

ABSTRACT

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BACTERIAL GHOSTS MODULATION OF INNATE IMMUNITY: IMMUNE  
RESPONSES DURING *CHLAMYDIA* INFECTION

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*Chlamydia trachomatis* (CT) is a pestilent infection affecting upwards of 90 million people worldwide. An efficacious vaccine is needed to control the morbidities and rising healthcare cost associated with genital CT infection. We have established that protection against *chlamydia* infection parallels with a high frequency of T helper Type 1 cells and the associated antibodies. The current study focuses on the induction of innate immune responses involved during *Chlamydia* infection by a *Vibrio cholera* ghost-based (VCG) vaccine vector. THP-1 cells were used for dose and kinetic experiments. HeLa cells were used for infectivity assays. Based on preliminary studies, we hypothesized that the induction of immune responses by a VCG-based vaccine involves multiple innate immune signaling. Multiplex assay was used to measure T helper Type I and Type II cytokine secretion by THP-1 monocytes (Mn) or macrophages (M $\phi$ ). Immunostimulatory cytokine secretion was significant when both cell morphologies were pulsed with VCG or VCG/murine splenocytes. We concluded that this secretion was significant enough to compliment that which would be secreted when THP-1 cells are

pulsed with *Chlamydia* elementary bodies alone, enhancing the innate immune response during infection. Cellular supernatants (conditioned media) containing Th1-type and Th2-type cytokines were used to culture *Chlamydia*-infected HeLa cell monolayers. Infected HeLa monolayers cultured in the conditioned media were significantly less infected (968 IFUs) versus HeLa monolayers cultured in Earle's minimum essential media (16,486 IFUs;  $p < 0.001$ ). We concluded that factors contained in conditioned media prevent and/or significantly reduce infection by *Chlamydia* and the development of inclusion forming units.

BACTERIAL GHOSTS MODULATION OF INNATE IMMUNITY:  
IMMUNE RESPONSES DURING *CHLAMYDIA* INFECTION

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*trachomatis*.....76

## LIST OF ABBREVIATIONS

3MB.....	3-methylbenzoate
ADCC.....	Antibody-dependent Cellular Cytotoxicity
AKT.....	Protein Kinase B
APC.....	Antigen Presenting Cells
APP.....	Actinobacillus pleuropneumoniae
ATP.....	Adenosine Triphosphate
BCA.....	Bicinchoninic acid
BG.....	Bacterial Ghosts
BHI.....	Brain Heart Infusion
C-Terminal.....	Carbon
CD.....	Cluster of Differentiation
CT.....	<i>Chlamydia trachomatis</i>
DAPI.....	4'-6-Diamidino-2-Phenylindole
DC.....	Dendritic Cells
DNA.....	Deoxyribonucleic Acid
EB.....	Elementary Bodies
ELISA.....	Enzyme-linked Immunosorbent Assay
FBS.....	Fetal Bovine Serum

FL.....	Fleet 3 Ligand
GTP.....	Guanosine Triphosphate
HEPES.....	4-(2-Hydroxyethylo)-1-Piperazinethanesulfonic Acid
HIV.....	Human Immunodeficiency Virus
HRP.....	Horseradish Peroxidase
IDO.....	Indoleamine-2,3-Deoxygenase
IFN $\gamma$ .....	Interferon gamma
IFU.....	Inclusion Forming Units
IgG.....	Immunoglobulin G
IL.....	Interleukin
Incs.....	Inclusions
IPTG.....	Isopropyl- $\beta$ -D-1-Thiogalactopyranoside
IRAK.....	Interleukin-1 receptor kinase
LB.....	Luria Broth
LD.....	Lipid Droplets
LGV.....	Lymphogranuloma Venerum
LPS.....	Lipopolysaccharide
M $\phi$ .....	Macrophage
MAPK.....	Map Kinase
MEM.....	Mininum Essential Medium
MeOH.....	Methanol
MHC.....	Major Histocompatibility Complex
Mn.....	Monocyte

MOI.....	Multiplicity of Infection
MOMP.....	Major Outer Membrane Protein
MoPn.....	Mouse <i>Pneumonitis</i>
MVB.....	Multi-Vesicular Body
MyD88.....	Myeloid Differentiation
N-terminal.....	Nitrogen
NFkB.....	Nuclear Factor kappa B
NK.....	Natural Killer
PAMP.....	Pathogen Associated Molecular Pattern
PBS.....	Phosphate Buffered Saline
PI3K.....	Phosphate Inositol-3-Kinase
PID.....	Pelvic Inflammatory Disease
PmpD.....	Polymorphic Membrane Protein D
PorB.....	Porin B
PRR.....	Pattern Recognition Receptor
RAW.....	Red and White
RB.....	Reticulate Bodies
RIPA.....	Radioimmunoprecipitation Assay
ROS.....	Reactive Oxygen Species
RPML.....	Roswell Park Memorial Institute
RT.....	Room Temperature
RT-PCR.....	Reverse Transcription Polymerase Chain Reaction
rVCG.....	Recombinant <i>Vibrio cholerae</i> Ghosts

SEBH.....	Sucrose Electroporation Buffer H
SPG.....	Sucrose Phosphate Glycerol
STI.....	Sexually Transmitted Infections
T3SS.....	Type 3 Secretion System
TLRs.....	Toll-like Receptors
TNF $\alpha$ .....	Tumor Necrosis Factor Alpha
TRIF.....	TIR-Domain Containing Adapter-Inducing Interferon- $\beta$
VCG.....	<i>Vibrio cholerae</i> Ghost

## CHAPTER I

### INTRODUCTION

*Chlamydia trachomatis* (CT) is an obligatory intracellular prokaryotic pathogen (1) that causes genital *Chlamydia* infection. CT causes the most common bacterial sexually transmitted infections (STI) in the United States and one of the ten most common STIs worldwide. Each year an estimated 90 million sexually transmitted *Chlamydia trachomatis* infections occur around the world with more than two-thirds of these cases occurring in developing countries (2). The sequelae arising from CT infection include pelvic inflammatory disease (PID), ectopic pregnancy, and infertility, and have substantial psychological, public health, and economic implications (2). Understanding the molecular mechanisms involved in the induction of *chlamydia* infection immune responses will help in developing effective long-term treatment (2-10).

*Chlamydia* infection enhances the risk of Human Immunodeficiency Virus (HIV)-1 acquisition and transmission (11), and as such, this combination increases the public health problem worldwide (8). Treatment of symptomatic infections does not prevent reinfection nor affect established pathology (8, 12-13). The potential severity of complications resulting from infections with *C. trachomatis*, particularly in women, coupled with enhanced HIV-1 transmission and inability to prevent reinfection with antibiotic treatment provide strong motivation to prevent *Chlamydia* infection through vaccination (3, 5, 7-8, 10, 14).



An efficacious chlamydial vaccine should induce broad-based, long-lasting immunity (3, 8). A better understanding of the correlates of long-lasting, cross protective immunity will support the development of better protective vaccine strategies. This understanding will play a major role in reducing the economic burden on the public healthcare system and the high treatment costs of infectious complications (2-3, 7-8). Previous studies conducted by Eko et al. (2004) suggest that achievement of long-term, cross-protective chlamydial immunity is possible and would require the appropriate delivery of specific multiple chlamydial antigens and effective immunomodulation (12). Studies of the molecular mechanisms involved in *Chlamydia* infection have examined T-cell control and the role of nitric oxide (15), and the role of dendritic cells and Toll-like receptor (TLR) 9 in innate immunity (16). TLRs are a family of type I transmembrane receptor proteins that are required for the recognition of various pathogen-associated molecular patterns (PAMPs) expressed by a diverse group of infectious microorganisms, resulting in the activation of host immune responses. In the present study, the involvement of TLR2 and TLR4 during *Chlamydia* infection was evaluated. The molecular involvement of TLR2 and TLR4 in *Chlamydia* infection has been studied more extensively than the other nine [human] TLRs, and the *Chlamydia trachomatis* ligand for TLR4 is well defined. However, the *Chlamydia trachomatis* ligand for TLR2 is not, as it recognizes a wide array of PAMPs in heterodimer complexes with TLR1 or TLR6. TLR2 and TLR4 are largely located on the cell surface. This study investigated TLR signaling in, and modulation of protective immunity against *Chlamydia* infection. Additionally, we

investigated the intrinsic structural properties of our *Vibrio cholera* ghost (VCG) vector and the presence of TLR ligands on the bacterial ghost envelope.

Recent studies in animal models of genital chlamydial disease revealed that early recruitment of dendritic cells and specific T helper type-1 (Th1) cells into the genital mucosae is crucial for reducing the severity of the acute phase of a cervico-vaginal infection and arresting ascending disease (6, 17). The CD4<sup>+</sup> Th1 and CD8<sup>+</sup> T cells and their cytokines, especially gamma interferon (IFN- $\gamma$ ), are crucial for anti-chlamydial immunity in mice (17). These immune effectors are therefore important for preventing major complications of genital chlamydial infection (6, 17). The recruitment of Th1 cells into genital mucosae after chlamydial infection has previously been demonstrated (17). This recruitment would require obligatory interactions among chemokines, chemokine receptors, and cell surface molecules such as the major histocompatibility complex (MHC), addressins, co-receptors, co-stimulatory and adhesion molecules (17). Factors that regulate the induction, recruitment, and persistence of immune effectors (especially Th1 cells) in the genital mucosal site control long-term genital mucosal immunity and are particularly important for anti-chlamydial immunity (17).

In this respect, studies in animal models and corroborating clinical findings in humans have revealed that an infected host develops immunity against *C. trachomatis* (18), which correlates with a strong T helper type-1 cell response and a complementary antibody response (18). Thus, a vaccine that elicits this profile of specific immune effectors may be protective (18).

The majority of successful vaccines have been derived from either inactivated organisms or live-attenuated organisms (19). One goal of vaccine research is to develop a single-dose, orally or nasally administered, recombinant vaccine that induces long-lasting mucosal and systemic immunity (2-3, 8). As recombinant proteins are poorly immunogenic (3), particularly when given by mucosal routes, their use in vaccines requires co-administration of an adjuvant (20). The recombinant *Vibrio cholera* ghost (rVCG) platform has been shown to be an effective carrier and delivery system for cloned CT proteins, eliciting chlamydial-specific immune responses following immunization (8, 12-13). In the present study, we evaluated the immunogenicity of our VCG vector by elucidating the immune response and signaling pathways elicited by the vector in the host. Furthermore, we observed how it may enhance the innate immune response during *Chlamydia* infection.

The purpose of this study is to examine immune activation of pathogen-associated molecular pattern recognition receptors (PRR) and elucidate the molecular mechanisms involved in the induction of immune responses during *Chlamydia* infection by a VCG-based vaccine vector. Studies were carried out to support our hypothesis that the induction of immune responses by a VCG-based vaccine involves multiple innate immune responses and signaling pathways.

## CHAPTER II

### LITERATURE REVIEW

#### **2.1 *Chlamydia trachomatis* biology**

*Chlamydia trachomatis* are coccoide, gram-negative microbial pathogens which are metabolically deficient in their ability to synthesize ATP and thus have an obligate intracellular life cycle. The pathogen is of the order *Chlamydiales* and currently has one family, the *Chlamydiaceae*, containing two genera, *Chlamydia* and *Chlamydophila* (21). Three species of *Chlamydia* exists: *C. trachomatis*, strictly a human pathogen, *C. psittaci*, a pathogen of lower mammals (22) *e.g.*, birds, and *C. muridarum*, a mouse/hamster pathogen.

*Chlamydia trachomatis* consists of 15 serovars that cause many human diseases, including ocular trachoma (serotypes A to C); urethritis, epididymitis, cervicitis, and salpingitis (serotypes D to K); and lymphogranuloma venereum (LGV; serotypes L1 to L3) (23). Serotypes A to K (trachoma biovar) primarily infect columnar epithelial cells of the mucous membranes, while serotypes L1 to L3 (LGV biovar) also proliferate in lymphatic tissue and cause a more systemic infection. The third biovar of *C. muridarum* is the murine biovar, consisting of two strains, MoPn, which causes mouse pneumonitis but does not infect humans, and SFPD, concurrent with a causative agent of proliferative ileitis, obtained from a hamster (127). In addition, three serogroups among the human biovars that appear to be independent of biovar have been defined: the B complex

(serotypes B, Ba, D, Da, E, L1, L2, and L2a), the C complex (serotypes A, C, H, I, Ia, J, K, and L3), and the intermediate group (serotypes F and G) (23).

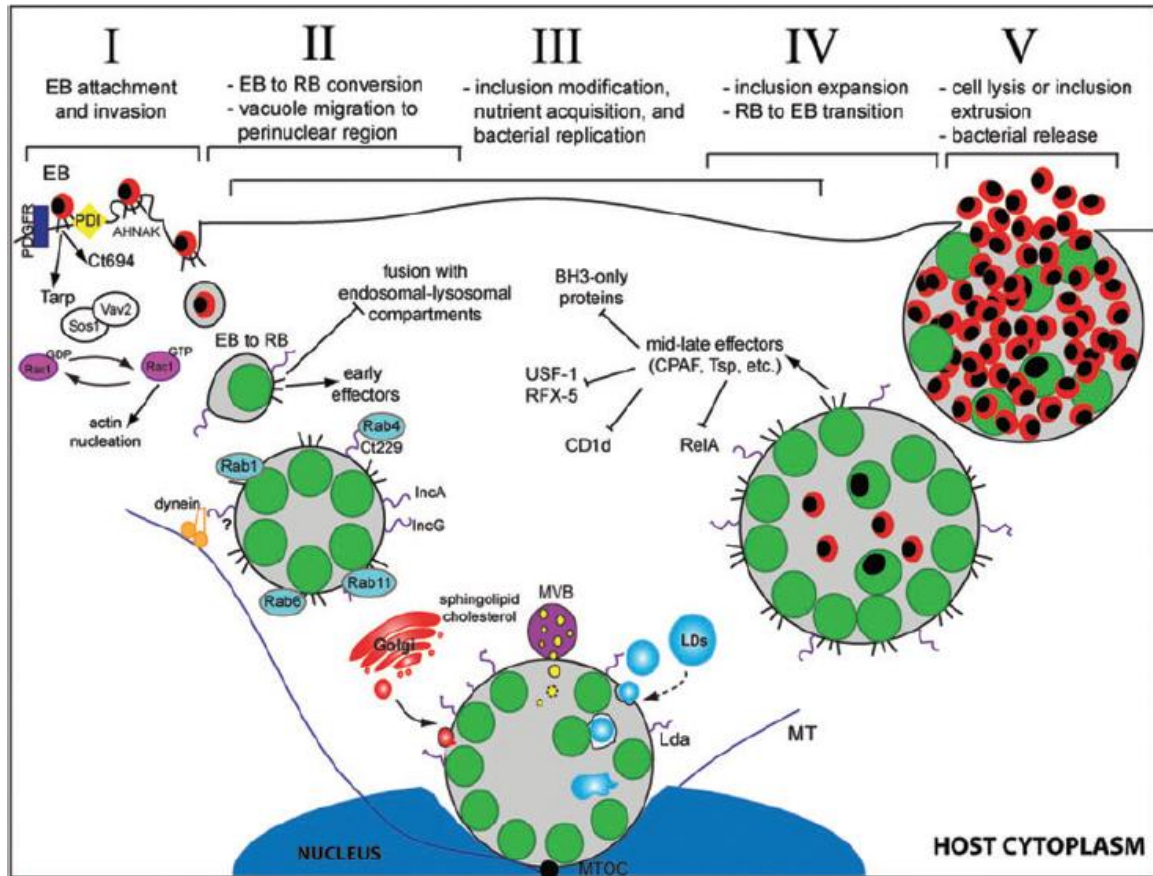
Serotype specificity is conferred by the major outer membrane protein (MOMP; the product of the *omp1* gene). MOMP constitutes ~60% of the protein mass of the chlamydial outer membrane and has been shown to have porin-like characteristics *in vitro* (23).

## **2.2 *Chlamydia trachomatis* infection and modulation of host cell functions**

*Chlamydia trachomatis* has a significant impact on host signaling pathways causing substantial changes to cellular transcription as well as cell damage (24). What distinguishes *Chlamydia* from other bacteria is their intracellular parasitism, responsible for the chronic and asymptomatic course of infection (25). Elucidating the molecular mechanisms *Chlamydia* infection engages to perpetuate the use of its effector proteins to “trick” host cell defenses will help in developing a system for long-term immunity that could reduce and/or eliminate reinfection.

*Chlamydiae* undergo a distinct developmental cycle, converting between two morphologically and functionally discrete forms - the elementary body (EB) and the reticulate body (RB). The basic cycle follows this sequence: (I) attachment and internalization; (II) EB to RB differentiation; (III) remodeling of the parasitophorous vacuole (“inclusion”) and bacterial replication; (IV) inclusion expansion and transition of RB into EB; and (V) release of bacteria from the host cell and infection of new target cells by EBs (fig. 1) (6). Studies based on expression analysis (*Belland et al., 2003*; *Nicholson et al., 2003*), heterologous type III translocation systems (*Fields et al., 2003*;

*Subtil et al., 2005*), function-based screens (*Sisko et al., 2006*) and bioinformatics (*Samudrala et al., 2009*) have yielded many candidate *Chlamydia* proteins that may participate in the manipulation of host cellular processes (13).



**Figure 1. Schematic illustration of the infectious cycle of *Chlamydia trachomatis*.** The *Chlamydia trachomatis* infectious cycle and modulation of host cell functions. *Chlamydia* infection begins when an elementary body (EB) binds to the host cell surface (I). At this stage effector proteins injected into the host via the type III secretion system (T3SS) facilitate bacterial entry. Following endocytosis, EBs transition to reticulate bodies (RBs) (II). New effectors are secreted and the bacterial vacuole is modified by bacterial inclusion membrane proteins (IncA) to limit fusion with the host degradative compartments while promoting contact with other host organelles and factors, including Rab proteins. The inclusion interacts with the Golgi apparatus, multivesicular bodies (MVBs) and lipid droplets (LDs). LDs can be directly translocated into the inclusion lumen for nutrient delivery (III). During infection, host cell death and immune defenses are inhibited. Approximately midway through the infectious cycle, bacterial replication becomes asynchronous and RBs re-differentiate into EBs (IV). Late in the cycle, the inclusion is packed with EBs and fills almost the entire cell volume. Eventually, the inclusion and host cell rupture releasing infectious EBs into the extracellular space for re-infection (V) (6).

The word *chlamys* is Greek for “cloak draped around the shoulder.” This describes how the intracytoplasmic inclusions caused by the bacterium are “draped” around the infected cell’s nucleus (26). Once established in this niche, the inclusion can selectively interact with organelles that provide factors essential for chlamydial development such as eukaryotic lipids, including sphingolipids (*Hackstadt et al., 1996; Moore et al., 2008*), cholesterol (*Carabeo et al., 2003*) and glycerophospholipids (*Wylie et al., 1997*). While sphingolipids and cholesterol isolated from bacteria are not modified, fatty acids in the Sn2-position of host glycerophospholipids are exchanged with *Chlamydia*-derived branched chain fatty acids (*Wylie et al., 1997; Su et al., 2004*). Lipid transport to the inclusion occurs through largely undefined pathways (13).

The *Chlamydia trachomatis* infectious cycle and modulation of host cell functions begins when an elementary body binds to the host cell surface. At this stage effector proteins injected into the host via the type III secretion system (T3SS) facilitate bacterial entry. Following endocytosis, EB reorganize into reticulate bodies. New effectors are then secreted and the bacterial vacuole is modified by bacterial inclusion membrane proteins (Incs) to limit fusion with the host degradative compartments while promoting contact with other host organelles including the nucleus. The inclusion interacts with the Golgi apparatus, multivesicular bodies (MVBs), and lipid droplets (LDs). LDs can be directly translocated into the inclusion lumen for nutrient delivery. During infection, host cell death and immune defenses are inhibited. Approximately midway through the infectious cycle, bacterial replication becomes asynchronous and RB re-differentiate into EB. Late in the cycle, the inclusion is packed with EB and fills almost the entire cell

volume. Eventually, the inclusion and host cell rupture releasing infectious EB into the extracellular space for reinfection (13).

### **2.3 Innate and Adaptive Immunity to *Chlamydia***

In the genital tract, *Chlamydia trachomatis* infects primarily epithelial cells and requires Th1 immunity for optimal clearance. Innate immunity plays a role in controlling chlamydial infections (27). Natural killer (NK) cells and neutrophils are the first cells that are recruited to the site of a chlamydial infection. These cells are important in innate immunity and have been implicated in the initial control of chlamydial infections. In fact, neutrophils are usually the first host cells recruited to an infectious site (28-29), and their likely role is to reduce the initial chlamydial infection and limit it from spreading (30). NK cells are known to be involved primarily in viral infections and cancer but have also been shown to be important in the early elimination of intracellular bacteria (31-32). Cytokine production by epithelial cells and dendritic cells (DC) has been implicated in NK cell IFN- $\gamma$  production during a chlamydial challenge. Cytokines (interleukin (IL)-12 and IL-18, respectively) produced by DC and human epithelial cells induce NK cells to secrete IFN- $\gamma$  (33). IFN- $\gamma$  is important for inhibiting the growth of *chlamydia* (34). Just as important, IFN- $\gamma$  is one of the main cytokines for the induction of a Th1 immune response. Furthermore, early IFN- $\gamma$  production by NK cells modulates DC to down-regulate the Th2 response, thereby allowing expression of strong Th1-mediated immunity, which has been shown to be essential for the resolution of *Chlamydia* infection (35). IFN- $\gamma$  down-regulation of the transferrin receptor (36), which is needed for the import of iron into the cell, may also inhibit *Chlamydia* growth by limiting the available



iron to the bacterium. Most *Chlamydia* species require tryptophan for survival (37).

IFN- $\gamma$  induces the expression of the cellular tryptophan-decycling enzyme indoleamine-2,3-dioxygenase (IDO), which degrades tryptophan.

