MODULATION OF HOST PROTEIN PHOSPHORYLATION UPON MYCOBACTERIAL INFECTION

by

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ABSTRACT

Tuberculosis, caused by *Mycobacterium tuberculosis*, remains the number one killer in the world today due to a single bacterial infection. *M. tuberculosis* primarily infects and resides within the human macrophage where it resists the host killing mechanisms.

Based on the hypothesis that mycobacteria actively interfere with host signaling pathways to promote their own survival, we have employed a novel technique in proteomics to screen for simultaneous changes in host protein phosphorylation upon infection. The human monocytic cell line, THP-1, was differentiated with PMA and used as a macrophage model for infection with live or heat-killed *M. bovis* BCG or treated with the cell wall glycolipid lipoarabinomannan (LAM), known as a potential mycobacterial virulence factor. An infection or treatment time of 24 hours was chosen to study late signaling events associated with the progression of the pathogen within the host cell, as opposed to early events, shared by most microbial and inert particles during early stages of their uptake. Cell lysates were analyzed by employing an array of 31 phospho-specific antibodies covering kinases and other signaling elements from the major eukaryotic signaling networks known to date. Based upon two separate screens, we have identified changes in host signaling pathways that have not previously been described in mycobacterial infection. Six host proteins involved in regulation of apoptotic pathways, cytoskeletal arrangement, calcium signaling and macrophage activation have been identified.

One of the major findings of the screens was an increased phosphorylation of the cytoskeletal protein α-adducin upon mycobacterial infection. α-Adducin binds to actin and spectrin and plays an important role in actin filament rearrangement in eukaryotic cells. Using classical Western blot techniques, we demonstrated that α-adducin undergoes

increased phosphorylation in cells infected with live bacteria compared to those infected with heat-killed bacteria. Furthermore, adducin phosphorylation increases as function of time to reach a maximum at 24 hours. Interestingly, the surface glycolipid LAM was shown to trigger a dose-dependent increase in α-adducin phosphorylation. Moreover, immunostaining experiments and fluorescence microscopy in combination with Western blots have shown that adducin translocates from the cell membrane to the cytosol upon infection. We have also shown that live mycobacteria, in contrast to dead mycobacteria, prevent the assembly of actin around phagosomes during infection.

These findings show an interesting and potentially important relationship between mycobacterial infection and α-adducin activity within the host cell. Given the importance of adducin in regulating actin rearrangements, mycobacterial-induced changes in α-adducin phosphorylation may be involved in the observed inhibition in phagosomal actin assembly around phagosomes containing live bacteria. Thus, α-adducin phosphorylation may play a role in the mechanisms allowing mycobacteria to survive within the host cell.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADF	Actin-depolymerizing-factor
Akt	V-Akt murine thymoma viral oncogene homolog 1
Arp	Actin related protein
ATCC	American type culture collection
AraLAM	Arabinose capped lipoarabinomannan
ATP	Adenosine triphosphate
Bad	Bcl-$_2$ antagonist of cell death
BCG	Bacille Calmette Guerin
Bcl-2	B-cell cll/lymphoma 2
Ca	Calcium
CREB	cAMP response element binding protein
CTIIA	Class II *trans*-activator
DNA	Deoxyribonucleic acid
ECL	Enhanced Chemiluminescence
EDTA	Ethylene-diamide-tetraacetic acid
EEA1	Early endosomal antigen1
EGTA	Ethylene glycol-bis(2-aminoethyl)-N,N,N¢,N¢-tetraacetic acid
ERK	Extracellular-signal regulated kinase
FACS	Fluorescence-activated cell sorting/cell-scanning
GM1	Ganglioside 1
GFP	Green fluorescence protein
GTP	Guanosine tri phosphate
GSK3β	Glycogen Synthase kinase 3β
HBSS	Hanks balanced salt solution
HLA-DR	Human leukocyte antigen isoform-DR
HIV	Human immunodeficiency virus
HRPO	Horseradish peroxidase
IL-1	Interleukin-1
IL-8	Interleukin-8
IL-10	Interleukin-10
INF-γ	Interferon gamma
KPSS	Kinexus phospho-site-screen
JAK	Janus kinase
JNK	c-Jun-N-terminal kinase
LAM	Lipoarabinomannan
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
ManLAM	Mannose capped LAM
MAPK	Mitogen activated protein kinase
Mcl-1	Myeloid cell leukemia 1
MD-2	MD-2 protein
MKK	MAPK kinase
MyD88	Myeloid differentiation primary response gene 88

NF-κB	Nuclear factor-κB
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NR1	NMDA receptor subunit1
OADC	Oleic acid-albumin-dextrose-catalase
PBS	Phosphate buffered saline
PGE_2	Prostaglandin E_2
PI3-K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKCϵ	PKC epsilon
PMA	Phorbol-12-myristate-13-acetate
PMSF	Phenylmethanesulfonyl fluoride
PPD	Purified protein derivative
Rab5	Ras associated protein 5
Rab7	Ras associated protein 7
RB	Retinoblastoma
RNA	Ribonucleic acid
SAPK	Stress activated protein kinase
SDS	Sodium dodecyl sulfate
STAT	Signal transducer and regulator of transcription
SH-2	Src homology 2
TACO	Tryptophan aspartate-containing coat protein
TB	Tuberculosis
Thr	Threonine
TNF	Tumor necrosis factor
TNFR1	TNF receptor 1
Tyr	Tyrosine
μg/ml	Micrograms per milliliter
U/ml	Enzymatic unit per milliliter
WHO	World health organization

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CHAPTER 1: INTRODUCTION

1.1 The epidemiology and history of tuberculosis

More than a century ago Robert Koch identified *Mycobacterium tuberculosis* as the causative agent of "consumption" (92), a disease that was first mentioned in the Greek literature around 460 BC, and described by Hippocrates as the most widespread disease of the time. It is currently believed that *M. tuberculosis* share a common ancestry with the group of mycobacteria comprising the *M. tuberculosis* complex (*M. tuberculosis, M. africanum, M. bovis, M. bovis* BCG, *M. microti and M. canettii*). These bacteria share a 99.9 % similarity on the genomic level but differ significantly in their pathogenicity and host preference (22). Genetic analysis has suggested that the strain of *M. tuberculosis* accountable for the majority of today's cases has arisen from a single clone of bacteria (22). This has led to the hypothesis that *M. tuberculosis* encountered an evolutionary bottleneck 15,000-20,000 years ago before it spread around the world (143).

Today tuberculosis (TB) is the number one cause of death in the world due to a single bacterial infection. It is responsible for 2-3 million deaths per year, primarily in the developing world (125). Additionally, more than seven million new cases of TB are reported annually and this number is estimated to reach ten million by 2015 (160). The dramatic rise in number of cases has been associated with an increased number of immunocompromised individuals due to the HIV epidemic, and additionally with the emergence of multidrug resistant strains, which leads to more severe infection. These strains no longer respond to the first line antibiotics, including isoniazid and rifampin, and pose a major threat for the world as a whole.

1.2 *Mycobacterium tuberculosis* as a human pathogen

Our immune system efficiently protects us from the majority of infectious organisms inhabiting the earth. However, our cohabitation with pathogenic organisms has allowed some of them to develop strategies aimed at circumventing the host defense mechanisms. One prime example is *M. tuberculosis*, which has survived as one of the most successful pathogens in human history.

1.2.1 Mycobacterium tuberculosis

M. tuberculosis belongs to the genus *Mycobacteria* and is classified as an acid-fast, gram-positive microorganism. The bacteria are non-motile, rod shaped organisms approximately 1.0 μm wide and 5-10 μm long. Species of *mycobacteria* are often classified into two groups depending on their growth rate. The slow growers such as *M. tuberculosis, M. bovis, M. avium* and *M. leprae* are associated with human or animal disease, whereas the fast growers such as *M. smegmatis* and *M. phlei* are non-pathogenic. The complexity and the different components of the mycobacterial cell envelope differentiate this species from most other prokaryotes. Three major components comprise the *M. tuberculosis* cell envelope: a plasma membrane, a covalently linked mycolic acid, arabinogalactan and peptidoglycan complex, and a polysaccharide-rich capsule-like material (37). As a consequence of the structural arrangement, the bacterial cell envelope has an unusually low permeability, which has been suggested to play a role in mycobacterial resistance to therapeutic agents (13).

1.2.2 Establishment of infection

The primary route of infection is via the respiratory tract where inhalation of as few as 1-3 bacilli can be sufficient to establish pulmonary tuberculosis (40). *M. tuberculosis* can also establish infection in a variety of human tissues, including bone and skin (40). The alveolar macrophages serve as the first line of cellular defense in the lung and engulf the bacilli through receptor-mediated phagocytosis [reviewed in (51)]. At this primary stage of infection, infected macrophages signal the presence of the pathogen to bystander cells, which leads to initiation of a protective immune response [reviewed in (90)]. A granuloma, consisting of inflammatory leukocytes, surrounds the site of infection and serves to wall off the bacteria and limit further dissemination of the disease. In approximately 90% of cases, infection merely results in a small granulomatous lesion and these individuals can remain asymptomatic and non-infectious for the rest of their lives. Only in immunocompromised individuals, such as newborns, the aged and HIV-positive patients does primary infection lead to active disease. However, even in immunocompetent individuals the protective immune response is sometimes unable to completely eradicate the bacteria. Some bacilli resist the host killing mechanisms and attain a state of dormancy that can last for decades (120). These individuals will remain hypersensitive to tuberculin (purified protein derivative, PPD) and are thought to represent as many as one third of the world's population. Weakening of the immune system due to old age or disease can result in reactivation of tuberculosis. The mechanisms behind reactivation remain unclear, but are seen in connection with a compromised immune response where the host is less capable of restraining the bacteria.

1.3 Host response to *Mycobacterium tuberculosis*

The first line of cellular defense against infection is represented by the host macrophage, the same cell in which the mycobacteria reside. Under normal circumstances, macrophage activation results in a series of events specifically designed to induce killing of engulfed microorganisms. These include: i) the gradual acidification of the phagosome due to the activity of a proton-ATPase pump located in the phagosomal membrane, ii) phagosome-lysosome fusion, which loads the resulting phagolysosome with proteolytic enzymes, iii) induction of reactive oxygen and nitrogen intermediates, and iv) antigen processing [reviewed in (55)]. The resulting acidic and otherwise lethal environment is effectively designed to eliminate invading microorganisms. Macrophages process and present antigens through MCH class I and class II pathways, activating CD8+ and CD4+ T-cell populations, respectively. Activation of T-cells is essential for the development of specific cellular immunity and comprise the second important part of the host defense against mycobacterial infection [reviewed in (90)].

Mycobacteria localize within membrane-bound endocytic vesicles, which allows for antigen presentation through the MCH class II pathway and subsequent activation of CD4+ cells. The ability of mycobacteria to also induce a strong MCH-class I restricted T-cell response was long elusive due to the seclusion of mycobacteria within membranous compartments. However, the mycobacterium-containing phagosome has been shown to be permeable to bacterial antigens and mycobacterial secretion of peptides into the cytosolic space enables antigen presentation through the MHC class I pathway (111, 147). Additionally, infected antigen presenting cells, approaching apoptotic cell death, release vesicle-bound mycobacterial antigens before they die (137). These vesicles are taken up by

bystander cells, such as dendritic cells, which process and present these antigens in MHC class I molecules (137). The CD4+ and the CD8+ both have distinct roles in the defense against mycobacterial infection (91). Upon activation, they are potent producers of IFN-γ and TNF-α, the principal stimulators of macrophage activation and granuloma formation (54, 56). The importance of CD4+ cells is typically seen in HIV-positive individuals, who significantly increase their chance of acquiring an active state of disease due to the systematic depletion of CD4+ cells from their immune system (78). Dendritic cells also express CD1 molecules, which show specificity for mycobacterial glycolipids (123, 153). This mechanism could thereby explain the involvement of the unconventional CD1-restricted T-cells in the protective immune response against mycobacterial infection. These cells can produce high levels of IFN-γ (140) in addition to having a direct cytolytic activity towards infected macrophages that leads to the killing of intracellular bacteria (144). Finally, T cells expressing the $\gamma\delta$-receptor are also involved in the immune response against *M. tuberculosis* (136) and have been shown to regulate granuloma formation (100). Together these cells create a complex set of stimuli that aid in the activation and recruitment of macrophages, as well as in the establishment and maintenance of granulomas. Although pathogenic mycobacteria face a vigorous immune response, they have found ways to circumvent the host killing machinery including reduction of MHC class II directed antigen presentation (67, 119).

1.4 Mycobacteria induce phagosomal maturation arrest

More than 30 years ago, Armstrong and Hart showed that phagosomes containing *M. tuberculosis* were resistant to fusion with the later stages of the endosomal-lysosomal pathway (6). Since then, it has been established that *M. tuberculosis,* the vaccine strain *M. bovis* BCG and *M. avium* all reside in compartments secluded from the terminal stages of the endocytic pathway (32, 41, 45, 161). This block in phagosomal maturation is today considered a hallmark of mycobacterial infection and is thought to represent one of the key mechanisms by which mycobacteria are able to avoid host killing and survive within macrophages.

Phagosomes containing viable, virulent mycobacteria have been shown to equilibrate to a pH of 6.2-6.3, a significantly higher pH than normally associated with endosomal compartments. In correlation with this observation, the proton ATPase responsible for acidification of the phagosome was found to be absent on mycobacterium-containing phagosomal membranes (146). Mycobacterial phagosomes have been shown to retain the actin binding protein TACO (for tryptophan-aspartate-containing coat protein, also known as coronin) (53) and show the absence of membrane markers typical for later stages of the endocytic pathway, such as the lysosomal protease Cathepsin D (32) and mannose-6-phosphate receptor (161). Moreover, mycobacterial infection is associated with impaired antigen processing (114), attenuated IFN-γ activation of the macrophage (139), and reduced production of reactive oxygen and nitrogen intermediates (26). The latter is seen in connection with inhibition of protein kinase C activity (26). Together, these events are characteristic to phagosomal maturation arrest and create an environment in which the tubercle bacilli can thrive and divide.

A closer examination of the mycobacterial phagosome has shown that it resembles early endosomes in terms of membrane composition and in its ability to fuse with other organelles of the endosomal pathway. Various plasma membrane and endosomal markers including the transferrin receptor, MHC-class II molecules, and the ganglioside GM1 are present in the membrane of phagosomes containing mycobacteria, indicating an ongoing fusion with the early parts of the endocytic pathway (132). Presence of the transferrin receptor ensures mycobacteria access to iron, which is essential for intracellular survival (135).

The process of phagosomal maturation requires the assembly of proper fusion machinery, which in part is determined by specific phagosomal coat proteins. The small GTPases, Rab5 and Rab7, play important roles in this process and are normally only transiently associated with, respectively, early and late endosomal compartments [reviewed in (46)]. Normally, Rab5 facilitates endocytosis and homotypic fusion between early endosomes while Rab7 regulates transport from early to late endosomes. The phagosomal maturation arrest caused by pathogenic mycobacteria has been associated with an inhibition of functions controlled by Rab5 and Rab7 (157). Deretic and colleagues (60) have in the past few years provided a series of direct evidence for mycobacterial involvement in the phagosomal maturation arrest. This group has identified two Rab5 effectors, PI3-kinase and the tethering protein early endosomal antigen1 (EEA1) as necessary for maturation of phagosomes into late endosomes (60). *M. tuberculosis* as well as the cell wall glycolipid, mannose-capped lipoarabinomannan (ManLAM) have been found to mediate the exclusion of EEA1 from the phagosomal membrane in correlation with reduced maturation (60). Furthermore, mycobacterial ManLAM-induced EEA1 exclusion is due to inhibition of the

sorting pathway from the *trans*-Golgi-network to phagosomes and is seen in connection with inhibition of a PI3-kinase dependent pathway (61). A block in this pathway can also explain the absence of phagosomal markers, such as the vacuolar ATPase proton pump, Cathepsin D and the mannose-6-phosphate receptor on the mycobacterial phagosome (61).

Calcium is an important regulator of signaling pathways involved in phagosome-lysosome fusion and has recently been shown to affect phagosomal maturation (106, 107). Macrophages infected with live *M. tuberculosis* demonstrated a decrease in the elevation of calcium normally associated with phagocytic uptake (106). Mycobacterial-induced inhibition of two principal calcium effectors, sphingosine kinase and CaMII kinase was associated with this observation (108). Furthermore, inhibition of calcium was associated with reduced acidification of the phagosomal compartment. Recently, it was shown that purified ManLAM from *M. tuberculosis* also serves as a potent inhibitor of calcium increase during the early stages of infection (156). Moreover, it was hypothesized that the inhibition of calcium increase is connected to the inhibition of EEA1-recruitment to phagosomal membranes (156). In support of this, several observations were made: 1) calmodulin, necessary for the activation of CaMII kinase, promotes the association of phagosomes with EEA1, 2) the effects of calmodulin are PI3-kinase dependent, and 3) calcium is necessary for the interaction of PI3-kinase with calmodulin. Consequently, LAM-induced inhibition of intracellular calcium increase abrogates the activity of PI3-kinase resulting in a block in the sorting pathway between the *trans*-Golgi-network and phagosomes (**Figure 1**). Together these reports provide compelling evidence to suggest active mycobacterial interference with host signaling pathways to prevent phagosomal maturation.

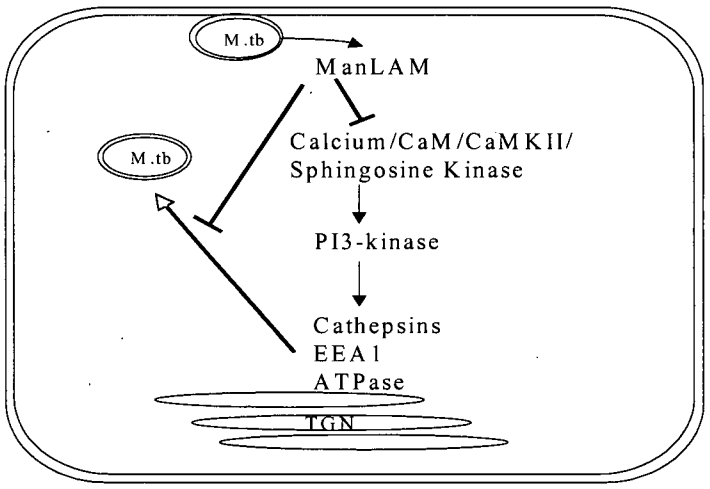

Figure 1: Mycobacterial inhibition of signaling pathways involved in phagosomal maturation arrest.

Cathepsins, EEA1 and the vacuolar ATPase are transported from the *trans*-Golgi-network to the phagosomal compartment during maturation of phagosomes (red arrow). This pathway is dependent on PI3K and EEA1. By inhibiting intracellular increase in calcium, *M. tuberculosis* inhibits a signaling cascade involving CaM, CaMKII and Sphingosine kinase, which control the PI3K-dependent delivery of cathepsins, EEA1 and the vacuolar ATPase from the TGN to the phagosomal compartment. ManLAM from *M. tuberculosis* is directly involved in the inhibition. CaM, calmodulin; CaMKII, Calcium/calmodulin dependent kinase II; PI3-kinase, phosphatidylinositol-3-kinase; TGN, *trans*-Golgi-network. The figure is adapted from (155).

1.5 Interference with MHC class II expression

One of the most important functions of the macrophage is to present fragments of the ingested organism on its cell surface. From there, the antigen will quickly be recognized by the $CD4^+$ T helper cells, induce cytokine secretion and subsequent activation of the immune system. Macrophage antigen processing, transportation, and final presentation of the antigen in combination with MHC class II molecules involve a complex process that initially is induced by INF-γ, the principal activator of macrophage resistance to intracellular pathogens [reviewed in (128)].

1.5.1 The interferon gamma signaling pathway

INF-γ regulates the expression of MCH class II molecules primarily at the level of transcription (128). Research over the past few years has elucidated the signaling pathway controlling the expression of MHC class II in macrophages. Binding of INF-γ to its receptor triggers dimerization or oligomerization of the receptor subunits, followed by phosphorylation of the receptor associated Janus kinases (JAKs), JAK1 and JAK2. Activation of JAK1 and JAK2 is followed by phosphorylation and activation of cytoplasmic STAT1α. Furthermore, phosphorylated STAT1α homodimerizes through interaction of the SH-2 domain on one molecule with phosphotyrosine on another. This is followed by translocation of the homodimer to the nucleus where it binds to specific γ-activation sequences. A variety of human genes have been shown to posses γ-activation sequences including Fcγ receptor type 1, guanylate binding protein-2, class II *trans*-activator (CTIIA), and indoleamine-2,3-dioxygenase (5, 31, 104), where CTIIA is the principal regulator of MHC class II expression (27). To achieve maximal transcriptional activity, STAT1α also requires phosphorylation of a serine residue (159). Phosphorylation at Ser^{727} may in part regulate the interaction of STAT1α with the CREB-binding protein/p300 family of transcriptional coactivators, which is necessary for transcriptional activation (164). Inhibition of MHC class II expression may allow *M. tuberculosis* residing within macrophages to avoid detection by $CD4^+$ T-helper cells, promoting persistence of infection.

1.5.2 Mycobacteria suppress MHC class II expression in macrophages

Macrophages infected with *M. tuberculosis* show reduced expression of MHC class II molecules (81, 117). Several researchers have proposed a model in which mycobacteria

interfere with the INF-γ signaling pathway to suppress the expression of MHC class II molecules. Ting *et al.* (148) showed that *M. tuberculosis* infection of human monocytes disrupts the association of STAT1α with components of the transcriptional machinery, the coactivators CREB-binding protein and p300, thereby blocking the transcription of γ-activating sequences. Using a different model system, Hussain *et al.* (86) showed that *M. avium* infection of mouse macrophages resulted in reduced expression of IFN-γ inducible genes. Here, the mechanism was shown to involve reduced phosphorylation of the members of the IFN-γ signaling pathway, including the INF-γ receptor, the JAKs, and STAT1 protein (86). *M. avium* infection was also shown to result in down regulation of the INF-γ receptor (86).

