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SHORT COMMUNICATION

Extracellular vesicles elicit protective immune responses against *Salmonella* infection

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Abstract

Small extracellular vesicles (sEVs) produced by antigen-presenting cells represent a novel mechanism of cell-to-cell communication. The sEVs have been shown to drive Th1-type adaptive immune responses against intracellular infections such as *Salmonella*. In this study, we have demonstrated that an administration of sEVs produced by *Salmonella*-infected macrophages to BALB/c mice that were then challenged with *Salmonella* infection decreased bacterial load in infected animals and led to protection against a lethal dose of *Salmonella*. Second, the same sEVs induced a robust production of IgA anti-*Salmonella* antibodies (Abs) in BALB/c mice, including IgA anti-OmpD Abs. These results show that the nanoscale sEVs stimulate adaptive immune responses against intracellular pathogens and that these sEVs can be used to provide animals with complete protection against lethal infection, such as the systemic bacterial infection in immunodeficient BALB/c mice.

KEYWORDS
extracellular vesicles, Salmonella infection, protective immune responses, IgA

1 | INTRODUCTION

Salmonella enterica causing potentially severe and life-threatening nontyphoidal septicaemia remains a threat to humans due to the lack of licensed vaccines and increased antibiotic resistance (Tennant et al., 2016). Salmonella causes an estimated 93.8 million cases of gastroenteritis and 155,000-681,000 deaths globally each year, and there are no FDA-approved vaccines to prevent this nontyphoidal infection in humans (G. N.-T. S. I. D. Collaborators 2019; Majowicz et al., 2010). Salmonellosis illnesses are acquired and spread through the consumption of water or food contaminated with faeces (Scharff, 2012). The host immune response against this pathogen is controlled by multiple regulatory mechanisms, where intercellular communication between infected and uninfected cells is accomplished by cell-cell interactions and long-distance communication (Rivera et al., 2016). One such mechanism of intercellular communication is via small extracellular vesicles (sEVs) (Raposo & Stoorvogel, 2013). These nanoscale sEVs transmit cargo to other cells, such as proteins, RNA, or metabolites (Thery et al., 2002), and have previously contributed to bacterial infection in vivo (Gioseffi et al., 2021). For instance, sEVs produced during the infection with the acid-fast bacterium *Mycobacterium tuberculosis* provide immune protection against this pathogen (Cheng & Schorey, 2013). The importance of sEVs in bacterial infection has been demonstrated using rab27a-deficient mice (Smith et al., 2017), which lessens the capacity to produce sEVs than wt mice. The deletion of rab27a correlates with an increased bacterial burden and decreased T cell activation, indicating the importance of sEVs in the T cell function in this infection model (Smith et al., 2017).

SIGNIFICANCE STATEMENT

- This study uncovered the ability of the host extracellular vesicles to elicit protective immune responses towards intracellular infection with *Salmonella*. These extracellular signalling mechanisms are expected to play essential functions in modulating adaptive immune responses to this pathogen. This contribution is significant because it is an essential step toward the rational design of vaccines toward reducing the health burden of *Salmonella*-derived gastroenteritis cases which accounts for between 155,000-and 681,000 deaths globally. There are currently no FDA-approved vaccines to prevent nontyphoidal salmonellosis in humans. Extracellular vesicles represent an attractive mechanism of antigen trafficking in the host, and our study focuses on identifying how the host extracellular vesicle pathway commands the immune response in *Salmonella* infection. This study fills the void currently limiting identifying and advancing a new preventative treatment for *Salmonella* infections.

In the previous study, sEVs derived from infected macrophages were found to carry bacterial antigens (Ags) from *S. enterica* Typhimurium-infected macrophages (Hui et al., 2021). The same sEVs were shown to stimulate bone-marrow-derived macrophages (BMDMs) and dendritic cells (DCs) to produce cytokines involved in T cell recruitment and priming (Hui et al., 2018). These sEVs polarize naïve macrophages towards the M1 phenotype in vitro, altering the functions of these Ag-presenting cells (APCs). Moreover, the sEVs accumulate in the mucosal tissues following their intranasal delivery (Hui et al., 2021), leading to the humoral responses marked by the production of anti-S. Typhimurium antibodies (Abs). These studies found that sEVs stimulate pathogen-specific T helper 1(Th1) cell responses marked by pathogen-specific $CD4^+$ T cells, but the protective effect of sEVs in preventing the systemic disease caused by *Salmonella* have never been demonstrated.

In the present study, vaccination with sEVs produced by *Salmonella*-infected macrophages prior to infection was associated with diminished bacterial loads in infected animals and resulted in the protection of mice against the lethal outcomes of salmonellosis in the immunodeficient BALB/c mice. Protective responses triggered by sEVs were present 4 weeks post-vaccination., which is the time when memory response to the vaccine was established. Nasal sEV delivery induced robust production of faecal anti-*Salmonella* secretory IgA (SIgA) Abs, and the nasal delivery of vesicles was required for these effects, as oral delivery was insufficient to lead to the IgA generation. Notably, we also confirmed the relationship between the vesicular cargo and specific Ab responses, since the anti-*Salmonella* IgA Abs included anti-OmpD IgA Abs, and OmpD has been identified in these sEVs, where OmpD is known to be a protective Ag (Domã-Nguez-Medina et al., 2020; Gil-Cruz et al., 2009; Schager et al., 2018). In conclusion, sEVs can be used as a possible strategy to prevent systemic infection with intracellular bacteria such as *Salmonella*.

2 | RESULTS

2.1 | Mucosal vaccination with sEVs stimulates secretory anti-*Salmonella* IgA generation

The intestinal humoral immune response is a significant contributor to protection against enteric bacterial infections such as *Salmonella*, where an adequate humoral immune response at mucosal surfaces is required to control infection. In the case of *Salmonella*, the locally produced secretory IgA (SIgA) Abs act as one of the primary defences against this bacterium. The sEVs produced by macrophages during *Salmonella* infection and delivered to BALB/c mice intranasally lead to Ag-specific IgA and IgG responses against *Salmonella* (Hui et al., 2021), and therefore, it is likely that sEVs can initiate the production of anti-*Salmonella* SIgAs. Previously characterized *Salmonella*-infected RAW264.7 macrophages were used as a source of sEVs (Figure 1A). The size of isolated sEVs falls within the expected size of exosomes (Figure 1B).