*Chlamydia*-specific antibodies play a role in *C. trachomatis* protective immunity (38-39), and numerous *C. trachomatis* proteins have been shown to induce antigen-specific antibodies (40). B cells may not be important for initial chlamydial infection but, instead, play an important role in the secondary memory response (41-42). There are several possible mechanisms by which B cells modulate immunity during reinfection which include, but are not limited to antibody-mediated neutralization and opsonization (43), antibody-dependent cellular cytotoxicity (ADCC), the formation of antigen-antibody complexes that bind Fc receptors on APC enhancing phagocytosis and antigen presentation to CD4<sup>+</sup> T cells (44).

Natural killer T cells (NK T cells) have a role in protective Th1 immunity against *Chlamydia* (45). These granular cytolytic lymphocytes are able to destroy infected and cancerous cells without prior sensitization and also secrete cytokines that are important in both innate and adaptive immunity. Furthermore, these cells have been implicated in regulating innate (macrophages, natural killer, and dendritic cells) and adaptive (B and conventional T cells) immune cells (46-48).

M $\phi$  migrate to chlamydial infection sites (49), phagocytose bacteria (50), and produce pro-inflammatory cytokines (51-52). *C. trachomatis* destruction inside M $\phi$  has been associated with host cell autophagy, a process by which cells degrade cytoplasmic proteins and organelles (53-55). M $\phi$  autophagy can enhance antigen presentation to T

cells (56). Furthermore, IFN- $\gamma$  has been shown to enhance both autophagy and up-regulation of MHC class II molecules in M $\phi$  (54, 57). Enhanced up-regulation of MHC molecules containing chlamydial antigens may induce T cells to initiate both cell-mediated and antibody immune responses against *Chlamydia*. In addition to efficiently eliminating *Chlamydia* and presenting the peptides to T cells, M $\phi$  may also have an effect on chlamydial infection by inducing T cell death and perpetuating a persistent infection.

DC are important for vaccine research because they are the critical links between innate and adaptive immunity (58). Antigen presenting cells (APC) such as DC and M $\phi$  are able to phagocytose chlamydial EBs in the extracellular space or engulf infected cells harboring RB. DC are known to be the quintessential APC. Immature DC are highly phagocytic, and after internalization of pathogens, they degrade the components and present the peptides to T cells via MHC receptors. This activates the T cells to initiate a cell-mediated and/or humoral immune response. The types of cytokines produced and antigens processed by DC and presented to CD4<sup>+</sup> T cells mediate the Th1/Th2 balance during a chlamydial infection (35).

In summation, cell-mediated immunity that activates M $\phi$ , NK cells, NK T cells, neutrophils, and mediators such as IL-12 and IFN- $\gamma$  is required for initial clearance. However, for protective immunity, both cell-mediated immunity and humoral immunity are needed, including antigen-specific T cells and antibodies that enhance phagocytosis and subsequent degradation and presentation of bacterial components by DC for a rapid Th1-mediated immune response (35).

## 2.4 Toll-like receptors (TLR)

TLRs belong to the family of pathogen-associated molecular pattern recognition receptors (PRRs) and are vital components of the host's immune system for sensing dangerous pathogens, and for initiating inflammatory and immune responses directed against these pathogens. The mechanism of signal transduction through TLRs is well characterized (59-66). There are two possible routes for mediation of signals received by TLRs depending on which of the two adapter molecules, myeloid differentiation factor 88 (MyD88) and TIR-domain containing adapter-inducing interferon- $\beta$  (TRIF), are involved. The importance of MyD88 and TRIF lies in the finding that each leads to a distinct profile of immune mediators that in turn determine the phenotype of the cells that are primarily responsible for the development of adaptive immune responses (67-70). MyD88 represents a common adaptor protein required for signaling of all TLRs identified to date, with the exception of TLR3 (71). TLR4 mediates through both the MyD88 and TRIF pathways, TLR3 signals through TRIF, and all the other TLRs mediate through MyD88 pathway. TLRs as PRRs are pivotal for maximizing pathogen detection (72). The TLR family includes a total of 11 receptors that are responsible for the recognition of highly conserved structural motifs that are essential for pathogen survival and are conserved across broad subclasses of microorganisms (73-74). These conserved microbial structural motifs are referred to as pathogen-associated molecular patterns (PAMPs) (75-78).

In this study we characterized the expression of MyD88 in response to our VCG vector in addition to downstream activation of nuclear factor kappa B.

## 2.5 Nuclear factor kappa B (NF-κB)

Nuclear factor kappa B (NF-κB) is a family of transcription factors with important gene regulatory functions in inflammation (79). This protein complex controls transcription of DNA and is found in almost all animal cell types (80-84). NF-κB plays a key role in regulating the immune response to infection (κ light chains are critical components of immunoglobulins) and is responsible for cytokine production and cell survival (85-89).

NF-κB is activated by known inducers which are highly variable and include reactive oxygen species (ROS), tumor necrosis factor alpha (TNFα), interleukin 1-beta (IL-1β), bacterial lipopolysaccharides (LPS), isoproterenol, cocaine, and ionizing radiation in response to harmful cellular stimuli (90).

NF-κB is a major transcription factor that regulates genes responsible for both the innate and adaptive immune response. Upon activation of either the T- or B-cell receptor, NF-κB becomes activated through distinct signaling components. Through a cascade of phosphorylation events, the kinase complex is activated and NF-κB is able to enter the nucleus to upregulate genes involved in T-cell development, maturation, and proliferation (91).

## 2.6 The roles of TLR and NF-κB during *Chlamydia* infection

The involvement of Toll-like receptors (TLRs) has been suggested in various inflammatory and autoimmune diseases (67-69). Binding of microbial ligands to TLRs elicit a sequence of molecular events that lead to activation of transcription factors such as nuclear factor κB (NF-κB) that control the organization of the proinflammatory

response. TLR mediated activation of NF- $\kappa$ B requires phosphorylation of its p65 subunit through a second pathway that involves Rho-GTPase Rac1, phosphoinositide-3 kinase (PI3-K) and AKT (75-78, 92).

TLR2 and TLR4 have been extensively studied and well characterized. The expression of these PRR upon stimulation by *Chlamydia* or bacterial ghosts was investigated in this study. The PAMPS that Toll-like receptors recognize include lipopolysaccharide (LPS), lipoprotein, nucleic acids and other phenotypic characteristics of bacterial pathogens.

The ligand for TLR4, LPS, is a cell wall component mainly found in Gram-negative bacteria. Bacterial lipoproteins are recognized by TLR2, as well as the ligands peptidoglycan, lipoarabinomannan, porins and others, from not only bacteria, but viruses or parasites (93). TLR2 and TLR4 are largely localized on the cell surface to recognize the aforementioned PAMPs (93).

TLR2- and TLR4-mediated MyD88-dependent signaling pathways lead to activation of NF- $\kappa$ B and MAPK, inducing gene expression of type I IFN. NF- $\kappa$ B also induces DNA transcription of IL-1 $\beta$ , tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), B7, tissue factor etc. in response to infection by bacterial pathogens.

## **2.7 Bacterial ghosts**

Expression of cloned PhiX174 gene *E* in Gram-negative bacteria results in lysis of the bacteria by formation of an *E*-specific transmembrane tunnel structure built through the cell envelope complex. Bacterial ghosts have been produced from a variety of bacteria including *Escherichia coli*, *Salmonella typhimurium*, *Salmonella enteritidis*,

*Vibrio cholerae*, *Klebsiella pneumoniae*, *Actinobacillus pleuropneumoniae*, *Haemophilus influenzae*, *Pasteurella haemolytica*, *Pasteurella multocida*, and *Helicobacter pylori*.

Such ghosts are used as non-living candidate vaccines and represent an alternative to heat or chemically inactivated bacteria. In recombinant ghosts, foreign proteins can be inserted into the inner membrane prior to *E*-mediated lysis via specific N-, or C-, or N- and C-terminal anchor sequences. The export of proteins into the periplasmic space or the expression of recombinant S-layer proteins vastly extends the capacity of ghosts or recombinant ghosts as carriers of foreign epitopes or proteins. Oral, aerogenic or parenteral applications of (recombinant) ghosts in experimental animals induced specific humoral and cellular immune responses against bacterial and target components including protective mucosal immunity. The most relevant advantage of ghosts and recombinant bacterial ghosts as immunogens is that no inactivation procedures that denature relevant immunogenic determinants are employed in the production of ghosts used as vaccines or as carriers of relevant antigens. The inserted target antigens into the inner membrane or into S-layer proteins are not limited in size (94).

Formation of a transmembrane tunnel structure with a diameter of 40-200 nm allows for the release of Gram-negative bacteria cytoplasmic material resulting in empty bacterial cells (ghosts) that retain all of the structural features of their outer envelope (95). In this study, the transmembrane lysis mechanism of PhiX174 gene *E* was used to produce VCG which was investigated as a non-living material vaccine adjuvant in modulating innate immune responses.

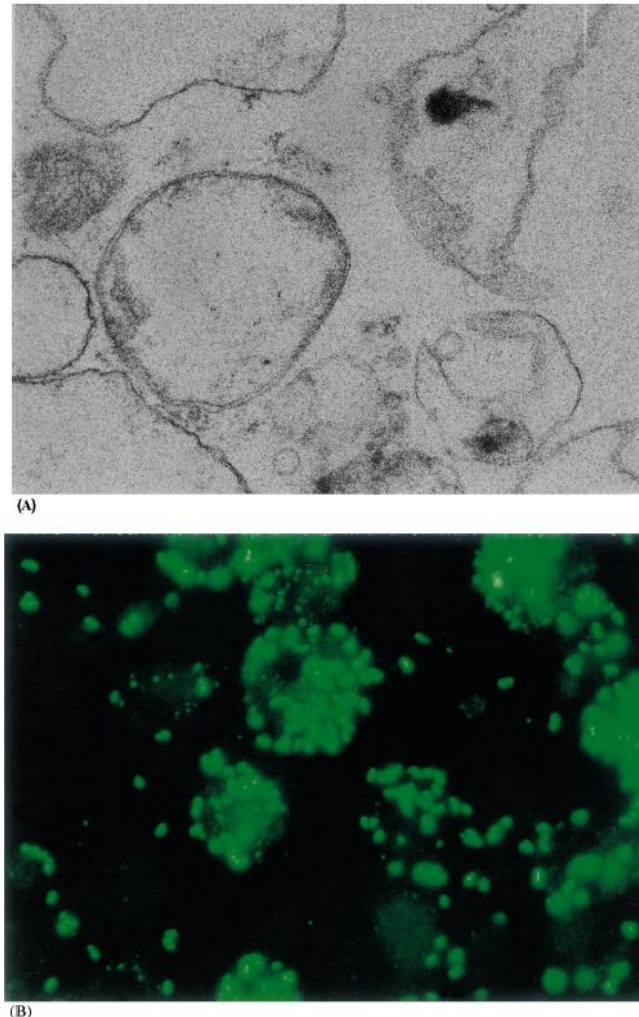
## 2.8 Bacterial ghosts as adjuvants

Adjuvants may be used to nonspecifically or specifically, such as in the case of bacterial ghosts, to potentiate the immune response to target antigens. Bacterial ghosts contain some well-known immune-stimulating compounds, such as LPS and peptidoglycan and specific receptor recognition properties of the envelope, contributing to effective uptake by macrophages and DC (96). The endotoxicity of the LPS does not limit the use of bacterial ghosts as vaccine candidates. Since LPSs and peptidoglycans are known adjuvants and bacterial uptake by macrophages is highly effective, immunization with low doses of bacterial ghosts will stimulate the innate immune system very efficiently, as seen in a protective mucosal response by aerosol application of ghosts in pigs, vibriocidal antibodies in mice, and humoral and cellular immunity to membrane anchored target proteins in rabbits and mice (97). Additionally, it is known that most epitopes found to be protective following vaccination are located on the bacterial surface (97). Comparable synthesis of inflammatory macrophage mediators, such as tumor necrosis factor (TNF)- $\alpha$  in the THP-1 cell line need a 100-fold higher dose of VCG than the corresponding amount of LPS (98-99). The bacterial ghosts stimulate the activation of cellular Th1 immune responses by increased interleukin (IL)-12, IL-18 and TNF- $\alpha$  production (96). Furthermore, the bacterial ghosts mediate the maturation of human DCs by decreasing the expression levels of CD83 and penetrate through matrigel-coated porous membrane (96). In addition, an exposure of ghosts to DCs results in a marked increase in their ability to activate T-cells. Thus, ghosts used as carriers of foreign proteins enhance the innate immune response against target antigens (96).

## **2.9 Phagocytosis of bacterial ghosts**

Haslberger et al. investigated the activation of the antigen presenting cells by bacterial ghosts in more detail by studying the uptake of bacterial ghosts in dendritic porcine cells and RAW macrophages and the induction of inflammatory mediators or mediators directing the innate immune response in THP-1 human macrophage cell line. A rapid uptake of bacterial ghosts in macrophages within 10-30 minutes was confirmed by electron microscopy (fig. 2) (98). DC are known to be phagocytic in specific immature states and Haslberger et al., observed significant uptake of bacterial ghosts within 2 hours. Their data suggested that bacterial ghosts effectively stimulate monocytes and macrophages for the induction of Th1 directed immune responses and dendritic cells treated with bacterial ghosts may serve as a promising vehicle for active immunization and immunotherapy *in situ* (98).





**Figure 2. Uptake of bacterial ghosts: Electron microscopy of mouse RAW macrophages.** Bacterial ghosts of *E. coli* O26:B6 have been added to the cell culture 20 min before. Still intact cell walls as well as degraded cell walls of phagocytosed cells can be seen (A). Fluorescence microscopy of porcine dendritic cells which have been cultures with FITC labeled *V. cholerae* ghosts. Fluorescence can be seen centralized inside the cells (B) (98).

## 2.10 Immune response elicited by bacterial ghosts

Mayr et al. investigated the activation of antigen presenting cells (APC) by BG by studying the *in vitro* uptake of VCG and *E. coli* BG in dendritic cells and RAW macrophages and the induction of inflammatory mediators in the THP-1 human macrophage cell line. What they observed was the synthesis of inflammatory mediators

such as TNF- $\alpha$  in the THP-1 cell line was stimulated by a hundred-fold higher dose of VCG than the corresponding amount of free LPS with no toxic side effects from the administration of BG but with significant humoral immune responses. Furthermore, the capability of BG to induce a T-cell-mediated immune response was studied following uptake of *Actinobacillus pleuropneumoniae* (APP) ghosts by primary APC in pigs. Specific T-cell responses were detected after *in vitro* re-stimulation of primed blood T cells with APP ghosts. Additionally they investigated uptake of APP BG by DC and subsequent DC activation. Following the internalization and processing of the antigens, increased expression of MHC class II molecules in APC was shown 12 h after their exposure to BG (100).

## CHAPTER III

### MATERIALS AND METHODS

#### 3.1 Chemicals and reagents

Mammalian cell culture media were donated by Dr. Joseph Igietseme, Centers for Disease Control and Prevention. Bacterial cell culture media were purchased from Becton Dickinson. Antibiotics (kanamycin and ampicillin; penicillin and streptomycin) were purchased from Sigma-Aldrich and Atlanta Biologicals, respectively. Agarose was purchased from Bio-Rad. Isopropyl  $\beta$ -D-thiogalactoside (IPTG), 3-methyl benzoate (3MB), lipopolysaccharide from *E. coli* 026:B6, protease inhibitor, and concavalin A were purchased from Sigma-Aldrich. Pam3CSK4 TLR2 ligand was purchased from InvivoGen. QIAprep Spin Mini- and Midiprep Kits were purchased from Qiagen. Pathfinder *Chlamydia* Culture Confirmation System was purchased from Bio-Rad. Pierce BCA Protein Assay was purchased from ThermoScientific. BD OptEIA™ Set Human TNF ELISA was purchased from BD Biosciences. Sucrose-phosphate-glycerol (SPG) media was donated by Dr. Qing He, Morehouse School of Medicine. Cycloheximide was donated by Dr. Joseph Igietseme, Centers for Disease Control and Prevention. Permout® was purchased from Fisher Scientific. Anti-human Toll-like receptor 4 (CD284) and anti-human Toll-like receptor 2 (CD282) antibodies were purchased from eBioscience Affymetrix, San Diego. MyD88 rabbit polyclonal antibody (385800), NF-kappa B p65 rabbit monoclonal antibody (701079), and goat anti-rabbit IgG HRP

(656120) were purchased from Life Technologies, Grand Island, NY. RNeasy<sup>®</sup> Mini Kit was purchased from Qiagen Sciences, Maryland, USA. DAPI counter stain was donated by Dr. Periakaruppan Nagappan, Center for Cancer Research and Therapeutic Development, Clark Atlanta University.

### **3.2 Mammalian and bacterial strains and growth mediums**

The monocytic leukemia cell line THP-1 (donated by Dr. Byron Ford, Morehouse School of Medicine and Dr. Joseph Igietseme, Centers for Disease Control and Prevention) was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 200 mM glutamine, 100 mM sodium pyruvate, 0.5% HEPES and penicillin/streptomycin (100 U/mL; 100 ug/mL) in a humidified atmosphere under 5% CO<sub>2</sub>, at 37°C.

The cervical cancer cell line HeLa (donated by Dr. Joseph Igietseme, Centers for Disease Control and Prevention) was maintained in Minimum Essential Medium (MEM) Earle's with 2mM L-glutamine, 10% heat-inactivated FBS, 1 mM sodium pyruvate, 0.5% fungizone, and 1.0% penicillin/streptomycin (100 U/mL; 100 ug/mL) in a humidified atmosphere under 5% CO<sub>2</sub>, at 37°C.

*Vibrio cholera* (V588 or V912) from lab stocks were cultured in brain heart infusion (BHI) media supplemented with 100 ug/mL ampicillin and 25 ug/mL kanamycin and grown in suspension at 37°C while shaking at 125 rpm. For induction of the *lac* promoter, isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) was added to growing cultures at 2.5 mM final concentration.

*E. coli* (O26:B6) from lab stocks were cultured in Luria broth supplemented with 0.2 ug/mL kanamycin and 0.2 ug/mL ampicillin and grown in suspension at 37°C while shaking at 250 rpm.

### **3.3 *Chlamydia trachomatis* SPP**

*Chlamydia trachomatis* serotypes D, E, and F were generated by propagating elementary bodies (EBs) in HeLa cells and the murine strain MoPN generated by propagating EBs in McCoy cells followed by purification of EBs over renografin gradients (generously donated by Dr. Joseph Igietseme, Centers for Disease Control and Prevention). IFUs was provided on the packaging.

### **3.4 Transformation of *V. cholerae* with chlamydial antigens**

*V. cholerae* (V588-pDKLO1) were maintained in BHI broth supplemented with kanamycin (25 ug/mL) at 37°C while shaking at 225 rpm to an  $A_{600}$  of 0.6 O.D. After cell proliferation overnight, the cell pellet was harvested by centrifugation at 2900 rpm in two centrifuge tubes and prepared to make competent. The pellet was washed in sucrose electroporation buffer H (SEBH) (137 mM sucrose, 1 mM HEPES, 10% glycerol) 3x. After washing, the pellet was vortexed to loosen then, 100 uL aliquots of the competent cells were introduced to Eppendorf tubes and stored at -80°C. To one of the aliquots, pKs-PmpD/PorB-FL was added and pulsed on a BioRad pulser for 4.8 secs at 200  $\Omega$  to transform the cells with the plasmid. Transformants were inoculated into BHI and incubated for 1 h at 37°C while shaking. After 1 h transformants were spread onto BHI agar and LB agar overnight at 37°C.

### 3.5 Production of bacterial ghosts

Production of the rVCG was carried out by gene *E*-mediated lysis. Briefly, bacteria were maintained in BHI broth supplemented with ampicillin (100 ug/mL) and kanamycin (25 ug/mL) at 37°C while shaking at 200 rpm to mid-log phase. At this point, IPTG (2.5 mM) was added to the growing culture to induce the *Lac operon* for gene expression. After 35 min of PmpD/PorB-FL induction, cell lysis was achieved by adding 3MB (5 mM) to induce expression of gene *E*. Production of the VCG was accomplished using the same protocol however, the broth did not require ampicillin. When lysis was complete, cellular cultures were harvested by centrifugation and the pellet resuspended in a low ionic buffer then washed with cold PBS. Harvested ghosts were resuspended in PBS then lyophilized and stored at room temperature until use.

### 3.6 Protein gel electrophoresis and Western blots

Three bacterial (*V. cholerae*) cell cultures containing pHs-PmpD/pDKLO1 were harvested and centrifuged at 4000 rpm for 15 min to pellet. To prepare samples for polyacrylamide gel electrophoresis, cellular pellets were resuspended in 1 mL cold RIPA buffer to release whole cell protein. Lysate was then transferred to 1.5 mL eppendorf tubes. To ensure an efficient whole cell protein suspension, the cultures were sonicated for 2 bursts of 10 sec at ~30 W (watts). Protein samples were diluted in 1X RIPA buffer at 1:1, 1:2 and 1:4 dilutions. Samples were electrophoresed at 75 V for 3 h.

Standard Western blotting procedure was used to confirm the identity of and quantify PmpD. The primary and secondary antibodies used to probe PmpD were anti-chlamydial PmpD and Goat-anti-Mouse IgG-HRP, respectively.

### 3.7 Infection of HeLa cell monolayer

*Chlamydia trachomatis* serovar D ( $1.2 \times 10^{10}$ /mL IFUs) and MoPn ( $2.2 \times 10^9$ /mL IFUs) (donated by Dr. Joseph Igietseme, Centers for Disease Control and Prevention) were aliquot to a MOI of 3. HeLa cells ( $30 \times 10^4$  cells/mL) maintained in MEM Earle's Salts supplemented with 10% FBS and 1% pen/strep were seeded in a 24-well culture plate with cover slips and set in a 37°C, 5% CO<sub>2</sub> jacketed incubator until confluent. Once confluent, the HeLa monolayer was treated with Serovar D or MoPn at a MOI of 3. Two wells were left untreated to serve as negative controls. After treatment introduction, the plate was centrifuged at room temperature (RT) for 1 h at 2200 rpm, then immediately set in a 37°C, 5% CO<sub>2</sub> jacketed incubator for 1 h. The *Chlamydia* treatments were gently aspirated, and 1 mL of warm cycloheximide media was introduced and the plate was returned to 37°C, 5% CO<sub>2</sub> jacketed incubator for 48 h.

