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Phagocytosis of *Mycobacterium Paratuberculosis* Causes
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PHAGOCYTOSIS OF *MYCOBACTERIUM PARATUBERCULOSIS* CAUSES UNIQUE GENE EXPRESSION PROFILES IN BOVINE MACROPHAGE CELLS

By

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ABSTRACT

PHAGOCYTOSIS OF *MYCOBACTERIUM PARATUBERCULOSIS* CAUSES UNIQUE GENE EXPRESSION PROFILES IN BOVINE MACROPHAGE CELLS

By

Brian C. Tooker

Mycobacterium avium subspecies *paratuberculosis* (*M. paratuberculosis*) is a facultative intracellular bacterium with the ability to survive and proliferate inside the phagocytic vesicles of macrophage cells. How *M. paratuberculosis* is able to survive in this hostile host environment is not well understood. In this study we hypothesized that phagocytosis of *M. paratuberculosis* would uniquely alter macrophage gene expression patterns relative to patterns observed following the phagocytosis of *E. coli* or latex beads and that these unique changes could contribute to the survival of *M. paratuberculosis*.

Using the techniques of DDRT-PCR and Northern blot hybridization we have begun to test this hypothesis by comparing patterns in bovine macrophage cell gene expression during no phagocytosis and phagocytosis of *E. coli*, *M. paratuberculosis* and latex beads. These combined analysis revealed that the Nucleolin Related Protein gene was not activated in macrophage cells following phagocytosis of *M. paratuberculosis* but was activated at high levels following phagocytosis of either *E. coli* or latex beads. Another gene, NADPH Dehydrogenase subunit 1 (ND1), was activated only at low levels following *M. paratuberculosis* phagocytosis but was strongly induced following phagocytosis of both *E. coli* and latex beads. Both of these genes have hypothesized roles in bacterial destruction and the failure to induce gene expression following phagocytosis may promote survival of *M. paratuberculosis* within bovine macrophages.

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LIST OF ABBREVIATIONS

ADCC = Antibody Dependant Cellular Cytotoxicity

AICC = Antibody Independent Cellular Cytotoxicity

AP = Anchored Primer

APC = Antigen Presenting Cell

ARP = Anchored Random Primer

ATCC = American Type Culture Collection

BCG = Bacillus Calmette-Guerin

BLAST = Basic Local Alignment Search Tool

BLASTX = Basic Local Alignment Search Tool Against Protein Databases

BRESI-1 = Breast Epithelial Stromal Interaction 1

BSA = Bovine Serum Albumin

CD = Cluster of Differentiation

cDNA = Copy Dioxyribose Nucleic Acid

CMI = Cell Mediated Immunity

CR = Complement Receptor

ddH_2O = Double Distilled Water

DDRT-PCR = Differential Display Reverse Transcriptase Polymerase Chain Reaction

DHT = Delayed Type Hypersensitivity

DNA = Deoxyribose Nucleic Acid

DTT = Dithiothreitol

EDTA = Ethylenediamine-N,N,N',N'-Tetraacetic Acid

EtOH = Ethanol

FBS = Fetal Bovine Serum

FR = Fibronectin Receptor

GAPDH = Glyceraldehyde-3-Phosphate Dehydrogenase

GM-CSF = Granulocyte-Macrophage Colony-Stimulating Factor

IL = Interleukin

INF = Interferon

iNOS = Inducible Nitrous Oxide Species

IPTG = Isopropylthio-β-Galactoside

IS = Insertion Sequences

KO = Knock Out

LAM = Lipoarabinomannan

LAMP = Lysosomal Associated Macrophage Protein

LB = Luria Broth

LPG = Lipophosphoglycan

LPS = Lipopolysaccharide

MetOH = Methanol

MHC = Major Histocompatibility Complex

MR = Mannose Receptor

NADPH = Nicotinamide Adenine Dinucleotide Phosphate Hydrogen

NaOAc = Sodium Acetate

NO = Nitrous Oxide

NRP = Nucleolin-Related Protein

NSF = N-ethylmaleimide-sensitive factor

PBMC = Peripheral Blood Mononuclear Cell

PBS = Phosphate Buffered Saline

PCR = Polymerase Chain Reaction

RNA = Ribose Nucleic Acid

RNI = Related Nitrogen Intermediates

ROS = Reactive Oxygen Species

SDS = Sodium Dodecyl Sulfate

SNAP = N-ethylmaleimide-Sensitive Factor Attachment Protein

SSC = Sodium Citrate and Sodium Chloride

tBLASTx = Basic Local Alignment Search Tool for Back Translated Protein Databases

TCR = T-cell Receptor

TE = Tris-EDTA

TGF = Transforming Growth Factor

TLR = Toll-like Receptor

TNF = Tumor Necrosis Factor

t-SNARE = Target N-ethylmaleimide-Sensitive Factor Attachment Protein Receptor

v-SNARE = Vesicle N-ethylmaleimide-Sensitive Factor Attachment Protein Receptor

X-gal = 5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside

CHAPTER ONE

A Review of Literature

I. Mycobacteria, *M. paratuberculosis* and Johne's Disease

A. An Overview of Mycobacteria and *M. paratuberculosis*

Intracellular Mycobacterial pathogens such as *Mycobacterium bovis* (bovine tuberculosis), *Mycobacterium leprae* (human leprosy), *Mycobacterium tuberculosis* (human tuberculosis) and *Mycobacterium avium* (common infectious agent in birds and mammals) are all closely related to the pathogenic *Mycobacterium avium* subspecies *paratuberculosis* (*M. paratuberculosis*). *M. paratuberculosis* infection was first clearly described in 1895 as "a peculiar case of tuberculosis" (Clarke et al., 1997) in a cow with chronic enteritis, diffuse thickening and corrugation of the intestinal mucosa and with acid-fast bacilli in intestinal lesions. As reviewed by Clarke et al. this original diagnosis, made over one hundred years ago by Johne and Frothingham, identified yet another Mycobacteria associated with disease (Clarke et al., 1997).

Mycobacteria in general are acid-fast, weakly Gram-positive bacilli 0.5-1.5μm in length, and require an organic source of iron to survive. *M. paratuberculosis* can be differentiated phenotypically from other Mycobacteria by an extremely slow growth in culture (up to 16 weeks) and a requirement for addition of Mycobactin J to the growth media. Genotypically *M. paratuberculosis* can be distinguished from its closest relative *M. avium* subspecies *avium* (*M. avium*) by DNA sequence. *M. avium* and *M. paratuberculosis* share a 99.6% similarity in DNA sequence. One marker often used by

researchers is presence of insertion sequences known as IS900 in the *M. paratuberculosis* genome. As stated above many Mycobacteria are pathogenic for humans, birds and mammals. *M. paratuberculosis* has been described as a facultative intracellular pathogen of bovine macrophages and as the causative agent of an inflammatory bowel disease now known as Johne's disease (Clarke et al., 1997).

B. General Overview of the Disease

Chronic weight loss, incurable diarrhea and eventual death are indicative of Johne's disease in cattle. The bacterium causes chronic granulomatous enterocolitis and regional lymphangitis and lympadenitis in cattle leading to typical clinical signs of progressive weight loss and eventual death, presumably from failure to absorb nutrients. *M. paratuberculosis* causes a persistent infection in intestinal macrophages leading to a local inflammatory immune response in infected hosts. Chronic inflammation leads to formation of granulomatous lesions at sites of infection and may play a role in the bacterium's ability to persist within host tissues by excluding fresh immune cells from sites of infection. Chronic inflammation may also be responsible for the majority of localized damage observed in chronic *M. paratuberculosis* infections. Clinically infected cattle shed the bacteria through milk, semen and feces. Bacterial shedding in feces is of principal importance since it allows passage to offspring and herd mates via the fecal to oral route.

Based on isolation and growth of the bacterium shed in feces of infected cattle (gold standard), prevalence of Johne's disease in dairy herds has been estimated from as low as 15-18% in parts of the United States and the United Kingdom (Braun RK, 1990) to as high as 54% in some regions of the United States (Johnson-Ifearulundu Y, 1999).

Within dairy herds that have tested positive for Johne's disease it is not uncommon to observe individual infection rates of 7-20%. Infection rates within infected herds will increase through horizontal transfer as healthy calves are exposed to fecal contamination and infected colostrum. Another cause for concern in the spread of Johne's disease is that *M. paratuberculosis* has been reported to infect fetuses *in utero* (Doyle et al., 1958; Seitz et al., 1989; Sweeney et al., 1992) when the dam is in either subclinical or clinical stages of disease.

Infection with *M. paratuberculosis* usually occurs within the first 6 months of a calf's life when it's immune system is not prepared to respond to Mycobacterial infections (Payne et al., 1961; Rankin et al., 1961). Exactly how the immune system becomes prepared for a Mycobacterial infection is not yet known, but may involve balancing between various T cell subsets and tissue distribution of immune cells. Due to the limits of specificity and sensitivity in commercially available tests that monitor peripheral blood cytokine profiles for diagnosis of Johne's disease, *M. paratuberculosis* infections can go undetected until there are observable signs of clinical disease. A long subclinical phase (up to 3 years) of *M. paratuberculosis* infection also makes the task of tracking and controlling Johne's disease extremely problematic.

II. Bacterial Survival

A. The Endocytic Pathway

Macrophages and other APC employ three main strategies for destruction of invading bacteria. All three of them are represented in the endocytic pathway [see Figure 2.1]. First, APCs can acidify the environment where bacteria are replicating. Secondly, the cells can sequester bacteria away from essential nutrients such as iron. Lastly, APCs

can fuse phagosomes containing ingested bacteria with lysosomes containing lysozyme and other killing enzymes thus exposing the bacteria to potent anti-bacterial molecules. Endocytosis, pinocytosis, macropinocytosis and the receptor-mediated endocytotic process called phagocytosis are all used by APC to envelope target bacteria.

Since phagocytosis is a receptor-mediated process, it is the most specific ingestion method available to APCs. Receptors used for phagocytosis of pathogenic bacteria are the mannose receptors (MR) (Astarie-Dequeker et al., 1999; Schlesinger et al., 1993), the complement receptors CR1, CR3 and CR4 (Schlesinger and Horwitz, 1991), Fc-γ receptors (Ernst et al., 1999), carbohydrate receptors (Ernst et al., 1999), fibronectin receptors (FR) (El-Etr and Cirillo, 2001) and Toll-like receptors (TLR) TLR4 and TLR2 (Means et al., 1999). All of these membrane bound receptors on APCs can bind pattern recognition domains on bacteria and initiate the process of phagocytosis. After binding bacteria through one of these receptors, APCs form an invagination or pit that surrounds the bacteria until it is completely sequestered from the extra-cellular space. Membrane bound vesicles that surround ingested bacteria are known as phagosomes.

Following internalization, phagosomes undergo a stepwise process of maturation that allows the original phagosome structure to fuse with important endocytic and exocytic vesicles. The purpose of these fusion events is ultimately to degrade phagocytozed targets into small peptide and lipid components for presentation as antigens in the context of major histocompatibility complex, MHC II (in the case of peptides) or CD1 (in the case of lipids). This presentation of bacterial antigens in the context of MHC II or CD1 will activate T cells to release pro-inflammatory cytokines (which can increase bacterial killing) or B cell expansion in an antigen specific fashion (which will increase

bacterial destruction through opsinization). The final stage in pathogen destruction occurs when late maturation vesicles (late phagosomes) fuse with lysosomes to create a phagolysosome, where bacteria are fully digested by antimicrobial agents and lysosomal enzymes.

Processing through the endocytic pathway has been hypothesized to occur via multiple fusion events with other vesicles (endosomes) in a process termed the "kiss and run" hypothesis [see Figure 2.1] (Duclos et al., 2000). The specificity of intracellular trafficking and fusion events are conferred by integral membrane proteins on both the phagosomes and endosomes, termed v-SNAREs and t-SNAREs for vesicle- and target-specific soluble-N-ethylmaleimide-sensitive factor (NSF)-attachment protein (SNAP), respectively. Binding of t-SNAREs with v-SNAREs from opposing vesicle membrane faces causes vesicles to be brought into close proximity to one another and ultimately to fuse (Rothman and Wieland, 1996). Several other host proteins are also involved in regulating vesicle trafficking, including the Rab family of small GTP-binding proteins with intrinsic GTPase activity (Pfeffer et al., 1994). The Rab family of proteins also figures prominently in another proposed model of endosome maturation known as the "fusion" model.

In the fusion model various Rab proteins are brought to maturing phagosomes via pre-packed endocytic vesicles (Novick and Brennwald, 1993). Acquisition of Rab proteins is said to "mature" phagosomes. As phagosomes mature, their ability to degrade bacteria also increases. It has also been theorized that vesicle maturation through the endocytic pathway may occur through a sequential acquisition process (Via et al., 1997) where endosomes must acquire Rab5 if they are to acquire Rab7. In this model,

cytoplasmic proteins, including Rab5 and Rab7, are recruited to the maturing phagosome. Rab protein recruitment brings about structural alteration or enhanced expression of receptor molecules on the surface of phagosomes, leading to a step-wise fusion process.

Regardless of the way phagosomes acquire Rab proteins, they appear to be necessary for vesicle maturation through the endocytic pathway (Pfeffer et al., 1994) and are believed to positively or negatively regulate the rates of SNARE complex assembly between endosomes (Novick and Zerial, 1997; Schimmoller et al., 1998). There are more than thirty known members of the Rab family (Lutcke et al., 1994; Zerial and Stenmark, 1993), including Rab5 and Rab7, which appear to control major fusion steps in the endocytic pathway [see Figure 2.1]. Both Rab5 and Rab7 have intrinsic GTPase activities similar to the Ras super-family of proteins (Chavrier et al., 1990; Simons and Zerial, 1993) and are most likely involved in phosphorylation/de-phosphorylation events related to membrane fusion. The absence of Rab5 on phagosomes has been shown to block fusion with early endosomes (Zerial et al., 1992) and stop entry of phagosomes into the endocytic pathway. (Horwitz and Maxfield, 1984) [see Figure 2.1 "D"].

If the endocytic pathway is functioning correctly, phagosomes acquire vesicular proton-ATPase (H^+-ATPase) pumps from early endosomes (Russell et al., 1995) either through vesicle fusion or the mechanism put forth in the "kiss and run" hypothesis. Acquisition of this integral membrane bound H^+-ATPase pump has the rather important job of acidifying early endosomes to initiate destruction of ingested bacteria. Having this H^+-ATPase pump present on early phagosomes, or at least acidification of the early phagosomes (endosomes) caused by this pump, may be mandatory for further phagosome maturation (Xu et al., 1994). The next step in vesicle maturation is either fusion of early

phagosomal (endosomal) vesicles with late endosomes or at least acquisition of proteins from late endosomes.

Maturing phagosomes begin to lose Rab5 and acquire Rab7 (Novick and Brennwald, 1993) [see Figure 2.1 "F"]. At this stage of maturation bacteria continue to be degraded by the low pH in early endosomes. Finally, the late phagosome (endosome) will lose integral Rab7 membrane proteins and fuse with a lysosome to form a phagolysosome (Deretic et al., 1997). The phagolysosomal environment will expose already partially degraded bacteria to hydrolytic enzymes, reactive oxygen species (ROS), reactive nitrogen intermediates (RNI) and inducible nitrogen oxide synthase (iNOS) that have inherent bactericidal properties. This last step in phagosomal (endosomal) maturation will fully degrade bacteria and is needed for presentation of bacterial antigens in the contest of MHC II and CD1 [see Figure 1.1].

B. Bacterial Survival Strategies

As described previously, APC are uniquely designed to destroy bacteria. After phagocytic uptake, APC can destroy bacteria with a combination of hydrolytic enzymes, ROS, RNI, iNOS and nutrient deprivation through sequestration in phagosomes and a process of step-wise destruction through the endocytic pathway. However, several intracellular bacteria have developed unique methods to evade and circumvent phagocytic destruction. Generally, bacterial survival mechanisms can affect the phagocytic host cell on either a global (throughout the cell) or local (limited to a specific bacterial phagosome) scale (Hackstadt et al., 2000). Although mechanisms vary for different bacteria, they all have in common the same goal, to circumvent the awesome destructive power of the endocytic pathway [see Table 1.1].

Salmonella typhimurium enters macrophage cells through the process of macropinocytosis. Entering macrophages through this non-receptor mediated process is believed to nullify generation of the oxidative burst used by macrophages to destroy many types of bacteria (Ernst et al., 1999) and could also avoid calcium mobilization necessary for some macrophage activation events. Uptake by macrophages also allows *Salmonella typhimurium* to avoid neutrophils, which are successful in degrading the bacterium. *S. typhimurium* is able to induce polymerization of the local host G-actin. This polymerized actin surrounds *S. typhimurium* containing vacuoles for as long as 16 hours and may help stabilize vesicles providing a framework that can be used to recruit endosomes for vesicle expansion (Amer and Swanson, 2002; Holden, 2002). *S. typhimurium* can also exclude host proteins from the vesicle it resides in by using a type III secretion system that may play a role in preventing formation of the NADPH oxidase complex on salmonella-containing vesicles (Holden et al., 2002). By utilizing these different strategies *S. typhimurium* is able to evade host immune responses and thrive within vesicles of macrophage cells.

