EXOSOMES RELEASED DURING INFECTION WITH BURKHOLDERIA THAILANDENSIS PLAY A PROTECTIVE ROLE FOR THE HOST THROUGH STIMULATION OF INNATE IMMUNE RESPONSES

by

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Exosomes released during infection with Burkholderia thailandensis play a protective role for the host through stimulation of innate immune responses

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

by

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Bachelor of Science
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DEDICATION

This is dedicated to my loving friends and family who have continually supported me. A special thanks goes to my mother, whose unconditional love and support throughout my life encourages me to never give up.

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I would like to thank Dr. Hakami for his mentorship, advice, and valuable insight over the past few years. I would also like to thank my committee members, Dr. van Hoek and Dr. Petricoin, for their help and support. In addition, I would like to thank our collaborators, Dr. Weidong Zhou and Dr. Petricoin with CAPMM for their assistance performing mass spectrometry analysis. I would also like to thank both previous and current members of the Hakami lab whose training, advice, and support has helped me immensely. Lastly, I would like to thank my family and friends for their ongoing support and encouragement.

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LIST OF ABBREVIATIONS

Burkholderia pseudomallei ..... Bp
Burkholderia thailandensis ..... Bt
Colony Forming Unit ..... CFU
Exosomes derived from uninfected cells ..... EXu
Exosomes derived from infected cells ..... EXi
Fetal Bovine Serum ..... FBS
Mass spectrometry ..... MS
Multiplicity of Infection ..... MOI
Phosphate Buffered Saline ..... PBS
Proteinase-K ..... PK
Roswell Park Memorial Institute ..... RPMI
Transmission electron microscopy ..... TEM
Yersinia pestis ..... Yp

ABSTRACT

EXOSOMES RELEASED DURING INFECTION WITH BURKHOLDERIA THAILANDENSIS PLAY A PROTECTIVE ROLE FOR THE HOST THROUGH STIMULATION OF INNATE IMMUNE RESPONSES

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George Mason University, 2018

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Exosomes are extracellular vesicles that play a central role in intercellular communication. They have recently attracted much attention as their significant function in a variety of disease, including infectious diseases, has become better understood. However, there are significant gaps in our knowledge regarding how exosomes derived from an infectious origin (EXi) can alter host innate immunity. Using our model of purifying exosomes from *Burkholderia pseudomallei* (Bp) infected cells, we aimed to better understand the role host exosomes play in the pathogenesis of Bp, a Category B priority pathogen that possess significant risk to public health and for which we currently have no effective countermeasures. We have shown that EXi induce human monocytes to differentiate into macrophage-like cells and stimulate the release of IL-2, IL-6, and TNF-α. Additionally, we have demonstrated that EXi promote increased bacterial clearance in treated naïve monocytes, and experiments are under way to elucidate the exact

mechanisms. We have also analyzed the protein complements of the EXi and EXu by mass spectrometry analysis. Collectively, our data support a model in which the EXi prime local and distal naïve cells to mount a more effective response against Bp. These findings provide insights into a previously unexplored aspect of host response to infection, which can aid in future development of novel vaccines or therapeutics against bacterial pathogens.

CHAPTER ONE - INTRODUCTION

History and Epidemiology

Burkholderia pseudomallei (Bp) is a gram negative, facultative intracellular bacterium that can be isolated from soil and water and is the causative agent of melioidosis (Kespichayawattana 2004). Melioidosis is endemic to Southeast Asia and Northern Australia and causes 20% of all community-acquired septicemias and 40% of sepsis-related mortality in northeast Thailand (Wiersinga 2006). Melioidosis is thought to be severely underreported in 45 countries where it is known to be endemic and is believed to be endemic to an additional 34 countries that have not reported the disease, potentially due to diagnostic, clinical, and laboratory limitations found in these countries (Limmathurotsakul 2016).

In addition, there is the threat of importation or accidental/intentional release events to non-endemic areas resulting in a melioidosis outbreak. In 1975, a melioidosis outbreak occurred in a Parisian zoo. The outbreak spread to other zoos in Paris and Mulhouse and to equestrian clubs throughout France, causing at least two known human deaths. The outbreak was thought to be caused by either the importation of infected horses from Iran or an infected panda donated by Mao Tse-Tung (Cheng 2005).

Although melioidosis is not endemic to the United States, our military and troops are at risk for exposure. After a fourteen day trip to Thailand, similar to the length of

many travelers, 13 of 34 (38%) previously unexposed healthy U.S. Marines had positive serology and asymptomatic Bp infection, with one developing severe melioidosis (Kronmann 2009). As the ease of global travel increases, the concern focuses on the risk posed by short-term travelers, including those who are in good health conditions.

In 2014, the Center for Disease Control investigated the release of Bp in Louisiana from a building holding experimentally infected mice to a primate facility, possibly through contaminated inner garments worn by staff (CDC 2015). A study by Limmathurotsakul predicted that even though Bp is not endemic to USA and Japan, both have suitable environments for establishment (2016). Southern parts of Florida, Louisiana, and Texas in the US, and Okinawa and Kagoshima in Japan, are the most suitable locations within these countries, suggesting that an accidental or intentional release or exposure could have long lasting impacts, especially within these two countries. Additionally, Bp does naturally occur in Puerto Rico, which further raises concerns about an accidental exposure (CDC 2017).

The global burden of melioidosis is anticipated to increase substantially as more locations are being farmed as well as the increased prevalence of diabetes mellitus globally (Newbold 2015, GBD 2015). It is estimated that global mortality due to melioidosis is 89,000 cases annually, which is comparable to measles (95,600 annually) and is higher than dengue fever (9,100-12,500) which is considered by many health organizations a higher priority than Bp (Christopher 2016, GBD 2015).

Little is understood about the mechanisms of host pathogen interactions during Bp infection. Bp is classified by the US Centers for Disease Control and Prevention as a

category B agent, meaning that it is a highly dangerous biodefense pathogen that can be easily disseminated. It is necessary that additional research be performed for future development of innovative countermeasures against Bp.

Clinical Overview and Current Treatment Options

The genus *Burkholderia* contains more than 30 species, with the most pathogenic being *B. pseudomallei* (Bp) and *B. mallei*, the causative agent of glanders in horses. The genus also contains *Burkholderia thailandensis* (Bt), which co-exists in the soil with Bp but rarely causes human infections. Structural similarities have allowed for Bt E264 to be an accepted model of Bp infection and disease progression at the BSL-2 level (Ngugi 2010, Haraga 2008, David 2015). Despite sharing some virulence mechanisms, Bt has a reduced virulence of 10^5-fold in comparison to Bp clinical strains (Brett 1998). Bt has a genome with more than 95% 16S rRNA homology with Bp and contains various homologs of Bp virulence factors such as T3SS components (Brett 1998, Haraga 2008). Bt E264 possesses a lipopolysaccharide with a similar carbohydrate structure to that of Bp (Ngugi 2010).

The main route of infection is through skin inoculation, often seen in agricultural work in developing countries (Limmathurotsakul 2013). However, it has also been shown that Bp can be inhaled during extreme weather conditions, such as typhoons and rains (Chen 2015, Cheng 2006). Additionally, in 2012, Thailand experienced an outbreak due to ingestion of Bp-contaminated tap water (Limmathurotsakul 2014). High rainfall, high temperatures, anthrosol (soil modified by human activities such as irrigated agriculture), and acrisol (clay-rich soil found in warmer climates) are strongly associated with the

presence of Bp (Limmathurotsakul 2014). However, no strong evidence has been presented that Bp is a contagious disease, and therefore standard infection control procedures performed by healthcare providers is sufficient. Those diagnosed with diabetes mellitus, chronic kidney disease, and excessive alcohol consumption are especially prone to contracting the illness and therefore are considered a high-risk population for Bp infection (Limmathurotsakul 2016).

Melioidosis can also affect a wide variety of animals, including sheep, goats, swine, horses, and cattle (Finkelstein 2018). Depending on the species, a variety of non-specific symptoms can be present, such as fever, heavy breathing, swelling of the joints, abortion, and even death. Infected animals can spread the bacteria through nasal secretions, milk, and feces. There is no clear documentation of a zoonotic transmission of Bp, and therefore, the risk is considered low.

Melioidosis infection can be challenging to diagnose because it presents a wide array of signs and symptoms. Pneumonia is the presenting illness in half of all cases (Wiersinga 2018). Diagnosis is based on both clinical and epidemiological features, with the main method being a bacterial culture (Wiersinga 2018). In addition, although the average incubation period is 9 days, it can range up to 62 years, which can hinder receiving timely treatment.

Bp is intrinsically resistant to many antibiotics. Patients require antibiotic therapy for 20 weeks, and recurrence of infection still remains a concern (Wiersinga 2006). In locations with strong medical infrastructure and ample resources, mortality from Bp infection is around 10% (Currie 2015). However, many endemic locations do not have

sufficient resources and therefore see mortality rates around 40% (Currie 2015). There are currently no effective vaccines to treat melioidosis, and since it is multi-drug resistant, even currently used therapeutics are not overly effective (Wiersinga 2006). Treatment is broken up into two phases. First, a 14-day intensive therapy treatment, consisting of intravenous ceftazidime or meropenem, is provided with the aim of preventing death from sepsis (Dance 2014). Next is the eradication phase, during which a minimum of 20 weeks of oral drugs, normally trimethoprim/sulfamethoxazole, is given with the aim of preventing relapse, which occurs in 5-25% of patients (Dance 2014). Bp infection has a reported latency ranging from 19-29 years, indicating that Bp can enter a dormant state and evade immune surveillance (Lazar 2009).

