Title
Immune Modulation by the Mycobacterium tuberculosis mce1 operon

Permalink
https://escholarship.org/uc/item/50h8t6xp

Author
Howsmon, Rebecca Anne

Publication Date
2009

Peer reviewed|Thesis/dissertation
Immune Modulation by the *Mycobacterium tuberculosis mce1* operon

by

Rebecca Anne Howsmon

B.S. (University of Washington) 2001

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Infectious Diseases and Immunity in the Graduate Division of the University of California, Berkeley

Committee in charge:

Professor Lee W. Riley, Chair
Professor Gregory Barton
Professor Dan Portnoy
Professor Eva Harris

Fall 2009
Immune Modulation by the *Mycobacterium tuberculosis mce1* operon.

Copyright (2009)

by

Rebecca Anne Howsmon
Abstract

Immune Modulation by the *Mycobacterium tuberculosis* mce1 operon.

by

Rebecca Anne Howsmon

Doctor of Philosophy in Infectious Diseases and Immunity

University of California, Berkeley

Professor Lee W. Riley, Chair

*Mycobacterium tuberculosis* (M.tb), the causative agent of tuberculosis, is a highly successful pathogen that establishes a persistent human infection. The hallmark of human host response to M.tb infection is the formation of a conglomeration of immune cells called a granuloma that is thought to limit bacterial replication and spread. M.tb is able to persist within this harsh environment. Research from our laboratory demonstrates a role for a 13-gene cluster called the mce1 operon in immune modulation within this environment that allows the bacilli to persist. We hypothesize that fluctuations in expression of the mce1 operon during the course of infection are involved in the stable maintenance of the granuloma, which is also protective for the mycobacteria.

A mce1 operon mutant strain of M.tb (Δmce1) is hypervirulent in a mouse model of infection. Infected mice are unable to mount a proper granulomatous response. *In vitro* studies with RAW macrophages and A549 epithelial cells infected with Δmce1 demonstrate that this is likely due to an aberrant early innate immune response. In this dissertation we used primary mouse bone marrow derived macrophages and dendritic cells to further corroborate these findings. We found that macrophage and dendritic cell
cytokine and chemokine production was impaired in response to infection with ∆mce1, and dendritic cells failed to upregulate costimulatory molecules that are essential for their role in linking the innate and adaptive immune responses. The cell envelope of ∆mce1 was shown previously to contain an accumulation of mycolic acids that are not present in wild-type M.tb. We also investigated whether M.tb alters its lipid profile to directly or indirectly modulate the immune response. Our data show that the accumulated lipids from ∆mce1 do not block TLR2 signaling, nor do they directly inhibit macrophage or dendritic cell activation. Instead, these lipids may alter the bacterial cell wall architecture in such a way as to induce other, yet uncharacterized innate immune signaling pathway. These observations, nevertheless, show that early events in the interaction of M.tb with host immune cells have dramatic consequences later, and ultimately determine the clinical fate of this interaction—persistence or active disease.
This dissertation is dedicated to:

My husband, Shawn Scofield,
for standing by my side through this long journey, for encouraging me to keep pushing
through to the end, and for celebrating my successes with me.

My mother, Cheryl Goslant,
for encouraging me to reach my highest potential while remaining true to myself.

My son, Jasper Denny Scofield,
for reminding me of the wonders of the world and the excitement of exploration and
discovery.
Acknowledgements

I am thankful for all the opportunities afforded to me by the Program in Infectious Diseases and Immunity, its exceptional faculty and the students that I have had the honor of interacting with. Dr. Terry Machen, Dr. Eva Harris and Dr. Dan Portnoy, thank you for your helpful discussions and your input with this project.

Thank you to Sarah Gilmore and Michael Schelle from the Bertozzi lab, for providing me with protocols for cell culture, and for TB preparation and lipid extraction.

To everyone in the Barton lab, in particular Laura Lau, Roman Barbalat and Maria Mouchess, thank you for supplying me with mice and reagents, for all of your technical advice and for your willingness to work with my schedule. Of course this was all possible due to Greg Barton’s generosity. Greg, thank you for your enthusiasm and interest, this project would not have been possible without your guidance.

It has been incredible to work with such a diverse group of people in the Riley lab. I am very grateful to my mentor, Lee Riley, for providing such an environment. Lee, it has been a privilege to work in your lab. Thank you for your support, for your big picture explanations and for allowing me the freedom to pursue this project in spite of its challenges.

As for my labmates, Nicki, Ryan and Amy, thank you for teaching me all that I know about working with TB. Olivera, Kathleen, Sara, Owen, Amador, Brooke and Melaine, you are an amazing group of people and I have thoroughly enjoyed working with all of you. Thank you for all the discussions, feedback and advice related to lab and life. Mamiko, I am forever indebted to you for all of your assistance with my experiments.
and for helping me to troubleshoot them. Thank you also for commiserating with me and for encouraging me to “try just one more time”. A special thank you to Sally for always being available when I needed someone to bounce ideas off of, and for opening the door to a new area of research with your mcel operon lipid discoveries.

The last six years have been some of the most formative of my life, both personally and professionally. I could not have accomplished all that I have without the support and encouragement of my wonderful family. Thank you all for being in my life and for making me smile each and every day. I particularly want to thank Russ and Donna for their warmth and generosity and for helping care for Jasper so that I could write. Emily, thank you for being such a great friend for all these years and for helping me to keep my goals in sight. Mom, thank you for being my sounding board, and for cheering me on through to the end. And finally, to Shawn, thank you for ensuring that I maintained a balance with school and life, for helping me in the lab when I was sure my arm would fall off if I had to aliquot any more samples, and most importantly, for all that you have endured so that I could accomplish this. I could have never finished this dissertation without your help.
# TABLE OF CONTENTS

I. Chapter 1: Introduction – Disease Outcome: A Balance of Immune Activation and Immune Inhibition by *Mycobacterium tuberculosis* .................................................. 1  
A. Introduction to Dissertation ...................................................................................... 2  
B. Immune Response to Infection ............................................................................... 4  
   1. Granuloma Formation .......................................................................................... 4  
   2. Cytokines ............................................................................................................. 7  
      a. TNFα ................................................................................................................. 7  
      b. IL-12 ................................................................................................................. 9  
      c. IFNγ .................................................................................................................. 10  
      d. IL-10 and TGFβ .............................................................................................. 11  
   3. Chemokines .......................................................................................................... 11  
   4. Toll-Like Receptors ............................................................................................. 13  
   5. Innate Immunity .................................................................................................. 17  
      a. Epithelial Cells ................................................................................................. 17  
      b. Macrophages and Dendritic Cells ................................................................. 18  
   6. Adaptive Immunity .............................................................................................. 23  
   7. Summary of Immunological Events ..................................................................... 25  
C. Pathogenesis .......................................................................................................... 26  
   1. Immune Evasion .................................................................................................. 26  
   2. Mycobacterial Cell Envelope ............................................................................... 28  
      a. Architecture ..................................................................................................... 28  
      b. Lipomannans .................................................................................................... 29  
   c. Mycolic Acids ...................................................................................................... 31  
   3. *Mycobacterium tuberculosis* mce operon .......................................................... 34  
D. Figures .................................................................................................................... 41  
E. Tables ...................................................................................................................... 53  
F. References ............................................................................................................... 54  

II. Chapter 2: Activation of the early innate immune response by the  
*Mycobacterium tuberculosis* mce1 operon ............................................................... 75  
A. Introduction ............................................................................................................. 76  
B. Results ..................................................................................................................... 78  
   1. Cytokine and Chemokine Expression Kinetics ...................................................... 78  
   2. Surface Molecule Expression ............................................................................. 80  
C. Discussion ............................................................................................................... 80  
D. Materials and Methods ......................................................................................... 86  
E. Figures ..................................................................................................................... 90  
F. References ............................................................................................................... 98  

III. Chapter 3: The mce1 operon mutant strain of *Mycobacterium tuberculosis* is not  
impaired in its ability to signal through Toll-like receptor 2 .................................. 102  
A. Introduction ............................................................................................................ 103  
B. Results .................................................................................................................... 105
List of Figures

1-1 Anatomy of the respiratory system
1-2 Immune cells in the conducting airways and alveoli
1-3 The three stages of *Mycobacterium tuberculosis* infection
1-4 Pathology of the lung granuloma after infection with *Mycobacterium tuberculosis*
1-5 Schematic representation of the *Mycobacterium tuberculosis* cell envelope
1-6 Molecular structures of the three subclasses of mycolic acid
1-7 Schematic representation of the *Mycobacterium tuberculosis mce* operons
1-8 Survival kinetics of Balb/c mice or C57BL/6 mice infected with *Mycobacterium tuberculosis* wild-type or Δmce1
1-9 Lungs of Balb/c mice infected with *Mycobacterium tuberculosis* wild-type or Δmce1
1-10 Kinetic analysis of TNFα, IL-6 and MCP-1 production by RAW macrophages infected with *Mycobacterium tuberculosis* wild-type or Δmce1
1-11 Survival kinetics of Balb/c mice infected with *Mycobacterium tuberculosis* wild-type or Δmce1R
1-12 Mass spectrometry analysis of surface lipid extracts from *Mycobacterium tuberculosis* wild-type or Δmce1

2-1 TNFα production by Balb/c bone marrow derived macrophages and dendritic cells infected with *Mycobacterium tuberculosis* wild-type or Δmce1
2-2 IL-12p70 production by Balb/c bone marrow derived macrophages and dendritic cells infected with *Mycobacterium tuberculosis* wild-type or Δmce1
2-3 MCP-1 production by Balb/c bone marrow derived macrophages and dendritic cells infected with *Mycobacterium tuberculosis* wild-type or Δmce1
2-4 IL-10 production by Balb/c bone marrow derived macrophages and dendritic cells infected with *Mycobacterium tuberculosis* wild-type or Δmce1
2-5 TNFα production by Balb/c and C57BL/6 bone marrow derived macrophages and dendritic cells infected with *Mycobacterium tuberculosis* wild-type or Δmce1
Surface expression of CD40 and CD86 by C57BL/6 bone marrow derived dendritic cells after infection with *Mycobacterium tuberculosis* wild-type or ∆mcel

Surface expression of CCR7 by C57BL/6 bone marrow derived dendritic cells after infection with *Mycobacterium tuberculosis* wild-type or ∆mcel

Median fluorescence intensity of CD40, CD86 and CCR7 expression on the surface of C57BL/6 bone marrow derived dendritic cells after infection with *Mycobacterium tuberculosis* wild-type or ∆mcel

TNFα production by C57BL/6 bone marrow derived macrophages and dendritic cells exposed to Pam3CSK4 or LPS after incubation with *Mycobacterium tuberculosis* mycolic acids

Schematic rational for using TLR2 deficient cells

TNFα production by C57BL/6 wild-type or TLR2 deficient bone marrow derived dendritic cells after infection with *Mycobacterium tuberculosis* wild-type or ∆mcel

Surface expression of CD40 by C57BL/6 wild-type or TLR2 deficient bone marrow derived dendritic cells after infection with *Mycobacterium tuberculosis* wild-type or ∆mcel

Surface expression of CD86 by C57BL/6 wild-type or TLR2 deficient bone marrow derived dendritic cells after infection with *Mycobacterium tuberculosis* wild-type or ∆mcel

Surface expression of CCR7 by C57BL/6 wild-type or TLR2 deficient bone marrow derived dendritic cells after infection with *Mycobacterium tuberculosis* wild-type or ∆mcel

Median fluorescence intensity after subtracting out uninfected control background values for CD40, CD86 and CCR7 surface expression by C57BL/6 wild-type or TLR2 deficient bone marrow derived dendritic cells after infection with *Mycobacterium tuberculosis* wild-type or ∆mcel

TNFα production by C57BL/6 bone marrow derived macrophages and dendritic cells after infection with native or delipidated *Mycobacterium tuberculosis* wild-type or ∆mcel

TNFα production by C57BL/6 wild-type or TLR2 deficient bone marrow derived macrophages and dendritic cells after exposure to *Mycobacterium tuberculosis* wild-type or ∆mcel surface lipid extracts
Thin-layer chromatography of petroleum ether extracts from *Mycobacterium tuberculosis* wild-type or \( \Delta mce1 \)
List of Tables

1-1 Macrophage and dendritic cell responses to Mycobacterium tuberculosis infection, or the specific mycobacterial PAMPs, and their dependence upon TLRs
Chapter 1 - Introduction

Disease Outcome: A Balance of Immune Activation and Immune Inhibition

by Mycobacterium tuberculosis
**INTRODUCTION TO DISSERTATION**

*Mycobacterium tuberculosis*, the causative agent of tuberculosis, infects more then one-third of the world’s population, accounting for approximately 2 million deaths per year, globally. While our efforts to eradicate this global scourge have resulted in a decrease in the incidence, prevalence and death rates, someone is still newly infected with *Mycobacterium tuberculosis* every second. We are, however, steadily working towards the goal of halving and reversing the incidence, and halving the prevalence and death rates by 2015, as compared to the 1990 baseline rates (WHO, 2009).

Upon infection with *Mycobacterium tuberculosis*, there are four potential outcomes to the host: 1) immediate clearance with no evidence of disease, 2) primary disease, 3) latent infection that never reactivates, or 4) latent infection with eventual reactivation (Humphreys I. R., 2006; Stewart G. R., 2003). Those individuals who are able to contain the bacteria have a 5-10% lifetime risk of developing reactivation tuberculosis (Comstock G. W., 1982).

Control of tuberculosis relies on prevention through Bacillus Calmette-Guerin (BCG) vaccination and the treatment of cases using a variety of drugs. While BCG is used in most countries outside of the United States, there is much controversy concerning its efficacy, with a range of efficacy estimates from 0-80% to prevent adult pulmonary tuberculosis (Fine P. E., 1998). Drug treatment in an immunocompetent patient takes a minimum of 6 months. Multi-drug resistant (MDR) and extreme drug resistant (XDR) strains are becoming ever more prevalent, which require longer and more complicated treatment course (Cohn D. L., 1990). Hence, there is a global push to develop novel vaccines to help prevent new infections as well as to block reactivation of latent
infections. In order to do so, continued research is needed to better understand the relationship between the host immune response and infection with *Mycobacterium tuberculosis*.

The hallmark of *Mycobacterium tuberculosis* infection is its ability to establish persistent infection. The molecular mechanisms involved in establishing and maintaining a persistent mycobacterial infection and the processes involved in reactivation remain to be determined. This is due in part to the complex relationship *Mycobacterium tuberculosis* has with the host. A fine balance between immune activation and immune inhibition must be attained in order for both the host and the bacteria to survive and to allow for transmission (Flynn J. L. and J. Chan, 2005). In this dissertation, the role of the *Mycobacterium tuberculosis mce1* operon in modulating the immune response for establishment of a persistent infection will be explored. The present chapter will 1) introduce the host innate and adaptive immune responses to infection with *Mycobacterium tuberculosis*, 2) describe the known mycobacterial mechanisms of pathogenesis, and 3) present the current knowledge on the *mce1* operon. Chapter 2 will establish a role for the *mce1* operon in activating the early innate immune response. Chapter 3 will 1) explore whether mycobacterial mycolic acids are able impair Toll-like receptor activation and 2) determine if the impaired immune response to ∆*mce1* is due to an inability to signal through TLR2. Chapter 4 will 1) address whether extractable surface lipids are responsible for the impaired immune response to ∆*mce1* and 2) determine if the extractable surface lipids from ∆*mce1* are directly inhibiting the innate immune response to infection. Finally, Chapter 5 will conclude this dissertation.
IMMUNE RESPONSE TO INFECTION

*Mycobacterium tuberculosis* is transmitted to a new, unsuspecting host via inhalation of aerosolized bacteria that are released into the air when a person with active disease coughs. Bacteria can remain in the air for hours after being released, and therefore the infectious patient need not be present for transmission to occur. Once in the host, the bacteria travel through the airway into the lungs, consisting of a series of dichotomous branches from the trachea to a honeycomb network of millions of alveoli (Figure 1-1) (Weibel E. R. and D. M. Gomez, 1962). The alveoli are covered in a dense network of capillaries and lymphatic vessels that allow for gas exchange and immune cell migration between the lung tissue and the blood (Itoh H., 2004; Weibel E. R. and D. M. Gomez, 1962). The alveolar sacs, as well as the conducting airways, consisting of the trachea, bronchi and bronchioles, are lined with a variety of immune cells in order to defend the host against particles, toxic gases and microorganisms that are constantly inspired into the respiratory system (Figure 1-2).

Granuloma Formation

In response to infection with *Mycobacterium tuberculosis*, the ultimate goal of the host immune system is to contain the bacteria within a protective granuloma, a conglomeration of innate and adaptive immune cells, that is thought to limit *Mycobacterium tuberculosis* growth by restricting bacterial access to oxygen and nutrients and exposing the bacilli to acidic pH and immune effectors, such as nitric oxide (Rustad T. R., 2009). There are three interrelated stages of *Mycobacterium tuberculosis* infection, consisting of 1) initial infection, 2) persistent infection and 3) acute infection
In the first stage the bacterium comes into contact with cells of the innate immune system, including epithelial cells, macrophages and dendritic cells (Figure 1-2). These cells express various pathogen recognition receptors (PRR), such as Toll-like receptors (TLR), nucleotide-binding oligomerization domain (NOD)-like receptors, mannose receptors and C-type lectins, that recognize conserved microbial structures, collectively called pathogen associated molecular patterns (PAMPs) (Jo E. K., 2008).

When the PAMPs bind to their respective receptor, an intracellular signaling cascade is initiated that results in transcriptional activation of a proinflammatory response. This response is defined by secretion of the cytokines TNFα, IL-1β, IL-12, IL-10, and TGFβ, and the chemokines MCP-1, IL-8, MIP-1α, MIP-1β and RANTES, which serve to further activate cells and to signal for additional immune cells to localize to the site of infection (Cooper A. M. and S. A. Khader, 2008).

Regulated events result in the recruitment of immune cells to specific locales in the granuloma (Tsai M. C., 2006). In particular, macrophages and dendritic cells form the foundation of the granuloma during the early stages of infection. Dendritic cell differentiation and successive activation of naïve CD4+ T cells in the lymph nodes initiates the persistent stage of infection. During this stage dendritic cells are also found in the periphery of the granulomas along with the recruited CD4+ T cells (Iyonaga K., 2002; Tsai M. C., 2006; Uehira K., 2002). While dendritic cells predominantly interact with and activate T cells within the lymph node, their presence within the granuloma allows for further expansion of T cells at the site of inflammation (Pedroza-Gonzalez A., 2004). Analysis of lungs from tuberculosis patients reveals that within the granuloma
macrophages are present in two microenvironments, one at the luminal side of the necrotic lesion in which macrophages and T cells are not colocalized, and another at the opposite side of the necrotic region in which the two cell types are able to interact (Kaplan G., 2003). Direct contact between macrophages and T cells, in the presence of interferon (IFN)-γ, activates an antimicrobial response by infected macrophages (Saito S. and M. Nakano, 1996; Sugawara I., 1998). This interaction is critical to the success of the granuloma and to controlling bacterial replication, as demonstrated in a study by Bean et al., in which mice disrupted in the TNF gene are unable to control *Mycobacterium tuberculosis* replication and succumb to massive tissue necrosis and death (Bean A. G., 1999). They demonstrate that macrophages are present and form the foundation of the granuloma, and T cells are activated, as shown by their ability to produce IFNγ, and localize to the perivascular and peribranchial regions of the lung, but the T cells fail to migrate to the inflamed tissue. Thus, granulomas fail in the absence of TNFα due to an inability of macrophages and IFNγ-secreting CD4+ T cells to interact (Bean A. G., 1999; Botha T. and B. Ryffel, 2003; Flynn J. L., 1995; Kindler V., 1989; Saunders B. M. and W. J. Britton, 2007).