The intracellular bacteria *Leishmania donovani* has also developed specific strategies to live within macrophages. Unlike *S. typhimurium*, *L. donovani* is believed to be ingested by phagocytosis not macropinocytosis and ultimately reside within phagolysosomes where it can tolerate the extremely low pH that would degrade most other bacteria (Desjardins et al., 1997; Murray et al., 1988). Maturation of *L. donovani* phagosomes however, is not rapid. *L. donovani* can express a bacterial derived protein called lipophosphoglycan (LPG) and introduce it throughout the surface of the bacteria's phagosome (Duclos and Desjardins, 2000). It is believed that this LPG protein

introduction delays maturation of *L. donovani* phagosomes until the bacteria expresses genes that will allow it to survive in the hostile environment of the phagolysosome (Bogdan and Rollinghoff, 1999; Duclos and Desjardins, 2000). This tactic of delaying phagosome maturation is not unique.

Legionella pneumophila also delays phagosome maturation for several hours (Amer and Swanson, 2002). In contrast to Leshmania however, *L. pneumophila* is not delaying phagosome maturation to gain time for expression of survival genes, but rather to avoid the endocytic pathway altogether. *L. pneumophila* phagosomes do not enter the endocytic pathway (Russell et al., 1995). Through an unknown molecular process, *L. pneumophila* phagosomes are able to fuse with early autophagosomes or early endosomes of the autophagocytic pathway (Baba et al., 1994). The autophagocytic pathway is principally involved in degradation of organelles and cellular components and is not designed to destroy bacteria. Once fused with early autophagosomes, *L. pneumophila* reside within a cellular milieu that will provide them with ample raw materials for growth (Dorn et al., 2002). *L. pneumophila* containing endosomes have also been observed to associate with rough endoplasmic reticulium and ribosomes (Hackstadt et al., 2000). This association may be yet another way for *L. pneumophila* to effectively bypass endocytic pathway destruction.

Phagosomes containing *Brucella abortus* have been shown to associate closely with rough endoplasmic reticulium (Amer and Swanson, 2002) and also infiltrate the autophagocytic pathway (Arenas et al., 2000). In addition to fusing with endosomes from the autophagocytic pathway, *B. abortus* also has the capacity to globally affect phagocytic cells by utilizing a type IV secretion system that translocates effector

molecules to the host cytoplasm (Foulongne et al., 2000). These effector molecules may slow or inhibit fusion of all vesicles, allowing *B. abortus* time to express genes that may be important for facilitating fusion with autophagocytic pathway endosomes.

Membranes of phagosomes containing *Toxoplasma gondii* are studded with proteins that originate from the parasite (Mordue and Sibley, 1997). Incorporation of parasitic proteins into phagosome membranes may allow *T. gondii* phagosomes to avoid or slow fusion with other endosomes and ultimately allow this parasite to hide from machinery that normally integrates phagosomes into the endocytic pathway (Dubremetz et al., 1998). *T. gondii* also encodes for enzymes that scavenge hydrogen peroxide, making *T. gondii* within phagosomes resistant to destruction by ROS (Murray et al., 1981). Finally, *T. gondii* are able to enter macrophage cells by utilizing β1-integrin receptors. Utilization of this class of receptors for uptake can protect the bacteria from destruction within phagolysosomes by avoiding macrophage activation (Solbach et al., 1991).

In contrast to previous examples, Listera, Rickettsia and Shigella are not able to control maturation of phagosomes, but have developed a distinct survival strategy. These three families of bacteria are able to lyse phagosomes and live within the cytoplasm of host cells (Gouin et al., 1999). Thus, they avoid destruction not by halting maturation of vesicles, but by simply refusing to stay where they are put by the host cell. In addition to being able to lyse phagosomes *Listera monocytogenes* (*L. monocytogenes*) has another important survival technique. *L. monocytogenes*, like Mycobacteria, are able to enter macrophages through selective use of receptors (Zimmerli et al., 1996). Normally when *L. monocytogenes* is phagocytized via CR3 receptors it is degraded, but when *L.*

monocytogenes enters through an unknown alternate receptor (possibly mannose receptor) it can survive and multiply within macrophages.

C. Mycobacterial Survival Within Macrophages

Mycobacteria, like other intracellular bacteria mentioned previously, are able to successfully evade host immune responses. They are able to accomplish this feat by utilizing one of several survival strategies outlined in Table 1.2 (Kaufmann et al., 1993). Many Mycobacteria, including *M. paratuberculosis*, can enter macrophages by interacting with the cell's complement receptors (CR) (Stokes et al., 1993; Tessema et al., 2001) most notably the CR3 (Malik et al., 2000). By entering macrophages through CRs it is believed that the Mycobacteria are able to interfere with macrophage Ca^{2+} signaling and through an unknown mechanism avoid generation of ROS or iNOS (Tessema et al., 2001) and are thus able to survive one of the most potent weapons available to macrophages in combating intracellular bacteria. Besides entering macrophages through receptors to avoid generation of ROS and iNOS, Mycobacteria also actively produce molecules and proteins that may promote survival in the hostile environment of a macrophage phagosome.

Mycobacteria produce a lipoprotein named lipoarabinomannan (LAM) as a major component of their cell walls. This lipoprotein has been shown to not only be associated with Mycobacterial phagosomes but also to be associated with endosomes that appear to bud off from Mycobacterial phagosomes (Xu et al., 1994). LAM is an active scavenger of potentially cytotoxic substances such as ROS (Tessema et al., 2001). This exo-endosomal role of LAM is complemented by a second ability to delay fusion of

Mycobacterial phagosomes with early endosomes and thus delay entry into the endocytic pathway (Amer and Swanson, 2002).

Phagosomes are dynamic structures that are constantly maturing and moving throughout the cell. Mycobacterial phagosomes differ from other phagosomes in several ways. First, the pH of Mycobacterial phagosomes stabilizes around 6.4, matching the average pH of perinuclear recycling endosomal compartments (Crowle et al., 1991; Ullrich et al., 2000). This pH stabilization allows Mycobacteria to avoid destruction within phagosomes even if it does fuse with early endosomes for entry into the endocytic pathway. Reduced acidification of Mycobacterial phagosomes may be related to reduced association of H^+-ATPases with these endosomes (Deretic and Fratti, 1999; Xu et al., 1994). Lack of acidification may also account for other differences between phagosomes that mature through the endocytic pathway and phagosomes that contain Mycobacteria.

Acidification of phagosomes after entry into the endocytic pathway appears to be required for maturation to the late endosomal stage (marked by acquisition of Rab7). Mycobacterial phagosomes fail to acquire Rab7, even in systems where Rab7 is over expressed (Clemens et al., 2000). Deretic et al. showed that Mycobacterial phagosomes actively exclude Rab7 from their membranes (Deretic et al., 1997) and not only maintain Rab5 association, but appear to recruit even more Rab5 to their membranes. The maintenance and recruitment of Rab5 to Mycobacterial phagosomes appears to affect acquisition of other markers for endosomal maturation such as the mannose 6-phosphate receptor (M6PR) (Clemens and Horwitz, 1995) and lysosomal associated membrane protein (LAMP) (Clemens and Horwitz, 1995; Xu et al., 1994). Thus, available evidence suggests that Mycobacterial phagosomes do not progress past an early endosomal stage

[see Figure 2.1 "?"] (Ernst et al., 1999). As shown above, phagosomal arrest is not unique to Mycobacteria but the arrest of Mycobacterial phagosomes at early endosomal stages limits intersection of Mycobacteria with MHC II and CD1 antigen presentation pathways (Ullrich et al., 2000). Interestingly, this limiting of association with antigen presentation pathways is abolished if macrophages are pretreated with IFN-γ (Ullrich et al., 2000) or with LPS and IFN-γ (Via et al., 1998).

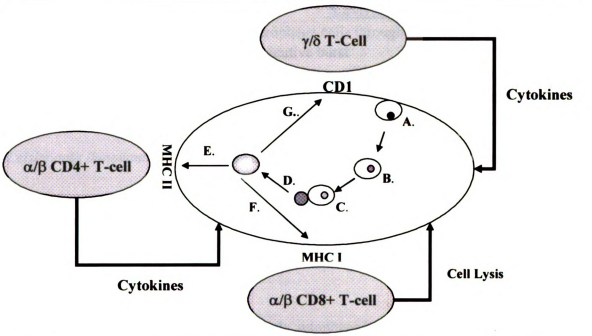
In addition to blocking acidification and endosomal maturation, Mycobacteria also appear to actively modify the expression of host proteins such as tryptophane aspartate-containing coat (TACO) protein and cellubrevin to avoid endocytic maturation. Mycobacterial phagosomes actively retain the host TACO protein (Ferrari et al., 1999). TACO has been implicated in the ability of phagosomes to recruit cholesterol to the membrane surface (Amer and Swanson, 2002). Recruitment of cholesterol appears to hide Mycobacterial phagosomes from cellular machinery used to introduce phagosomes into the endocytic pathway. Cellubrevin is a v-SNARE that is common to macrophage phagosomes and is part of the cellular machinery controlling fusion between vacuoles. In phagosomes containing live Mycobacteria, Cellubrevin is degraded (Fratti et al., 2002). How Mycobacteria are able to induce destruction of this protein is not known at this time, but this recent finding is yet another potential mechanism by which Mycobacteria alter and adapt to survive their chosen environment, the macrophage phagosome.

SNARE and Rab proteins control fusion events between phagosomes and endosomes which move along actin filament networks throughout the cell (Blocker et al., 1998; Blocker et al., 1997; Blocker et al., 1996; Swanson et al., 1992). Virulent Mycobacteria are able to disrupt this network of filamentous proteins (Guerin and de

Chastellier, 2000). Disruption of the actin cytoskeleton does not appear to be a way for Mycobacteria to evade entry into the endocytic pathway, as this disruption of the cytoskeleton occurs approximately 1 day post-phagocytosis. This disruption could be a way for Mycobacteria to slow or prevent induction of nitric oxide synthetase (Fernandes et al., 1996) by interrupting fusion of NADH dehydrogenase containing vesicles to phagosomes or inhibiting fusion with other vesicles that may be deleterious to the bacterium.

In summary, Mycobacteria and *M. paratuberculosis* specifically, are able to evade the host immune response by residing within non-maturing macrophage phagosomes. Once ingested, *M. paratuberculosis* appear to immediately affect the macrophage environment. These effects have been shown to halt vesicular maturation, disrupt the actin network, and actively recruit various proteins to bacterial phagosomes. In the study we report here, we hypothesized yet another mechanism by which *M. paratuberculosis* survives in bovine host macrophage cells, whereby phagocytosis of *M. paratuberculosis* leads to a gene expression profile in the host cells that permits survival of the bacterium. To begin to test this hypothesis, we used DDRT-PCR as a primary screening tool to compare and contrast gene expression profiles of resting bovine macrophages and bovine macrophages that were allowed to phagocytize *M. paratuberculosis*, *E. coli* (as a bacteria that is readily degraded), or latex beads (as a positive control for phagocytosis without destruction) for 60 minutes. Differential expression of several key host genes were confirmed by Northern blot analysis and associated with prolonged survival of *M. paratuberculosis* relative to *E. coli*. Identification and functions of these candidate genes of *M. paratuberculosis* survival in bovine macrophages cells are discussed.

Figure 1.1 – Antigen Presentation Following Phagocytosis.



*Bacteria are brought into professional antigen presenting cells (APC) by the receptor-mediated process of phagocytosis (A.). The phagosome is then gradually acidified as it matures through a step-wise fusion process in the endocytic pathway (B.). The late phagosome containing the now semi-degraded bacteria fuses with a lysosome (C.). The lysosome contains ROS, iNOS and RNI along with hydrolytic enzymes that finally finish degrading the bacteria (D.). Once bacteria are fully degraded they can be presented to sub-populations of T cells for several different results. If antigens are presented to $CD8^+$ /KEYWORD><KEYWORD>GTP-Binding Proteins/*metabolism</KEYWORD><KEYWORD>Genes, Structural, ial antigens are presented to $CD4^+$ T cell populations in the context of MHC II (E.) the APC are bombarded with cytokines that can induce or suppress APC activation. Finally, APC can present glycolipids to γδ T cell subpopulations in the context of non-traditional MHC CD1 molecules (G.).*

Table 1.1 – Intracellular Bacteria Survival Strategies.

Bacteria	Survival Strategy
Salmonella typhimurium	Enters macrophage cells through macropinocytosis avoiding oxidative burst Induce polymerization of the local host G-actin Exclude host proteins from the vesicle by using a type III secretion system
Leishmania donovani	Tolerate the extremely low pH of the phagolysosome Delay phagosome maturation with LPG
Legionella pneumophila	Delay phagosome maturation Phagosomes fail to acidify Fuse with early autophagosomes, avoiding the endocytic pathway
Brucella abortus	Fuse with early autophagosomes, avoiding the endocytic pathway Utilize a type IV secretion system to slow or inhibit fusion of host vesicles
Toxoplasma gondii	Phagosomes are derived from proteins that originate from the bacteria, not proteins from the host cell Encodes for enzymes that scavenge hydrogen peroxide Enter macrophages by the β1- integrin receptors which appears to protect against lysosomal fusion
Listera monocytogenes	Lyse the phagosome and live in the cytoplasm Enter macrophages through receptors that do not induce activation
Rickettsia	Lyse the phagosome and live in the cytoplasm
Coxiella burnetti	Tolerate the extremely low pH of the phagolysosome
Shigella	Lyse the phagosome and live in the cytoplasm

This table puts forth several intracellular bacteria that are either obligate or opportunistic pathogens of cells in the immune system and several ways that they are able to avoid, halt, confuse or evade the immune response in the host. This table is by no means a complete listing of intracellular bacteria or their strategies, it is here only as a comparative tool.

Table 1.2 – Survival Strategies of Mycobacteria.

	Survival Strategy
Mycobacteria	Encode for the protein LAM, that scavenges potentially cytotoxic substances (ROS).
	Recruit cholesteral to the phagosome to block lysosomal fusion machinery.
	Disrupt the actin network in a local fashion which may hinder endosomal maturation.
	Selectively degrad essential endosomal fusion proteins such as Cellubrevin (v-SNARE) which may retard the endosomes ability to mature.
	Actively repel Rab7 acquistion to phagosomes which possibly inhibits entry into the later stages of the Endocytic pathway.
	Exclude the stable incorporation of the endosomal membrane vesiclear H+-ATP pump and thus reducing acidification.

Outlining some specific actions taken by Mycobacteria after entry into host macrophage cells appears to confer at least a temporary survival advantage to Mycobacteria within early endosomal compartment. Whether or not any of these actions actually are responsible for Mycobacterial survival within host macrophage is not yet known.

CHAPTER TWO

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CHAPTER TWO

Survival Tactics of *M. paratuberculosis* in Bovine Macrophage Cells

I. Abstract

Mycobacterium avium subspecies *paratuberculosis* (*M. paratuberculosis*) is a facultative intracellular bacterium with the ability to survive and proliferate inside the vesicles of macrophage cells. How *M. paratuberculosis* and other Mycobacteria survive in this hostile environment is not well understood. Present research findings can be divided into three possibilities: (1) Mycobacteria may interfere with host protein expression and trafficking to stop vesicle maturation, (2) Mycobacteria may express proteins that interfere with macrophage activation in a more direct manner, or (3) Mycobacteria may enter macrophages in such a way as to avoid the normal process of activation via Toll-like receptors and other, as yet unknown mechanisms. Research thus far has predominately centered on possible macrophage/Mycobacteria protein interactions.

To more completely define how Mycobacteria interfere with the process of phagosome maturation our group has recently taken a functional genomics approach, allowing the macrophage to "tell" us what host genes may be affected by phagocytosis of Mycobacteria. We used DDRT-PCR to examine differences in macrophage cell gene expression during phagocytosis of *E. coli* and *M. paratuberculosis*. Macrophage cells not exposed to any phagocytosis target and in the process of phagocytosing latex beads were used as negative and positive controls, respectively. To date, we have identified 380

DDRT-PCR amplicons corresponding to transcripts whose expression profiles appear to be altered during the general process of phagocytosis. Dot-blot and Northern blot hybridizations with a subset of these amplicons were performed to confirm results observed with DDRT-PCR. Our preliminary results indicate that macrophage gene expression profiles change dramatically following phagocytosis and that gene expression profiles following phagocytosis of *M. paratuberculosis* are different than those following phagocytosis of *E. coli* or latex beads.