After 48 h, the cycloheximide media was gently aspirated. Cells were gently washed 2x with PBS (6.0 - 7.5 pH). Infected cells were fixed with 1 mL of cold methanol (MeOH) and set in 4°C for 10 min. After fixation the MeOH was aspirated and cells were washed 1x with PBS. To stain inclusion bodies, Pathfinder<sup>®</sup> *Chlamydia* Culture Confirmation System was introduced to each treated well and set in the dark for 2 h. After 2 h the plate was removed from the dark, and working in a germ-free darkened culture hood to protect the plate from intense light, the cover slips were removed from the wells and rinsed twice in dH<sub>2</sub>O then inverted onto a microscope slide containing Permunt<sup>®</sup> and viewed under fluorescent microscopy for detection and enumeration of IFUs.

### 3.8 *Chlamydia*-infected HeLa cell viability assay

HeLa 229 ( $60 \times 10^4$  cells/mL), endocervical epithelial cells, were infected with *Chlamydia* elementary bodies of strain MoPn or serovar F, then cultured in the supernatant from THP-1 macrophages pulsed by murine splenocytes and VCG. HeLa cell monolayers were infected with MoPn or serovar F at an MOI of 5. Two HeLa cell monolayers were pre-treated with DEAE-dextran (45 ug/mL) for 15 min at 37°C prior to infection. *Chlamydia* infected HeLa cell monolayers were incubated overnight. 4 h post infection (PI) HeLa monolayers were observed by phase microscopy for cytotoxicity. HeLa cells were identified for rounding, detachment and lysis vs. uninfected controls. The following morning CT-infected media was removed by pipet and cell monolayers were washed 3x with MEM Earle's Salts media. 1 mL of conditioned media was introduced to each CT-infected HeLa cell monolayer and returned to the humidified incubator under 37°C, 5% CO<sub>2</sub> for 24 h. MEM Earle's Salts media was added to uninfected HeLa cell monolayers, serving as our negative control. Cell viability was assayed by microscopy and counting cells using a cellometer (Nexcelom).

### 3.9 Determination of cytotoxicity of VCG on THP-1 cells

THP-1 monocytes (Mn) were maintained in RPMI 1640 medium supplemented with 10% FBS, 200 mM glutamine, 100mM sodium pyruvate, 1% penicillin/streptomycin (100 U/mL; 100ug/mL), and 0.5% HEPES (complete medium) in a humidified atmosphere under 5% CO<sub>2</sub>, at 37°C overnight. The following day 3 mL aliquots of THP-1 Mn ( $4.2 \times 10^7$  cells/mL) were seeded in a 6-well plate then pulsed in a dose-dependent manner with VCG at 20 ug/mL, 15 ug/mL, 10 ug/mL, 5 ug/mL or 1



ug/mL for 3 h. LPS (1 ug/mL) and RPMI 1640 served as positive and negative controls, respectively. After this, treated cellular pellets were collected by centrifugation at 1500 rpm for 10 min at 4°C, then resuspended in VCG-free medium and returned to the incubator overnight. The following day, 20 uL of each culture was aliquot into a cellometer chamber slide and counted using a Cellometer (Nexcelom).

### **3.10 Dose and kinetic experiments**

THP-1 monocytes (Mn) were maintained in RPMI 1640 medium supplemented with 10% FBS, 200 mM glutamine, 100mM sodium pyruvate, 1% penicillin/streptomycin (100 U/mL; 100ug/mL), and 0.5% HEPES (complete medium) in a humidified atmosphere under 5% CO<sub>2</sub>, at 37°C overnight. To measure the secretion levels of tumor necrosis factor (TNF) elicited by VCG, THP-1 Mn were treated with VCG at 20, 15, 10, 5, or 1 ug/mL for 24, 48, or 72 h in a humidified atmosphere under 5% CO<sub>2</sub>, at 37°C in an initial experiment. Then, THP-1 Mn were treated with VCG at 10, 5, or 1 ug/mL for 6, 12, or 18 h. THP-1 Mn incubated in the presence of RPMI 1640 complete media alone or *E. coli* O26:B6 LPS (1 ug/mL) served as negative and positive controls, respectively. The level of TNF secreted was determined by TNF ELISA (BD Biosciences).

### **3.11 Cytokine secretion by THP-1 Mn or Mφ following pulse by VCG**

THP-1 monocytes (Mn) were maintained in RPMI 1640 supplemented with 10% FBS, 200 mM glutamine, 100mM sodium pyruvate, 1% penicillin/streptomycin (100 U/mL; 100ug/mL), and 0.5% HEPES (complete medium) in a humidified atmosphere under 5% CO<sub>2</sub>, 37°C incubation. Mn (suspension cells) were differentiated into adherent

macrophages (M $\phi$ ) by treatment with 160 nM PMA (phorbol 12-myristate 13-acetate) for 1 h. After 1 h the activated monocytes were pelleted by centrifugation at 1500 rpm for 10 min to remove PMA treatment then resuspended in PMA-free complete media. 1 mL PMA-free cell culture aliquots were put into ten wells of a 24-well plate. Mn cellular culture was aliquot (1 mL) into ten wells of the same 24-well plate and returned to the incubator overnight (o/n). The next day THP-1 Mn ( $2.7 \times 10^6$  cells/mL) and M $\phi$  ( $1.2 \times 10^6$  cells/mL) were treated with VCG (10  $\mu$ g/mL), VCG (10  $\mu$ g/mL) and IL-10 (5  $\mu$ g/mL), or LPS (1  $\mu$ g/mL). LPS served as a positive control; RPMI 1640 complete medium, served as a negative control. IL-10 was co-pulsed with VCG as a negative control to inflammatory cytokine secretion. Mn and M $\phi$  were pulsed for 24 h in a humidified atmosphere under 5% CO<sub>2</sub>, at 37°C. Following the stimulation period the contents of each well were carefully transferred to its own 1.5 mL Eppendorf tube and centrifuged at 1500 rpm for 10 min at 4°C to collect the cellular supernatant (soups). Soups were kept at -20°C until assayed by Multiplex technology (Quansys Biosciences, Logan, UT).

### **3.12 Isolation, culture and activation of murine splenocytes**

Spleens from five 14 week old C57BL/6 female nude mice were removed and processed to isolate murine splenocytes. Mice were euthanized by CO<sub>2</sub> suffocation. Spleens were removed and placed in chilled PBS with 0.5% FBS. To isolate murine splenocytes, spleens (one at a time, using a new strainer each time) were placed in a 100  $\mu$ m cell strainer placed atop a 50 mL centrifuge tube containing 5 mL RPMI 1640 complete media supplemented with 10% FBS and 1% pen/strep. Each spleen was pressed

with a rubber policeman until most of the tissue had passed through. The strainer and policeman were then rinsed with 1 mL RPMI 1640 complete media to ensure all cells passed through the strainer. Once all the spleens were processed, the cellular suspension was collected by pipet and passed through a 70  $\mu$ m cell strainer atop a 50 mL centrifuge tube. The cellular suspension was again collected by pipet and transferred to a 15 mL centrifuge tube and centrifuged at 90g for 5 mins to collect the cellular pellet. Cellular pellet was then resuspended in RPMI 1640 complete medium, placed in a culture flask and set in a humidified incubator under 37°C, 5% CO<sub>2</sub> for 2.5 days. To activate murine splenocytes, 1  $\mu$ L PMA (160 nM) plus 10  $\mu$ L concavalin A (1 %) was added to 10 mL of murine splenocytes ( $1.2 \times 10^6$  cells/mL) and returned to humidified incubator under 37°C, 5% CO<sub>2</sub> for 24 h.

### **3.13 Co-culture of activated THP-1 Mn or M $\phi$ with murine splenocytes**

[Activated] murine splenocytes culture was removed from incubation and counted using Trypan blue exclusion with a hemacytometer. For efficient co-culture with THP-1 Mn or activated M $\phi$ , 225  $\mu$ L of murine splenocytes ( $1.6 \times 10^6$  cells/mL) (8:1) were added to a 96-well plate that already contained THP-1 Mn or activated M $\phi$  designated treatment wells. Plate was returned to the humidified incubator under 37°C, 5% CO<sub>2</sub> for 48 h. After 48 h co-cultures and controls were carefully removed by pipet, put into 1.5 mL eppendorf tubes and centrifuged at 500 rpm for 10 mins to collect cellular supernatants. Cellular supernatants were introduced with 1 – 2  $\mu$ L protease inhibitors and stored in - 20°C until assayed for cytokine concentration.

### 3.14 Multiplex assay

Multiplex assay was contracted out to Quansys Bioscience, Logan, UT. All samples were tested for IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17, IL-23, IFN $\gamma$ , and TNF $\alpha$  by Quansys Biosciences (Logan, UT) Q-Plex Array<sup>TM</sup> kits for human cytokines. Samples were thawed on ice and diluted into Quansys Human Sample Dilution Buffer prior to loading into preparatory polypropylene low-binding 96-well plates. Standard ELISA incubation steps apply such as initial sample incubation, washing, secondary antibody incubation, washing, incubation with the label and measurement are involved. The label and reporting system used in a Q-Plex Array<sup>TM</sup> is chemiluminescent. The total protein concentration was determined via absorbance at 280 nm and an extinction coefficient of 1 using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington DE).

### 3.15 Immunofluorescence

THP-1 M $\phi$  ( $11 \times 10^4$  cells/mL) were seeded onto an 8-well glass Chamber Slide<sup>TM</sup> (Lab-Tek<sup>®</sup>) then introduced with 4% formalin for 15 min in a humidified incubator under 5% CO<sub>2</sub>, at 37°C to fix the monolayer. After 15 min, the fixative was carefully removed by pipet, and the cell monolayers were washed 3x with PBS for 5 min each. To probe for TLR expression, anti-human Toll-like receptor 4 (CD284) (1 ug) or anti-human Toll-like receptor 2 (CD282) (1 ug) (eBioscience Affymetrix, San Diego) antibody was added at a concentration of 1:100 or 1:200 and incubated in a humidified incubator under 5% CO<sub>2</sub>, at 37°C for 4 – 5 h. After incubation with the respective antibody, CD284 or CD282 was carefully removed and cellular monolayers were washed

3x with PBS for 5 min each. DAPI counterstaining was introduced to the monolayers (for nuclei staining), covered with a coverslip and sealed with clear nail polish to secure for viewing under fluorescent microscopy.

THP-1 Mn ( $1 \times 10^6$  cells/mL), maintained in RPMI 1640 complete media, were seeded into a 24-well plate and pulsed by VCG (10 ug/mL), LPS (1 ug/mL), or EB (MOI 2.5). RPMI 1640 complete media served as a negative control, and LPS and EB served as positive controls. THP-1 Mn were pulsed for 6 h in a humidified incubator under 5% CO<sub>2</sub>, at 37°C. After the treatment period, the contents of each well was transferred to its own 1.5 mL Eppendorf tube and centrifuged at 1500 rpm for 10 min to collect the cellular pellet. Each pellet was resuspended in 1 mL RPMI 1640 complete media. 50 uL of each was introduced to an 8-well glass Chamber Slide™ (Lab-Tek®) and set in the incubator for 5 min. Then, 100 uL of 4% formalin fixative was added to each well and returned to the incubator for 15 min. After 15 min, the fixative was removed by aspiration and each well washed 3x with PBS for 5 min each. Then, under dimly lit conditions, 1 ug CD282 (TLR2) AlexaFluor® 488 antibody or 1 ug CD284 (TLR4) AlexaFluor® 488 antibody (Affymetrix eBiosciences) was introduced to each designated sample well and returned to the incubator for 2 h. After 2 h, the antibody was washed off 3x with PBS for 5 min each. DAPI counterstain was introduced to the slide (for nuclei staining) and covered with a coverslip then sealed with clear nail polish and set in the dark for 20 min. TLR expression was viewed under fluorescent microscopy.

### 3.16 Characterization of MyD88 and NF-kappa B

THP-1 Mn were maintained in RPMI 1640 medium supplemented with 10% FBS, 200 mM glutamine, 100mM sodium pyruvate, 1% penicillin/streptomycin (100 U/mL; 100ug/mL), and 0.5% HEPES (complete medium) in a humidified atmosphere under 5% CO<sub>2</sub>, at 37°C overnight. The following day, to induce differentiation into adherent Mφ, the culture was treated with PMA (160 nM) for 1 h. After 1 h, the cellular pellet was collected by centrifugation at 1500 rpm for 10 min to remove PMA treated media. The pellet was resuspended in 1 mL PMA-free media then brought to a final volume of 25 mL. 1 mL (5.24 x 10<sup>6</sup> cells/mL) aliquots of the activated Mn cell culture were seeded into the wells of a 24-well plate and returned to the incubator for 8 h. After 8 h, activated Mφ were pulsed with VCG (10 ug/mL), Pam3CSK4 (250 ng/mL), a TLR2 agonist, MoPn elementary bodies (MOI 10), LPS (1 ug/mL), or RPMI 1640, which served as a negative control, for 4 – 6 h in a humidified atmosphere under 5% CO<sub>2</sub>, at 37°C. After the treatment period the cellular pellet was collected by centrifugation and prepared for protein concentration analysis using Pierce BCA Protein Assay (ThermoScientific) following manufacturer's instructions. Briefly, cellular pellet was washed 3x in PBS. Then to lyse the cells, the pellet was resuspended in lysis buffer (RIPA buffer, PMSF, protease inhibitor, β-mercaptoethanol, and DTT), vortexed for 30 sec, set in -80°C for 10 min, then set on ice to thaw. This was repeated twice. After, the lysate was centrifuged at 4°C at 10,000 rpm for 5 mins. Then the lysate was diluted 1:8 (v/v) with working reagent, incubated at 37°C for 30 min, cooled at room temperature then protein concentration was read on a SpectraMAX 190 at 562 nm. 15ug of each sample contained

enough protein to prepare the samples for Western blot analysis and characterization of NF- $\kappa$ B transcription factor or MyD88 adaptor protein. Blot was developed by chemiluminescence and viewed using the LAS4000 Imager.

### **3.17 Statistical analysis**

Where applicable, data were analyzed by a paired Student's t-test or ANOVA using GraphPad Prism software. The level of significance was judged at  $p < 0.05$ .

## CHAPTER IV

### RESULTS

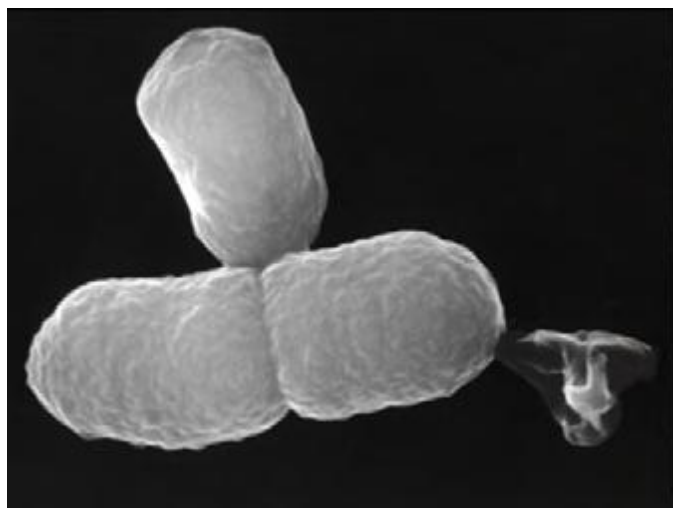
#### **4.1 Transformation of *Vibrio cholerae***

Competent *Vibrio cholerae* (V588-pDKLO1) cells were successfully transformed with pKs-PmpD/PorB-FL. Transformation of plasmid DNA into wild type *Vibrio cholerae* strains was possible by electroporation. Transformants were plated on BHI agar and LB agar and incubated at 37°C overnight. The following morning all cultures had colony growth relative to the volume of transformants plated.

#### **4.2 PhiX174 gene *E*-mediated lysis of *V. cholerae* and construction of the vaccine vector**

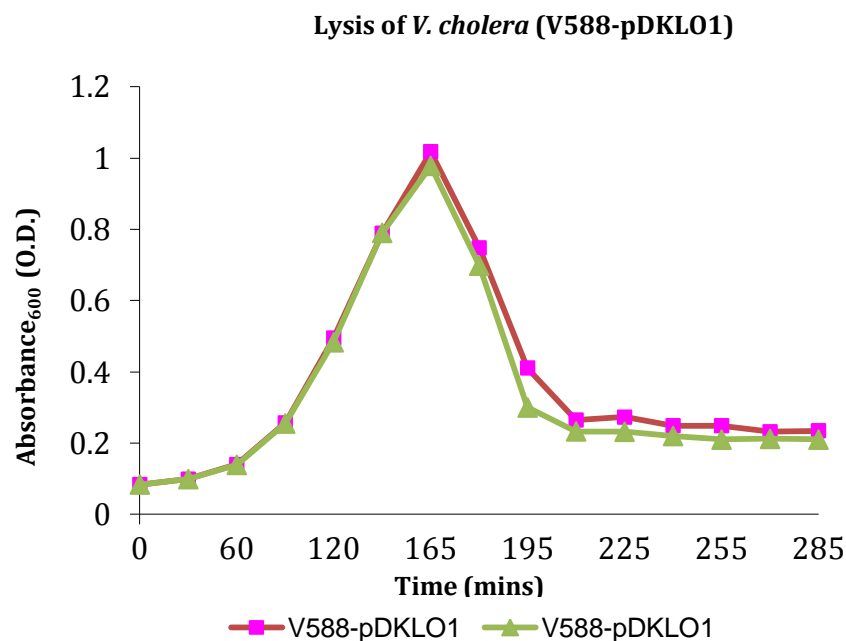
The protein *E*-mediated lysis system is derived from the *E. coli*-specific bacteriophage PhiX174. It exerts its lytic function by the fusion of inner and outer membranes and transmembrane tunnel formation. After controlled expression of subcloned gene *E* during the exponential growth phase of the bacteria, which includes all cell surface structures and appendices, none of the bacteria morphology is affected by the lysis event and the peptidoglycan is not degraded (101). The lysed bacteria (ghosts) resemble structurally their living counterparts (fig. 3).



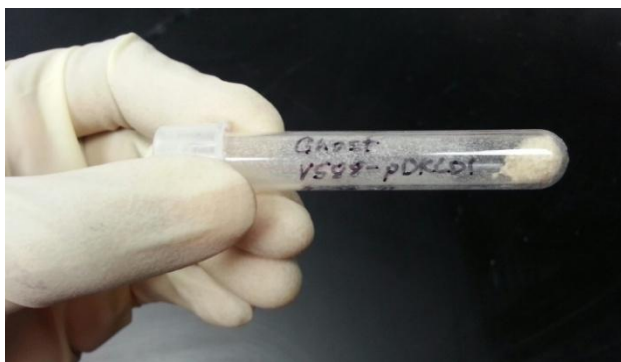


**Figure 3. Protein E-mediated lysis of Gram-negative bacteria.** Bacterial ghost system. Inducible expression of gene *E* causes the fusion of inner and outer membranes of the bacterial cells and forms an intermembrane tunnel. The empty BG envelope is devoid of cytoplasmic content, whereas the inner and outer membrane structures including LPS and peptidoglycan are preserved and remain intact (104).

*Vibrio cholerae* V588 harboring the membrane targeting plasmid pKs was co-transformed with the lysis plasmid pDKLO1. Cultures were normally grown at 28°C. Transcription of gene *E* from plasmid pDKLO1 was directed by the activity of thermosensitive repressor coded on the plasmid. Temperature increase of exponentially growing *Vibrio cholerae* to 42°C led to rapid de-repression and protein *E* induction. The extent and rate of lysis was quantified from the decrease in turbidity/unit of time and monitored by measuring the absorbance of the lysing culture at 600 nm (fig. 4). At the end of lysis, the lysed cells (VCG) were harvested by centrifugation (330g, 4°C, 10 min, Beckman Coulter GS-6R), washed 3x in 50 mL ice-cold PBS, and resuspended in 2 mL ice-cold PBS/sorbitol. For long-term storage, VCG were lyophilized and kept at room temperature (fig. 5).



**Figure 4. Production of *Vibrio cholerae* ghosts.** *V. cholerae* strain V588 were transformed with the lysis plasmid pDKLO1. The transformed bacterial cells were grown at 37°C in brain heart infusion broth containing Kanamycin (25 ug/mL) to an optical density of 1.0 ( $A_{600}$ ). Cell lysis was achieved by the addition of 3-methyl-benzoate (5 mM) to induce gene *E* expression (12).



**Figure 5. Lyophilized *Vibrio cholerae* ghosts.** For long-term storage, lyophilized VCG are kept at room temperature.

#### 4.3 Characterization of Polymorphic Membrane Protein D (PmpD)

Western blot analysis of PmpD chlamydial protein (fig. 6) with monoclonal anti-chlamydial PmpD antibodies revealed that the 67 kDa protein was expressed following

IPTG induction in the VCG. The total cellular protein of *V. cholerae* V588 cultures transformed with the expression plasmid containing PmpD revealed the appearance of PmpD-specific bands (fig. 6) visualized by use of anti-PmpD monoclonal antibody, HRP-conjugated Goat-anti-Mouse IgG antibodies and BCIP/NBT as substrate.