Next, the sEVs were delivered by the intranasal (IN) or oral routes to BALB/c mice in three doses (Figure 1C) to test the ability of sEVs to induce anti-*Salmonella* SIgA Abs. The Δ*aroA Salmonella* was used as a positive vaccination control as an auxotrophic vaccine strain delivered orally (Stocker et al., 1983). PBS or sEVs from uninfected RAW264.7 cells, the two latter control treatments, were IN delivered. Fresh faecal pellets were collected from individual mice weekly and assessed for the presence of SIgA anti-*Salmonella* endpoint titres. Only mice receiving oral Δ*aroA Salmonella* or IN sEVs from infected macrophages produced detectable SIgA anti-*Salmonella* Abs (Figures 2A, S1A, S1B). The mice vaccinated orally with sEVs failed to produce any SIgA anti-*Salmonella* Abs. The mice vaccinated with sEVs from uninfected cells also lacked SIgA Abs. As expected, immunization with Δ*aroA Salmonella* led to a robust SIgA anti-*Salmonella* Ab production and PBS had no IgA response. Both oral Δ*aroA Salmonella* and IN sEVs elicited serum IgG anti-*Salmonella* Abs (Figure S1C). In summary, sEVs derived from infected macrophages delivered by the IN route promote IgA directed against *Salmonella* Ags. Our sEV vaccination regimen induces memory B cell responses since the faecal Ab titres represent the daily production of IgA generated. Since we measured SIgA Ab titres for at least 12 weeks, these must be derived from memory B cells.

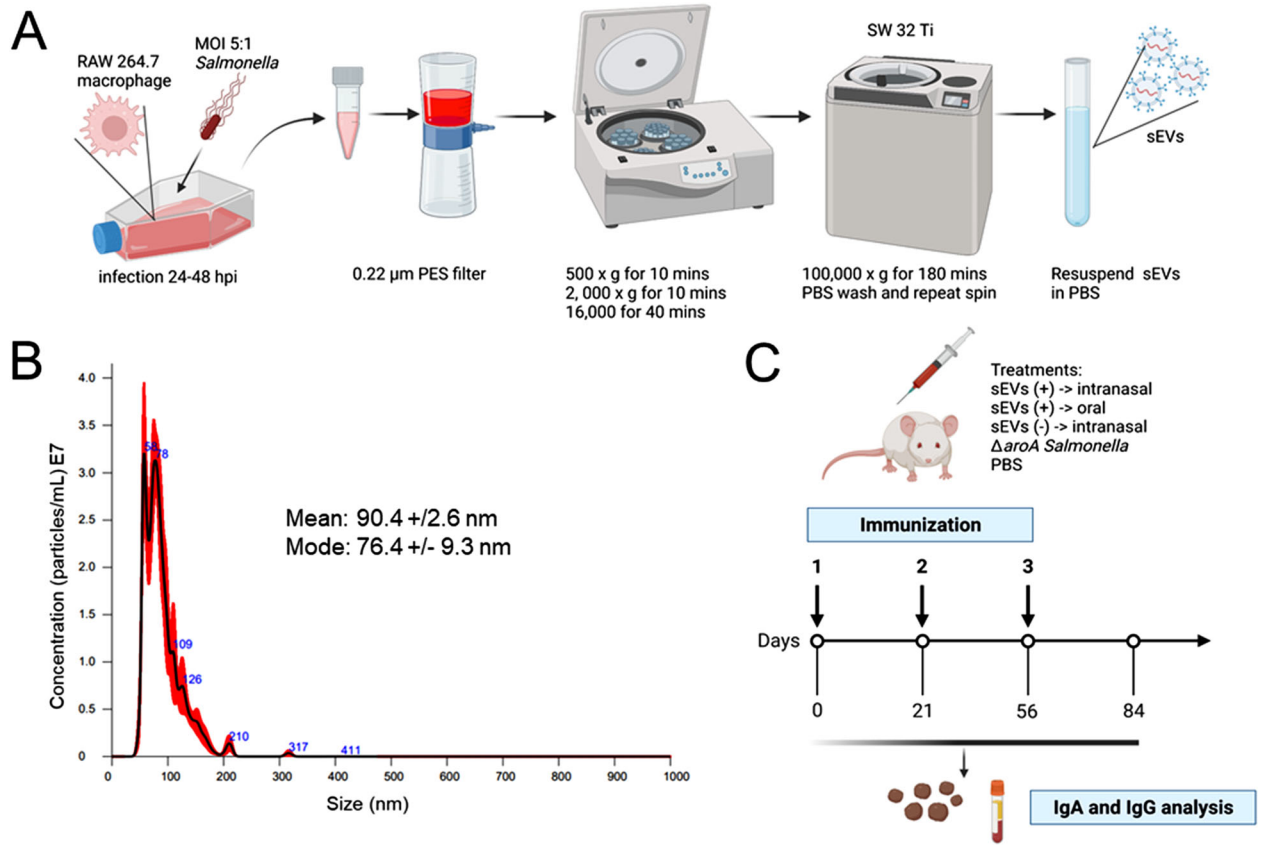

FIGURE 1 (A). Schematic of the sEV isolation. RAW-264.7 cells were infected with *S.* Typhimurium for 24–48 h and isolated by differential ultracentrifugation to obtain sEVs. (B). NanoSight Tracking Analysis of sEVs derived from RAW264.7 macrophages infected with *S.* Typhimurium. (C). Schematic of the immunization regimen for IgA and IgG studies