The lack of effective therapeutics and the ability of Bp to be weaponized and spread through aerosol transmission highlight the need for a better understanding of Bp and more efficient and cost-effective treatment options.

Molecular Properties and Host-Pathogen Interactions

The genome of Bp, strain K96243, has been sequenced and has been found to be comprised of two chromosomes, one 4.07 Mb and one 3.17 Mb (Wiersinga 2006). The larger chromosome contains many genes that control cell growth and metabolism, whereas the smaller chromosome contains genes that are associated with adaptation and survival. Approximately 6% of the genome is made up of putative genomic islands that are thought to have been acquired through horizontal gene transfer (Wiersinga 2006). All three Type 3 Secretion systems reside on chromosome 2 (Holden 2004).

Bp secretes N-acyl-homoserine lactones (AHL), which are signaling molecules involved in the quorum sensing machinery that is used to coordinate attacks against the host environment and for biofilm formation (Liu 2011, Klaus 2018). Bp also possesses multiple secretion systems, which are evolutionary apparatuses that enable the transport of proteins across cellular membranes and respond to the environment. The Type III secretion system (T3SS) in particular aids in host cell invasion and escape from endocytic vesicles. The T3SS is comprised of a molecular "syringe" made of a filamentous needle that is deployed upon close contact with a host cell, and translocates effector proteins into the surroundings or within the cell (Vander Broek 2017, Sun 2010). Attachment to epithelial cells is thought to be mediated by type IV pili, which are hair-like structures on the bacterial surface (Essex-Lopresti 2005). The type IV pilin protein PilA (encoded by *pilA*), a subunit type IV pili needed for adhesion to epithelial cells, could potentially play a role in internalization as well (David 2015). Flagella allow for close contact of Bp and mucosal membranes, but flagella are not thought to be a major adhesion factor for Bp attachment to mammalian cells (Chuaygud 2008). However, two type V secretions adhesion proteins, BoaA and BoaB, are known to enhance adherence (Wiersinga 2018, Balder 2010).

Typically, Bp first enters and replicates in epithelial cells of the mucosal surfaces or enters through broken skin and then spreads to both phagocytic and non-phagocytic cells. Following endocytosis, Bp is able to survive the endocytic vesicle by an ecotin, a periplasmic serine protease inhibitor homologue, which is involved in resisting degradation by lysosomal enzymes (Ireland 2014). Due to its lipopolysaccharides (LPS)

and capsule, Bp is resistant to lysosomal defensins and cationic peptides, which enables continued survival within cells (Lazar 2009). Bp is then able to escape from endocytic vacuoles in the cytosol by lysing the endosome membrane using its T3SS, T6SS, and T2SS (Stevens 2002, Wiersinga 2018). This bacterium is resistant to some host antimicrobial peptides, and interferes with the synthesis of iNOS, which is known to have an important role in the killing of intracellular bacteria (Allwood 2011, Pruksachartvuthi 1990). However, macrophages activated by IFNγ display improved killing of *B. pseudomallei*, which is thought to be due to an increased activation of inducible nitric oxide synthesis (Miyagi 1997).

Following vesicular escape, T3SS effector protein BopA and translocator protein BipD further block sequestration in endocytic vesicles (Gaylov 2010). BopA prevents microtubule-associated protein 1A/1B light chain 3B (LC3) – associated autophagy through a partially unknown mechanism (Cullinane 2008). Bp is capable of inducing apoptosis in both phagocytic and non-phagocytic cells in a strain dependent manner (Kespichyayawattana 2000). Some strains cause apoptosis, others cause pyroptosis, and others cause neither (Kespichyayawattana 2000).

Once free in the cytoplasm, Bp continues to replicate. In addition, Bp induces the formation of actin-based protrusions and can move via continuous polymerization of host cell actin at polar ends, a process which is regulated by the autotransporter BimA (Stevens 2002). The neighboring cells can then phagocytose these protrusions, which allows Bp to spread to a new cell without being exposed to antibiotics (Kespichayawattana 2000). Once in the new cell, Bp will once again escape from the

secondary vacuoles and begin multiplying intracellularly. Intercellular spread results in cell fusion to form multinuclear giant cells (MNGC), which is a hallmark of melioidosis infection (Lazar 2009). T6SS and type IV secretion system (VgrG-5) are essential for the process of cell fusion and MNGC formation (Lazar 2009).

Toll-like receptors (TLRs), including TLR2, TLR4, and TLR5, located on cell surfaces recognize pathogen-associated molecular patterns, such as LPS and flagella. This mediates nuclear factor-kB (NFkB) induced activation of the immune response, which in turn releases pro-inflammatory cytokines, such as IL-1β, IL-12, and TNF (Wiersinga 2007). Ultimately, neutrophils, dendritic cells, B cells, and T cells are recruited toward the site of infection, and the complement and coagulation cascades are activated (Wiersinga 2018).

Exosome Overview

Exosomes are small (30-120 nm) extracellular vesicles with a density of around 1.11 g ml^{-1} that are released by cells (Tauro et al 2012). They are a uniform spherical shape or cup-shaped, depending on the electron microscopy method used for visualization (Luga et al. 2012, Conde-Vancells et al. 2008). Exosomes have a lipid bilayer membrane and are derived from the late endosome of the cell and secreted extracellularly. Secreted exosomes are internalized by recipient cells via endocytosis (Tian et al 2010). The uptake of exosomes can trigger a variety of downstream effects. For example, they are known to directly affect gene transcription and translation, influence signaling cascades to alter transcript and protein modifications, and regulate protein localization (Pant et al 2012). They are known to act a carrier of biological

molecules, such as RNA species, proteins, and lipids, and are decorated with various surface membrane proteins (Tauro et al 2012). Exosomal content is thought to vary depending on the cellular origin, but common markers do exist to identify exosomes, such as CD63, Alix, or TSG101 (Logozzi 2009).

Initially thought to simply be part of the waste removal process for cells, exosomes have garnered a huge amount of interest in recent years as their important functions in intercellular communication and in different types of diseases, including infectious diseases, have been demonstrated (Fleming 2014). For example, exosomes from immune and non-immune cells are known to be involved in immune modulation processes (Robbins 2014). In addition, exosomes play a meaningful role in enhanced infectivity during viral infections and induction of pathogenesis (Fleming 2014). Both pathogen-derived and host-derived molecules are found to be packaged within exosomes, although the exact mechanisms are still uncertain.

Exosomes and Bacterial Infection

There is a considerable lack of understanding surrounding how bacterial infections alter host exosome release. Most studies have focused on *Mycobacterium tuberculosis* (Mtb), with most other pathogens that fall under the umbrella of infectious diseases remaining largely unelucidated. Mice that have inhaled exosomes released from Mtb-infected macrophages produce increased levels of TNF-α and IL-12, as well as recruit macrophages and neutrophils to the lungs, which suggests that these exosomes have the potential to recruit non-infected cells to promote a more effective immune response (Bhatnagar et al. 2007b).

Both cell signaling and gene activation events can be modulated by exosomes (Rodrigues 2018). Exosomes derived from Mtb infected cells (EXi-Mtb) can activate MAPK signaling pathways and NF-kB pathways through altered TLR-interactions (Bhatnagar et al. 2007a). Both pathways are of great interest in any infection process, as they play key roles in the regulation of numerous immune response genes. EXi-Mtb released from macrophages can stimulate non-infected macrophages to secrete chemokines to induce the migration of naïve T-cells and macrophages (Singh 2012). Macrophages infected with *M. avium* and *M. smegmatis* exhibited increased exosomal expression of HSP70, which promotes macrophage activation and TNF-α expression (Anand 2010). Conversely, EXi-Mtb contain glycoprotein lipoarabinomannan (LAM) that inhibits T cell receptor signaling and T cell activation responses, which is thought to activate immune suppressive mechanisms that promote the survival and dissemination of Mtb (Yang 2012, Mahon 2012). EXi-Mtb may influence innate and acquired immunity, but additional research is required to fully understand the EXi-induced effects on the overall immune system.

However, the role of exosomes during infections with highly pathogenic biodefense agents remains significantly unexplored. Previous research from the Hakami lab has found that exosomes obtained from *Yersinia pestis* (Yp)-infected cells (EXi) induce the following specific phenotypes in naive (uninfected and untreated) monocytes: a) induction of differentiation to macrophages as demonstrated by a significantly prolonged G1 phase of the cell cycle, increased attachment, and appearance of CD68 marker; b) induction of significantly improved capacity for bacterial clearance; c)

significant release of the inflammatory cytokines IL-6, IL-8, and IL-10. Interestingly, these phenotypes are identical to when naive monocytes become infected with Yp bacteria, suggesting that EXi prime their recipient cells to mount an immune response as if they have become infected so that they can eliminate Yp when they reach the bacteria. Here, we study whether these EXi-induced phenotypes observed for Yp infection are disease specific or if exosomes are also able to confer protective immunity in cells infected with Bp.