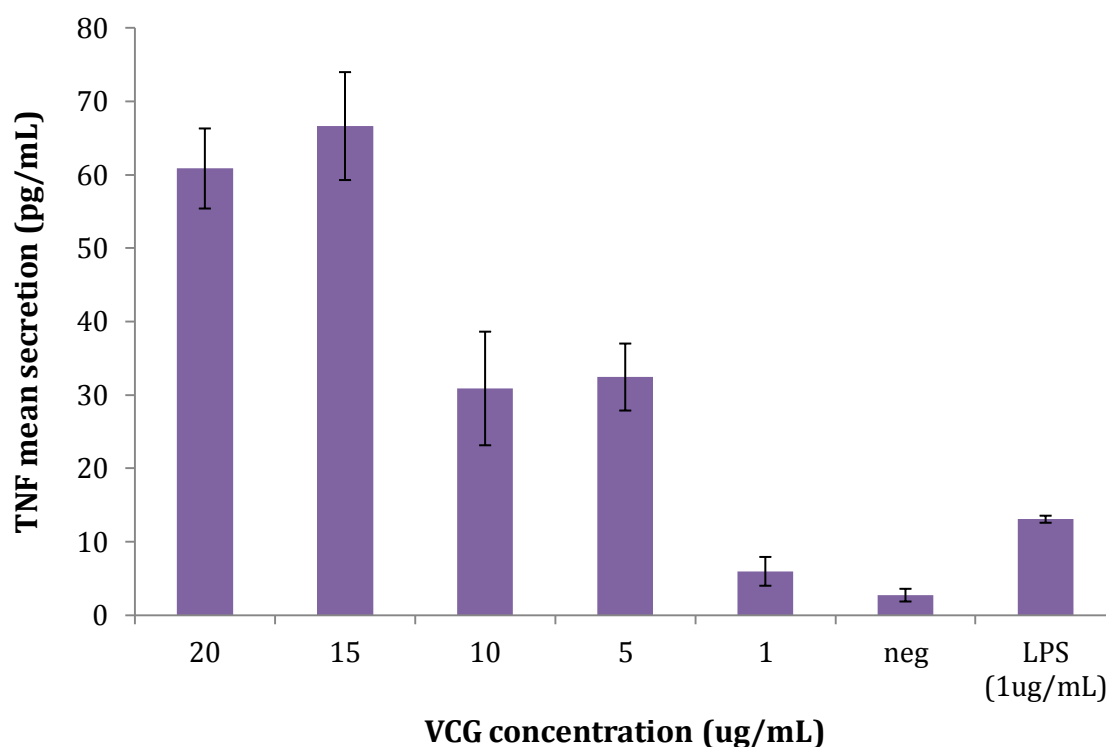
**Figure 6. Western blot of Chlamydial Polymorphic Membrane Protein D (PmpD).** Characterization of PmpD,, a 67 kDa protein, expression on *V. cholerae* bacterial ghost.

#### 4.4 Immunogenicity of *Vibrio cholerae* ghost vaccine vector

##### *TNF ELISA*

VCG, used as an adjuvant, will stimulate a signaling pathway and elicit its own immune response. As an initial experiment to determine immunogenicity, TNF $\alpha$  secretion was measured by Human TNF ELISA set (BD OptEIA™). TNF is one of a few cytokines that stimulate the acute phase reaction following infection by pathogens. Other acute phase cytokines include IL-1 $\beta$ , IL-4, IL-6, and IL-8. THP-1 Mn were pulsed by VCG at varying concentrations (20, 15, 10, 5 or 1  $\mu$ g/mL) for 1 – 3 days. After 24 h TNF secretion was only slightly developing (fig. 7). Leading us to believe 1) the dosage was insufficient; or 2) the kinetics for VCG takes longer. TNF secretion from the positive control should have been much higher, and we expect the paucity of the TNF secretion

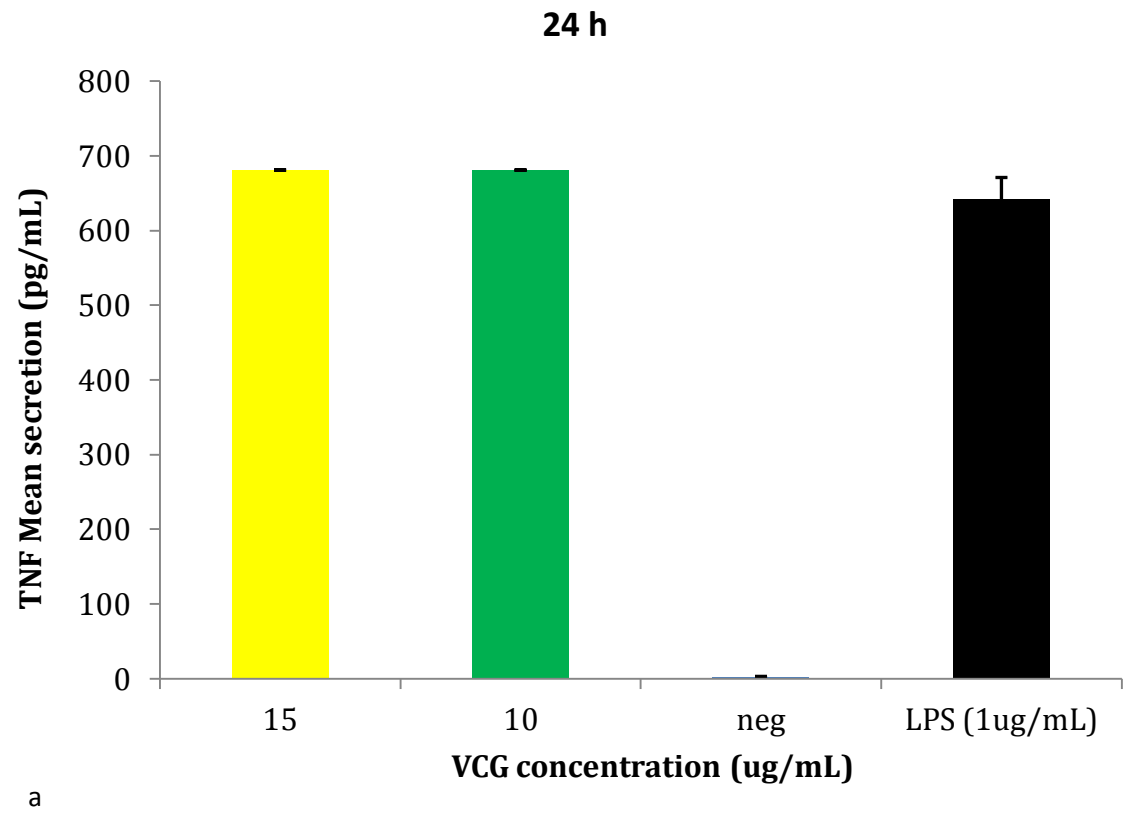
was due to experimental error. From these results we extended our time-course to include 48 h and 72 h stimulation of THP-1 cells with only 10 or 15 ug/mL VCG since these two dosages elicited significant initial TNF secretion.



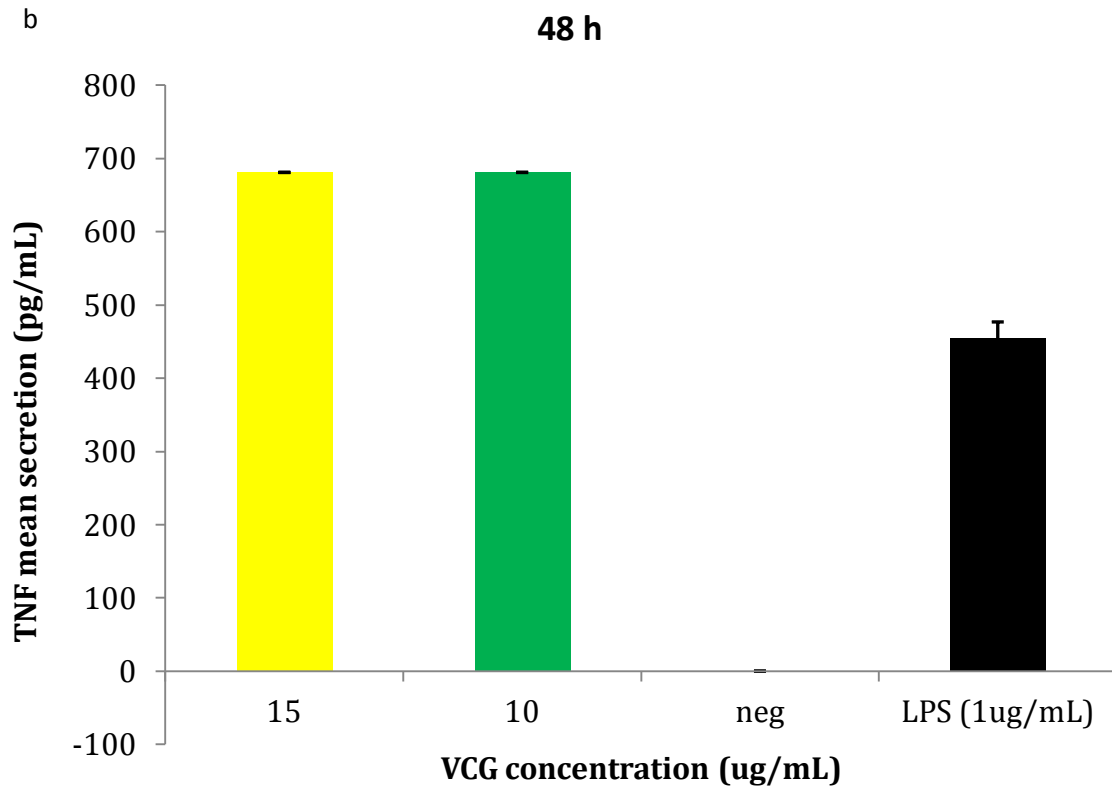
**Figure 7. Graph of tumor necrosis factor alpha (TNF $\alpha$ ) secretion (24 h).** THP-1 monocytes (Mn) were pulsed by VCG at various dosages and lengths of time to determine the optimal dose and pulse duration for TNF secretion to peak. This initial data was inconclusive.

We have seen ghosts “clump” together when resuspended in cell culture medium in preparation for stimulating cells. We speculate that this results in the VCG particles being too large for cell surface receptors to bind and engulf. As such, going forward we prepared the VCG stock solution in 1:1 PBS:FBS then vortexed briefly to distribute bacterial ghosts before aliquots were introduced to experimental cultures. The

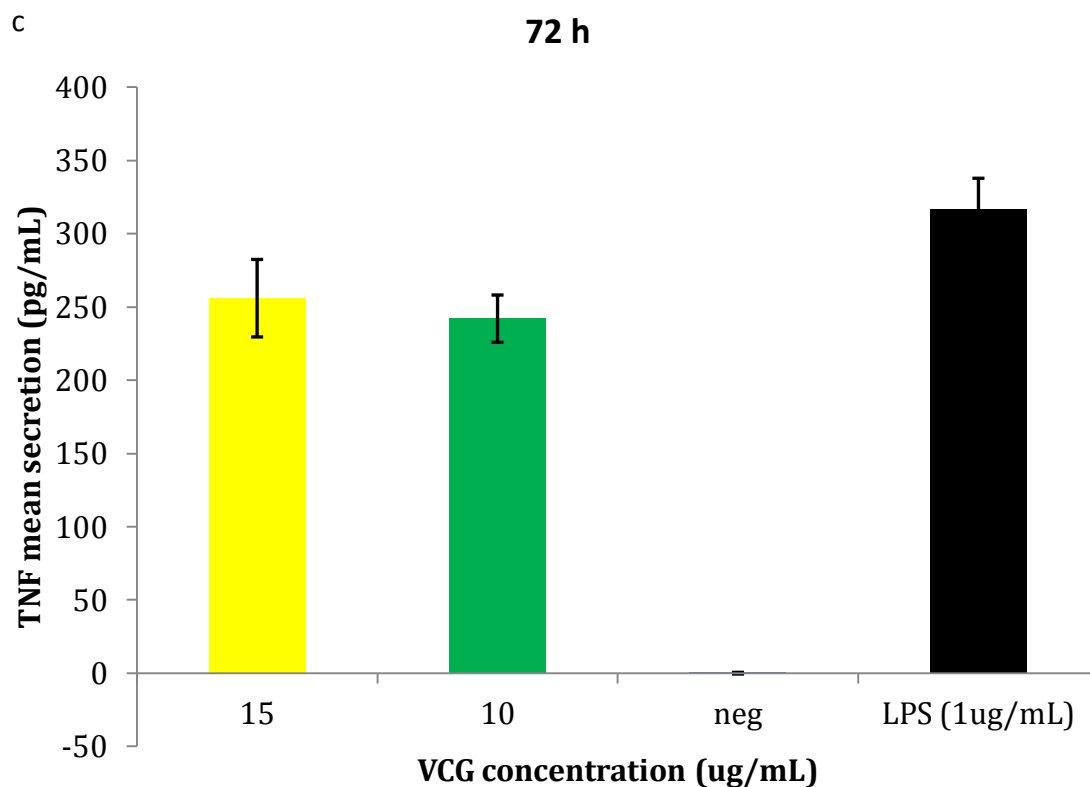
concentration of the FBS protein significantly reduced clumping, leaving ghosts that the cell could more efficiently uptake. TNF secretion after 24, 48 and 72 h was measured using human TNF ELISA set (fig. 8a – c). After 24 h and 48 h stimulation of THP-1 cells with 10 or 15 ug/mL VCG, TNF secretion was 681 pg/mL (figs. 8a & 8b). After 72 h stimulation of THP-1 cells with 10 or 15 ug/mL VCG, TNF secretion was 242 pg/mL and 256 pg/mL, respectively (fig. 8c). 24 h secretion of TNF by LPS (positive control) treated THP-1 Mn was lower compared to THP-1 Mn treated by ghosts (at 10 or 15 ug/mL). This could indicate that soluble LPS (1 ug/mL) is toxic to the cells at this concentration and/or time-course therefore, leading to a reduction in TNF concentration (fig. 8a).



**Figure 8a-c. TNF secretion preliminary data.** Following 24 h and 48 h pulse of THP-1 Mn by VCG (15 ug/mL or 10 ug/mL), secretion of the proinflammatory cytokine TNF $\alpha$  was 681 pg/mL (a & b). TNF $\alpha$  secretion after 3 days was significantly less (c). LPS (1 ug/mL) serves as the positive control. RPMI 1640 complete medium served as the negative (neg) control.



**Figure 8a-c. TNF secretion preliminary data.** Following 24 h and 48 h pulse of THP-1 Mn by VCG (15 ug/mL or 10 ug/mL), secretion of the proinflammatory cytokine TNF $\alpha$  was 681 pg/mL (a & b). TNF $\alpha$  secretion after 3 days was significantly less (c). LPS (1 ug/mL) serves as the positive control. RPMI 1640 complete medium served as the negative (neg) control.

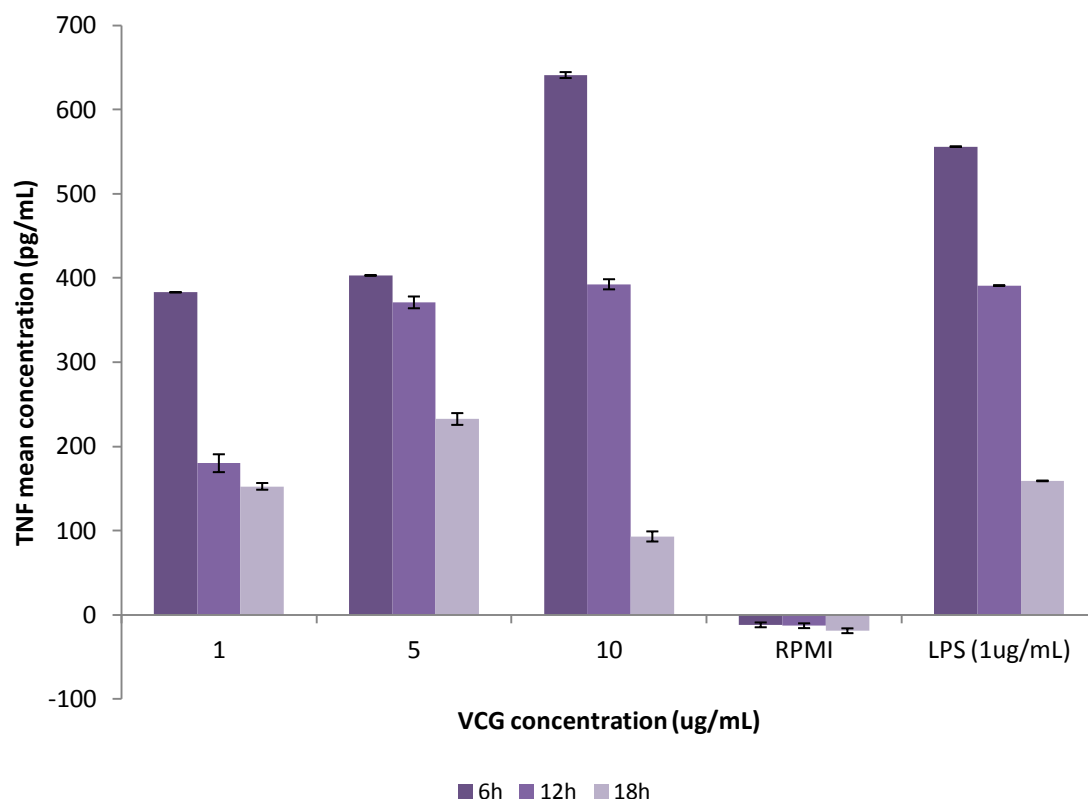


**Figure 8a-c. TNF secretion preliminary data.** Following 24 h and 48 h pulse of THP-1 Mn by VCG (15 ug/mL or 10 ug/mL), secretion of the proinflammatory cytokine TNF $\alpha$  was 681 pg/mL (a & b). TNF $\alpha$  secretion after 3 days was significantly less (c). LPS (1 ug/mL) serves as the positive control. RPMI 1640 complete medium served as the negative (neg) control.

Following these results we hypothesized that that optimal secretion of the proinflammatory cytokine TNF-alpha by THP-1 cells pulsed with VCG occurs <24h. Therefore, THP-1 monocytes were pulsed with VCG (1, 5 or 10 ug/mL) for 6, 12 and 18 h and the concentration of TNF secretion was measured (fig. 9) by ELISA. Our results indicated that the optimal dose of VCG is 10 ug/mL and stimulation of THP-1 Mn for TNF $\alpha$  to reach its peak is 6 h. After 6 h, TNF $\alpha$  secretion decreases, which would confirm



our hypothesis that optimal secretion of the proinflammatory cytokine TNF-alpha by THP-1 cells pulsed with VCG is <24h.



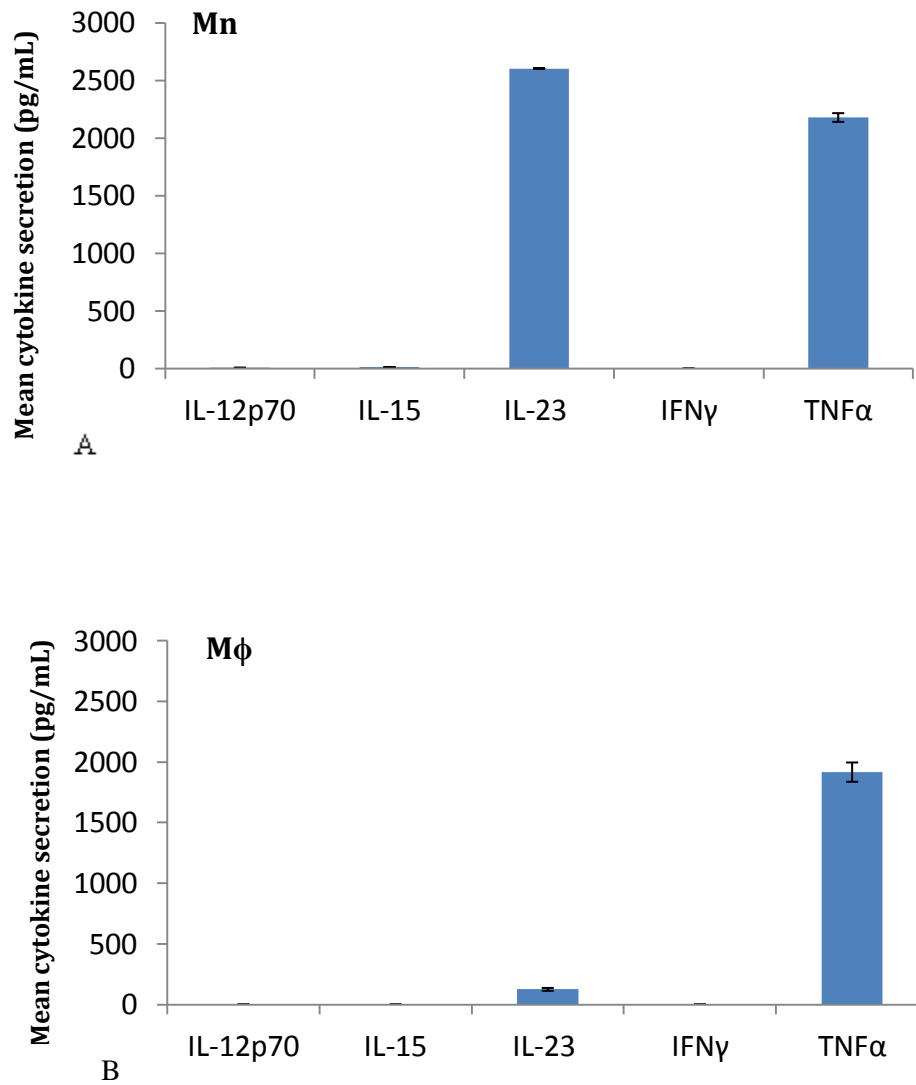
**Figure 9. TNF peak production.** THP-1 Mn were pulsed with VCG (1, 5, or 10 ug/mL) for 6, 12 or 18 h. The optimal dosage of VCG was determined to be 10 ug/mL to elicit peak production of TNF. Optimal pulse time is between 4 - 6 h. These conditions were used throughout the research to determine immune responses elicited by VCG.