Results from several groups indicate that the IFN-γ signaling pathway from receptor binding to STAT phosphorylation, dimerization and nuclear translocation remains unaffected and that the inhibitory effect exerted by the bacteria on MHC class II expression takes place at a later stage (66, 81, 118). Several post-transcriptional regulatory mechanisms are involved in the expression of functional class II molecules and have been proposed as mycobacterial targets. Hmama *et al.* (81) showed that *M. tuberculosis* infection of the human monocytic cell line, THP-1 inhibited MHC class II expression by blocking the transport and processing of MCH class II molecules through the enodosomal/lysosomal system. They found that infection neither impaired the induction of MHC class II and CTIIA gene expression by INF-γ, nor the activation of the JAK-STAT signaling pathway (81). These results were seen in macrophages exposed to viable *M. tuberculosis*, whereas inhibition of class II expression was significantly less when cells were exposed to heat-killed bacteria. In contrast, reduced mRNA levels of the MHC class II component HLA-DR was observed in *M. tuberculosis*

infected THP-1 cells (66). Attempts to elucidate the mycobacterial factors responsible for MHC class II inhibition have identified the 19-kDa-lipoprotein as a likely candidate. Whole bacteria (*M. tuberculosis*) and purified 19-kDa lipoprotein inhibited the IFN-γ-induced expression of CTIIA and MHC class II mRNA without interfering with STAT1 phosphorylation or translocation to the nucleus (118). Interestingly, the inhibitory effect was time-dependent and only effective after 16-hours of 19-kDa-lipoprotein stimulation.

Discrepancies between studies indicate that mycobacteria could employ several mechanisms capable of interfering at different points in the signaling machinery of the host. However, the use of different model systems, such as human macrophages versus murine macrophages, could also be responsible for the differences between the studies mentioned above.

1.6 MAPK pathways are activated during mycobacterial infection

Mitogen activated protein kinases (MAPKs) comprise a large family of eukaryotic kinases that play important roles in a wide range of cellular functions including differentiation, proliferation, cell fate and inflammatory responses [reviewed in (89)]. Three distinct subfamilies have been identified: i) p42 and p44 extracellular signal-regulated regulated kinases 1 and 2 (ERK 1 and 2), ii) p38 MAPK, and iii) p46 and p54 stress activated protein kinase (SAPK, also known as c-Jun-N-terminal kinase (JNK)). Although, there is considerable co-operation between these kinases, the ERK 1/2 pathway is generally stimulated by growth factors, mitogens and tumor promoters, whereas cellular stresses and inflammatory stimuli activate JNK and p38 MAPK. Activation of the MAPKs requires dual phosphorylation of a highly conserved Thr-XXX-Tyr motif, which is brought about by their

upstream kinase, the mitogen-activated protein kinase kinase (MKK). Upon activation, MAPKs translocate to the nucleus where they directly bind to transcription factors and induce transcription of response genes (89).

Studies undertaken to investigate the role of MAPK in mycobacterial infection have agreed on a principal role for these kinases in the control of cytokine and chemokine production by macrophages (19, 127, 151). The importance of these mediators in the activation and recruitment of inflammatory leukocytes to the site of infection, gives the MAPKs an important function in the control of mycobacterial infection. MAPKs have also been implicated in the macrophage's production of nitric oxide (NO), another anti-mycobacterial agent. Employing specific inhibitors, Chan *et al.* (25) found that activation of ERK and JNK by their upstream kinases, MEK1 and MKK7 respectively, were involved in NO production following a simultaneous stimulation by ManLAM and INF-γ. The availability of specific pharmaceutical inhibitors has allowed for a more thorough dissection of the individual pathways as well as how mycobacterial infection affects the activation status of these kinases. However, there is still a very limited amount of information available on the role of JNK, which is in part due to the lack of a specific inhibitor of this kinase.

Mycobacterial infection leads to an early activation of all three MAPK subfamilies in host cells of both human and murine origin (19, 127, 151). This is not specific to mycobacterial exposure, as other non-mycobacterial antigens, such as LPS, induce a similar response, indicating a general role for this family of kinases in the response to inflammatory stimuli. The pathway through which these kinases are activated are known to depend on the macrophage CD14 receptor, as the presence of anti-CD14 antibodies block phosphorylation of all three kinases, suggesting a pathway in common with LPS (124).

Several groups have taken advantage of the difference in virulence between two morphotypes of *M. avium* to investigate MAPK dynamics in macrophages (19, 21, 151). Whereas both the virulent and avirulent strain induce an initial activation of all three subfamilies, the virulent strain seems to induce less activation or completely inhibit the activation over time (151). Comparing the two strains, these studies have in general shown that the virulent strain induces less production of inflammatory cytokines than the avirulent strain, correlating with decreased activation of MAPKs.

Several studies have indicated that p38 MAPK acts in favour of the infecting bacteria. Chan *et al.* (25) showed that in contrast to ERK, p38 MAPK had an inhibitory effect on NO production. Infection of murine bone marrow-derived macrophages with *M. avium* demonstrated that activation of p38 MAPK was necessary for optimal growth of the virulent phenotype. Upon examination of this phenomenon, it was found that the p38 MAPK-dependent production of prostaglandin E_2, a mediator of macrophage down-regulation, was critical for survival of the bacteria (151). The same study found an inhibitory effect of p38 MAPK on ERK activation, resulting in diminished secretion of TNF-α. Additionally, it has been found that IL-10 secretion, another potent down-regulator of macrophage activation, is dependent on p38 activation (127). As described above, accumulating evidence suggests a role for the tethering protein EEA1 in phagosomal maturation (60). Mycobacterium-induced p38MAPK activation reduces the acquisition of EEA1 to phagosomal membranes and is linked to a block in the binding of EEA1 to Rab5 (62). Together these results point to a regulatory role of p38 MAPK that could be exploited by the bacteria to down-regulate defense mechanisms.

1.7 Apoptosis in host defense against mycobacteria

It has been well established that macrophages undergo increased rates of apoptosis as a result of mycobacterial infection (96, 130, 133). *In vitro* infection of both monocytes and alveolar macrophages results in apoptosis, and alveolar macrophages recovered from patients with pulmonary tuberculosis show an increased frequency of apoptosis (93, 94, 122).

A role for TNF-α in the induction of host cell apoptosis has been proposed. Although TNF-α is involved in the protective immune response in mice (56), its specific role in human tuberculosis remains less understood. Using alveolar macrophages, Keane *et al.* (93) showed that production of TNF-α is necessary for the induction of host cell apoptosis in response to *M. tuberculosis* infection.

The role of apoptosis in mycobacterial infection has been somewhat controversial and has prompted questions as to whether the host or the bacterium would benefit from such a process. It could be argued that mycobacteria enclosed within a macrophage undergoing apoptosis will be more efficient in entering other macrophages (16). However, growing evidence supports a role of host cell apoptosis in the active defense against mycobacterial infection. Fratazzi *et al.* (58) reported how apoptosis prevents spreading of mycobacterial infection by packaging the mycobacteria within apoptotic bodies. Furthermore, sequestering of mycobacteria inside these apoptotic bodies makes them available for ingestion by fresh phagocytes, which are actively recruited to sites of mycobacterial-induced inflammation *in vivo* (138). In addition to depriving bacilli of an intracellular environment that facilitates growth, there is evidence that ingestion of bacilli contained in apoptotic bodies by freshly added macrophages results in enhanced killing (59). This was clearly supported in a recent report by Shiable *et al.* (137) who, as previously mentioned, showed that mycobacterial-

induced apoptosis of macrophages serve an important function in host defense by enabling antigen presentation by bystander cells and enhanced activation of inflammatory leukocytes.

Taken together, these results largely support apoptosis as a host defense mechanism against mycobacterial infection. However, since survival within the macrophage most likely serve a role in protecting the mycobacteria from other more effective host responses, it is not surprising to find that *M. tuberculosis* has developed strategies to block host cell apoptosis.

1.7.1 Mycobacterium tuberculosis inhibits host cell apoptosis

Keane *et al.* (94) showed that attenuated mycobacterial strains such as H37Ra and *M. bovis* BCG are significantly more potent inducers of apoptosis than the corresponding virulent strains, H37Rv and wild type *M. bovis*. The same group had previously shown that mycobacterial-induced apoptosis was triggered in a TNF-α dependent manner (93). By adding anti-TNF-α antibody, they were able to inhibit host cell apoptosis, suggesting a similar mechanism might be employed by *M. tuberculosis* to inhibit host cell apoptosis. IL-10 stimulation leads to shedding of soluble TNFR2, which is known to neutralize TNF-α bioactivity (10). In accordance with this, it was shown that induction of IL-10 by *M. tuberculosis* led to the inhibition of TNF-α and subsequent inhibition of host cell apoptosis (9).

Apoptosis can also be inhibited or promoted by members of the Bcl-$_2$ family (98, 126). Klinger *et al.* (96) showed that a down regulation of Bcl-$_2$ accounted for the onset of apoptosis in mononuclear phagocytes infected with *M. bovis* BCG. Consistent with a model in which the Bcl-$_2$ family regulates apoptosis in macrophages infected with mycobacteria, Maiti *et al.* (105) proposed a counteracting mechanism exerted by virulent mycobacteria to

promote host cell survival. They found that exposing human monocytes to purified mannose-capped LAM of *M. tuberculosis* induced phosphorylation of the pro-apoptotic member Bad. This was brought about in a phosphoinositol-3-kinase (PI3-K) dependent manner and consequently led to the inhibition of apoptosis (105). Another mechanism for evasion of apoptosis involved up regulation of the anti apoptotic factor Mcl-1 and was observed in both THP-1 cells and MDMs infected with the virulent *M. tuberculosis* strain H37Rv (141). The mycobacterial 19-kDa liporotein has recently been identified as a factor responsible for the inhibition of macrophage apoptosis (102).

1.8 Mycobacterial mediators of host cell interactions

It is now well established that mycobacteria have the ability to actively interfere with the signaling machinery of the host to counteract host defense mechanisms and promote bacterial survival. The question arises as to which mycobacterial mediators account for these effects.

Lipids are an abundant feature of the mycobacterial cell wall and make ideal mediators for subverting host microbicidal mechanisms. Several reports indicate that the mycobacterial phagosome membrane is disrupted in a manner that may allow for entry of the organism or its products into the host cell cytosol (112, 147). Indeed, analysis of infected macrophages revealed that lipid-containing moieties are actively trafficking out of the mycobacteria-containing phagosome (14). The cell wall glycolipid, ManLAM, of *M. tuberculosis,* has long been considered a virulence factor. As discussed above, several lines of evidence have implicated ManLAM to be involved in a wide array of immunomodulatory functions. In addition to a direct role in phagosomal maturation arrest

(61), ManLAM has been implicated in: i) inhibition of IFN-γ signaling, ii) scavenging of oxygen free radicals, and iii) inhibition of protein kinase C (26). Other glycolipids of the mycobacterial cell wall have also been shown to inhibit macrophage microbicidal functions. As outlined above, the 19-kDa-lipoprotein of *M. tuberculosis* has been found to inhibit several functions associated with host defense such as MHC class II expression and antigen processing (117) and apoptosis (102). The 19-kDa lipoprotein of *M. tuberculosis* belongs to a family of bacterial lipoproteins characterized by a distinctive N-terminal lipo-amino acid, N-acyl S-diacylglycerol. Although diacylation abrogates the effect ManLAM, the same is not true for the 19-kDa lipoprotein where the polypeptide component is sufficient for the inhibitory mechanisms to occur (102). Both ManLAM and 19-kDa-lipoprotein have been shown to bind specifically to cell surface expressed host receptors, the toll-like receptors (113).

Sequencing of the *M. tuberculosis* genome revealed the presence of eukaryotic-like protein kinases and protein phosphatases. An analysis of this family of phosphoproteins showed that at least six of them could be phosphorylated *in vitro* (8). This clearly indicates the presence of functional protein kinases in *M. tuberculosis.* An earlier report demonstrated the existence of a major 55kDa tyrosine phosphorylated protein (30) and so the presence of a functional tyrosine kinase in the *M. tuberculosis* genome was predicted. Surprisingly, no such kinase was identified. This poses the question as to the source and function of these tyrosine-phosphorylated proteins. If the virulence of *M. tuberculosis* is dependent on the activity of mycobacterial phospho-proteins, secretion of these proteins into the phagosome, and possibly through the phagosomal membrane into the host cell cytoplasm, is likely.

Supporting this hypothesis is the presence of a protein tyrosine phosphatase in the culture medium of *M. tuberculosis* (36).

1.9 Concluding remarks

The macrophage represents a highly specialized cell of the immune system, yet fails to eliminate the *M. tuberculosis* bacillus, one of the most successful pathogens of our time. Through years of cohabitation and mutual interaction, *M. tuberculosis* has managed to establish itself within the macrophage, avoiding the host killing mechanisms.

The ability of *M. tuberculosis* to infect and persist within these specialized cells could be viewed as two principal mechanisms employed by the bacteria. Primarily, this is a result of the bacteria's ability to adapt to a changing environment and to utilize the sources available to it within the host. Sequencing of the *M. tuberculosis* genome (33) and post-genomic research has allowed for the identification of genes important in growth and survival (134) and has significantly contributed to our understanding of the interaction between *M. tuberculosis* and its host environment. Mycobacterial proteins such as respiratory enzymes, stress-related products, metabolic enzymes and proteins involved in fatty-acid metabolism have been identified as critically important for the survival of the bacteria within the host cell [reviewed in (83)]. Moreover, a critically important part of the mycobacterial survival lies in its ability to respond to the environment in which it resides. However, another and probably equally important part lies in the ability of mycobacteria to actively interfere with and manipulate host defense mechanisms.

Cell signaling controls virtually every cellular event and is regulated by two principal classes of enzymes; protein kinases and protein phosphatases. These enzymes represent key

control points within the host cell and possible targets for mycobacterial interference. It is intriguing to hypothesize a model of interference with host cell signaling machinery in which mycobacterial mediators in form of lipoproteins, protein kinases and protein phosphatases are transported out of the phagosomal membrane, into the host cell cytoplasm.

The past decades' advances within immunology, microbiology and biotechnology has accelerated our knowledge and taken us several steps further in our understanding of the interaction between *M. tuberculosis* and its host cell. However, the knowledge we have must be seen in the light of what methods we use to achieve it. The experimental work in macrophages employs a variety of cell lines and primary cells of murine and human origin. Results from different research laboratories have revealed considerable differences in responses to stimuli between primary cells and cell lines and also between the different cell lines. For example, LPS stimulation causes activation of ERK in the mouse macrophage cell line RAW 264.7 but not in P388D1 mouse macrophages (11). These differences especially apply to the study of signaling pathway where differences between cell lines often are manifested through variation in signaling pathways.

1.10 The focus of this thesis

Hypothesis: Mycobacterial infection modulates key elements in macrophage signaling that may lead to interruption of phagosome maturation.

This thesis will further examine the interactions between *M. tuberculosis* and its host macrophage. The focus is on late signaling events and I have chosen to examine macrophages after an infection time of 24 hours. Several inhibitory functions exerted by mycobacteria on host defense have been shown to rely on time-dependent mechanisms. By

examining cells after 24 hours of infection, I am excluding signaling events associated with the initial phagocytic uptake of bacteria, which are more or less common events shared by most of the microbial and inert particles. Instead, I am focusing on later events at a time when the bacteria are established in their host cell environment to a greater extent than earlier. The initial aim of this research project is to identify key host proteins that are modified upon mycobacterial infection. *M. bovis* BCG has been used as a model system to infect the human monocytic cell line, THP-1.

The second part of this research project includes the selection of a key host protein identified from the first experiment. Further experiments have been conducted on the function and role of this protein in mycobacterial infection.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

RPMI 1640, DMEM, HBSS, protease inhibitors and phosphatase inhibitors were obtained from Sigma Chemical Co. (St. Louis, MO). Anti-phospho adducin was obtained from Upstate Biotechnology (Lake Placid, NY), anti-adducin was obtained from Santa Cruz (Santa Cruz, CA), and HRPO-conjugated anti-rabbit secondary antibody was obtained from Bio-Rad Laboratories (Mississauga, ON, Canada). Alexa-conjugated anti-rabbit and anti-mouse secondary antibodies were from Molecular Probes (Eugene, OR). Endotoxin-free LAM was generously provided by Dr. J. Belisle (Colorado State University, Ft. Collins, CO, through TB Research Materials and Vaccine Testing contract (NIH, NIAID NO1-A1-75320). The LAM used was mannose capped and derived from the virulent H37Rv strain of *M. tuberculosis.*

2.2 Methods

2.2.1 Culturing of Mycobacterium bovis BCG

M. bovis BCG organisms (American type culture collection # 35734) were grown in Middlebrook 7H9 broth (Difco, Detroit, MI) supplemented with 10% (vol/vol) OADC (oleic acid, albumin and dextrose solution, Difco), and 0.05% (vol/vol) Tween 80 (Sigma) at 37°C to an A_{600} of 0.5 on a rotating platform (50 rpm). Bacteria were harvested by centrifugation (5 min, 6,000 x *g*) at 4°C and pellets were resuspended in fresh media plus 10% glycerol, aliquoted and kept at -70°C for later use. Frozen stocks were thawed and replenished in fresh media and grown for 2-4 days before they were used for cell infection. Due to the tendency of mycobacteria to clump together, BCG cultures were passed through a sterile 6cc syringe

filled to ~ 1ml with glass fiber wool prior to infection. Heat-killed BCG was prepared by heating desired volume of bacteria in culture medium at 80°C for 30 minutes. *M. bovis* BCG carrying a plasmid constitutively expressing the green fluorescent protein (GFP) (35) was used for the majority of experiments. *M. bovis* BCG GFP was grown Middelbrook 7H9 broth as described with the addition of 50 μg hygromycin per ml. THP-1 cells were infected with either growing cultures of *M. bovis* BCG or *M. bovis* BCG GFP or the equivalent frozen stocks. The frozen stocks were "boosted" for three hours before infection: stocks were thawed in a 37°C water bath, then spun down to remove the glycerol containing media before the bacteria were re-suspended in fresh media. The cultures were then incubated with agitation for 3 hours before infection.

2.2.2 Infection of THP-1 cells and treatment with lipoarabinomannan

The monocytic cell line THP-1 (ATCC, Rockville, MA) was cultured in RPMI 1640 supplemented with 10% FCS (Hyclone, Logan, UT), 2 mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultures were maintained at 2-6 x 10^5 cells/ml in 75 cm^2 culture flasks (Corning Inc. Corning, NY) with the addition of fresh media every 2-3 days and count/viability was monitored using trypan blue exclusion and hemocytometry. Cells were seeded at a density of 10^5 per cm^2 in 10 cm diameter culture dishes (Corning Inc. Corning, NY) and allowed to adhere and differentiate overnight in the presence of PMA (20 ng/ml) at 37°C in a humidified atmosphere of 5% CO_2. J774 macrophages (ATCC, Rockville, MA) were cultured in DMEM supplemented with 1% HEPES and non-essential amino acid mixture in addition to 10% FCS, 2mM L-glutamine, penicillin (100U/ml) and streptomycin (100 μg/ml). Prior to infection cells were washed 3 times with pre-warmed

HBSS and adherent monolayers were exposed to live or killed (80°C/30 min) BCG at a multiplicity of infection of 50 to 1 in respective media containing 1% L-glutamine, 10 % human serum (PPD negative) and no antibiotics. After 3 hours incubation at 37°C and 5% CO_2, cells were washed twice with HBSS to remove non-ingested bacteria and reincubated in complete medium at 37°C, 5% CO_2 for the indicated time periods. Alternatively, differentiated THP-1 cells or J774 macrophages were treated with mannose-capped lipoarabinomannan (ManLAM) at a concentration of 1μg/ml. The infection rate was verified on cells adherent to tissue culture-treated coverslips (Fisher Scientific, Nepean, ON, Canada) in 24-well plates. After phagocytosis, cells were fixed for 15 min at 37°C with 2.5% paraformaldehyde/HBSS then washed three times with HBSS and once with distilled water. Coverslips were then examined using an epifluorescence microscope (Zeiss Axioplan II) and images taken using CCD camera and Empix software. Phagocytosis was also evaluated by FACS analysis of cell loaded with GFP-BCG.

2.2.3 Kinome Analysis by Kinetworks Phospho Site Screen (KPSS) Assay

Two separate Kinetworks Phospho Site Screen (KPSS) (Kinexus, Vancouver, Canada) analyses were performed. In the initial screen, THP-1 cells were infected with live or heat-killed *M. bovis* BCG, and in the second screen THP-1 cells were infected with live *M. bovis* BCG or treated with purified ManLAM from H37Rv *M. tuberculosis* at a concentration of 1μg/ml. Untreated, PMA differentiated THP-1 cells were used as a control in both experiments. The KPSS analyses were performed as previously described (163), and according to the instructions of the manufacturer (Kinexus). Essentially, cells were homogenized at 4°C in a buffer containing 20 mM Tris-HCl, pH 7.0; 2 mM EGTA; 5 mM EDTA; 30 mM sodium fluoride; 40 mM β-glycerophosphate (pH 7.2), 2 mM sodium

orthovanadate, 10 mM sodium pyrophosphate and 1 mM phenylmethylsulfonyl fluoride. The resulting lysates were spun down at 100000 x *g* in a Beckman Table Top GS 6R ultracentrifuge. The protein content was estimated by using the Bradford assay (Bio-Rad Laboratories, Mississauga, Ont.). Three hundred micrograms of total protein for each sample were diluted with four times Laemmli sample buffer (50% glycerol, 125nM Tris-HCl, pH 6.8, 4% SDS, 0.08% bromophenol blue, and 5% β-mercaptoethanol) and boiled for 4 minutes. Samples were shipped to Kinexus for further analysis using the KPSS screen. Here, each sample was divided equally onto a 20-lane Immunetics mutiblotter. Each channel was probed with up to three primary antibodies from an array of 31 phospho-specific antibodies, selected so as to avoid overlapping cross-reactivity with target protein. The blots were developed with ECL Plus reagent (Amersham Biosciences, Arlington Heights, Ont.) and signals were captured with Fluor-S-MultiImager and quantified with Quantity One software (Bio-Rad Laboratories, Mississauga, Ont.).

Changes in phosphorylation were measured based on the band intensity for individual phospho-proteins. Evaluation of the screening procedure has determined that a change in signal (phosphorylation) of up to 25% can arise from experimental variation (Kinexus). Therefore, only changes in phosphorylation of greater than 25 % between control and treated cells have been considered significant.