It is still unknown which specific Ags present in the sEVs lead to the potent Ab response, including SIgA response. One of the aims of this study is to link the content of the sEVs to the specific Ab responses in animals receiving these sEVs. One of the top candidates detected in the sEVs from our previous study was OmpD (Hui et al., 2021), and immunity to this Ag confers immune protection (Gil-Cruz et al., 2009). OmpD is detected at high levels in the sEVs relative to total *Salmonella* Ag preparation, based on a high percentage of protein sequence coverage of OmpD in the sEVs (Figure 2B, C, D). The frequency of anti-OmpD IgA induced by mice vaccinated with sEVs, Δ*aroA Salmonella*, or PBS control was also evaluated. The sEV-vaccinated mice exhibited the greatest level of anti-OmpD IgA Abs among the tested groups (Figure 2E) and a significantly higher abundance of OmpD Abs than Δ*aroA Salmonella* (Figure 2F), showing that the OmpD carried by sEVs stimulates the specific IgA response in mice. Moreover, while Δ*aroA Salmonella* led to improved anti-*Salmonella* IgA titres in comparison to the sEV-based immunization, it approached antibody titres caused by live mutant, and there were significantly more anti-OmpD IgAs in mice dosed with sEVs compared to PBS-treated mice. In contrast, the titres of OmpD IgAs in Δ*aroA Salmonella*-vaccinated animals were not significantly more than the PBS-treated mice.

2.2 | Mucosal administration of sEVs protects BALB/c mice against *Salmonella* challenge

Next, the efficacy of IN sEVs in protecting mice from *Salmonella* infection was examined compared to two control treatments (Figure 3A). Groups of BALB/c mice were IN vaccinated with sEVs or PBS, or vaccinated orally with Δ*aroA Salmonella*, rested for 4 weeks, and challenged with a lethal dose of *Salmonella* (4.5×10^6 CFUs/mouse). The protective responses triggered by sEVs were tested at the 4 weeks post-vaccination. Four days after the challenge, several mice (n = 3) from each group were euthanized and analysed for the extent of salmonellae colonization of the liver and changes to the small intestine. The remaining mice were observed over 14 days to follow survival rates for each treatment group (n = 7). Mice immunized with sEVs derived from infected macrophages and mice immunized with Δ*aroA Salmonella* were completely protected from death compared to PBS control (Figure 3B). Moreover, the bacterial burden measured as CFU/g tissue showed a significantly reduced bacterial load in livers of BALB/c mice immunized with sEVs (by ~1 log) or Δ*aroA Salmonella* (by approximately 1-log) compared to control