Current Bp Vaccines and Prospects of Using Exosomes as Novel Protective Vehicles

Current Bp vaccine prospects are largely considered for protection within the biothreat context, but vaccines could be a cost-effective intervention in developing tropical countries, especially if used within the high-risk populations. Vaccines being developed for biodefense purposes would need to protect against inhalational exposure, which has proven to be very challenging. Live-attenuated vaccines are currently being studied and have the potential advantage of being able to induce long-term protection against Bp, but it may prove more difficult to license this type of vaccine as development of a latent infection is still possible (Peacock 2012). Few studies are being performed with inactivated whole cell vaccines, which present a wide variety of antigens to the immune systems and is therefore beneficial considering the genetic and immunological diversity of Bp. However, the main disadvantages are undesirable short-term side effects, largely due to LPS (Peacock 2012). Sub-unit vaccines are found to have increased safety levels and can evoke immune responses only to the protective antigen rather than to the entire microorganism. They also have a high potential for large scale production.

However, no reports of the ability for these vaccines to protect against an inhalation challenge are available (Peacock 2012). Experimental evidence indicates that combining bacterial polysaccharides (LPSs or other capsular polysaccharides) with well-defined protein antigens can generate substantial protection against Bp infection (Muruato 2016). Lastly, two reports have focused on the protection provided by immunization with DNA vaccines encoding the Bp flagellar subunit gene, *fliC* (Chen 2006a, Chen 2006b). In general, naked DNA virus vaccines against infectious diseases have been weakly immunogenic in humans even though they promote a vigorous immune response in mice (Liu 2010).

A multivalent vaccine containing numerous immunogenic bacterial components will probably be necessary to achieve complete protection (Wiersinga 2018). Although therapeutics costs have reduced over the last decade, melioidosis treatment remains unaffordable for many endemic locations in developing countries, and the likelihood of new or cheaper options in the immediate future is bleak. This, plus considering the absence of an effective, licensed vaccine and that Bp shows resistance to a number of antibiotics highlights the need for safe, economical, and effective countermeasures for both inhalation and inoculation exposures. Host-derived exosomes not only offer a new venue for drug delivery and therapeutics but they also possess the potential to be used as a vector for vaccinations. Exosomes are capable of acting as a delivery system for proteins and genetic material with high efficacy to various parts of the organism, including across the blood brain barrier (Batrakova 2016, Andaloussi 2013).

This research investigates the potential of exosomes released from Bp infected cells to serve as a novel therapeutic, particularly an immunostimulant, against Bp infection. Here, we demonstrate that exosomes from Bp-infected cells (Bp-EXi) are able to alter the phenotypes and immune responses of recipient cells in a protective manner. Since exosomes are released naturally from infected cells during the course of infection, they are less likely to exert toxicity or adverse immunological effects.

CHAPTER TWO – METHODS

Cell Lines

The human monocytic cell line U937 and human epithelial cell line HeLa were used in this study. All cell types were grown in RMPI 1640 (Lonza) supplemented with 10% Fetal Bovine Serum (VWR) and 2mM L-glutamine (VWR).

Bacterial Strains and Cultures

B. thailandensis E264 (Bt) was used in this study. For infections, Bt E264 was streaked onto Luria-Bertani (LB) plates (Thermofisher Scientific) from a frozen stock and grown at 37ºC. To prepare a bacterial culture for infecting cells, a single colony used to inoculate LB broth for overnight growth at 37ºC using a shaking incubator. Overnight cultures were quantified using OD_{600nm} (we have established that for Bt an OD_{600nm} reading of 1 is equivalent to $4x10^7$ CFU/ml).

Infection with *Burkholderia thailandensis* (Bt)

Exosome free media (EFM) was prepared as described by performing a 3 hour 120,000xg centrifugation of FBS diluted 1:1 in RPMI 1640 (Eitan 2015). Culture media of the cells was replaced with EFM 24 hours prior to infection. For infections, cells were incubated with Bt at MOI of 1 for 45 minutes at 37ºC (Figure 7). After infection, cells were washed and resuspended in PBS (Invitrogen). Cell cultures were then treated with 75 μg/mL of kanamycin for 1 hour at 37ºC to eliminate extracellular bacteria. They were subsequently

incubated in media containing a maintenance concentration of kanamycin until exosome harvest.

Exosome Purification

Exosomes were purified from culture supernatants at 48 hours post-infection by differential centrifugation followed by sucrose density gradient purification (Figure 8). Initial purification spins consisted of two 2-hour, 117,000xg spins for crude exosome pellet isolation. A step-wise gradient was created by layering sucrose solutions of differing molarities diluted in PBS (Invitrogen) and semi-purified exosome were layered on top. Gradients were spun for 3 hr at 117,000xg at 4ºC. The fractions of interest were harvested, diluted further in PBS, and pelleted at ultraspeed at 4ºC. The pellets produced were resuspended in PBS containing 1x Halt protease inhibitor cocktail (Thermo-fisher Scientific), filter sterilized using a 0.22um syringe filter (Thermo-Fisher Scientific), and stored at -80ºC. Each sample was then probed for CD63 marker by Western blot and quantified by both BCA analysis (Pierce) and analysis by Zetaview prior to subsequent use.

Western Blot Analysis

The following primary antibodies were used: mouse anti-CD63 (EMD Millipore), rabbit anti-CD68 (Abcam), mouse anti-TG101 (BD Biosciences), rabbit anti-LC3A/B (Cell Signaling) and mouse anti-actin (Abcam). Thirty microliters of cell lysate in 4x LDS lysis buffer were heated at 70ºC for 5 minutes before separation on NuPAGE 4-12% Bis-Tris gels (Novex). Proteins were dry transferred using iBlot 2 gel transfer nitrocellulose stacks (Thermofisher Scientific). After transfer, membrane was blocked for 1 hour at room

temperature in 5% non-fat dried milk in TBS + 0.1% tween-20 (TBS-T) or 5% BSA in TBS-T. Membranes were then incubated for 18 hours at 4ºC with primaries diluted according to manufacturer's instructions. The membranes were washed five times for five minutes each with TBS-T. Membranes were then incubated for 1 hour at room temperature with either goat anti-mouse HRP-conjugated secondary antibody (Cell Signaling), or goat anti-rabbit HRP-conjugated secondary antibody (Thermofisher Scientific). After five, 5-minute washes with TBS-T, protein bands were visualized using SuperSignal West-Femto Maximum Sensitivity Substrate (Pierce). Images and quantification of the blots were obtained using Chemidoc XRS System (BioRad).

Electron Microscopy

Ten microliters of each sucrose gradient-purified exosome preparation was placed on a copper TEM grid. After 10 seconds of absorption, the grid was negatively stained with 2% uranyl acetate. The copper grid was blotted and examined with a Hitachi TEM, operating at 80kV.

LC-MS/MS Analysis

The samples were lysed in 8 M urea with protease inhibitor. After centrifugation, the supernatant was transferred to a clear tube, and protein concentration was measured by Bradford assay. Ten micrograms of protein from each sample were reduced by 10 mM dithiothreitol (DTT) for 15 minutes at 37°C, alkylated by 50 mM iodoacetamide for 10 minutes at room temperature, and digested by trypsin at 37 °C for 4 hours in a buffer containing 2 M urea and 50 mM ammonium bicarbonate. The digestion mixture was then acidified by adding glacial acetic acid to a final concentration of 2% and desalted by

ZipTip (Millipore). The tryptic peptides were analyzed by high sensitive nanospray liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) using an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific). After sample injection, peptides were eluted using a linear gradient of 5% mobile phase B (0.1% formic acid, 80% acetonitrile) to 50% B in 150 minutes at 300 nL/minute, then to 100% B for an additional 5 minutes. The Orbitrap Fusion was operated in a data-dependent mode in which each full MS scan (100,000 resolving power) was followed by multiple MS/MS scans at fastest speed. Tandem mass spectra were searched against the NCBI human protein and Burkholderia thailandensis database using Proteome Discoverer (version 2.1) with full tryptic cleavage constraints, static cysteine alkylation by iodoacetamide, and variable methionine oxidation. Mass tolerance for precursor ions was 3 ppm and mass tolerance for fragment ions was 0.5 Da. The search results were filtered by high stringent criteria with 1% false discovery rate.

Cell Growth Rate Assay

Seeded cells were either left untreated or infected with Bt (as described above), or treated with 10µg of appropriate sucrose gradient-purified exosomes (Figure 9). At 0, 24, 48, and 72 hour post treatment, cell concentration and viability were assayed by trypan blue staining. Cell growth rate over a 24 hour period was calculated using the following formula:

$$[\text{Cell Concentration}_{present}\text{-Cell Concentration}_{past}]/\text{Cell concentration}_{past}$$

Cell Attachment Assay

U937 cells were either left untreated or were treated with 10μg of sucrose gradient-purified EXu or EXi (Figure 9). Untreated cells infected with Bt were also included. After 24 hours, the culture supernatant was removed and the wells were gently washed with PBS. The remaining adherent cells were trypsinized and quantified by trypan blue staining.

Cytokine Analysis

U937s were treated with 10μg of either sucrose gradient-purified EXu or EXi at 0 hour (Figure 9). Untreated cells that were infected with Bt as described above (positive control) and untreated and uninfected cells (baseline control) were also included. At 6, 12, and 24 hours post treatment, a specific volume of the cell culture supernatant was harvested from each well and the cells were pelleted out by centrifugation at 800xg for 5 minutes at room temperature. The resulting supernatants were then prepared for cytokine analysis using the Aushon Cirscan System (Aushon Biosystems), following the manufacturer's instructions. Three biological repeats of the cytokine measurements were performed using a 10-plex Aushon Ciraplex Human Cytokine Array for IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, and TNF-α (Aushon Biosystems) following the manufacturer's protocol. Each measurement was done in technical duplicates.