II. Introduction

Macrophages are phagocytic cells that ingest bacteria and other particulate matter by the receptor mediated process called phagocytosis. A vesicle is formed around the bacteria, which then become internalized by the macrophage cell. Following internalization, vesicles containing bacteria undergo a stepwise process of maturation that allows the original structure to fuse with important endocytic and exocytic vesicles for ultimate destruction of the phagocytized bacteria [see Figure 2.1]. The final stage in pathogen destruction occurs when late maturation vesicles (late phagosomes) fuse with lysosomes to create a phagolysosome where the bacteria are digested by reactive oxygen and lysosomal enzymes. Mycobacteria (Crowle et al., 1991) and other intracellular bacteria, such as *L. pneumophila* (Baca et al., 1994) and *Coxiella burnetti* (Feng et al., 1995) have developed various strategies to avoid degradation in the macrophage following phagocytosis. Though it is not clear what molecular mechanisms might contribute to the intracellular survival of various pathogens, recent studies on mycobacterial survival in the phagocytic/endocytic pathway have revealed that

Mycobacteria block vesicle maturation. By doing so, the Mycobacteria avoid phagosome-lysosome fusion and subsequent bacterial destruction (Via et al., 1997).

As shown in Figure 2.1, processing through the endocytic pathway may occur through multiple fusion events with other vesicles (endosomes). Several protein markers for vesicle maturation, called Rab proteins, have been identified as members of the superfamily of GTP-binding proteins (Pfeffer et al., 1994). In the fusion model of vesicle maturation, various Rab proteins are brought to the maturing phagosome via pre-packed endocytic vesicles (Novick and Brennwald, 1993). It has also been theorized that vesicle maturation through the endocytic pathway may occur through a sequential acquisition process (Via et al., 1997). In this model, the recruitment of various cytoplasmic proteins, including Rab5 and Rab7 may be brought about through structural alteration or enhanced expression of receptor molecules on the phagosome surface.

Regardless of the way phagosomes acquire Rab proteins, they appear to be necessary for vesicle maturation through the endocytic pathway (Pfeffer et al., 1994). There are more than thirty known members of the Rab family (Lutcke et al., 1994; Zerial and Stenmark, 1993) including Rab5 and Rab7, which control the major fusion steps in the endocytic pathway [see Figure 2.1]. Both Rab5 and Rab7 have GTPase activities similar to the Ras super-family of proteins (Chavrier et al., 1990; Simons and Zerial, 1993) and are most likely involved in phosphorylation/de-phosphorylation events related to membrane fusion. The presence of Rab5 on both phagosomes and early endosomes promotes both endosomal fusion with the newly formed phagosomes (Zerial et al., 1992) and entry of the phagosomes into the endocytic pathway (Horwitz and Maxfield, 1984) [see Figure 2.1 "D"]. Fusion of early endosomal vesicles with late endosomes and

lysosomes, required for bacterial killing, is characterized by acquisition of Rab7 (Novick and Brennwald, 1993) [see Figure 2.1 "F"].

Mycobacteria-containing vesicles acquire Rab5 in the normal fashion (Gorvel et al., 1991). However, Mycobacteria-containing phagosomes fail to acquire Rab7 and thus do not enter the later phases of the endocytic pathway (Via et al., 1997). As a result, these phagosomes do not become acidified or fuse with lysosomes, thus promoting survival of the Mycobacteria. Inhibition of the later phases of the endocytic pathway may thus contribute to the proliferation of phagocytized Mycobacteria in macrophages [see Figure 2.1 "?"]. Virtually nothing is known regarding the molecular mechanisms by which Mycobacteria inhibit Rab7 recruitment to phagosomes or stop progression to the terminal phagosome-lysosome fusion event.

Given the facts that phagocytosis is a receptor-mediated event and that various proteins marking maturation through the endocytic pathway belong to a family of proteins involved in signal transduction, we reasoned that there might be critical gene expression events that are interrupted or miss-directed following phagocytosis of Mycobacteria. To test this hypothesis, we used a functional genomics approach to study gene expression profiles in macrophage cells following phagocytosis of Mycobacteria and other infectious and non-infectious agents. Our preliminary differential display reverse transcriptase polymerase chain reaction (DDRT-PCR) results suggest that phagocytosis does cause profound changes in macrophage gene expression, and that macrophage gene expression profiles are different following phagocytosis of Mycobacteria compared with phagocytosis of *E. coli* or latex beads.

III. Materials and Methods

A. Cell Culture

Bacterial Cultures

E. coli-DH5α were grown in Luria Broth media at 37°C with agitation for eighteen hours. The bacteria were serially diluted and counted using a bacterial hemocytometer.

M. paratuberculosis cells were obtained from the American Type Culture Collection (ATCC #19698) and grown at 37°C in Middlebrooks 7H9 media with 10% Middlebrooks OADC enrichment and Mycobactin J (Allied Monitor, Lexana KS) at 2mg/L for 12-16 weeks. *M. paratuberculosis* were serially diluted and counted on a bacterial hemocytometer.

Bovine Macrophage Cells

The BOMAC cell line (Stabel and Stabel, 1995), a generous gift from Dr. J. Stabel USDA-ARS-NADC, was used in all experiments. BOMAC cells were grown in 75-mm^2 flasks containing RPMI-1640 with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100U/ml penicillin, and 100μg/ml streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 39°C until a confluent monolayer (approximately $2x10^6$ cells) was achieved.

B. Phagocytosis and RNA Collection in BOMAC Cells

Cell culture medium was aspirated from the BOMAC cells and 5ml of 0.025% trypsin, 1mM EDTA added to each $75mm^2$ flask. The flasks were then incubated for 5 minutes at 39°C to facilitate separation of the BOMAC cells from the substratum. Trypsin/EDTA was quenched with three volumes of growth medium and cells were

washed into the supernatant by repeated pipetting. Cells were counted using a hemocytometer and then added to six-well plates at a concentration of $2.0x10^5$ cells/well. Cells were allowed to adhere to substratum in the wells overnight in 4ml of growth medium/well. After overnight incubation, growth medium was removed from the six-well plates by vacuum and the BOMAC cells were washed 3 times with sterile phosphate buffered saline (PBS) to remove any non-adherent cells. Next, 1ml of fresh growth medium was added to each well to cover the BOMAC cells. The various phagocytosis targets; *E. coli*, *M. paratuberculosis*, and latex beads were counted and resuspended at $2.0x10^7$ cells or beads per ml of sterile PBS. To initiate phagocytosis, 100µl of the various target solutions were added to separate wells at a final concentration of $2.0x10^6$ cells or beads/well (10:1, target: cell ratio). One set of wells received only 100µl of PBS only as a no phagocytosis control. Plates were centrifuged for 10 minutes at 3000xg to facilitate interaction between the cells and the various phagocytosis targets. Plates were then incubated for one hour at 39°C in a humidified atmosphere of 5% CO_2 and 95% air. Phagocytosis was arrested after 60 minutes by aspirating the medium and washing 3 times in ice cold PBS. RNA was harvested using either a Qiagen RNeasy Maxi Kit according to the manufacturer's instructions (Qiagen, Valencia, CA) or by using Trizol, also according to the manufacturer's instructions (Invitrogen Life Technologies, Inc., Gaithersburg, MD). RNA from the four treatment groups was stored at -80°C under ethanol until used.

C. Differential Display RT-PCR

RNA from the various phagocytosis treatments was diluted in RNase-free ddH_2O to yield a working concentration of 100ng/µl. Differential display RT-PCR was

preformed using the Hieroglyph Kit (Beckman Coulter, Inc., Fullerton, CA), essentially according to the protocol provided by the manufacturer. Briefly, an anchored primer (AP) 5'-T7-TTTTTTTTTTXX (where "XX" is a combination of any two base pairs except TT, TC, TA and TG) was used to synthesize 1st strand cDNA using reverse transcription (RT) at 50°C for 50 minutes. RT reactions were stopped by heating samples to 70°C for 15 minutes. The 1st strand cDNA reaction was then split into four separate pools for amplification of cDNA subsets by polymerase chain reaction (PCR). PCR amplifications were preformed in the presence of 0.125μCi [α-^{33}P] dATP/reaction along with 0.2μM AP, 0.2μM anchored random primer (ARP, a random 10-mer with M13-Reverse primer sequences appended at the 5' end), and 20μM dNTP mix. PCR amplifications were performed using standard conditions: 95°C, for 2 min., [92°C, 15 sec; 50°C, 30 sec; 72°C, 2 min.]^{4} [92°C, 15 sec; 60°C, 30 sec; 72°C, 2 min.]^{25}, with a final hold at 4°C. Amplified cDNA fragments (amplicons) were separated by electrophoresis on a 5.2% Bis-acrylamide gels at 45 watts constant power. For each reaction, two separate gels were run, one for 5 hours and a second for 8 hours, to facilitate separation of a wide range of fragment sizes. The gel was then dried to a piece of Whatman 3-mm paper (Whatman Ltd., Maidstone, UK) and exposed to X-ray film for 48 hours.

D. Excision and Re-amplification of Suspect cDNA amplicons.

Labeled cDNA fragments (amplicons), amplified using the same AP/ARP primer set, that showed differences in intensity across the four different phagocytosis treatments were excised from the gel and eluted into 100μl TE pH 7.5. Impurities were removed using Sephadex G-50 exclusion chromatography (Amersham Pharmacia Biotech., Buckinghamshire, UK), contained in a 96-well spin plate format (Millipore Corp.,

Bedford, MA). Re-amplification of excised and eluted cDNA fragments was accomplished by transferring 5-10µl of the purified eluate into a PCR reaction tube containing 20µM dNTP mix, 0.2µM T7 primer, 0.2µM M13-Reverse primer, PCR reaction buffer (200mM Tris-HCl (pH 8.4), 500mM KCl), and Taq DNA polymerase (Invitrogen Life Technologies, Inc., Gaithersburg, MD). Conditions for PCR thermocycling were identical to those described above for DDRT-PCR. The quality and amount of each amplicon DNA was evaluated by gel electrophoresis in 1.0% or 2.0% agarose/TAE gels.

E. Dot-Blot Hybridization

Suspect cDNA amplicons were amplified as noted above, denatured in 0.4N NaOH and blotted to nylon membranes (Hybond XL, Amersham Pharmacia Biotech., Buckinghamshire, UK) using a 96-well vacuum dot-blot apparatus (Bio-Rad Corp. Hercules, CA). Spotted cDNA fragments were UV cross-linked and dot-blots stored at –20°C until utilized. To probe the dot-blots, 5µg of total cellular RNA isolated from negative control (NC) and *M. paratuberculosis* (M) treatments were used to prime separate first-strand cDNA reactions in the presence of [α-^{32}P] dCTP. Prior to hybridization, dot-blot membranes were blocked for 2 hours at 65°C with 4mg of heat-denatured (10 minutes at 95°C) herring sperm DNA in 5ml of Perfect Hyb Solution (Sigma Chemical Co., St. Louis, MO). Blocking solution was decanted and $2x10^6$ cpm/ml of heat-denatured probe in 5ml of Perfect Hyb solution was added to the dot-blot membranes. The membranes were then hybridized overnight (16-18 hours) at 65°C. Following hybridization, the membranes were washed twice at room temperature in 2X SSC, 0.1% SDS and twice more at 65°C in 0.1X SSC and 0.1% SDS to remove unbound

probe. Radioactivity remaining associated with the membranes was visualized and quantitated using a Phosphoimager and Quantity-One software (Bio-Rad Corp., Hercules, CA).

F. Northern Blot Analysis

Total RNA from each treatment (10μg/lane) was separated by gel electrophoresis on an 11x14cm 1% denaturing formaldehyde/agarose gels. Electrophoresis conditions were 60V for 5 hours. Separated RNA was then transferred to nylon (Hybond XL, Amersham Pharmacia Biotech., Buckinghamshire, UK) membranes using the "wicking technique" in 20X SSC. UV cross-linking was preformed to fix transferred RNA to the membranes. Final membranes were stored at –20°C and allowed to thaw before being probed with radiolabeled DDRT-PCR amplicons. The suspect DDRT-PCR cDNA amplicons were radioactively labeled using a random prime labeling kit (Promega Corp., Madison, WI) in the presence of [α-^{32}P] dCTP and used for Northern blot hybridizations. Prior to hybridization, membranes were blocked for 2 hours at 65°C with 4mg of heat-denatured (10 minutes at 95°C) herring sperm DNA in 5ml of Perfect Hyb Solution (Sigma Chemical Corp, St. Louis, MO). Blocking solution was decanted and $2x10^6$ cpm/ml of heat-denatured probe in 5ml of Perfect Hyb solution was added to the Northern blot membranes. Membranes were hybridized with radiolabeled probe overnight (16-18 hours) at 65°C. Following hybridization, membranes were washed twice at room temperature in 2X SSC, 0.1% SDS and twice at 65°C in 0.1X SSC and 0.1% SDS to remove unbound probe. Washed membranes were exposed to X-ray film for 24-48 hours.

IV. Results

A. Identification of Differentially Expressed Transcripts in Bovine Macrophage Cells.

To begin testing the hypothesis that phagocytosis of Mycobacteria by bovine macrophage cells would lead to gene expression profiles different than those following phagocytosis of *E. coli* or latex beads, we employed DDRT-PCR. In an effort to limit differences attributable to the source of macrophages or their viability, we initiated this study using an immortalized bovine peritoneal macrophage cell line, called BOMAC ((Stabel and Stabel, 1995), a generous gift of Dr. J. Stabel, USDA-ARS-NADC). The functional macrophage characteristics of these cells are well established (Stabel and Stabel, 1995). In general, BOMAC cells are similar to primary bovine macrophages in terms of phagocytosis, cellular cytotoxicity, production of oxygen radicals, and production of key cytokines following activation. In preliminary studies using differential staining and light microscopy, viable *E. coli* placed in contact with BOMAC cells as described in Materials and Methods were readily internalized and degraded. In contrast, *M. paratuberculosis* were not readily degraded following internalization by BOMAC cells (data not shown). Subsequent time course analyses showed that most internalized *E. coli* were completely degraded within 60 minutes of initial macrophage contact. In contrast, internalized *M. paratuberculosis* remained visible as acid-fast bacilli within vesicles of BOMAC cells for at least 96 hours post initial contact (data not shown).

To begin screening bovine macrophage cells for differentially expressed transcripts by DDRT-PCR, cells were seeded onto 8 separate six-well plates (2 plates per treatment category). Phagocytosis targets were prepared and diluted as described in

Materials and Methods. Diluted targets were added to appropriate plates and briefly centrifuged to improve contact between phagocytosis targets and cells. All plates were incubated for 60 minutes at 39°C in a humidified atmosphere of 5% CO_2 and 95% air. Brief rinsing in ice-cold PBS halted phagocytosis and total cellular RNA was extracted as described in Materials and Methods.

RNA expression profiles from the three phagocytic treatment groups [*E. coli* (E), *M. paratuberculosis* (M), and latex beads (PC)] were compared to each other and to RNA expression profiles of the no phagocytosis group (NC) by subjecting extracted RNA to the DDRT-PCR protocol. By comparing RNA expression profiles across the four treatments, it was possible to observe differential gene expression during the general process of phagocytosis and differential gene expression during phagocytosis of specific phagocytic targets. Because the general process of phagocytosis is not well defined in the bovine or any other system, DDRT-PCR amplicons representing transcripts that appeared to change expression levels across any of the four treatments were marked for isolation. Amplicons were eluted, re-amplified, and cataloged as to how their gene expression profiles changed (repressed or induced) with respect to the various phagocytosis treatments. To date, we have isolated 380 amplicons with observed differences across one or more of our 4 treatment groups. Selected amplicons were eluted and re-amplified for validation of expression differences by a combination of dot-blot and Northern blot hybridizations.

B. Validation of Differential Gene Expression by Dot-Blot Hybridization.

To validate expression differences observed by DDRT-PCR, dot-blot analysis was preformed on 30 selected amplicons. Selected amplicons were spotted in quintuplicate

onto a nylon membrane. Membranes also contained 8 GAPDH spots to account for blot-to-blot variation and to allow normalization between blots. Each membrane also contained 10 blanks to allow for quantification of background. DNAs were UV cross-linked to the membranes and probed with [α-^{32}P] labeled 1st strand cDNA as described in Materials and Methods. In the first set of comparisons, labeled cDNA derived from the no phagocytosis cell RNA (NC) was used to probe one blot while labeled cDNA derived from the *M. paratuberculosis* phagocytosis treatment cells (M) was used to probe a duplicate blot. In this comparison, several selected amplicons, such as 5-1-1, and 5-1-9, hybridized to transcripts that showed statistically significant differences in expression levels ($p < 0.05$) between the two treatments. However, other amplicons, such as 5-2-10, and 5-4-2 hybridized to transcripts that showed no significant differences in expression level between the NC and M treatments. Based on results of Northern blot hybridization studies described below, additional dot-blot validations will be performed with the same set of amplicons, but will compare cDNA derived from cells following phagocytosis *of M. paratuberculosis* versus cDNA derived from cells following phagocytosis of *E. coli*.