2.2.4 Western analysis of adducin

PMA differentiated THP-1 cells were rested and infected with live or heat-killed BCG or challenged with purified ManLAM (concentration range, 0.01 to 1.0 μg/ml). Cells were then incubated for 2, 12 or 24 hours with bacteria or ManLAM. THP-1 cells were rested for indicated time periods in PMA free media. PMA stimulation of cells was done by

exposing THP-1 cells to 15 μM PMA for 15 minutes. Serum starvation was done by incubating cells in serum-free medium for indicated time periods. Cell extract were prepared as described for the KPSS assay and protein samples were loaded on to a 7.5 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) minigel and run at constant voltage (200V) for about 45 min. Prestained cocktail proteins from Fermentas Inc. (Burlington, ON) were used as molecular weight reference markers. The SDS-PAGE gel was equilibrated in transfer buffer (40 mM glycerol, 20 % methanol, 2 mM SDS, 50 mM Tris) and proteins were transferred onto a nitrocellulose membrane with a semidry transfer apparatus (Bio-Rad) set to 41mA for 1 hour and 15 minutes. Immediately after transfer, the nitrocellulose membrane was placed in blocking solution (5 % non-fat dry milk, TBS-Tween) and left on a rocker for 1 hour at room temperature. The blot was probed with primary antibody (anti-phospho-adducin) at a dilution of 1:2000 and incubated overnight at 4°C. Next, the blot was washed three times in TBS-Tween and incubated with secondary HRPO-conjugated goat anti-mouse or goat anti-rabbit antibody for 45 minutes at room temperature. The blot was washed three more times in TBS-Tween and developed by enhanced chemiluminescent. Densitometry analyses were done with Un-Scan-It software (Silk Scientific Corporation).

2.2.5 Fluorescence microscopy analysis of adducin and actin in infected THP-1 cells

THP-1 cells were PMA differentiated overnight onto cover slips in a 24-well plate, washed and rested for 24 hours prior to infection. Cells were untreated or infected for 0, 2, 12 or 24 hours. After completed infection, adherent cells were washed with PBS and fixed for 20 min in 2.5% paraformaldehyde solution diluted in PBS and washed twice with PBS. The

following method was used for intracellular staining of phospho-adducin. All antibodies were diluted in RPMI containing 10% normal serum to minimize non-specific binding and 0.05 % sodium azide to kill possible uningested bacteria. Cells were permeabilized with 0.2 % saponine (Sigma Chemical Co., St. Louis, MO) for 5 min, washed twice in PBS, and incubated with primary anti-phospho-adducin antibody, diluted 1:100, for 20 min. Cells were then washed twice with PBS and incubated for 20 min. with an Alexa 546 (red)-conjugated goat α-rabbit secondary antibody (Molecular Probes) used at a 1:100 dilution. Alexa 546 (red)-conjugated phalloidin (Molecular Probes) was used to stain for filamentous actin in untreated and infected cells. Cells were fixed and washed as described above. Cells were permeabilized with 0.2 % saponine for 5 min, washed twice and incubated in PBS containing 1% BSA for 20 min. to minimize non-specific binding. Cells were washed again and incubated for 20 min. with alexa-conjugated phalloidin diluted 1:100 in PBS. All cover slips were then washed twice in PBS and once in distilled H_20 before they were inverted on a glass slide in a drop of Fluorsave Reagent (Calbiochem). Coverslips were then examined using an epifluorescence microscope (Zeiss Axioplan II) and images taken using CCD camera and Empix software.

CHAPTER 3: RESULTS

3.1 Mycobacterial infection modulates host protein phosphorylation

As described in Chapter 1, several researchers have proposed mycobacterial interference with host signaling machinery as a mechanism by which mycobacteria are able to circumvent the host defense (61, 106, 157). In this setting, determining how the phosphorylation level and thereby the activity of host proteins is modified upon infection will provide important insight as to how the signaling machinery is affected by pathogenic mycobacteria. So far 510 protein kinases have been identified in the human genome (99), and considering the numerous possibilities by which mycobacteria can interfere with various signaling pathways, it becomes important to investigate the effect of mycobacteria on host signaling as interactions with a network of signaling pathways rather than focusing on single events.

Several large-scale analyses of protein profiles identifying post-translational modifications such as phosphorylation are available today. Commonly used techniques include mass spectrometry and 2D gel electrophoresis. However, protein kinases are present in low abundance, often in amounts that are not detectable by the highly sensitive stain procedures available today. We have employed a novel technique in proteomics, termed kinome analysis that uses high affinity and specific antibodies to quantitatively detect single amino acid phosphorylations (121). The purpose of this experiment was to identify key host proteins that are potentially targeted by mycobacteria and their products. This multi-phospho protein analysis employs an array of 31 known phospho-specific antibodies covering key kinases and other signaling proteins from the eukaryotic proteome.

Cells were subjected to a simultaneous screen for the phosphorylation status of 31 host phospho-proteins. Two separate screens were performed; first, THP-1 cells were infected with either live or heat-killed *M. bovis* BCG (**Figure 2**), and secondly, THP-1 cells were infected with live bacteria or treated with purified ManLAM (**Figure 3**). Untreated, differentiated THP-1 cell were used as control in both screens. As seen in **Figure 2**, numerous proteins displayed significant changes in phosphorylation. These include alpha-adducin (α-adducin), N-methyl-D-aspartate glutamate receptor subunit1 (NR1), oncogene Jun (c-Jun), c-Jun-N-terminal kinase (JNK, also known as stress-activated protein kinase (SAPK)), retinoblastoma (RB), Glycogen Synthase kinase 3β (GSK3β), and protein kinase C epsilon (PKCϵ).

To examine if some of the observed changes in phosphorylation could be due to the effect of the cell wall component, ManLAM, a second screen was applied to cells infected with live bacteria and cells treated with purified ManLAM from the H37Rv strain of *M. tuberculosis* (**Figure 3**). Similarly to the first screen, cells were infected with *M. bovis* BCG or treated with ManLAM for 24 hours prior to cell lysis and protein extraction. As observed in the first screen, α-adducin, c-Jun, JNK, GSK3β and NR1 showed increased phosphorylation in cells exposed to live mycobacteria as compared to cells infected with heat-killed bacteria and control untreated cells. Additionally, phosphorylation of PKCϵ was completely attenuated in cells infected with live bacteria for both screens. The changes in phosphorylation for α-adducin, c-Jun, JNK, and GSK3β were reproduced in cells exposed to ManLAM. This was not the case for NR1 and PKCϵ, suggesting that another mycobacterial factor acts on these proteins.

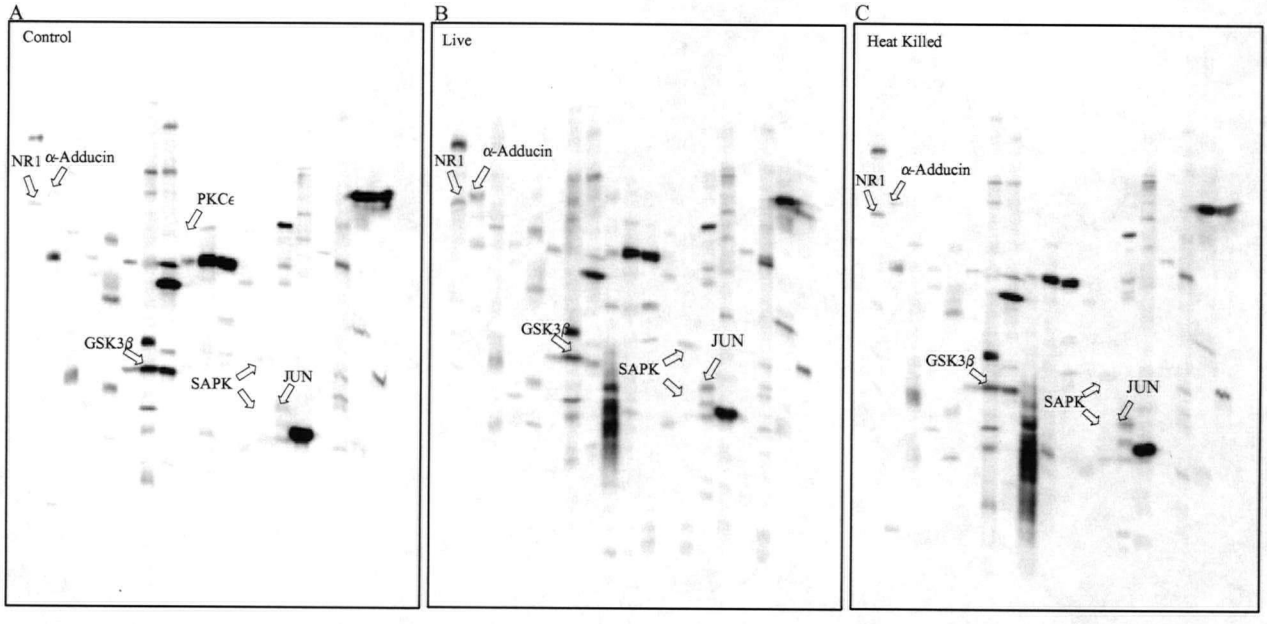

Figure 2: Multiphosphoprotein analysis of THP-1 cells infected with live and heat-killed *M. bovis* BCG.

Each sample is represented as a phosphoprotein fingerprint. Accurate intensity values for each band are the accumulated signal obtained over a given scan time for each blot. These are shown as numerical values in Table 1. The data shown here represent protein bands visualized as a snapshot of the scan time and may therefore not represent exact band intensities. The three gels represent: (A) untreated THP-1 cells, (B) THP-1 cells infected with live *M. bovis* BCG, and (C) THP-1 cells infected with heat-killed *M. bovis* BCG. Each lane was probed with one or more antibodies. The proteins indicated by arrows are discussed in the text. Lanes 1 and 21 in each panel contained molecular size standards. Antibodies against the phosphorylated proteins were as follows: lanes 2, NR1 (S^{896}); lanes 3, adducin (S^{724}) and CDK1 (Y^{15}); lanes 4, CREB (S^{133}); lanes 5, ERK1/2 (T^{202}/Y^{204}) (T^{183}/Y^{185}) and p70 S6K (T^{389}); lanes 6, RSK (T^{360}/S^{364}), RAF1 (S^{259}), and MEK1/2 (S^{217}/S^{221}); lanes 7, GSK3α (S^{21}), GSK3β (S^{9}), and PKB (T^{308}); lanes 8, GSK3α (Y^{279}/Y^{216}); lanes 9, PKR1 (T^{451}); lanes 10, PKCϵ (S^{719}); lanes 11, PKCα (S^{567}); lanes 12, PKCα/β (T^{638}) and SRC (Y^{529}); lanes 13, PKCδ (T^{505}); lanes14, SAPK (T^{183}/Y^{185}); lanes 15, MSK 1/2 (S^{376}) and JUN (S^{73}); lanes 16, JAK2 (Y^{1007}/Y^{1008}) and p38MAPK (T^{180}/Y^{182}); lanes 17, STAT1 (S^{701}); lanes 18, STAT3 (S^{727}); lanes 19, RB (S^{780}); lanes 20, RB (S^{807}/S^{811}).

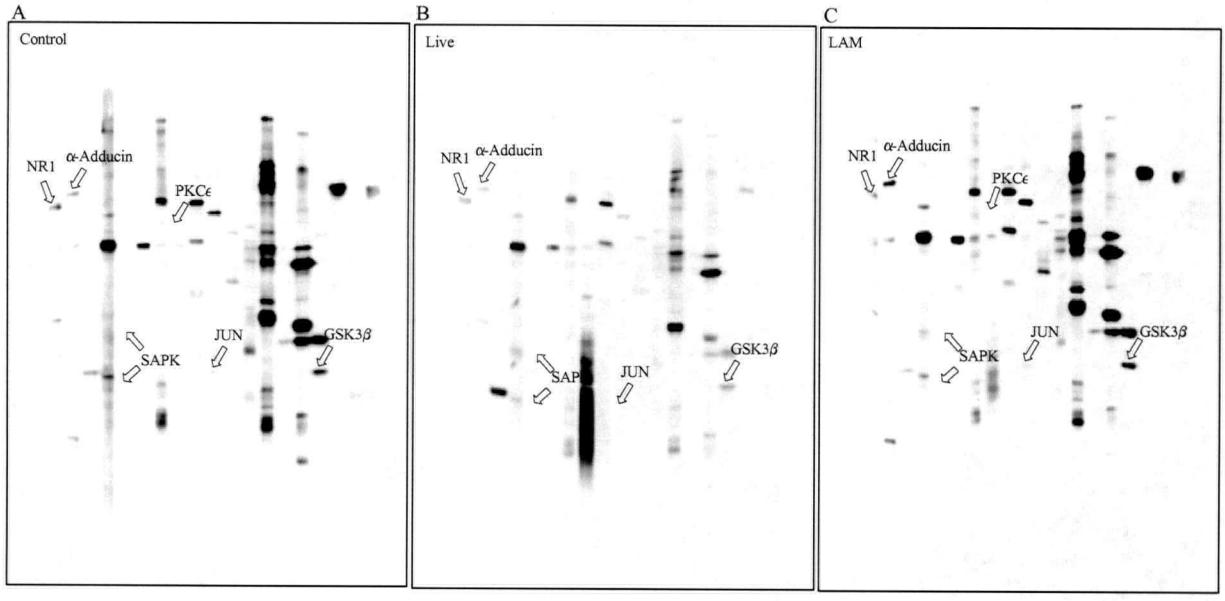

Figure 3: Multiphosphoanalysis of THP-1 cells infected with *M. bovis* BCG or exposed to ManLAM.

As for Figure 2, accurate intensity values for each band are the accumulated signal obtained over a given scan time for each blot. These are shown as numerical values in Table 1. The data shown here represent protein bands visualized as a snapshot of the scan time and may therefore not represent exact band intensities. The second screen, presented here, shows three separate blots: (A) untreated THP-1 cells, (B) THP-1 cells infected with *M. bovis* BCG, and (C) THP-1 cells treated with purified ManLAM. The proteins indicted by arrows are discussed in the text. Lanes 1 and 21 in each panel contained molecular size standards. Antibodies against the phosphorylated proteins were as follows: lanes 2, NR1 (S^{896}); lanes 3, α-adducin (S^{724}), γ-adducin (S^{662}), CDK1 (Y^{15}), and SRC (Y^{529}); lanes 4, p38MAPK (T^{180}/Y^{182}) and STAT5 (Y^{694}); lanes 5, SRC (Y^{418}) and PKCα (T^{567}); lanes 6, RSK (T^{360}/S^{364}) and SAPK (T^{183}/Y^{185}); lanes 7, PKCβ (T^{368}), MEK3 (S^{189}/T^{193}), and MEK6 (S^{207}/T^{211}); lanes 8, ERK1 (T^{202}/Y^{204}), ERK2 (T^{185}/Y^{204}), and p70 S6K (T^{389}); lanes 9, PKCε (S^{719}) and SMAD1 (S^{463}/S^{465}); lanes 10, STAT3 (S^{727}); lanes 11, JUN (S^{73}); lanes 12: RAF1 (S^{259}) and STAT1 (S^{701}); lanes 13, CREB (S^{133}), PKBα (T^{308}), and PKCδ (T^{505}); lanes 14, PKBa (S^{473}); lanes 15, GSK3a (S^{21}), GSK3 beta (S^{9}) and MSK1/2 (S^{376}); lanes 16, PKR1 (T^{451}); lanes 17, GSK3a (Y^{279}) and GSKb (Y^{216}); lanes 18, RB (S^{780}); lanes 19, MEK1/2 (S^{221}/S^{225}); lanes 20, RB (S^{807}/S^{811}).

The two screens were compared in terms of the relative fold-change in phosphorylation between live infection and untreated control cells (**Table 1**) (determined as trace quantity for control cells subtracted from the trace quantity for each protein detected). Values for cells infected with heat-killed bacteria and cells treated with ManLAM are shown as fold changes relative to their respective control cells (**Table 1**). As described in materials and methods, changes in phosphorylation were measured based on the band intensity for individual phospho-proteins. Evaluation of the screening procedure has determined that a change in signal (phosphorylation) of up to 25% can arise from experimental variation (Kinexus). Therefore, only changes in phosphorylation of greater than 25 % between control untreated cells and cells infected with live bacteria have been considered significant.

Five host signaling proteins: NR1, JNK, c-Jun, GSK3β, and α-adducin, (**Figure 2, 3 and Table 1**) showed a greater than 25% increase in phosphorylation over control untreated cells for both screens: NR1 S896 phosphorylation showed an average increase of 93%, two isoforms of SAPK showed an average increase of 155% and 236% respectively, c-Jun showed an average increase of 108%, GSK3β Y216 increased on average 57%, and α-adducin phosphorylation increased with an average of 75%. Conversely, PKCϵ showed no detectable signal for cells infected with live bacteria in either screen as compared to untreated control cells and cells exposed to ManLAM.

Protein		Epitope(s)	Signal[a]			
Full name	Abbreviation		Control	Fold change		
				Live avg	LAM	HK
Alpha adducin	α-Adducin	S724	1	1.75[b]	2.62	0.38
Gamma adducin	γ-Adducin	S662	1	0.00	2.15	ND[c]
Cyclic AMP response element binding protein	CREB	S133	1	0.00	1.00	0.86
Cyclin-dependent kinase 1 (Cdc2)	CDK1	Y15	1	0.37	2.07	0.00
Double-standard RNA dependent protein kinase	PKR	T451	1	1.16	1.88	0.57
Extracellular regulated kinase 1	ERK1	T202/Y204	0	0.51	0.00	1.01
Extracellular regulated kinase 1	ERK2	T185/Y204	0	0.65	0.00	0.79
Glycogen synthase kinase 3 alpha	GSK3α	Y279	1	1.29	1.44	0.82
Glycogen synthase kinase 3 alpha	GSK3α	S21	1	0.92	1.79	0.36
Glycogen synthase kinase 3 beta	GSK3β	Y216	1	1.57[b]	1.52	0.65
Glycogen synthase kinase 3 beta	GSK3β	S9	0	0.00	0.00	0.36
Mitogen-activated protein kinase 1/2	MEK1/2	S221/S225	1	1.85	1.69	0.39
Mitogen-activated protein kinase 3	MEK3	S189/T193	0	0.00	0.00	ND
Mitogen-activated protein kinase 6	MEK6	S207/T211	0	0.00	0.00	ND
Mitogen- and stress-activated protein kinase 1/2	MSK1/2	S376	1	1.46	2.69	0.48
Mitogen- and stress-activated protein kinase 1/2	MSK1/2	S376	1	2.71	4.01	0.33
N-Methyl-D-aspartate glutamate receptor subunit 1	NR1	S896	1	1.93[b]	1.24	0.91
Oncogene JUN	JUN	S73	1	2.08[b]	1.30	1.21
Oncogene Raf 1	RAF1	S259	1	1.28	3.01	0.20
Oncogene Raf 1	RAF1	S259	1	1.78	4.71	0.28
Oncogene SCR	SRC	Y529	1	3.18	1.35	0.83
Oncogene SCR	SRC	Y418	0	0.00	0.00	ND
p38 alpha mitogen-activated protein kinase	p38MAPK	T180/Y182	1	4.91	1.10	0.74
Protein kinase B alpha (Akt1)	PKBα	S473	0	0.00	0.00	ND
Protein kinase B alpha (Akt1)	PKBα	T308	1	1.08	1.60	0.63
Protein kinase C alpha	PKCα	S657	1	1.61	1.93	0.55
Protein kinase C alpha/beta	PKCα/β	T638/641	1	1.69	2.82	0.51
Protein kinase C delta	PKCδ	T505	1	1.28	2.59	0.43
Protein kinase C epsilon	PKCϵ	S719	1	0.00[b]	1.21	0.00
Retinoblastoma 1	RB	S780	1	0.82	1.85	0.64
Retinoblastoma 1	RB	S807/S811	1	0.35	2.05	0.54
Ribosomal S6 kinase 1	RSK1	T360/S364	0	0.53	0.00	0.31
S6 kinase p70	p70 S6K	S398	1	2.40	3.93	0.57
S6 kinase p70	P70 S6K	S389	1	2.60	4.27	0.71
Signal transducer and activator of transcription 1	STAT1	T701	1	2.05	3.02	0.80
Signal transducer and activator of transcription 3	STAT3	S727	1	1.93	2.59	0.00
Signal transducer and activator of transcription 5	STAT5	Y694	0	0.00	0.00	ND
SMA- and MAD-related protein 1	SMAD1	S463/465	0	0.00	0.00	ND
Stress activated protein kinase (JNK)	SAPK	T183/Y185	1	2.55[b]	3.07	0.68
Stress activated protein kinase (JNK)	SAPK	T183/Y185	1	3.36[b]	1.85	0.80

Table 1: Comparison of phosphorylated host proteins

[a] The trace quantity of each protein band is measured by the area under its intensity profile curve and corrected for the individual scan times (recorded time before saturation occurs). Values for the control samples have been set to 1 or 0. A value 0 indicates that no immunoreactive signal was detected for this protein in either of the two screens. Live average is the average value, expressed as fold change, for the difference in phosphorylation between live infection and respective control samples for both screens. Values for LAM and heat killed bacteria (HK) show the fold change relative to their respective control samples.

[b] Proteins that showed a similar and grater-than-25% change in phosphorylation for both screens.

[c] ND, not determined.

3.2 Western analysis of adducin phosphorylation in macrophages

Among the six host proteins that were shown to undergo significant changes in phosphorylation, α-adducin was chosen for further analysis. It was chosen due to its novelty, as it had not yet been investigated as a mycobacterial target protein in such a setting. Furthermore, α-adducin is a cytoskeletal-associated heterodimeric protein with an essential role in the rearrangement of the actin filament network (109). Several pathogens specifically target the host actin network as part of their ability to survive intracellularly (72). Also mycobacteria have been shown to interfere with the host's actin cytoskeleton although no specific mechanism has been delineated (74). In this context, it is interesting to analyze adducin activity as it may provide a link between mycobacterial infection and actin filament dynamics in the host cell. Our results from the Kinome analysis showed a 75% average increase in α-adducin phosphorylation in cells infected with live bacteria compared to cells exposed to heat-killed bacteria and control untreated cells. ManLAM-treated cells showed a 162 % increase in α-adducin phosphorylation compared to control cells.

The purpose of the following series of experiments was to establish a system for the analysis of adducin expression. We have used two different macrophage cell lines, of human and murine origin respectively, and two bacterial strains to construct the best system to study the activity of adducin.

3.2.1 Effects of PMA differentiation and serum starvation on adducin phosphorylation in THP-1 cells: a comparison with the murine macrophage cell line, J774

PMA (phorbol-12-myristate-13-acetate) is a commonly used agent for differentiation of monocytic cell lines and a known stimulator of a variety of signaling cascades (39, 158) in monocytic cells. Furthermore, PMA is used as a positive control to stimulate adducin

phosphorylation in different cells lines (12). Since PMA is also used to induce the differentiation of THP-1 cells to a macrophage-like cell line, it was necessary to determine the specific effects of PMA on adducin phosphorylation in macrophages. **Figure 4** shows phosphorylation of THP-1 cells and J774 macrophages exposed to PMA, either through overnight differentiation or through 15 min. stimulation. The effect of three hour serum starvation was also tested in this experiment.

