MEMBRANE VESICLES AS A VACCINE PLATFORM FOR SALMONELLA

A Senior Scholars Thesis

by

MEGAN LEE HINKLEY

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2010

Major: Biochemistry

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Approved by:

Research Advisor: Robert Alaniz
Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

Membrane Vesicle as a Vaccine Platform for Salmonella. (April 2010)

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Salmonella species cause substantial morbidity, mortality, and disease burden globally with multiple clinical syndromes. A protective host defense to *Salmonella* infection requires both T-cell (cell-mediated) and B-cell (humoral) immune response. Current vesicle vaccine platforms include inactivated, non-replicating, or synthetic liposomes which do not produce a robust immune response because they lack foreign distinctness and complexity. Membrane vesicles (MVs), released in a conserved process by Gram-negative bacteria, are discrete, spherical nano-particles that are composed of outer membrane and periplasmic constituents including lipopolysaccharides and proteins. MVs are promising vaccine candidates for the following reasons: their intrinsic adjuvant properties, genetically malleability via the parental bacterial strain that can alter vaccine efficacy, a minimal production cost, and they are stable in powder form. As biologically derived but nonreplicating particles they are important in the vaccine strategy for immunocompromised populations. My findings reveal MVs posses important

inflammatory properties that demonstrate a viable vaccine platform potential for *Salmonella* infection.

DEDICATION

This thesis is dedicated to

George Spencer Mickum

Completing this research project has been an adventure that I couldn't imagine finishing without your unwavering love and support. Plus, it's a good preview of our lives in graduate school, yet another endeavor we will embark on together. You are my rock.

ACKNOWLEDGMENTS

First of all, I would like to thank Dr. Robert Alaniz because without his guidance I may not have discovered how truly rewarding biomedical research can be. Under his supervision I have grown from a student worker to a Research Scholar, something I could not have accomplished on my own. Thanks to his wealth of knowledge and dedication to teaching I feel confident in my ability to continue in graduate school with success. He is an inspiring person to work with and everything a student could ask for in a mentor. I'm going to keep my "Science is Fun" button forever.

I would also like to recognize Kristina Ryden, Jane Miller, and the members of the Alaniz lab for the constant support and encouragement. Kristina was never too busy to show me a technique or teach me how to fix the Aria every single time I broke it. She is not only a valued colleague but a very dear friend. It's impossible to have a bad day when working along side her, especially since she shares my ridiculous organizational tendencies. I will miss her, and the lab, very much.

My family has been a constant lifeline during my college years. My sister, having experienced many of the same trials and tribulations, was never without the advice I always seemed to need. Clare, I love you and look forward to the day when we can work side by side. My parents, Dave and Tawnie, have been endlessly supportive in

everything I do. From the very beginning they have been role models, friends, and parents. No one could do it better and they gave me the strength I needed to succeed.

NOMENCLATURE

APC	Antigen presenting cell
DC	Dendritic cell
IFN	Interferon
IL	Interleukin
LB	Luria-bertani broth
LPS	Lipopolysaccharides
MLN	Mesenteric lymph nodes
MV	Membrane vesicle
Mϕ	Macrophage
PMN	Polymorphonuclear cell
STM	*Salmonella typhimurium*
TFF	Tangential flow filtration
TNF	Tumor necrosis factor
TSB	Tryptic soy broth

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CHAPTER I

INTRODUCTION

Microbes are ubiquitous in the world, and some cause disease. Yet despite the constant exposure, illness does not persistently occur. This is due to the immune system, a collection of specialized cells and tissues that use specific biological structures and mechanisms in order to protect against disease by identifying then killing pathogens. In vertebrate animals there are two arms of the immune system that protect the body from infection, the innate branch and the adaptive branch.

Innate host defenses

Innate host defenses are genetically encoded and do not improve over time or after multiple exposures. Innate immunity includes physical barriers, like the skin and epithelial cells that line mucosal surfaces, which block the entrance of microbes (13). Mucosal membranes line the respiratory, digestive, and urogenital tracks, and secrete antimicrobial chemicals and enzymes, like the small cationic peptides defensins, an antimicrobial protein against various microorganisms (4, 5). Besides physical and microbicidal barriers, the innate immune system also uses non-specific effector cells that act rapidly against invaders recognized as foreign. One such example of a non-specific effector cells are macrophages, which reside in almost all the tissues of the body and are

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This thesis follows the style of the Journal of Immunology.

often the first immune cells to encounter invading pathogens. Mϕ engulf and kill pathogens in a process called phagocytosis, help induce inflammation by signaling and recruiting other effector cells, and present antigen to activate the adaptive immune system (7, 13).