Despite the robust immune response to *Mycobacterium tuberculosis*, the majority of infected individuals do not completely eradicate the bacteria, but instead contain the bacteria in a persistent/latent state for life. Maintenance of the granuloma is ongoing throughout the course of a mycobacterial infection, lending to continual changes in granuloma size, structure and composition (Rhoades E. R., 1997). When the immune system wanes, such as with HIV coinfection, bacterial replication is no longer controlled and increased cell death results (Lawn S. D., 2002). This represents the third, acute stage
of infection, also known as reactivation. During this stage the center of the granuloma is said to “decay” into a necrotic caseating lesion, allowing the reactivated bacteria to spill into the airway and be coughed out into the environment (Figure 1-4) (Russell D. G., 2007). Individuals with reactivated tuberculosis are typically unaware that they were ever infected with *Mycobacterium tuberculosis*, so by the time they have been diagnosed as having active tuberculosis, it is likely that they will have already infected others. Therefore, individuals with persistent/latent *Mycobacterium tuberculosis* infection serve as the main reservoir for new tuberculosis cases.

**Cytokines**

Infection with *Mycobacterium tuberculosis* induces MAPK and host-cell signaling cascades, which result in the production of proinflammatory cytokines (Koul A., 2004). These secreted molecules can act in an autocrine or paracrine fashion to activate and/or recruit additional immune cells to the site of infection.

**TNFα**

Tumor necrosis factor (TNF) - α plays a critical and complex role during *Mycobacterium tuberculosis* infection, by both enhancing and controlling the immune response. It signals through TNF receptors (TNFR) 1 and 2 to activate the transcription factor, NFκB. This in turn aids in the formation of the granuloma by activating expression of adhesion molecules by local endothelial cells, and of the chemokines MCP-1, IL-8 and RANTES by innate immune cells (Bean A. G., 1999; Mulligan M. S., 1993; Saukkonen J. J., 2002; Strieter R. M., 1990; Tessier P. A., 1997). Secretion of these
chemokines provides a gradient that guides cellular migration from the blood to the source of the infection and thus maintains the integrity of the granuloma. In the absence of chemokine activation, such as with a mutant strain of mice that are deficient in TNFα, granuloma formation is impaired in response to low-dose aerosol infection with *Mycobacterium tuberculosis* (Bean A. G., 1999; Flynn J. L., 1995). Thus, bacterial replication is not controlled and mutant mice succumb prematurely to infection. It was later discovered that this impaired response to *Mycobacterium tuberculosis* infection in the mutant mice is also due to an uncontrolled “type 1 syndrome” characterized by uncontrolled expansion of activated T cells, an increased frequency of mycobacterium-specific T cells and heightened levels of IL-12 and IFNγ (Zganiacz A., 2004). Therefore, in addition to its role in promoting inflammation, TNFα is also a critical negative regulator of the adaptive type 1 response.

Signaling through TNFR1 and 2 by TNFα, is also directly involved in mycobacterial killing, either by inducing nitric oxide production by infected macrophages or by inducing macrophage apoptosis via activation of a caspase cascade that results in DNA cleavage, and hence cell death (Ding A. H., 1988; Flesch I. E. and S. H. Kaufmann, 1990; Micheau O. and J. Tschopp, 2003; Rojas M., 1999). In a controlled environment, TNFα - mediated killing is beneficial to the host, but excess TNFα is associated with overwhelming tissue damage, and therefore, the concentration of TNFα during *Mycobacterium tuberculosis* infection directly determines whether it is protective or destructive (Bekker L. G., 2000).

As stated previously, immune control of *Mycobacterium tuberculosis* infection requires a balance between immune activation and immune inhibition. TNFα appears to
play a role in both aspects, and therefore it is not surprising that in an aerosol infection model of drug induced latent and reactivated murine tuberculosis, using rifampicin and isoniazid, Botha and Ryffel show that in addition to its importance in controlling the early stages of infection, TNFα is also critical for the maintenance of latent infection and for protection against reactivated tuberculosis (Botha T. and B. Ryffel, 2003).

**IL-12**

The cytokine interleukin (IL) -12, also know as IL-12p70, is a heterodimeric protein consisting of a p40 and a p35 subunit. IL-12p40 can be secreted independently of IL-12p70, but IL-12p35 is only secreted when the p40 subunit is produced by the same cell (Trinchieri G., 2003). As such, the IL-12 membrane receptor is a complex of two chains, IL-12Rβ1 and IL-12Rβ2. IL-12Rβ1 recognizes only IL-12p40, whereas IL-12Rβ2 recognizes either IL-12p70 or IL-12p35.

IL-12 plays a key role in relaying information from the innate immune system to the adaptive immune system by selectively activating a T-helper 1 (Th1) response during *Mycobacterium tuberculosis* infection, that is characterized by the production of IFNγ by activated T cells (Cooper A. M., 1997; Schmitt E., 1994). In the absence of IL-12, *Mycobacterium tuberculosis* infected mice are unable to make significant levels of IFNγ and hence IFNγ-dependent activation of macrophages is impaired resulting in increased bacterial loads in the lung, liver and spleen (Cooper A. M., 1997). In addition, numerous studies in human patients with deficiencies in the IL-12 receptor demonstrate severe mycobacterial infections that are cleared following treatment with antibiotics.

**IFN$_\gamma$**

Interferon (IFN)- $\gamma$ is predominantly produced by activated CD4+ T cells and is involved in activating an anti-mycobacterial response by macrophages (Sugawara I., 1998). During infection, direct interaction with TNF$\alpha$ and/or microbes primes macrophages for full scale activation by IFN$_\gamma$ (Saito S. and M. Nakano, 1996). IFN$_\gamma$ secretion activates the macrophage Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway leading to induction of nitric oxide synthase (NOS) -2, which generates the anti-mycobacterial effector molecules nitric oxide (NO) and reactive nitrogen intermediates (RNI) (Schaible U. E., 1998; Shtrichman R. and C. E. Samuel, 2001). While these effector molecules do play an important role in controlling bacterial replication, they are not solely responsible for combating infection with *Mycobacterium tuberculosis*, as demonstrated with NOS-2 gene disrupted mice that are able to control bacterial replication similar to wild-type mice (Cooper A. M., 2000). IFN$_\gamma$, however, is essential for controlling mycobacterial replication; in addition to its role in activating macrophage reactive oxygen and nitrogen intermediates, it has also been shown to promote phagosomal acidification for mycobacterial degradation (Schaible U. E., 1998)
IL-10 and TGF-β

To counter the proinflammatory response during *Mycobacterium tuberculosis* infection, the anti-inflammatory cytokines IL-10 and Transforming Growth Factor (TGF)-β, are also secreted (Hope J. C., 2004; van Crevel R., 2002). Both of these cytokines are involved in directly inhibiting T cell activation, and subsequently IFNγ production, by preventing transcriptional activation of IL-2, the cytokine involved in T cell proliferation (Hirsch C. S., 1996; Rojas M., 1999). They have also been shown to indirectly prevent T cell activation by downregulating expression of costimulatory molecules on antigen presenting cells, and through inhibition of IL-12 activity (Rojas M., 1999; Schmitt E., 1994). The production of IL-10 and TGFβ in response to *Mycobacterium tuberculosis* is beneficial to the host in that down regulation of the immune response would limit tissue injury by inhibiting excess inflammation (Sharma S. and M. Bose, 2001). From the bacteria’s perspective, induction of these anti-inflammatory cytokines allows for uninhibited bacterial replication.

Chemokines

Chemokines are small 8-10 kDa chemoattractant proteins that stimulate cellular migration across the endothelium into tissues. Immune cells, such as dendritic cells and T cells, can alter expression of their chemokine receptors in response to activation, allowing them to serve different roles during inflammation (Foti M., 1999; Qin S., 1998; Sallusto F., 1998; Sallusto F., 1999). The chemokines MCP-1 (CCL2), IL-8 (CXCL8), MIP-1α (CCL3), MIP-1β (CCL4) and RANTES (CCL5) are expressed by various immune cells during *Mycobacterium tuberculosis* infection, including epithelial cells, macrophages and
dendritic cells (Lin Y., 1998; Moller A. S., 2003; Saukkonen J. J., 2002; Strieter R. M., 1990). The chemokine gradients produced allow cells expressing the chemokine receptors (CCR) 1, 2 and 5 to localize to the site of inflammation. Chemokines can be induced directly via mycobacterial products or indirectly by TNFα, as described previously. Due to the significance of TNFα on chemokine production, experimental reduction of TNFα in mice infected with *Mycobacterium tuberculosis*, leads to modifications in chemokine production as well. The resulting disorganized granuloma is likely caused by cells not being held within the structure, so chemokine secretion by immune cells is seen throughout the lungs, rather than in a localized area (Algood H. M., 2003). CCR2, in particular, plays a significant role during infection with *Mycobacterium tuberculosis*. Mice disrupted in CCR2 expression are unable to control bacterial replication in the lungs and thus die faster than wild-type mice. This is due to a defect in both macrophage and dendritic cell recruitment to the lungs, which later causes a significant reduction in the number of activated dendritic cells that migrate to the lymph node. The defective innate response consequently results in diminished T cell activation and IFNγ production in the infected lungs (Peters W., 2004; Peters W., 2001).

Due to the redundant function of chemokines and their receptors, it is difficult to extrapolate the exact function of individual chemokines during the course of infection. Additionally, because cellular trafficking is dependent upon a chemokine gradient, assessment of chemokine concentration does not necessarily provide insight into the density of migratory cells. To demonstrate this point, constitutive expression of MCP-1 at high or moderate levels in mice during *Mycobacterium tuberculosis* infection, shows the mice to be more susceptible to disease than wild-type mice (Gu L., 1997; Rutledge B. J.,
This is likely due to a desensitization of CCR2 (the MCP-1 receptor) and/or the lack of a concentration gradient that impairs directed cellular migration. Similarly, mice disrupted in MCP-1 or CCR2 expression are also impaired in their ability to control infection with Mycobacterium tuberculosis (Lu B., 1998; Peters W., 2001; Rutledge B. J., 1995). The MCP-1 disrupted mice are actually better able to control infection than CCR2 disrupted mice, which the researchers explain is likely due to the existence of alternative CCR2 ligands that could compensate for the lack of MCP-1 (Lu B., 1998).

In addition to the inducible chemokines discussed, some chemokines are constitutively produced. Epstein-Barr virus–induced molecule 1 ligand chemokine (ELC/CCL19) and secondary lymphoid tissue chemokine (SLC) are expressed by stromal cells and high endothelial venules of the lymph node (Gunn M. D., 1999). These chemokines are recognized by CCR7 and binding allows cells to localize to the T cell zone of the lymph node (Ebert L. M., 2005).

**Toll-Like Receptors**

Toll-like receptors (TLRs) are transmembrane receptors that recognize conserved microbial structures called pathogen associated molecular patterns (PAMPs). This pathogen specificity allows innate immune cells to distinguish between self and non-self particles, protecting the host from inducing an autoimmune response. Binding of the TLRs by the PAMPs initiates an intracellular signaling casacade that leads to immune activation. To date, ten human and twelve mouse TLRs have been identified (Chaturvedi A. and S. K. Pierce, 2009). Both humans and mice express TLRs 1-9. Humans, but not
mice, also express TLR10, and mice, but not humans, express TLRs 11-13 (Chaturvedi A. and S. K. Pierce, 2009).

TLRs differ from one another in the cell types in which they are expressed, their ligand specificity, the signaling adaptors they utilize, and the cellular responses they induce (Iwasaki A. and R. Medzhitov, 2004). The discovery that TLR4 specifically recognizes bacterial lipopolysaccharide (LPS), initiated a wave of research to unveil the ligand specificity for the other TLRs (Poltorak A., 1998). Our current knowledge is that bacterial lipoproteins and lipoteichoic acids are detected by TLR2, which forms heterodimers with TLR1 or TLR6, double-stranded RNA is detected by TLR3, LPS is detected by TLR4, flagellin is detected by TLR5, single-stranded viral RNA is detected by TLR7 and TLR8, and unmethylated CpG DNA of bacteria and viruses is detected by TLR9 (Chaturvedi A. and S. K. Pierce, 2009; Iwasaki A. and R. Medzhitov, 2004).

The cytoplasmic region of all TLRs contains a conserved Toll/interleukin-1 receptor (TIR) domain that recruits the adaptor proteins MyD88, TIRAP, TRIF, or TRAM (O'Neill L. A. and A. G. Bowie, 2007). The adaptors then recruit downstream kinases and signaling molecules, which activate transcription factors such as Nuclear Factor-κB (NFκB), AP-1, Interferon regulatory factor (IRF)-3 and the MAP kinase (MAPK) family, initiating transcription of genes involved in the proinflammatory response (Jenkins K. A. and A. Mansell, 2009). MyD88 is a common adaptor protein utilized by all TLRs, except TLR3, as well as the IL-1 and IL-18 receptors. TLR4 is unique in that it can induce signaling by either a MyD88 dependent or independent pathway (Kopp E. and R. Medzhitov, 2003).
Studies with mice disrupted in MyD88 and infected with *Mycobacterium tuberculosis* demonstrate that these mice are unable to form proper granulomas and thus cannot control bacterial replication. Bone marrow derived macrophages and dendritic cells from MyD88 disrupted mice have reduced production of TNFα and IL-12p40 in response to *Mycobacterium tuberculosis* infection, but expression of the costimulatory molecules CD40 and CD86 is still upregulated. This explains why similar numbers of CD4+ and CD8+ T cells, as well as IFNγ production, in both wild-type and MyD88 disrupted mice is seen *in vivo*. Therefore, one can conclude that a MyD88 dependent innate immune response to *Mycobacterium tuberculosis* is essential for combating infection, but activation of the adaptive immune response is MyD88 independent (Fremond C. M., 2004; Scanga C. A., 2004).

Due to the wide array of TLR agonists within the cell envelope of *Mycobacterium tuberculosis*, there is conflicting evidence as to the exact immunological response generated by the individual molecules (Table1-1). In addition, macrophages and dendritic cells can respond differently to the same agonist – TLR interaction. Thus far, studies have shown that the majority of the *Mycobacterium tuberculosis* cell envelope lipoproteins bind to TLR2, and while studies in which TLR4 is disrupted demonstrate a role for TLR4 in recognition of *Mycobacterium tuberculosis*, no TLR4 ligands have yet been identified (Abel B., 2002; Aliprantis A. O., 1999; Gilleron M., 2003; Jones B. W., 2001; Quesniaux V. J., 2004; Uehori J., 2003).

Binding of TLR2 or TLR4 by *Mycobacterium tuberculosis* predominantly induces the expression of the proinflammatory cytokines TNFα and IL-12 by macrophages and dendritic cells (Gilleron M., 2003; Jones B. W., 2001; Pecora N. D., 2006; Quesniaux V.
TLR2 activation has also been shown to direct an antimicrobial response against intracellular *Mycobacterium tuberculosis* (Thoma-Uszynski, S., 2001). Expression of the costimulatory molecules CD40, CD80 and CD86 by macrophages appears to be mediated via binding to TLR2, but there is still some discrepancy as to which TLR, if any, is involved in inducing CD40, CD80 and CD86 expression by dendritic cells (Jang S., 2004). Pompei et al. demonstrate that maturation of dendritic cells is dependent upon TLR9 and Bafica et al. show that TLR9 works in concert with TLR2 for dendritic cell activation (Bafica A., 2005; Pompei L., 2007). However, as previously stated, Fremond et al. and others have found that costimulatory molecule expression is independent of MyD88 and Jang et al. found that dendritic cell maturation is independent of TLR2 and TLR4 (Fremond C. M., 2007; Fremond C. M., 2004; Jang S., 2004; Schnare M., 2001). Studies with the *E. coli* TLR4 agonist, lipopolysaccharide (LPS) have also found upregulation of costimulatory molecules to be MyD88 independent, but dependent upon IFNβ, and BCG peptidoglycan and cell wall skeleton (CWS) have been shown to induce dendritic cell maturation in a TNFα dependent manner (Hoebe K., 2003; Tsuji S., 2000; Uehori J., 2003). In the latter examples, one could argue that dendritic cell maturation is indirectly dependent upon TLR signaling, since TNFα production is dependent upon BCG peptidoglycan or CWS binding to TLR2 and TLR4 (Uehori J., 2003).

The IL-1 receptor (IL-1R), which also utilizes the MyD88 adaptor protein for kinase signaling, and Nucleotide-binding oligomerization domain protein (NOD) 2, an intracytoplasmic sensor for peptidoglycans, have both been shown to participate with TLRs in recognition of *Mycobacterium tuberculosis* (Ferwerda G., 2005; Fremond C. M.,
2007; Fritz J. H., 2006; Gandotra S., 2007). While IL-1R disrupted mice are highly susceptible to *Mycobacterium tuberculosis* infection, NOD2 appears to be dispensable for control of *Mycobacterium tuberculosis in vivo* (Fremond C. M., 2007; Gandotra S., 2007).

**Innate Immunity**

**Epithelial Cells**

The most predominant cells in the lungs are type I and type II epithelial cells that are linked by tight junctions, intermediate junctions, gap junctions and desmosomes to form a physical barrier between the lumen and underlying tissues (Mercer R. R., 1994). They constitutively secrete the chemokines IL-8 and MCP-1, to allow for steady state recruitment of immune cells into the lungs, and secrete the cytokines M-CSF and GM-CSF, to allow for the differentiation of incoming monocytes into macrophages and dendritic cells, respectively (Cox G., 1992; Nicod L. P., 1999; Pechkovsky D. V., 2005). Since nonpathogenic particles are constantly being introduced into the lungs, epithelial cells secrete the lipoprotein, surfactant, which serves to inhibit activation of the innate and adaptive immune cells, protecting the host from any unnecessary inflammatory destruction of the lung tissues (Hussain S., 2004). However, upon sensing microbes, epithelial cells produce the cytokines IL-1, IL-6 and TNFα and the chemokines IP-10 and ICAM-1, which release the immune cells from inhibition and allow for recruitment of activated T cells into the lungs (Mayer A. K. and A. H. Dalpke, 2007; Pechkovsky D. V., 2005).
It was generally thought that alveolar macrophages constitute the first line of defense against *Mycobacterium tuberculosis* infection, however, studies have now demonstrated that *Mycobacterium tuberculosis* can enter and replicate within alveolar epithelial cells (Bermudez L. E. and J. Goodman, 1996; Lin Y., 1998). Since the number of alveolar epithelial cells far exceeds the number of alveolar macrophages in the lungs (Crystal R.J., 1991; Schneeberger E. E., 1991), it is hypothesized that *Mycobacterium tuberculosis* interacts with alveolar epithelial cells prior to encountering any other innate immune cell in the lung. Mycobacteria infected alveolar epithelial cells produce IL-8 and MCP-1, likely due to intracellular growth (Lin Y., 1998).