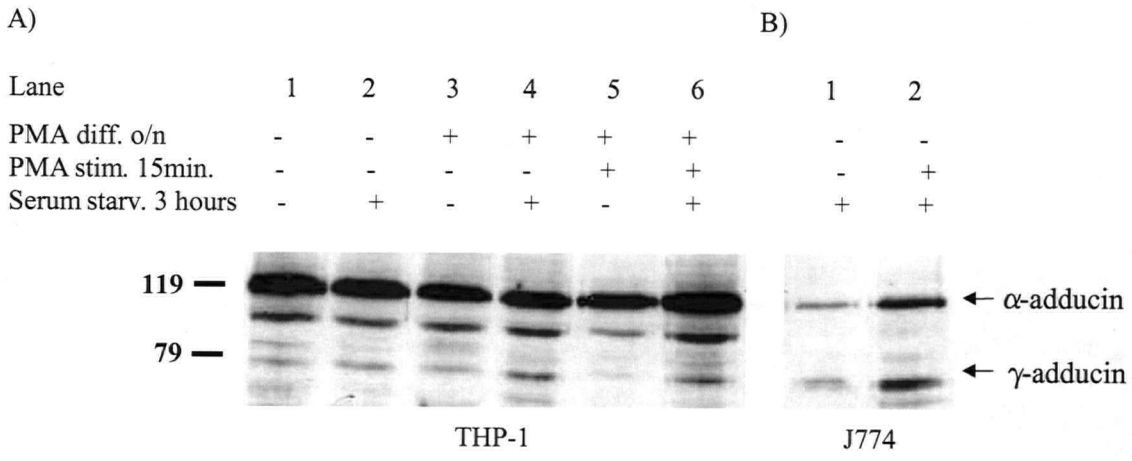

Figure 4: Effect of PMA and serum starvation on adducin phosphorylation: THP-1 cells compared to J774 cells.

(A) THP-1 cells were undifferentiated (lane 1 and 2), differentiated with PMA overnight (lane 3 and 4) or differentiated overnight and stimulated with 1 μM PMA for 15 min. (lane 5 and 6). Cells were serum starved for 3 hours (lane 2, 4, and 6) or not (lane 1, 3, and 5). (B) J774 macrophages were serum starved for 3 hours and left untreated (lane 1) or stimulated with 1μM PMA for 15 min. (lane 1).

Undifferentiated cells (monocytes) as well as cells differentiated overnight with PMA showed a high basal level of adducin phosphorylation. PMA stimulation for 15 min did not significantly change the level of phosphorylation (**Figure 4A**). The level of adducin phosphorylation was already high in THP-1 cells differentiated overnight with PMA, making it difficult to assess whether an additional 15 min. of PMA stimulation had an effect. The same was true for the effect of serum starvation. Three hour serum starvation did not seem to have any effect on adducin phosphorylation, but could not be properly assessed due to the already high level of phosphorylation.

To decrease the phosphorylation of adducin, THP-1 cells were washed and rested in fresh, PMA-free media for 24 hours and 48 hours after overnight PMA differentiation. This treatment demonstrated that the level of adducin phosphorylation was significantly reduced in cells rested for 48 hours compared to unrested cells (**Figure 5**). Again, the effect of 12 hours serum starvation was tested and shown not to have a significant effect on the level of adducin phosphorylation. Based on these results, a 48-hour rest period, and no serum starvation, after PMA differentiation and prior to treatment, was used in further experiments.

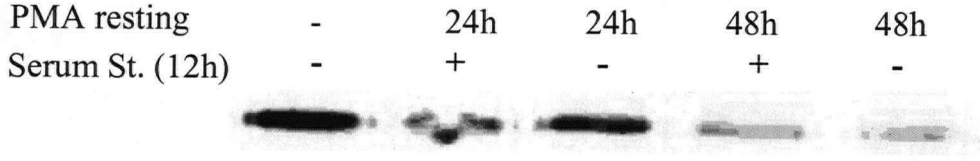

Figure 5: Effect of PMA-differentiation on α-adducin phosphorylation in THP-1 cells.

THP-1 cells differentiated with PMA overnight (0h rest), show a high level of α-adducin phosphorylation. Resting of cells for 24 or 48 hours significantly decreases the level of α-adducin phosphorylation.

To more accurately assess the effect of PMA on adducin phosphorylation in THP-1 cells, cells were rested for 48 hours in PMA-free media prior to PMA stimulation. Cells were then stimulated with increasing concentrations of PMA for 15 min. As demonstrated in **Figure 6,** a concentration of 0.1 μM was sufficient to induce phosphorylation of adducin.

Figure 6: Effect of PMA-stimulation on adducin phosphorylation in THP-1 cells
Cells were differentiated overnight with PMA and allowed to rest in PMA-free media for 48 hours prior to PMA stimulation.

PMA stimulation in J774 macrophages significantly induced the level of phosphorylation as compared to unstimulated cells (**Figure 4B**). J774 macrophages express both α- and γ-adducin whereas THP-1 cells have a much lower expression of γ-adducin than α-adducin. Additionally, the antibody we have used was more specific in J774 macrophages as compared to THP-1 cells. Based on these results, we thought of using J774 macrophages as an infection model. However, several experiments where J774 macrophages were infected with live and heat-killed *M. bovis* BCG GFP could not reproduce the initial results from the KPSS 1.0 screen (**Figure 7**). No change in the phosphorylation status between cells infected with live and heat-killed bacteria could be detected. From these results it was decided to continue using THP-1 cells as the host cells for mycobacterial infections.

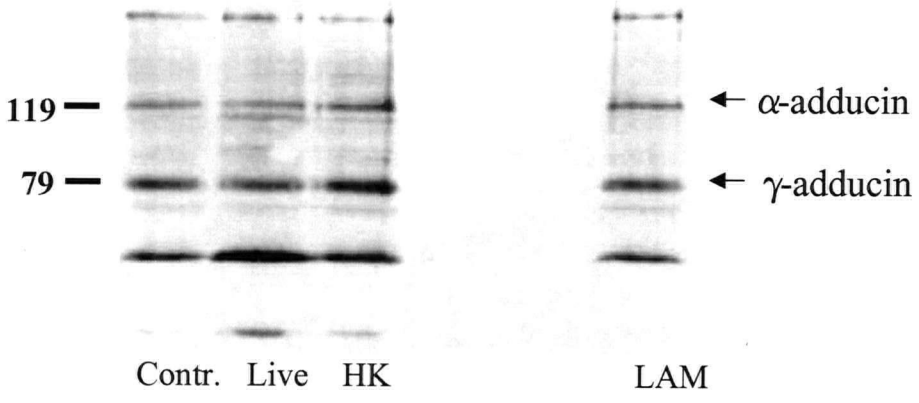

Figure 7: Adducin phosphorylation in J774-macrophages.

Infection of J774 macrophages with *M. bovis* BCG did not significantly induce the level of adducin phosphorylation compared to control and cells infected with heat-killed bacteria. Stimulation with ManLAM (1μg/ml) did not enhance adducin phosphorylation.

3.2.2 Different strains of M. bovis BCG have varying effect on adducin phosphorylation

We chose to use the BCG strain harboring a plasmid expressing the green fluorescence protein (GFP) (35) for two main reasons. First, BCG GFP allows us to locate the organism within the host cells and simplifies the verification and quantification of infection. Second, these bacteria enable us to easily examine colocalization between mycobacterium-containing phagosomes and stained proteins of interest using fluorescence microscopy. In addition, GFP-expressing bacteria are resistant to the antibiotic hygromycin, allowing for the addition of hygromycin to the culture media. This reduces the chances of contamination with environmental bacteria upon long-term incubation. Additionally, we have noted that THP-1 cells infected with growing cultures of *M. bovis* BCG GFP displayed a higher level of adducin phosphorylation than that of growing *M. bovis* BCG or boosted cultures of the two different strains (**Figure 8**). The formation of the GFP fluorophore

involves an auto catalytic reaction that produces hydrogen peroxide as a by-product (35). As a factor of cellular stress, the presence of hydrogen peroxide in GFP expressing bacteria constitutively stimulates stress-response genes and may confer higher survival potential for these bacteria inside macrophages. Thus, the higher level of adducin phosphorylation may be a result of these bacteria coping better within the macrophage, and consequently they are more able to interfere with host cell signaling.

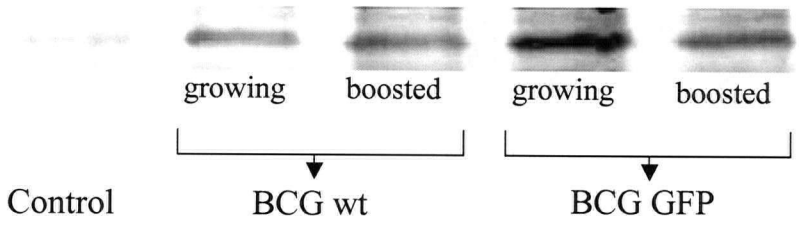

Figure 8: α-Adducin phosphorylation in THP-1 cells infected with different strains of *M. bovis* BCG.

PMA differentiated THP-1 cells were infected with growing or boosted cultures of *M. bovis* BCG (BCG wt) or *M. bovis* BCG GFP (BCG GFP) for 24 hours.

3.2.3 Effects on α-adducin phosphorylation in THP-1 cells infected with live or heat-killed M. bovis BCG GFP or exposed to lipoarabinomannan

Classical Western analysis was used to verify the results obtained from the Kinome analysis. THP-1 cells were treated as described in materials and methods and infected with either live or heat-killed *M. bovis* BCG GFP or exposed to purified ManLAM from *M. tuberculosis* strain H37Rv. Infection or treatment of cells for 24 hours confirmed our screening results and showed that α-adducin phosphorylation is induced in THP-1 cells infected with live but not heat-killed *M. bovis* BCG GFP. α-Adducin phosphorylation was

also induced in cells exposed to purified ManLAM form *M. tuberculosis* (**Figure 9A**). To examine the relationship between time and phosphorylation upon infection, cells where left untreated or infected for 2, 12 and 24 hours, respectively. Results showed that adducin phosphorylation increased as a function of time to reach a maximum at 24 hours (**Figure 9B**). To further investigate the effect of ManLAM on the phosphorylation of α-adducin, cells were treated with increasing concentrations of ManLAM. We found that a concentration as low as 0.01μg/ml was sufficient to induce an increase in phosphorylation and that adducin phosphorylation was related to ManLAM treatment in a dose-dependent manner (**Figure 9C**).

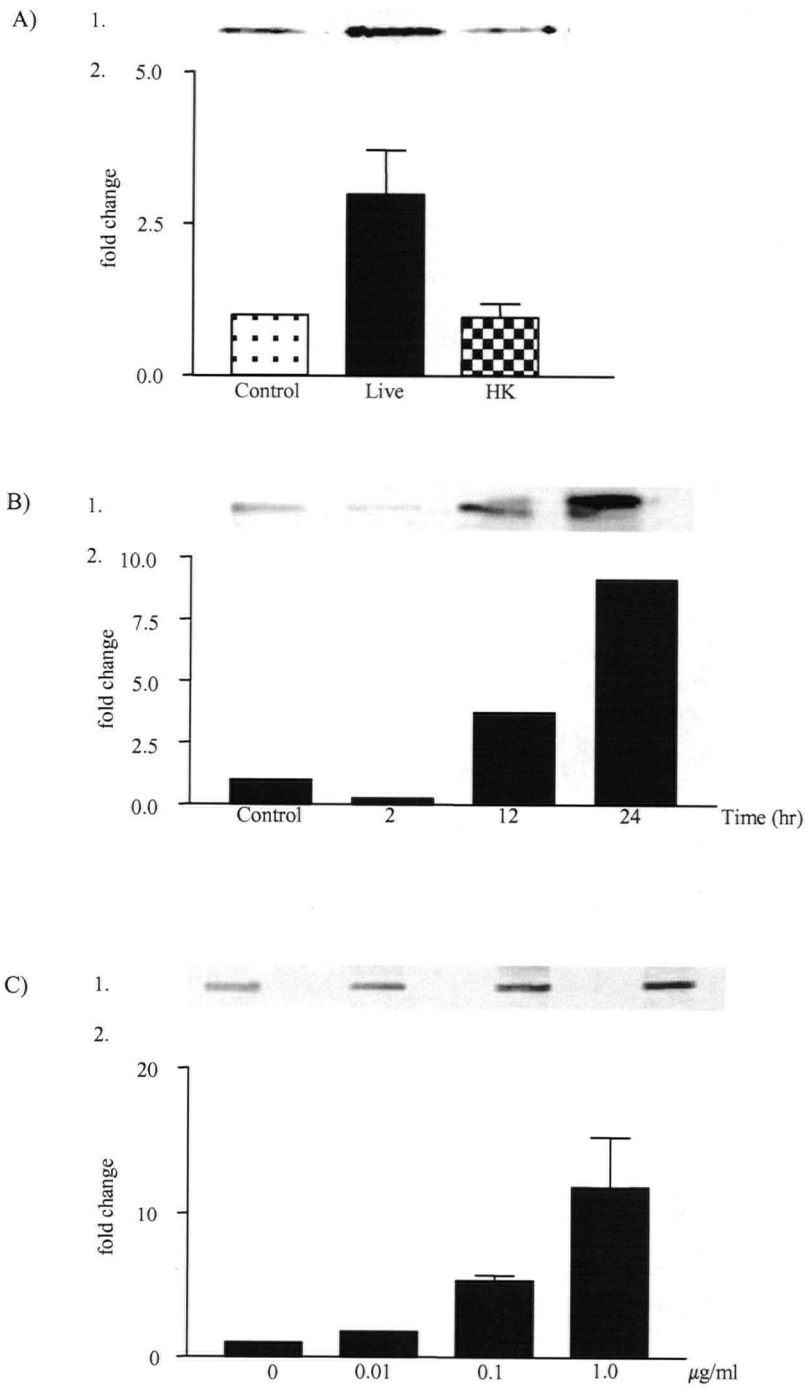

Figure 9: Western analysis of α-adducin phosphorylation in THP-1 cells.

(A) α-Adducin is phosphorylated in cells infected with BCG but not in cells infected with heat-killed bacteria. Panel 1, Western blot of THP-1 cells infected with live and heat-killed (HK) BCG for 24 hours; panel 2, densitometry analysis of panel 1. (B) Time-dependent phosphorylation of α-adducin by *M. bovis* BCG infection. Panel 1, Western blot of THP-1 cells infected with BCG for 2, 12 or 24 hours; panel 2, densitometry analysis of panel 1. (C) α-Adducin phosphorylation by purified ManLAM. Panel 1, Western blot of THP-1 cells treated with increasing concentrations of purified ManLAM for 24 hours; panel 2, densitometry analysis of panel 1. Error bars represent standard error of at least three independent experiments. Equal loading of protein was verified by India ink staining.

3.2.4 Adducin translocates from membrane to cytosol upon phosphorylation

Adducin is a substrate for several protein kinases including protein kinase A (PKA), protein kinase C (PKC) and Rho-kinase (64, 109). Several phosphorylation sites are available for phosphorylation, and, depending on the upstream kinase acting on adducin, the actin-binding properties of adducin are either enhanced or abolished. Adducin carries a MARCKS-like domain in its C-terminal region. The MARCKS-family of proteins is phosphorylated in a protein kinase C (PKC) dependent manner (1). The α-adducin phosphorylation site detected in our screens, serine 724, lies within the MARCKS-like domain and has previously been shown to be a downstream target for PKC (109). Several studies have shown that phosphorylation of adducin in the MARCKS domain leads to dissociation of phospho-adducin from the cytoskeleton and subsequent translocation to cytosol (12, 68). To examine if the observed increase in phosphorylation was related to a translocation of adducin from the cytoskeletal fraction to the cytosolic fraction, a triton-X fractionation of the cell lysate was used. Pellet and supernantant were brought to equal volumes in sample buffer and equal volumes were loaded onto a 7.5% polyacrylamide gel. Probing with anti-phospho-adducin antibody showed that only the supernatant fraction of infected cells contained phosphorylated α-adducin (**Figure 10A**). Probing of the same samples with anti-adducin showed a translocation of adducin from insoluble fraction to soluble fraction upon infection (**Figure 10B**).

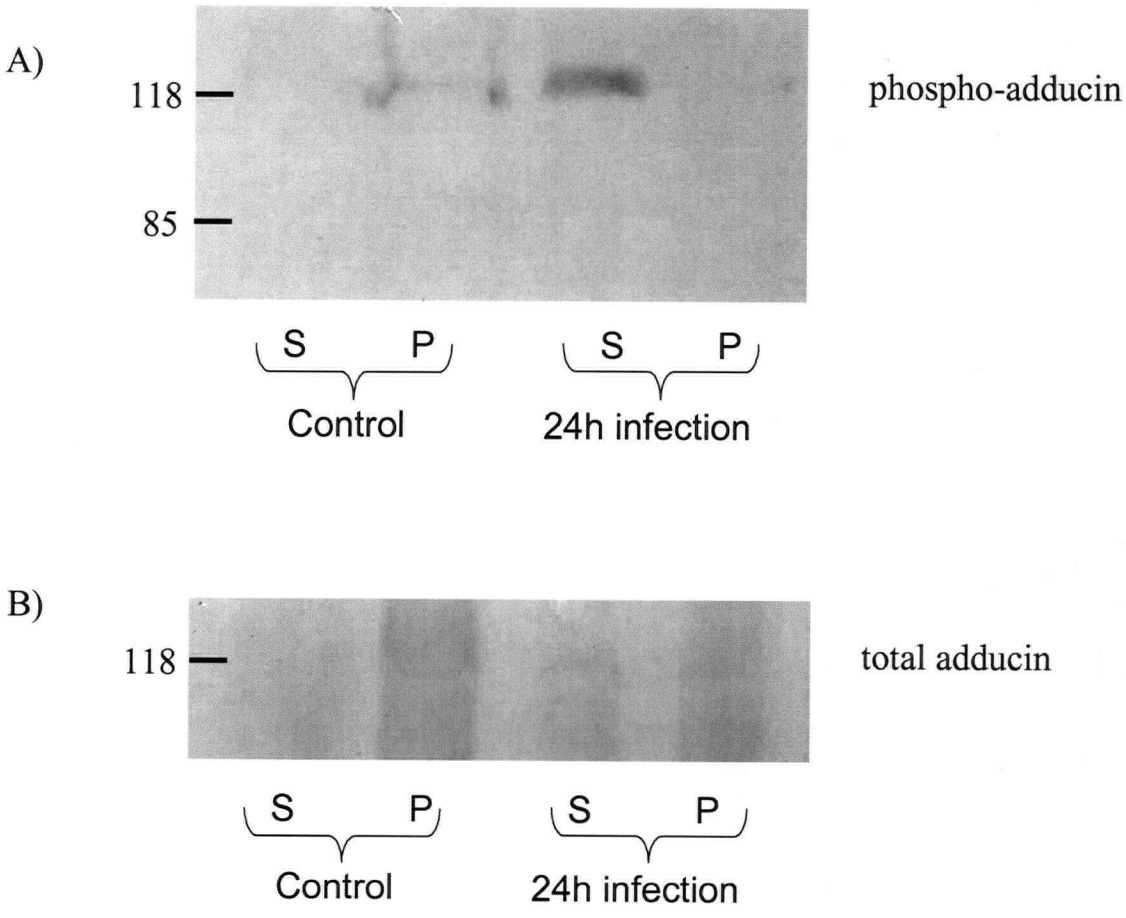

Figure 10: α-Adducin translocates from membrane fraction to cytosol upon infection.

PMA differentiated THP-1 cells were infected or not with *M. bovis* BCG for 24 hours. Soluble (S) and insoluble (P) fraction were loaded in equal amounts and probed with anti-phospho-adducin (A) or total adducin (B).

3.3 Fluorescence microscopy analysis of adducin distribution in infected cells

As our Western analysis of adducin indicate that α-adducin translocates from membrane fraction to cytosol upon infection, immunostaining of adducin was used to determine the subcellular location of adducin in control and infected cells. Cells were differentiated on cover slips, left uninfected or infected with live or heat-killed *M. bovis* BCG for 12 or 24 hours. Permeabilized cells were stained with anti-phopho-adducin and flourescent-conjugated secondary antibody (**Figure 11**). Untreated control cells showed a punctuate pattern of adducin staining mainly localized at the outskirts of the cell. Cells infected with live bacteria showed an increasing distribution of phosphorylated adducin in the cytosolic fraction as a function of time, with highest distribution at 24 hours post infection. In contrast, cells infected with heat-killed bacteria displayed a pattern of phospho-adducin distribution resembling that for control untreated cells. These results are in agreement with our Western analyses, and shows that: 1) live but not heat-killed bacteria induce an increased phosphorylation of adducin compared to control untreated cells, 2) phosphorylation increases as a function of time, and 3) an increased amount of phosphorylated adducin appears in the cytocolic fraction of infected cells.

Actin filaments along with actin binding proteins have been found in association with the phagosomal membrane during phagocytosis and subsequent phagosomal maturation (42). Our results, as seen in **Figure 11**, show phospho-adducin evenly scattered in the cytosol with no observed co-localization between the bacteria and phosphorylated adducin at any time point. As of yet, no specific function has been assigned to phosphorylated adducin once detached from the spectrin-actin junctions. For example, platelet activation was associated with phosphorylation of adducin and subsequent proteolysis of adducin by the protease

calpain (68). Similarly, adducin was proteolysed in a caspase 3-dependent manner upon phosphorylation in epithelial cells (154). These reports indicate that adducin may be active only in association with the actin-spectrin network. Thus, the finding that phosphorylated adducin does not colocalize with the phagosomal membrane was not unexpected. However, adducin in its unphosphorylated form may be part of the numerous actin-binding proteins present on phagosomes in connection with actin. Unfortunately we have been limited in our immunohistochemistry analyses of adducin due to the lack of a total-adducin antibody suitable for intracellular staining, and so have not been able to investigate this possibility.

