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Altered host immune responses to membrane vesicles from *Salmonella* and Gram-negative pathogens

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ABSTRACT

Membrane vesicles (MVs), discrete nano-structures produced from the outer membrane of Gram-negative bacteria such as *Salmonella enterica* Typhimurium (*S.* Typhimurium), strongly activate dendritic cells (DCs), contain major antigens (Ags) recognized by *Salmonella*-specific B-cells and CD4+ T-cells, and provide protection against *S.* Typhimurium challenge in a mouse model. With this in mind, we hypothesized that alterations to the gene expression profile of bacteria will be reflected in the immunologic response to MVs. To test this, we assessed the ability of MVs from wild-type (WT) *S.* Typhimurium or a strain with a phenotype mimicking the intracellular-phase of *S.* Typhimurium (PhoP^c) to activate dendritic cells and initiate a strong inflammatory response. MVs, isolated from wild-type and PhoP^c *S.* Typhimurium ($_{WT}$MVs and $_{PhoPc}$MVs, respectively) had pro-inflammatory properties consistent with the parental bacterial strains: $_{PhoPc}$MVs were less stimulatory for DC activation *in vitro* and were impaired for subsequent inflammatory responses compared to $_{WT}$MVs. Interestingly, the reduced pro-inflammatory properties of $_{PhoPc}$MVs did not completely rely on signals through TLR4, the receptor for LPS. Nonetheless, both $_{WT}$MVs and $_{PhoPc}$MVs contained abundant immunogenic antigens capable of being recognized by memory-immune CD4+ T-cells from mice previously infected with *S.* Typhimurium. Furthermore, we analyzed a suite of pathogenic Gram-negative bacteria and their purified MVs for their ability to activate DCs and stimulate inflammation in a manner consistent with the known inflammatory properties of the parental strains, as shown for *S.* Typhimurium. Finally, analysis of the potential vaccine utility of *S.* Typhimurium MVs revealed their capacity to encapsulate an exogenous model antigen and stimulate antigen-specific CD4+ and CD8+ T-cell responses. Taken together, our results demonstrate the dependence of bacterial cell gene expression for MV immunogenicity and subsequent *in vitro* immunologic response, as well as their potential utility as a vaccine platform.

1. Introduction

Q3 The increased incidence of emerging infectious pathogens compounded with an emergence of antibiotic resistant pathogens emphasizes the necessity for continued vaccine innovation [1,2]. Exacerbating the need for novel vaccines is the continuing threat from biothreat agents for which there is no available vaccination [3]. While single- or multi-subunit vaccines are "encouraged" from a targeted approach and regulatory perspective, these vaccines do not always provide the level of protective immunity observed after natural infection or by immunization with live attenuated vaccines [4]. In this effort, attenuated microbial vaccine vectors are intensively studied as an alternate strategy to induce prophylactic immunity to infection [5]. Of these, *Salmonella enterica* Typhimurium, a Gram-negative facultative intracellular bacterial pathogen that causes gastroenteritis in humans and livestock, and systemic disease in mice, is a leading bacterial vector being tested in strategies for inducing protective immunity to a variety of pathogens [6–9].

The infectious cycle of *S.* Typhimurium within the intestine is characterized by the coordinated expression of two distinct *Salmonella* pathogenicity islands (SPI): SPI-1, responsible for induction of bacterial uptake by host cells, and SPI-2, which manipulates the host cell and establishes the intracellular replicative niche. Both SPIs encode a type-3 secretion system

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(T3SS) and numerous effector molecules whose expression is spatially and temporally regulated [10]. For SPI-2, the PhoP–PhoQ regulon controls multiple genes that regulate the surface modifications *S.* Typhimurium to transition to the intracellular niche [11,12]. *S.* Typhimurium under SPI-1 or SPI-2 show a distinct gene expression profile and outer membrane composition and pro-inflammatory properties [13–15]. PhoP-dependent molecular modifications of membrane LPS are known to increase bacterial resistance to antimicrobial peptides and lower the inflammatory bacterial LPS signature [16]. However, non-LPS outer membrane modifications also occur in a PhoP-dependent manner [17]. Indeed, *S.* Typhimurium genetically engineered to have a constitutively active PhoP ($PhoP^c$) mimic the intracellular-phase phenotype, are increased in resistance to antimicrobial peptides and have an LPS with reduced pro-inflammatory properties [18–21].

Membrane vesicles (MVs) are discrete non-somatic nanoparticles (50–250 nm diameter) derived from the bacterial periplasm and outer membrane [22–26]. Although produced by all Gram-negative bacteria investigated thus far, the mechanisms regulating MV production and formation are only now being clarified [25–29]. Gram-negative MVs contain several compounds recognized by host microbial pattern-recognition receptors [26,30–32]. Our group and others have suggested that MVs are the biologically relevant form of pathogen-associated molecular pattern (PAMP) complexes recognized by host defenses *in vivo* [9,33–35]. Additional work has demonstrated the utility of *S.* Typhimurium MVs as a strong inducer of immunity against *S.* Typhimurium, but also as a carrier for antigens against other pathogens [36–38] when genetically expressed in the parental bacteria.

Given the distinct gene expression profiles of *S.* Typhimurium during the course of an infection [10,39], and the highly immunogenic response to MVs, we hypothesized that MVs from different *S.* Typhimurium expression profiles would result in altered host immune responses. To test this, we measured the stimulatory capacity of MVs from wild-type (WT) or a strain constitutively expressing an intracellular phenotype ($PhoP^c$) in TLR4-sufficient or TLR4-deficient DC cultures. Additionally, we identified a robust *Salmonella*-specific T-cell recall response to $_{WT}$MVs or $_{PhoPc}$MVs and identified the potential for *S.* Typhimurium MVs to serve in antigen delivery. Finally, the range of DC activation and inflammatory induction from other Gram-negative pathogen MVs was reported.