Adaptive host defenses

The adaptive branch of the immune system responds later after the innate defenses. Although innate responses control infection, clearing an infection lies with the adaptive immune response. Adaptive immunity is often referred to as "specific immunity" because it responds to a specific foreign cell or protein. The components that immune cells respond to are termed epitopes and are unique to the pathogen. The adaptive immune response is also capable of remembering each invader it encounters by keeping a collection of memory cells to specific epitiopes that respond faster and in greater magnitude (2). The adaptive response is closely linked to the innate response through cell-cell interactions of adaptive effector cells and APCs, which are required to activate the adaptive effector cells. This is the hallmark branch of the immune system and can be further divided into cell-mediated immunity or humoral immunity. Cell mediated immunity involves the T lymphocytes whose functions fall into three broads classes: cytotoxic, helper, and regulatory. Cytotoxic T cells kill host cells infected with intracellular pathogens, helper T cells are necessary to activate B cells, and regulatory T cells help control immune responses. B lymphocytes make up the humoral response and are responsible for the production of antibodies. Antibodies help in the destruction of

foreign invaders and also provide us with long-term protection after clearing the infection. B cells have unique surface receptors called the B cell receptors that are specific to different epitopes on pathogens. When a pathogenic antigen binds to the B cell receptor, the cell proliferates, differentiates into a plasma cell, and produces antibodies with identical antigen specificity (7, 13).

Salmonella infection

Salmonella enterica is a rod shaped, Gram-negative, facultative intercellular bacterium belonging to the *Enterbacteriaceae* family (17). *Salmonella* causes as many as 1.3 billion cases of disease per year worldwide and as many as 200,000 deaths. In humans, multiple clinical syndromes include typhoid fever (enteric fever), enterocolitis, diarrhea, and bacteremia (3). Over 2,500 serovars, or classification groups based on surface antigens, have been identified. However, *typhi* and *paratyphi,* which cause typhoid fever, are exclusively pathogenic for humans (17). Our current understanding of typhoid pathogenesis comes from infecting susceptible mice with the serovar *typhimurium* that produces a disease with typhoid-like symptoms (3).

Protective immunity to *Salmonella*

In mice, the first cells that encounter *Salmonella* are intestinal epithelial cells, DCs, and Mϕs (1, 15). Interaction with these cells leads to production and release of pro-inflammatory cytokines and chemokines leading to a large influx of immune effector cells such as PMNs, DCs, and Mϕs to the infected area. Some of the critical cytokines

for producing a robust immune response to STM include IFN-γ, TNF-α, and IL-12. TNF-α, in particular, activates macrophages ands enhances their toxic nitric oxide production to increase overall bacterial killing (3). Pathogens may overwhelm the innate immune system and need adaptive immunity for host defense (Murphy). In previous studies, it has been determined that a protective host defense to *Salmonella* infection requires both T-cell (cell-mediated) and B-cell (humoral) immune response (1, 17).

Current vaccine platforms

A quality vaccine must develop a robust, protective immune response, stimulate both B and T cells, provide long lasting immunity, and have minimal adverse side effects (7). Current vaccine models that utilize live pathogens have been attenuated by random or directed mutagenesis. Some strains used include aroA-null, phoP-null, and waaL-null. The aroA-null mutant is deficient in an aromatic amino acid, the phoP-null mutant locks the bacterium in its intracellular conformation, and waaL-null mutants lack the O antigen in the LPS. Mutagenesis only works for pathogens that are known to have immunogenic properties. To date, around 100 genes are associated with virulence in *Salmonella typhimurium,* but there remains no vaccine. This is due to the fact that not all mutations create stable attenuated phenotypes. Some mutations do not attenuated the pathogen enough for safe administration, while others are so attenuated that they are unable to induce protection against a live, virulent *Salmonella* challenge. Historically, vesicle vaccines include non-replicating or synthetic liposomes. However, liposome vaccines do not produce a robust immune response because they lack foreign distinctness and

complexity (16).