C. Confirmation of Differential Expression by Northern Blot Hybridization

Thus far DDRT-PCR derived suspect amplicons 5-1-1, 5-2-10, 3-1-4, and 5-4-2 have been used to probe Northern blots containing total RNA from the three phagocytic treatment groups and from macrophages not undergoing phagocytosis. Based on results of Northern blot hybridizations, amplicon 5-1-1 appears to represent a transcript that is expressed in macrophage cells not exposed to phagocytosis targets, but is down regulated following exposure to all three phagocytosis targets. This result agrees with expression differences between the no phagocytosis and *M. paratuberculosis* phagocytosis treatment

groups observed in DDRT-PCR [see Figure 2.2] and validated by dot-blot analysis. As suggested by initial dot-blot analysis, Northern blot hybridization using amplicons 5-2-10, 3-1-4, and 5-4-2 as probes revealed that there was indeed little or no difference in the expression of the corresponding transcripts between the no phagocytosis and *M. paratuberculosis* phagocytosis treatments [see Figure 2.3]. These amplicons all seem to represent macrophage transcripts whose expression is up regulated following exposure of macrophage cells to either *E. coli* or latex beads, but not following exposure to *M. paratuberculosis*.

V. Discussion

Failure of phagosomes containing Mycobacteria to progress through the endocytic pathway promotes survival and proliferation of these pathogens within the host macrophage. Despite a growing body of literature on movement of phagosomes through the endocytic pathway and discovery of various proteins critical for this process, the precise molecular mechanisms responsible for failure of Mycobacteria containing phagosomes to mature are largely unknown. Efforts to prevent or control mycobacterial infections in humans and animals depend upon knowledge of mycobacterial survival mechanisms and on understanding the host response to this diverse group of pathogens. Our group has developed a working hypothesis that various gene expression events, critical for maturation of phagosomes and proper macrophage activation are suppressed in macrophages following phagocytosis of Mycobacteria.

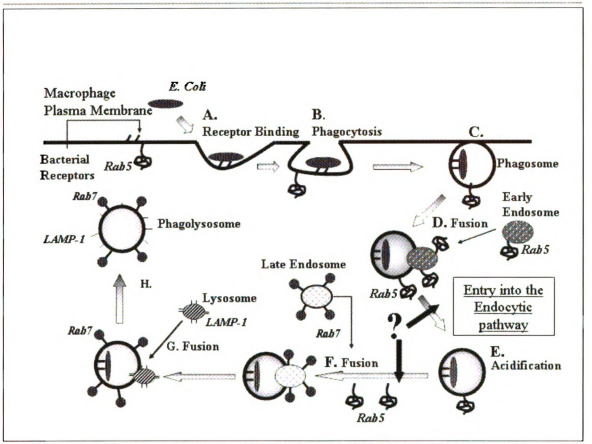
As a first step in testing this hypothesis we have employed DDRT-PCR to evaluate gene expression profiles in a macrophage cell line exposed to *M. paratuberculosis* and 2 other phagocytic targets (*E. coli* and latex beads). Using this

approach, we have been able to directly compare the expression patterns from our treatment groups across thousands of genes as DDRT-PCR amplicons. To date, over 380 DDRT-PCR amplicons that appeared to be differentially expressed across one or more of the four treatments (NC, E, M, or PC) have been isolated and re-amplified for further study and confirmation of differential expression. Because DDRT-PCR is known to yield a high number of false positives, 30 amplicons representing transcripts with the most obvious differences in expression pattern were selected for verification of DDRT-PCR results by dot-blot analyses and Northern blot hybridization. In an initial dot-blot analysis comparing the NC and M treatments, one amplicon (5-1-1) showed clear down-regulation following macrophage phagocytosis of Mycobacteria. This result confirmed results of DDRT-PCR, which indicated that the transcript corresponding to amplicon 5-1-1 was down-regulated following phagocytosis of either *M. paratuberculosis*, *E. coli*, or latex beads. Northern blot hybridization using amplicon 5-1-1 as probe subsequently confirmed both DDRT-PCR and dot-blot analyses. Thus, amplicon 5-1-1 appears to represent a transcript whose expression is down regulated by the general process of phagocytosis. While interesting, this gene may not be involved in Mycobacterial survival in macrophage cells. In contrast transcripts corresponding to 3 other amplicons (3-1-4, 5-2-10 and 5-4-5) exhibit very low expression levels in both macrophages not undergoing phagocytosis and macrophages following phagocytosis of Mycobacteria, but are expressed at high levels in macrophages following phagocytosis of *E. coli* or latex beads. We conclude therefore that expression of genes represented by these amplicons is up regulated in macrophages following phagocytosis of *E. coli* or latex beads, but is specifically held at low levels following phagocytosis of *M. paratuberculosis*. It is

interesting to speculate that failure to activate genes such as those represented by amplicons 3-1-4, 5-2-10, and 5-4-5 may be at least partially responsible for the ability of *M. paratuberculosis* to survive in macrophages.

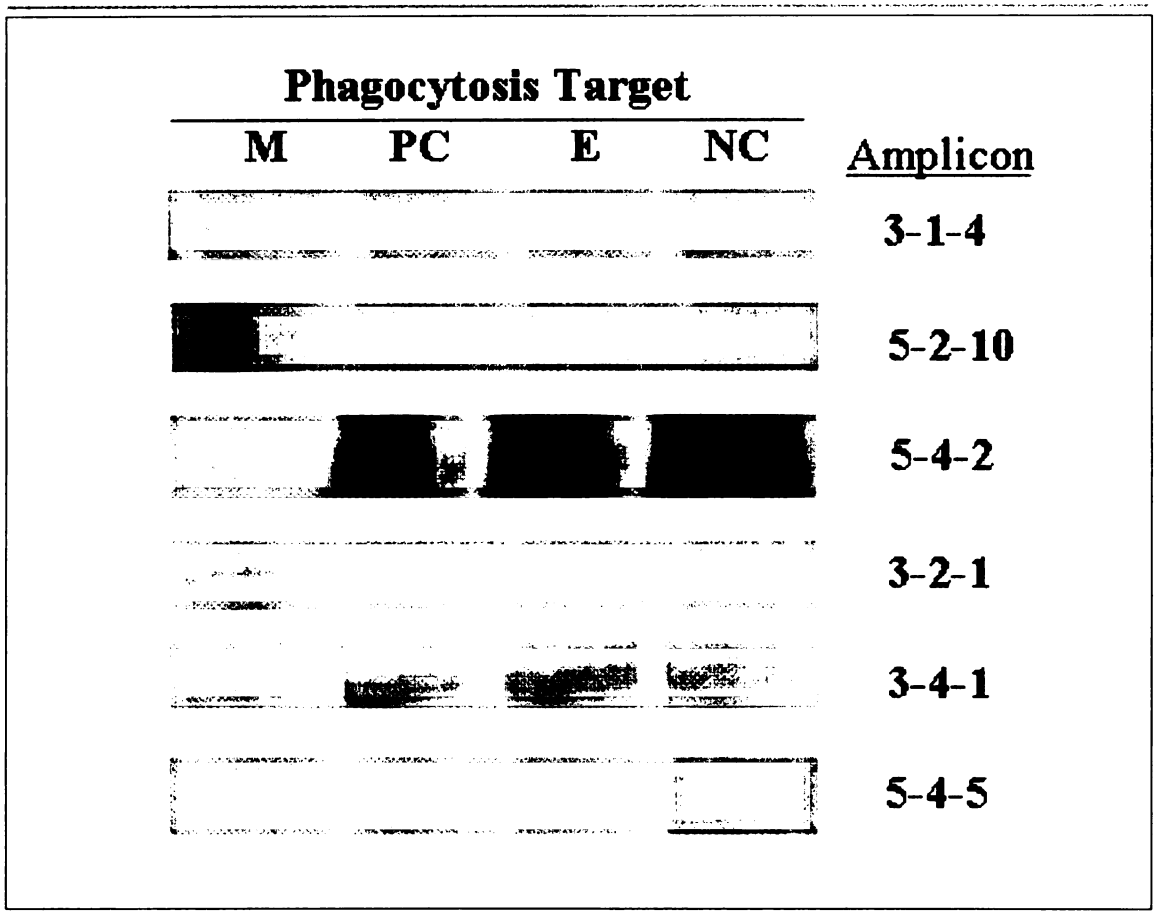
Of the remaining 30 amplicons selected for dot-blot analysis, 10 have failed to show clear differential expression in macrophages not undergoing phagocytosis and macrophages following phagocytosis of *M. paratuberculosis*. However, it is possible that these amplicons represent transcripts whose expression pattern is similar to 3-1-4, 5-2-10, and 5-4-5. That is, expression profiles of the remaining amplicon transcripts are similar between untreated macrophage cells and macrophage cells following phagocytosis of *M. paratuberculosis*, but distinct from cells following phagocytosis of *E. coli* or latex beads. Additional dot-blot validation studies to ascertain this possibility are in progress. Whatever the outcome from these studies results presented herein validate our hypothesis, that the general process of phagocytosis has a profound effect on macrophage gene expression profiles and that gene expression profiles of bovine macrophage cells following phagocytosis of *M. paratuberculosis* are distinct from those of macrophage cells following phagocytosis of *E. coli* or latex beads. Although it still remains to be seen if results presented here carry over to primary bovine macrophages and monocytes, this work clearly demonstrates that DDRT-PCR is an effective tool for identifying differentially expressed macrophage genes. Identification of such genes offers an opportunity to discover different pathways used by or activated by various pathogens as they enter macrophages and are either destroyed through the process of phagosome maturation or persist through interference with this normal first line of host defense.

Figure 2.1 - The Endocytic Pathway (The Fusion Model).



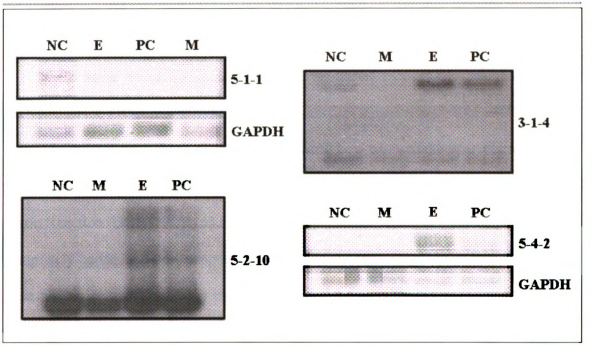
Through this pathway degradable bacteria such as E. coli, are destroyed by the stepwise fusion of phagosomes with early endosomes, late endosomes, and lysosomes. Various steps within the endocytic pathway are denoted by capital letters. Receptor-mediated uptake of E. coli by phagocytosis, results in formation of a phagosome ***(A-C).*** *Fusion of the phagosome with early endosomes results in a vesicle that now possesses external markers of the early endosome, including abundant Rab5* ***(D)****. Acquisition of Rab5 may be viewed as signaling entry of the vesicle into the endocytic pathway. Presence of high levels of Rab5 on early phagosomes coincides with (and may be directly related to) initiation of acidification in the vesicle, which aids in the degradation of phagocytized bacteria* ***(E)****. Fusion of the early phagosome with late endosomes marks the next stage of vesicle maturation and is characterized by acquisition of Rab7 and loss of Rab5* ***(F)****. Presence of Rab7 is absolutely required for subsequent interaction of the phagosome with lysosomes. Fusion of the late phagosome with lysosomes marks the final stage of vesicle maturation, and is characterized by the acquisition of LAMP-1 and LAMP-2* ***(G)****. .</AUTHOR><AUTHOR>Saama, P. M.</AUTHOR><AUTHOR>Bauman, D. E.</AUTHOR><AUTHOR>Boisclair, Y. R.</AUTHOR><AUTHOR>**Burt**on, J. L.</AUTHOR><AUTHOR>Collier, R. J.</AUTHOR><AUTHOR>DePend proliferate inside macrophage phagosomes. How Mycobacteria might accomplish this is not well understood, but is thought to occur at the stage of initial acidification (question mark and solid arrows).*

Figure 2.2 - DDRT-PCR Suspect Amplicons.



Representative DDRT-PCR amplicons selected for further study on the basis of apparent differential expression across the four treatment scenarios. ^{33}P-labeled amplicons were separated by electrophoresis and exposed to X-ray film for visualization. Selected amplicons 3-1-4, 5-2-10, and 3-2-1 show an apparent increase in macrophage gene expression during phagocytosis of M. paratuberculosis (M) when compared to either the E. coli phagocytosis (E) or the no phagocytosis control (NC) treatments. Antithetically, a marked depression in macrophage gene expression for amplicons 5-4-2, 3-4-1 and 5-4-5 is apparent during M. paratuberculosis phagocytosis (M) when compared to the (NC) no phagocytosis treatment. In the cases of amplicons 5-4-2 and 3-4-1 the depression is further seen when the M. paratuberculosis treatment (M) is compared against either of the other two phagocytosis treatments of E. coli (E) or latex beads (PC).

Figure 2.3 - Northern Blot Hybridization with Amplicon Probes.



Northern blot hybridization using selected DDRT-PCR amplicon DNAs as probes. 10μg of total cellular RNA from each of the four treatments was separated by gel electrophoresis on 1% denaturing formaldehyde/agarose gels and transferred to nylon membranes. Membranes were hybridized to the indicated ^{32}P-labeled DDRT-PCR amplicons, washed and exposed to X-ray film. Each membrane was stripped of radioactivity and re-probed with ^{32}P-labeled GAPDH amplicon as an RNA loading control. Amplicon 5-1-1 hybridized to a macrophage gene transcript that appears to be expressed when the macrophage cell is not undergoing phagocytosis (NC) and whose expression is depressed across all the phagocytic treatments [(M), (E), and (PC)]. In contrast, probes 5-2-10, 3-1-4 and 5-4-2 hybridized to macrophage gene transcripts whose expression levels are up-regulated during the phagocytosis of either E. coli *(E) or latex beads (PC) but which remain at low levels during the phagocytosis of* M. paratuberculosis *(M).*

CHAPTER THREE

The Effect of Different Phagocytic Targets on Bovine Macrophage Gene Expression

I. INTRODUCTION

In the previous chapter our group explained how we used the open platform functional genomics approach of Differential Display Reverse Transcription Polymerase Chain Reaction (DDRT-PCR) as a high throughput initial screen in an attempt to more completely understand how phagocytosis of *M. paratuberculosis* affects host macrophage gene expression. As previously stated, this approach allows the macrophage to "tell" us which genes may have an increased or decreased expression due to the phagocytosis of *M. paratuberculosis*. In the previous chapter, initial screening using DDRT-PCR revealed apparent macrophage gene expression differences dependent upon phagocytic stimuli. This has led to the hypothesis that these differences in gene expression, mostly between *E. coli* and *M. paratuberculosis*, may be important for survival of *M. paratuberculosis* in bovine macrophage cells. In this chapter we expand upon several specific points, such as bacterial survival in BOMAC cells, conformation of observed differences in gene expression across phagocytic treatments, cloning and sequencing of DDRT-PCR derived amplicons, and identification of differentially expressed genes via database searches.

An initial goal of our group was to clarify apparent differences in host gene expression after phagocytosis of *M. paratuberculosis* or *E. coli* by the immortalized bovine macrophage cell line BOMAC (Stabel and Stabel, 1995). A major difference

between the two bacteria is that *M. paratuberculosis* can survive within normal bovine macrophages while *E. coli* are an example of bacteria that are destroyed following phagocytosis. To evaluate survival of *M. paratuberculosis* and *E. coli* in BOMAC cells, an experimental procedure utilizing quantative real time PCR (Q-RT-PCR) was designed. This procedure covered time points from time zero to 96 hours post introduction of each of the bacterial phagocytic stimuli. Genomic DNA from both treatments (*E. coli* and *M. paratuberculosis*) was collected and subjected to real time PCR amplification for accurate quantification of bacterial genomic DNA present in each sample relative to amounts of BOMAC genomic DNA. Ratios of bacterial to macrophage DNA should become smaller with successful destruction of bacteria or stay relatively constant if bacteria survive within BOMAC cells. Two potential caveats are 1) that decreased cell survival could parallel decrease bacterial survival and therefore, the ratio of bacterial to BOMAC DNA would stay constant yielding a false report of bacterial survival when in fact there is bacterial destruction and 2) performing real time PCR will not conclusively show whether the bacteria are alive or dead. In unpublished data our lab has shown by microscopy that *M. paratuberculosis* is taken up by BOMAC cells and remains intact for at least 96 hours following phagocytosis [see Appendix Three: Figure A3.1]. Also work presented by Dr. Raúl G. Barletta's group at the University of Nebraska showed that two stains of *M. paratuberculosis* (one wild type K-10 and another K-10 transformed with a plasmid encoding for GFP) were able to survive and replicate for up to three days within both monocytes derived macrophages (MDM) and BOMAC cells [see Appendix Three: Figure A3.2]. By using Q-RT-PCR our group is confirming these survival results.

