture onto TSB agar plates, followed by colony counting after an overnight incubation at 37°C. As negative controls, wells with bacteria and baby rabbit complement were added. The proportion of killed bacteria (per well) was calculated using the formula [1 - (number of CFU in the test well/number of CFU in the negative control well)] × 100. The standard differences between the mice in each group were shown by the error bars. Data are presented as the means ± SEM ($n = 3$). Statistical differences were assessed by a two-way ANOVA test followed by Tukey's multiple comparison test; superscript letters a indicate $P < 0.05$ for comparison with the OMV vector group. (**E**, **F**) Adherence inhibition assay. The INT407 cells were suspended in DMEM medium added with 10% FBS (without penicillin-streptomycin) and plated in 24-well plates (~ 5×10^5 cells/well). The wild-type *E. coli* O111 (**E**) or O157 (**F**) strain of 1×10^7 CFU was suspended in 1 ml DMEM medium or 800 µl of DMEM medium supplemented with 200 µl of previously heat-inactivated pooled serum (1:5) from the relevant group. The mixtures were then incubated at 37°C for 30 min. After that, the treated bacteria of 1×10^6 CFU/100 µl were used to infect INT407 cells for 2 h at 37°C with 5% CO_2. Three washes with PBS eliminated non-adherent bacteria, while adherent bacteria were retrieved using lysis with 0.1% SDS, serially diluted in PBS, and subsequently plated on MacConkey agar for quantification. The results were calculated as the percentage of adhering bacteria relative to the initial quantity of introduced bacteria, normalized to the proportion of bacterial adhesion when incubated in DMEM medium (without serum). The standard differences between the mice in each group were shown by the error bars. Data are presented as the means ± SEM ($n = 3$). Statistical differences were assessed by a one-way ANOVA test followed by Bonferroni's multiple comparison test; superscript letters a indicate $P < 0.05$ for comparison with the OMV vector group, while ns means no significant difference

also set as negative controls, and no significant fluorescent signal was displayed (data not shown). These results imply that the serum antibodies specific to the O-antigen of *E. coli* O111 and/or O157 elicited by OMV from engineered *S.* Typhimurium possess significant biological functionality.

In vitro killing of wild-type *E. Coli* by sera from mice immunized with OMV displaying *E. Coli* O-antigen

To further investigate the capacity of engineered *S.* Typhimurium OMV displaying single or mixed *E. coli* O-antigen to induce functional antibodies, the serum samples taken on day 49 after primary immunization were applied to conduct *E. coli*-specific serum bactericidal antibody assay (SBA). As shown in Fig. 5C, not

only did sera from the OMV1 (O111)-immunized group display SBA killing to wild-type *E. coli* O111, but sera from the OMV3 (O111&O157)-immunized group also showed similar SBA killing to the wild-type *E. coli* O111. In addition, sera from OMV2 (O157)-immunized mice responded similarly to the wild-type *E. coli* O157 as sera from OMV3 (O111&O157)-immunized mice responded to the wild-type *E. coli* O157 (Fig. 5D). SBA killing was negligibly detected in dilution of 1:2 sera obtained from female mice inoculated with OMV vector (empty-plasmid control) (Fig. 5C and D).

Sera from mice immunized with OMV displaying *E. Coli* O-antigen mediated adherence inhibition

One of the most crucial characteristics for pathogenic *E. coli* to colonize the gut mucosa is the capacity to adhere to intestinal epithelial cells [1, 38]. Hence, it was assessed whether sera from mice immunized with OMV displaying single or mixed *E. coli* O-antigen could prevent the adhesion of the wild-type *E. coli* O111 and O157 strains to INT407 cells. The sera from the experimental group were able to significantly reduce the adhesion of wild-type *E. coli* O111 and/or O157 to the INT407 cells compared with the control group (Fig. 5E and F). This observation supports the possible role of *E. coli* O-antigen-specific IgG antibodies in inhibiting the adhesion of wild-type *E. coli* to intestinal epithelial cells.

The protection conferred by OMV displaying mixed O-antigens against wild-type *E. Coli* strain

To assess the protective efficacy induced by OMV displaying mixed *E. coli* O-antigen, survival evaluation was performed in a peritonitis mice model induced by *E. coli*. The female mice in four experimental groups (1 OMV1 group, 1 OMV2 group, and 2 OMV3 groups) and four control groups (2 PBS control groups, 2 OMV vector control groups) were challenged intraperitoneally with 5.6×10^7 CFU/100 µl of wild-type *E. coli* O111, or 5.4×10^6 CFU/100 µl of wild-type *E. coli* O157, respectively, and the survival rate was monitored. This high dose challenge led to a 100% death in mice inoculated with PBS and OMV vector (Supplementary Table 2, Fig. 6A and B). However, OMV1 (O111) and OMV3 (O111&O157) were able to confer 100% and 87.5% protection rates in mice when challenged with wild-type *E. coli* O111, respectively, which were significantly higher than that in PBS and OMV vector groups (Fig. 6A). Moreover, the survival rates of mice inoculated with OMV2 (O157) or OMV3 (O111&O157) were 87.5% and 75%, respectively, when challenged with wild-type *E. coli* O157 (Fig. 6B). The challenge results in immunized male mice were consistent with those in female mice (Supplementary Fig. 3).