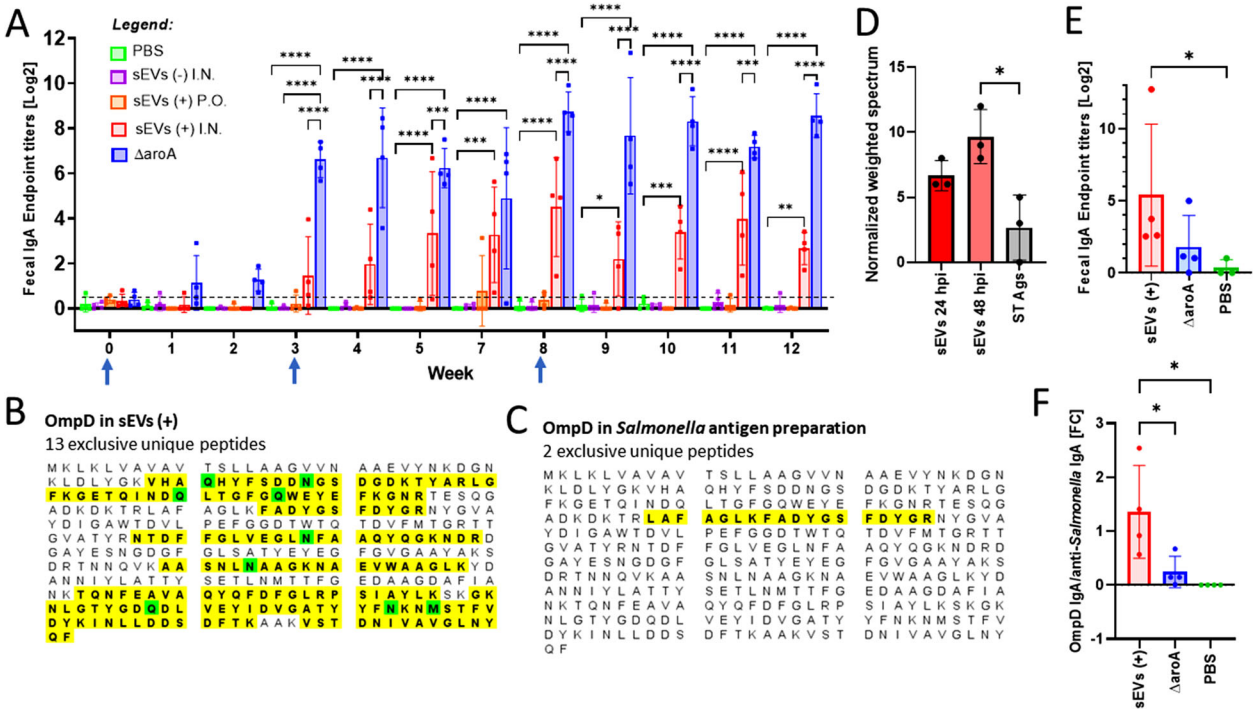

FIGURE 2 The sEVs from infected macrophages promote anti-*Salmonella* IgA generation in BALB/c mice intranasally administered with these sEVs. (A). Mice were administered with sEVs from infected macrophages [sEVs (+)] intranasally (I.N) or per-orally (P.O) or were alternatively given sEVs from uninfected macrophages [sEVs (-)] by I.N. route, where all administrations were given in week 0, 3, and 8 (n = 4). In addition, Δ*aroA Salmonella* or PBS were used as positive and negative controls, respectively. The faecal samples were collected throughout the 12 weeks, and ELISA test established the titres of anti-Salmonella IgA. The arrows indicate the dates when the immunizations were given. (B)-(C). The sEVs generated during *Salmonella* infection contain OmpD antigen. A protein sequence coverage of OmpD was identified by Orbitrap Mass Spectrometer in sEVs generated by RAW264.7 cells at 48 hpi (B) or total *Salmonella* antigen preparation (C). An equal amount of digested protein material (2 μg) was used for analysis. The identified amino acids in each sample are shown in yellow, and any post-translational modifications are shown in green. (D). Normalized weighted spectral count of OmpD antigen in sEVs generated by RAW 264.7 cells at 24 hpi and 48 hpi, compared to an equal amount of *Salmonella* antigen preparation (ST Ags; n-3). (E)-(F). The sEVs generated during *Salmonella* infection promote anti-OmpD IgA generation in vaccinated animals. BALB/c mice (n = 4) were immunized with sEVs from infected macrophages [sEVs (+)], negative control (PBS) or Δ*aroA Salmonella*, and titter ELISA quantified the titter of faecal anti-OmpD Abs (IgA) at week 5 (D). The data were also shown as a fold change (FC) of anti-OmpD Abs amongst total anti-*Salmonella* IgAs (E)

(Figure 3C). Moreover, mice immunized with sEVs derived from infected macrophages had significantly lower CFU/mg load of *S.* Typhimurium in tissue than mice immunized with sEVs derived from uninfected macrophages (Figure 3D). Mice immunized with sEVs and Δ*aroA* displayed significantly better clinical outcomes as measured via dehydration scores (Figure 3E) compared with PBS control. Histological evaluation of tissue sections of the ileum from infected mice revealed that the tissues from PBS-dosed, *Salmonella*-infected mice showed destruction of the villi and massive cellular infiltration (Figure S2). In contrast, there is little to no tissue destruction or inflammation in sEV- and Δ*aroA-Salmonella*- vaccinated mice, displaying intact villi and epithelial barriers. Interestingly, Δ*aroA-Salmonella*-vaccinated mice tissues showed some crypt hyperplasia, while none was observed in sEV-vaccinated mice. While we have not used a streptomycin-treated mouse model (Barthel et al., 2003), the UK-1 *Salmonella* strain is able to colonize the ileum much better than 12023 *Salmonella* strain even in the absence of streptomycin (Sanapala et al., 2018).

3 | DISCUSSION

The sEVs constitute an essential vehicle of cell-to-cell communication (Robbins & Morelli, 2014), yet the ability of sEVs to elicit protective responses against Gram-negative infections affecting immunological memory remains unclear. We have previously demonstrated that mucosal administration of mice with sEVs derived from APCs stimulates Th1-type responses against *Salmonella* by stimulating Ag-specific CD4+ T lymphocytes (Hui et al., 2021). Here, the sEVs were tested in their capacity to protect animals from infection using a lethal typhoid fever model of *Salmonella* infection in BALB/c mice. The sEVs are found to stimulate SIgA anti-*Salmonella* Ab production in mice IN-vaccinated with sEVs from infected macrophages, including anti-OmpD IgA. These same sEVs protect mice against bacterial colonization, disease symptoms, and lethality. The data