Bacterial Clearance and Uptake Assay

U937 cells in EFM were pretreated with 10μg of either sucrose gradient-purified EXu or EXi, or were left untreated for 24 hours (Figure 9). Cells were then infected at MOI of 1.

Internalized bacteria were recovered and quantified by CFU count at 0 and 24 hours post infection.

Proteinase K Treatment of Exosomes

Exosomes were treated with proteinase K (Sigma) and incubated at 37ºC for 1 hr. Exosomes were then washed with PBS and pelleted at ultraspeed at 4ºC. Exosome pellet was resuspended in sterile PBS containing 1x Halt Protease inhibitor (Thermofisher Scientific), filter sterilized using a 0.22µm syringe filter, and stored at -80ºC.

Statistical Analysis

Statistical analysis was performed using R studio version 1.0.153. All data sets were initially tested for normality and homogeneity of variance using Shapiro-Wilk and Bartlett's test. Parametric data was further tested using one-way ANOVA with Dunnet's post-test to analyze significance. When applicable, student's t-test was performed. Kruskal-Wallis with Dunn's post-test was used to test for significance of non-parametric data.

CHAPTER THREE - RESULTS

Validation and Characterization of Exosomes Released by Bt-Infected Cells (EXi)

Exosome purifications of untreated (EXu) and Bt-infected U937 cells (EXi) were performed in parallel as outlined in Supplementary Figure 2. In summary, a crude exosome pellet was harvested from cell culture supernatant through differential centrifugation and then further purified by sucrose density gradient separation. Sucrose gradient purified fractions were then probed for exosomal marker CD63 expression (Figure 1A). Consistent with previous literature demonstrating exosomes have roughly a 1.11 g ml^{-1} density, exosomes obtained from Bt-infected U937 cells were found to migrate to the 1.103 g ml^{-1} and 1.149 g ml^{-1} density fractions. These two fractions were then combined to obtain a single preparation. Protein quantification was performed on exosomes samples (Figure 1B). We recover equivalent protein levels from EXu and EXi preps, with an average of $5.5\pm0.9x10^2$ μg/mL for EXu and 5.3 ± 0.6 $x10^2$ μg/mL for EXi. This indicates that Bt infection does not alter exosome production or release. We have also demonstrated that total protein quantification is a viable means of quantifying our exosomes samples. In Figure 1B, CD63 signal was normalized to loaded protein, which produce equivalent band intensities for EXu and EXi preps when compared by western blot. Zetaview analysis further validates that EXu and EXi samples are equivalent in concentration, with EXu possessing an average of $1x10^9$ particles/ml and EXi containing

$9x10^8$ particles/ml on average (Figure 1B). TEM analysis of sucrose gradient purified EXu and EXi preps reveal intact vesicles ranging 30-120nm in diameter, which is fully consistent with the accepted size of exosomes based on a large compendium of previous published reports (Figure 1C) (e.g., Tauro et al 2012). Zetaview analysis further validates that EXu and EXi samples are equivalent in size, with an average size of $1.3\pm0.02x10^2$ nm for EXu and $1.2\pm0.02x10^2$ nm for EXi (Figure 1C). Figure 1D demonstrates that the zetapotential for EXu is $-2.8\pm0.02x10^2$ mV and EXi is $-3.1\pm0.01x10^2$ mV. This magnitude of charge will prevent the exosomes from precipitating or aggregating, as the exosomes have enough charge to repel each other which will in turn increase stability of the individual particles. Mass spectrometry analysis was performed to identify bacterial and host proteins selectively packaged into the EXi. Glutamate synthase, NADH dehydrogenase, and hypothetical protein BTH_I3137 were identified with one spectral hit in one of three biological replicates. Therefore, additional experimentation is needed to elucidate the cause of the low spectral hit counts of the bacterial proteins and the inconsistency in proteins present between the preparations. Likely causes are low levels of these bacterial proteins that makes detection difficult, in which case the application of additional front end fractionation may be tested, or that these bacterial proteins may be getting randomly packaged into the EXi.

EXi Induce Human Monocytes to Differentiation into a Macrophage-like State

Other groups have reported that exosomes released from virally infected cells cause decreased viability within recipient cells (Pleet 2016, Ahsan 2016). We do not observe a change in viability when U937s cell are treated with EXu or EXi (Figure 2A).

However, we do see a 2.5-fold decrease in growth rate of EXi treated cells when compared to untreated or EXu treated cells (Figure 2B). This growth defect mimics the decrease in growth rate also seen in Bt-infected cells.

We next analyzed whether the EXi-induced growth defect is also observed in non-immune cells. EXi treated HeLa cells and Bt-infected HeLa cells were found to have a significant growth decrease between 0-24 hours, which is similar to the effect seen in immune cells (Figure 2C). This also suggests that exosome produced by immune cells can significantly affect non-immune cells, which would allow infected cells to be able to communicate with both local and distal cells.

U937 cells begin differentiating into macrophages by initially demonstrating enhanced attachment (Ferreira 1991). Since EXi was not causing changes in viability but growth rate was being affected, we explored whether EXi was inducing the differentiation of monocytes into macrophages. We analyzed whether EXi treatment was causing increased rates of attachment in recipient cells. Figure 3A shows that treatment with EXi causes a $1.6\pm0.1x10^{1}$% increase in attachment as compared to untreated or EXu treated cells ($6.7\pm0.1x10^{1}$% and $6.8\pm0.01x10^{1}$% respectively). Similar to growth rate, the EXi treated cells also demonstrate a phenotype similar to infected cells, which had a $1.7\pm0.1x10^{1}$% increase in attachment. We next investigated further whether EXi-treated U937 cells express known macrophage markers. We analyzed whether expression of CD68, a well-known macrophage differentiation marker, is increased in the attached cells. PMA, a potent inducer of macrophage differentiation, was included as a positive control. Similar to PMA treatment, cells that attached in response to EXi or infection had

significantly increased levels of CD68 expression (Figure 3B). Taken together, these results demonstrate that consistent with Bt infection, EXi treatment induces recipient cells to differentiate from monocytes to a macrophage-like state.

To address whether the observed differentiation phenotype is due to surface proteins on the exosomes, we treated exosomes with proteinase K (PK), an enzyme that cleaves external protein moieties. TEM analysis of our PK-EXu and PK-EXi CD63+ exosomes revealed intact, circular vesicles 50-100nm in diameter. (Figure 5A). Therefore, Proteinase K treatment does not alter exosome integrity, as vesicles are intact and retain their size and morphology post treatment. We investigated whether if the EXi-induced growth defect is also observed with PK-EXi. Figure 5B shows that a similar decreased growth rate is observed following treatment with PK-EXi or infection with Bt. This suggests that external protein moieties are not essential for this phenotype, and indicates an additional entity, such as miRNA, may be responsible for inducing the observed phenotype.

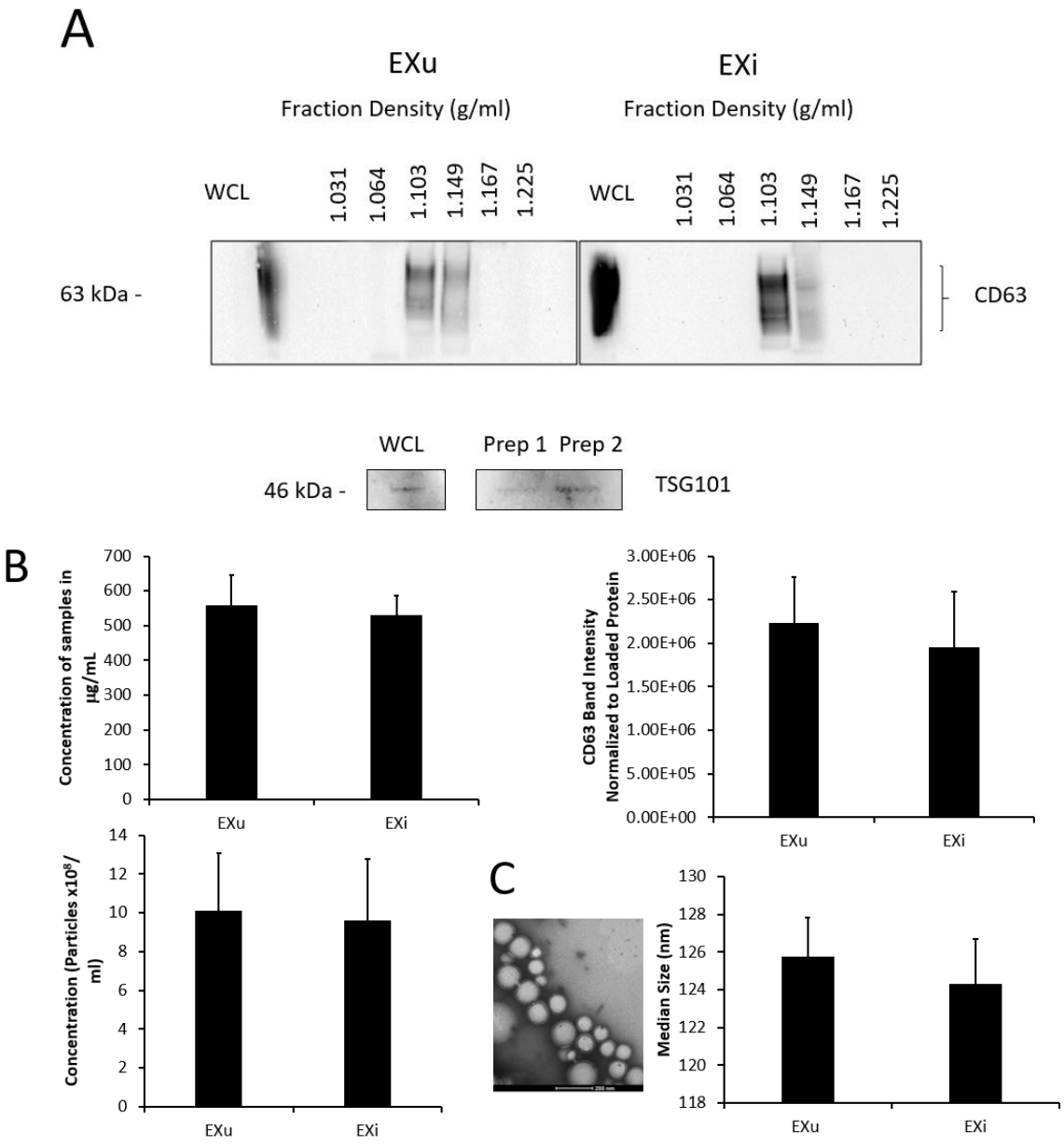
EXi Treatment Induces Innate Immune Response and Increased Bacterial Clearance