We also assess the clearance of wild-type *E. coli* O111 and/or O157 in infected mice conferred by OMV displaying single or mixed *E. coli* O-antigen via a streptomycin-pretreated mouse model [34]. Female mice were provided unrestricted access to water added with streptomycin (5 g/l) for three days (days 32 after the initial immunization) preceding infection and then received 1×10^9 CFU/20 µl *E. coli* strain (days 35 after the initial immunization) by oral infection route. The number of streptomycin-resistant *E. coli* in fecal shedding decreased from approximately 10^7 CFU/g to below the lower limit of detection following 3 days of streptomycin treatment (data not shown). Immunized mice exhibited significantly reduced fecal shedding of *E. coli* O111 and/or O157 strains relative to mice belonging to PBS groups on days 9 and 12 post-infection, although infected *E. coli* strains were still detectable in fecal shedding during the experimental period (Fig. 6C and D). The combined results demonstrated that mice inoculated with OMV displaying single or mixed *E. coli* O-antigen successfully generated a protective immune response against *E. coli* O111 and/or O157 strains (Fig. 6 and Supplementary Fig. 3).

OMV displaying mixed O-antigens provided long-term protection against wild-type *E. Coli* infection

To ensure the effectiveness of a vaccine, it is crucial to stimulate and establish long-lasting memory immunity. Hence, we conducted a study to examine the long-term immune responses induced in female mice following OMV displaying single or mixed serotypes immunization via the intraperitoneal injection. The IgG and IgG isotype antibody levels in the serum of immunized mice were quantified on the 120th day following the initial immunization. Considerable levels of antibodies were still observable in all experimental groups even 120 days after vaccination, indicating that OMV1 (O111), OMV2 (O157), which represent the O-antigens of *E. coli* O111 or O157, respectively, and OMV3 (O111&O157), which show the mixed O-antigens of *E. coli* O111 and O157, were able to elicit a long-term immune response (Fig. 7A). Notably, the mice vaccinated with OMV2 (O157) and OMV3 (O111&O157) produced a similar level of anti-*E. coli* O157 LPS IgG1 and IgG2a immune response on day 120 post-inoculation (Fig. 7B and C). These results implied that OMV1 (O111) and OMV3 (O111&O157) induced a balanced Th1/Th2-type immune response against *E. coli* O111 LPS throughout the experiment, whereas OMV2 (O157) and OMV3 (O111&O157) balanced Th1/Th2-type immune response against *E. coli* LPS O157 over time. When mice immunized with OMV1 (O111) and OMV3 (O111&O157) were challenged intraperitoneally with wild-type *E. coli* O111 at a dose of 4.5×10^7 CFU/100 µl, the results showed that all OMV was able to provide significant protection in the mice relative to the control groups (Fig. 7D). All of the mice in the control group died when mice were challenged with