**Macrophages and Dendritic Cells**

Pulmonary macrophages and dendritic cells are generated from constitutively circulating monocytes, which originate from myeloid progenitor cells in the bone marrow (Gordon S. and P. R. Taylor, 2005; Skold M. and S. M. Behar, 2008). Despite the fact that they are derived from the same precursor cells and are both antigen presenting cells, able to present processed antigens to activate an antigen specific T cell response, macrophages and dendritic cells serve different, yet complimentary roles during infection with *Mycobacterium tuberculosis* (Giacomini E., 2001; Wolf A. J., 2007).

Alveolar macrophages are the predominant cell type in the alveolar space during steady state (Martin T. R. and C. W. Frevert, 2005). They are generated from a reservoir of monocyte derived macrophages that accumulate in the lung tissues (Landsman L. and S. Jung, 2007). During non-inflammatory conditions, alveolar macrophages clear dying cells and particles and are maintained in an inactive state by epithelial cell secretion of
surfactant, as mentioned previously, as well as by TGFβ which is bound by alveolar epithelial cells (Takabayshi K., 2006). As with surfactant, TGFβ mediated inhibition is lost upon exposure of the cells to pathogens.

Due to their predominance in the alveoli, macrophages are one of the first cells to detect the presence of *Mycobacterium tuberculosis* in the lungs. Phagocytosis and entry of *Mycobacterium tuberculosis* is mediated via binding of the bacteria to complement receptors (CR) and mannose receptors (MR) on the surface of macrophages (Hirsch C. S., 1994; Schlesinger L. S., 1993; Schlesinger L. S., 1990). Typically, the microbe containing phagosome undergoes maturation and fuses with lysosomes; the resulting phagolysosomes contain hydrolytic enzymes that digest the engulfed bacteria (Cohn Z. A., 1963). *Mycobacterium tuberculosis*, however, is able to reside within the phagosome where it is able to thrive and replicate despite the antimicrobial responses of the host cells (Koul A., 2004).

Dendritic cells were first discovered in 1973 by Steinman and Cole, upon examination of cells from mouse peripheral lymphoid organs (Steinman R. M. and Z. A. Cohn, 1973). Dendritic cells are so called because of their characteristic long cytoplasmic processes, or dendrites. They are distinguished from other known cell types based on their morphology and tissue distribution, and are found to make up a small percentage of nucleated cells in the tissues (Steinman R. M. and Z. A. Cohn, 1973). In order to better study these unique cells, researchers have developed *in vitro* protocols to differentiate mouse bone marrow cells and human monocytes into dendritic cells using the cytokine GM-CSF (Inaba K., 1992; Sallusto F. and A. Lanzavecchia, 1994).
In the lungs, 80% of dendritic cells are found in the subepithelial regions while the remaining 20% are located in the interstitial spaces (Gonzalez-Juarrero M. and I. M. Orme, 2001; Jahnsen F. L., 2006). 1-5% of intraepithelial dendritic cells are found to extend their dendrites between the epithelial cells, without disturbing the epithelial barrier, in order to sample the antigens in the airway lumen (Gehr P., 2006; Jahnsen F. L., 2006). Interaction with antigen, with or without inflammation, signals for dendritic cells to migrate to lymph nodes where they can relay information to the adaptive immune cells (Jahnsen F. L., 2006; Jakubzick C., 2006; Vermaelen K. Y., 2001). Airway dendritic cell numbers are replenished by dendritic cell precursors in a CCR2 dependent manner (Jakubzick C., 2006; Osterholzer J. J., 2005). While macrophages are also able to migrate to the lymph node after antigen uptake, they appear to be less efficient than dendritic cells at transporting particles (Jakubzick C., 2006).

In order for dendritic cells to migrate from the lungs to the local lymph nodes and subsequently interact with naïve T cells, they must undergo a dramatic differentiation whereby they transition from “immature” to “mature” dendritic cells. Immature dendritic cells are characterized by their high proliferation rate, low motility with high expression of CCR1, CCR2 and CCR5, high capacity for antigen uptake and processing and low surface expression of the costimulatory molecules Major Histocompatibility Complex (MHC) class II, CD40, CD80 and CD86 (Foti M., 1999; Pierre P., 1997; Sallusto F., 1999; Winzler C., 1997). Inflammatory mediators, such as TNFα, uptake of particles and binding of PAMPs, can all activate maturation of dendritic cells, resulting in lower proliferation rates, high motility with downregulated expression of CCR1, CCR2 and CCR5, and upregulated expression of CCR7, lowered ability to uptake and process
antigen and upregulated expression of costimulatory molecules (Buettner M., 2005; Foti M., 1999; Pierre P., 1997; Rescigno M., 1999; Sallusto F., 1999; Winzler C., 1997). IL-12 production, the critical cytokine involved in promoting CD4+ T cells to become Th1 effector cells, is also upregulated, but only by those dendritic cells that come into direct contact with PAMPs (Sporri R. and C. Reis e Sousa, 2005). It is speculated that indirectly activated dendritic cells (inflammatory mediators or particle uptake) likely present self antigen for induction of tolerance, an important feature of the immune system that prevents destruction of self tissues (Sporri R. and C. Reis e Sousa, 2005).

Numerous studies have been performed with *Mycobacterium tuberculosis* infected mice or *in vitro* mouse and human cells to determine the roles macrophages and dendritic cells play during infection. It has been found that both macrophages and dendritic cells secrete the cytokines TNFα, IL-1 and IL-10, and the chemokines MCP-1 and RANTES in response to *Mycobacterium tuberculosis* infection (Giacomini E., 2001; Henderson R. A., 1997; Hickman S. P., 2002; Peters W. and J. D. Ernst, 2003). However, macrophages produce substantially more TNFα and MCP-1 while dendritic cells are the predominant producers of IL-12 (Bodnar K. A., 2001; Giacomini E., 2001; Jang S., 2008). In addition, *Mycobacterium tuberculosis* induces dendritic cell maturation, i.e. upregulated expression of MHC class II, CD40, CD80 and CD86, whereas expression of these molecules on the surface of macrophages is not significantly increased with infection (Bodnar K. A., 2001; Giacomini E., 2001; Henderson R. A., 1997). Instead, macrophages appear to constitutively express CD40, CD80 and CD86, yet are poor T cell stimulators (Soler P., 1999).
An in-depth look at the specific cell types infected with *Mycobacterium tuberculosis* as well as their tissue localization after aerosol infection of mice with bacteria expressing green fluorescent protein (GFP), demonstrate that dendritic cells are the predominantly infected cells in the lungs, even though they represent only a small percentage (6.8%) of the total cells in the lungs (Wolf A. J., 2007). In addition, dendritic cells are the predominant infected cell type in the lymph node at days 14 to 28 post infection, with a peak at day 21, corresponding to activation of the adaptive immune response, that is followed by a drastic decline in the number of infected lymph node dendritic cells. The number of infected dendritic cells in the lungs, however, continues to increase, demonstrating that dendritic cell migration to the lymph node is only transient. Infected macrophages are present in the lung and the lymph node as well, but at significantly lower numbers than dendritic cells. Comparison of the number of bacteria per cell in macrophages versus dendritic cells, demonstrates that approximately 60% of the macrophages analyzed but only 32% of dendritic cells have five or more intracellular bacteria. These data coincide with other studies showing that *Mycobacterium tuberculosis* is unable to replicate in dendritic cells (Buettner M., 2005; Tailleux L., 2003). It has been suggested that the dissimilar intracellular behavior of *Mycobacterium tuberculosis* in macrophages and dendritic cells may be due to differences in the receptors involved in bacterial uptake; macrophages predominantly use MRs and CRs, whereas DC-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN) has been found to be the major *Mycobacterium tuberculosis* receptor on dendritic cells, recognizing mannose-rich molecules within the mycobacterial envelope, such as ManLAM (Herrmann J. L. and P. H. Lagrange, 2005; Jo E. K., 2008; Tailleux L., 2003).
Taken together, these studies demonstrate that macrophages are predominantly responsible for controlling the inflammatory response to *Mycobacterium tuberculosis* infection in the lungs, as well as for providing an intracellular environment for bacterial growth, whereas dendritic cells are the principal cell type involved in linking the innate and adaptive immune responses by transporting *Mycobacterium tuberculosis* from the lung to the lymph node for activation of a Th1 response. To substantiate these ideas, *in vivo* cell depletion studies show that in the absence of resting macrophages, *Mycobacterium tuberculosis* numbers are decreased whereas selective elimination of activated macrophages leads to increased bacterial replication (Leemans J. C., 2005). Hence, resting macrophages provide a hiding place for bacteria to replicate, whereas activated macrophages inhibit bacterial growth. Importantly, this study also shows that fewer granulomas are present in the lungs of mice depleted of activated macrophages, providing further evidence that macrophages are the key cell type involved in granuloma formation. Transient deletion of dendritic cells, however, leads to a decreased T cell response that returns to normal when dendritic cells are replenished. Despite this, the immune response is not able to compensate for the early immune impairment and the burden of *Mycobacterium tuberculosis* is higher than in untreated mice (Tian T., 2005).

**Adaptive Immunity**

The adaptive arm of the immune response to *Mycobacterium tuberculosis* infection involves the actions of CD4+ and CD8+ T lymphocytes. While the innate response is able to limit bacterial replication during the early stages of infection, the adaptive immune response is essential for containing the bacteria during later stages of
infection and for preventing reactivation. Both CD4+ and CD8+ T cells are essential for controlling infection with *Mycobacterium tuberculosis*, but CD4+ T cells serve a much greater role and are found in greater numbers within the lung granuloma (Flynn J. L. and J. Chan, 2001; Gonzalez-Juarrero M. and I. M. Orme, 2001; Mogues T., 2001; Serbina N. V., 2001). This is best demonstrated in HIV + patients, where the reduction in CD4+ T cells coincides with reactivation tuberculosis as well as an increased susceptibility to infection with *Mycobacterium tuberculosis*.

Control of mycobacterial infection by CD4+ and CD8+ T cells is mediated through production of IFN$_\gamma$. While both cell types produce IFN$_\gamma$, it has been shown that IFN$_\gamma$ production by CD8+ T cells cannot substitute for the CD4+ T cell contribution of this cytokine (Caruso A. M., 1999; Serbina N. V., 2001). As discussed previously, IFN$_\gamma$ induces a mycobacteriocidal response in infected macrophages.

Naïve CD4+ T cells are activated by dendritic cells after undergoing pathogen induced maturation. Mature dendritic cells present processed antigen, via MHC class II, to the T cell receptor (TCR), initiating a series of interactions between the two cells. MHC class II-TCR binding upregulates expression of the costimulatory molecule CD40 ligand (CD40L) that binds the CD40 receptor (CD40) on the dendritic cell (Cayabyab M., 1994). The CD40L-CD40 interaction initiates T cell proliferation by activating T cell IL-2, as well as enhances production of IL-12 by dendritic cells (Demangel C., 2001; Florido M., 2004; Snijders A., 1998). The costimulatory molecules CD80 (B7.1) and CD86 (B7.2), expressed on the surface of mature dendritic cells, bind CD28 on the surface of the CD4+ T cell which induces expression of the IL-12 receptor (IL-12R) by T cells (Park W. R., 2001). Binding of IL-12 to the IL-12R triggers signal transducers and
activators of transcription (STAT) 4 mediated gene transcription that selectively skews the CD4+ T cell to become an IFNγ producing Th1 cell (Manetti R., 1993; Rogge L., 1997). In order for the CD4+ T cell to localize to the site of infection and interact with infected macrophages, activation causes expression of CCR7 to be downregulated and expression of CCR1 and CCR5 to be upregulated (Qin S., 1998; Sallusto F., 1998).

Phagocytosis of *Mycobacterium tuberculosis* by macrophages and dendritic cells causes the bacteria to reside within a membrane-bound phagosome, inhibiting bacterial degradation by the MHC class I processing machinery, located in the cytoplasm, for antigen presentation to CD8+ T cells. However, it has been found that dendritic cells, but not macrophages, have the unique ability to retrograde transport phagocytosed apoptotic blebs, resulting from induced cell death, into the cytosol, termed “cross presentation” (Albert M. L., 1998). In this way dendritic cells can activate CD8+ cytotoxic T cells (CTL) which actively kill cells harboring intracellular bacteria (Albert M. L., 1998; Albert M. L., 1998).

**Summary of Immunological Events**

Upon inhalation of *Mycobacterium tuberculosis*, the bacteria enter or are phagocytosed by airway epithelial cells, macrophages and dendritic cells. Infection of these cells causes activation of proinflammatory cytokines and chemokines to signal for additional immune cells to localize to the site of infection, for bacterial clearance and to initiate granuloma formation. Interaction of dendritic cells with *Mycobacterium tuberculosis* activates dendritic cell maturation, and hence migration to the local lymph node for interaction with and skewing of naïve CD4+ T cells to become IFNγ producing
Th1 cells. The activated CD4+ T cells then migrate to the site of infection where they induce the macrophage antimicrobial response and further contribute to the establishment of a persistent infection within the granuloma.

PATHOGENESIS

Immune Evasion

Stimulation of the immune response by *Mycobacterium tuberculosis* induces an immediate proinflammatory response that is later intercepted by various host and bacterial proteins. The host processes that are inhibited include phagosomal-lysosomal fusion, antigen presentation, stimulation of bactericidal responses and apoptosis (Koul A., 2004).

As described previously, acidification of the phagosomal environment through fusion with lysosomes, is one of the initial mechanisms utilized by macrophages to control bacterial replication. The mycobacterial cell envelope lipid, ManLAM, inhibits intracellular Ca\(^2+\) signaling pathways, which would otherwise lead to phagosomal maturation (Armstrong J. A. and P. D. Hart, 1975; Jaconi M. E., 1990). In addition, secretion of protein kinase G (PKG) by *Mycobacterium tuberculosis* interferes with delivery of the bacteria into lysosomes and thus prevents bacterial killing (Walburger A., 2004).

The indirect effect of inhibiting phagosomal-lysosomal fusion is interference with the antigen processing pathway, so macrophages and dendritic cells are unable to present mycobacterial antigen to T cells via MHC class II. Mycobacterial lipoproteins can also directly interfere with the presentation of antigens by downregulating expression of MHC
class II molecules, or inhibiting MHC class II antigen processing by infected cells (Gehring A. J., 2004; Noss E. H., 2001; Pai R. K., 2003; Pecora N. D., 2006).

In addition to the induction of proinflammatory cytokines, recognition of *Mycobacterium tuberculosis* by innate immune receptors, such as binding of ManLAM to DC-SIGN, also induces the anti-inflammatory cytokine IL-10, which limits the proinflammatory response (Geijtenbeek T. B., 2003). Binding to DC-SIGN also impairs dendritic cell maturation, which allows the bacteria to avoid clearance through recognition by T cells (Tailleux L., 2003).

Bacteria that are able to circumvent the innate response must then be able to evade the adaptive response, in particular IFNγ induced reactive nitrogen (RNI) and oxygen (NO). Mycobacterial binding of complement receptors blocks the production of harmful reactive oxygen intermediates by inhibiting the recruitment of NADPH oxidase to phagosomes (Melo M. D., 2000). Additionally, it has been shown that the mycobacterial cell envelope lipid, trehalose dimycolate (TDM), blocks JAK/STAT signaling and thus inhibits IFNγ induced bacteriocidal activity (Imai K., 2003). Engagement of TLR2 by mycobacterial peptidoglycan or lipoproteins is another bacterial mechanism that is involved in inhibiting production of reactive nitrogen (Banaiee N., 2006; Uehori J., 2003). Interestingly, NO has also been shown to act as a signal for *Mycobacterium tuberculosis* to activate a dormancy transcriptome that allows the bacteria to protect itself within the phagosome. The genes induced are involved in degrading fatty acids, DNA repair, cell envelope remodeling and production of siderophores to acquire iron (Rogerson B. J., 2006; Schnappinger D., 2003).
To ensure its survival, *Mycobacterium tuberculosis* also uses means to prevent apoptosis of infected cells. Similar to its role in inhibiting phagosomal-lysosomal fusion, ManLAM manipulates Ca\(^{2+}\) signaling pathways to prevent apoptosis (Koul A., 2004; Rojas M., 2000). Binding of TLR2 also allows *Mycobacterium tuberculosis* to subvert apoptosis (Loeuillet C., 2006). In addition, an indirect effect of IL-10 mediated control of the proinflammatory response is prevention of TNF\(\alpha\) induced apoptosis. IL-10 induces the release of the soluble TNF receptor type 2 (TNFR2) protein, which forms an inactive complex with TNF\(\alpha\) (Balcewicz-Sablinska M. K., 1998; Durrbaum-Landmann I., 1996).

Another potential means of evading the immune response by *Mycobacterium tuberculosis* is by infecting undifferentiated monocytes. *In vitro* experiments demonstrate that infection with *Mycobacterium tuberculosis* prevents GM-CSF and IFN\(\alpha\) induced monocytes from differentiating into dendritic cells (Mariotti S., 2004). Instead, the infected monocytes differentiate into macrophage-like cells that are unable to activate effector T cells, due to a lack of IL-12, and impaired expression of costimulatory molecules (Mariotti S., 2004). While the exact mechanism is unknown, the authors propose that interference with monocyte differentiation would favor the generation of macrophages, and thus provide an environment for *Mycobacterium tuberculosis* replication, as well as reduce the number of available dendritic cells for activating an adaptive immune response. In this way *Mycobacterium tuberculosis* would elude the immune response and facilitate persistence.

**Mycobacterial Cell Envelope**

*Architecture*
"Mycobacterium tuberculosis" is covered by a rigid and hydrophobic cell envelope that is composed of a high proportion of lipids and associated lipid complexes (Figure 1-5) (Bhowruth V., 2008; Schaible U.E and F. Winau, 2006). The lipid composition of the cell envelope serves to protect the bacterium from potentially harmful water-soluble substances in the environment, and is responsible for the intrinsic resistance to many antibiotics, but porins allow the bacterium to access essential nutrients (Draper P. and M. Daffe). In addition, the cell wall skeleton and individual lipid molecules are able to modulate the immune response by binding to host cell receptors (Table 1-1) (Quesniaux V. J., 2004). Complete Freund’s adjuvant (CFA), for example, is a commonly used immuno-adjuvant containing dead "Mycobacterium tuberculosis" cells suspended in mineral oil (Freund J., 1956).

The core of the cell envelope is made up of a plasma membrane, which interacts with a peptidoglycan (PG) layer, and arabinogalactan, which is connected to PG via a phosphodiester linkage. Various lipids are associated with this core: lipomannans (LM) are anchored to the plasma membrane, while pthiocerol dimycocerosate (PDIM), sulfolipids (SL) and mycolic acids (MA) all interact with arabinogalactan (Figure 1-5) (Dao D. N., 2008).

**Lipomannans**

Lipomannan is composed of a mannan core attached to phosphatidyl inositol mannoside (PIM). LM from both pathogenic and nonpathogenic mycobacteria interacts with TLR2 on host cells to induce TNFα, IL-12p40, NO production and apoptosis (Dao D. N., 2004; Quesniaux V. J., 2004). The biological activity of LM was found to be
dependent upon the presence of the mannan core (Dao D. N., 2004). In addition, PIM was also found to induce TNFα secretion by macrophages via binding to TLR2 (Gilleron M., 2003; Jones B. W., 2001).