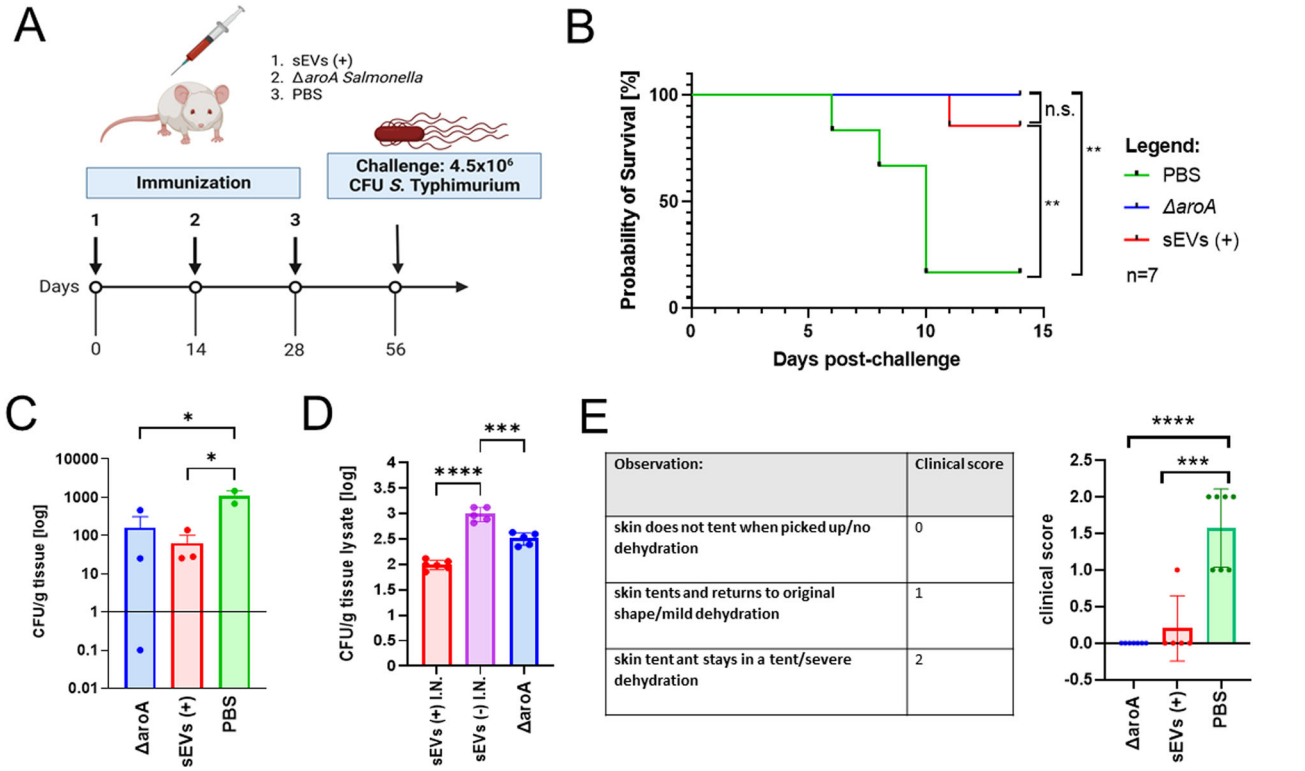

FIGURE 3 The macrophage-derived sEVs produced during *Salmonella* infection protect against the challenge with a lethal dose of *Salmonella*. (A). Mice administered with sEVs from infected macrophages [sEVs (+)], Δ*aroA Salmonella*, or PBS were rested for 4 weeks and challenged with *Salmonella* (4.5×10^6 CFU/mouse). (B). The mice were observed over 14 days to establish the survival probability amongst the groups (n = 7). Kaplan-Meier survival curve is shown. (C). Four days after the challenge, mice from each group were euthanized and analysed for bacterial load in the liver measured as CFU/mg tissue. A one-way ANOVA test with Tukey's multiple correction test was used to establish statistical significance. (D). Mice administered with sEVs from infected macrophages [sEVs (+)], uninfected macrophages [sEVs (-)], or Δ*aroA Salmonella* were rested for 4 weeks and challenged with *Salmonella*, after which mice were euthanized and analysed for bacterial load in the liver measured. A one-way ANOVA test with Tukey's multiple correction test was used to establish statistical significance. (E). The clinical score for observed mice was analysed using the criteria described in the accompanying table and displayed as the mean score for each condition evaluated. A one-way ANOVA test with Tukey's multiple comparison test was used to establish statistical significance

presented here demonstrate the feasibility of using sEVs as a cell-free vaccine strategy against bacterial infections since sEVs stimulated specific protective responses against intracellular infection and led to positive outcomes in the infected mice.

The humoral immunity to *Salmonella* infection constitutes one of the critical elements of the host immune response to this intracellular infection, next to cell-mediated immunity. For *Salmonella* infection, SIgA Abs are critical since these Abs can restrict the pathogen in the mucosal environment where the infection is often initiated. It has been shown that the oral administration of these SIgA Abs reduces bacterial entry into Peyer's patches (Bioley et al., 2017; Corthésy et al., 2018). However, the previously tested concentrations of these orally delivered IgA Abs were not practical for clinical applications. In our study, the IN sEV administration was sufficient to stimulate a robust SIgA production against *Salmonella*, which strongly suggests that the sEVs act as adjuvant and delivery vehicles to ultimately generate protective IgA Abs. Interestingly, oral delivery of sEVs failed to stimulate the generation of IgA Abs against *Salmonella*. It remains unclear why the delivery route is crucial in stimulating a SIgA response. Perhaps the sEVs cannot survive stomach digestion, although previous studies have shown that orally delivered sEVs (exosomes) can be used to deliver substances and can resist digestion (Agrawal et al., 2017; Aqil et al., 2016; Munagala et al., 2017; Vashisht et al., 2017). One possibility is that the delivery route can affect sEV trafficking to specific compartments (Wiklander et al., 2015) (Kang et al., 2021), which biodistribution studies testing various delivery conditions could address. Mucosal delivery by IN has been previously shown to be an effective delivery route for the *Salmonella* vaccine to induce specific Ab and T cell responses in the gut (Corthésy & Bioley, 2017). Furthermore, while an expected increase in SIgA Abs was observed following the second sEV dose at 3 weeks, no dose effect was observed in SIgA Abs following the third sEV dose at 5 weeks. The SIgA titres are maintained over 12 weeks, indicating a memory B cell response. In contrast to serum IgGs, the SIgA titres tested in mucosal secretions represent the SIgA produced daily. The intestinal IgA response typically requires a high threshold for induction and has a long half-life (>16 weeks) (Mantis et al., 2011).

Apart from the delivery route of the immunogen, a good Ag choice is also essential since Ag can either generate a protective Ab response or a non-protective one in the case of immunodominant Ag that stimulates the generation of Abs that recognize

Ags not accessible on the cell surface, for example, such as OmpA (Singh et al., 2003). Thus, the hypothesis that sEVs generate protective humoral anti-bacterial responses was tested, and specific IgA Abs against known protective Ag were analysed. OmpD was selected because it was previously identified as cargo in sEVs described in this study (Hui et al., 2021), and others have shown OmpD to be a protective Ag (Domã-Nguez-Medina et al., 2020; Gil-Cruz et al., 2009). Indeed, the administration of sEVs led to anti-OmpD SIgA Abs in the sEV vaccinated mice. This novel finding suggests that sEVs might exert a protective humoral response in these animals, although the protective function of OmpD-specific SIgA induced by sEVs has not been experimentally demonstrated in this study, and requires further examination. Moreover, previous work showed that OmpD-specific IgG1 rather than the IgG2a (related to the Th1 cell response) plays an important role in protection against *S*. Typhimurium infection, at least using a model where OmpD porin has been delivered to mice by intraperitoneal injection in the absence of adjuvants (Zhang et al., 2017). Since the EVs analysed in this study have been previously shown as both the delivery agent and adjuvant driving Th1-type immunity (Hui et al., 2021), the Ag-specific humoral and cellular responses responsible for the protection offered by sEVs need to be further studied.