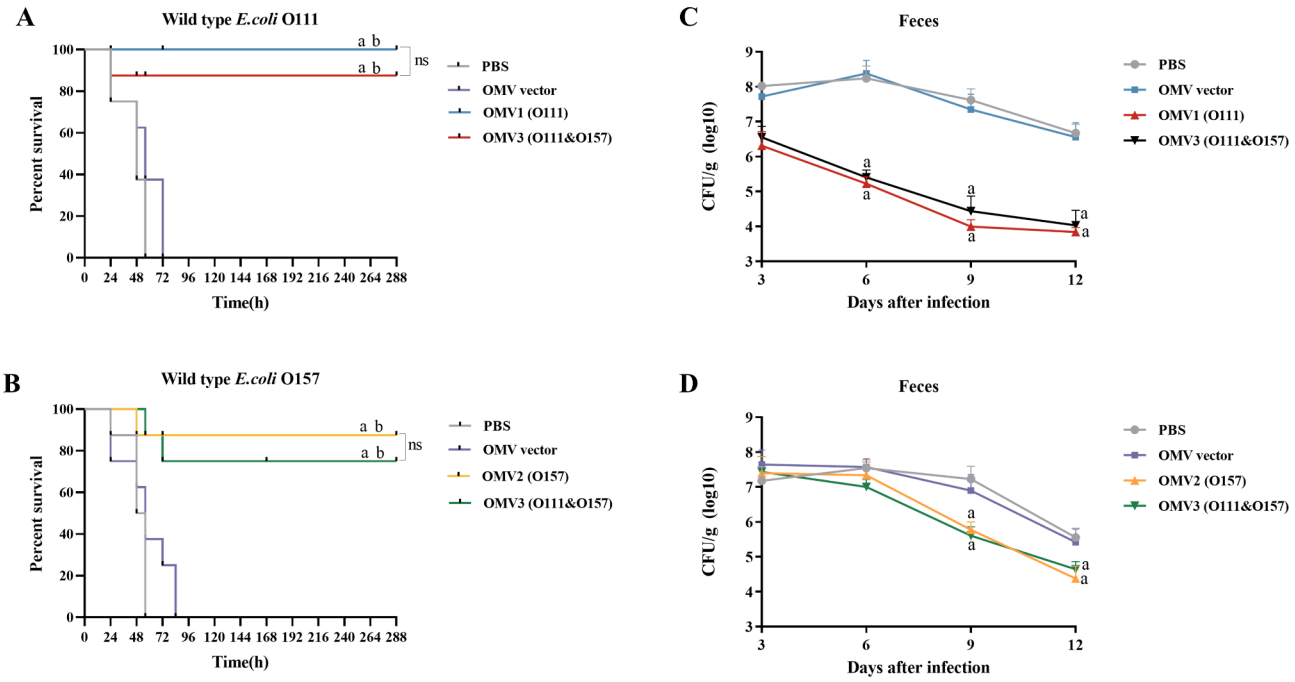

Fig. 6 Survival of female mice challenged with *E. coli* O111 or O157 strains after immunization with OMV delivering *E. coli* O111, O157, or mixed O-antigens. The immunized female mice underwent an intraperitoneal challenge with 5.6×10^7 CFU/100 µl of wild-type *E. coli* O111 strain (**A**) or 5.4×10^6 CFU/100 µl of wild-type *E. coli* O157 strain (**B**) at week 8 after the initial immunization, respectively. Following the challenge, mortality was recorded for 12 days. Data from two independent experiments were summarized ($n=8$). Mouse survival differences were analyzed using the log-rank test, with the Kaplan-Meier survival curve used for monitoring; superscript letters a and b indicate $P<0.05$ for comparisons with the PBS and OMV vector groups, respectively, while ns means no significant difference. (**C**. **D**) Female mice were provided unrestricted access to water added with streptomycin for three days preceding infection to clean the intestinal flora and were orally infected with 20 µl of a bacterial suspension comprising about 1×10^9 CFU of the *E. coli* O111 (**C**) or O157 (**D**), which is a non-lethal dose. Following the infection, water with added streptomycin was given back and fecal shedding was collected on days 3, 6, 9, and 12. Fecal pellets were weighed and homogenized in PBS (0.1 g/ml). The quantification of bacteria per 1 g of feces was performed by dropping serially diluted or undiluted fecal homogenate on the MacConkey agar plate containing 25 µg/ml streptomycin. Following the challenge, mortality was recorded for 12 days. Data from two independent experiments were summarized ($n=6$). The standard differences between the mice in each group were shown by the error bars. Data are presented as the means ± SEM. Statistical differences were assessed by a two-way ANOVA test followed by Tukey's multiple comparison test; superscript letters a indicate $P<0.05$ for comparison with the PBS

wild-type *E. coli* O157 at a dose of 5.1×10^6 CFU/100 µl; Meanwhile, although OMV3 (O111&O157) provided a lower level of protection than that conferred by OMV2 (O157), it prolonged the survival time of mice (Fig. 7E).

The physical mixtures of three different OMV provided protection in mice against infection of multiple wild-type *E. Coli* strains

The multitude of pathogenic *E. coli* serotypes poses a challenge for the development of *E. coli* vaccines. To cover more STEC prevalent serotypes, DNA fragments of O-antigen operons involved in the biosynthesis of the *E. coli* O26, O45, O145, and O103 O-antigen (Fig. 2C) were also integrated with the pSW047 or pSW048 DNA fragments to generate the plasmids of pSW051, pSW052, pSW053, pSW054, respectively (Table 1). Subsequently, both pSW051 and pSW052, as well as both pSW053 and pSW054 were introduced into the strains SW125 (Δ*rfbP* Δ*fliC* Δ*fljB* Δ*pagP81*::P_{lpp}*lpxE* Δ*tolR* Δ*asd*), respectively, to obtain the corresponding engineered *S.* Typhimurium strains, which were used to isolate and purify OMV (Table 1). Therefore, dual O-antigens from both *E. coli* O26 and O45, and both O145 and O103 were also exhibited on engineered *S.* Typhimurium OMV in addition to O111 and O157, respectively, resulting in three OMVs displaying three mixed *E. coli* O-antigens (Table 1, Supplementary Fig. 4). These three OMVs were physically mixed in PBS and administered intraperitoneally to six groups of female mice on days 0 and 14 with a dose containing 1 µg of each OMV in 100 µl PBS. As expected, mice in the experimental group exhibited significantly elevated antibody levels specific to six *E. coli* O-antigens on day 49 following the first immunization (*E. coli* O26, O45, O111, O157, O145, and O103) in serum compared to the control group (Supplementary Fig. 5). The immunized mice were intraperitoneally challenged with six different wild-type *E. coli* to assess the protection rate of OMV at 8 weeks following the first immunization. According to the results of the minimum lethal dose assay, the challenge doses for wild-type *E. coli* O26,