Glycosylation of LM with arabinan gives rise to LAM, and further capping with mannose or phosphoinositol results in the production of ManLAM and PILAM, respectively. While LAM, ManLAM and PILAM are all derived from the precursor LM, the outcome of their interaction with host cells differs quite drastically. Similar to LM, PILAM induces IL-12p40 expression and apoptosis of macrophages in a dose-dependent manner, whereas LAM and ManLAM inhibit IL-12 production and apoptosis (Dao D. N., 2004). Dao et al. propose that the arabinan core in LAM and ManLAM masks the activity of mannan in LM and that conformational changes from the phosphoinositol residues in PILAM may expose the hidden mannan for binding to TLR2.

Direct inhibition of the proinflammatory response by ManLAM is mediated by binding of the mannose cap to the host cell mannose receptor (MR). This interaction induces the expression of IL-1 receptor-associated kinase M (IRAK-M), a negative regulator of TLR signaling (Pathak S. K., 2005). Indirectly, ManLAM can also suppress inflammation by inducing IL-10 and TGF-β production via binding to DC-SIGN, which inhibits macrophage and CD4+ T cell activation (Chujor C. S., 1992; Dahl K. E., 1996; Geijtenbeek T. B., 2003; Takeuchi M., 1998).

While LM and PILAM are proinflammatory mediators, and ManLAM is an anti-inflammatory mediator, LAM appears to behave somewhere in between. As stated, LAM plays a role in inhibiting IL-12 production and apoptosis, but it has also been shown to be a potent inducer of TNFα and cellular chemotaxis (Bernardo J., 1998). Since all classes
of lipomannans coexist in the mycobacterial cell envelope, it has been proposed that a ratio of the different classes may be important for *Mycobacterium tuberculosis* to influence the outcome of infection (Dao D. N., 2004).

*Mycolic Acids*

Mycolic acids are long chain fatty acids, containing 70-90 total carbon atoms. They make up the majority of the lipids in mycobacteria and comprise 40-60% of the dry weight of *Mycobacterium tuberculosis*, thus they are a key component of the mycobacteria resistance phenotype (Barry C. E., 3rd, 2001; Bhowruth V., 2008). Biosynthesis of mycolic acids is controlled by at least two elongation systems, Fatty Acid Synthase I (FAS-I), which is responsible for de novo fatty acid biosynthesis, and FAS-II that extends fatty acids to form mature mycolic acids (Bhowruth V., 2008).

Mycolic acids are composed of an $\alpha$-branch and a longer meromycolate branch. The meromycolate branch has two positions, one proximal and one distal from the carboxyl end, that can contain a double bond or cyclopropane. At the distal end, the double bond and the cyclopropane can be converted from a *cis* to a *trans* structure, whereas at the proximal end, the presence of a double bond, methoxy group or keto group determines the class of mycolic acid (Figure 1-6) (Brennan P. J., 1989; Lu B., 1998). Typically 70% of the cell envelope mycolic acids are $\alpha$-mycolic acids, with methoxy- and keto-mycolic acids each making up 10-15%. While limited in number compared to $\alpha$-mycolates, keto-mycolates are essential for growth within the host cell (Yuan Y., 1998). The number of *trans*-mycolates, the length of the mycolates, and *cis*-cyclopropanation have all been shown to contribute to enhancement of the mycobacterial cell envelope.
impermeability (Liu J., 1996). A mutant strain of *Mycobacterium marinum* that is disrupted in the β-ketoacyl-acyl carrier protein synthase B (*kasB*) gene, whose expression is required for fatty acid elongation, is defective in its ability to form full length mycolic acids, resulting in mycolic acids that are 2-4 carbons shorter than wild-type mycolic acids (Gao L. Y., 2003). The number of keto-mycolic acids in the cell envelope of the mutant bacteria is significantly reduced, accompanied by a compensatory increase in α and methoxy-mycolic acids. The resulting bacterial cell wall is dramatically more permeable, causing the mutant bacteria to be more susceptible to antibiotics and to the intracellular defense molecules defensin and lysozyme, and yields the bacteria unable to inhibit phagosomal-lysosomal fusion. These results demonstrate that even small alterations in mycolic acid length or composition can significantly affect overall packing, and ultimately, permeability and mycobacterial virulence.

Trehalose dimycolate (TDM), also known as “cord factor”, is a glycolipid made up of two mycolic acid molecules linked with trehalose by the hydroxyl groups of carbons 6 and 6’ (Noll H., 1956). TDM and trehalose monomycolate make up 6% of the lipid population in mycobacteria and TDM has been implicated as the major contributor to the characteristic tight bundles, or “cords” of bacilli in close parallel arrangement, formed by virulent mycobacteria (Allgower M. and H. Bloch, 1949). Not only does TDM help to make up the cell envelope structure, but it has also been shown to be the principle biologically active lipid in the cell envelope of BCG (Geisel R. E., 2005). TDM inhibits calcium induced fusion between phagosomal and lysosomal membranes, and is a potent inducer of IL-1β, IL-6, TNFα and MCP-1 via a TLR2 and TLR4 independent, but MyD88 dependent mechanism (Geisel R. E., 2005; Ryll R., 2001; Spargo B. J., 1991).
Multiple studies have shown that the chemical structure of the mycolic acids that make up TDM are critical to its inflammatory inducing phenotype (Dao D. N., 2008; Rao V., 2005; Rao V., 2006). The pcaA gene of *Mycobacterium tuberculosis* encodes an S-adenosyl methionine-dependent methyltransferase that catalyzes the proximal cis-cyclopropanation of α-mycolate. A pcaA mutant strain of *Mycobacterium tuberculosis* is hypoinflammatory in a mouse model of infection, with a reduced TNFα response by infected macrophages during the early phase of infection (Rao V., 2005).

Cyclopropane-mycolic acid synthase 2 (*cmaA2*) is required for the synthesis of the trans cyclopropane rings of both the methoxy-mycolates and keto-mycolates. Deletion of *cmaA2* abolishes trans-cyclopropanation of both methoxy- and keto-mycolates while α-mycolates are unaffected (Glickman M. S., 2001). Mice infected with the mutant strain of *cmaA2* die earlier than mice infected with wild-type *Mycobacterium tuberculosis*, due to excessive immune activation (Rao V., 2006). Thus, while cis-cyclopropanation of TDM is proinflammatory, trans-cyclopropanation is anti-inflammatory.

In contrast to the ability of TDMs to induce proinflammatory cytokines and chemokines, they have also been shown to differentially repress IL-12p70 production by infected macrophages, but not by dendritic cells (Dao D. N., 2008; Geisel R. E., 2005; Korf J., 2005). This phenotype is dependent upon the *mmaA4* gene which encodes a methyl transferase required for introducing the distal oxygen-containing modifications of methoxy- and keto-mycolates. Taken together these studies demonstrate that TDM alone is not responsible for the virulence of *Mycobacterium tuberculosis*, but the mycolic acid structure and relative abundance of the different subclasses of mycolic acid directly
affects the outcome of infection. This is highlighted in the fact that TDM has also been isolated from nonpathogenic mycobacteria, but these mycobacteria lack cyclopropane modification of mycolic acids (Mompon B., 1978).

TDM can be released from the cell envelope into the endocytic network of the host cell and thus individual lipids can directly affect *Mycobacterium tuberculosis* virulence (Rhoades E., 2003). It is important to note however, that due to the density of mycolic acids within the cell envelope, structural modifications and alterations in mycolic acid subclass composition would also affect cell envelope fluidity and permeability, thus affecting virulence indirectly (Barry C. E., 3rd, 2001).

**Mycobacterium tuberculosis** mce operon

The *mce* operon was first discovered in 1993 by Arruda et al., upon identification of a *Mycobacterium tuberculosis* DNA fragment, that when cloned into a nonpathogenic strain of *E. coli*, conferred upon the bacterium the ability to enter nonphagocytic HeLa cells (Arruda S., 1993). Thus this DNA fragment was named *mce* for mammalian cell entry. Once the genome of *Mycobacterium tuberculosis* became available, it was determined that this *mce* gene was part of an 8-gene operon and that the *Mycobacterium tuberculosis* genome contains four homologous operons (*mce1*-4) of similar organization (Cole S. T., 1998). Each operon is comprised of six *mce* genes (*mceA*-F) and two upstream genes, *yrbEA* and *yrbEB*, encoding integral membrane proteins (Figure 1-7) (Cole S. T., 1998).

The active domain for entry into HeLa cells was found to be located within the protein product of the *mce1A* gene (Casali N., 2002; Chitale S., 2001), and Mce1A was
shown to induce the internalization of bacteria and inert particles into A549 epithelial cells (Kohwiwattanagun J., 2007). In order to further decipher the role of mce1A during infection, an mce1A mutant strain of Mycobacterium tuberculosis was constructed by homologous recombination (Shimono N., 2003). Immunoblot analyses demonstrate that mutation of mce1A results in the loss of expression of the downstream mce1 operon genes. This same polar effect is seen with a yrbE1B mutant, and thus, these mutant strains are designated mce1 operon mutant or Δmce1.

Balb/c mice infected with the Δmce1 strain all die by 41 weeks post infection, whereas all mice infected with the wild-type strain of Mycobacterium tuberculosis (H37Rv) are still alive at 41 weeks (Figure 1-8) (Shimono N., 2003). In Balb/c mice this hypervirulent phenotype of Δmce1 appears (Lima P., 2007; Shimono N., 2003) to be due to uncontrolled bacterial replication in the lungs, but when using a more resistant mouse strain, C57BL/6, it was found that bacillary load does not correlate with mouse mortality (Lima P., 2007). Further analysis of the lungs of the Δmce1 infected Balb/c and C57BL/6 mice showed diffuse, poorly circumscribed and coalescing granulomas, compared to granulomas with sharp circumscribed boarders of the wild-type infected mice. Histologically, lungs of mice infected with the wild-type bacteria have a higher density of lymphocytes and fewer coalescing granulomas (Lima P., 2007; Shimono N., 2003). Significantly lower levels of TNFα are produced by Balb/c peritoneal macrophages and RAW macrophages infected with Δmce1, as compared to wild-type infected cells (Figure 1-10). Similarly, decreased levels of MCP-1 and IL-8 are produced by A549 epithelial cells and decreased levels of MCP-1, IL-6 and NO are produced by RAW macrophages in response to Δmce1 as compared to wild-type infected cells, even with pretreatment of
cells with IFNγ (Sequeira P., 2007; Shimono N., 2003). The complemented strain of
mce1 restores the proinflammatory response by macrophages (Shimono N., 2003). Thus,
the products of the mce1 operon appear to be directly or indirectly involved in inducing a
host immune response for development of a protective granuloma that is important for
establishment of a persistent infection by Mycobacterium tuberculosis.

In contrast to Δmce1, mutant strains of the mce2, mce3 and mce4 operons appear
to be attenuated in mice; Δmce2, Δmce4 or Δmce3/4 infected mice survived significantly
longer than mice infected with wild-type Mycobacterium tuberculosis (Marjanovic O.,
2008; Senaratne R. H., 2008). Mice infected with Δmce2 have similar bacterial numbers
in the lungs as wild-type infected mice, whereas Δmce4 or Δmce3/4 infected mice have
significantly fewer bacteria in the lungs. Additionally, little (Δmce2) or no difference
(Δmce4 or Δmce3/4) in cytokine or chemokine production by RAW macrophages was
seen in response to the mutant strains, as compared to wild-type (Marjanovic O., 2008;

Expression of three of the four operons is controlled by unique negative
transcriptional regulators located proximally to their respective operon (Casali N., 2006;
Santangelo M. P., 2002; Vindal V., 2008). Expression of the mce1 operon is controlled
by mce1R (Casali N., 2006). Mice infected with a mutant strain of mce1R (Δmce1R) die
sooner, have higher cytokine and chemokine expression in the lungs, greater bacteria
numbers in the lung, and consequently have 50% less alveolar space than wild-type
infected mice (Figures 1-9 & 1-11) (Uchida Y., 2007). Comparison of the outcome to
infection with Δmce1R versus Δmce1 demonstrates two extremes: a diminished host
immune response with death occurring after 25 weeks of infection with Δmce1, versus a
rapid infiltration of macrophages and lymphocytes leading to obliteration of alveolar airspaces before the adaptive immune response sets in, with Δmce1R (Uchida Y., 2007).

Casali et al. showed that expression of the mce1 operon fluctuates throughout the course of natural infection. Using real time PCR (RT-PCR), they demonstrate that mce1A expression is detected at 8 weeks but not 4 weeks post infection in wild-type infected mouse lung granuloma macrophages. FadD5, mce1A and mce1F expression is found to be rapidly repressed intracellularly, a 10-fold reduction by 4 hours post infection, in RAW macrophages, with mce1A expression levels increasing in a time dependent manner, as compared to expression in standard growth media (Casali N., 2006). Harboe et al. also found that the mce1A-mce1F genes are expressed in actively growing Mycobacterium tuberculosis cultures (Harboe M., 1999). Additionally, Sassetti and Rubin found that the mce1 operon appears to be most important during early infection, while the mce4 operon is more important at later stages of infection (Sassetti C. M. and E. J. Rubin, 2003). Therefore, despite the sequence similarity between mce operons 1-4, each operon appears to play a unique role during Mycobacterium tuberculosis infection. In particular, the differential expression of the mce1 operon is potentially involved in modulating the early host immune response for establishment of a persistent infection.

Mycobacterium tuberculosis encounters a variety of stimuli within the host during infection. Numerous studies have analyzed the transcriptome of Mycobacterium tuberculosis intracellularly and found that the bacterium actively senses its environment and responds by inducing the expression of genes involved in DNA repair and cell wall synthesis (Cappelli G., 2006; Cappelli G., 2001; Rengarajan J., 2005; Schnappinger D., 2003). The Cappelli group found that one-fifth of the upregulated Mycobacterium
*Mycobacterium tuberculosis* genes belong to the “cell wall and cell processes” group (Cappelli G., 2006). Of these, 50% were membrane proteins, suggesting that the antimicrobial phagosomal environment targets the bacterial cell envelope. As stated previously, the composition of the *Mycobacterium tuberculosis* cell wall is 60% lipid, and intraphagosomal *Mycobacterium tuberculosis* has been shown to rely on β-oxidation of fatty acids as an energy and carbon source (Bhowruth V., 2008). In addition, cell wall lipids are known virulence factors involved in modulating the host immune response, and therefore, induction of genes involved in lipid biosynthesis and degradation are important for remodeling the cell envelope and thus for *Mycobacterium tuberculosis* survival (Table 1-1) (Cappelli G., 2006; Cappelli G., 2001; Dubnau E., 2002; Schnappinger D., 2003).

Bioinformatic examination of the *mce* operons suggests that the operons encode ATP-binding cassette (ABC) transporters involved in uptake of substrates, possibly fatty acids (Casali N. and L. W. Riley, 2007; Joshi S. M., 2006). The MceA-F proteins from all four operons show homology to substrate binding proteins, which correlates with results from electron microscopy that reveal localization of Mce1A to the bacterial surface, and Western immunoblot analysis of subcellular fractions of *Mycobacterium tuberculosis* probed with Mce1 antibodies demonstrate that the Mce proteins localize to the cell wall fraction (Cantrell S., 2005; Chitale S., 2001). The four genes downstream of the *mce1* operon, as well as the two genes downstream of the *mce3* and *mce4* operons, termed “mas” for *mce*-associated, are suggested to be involved in ligand binding due to their domain architecture (Casali N. and L. W. Riley, 2007). Further, a gene upstream of the *mce1* operon (*mceG*), which encodes an ATPase (Mkl), has been shown to interact with the *mce1* and *mce4* loci, suggesting that the MceG protein associates with the YrbEA and
YrbEB proteins to generate the energy required by all four mce operons, for substrate transport (Casali N. and L. W. Riley, 2007; Dassa E. and P. Bouige, 2001; Joshi S. M., 2006).