#### *Cytokine secretion by THP-1 cells primed with IL-10 is down-regulated*

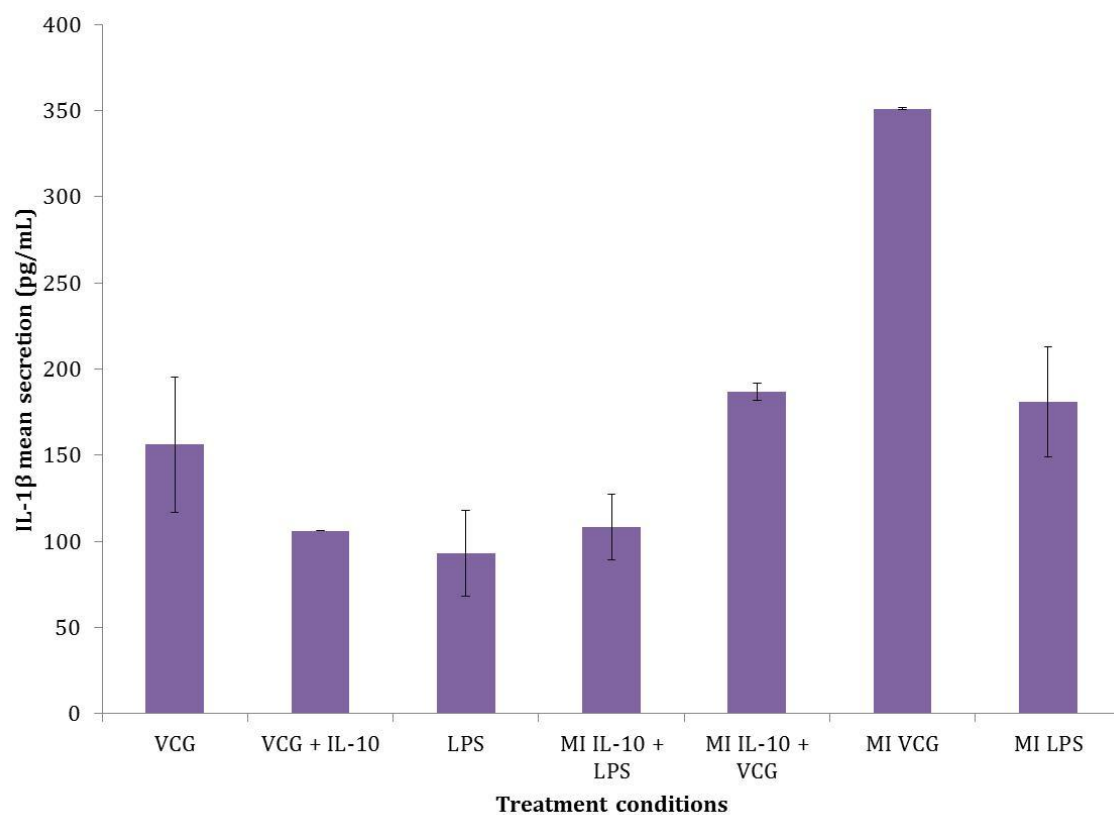
The identification and characterization of cytokines secreted from cells involved in innate immunity can broaden our understanding of immune cell function. The human myelomonocytic cell line, THP-1, has two morphologies – monocytes, which grow in suspension and monocyted-derived macrophages which are adherent. The two THP-1 cell morphologies were pulsed with VCG (10 ug/mL). Both were co-pulsed with IL-10

(5 ug/mL), an anti-inflammatory cytokine. Cellular supernatants were assayed via Quansys Biosystems Q-Plex Array<sup>TM</sup> technology; a traditional ELISA technology performed in micro-scale and multiplexed allowing for the simultaneous measurement and detection of multiple proteins.

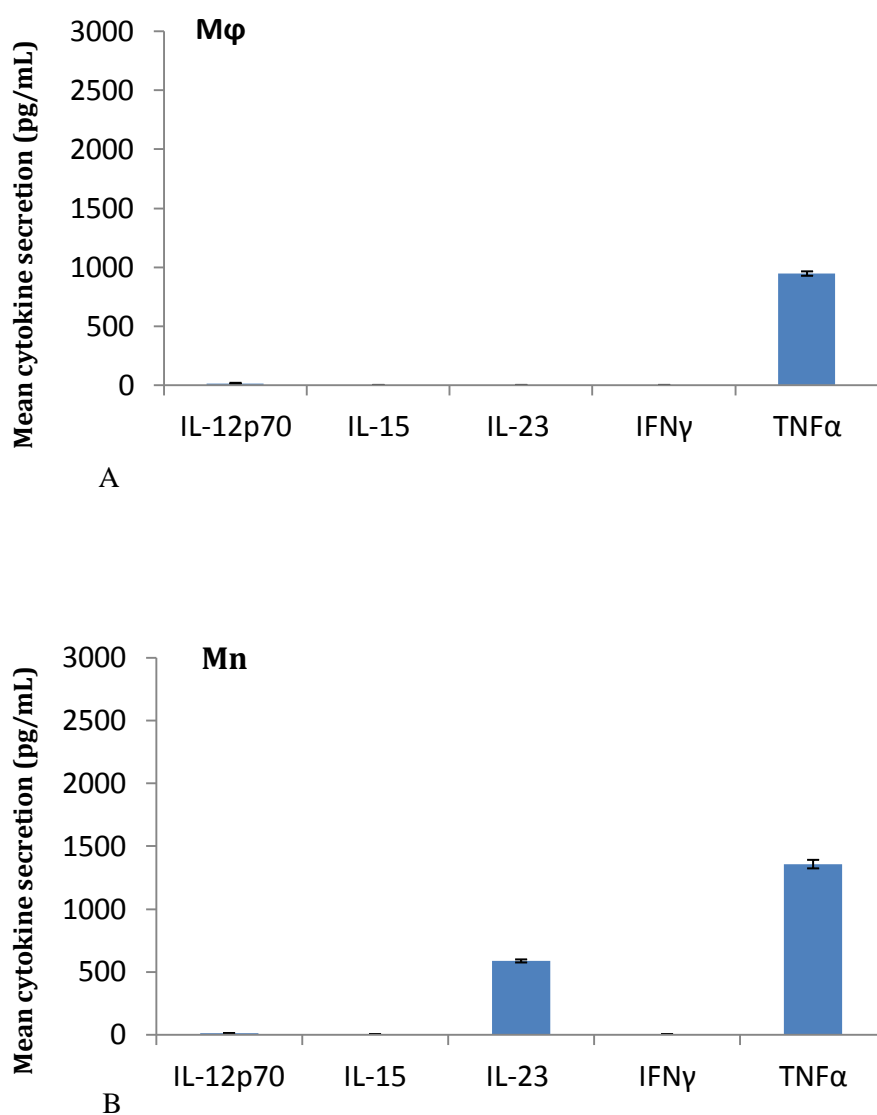
Th1-type cytokine secretion of IL-21p70, IL-15, IL-23, IFN $\gamma$ , and TNF $\alpha$  by THP-1 Mn was greater than secretion of the same cytokines by THP-1 M $\phi$  (figs. 10a and 10b), except for IL-1 $\beta$  secretion which was twice as high by THP-1 M $\phi$  (fig. 11). When primed by IL-10, cytokine secretion by THP-1 Mn > THP-1 M $\phi$  (figs. 12a and 12b), except for IL-1 $\beta$  (fig. 11) and IL-12p70. TNF $\alpha$  secretion was higher by THP-1 Mn than by THP-1 M $\phi$ , primed (figs. 12a and 12b) and unprimed (figs. 10a and 10b).



**Figure 10. T helper 1-type cytokine secretion by THP-1 monocytes or macrophages.** Th1-type cytokine secretion by Mn (a) was greater than by Mφ (b) when pulsed by VCG. Multiplex assay was employed to determine analyte concentration.



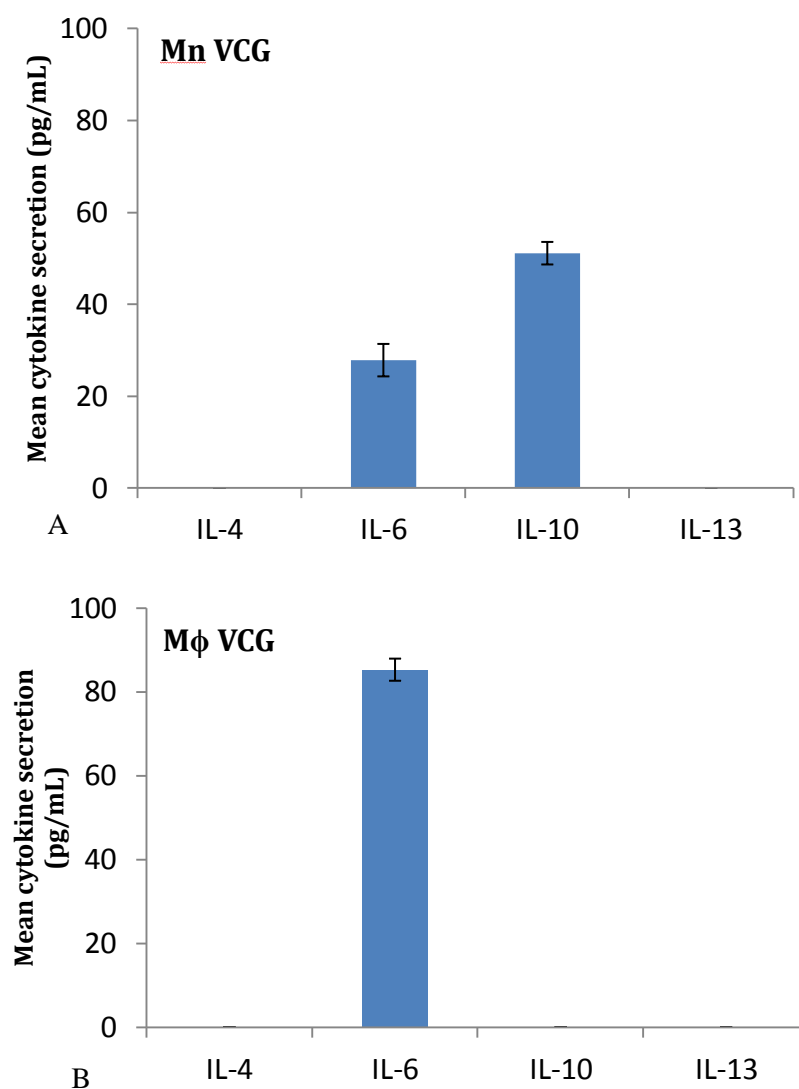
**Figure 11. IL-1 $\beta$  secretion.** IL-1 $\beta$  secretion under all treatment conditions. Secretion by THP-1 M $\phi$  was double that by Mn following pulse with VCG (10 ug/mL). Multiplex assay was employed to determine analyte concentration. IL-1 $\beta$  is an acute phase cytokine that plays a role in mediating the inflammatory innate immune response.



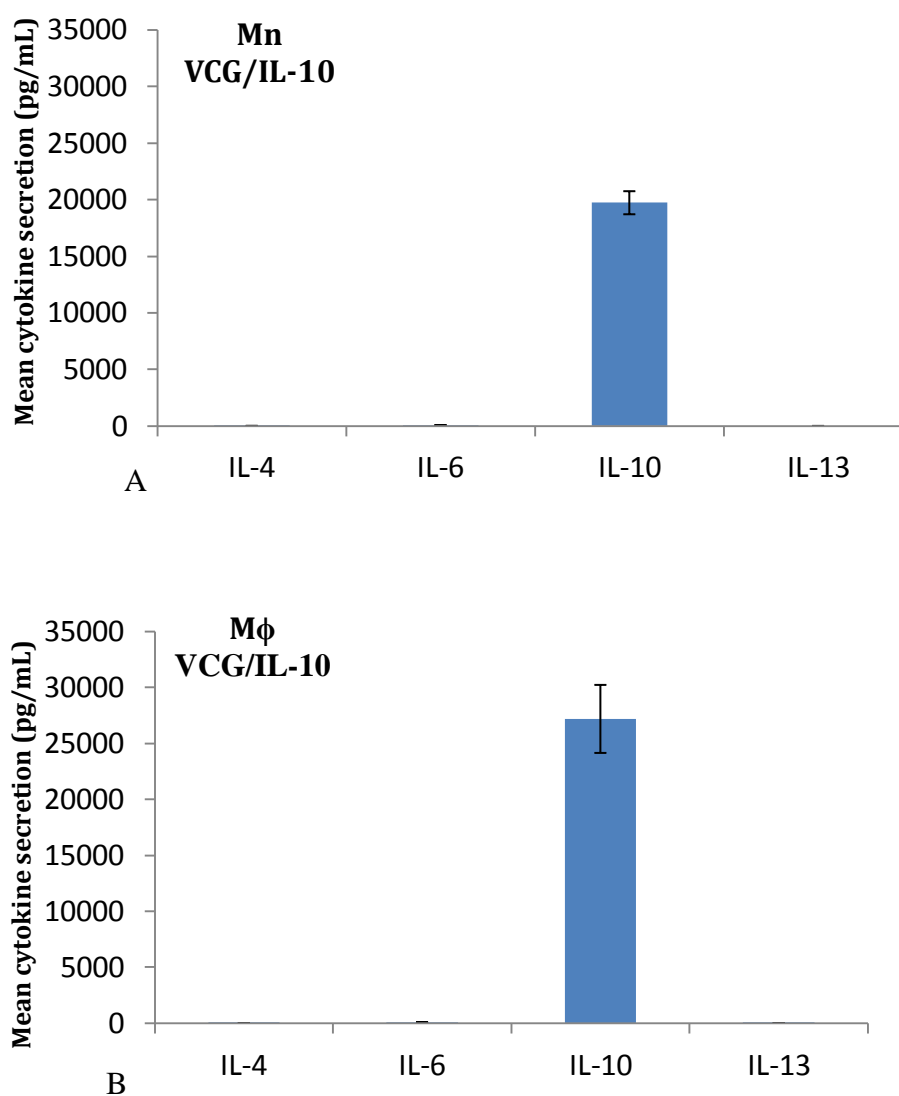
**Figure 12. THP-1 Mn or M $\phi$  primed with IL-10 and pulsed with VCG (10 ug/mL).** Th1-type cytokine secretion by Mn (a) was greater than by M $\phi$  (b) when the cells were primed with IL-10. The only exceptions were secretion by IL-1 $\beta$  and IL-12p70. Multiplex assay was employed to determine analyte concentration.

Th2-type cytokine secretion of IL-4, IL-6, IL-10, and IL-13 varied based on cell morphology and cell surface markers. Secretion of IL-4 by THP-1 Mn or THP-1 M $\phi$

differed by only 0.4 pg/mL. THP-1 M $\phi$  secreted IL-6 nearly three times that by THP-1 Mn. IL-10 secretion by THP-1 Mn was 51.1 pg/mL, whereas M $\phi$  secretion of this cytokine was negligible (figs. 13a and 13b). Secretion of IL-10 by cells primed with IL-10 was significant (fig. 14). THP-1 M $\phi$  secretion of IL-10 was higher (27203 pg/mL) vs. THP-1 Mn secretion of IL-10 (19730 pg/mL) (figs. 14a and 14b, respectively). This result is very much likely due to the treatment conditions.



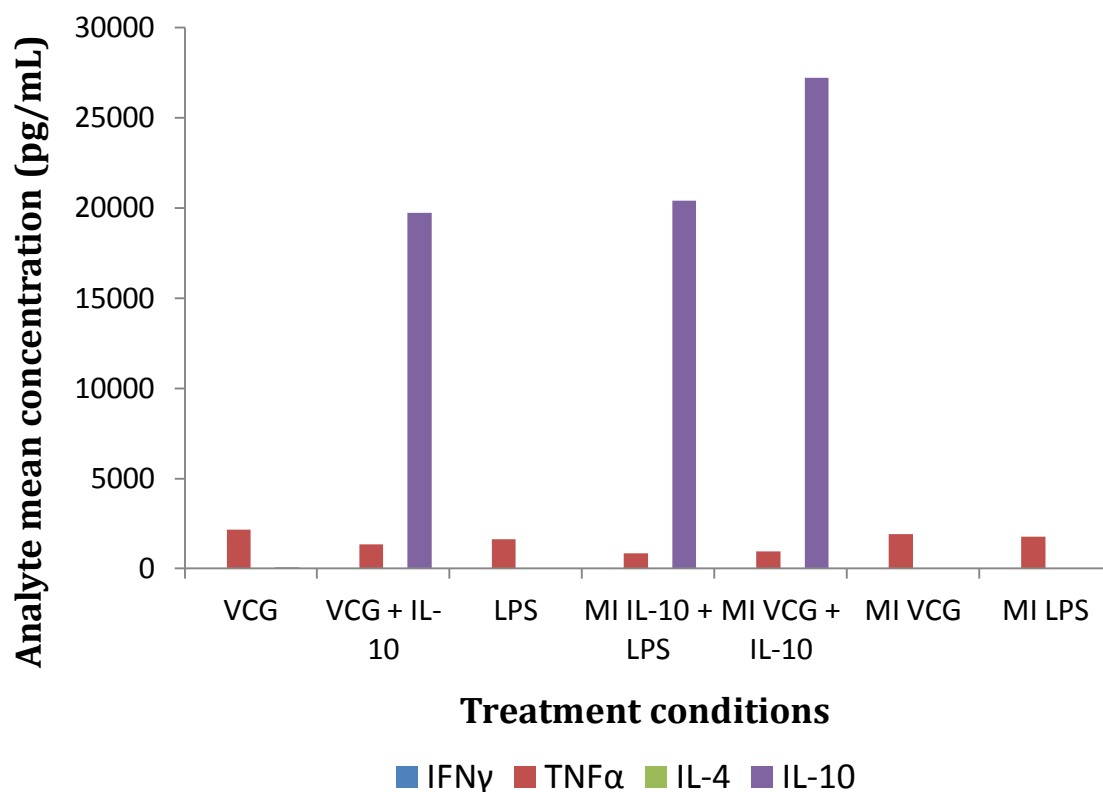
**Figure 13. T helper 2-type cytokine secretion by THP-1 monocytes or macrophages.** Secretion of IL-6 was modest by both Mn or MΦ. Secretion of IL-10 by Mn was 51 pg/mL (a) but negligible by MΦ (b) likely due to the phenotype of the cell morphologies. Mn express the marker for IL-10; MΦ do not express the marker for IL-10. Multiplex assay was employed to determine analyte concentration.



**Figure 14. IL-10 concentration by IL-10 primed VCG-pulsed THP-1 Mn or Mφ.** IL-10 concentration is that which was used to pulse the cells, not secretion by Mn (a) or Mφ (b). Multiplex assay was employed to determine analyte concentration.

Th1-type (IFN $\gamma$  and TNF $\alpha$ ) and Th2-type (IL-4 and IL-10) cytokines is summarized by mean concentration of analyte (pg/mL) (fig. 15).





**Figure 15. T helper 1 and T helper 2 cytokines.** Th1 (IFN $\gamma$  and TNF $\alpha$ ) and Th2 (IL-4 and IL-10) cytokines mean concentration following VCG-pulse of THP-1 monocytes or macrophages. When IL-10 was added to treatment, TNF $\alpha$  secretion was attenuated. Conversely, IL-10 concentration was a result of that added to the culture. Multiplex assay was employed to determine analyte concentration.

*Cytokine secretion following co-pulse Murine splenocytes and THP-1 Mn or THP-1 M $\phi$*

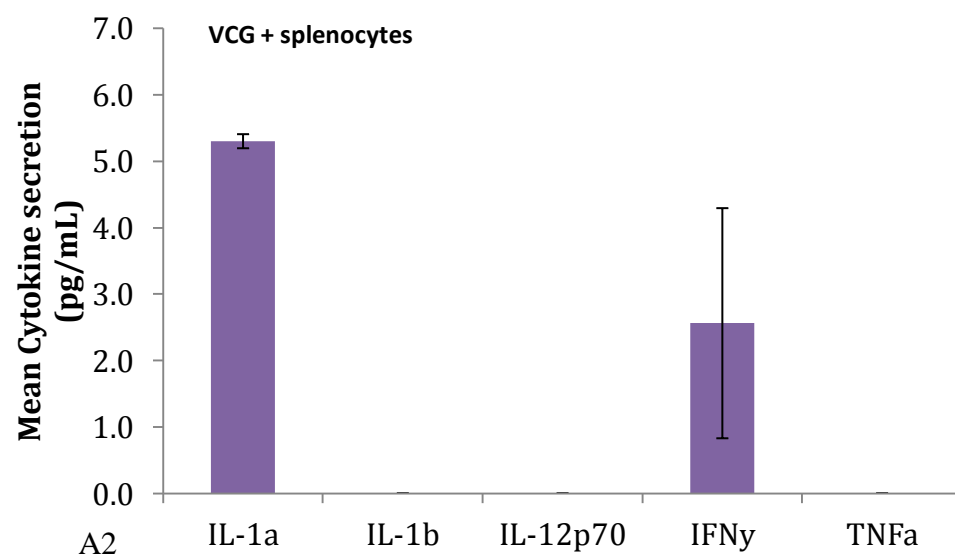
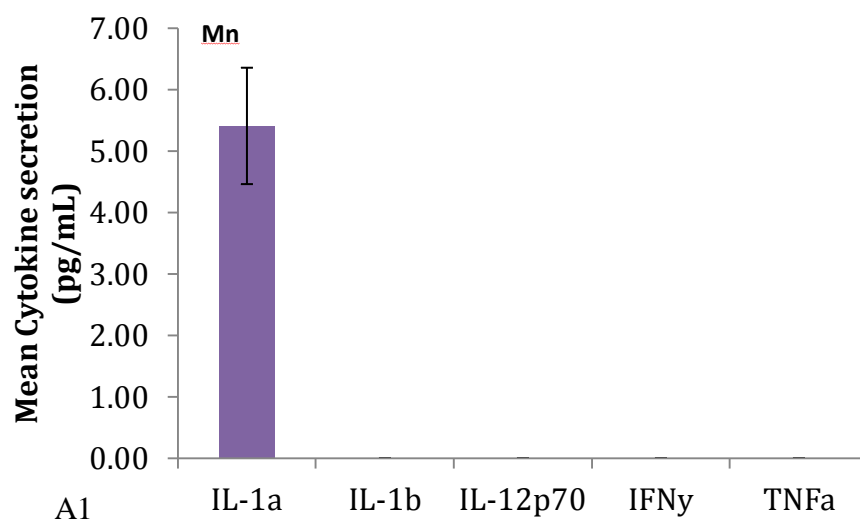
THP-1 Mn or M $\phi$  were pulsed by VCG (10  $\mu$ g/mL) and *Chlamydia* EBs serotype F (MOI 5), then co-cultured with activated murine splenocytes (isolated from 14 week old C57BL/6 female nude mice). The cellular supernatants were assayed for splenocytes cytokine production by Quansys Biosciences (Logan, UT).