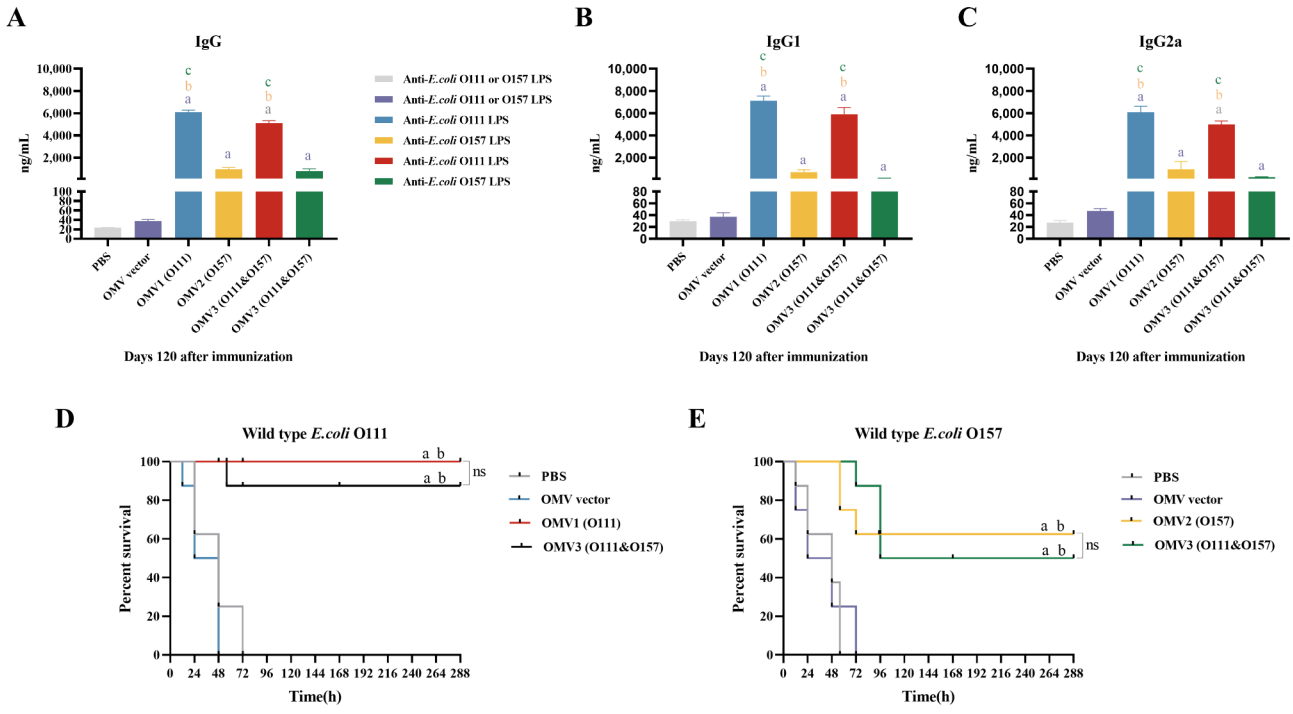

Fig. 7 The assessment of long-term immune responses induced by OMV delivering *E. coli* O111, O157, or mixed O-antigens. (**A**, **B**, **C**) Serums of immunized female mice were gathered at day 120 post-initial vaccination. Quantitative ELISA was applied to analyze specific IgG (**A**) against LPS from *E. coli* O111 or O157, as well as the subtypes IgG1 (**B**) and IgG2a (**C**). The results displayed the precise levels of antibodies, as measured by a standard curve, in mice intraperitoneally inoculated with the OMV delivering *E. coli* O-antigen at the scheduled weeks. The standard differences between the mice in each group were shown by the error bars. Data are presented as the means ± SEM. Statistical differences were assessed by a one-way ANOVA test followed by Bonferroni's multiple comparison test; superscript letters a, b, and c indicate $P < 0.05$ for comparisons with *E. coli* LPS-specific IgG antibody levels in the OMV vector group, *E. coli* O157 LPS-specific IgG antibody levels in the OMV2 group, and *E. coli* O157 LPS-specific IgG antibody levels in the OMV3 groups, respectively. (**D**, **E**) The immunized mice underwent an intraperitoneal challenge with 4.5×10^7 CFU/100 µl of wild-type *E. coli* O111 strain (**D**) or 5.1×10^6 CFU/100 µl of wild-type *E. coli* O157 strain (**E**) at week 8 after the initial immunization, respectively. Following the challenge, mortality was recorded for 12 days. Data from three independent experiments were summarized ($n = 8$). Mouse survival differences were analyzed using the log-rank test, with the Kaplan-Meier survival curve used for monitoring; superscript letters a and b indicate $P < 0.05$ for comparisons with the PBS and OMV vector groups, respectively, while ns means no significant difference

O45, O111, O157, O145, and O103 were 8×10^8 CFU, 1.5×10^8 CFU, 5×10^7 CFU, 5.3×10^6 CFU, 6.5×10^7 CFU, and 3.8×10^8 CFU in 100 µl, respectively (Supplementary Table 2). The protection results showed that physically mixed OMV conferred effective protection to mice against each corresponding infection with wild-type *E. coli* (Fig. 8).