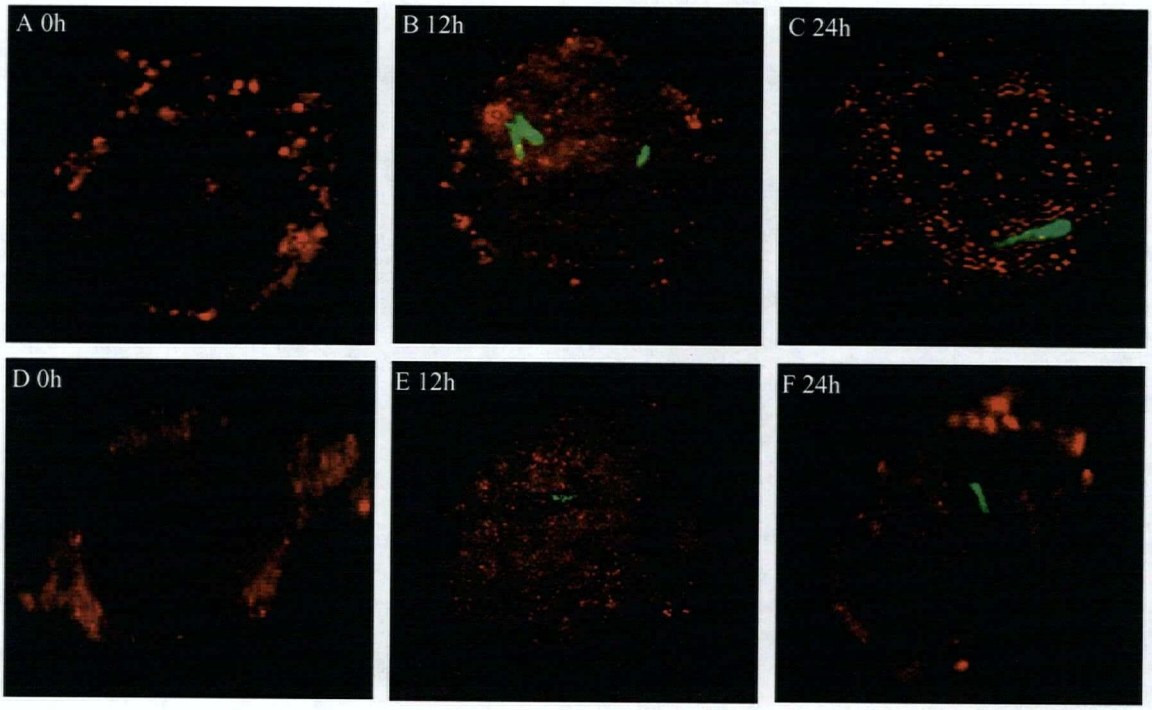

Figure 11: Immunostaining of phosphorylated adducin in infected cells.

THP-1 cells were left uninfected, (A) and (D), infected with live (B) and (C), or heat-killed, (E) and (F), M. bovis BCG for the indicated time periods. Phosphorylated adducin is shown in red. Green represents *M. bovis* BCG expressing the green fluorescent protein (GFP).

3.4 Fluorescence microscopy analysis of actin filaments in infected cells

Due to the role of adducin in actin filament organization, we decided to investigate actin filament formation in cells infected with live and heat-killed bacteria. Actin is a major target for several pathogenic organisms, which in various ways manipulate its structure and dynamics to their own advantage (72). Only a few years ago, the first evidence was provided that also pathogenic mycobacteria may take advantage of the host's actin network for their intracellular survival (73, 74). Phalloidin, produced by the mushroom *Amanita Phalloides,* is extensively used for intracellular staining of actin. It specifically binds to and stabilizes filamentous actin, preventing depolymerization.

3.4.1 Live M. bovis BCG inhibits phagosomal actin assembly.

We infected THP-1 cells adherent to coverslips with live or heat-killed *M. bovis* BCG for 24 hours (**Figure 12).** *M. bovis* BCG expressing GFP allows for easy localization of bacteria within the host cells. Red fluorescence conjugated phalloidin was used to stain for actin filaments. Actin was seen throughout the cell either as thin filaments or in a punctuate pattern indicative of adhesive structures (not shown). The concentration of actin was highest near the plasma membrane. Infection of THP-1 cells with live bacteria showed no significant change in the distribution of actin. No colocalization between with actin and mycobacteria-containing phagosomes was observed (**Figure 12A**). In stark contrast, infection with heat-killed bacteria showed that a significant proportion of engulfed bacteria were coated with actin (**Figure 12B).** This is in keeping with the understanding that actin has a functional role at all stages of phagosomal maturation (48). These results show that live mycobacteria are able to inhibit the assembly of actin filaments surrounding the phagosome.

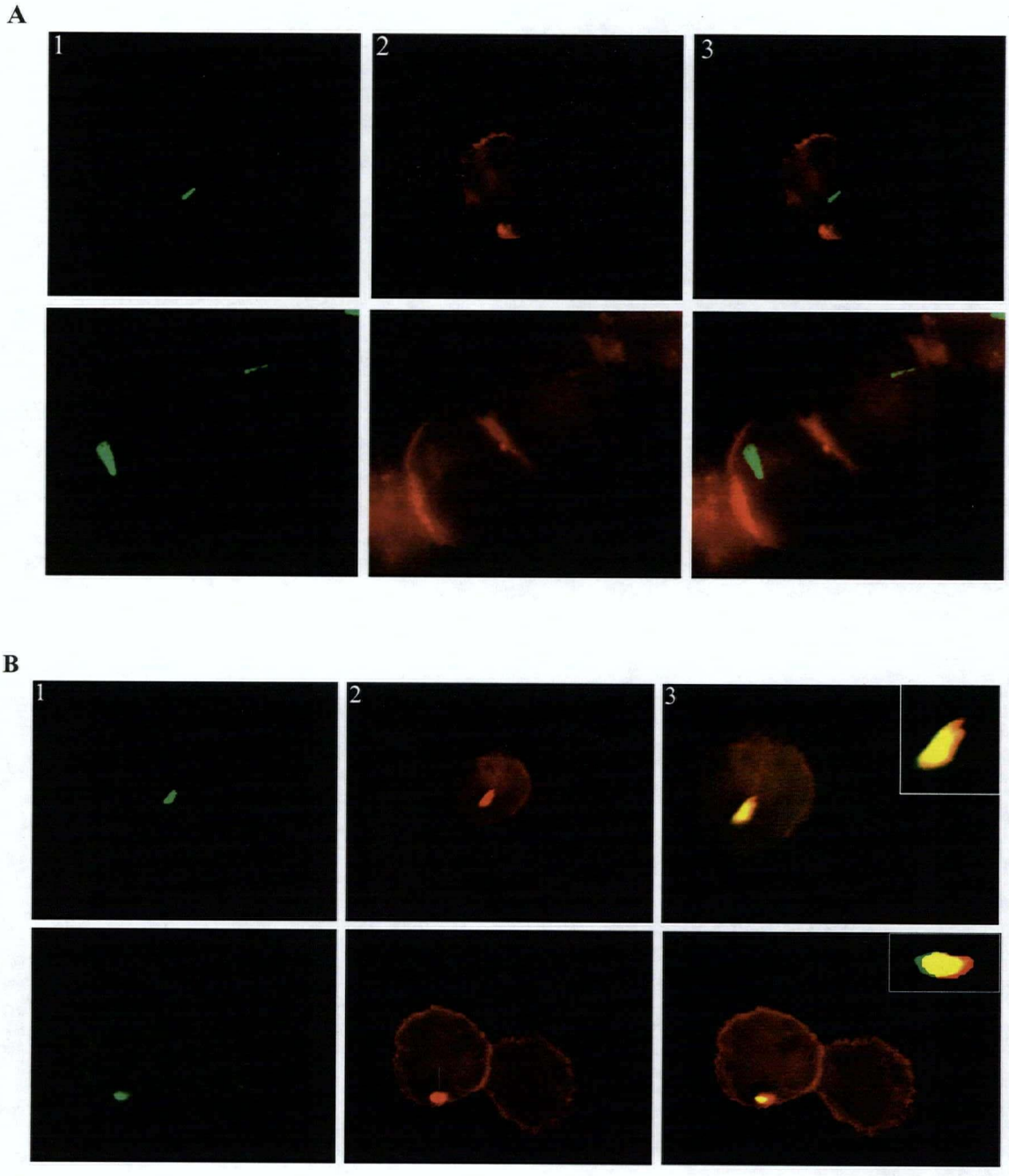

Figure 12: Actin assembly in infected macrophages.

(A) THP-1 macrophages were infected with live *M. bovis* BCG GFP for 24 hours. Panel 1 and 2 show GFP expressing *M. bovis* BCG and Alexa-phalloidin staining of infected cell, respectively; panel 3 shows panel 1 and 2 in combination. (B) THP-1 macrophages were infected with heat-killed *M. bovis* BCG GFP for 24 hours. Panel 1 and 2 show GFP expressing *M. bovis* BCG and Alexa-phalloidin staining of infected cell, respectively; panel 3 shows panel 1 and 2 in combination. Panel 3 insets show area of colocalization enlarged. Pictures are shown in duplicates. All experiments are done in triplicates.

CHAPTER 4: DISCUSSION

4.1 Modulation of key host proteins upon mycobacterial infection

4.1.1 THP-1 macrophages and M. bovis BCG as a model system for the study of mycobacterial infection

M. bovis BCG organisms were used in this project to infect the human monocytic cell line, THP-1. PMA treatment induces differentiation of THP-1 cells into a macrophage-like cell line that displays most of the human monocyte-derived macrophage phenotypes with regard to morphology, phagocytic potential, cytokine secretion, and expression of surface markers and receptors (7).

M. bovis Bacille Calmette Guérin (BCG) was established by the two French researchers, Calmette and Guérin (15) in an attempt to develop an effective vaccine against the human pathogen *M. tuberculosis.* A strain of the bovine pathogen *M. bovis* was passaged 230 times *in vitro* between 1908 and 1921, and the resulting strain was thought to have retained its immunogenic potential in combination with reduced virulence (15).

Today *M. bovis* BCG is widely used as a model system to study the pathogenesis of *M. tuberculosis.* Although *M. bovis* BCG is a deletion version of *M. tuberculosis* (15) it retains its ability to survive intracellularly and it successfully prevents phagosomal maturation (79, 157). This makes THP-1 cells and *M. bovis* BCG a suitable model system for the study of interactions between mycobacteria and the host cell.

4.1.2 Analysis of host protein phosphorylation upon mycobacterial infection

The first part of this research project was aimed at identifying key host proteins that are modified upon mycobacterial infection. To achieve this, we employed a unique multi-phospho analysis, using an array of 31 phospho-specific antibodies covering kinases and

other signaling proteins from major eukaryotic networks known to date. The advantage of this technique lies in its ability to simultaneously and quantitatively track single amino acid phosphorylations of up to 100 phospho-proteins. We have chosen to investigate cell signaling after an infection period of 24 hours, thereby excluding early events associated with initial phagocytotic uptake of the bacteria. We rationalize that at this timepoint, the bacteria have established themselves in their host environment to a greater extent, offering an opportunity to assess the host response towards bacteria residing within phagosomes.

Two separate screens were performed: first, THP-1 cells were infected with either live or heat-killed *M. bovis* BCG (**Figure 2**); secondly, THP-1 cells were infected with live bacteria or treated with purified mannose capped lipoarabinomannan, referred to as ManLAM (**Figure 3**). Untreated, differentiated THP-1 cells were used as control in both screens. ManLAM is expressed by both *M. tuberculosis* and *M. bovis* BCG and differs in terms of structure and immunogenicity from the LAMs expressed by avirulent strains of mycobacteria (116). ManLAM from BCG and *M. tuberculosis* have been found to inhibit the production of proinflammatory cytokines such as IL-12 and TNF-α (97), in addition to playing a more direct role in phagosomal maturation arrest (61), suggesting a role for ManLAM in the persistence of virulent mycobacteria within macrophages.

This discussion is limited to proteins that for each screen showed more than a 25% change in phosphorylation between infected and control cells. As seen in Table 1, none of the changes in phosphorylation induced by ManLAM were reproduced in cells infected with heat-killed bacteria. Since common purification methods for LAM involve heat-killing of *M. tuberculosis* (77), one would assume that ManLAM is unaffected by heat treatment and that heat-killed bacteria retain LAM in its original structure. However, several factors could

explain the differences in host cell response between the effects of ManLAM in its purified form and ManLAM in heat-killed bacteria. First, mycobacteria may shed LAM as a result of heat treatment, reducing the amount of LAM encountered by the host cell. Second, heat may induce conformational changes of bacterial surface molecules that can hinder interaction of LAM with host cell receptors. Thirdly, heat treatment, followed by killing of BCG most probably alters the active release of ManLAM once the bacteria reside within the host cell. Active release of ManLAM by live bacteria causes exposure of the whole molecule of ManLAM to the host cell. It is possible that the lipid moiety of ManLAM, normally embedded in the mycobacterial cell wall, is required for the biological functions of ManLAM in its interaction with host cell signaling elements. Lipopolysaccharide (LPS), which is present on the surface of gram-negative bacteria, is an example of a molecule that shows structural similarity to LAM. The endotoxic activity of LPS is predominantly associated with its free form and particularly the lipid component, lipid A, which needs to be exposed for full activity (3).

4.1.3 Six host proteins show significant change in phosphorylation upon mycobacterial infection

Five host signaling proteins, i.e., NR1, SAPK, c-Jun, GSK3β, and α-adducin, (**Figure 2, Figure 3 and Table 1**) showed more than 25% increase in phosphorylation over that for the control untreated cells in both screens. Conversely, the basal level of PKCϵ phosphorylation was completely attenuated in cells infected with either live or killed bacteria indicating a possible deactivation of this protein upon infection.

NR1 is a principle subunit of the N-methyl-D-aspartate (NMDA) receptors, which represent a major class of glutamate-gated ion channels in the central nervous system (115).

These receptors are highly permeable to calcium and regulate intracellular levels of calcium in neuronal cells as well as in non-neuronal cell lines when transfected into these (29, 70, 71, 152). A complex regulatory machinery involving a number of protein kinases and phosphatases controls the function of NMDA receptors in neurons [reviewed in (103)]. The S^{896} of the NR1 subunit was phosphorylated upon infection with BCG, and has been identified as a specific phosphorylation site for PKC (149). PKC phosphorylation of the NR1 subunit has been shown to both enhance and inhibit NMDA receptor currents depending on cell type and compositional variation of the receptor itself (69). In our system, cells infected with live bacteria showed a twofold average increase in the phosphorylation of NR1 S^{896} above levels for cells infected with heat-killed bacteria and cells exposed to LAM (**Table 1**). The possible role of NR1 expression in THP-1 cells is not clear; to our knowledge, NMDA receptors are not expressed in THP-1 cells or any other cell from the haemopoietic cell linage. Our results indicate that an NR1-homologue could be present in THP-1 cells, possibly constituting an NMDA-related receptor. If so, this receptor might have a similar function to that described in neurons, regulating levels of intracellular Ca^{2+} in macrophages. *M. tuberculosis* has been shown to inhibit calcium signaling in human macrophages correlating with reduced phagosomal maturation and enhanced intracellular survival of the bacteria (106). It is therefore tempting to speculate that mycobacterial interference with a potential NMDA-related receptor could be a contributing factor to the inhibition of host Ca^{2+} signaling upon infection.

SAPK showed a 2-3-fold increase in phosphorylation in cells infected with live bacteria and in cells exposed to ManLAM over heat-killed and untreated cells (**Table 1**). SAPK is a member of the mitogen-activated protein kinase (MAPK)-family and is encoded

by three genes: *SAPK1, SAPK2, and SAPK3* (75). All *SAPK* genes are expressed as 46kDa and 54kDa protein kinases (75) and both SAPK-isoforms showed a similar increase in phosphorylation in our system. Several transcription factors, including ATF-2, Ets and c-Jun have been identified as downstream targets for SAPK [reviewed in (87)]. Specifically, SAPK has been found to bind the c-Jun transactivation domain and phosphorylate it on S^{63} and S^{73}, thereby enhancing transcriptional activity (47, 80). c-Jun is a central component of activator protein 1 complexes (28), which upon transcriptional activation have been associated with a variety of cellular functions including cell proliferation, tumourigenesis and apoptosis [reviewed in (50)]. In this study we show increased phosphorylation of c-Jun S^{73} in cells infected with live bacteria, as well as in cells exposed to ManLAM. Activation of SAPK upon mycobacterial infection has been shown as an early response in several model systems, often in concert with p38 MAPK and ERK 1/2 activation (19, 151). Even though a regulatory role of the JNK signaling pathway has been suggested for the production of nitric oxide in mouse macrophages (25), the role of SAPK in mycobacterial infection remains unclear. Our results are the first to indicate that LAM triggers a signaling cascade leading to activation of SAPK and its downstream effector c-Jun.

The phospho-protein GSK3β showed an average increase of 57% for Y^{216} phosphorylation in THP-1 cells infected with live bacteria and in cells exposed to LAM. GSK3 has been shown to play an essential role in the regulation of cell fate in both a pro- and anti-apoptotic manner. For example, GSK3β gene disruption in mice caused hepatocyte apoptosis and correlated with impaired anti-apoptotic NF-κB signaling (82). In contrast, PI3-kinase mediated activation of protein kinase B (Akt), was found to induce cell survival by inhibiting GSK3 (38). In agreement with the latter observation, Akt has been shown to inhibit

GSK3β activity through phosphorylation of its Ser^{9} residue (49, 57). Conversely, phosphorylation of GSK3β Y^{216} is critical for full activation of the enzyme (85), and has been shown to induce apoptosis (18). Our results demonstrate activation of GSK3β through increased phosphorylation of Y^{216} upon infection with live bacteria and exposure to ManLAM. Furthermore, our results showed no significant activation of Akt or Ser^{9} phosphoryltation of GSK3β in infected cells (**Table 1**), suggesting that mycobacterial infection primes THP-1 cells for apoptosis via activation of GSK3β while the anti-apoptotic pathway remains silent.

The two phosphoscreens showed no phosphorylation of PKCϵ in cells infected with live bacteria. In contrast, control cells and cells exposed to ManLAM showed substantial PKCϵ phosphorylation. The function of PKC and the specificities of the different isoforms in macrophage signaling and mycobacterial infection are still unclear. However, PKCϵ has been shown to be important in macrophage activation and defense against bacterial infection (24). Macrophages from PKCϵ-/- mice showed severely attenuated response to lipopolysaccharide and INF-γ, characterized by a dramatic decrease in the generation of nitric oxide, TNF-α, and IL-1β (24). Our results indicate an attenuation of PKCϵ activity, which could be regarded as a strategy employed by mycobacteria to avoid activation of the macrophage.

A major finding in this study is the phosphorylation of the cytoskeletal protein α-adducin (**Table 1**). Phosphorylation of α-adducin showed an average increase of 75 % in cells infected with live bacteria compared to those infected with heat-killed bacteria. Owing to its status as a unique protein that has not previously been investigated with respect to mycobacterial infection, α-adducin was selected for further analysis (see below).

Changes in phosphorylation seen for SAPK, c-Jun, α-adducin and GSK3β upon infection with *M. bovis* BCG have been reproduced in cells exposed to ManLAM, suggesting ManLAM to be the mediating factor. In contrast, changes in phosphorylation of PKCε and NR1 were not reproduced in cells exposed to ManLAM, indicating that another bacterial component is responsible. In murine macrophages, phagosomes containing *M. bovis* BCG were shown to be permeable to dextrans as large as 70 kDa (147) and a number of mycobacterial surface proteins are released from phagosomes into subcellular compartments (14). Thus, the notion that mycobacteria are capable of releasing proteins into the host cell cytoplasm led us to hypothesize that mycobacteria actively interfere with host signalling elements to promote their own survival. With the presence of genes for 11 eukaryotic-like protein serine kinases and four protein phosphatases in the genome of *M. tuberculosis* (8), it is tempting to speculate that these proteins might be exported intracellulary and interfere with signal transduction cascades within the host cell. Eukaryotic-like protein kinases and phosphatases have previously been implicated in the virulence of other pathogens such as *Yersinia pseudotuberculosis* and *Salmonella entericia* serovar Typhimurium, both of which translocate bacterial proteins into the host cell cytoplasm, resulting in disruption of the host cell cytoskeleton (63, 76). Interestingly, a mycobacterial phosphatase, PtpA is present in the mycobacterial genome without a corresponding substrate within the bacterium (36). Additionally, PtpA was found in the culture medium of growing bacteria, suggesting that this phosphatase may be secreted (36). These observations further support the hypothesis of cross-interaction between bacterial and host cell signalling elements.

4.2 Adducin

Adducin was first isolated from human erythrocyte membranes (65) and has since then been detected in all tissues examined including brain, kidney, testes, liver, lung, and also in a variety of cell lines. Adducin is expressed as a heterodimeric protein comprised of either α and β subunits or α and γ subunits [reviewed in (110)]. α- and γ-adducin are ubiquitously expressed whereas the beta isoform shows a more restricted pattern of expression. All three subunits are related in amino acid sequence and organization and contain three distinct domains: a 39-kDa amino terminal head domain, a 9 kDa "neck" domain, and a carboxy-terminal tail domain **(Figure 13a).**

Adducin forms a ternary complex with spectrin and F-actin where adducin functions to recruit spectrin to ends of actin filaments, bundle actin filaments and cap the barbed (fast growing) ends of filamentous actin **(Figure 13b)**. These functions may represent independent aspects of adducin activity (110). Compositional studies have shown that the head domains are in contact to form a globular core with interacting tail domains extended away from the core. Adducin binds spectrin-actin-complexes via its tail domains and the interaction has been shown to involve beta-spectrin **(Figure 13c)** (84). A highly conserved myristolated alanine-rich C kinase (MARCKS)-related domain (1, 20) is present in the C-terminal of all three subunits **(Figure 13a)**. The MARCKS-like domain is required for interactions of adducin with spectrin and actin and contains a major phosphorylation site for PKC and PKA as well as a biding site for calcium/calmodulin (109). Adducin is also phosphorylated by Rho-kinase. Together these proteins regulate adducin's interaction with spectrin and actin. *In vitro* and *in vivo* phosphorylation by PKC inhibits adducin's actin capping activities and its ability to recruit spectrin to ends and sides of actin filaments (109). Although

phosphorylation by PKA has been shown to have a similar effect on adducin activities *in vitro*, there is evidence to suggest that PKA do not phosphorylate adducin *in vivo* (109). PKC phosphorylation also inhibits binding of calcium/calmodulin to adducin. In contrast to the effects of PKC activity towards adducin, phosphorylation of adducin by Rho kinase enhances the actin binding activity of adducin and has been seen in connection with cell motility and membrane ruffling (64).