Since the macrophage-derived sEVs can elicit both cell-mediated Th1-type responses (Hui et al., 2021) and humoral (this study) Ag-specific responses against *Salmonella*, it was expected that the sEVs also provide protection in the murine model of *Salmonella*, which in wt BALB/c mice causes a systemic infection that resembles typhoid fever caused by *S. enterica* Typhi in humans. In the *Salmonella* challenge study, BALB/c mice vaccinated with sEVs derived from infected macrophages conferred protection, evident by reduced salmonellae colonization of the liver and against lethal challenge. The immunity elicited by these sEVs is likely long-lasting as the infection commenced 4 weeks after the last vaccination. The only precedent for using host-derived sEVs as a vaccination strategy in bacterial infection was the use of exosomes in acid-fast *Mycobacterium tuberculosis* infection (Cheng & Schorey, 2013; Smith et al., 2017), in which case the sEVs derived from cells exposed to *M. tuberculosis* culture filtrate proteins did partially protect the animals against a low-dose of aerosolized *M. tuberculosis* infection (Cheng & Schorey, 2013). Moreover, sEVs from *M. tuberculosis*-infected macrophages decreased *M. tuberculosis* infection in mice when combined with moxifloxacin, but only minimally without adding moxifloxacin (Cheng & Schorey, 2019). The protective role of sEVs in adaptive immunity against other bacterial infections has not yet been examined. However, it is known that sEVs carry bacterial DNA to bystander cells, leading to stimulation of the cGAS-STING pathway in case of *Listeria* infection, having detrimental effects on the host (Nandakumar et al., 2019). Moreover, decoy exosomes can protect against bacterial toxins, such as *Staphylococcus aureus* toxins, where sEVs act as capturing devices (Keller et al., 2020).

Overall, the data presented here demonstrate that sEVs can induce Ag-specific adaptive immune responses against *Salmonella* and protect the animals from the lethal infection dose by inducing immunological memory response toward specific *Salmonella* Ags, for instance, based on the fact that our sEV vaccination regimen induces higher titres of anti-OmpD SIgAs compared to live vaccines that do not induce appreciative levels of these Abs. EVs can traffic select bacterial Ags, such as OmpD, reflected by the cargo and immunogenicity of these sEVs leading to the production of IgA anti-OmpD Abs. However, the neutralizing role of these IgAs has not been directly demonstrated in this study. These immunogenic sEVs generate immune responses that protect animals against a lethal dose of *Salmonella*. While engineered EVs expressing immunodominant Ags are promising candidates for vaccine development, native EVs, such as the ones presented here, have some limitations since there might be batch-to-batch variations of the EV contents in vesicles obtained in this manner. Therefore, such sEVs should be optimized before any clinical use is possible. Other IN delivery strategies of *Salmonella* vaccines include the use of outer membrane vesicles (OMVs) produced by bacteria (Liu et al., 2016) as well as microbubbles (Corthésy & Bioley, 2017). Compared to these strategies mentioned above, sEVs produced by the host might offer some advantages. One such example is the tropism of the sEVs to specific tissues that depends on the source of the sEV [reviewed in (Edelmann & Kima, 2022)]. Another example is the presence of host molecules on sEVs that affect the type of immune (Th1- versus Th2-type) responses and hence act as a specialized adjuvant (Tkach et al., 2017).

In conclusion, sEVs derived from antigen-presenting cells modulate host immune responses and are promising vaccine development candidates. Since there are no FDA-approved vaccines for nontyphoidal salmonellae in humans, a novel vaccine against *Salmonella* based on sEVs would be a valuable addition. While this study's strength is testing the efficacy of the sEVs in the pre-clinical model, more extensive work in other models should be used in the future since the murine model presented here reflects the typhoid fever disease primarily, rather than the gastrointestinal disease caused by *S. enterica* in humans. Moreover, using engineered EVs expressing select immunodominant antigens would be a preferable approach for vaccine development. However, we are not yet sure of the specific constituents of the native sEVs studied here and in our previous studies (Hui et al., 2018; Hui et al., 2021) that drive protective Th1 cell responses in the immunized animals, which could be of both bacterial or host origin and hence the engineering of such sEVs is still problematic. Finally, we also need to understand the roles of IgA Abs generated in mice vaccinated with sEVs, such as the neutralizing function of these IgA Abs (Corthésy et al., 2018). Addressing these questions can fill a need to identify the role of sEVs in the adaptive immune responses against intracellular pathogens to build a mechanistic framework for developing subunit vaccines for *Salmonella* to overcome the limitations of existing vaccines.

TABLE 1 Key resources

Resource	Source	Identifier	Additional Information
RAW264.7 macrophages	ATCC	TIB-71	
Oral Gavage Needles 344058	Cadence Science	7902	Reusable 20G x 1.5″ 2.25 MM Straight
Ultracentrifuge tubes	Beckman	344058	Open top 38.5 mL
DMEM Media	Gibco	11971-025	High glucose, no phosphate
Protease Inhibitor Cocktail	Pierce	A32963	
IgG secondary antibody	Invitrogen	31430	
IgA secondary antibody	Invitrogen	62-6720	
Nunc MaxiSorp 96-well flat-bottom plate	Thermo Scientific	439454	Polystyrene, binding capacity of 600–650 ng IgG/cm2
Sodium Azide (NaN_3)	Sigma	110H0269	
ABTS	ThermoFisher Scientific	00-2024	
BSA	Fisher Bioreagents	1600-100	
Salmonella enterica Serovar Typhimurium Strain UK-1	Gift from Roy Curtiss III Lab	ATCC 68169	
Salmonella enterica Serovar Typhimurium Strain UK-1 Δ*aroA*	Gift from Roy Curtiss III Lab		
OmpD Antigen	MyBioSource	MNS1220404	

4 | MATERIALS AND METHODS

All key resources are listed in Table 1.

4.1 | Eukaryotic and bacterial cell culture and infection conditions

RAW264.7 macrophages (ATCC# TIB-71, ATCC, USA) were cultured in DMEM media which were supplemented with 10% foetal bovine serum (FBS) and 1% Penicillin/Streptomycin (Life Technologies Inc., USA). The cells were grown into 80% confluency and then seeded at 2.1×10^6 cells per one T-75 flask 1 day before infections. One hour before infection, RAW264.7 cells were washed with phosphate-buffered saline (PBS) and incubated in incomplete DMEM media.

Salmonella enterica serovar Typhimurium, strain UK-1 χ3761(wild-type), and χ9909(ΔaroA vaccine strain) were a generous gift from Dr. Roy Curtiss III, University of Florida). The bacteria were grown at 37°C in lysogeny broth (LB) media and at 200 rpm overnight. For infections, the overnight bacterial cultures were diluted in LB media to reach the optical density at 600 nm (OD_{600}) of 0.05. Such cultures were grown until the mid-logarithmic phase when OD600 reached 0.50. Bacteria were then pelleted by centrifugation at 6,000 x g for 5 min, followed by a wash in PBS.