Differential gene expression in cultured macrophage cells was originally assayed by Northern blot hybridizations with DDRT-PCR derived amplicons [see Chapter 2]. Results obtained from these hybridizations were not conclusive. Multiple hybridization bands were observed on some Northern blots preformed using amplified DDRT-PCR derived amplicons as probes. Cloned DDRT-PCR amplicons were used as probes in further Northern blot hybridizations to remove the possibility of multiple genes represented in amplicon stocks. The benefits of amplicon cloning are three fold: first, cloning DDRT-PCR derived amplicons into stable plasmid vectors provided a permanent stock of DDRT-PCR amplicons. Second, cloning allowed purification of target amplicons that may have been contaminated by undesired bands during removal from DDRT-PCR gels. Finally, cloning allowed DNA sequence analysis of amplicon DNAs that represented genes whose expression differed across the phagocytic treatments. Sequence information in turn, allowed putative identification of several DDRT-PCR derived amplicons through BLASTN, BLASTX, and tBLASTx database searches.

At this time very little is known regarding molecular mechanisms that may affect maturation of *M. paratuberculosis* phagosomes within bovine macrophages. From work preformed in mouse and other systems a family of well-characterized proteins marking maturation through the endocytic pathway has been identified but their role in phagosome maturation is still unclear. In Chapter 2, our group put forth the hypothesis that *M. paratuberculosis* survival in bovine macrophages brings about a change in macrophage gene expression profiles relative to profiles of macrophages during the phagocytosis of bacteria, such as *E. coli*, that are successfully killed and degraded (Tooker et al., 2002) or stimuli such as latex beads that are non-degradable, but whose phagosomes undergoes

full maturation through the endocytic pathway. In this chapter we identify the genes discovered in Chapter 2 using DDRT-PCR and quantify differences in gene expression using Northern blot hybridizations and scanning densitometry with cloned amplicons as probes.

II. METHODS AND MATERIALS

A. Cell Culture

Bacterial and BOMAC cell culture was preformed as outlined in Chapter 2 Methods and Materials: page 23 [see Chapter2].

B. Bacterial Survival

Quantifying Bacterial Survival Using Quantitative Real Time Polymerase Chain Reaction (Q-RT-PCR)

BOMAC and bacterial cells were prepared for the time course experiment as outlined in Chapter 2 Methods and Materials: page 23-24 [see Chapter 2]. To begin the time course experiment, 10µl (~$2.0x10^6$) of bacteria or 10µl of sterile room temperature PBS was added to BOMAC cells in 6 well plates and mixed gently. Plates containing BOMAC cells and either phagocytic target (*M. paratuberculosis* or *E. coli*) or PBS as a negative control, were incubated at 39°C with 5% CO_2 and 95% humidified air for one hour. After incubation, medium was removed and the wells vigorously washed 3 times with sterile room temperature PBS to remove non-adherent bacteria (de Chastellier et al., 1995). At this time, designated T=0 hr, the first time point was collected by the following protocol while 5ml complete RPMI was added to remaining wells and incubated at 39°C with 5% CO_2 and 95% humidified air until subsequent collection

times. For time points after 24 hours (48, 72 and 96 hours), medium was removed by vacuum and 5ml of complete RPMI media added to each well every 24 hours.

At collection times, growth medium was removed from wells and 500µl of sterile 4°C PBS added to each well. Cells were mechanically dislodged into PBS by scraping and removed to 1.5ml Eppendorf tubes. Tubes containing BOMAC cells with bacteria were centrifuged at 1600xg for 5 minutes to pellet cells. Once pelleted, supernatants were removed by pipette and cells resuspended in 50µl Buffer AP1 from a Qiagen DNA Extraction Kit for Plants (for BOMAC cells treated with *M. paratuberculosis*) or 50µl Cell Lysis Solution from the Promega Wizard Genomic DNA Preparation Kit (for BOMAC cells treated with *E. coli*). Our group has observed that Mycobacteria residing within phagosomes are resistant to cell lysis (unpublished observation). Due to this fact, *M. paratuberculosis* time course samples were treated more aggressively than the *E. coli* time course samples.

BOMAC cells challenged with *M. paratuberculosis* were further subjected to three rounds of freeze/thaw (freezing in an ethanol-dry ice bath and boiling in water). Next, 1µl of 100mg/ml RNase A was added to each tube and cells incubated at 65°C for 10 minutes with tube inversion every 3 minutes to mix. After incubation 30µl of Buffer AP2 was added to each tube and cells incubated on ice for 5 minutes. Next, each lysate was added to a shredder column provided in the Qiagen DNA Extraction Kit for Plants and centrifuged at 14,000xg for 2 minutes. Flow through from each shredder column was mixed with 1.5 volumes (~200µl) of Buffer AP3/E and mixed gently by pipetting. This mixture was added to a spin column and centrifuged at 8,000xg for one minute. Flow through was discarded and 500µl of Buffer AW added to each column. Columns were

then centrifuged for 2 minutes at 14,000xg. Spin columns were moved to new 1.5ml Eppendorf tubes and 50µl of 65°C Buffer AE added to each spin column filter. Columns were incubated for 5 minutes with Buffer AE and centrifuged at 8,000xg for one minute. The previous step was repeated and eluted genomic DNA stored at 4°C until utilized.

BOMAC cells challenged with either *E. coli* or the negative phagocytic control of PBS were subjected to 10 minutes of incubation in cell lysis solution at room temperature and then centrifuged for 2 minutes at 13,000xg to pellet cells. Supernatants were removed and cells suspended in 60µl of Nuclei Lysis Solution/tube. Following suspension, 1µl of RNase A was added to each tube at 100mg/ml and solutions mixed by inversion 3 times throughout a 10 minute incubation at 65°C. Solutions were allowed to cool to room temperature for ~5 minutes and 20µl of Protein Precipitation Solution added to each tube. Solutions were vortexed for 20 seconds and incubated on ice for 5 minutes. Next, solutions were centrifuged for 4 minutes at 13,000xg and supernatants removed to new 1.5ml Eppendorf tubes containing 120µl of isopropanol. Supernatant and isopropanol were gently mixed by inversion and centrifuged at 13,000xg for one minute to pellet DNA. Following centrifugation, supernatants were decanted and DNA washed with 250µl of 70% ethanol by gentle inversion. DNA in 70% ethanol was centrifuged at 13,000xg for one minute to pellet DNA and ethanol removed by pipette. Tubes were inverted and allowed to air dry for 10 minutes at room temperature. DNA was then suspended in 100µl of Rehydration Solution/tube and incubated at 65°C for 60-120 minutes. Genomic DNA was quantified by spectrophotometry and visualized by electrophoresis on 1% argrose gels. After rehydration, DNA was stored at 4°C until utilized.

Quantitative real time PCR (Q-RT-PCR) was performed using primers [see Table 3.1] specific for genomic DNA sequences from *E. coli* (16S ribosomal), *M. paratuberculosis* (IS900) and *bos taurus* (GAPDH). As bacteria are destroyed within BOMAC cells, their DNA will degrade. This destruction of bacterial DNA will lead to fewer templates, either 16S or IS900, for real time PCR primers in proportion to the total amount of genomic DNA collected (BOMAC and bacterial DNA). Conversely if the bacteria are able to avoid destruction within BOMAC cells, bacterial DNA will remain intact and continue to be used as template for real time PCR. Primers were designed using Primer Express Version 2.0 (Applied Biosystems, Foster City, CA) [see Table 3.1] and real time PCR was preformed by the following protocol.

In duplicate wells, purified genomic DNA from each time point (0, 1, 12, 24, 48, 72, and 96 hours) was used as template in amplification of GAPDH and either IS900 or 16S ribosomal DNA (treatment dependant). Amplification was preformed in an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using the default 2-step amplification procedure and 2X SYBR Green Master Mix (Applied Biosystems Foster City, CA) in 50µl reaction volumes with 300nM of each primer set and 30-100ng of mixed genomic DNA (BOMAC and bacteria). After amplification cycles to threshold (Ct) were converted to amounts of starting template by comparison to standard curves generated for GAPDH, IS900 and 16S ribosomal DNA and solving the equation $y=mx+b$ (where m is the slope of the linear standard curve, b is the y-intercept of that linear line in Ct, x is the $\log_2$ of the template concentration and y is the Ct of the sample) for the template concentration. Once total amount of template material had been determined, a simple ratio of bacteria DNA (16S or IS1900) to BOMAC DNA (GAPDH)

determined amounts of bacterial genomic DNA relative to amounts of BOMAC genomic DNA. As stated above this ratio becomes smaller as bacteria are being destroyed by phagocytosis within BOMAC cells, but remains constant if there is no bacterial destruction or if amounts of bacterial and BOMAC starting templates decrease at equal levels.

C. Cloning of Amplicons

DDRT-PCR amplicons suspected of representing differentially expressed transcripts (see Chapter 2) were ligated into pGEM-T Easy plasmids using the pGEM-T Easy System I (Promega Corp., Madison WI) that inserts DNA into a *lacZ* reporter gene. Briefly, 5µl of PCR amplified amplicon (~100ng) was combined with 2X ligation buffer (60mM Tris-HCl [pH 7.8], 20mM $MgCl_2$, 20mM DTT, 2mM ATP and 10% PEG [polyethylene glycol-8000]), 50ng of pGEM-T Easy plasmid vector, and 1U T4 DNA ligase (in 10mM Tris-HCl [pH 7.4], 50mM KCl, 1mM DTT, 0.1mM EDTA and 50% glycerol). This mixture was incubated for 2 hours at room temperature (~25°C). Newly prepared plasmids with inserted amplicons were then used to transform competent *E. coli*-DH5α.

E. coli-DH5α, were removed from –80°C and allowed to thaw on ice. 50µl aliquots (one for each plasmid) of bacteria were added to sterile ice chilled 1.5ml Eppendorf tubes. One quarter of the plasmid ligation reaction (5µl) was added to competent *E. coli* and mixed gently by pipette. Transformation mixtures were then incubated for 30 minutes on ice followed immediately by a heat shock of 42°C for 45 seconds. Heat shock was directly followed by incubation for 2 minutes on ice (cold

shock). After incubation, 900µl of room temperature SOC medium was added to each Eppendorf tube containing the newly transformed cells.

Transformed *E. coli* were incubated at 37°C for 60 minutes with 250rpm agitation, as recommended by the manufacturer, to allow expression of plasmid encoded ampicillion resistance (Amp^r) used as a selectable marker. Following incubation, bacteria were centrifuged at 1000xg to pellet cells and growth media decanted. Bacteria were suspended in 100µl of fresh room temperature SOC media and immediately spread onto 60mm^2 LB agar plates with 100µg/ml ampicillion, 20mM IPTG (isopropylthio-β-galactoside), and 0.5ng X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). Plates were covered and allowed to dry at room temperature (approximately 10 minutes), then inverted and incubated overnight at 37°C.

After overnight incubation, 10-20 colonies from each amplicon were picked utilizing blue/white screening (blue colonies arise from *E. coli* transformed with plasmids that retain a functional *lacZ* gene and therefore have no inserted amplicon). Individual colonies were added to 5ml of LB media with 100µg/ml ampicillion and grown overnight at 37°C with agitation. After overnight growth, 1ml of the culture was retained at -80°C as a glycerol stock. Remaining transformed bacteria were lysed for plasmid recovery. Recovery of plasmids was preformed using the Promega Wizard Plus Miniprep DNA Purification System (Promega Corp., Madison WI).

Briefly, bacterial cultures were centrifuged at 1600xg to pellet cells and facilitate removal of growth media. After removing growth media by vacuum, bacteria were resuspended in 200µl of Cell Resuspension Solution/culture and transferred to 1.5ml Eppendorf tubes. Cell Lysis Solution (200µl) was then added to each tube and tubes

inverted 4-6 times to facilitate cell lysis. Cell lysis was brought to a halt by the addition of 200µl of Neutralization Solution and inversion 4-6 times. Tubes were centrifuged at 13000xg for 20 minutes to separate cell lysate from bound protein. After centrifugation cell lysate was loaded onto a DNA binding column with 1.0ml of DNA Binding Resin and incubated for 60 seconds at room temperature. Lysate mixture was pulled through the DNA binding column with vacuum and each column washed with 2.5ml of Column Wash Solution. Columns were removed from vacuum and transferred to a 1.5ml Eppendorf tubes and centrifuged at 13000xg for 2 minutes to remove residual Column Wash Solution. After centrifugation, columns were transferred to new 1.5ml Eppendorf tubes and 50µl of 65°C double distilled water (ddH_2O) added to each column to elute DNA. Columns and water were incubated for 60 seconds at room temperature followed by centrifugation at 13,000xg for 30 seconds. Insertion of DDRT-PCR derived amplicons was checked by restriction digest and PCR amplification. Quality of plasmid and insert DNA was observed by gel electrophoresis on 1% agarose gels.

D. DNA Sequencing

Cloned, DDRT-PCR derived amplicons were sequenced using an ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City CA) according to the manufacture's protocol. Briefly, plasmids containing amplicons were purified from *E. coli*-DH5α as stated above and between 250-500ng of plasmid DNA was mixed with 8.0µl of Terminator Ready Reaction Mix (A-Dye Terminator, C-Dye Terminator, G-Dye Terminator, T-Dye Terminator, dTTP, dITP, dCTP, dATP, thermal stable pyrophosphatase, $MgCl_2$ and AmpliTaq DNA Polymerase FS), 0.16µM SP6 primer, and 0.16µM M13-Forward primer. Once mixed reactions were subjected to the

following thermalcycling conditions: [96°C, 10 seconds; 50°C, 5 seconds; 60°C, 4 minutes][25]. The now fluorescently labeled amplicons were purified with the following protocol.

Sequencing reactions were transferred from 0.2ml thin walled PCR tubes to 1.5ml Eppendorf tubes where 50µl of ethanol and 2.0µl 3M sodium acetate were added to each reaction. This mixture was vortexed and placed at -20°C for 2 hours to precipitate labeled DNA. After precipitation, DNA was centrifuged at 13000xg for 30 minutes at 4°C to pellet DNA. Supernatants were decanted and pelleted DNA rinsed with 250µl of 70% ethanol. DNA was centrifuged at 13000xg for 5 minutes at 4°C and supernatants decanted. Next, DNA was centrifuged under vacuum for 5 minutes until dry. Finally 6µl of loading buffer (25mM EDTA [pH 8.0] with 50mg/ml Blue Dextran and formamide at a ratio of 1:5) was added and DNA suspended by a combination of heating to 95°C for 2 minutes and vigorous vortexing.

Sequencing gels were prepared using the protocol described in Chapter 2 for DDRT-PCR amplicon separation gels. Briefly, a 0.2mm thick 5.2% Bis-acrylamide gel was poured between sequencing grade glass plates and allowed to polymerize. Once, polymerized sequencing gels were placed into an ABI 373 Automatic DNA Sequencing Apparatus (Applied Biosystems, Foster City CA) and 1X TBE (Tris-HCl, Boric Acid and EDTA) buffer added to the top and bottom buffer chambers. Finally, DNA sequencing samples were heated to 95°C for 4 minutes and loaded into wells on the sequencing gel. The 373 Sequencing Apparatus was then activated and run overnight (16 hours) where a combination of laser excitement of fluorescent dyes and computer recording yielded DNA sequences for the various amplicons.

E. Sequence Analysis and Identification

Amplicon sequences were visualized and edited using Chromas Software (Technelysium, Queensland, Australia). After editing, DNA sequences were compared to known nucleotide sequences available in the NCBI public databases (Genbank NR) using BLASTN (Basic Local Alignment Research Tool for Nucleotides) analysis to discover probable identifications. Amplicon sequences were also translated into protein sequences (in all six frames) and compared to known protein sequences on the NCBI PIR database by using BLASTX (Basic Local Alignment Research Tool for Protein) to determine if translated DNA sequences showed any similarity to proteins in public databases. Finally, DNA sequences were compared to a database of proteins that had been back translated into DNA using the tBLASTx search tool.

F. Northern Blot Hybridization

Total BOMAC RNA was collected from each of the four phagocytic treatments and 5-10μg of RNA for each treatment (depending on the gel) mixed with equal volumes of formamide containing loading buffer and heated at 65°C for 10 minutes to denature RNA. RNA was loaded into the wells of 1% denaturing formaldehyde/agarose gels and electrophoretically separated. Electrophoresis conditions were 60-80V for 3-5 hours (again depending on the gel). Total BOMAC RNA and Millennium RNA Sizing Ladders (Ambion Inc., Austin, TX) were run in the first and second lanes of the gel, respectively. After electrophoresis, these two lanes were excised from the gel and stained with ethidum bromide. Distances traveled through the denaturing argrose gel were measured for each size standard. This data was used to plot distance vs. size (standard plot). When

Northern hybridizations were preformed, distances to DDRT-PCR amplicon hybridization bands were compared to the standard plot for hybridization band size.