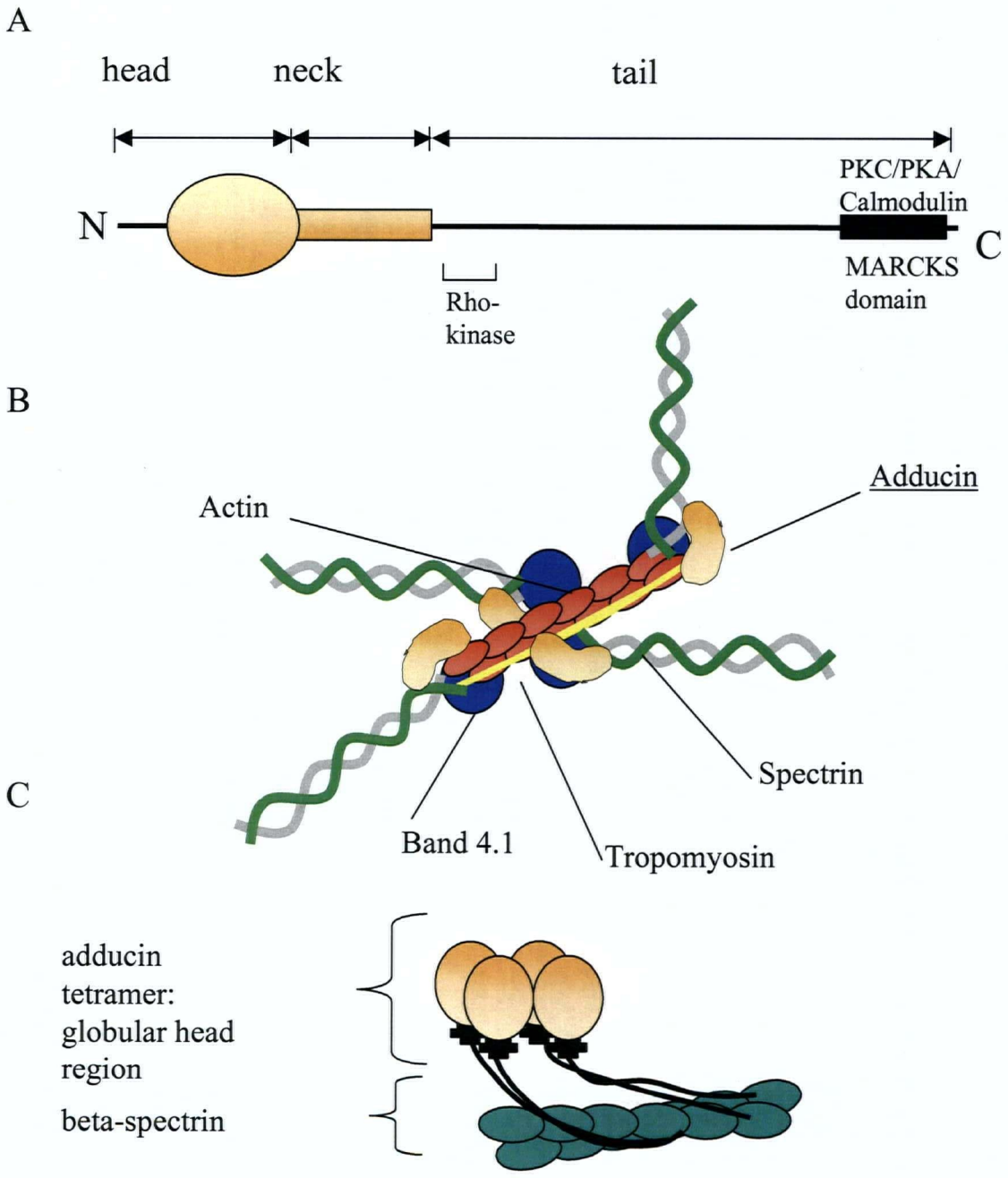

Figure 13: Structure and function of adducin.

(A) All three subunits of adducin are related in amino acid sequence and organization and contain three distinct domains: a 39-kDa amino terminal head domain, a 9 kDa "neck" domain, and a carboxy-terminal tail domain. The C-terminal contains a highly conserved myristolated alanine-rich C kinase (MARCKS)-related domain, which is the phosphorylation site for protein kinase A and C as well as the binding site for calmodulin. (B) Adducin forms a tetramer with actin and spectrin and is bound to the actin-spectrin complex via its MARCKS domain. Tropomyosin and Band 4.1 are cytoskeletal proteins. (C) Compositional studies have shown that the head domains are in contact to form a globular core with interacting tail domains extended away from the core. Adducin binds spectrin-actin-complexes via its tail domains and the interaction has been shown to involve beta-spectrin. Figure A and C are adapted from (110). Figure B is adapted from (2).

4.2.1 Adducin expression in human THP-1 cells

Western blot analyses using commercially available antibodies (Upstate biotechnology, Santa Cruz) have been applied to study the activity of adducin in THP-1 cells. The results obtained showed that THP-1 cells express α- (120 kDa) and γ-adducin (80 kDa). α-adducin was shown to undergo increased phosphorylation upon infection as identified by the Kinome analysis. Therefore, our experiments have been focused on the expression of this isoform.

Undifferentiated THP-1 cells in culture show a high level of α-adducin phosphorylation, as do cells differentiated overnight with PMA (**Figure 4**). Undifferentiated THP-1 cells are in a state of continuous proliferation and cell division, which require a dynamic actin cytoskeleton (101). This could explain the observation that a large proportion of adducin resides in a phosphorylated state. The high level of adducin phosphorylation in differentiated cells could be a direct cause of PMA stimulation. However, since 100 nM PMA is necessary to induce a moderate increase in adducin phosphorylation (**Figure 6**), it seems unlikely that the concentration of PMA used for differentiation (~30nM) is responsible for the high levels seen at 16-24 hours after PMA stimulation. A more probable explanation is the process of differentiation itself. As THP-1 cells differentiate into macrophages they undergo a dramatic change in morphology. Differentiation is accompanied by development of various adhesive structures to allow attachment and adherence to the underlying surface, spreading, and development of protrusions such as pseudopodia (44). These changes are accompanied by an extensive reorganization of actin filament networks and provide a likely explanation for the high level of adducin phosphorylation observed in THP-1 cells after overnight differentiation. Phosphorylation-induced inhibition of spectrin recruitment, actin

capping, and actin bundling activities of adducin would allow for elongation and reorganization of the actin filaments. These activities are most likely necessary for the morphological changes to occur both in dividing and differentiating cells. Furthermore, gene expression studies of the leukemic cell line HL-60 showed an up-regulation of adducin during differentiation of these cells into a granulocytic lineage (142). This report is supportive of our results indicating a functional role of adducin during the differentiation of monocytes into macrophages. We show that resting of differentiated THP-1 cells reduces the level of phosphorylation over time, indicating a more stationary cytoskeleton at this point.

4.2.2 Mycobacterial infection induces phosphorylation of adducin

Phosphorylation of α-adducin showed an average increase of 75% in cells infected for 24 hours with live bacteria compared to those infected with heat-killed bacteria. Western blot analyses using identical antibodies to those used in the screens were used to further examine adducin phosphorylation in THP-1 cells. The results from the two phospho-screens were confirmed and showed that α-adducin is phosphorylated in THP-1 cells infected with live *M. bovis* BCG, but not in cells infected with heat-killed bacteria. Furthermore, α-adducin phosphorylation increases with time to reach a maximum at 24 hours. Treatment of cells with purified ManLAM showed an increase in α-adducin phosphorylation. Further investigation showed that ManLAM induces phosphorylation in a dose-dependent manner detectable at a concentration as low as 0.01μg/ml (**Figure 9**). The α-adducin S^{724} residue, detected in our screens, is located within the MARCKS domain, suggesting that PKC could be the upstream kinase acting on α-adducin. To support this hypothesis we have observed an increased phosphorylation of α-adducin upon treatment of cells with the PKC-activator, PMA

(**Figure 6**), and that adducin translocates from the cytoskeletal fraction to cytosol upon phosphorylation (**Figure 10**). These observations are typical outcomes of PKC-mediated phosphorylation of adducin (12). However, we did not detect a significant increase in phosphorylation for any of the PKC isoforms tested in our screens. It might be that another kinase is involved in adducin phosphorylation in macrophages. Nevertheless, the lack of PKC phosphorylation may be due to a deactivation of upstream kinases at the 24 hour timepoint tested and cannot necessarily rule out the involvement of PKC in our system.

As described above, phosphorylation of adducin by PKC inhibits the principal activities of adducin i.e. capping of the fast growing end of actin filaments, recruitment of spectrin to ends of actin filaments, and bundling of actin filaments (109). PKC-induced dissociation of phosphorylated adducin from actin filaments may be involved in exposing barbed ends to allow for actin nucleation (12). Additionally, inhibition of actin bundling and spectrin recruitment may facilitate a structural reorganization of actin filament networks.

In a pathogenic setting, cytoskeletal dynamics play a crucial role in facilitating fusion events between early endosomes, phagosomes and other organelles of the endocytic pathway [reviewed in (17)]. Although the participation of actin filaments in phagocytosis of microorganisms has been well characterized and studied in several model systems, the involvement of actin and actin-binding proteins in the maturation and fusion events of phagosomes is still unclear. Nevertheless, several lines of evidence assign an important role for actin filaments in the post phagocytotic fate of nascent phagosomes, at all stages of phagosomal maturation. For example, treatment with the actin-depolymerizing agent Cytochalasin D has been shown to inhibit phagosomal transport and fusion events along the endosomal pathway (88, 150). Furthermore, actin, along with several actin-binding proteins,

has been found in association with mature phagosomes (42, 95, 145). Unambiguous evidence for the role of actin in phagosomal motility was provided by video microscopy in murine macrophages, which showed the formation of actin-rich rocket tails behind bead-containing phagosomes (162). Studies employing latex bead-containing phagosomes have greatly contributed to our understanding of actin in phagosome biogenesis. This system has shown that phagosomes assemble actin *de novo* in such a way that the fast growing end is localized at the membrane while the slow growing end points away from the membrane (42, 43). This provides the right polarity for fusion partners, such as lysosomes, to move along these actin tracks. With the help of myosin, organelles can bind to and move along actin filaments (131).

The actin network of phagocytic cells has been identified as a target for a wide range of pathogenic bacteria, including *Salmonella, Shigella, Listeria,* and *Legionella* [reviewed in (72)]. These pathogens manipulate the actin cytoskeleton in several ways to either enhance their uptake into mammalian cells or to facilitate their own movement through host cell cytoplasm and eventually into neighboring cells. As described earlier, adducin phosphorylation may be involved in exposing actin filament barbed ends (12). Consequently, adducin may be involved in the establishment of assembly sites for actin nucleation. Three intracellular pathogens, namely *Listeria, Shigella,* and enteropathogenic *Escherichia coli* (EPEC), specifically manipulate signaling pathways controlling actin nucleation. These pathogens have independently developed strategies that at some point intersect the signaling pathways involved in the activation of the Arp2/3 complex [reviewed in (72)]. This is a seven-protein complex that upon activation can bind to sides of preexisting filaments or to barbed ends and initiate the nucleation of daughter strains (23, 52). This mechanism is manipulated by these pathogens to either enhance their uptake into the host cell or to

facilitate actin based motility within the host cell (72). There are currently three major mechanisms defined for initiating actin assembly in mammalian cells. One involves the removal of capping proteins to expose barbed ends. Another depends on the activity of the ADF/cofilin family of proteins, which by severing of actin filaments expose free barbed ends. The third mechanism involves the Arp2/3 complex (34). The extent by which intracellular pathogens have been shown to manipulate the host actin cytoskeleton, would suggest that also pathogenic species of mycobacteria might take advantage of such a mechanism. In support of this, adducin phosphorylation can, similarly to Arp2/3 activation, provide a site for actin nucleation.

We have shown that live *M. bovis* BCG prevents actin accumulation around their phagosome (**Figure 12**). This finding strongly supports the hypothesis that mycobacteria interfere with the host's actin filament network upon infection. In support of this finding, virulent strains of *M. avium* have earlier been shown to disrupt the host actin network, correlating with a delay in the acquisition of endocytic markers by phagosomes containing mycobacteria (73). In this case, it was shown that disruption of the actin network occurred after 24 hours of infection and was maintained up to 15 days following infection. The pattern of cytoskeletal disruption was found to resemble one in which cells are treated with the actin-depolymerizing agent, Cytochalasin D. This chemical, when used in appropriate amounts, inhibits actin polymerization by decreasing the addition of monomeric actin molecules to the barbed end of actin filaments. Guerin and de Chastellier (74) proposed that actin filaments, along with their associated proteins may function as a network surrounding nascent and maturing phagosomes. Here, organelles of the endocytic machinery are brought into proximity to facilitate fusion events and intermingling of contents between compartments. By

disrupting or reorganizing such a network, invading mycobacteria can possibly prevent fusion with lysosomes and inhibit the ongoing intermingling of contents between compartments. Thus, manipulation of actin filament dynamics, including both polymerization and depolymerization, is likely to play a role in phagosomal maturation. In this context, adducin phosphorylation, mediated by mycobacterial factors such as lipoarabinomannan, could possibly lead to a reorganization of the actin cytoskeleton enabling exclusion of the phagosome from the endocytic pathway.

Additional note:

While writing this thesis, a paper was published demonstrating that macrophages containing live pathogenic mycobacteria, including *M. bovis* BCG, inhibited actin assembly on their phagosomes (4). In contrast, phagosomes containing non-pathogenic or killed pathogenic mycobacteria assembled actin on their phagosomes in a manner very similar to that observed for phagosomes containing latex beads (4, 48). Furthermore, this paper demonstrated that several lipids were able to activate and restore actin assembly resulting in phagosomal maturation and enhanced killing of pathogenic mycobacteria. This report has clearly assigned a critical role for actin in phagosomal maturation and shown that by inhibiting phagosomal actin assembly, pathogenic mycobacteria prevent maturation of their phagosome. These findings strongly support the findings from this thesis.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions

5.1.1 Kinome Analysis

Our ultimate goal in this set of experiments was to identify key host proteins that are modified upon mycobacterial infection. In accordance with this aim, we have presented a unique set of results based on a simultaneous screen of key host proteins upon mycobacterial infection of a human macrophage-like cell line. We have shown changes in phosphorylation of host proteins that are novel to the field of cellular mycobacteriology. **Figure 14** summarizes the findings with accompanying hypotheses from the kinome analysis. Some of the signaling proteins shown to be activated, such as SAPK, c-Jun, and GSK3β have previously been implicated in the regulation of apoptotic pathways. As described, it has been well established that macrophages infected with mycobacteria have increased rates of apoptosis *in vitro* (59, 129, 130). The observation that virulent strains induce less apoptosis than avirulent and attenuated strains (10, 94) has reinforced the conception that apoptosis functions as a host cell defense mechanism in mycobacterial infection and that virulent strains have developed strategies to promote host cell survival. *M. bovis* BCG was recently shown to induce apoptosis in THP-1 cells (129). In agreement with this, activation of the JNK-c-Jun signaling pathway and GSK3β could be interpreted as pro-apoptotic signaling as part of the host cell defense (A). On the other hand, we have presented evidence to suggest an active attenuation of macrophage defense through mycobacterial inhibition of PKCϵ (B) and possible interference with host Ca^{2+}-signaling (C). Furthermore, we propose a potential mechanism by which mycobacteria interfere with the actin cytoskeleton as a means to exclude the phagosome from the endocytic pathway (D). Experiment designed to analyze

downstream effects of the reported phosphorylation events, will allow us to better understand the ultimate outcome of these events. Taken together, our results present evidence in accordance with previous reports indicating that a number of host signaling pathways are modulated upon mycobacteria infection. Whether this is due to a single modulation event affecting several downstream signaling pathways or direct modulation of different signaling cascades, remains to be elucidated.

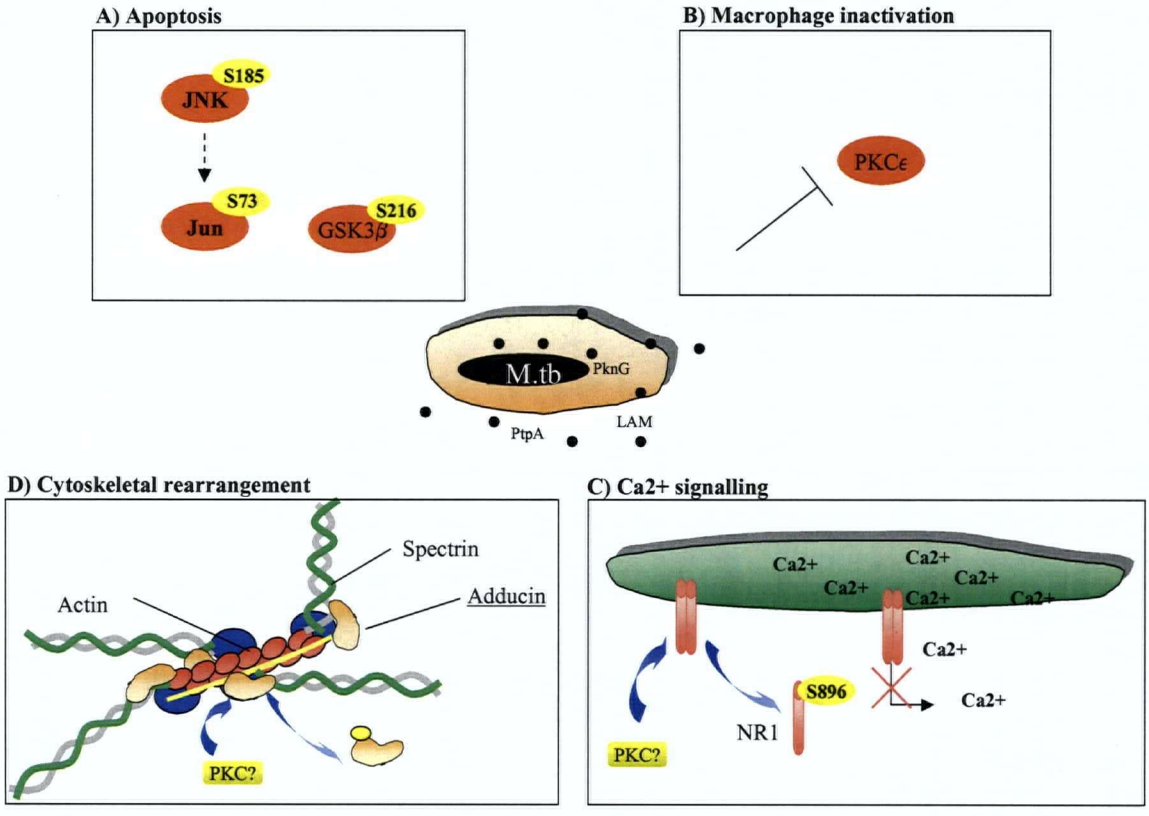

Figure 14: Schematic representation of proposed events during mycobacterial infection.

(A) Phosphorylation of SAPK, c-Jun, and GSK3β primes the host cell for apoptosis, which can be regarded as a mechanism of host defense towards infection. (B) In contrast, infection may lead to deactivation of host defense through the phosphorylation of PKCϵ. (C) Phosphorylation of NR1 may lead to interference with macrophage calcium signaling and (D) adducin phosphorylation potentially leads to rearrangement of the actin filament network. Some of the identified phosphorylation sites suggest that PKC may act as an upstream kinase (A) and (B). Our results have indicated ManLAM as a mycobacterial mediator of these phosphorylation events. Other mediators could be mycobacterial kinases, such as PtpA and PknG.

5.1.2 Adducin phosphorylation in mycobacterial infection

The cytoskeletal protein adducin was chosen for further studies due to its central role in cytoskeletal organization and because of its novelty as a mycobacterial target protein. In its unphosphorylated form adducin caps actin filament barbed ends, bundles actin filaments, and recruits spectrin to sides and ends of actin filaments (109). We are the first group to define a role for adducin in a macrophage-like cell line. Our results indicate that mycobacteria may take advantage of the functions controlled by adducin upon infection of macrophages. Adducin phosphorylation may have a variety of functions in the macrophages. As described, phosphorylation of adducin may be implicated in establishment of actin nucleation sites. These are sites from which monomeric actin assembles to form elongated strands. Two intracellular pathogens, *Shigella* and *Legionella* both take advantage of such a mechanism to facilitate their movement through host cell cytoplasm and further on into neighboring cells. Actin assembly could possibly be utilized by mycobacteria to guide the movement of its phagosome within the host cytosol or to control the transportation of component to and from the phagosome. Phosphorylation-induced inhibition of adducin's activities such as actin- capping and -bundling, and spectrin-recruitment facilitates reorganization of the actin cytoskeleton. As Guerin and de Chastellier (73) demonstrated, cytoskeletal disruption may serve as a means to control the endocytic fusion machinery and prevent the acquisition of endosomal markers.

Because the cytoskeletal network is such a prominent target for a wide range of other intracellular pathogens, it is of specific interest to find that mycobacteria may also share this function. We have presented evidence to suggest that live mycobacteria target the host's actin filament network to prevent the assembly of actin around phagosomes. This observation was

recently verified and published by a different group (4). They showed that *M. tuberculosis* as well as *M. bovis* BCG, in contrast to non-pathogenic strains, such as *M. smegmatis,* inhibited actin nucleation on the phagosomal membrane. Furthermore, triggering of actin assembly on the same phagosomes correlated with enhanced mycobacterial killing.

5.2 Future Directions

5.2.1 Kinome Analysis

Further research should be devoted to examining the specific role of JNK, c-Jun, GSK3β, PKCϵ and NR1 in mycobacterial infection. Our results from the Kinome analysis have identified these proteins as potential mycobacterial targets. More extensive analyses, including traditional Western blot essays, are required to determine their pathways and their implication in mycobacterial infection.

5.2.2 Adducin

5.2.2.1 Adducin activity in macrophages: phosphorylation by ManLAM versus AraLAM

We have shown that ManLAM induces the phosphorylation of adducin and that phosphorylated adducin translocates from the cytoskeleton to cytosol upon infection. This pattern of intracellular distribution is similar to that observed in earlier reports (12) and indicates that adducin, once phosphorylated, dissociates from the cytoskeleton upon infection. It is of primary importance to determine the intracellular location of unphosphorylated adducin in association with the macrophage cytoskeleton, since this has not been investigated in macrophages before. This can be done by intracellular staining of adducin in combination with actin and spectrin filaments.

Furthermore, it would be of interest to determine whether adducin is a part of the actin-binding proteins found in association with the phagosomal membrane. This could be done through phagosomal purification followed by Western analysis employing adducin-specific antibodies. Again, intracellular staining of adducin in combination with a specific stain for a phagosomal membrane protein could also determine this.

We have shown that ManLAM induces the phosphorylation of adducin. To confirm that this effect is specific to ManLAM, cells exposed to arabinose-capped LAM (AraLAM) from non-virulent mycobacterial species can be compared to ManLAM-treated cells. This would establish if phosphorylation of adducin is specific to pathogenic mycobacteria. Intracellular staining can be used to further examine the intracellular distribution of adducin upon infection and treatment with ManLAM versus AraLAM.