Discussion

Several polysaccharide vaccines, such as pneumococcal conjugate vaccine (PCVs) [39], Haemophilus influenzae type b (Hib) vaccine [40], meningococcal conjugate vaccine (MenACWY) [41], meningococcal serogroup B (MenB) vaccine [41], and typhoid conjugate vaccine [9], have been approved for the prevention of infections developed from specific bacterial pathogens. The successful development of these polysaccharide vaccines has demonstrated the feasibility and effectiveness of polysaccharide antigens located on the bacterial surface as vaccine targets and the potential of polysaccharides to induce resultful adaptive immune responses in the organism when it is attached to appropriate carriers [42]. OMV has been demonstrated in many studies to be a promising antigen carrier due to its natural composition, immunogenicity, and power to trigger both innate and adaptive immune responses [43–45]. The *rfbP* gene is responsible for transferring and linking Galactose-1-phosphate (Gal-1-P) to undecaprenyl-phosphate (Und-P), resulting in the formation of Und-PP-Gal in *Salmonella*, and the deletion of this gene completely prevented *Salmonella* from synthesizing its own O-antigen [35, 36], which contribute to the synthesis of heterologous O-antigen in *Salmonella*, and can be linked to the lipid A core on the *Salmonella* surface via the *Salmonella waaL* gene. In addition, the *fliC*, *fljB*, and *tolR* mutations were also introduced into *Salmonella*, which is conducive to increasing OMV production [25, 46]. Our prior research demonstrated that LpxE has the ability to specifically eliminate the