We next analyzed whether EXi is capable of inducing an innate immune response through cytokine release, and if any potential responses are due to surface proteins. We performed quantitative analysis of 10 human proinflammatory cytokines: IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, and TNF-α. Compared to uninfected and EXu-treated cells, EXi-treated cells produce 2 to 3.5-fold increased levels of IL-2, IL-6, and TNF-α (Figure 4A). Bt-infected cells also demonstrate a similar cytokine profile as

the EXi-treated cells, suggesting that the EXi are able to raise a similar immune response to bacterial infection. To analyze whether this induction of innate immune response depends on exosomal surface proteins, cytokine response from naïve U937s cells treated with Proteinase-K treated exosomes was tested. We found that PK-EXi-treated cells show a similar profile of proinflammatory cytokines releases when compared to EXi-treated cells, suggesting that surface proteins are also not involved in inducing cytokine response (Figure 4B).

Given that EXi is able to induce macrophage-like differentiation and innate immune response, we analyzed whether bacterial uptake or growth is affected by the EXi. Cells were pretreated with EXu, EXi, or were left untreated 24 hours prior to infection. At zero hours, all treatment groups showed equivalent levels of bacterial uptake. However, at 24 hours, cells pre-treated with EXi showed an almost 10-fold decrease in intracellular bacterial numbers, demonstrating a significantly increased capability to overcome intracellular bacterial growth. Studies have shown that induction of autophagy in Bp-infected cells allows for decreased levels of intracellular bacteria (Cullinane 2008). Preliminary data indicates an increase in LC3-2/actin ratio and therefore, an upregulation of autophagy in EXi treated attached cells (Figure 5D). Further experimentation is required to confirm if this observed upregulation of autophagy is statistically significant.

CHAPTER FOUR - FIGURES



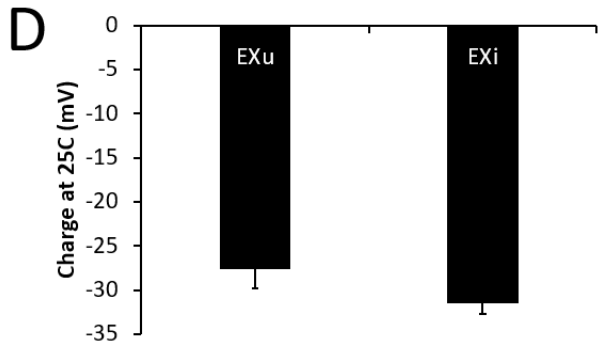


Figure 1: Validation and Characterization of Purification of Exosomes from U937s. *(A)* EXi released from Bt-infected U937 monocytes were harvested by serial centrifugation and further purified by sucrose density gradient. EXu were purified in parallel from uninfected U937s following the same procedure. Recovered sucrose fractions were washed and probed for known exosomal markers CD63 (top) and TSG101 (bottom) using whole cell lysate (WCL) as a control. *(B)* Total protein content of sucrose gradient-purified EXu and EXi preparations were measured by BCA (left) (means ± SEM, n=3, p = 0.9, student's t-test). Western blot analysis of equivalent levels of sucrose gradient-purified EXu and EXi based on total protein amounts demonstrate identical CD63 band intensities (right) (means ± SEM, n=3, p = 0.78, student's t-test). Exosome concentration was measured by Zetaview analysis (bottom) (means ± SEM, n=3, p = 0.1, student's t-test). *(C)* TEM analysis was performed to examine size and morphology of sucrose gradient-purified CD63+ exosome samples (left). Zetaview analysis was performed to measure exosome diameter (right) (means ± SEM, n=3, p = 0.1, student's t-test). *(D)* Zetapotential, or charge, of EXu and EXi were measured (means ± SEM, n=3, p = 0.2, student's t-test).

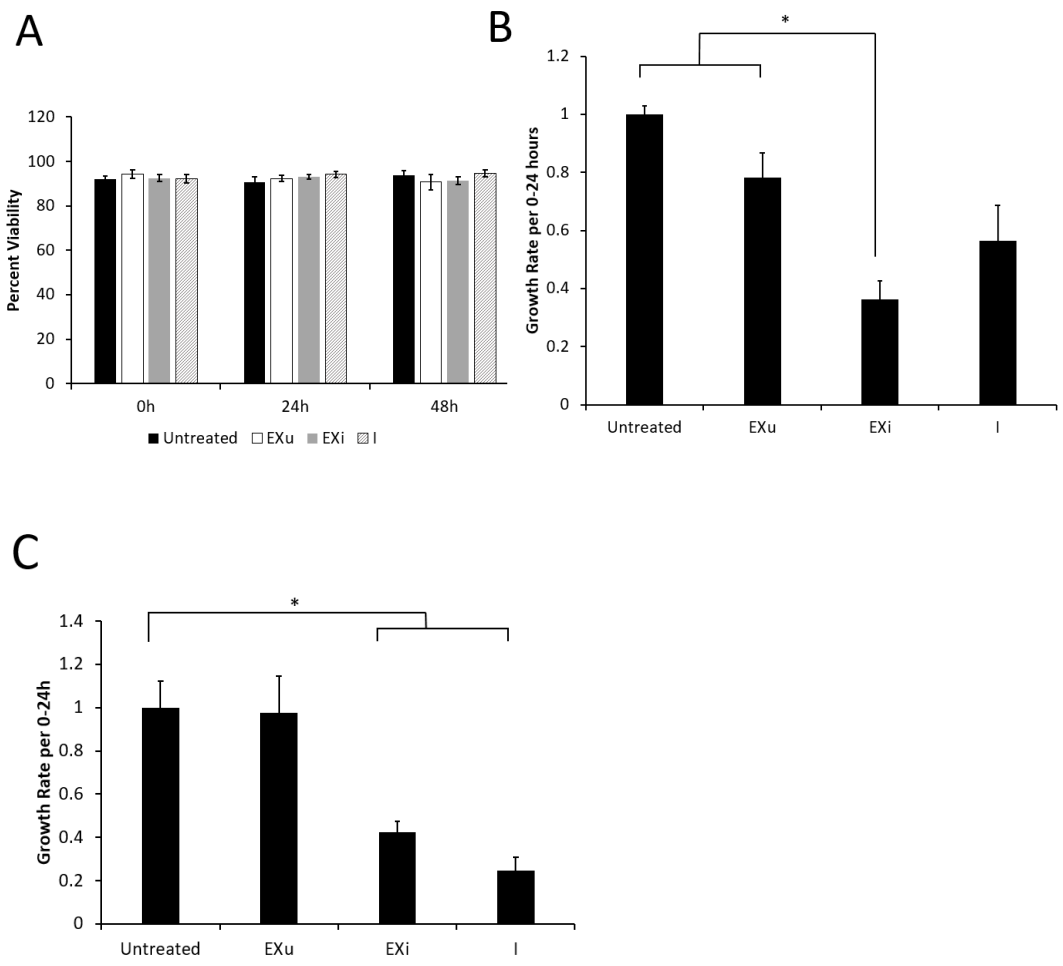


Figure 2: Cell Viability and Growth Analysis. *(A)* Naïve U937 cells were treated with either sucrose gradient-purified EXu or EXi, or were left untreated, or were infected with Bt. Cell viability was assayed by AO/PI staining at 0h, 24h, and 48h (means ± SEM, n=4, p=0.91, ANOVA). *(B)* Naïve U937 cells were treated with sucrose gradient-purified EXu or EXi, left untreated, or infected with Bt. Cell growth rate was quantified from 0-24 hours post treatment (means ± SEM, n=4, *p<0.05, ANOVA). *(C)* Naive HeLa epithelial cells were treated with sucrose gradient-purified EXu or EXi, left untreated, or infected

with Bt. Cell growth rate was quantified from 0-24 hours post treatment (means ± SEM, n=4, *p<0.05, Kruskal Wallis).