In our laboratory, thin layer chromatography (TLC) and mass spectrometry analysis of the lipid composition of Δmce1 revealed an accumulation of free mycolic acids on the surface of Δmce1 that is not seen with wild-type or the complemented strain of Mycobacterium tuberculosis, suggesting that the mce1 operon is involved in importing mycolic acids (Figure 1-12) (Cantrell S., 2005). FadD5, a putative fatty acyl-CoA synthetase that is only present in the mce1 operon, is involved in fatty acid degradation (Trivedi O. A., 2004). Our laboratory has shown that a fadD5 mutant strain of Mycobacterium tuberculosis is able to grow normally in minimal media supplemented with various fatty acid, but when mycolic acids are used as the sole carbon source, the fadD5 mutant does not grow as well as wild-type bacteria, thus providing further evidence that the mce1 operon is involved in importing or contributing to the metabolism of mycolic acids (Dunphy K., 2008). In contrast to the mce1 operon, analysis of Δmce2 revealed a potential role for the mce2 operon in transporting sulfolipids (SL-1), and Paney and Sassetti have shown the mce4 operon to be the major cholesterol import system of Mycobacterium tuberculosis (Marjanovic O., 2008; Pandey A. K. and C. M. Sassetti, 2008). Taken together it appears that the four operons are responsible for transporting different substrates during the course of infection (Sassetti C. M. and E. J. Rubin, 2003). It is hypothesized that Mycobacterium tuberculosis requires these different import systems as a way to circumvent the nutrient poor environment of the phagosomal compartment and of the granuloma.
Altered concentrations of the three different forms of mycolic acids is found within the cell envelope of the $\Delta mce1$ strain, with methoxy-mycolic acid predominating (~50%) and $\alpha$-mycolic acids significantly reduced (~40%), whereas keto-mycolic acid concentration is unchanged (~10%), as compared to the lipid composition of the wild-type strain of *Mycobacterium tuberculosis* (Cantrell S., 2005). Studies have shown that modifications of cell wall mycolic acids can have profound effects on the host immune response, but since the cell wall is composed of a variety of important virulence factors, any disruption to the integrity of the cell wall or masking of these virulence factors could also alter the host response to the bacterium (Dao D. N., 2008; Rao V., 2005; Rao V., 2006). Therefore, it is interesting to speculate whether the aberrant immune response to $\Delta mce1$ is due to an indirect or direct inhibition of the immune response and whether this is caused by the altered distribution of the three forms of mycolic acid and/or the accumulation of mycolic acids on the surface of *Mycobacterium tuberculosis*. These ideas will be discussed in the following chapters of this dissertation. The next chapter will provide evidence for a role of the *mce1* operon in activating the early innate immune response, an essential step in granuloma formation and establishment of persistence by the bacterium.
Figure 1-1: Anatomy of the respiratory system showing the trachea, bronchi, both lungs and their lobes and airways. Inset shows bronchioles, alveoli, artery, and vein and the capillary network covering each alveoli (Winslow Terese, 2006).
Figure 1-2: Immune cells in the conducting airways (a) and alveoli (b) capture inspired antigen. Mature dendritic cells migrate through the afferent lymphatics to the draining lymph nodes and present processed antigen to naive T cells (c). Activated T cells proliferate and migrate through the efferent lymphatics and into the blood for localization to the infected lung (d), or to other mucosal sites (e) (Holt P. G., 2008).
Figure 1-3: The three stages of Mycobacterium tuberculosis infection. Stage 1: bacteria interact with innate immune cells for induction of a proinflammatory response and formation of the granuloma foundation. During this stage bacteria continue to replicate relatively uninhibited. Stage 2: induction of a Th1 response results in granuloma maturation and control of bacterial replication which allows for long term persistence of the bacteria in the host. Stage 3: bacterial reactivation leads to acute infection (Zahrt T. C., 2003)
Figure 1-4: Pathology of the lung granuloma after infection with *Mycobacterium tuberculosis*. Bacteria infect epithelial cells, macrophages and dendritic cells and induce a proinflammatory response leading to the recruitment of additional immune cells, from local blood vessels, for containment of the bacteria within a granuloma. The granuloma consists of a core of infected macrophages surrounded by recruited immune cells, and an outer layer of lymphocytes in association with a fibrous cuff. When the immune system wanes, the granuloma “decays” into a necrotic caseating lesion, allowing the reactivated bacteria to spill into the airway and be coughed out into the environment (Russell D. G., 2007).
Figure 1-5: Schematic representation of the *Mycobacterium tuberculosis* cell envelope (Riley L. W., 2006).
Figure 1-6: Molecular structures of the three subclasses of mycolic acid (Ryll R 2001).
Figure 1-7: Schematic representation of the *Mycobacterium tuberculosis* mce operons (Casali N. and L. W. Riley, 2007).
Fig. 1-8: Survival kinetics of Balb/c mice infected i.v. via tail vein (A) or C57BL/6 mice infected via aerosol (B), with *Mycobacterium tuberculosis* wild-type, Δmce1 or the complemented strain. Results are shown starting with 10 mice per group (Lima P., 2007; Shimono N., 2003).
Figure 1-9: Lungs of BALB/c mice infected i.v. via tail vein with wild-type *Mycobacterium tuberculosis* Erdman (A) or ∆mce1 (B) after 32 weeks, or wild-type H37Rv (C), or ∆mce1R (D) after 4 weeks (Shimono N., 2003; Uchida Y., 2007).
Figure 1-10: Kinetic analysis of TNFα (A), IL-6 (B), and MCP-1 (C) production by RAW macrophages infected with H37Rv wild-type (Δ) or Δmce1 (ο) strains at a multiplicity of infection (MOI) of 1:1. Controls included uninfected RAW cells (lower □) or lipopolysaccharide-treated cells (upper □) (Shimono N., 2003).
Figure 1-11: Survival kinetics of Balb/c mice infected i.v. via tail vein with Δmce1R (A) or wild-type (B) *Mycobacterium tuberculosis* at different doses (5 × 10^4, 5 × 10^5, or 5 × 10^6 per mouse). Results are shown starting with 10 mice per group (Uchida Y., 2007).
Figure 1-12: Mass spectrometry analysis of surface lipid extracts from wild-type, Δmce1 or the complemented strain of *Mycobacterium tuberculosis* (Cantrell S., 2005).
<table>
<thead>
<tr>
<th>TLR</th>
<th>Ligand</th>
<th>Response</th>
<th>Cell type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lipoproteins</td>
<td>CD40, CD86, MHC class II, TNF, IL-12, IL-10</td>
<td>DC</td>
<td>Pecora ND, 2006; Hertz CJ, 2001</td>
</tr>
<tr>
<td></td>
<td>Lipoproteins</td>
<td>IL-10, TNF</td>
<td>MO</td>
<td>Pecora ND, 2006</td>
</tr>
<tr>
<td></td>
<td>Lipoproteins</td>
<td>inhibit antigen processing and MHC class II expression</td>
<td>MO</td>
<td>Pecora ND, 2006; Gehring AJ, 2004</td>
</tr>
<tr>
<td></td>
<td>LM</td>
<td>TNF, IL-12p40, NO, CD80, CD86</td>
<td>MO</td>
<td>Quesniaux VJ, 2004</td>
</tr>
<tr>
<td></td>
<td>LM, PILAM</td>
<td>IL-12p40 and apoptosis</td>
<td>MO</td>
<td>Dao DN, 2004</td>
</tr>
<tr>
<td></td>
<td>Peptidoglycan</td>
<td>blocks IFN activation</td>
<td>MO</td>
<td>Banaiee N, 2006; Uehori J, 2003</td>
</tr>
<tr>
<td></td>
<td>Mtb cell wall fractions</td>
<td>TNF</td>
<td>MO</td>
<td>Underhill DM, 1999</td>
</tr>
<tr>
<td></td>
<td>Mtb</td>
<td>TNF, IL-12p40</td>
<td>MO</td>
<td>Uehori J, 2003</td>
</tr>
<tr>
<td></td>
<td>Mtb</td>
<td>IL-12p40</td>
<td>MO</td>
<td>Pompei L, 2007</td>
</tr>
<tr>
<td></td>
<td>Mtb</td>
<td>CD80, CD86, IL-10</td>
<td>MO</td>
<td>Jang S, 2004</td>
</tr>
<tr>
<td></td>
<td>Peptidoglycan</td>
<td>TNF</td>
<td>DC</td>
<td>Uehori J, 2003</td>
</tr>
<tr>
<td></td>
<td>BCG CWS</td>
<td>TNF</td>
<td>DC</td>
<td>Tsuji S, 2000</td>
</tr>
<tr>
<td></td>
<td>Mtb</td>
<td>IL-12p40, TNF</td>
<td>MO</td>
<td>Reiling N, 2002</td>
</tr>
<tr>
<td></td>
<td>PIM</td>
<td>TNF, IL-12</td>
<td>MO</td>
<td>Gilleron M, 2003; Jones BW, 2001</td>
</tr>
<tr>
<td></td>
<td>Mtb</td>
<td>CD80, CD86, IL-12p40</td>
<td>DC</td>
<td>Bafica A, 2005; Jang S, 2004</td>
</tr>
<tr>
<td></td>
<td>Mycolic acid</td>
<td>IL-12</td>
<td>MO</td>
<td>Korf J, 2005</td>
</tr>
<tr>
<td></td>
<td>TLR9</td>
<td>Mtb</td>
<td>DC maturation, IL-12p40</td>
<td>DC</td>
</tr>
<tr>
<td></td>
<td>TLR9 in collaboration with TLR2</td>
<td>Mtb</td>
<td>DC maturation, IL-12p40</td>
<td>DC</td>
</tr>
<tr>
<td></td>
<td>MyD88 independent</td>
<td>Mtb</td>
<td>CD40, CD80, CD86, MHC class II</td>
<td>DC</td>
</tr>
<tr>
<td></td>
<td>MyD88 dependent</td>
<td>Mtb</td>
<td>TNF, IL-12p40, NO</td>
<td>MO, DC</td>
</tr>
</tbody>
</table>

**Table 1-1**: Macrophage (MO) and dendritic cell (DC) responses to *Mycobacterium tuberculosis* infection (Mtb), or the specific mycobacterial PAMPs, and their dependence upon TLRs.
REFERENCES


Dunphy, K. 2008.


Ferwerda, G., S. E. Girardin, B. J. Kullberg, L. Le Bourhis, D. J. de Jong, D. M. Langenberg, R. van Crevel, G. J. Adema, T. H. Ottenhoff, J. W. Van der Meer,


Marjanovic, O. 2008.


71


Winslow, T. 2006.


Chapter 2

Activation of the early innate immune response by the *Mycobacterium tuberculosis* mce1 operon
INTRODUCTION

The hallmark of *Mycobacterium tuberculosis* infection is the formation of a protective granuloma (Russell D. G., 2007). As described in Chapter 1, granuloma formation occurs in two stages; stage one takes place during early/initial infection with the recruitment of innate immune cells to the site of infection for development of the granuloma foundation (Zahrt T. C., 2003). Macrophages and dendritic cells are the primary innate immune cells involved in this process, and have been shown to play complementary roles during the immune response to *Mycobacterium tuberculosis* infection (Giacomini E., 2001). Stage two occurs late in infection upon migration of activated CD4+ T cells to the inflammatory site and their subsequent interaction with infected macrophages (Kaplan G., 2003; Sugawara I., 1998). Within the mature granuloma, bacterial replication is controlled and a persistent infection is established (Bean A. G., 1999; Saito S. and M. Nakano, 1996).

During early infection, interaction with *Mycobacterium tuberculosis* induces macrophages and dendritic cells to secrete a diverse set of cytokines and chemokines, such as TNFα, IL-12, IL-10 and MCP-1 (Giacomini E., 2001; Henderson R. A., 1997; Hickman S. P., 2002; Hope J. C., 2004; Peters W. and J. D. Ernst, 2003; Schaible U.E and F. Winau, 2006). These molecules serve to signal for additional immune cells to localize to the site of infection and to initiate granuloma formation. Therefore, the activation status of macrophages and dendritic cells can be determined *in vitro* through assessment of cytokine and chemokine concentrations in the extracted supernatants. In addition, interaction with *Mycobacterium tuberculosis* induces dendritic cells to undergo a dramatic differentiation that leads to the upregulation of the costimulatory molecules.
CD40 and CD86, as well as the chemokine receptor, CCR7, on the surface of mature dendritic cells (Henderson R. A., 1997). These molecules are all important for dendritic cells to interact with and activate naïve T cells. Hence, dendritic cell activation can also be assessed by examining expression of these surface molecules.

Previous studies from our laboratory have shown that Balb/c mice infected with a strain of *Mycobacterium tuberculosis* that is disrupted in the *mce1* operon (Δ*mce1*) all die sooner than those infected with the wild-type strain of *Mycobacterium tuberculosis* (H37Rv), and hence Δ*mce1* is considered to be hypervirulent (Shimono N., 2003). It was initially thought that the decreased survival of Δ*mce1* infected mice was due to uncontrolled bacterial replication. However in a more resistant mouse strain, C57BL/6, it was found that bacillary load does not correlate with mouse mortality (Lima P., 2007). Instead, improper granuloma formation in the lungs of the Δ*mce1* infected Balb/c and C57BL/6 mice appears to be more important.

As discussed in Chapter 1, granuloma formation is critical to the control of infection, and hence to the survival of the host. However, because of the profound effect on granuloma formation associated with disruption of the *mce1* operon, we hypothesized that granuloma formation itself may be important for the survival of *M. tuberculosis* in the host. Since proper granuloma formation is dependent upon activation of the innate immune cells during the early stage of infection, we initially performed *in vitro* studies with RAW 264.7 macrophages and A549 epithelial cell lines in order to get a clue about the role of the *mce1* operon in initiating this process. Infection of either cell type with Δ*mce1*, demonstrated a reduced cytokine and chemokine response, as compared to the response to infection with wild-type H37Rv, suggesting that if this occurred *in vivo*, it
could have downstream consequences in the formation of granulomas (Sequeira P., 2007; Shimono N., 2003).

Macrophages were originally chosen for these *in vitro* studies due to their importance in controlling the early inflammatory response to *Mycobacterium tuberculosis* infection in the lungs, and their ability to kill intracellular bacteria upon interaction with activated CD4+ T cells (Giacomini E., 2001; Pedroza-Gonzalez A., 2004; Wolf A. J., 2007). While use of the RAW 264.7 cell line allowed us to get a general idea of the host response to infection, we felt that the use of primary cells would provide us with a more accurate picture of how individual cell types respond *in vivo*. Therefore, the experiments in this dissertation involve the use of mouse bone marrow derived macrophages.

In addition to the impaired ability of Δmcel infected mice to form granulomas, histological examination of the mutant infected mice revealed reduced lymphocyte migration to the lungs (Shimono N., 2003). Dendritic cells are efficient antigen-present cells (APCs) that have been shown to transport bacteria from the lungs to the lymph node in order to activate naïve T cells and induce a Th1 type of adaptive immune response (Pedroza-Gonzalez A., 2004; Tailleux L., 2003; Wolf A. J., 2007). Therefore, in the following experiments, we also investigated the response of mouse bone marrow derived dendritic cells to infection with Δmcel in order to determine if the reduced lymphocyte presence in the lungs was due to a lack of dendritic cell activation.

**RESULTS**

**Cytokine and Chemokine Expression Kinetics**
In order to determine whether expression of the \emph{mce1} operon is essential for activation of the innate immune response, we infected Balb/c mouse bone marrow derived macrophages and dendritic cells with H37Rv (wild-type) or the \emph{mce1} operon mutant strain of \emph{Mycobacterium tuberculosis} (\emph{Δmce1}). Comparison of macrophage and dendritic cell production of the pro-inflammatory cytokines, TNFα (Figure 2-1) and IL-12 (Figure 2-2), and the chemokine, MCP-1 (Figure 2-3), in response to the two bacterial strains was assessed by ELISA. The data demonstrated that, similar to what was seen previously from our laboratory, \emph{Δmce1} induced significantly lower levels of TNFα, IL-12, and MCP-1 from both cell types, as compared to cells infected with H37Rv.

In order to determine if the reduced pro-inflammatory response to \emph{Δmce1} was due to an enhanced anti-inflammatory response, we tested the cell supernatants for production of IL-10 (Figure 2-4). Similar to what was seen with the pro-inflammatory cytokines, decreased levels of IL-10 were seen from both cell types in response to \emph{Δmce1}, as compared to cells infected with H37Rv. Therefore, IL-10 is not responsible for the impaired pro-inflammatory response.

The remaining experiments for this dissertation used cells derived from C57BL/6 mouse bone marrow, and therefore we wanted to confirm that mouse background did not affect the differential response seen with the wild-type versus the mutant strain of \emph{Mycobacterium tuberculosis}. Macrophages and dendritic cells derived from Balb/c or C57BL/6 mouse bone marrow were assessed for production of TNFα. As shown in Figure 2-5, both Balb/c and C57BL/6 mouse macrophages and dendritic cells secreted an abundance of TNFα in response to infection with H37Rv and were impaired in their
response to infection with \textit{Amce1}. Therefore, the observed differences in the cytokine responses induced by the bacterial strains were not due to mouse strain differences.

\textbf{Surface Molecule Expression}

To determine whether expression of the \textit{mce1} operon is important for \textit{Mycobacterium tuberculosis} to induce dendritic cell maturation and migration potential, we examined expression levels of the costimulatory molecules, CD40 and CD86, and of the chemokine receptor, CCR7, by \textit{Amce1} infected dendritic cells. Comparison of the histograms in Figure 2-6 shows that surface expression of CD40 and CD86 by dendritic cells in response to infection with \textit{Amce1}, is slightly lower as compared to cells infected with H37Rv. CCR7 expression, however, appears to be identical in response to infection with either \textit{Amce1} or H37Rv (Figure 2-7). Evaluation of median fluorescence intensity supports these conclusions, although the difference in median fluorescence intensity for CD40 expression was statistically significant and that for CD86 was not.

\textbf{DISCUSSION}

Our laboratory has shown that in the absence of \textit{mce1} operon expression, \textit{Mycobacterium tuberculosis} is unable to establish a persistent infection due to an inability to initiate a granulomatous response in the host (Shimono N., 2003). The data suggests that the host response is altered from the onset of infection. However the underlying mechanism for this response involving individual cell types was still unknown (Sequeira P., 2007; Shimono N., 2003). Due to the well-established and distinct roles macrophages and dendritic cells play during the early stages of infection with
Mycobacterium tuberculosis, we chose to investigate how expression of the mce1 operon affects their activity in vitro.

We found that secretion of TNFα, IL-12, IL-10 and MCP-1 by mouse bone marrow derived macrophages and dendritic cells was significantly reduced in response to ∆mce1 (Figures 2-1, 2-2, 2-3, 2-4). This is in agreement with previous studies in our laboratory using RAW 264.7 and A549 epithelial cell lines (Sequeira P., 2007; Shimono N., 2003). Therefore, we can conclude that expression of the mce1 operon is essential for robust cytokine and chemokine production in response to infection with Mycobacterium tuberculosis, and, therefore, proper granuloma formation.

Interestingly, the levels of TNFα production by dendritic cells and macrophages in response to wild-type H37Rv was relatively equal (Figure 2-1). However, IL-12 and IL-10 production by macrophages was approximately half of that produced by dendritic cells (Figures 2-2 and 2-4) and macrophage MCP-1 levels were greater than that produced by dendritic cells (Figure 2-3). These results are in agreement with the different roles macrophages and dendritic cells are known to play in response to Mycobacterium tuberculosis infection (Giacomini E., 2001; Wolf A. J., 2007). Elevated levels of TNFα and MCP-1 by macrophages allow them to control the inflammatory response and inhibit bacterial replication (Bean A. G., 1999; Flesch I. E. and S. H. Kaufmann, 1990; Flynn J. L., 1995; Majumder N., 2008; Saukkonen J. J., 2002; Tessier P. A., 1997), while the higher levels of IL-12 produced by dendritic cells are important for them to interact with naïve T cells and selectively induce a Th1 response (Cooper A. M., 1997; Khader S. A., 2006; Schmitt E., 1994). The increased levels of IL-10 produced by dendritic cells is in line with what other researchers have found, and is of little significance since dendritic
cells appear to be insensitive to IL-10 mediated inhibition of IL-12 (Hickman S. P.,
2002).

Dendritic cells are known for their role in migrating to the lymph nodes to interact
with and activate naïve T cells via binding of the dendritic cell costimulatory molecules
CD40 and CD86, with the T cell surface molecules CD40L, and CD28, respectively
(Cayabyab M., 1994; Khader S. A., 2006; Park W. R., 2001). The activated CD4+ T
cells, in turn, trigger an antimicrobial response from infected macrophages at the site of
infection. Therefore, to address whether the impaired response to infection with $\Delta mce1$
in the mouse lungs is due to an inability of $\Delta mce1$ infected dendritic cells to activate T cells,
we determined the level of surface expression of the costimulatory molecules CD40 and
CD86, using fluorescent antibodies. Flow cytometry results demonstrate that dendritic
cell differentiation is impaired, albeit modestly, in cells infected with $\Delta mce1$ as compared
to those infected with H37Rv (Figure 2-6). While there is still debate within the literature
as to whether dendritic cell cytokine production and costimulatory molecule expression
are induced via the same signaling pathway(s), a growing body of evidence suggests that
TNF\(\alpha\), and not direct contact with antigens, induces dendritic cell maturation (Fremond
C. M., 2004; Tsuji S., 2000; Uehori J., 2003). Therefore, the subtle differences in
costimulatory molecule expression versus the drastic differences in cytokine production
we saw with dendritic cells infected with H37Rv compared to infection with $\Delta mce1$,
could be due to basal levels of TNF\(\alpha\) production that are skewing the flow cytometry
results. In addition, dendritic cells are quite sensitive to manual manipulation, and
expression of costimulatory molecules can be modestly upregulated with simple pipetting
(as seen with personal experiments comparing dendritic cell differentiation techniques,
Data not shown). Therefore, the in vitro methods used in these experiments could be subduing any dramatic impairment in upregulation of dendritic cell costimulatory molecule expression in response to infection with Δmce1. Despite these potential complications, and since all the cells were treated similarly, the statistically significant reduced expression of CD40 observed in this experiment, and the reduced expression of CD86 we saw in a previous experiment (data not shown) in response to Δmce1 infection, suggests that expression of the mce1 operon is not only essential for cytokine activation, but also for induction of dendritic cell costimulatory molecule expression.