RAW264.7 cells were infected with *S.* Typhimurium UK-1 at a multiplicity of infection (MOI) of 5:1. Two hours post-infection, culture media were replaced with DMEM containing 100 μg/mL gentamicin for 1 h and then replaced with DMEM supplemented containing 20 μg/mL gentamicin and sEV-free heat-inactivated FBS for the remaining time of infection. At 24- and 48-h post-infection, the cell culture supernatants were collected and used to purify sEVs.

4.2 | Isolation of sEVs

Cell culture supernatant was collected and resuspended in PBS containing protease inhibitor cocktail (EDTA-free; Roche, USA), followed by filtration using a 0.22-micron polyethersulfone (PES) filter. Such supernatant was centrifuged for 10 min at 500 × g, then for 10 min at 2,000 × g, and 40 min at 16,000 × g to remove debris. The supernatant from these spins was then centrifuged for

180 min at 100,000 × g using SW32 Ti rotor and Optima XPN ultracentrifuge (Beckman, USA). The pellets containing sEVs were washed once by PBS, then centrifuged at 100,000 × g. The sEV pellets were resuspended in 37.5 mL of sterile PBS supplemented with a protease inhibitor cocktail (Roche, USA). The samples were verified not to contain bacteria by culturing them in cell culture media and growing them overnight.

The sEVs were analysed by using a BCA protein assay to establish the concentration of the protein. Moreover, the sEV hydrodynamic size and concentration were characterized using Nanoparticle Tracking Analysis (NTA, NanoSight LM10). The EV samples were diluted in PBS to reach a concentration of 1.0×10^8 to 9.0×10^8 particles/mL. The PBS used to dilute the sEV samples were analysed in the NanoSight to ensure a lack of contamination. Once the desired concentration was reached, the sample was injected into the chamber of the NanoSight, and particle size distribution was analysed by Nanoparticle Tracking Analysis (NTA). The mean square displacement of scattering species that cross the path of a sheet laser was measured. The hydrodynamic diameter of objects is calculated by using the Stokes-Einstein equation. The measurement of many scatters yields direct measurements of the hydrodynamic distribution and concentration of particulates in the sample. Data were analysed and graphed in IGOR Pro 7(WaveMetrics Inc.).

4.3 | OmpD spectral analysis

The content of sEVs was analysed from a previous study (Hui et al., 2021) regarding the spectral count of OmpD antigen in sEVs isolated at 48 hpi compared to total antigen preparation.

4.4 | Intranasal immunization

Female 6- to 8-week-old female BALB/c mice (Charles River Laboratories, Inc., Worcester, MA) were used for experiments. The experimental procedures were compliant with institutional animal health and well-being policies, approved by the Institutional Animal Care Use Committee (IACUC). Animals were maintained at the University of Florida Animal Care Services in individual cages under HEPA-filtered barrier conditions. Animals were nasally vaccinated on days 0, 21, and 56 for cohort 1(n = 4) and days 0, 14, and 28 for cohort 2(n = 10) with 20 μg of sEVs derived from RAW264.7 cells infected with wild-type S. Typhimurium. The dose tested in this study (20-μg sEVs/mouse) was sufficient to generate these Th1 cell responses in BALB/c mice (Hui et al., 2021). As a positive control, animals were subjected to sodium bicarbonate pre-treatment and immunized with the live attenuated *S.* Typhimurium UK-1 ΔaroA21419(Δ*aroA*) given orally at 5×10^9 CFUs (cohort 1, n = 4; cohort 2, n = 10). The CFU of *S.* Typhimurium UK-1 ΔaroA21419 given to the animals was confirmed by serial dilution of inoculum on agar. As a negative control, animals were given PBS intranasally (cohort 1 n = 4, cohort 2 n = 10). During the procedure of intranasal immunization, each mouse was anesthetized using isoflurane and given 30 μL of PBS-suspended sEVs or PBS using a micropipette to administer dropwise into the external nares of mice (10 μL/nare at each time). Cohorts 1 and 2 were almost identical since, in each case, the animals received three doses of sEVs containing the same amount of sEVs, but the purpose of cohort 1 was to analyse the IgA and IgG responses, while the purpose of cohort 2 was to analyse the IgA responses as well as the animal survival, clinical scores, and bacterial tissue load.

4.5 | *Salmonella* Ag preparation

Soluble NaOH-treated *Salmonella* Ag was derived to that previously described (Hui et al., 2021). *Salmonella* was grown overnight at 37°C a 200 rpm with shaking until the stationary phase. Cells were pelleted at 13,000 x g at 4°C, washed twice with PBS containing 5 mM EDTA, and the cells were pelleted by centrifugation after each wash. Cells were resuspended in PBS to a concentration of 2e+10 CFU/mL. The cells were sonicated using Sonifier Cell Disruptor (Heat Systems-Ultrasonics Inc., Plainview, NY), and the debris was pelleted by centrifuging the sample 13,000 x g at 4°C. The sample was sterilized through a 0.22-μm PES (polyethersulfone) filter (Genesee Scientific, El Cajon, CA). For the optional NaOH inactivation of LPS, 10 M NaOH was added to the antigen preparation to a final concentration of 0.25 M NaOH and incubated at 37°C for 3 h. Phenol red was added to monitor the alkaline pH. Hydrochloric acid was titrated to adjust pH to neutral and measured using pH paper strips.