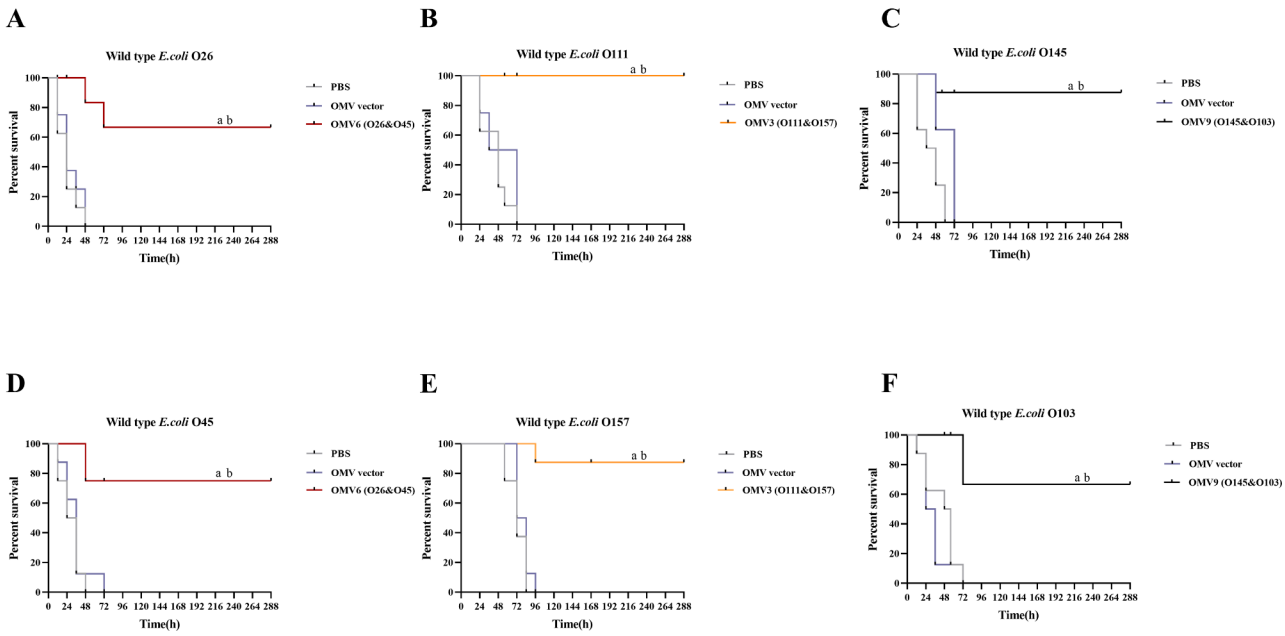

Fig. 8 The evaluation of protection induced by the physical mixtures of three different OMVs against infection with multiple *E. coli* strains. These three OMVs, which display *E. coli* O26 and O111, and O145 and O103 in addition to O111 and O157, respectively, were physically mixed in PBS and administered intraperitoneally to six groups of female mice on days 0 and 14 with a dose containing 1 μg of each OMV in 100 μl PBS. The immunized mice underwent an intraperitoneal challenge with wild-type *E. coli* O26 (**A**), O45 (**D**), O111 (**B**), O157 (**E**), O145 (**C**), and O103 (**F**) were 8×10^8 CFU/100 μl, 1.5×10^8 CFU/100 μl, 5×10^7 CFU/100 μl, 5.3×10^6 CFU/100 μl, 6.5×10^7 CFU/100 μl, and 3.8×10^8 CFU/100 μl, respectively, at week 8 after the initial immunization. Following the challenge, mortality was recorded for 12 days. Data from six independent experiments were summarized ($n=8$). Mouse survival differences were analyzed using the log-rank test, with the Kaplan-Meier survival curve used for monitoring; superscript letters a and b indicate $P<0.05$ for comparisons with the PBS and OMV vector groups, respectively

1′-phosphate group from lipid A of *Salmonella*, resulting in a product that closely resembles monophosphoryl lipid A (MPL) [17]. In this study, to decrease the endotoxic activity of engineered *S.* Typhimurium OMV, the combination mutations, including Δ*pagP81*::P_{lpp}*lpxE*, were introduced into the mutant of SW121 (Δ*rfbP* Δ*fliC* Δ*fljB*), resulting in SW124 (Δ*rfbP* Δ*fliC* Δ*fljB* Δ*pagP81*::P_{lpp}*lpxE* Δ*tolR*) (Table 1). This engineered *S.* Typhimurium was used as a vector to produce OMV under normal growth to deliver O-antigen from *E. coli* and assessed its capacity to activate humoral and mucosal immune responses.

The cluster of genes involved in the synthesis of O-antigen is usually located between the *galF* and *gnd* genes in the genome of Gram-negative bacteria and consists mainly of genes for the synthesis of monosaccharides involved in the synthesis of ribonucleotide sugar precursors, genes for glycosyltransferases (GT) that transport ribonucleotide sugar precursors and participate in the synthesis of the corresponding glycosidic bonds, and genes involved in the subsequent O-antigen flipping, elongation, and length control [47, 48]. By analyzing the O-antigen synthesis gene clusters as well as the chemical structures of *S.* Typhimurium and *E. coli*, we found that genes related to rhamnose and/or mannose synthesis are prevalent in the O-antigen synthesis gene clusters of these bacteria. Therefore, the monosaccharide synthesis genes such as rhamnose and/or mannose in the *E. coli* O-antigen synthesis gene cluster were deleted, and the glycosyltransferase genes, the genes involved in the subsequent O-antigen flipping, elongation, and length control, and the genes related to the synthesis of the rare sugars required for certain serotypes were retained since the same rhamnose and mannose monosaccharide synthesis genes existed in the *Salmonella* (Fig. 2). The results of silver-staining profiles and western blot showed that engineered *Salmonella*, containing the plasmid for *E. coli* O-antigen synthesis, and its OMV exhibit a typical ladder LPS banding pattern and were recognized by antisera against corresponding *E. coli* O-antigen, indicating that the fragment of the *E. coli* O-antigen gene cluster could be sufficient to synthesize the corresponding O-antigen polysaccharide in *Salmonella* (Fig. 3). *Salmonella* or other Gram-negative bacteria are widely used as platforms to develop effective polysaccharide vaccines [49–51]. The entire polysaccharide gene clusters were usually cloned into a vector for this purpose, in this study, only partial gene clusters including GT genes and polysaccharide processing genes were cloned to synthesize the

O-antigen polysaccharide of *E. coli*, implying that the sugar synthesis genes in *Salmonella* can be used for heterologous polysaccharide synthesis. This strategy allows for shortening the length of the polysaccharide synthesis gene cluster and reduces the difficulty of cloning a large DNA gene cluster into the plasmid.