Our results suggest that adducin is phosphorylated in a PKC dependent manner. To examine the involvement of PKC, adducin phosphorylation can be determined after co-immunoprecipitation using an *in vitro* [γ-^{32}P] PKC activity assay and specific reaction with anti-phospho-adducin antisera. Western blot assays employing inhibitors specific for different PKC isoforms can also provide information about the specificity of the upstream kinase responsible for adducin phosphorylation. These inhibitors can also be used in combination with intracellularly stained ManLAM-treated cells and confocal microscopy to confirm the involvement of PKC.

5.2.2.2 Phagosomal actin assembly and maturation: role of adducin

Currently, the findings that mycobacteria induce the phosphorylation of adducin and that live mycobacteria inhibit phagosomal actin assembly, stand as two independent results.

An obvious future directive will be to establish a potential connection between the two events. Since ManLAM has been identified as the mycobacterial factor responsible for adducin phosphorylation in THP-1 cells upon infection, it would be of interest to determine whether ManLAM is also responsible for the inhibition of phagosomal actin assembly. Cells exposed to latex beads coated with ManLAM, followed by intracellular staining would establish whether ManLAM is directly involved in this process.

To establish a function of adducin in phagosomal biogenesis, a loss-of-function experiment, over expressing a mutant form of adducin (dominant negative), may provide information about the specific function of adducin in macrophages in relation to mycobacterial infection. This system can then be used to assess whether adducin activity is involved in phagosomal maturation. Infection of the two cell lines, mutant versus wild type, with pathogenic mycobacteria, would be followed by an analysis of phagosomal maturation and actin assembly as described above.

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APPENDIX 1: Hestvik AL, Hmama Z, Av-Gay Y. 2003. Kinome analysis of host response to mycobacterial infection: a novel technique in proteomics. Infec. Immun. **71**: 5514-5522.

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Kinome Analysis of Host Response to Mycobacterial Infection: a Novel Technique in Proteomics

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An array of mammalian phospho-specific antibodies was used to screen for a host response upon mycobacterial infection, reflected as changes in host protein phosphorylation. Changes in the phosphorylation state of 31 known signaling molecules were tracked after infection with live or heat killed *Mycobacterium bovis* BCG or after incubation with the mycobacterial cell wall component lipoarabinomannan (LAM). Mycobacterial infection triggers a signaling cascade leading to activation of stress-activated protein kinase and its subsequent downstream target, c-Jun. Mycobacteria were also shown to inhibit the activation of protein kinase C ε and to induce phosphorylation of proteins not yet known to be involved in mycobacterial infection, such as the cytoskeletal protein α-adducin, glycogen synthase kinase 3β, and a receptor subunit involved in regulation of intracellular Ca^{2+} levels. The mycobacterial cell wall component LAM has been identified as a trigger for some of these modulation events.

Mycobacterium tuberculosis is responsible for 2 million to 3 million deaths every year and persists as the leading cause of deaths worldwide due to a single bacterial agent (76). Residing within the host macrophage, *M. tuberculosis* is able to circumvent the host killing machinery and maintain a state of dormancy that can last for decades (62). Research over the past decades has described *M. tuberculosis* as a pathogen that is able to adapt to a dynamic and changing host environment (for a review, see reference 45) as well as to actively interfere with and modulate the host killing machinery (53, 68). However, *M. tuberculosis* is one of the most successful human pathogens of our time, and the mechanism permitting its survival within the host macrophage still remains largely elusive.

Since its discovery more than 30 years ago, a hallmark of mycobacterial infection has been the prevention of phagolysosome fusion (3), enabling the tubercle bacilli to survive and replicate secluded from the endocytic pathway. Additionally, mycobacterial infection is associated with inhibited antigen processing and attenuated gamma interferon activation of the macrophage (15, 43). These cellular events are tightly controlled at the level of signal transduction and are critical for successful clearance of pathogens from the macrophage.

Mycobacterial interference with host signaling pathways has been proposed to be the principal mechanism developed by the bacteria to establish a successful infection. A wide range of host proteins and pathways have been suggested to act as mycobacterial targets, including mitogen-activated protein kinase (MAPK) signaling pathways, JAK-STAT signaling pathways, Ca^{2+} signaling, NF-κB signaling, and protein kinase C (PKC) signaling (15, 47, 57, 61, 65, 73). These pathways control cellular functions such as cell proliferation, apoptosis, cytokine release, and gene regulation and are an indication of the complexity and scale with which mycobacterial infection might interfere with and possibly counteract host defense mechanisms. The identification of at least 510 protein kinases in the human genome (54) is evidence of the vastness of the eukaryotic signaling network. In such a setting it becomes important to investigate signal transduction as a network of phosphorylation events involving several pathways, rather than focusing on single events.

Large-scale analysis of signaling networks can be performed by using a novel technique in proteomics termed kinome analysis (63). This technique involves the use of an array of phospho-specific antibodies covering kinases and other signaling proteins from major eukaryotic signaling networks known to date. We have used this technique as a tool to identify changes in protein phosphorylation in a human macrophage cell line upon infection with *Mycobacterium bovis* BCG. We have discovered changes in host signaling pathways that have not previously been described for mycobacterial infection. This includes host proteins involved in regulation of apoptotic pathways, cytoskeletal arrangement, Ca^{2+} signaling, and macrophage activation.

MATERIALS AND METHODS

Materials. RPMI 1640, Hanks balanced salt solution (HBSS), protease inhibitors, and phosphatase inhibitors were obtained from Sigma Chemical Co. (St. Louis, Mo.). Anti-phospho-adducin was from Upstate Biotechnology (Lake Placid, N.Y.), and horseradish peroxidase-conjugated anti-rabbit secondary antibody was from Bio-Rad Laboratories (Mississauga, Ontario, Canada). Endotoxin-free lipoarabinomannan (LAM) was generously provided by J. Belisle (Colorado State University, Fort Collins). The LAM used was mannose capped and derived from the virulent H37Rv strain of *M. tuberculosis*.

Culturing of *M. bovis* BCG. *M. bovis* BCG (ATCC 35734) carrying a plasmid constitutively expressing the green fluorescent protein (19), was used in all experiments. Bacteria were grown in Middlebrook 7H9 broth (Difco, Detroit, Mich.) supplemented with 10% (vol/vol) oleic acid-albumin-dextrose solution (Difco), 0.05% (vol/vol) Tween 80 (Sigma), and 50 μg of hygromycin per ml at 37°C to an A_{600} of 0.5 on a rotating platform (50 rpm). Bacteria were harvested by centrifugation (5 min, 6,000 × *g*) at 4°C, and the pellets were resuspended in fresh medium plus 10% glycerol, aliquoted, and kept at −70°C for later use.

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Frozen stocks were thawed, replenished in fresh medium, and grown for 2 to 4 days before they were used for cell infection.

Infection of THP-1 cells and treatment with LAM. The monocytic cell line THP-1 (American Type Culture Collection, Manassas, Va.) was cultured in RPMI 1640 supplemented with 10% fetal calf serum (HyClone, Logan, Utah), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were seeded at a density of 10^5 per cm^2 in 10-cm-diameter culture dishes (Corning Inc., Corning, N.Y.) and allowed to adhere and differentiate in the presence of phorbol myristate acetate (PMA) (20 ng/ml) at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. The cells were then washed three times with HBSS, and adherent monolayers were exposed to live or killed (80°C, 30 min) BCG at a multiplicity of infection of 50 to 1 in RPMI containing 1% L-glutamine, 10% human serum (purified protein derivative negative), and no antibiotics. After 3 h of incubation at 37°C and 5% CO_2, the cells were washed twice with prewarmed HBSS to remove noningested bacteria and reincubated in complete medium at 37°C and 5% CO_2 for the indicated time periods. Alternatively, differentiated THP-1 cells were treated with mannose-capped lipoarabinomannan (ManLAM) at a concentration of 1 μg/ml. The infection rate was verified on cells adherent to tissue culture-treated coverslips (Fisher Scientific, Nepean, Ontario, Canada) in 24-well plates. After phagocytosis, cells were fixed for 15 min at 37°C with 2.5% paraformaldehyde–HBSS and then washed three times with HBSS and once with distilled water. Coverslips were then examined with an epifluorescence microscope (Zeiss Axioplan II), and images were taken with a charge-coupled device camera and Empix software.

Kinome analysis by KPSS assay. Two separate Kinetworks Phospho Site Screen (KPSS) (Kinexus, Vancouver, Canada) analyses were performed. In the initial screen, THP-1 cells were infected with live or heat-killed *M. bovis* BCG, and in the second screen, THP-1 cells were infected with live *M. bovis* BCG or treated with purified ManLAM from *M. tuberculosis* H37Rv at a concentration of 1 μg/ml. Untreated, PMA-differentiated THP-1 cells were used as a control in both experiments. Kinome analyses (KPSS) were performed as previously described (78) and according to the instructions of the manufacturer (Kinexus). In brief, cells were homogenized at 4°C in a buffer containing 20 mM Tris-HCl (pH 7.0), 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 40 mM β-glycerophosphate (pH 7.2), 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, and 1 mM phenylmethylsulfonyl fluoride and spun down at 100,000 × *g* in a Beckman GS 6R tabletop ultracentrifuge. Protein content was estimated by using the Bradford assay (Bio-Rad). Three hundred micrograms of total protein for each sample was divided equally on a 20-lane Immunetics mutiblotter. Each channel was probed with up to three primary antibodies from an array of 31 phospho-specific antibodies, which were selected so as to avoid overlapping cross-reactivity with target protein. The blots were developed with ECL Plus reagent (Amersham Biosciences), and signals were captured with a Fluor-S-MultiImager and quantified with Quantity One software (Bio-Rad).

Western analysis of adducin. PMA-differentiated THP-1 cells were infected with live BCG or challenged with purified LAM (concentration range, 0.01 to 1.0 μg/ml). The cells were then incubated for 2, 12, or 24 h. The blots were probed with anti-phospho-adducin and horseradish peroxidase-conjugated anti-rabbit antibodies and developed by enhanced chemiluminescence.

RESULTS AND DISCUSSION

In this study we have examined the effect of mycobacterial infection on human macrophage signal transduction, reflected as changes in the phosphorylation profiles of 31 known signaling molecules. *M bovis* BCG organisms were used in this study to infect the human monocytic cell line THP-1, which is one of the common human cell lines used in mycobacterial infection studies. PMA treatment induces differentiation of THP-1 cells into a macrophage-like cell line that displays most of the human monocyte-derived macrophage phenotypes with regard to morphology, expression of membrane receptors, cytokine secretion, and induction of several proto-oncogenes (for a review, see reference 4).

Although genotypically *M. bovis* BCG is a deletion version of *M. tuberculosis* (8), it retains the ability to survive intracellularly (59) and it successfully prevents macrophage phagosome maturation (41, 75). Therefore, *M. bovis* BCG is a suitable model for the study of interactions between mycobacteria and the host cell. We have chosen to investigate events occurring after 24 h, thereby excluding early events associated with initial phagocytotic uptake of the bacteria. We rationalize that at this time point, the bacteria have established themselves in their host environment to a greater extent than earlier, offering an opportunity to assess the host response to bacteria residing within phagosomes.

A unique multiphosphoprotein analysis, termed kinome analysis (63), was used as a tool to identify changes in phosphorylation of key host proteins upon infection. The advantage of this technique is the ability to quantitatively track single amino acid phosphorylations. Cells were subjected to a simultaneous screen for the phosphorylation status of 31 host phosphoproteins. Two separate screens were performed; first, THP-1 cells were infected with either live or heat-killed *M. bovis* BCG (Fig. 1), and second, THP-1 cells were infected with live bacteria or treated with purified ManLAM (Fig. 2). Untreated, differentiated THP-1 cell were used as controls in both screens. ManLAM is expressed by both *M. tuberculosis* and *M. bovis* BCG and differs in terms of structure and immunogenicity from LAMs expressed by avirulent strains of mycobacteria (23). ManLAM from BCG and *M. tuberculosis* has been found to inhibit the production of proinflammatory cytokines such as interleukin-12 and tumor necrosis factor alpha (53). Experiments using latex beads coated with ManLAM have further supported a role for this molecule in the persistence of virulent mycobacteria within macrophages. In this manner, ManLAM was recently shown to interfere with a phosphatidylinositol 3-kinase-dependent pathway between the trans-Golgi network and the phagosomal compartment, inhibiting the acquisition of lysosomal markers and resulting in decreased phagosomal maturation (31).

Changes in phosphorylation were measured based on the band intensity for individual phosphoproteins. Any change in phosphorylation of greater than 25% between control and treated cells was considered significant. This is justified by the high sensitivity of the screens in determining the phosphorylation state, as well as the level of each phosphoprotein present within the cells. According to the manufacturer (Kinexus), a change in phosphorylation of less than 25% could be due to experimental variation. As seen in Fig. 1, several proteins displayed significant changes in phosphorylation. These include α-adducin, the *N*-methyl-D-aspartate (NMDA) glutamate receptor subunit NR1, the c-Jun oncoprotein, stress-activated-protein kinase (SAPK) (also known as c-Jun N-terminal kinase), retinoblastoma protein (RB), and glycogen synthase kinase 3β (GSK3β). To examine whether the observed phosphorylation changes could be due to an effect of mycobacterial cell wall components, a second screen was applied to ManLAM-treated cells along with cells infected with live bacteria (Fig. 2). As observed in the first screen, α-adducin, c-Jun, SAPK, GSK3β, and NR1 showed increased phosphorylation in THP-1 cells exposed to live mycobacteria. Interestingly, most of these results were also seen for cells exposed to purified ManLAM.

The two screens were compared in terms of the relative fold change in phosphorylation between live infection and untreated control cells (Table 1). This discussion is limited to proteins that for each screen show more than a 25% change in phosphorylation between infected and control cells. Values for

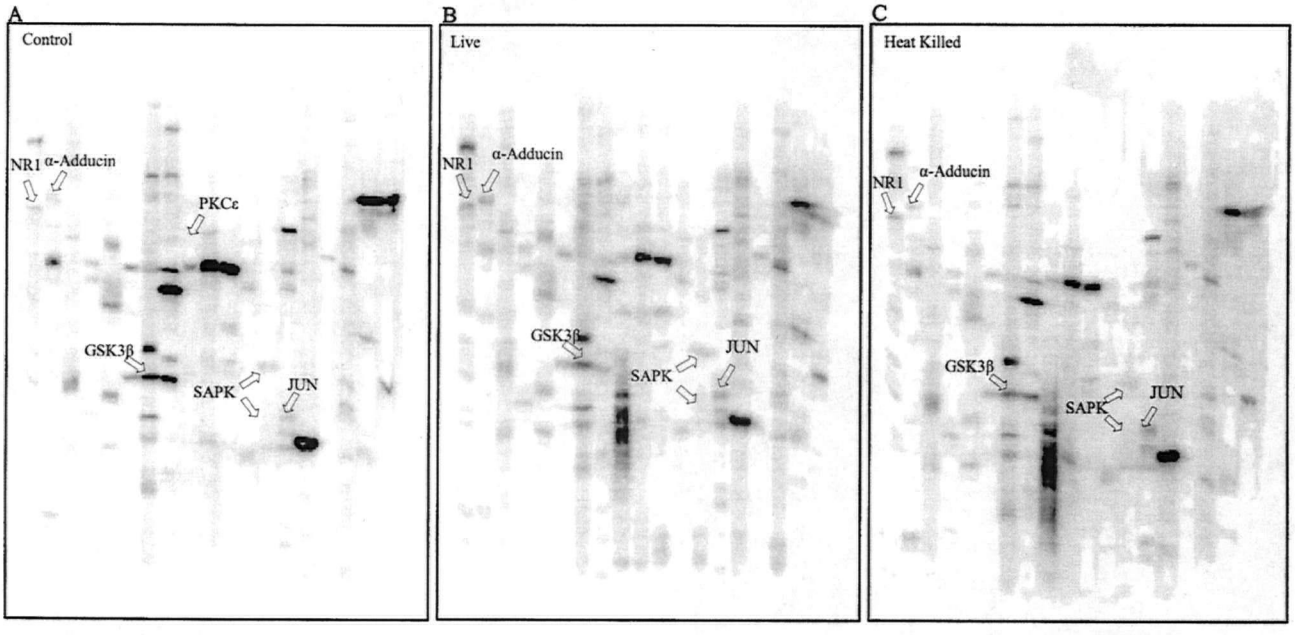

FIG. 1. Multiphosphoprotein analysis of THP-1 cells infected with live and heat-killed *M. bovis* BCG. Each sample is represented as a phosphoprotein fingerprint. Accurate intensity values for each band are the accumulated signal obtained over a given scan time for each blot. These are shown as numerical values in Table 1. The data shown here represent protein bands visualized as a snapshot of the scan time and may therefore not represent exact band intensities. The three gels represent untreated THP-1 cells (A), THP-1 cells infected with live *M. bovis* BCG (B), and THP-1 cells infected with heat-killed *M. bovis* BCG (C). Each lane was probed with one or more antibodies. The proteins indicated by arrows are discussed in the text. Lanes 1 and 21 in each panel contained molecular size standards. Antibodies against the phosphorylated proteins were as follows: lanes 2, NR1 (S^{896}); lanes 3, adducin (S^{724}) and CDK1 (Y^{15}); lanes 4, CREB (S^{133}); lanes 5, ERK1/2 (T^{202}/Y^{204}) (T^{183}/Y^{185}) and p70 S6K (T^{389}); lanes 6, RSK (T^{360}/S^{364}), RAF1 (S^{259}), and MEK1/2 (S^{217}/S^{221}); lanes 7, GSK3α (S^{21}), GSK3β (S^{9}), and PKB (T^{308}); lanes 8, GSK3α (Y^{279}/Y^{216}); lanes 9, PKR1 (T^{451}); lanes 10, PKCε (S^{719}); lanes 11, PKCα (S^{567}); lanes 12, PKCα/β (T^{638}) and SRC (Y^{529}); lanes 13, PKCδ (T^{505}); lanes 14, SAPK (T^{183}/Y^{185}); lanes 15, MSK 1/2 (S^{376}) and JUN (S^{73}); lanes 16, JAK2 (Y^{1007}/Y^{1008}) and p38MAPK (T^{180}/Y^{182}); lanes 17, STAT1 (S^{701}); lanes 18, STAT3 (S^{727}); lanes 19, RB (S^{780}); lanes 20, RB (S^{807}/S^{811}).

cells infected with heat-killed bacteria and cells treated with ManLAM are shown as fold changes relative to the respective control cells (Table 1). As seen in Table 1, none of the changes in phosphorylation induced by ManLAM were reproduced in cells infected with heat-killed bacteria. Since common purification methods for LAM involve heat killing of *M. tuberculosis* (39), one would assume that ManLAM is unaffected by heat treatment and that heat-killed bacteria retain LAM in its original structure. However, several factors could explain the differences in host cell response to ManLAM in its purified form and ManLAM in heat-killed bacteria. First, mycobacteria may shed LAM as a result of heat treatment, reducing the amount of LAM encountered by the host cell. Second, heat may induce conformational changes of bacterial surface molecules that can hinder interaction of LAM with host cell receptors. Third, heat treatment followed by killing of BCG most probably alters the active release of ManLAM once the bacteria reside within the host cell. Active release of ManLAM by live bacteria causes exposure of the whole ManLAM molecule to the host cell. It is possible that the lipid moiety of ManLAM, which is normally embedded in the mycobacterial cell wall, is required for the biological functions of LAM in its interaction with host cell signaling elements. Lipopolysaccharide, which is present on the surface of gram-negative bacteria, is an example of a molecule that shows structural similarity to LAM. The endotoxic activity of lipopolysaccharide is predominantly associated with its free form and particularly the lipid component, lipid A, which needs to be exposed for full activity (2).

Five host signaling proteins, i.e., NR1, SAPK, c-Jun, GSK3β, and α-adducin (Fig. 1 and 2 and Table 1) showed more than a 25% increase in phosphorylation over that for the control untreated cells in both screens. Conversely, the basal level of PKCε phosphorylation was completely attenuated in cells infected with either live or killed bacteria, indicating a possible deactivation of this protein upon infection.