Upon interaction with Mycobacterium tuberculosis, dendritic cells downregulate expression of CCRs 1, 2 and 5 and upregulate expression of CCR7 so that they can localize to the local lymph node and interact with naïve T cells (Dieu M. C., 1998; Vecchi A., 1999). Therefore, to address whether the reduced lymphocyte migration seen in the lungs of Δmce1 infected mice could be related to an impaired ability of Δmce1 infected dendritic cells to migrate to the lymph node, we assessed Δmce1 infected dendritic cells for expression of CCR7. No difference in CCR7 expression by dendritic cells was seen in response to infection with Δmce1, as compared to infection with H37Rv (Figure 2-7, 2-8). Recent work by Anis et al. shows that uninfected and BCG infected dendritic cells express similar levels of CCR7, but in a transwell assay, BCG infected dendritic cells migrate toward CCL21 in greater numbers than do uninfected dendritic cells (Anis M. M., 2008). Therefore, expression of CCR7 alone does not directly correlate with the migration potential of dendritic cells, and future chemotaxis assays need to be performed in order to determine whether expression of the mce1 operon is important for dendritic cell migration.
The essential role of the innate immune response in controlling infection with *Mycobacterium tuberculosis* is highlighted in studies where either macrophages or dendritic cells are eliminated *in vivo*. Chemical depletion of activated mouse pulmonary macrophages significantly impairs the host response to infection, as demonstrated by the increased numbers of bacteria in the lungs and spleen, the decreased numbers of granulomas and the reduced levels of TNFα in the lungs, despite the fact that CD4+ T cell numbers remain unaffected (Leemans J. C., 2005). Transient diphtheria toxin depletion of mouse dendritic cells during *Mycobacterium tuberculosis* infection leads to a significant reduction in CD4+ T cell numbers and an increase in bacterial burden (Tian T., 2005). Repopulation of the dendritic cells returns the levels of CD4+ T cells to that of untreated mice. However, the bacterial burden in the treated mice remains higher than in untreated mice. Together these two studies demonstrate that early events in the immune response to *Mycobacterium tuberculosis* infection has profound consequences on the outcome of infection and disease, and that while CD4+ T cells are important for controlling infection, they cannot reverse any defects set in motion during the initial innate response.

Granuloma formation, in response to *Mycobacterium tuberculosis* infection, is initiated by the host for containment of the bacteria within the lungs and to control bacterial replication. The genome of *Mycobacterium tuberculosis*, however, comprises an array of genes involved in modulating the host response in order to establish a persistent infection (Stewart G. R., 2003). Previous studies from our laboratory provide evidence that the *mce1* operon of *Mycobacterium tuberculosis* is also involved in modulating the immune response, and that disruption of the *mce1* operon leads to improper granuloma
formation in a mouse model of infection (Casali N., 2006; Shimono N., 2003). Using primary cells derived from mouse bone marrow, we demonstrate in this chapter that expression of the mce1 operon is essential for macrophage and dendritic cell activation in response to infection with *Mycobacterium tuberculosis*. These observations, together with our previous data, suggest that the mce1 operon is involved, either directly or indirectly, in initiating an early innate immune response that ultimately affects the outcome of *Mycobacterium tuberculosis* infection.
MATERIALS AND METHODS

Growth and Maintenance of Mycobacterial Strains

Mycobacterial strains were grown in Middlebrook 7H9 broth (Difco, MD) containing 10% ADC and 0.2% glycerol (Fisher Scientific, NJ). Strains were grown to mid-log phase and then passaged or pelleted for further processing.

Differentiation of Mouse Bone Marrow Cells

Balb/c or C57BL/6 mice were anesthetized with isoflurane (Phoenix Pharmaceutical, MO) for 1-2 minutes. Femurs and tibias were removed from each mouse, after spinal dislocation. Cells were extracted from the bone marrow with a mortar and pestle and transferred to a 15 ml conical tube (VWR) in RPMI complete media (RPMI 1640 + L-glutamine, 10% FBS, 1% sodium pyruvate, 1% HEPES Buffer, 0.1% β-mercaptoethanol). Cells were pelleted at 1200 rpm for 5 minutes at 23°C. Red blood cells were lysed by resuspending cells in 1 ml ACK lysing buffer for 1-2 minutes. Lysis was stopped with 10 ml RPMI complete media. Cells were pelleted again and resuspended in 10 ml RPMI complete media. Cell debris was removed by straining the suspension through a 40 um cell strainer. Cells were then washed 3 times in RPMI complete media. Cells were resuspended in 10 ml RPMI complete media and counted with a hemocytometer.

Macrophages

Cells were cultured in RPMI complete media supplemented with M-CSF (30%) and Penicillin/Streptomycin (1:100). Cells were plated at 5 x 10^6 cells per 15 ml of RPMI
complete media in 25 cm non-tissue culture treated Petri dishes (VWR) in a 5% CO₂ humidified incubator at 37°C. On day 4 of incubation, all media was removed and replaced with 15 ml of fresh RPMI complete media supplemented only with M-CSF. Macrophages were differentiated by day 6.

_Dendritic Cells_

Cells were cultured in RPMI complete media supplemented with GM-CSF (1:50) and Penicillin/Streptomycin (1:100). Cells were plated at 7 x 10⁵ cells/ml in 24-well non-tissue culture treated plates (BD Falcon, CA) in a 5% CO₂ humidified incubator at 37°C. On day 2 and day 4 of incubation, 500 ul of media from each well was removed and replaced with 500 ul fresh RPMI complete media supplemented only with GM-CSF. Dendritic cells were differentiated by day 6.

_In Vitro Infections_

On day 6 of incubation, macrophages and dendritic cells were removed from the plates by pipetting, followed by addition of cold PBS for 5 minutes to remove adherent cells. Cells were pelleted at 1200 rpm for 5 minutes and resuspended in 10 ml RPMI complete media. Each cell type was then counted with a hemocytometer and plated, in triplicate, at 1 x 10⁶ cells/ml in 24-well tissue culture treated plates.

Mid-log phase H37Rv or Δmce1 strains of *Mycobacterium tuberculosis* were pelleted at 500 rpm for 5 minutes to remove large clumps of bacteria. The supernatant, containing single cell suspension bacteria, was removed and pelleted at 3500 rpm for 5 minutes. The bacteria were washed two times in 0.05% PBS-Tween 80. The bacteria
were resuspended in 6 ml of 0.05% PBS-Tween 80 and an OD$_{600}$ was performed to equilibrate all strains. Serial dilutions of bacteria were plated on Middlebrook 7H11 agar (Difco, MD) plates containing 10% OADC and 100 ug/ml cycloheximide (VWR). Colony forming units (CFU) for each strain were determined to confirm multiplicity of infection (MOI).

Cells were then infected with *Mycobacterium tuberculosis* at an MOI of 5 to 7. LPS (1ug/ml) (Sigma, MO) and Pam3CSK4 (1ug/ml) (InvivoGen, CA) were used as positive controls to stimulate the cells, and PBS-Tween 80 infected cells served as a negative control for each experiment.

Dendritic cells used for surface molecule expression experiments were not removed from the plates on day 6 of incubation, but instead were left untouched so as to eliminate potential activation from over pipetting. Instead, 800 ul of old media was removed from each well and replaced with 800 ul of fresh RPMI complete media. Cells were infected with strains of *Mycobacterium tuberculosis* as above.

**Enzyme-linked Immunosorbent Assay (ELISA)**

At 4, 24, 48 and 72 hours post infection, supernatants were removed from each well and filtered through a 0.2 um filter (Fisher). Supernatants were analyzed for cytokine and chemokine production with ELISA kits (eBioscience, CA). Supernatants were stored at -80°C.

**Flow Cytometry**
At 24 hours post infection, dendritic cells were removed from the wells. Cold PBS (1 ml) was added to each well and incubated for 5 minutes to remove adherent cells. All cells were transferred to 1.5 ml tubes. Cells were pelleted at 1200 rpm for 5 minutes and resuspended in 25 ul of 2% FBS in PBS to block cells from any nonspecific binding. Following a 30 minute incubation at 4°C, another 25 ul of 2% FBS in PBS containing a combination of the fluorescent antibodies (eBioscience, CA): CD40-PE, CD86-PECy5, CD11c-PECy7 or CCR7-PE, CD11c-PECy7, was added. Cells were incubated at 4°C (CD40/CD86/CD11c) or 37°C (CCR7/CD11c), for 30 minutes. Cells were washed in PBS and then fixed overnight at 4°C in 4% paraformaldehyde for removal from the BSL3 laboratory. Cells were pelleted and resuspended in 500 ml PBS and run on an FC-500 flow cytometer. Data were analyzed by Flow-Jo. CD11c was used as a marker for dendritic cells.

Statistics

Comparison of mean values was assessed by the Student’s t test. Differences were considered significant at p ≤ 0.05.
Figure 2-1: TNFα production by Balb/c bone marrow derived macrophages (A) and dendritic cells (B) infected with H37Rv (○), Δmce1 (◼), LPS (△) or left uninfected (◊). Data are representative of three independent experiments. p ≤ 0.05 (*).
Figure 2-2: IL-12p70 production by Balb/c bone marrow derived macrophages (A) and dendritic cells (B) infected with H37Rv (○), Δmcel (□), LPS (Δ) or left uninfected (◊). Data are representative of three independent experiments. p ≤ 0.05 (*).
Figure 2-3: MCP-1 production by Balb/c bone marrow derived macrophages (A) and dendritic cells (B) infected with H37Rv (○), Δmce1 (□), LPS (Δ) or left uninfected (◊). Data are representative of three independent experiments. p ≤ 0.05 (*).
Figure 2-4: IL-10 production by Balb/c bone marrow derived macrophages (A) and dendritic cells (B) infected with H37Rv (○), Δmce1 (□), LPS (Δ) or left uninfected (◊). Data are representative of three independent experiments. p ≤ 0.05 (*).
Figure 2-5: TNFα production by Balb/c and C57BL/6 bone marrow derived macrophages (A) and dendritic cells (B) infected with H37Rv (grey), Δmce1 (black), or left uninfected (white). Data are representative of three independent experiments. p ≤ 0.05 (*).
Figure 2-6: Surface expression of CD40 (A) and CD86 (B) by C57BL/6 bone marrow derived dendritic cells 24 hours after infection with H37Rv, Δmce1, Pam3CSK4 or LPS (open regions) as compared to uninfected controls (shaded regions). Histograms show data from two independent experiments.
Figure 2-7: Surface expression of CCR7 by C57BL/6 bone marrow derived dendritic cells 24 hours after infection with H37Rv, Δmce1, Pam3CSK4 or LPS (open regions) as compared to uninfected controls (shaded regions). Histograms show data from two independent experiments.
Figure 2-8: Median fluorescence intensity (MFI) of CD40, CD86 and CCR7 expression on the surface of C57BL/6 bone marrow derived dendritic cells 24 hours after infection with H37Rv (grey), ∆mce1 (black), Pam3CSK4 (diagonal stripes), LPS (vertical stripes) or left uninfected (white). Data are representative of two independent experiments. p ≤ 0.05 (*).
REFERENCES


Chapter 3

The mce1 operon mutant strain of Mycobacterium tuberculosis is not impaired in its ability to signal through Toll-like receptor 2.
INTRODUCTION

*Mycobacterium tuberculosis* is an obligate intracellular pathogen, whose replication and survival are dependent upon uptake by available innate immune cells. The core of the *Mycobacterium tuberculosis* induced granuloma is made up of recruited macrophages in close proximity to one another which provides an ideal residence for the bacteria and protects the host from bacterial dissemination (Iyonaga K., 2002; Tsai M. C., 2006; Uehira K., 2002). In the face of continuous cell turnover, a regular influx of macrophages is necessary to maintain the granuloma foundation (Saunders B. M., 1999). In addition, T cell activation and recruitment to the granuloma is essential for controlling bacterial replication (Bean A. G., 1999).

As described in Chapter 1, activation of the innate immune response during the initial stages of *Mycobacterium tuberculosis* infection is predominantly dependent upon binding of mycobacterial ligands to Toll-like receptor (TLR) 2 and/or TLR4 (Table 1-1), expressed on the surface of macrophages and dendritic cells. In the absence of TLR2 or of the TLR adaptor protein, MyD88, granuloma formation in response to *Mycobacterium tuberculosis* infection is disrupted in mouse lungs, and the mice die significantly earlier than wild-type mice due to an inability to control bacterial replication (Drennan M. B., 2004; Fremond C. M., 2004). In contrast, TLR4 deficient mice are able to mount an early granulomatous response in the lungs, but eventually succumb to infection (Abel B., 2002). Therefore, TLR2 signaling is critical during early infection, whereas TLR4 signaling appears to be important in the later, chronic stages of infection.
The results from Chapter 2 of this dissertation, as well as previous reports from our laboratory, demonstrate that expression of the *Mycobacterium tuberculosis* mce1 operon is essential for induction of the early innate immune response (Sequeira P., 2007; Shimono N., 2003). Similar to the results from the MyD88 or TLR2 mutant studies, mice infected with Δmce1 have fewer and more diffuse granulomas than mice infected with the wild-type strain of *Mycobacterium tuberculosis* (Shimono N., 2003), and in vitro Δmce1 infected macrophages and dendritic cells are impaired in their ability to secrete TNFα (Chapter 2). Due to these similarities, we hypothesize that the impaired immune response to Δmce1 is due to an inability of the mutant bacteria to activate TLR signaling.

While the exact function of the mce1 operon is still unknown, bioinformatics analysis suggests that it is involved in lipid import (Casali N. and L. W. Riley, 2007). This is corroborated with evidence from our laboratory showing an accumulation of free mycolic acids in the surface lipid extracts from the mce1 operon mutant strain (Δmce1) that is not present in the surface lipid extracts from wild-type bacteria (Cantrell S., 2005). It is unclear, however, whether these excess mycolic acids are within the cell envelope or on the surface of the bacteria.

The *Mycobacterium tuberculosis* cell envelope is composed of a dense network of lipids including lipomannan (LM), phosphatidyl inositol mannoside (PIM), phosphoinositol lipoarabinomannan (PILAM), peptidoglycan (PG), and the 19 kDa lipoprotein, that have all been found to induce a proinflammatory response via binding to TLR2 (Gilleron M., 2003; Jo E. K., 2008; Jones B. W., 2001; Quesniaux V. J., 2004; Uehori J., 2003). Interestingly, one of the mce1 operon-encoded proteins is a lipoprotein (Mce1E), which may induce TLR2 signaling. While TLR4 has been shown to play a role
in the host response to *Mycobacterium tuberculosis* infection, no ligands have yet been found.

Fremond et al. demonstrated that TLR signaling is required for secretion of TNFα by macrophages and dendritic cells (Fremond C. M., 2004), and in Chapter 2 we showed that mouse bone marrow derived macrophages and dendritic cells produced dramatically different levels of TNFα in response to infection with wild-type *Mycobacterium tuberculosis* versus infection with ∆mce1. Since recognition of mycobacterial cell envelope lipids by TLR2 is such a key component of the early innate response to *Mycobacterium tuberculosis*, and hence of granuloma formation, we assessed whether the aberrant immune response to ∆mce1 is due to the accumulated free mycolic acids on ∆mce1 blocking the mycobacterial TLR2 ligands from binding to and activating TLR2 signaling.

RESULTS

**Mycolic Acids Diminish TLR Activation**

In order to test whether mycolic acids can block TLR signaling, bone marrow derived macrophages and dendritic cells were incubated with different concentrations of purified *Mycobacterium tuberculosis* mycolic acids for 30 minutes prior to stimulation with the TLR2 agonist Pam3CSK4 or the TLR4 agonist LPS. There was a significant reduction in TNFα production, by both macrophages and dendritic cells, in response to LPS stimulation when the cells were pre-incubated with 125ng/ml purified *Mycobacterium tuberculosis* mycolic acids (Figure 3-1). In macrophage, but not dendritic cells, secretion of TNFα was also reduced in response to the agonist Pam3CSK4 after
pre-incubation with mycolic acids. Mycolic acids alone did not stimulate TNFα production at any concentration. While earlier time points were assessed (data not shown), the impaired TNFα production was only demonstrated at 48 ours post infection for macrophages, and 72 hours post infection for dendritic cells.

**The Impaired Response to Δmce1 is Independent of TLR2**

To further address whether the aberrant immune response to Δmce1 is due to impaired TLR2 signaling, dendritic cells were derived from C57BL/6 wild-type or TLR2 deficient (TLR2 -/-) mouse bone marrow and infected with either wild-type (H37Rv) or the mce1 operon mutant strain of *Mycobacterium tuberculosis*. The rational is that if the impaired immune response to Δmce1 is due solely to the accumulated mycolic acids blocking mycobacterial ligands from binding to and activating TLR2 signaling, then a similar impaired immune response should be seen by TLR2 deficient cells in response to infection with H37Rv (Figure 3-2). In addition, if this hypothesis is correct, then the response of wild-type or TLR2 deficient cells to infection with Δmce1 should be identical.

At 24 hours post infection, a marked reduction in TNFα production was seen in response to H37Rv and Δmce1 infection from dendritic cells derived from TLR2 deficient mice, as compared to the levels from wild-type cells (Figure 3-3). These results demonstrate that Δmce1 does not appear to be impaired in its ability to activate TLR2 signaling. Stimulation with Pam3CSK4 was used as a control, which induced little to no TNFα production in TLR2 deficient dendritic cells. In contrast, LPS was used as a control for TLR4 signaling and was found to induce robust TNFα production, regardless
of the mouse background. Bone marrow derived macrophages were also used in these studies, but due to low cell counts after differentiation, the number of available macrophages was reduced by half. We attempted to complete these experiments using fewer cells, but the data were inconclusive (data not shown).

There is controversy over whether induction of dendritic cell maturation by *Mycobacterium tuberculosis* is dependent upon TLR signaling. However since our results from Chapter 2 demonstrated that dendritic cell maturation was impaired in response to Δ*mce1*, we wanted to examine whether this was potentially due to an inability of the mutant strain of bacteria to activate TLR2 signaling. We found that expression of the costimulatory molecule, CD40, and of the chemokine receptor, CCR7, on the surface of TLR2 deficient dendritic cells was slightly less than that for wild-type dendritic cells, in response to infection with H37Rv, Δ*mce1* or Pam3CSK4, whereas the absence of TLR2 appeared to have no effect on CD40 expression in response to the TLR4 agonist LPS (Figures 3-4 & 3-6). Expression of CD86 on the surface of TLR2 deficient dendritic cells was also impaired in response to Δ*mce1* and Pam3CSK4, but CD86 expression was relatively equal for both wild-type and TLR2 deficient dendritic cells in response to infection with H37Rv or LPS (Figure 3-5). According to the histograms of the uninfected cells (shaded regions), the background expression of the surface molecules by the wild-type dendritic cells appeared to be different from that of the TLR2 deficient cells, which could account for the minor differences seen between the two cell types in response to the various stimulants. In order to ensure that our interpretation of the histogram data was accurate, we attempted to calculate the attributable effect the absence of TLR2 had on the cellular response to infection with H37Rv, Δ*mce1*, Pam3CSK4 or LPS by subtracting out
the median fluorescence intensity of the uninfected cells from those of the stimulated cells, for each cell type (Figure 3-7). TLR2 deficient cells were indeed found to be impaired in their ability to induce expression of CD40 and CCR7 in response to H37Rv, Δmcel or Pam3CSk4, compared to the levels seen in wild-type cells. For CD86 expression, however, it appeared that the only significant difference between wild-type and TLR2 deficient cells was in response to the TLR2 agonist Pam3CSK4 that was used as a control. In response to infection with H37Rv, Δmcel or LPS, expression of CD86 by wild-type and TLR2 deficient dendritic cells was relatively equal.