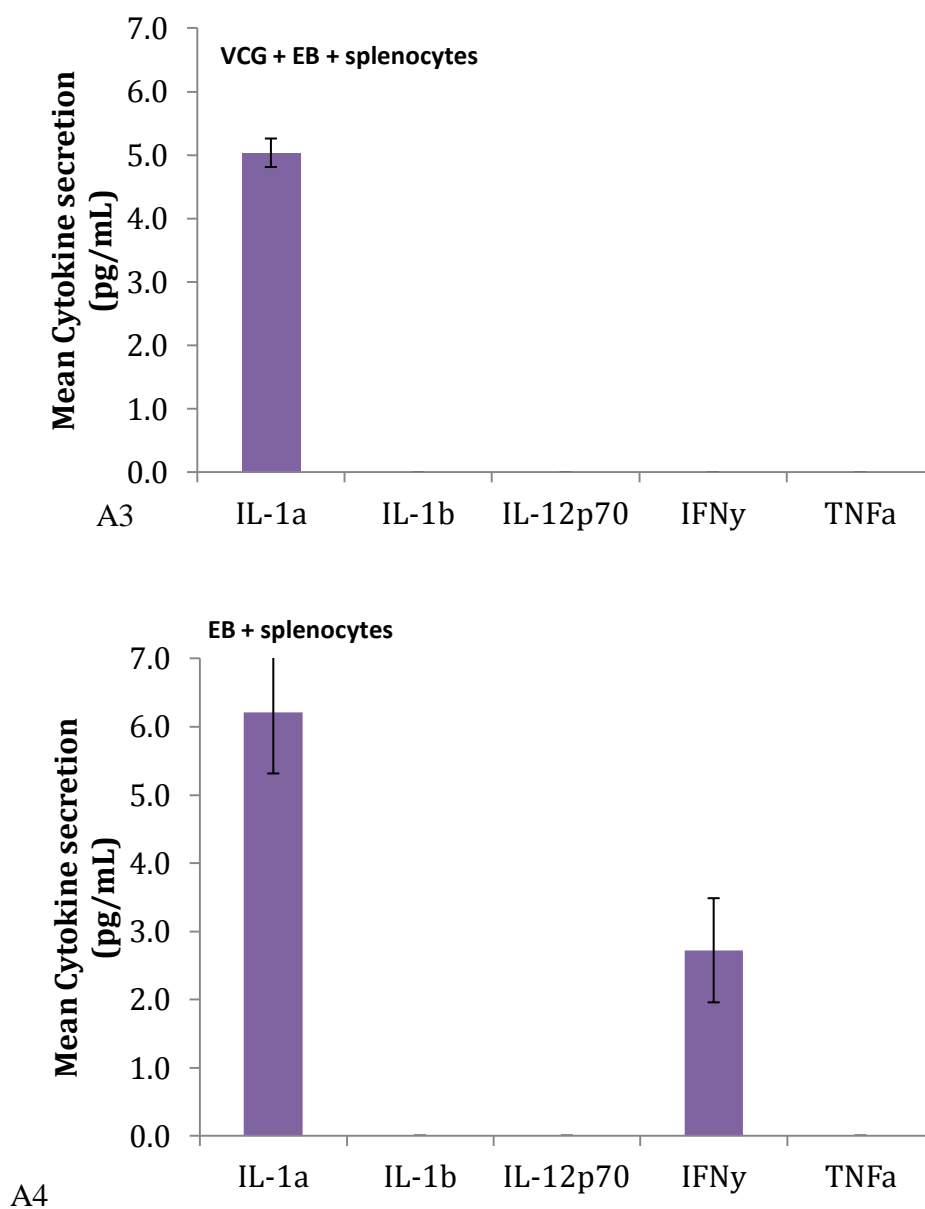
THP-1 cells have been shown to have antigen presentation character (105) however, this depends on their morphological state. 21 markers are expressed on both

THP-1 monocytes and macrophages, 23 markers uniquely expressed on THP-1 monocytes, and 20 markers uniquely expressed on THP-1 macrophages (106). Upon stimulation with cytokines, THP-1 cells have been demonstrated to acquire dendritic cells (DC) properties (107-109).

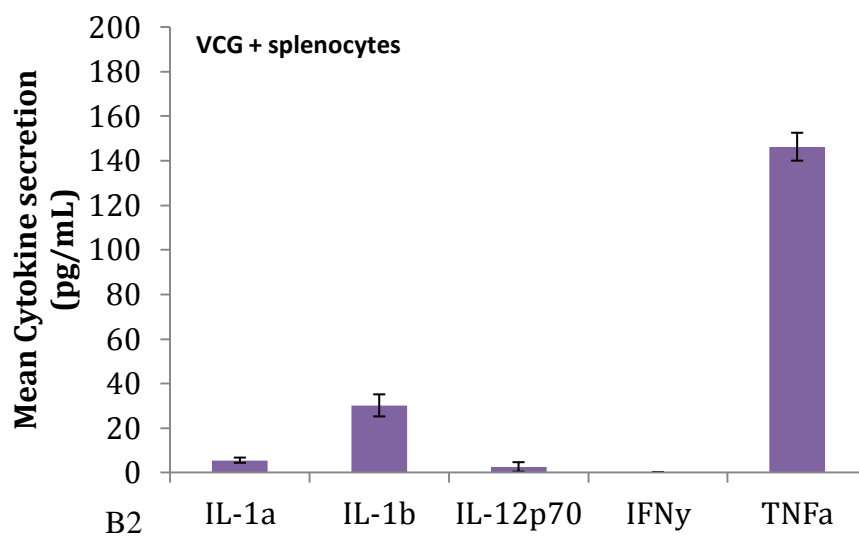
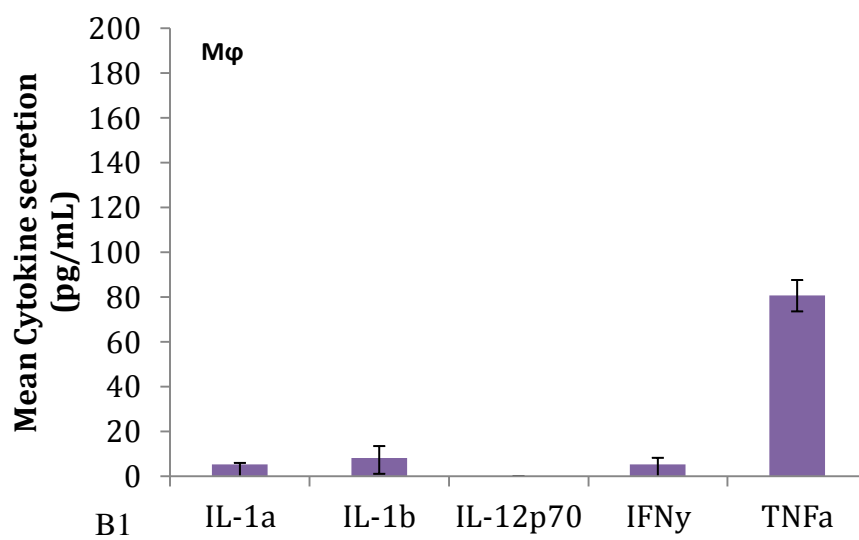
Multiplex assay was contracted out to Quansys Bioscience, Logan, UT. The samples were tested for IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-17, IL-23, IFN $\gamma$ , and TNF $\alpha$  by Quansys Biosciences (Logan, UT) Q-Plex Array<sup>TM</sup> kits for human cytokines. The total protein concentration for splenocytes cytokine production was determined via absorbance at 280 nm and an extinction coefficient of 1 using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington DE).

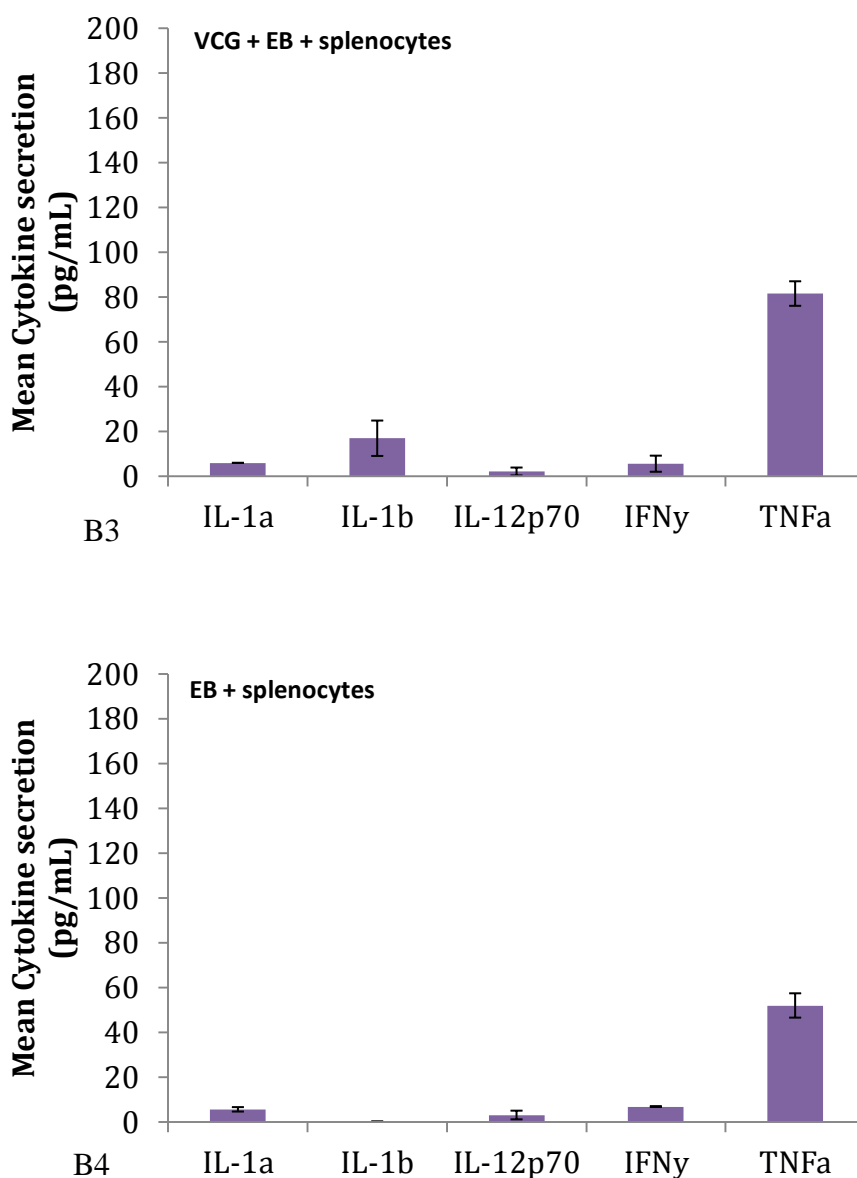
T helper 1-type (IL-1 $\alpha$ , IL-1 $\beta$ , IL-12p70, IFN $\gamma$  and TNF $\alpha$ ) (figs. 16a and 16b) or Th2-type (IL-4, IL-6, IL-10 and IL-17) (figs. 16c and 16d) cytokines fluctuated depending upon the morphology of the presenter (Mn or M $\phi$ ). IFN $\gamma$ , a cytokine previously reported [by our lab] as having high titer in *Chlamydia* infected mice vaginal washes (3), was secreted at a modest level by splenocytes when co-cultured with VCG-pulsed or EB-pulsed THP-1 Mn (fig. 16a). The same was true for VCG+EB-pulsed or EB-pulsed M $\phi$  (fig. 16b). IL-1 $\alpha$  was secreted by splenocytes at the same level when co-cultured with Mn or activated M $\phi$  (figs. 16a and 16b).





**Figure 16. T helper 1-type and T helper 2-type cytokines.** Th1-type cytokine, IFN $\gamma$ , was only measureable when Mn were pulsed with EB or VCG. But when EB and VCG were in treatment together, secretion of IFN $\gamma$  was negligible. We expected IFN $\gamma$  secretion to increase (a1-a4). M $\phi$  secretion of Th1-type cytokines was much more robust (b1-b4). Th2 cytokine, IL-6 secretion was constant in all treatment conditions with Mn (c1-c4). Th2-type cytokines IL-6, IL-10 and IL-17 secretion fluctuated depending on the treatment conditions (d1-d4).

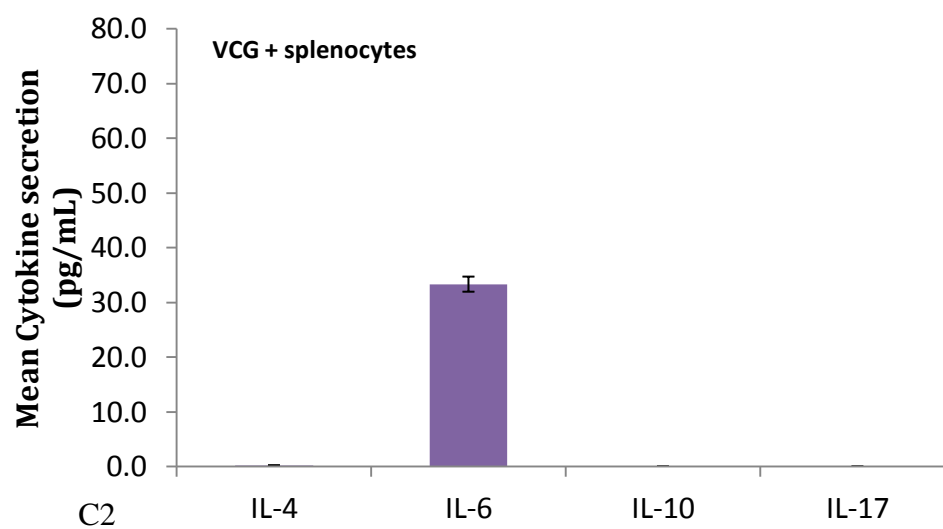
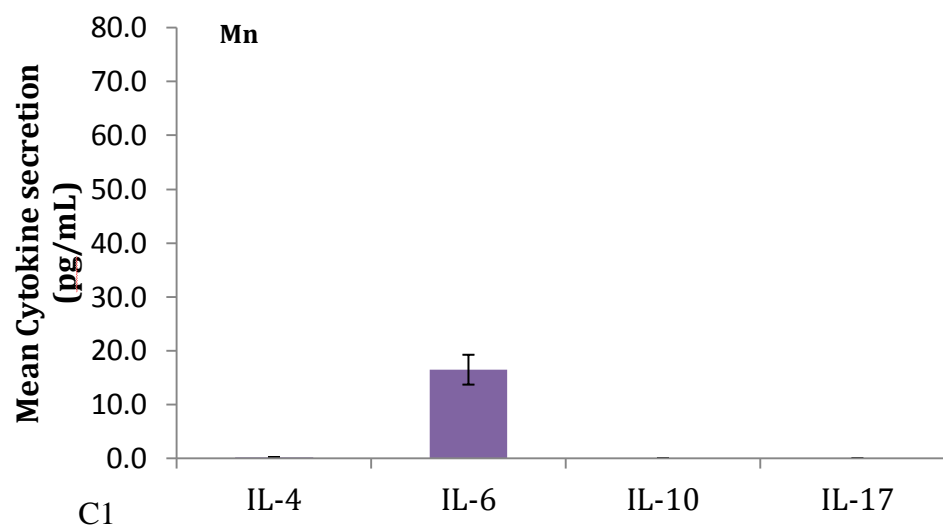




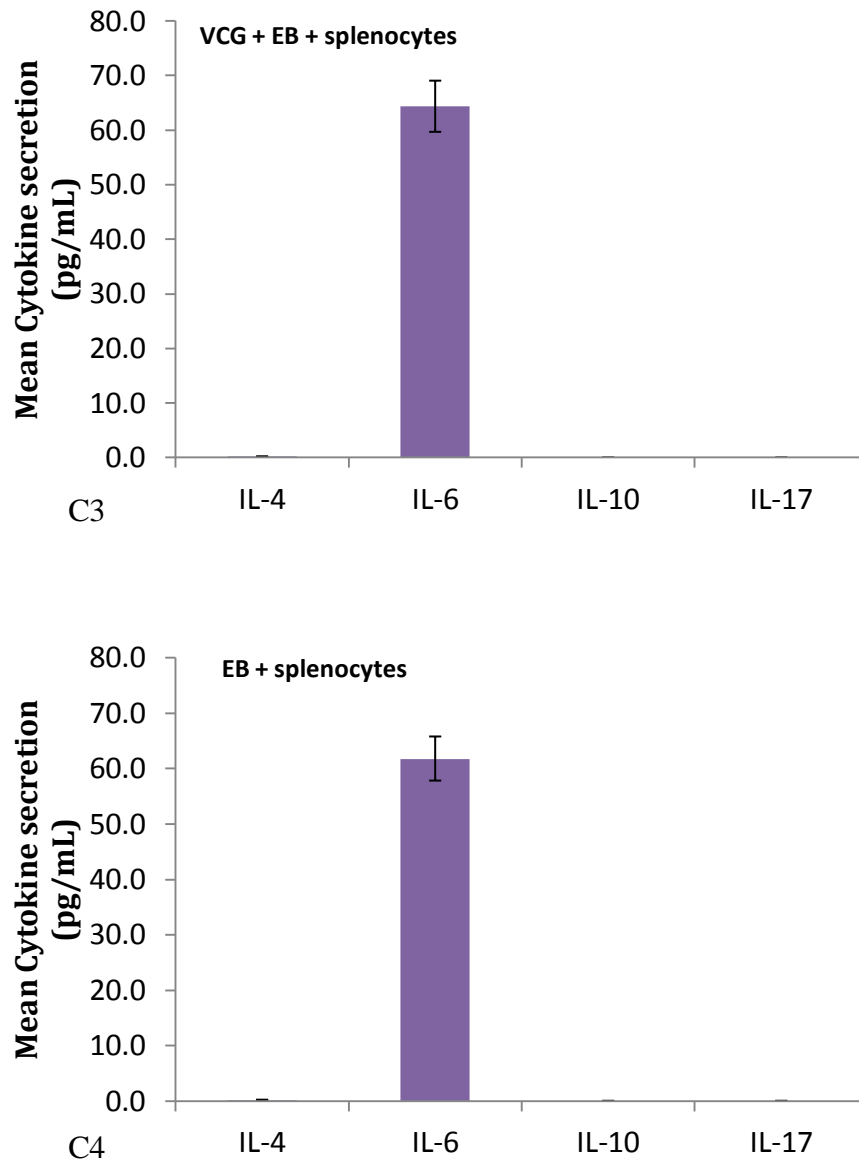
**Figure 16. T helper 1-type and T helper 2-type cytokines.** Th1-type cytokine, IFN $\gamma$ , was only measureable when Mn were pulsed with EB or VCG. But when EB and VCG were in treatment together, secretion of IFN $\gamma$  was negligible. We expected IFN $\gamma$  secretion to increase (a1-a4). M $\phi$  secretion of Th1-type cytokines was much more robust (b1-b4). Th2 cytokine, IL-6 secretion was constant in all treatment conditions with Mn (c1-c4). Th2-type cytokines IL-6, IL-10 and IL-17 secretion fluctuated depending on the treatment conditions (d1-d4).