Separated treatment RNAs, which remained on the remainder of the denaturing gel, were transferred to nylon membranes (Hybond XL, Amersham Pharmacia Biotech., Buckinghamshire, UK) using the overnight (15-18 hours) "wicking technique" in 20X SSC (Sodium Citrate and Sodium Chloride) (Ausubel et al., 1987). Following transfer, UV cross-linking was preformed to fix RNA to membranes. Final membranes were stored at –20°C until utilized. Before probing blots with radiolabeled [α-^{32}P] cDNA probes, Northern blots were removed from –20°C and allowed to soak in room temperature 6X SSC for 15 minutes to reduce background.

Northern blot hybridization probes were derived from amplicons eluted from DDRT-PCR gels [see Chapter 2] and then cloned [see above]. Cloned amplicons were PCR amplified using the following protocol: 50ng of plasmid was mixed with 10X PCR Buffer (200mM Tris-HCl [pH 8.4], 500mM KCl), 2μM SP6 primer, 2μM M13-Forward primer, 0.5mM dNTP mix (dATP, dTTP, dGTP, and dCTP) and 5U Taq Polymerase and the following conditions: 95°C, for 10 minutes, [94°C, 60 seconds; 56°C, 60 seconds; 72°C, 60 seconds]^{30} 72°C, 10 minutes. Next, amplicons were radioactively labeled using a Random Prime Labeling Kit (Invitrogen Life Technologies, Inc., Gaithersburg, MD) as described by the manufacturer but with some modifications. Briefly, 100ng of PCR amplified DNA (plasmid insert) was denatured at 95°C for 10 minutes and mixed with 50μCi [α-^{32}P] dCTP, 20μM dTTP, 20μM dATP, 20μM dGTP, 15μl of Random Primers Buffer Mixture (0.67M HEPES, 0.17M Tris-HCl, 17mM MgCl2, 33mM 2-mercaptoethanol, 1.33mg/ml BSA, 18 units/ml O.D. 260 oligodeoxyribonucleotide

primers (hexamers), pH6.8) and 3U Klenow Fragment. Mixture was allowed to incubate for 2 hours at room temperature and reactions halted by the addition of EDTA to a final concentration of 0.2M. The radiolabeled probe was separated from unincorporated [α-^{32}P] dCTP by a 30 second centrifugation at 2000xg through a Sephadex G-50 exclusion chromatography column (Amersham Pharmacia Biotech., Buckinghamshire, UK). The radioactive probes were then used for Northern blot hybridizations.

Prior to hybridization membranes were blocked in a hybridization oven for 2 hours at 68°C with 1mg of heat-denatured (10 minutes at 95°C) sheared herring (or salmon) sperm DNA in 5ml of Perfect Hyb Solution (Sigma Chemical Corp, St. Louis, MO). Blocking solution was decanted and $2x10^6$- $2x10^7$cpm of heat-denatured probe with 0.5mg heat-denatured sheared herring (or salmon) sperm DNA in 5ml of Perfect Hyb Solution added to Northern blot membranes. Membranes were allowed to hybridize with radiolabeled probes overnight (16-18 hours) at 65-68°C (depending on the Northern blot). Following hybridization, membranes were removed from the radioactive hybridization solution and washed twice at room temperature in 2X SSC, 0.1% SDS and then twice at 65°C in 0.1X SSC, 0.1% SDS to remove unbound probe. Washed membranes were exposed to X-ray film with intensifying screens and incubated at –80°C for 24-48 hours prior to development using a Futura 200E Automatic X-ray Film Processor (Fischer Industries Inc., Geneva IL).

IV. Results

A. Bacterial Survival

Quantifying amounts of bacterial DNA utilizing Q-RT- PCR and then normalizing those amounts to amounts of BOMAC DNA in each time point sample

yielded two dramatically different curves (one for GAPDH and one for bacterial 16S or IS900 amplification) even though the quantities of DNA obtained from each time point across both treatments were strikingly similar [see Figure 3.1]. Table 3.2 contains relative mean values for *E. coli* DNA normalized to amounts of BOMAC DNA present within each time points from zero to 96 hours post introduction and corresponding standard errors for each relative mean value. Data from this table are illustrated in Figure 3.2 where the x-axis represents time of incubation until collection and the y-axis represents relative mean normalized DNA quantities. Data presented in Table 3.2 and Figure 3.2 suggests that *E. coli* are undergoing destruction as soon as one hour after introduction and fully degraded within BOMAC cells by 12 hours post introduction of bacteria. At all collection time points amounts of *E. coli* DNA were determined to be significantly lower by students t-test ($p > 0.01$) than levels observed at time point zero.

M. paratuberculosis are successfully ingested by BOMAC cells [see Appendix Three: Figure A3.1] but in contrast to *E. coli*, *M. paratuberculosis* are not degraded by BOMAC cells within the 96-hour time course [see Table 3.3, Appendix Three: Figure A3.2]. When data from Table 3.3 were represented graphically, [see Figure 3.3] it was observed that relative amounts of GAPDH normalized *M. paratuberculosis* DNA fluctuated over the time course but that overall amounts of DNA did not diminish significantly throughout 96-hour post treatment. This failure of BOMAC cells to degrade *M. paratuberculosis* following phagocytosis is consistent with observations of *M. paratuberculosis* survival within bovine peritoneal macrophages (Tessema et al., 2001).

B. DNA Sequencing, Sequence Analysis and Identification

Direct DNA sequencing and BLASTN analysis of cloned amplicons [Appendix 2] representing differentially expressed genes were used to determine putative gene identification and function [see Table 3.5]. DNA sequence of amplicon 3-1-4 (upregulated by *E. coli* and latex beads but not *M. paratuberculosis*) was highly similar to that of a *Rattus norvegicus* nucleolin-related protein (NRP) mRNA (Genbank NM_022621) with 90% similarity over 379bp and a corresponding expectation value of e^{-131}. DNA sequence of amplicon 5-4-2 was 87% similar to a hypothetical Homo sapiens protein, MGC14817, mRNA (Genbank NM_032338) over 369bp and a corresponding expectation value of e^{-104}. Sequence derived from amplicon 4-1-6 yielded an 85% similarity over 145bp to Homo sapiens mRNA encoding epithelial stromal interaction 1 protein (BRESI-1) with an expectation value of 4x e^{-24} (Genbank AF396928.). DNA sequence for amplicon 5-2-10 was highly similar to the bovine sequence for Nicotinamide adenine dinucleotide dehydrogenase (F NADH dehydrogenase) subunit 1 (Genbank AF493542). This identification yielded an expectation value of zero and 100% similarity over 339bp. Further DNA sequence comparisons were preformed utilizing the BLASTX program available from NCBI that translates DNA sequence into protein sequences in all 6 open reading frames (3 forward and 3 reverse) [see Table 3.5].

Comparison of translated DDRT-PCR derived amplicon sequences with the NCBI protein database (PIR) confirmed identifications obtained utilizing DNA nucleotide BLASTN. Translated sequences from amplicons 3-1-4 and 5-2-10 again showed similarity to the nucleolin related protein (Genbank AAH05460.1) and NADH dehydrogenase subunit I protein (Genbank NP_008095), respectively, while amplicons 5-

4-2 and 4-1-6 showed similarity to the hypothetical protein MGC1487 (Genbank NP_115714) and BRESI-1 protein (Genbank AAH23660).

As a final confirmation of amplicon identities, another type of BLAST program was utilized to compare amplicon sequences to the NCBI (Genbank NR) database [see Table 3.5]. The tBLASTx program performs a back translation of all protein sequences in the NCBI database. This database of back translated DNA sequences are then compared to query DNA sequences, in this case DDRT-PCR amplicons, for probable identification. Results from the tBLASTx are outlined in Table 3.5, and were identical to those obtained by both nucleotide BLAST and BLASTX procedures.

C. Northern Blot Hybridization

Probing of Northern blots made from total BOMAC cell RNA from the three phagocytic treatments (M, E and PC) and the no phagocytosis control (NC) with each cloned amplicon insert resulted in [see Figure 3.5, 3.6, 3.7 and 3.8] a single band in each treatment which hybridized to corresponding RNA transcripts. Band intensities were determined using densitometry and normalized to corresponding treatment GAPDH transcript intensities to account for potential RNA loading differences. Phagocytic treatment gene expression levels were then calibrated to no phagocytosis (NC) control levels for comparison across the three phagocytic treatments.

Amplicon 3-1-4 [see Figure 3.4] yielded a single band with hybridization to an RNA transcript of ~3.1Kb. Transcript levels (represented by amplicon 3-1-4) in BOMAC cells exposed to *M. paratuberculosis* (M) were similar to levels observed in negative control or non-challenged BOMAC cells (NC) [see Table 3.4]. Conversely, BOMAC cells exposed to latex beads (PC) or *E. coli* (E) increased expression of the 3-1-

4 transcript, by 8-fold and 10.5-fold respectively, over expression levels observed in negative control BOMAC cells (NC). Direct comparison of normalized 3-1-4 transcript levels from treatments M and E yields a greater then 10.6-fold higher level of expression in BOMAC cells following phagocytosis of *E. coli* (E) relative to similar cells following phagocytosis of *M. paratuberculosis* (M). We conclude that amplicon 3-1-4 represents a subset of genes whose expression levels are up regulated following phagocytosis of *E. coli* and latex beads but not *M. paratuberculosis* [see Figure 3.4].

Amplicon 5-2-10 [see Figure 3.5] hybridized to distinct RNA transcripts of 1.1Kb in RNA from each of the four treatments. Amplicon 5-2-10 transcript levels in BOMAC cells following phagocytosis of *M. paratuberculosis* were slightly higher (1.7-fold) then levels observed in negative control or non-challenged BOMAC cells (NC) [see Table 3.4]. In contrast, BOMAC cells exposed to latex beads (PC) or *E. coli* (E) yielded 5-2-10 expression levels, 5.7-fold and 10.3-fold respectively, higher then levels observed in negative control or non-challenged BOMAC cells (NC). Direct comparison of amplicon 5-2-10 transcript levels in M and E treatments showed 5.8-fold higher expression levels in BOMAC cells following phagocytosis of *E. coli* (E) relative to similar cells following phagocytosis of *M. paratuberculosis* (M). We conclude that amplicon 5-2-10 represents genes whose expression is increased following phagocytosis of all three phagocytic stimuli, but expression levels are markedly higher with either *E. coli* or latex beads then with *M. paratuberculosis*.

Northern blot hybridizations for amplicon 5-4-2 hybridized to an RNA transcript of 750bp in RNA from each of the four treatments [see Figure 3.6]. Amplicon 5-4-2 transcript levels in BOMAC cells exposed to *M. paratuberculosis* (M) were 5.5-fold

lower then levels observed in negative control or non-challenged BOMAC cells (NC) [see Table 3.4]. Conversely, BOMAC cells exposed to latex beads (PC) or *E. coli* (E) have only nominal (1.4-fold and 1.5-fold respectively) increases in expression of the 5-4-2 transcript relative to negative control BOMAC cells (NC). Direct comparison of normalized transcript 5-4-2 levels from treatments M and E yields a greater then 8.5-fold higher expression level in BOMAC cells following phagocytosis of *E. coli* (E) relative to similar cells following phagocytosis of *M. paratuberculosis* (M) [see Table 3.4]. We conclude that expression of the gene represented by amplicon 5-4-2 is specifically repressed following phagocytosis of *M. paratuberculosis* (M), but not latex beads (PC) or *E. coli* (E).

Finally, amplicon 4-1-6 hybridized to an RNA transcript of ~1.6Kb in RNA from the four treatments [see Figure 3.7]. Amplicon 4-1-6 transcript levels were suppressed in all three phagocytic treatments [see Table 3.4]. Interestingly, 4-1-6 transcript levels in BOMAC cells following phagocytosis of *M. paratuberculosis* (M) were 3.3-fold lower then levels observed in BOMAC following phagocytosis of *E. coli* (E). Amplicon 4-1-6 therefore represents genes that appear to be repressed following the general process of phagocytosis although levels of repression across the three phagocytic treatments appear to be stimuli specific.

V. Discussion

M. paratuberculosis is the casual agent of Johne's disease in ruminants and responsible for substantial world wide economic losses ($200 million/ year) to the dairy and beef industries worldwide (Jones et al., 1989). Understanding how this bacterium is able to suppress or redirect host immune responses and specifically how it is able to

survive within the hostile environment of host macrophage phagosomes may aid development of treatments for this disease in cattle as well as similar diseases in other animals, possibly including humans afflicted with Crohn's disease, leprosy or tuberculosis.

As outlined in Chapter 2 our group used DDRT-PCR as a primary screening tool to examine differences in gene expression 60 minutes after introduction of three phagocytic stimuli representing: 1) bacteria that are readily degradable (*E. coli*); 2) bacterium that are not degraded (*M. paratuberculosis*); and 3) latex beads as a positive control for macrophage phagocytosis but not macrophage phagocytic destruction and PBS as a negative control for phagocytosis. By using the immortalized bovine macrophage cell line BOMAC in all experiments we eliminated levels of variability that would be introduced by repeated harvesting and *ex vivo* stimulation of monocyte derived macrophages (MDM). This cell line exhibits many well-characterized properties of macrophages including the ability to phagocytize particles (Stabel and Stabel, 1995). For BOMAC cells to be useful in this study it was necessary to demonstrate that BOMAC cells also behaved like primary bovine macrophages during phagocytosis of bacteria such as *E. coli* and *M. paratuberculosis*.

Utilizing quantitative real time PCR (Q-RT-PCR) it was possible to quantify amounts of bacterial DNA relative to total DNA (bacterial and BOMAC) and thus relative amounts of intact bacteria, by using bacterial DNA as a template for amplification. Primers were designed to flank DNA regions specific for *E. coli* (16S), *M. paratuberculosis* (IS900) and the BOMAC cell line (GAPDH). At time points along the

96-hour incubation, challenged BOMAC cells were harvested and total genomic DNA collected and subjected to real time PCR for detection of bacterial DNA.

Based on Q-RT-PCR analysis of IS900 elements, *M. paratuberculosis* putatively survived for at least 96 hours within BOMAC phagosomes, while amounts of *E. coli* DNA were determined significantly reduced by students t-test ($p > 0.01$) within 60 minutes after bacterial introduction. By 12 hours following introduction of bacteria, *M. paratuberculosis* DNA was still at levels similar to the starting concentration but *E. coli* DNA was reduced to levels of zero. These data support that BOMAC cells are able to readily phagocytize both *E. coli* and *M. paratuberculosis* and that *E. coli* are readily destroyed by BOMAC cells while *M. paratuberculosis* remains intact. *M. paratuberculosis* DNA levels are not an artifact of BOMAC destruction (see caveat to bacterial survival) as there were similar concentrations of total genomic DNA obtained at each time point throughout the time course.

Of the more then 380 amplicons derived by DDRT-PCR, only 6 were cloned into plasmid vectors and used as probes for Northern blot hybridizations. This low number of cloned amplicons is due to an inability to amplify DDRT-PCR derived amplicons to sufficient levels where ligation into plasmids was possible. Of the six amplicons that were successfully cloned into plasmid vectors, four were sequenced and used for Northern blot hybridization probes.

Amplicon 3-1-4 was identified as the gene that encodes for the nucleolin-related protein (NRP). Following a 60 minute challenge with *E. coli* or latex beads, NRP gene expression was up regulated greater then 10 fold over gene expression observed in resting BOMAC cells. Conversely in *M. paratuberculosis* challenged cells NRP gene expression

remained at levels consistent with gene expression levels in resting BOMAC cells. NRP is an interesting but not well characterized gene product. As its name implies it is related to the better characterized nucleolin which shares with NRP an ability to bind calcium (Sorokina and Kleinman, 1999). This ability to bind calcium is of primary import in how NRP expression may affect bacterial destruction through the endocytic pathway and generation of oxygen metabolites.

Calcium has many different roles within cells. It has been proposed as a second messenger in the signal transduction relevant to the generation of oxygen metabolites by phagocytic cells (Forehand et al., 1989; Korchak et al., 1988; Tse and Tse, 1999). In fact, increases in cytosolic calcium concentration are essential for activation of the phagocyte respiratory burst, production of nitric oxide, secretion of microbicical granule constituents and synthesis of pro-inflammatory mediators including TNF-α (Denlinger et al., 1996; Kim-Park et al., 1997; Tapper et al., 1996; Watanabe et al., 1996). Calcium is essential for destruction of *M. tuberculosis* (Majeed et al., 1998).

Activation of macrophages has been shown to coincide with a brisk increase in intracellular calcium levels (Marodi et al., 1993). Degradable bacteria such as heat killed *M. tuberculosis* induce a rapid and significant rise in macrophage calcium concentrations while macrophages that have ingested viable *M. tuberculosis* do not have increase in calcium concentrations (Malik et al., 2000). This lack of increase in intracellular calcium concentration associates with mycobacterial survival within macrophages (Malik et al., 2000).