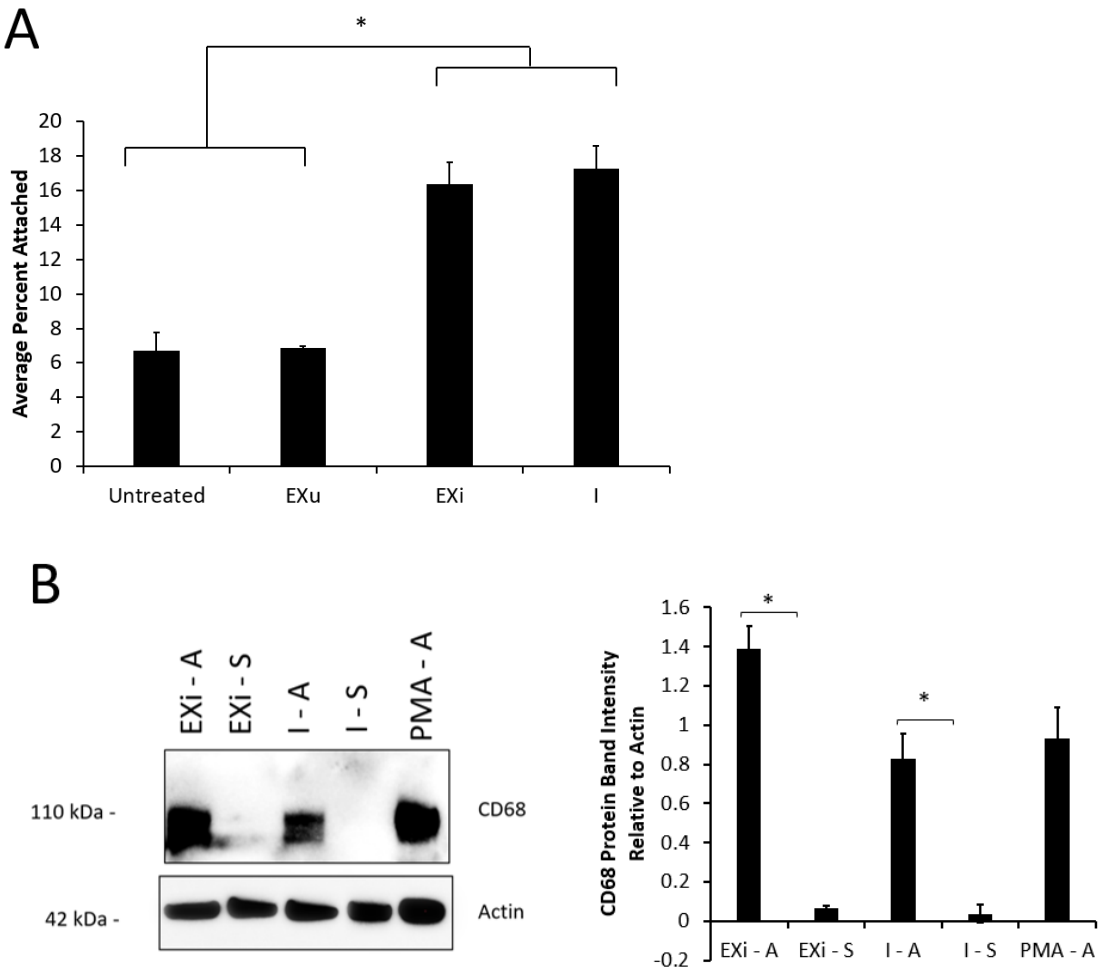


Figure 3: Macrophage Differentiation Analysis. *(A)* Naïve U937 cells were treated with sucrose gradient-purified EXu or EXi, left untreated, or infected with Bt. After 24 hours, the cells in suspension were removed and the number of adherent cells was quantified by trypan blue assay (means ± SEM, n=5, *p<0.05, ANOVA). *(B)* Naïve U937

cells were treated with either sucrose gradient-purified EXu or EXi, left untreated, or infected with Bt. Treatment with PMA, a potent inducer of monocyte to macrophage differentiation, was included as an additional control. After 24 hours, suspension cells (S) and attached cells (A) were probed for the presence of macrophage marker CD68. Equivalent protein levels were loaded (left). Band intensity was quantified relative to actin (right) (means ± SEM, n=5, *p<0.05, ANOVA).

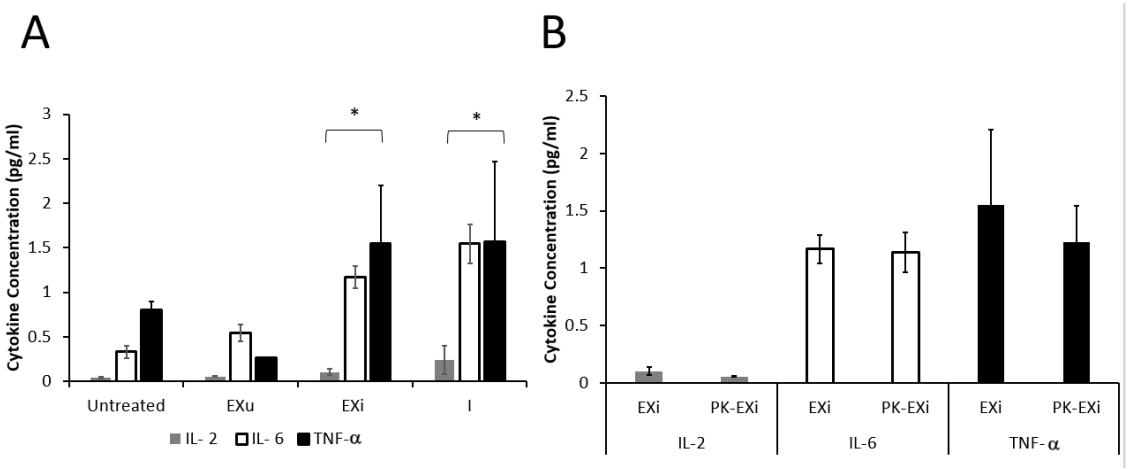


Figure 4: Immune Response Analysis. *(A)* Naïve U937 cells were treated with sucrose gradient-purified EXu or EXi, left untreated, or were infected with Bt. Culture supernatants were harvested at 6h, 12h, and 24h post treatment for quantitative multiplex probing of 10 human proinflammatory cytokines using the Aushon Cirascan platform. IL-2, IL-6 and TNF-α were the cytokines identified from this panel that were significantly affected by EXi treatment (means ± SEM, n=3, *p<0.05, Kruskal Wallis with Dunn's post hoc). *(B)* Naïve U937 cells were treated with either sucrose gradient-purified EXu or EXi, or sucrose gradient-purified EXu or EXi that had been treated with proteinase-K

(PK-EXu or PK-EXi). Culture supernatants were harvested at 6h, 12h, and 24h post treatment for quantitative multiplex probing of 10 human proinflammatory cytokines using the Aushon Cirascan platform (means ± SEM, n=3, p=0.4 (IL-2), p=0.87 (IL-6), p=0.81 (TNF-α), Kruskal Wallis with Dunn's post hoc).