4.6 | IgA ELISA

Stool samples were collected from experimental animals once a week. The stool samples were weighed, and 25% weight/volume of faecal slurry solution (0.01% sodium azide, 1% protease inhibitor, PBS) was added. The samples were homogenized by vortexing for 15 min. Nunc MaxiSorp flat-bottom 96-well plates (Thermo Fisher Scientific, Waltham, MA) are polystyrene, high-binding capacity ELISA plates suitable for capturing immunoglobulins at a concentration of ~600-650 ng IgG/cm2. The ELISA plates

were coated with 2 μg *Salmonella* antigen per well and incubated overnight. Alternatively, 0.1 μg per well of OmpD recombinant protein (MyBiosource) was added to the wells. Wells were blocked for 2 h by using 1% bovine serum albumin at 37°C, followed by washing three times with PBS containing 0.2% Tween-20 and one wash with PBS alone. The stool samples were serially diluted and added to the well, followed by overnight incubation at 4°C. After the wash steps described above, the secondary Ab was added, goat anti-mouse IgA, cross adsorbed-HRP (Invitrogen, Rockford, IL). The Ab was incubated with samples at 37°C for 90 min. 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) substrate (Life Technologies) was added, and the absorbance was read at 5- and 15- min intervals for 60 min total at 415 nm in Cytation 3 plate reader (Biotek, USA). Endpoint titres were defined as the reciprocal of the dilution giving absorbance 0.1 U above negative control. To calculate the frequency of the anti-OmpD Abs amongst all anti-*Salmonella* IgAs, the values were reflected as fold change of total anti-*Salmonella* IgAs. The data were analysed by multiple Mann-Whitney U tests with Bonferroni correction for multiple comparisons.

4.7 | IgG ELISA

Experimental animals were bled bi-weekly between the doses of sEVs and collected blood in Eppendorf tubes. Blood samples were centrifuged for 10 min at 10,000 × g at 4°C. The serum in the supernatant was separated from the pellet and used for ELISA to determine mouse Ab endpoint titres. Nunc MaxiSorp 96 well plates (Thermo Fisher Scientific, Waltham, MA) were coated with 2 μg NaOH-treated (LPS-cured) *Salmonella* Ag per well and incubated overnight. Wells were blocked for 2 h by using 1% bovine serum albumin at 37°C, followed by washing three times with PBS containing 0.2% Tween-20 and one wash with PBS alone. Serially diluted sera were added to the well, followed by overnight incubation at 4°C, followed by washing three times with PBS containing 0.2% Tween-20 and one wash with PBS. The secondary Ab goat anti-mouse IgG, human absorbed-HRP (Invitrogen, Rockford, IL) was then added, and the rest of the protocol was performed as described for IgA ELISA.

4.8 | Mouse challenge with *Salmonella*

Four weeks after the last dose of sEVs was given, the mice were pretreated with 50 μl of 0.3 M sodium bicarbonate and challenged with 4.5×10^6 CFU *S.* Typhimurium given by oral gavage. Ten mice were followed over 14 days to assess survival and analysed for statistical differences by log-rank tests. Three mice were challenged and sacrificed on day 4 for CFU plating. Animals were monitored at least twice a day. The moist chow/gel diet/napa nectar was provided for sick animals (Body Condition Score, BCS = / > 2). For dehydrated animals, 1 mL of normal saline was administered twice daily. Animals that reached body score BCS = // < 2 were euthanized. Some figures were prepared using BioRender.com.

4.9 | Mouse dehydration scoring

Mice were assessed twice daily following challenge for body condition scores and hydration status. Mice were given a score of either 0 for no dehydration, 1 for mild dehydration, and 2 for severe dehydration based on body condition, weight loss, and skin tenting. All cages with any mice scoring a 1 or 2 were supplemented with moist chow, and the mice scoring a 1 or 2 were rehydrated with saline.

4.10 | CFU determination in liver

Tissues, including liver, were harvested from each mouse. The organs were weighed and homogenized for 5 min using 5-mm stainless steel beads and TissueLyser II (Qiagen). The homogenates were then serially diluted in sterile PBS and spread plated on LB agar to establish the number of colony-forming units (CFUs) per gram of tissue. Samples were diluted and plated with three technical replicates.

4.11 | Histology

As explained above, 6 to 8-week-old groups of female BALB/c mice (n = 3) were subjected to immunization with sEVs and challenged with a lethal dose of *Salmonella*. The intestine of each mouse was dissected, washed thoroughly in PBS, fixed in 10% formalin, and embedded in paraffin. The Molecular Pathology Core carried out histochemistry preparation at the University of Florida. The intestines were rolled into Swiss rolls, and for haematoxylin and Eosin staining, tissue sections were deparaffinized with xylene and rehydrated in an ethanol solution, followed by staining with haematoxylin (Richard-Allan Scientific, 7212). The bluing reagent was added (Richard-Allan Scientific, 7301), followed by a wash in 80% ethanol and eosin staining (Richard-Allan Scientific, 71311). The stained slides were dehydrated in a graded ethanol series, dipped in xylene, and covered with coverslips.

Slides were scanned using an Aperio CS2 Scanscope (Leica/Aperio, Vista, CA). Images were taken using Aperio ImageScope v12.4.3.

4.12 | Statistical analysis

Kaplan-Meir Curve was used to visualize the survival of the animals in the challenge study, where the log-rank tests were used to analyse the significance of the changes in samples. One-Way ANOVA tests with multiple comparisons were used for the Ab titres. All statistical analyses were performed by using Graph Pad Prism Version 9.3.1.

AUTHOR CONTRIBUTIONS

Lisa E Emerson: Formal analysis; Investigation; Methodology; Writing – original draft. Hailey Barker: Formal analysis; Methodology; Visualization; Writing – original draft. Terri Tran: Investigation. Samantha Barker: Investigation. Samantha Enslow: Investigation. Mark Ou: Formal analysis; Investigation. Carol Hoffman: Investigation; Methodology. Melissa Jones: Conceptualization; Formal analysis; Writing – original draft. Mariola J. Edelmann: Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Visualization; Writing – original draft.

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CONFLICT OF INTEREST

No competing interests were associated with this study.

DATA AVAILABILITY STATEMENT

The proteomic data have been deposited to Proteome Exchange and are available as the dataset: http://doi.org/10.6019/PXD024838

ETHICS APPROVAL STATEMENT

The experimental procedures were compliant with institutional animal health and well-being policies, approved by the Institutional Animal Care Use Committee (IACUC).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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