2. Materials and methods

2.1. Bacterial strains, antigens, and membrane vesicles

Bacterial strains used in this study were *S.* Typhimurium strain ST14028 (wild-type), ST14028 (*pho-24 pmrA505* ($PhoP^c$) [40] from Dr. Samuel Miller, University of Washington, were used for preparing Ags and MVs. *Yersinia pseudotuberbulosis* (RA090, YpIII pYV+, originally from Dr. Joan Mecsas, Tufts University, USA) [41], *Yersinia pestis* BSL2-approved (RA091, KIM8 pgm−, pCD1+, pPCP1−, pMT1+, from Dr. James Bliska, SUNY Stonybrook, USA), and *Francisella novicida* (RA089, U112 strain, from Dr. Francis E. Nano, University of Victoria, Canada) [42] were used for preparing Ags and MVs. Somatic wild-type and $PhoP^c$ *Salmonella* Ags were prepared as before [34]. The vaccine strain *S.* Typhimurium SL3261 (an *aroA* derivative of SL1344) [43] was used to establish *S.* Typhimurium immunocompetent mice. For somatic Ags of non-Salmonella bacteria, cultures were prepared as follows: RA090 and RA089 were grown in TSB overnight with aeration at room-temperature or 37 °C, respectively; RA091 was grown in 1% tryptone (Difco) overnight at room-temperature with aeration. Bacteria were heat-inactivated for 1 h at 65 °C. Membrane vesicles (MVs) were isolated from bacteria as grown for somatic Ag with the exception the supernatant was from log-phase cultures. Briefly, bacterial cells were removed by centrifugation and the supernatant was filtered through a 0.22 μm pore-size filter (Corning, Inc.). A tangential flow filtration (TFF) system (MiniMate, Pall, Port Washington, NY, USA) with 100 kDa molecular weight cut-off cassettes was used to enrich the MVs from the cell-free supernatant according to the manufacturers instructions. Enriched MVs were purified and concentrated by ultracentrifugation (100,000 × *g*, 1–2 h, Beckman) and reconstituted in sterile, non-pyrogenic water, an aliquot was lyophilized to determine total dry weight and stored at 4 °C in a 1 mg/ml working concentration in PBS. In experiments where the model Ag purified chicken ovalbumin (OVA, Sigma) was encapsulated, MVs were purified as above. Dehydrated MVs were rehydrated with OVA (200 mg/ml) to a standard MV concentration (1 mg/ml) resulting in $_{OVA}$MVs. $_{OVA}$MVs were used at a 25 μg/ml based on MV dry-weight; this corresponded to an encapsulated OVA concentration of 7 μg/ml determined by Western (data no shown). To remove excess OVA, $_{OVA}$MVs were washed twice with H_2O, ultracentrifugated, and reconstituted in PBS at 1 mg/ml. H-2^b MHC-I- and II-restricted stimulating peptides of OVA, OVA (257–264) and OVA (323–339) respectively, were purchased from AnaSpec (Freemont, CA, USA) and used at a final concentration of 1 μg/ml. Synthetic liposomes (poly(lactic-co-glycolic acid) (PLGA)) were purchased from Avanti Lipids (Alabaster, AL, USA).

2.2. Mice and cell culture

Six to eight-week-old female C3H/HeJ and C3H/HeN mice were obtained from The Jackson Laboratories (Bar Harbor, ME) and used at 6–14 weeks of age. Transgenic OT-I and OT-II mice with T-cells specific for the H-2^b MHC-I- and II-restricted epitopes of OVA, respectively, were purchased from Jackson Laboratories. All mice were housed in specific pathogen-free conditions and cared for in accordance with Texas A&M Health Science Center Institutional Animal Care and Use Committee guidelines. Immune mice, generated by oral infection with 10^9 *S.* Typhimurium SL3261 by gavage with feeding needles ($22 \times 1^{1/2}$ with $1^{1/4}$ mm ball, No. 7920, Popper & Sons, Inc., New Hyde Park, NY), were used at 70–100 days post immunization. Murine host cells were cultured as described [33]. 10% FCS (RP-10) was used to culture T-cells, whereas 5% FCS (RP-5) was used for DCs which were derived from C3H/HeN or C3H/HeJ (LPS hyporesponsive, [44]) bone marrow cultured for 7 days in RP-5 plus GM-CSF (20 ng/ml) [45].

2.3. DC and T-cell activation, flow cytometry and intracellular cytokine staining

For detection of DC maturation, 2.5×10^5 DCs, resuspended in cold PBS plus 0.5% (w/v) bovine serum albumin (Sigma Aldrich, St. Louis, MO) (PBSA), were co-incubated with the indicated concentrations of bacterial Ags for 18–24 h at 37 °C in 5% CO_2. Afterwards, DCs were surface stained in the presence of Fc block (clone 2.4G2) with phycoerythrin (PE)-labeled antibodies to murine CD11c (clone HL3) and biotin-conjugated antibodies specific for MHC class II (I-E^k, clone 14-4-4S), CD86 (clone B7-2), or CD40 (clone 1C10) plus streptavidin–allophycocyanin (SA-APC). T-cell and DC activation were measured by production of cytokines in response to bacterial Ags and detected by intracellular cytokine staining (ICS) [46]. Briefly, 4×10^6 splenocytes (in RP-10) collected directly from *Salmonella*-immune mice or OT-I and OT-II mice and from naïve mice or 2.5×10^5 DCs (in RP-5) were co-incubated with the

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