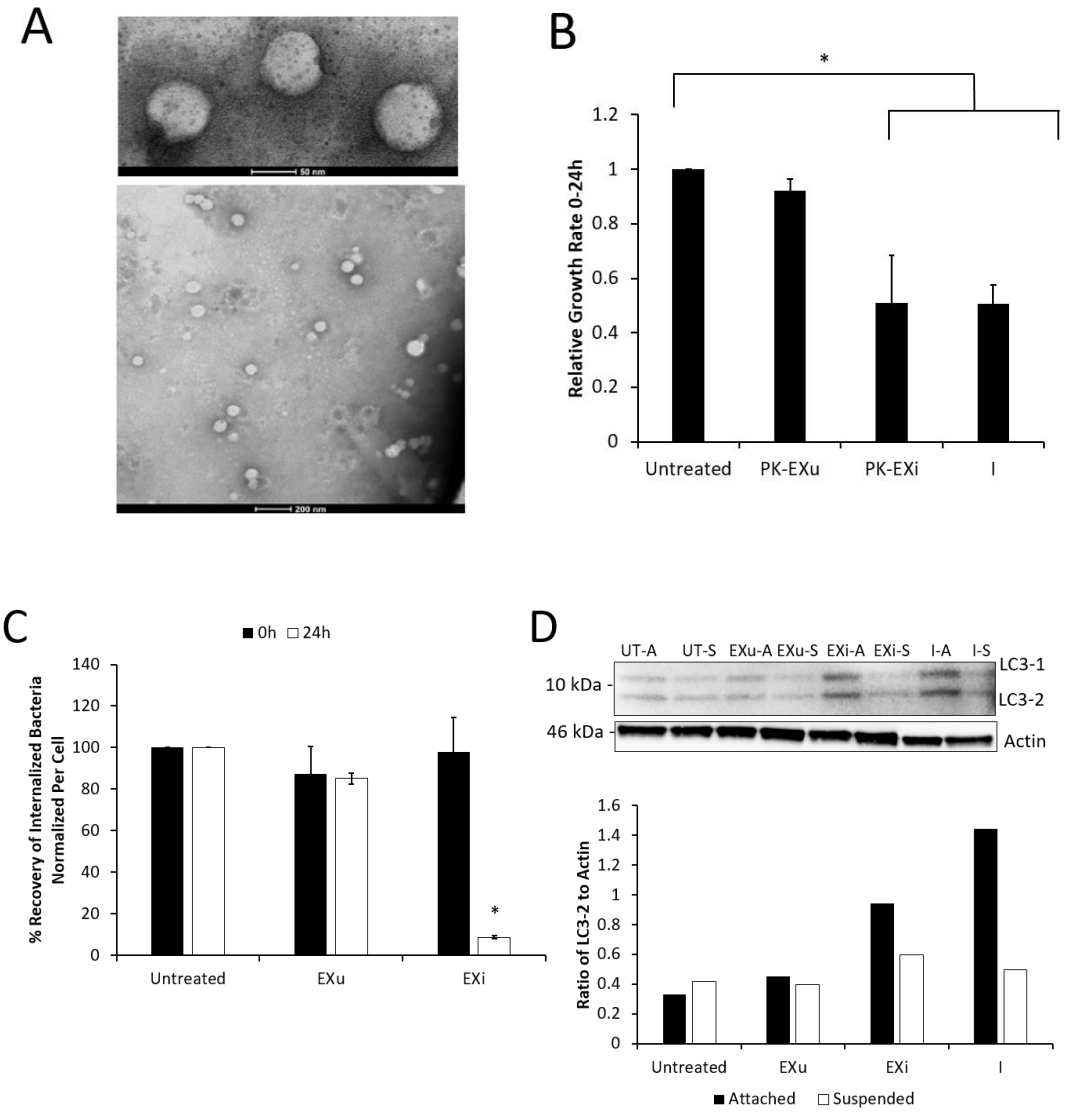


Figure 5: Mechanisms by which EXi mediate their effects. *(A)* Sucrose gradient-purified exosomes were treated with proteinase-K (PK) to remove external surface proteins. TEM analysis was performed to determine size and morphology of PK-treated exosome samples. *(B)* U937 cells were treated with either PK-EXu or PK-EXi, left untreated, or were infected with Bt. Cell growth rate was quantified from 0 to 24h post treatment by AO/PI staining (means ± SEM, n=3, *$p<0.05$, Kruskal Wallis). *(C)* Naïve U937s were treated either with sucrose-gradient purified EXu or EXi or were left untreated. After 24 hours, cells still in suspension were removed. Adherent cells were subsequently infected with Bt. Internalized bacteria were quantified by CFU counts at both 0 hour and 24 hour post infection and percent recoveries of internalized bacteria relative to untreated values were measured (means ± SEM, n=2, *$p<0.05$, ANOVA). *(D)* U937 cells were treated with EXu, EXi, were infected, or left untreated. After 24 hours, adherent and cells in suspension were lysed and probed for LC3 (n=1).

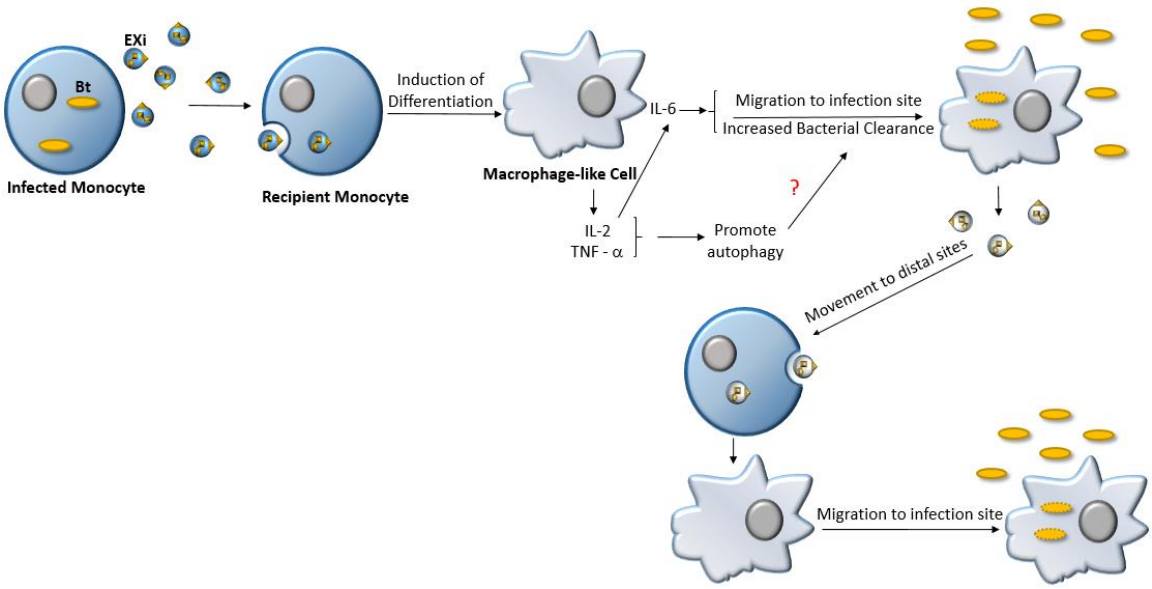


Figure 6: Proposed Model.

CHAPTER FIVE - SUPPLEMENTARY FIGURES

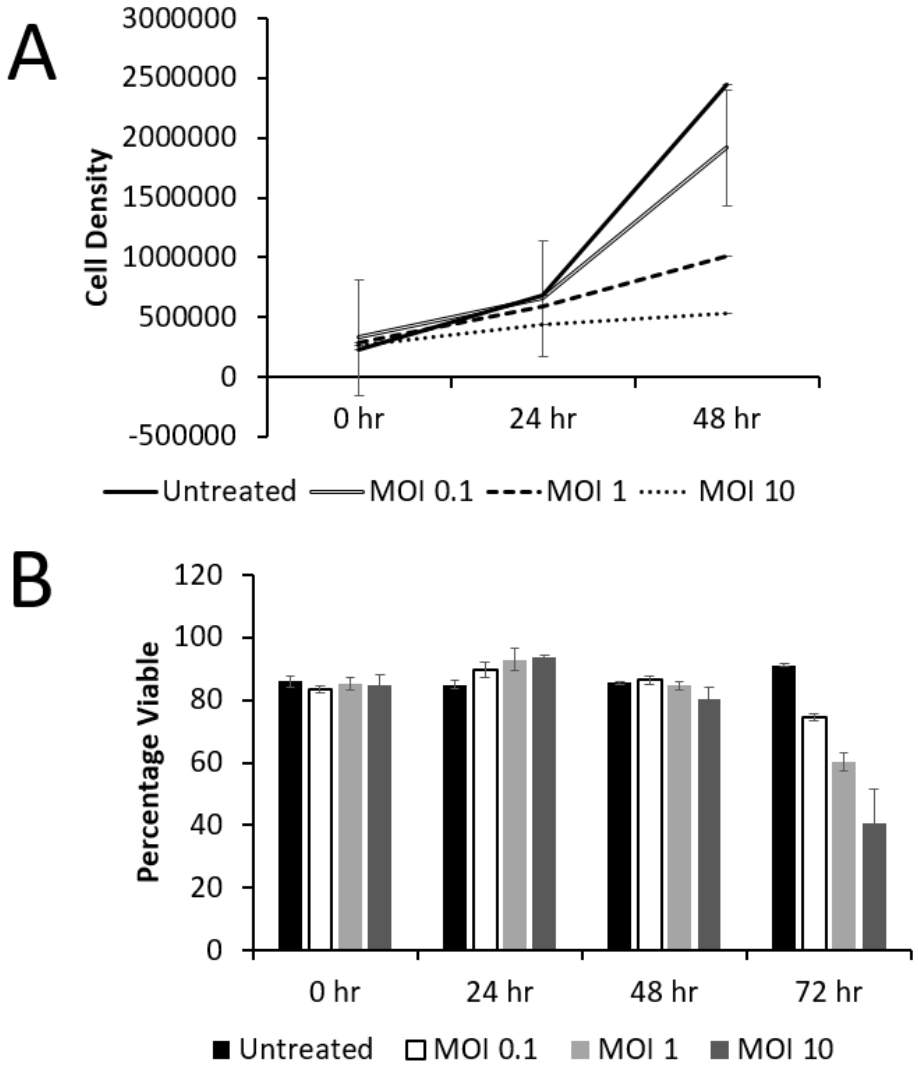


Figure 7: Supplementary - Bt Infection Parameters Optimization.

(A) Naïve U937 cells were left untreated or infected at MOI of 0.1, 1, or 10. Cell density was measured by trypan blue staining at 0 hour, 24 hour, 48 hour, and 72 hour post

infection (means ± SEM, n = 5). *(B)* Naïve U937 cells were either left untreated or infected at MOI of 0.1, 1, or 10. Cell viability was measured by trypan blue staining at 0 hour, 24 hour, 48 hour, and 72 hour post infection (means ± SEM, n = 5).