While O-antigen is widely acknowledged as an effective protective antigen [52–55], the majority of vaccine formulations targeting STEC infections focused on secreted protein toxins including Stx2A, Stx2B, and/or Stx1B, and this preference stems from the fact that O-antigen-based vaccines may offer less efficacy against heterologous serotypes due to the specificities dictated by the O-antigen [1]. For instance, currently, licensed STEC vaccines like Epitopix SRP and Econiche exhibit efficacy exclusively against serotype O157 infection in cattle, however, they fail to confer cross-protection against prevalent non-O157 serotypes such as O26, O45, O103, O111, and O145 [9, 10]. A vaccine primarily targeting Stx2A, Stx2B, and/or Stx1B Shiga toxin subunits may exhibit limitations due to their narrow serotype-specific immunity, variability in Shiga toxin expression among STEC strains, and potential incomplete protection against STEC infections. The primary objective of the study was not only to develop a useful vaccine for preventing and managing infection caused by a single serotype of STEC but also to develop a vaccine design strategy by utilizing OMV that can confer protection against multiple prevalent STEC strains. Therefore, the dual plasmid system was constructed and introduced to the engineered *S.* Typhimurium, which was designed for producing OMV with low endotoxic levels and displaying mixed O-antigens of *E. coli* in this study. Compared to the fusion of O-antigen gene clusters to the chromosomes of bacteria, the dual plasmid system used in this study ensures that both plasmids can be independently maintained and propagated to simultaneously synthesize dual heterologous O-antigens in bacteria. Our results demonstrated that *Salmonella* carrying the dual-plasmid system was able to synthesize mixed O-antigens simultaneously (Fig. 3). Notably, there may be hybrid O-antigens on the surface of engineered *Salmonella* containing the dual plasmid system, in addition to the expected two O-antigens. Gasperini et al., introduced various OAg-modifying enzymes to a scaffold strain of *S. flexneri*, either separately or in combinations, and synthesized a hybrid O-antigen [52]. Indeed, all *S. flexneri* serotypes, except serotype 6, share a conserved O-antigen backbone, which can be modified with various O-antigen-modifying enzymes of *S. flexneri* to yield specific polysaccharide epitopes [56]. Unlike *S. flexneri*, *E. coli* possesses a significantly distinct O-antigen structure; therefore, this process may potentially lead to mutual interference when two different *E. coli* O-antigens are synthesized in *Salmonella* at the same time. This is the reason why no more than two O-antigens of *E. coli* were simultaneously synthesized in *Salmonella*.

In this study, although OMV1 (O111), OMV2 (O157), and OMV3 (O111&O157) elicited a significant increase in the level of *E. coli* O111 and/or O157 LPS-specific IgG compared to the PBS and OMV vector control groups, the *E. coli* O111 LPS-specific antibody level of IgG in the OMV1 (O111) and OMV3 (O111&O157) groups was significantly higher than the *E. coli* O157 LPS-specific antibody level of IgG in the OMV2 (O157) and OMV3 (O111&O157) groups (Fig. 4A, B and C and Supplementary Fig. 2A, 2B, C). Moreover, OMV3 (O111&O157) stimulated similar levels of *E. coli* O111-LPS-specific IgG1 and IgG2a subtypes in mice, and *E. coli* O157-LPS-specific levels of IgG1 and IgG2a subtypes remained IgG2a-dominant (Fig. 4D, E and F and Supplementary Fig. 2D, 2E). This may be caused by the different structures of *E. coli* O111 and *E. coli* O157 O-antigens, as polysaccharide-induced immune responses have been previously reported to be closely related to their structures [57]. The 3,6-dideoxyhexoses have attracted significant interest among the numerous monosaccharide residues present in the O-antigen of bacteria LPS, owing to their highly immunogenic properties [58]. Only five of the eight possible stereoisomers of 3,6-dideoxyhexoses are found in nature, and these are colitose, abequose, tyvelose, ascarylose, and paratose [58]. Colitose is found at the ends of *E. coli* O111 O-antigens, where it functions as a unique antigenic element of LPS (Fig. 2). Colitose was also regarded as a crucial constituent of particular ligands for specific LPS-binding lectins, such as horseshoe crab tachylectin-4, which selectively binds to *E. coli* O111 LPS [59]. This reminds us of the importance of considering the effect of O-antigen structure on the immune response when applying OMV vectors to deliver O-antigens in the future. The number of *E. coli* O111 or O157 LPS molecules on the surface of OMV3 (O111&O157) may be less than the number of the correspondent LPS molecules on the surfaces of OMV1 (O111) or OMV2 (O157). But in fact, our results showed that the mice immunized with OMV3 (O111&O157) produced similar levels of humoral immune responses compared to the mice immunized with OMV1 (O111) or OMV2 (O157) (Fig. 4, Supplementary Fig. 2). The levels of IgA antibodies specific to *E. coli* O111 and/or O157 O-antigen in serum, vaginal wash, or fecal shedding elicited by OMV1 (O111), OMV2 (O157), and OMV3 (O111&O157) at 5 weeks following primary immunization were failed to detect by ELISA, indicating OMV was ineffective in provoking mucosal immune response in this study (Supplementary Fig. 1, Supplementary Fig. 2F). We speculate that these results may be associated with the route of inoculation in this study, and there are studies have reported that OMV failed to trigger an

IgA immune response via vaccination intraperitoneally, which is consistent with the results in this study, whereas high levels of specific IgA antibodies were induced by inoculation intranasally in mice [44, 60]. The results of the long-term immune responses demonstrated that the OMV designed to display mixed O-antigens can effectively elicit a sustained humoral immune response and establish immunological memory in mice (Fig. 7).