IL-4 and IL-6 showed measureable secretion by monocytes in all treatment condition (fig. 16c). IL-6 secretion was about the same when Mn were pulsed by

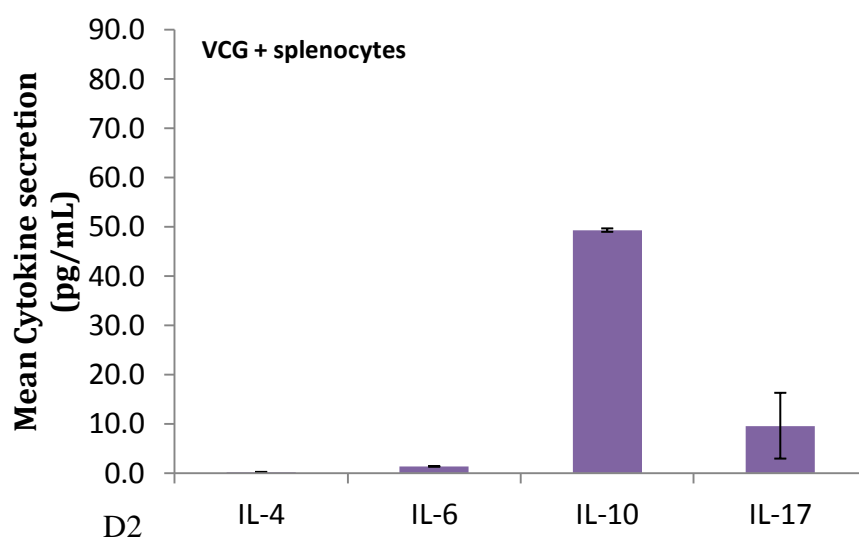
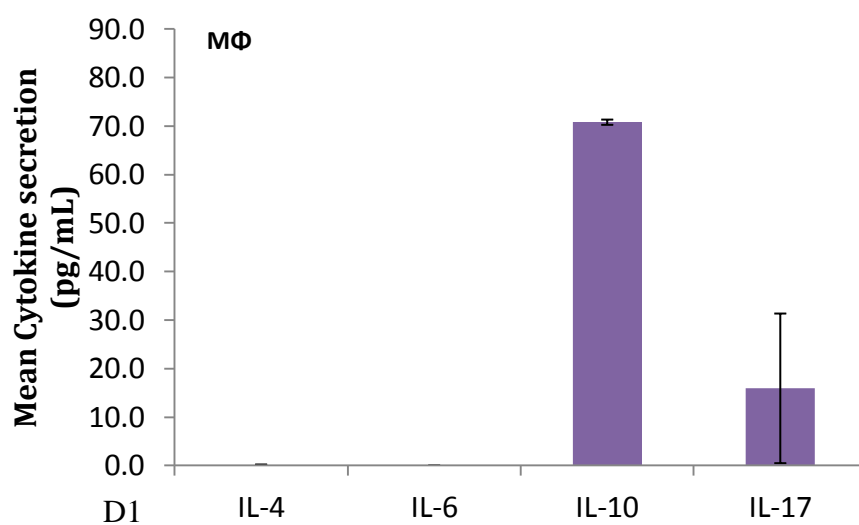
VCG/EBs/splenocytes or EBs/splenocytes - ~60 pg/mL. Secretion of IL-6 was only ~30 pg/mL when Mn were co-pulsed by VCG/splenocytes (fig. 16c). IL-6, IL-10, and IL-17 showed measureable secretion by macrophages under all treatment conditions (fig. 16d). IL-6 secretion was about the same (~80 pg/mL) when M $\phi$  were co-pulsed with VCG/EBs/splenocytes or EBs/splenocytes. IL-10 secretion was attenuated when EBs were in treatment, averaging 15 pg/mL vs. 50 pg/mL when not in treatment. IL-17 secretion was constant, but fluctuated (10 – 30 pg/mL) depending on the treatment condition.

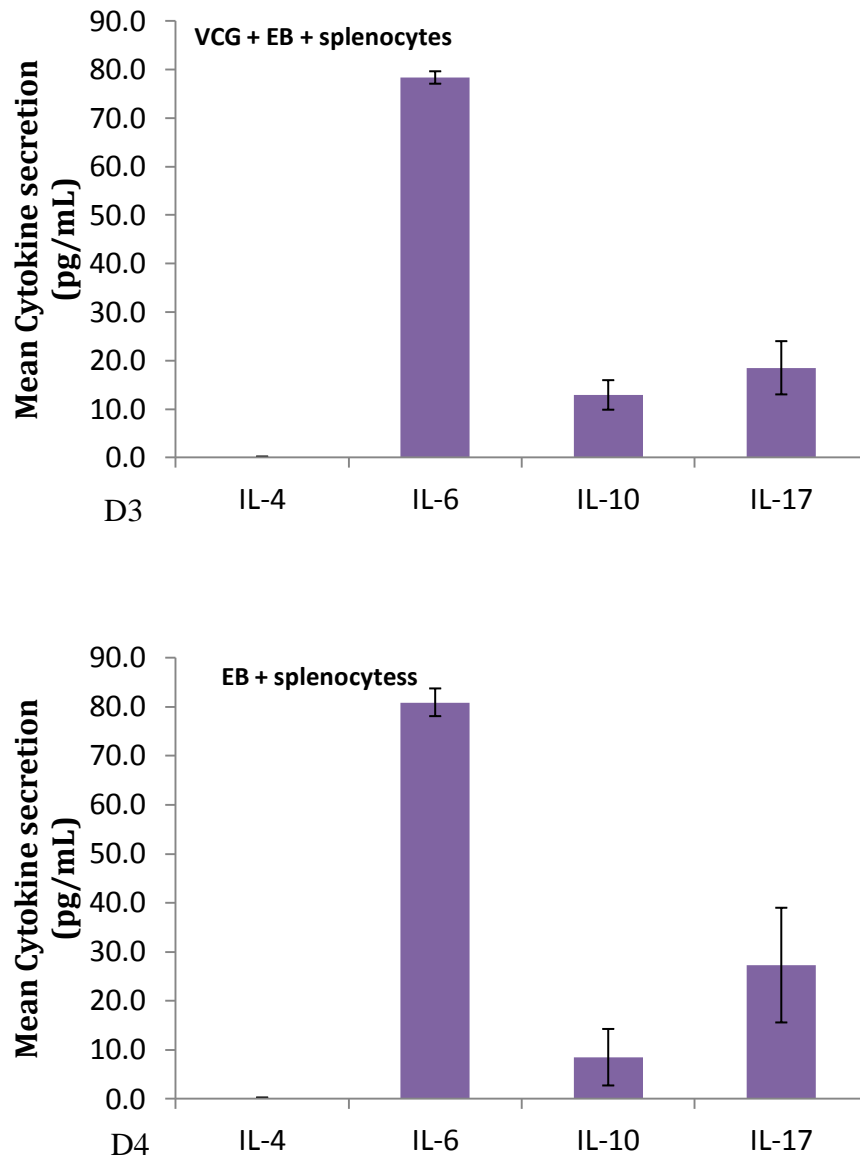






**Figure 16. T helper 1-type and T helper 2-type cytokines.** Th1-type cytokine,  $\text{IFN}\gamma$ , was only measurable when Mn were pulsed with EB or VCG. But when EB and VCG were in treatment together, secretion of  $\text{IFN}\gamma$  was negligible. We expected  $\text{IFN}\gamma$  secretion to increase (a1-a4). M $\phi$  secretion of Th1-type cytokines was much more robust (b1-b4). Th2 cytokine, IL-6 secretion was constant in all treatment conditions with Mn (c1-c4). Th2-type cytokines IL-6, IL-10 and IL-17 secretion fluctuated depending on the treatment conditions (d1-d4).

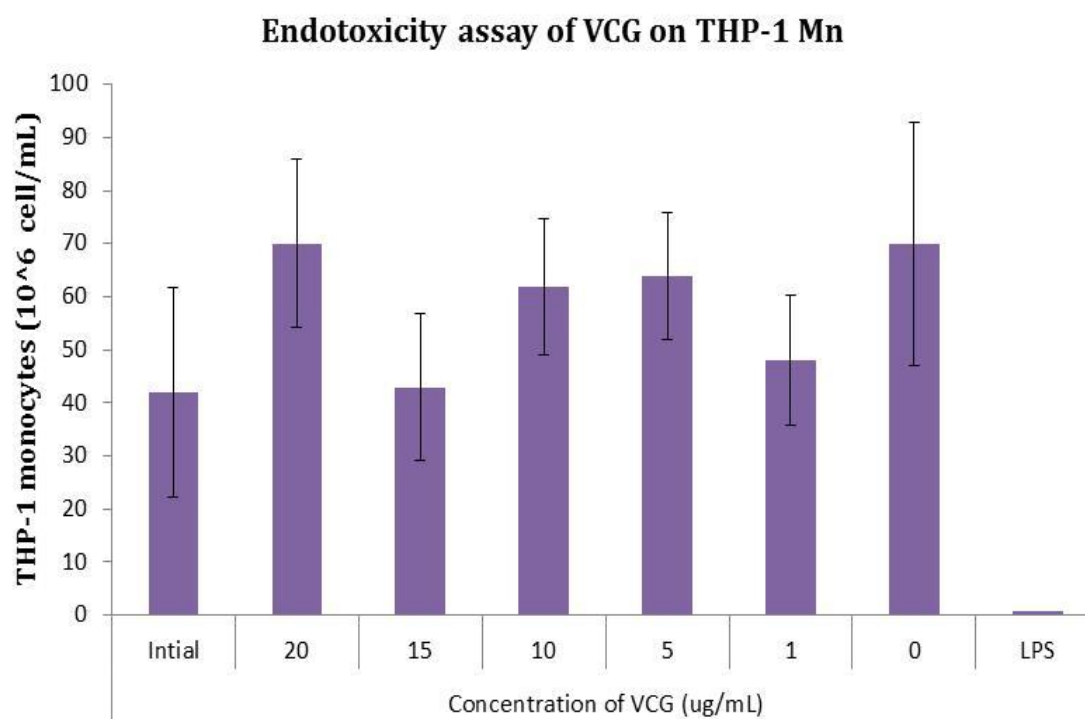




**Figure 16. T helper 1-type and T helper 2-type cytokines.** Th1-type cytokine,  $\text{IFN}\gamma$ , was only measureable when Mn were pulsed with EB or VCG. But when EB and VCG were in treatment together, secretion of  $\text{IFN}\gamma$  was negligible. We expected  $\text{IFN}\gamma$  secretion to increase (a1-a4). M $\phi$  secretion of Th1-type cytokines was much more robust (b1-b4). Th2 cytokine, IL-6 secretion was constant in all treatment conditions with Mn (c1-c4). Th2-type cytokines IL-6, IL-10 and IL-17 secretion fluctuated depending on the treatment conditions (d1-d4).

#### 4.5 Endotoxicity of *Vibrio cholerae* ghost vaccine vector

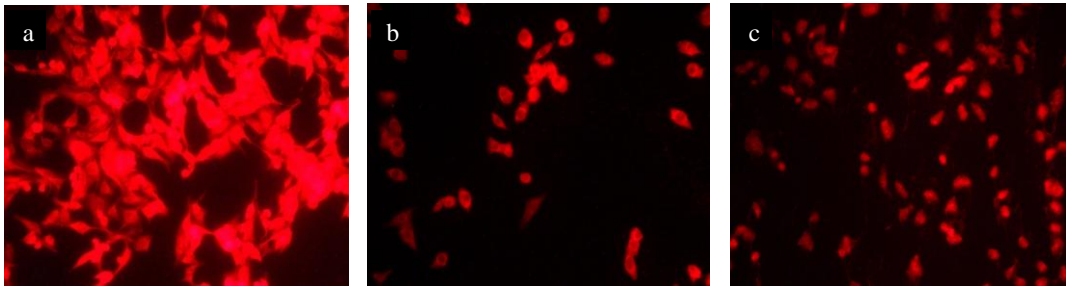
To confirm that the bacterial ghost system, specifically *V. cholerae* ghost, is a viable, non-toxic therapy for antigen and drug delivery, THP-1 monocytes were pulsed for 3 h with varying concentrations (20 ug/mL, 15 ug/mL, 10 ug/mL, 5 ug/mL or 1 ug/mL) of VCG; LPS (1 ug/mL) or RPMI 1640 complete media serving as a positive or negative controls, respectively (fig. 17). Cells continued to proliferate normally following pulse with VCG, indicating that our vector is not toxic to the cells at any dosage. Proliferation overnight was as much as 60% (20 ug/mL VCG).



**Figure 17. VCG are not toxic to THP-1 cells.** THP-1 monocytes pulsed by various concentrations of VCG exhibited no stress, and proliferation progressed normally.

#### 4.6 Enumeration of *Chlamydia* inclusion forming units (IFUs)

HeLa cells were treated with two different strains of *chlamydia* in an effort to determine if either was more toxigenic than the other and to detect and enumerate inclusion forming units under fluorescent microscopy (fig. 18). The human strain of *Chlamydia trachomatis*, serovar D, developed fewer IFUs (fig. 18b). Conversely, MoPn (*Chlamydia pneumonia*), developed more IFUs (fig. 18c) at equitable MOI. This result indicates that the toxigenic effect of MoPn may be greater (fig. 19).



**Figure 18. Immunofluorescence of HeLa infectivity assay.** Uninfected HeLa (a), serovar D infected (b) and MoPn infected (c) HeLa cells. Infectivity by MoPn was greater by 34.2% than serovar D.

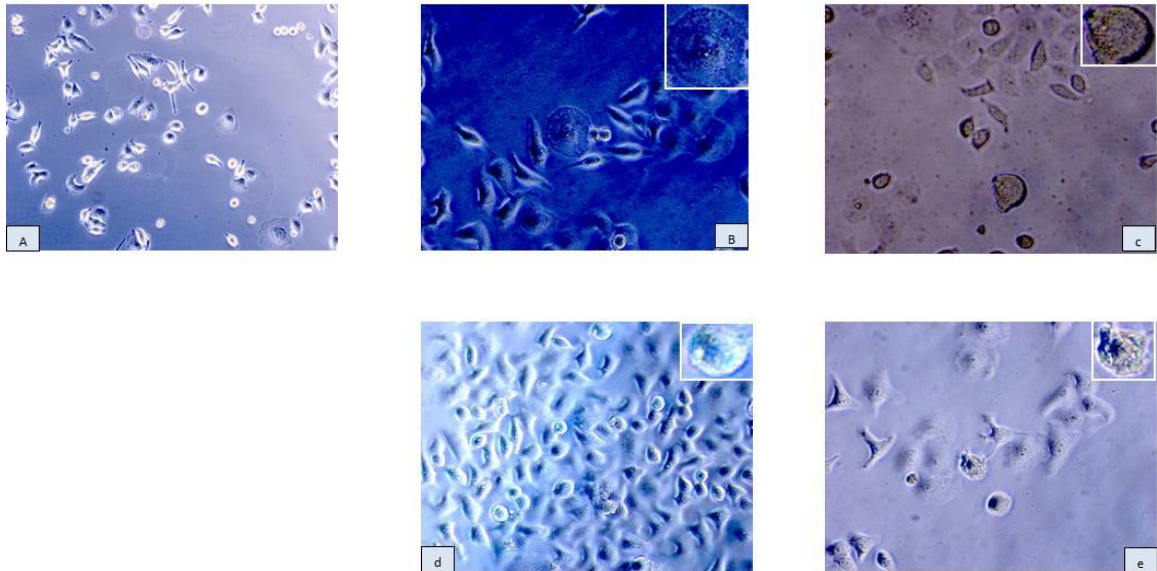


**Figure 19. Quantitative enumeration of *chlamydia* inclusion forming units (IFUs).** HeLa cells were infected with *Chlamydia trachomatis* serovar D (MOI 10) or *Chlamydia pneumoniae* (MoPn) (MOI 10). After infectivity, inclusion forming units were enumerated. Infectivity by MoPn was 34.2% > serovar D.

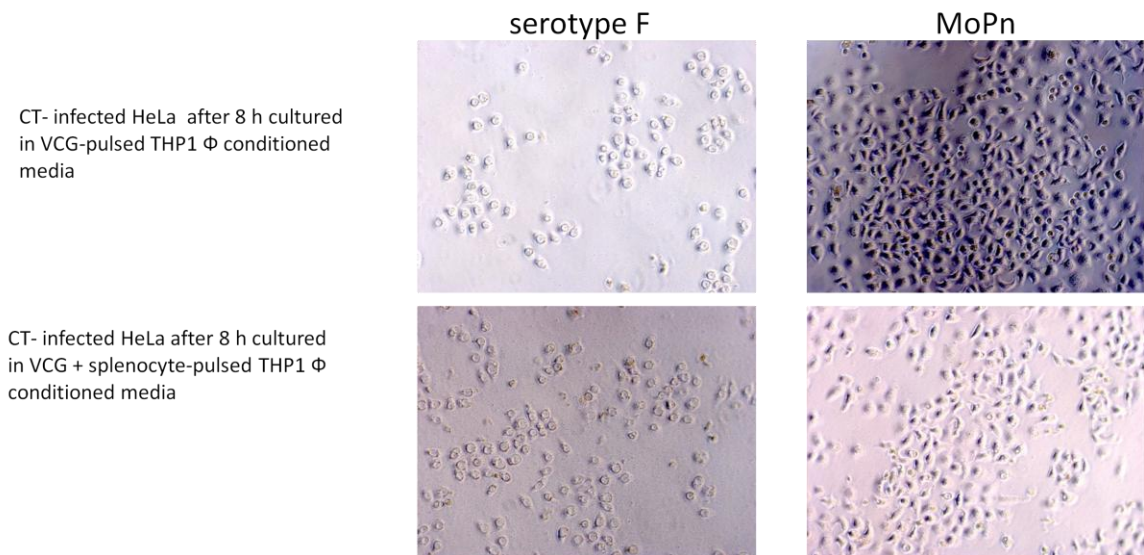
The [serovar D] infected HeLa cells appeared more distressed with marked cell rounding (figs. 18b and 18c), morphological changes not observed in untreated HeLa cells which were elongated with sharp points (fig. 18a). MoPn and Serovar D initiated entry and successfully infected the cells, forming Incs. MoPn had higher levels of infection at the same MOI than serovar D based on the physical counts and immunofluorescent microscopy (figs. 18b and 18c). 99% of the cells treated with serovar D or MoPn were successfully infected and is quantitatively illustrated in figure 19. HeLa cells treated with serovar D had  $1.19 \times 10^3$  IFUs/mL; HeLa cells treated with MoPn had  $3.48 \times 10^3$  IFUs/mL; MoPn infectivity was 34.2% > serovar D.

#### **4.7 Cytokines secreted by murine splenocytes are toxic to *Chlamydia*-infected HeLa cells**

To determine if the factors secreted by murine splenocytes co-cultured with VCG-pulsed THP-1 macrophages were toxic to *Chlamydia*-infected HeLa cells, CT-infected HeLa monolayers were cultured in the supernatants from the culture-stimulated THP-1 cells. The CT-infected HeLa cells were visibly stressed 4 h post infection (PI) (figs. 20b-e) vs. uninfected (fig. 20a) as observed by phase microscopy. Cell morphology was rounded or detached in stressed cells. Two of the monolayers (figs. 20c-e) were pretreated with DEAE-dextran to induce cell sensitization (103). This was done to compare the efficiency of EB uptake by the HeLa cells. The toxic effect of the factors contained in the supernatants from the culture-stimulated THP-1 cells was evident 8 h post-culture (fig. 21) and consistent among both *Chlamydia* strains. The toxigenic effect of the factors was evidenced by cell viability of uninfected vs. infected cells (DEAE-treated or not) (fig. 22) by counting post-treatment-culture using a cellometer (Nexcelom). MoPn-infected HeLa cell viability decreased by 99.65%, whereas serovar F-infected HeLa cell viability decreased by 92.5% after 24 h. DEAE-dextran (DD) pretreated MoPn-infected HeLa cells were 10% viable vs. DD-pretreated serovar F-infected HeLa which were 1.3% viable (fig. 22) after 24 h.

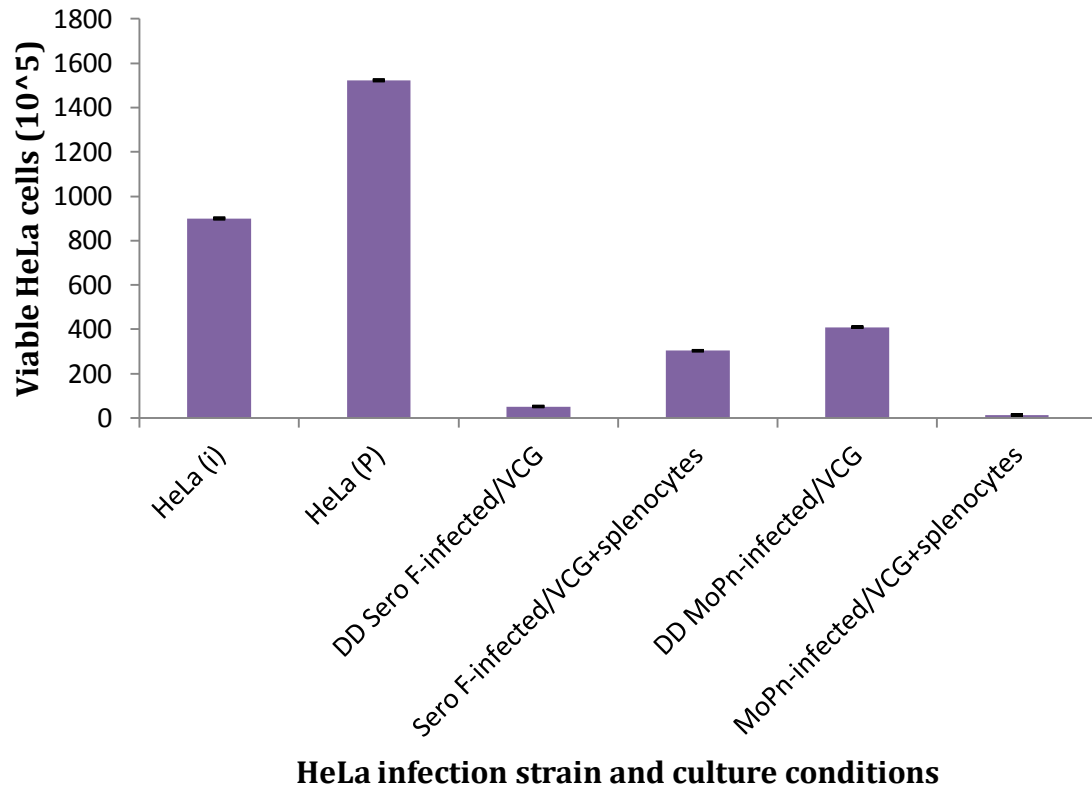


**Figure 20. *Chlamydia* infected HeLa cell monolayers 4 h post infection (PI).** Uninfected (a), DEAE-dextran pretreated serovar F-infected (b), Serovar F-infected (c), DEAE-dextran pretreated MoPn-infected (d) MoPn-infected (e). HeLa cells exhibited stress (“ballooning”) following infection by *Chlamydia* strains. DEAE-dextran increases sensitivity for microbial entry.



**Figure 21. *Chlamydia* infected HeLa cell monolayers after 8 h culture in conditioned media.** Effects of immunostimulatory factors secreted by VCG-pulsed THP-1 cells was evidenced by cell morphological alterations with marked cell rounding (in serotype F infected) characterized by a distinct “ballooning” appearance (103). MoPn was more toxicogenic at the same MOI.