Membrane vesicles

Membrane vesicles (MVs) have been investigated for decades, but the physiological significance and utility has not been appreciated until recently (4). MVs are discrete, spherical nano-particles that are composed of outer membrane and periplasmic constituents including lipopolysaccharides (LPS) and proteins. The release of vesicles is a conserved process in Gram-negative bacteria, including many pathogens such as *Salmonella*, and they function as an alternate secretion pathway (1, 11). MV production *in vivo* may be one method by which bacteria interact with eukaryotic cells. They are interesting candidates for vaccines due to their intrinsic adjuvant properties and genetically malleability via the parental bacterial strain which can be used to alter vaccine properties. MVs also involve a minimal production cost/complexity and are stable in powder form. MVs are biologically derived but are non-replicating particles, which makes them important in the vaccine strategy for immunocompromised populations that cannot receive live attenuated vaccines without complications. In previous studies, MV vaccination in mice induced pathogen-specific B-cell and T-cell host defenses and protective immunity against subsequent live pathogen challenge (1).

CHAPTER II

METHODS

Bacterial strains and cultures

The bacterial strains used in this study are both *S. typhimurium* STM14028 strains received from Dr. Andrews-Polymenis. RA100 is a wild-type virulent strain and RA133 is a del-STM3713::KanR (waaL-null with kanamycin resistance). STM strains were grown in LB, TSB, or on LB agar plates at 37°C.

Tissue cultures

J774-A1 Mϕs were maintained in a humidified incubator in an atmosphere of 5% CO_2 at 37°C and cultured in RPMI complete medium supplemented with 5% fetal calf serum (FCS; Gibco-Invitrogen), 2-mercapthoethanol (50 uM), Pen/Step antibiotics (100ug/mL), and Gentamicin antibiotics (50ug/mL).

Glassware preparation

All glassware used for the isolation and purification of membrane vesicles was acid-washed in 5% phosphate free Contrad detergent and rinsed thoroughly in distilled water. All interior surfaces were rinsed with 1N HCl for 10 minutes and rinsed thoroughly in distilled water. Media was made in the Fernback flasks with Millipore water, autoclaved, and stored 1-4 days with an airtight seal until use. Sorvall centrifuge bottles and glass vacuum filter bottles were autoclaved and stored with sealed caps.

***Salmonella* antigens**

Heat inactivated (HKSTM) antigens were prepared from10 mL LB cultures grown overnight at 37°C with shaking (200 rpm). HKSTM were pelleted by centrifugation (14,000 rpm for 15 min at 25°C) and resuspended to $2x10^9$ CFU/mL in Millipore water, aliquoted, and stored at -20°C. The following antigens were previous prepared by Kristina Ryden or other lab alumni: phorbol myristate acetate (PMA) at working concentration 20ng/mL stored at -20°C, concanavalin A from *Canavalia ensiformis* (ConA) at working concentration 0.1ug/mL stored at -20°C, and lipopolysaccharide (LPS) at 1mg/mL stored at 4°C.

Isolation and purification of MVs (Figure 1)

Vesicles were harvested from culture supernatants of bacteria grown to mid-log phase. A 35 mL TSB in a 50 ml conical was inoculated with a single colony and incubated overnight at 37°C with shaking (200 rpm) and used to inoculated 1 L TSB (acid-washed Fernbach flask) and incubated 4 hours at 37°C with shaking (200 rpm) to an OD_{600} of ~3. Bacteria were pelleted by centrifugation (9,000 x g for 30 min at 4°C) and the supernatant was decanted and passed through a 0.45 um filter. The supernatant was concentrated by either Amicon Ultra Millipore filters (100K cutoff) and centrifugation (2500 rpm for 10 min/spin at 4°C) or TFF (100K cassette) using gas sterilized tubing and reservoir. Vesicles were collected by ultracentrifugation (150,000 x g for 2 h at 4°C), lyophilized, and resuspended to 1.0 mg/mL in Millipore water and stored at 4°C.