LPS is composed of lipid A, core oligosaccharide, and O-antigen (distal polysaccharide), with the O-antigen responsibilities as a viable vaccination target; however, it is a poor vaccine antigen that can be chemically or biologically conjugated to suitable carrier proteins (such as tetanus toxoid, cross-reactive material 197, recombinant exoprotein A of Pseudomonas aeruginosa, et al.,) to enhance immunogenicity [50, 61]. In this study, the heterologous O-antigen was covalently linked to the lipooligosaccharides (LOS) of *S.* Typhimurium and anchored to the outer membrane of this bacteria, which is similar to the native state of the O-antigen. Although the mechanism of the adaptive immune response induced by polysaccharide-protein complexes is currently elucidated to some extent, the mechanism of the adaptive immune response induced when O-antigen is covalently linked to LOS and anchored to the bacterial outer membrane is unknown [57, 62, 63]. Notably, our animal results showed that OMV derived from engineered *S.* Typhimurium by intraperitoneal injection not only induced robust humoral immunity but also provided effective protection against wild-type *E. coli* infection in mice, as well as long-lasting immunity, indicating OMV displaying heterologous O-antigens is capable of eliciting a T cell-dependent immune response for the O-antigen (Figs. 4, 6 and 7). This provides an important reference for the development of vaccines based on the O antigen of pathogenic Gram-negative bacteria.

Animal models commonly used to study *E. coli* infections include mice, rats, chickens, rabbits, pigs, and non-human primates. Of these, mice are one of the most commonly used animal models. In this study, we first administered different concentrations of wild-type *E. coli* intraperitoneally to determine the minimum lethal dose of each wild-type *E. coli* in mice and then challenged the immunized mice based on the minimum lethal dose to assess the immune protection conferred by OMV derived from engineered *S.* Typhimurium (Supplementary Table 2). The wild-type *E. coli* was cultured in a TSB medium due to the expression of some virulence factors of *E. coli* being low or not expressed in the LB medium. Animal experimental data demonstrated that our OMV designed to exhibit mixed O-antigens could provide protection against wild-type *E. coli* infection (Fig. 6A and B and Supplementary Fig. 3A, 3B). The challenge results in the streptomycin-pretreated mouse model further prove that OMV displaying single or mixed O-antigen confers effective protection (Fig. 6C and D), although the OMV fails to elicit mucosal immune responses (Supplementary Fig. 1, Supplementary Fig. 2F). Safety is a priority when vaccines are designed and developed, therefore inactivated or recombinant subunit vaccines are always preferably selected, however, inactivated or subunit vaccine preparation are not suitable for preventing infection of all pathogens. OMV could be utilized as an optimal carrier that achieves an effective balance between safety and immunogenicity. Many studies reported that the OMV produced by bacteria during normal growth has no inherent cytotoxicity except at high doses [44, 64] and can also be engineered to decrease the interaction of lipid A and TLR4 and endotoxic activity while maintaining their good immunogenicity [17]. The results of the current study showed that throughout the whole vaccination scheme with two doses of administration, no deaths or instances of disheveled fur or weight loss were observed in mice. Furthermore, both OMV displaying mixed O-antigens and OMV displaying a single O-antigen were able to provide effective protection while mice received immunization of a low dose of 1 μg (Fig. 6, Supplementary Fig. 3). Three mixed OMVs induced significant antibody levels specific to six O-antigens (*E. coli* O26, O45, O111, O157, O145, and O103) in serum compared to the control group and conferred effective protection (Fig. 8), indicating that mixed multiple polysaccharides in OMV could be a multivalent vaccine to cover the most prevalent serotypes of *E. coli*.

In conclusion, we have designed an OMV vaccine candidate with low endotoxic activity, which is derived from engineered *S.* Typhimurium and displayed mixed *E. coli* O-antigens. We have demonstrated that the OMV not only induces robust humoral immunity but also confers significant protection to mice against the infection of multiple wild-type STEC. The OMV strategy shows great promise due to its benefits of ease of production, low manufacturing costs, and high immunogenicity and protection efficacy.

Supplementary Information

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Supplementary Material 1
Supplementary Material 2
Supplementary Material 3
Supplementary Material 4
Supplementary Material 5
Supplementary Material 6
Supplementary Material 7
Supplementary Material 8

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Author contributions
XP, QL, and QK initiated the research. XP led the design of in vitro and in vivo experiments, data acquisition and analysis, and manuscript preparation. YL, WJ, XY, MH, HX, and MR aided in data acquisition; QL and QK participated in experimental design, data analysis, and manuscript preparation and revision. All authors contributed to the article and approved the submitted version.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Animal experiments were conducted in the Laboratory Animal Center of Southwest University after the Review Form for Laboratory Animal Welfare and Ethics submitted by us was approved by IACUC (No. IACUC-20191120-02). We usually make an appointment in the online system with our registered personal information and carry out animal experiments after approval, and there is no license number for each animal experiment.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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