Virulent *M. tuberculosis* are able to inhibit complement receptor mediated calcium signaling. This alteration of macrophage activation contributes to inhibition of

phagosome-lysosome fusion and promotion of intracellular mycobacterial survival. This lack of initiation of calcium signaling may be a general property of pathogenic *M. tuberculosis* and possibly other pathogenic Mycobacteria such as *M. bovis* and *M. paratuberculosis* (Malik et al., 2000). Not only the role of calcium but also the role of calcium binding proteins has been investigated in bacterial destruction.

Both annexin and calmodulin are proteins that have the ability to bind calcium and have been shown to play roles in bacterial destruction through the endocytic pathway. Annexin translocation brings calcium to phagosomes inducing phagosome/ lysosome fusion (Majeed et al., 1998). Calmodulin also associates with phagosomes and is associated with phagosomes that contain virulent *M. tuberculosis* at levels that are lower then levels observed with heat killed *M. tuberculosis* (Malik et al., 2001).

To date NRP has not been investigated for any role in endosome maturation or bacterial destruction, but NRP does have many characteristics that make it an ideal candidate for further study. NRP has been shown to associate with the membrane cytoskeleton in the presence of calcium (Sorokina and Kleinman, 1999). This association may affect stabilization of the cytoskeleton and maturation of phagosomes through the endocytic pathway (Sorokina and Kleinman, 1999). Probably the most significant piece of evidence for NRP involvement in Mycobacterial destruction is described in a paper by Garcia et al. in 2000. In this paper, Mycobacterial infection decreased the amount of "phosphonucleolin" while dead bacilli or LPS showed no diminishing effects (Garcia et al., 2000). This "phosphonucleolin" shows a remarkable similarity to NRP through BLAST analysis and it is possible that Garcia et al. were in fact measuring NRP levels. These data would be consistent with results presented in this paper. Taken together

available data suggests calcium and various calcium binding proteins, such as NRP, are important in destruction of virulent Mycobacteria such as *M. paratuberculosis*.

Expression of the NADH dehydrogenase subunit 1 gene (amplicon 5-2-10) failed to increase above negative control levels during challenge with *M. paratuberculosis*. However, stimulation with *E. coli* increased NADH dehydrogenase subunit 1 gene expression greater than 5 fold. The NADH dehydrogenase subunit 1 gene encodes for a protein that has roles in energy metabolism as well as substrate modification (Yagi and Matsuno-Yagi, 2003; Yano et al., 2002) by catalyzing the initial reaction of the electron transport chain, the reaction of NADH to NAD^+.

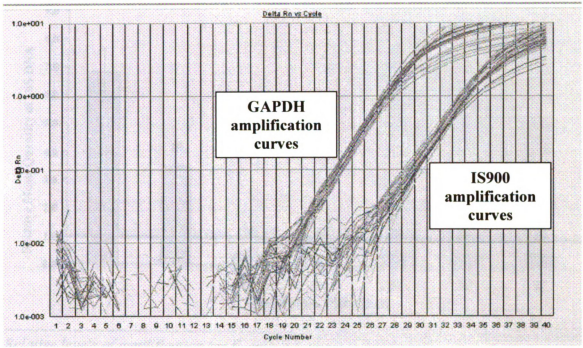
Ragno and colleagues investigated the effect of *M. tuberculosis* on murine macrophage gene expression using DDRT-PCR and observed a change in gene expression that parallels results our group observed for NADH dehydrogenase subunit 1. Data presented by Rango et al. indicated a down-regulation in expression of cytochrome c oxidase subunit VIIc when murine macrophages were challenged with virulent *M. tuberculosis* relative to gene expression levels observed with heat killed *M. tuberculosis*, *M. tuberculosis* BCG or latex beads (Ragno et al., 1997; Ragno et al., 1998). As reviewed by Weiss et al. (Weiss et al., 1991) cytochrome c oxidase catalyzes the terminal reaction in the electron transport chain while NADH dehydrogenase catalyzes the initial reaction.

Interfering with the electron transport chain by suppressing gene expression in the case of NADH dehydrogenase subunit 1 or down-regulating cytochrome c oxidase would allow survival of intracellular Mycobacteria by interfering with an infected macrophage's ability to generate reactive oxygen species, which are important for antimicrobial defense

in macrophages. In this case the NADH dehydrogenase protein, along with the rest of the electron transport chain proteins are embedded in the phagosomal and plasma membranes. Alternatively, suppression of NADH dehydrogenase gene expression could result in altered susceptibility to apoptosis a key feature of Mycobacterial survival. NADH dehydrogenase is imbedded in the mitochondrial cell membrane. Modifying the properties of mitochondrial membranes in infected macrophages may disrupt the macrophages ability to undergo apoptosis (Ragno et al., 1997; Ragno et al., 1998).

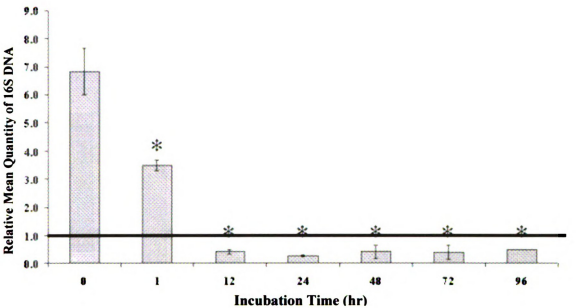
The exact role of gene expression modulation during *M. paratuberculosis* phagocytosis is not yet known. It is possible that *M. paratuberculosis* actively induces changes in gene expression within macrophages and these changes allow for bacterial survival or that there are factors such as bacterial cell wall components responsible for these changes in gene expression. By studying how *M. paratuberculosis* and other Mycobacteria affect their chosen niche we will gain insight on how this intracellular bacteria is able to live in this hostile environment. Continuing work will reveal how and why these changes come about on a molecular level.

Figure 3.1 – Bacterial Survival Quantified by Real Time PCR.



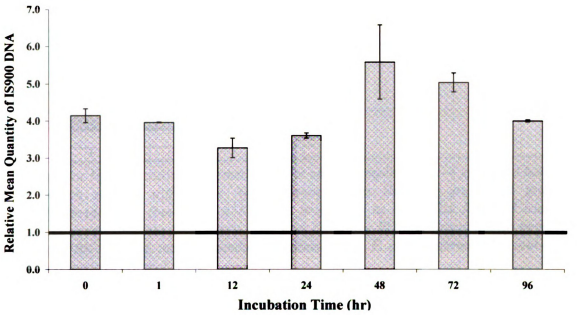
The Real Time PCR amplification plot shown above clearly shows two distinct groups of DNA amplification. The curves ion the center of the picture is those generated using the primers designed to amplify GAPDH from the BOMAC genomic DNA. While the curves on the right side of the figure are from primers designed to flank the specific M. paratuberculosis DNA IS900 sequence. A similar set of curves were generated using E. coli challenged BOMAC (GAPDH and 16S respectively).

Figure 3.2 – Survival of *E. coli* in BOMAC Cells.



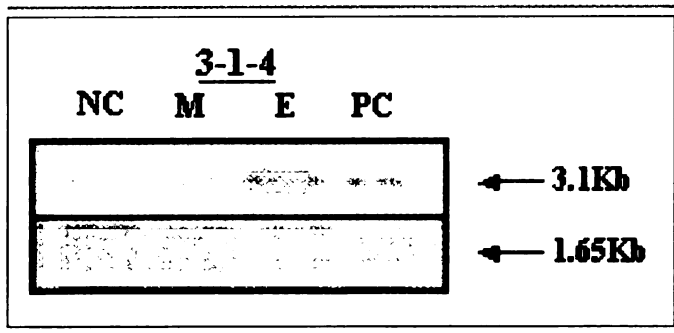
Relative levels of amplification for E. coli 16S ribosomal DNA decreases significantly by students t-test (*) ($p > 0.01$) across all time points (1, 12, 24, 48, 72 and 96 hours) when compared to time point zero hour. Relative amounts of GAPDH (BOMAC template) remain constant across all time points (line) and thus this decrease in template ratio implies decreasing levels of E. coli across the time course.

Figure 3.3 – Survival of *M. paratuberculosis* in BOMAC Cells.



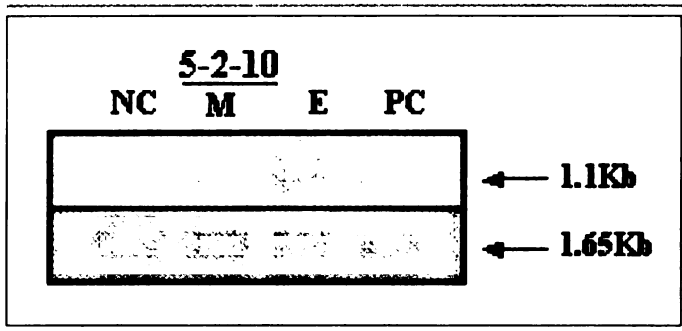
Amounts of M. paratuberculosis IS900 template DNA relative to BOMAC template DNA appear to decrease slightly by 12 hours but increase again to levels higher then starting levels by 48 hours. By 96 hours post introduction, relative levels of IS900 template DNA return to levels observed at time point zero implying survival of M. paratuberculosis following phagocytosis by BOMAC cells. Relative levels of GAPDH (BOMAC template) are constant across all time points (line). Therefore, changes in ratios reflect changes in M. paratuberculosis concentration.

Figure 3.4 - Northern Blot Hybridization Utilizing the Cloned Amplicon 3-1-4.



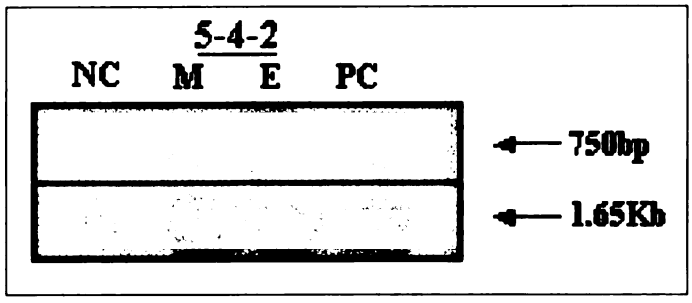
Northern Blot Hybridization preformed with the cloned amplicon 3-1-4 yielded a band at 3.1Kb which showed differential hybridization across the four treatment groups. GAPDH hybridization (band at 1.65Kb) was preformed to normalize for RNA loading differences across the four treatments. The four lanes are BOMAC treated with negative control of PBS (NC), M. paratuberculosis treatment (M), E. coli treatment (E), and latex beads as a positive control (PC).

Figure 3.5 – Northern Blot Hybridization Utilizing the Cloned Amplicon 5-2-10.



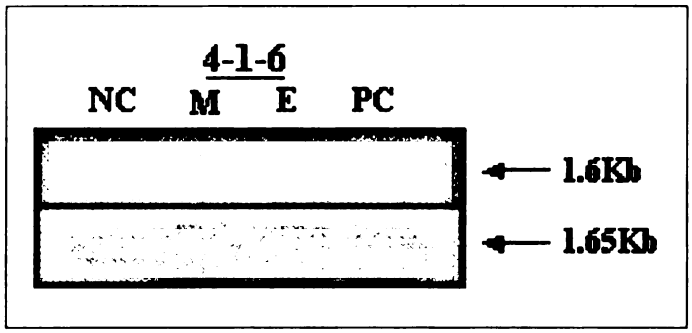
Northern Blot Hybridization preformed with the cloned amplicon 5-2-10 yielded a band at 1.1Kb which showed differential hybridization across the four treatment groups. GAPDH hybridization (band at 1.65Kb) was preformed to normalize for RNA loading differences across the four treatments. The four lanes are BOMAC treated with negative control of PBS (NC), M. paratuberculosis treatment (M), E. coli treatment (E), and latex beads as a positive control (PC).

Figure 3.6 - Northern Blot Hybridization Utilizing the Cloned Amplicon 5-4-2.



Northern Blot Hybridization preformed with the cloned amplicon 5-4-2 yielded a band at 750bp which showed differential hybridization across the four treatment groups. GAPDH hybridization (band at 1.65Kb) was preformed to normalize for RNA loading differences across the four treatments. The four lanes are BOMAC treated with negative control of PBS (NC), M. paratuberculosis treatment (M), E. coli treatment (E), and latex beads as a positive control (PC).

Figure 3.7 – Northern Blot Hybridization Utilizing the Cloned Amplicon 4-1-6.



Northern Blot Hybridization preformed with the cloned amplicon 4-1-6 yielded a band at 1.6Kp which showed differential hybridization across the four treatment groups. GAPDH hybridization (band at 1.65Kb) was preformed to normalize for RNA loading differences across the four treatments. The four lanes are BOMAC treated with negative control of PBS (NC), M. paratuberculosis treatment (M), E. coli treatment (E), and latex beads as a positive control (PC).

Table 3.1 – Real Time PCR Primers.

Primer Name	Sequence	Fragment Size (bp)
GAPDH-FORWARD	5'-GCA TCG TGG AGG GAC TTA TGA	61
GAPDH-REVERSE	5'-GGG CCA TCC ACA GTC TTC TG	
16S-FORWARD	5'-CGT GTT GTG AAA TGT TGG GTT AAG	63
16S-REVERSE	5'-CCG CTG GCA ACA AAG GATA	
IS900-FORWARD	5'-TGG TAG CCA GTA AGC AGG ATC A	60
IS900-REVERSE	5'-TGG AAC GCG CCT TCG A	

This table contains the forward and reverse sequences for all three sets of primers designed and employed for real time PCR bacterial survival (GAPDH for BOMAC cells, 16S for E. coli, and IS900 for M. paratuberculosis.

Table 3.2 – Quantification of *E. coli* Survival Using Real Time PCR.

Time (hr)	Mean Normalized DNA	Stderr
0	6.82	0.8351
1	3.48	0.2014
12	0.41	0.0879
24	0.27	0.0421
48	0.42	0.2521
72	0.40	0.2700
96	0.47	0.0000

Normalized amplification data obtained from Real Time PCR analysis using primers designed to amplifiy genomic DNA from the GAPDH region in BOMAC cells and 16S ribosomal DNA from E.coli.

Table 3.3 – Quantification of *M. paratuberculosis* Survival Using Real Time PCR.

Time (hr)	Mean Normalized DNA	Stderr
0	4.14	0.1898
1	3.95	0.0115
12	3.27	0.2649
24	3.60	0.0694
48	5.57	0.9943
72	5.02	0.2540
96	3.99	0.0288

Normalized Normalized amplification data obtained from Real Time PCR analysis using primers designed to amplify genomic DNA from the GAPDH region in BOMAC cells and IS900 DNA from M. paratuberculosis.

Table 3.4 – Northern Blot Hybridization Information Table.

Clone	Normalized M Treatment Values Fold Difference	Normalized M Treatment Values Result	Normalized E Treatment Values Fold Difference	Normalized E Treatment Values Result	Normalized PC Treatment Fold Difference	Normalized PC Treatment Values Result
3-1-4	-1.01	Failure to activate	10.49	Highly activated	7.93	Highly activated
5-2-10	1.78	Activation at low levels	10.31	Highly activated	5.66	Highly activated
5-4-2	-5.54	Suppressed	1.53	Activation at low levels	1.40	Activation at low levels
4-1-6	-6.70	Suppressed	-2.01	Suppressed	-11.87	Suppressed

Summation of gene expression levels obtained from Northern hybridizations confirms stimuli dependent expression of the four DDRT-PCR derived amplicons. Genes represented by amplicon 3-1-4 show no gene expression activation following M. paratuberculosis phagocytosis but high levels of activation following either E. coli or latex bead phagocytosis. Genes represented by amplicon 5-2-10 show little gene expression activation following M. paratuberculosis phagocytosis but high levels following E. coli or latex bead phagocytosis. Amplicon 5-4-2 represents genes whose expression appears to be specifically suppressed following M. paratuberculosis phagocytosis and activated at low levels following E. coli or latex bead phagocytosis. Finally, amplicon 4-1-6 is represenative of genes whose expression is suppressed following phagocytosis of any of the phagocytic stimuli (M. paratuberculosis, E. coli or latex beads) but the levels of suppression vary across treatment.

Table 3.5 – DNA Sequence BLAST Analysis.