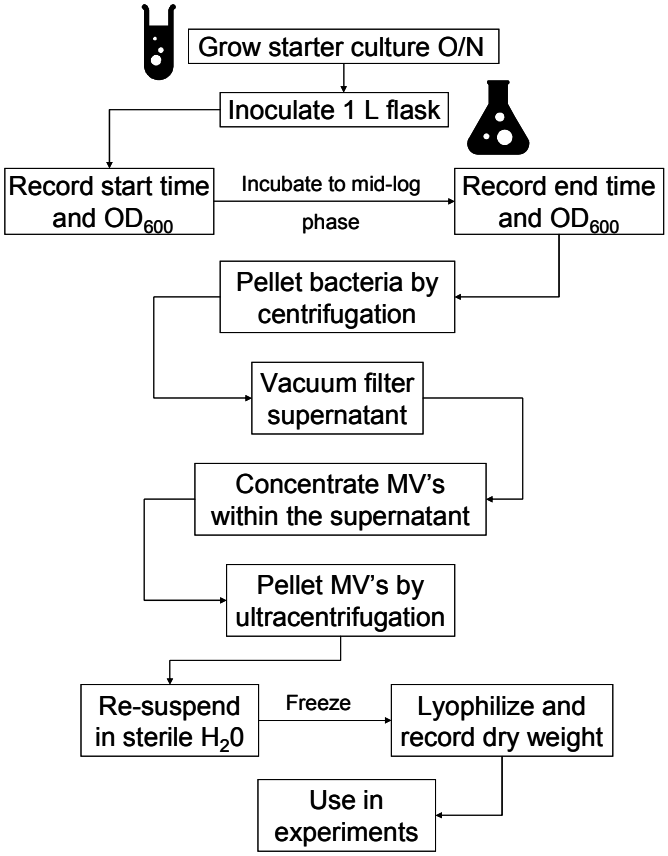

Figure 1. MV Isolation Flow Chart. This depicts the isolation and purification of MVs developed during the course of this project.

Particle analysis of MVs

Concentrated MVs were lyophilized once or twice and reconstituted to 1mg/mL. The first dilution of 1:10 used 150uL MV sample and 1500uL Millipore ultra pure H_2O. The second dilution of 1:3 used 1mL from the 1:10 sample and 3mL Millipore ultra pure H_2O. The particle analysis requires a 3mL sample taken from the final dilution. The samples were analyzed in the Slahtz lab in the Chemical Engineering Department.

Flow cytometry and intracellular cytokine staining (ICS)

Production of cytokines by J774-A1 Mϕs was detected by ICS using a method previous optimized by the Alaniz lab. 2 x 10^5 macrophages (in RPMI-C) were coincubated with the indicated titrations of HKST, MVs, LPS, ConA, or PMA in single wells of 96-well U-bottom tissue culture plates. Plates incubated at 37°C in 5% CO_2 for 4 or 24 hrs. GolgiPlug was added (1/1000 dilution) for the final 4 hr in 24 hr stimulations or at the beginning for 4 hour stimulations. After stimulation, cells were washed twice in cold PBS plus 0.5% (w/v) BSA (Sigma Aldrich) (PBSA), and surface stained with A488-labeled Abs to murine CD11b. Stained cells were fixed on ice with 2% paraformaldehyde and permeabilized with Perm/Wash buffer, followed by staining with PE-labeled Abs to murine TNF-α and APC-labeled Abs to murine IL-12. Cells were resuspended in PBSA and stored at 4°C in the dark until analysis. Unstained, unstimulated cells were used as negative controls. Data were acquired on a FACS Aria and analyzed using FlowJo Software.

Mouse immunization

Female C57Bl6 mice were used at 17- 18 weeks of age. Mice were immunized intraperitoneal with 50-100ng of RA100 MVs and the control group received PBS. Three days post injection 1 mouse was taken from each group for a cytokine harvest. Organs from individual mice (spleen, liver, Peyer's patches, MLN, and blood) were homogenized in sterile 1x-NP40 using a motorized homogenizer (Omni International). Homogenates were spun down (1400 rpm for 10 min at 25°C) and filter sterilized using a

0.2 um syringe filter and 25 gauge needle. Samples were stored incorrectly at 4^{o}C for several days before being moved to -20^{o}C. All mice were housed in specific pathogen-free conditions and cared for in accordance with Animal Care and Use Committee guidelines.

CHAPTER III

RESULTS

Salmonella MVs are nano-scale particles

When evaluated in the particle analyzer, MVs lyophilized once had a mean diameter of 179.9nm and a range of 50-500nm (Figure 2). The MVs lyophilized twice showed two distinct ranges of diameters. The first ranged 158.11-666.76nm and the second grouping ranged from 3.749-11.858um. Multiple lyophilizations must cause the individual MVs to fuse together and form much larger vesicles. This indicated that single lyophilization proved to be the best method of retaining the MVs natural form while treating them as a vaccine product.