NR1 is a principle subunit of the NMDA receptors, which represent a major class of glutamate-gated ion channels in the central nervous system (60). These receptors regulate intracellular levels of Ca^{2+} in neuronal cells and also in nonneuronal cell lines when these cells are transfected with recombinant receptors (17, 34, 35, 74). A complex regulatory machinery involving several protein kinases and phosphatases controls the function of NMDA receptors in neurons (for a review, see reference 55). S^{896} of the NR1 subunit is phosphorylated upon infection with BCG and has been identified as a specific phosphorylation site for PKC (71). Controversy exists with regard to the consequences of PKC phosphorylation of the NR1 subunit. PKC phosphorylation has been shown to both enhance and inhibit NMDA receptor currents, depending on cell type and compositional variation of the receptor itself (for a review, see reference 33). In our system, cells infected with live bacteria show a twofold average increase in the phosphorylation of

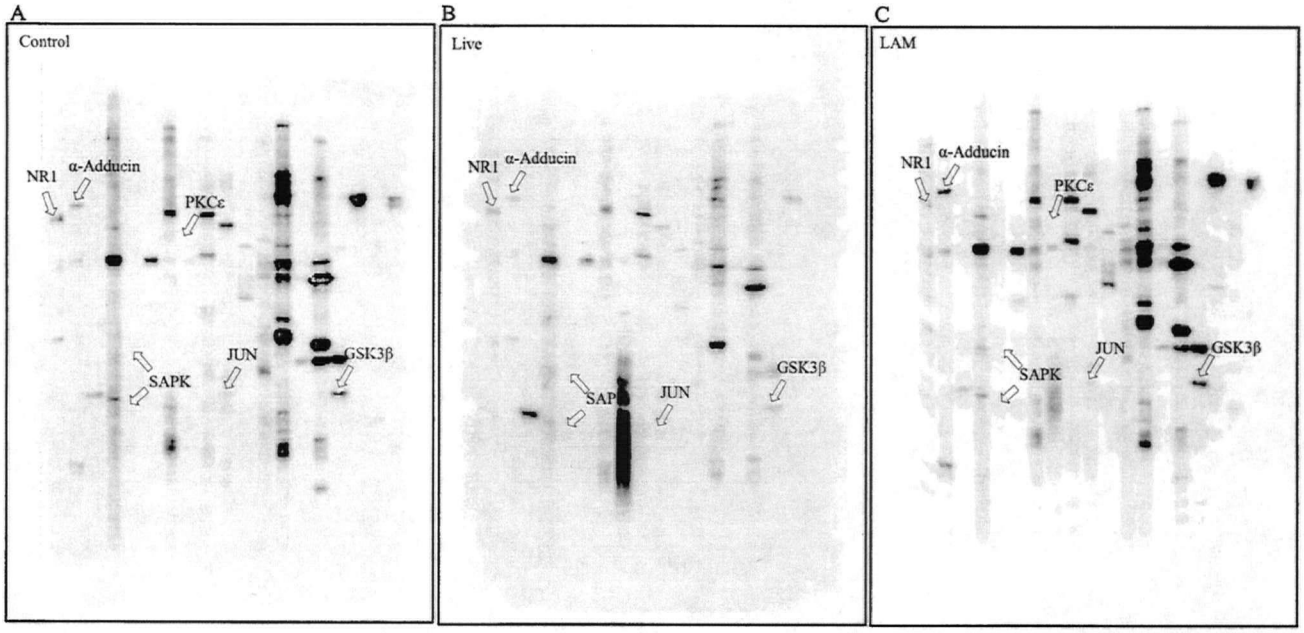

FIG. 2. Multiphosphoprotein analysis of THP-1 cells infected with *M. bovis* BCG or exposed to ManLAM. As for Fig. 1, accurate intensity values for each band are the accumulated signal obtained over a given scan time for each blot. These are shown as numerical values in Table 1. The data shown here represent protein bands visualized as a snapshot of the scan time and may therefore not represent exact band intensities. The second screen, presented here, shows three separate blots: untreated THP-1 cells (A), THP-1 cells infected with *M. bovis* BCG (B), and THP-1 cells treated with purified ManLAM (C). The proteins indicated by arrows are discussed in the text. Lanes 1 and 21 in each panel contained molecular size standards. Antibodies against the phosphorylated proteins were as follows: lanes 2, NR1 (S^{896}); lanes 3, adducin (S^{724}), gamma (S^{662}), CDK1 (Y^{15}), and SRC (Y^{529}); lanes 4, p38MAPK (T^{180}/Y^{182}) and STAT5 (Y^{694}); lanes 5, SRC (Y^{418}) and PKCα (T^{567}); lanes 6, RSK (T^{360}/S^{364}) and SAPK (T^{183}/Y^{185}); lanes 7, PKCβ (T^{368}), MEK3 (S^{189}/T^{193}), and MEK6 (S^{207}/T^{211}); lanes 8, ERK1 (T^{202}/Y^{204}), ERK2 (T^{185}/Y^{204}), and p70 S6K (T^{389}); lanes 9, PKCε (S^{719}) and SMAD1 (S^{463}/S^{465}); lanes 10, STAT3 (S^{727}); lanes 11, JUN (S^{73}); lanes 12, RAF1 (S^{259}) and STAT1 (S^{701}); lanes 13, CREB (S^{133}), PKBα (T^{308}), and PKCδ (T^{505}); lanes 14, PKBα (S^{473}); lanes 15, GSK3α (S^{21}), GSK3β (S^{9}), and MSK1/2 (S^{376}); lanes 16, PKR1 (T^{451}); lanes 17, GSK3α (Y^{279}) and GSKβ (Y^{216}); lanes 18, RB (S^{780}); lanes 19, MEK1/2 (S^{221}/S^{225}); lanes 20, RB (S^{807}/S^{811}).

NR1 S^{896} above levels for cells infected with heat-killed bacteria and cells exposed to ManLAM (Table 1). The possible role of NR1 expression in THP-1 cells is not clear; to our knowledge, NMDA receptors have not yet been described for THP-1 cells or any other cells from the hemopoietic cell linage. Our results indicate that an NR1 homologue could be present in THP-1 cells, possibly constituting an NMDA-related receptor. If so, this receptor might have a function similar to that described for neurons, i.e., regulation of intracellular Ca^{2+} levels. Given that *M. tuberculosis* is able to inhibit Ca^{2+} signaling in human macrophages, correlating with its intracellular survival (56), it is tempting to speculate that mycobacterial interference with a potential NMDA-related receptor could be a mediating factor for the inhibition of host Ca^{2+} signaling upon infection.

SAPK showed a two to threefold increase in phosphorylation in cells infected with live bacteria or exposed to ManLAM compared to heat-killed and untreated cells (Table 1). SAPK is a member of the MAPK family and is encoded by three genes, *SAPK1*, *SAPK2*, and *SAPK3* (37). All SAPK genes are expressed as 46- and 54-kDa protein kinases (37), and both SAPK isoforms showed a similar increase in phosphorylation in our system. Several transcription factors, including ATF-2, Ets, and c-Jun have been identified as downstream targets for SAPK (for a review, see reference 48). Specifically, SAPK has been found to bind the c-Jun transactivation domain and phosphorylate it on S^{63} and S^{73}, thereby enhancing transcriptional activity (25, 42). c-Jun is a central component of activator protein 1 complexes (16), which upon transcriptional activation have been associated with a variety of cellular functions, including cell proliferation, tumorigenesis, and apoptosis (for a review, see reference 28). In this study we show increased phosphorylation of c-Jun S^{73} in cells infected with live bacteria, as well as in cells exposed to ManLAM. Activation of SAPK upon mycobacterial infection has been shown to be an early response in several model systems, often in concert with p38 MAPK and ERK 1/2 activation (11, 73). Even though a regulatory role of the SAPK signaling pathway has been suggested for the production of nitric oxide in mouse macrophages (14), the role of SAPK in mycobacterial infection remains unclear. Our results are the first to indicate that LAM triggers a signaling cascade leading to activation of SAPK and its downstream effector c-Jun.

The phosphoprotein GSK3β showed an average increase of 57% for Y^{216} phosphorylation in THP-1 cells infected with live bacteria and in cells exposed to LAM. GSK3 has been shown to play an essential role in the regulation of cell fate in both pro- and antiapoptotic manners. For example, GSK3β gene disruption in mice caused hepatocyte apoptosis and correlated with impaired antiapoptotic NF-κB signaling (44). In contrast, phosphatidylinositol 3-kinase-mediated activation of protein kinase B (Akt) was found to induce cell survival by inhibiting

TABLE 1. Comparison of phosphorylated host proteins

Protein		Epitope(s)	Signal[a]			
Full name	Abbreviation		Control	Fold change		
				Live avg	LAM	HK
Alpha adducin	α-Adducin	S724	1	1.75[b]	2.62	0.38
Gamma adducin	γ-Adducin	S662	1	0.00	2.15	ND[c]
Cyclic AMP response element binding protein	CREB	S133	1	0.00	1.00	0.86
Cyclin-dependent kinase 1 (cdc2)	CDK1	Y15	1	0.37	2.07	0.00
Double-standard RNA-dependent protein kinase	PKR	T451	1	1.16	1.88	0.57
Extracellular regulated kinase 1	ERK1	T202/Y204	0	0.51	0.00	1.01
Extracellular regulated kinase 2	ERK2	T185/Y204	0	0.65	0.00	0.79
Glycogen synthase kinase 3 alpha	GSK3α	Y279	1	1.29	1.44	0.82
Glycogen synthase kinase 3 alpha	GSK3α	S21	1	0.92	1.79	0.36
Glycogen synthase kinase 3 beta	GSK3β	Y216	1	1.57[b]	1.52	0.65
Glycogen synthase kinase 3 beta	GSK3β	S9	0	0.00	0.00	0.36
Mitogen-activated protein kinase kinase 1/2	MEK1/2	S221/S225	1	1.85	1.69	0.39
Mitogen-activated protein kinase kinase 3	MEK3	S189/T193	0	0.00	0.00	ND
Mitogen-activated protein kinase kinase 6	MEK6	S207/T211	0	0.00	0.00	ND
Mitogen- and stress-activated protein kinase 1/2	MSK1/2	S376	1	1.46	2.69	0.48
Mitogen- and stress-activated protein kinase 1/2	MSK1/2	S376	1	2.71	4.01	0.33
N-Methyl-D-aspartate glutamate receptor subunit 1	NR1	S896	1	1.93[b]	1.24	0.91
Oncogene JUN	JUN	S73	1	2.08[b]	1.30	1.21
Oncogene Raf 1	RAF1	S259	1	1.28	3.01	0.20
Oncogene Raf 1	RAF1	S259	1	1.78	4.71	0.28
Oncogene SRC	SRC	Y529	1	3.18	1.35	0.83
Oncogene SRC	SRC	Y418	0	0.00	0.00	ND
p38 alpha mitogen-activated protein kinase	p38MAPK	T180/Y182	1	4.91	1.10	0.74
Protein kinase B alpha (Akt1)	PKBα	S473	0	0.00	0.00	ND
Protein kinase B alpha (Akt1)	PKBα	T308	1	1.08	1.60	0.63
Protein kinase C alpha	PKCα	S657	1	1.61	1.93	0.55
Protein kinase C alpha/beta	PKCα/β	T638/641	1	1.69	2.82	0.51
Protein kinase C delta	PKCδ	T505	1	1.28	2.59	0.43
Protein kinase C epsilon	PKCε	S719	1	0.00[b]	1.21	0.00
Retinoblastoma 1	RB	S780	1	0.82	1.85	0.64
Retinoblastoma 1	RB	S807/S811	1	0.35	2.05	0.54
Ribosomal S6 kinase 1	RSK1	T360/S364	0	0.53	0.00	0.31
S6 kinase p70	p70 S6K	T389	1	2.40	3.93	0.57
S6 kinase p70	p70 S6K	T389	1	2.60	4.27	0.71
Signal transducer and activator of transcription 1	STAT1	S701	1	2.05	3.02	0.80
Signal transducer and activator of transcription 3	STAT3	S727	1	1.93	2.95	0.00
Signal transducer and activator of transcription 5	STAT5	Y694	0	0.00	0.00	ND
SMA- and MAD-related protein 1	SMAD1	S463/465	0	0.00	0.00	ND
Stress-activated protein kinase (JNK)	SAPK	T183/Y185	1	2.55[b]	3.07	0.68
Stress-activated protein kinase (JNK)	SAPK	T183/Y185	1	3.36[b]	1.85	0.80

[a] The trace quantity of each protein band is measured by the area under its intensity profile curve and corrected for the individual scan times (recorded time before saturation occurs). Values for the control samples have been set to 1 or 0. A value 0 indicates that no immunoreactive signal was detected for this protein in either of the two screens. Live average is the average value, expressed as fold change, for the difference in phosphorylation between live infection and respective control samples for both screens. Values for LAM and heat-killed bacteria (HK) show the fold change relative to their respective control samples.

[b] Proteins that showed a similar and greater-than-25% increase in phosphorylation for both screens.

[c] ND, not determined.

GSK3 (22). In agreement with the latter observation, Akt has been shown to inhibit GSK3β activity through phosphorylation of its Ser^9 residue (27, 29). Conversely, phosphorylation of GSK3β Y^{216} is critical for full activation of the enzyme (46) and has been shown to induce apoptosis (10). Our results demonstrate activation of GSK3β through increased phosphorylation of Y^{216} upon infection with live bacteria and exposure to ManLAM. Furthermore, our results showed no significant activation of Akt or Ser^9 phosphorylation of GSK3β in infected cells (Table 1) suggesting that mycobacterial infection primes THP-1 cells for apoptosis via activation of GSK3β, while the antiapoptotic pathway remains silent.

The two phosphoscreens showed no phosphorylation of PKCε in cells infected with live bacteria. In contrast, control cells and cells exposed to LAM showed substantial PKCε phosphorylation. The function of PKC and the specificities of the different isoforms in macrophage signaling and mycobacterial infection are still unclear. However, PKCε has been shown to be important in macrophage activation and defense against bacterial infection (13). Macrophages from $PKC\varepsilon^{-/-}$ mice showed severely attenuated responses to lipopolysaccharide and gamma interferon, characterized by a dramatic decrease in the generation of nitric oxide, tumor necrosis factor alpha, and interleukin-1β (13). Our results indicate attenuation of PKCε activity, which could be regarded as a strategy employed by mycobacteria to avoid activation of the macrophage.

A major finding in this study is the phosphorylation of the cytoskeletal protein α-adducin (Table 1). Phosphorylation of α-adducin showed an average increase of 75% in cells infected with live bacteria compared to those infected with heat-killed

bacteria. Owing to its status as a unique protein that has not previously been investigated with respect to mycobacterial infection, α-adducin was selected for further analysis. Western blot analysis of α-adducin phosphorylation confirmed our screening results and showed that α-adducin is phosphorylated in THP-1 cells infected with live *M. bovis* BCG but not in cells infected with heat-killed *M. bovis* BCG (Fig. 3A). Furthermore, adducin phosphorylation increased with time, to reach a maximum at 24 h (Fig. 3B). Treatment of cells with purified ManLAM caused an increase in α-adducin phosphorylation, and further investigation showed that ManLAM induces phosphorylation in a dose-dependent manner (Fig. 3C). We found that a concentration of as low as 0.01 μg/ml was sufficient to induce phosphorylation of α-adducin.

Adducin is expressed as a tetramer of either α/β or β/γ subunits (26, 50). A myristolated alanine-rich C kinase-related domain (1, 12), present in the C termini of the adducin subunits, constitutes the major phosphorylation site for PKC (58). The S^{724} of α-adducin detected in our screens lies within the myristolated alanine-rich C kinase domain, suggesting PKC as an upstream kinase acting on α-adducin. In support of this, we observed increased phosphorylation of α-adducin upon treatment of cells with the PKC activator PMA (results not shown).

Phosphorylation of adducin by PKC has been shown to interfere with organization of the actin filament network through inhibition of actin capping and prevention of spectrin recruitment to the ends of actin filaments (58). In a pathogenic setting, the cytoskeletal network, including microtubules, microfilaments, and actin filaments, plays a crucial role in facilitating fusion between early endosomes, phagosomes, and other organelles of the endocytic pathway (for a review, see reference 9). Although the participation of actin filaments in phagocytosis of microorganisms has been well characterized and studied in several model systems, the involvement of actin and actin-binding proteins in the maturation and fusion of phagosomes is still unclear. Nevertheless, several lines of evidence assign an important role for actin filaments in the postphagocytotic fate of nascent phagosomes. For example, treatment with the actin-depolymerizing agent cytochalasin D has been shown to inhibit phagosomal transport and fusion events along the endosomal pathway (49, 72). Furthermore, actin, as well as several actin-binding proteins, has been found in association with mature phagosomes (24, 52, 67). Unambiguous evidence for the role of actin in phagosomal motility was provided by video microscopy of murine macrophages, which showed the formation of actin-rich rocket tails behind latex bead-containing phagosomes (77). These reports suggest a role for the actin filament network beyond the initial phagocytic uptake, during phagosomal motility and processing.

The actin network of phagocytic cells has been identified as a target for a wide range of pathogenic bacteria, including *Salmonella*, *Shigella*, *Listeria*, and *Legionella* (18, 21, 40, 70). These pathogens have been shown to manipulate the actin cytoskeleton in several ways to either enhance their uptake by mammalian cells or facilitate their own movement through host cell cytoplasm and eventually into neighboring cells. Therefore, it can be hypothesized that pathogenic species of mycobacteria would take advantage of such a mechanism. Indeed, virulent strains of *Mycobacterium avium* have been shown to disrupt the host actin network, correlating with a

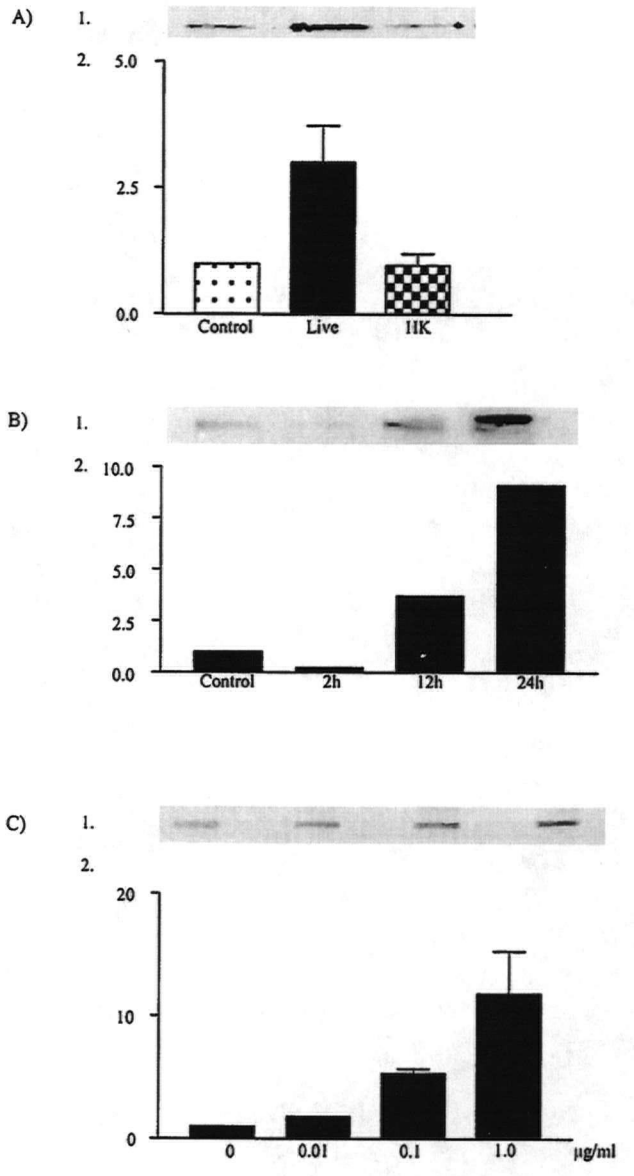

FIG. 3. Western analysis of adducin phosphorylation in THP-1 cells. (A) Adducin is phosphorylated in cells infected with BCG but not in cells infected with heat-killed bacteria. Panel 1, Western blot of THP-1 cells infected with live or heat-killed (HK) BCG for 24 h; panel 2, densitometry analysis of panel 1. (B) Time-dependent phosphorylation of adducin by *M. bovis* BCG infection. Panel 1, Western blot of THP-1 cells infected with BCG for 2, 12, or 24 h; panel 2, densitometry analysis of panel 1. (C) Adducin phosphorylation by purified ManLAM. Panel 1, Western blot of THP-1 cells treated with increasing concentrations of purified ManLAM; panel 2, densitometry analysis of panel 1. Error bars represent standard errors from at least three independent experiments. Equal loading of protein was verified by India ink staining.

delay in the acquisition of endocytic markers by mycobacterium-containing phagosomes (36). It was shown that disruption of the actin network occurred at 24 h postinfection and was maintained up to 15 days following infection. In agreement with this observation, our results also demonstrate a time-dependent mechanism for the phosphorylation of adducin,

with the highest phosphorylation occurring at 24 h postinfection (Fig. 3B)

As proposed by Guerin and de Chastellier (36), actin filaments, along with their associated proteins, may function as a network surrounding nascent and maturing phagosomes. Here, organelles of the endocytic machinery are brought into proximity to facilitate fusion events and intermingling of contents between compartments. By disrupting such a network, invading mycobacteria can possibly prevent exchange of content between organelles and inhibit eventual fusion with lysosomes. In this context, adducin phosphorylation, mediated by mycobacterial factors such as LAM, could possibly lead to the disruption or reorganization of the actin cytoskeleton, enabling exclusion of the phagosome from the endocytic pathway.

Changes in phosphorylation seen for SAPK, c-Jun, α-adducin, and GSK3β upon infection with *M. bovis* BCG have been reproduced in cells exposed to ManLAM, suggesting ManLAM to be the mediating factor. In contrast, changes in phosphorylation of PKCε and NR1 were not reproduced in cells exposed to ManLAM, indicating that another bacterial component is responsible. In murine macrophages, phagosomes containing *M. bovis* BCG were shown to be permeable to dextrans as large as 70 kDa (69), and a number of mycobacterial surface proteins are released from phagosomes into subcellular compartments (7). Thus, the notion that mycobacteria are capable of releasing proteins into the host cell cytoplasm led us to hypothesize that mycobacteria actively interfere with host signaling elements to promote their own survival. With the presence of genes for 11 eukaryotic-like protein serine kinases and four protein phosphatases in the genome of *M. tuberculosis* (5), it is tempting to speculate that these proteins might be exported intracellulary and interfere with signal transduction cascades within the host cell. Eukaryotic-like protein kinases and phosphatases have previously been implicated in the virulence of other pathogens such as *Yersinia pseudotuberculosis* and *Salmonella enterica* serovar Typhimurium, both of which translocate bacterial proteins into the host cell cytoplasm, resulting in disruption of the host cell cytoskeleton (32, 38). Interestingly, a mycobacterial phosphatase, PtpA, is present in the mycobacterial genome without a corresponding substrate within the bacterium (20), further supporting the hypothesis of cross-interaction between bacterial and host cell signaling elements.

In conclusion, we have presented a unique set of results based on a simultaneous screen of key host proteins upon mycobacterial infection of a human macrophage-like cell line. We have shown changes in phosphorylation of host proteins that are novel to the field of cellular mycobacteriology. As described above, some of the signaling proteins shown to be activated, such as SAPK, c-Jun, and GSK3β, have previously been implicated in the regulation of apoptotic pathways. It has been well established that macrophages infected with mycobacteria have increased rates of apoptosis in vitro (30, 64, 66). The observation that virulent strains induce less apoptosis than avirulent and attenuated strains (6, 51) has reinforced the conception that apoptosis functions as a host cell defense mechanism in mycobacterial infection and that virulent strains have developed strategies to promote host cell survival. *M. bovis* BCG was recently shown to induce apoptosis in THP-1 cells (64). In agreement with this, activation of the JNK–c-Jun signaling pathway and GSK3β could be interpreted as proapoptotic signaling as part of the host cell defense. However, since we have not analyzed downstream effects of the reported phosphorylation events, we cannot draw any final conclusions regarding the ultimate outcome of these events. On the other hand, we have presented evidence to suggest an active attenuation of macrophage defense through mycobacterial inhibition of PKCε and possible interference with host Ca^{2+} signaling. Furthermore, we propose a potential mechanism by which mycobacteria interfere with the actin cytoskeleton as a means to exclude the phagosome from the endocytic pathway. Taken together, our results present evidence in accordance with previous reports indicating that a number of host signaling pathways are modulated upon mycobacterial infection. Whether this is due to a single modulation event affecting several downstream signaling pathways or to direct modulation of different signaling cascades remains to be elucidated. Further research into these pathways will lead to increased knowledge about the complex interplay between mycobacteria and the host cell.

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