**DISCUSSION**

In order for *Mycobacterium tuberculosis* to maintain a persistent infection in the host, a balance between immune activation and immune inhibition must be attained (Flynn J. L. and J. Chan, 2005). Thus, the mycobacterial cell envelope has evolved to contain a diverse set of immunostimulatory and immunoinhibitory lipids (Quesniaux V. J., 2004). Research from our laboratory has revealed a potential role for the mcel operon in modulating the immune response during *Mycobacterium tuberculosis* infection (Shimono N., 2003). We have found that expression of the mcel operon fluctuates during the course of infection, being highly expressed extracellularly and downregulated intracellularly (Casali N., 2006). In order to understand how this affects the host response, we compare the cellular responses to infection with wild-type bacteria (*mcel operon expressed*) versus infection with the *mcel operon mutant strain (*mcel operon is not expressed*).
Thus far we have determined that when the \textit{mce1} operon is not expressed (\textit{\Delta \textit{mce1}}) immune activation is impaired (Sequeira P., 2007; Shimono N., 2003; Uchida Y., 2007). We have also shown that the cell envelope of \textit{\Delta \textit{mce1}} contains an abundance of free mycolic acids that are not present in the cell envelope of the wild-type bacteria (Cantrell S., 2005). Research has demonstrated that the integrity of the mycobacterial cell envelope is critical for establishment of a persistent infection, and hence for granuloma formation (Rao V., 2005). This is due in part to the abundance of immunomodulatory lipids within the cell envelope. The majority of the immunostimulatory lipids have been shown to signal through TLR2 on macrophages and dendritic cells (Gilleron M., 2003; Jo E. K., 2008; Jones B. W., 2001; Quesniaux V. J., 2004; Uehori J., 2003).

A number of parallels can be seen in comparing mice infected with the \textit{mce1} operon mutant and TLR2 deficient mice infected with wild-type \textit{Mycobacterium tuberculosis}. In both situations the mice succumb to infection due to an inability to initiate an effective innate immune response (Drennan M. B., 2004; Shimono N., 2003). Therefore, we sought to determine whether the aberrant immune response to \textit{\Delta \textit{mce1}} was due to the accumulated free mycolic acids impairing TLR2 signaling.

We found that preincubation of mouse bone marrow derived macrophages and dendritic cells with \textit{Mycobacterium tuberculosis} mycolic acids reduced the ability of the TLR2 agonist, Pam3CSK4, and the TLR4 agonist, LPS, to induce TNF\textalpha{} (Figure 3-1). Interestingly, the diminished response only occurred in macrophages at 48 hours post infection, and by dendritic cells at 72 hours post infection. This leads us to believe that the mycolic acids are not blocking the receptors, as we would assume that this would have resulted in an earlier impaired response to the TLR agonists. Instead, the mycolic
acids could be actively downregulating expression of the TLRs, similar to what was shown by Nilsen et al. with RAW 264.7 macrophages incubated with an anti-TLR2 antibody (Nilsen N., 2004). They found that incubating the cells with the antibody for one hour does not affect the cellular response to the TLR2 ligand Pam3CSK4. However, after overnight treatment of cells with the anti-TLR2 antibody, a significant reduction in TNFα release is seen in response to stimulation with Pam3CSK4. In our experiments cells were incubated with mycolic acids for 30 minutes before stimulation with the TLR agonists, but the excess mycolic acids were not removed before addition of the TLR agonists. Therefore mycolic acids could have potentially competed with the TLR agonists over the course of the experiment. The fact that Nilsen et al. were able to see the reduced response to Pam3CSK4 at 24 hours as opposed to our results that did not show a diminished response until 48 and 72 hours after stimulation, could be due to differences in inhibitory activity of the anti-TLR2 antibody versus that of the mycolic acids. Another noteworthy observation from our data was that TNFα production by macrophages, but not dendritic cells, was impaired in response to Pam3CSK4 after incubation with mycolic acids. While this may be due to experimental error, it is also possible that dendritic cells express higher levels of TLR2 than macrophages, and thus the concentration of mycolic acids used was not sufficient to affect the TLR2 response. Further experiments need to be performed in order to determine the mechanism involved in mycolic acid dependent impairment of TLR activation.

Previous research from our laboratory demonstrated that TLR4, but not TLR2 mediated signaling is intact in the \textit{mce1} operon mutant, with only the wild-type strain of \textit{Mycobacterium tuberculosis} able to signal via TLR2 (Morici L., 2003). Additionally it
was shown that incubation of A549 epithelial cells with anti-TLR2 antibodies prior to infection with wild-type *Mycobacterium tuberculosis* results in a decreased cytokine and chemokine response that is similar to that seen with A549 cells infected with Δ*mce1* without prior incubation with antibodies (Sequeira P., 2007). Therefore, despite the fact that our experiments demonstrated that mycolic acids were able to diminish both TLR2 and TLR4 signaling, we decided to focus on impaired TLR2 activation as the main mechanism involved in the aberrant immune response to Δ*mce1*.

Chapter 2 demonstrated that expression of the *mce1* operon is critical for robust activation of the early innate immune response to *Mycobacterium tuberculosis* infection; recognition of *Mycobacterium tuberculosis* by TLR2 has been implicated as the predominant mechanism in activating the early innate immune response (Abel B., 2002; Drennan M. B., 2004). Using dendritic cells derived from TLR2 deficient mouse bone marrow, we demonstrated that TNFα production in response to infection with H37Rv and Δ*mce1* was impaired in the absence of TLR2 (Figure 3-3). While our rationale for these experiments and previous conclusions from our laboratory stated that if the proinflammatory response to H37Rv was impaired in the absence of TLR2 to the same degree as the response to infection with Δ*mce1* in the presence of TLR2, then we could conclude that the aberrant immune response to Δ*mce1* was due to impaired TLR2 activation. However, when we compared the TNFα response of wild-type and TLR2 deficient cells after infection with Δ*mce1*, we see that TLR2 deficient cells are further reduced in their ability to secrete TNFα (Figure 3-3), suggesting that the aberrant immune response to Δ*mce1* is not solely due to an inability to activate TLR2 signaling, but that additional mechanisms of immune inhibition must be occurring. This contradicts
the previous findings from our laboratory, but in those experiments the human kidney epithelial cell line, Hek293 was used (Morici L., 2003). Here we used primary mouse bone marrow derived dendritic cells that are likely to provide a more accurate picture of the in vivo response to infection.

As addressed in Chapter 1, there is conflicting evidence in the literature as to whether expression of the costimulatory molecules CD40 and CD86 during dendritic cell activation is dependent upon TLR activation. Some argue that their expression is MyD88 independent (Fremond C. M., 2007; Fremond C. M., 2004; Jang S., 2004; Schnare M., 2001), while others argue in favor of their expression being TLR2 and/or TLR9 dependent (Bafica A., 2005; Jang S., 2004; Pompei L., 2007). The data presented here are similar to our results with TNFα, in that expression of CD40 and of CCR7 by TLR2 deficient cells was reduced in response to infection with H37Rv or Δmce1 (Figure 3-4), as compared to infection of wild-type cells. Therefore, dendritic cell expression of CD40 and CCR7 appeared to be TLR2 dependent. Additionally, while Δmce1 has a reduced ability to upregulate expression of CD40 and CCR7 on wild-type cells, as compared to infection with H37Rv, this is not exclusively due to impaired TLR2 activation.

With regards to our results with dendritic cell expression of CD86, we saw no difference in expression between wild-type and TLR2 deficient cells in response to H37Rv or Δmce1. However as discussed in Chapter 2, comparison of CD86 expression in response to H37Rv versus Δmce1 infection showed a reduced level of expression from dendritic cells infected with Δmce1 (Figure 3-5). It is unclear why CD40 expression is TLR2 dependent while CD86 expression appears to be TLR2 independent, especially considering expression of both costimulatory molecules has been shown to be regulated
by the common transcription factor, NFκB (Benveniste E. N., 2004; Yoshimura S., 2001). As discussed in Chapter 2, our only assumption is that this discrepancy is due to experimental error.

In conclusion, we have shown that while purified *Mycobacterium tuberculosis* mycolic acids are able to diminish TLR2 and TLR4 activation by the agonists Pam3CSK4 and LPS, respectively, the impaired immune response to Δ*mce1* does not appear to be due to the accumulated mycolic acids blocking mycobacterial TLR2 ligands from binding to and activating TLR2 signaling. However, because the cell envelope lipid composition of the wild-type and *mce1* operon mutant bacteria are so different, we believe that a relationship exists between the inability of Δ*mce1* to induce a robust immune response and its altered lipid profile. The next chapter will address whether the accumulated free mycolic acids on Δ*mce1* are directly or indirectly inhibiting immune activation, independent of TLR2 signaling.
MATERIALS AND METHODS

Growth and Maintenance of Mycobacterial Strains

Mycobacterial strains were grown in Middlebrook 7H9 broth (Difco, MD) containing 10% ADC and 0.2% glycerol (Fisher Scientific, NJ). Strains were grown to mid-log phase and then passaged or pelleted for further processing.

Differentiation of Mouse Bone Marrow Cells

Wild-type or TLR2 deficient (TLR2-/-) C57BL/6 mice were anesthetized with isoflurane (Phoenix Pharmaceutical, MO) for 1-2 minutes. Femurs and tibias were removed from each mouse, after spinal dislocation. Cells were extracted from the bone marrow with a mortar and pestle and transferred to a 15 ml conical tube (VWR) in RPMI complete media (RPMI 1640 + L-glutamine, 10% FBS, 1% sodium pyruvate, 1% HEPES Buffer, 0.1% β-mercaptoethanol). Cells were pelleted at 1200 rpm for 5 minutes at 23°C. Red blood cells were lysed by resuspending cells in 1 ml ACK lysing buffer for 1-2 minutes. Lysis was stopped with 10 ml RPMI complete media. Cells were pelleted again and resuspended in 10 ml RPMI complete media. Cell debris was removed by straining the suspension through a 40 um cell strainer. Cells were then washed 3 times in RPMI complete media. Cells were resuspended in 10 ml RPMI complete media and counted with a hemocytometer.

Macrophages

Cells were cultured in RPMI complete media supplemented with M-CSF (30%) and Penicillin/Streptomycin (1:100). Cells were plated at 5 x 10^6 cells per 15 ml of RPMI
complete media in 25 cm non-tissue culture treated Petri dishes (VWR) in a 5% CO₂ humidified incubator at 37°C. On day 4 of incubation, all media was removed and replaced with 15 ml of fresh RPMI complete media supplemented only with M-CSF. Macrophages were differentiated by day 6.

**Dendritic Cells**

Cells were cultured in RPMI complete media supplemented with GM-CSF (1:50) and Penicillin/Streptomycin (1:100). Cells were plated at 7 x 10⁵ cells/ml in 24-well non-tissue culture treated plates (BD Falcon, CA) in a 5% CO₂ humidified incubator at 37°C. On day 2 and day 4 of incubation, 500 ul of media from each well was removed and replaced with 500 ul fresh RPMI complete media supplemented only with GM-CSF. Dendritic cells were differentiated by day 6.

**In Vitro Infections**

On day 6 of incubation, macrophages and dendritic cells were removed from the plates by pipetting, followed by addition of cold PBS for 5 minutes to remove adherent cells. Cells were pelleted at 1200 rpm for 5 minutes and resuspended in 10 ml RPMI complete media. Each cell type was then counted with a hemocytometer and plated, in triplicate, at 1 x 10⁶ cells/ml in 24-well tissue culture treated plates.

Mid-log phase H37Rv or Δmce1 strains of *Mycobacterium tuberculosis* were pelleted at 500 rpm for 5 minutes to remove large clumps of bacteria. The supernatant, containing single cell suspension bacteria, was removed and pelleted at 3500 rpm for 5 minutes. The bacteria were washed two times in 0.05% PBS-Tween 80. The bacteria
were resuspended in 6 ml of 0.05% PBS-Tween 80 and an OD$_{600}$ was performed to equilibrate all strains. Serial dilutions of bacteria were plated on Middlebrook 7H11 agar (Difco, MD) plates containing 10% OADC and 100 ug/ml cycloheximide (VWR). Colony forming units (CFU) for each strain were determined to confirm multiplicity of infection (MOI).

Cells were then infected with *Mycobacterium tuberculosis* at an MOI of 5 to 7. LPS (1ug/ml) (Sigma, MO) and Pam3CSK4 (1ug/ml) (InvivoGen, CA) were used as positive controls to stimulate the cells, and PBS-Tween 80 infected cells served as a negative control for each experiment.

Dendritic cells used for surface molecule expression experiments were not removed from the plates on day 6 of incubation, but instead were left untouched so as to eliminate potential activation from over pipetting. Instead, 800 ul of old media was removed from each well and replaced with 800 ul of fresh RPMI complete media. Cells were infected with strains of *Mycobacterium tuberculosis* as above.

**Mycolic Acid Blocking**

Cells were prepared as above. Prior to infection with LPS or Pam3CSK4, cells were incubated with 125 ng/ml, 50 ng/ml or 20 ng/ml *Mycobacterium tuberculosis* mycolic acids in DMEM (Sigma, MO) for 30 minutes. LPS and Pam3CSK4 alone were used as positive controls. Mycolic acids in DMEM, DMEM alone, or uninfected cells served as negative controls.

**Enzyme-linked Immunosorbent Assay (ELISA)**
At 4, 24, 48 and 72 hours post infection, supernatants were removed from each well and filtered through a 0.2 um filter (Fisher). Supernatants were analyzed for cytokine and chemokine production with ELISA kits (eBioscience, CA). Supernatants were stored at -80°C.

**Flow Cytometry**

At 24 hours post infection, dendritic cells were removed from the wells. Cold PBS (1 ml) was added to each well and incubated for 5 minutes to remove adherent cells. All cells were transferred to 1.5 ml tubes. Cells were pelleted at 1200 rpm for 5 minutes and resuspended in 25 ul of 2% FBS in PBS to block cells from any nonspecific binding. Following a 30 minute incubation at 4°C, another 25 ul of 2% FBS in PBS containing a combination of the fluorescent antibodies (eBioscience, CA): CD40-PE, CD86-PECy5, CD11c-PECy7 or CCR7-PE, CD11c-PECy7, was added. Cells were incubated at 4°C (CD40/CD86/CD11c) or 37°C (CCR7/CD11c), for 30 minutes. Cells were washed in PBS and then fixed overnight at 4°C in 4% paraformaldehyde for removal from the BSL3 laboratory. Cells were pelleted and resuspended in 500 ml PBS and run on an FC-500 flow cytometer. Data were analyzed by Flow-Jo. CD11c was used as a marker for dendritic cells.

**Statistics**

Comparison of mean values was assessed by the Student’s *t* test. Differences were considered significant at *p* ≤ 0.05.
Figure 3-1: TNFα production by bone marrow derived macrophages (A) and dendritic cells (B) 48 hours and 72 hours, respectively, after exposure to Pam3CSK4 or LPS. 30 minutes prior to stimulation, cells were incubated with or without 125 ng/ml, 50 ng/ml or 20 ng/ml of purified Mycobacterium tuberculosis mycolic acids. Data are representative of three independent experiments. p≤ 0.05 (*).
Figure 3-2: Schematic rational for using TLR2 deficient cells (TLR2-/-) in comparison to wild-type (WT) cells to test whether the impaired response to ∆mce1 is due solely to the accumulated mycolic acids blocking mycobacterial TLR2 ligands.
Figure 3-3: TNFα production from wild-type (grey) or TLR2 deficient (diagonal stripe) mouse bone marrow derived dendritic cells 24 hours after infection with H37Rv, Δmce1, Pam3CSK4, LPS, or left uninfected. Data are representative of three independent experiments.
Figure 3-4: Surface expression of CD40 by C57BL/6 wild-type or TLR2 deficient (TLR2-/-) bone marrow derived dendritic cells 24 hours after infection with H37Rv, Δmce1, Pam3CSK4 or LPS (open regions) as compared to uninfected controls (shaded regions). Histograms show data from two independent experiments.
Figure 3-5: Surface expression of CD86 by C57BL/6 wild-type or TLR2 deficient (TLR2-/-) bone marrow derived dendritic cells 24 hours after infection with H37Rv, Δmce1, Pam3CSK4 or LPS (open regions) as compared to uninfected controls (shaded regions). Histograms show data from two independent experiments.
Figure 3-6: Surface expression of CCR7 by C57BL/6 wild-type or TLR2 deficient (TLR2-/-) bone marrow derived dendritic cells 24 hours after infection with H37Rv, Δmce1, Pam3CSK4 or LPS (open regions) as compared to uninfected controls (shaded regions). Histograms show data from two independent experiments.
Figure 3-7: Median fluorescence intensity (MFI) after subtracting out uninfected control background values for CD40, CD86 and CCR7 surface expression by wild-type (grey) or TLR2 deficient (diagonal stripe) bone marrow derived dendritic cells 24 hours after infection with H37Rv, Δmce1, Pam3CSK4 or LPS. Data are representative of two independent experiments. p≤ 0.05 (*).
REFERENCES


Chapter 4

The excess surface lipids of $\Delta mce1$ do not directly inhibit innate immune activation.
INTRODUCTION

*Mycobacterium tuberculosis* is structurally distinguished from gram positive and gram negative bacteria based on its unique cell envelope containing a dense hydrophobic lipid layer (Glickman M. S. and W. R. Jacobs, Jr., 2001). This lipid layer accounts for many of its intrinsic properties, such as its ability to form cords, survive in a variety of harsh environments and resist antibiotic treatment (Barry C. E., 3rd, 2001). In addition, the cell envelope contributes significantly to its pathogenesis. Differential immune responses to various clinical isolates of *Mycobacterium tuberculosis* have been attributed to unique lipid profiles (Manca C., 1999; Reed M. B., 2004). In particular, phenolic glycolipid (PGL) produced by the W-Beijing family of *Mycobacterium tuberculosis* is responsible for the strains’ hypervirulence, due to its ability to inhibit the innate immune response (Reed M. B., 2004).

Mycolic acids are the most predominant lipid within the cell envelope and are present as individual molecules covalently linked to the cell wall or noncovalently attached in the form of trehalose dimycolate (TDM) (Barry C. E., 3rd, 2001; Bhowruth V., 2008; Glickman M. S., 2001; Noll H., 1956). *Mycobacterium tuberculosis* mycolic acids and TDM have both been shown to induce an inflammatory response in mice leading to granuloma formation (Geisel R. E., 2005; Korf J., 2005; Perez R. L., 2000). However, alterations in the distribution of the three types of mycolic acids (α, methoxy and keto) within the cell envelope has been shown to modify the immune response to infection (Dao D. N., 2008; Rao V., 2005; Rao V., 2006). Of particular interest is a mutant strain of *Mycobacterium tuberculosis* that is impaired in the pcaA gene, encoding a methyltransferase that catalyzes the proximal cis-cyclopropanation of α-mycolate.
(Glickman M. S., 2000). A number of similarities can be found in comparing the outcome of infection with the *pcaA* mutant and our *mce1* operon mutant. Both mutants are impaired in their ability to induce an early innate immune response resulting in aberrant granuloma formation (Rao V., 2005; Shimono N., 2003). In addition, the distribution of mycolic acid subclasses within the surface lipid extracts of both mutants is altered, with a reduction in the number of α- mycolates (Cantrell S., 2005; Rao V., 2005). Using a petroleum ether extraction method, Rao et al. demonstrated that the surface lipid extracts from the *pcaA* mutant strain are directly responsible for the hypoinflammatory response.