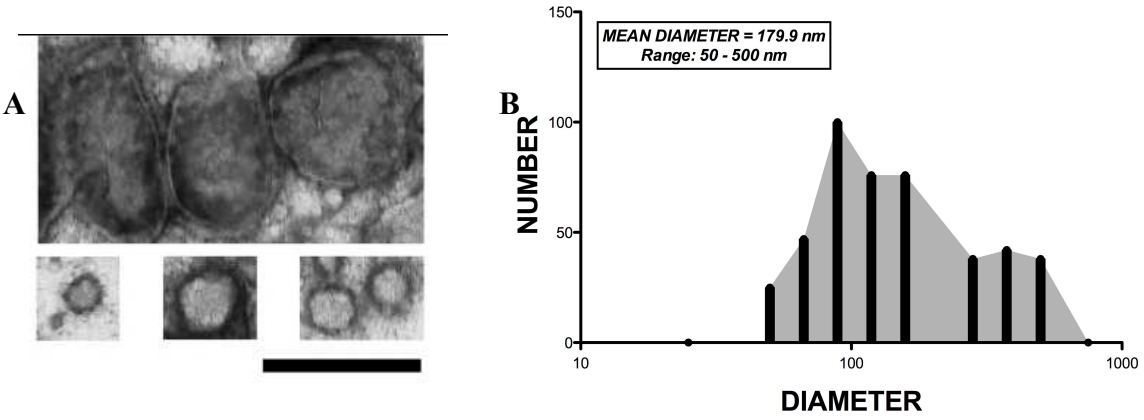

Figure 2. MV Particle Size Analysis, Electron Micrograph. MVs were isolated from RA100 a wild type strain of *S. typhimurium* 14028. (**A**) Electron micrograph obtained from: Alaniz, R.C., B.L. Deatherage, J.C. Lara, and B.T. Cookson. 2007. Membrane vesicles are immunogenic facsimiles of *Salmonellae typhimurium* that potentially activate DCs, prime B and T cell responses, and stimulate protective immunity *in vivo*. *J Immunol* 179: 7692-7701; bar = 250nm. (**B**) Particle size analysis of a 150uL aliquot of MVs, lyophilized once, and diluted (1:30) in Millipore ultra pure H_2O to a volume of 3mLs each.

Salmonella MVs activate Mϕs *in vitro*

Mϕs reside tissues of the body and are often the first immune cells to encounter pathogens that invade the tissues. Mϕs engulf and kill pathogens in a process called phagocytosis, help induce inflammation by signaling and recruiting other effector cells and present antigen to activate the adaptive immune system (1, 7). After MVs were incubated with J774-A1 Mϕs, large amounts of TNF-α were produced. TNF-α is an important mediator in the inflammation by helping regulate the immune response and activating the acute phase response (7). IL-12 was not produced in any significant amount by the MVs or any other stimulants.

Salmonella MVs are potent stimulators of Mϕs *in vitro*

MVs induce Mϕs to produce TNF-α, indicating they can activate pro-inflammatory pathways in macrophages. Compared to unstimulated cells, the MVs consistently activated over 80% of the cells to produce large amounts of TNF-α (Figure 3). LPS and HKSTM effectively activated macrophages but lost activity quicker after titration at 1:25 and 1:125 respectively. The MVs retained activation properties through a dilution range of 1:10,000 indicating they are capable of stimulating host immune cells over broad concentrations. For Mϕs stimulation experiments comparing the MVs and other antigens, 1:5 dilutions gave the clearest relationship of activating properties.