Clone Name	BLASTN Identification	e-value	BLASTX Identification	e-value	tBLASTx Identification	e-value	Probable Function
3-1-4	Rattus norvegicus nucleolin-related protein (NRP), mRNA	e-131	Nucleolin [Mus musculus]	0.002	Rattus Norvegicus Nucleolin-Related Protein (NRP), mRNA	1.00E-31	Caclium Binding/ Sequestering
5-2-10	Nicotinamide adenine dinucleotide subunit I, reduced from (NADPH)	0.0	NADH Dehydrogenase Subunit 1 [Bos taurus]	8.00E-43	Bos Taurus Isolate F NADH Dehydrogenase Subunit 1 (ND1), NADH	1.00E-74	Substrate Modification (electron transport chain), NO induction
5-4-2	Homo sapiens hypothetical protein MGC14817 (MGC14817), mRNA	e-104	Hypothetical Protein MGC14817 [Homo sapiens]	5.00E-31	Homo Sapiens Hypothetical Protein (MGC14817), mRNA	5.00E-49	Unknown
4-1-6	Homo sapiens putative epithelial stromal interaction 1 (BRESI-1), mRNA	4.00E-24	Similar to Epithelial Stromal Interaction 1 (breast) [Homo sapiens]	3.00E-22	Homo Sapiens Epithelial Stromal Interaction 1 (breast) (EPSTI1)	2.00E-43	Unknown

Summation of BLASTN, BLASTX and tBLASTx sequence analysis with probable gene product function. DDRT-PCR amplicon identifications showed the highest amount of similarity (low e-value) when DDRT-PCR nucleotide sequences were directly compared to the NCBI nucleotide data base (BLASTN). For further conformation, DDRT-PCR amplicons were translated in all 6 open reading frames and compared to the NCBI non-redundent protein data base yielding results similar to those obtained using NBLAST. Final conformation of DDRT-PCR derived amplicon identities was obtained by comparing amplicon sequences to NCBI submitted protein sequences that had been reverse translated to nucleotide sequences. Overall, DDRT-PCR amplicon identities matched across the three procedures (NRP, NADH Dehydrogenase subunit I, Hypothetical protein MGC14817 and BRESI-1 respectively).

CHAPTER FOUR

GENEREAL CONCLUSIONS AND DISCUSSION

The process of phagocytosis is essential for an effective host immune response to intracellular bacteria. Although much is known about the general interactions between *M. paratuberculosis* and host macrophages, the molecular mechanisms used by this pathogen to survive are still largely unknown. Intracellular bacteria such as *M. paratuberculosis* are only one of a large number of bacteria that have developed unique and varied strategies to circumvent, redirect, shutdown or stop the process of phagocytosis in resting macrophage cells. By avoiding destruction within macrophage phagolysosomes, Mycobacteria in general and *M. paratuberculosis* specifically, have found a niche in which they can survive and replicate. The hypothesis put forth in this study is that phagocytosis of bacteria that resist destruction, such as *M. paratuberculosis*, would yield observable differences in the pattern of host cell gene expression when compared to gene expression patterns of cells following the phagocytosis of bacteria that are easily degraded.

By utilizing an "open platform" genomic tool such as DDRT-PCR it was possible to identify genes that have differences in expression among treatments involving the phagocytosis of *M. paratuberculosis*, *E. coli*, latex beads or PBS as a negative control for phagocytosis. This functional genomic tool was responsible for the discovery of genes that fail to activate specifically during the phagocytosis of *M. paratuberculosis*, such as genes encoding both the nucleolin related protein and the NADPH subunit I protein.

Also, DDRT-PCR led to the discovery of what appeared to be suppression of gene expression in two other genes. When levels of gene expression during phagocytosis of *M. paratuberculosis* for either of the hypothetical proteins MGC14817 or BRESI-1 are compared to levels of gene expression in no phagocytosis control cells, it is apparent that there is a specific reduction in gene expression following phagocytosis of *M. paratuberculosis*. Discovery of differences in gene expression for these four genes during the process of phagocytosis is significant and opens possible new pathways of research. Despite considerable progress reported here, DDRT-PCR as a technique for comparative gene expression analysis has many major drawbacks.

At the time that Chapter 2 was published in Veterinary Immunology and Immunopathology, our group had excised approximately 380 amplicons from DDRT-PCR gels. By continuing work, our group excised over 450 amplicons from DDRT-PCR gels. Of these 450 plus amplicons approximately 380 amplified successfully. Of these, it was possible to clone 6 amplicons into plasmid vectors. When utilizing the DDRT-PCR protocol, oligonucletide primers and Taq polymerase are utilized to amplify fragments of expressed genes by PCR. By directly comparing levels of gene amplification across the phagocytic treatments it is possible to visualize bacterial dependent differences in macrophage gene expression.

In macrophages, the process of phagocytosis and bacterial destruction can occur rapidly following receptor binding. In this series of experiments a 60 minute time point was utilized to observe initial differences in host macrophage gene expression patterns that may be important to bacterial destruction. Utilizing DDRT-PCR it is possible to observe high levels of both false positive and false negatives (Motlik et al., 1998; Ragno

et al., 1997; Rivera-Marrero et al., 1998; Sung and Denman, 1997). This drawback was understood when our group decided the use DDRT-PCR as an "open platform" genomic tool and we therefore confirmed all observed differences in gene expression by quantifying gene expression in each treatment by Northern hybridization.

Four of the DDRT-PCR derived genes; nucleolin related protein gene, NADH dehydrogenase subunit 1 gene, MGC14817 gene and BRESI-1 gene, differed in their levels of expression between the two phagocytic treatments. Both the hypothetical gene MGC14817 and the BRESI-1 gene have no known function and it is not known how their changes in expression across the phagocytic treatments may impact the survival of *M. paratuberculosis*. It is, however, possible to hypothesize the possible effect that differences in expression of the other two genes may have on *M. paratuberculosis* survival.

Failure to activate the gene for the nucleolin related protein may interfere with intracellular calcium signaling or movement of *M. paratuberculosis* containing vesicles along the macrophages cytoskeleton. Lower levels of NADH dehydrogenase subunit 1 gene expression following *M. paratuberculosis* phagocytosis may cripple the macrophages ability to induce an effective ROS response or reduce the macrophages ability to undergo apoptosis.

This series of experiments has shown that there are indeed vast differences in host macrophage gene expression following the phagocytosis of *M. paratuberculosis*, when compared to either non-challenged macrophages, macrophages challenged with *E. coli* or latex bead challenged macrophages. These differences in gene expression may lead to the investigation of new cellular pathways and hopefully a better understanding of the

molecular mechanisms underlying the ability of *M. paratuberculosis* to survive within bovine macrophages.

CHAPTER FIVE

FUTURE RESEARCH

Discovery of genes that have different expression patterns depending on the type of targets phagocytized was the main goal of this series of experiments. Future research into this area might consist of two main thrusts. The first thrust could be an extension of the current set of experiments.

Even though there are host genes with differences in expression during the phagocytosis of *M. paratuberculosis* when compared to *E. coli* or latex beads, this does not mean that these genes are important for survival of the Mycobacteria within macrophage cells. It is possible that these genes could have very little to do with Mycobacterial survival within macrophages. Therefore, the next stage of research should test the hypothesis that observed gene expression differences are important for survival of *M. paratuberculosis* in host macrophages. To test this hypothesis, overexpression studies, gene knock out studies and protein expression studies should be preformed in primary bovine macrophages and BOMAC cells.

One fairly elegant experiment that could be preformed would involve knocking out the host genes one at a time by various techniques such as RNAi, and exposing the cells to bacteria that are usually quickly degraded, like *E. coli* or killed *M. paratuberculosis*. If these bacteria are able to remain in macrophages without being degraded in phagolysosomes, then the knocked-out gene would appear to be important for *M. paratuberculosis* survival (remember that all four genes discovered were either

down-regulated in gene expression or failed to have any gene expression levels above the negative control during the process of *M. paratuberculosis* phagocytosis). The second thrust that should be undertaken would not involve the genes found in this series of experiments but the attempt to identify more genes that have differences in their expression across the same type of treatment groups.

This attempt at identification could make use of Michigan State University's Center for Animal Functional Genomics (CAFG) high-density bovine specific cDNA microarrays that are now available (Suchyta et al., 2003). By utilizing these bovine specific arrays it would be possible to run experiments designed to determine the effect of *M. paratuberculosis* specifically on host cells. The test treatments could be expanded to include other virulent forms of Mycobacteria such as *M. bovis* or non-virulent Mycobacteria such as *M. bovis* (BCG) and *M. avium*. Other intracellular bacteria could also be used to determine if there are genes that are affected by intracellular bacteria in general. The possibilities of comparison are only limited to funding, the imagination of the investigator and availability of sample. The main focus of these microarray experiments would not even need to be as broad as finding out of host genes are differentially expressed with *M. paratuberculosis* phagocytosis they could be used to elucidate pathways that may be affected during the phagocytosis of *M. paratuberculosis*. This approach would yield data that is much more relevant to disease management and also to an overall understanding of how the bacterium affects its environment.

APPENDIX

APPENDIX ONE

CHAPTER TWO PUBLICAION PREMISSION

April 14, 2003 Our ref: TTookerMSUML4-03

Brian C. Tooker
Michigan State University
1310 University Village, Apt. A.
East Lansing, MI 48823
E-mail: Tookerbr@msu.edu

Dear Dr. Tooker:

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APPENDIX TWO

DNA SEQUENCE INFORMATION

Amplicon 3-1-4

GATTAGCGGATAACAATTTCACACAGGACGACTCCAAGAAAATGGCTCCTCC

CCCAAAGGAGGTAGAAGAAGATAGTGAAGATGAGGAAATGTCTGAAGATGA

AGACGATGAGAGCAGTGGAGAAGAGGTTGTTATCCCTCAGAAAAAAGGCAA

GAAGGCTGCCACAACTCCAGCAAAGAAATGGTGGTTTCCCCAACAAAAAAG

GTTGCAGTTGCCACACCAGCAAAGAAAGCAGTTGTCACCCCTGGCAAAAAGG

CAGCAGCTATGCCAGCCAAGAAGACAGTTACACCTGCCAAAGCAGTGGTTAC

ACCTGGCAAAAAGGGAGCCACCCCAGCAAAGCAGTGGTAGCAACCTCTGGT

AAGAAGGAGCAGCCACCCCAGGCAAGGGAGCAAAGAACGGCAAGAATGCC

Amplicon 5-2-10

TTTTTTTTTTTCATAGTAAGTGTATTAGTTGGTCATAGCGAAATCGAGGGTAG

GATGCTCGGATTCATAGGAAGGATATTGTGAGCAGTAGGGATTTAATGGTAA

AATTGATTGTGTAGAGTTCTGGTATGTGTGGATTGTGGGATGTTCCTAGGAAT

AAAATTGCTGTAAGATATTTATTATGATAATATTTGCGTACTCTGCTATGAAG

AAGGAGGGCAAATGGTCCTGCTGCATATTCTACGTTGAAGCCCGAGACTAGC

TCTGATTCTCCTTCAGTTAAATCAAATGGAGCTCGGTTTGTTTCTGCTAGTGTT

GAGATAAATCATATTATTGCTAGAGGCCATGCTAGC

Amplicon 5-4-2

GATTAGCGGATAACAATTTCACACAGGAGCTAGCAGACTTAAAAGTATTCTT

AAAATAGATGGTGATGTTTTAATGAAAGACGTTCAAGAGATAGCAACTGTGG

TGGAACCCAAACATTGTCAAGAGAAAACGCAGTGTGTGGTGAAGGATGAAA

CAGATGACATGAAAATGGAGACTGATATTAAGAGAAACAAAAAGACTCTTCT

AGACCAGCATGGGCAGTACCCCATATGGATGGACCAGAGGCAAAGGAAAAG

GCTGAAAGCAAAGCGAGAGAGAAGGAAGGGAAAAAGCAAAGTtTAAGCCAT

GAAGGCAGCAAAGGGTTTGACCTGGTAGACTCTAAAAACTTGAAAAATGCAA

CATGAGACAAATCTTTGATTAGAATGTATACATGGATTTCAGCTTCCACCAAA

TGAAAAGTTTATTTCCATTTTTTAACTTGGCCTTTTTCTTC

Amplicon 4-1-6

TTTTTTTTTTGTCACATTTGTAGCCTGAATTAAGAAAATAGGGATACAAATCT

AAAGGTGCCAGAGCTTCCCCAAACCTGATCTGAGAAGCAGAAAAAAATTAGC

TGCTAGTTTATAGAGACAGACAGGCAGGCAGCAGCCACAGCTCTCACTAACC

TGAAAGCATCAAGTGACTCCCTGCTATTCTAGCTTACTAACATGGCTGTAATT

ATCCTTCCAATAAGAGGCAAAACCAATTAGAAAAATAATGCAGCATTTCACT

AGCAGTAGAGATTAAATATGAGTTCAGTAATCTACGCCTACAAATATGCATA

AATAATGACAGGGAGAAGAAACCAGTTATCCTCAGCCGCTCAGCTGAGTGGG

TGGGTAGTTGGAGTCAAGGTAAAAAACAGTGAGGCTGAACAAAGCTGCAAG

AAAGAAAAACATCTCGTGTGGTTTTTCGAGGTCAGTTGACAAAGGCCAGGCA

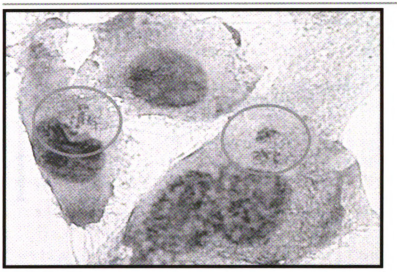
TAAGCAAATATTTCTCATGTATCCCAGCTGTTACCACTATTCATATTCCAATA

ACCACCAAAGTGCTGGAGGCCACCTGGTTGACTTTTGCCTTGGAGTCGTCC

APPENDIX THREE

M. PARATUBERCULOSIS INGESTION AND SURVIVAL IN BOMAC CELLS

Figure A3.1 – Survival of *M. paratuberculosis* within BOMAC Cells.



Microscope picture at 1000x shows M. paratuberculosis stained acid fast (circled) surviving within Hematoxylin stained BOMAC cells 96 hours after phagocytosis.

Figure A3.2 – Survial of *M. paratuberculosis* strains within BOMAC and Monocyte Derived Macrophages (MDM).

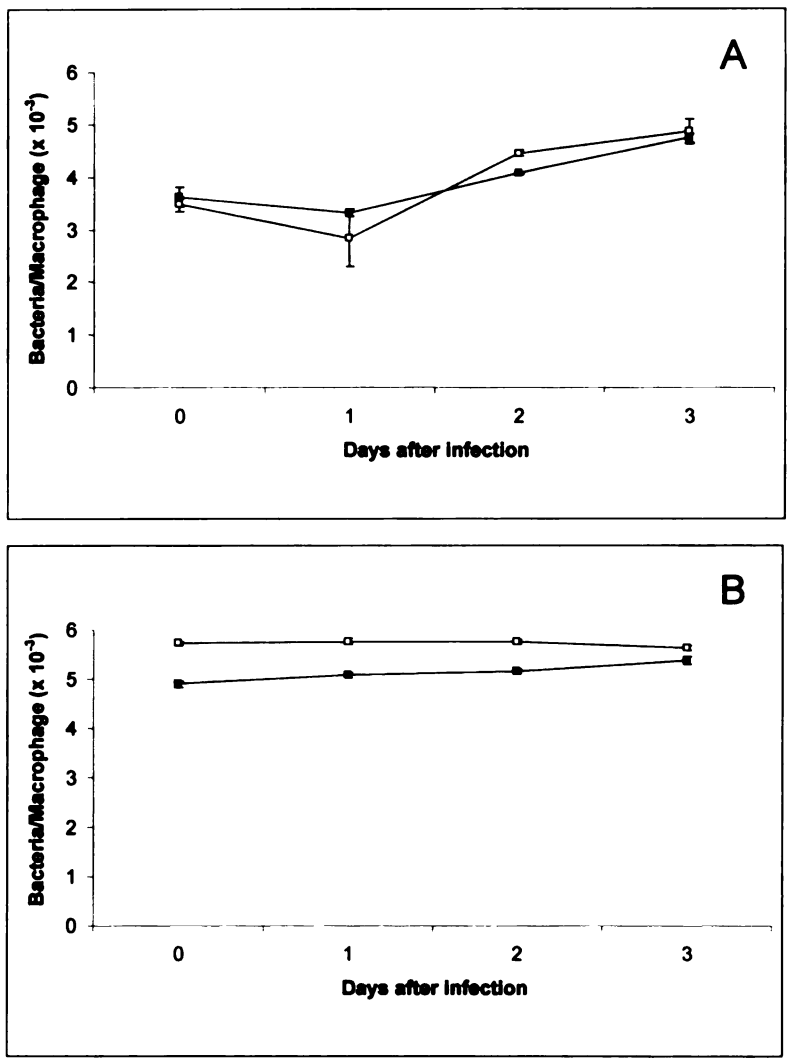


Figure used by permission of N. Beth Harris and Raúl G. Barletta, *Panel A: primary bovine monocytes (MDM). Panel B: BOMAC cells. Open squares represent the wild-type M. paratuberculosis strain K-10 and filled squares are M. paratuberculosis strain K-10 transformed with plasmid pWES4. Cell monolayers were infected with M. paratuberculosis at an moi of 10 bacilli per cell and incubated for 2 h. After removing nonadherent bacteria, monolayers were lysed at the time periods shown and the numbers of viable intracellular bacteria determined by plate counts. Results are shown as the means ± standard deviations of three independent experiments.*

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