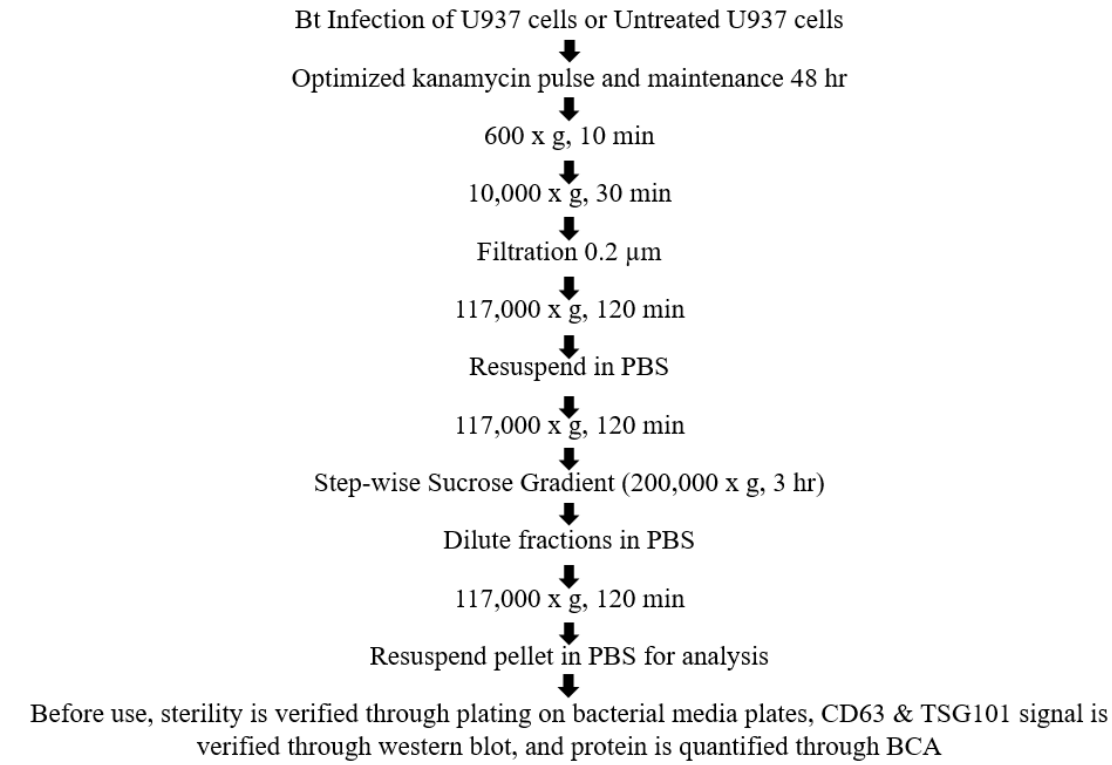


Figure 8: Supplementary - Flow chart of exosome purification. . Flow chart demonstrating the procedure for exosome purification from culture supernatants of Bt-infected cells. Serial differential centrifugation and filtration steps are followed by sucrose density gradient purification. For each purification, exosomes from uninfected cells were harvested and purified in parallel using the identical procedure.

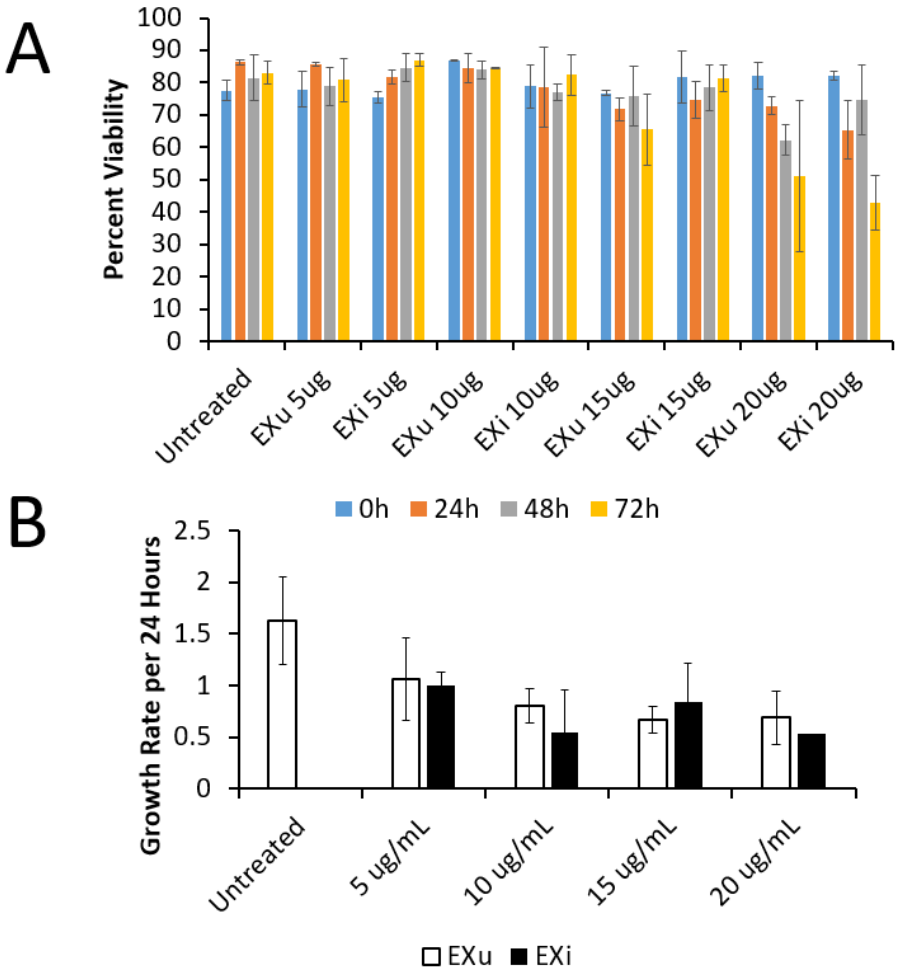


Figure 9: Supplementary - Exosome Treatment Optimization. *A)* U937 cells were treated with increasing doses of sucrose gradient-purified EXu or EXi and cell viability and growth were measured by trypan blue staining at 0h, 24h, 48h, and 72h (means ± SEM; n = 3). *B)* U937 cells were incubated with increasing amounts of sucrose gradient-purified EXu or EXi. Growth rate was measured by trypan blue staining at 0h and 24h (means ± SEM; n = 3).

CHAPTER SIX - DISCUSSION

Exosomes have recently garnered intense scientific interest as the considerable importance of their communication role has become established for many different diseases, including several infectious diseases (Kourembanas 2014, Fleming 2014). Studies of exosomes have been performed for only a few pathogenic bacteria, with the most extensive studies being performed with *Mycobacterium* species, and in all cases, exosomes have been proven important (Fleming 2014). In addition to expanding our understanding of the role exosomes play in additional bacterial infections, a comprehensive analysis and understanding of how exosomes modulate host innate immune system is necessary.

Using infection of human monocytes with Bp as a model, we have begun addressing these largely unelucidated questions. EXi derived from *Mycobacterium* infection are known to induce proinflammatory cytokine responses as well as macrophage activation (Bhatnagar 2007a, Bhatnagar 2007b). Based on our findings, we propose that released EXi prime naïve uninfected cells to mount immune responses in order to more effectively fight off Bp when they encounter the bacteria. According to our proposed model (Figure 6), Bt-infected monocytes release exosomes (EXi), which contain both host and bacterial proteins, into the extracellular environment to be taken up by naïve monocytes at either local or distal sites. Following uptake by the recipient

monocytes, EXi induce differentiation of the monocytes to macrophage-like cells that are capable of inducing innate immune responses. This includes promoting the release of IL-2, IL-6 and TNF-α in a signaling process that is not dependent on exosomal surface proteins. IL-2 is known to induce IL-6 production in human monocytes and macrophages (Musso 1992). IL-6 is thought to promote macrophage migration to the site of infection since the production of this cytokine has been shown to recruit nearby immune cells in response to infection (Kaplanski 2003). In addition, IL-6 has been shown to impact bacterial uptake and killing processes (Pechkovsky 1996, Kurtz 2013, Dann 2008). Differentiated macrophages are then capable of increased bacterial clearance, potentially through the function of IL-6 and/or increased levels of autophagy since both IL-2 and TNF-α are known to upregulate autophagy (El-Darawish 2017, Harris 2011, Lotz 2012). Therefore, according to our proposed model, exosomes released by Bt-infected cells travel to both local and distal sites to induce differentiation of monocytes, which can then be recruited to the site of infection to assist in bacterial clearance.

Future directions will include further investigation of the potential link between autophagy and bacterial clearance. Experiments are further validating whether LC3-II/actin ratio is increased by EXi treatment, as it is known that induction of autophagy effectively decreases intracellular survival of Bp (Cullinane 2008). In addition, plans are under way to perform RPMA analysis of changes in signaling pathways in response to EXi treatment, in order to better understand the cellular mechanisms that are modulated. In addition, future experiments will also be performed using the fully virulent (BSL-3) strain of *B. pseudomallei*.

Our study is very significant and contributes insight into bacterial manipulation of host exosomes. The molecular composition and functional mechanisms of exosomes released during Bp infection is poorly understood. Not only does this study provide characterization analysis of EXi purified from Bp-infected cells, but it also allows for a greater understanding of host-pathogen interactions that are mediated through exosomal exchange. The absence of a competent, effective therapeutic treatment against Bp highlights the need for a cost-effective and efficient vaccine for human use. Based on our results, exosomes carry the potential to be a vaccine component used for increased immunostimulation, and further studies are warranted to evaluate this potential. Interestingly, exosomes have been used as a drug delivery system with high therapeutic efficacy and low levels of toxicity and immunogenicity (Andaloussi et al., 2013; Batrakova and Kim, 2016). The data presented here suggest that exosomes from Bp-infected cells may alter the dynamics of the neighboring cells and can potentially interfere with the disease pathology. Therefore, further research is also needed to determine the potential for exosomes released from Bp-infected cells to serve as a novel therapeutic.

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