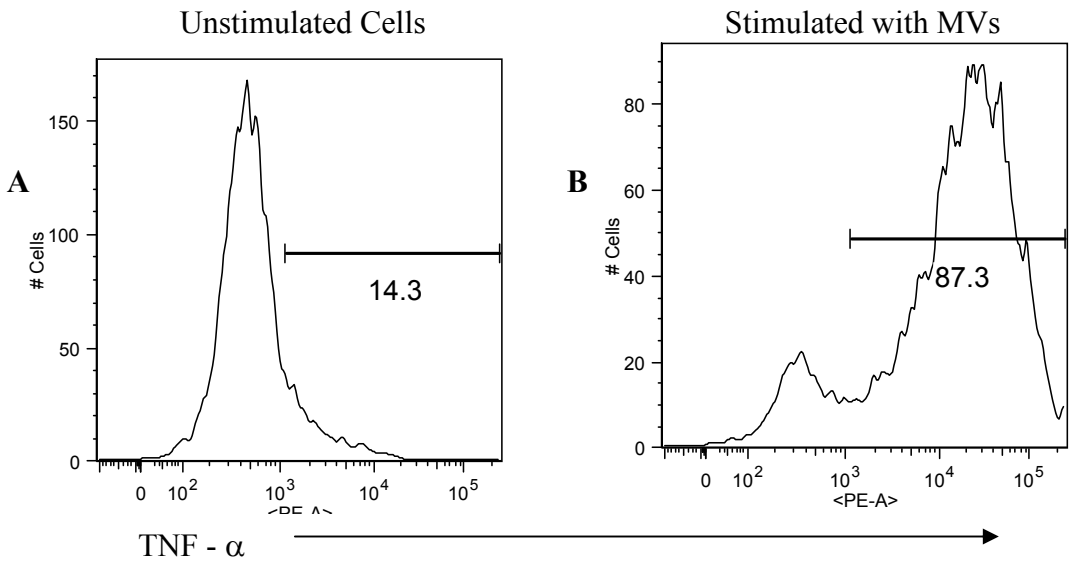

Figure 3. Macrophage Stimulation with MVs. This figure shows the TNF-α production in murine J774 Mϕs after a 4-hour stimulation with (**B**) MVs (50 μg/ml) or (**A**) no stimulents as a negative control. Percentages indicate proportion of J774 cells producing TNF-α of total cells.

MVs activate the immune system *in vivo*

MVs are composed of outer membrane and periplasmic constituents including LPS and proteins suggesting they can be recognized by the innate immune response and adaptive immune response specific for *Salmonella* (1, 4). To test this, 10 mice were intraperitoneal injected with 100ng of wild type (RA100) MVs in the initial immunization. Five days post injection; the mice appeared listless, hunched, and moribund. By day 5 end 8 of the 10 succumbed to their immunization. By day 14 only one mouse remained. This indicates the 100 ng dose induces a robust yet lethal pro-inflammatory response and suggested lower doses would be better tolerated.

Due to high mouse deaths the control group mice were taken for a new immunization: 3 mice received 75ng MVs, 3 mice received 50ngs MVs, and the rest remained naive. Three days post injection, one of the 75ng vaccinated mouse died. One mouse from each group was sacrificed for a cytokine harvest. Several organs in each mouse showed increased amount of TNF-α produced when compared to the naïve mouse. The results can be found in Table 1. By day 5 post injection, both the 50ng and 75ng immunized mice succumbed.

Table 1. Cytokine Harvest Results

	50 ng Mouse	75 ng Mouse
Organs	TNF-α (pg/mL)	
Peyer's Pathches	439.5	213.9
MLN	211.4	115.75
Spleen	64.5	----

Pro-inflammatory properties and mutant strains

Based on the overwhelming potency of the wild type MVs *in vivo*, I hypothesized that MVs with reduced pro-inflammatory properties would allow for better outcomes after mouse immunization. Attentions were directed towards the waaL-null mutant, a necessary enzyme for the creation of the O antigen in LPS. LPS is a complex glycolipid located in the outer membrane of Gram-negative bacteria that contributes to permeability-barrier properties and stability of the outer membrane (14). LPS molecules can be subdivided into three structurally distinct regions. The hydrophobic lipid A forms

the membrane lipid portion and is responsible for the endotoxic properties of LPS. The core oligosaccharide (core OS) serves as a link between lipid A and the outer polysaccharide repeating unit known as O antigen. O antigen plays a direct role in virulence, contributing to the resistance of gram-negative bacteria to complement-mediated serum killing (8).

The ligation of O antigen polysaccharide to lipid A-core oligosaccharide is a late step in the formation of LPS (10). WaaL proteins are ligases and currently the only protein known to be involved in the addition of the O anitgen. WaaL proteins in *Escherichia coli* and *Salmonella* share highly conserved predicted membrane topology and overall secondary structure (6). WaaL mutants produce rough LPS, meaning they lack O antigen repeat units (12). I hypothesized that the RA133 MVs would be less likely to overwhelm the immune system and cause death *in vivo*, but still be immunogenic due to the presence of other surface antigens. Comparisons of the two strains can be found in Table 2.