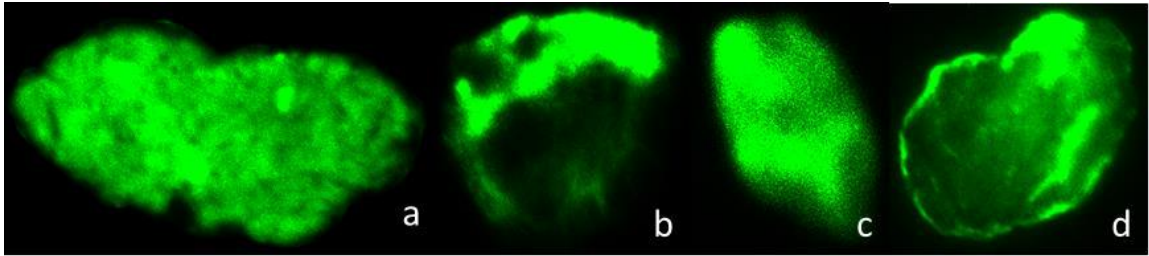




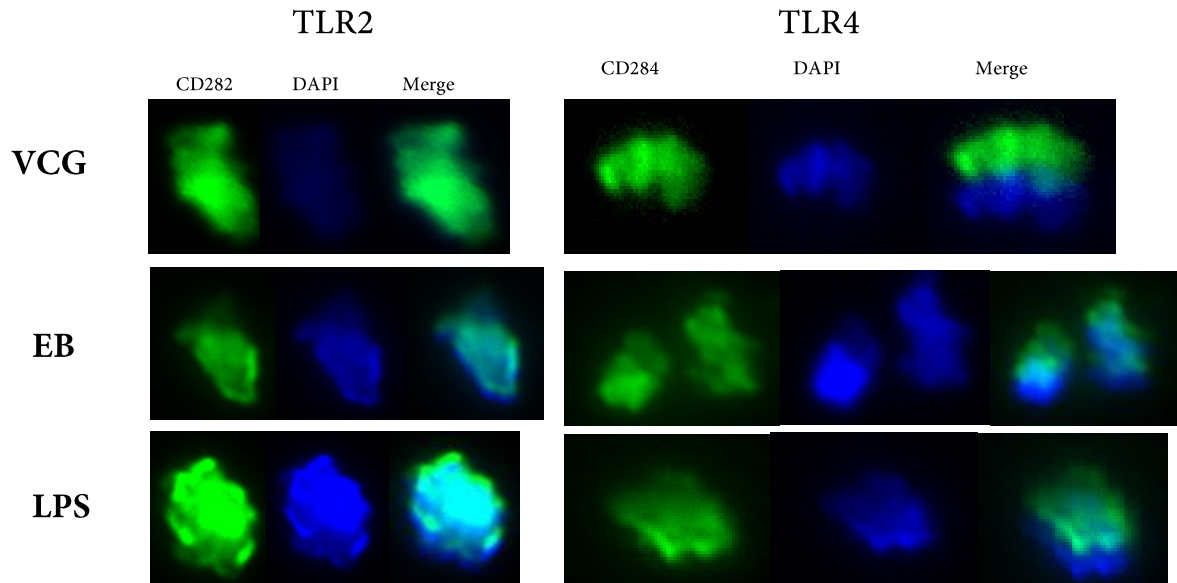
**Figure 22. HeLa cell viability following culture in conditioned media.** The toxigenic effect of immunostimulatory factors contained in conditioned media was sufficient enough to conclude that they are *Chlamydia*-specific across all treatment conditions. (i) Initial; (P) Post; (DD) DEAE-dextran.

#### 4.8 Toll-like receptor expression on THP-1 monocyte and macrophage cell surfaces

THP-1 Mn and M $\phi$  were stained (green) with antibodies against human TLR2 (hTLR2) or hTLR4 to view receptor expression by immunofluorescence (40x). Cell surface expression of these PRRs is well represented (figs. 23a-23d and fig. 24) on monocytes, but slightly less by macrophages.



**Figure 23. TLR expression on THP-1 Mn and Mφ.** THP-1 Mn or Mφ were stained with antibodies against TLR2 or TLR4 and viewed under fluorescence microscopy. TLR2 expression on Mn (a); TLR2 expression on Mφ (b); TLR4 expression on Mn (c); TLR4 expression on Mφ (d). Expression on Mn was ubiquitous vs. Mφ where expression was ~40-60%.



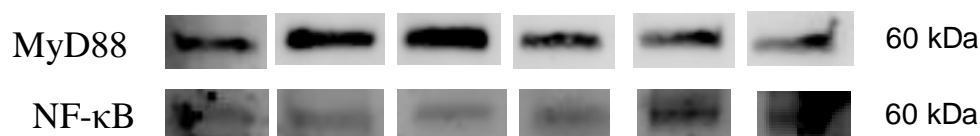
**Figure 24. TLR expression on THP-1 Mn.** TLRs on THP-1 Mn (pulsed by VCG, EB, or LPS) were stained (green) using antibodies against hTLR2 or hTLR4. Nuclei were stain with DAPI (blue). Images were taken at 40X magnification.

THP-1 Mn and Mφ (fig. 23) were stained for TLR expression then THP-1 Mn that had been pulsed by VCG, EBs, or LPS (fig. 24) were stained for TLR expression. TLR expression was comparable, but higher in “nude” cells. Nuclei were stained with DAPI (blue). Images were taken at 40X magnification using Axiovision software 4.8.2 with a Zeiss Axio Imager.z1 fluorescence microscope at ex=470 nm for FITC and ex=358 nm

for DAPI. Images demonstrate the expression of TLR2 and TLR4 (green) on the cell surface. TLR expression was greatest on cells pulsed by VCG, and slightly less on cells pulsed by either EBs or LPS (fig. 24).

#### **4.9 *Vibrio cholerae* ghosts induce immune signaling through MyD88 recruitment and NFκB p65 activation in THP-1 monocytes and macrophages**

THP-1 Mφ have cell surface receptors that recognize intrinsic properties contained on the bacterial ghost envelope that are not denatured during the production of the ghosts. Cells were pulsed with VCG (10 ug/mL) for 4 – 6 h, then the cell lysate was analyzed by Western blot for protein expression of NFκB p65 or MyD88. NFκB p65 belongs to the Rel family of transcription factors. In most cells, NFκB p65 homo and heterodimers are retained in an inert, non-DNA-binding form in the cytosol by inhibitory molecules (*e.g.*, IκB proteins). Activation of NFκB p65 occurs predominantly through phosphorylation and degradation of IκB proteins following cell surface receptor stimulation. Purified NFκB p65 ABfinity™ Recombinant Rabbit Monoclonal Antibody was used to detect the NFκB p65 protein (fig. 25) which has a band detected at ~60 kDa developed by chemiluminescence. Lanes 1 - 6 all showed expression of the transcription factor. Lane 1 represents lysate of cells pulsed by VCG alone. Lane 2 represents lysate of cells pulsed by VCG and EBs. Lane 3 represents lysate of cells pulsed by EBs alone. Lane 4 represents lysate from cells pulsed by Pam3CSK4 (a TLR2 ligand). Lane 5 represents lysate from cells pulsed by LPS (TLR4 ligand). Lane 6 represents lysate from our negative control where cells remained untreated in RPMI 1640 culture medium (fig. 25).



**Figure 25. Characterization of NFκB p65 and MyD88 by Western blot analysis.** THP-1 macrophages were pulsed under different conditions: VCG; VCG + EBs; elementary bodies (EBs); Pam3CSK4, a TLR2 ligand; LPS, the TLR4 ligand or RPMI 1640 as a negative control. Expression of NFκB p65 was detected at ~60 kDa when THP-1 MΦ were pulsed by VCG or LPS. MyD88 dimer was detected at 60 kDa under all treatment conditions and constitutively in the negative control, RPMI 1640.

MyD88 is known to function as an adaptor protein in the interleukin (IL)-1 signaling pathway for the association of IRAK (interleukin-1-receptor associated kinase) with the IL-1 receptor (*123-125*). MyD88 contains a characteristic N-terminal death domain that is essential for NFκB activation (*126*). MyD88 also plays an important role in the inflammatory response induced by endotoxin (*127*). Rabbit anti-MyD88 (Invitrogen) was used to detect the MyD88 dimer (fig. 25) at ~60kDa developed by chemiluminescence. Lanes 1 - 6 all showed expression of the transcription factor. Lane 1 represents lysate of cells pulsed by VCG alone. Lane 2 represents lysate of cells pulsed by VCG and EBs. Lane 3 represents lysate of cells pulsed by EBs alone. Lane 4 represents lysate from cells pulsed by Pam3CSK4 (a TLR2 ligand). Lane 5 represents lysate from cells pulsed by LPS (TLR4 ligand). Lane 6 represents lysate from our negative control where cells remained untreated in RPMI 1640 culture medium (fig. 25).

## CHAPTER 5

### DISCUSSION

Undeterred by the obvious limitations of their obligate intracellular niche, *Chlamydiae* are extraordinarily successful pathogens that infect and cause disease in animals at nearly every level of taxonomy. In humans, chlamydial infections are the leading cause of preventable blindness and sexually transmitted infections and can also cause respiratory infections and other chronic disease sequelae. Most disturbing, *Chlamydia trachomatis* poses a significant risk in women of child-bearing age, often leading to pelvic inflammatory disease, ectopic pregnancy, and infertility.

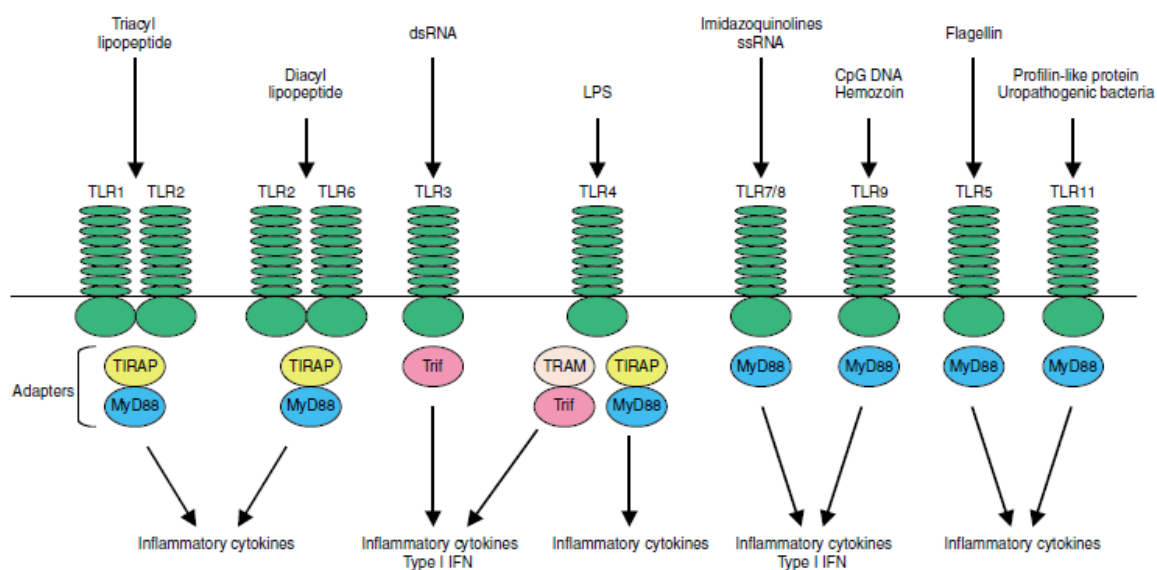
This study sought to elucidate some of the molecular mechanisms involved in the induction of immune responses to *chlamydia* infection. To do this we employed a veteran vaccine candidate vector, *Vibrio cholerae* ghosts, and investigated the immune response induced when using VCG as an adjuvant and the immune mediated signaling pathways activated following VCG stimulation. Furthermore, we sought to determine MyD88-dependent mechanisms involved in VCG-mediated adaptive immunity and what functional role NF- $\kappa$ B plays in immune induction.

Developing a long-term, cross-protective prophylactic against *Chlamydia* has been a challenge for many researchers for many years. The primary reason is this pathogen's immune evasive development cycle once inside the host. From the time the

pathogen attaches to its host, it is secreting proteins that fool its host into believing it is “friendly”. Although, the host isn’t always fooled, as depending on the health of the host, the immune response could be efficient enough to recognize the threat and produce a variety of immunomodulatory molecules. One of these molecules, known as cytokines, is IFN $\gamma$ , which induces several antimicrobial mechanisms, including the breakdown of tryptophan, an amino acid required for the synthesis of protein by the pathogen. Although, this is usually just a temporary “fix” as the pathogen will morph into a persistent state until the “coast is clear” for it to re-enter its infectious cycle. The effectiveness of *Chlamydia trachomatis* prevention is through antigen presentation. The goal is to develop a prophylactic that prevents attachment of the pathogen.

Traditional approaches to vaccine development have been ineffectual, mainly because we have yet to gain a thorough understanding of *Chlamydia* immunobiology. The current vaccine against *Chlamydia* does not provide long-term protection. It has been suggested that a multi-subunit prophylactic (3, 110) in the form of a vaccine would provide a protective advantage over current treatments. And although promising results have been observed in murine models, none of these are headed to clinical trial in humans. Currently infections caused by *C. trachomatis* are treated with antimicrobial agents, which can affect the host’s bacterial ecology hindering the natural course of immunity.

The innate immune system contains germline-encoded pattern recognition receptors (PRRs); among them are TLR2 and TLR4. They detect pathogens threat and trigger prompt responses against them (111). These signaling molecules respond to bacterial lipoproteins and have been implicated in innate immunity and inflammation (112). TLR2 is expressed by the myeloid lineage, including macrophage and dendritic cells (113). TLR4, along with its adapter proteins, is responsible for LPS recognition and signaling (114). Lipoproteins are well-defined molecular patterns of *Chlamydia trachomatis* as well as a large variety of pathogenic microorganisms with the capability to stimulate antigen presenting cells (APCs). They are strong cytokine inducers and have become a basis for vaccine development, making them key molecules in innate and adaptive immunity (115). There are two possible routes for mediation of signals received by TLRs depending on which of the two adapter molecules, myeloid differentiation factor 88 (MyD88) and TIR-domain containing adapter-inducing interferon- $\beta$  (TRIF), are involved. The importance of MyD88 and TRIF lies in the finding that each leads to a distinct profile of immune mediators that in turn determine the phenotype of the cells that are primarily responsible for the development of adaptive immune responses (67-70). An illustration of these immune responses is shown in figure 26 (128). Upon stimulation with various TLR ligands followed by recruitment of MyD88 or TRIF to the receptor complex, several phosphorylation events downstream allow NF- $\kappa$ B to be released into the nucleus and bind to the  $\kappa$ B transcription site on DNA for NF- $\kappa$ B responsive gene transcription (117).



**Figure 26. TLR-mediated immune responses.** TLR2 in concert with TLR1 or TLR6 discriminates between the molecular patterns of triacyl- and diacyl-lipopeptide, respectively. TLR3 recognizes dsRNA. TLR4 recognizes bacterial LPS. TLR7/8 mediates recognition of imidazoquinolines and ssRNA. TLR9 recognizes CpG DNA of bacteria and viruses. TLR5 recognizes bacterial flagellin. TLR1/2 and TLR2/6 utilize MyD88 and TIRAP/MAL as essential adapters. TLR3 utilizes Trif. TLR4 utilizes four adapters, including MyD88, TIRAP/MAL, Trif and TRAM. TLR7/8, TLR9, TLR5 and TLR11 use only MyD88. The MyD88-dependent pathway controls inflammatory responses, while Trif mainly mediates type I IFN responses. In addition, TLR7/8 and TLR9 induce type I IFN in a MyD88-dependent manner in pDCs (128).

Adaptive immunity to *Chlamydia* infection can be elicited by noncognate stimuli, although poorly understood. The adaptive immune response to infection produces a large population of antigen-specific effector T cells with appropriate functional activities to combat invading microbes (118). Innate immune response during an extended *Chlamydia* infection drives T cell clonal expansion whereby effector T cells can later be activated rapidly by a variety of noncognate stimuli. Long-term, protective immunity is critical for *Chlamydia* infection clearance because of the severity of the resulting



sequelae. Moreover, a robust T helper 1 response along with complimentary antibody titer is required for swift, and efficient clearance and to down-regulate inflammation.

It is worth mentioning the contribution hormones make in combating microbial infections. Unique immunological features of the female genital tract (FGT) mucosa differentiate from the common mucosal immune system of organs such as the lungs or intestine. The FGT mucosa lacks organized lymphoepithelial structures, but contains discrete lymphoid aggregates. Sex hormones create a balance between tolerance to infection and immunity to infection. In addition to the sex hormones, the menstrual cycle influences the immune response. The mechanisms that promote protective immunity in the FGT (119) are regulated by changes in the female sex hormones. However, it is less understood how female sex hormones affect the innate immune response of the FRT (female reproductive tract) epithelial cells, the initial cell type targeted by *Chlamydia* (120).

The roles that TLRs, MyD88 and NF- $\kappa$ B play in protective immunity and the molecular mechanisms involved following immunization by a VCG-based vaccine and that activate these molecules was demonstrated by the identification of intrinsic ligands on the VCG envelope. The bacterial ghost platform has been studied for decades. The advantages of this system are far reaching for vaccine development and its use in cancer and infectious disease therapies. The disadvantages however, are that 1) the levels of immune effectors produced are insubstantial to confer either sterilizing or long-term immunity and, 2) it is necessary to understand the local factors that regulate immunity at

mucosal sites of infection in order to determine the most appropriate route of immunization that will optimize protective immunity against *Chlamydia* (121).

As an adjuvant, VCG offer some advantages for boosting the immune response. However, the mechanism(s) by which this system is used for immunization to stimulate immunity must induce long-term stimulation of both the humoral and cell-mediated immunity.

The bacterial ghosts envelope maintain highly conserved structures which immune cells recognize – termed pathogen associated molecular patterns (PAMPs) – *e.g.*, lipopolysaccharides and outer membrane protein A (OmpA). *Vibrio cholerae* ghosts, the system used in this study, is a Gram-negative bacterium, and like *Chlamydia trachomatis*, both have similar outer membrane structure (Table 1). We rationalized that the target epithelial cells have Toll-like receptors that can be stimulated by VCG bacterial vector. Furthermore, this epithelial cell stimulation leads to an induction of a VCG-specific immune response and signaling pathway, which in turn can enhance the immune response to *Chlamydia* infection. Adjuvanticity is an innate property with immunopotency; just one of the important reasons for utilizing this vaccine system. Additionally, there is no size limitation of the foreign protein moieties – multiple antigenic determinants can be presented simultaneously; bacterial ghosts can be produced inexpensively in large quantities; recombinant ghosts are stable for long periods of time and do not require cold chain storage; inactivation procedures that denature relevant immunogenic determinants are not employed in the production of ghosts used as vaccines

or as carriers of relevant antigens; and, the recombinant proteins are inserted into a highly immune stimulatory environment (122).

**Table 1. Comparison of structure and property similarity between *V. cholerae* and *C. trachomatis* (116).**

	<i>C. trachomatis</i>	<i>V. cholerae</i>
Property		
Number of serotypes	18	4
Size	0.20–1.3µm	1-3 x 0.5-0.8µm
Morphology	rod-shaped	comma-shaped
Natural host	human	human, water birds, shellfish, fish and herbivores
Infection route	contact; epithelial cells	contact; epithelial cells
Lipopolysaccharides	Yes	Yes
Antibiotic sensitivity	Yes	Yes
Survival outside of host	No	Yes

## CHAPTER 6

### CONCLUSION

*Chlamydia trachomatis* is an obligate intracellular bacterium that causes *Chlamydia* infection annually in an estimated 4 million people in the United States and an estimated 92 million people worldwide. It is the most common sexually transmitted infection caused by bacteria in the United States. Treating the infection has been limited to antibiotic therapy and a vaccine - neither of which provides long-term, protective immunity against reinfection. Most chemotherapeutic molecules, such as antibiotics, are designed to target bacteria. However, this therapy does not discriminate between the *Chlamydia* pathogen and the normal human flora (which is often crucial to keep the body healthy). Therefore, these studies on *Vibrio cholerae* ghosts as an adjuvant to treating *Chlamydia* infections may contribute to our current knowledge of the immunomodulatory response during infections and treatments which may improve therapeutics that prevent and manage its growth and spread. The bacterial ghost platform is well studied and offers a promising therapeutic approach that is not only low-cost, but also free of any side effects. Our overall hypothesis is that the effectiveness of CT prevention is via antigen presentation. Previous studies conducted by our lab to identify molecular mechanisms involved

in the induction of *Chlamydia* infection immune responses have investigated the mechanism of T cell control and the role of nitric oxide (123), and integrin-mediated epithelial-T cell interaction with nitric oxide (124). In the current study we sought to elucidate the molecular mechanisms involved in the induction of *Chlamydia* infection immune responses by a *V. cholerae* ghost-based vaccine vector, with our goal being to determine the involvement of MyD88-dependent TLR signaling and evaluate the functional role of NF- $\kappa$ B signaling in the immune response induced by our VCG-based vaccine vector. Our results showed that the *V. cholerae* ghost vaccine vector induced a molecular pathway leading to the production of immunomodulatory factors capable of enhancing the immune response to *Chlamydia* infection. Immune factors produced from pulsing THP-1 monocytes or macrophages with VCG were comparable too or greater than those immune factors produced by *Chlamydia* EBs. Additionally, the signaling pathways VCG activated – PI3K and MAPK – are also activated during *Chlamydia* infection. This was evidenced by gene expression of TLR2 and TLR4, and subsequent protein expression of MyD88 and NF- $\kappa$ B. MyD88-dependent mechanisms involved in VCG-mediated adaptive immunity were evidenced by the immunomodulatory response, specifically the activation of NF- $\kappa$ B followed by the production of IFN $\gamma$  and IL-12p70 through TLR signaling pathways. IFN $\gamma$

is able to enhance the phagocytic capability of M $\phi$ , which may promote engulfment and elimination of CT. The molecular mechanisms observed as a result of investigating the adjuvant properties of VCG could provide a rational complementary approach for protective immunity against *Chlamydia* infection.

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