Table 2. Comparison of Mutant STM Strains

Stock #	Strain	Phenotype: Genotype	Source
RA100	*S. typhimurium* STM14028 (ATCC)	wild type: virulent	Dr. Andrews-Polymenis
RA133	*S. typhimurium* STM14028	del-STM3713::KanR (waaL-null, kanamycin resistant)	Dr. Andrews-Polymenis

Wild type and waaL-null *Salmonella* MVs both activate macrophages *in vitro*

Initial comparison data of the wild type and mutant HKSTM and MVs showed that both strains are potent activators of TNF-α production in J774-A1 Mϕs. Similar to the wild type MVs, RA133 MVs activated over 60% of the cells up to a dilution of 1:10,000 and retain activating properties over more concentrations than the RA133 HKSTM (Figure 4). There was not a distinct difference in Mϕ activation between the wild type strain and RA133.

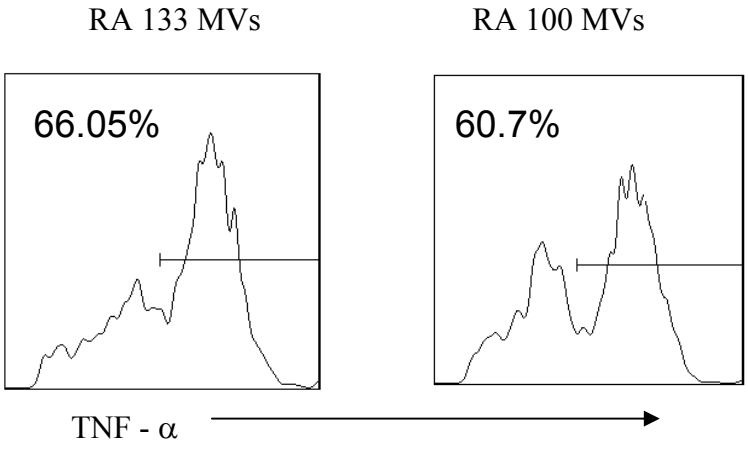

Figure 4. RA 133 and RA 100 MV Comparison. This figure shows the TNF-α production in murine J774 Mϕs after a 4-hour stimulation with RA 133 MVs (50 μg/ml) and RA 100 MVs (50 μg/ml). Percentages indicate proportion of J774 cells producing TNF-α of total cells.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Summary

The study of MVs spans decades and several groups have demonstrated their biological function as an alternate secretion system (11). However, more study is needed as the mechanisms for MV regulation, the MV role in bacterial pathogenesis, and the outcome of MV interactions with host defenses are still unclear. This is especially important in regards to pathogens such as *Salmonella,* which interact with the host immune system at multiple stages of infection (9). This project demonstrated some of the outcomes from interaction between the MVs and the host immune response.

Some the first cells that encounter *Salmonella* during a course of infection are the intestinal epithelial cells, DCs, and Mϕs (3). The MVs are composed of outer membrane and periplasmic constituents that can be recognized as pathogen associated molecular patterns by the host immune system (1, 4). Interactions with these cells produce pro-inflammatory cytokines and chemokines that cause a large influx of immune effector cells which, in turn, are responsible for producing a robust immune response in the *Salmonella* infection. A good vaccine for *Salmonella* would induce protective immunity without the active infection from a virulent bacterium.

Conclusions

I had hypothesized that MVs would be potent stimulators of Mϕs due to their composition of out membrane constituents and that MVs would induce a pro-inflammatory response *in vitro* and *in vivo* which could be applicable to a vaccine platform.

The ability of MVs to induce a pro-inflammatory response was tested by incubating the MVs with J774-A1 Mϕs *in vitro*. Both wild-type MVs and waaL-null mutant MVs consistently active ted a significant percentage of the Mϕs to produce large amounts of TNF-α (Figures 3, 4). This demonstrates that both strains of MVs are stimulants for a protective immune response. The MVs also retained activation properties through a dilution of 1:10,000 indicating they are capable of stimulating host immune cells over broad concentrations and in very minute amounts. This shows that MVs can persist without the use of an adjuvant and are sufficient to robustly activate immune effector cells.

The pro-inflammatory capabilities of MVs were also tested *in vivo* by immunizing C57Bl6 mice in doses of 100 ng, 75 ng, and 50 ng MVs per mouse. In the course of several days the mice were visibly affected by the immunization and eventually succumbed as a result from the MV injection. The cytokine harvest showed elevated levels of TNF-α in immunized mice. This shows that the MV immunization produced robust yet lethal pro-inflammatory response. A dosage titration would better evaluate

tolerance levels for future studies. Also, other routes of injection should be considered due to the systemic response of intraperitoneal injections.

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