In Chapter 3 we demonstrated that the impaired immune response to infection with Δmce1 is not due to the excess mycolic acids blocking mycobacterial TLR2 ligands. The mycolic acids used in the experiment were commercially obtained. Since the relative proportion of mycolic acid class (α, keto-, and methoxy-) can vary according to its source, in this chapter, we used techniques similar to Rao et al. in an attempt to determine if the excess mycolic acids within the surface lipid extracts of Δmce1 are directly inhibiting the innate immune response.

**RESULTS**

**Mycobacterial Delipidation**

To determine if the excess mycolic acids found in the surface lipid extracts of Δmce1 are directly involved in preventing immune activation during infection we compared the response of bone marrow derived macrophages and dendritic cells to infection with delipidated H37Rv or Δmce1. Bacteria were delipidated using a petroleum
ether extraction method that has been shown to remove *Mycobacterium tuberculosis* cell envelope surface lipids while retaining the viability of the bacteria (Bloch H., 1950; Indrigo J., 2002; Silva C. L., 1985). Macrophages and dendritic cells were infected with native or delipidated H37Rv or Δ*mce1*. No statistically significant differences were seen in the production of TNFα by either cell type in response to infection with native H37Rv or Δ*mce1* as compared to infection with delipidated H37Rv or Δ*mce1* (Figure 4-1). However, TNFα production by both cell types in response to infection with Δ*mce1* was significantly lower than the response to infection with H37Rv, regardless of the presence or absence of the surface lipids.

**Cellular Response to Extractable Lipids**

It is possible that the macrophages and dendritic cells did not demonstrate any alterations in their response to the delipidated bacteria as compared to infection with the native bacteria because the delipidation process failed. However, since the bacteria remain viable after delipidation it is also possible that during the 24 hour incubation period newly generated lipids were able to alter the immune response. To eliminate these potential issues and to analyze the direct contribution of the extractable lipids to the Δ*mce1* phenotype, we exposed bone marrow derived macrophages and dendritic cells to the extracted lipids from H37Rv or Δ*mce1*. We found that TNFα production by macrophages and dendritic cells was similar in response to the lipids from H37Rv and to the extracted lipids from Δ*mce1* (Figure 4-2). To determine if the TNFα response to the extracted lipids is dependent upon TLR2 signaling, we compared the response of wild-type cells and TLR2 deficient cells to the extracted lipids. TLR2 deficient macrophages
responded similarly to wild-type cells, but TLR2 deficient dendritic cells appeared to produce reduced levels of TNFα in response to H37Rv and Δmce1 lipids. To confirm that the reagents used in the lipid extraction process were not causing any background effects, we also exposed the cells to a mixture of petroleum ether, chloroform and methanol, or to isopropanol. There was no significant response to either of these solutions.

**DISCUSSION**

Establishment of a persistent infection by *Mycobacterium tuberculosis* requires the bacteria to survive a diverse set of conditions encountered during infection, including surviving the acidic environment of the macrophage phagosome and the nutrient poor environment of the granuloma (Mehrotra J. and W. R. Bishai, 2001). In order to adapt to these changing environments, *Mycobacterium tuberculosis* must alter its gene expression in response to environmental signals. Analysis of the transcriptional response of *Mycobacterium tuberculosis* within murine macrophages demonstrates that bacteria induce expression of genes involved in fatty acid biosynthesis and mycolic acid modification for use as carbon and energy sources and for repair of the cell envelope, respectively (Schnappinger D., 2003). These findings are similar to the expression profiles of bacteria isolated from human lungs of patients suffering from extensive tuberculosis lung disease (Chen E. S. and D. R. Moller, 2007). Using DNA microarray, the investigators were able to determine that twice the proportion of lipid biosynthesis genes and a series of fatty and mycolic acid modification genes are upregulated *in vivo* within the granuloma, pericavity, and distant lung, as compared to the gene expression of
bacteria grown in vitro. They propose that *Mycobacterium tuberculosis* modifies the lipid and cell wall components in order to evade the host immune response.

Expression of the *mcel* operon is under the control of the negative transcriptional regulator, *mcelR* (Casali N., 2006). In contrast to the hypoimmune response to infection with the *mcel* operon mutant, mice infected with an *mcelR* mutant, in which the *mcel* operon is overexpressed, die sooner than those infected with wild-type H37Rv due to an overabundance of inflammation in the lungs (Uchida Y., 2007). It has been demonstrated that expression of the *mcel* operon fluctuates during the course of infection, and appears to be most important when *Mycobacterium tuberculosis* is extracellular, as demonstrated by a rapid repression of *fadD5*, *mcelA* and *mcelF* expression inside RAW macrophages (Casali N., 2006). Our studies with the *mcel* operon mutant strain are an attempt to decipher the role of the *mcel* operon in this downregulated intracellular state.

Incubation of mycobacteria with petroleum ether removes the free lipids found in the outermost region of the cell envelope (Brennan P. J., 1989). The predominant lipids in these extracts are TDM with small quantities of free mycolic acids (Indrigo J., 2002; Silva C. L., 1985). Previous results from our laboratory show that an excess number of mycolic acids are readily removed from the *mcel* operon mutant strain of *Mycobacterium tuberculosis*, that are not present in the lipid extracts of wild-type H37Rv bacteria (Cantrell S., 2005). We hypothesize that this altered lipid profile of ∆*mcel* allows *Mycobacterium tuberculosis* to survive the harsh intracellular environment during infection, contributing to the hypervirulent phenotype we demonstrated previously in a mouse model of infection (Shimono N., 2003).
In order to determine if the free mycolic acids within the lipid extracts of Δmce1 are directly inhibiting the immune response during infection, we analyzed the response of bone marrow derived macrophages and dendritic cells to infection with delipidated bacteria or after exposure to extracted bacterial lipids. The data presented here demonstrate that delipidation of Δmce1 does not restore the cytokine response of infected macrophages and dendritic cells to that of wild-type H37Rv infected cells, suggesting that the excess mycolic acids present in the surface lipid extracts of Δmce1 are not directly inhibiting the immune response (Figure 4-1). However, Rao et al. did not show any difference in TNFα production in response to infection with native or delipidated bacteria deficient in the pcaA gene (Rao V., 2005). They were only able to demonstrate that the pcaA mutant-extractable lipids directly mediate the hypoinflammatory phenotype through lipid transfer experiments. We attempted to repeat these lipid transfer experiments with our mce1 operon mutant, but we were not successful. However, since we did not see a decrease in TNFα production by cells infected with wild-type H37Rv after treatment with petroleum ether, such as was seen by Rao et al. and Indrigo et al., it is likely that our delipidation efforts failed, and thus the data are inconclusive (Indrigo J., 2002; Rao V., 2005).

Despite this, we know that we were successful in extracting some mycobacterial lipid components since thin-layer chromatography (TLC) analysis of the lipid extracts did show additional lipids in the Δmce1 extracts that were not present in the extracts from wild-type H37Rv bacteria (Figure 4-3), as shown previously (Cantrell S., 2005). However, no difference was seen in the TNFα response to the extracted lipids from wild-type H37Rv as compared to the extracted lipids from Δmce1 (Figure 4-2). This
is in agreement with studies done previously in our laboratory, where polar and apolar fractions of mycobacteria were tested for their ability to induce RAW macrophage cytokine production (Cantrell S., 2005). We chose to repeat the assay here because the previous study had added lipids directly to the cell media, which has been found to be insufficient for inducing any detectible responses (Rao V., 2005). In the present experiments, lipids were coated onto the surface of tissue culture plates, followed by the addition of macrophages or dendritic cells. The similar response to wild-type H37Rv and Δmcel extracted lipids was true for both wild-type and TLR2 deficient macrophages and dendritic cells, further demonstrating that the lipid extracts from Δmcel are not directly responsible for the hypoinflammatory response during infection.

While we showed in Chapter 3 that the impaired immune response to Δmcel is not an indirect effect of TLR2 ligand blocking by the excess mycolic acids, it is still possible that this altered lipid profile is disrupting the cell envelope architecture in such a way as to block other innate immune signaling pathways. As described previously, the integrity of the cell envelope is imperative to mycobacterial virulence (Gao L. Y., 2003; Liu J., 1996). Rao et al. demonstrated that alterations in the structure of mycolic acids, in particular those that make up TDM, can impair the immune response to Mycobacterium tuberculosis infection (Rao V., 2005). Since TDM induces a granulomatous response in a manner that is TLR2 and TLR4 independent, but MyD88 dependent, future studies looking at MyD88 activation during infection with Δmcel will help to unravel the mechanism involved in the impaired immune response to the mcel operon mutant.
MATERIALS AND METHODS

Growth and Maintenance of Mycobacterial Strains

Mycobacterial strains were grown in Middlebrook 7H9 broth (Difco, MD) containing 10% ADC and 0.2% glycerol (Fisher Scientific, NJ). Strains were grown to mid-log phase and then passaged or pelleted for further processing.

Differentiation of Mouse Bone Marrow Cells

Wild-type or TLR2 deficient (TLR2-/-) C57BL/6 mice were anesthetized with isoflurane (Phoenix Pharmaceutical, MO) for 1-2 minutes. Femurs and tibias were removed from each mouse, after spinal dislocation. Cells were extracted from the bone marrow with a mortar and pestle and transferred to a 15 ml conical tube (VWR) in RPMI complete media (RPMI 1640 + L-glutamine, 10% FBS, 1% sodium pyruvate, 1% HEPES Buffer, 0.1% β-mercaptoethanol). Cells were pelleted at 1200 rpm for 5 minutes at 23°C. Red blood cells were lysed by resuspending cells in 1 ml ACK lysing buffer for 1-2 minutes. Lysis was stopped with 10 ml RPMI complete media. Cells were pelleted again and resuspended in 10 ml RPMI complete media. Cell debris was removed by straining the suspension through a 40 um cell strainer. Cells were then washed 3 times in RPMI complete media. Cells were resuspended in 10 ml RPMI complete media and counted with a hemocytometer.

Macrophages

Cells were cultured in RPMI complete media supplemented with M-CSF (30%) and Penicillin/Streptomycin (1:100). Cells were plated at 5 x 10^6 cells per 15 ml of RPMI
complete media in 25 cm non-tissue culture treated Petri dishes (VWR) in a 5% CO₂ humidified incubator at 37°C. On day 4 of incubation, all media was removed and replaced with 15 ml of fresh RPMI complete media supplemented only with M-CSF. Macrophages were differentiated by day 6.

**Dendritic Cells**

Cells were cultured in RPMI complete media supplemented with GM-CSF (1:50) and Penicillin/Streptomycin (1:100). Cells were plated at 7 x 10⁵ cells/ml in 24-well non-tissue culture treated plates (BD Falcon, CA) in a 5% CO₂ humidified incubator at 37°C. On day 2 and day 4 of incubation, 500 ul of media from each well was removed and replaced with 500 ul fresh RPMI complete media supplemented only with GM-CSF. Dendritic cells were differentiated by day 6.

**Surface Lipid Extraction**

Mid-log phase H37Rv or Δmce1 strains of *Mycobacterium tuberculosis* were pelleted at 500 rpm for 5 minutes to remove large clumps of bacteria. The supernatant, containing single cell suspension bacteria, was removed and pelleted at 3500 rpm for 5 minutes. The bacteria were washed two times in 0.05% PBS-Tween 80. The bacteria were resuspended in 6 ml of 0.05% PBS-Tween 80 and an OD₆₀₀ was performed to equilibrate all strains. Serial dilutions of native and delipidated bacteria were plated on Middlebrook 7H11 agar (Difco, MD) plates containing 10% OADC and 100 ug/ml cycloheximide (VWR). Colony forming units (CFU) for each strain were determined to confirm multiplicity of infection (MOI).
The bacterial suspension was divided: 1 ml of bacteria was saved as “native bacteria” and the remaining 5 ml of bacteria was processed for delipidation. Bacteria were delipidated using a previously published petroleum ether extraction method (Indrigo J., 2002). Bacteria were transferred to a glass vial (Fisher, NJ) and pelleted at 3500 rpm for 5 minutes. The pellet was resuspended in 2 ml of petroleum ether, vortexed for 2 minutes, incubated at room temperature for 5 minutes, and centrifuged at 3500 rpm for 5 minutes. The upper petroleum ether layer was removed and transferred to a new glass vial. The delipidation process was repeated and all extracts were combined. To remove the lipid extracts from the Biosafety Level 3 (BSL3) Laboratory, 8 ml of chloroform:methanol (2:1) was added to the 4 ml of extracted lipids. Reagents without bacteria (petroleum ether and chloroform:methanol) were used as a control for the delipidation process. The delipidated bacteria were washed two times in 0.05% PBS-Tween 80 then resuspended in 3 ml 0.05% PBS-Tween 80. An OD$_{600}$ was performed to equilibrate native and delipidated bacteria from all strains.

In Vitro Infections

On day 6 of incubation, macrophages and dendritic cells were removed from the plates by pipetting, followed by addition of cold PBS for 5 minutes to remove adherent cells. Cells were pelleted at 1200 rpm for 5 minutes and resuspended in 10 ml RPMI complete media. Each cell type was then counted with a hemocytometer and plated, in triplicate, at 1 x 10$^6$ cells/ml in 24-well tissue culture treated plates.

Cells were infected with native or delipidated *Mycobacterium tuberculosis* at an MOI of 5 to 7. LPS (1ug/ml) (Sigma, MO) and Pam3CSK4 (1ug/ml) (InvivoGen, CA)
were used as positive controls to stimulate the cells, and PBS-Tween 80 infected cells served as a negative control for each experiment.

Dendritic cells used for surface molecule expression experiments were not removed from the plates on day 6 of incubation, but instead were left untouched so as to eliminate potential activation from over pipetting. Instead, 800 ul of old media was removed from each well and replaced with 800 ul of fresh RPMI complete media. Cells were infected with strains of *Mycobacterium tuberculosis* as above.

**Exposure to Extracted Lipids**

This procedure was adapted from Rao et al (Rao, 2006). The solutions of extracted lipids from wild-type and Δ*mce1*, and “reagents only” controls were each divided into two glass vials (one for TLC and the other for use in the cellular exposure assays) and evaporated under nitrogen. For the cellular exposure assays, the lipids were resuspended in 1 ml of isopropanol, sonicated for 5 minutes in a bath sonicator, incubated at 60°C for 10 minutes, and sonicated again for 5 minutes. The lipids in solution were layered into 24-well tissue culture plates, at 60 ul per well, and allowed to evaporate overnight. Control wells were layered with isopropanol only, or left uncoated. Cells were exposed to the coated wells at a concentration of 1 × 10⁶ cells in 500 ul of RPMI complete media and incubated at 37°C.

**Thin-layer Chromatography**

Extracted lipids and “Reagents only” controls were processed as above. For TLC, the lipids were resuspended in 100 ul of chloroform. Lipids were spotted 1 cm from the
bottom and 0.5 cm from the left edge of a 5 x 7.5 cm Silicon gel 60 F254 aluminum TLC plate (EMD, NJ) using 5 ul Accupette Pipets (Dade Diagnostics, FL). Chloroform:methanol:water (100:14.0:0.8) v/v was added to the TLC chamber and equilibrated with filter paper. One direction TLC was performed.

**Enzyme-linked Immunosorbent Assay (ELISA)**

At 24 and 48 hours post infection, supernatants were removed from each well and filtered through a 0.2 um filter (Fisher). Supernatants were analyzed for cytokine and chemokine production with ELISA kits (eBioscience, CA). Supernatants were stored at -80°C.

**Statistics**

Comparison of mean values was assessed by the Student’s $t$ test. Differences were considered significant at $p \leq 0.05$. 
Figure 4-1: TNFα production by mouse bone marrow derived macrophages (A) and dendritic cells (B) 24 (black) or 48 (horizontal strip) hours after exposure to H37Rv or Δmce1 native or delipidated bacteria. Reagents for lipid extraction (petroleum ether and chloroform:methanol), isopropanol, or uncoated wells were used as controls. Data are representative of three independent experiments.
Figure 4-2: TNFα production by wild-type (grey) or TLR2 deficient (diagonal stripe) mouse bone marrow derived macrophages (A) and dendritic cells (B) 24 hours after exposure to H37Rv or Δmce1 surface lipid extracts. Reagents for lipid extraction (petroleum ether and chloroform:methanol), isopropanol, or uncoated wells were used as controls. Data are representative of three independent experiments.
Figure 4-3: Thin-layer chromatography of petroleum ether extracts from wild-type H37Rv (Rv) or the mcel operon mutant strain (KO) of *Mycobacterium tuberculosis*. Reagents used for delipidation and bacterial killing (Reagents) were used as a control.
REFERENCES


Chapter 5

Conclusions
DISSERTATION CONCLUSIONS

In 1882 Robert Koch identified *Mycobacterium tuberculosis* as the bacterium responsible for causing tuberculosis. To date we are still battling with this worldwide scourge and struggling to unravel the mechanisms it employs in establishing long-term persistence within its host. Key to its success as a pathogen is its ability to interact with and modulate the host immune response. A better understanding of this relationship will facilitate the development of an effective cure for this disease.

The genome of *Mycobacterium tuberculosis* contains four *mce* operons. The exact function of these operons has yet to be identified, but ongoing research is providing strong evidence that they all function as lipid importers, with each operon potentially transporting a unique lipid. Our laboratory has shown that expression of the *mce1* operon is important for *Mycobacterium tuberculosis* to establish a persistent infection in mice. A *mce1* operon mutant strain of *Mycobacterium tuberculosis* is hypervirulent in mice due to an impaired host immune response. This dissertation elaborates these findings by showing that expression of the *mce1* operon is critical for inducing the early innate immune response to infection, a critical step in establishing the foundation of the granuloma. We also attempted to reveal the mechanism by which the *mce1* operon mutant is impaired in its ability to induce the innate immune response. Rather then identifying the precise method, we were able to eliminate two possibilities, thus moving us closer to uncovering the role of the *mce1* operon in *Mycobacterium tuberculosis* pathogenesis.

The Role of the *mce1* operon in Activating Innate Immunity
Previous research from our laboratory revealed that RAW macrophage and A549 epithelial cell lines are impaired in their ability to mount a robust cytokine and chemokine response to in vitro infection with the mce1 operon mutant. In addition to the important roles these two cell types play during the early stages of infection with *Mycobacterium tuberculosis*, we have discussed in detail the significance of dendritic cells in inducing a granulomatous response, in particular their role in linking the innate and adaptive immune responses. Therefore, a major aim of this dissertation was to determine if the inability of Δmce1 infected mice to form proper granulomas in the lungs was due to a failure of Δmce1 to activate dendritic cell maturation. Due to the complimentary roles macrophages and dendritic cells play during the early innate response to *Mycobacterium tuberculosis*, we also compared the response of these two cell types to infection with the wild-type strain of *Mycobacterium tuberculosis* and with the mce1 operon mutant. In Chapter 1 we demonstrated that mouse bone marrow derived macrophage and dendritic cell activation/maturation was impaired in response to infection with Δmce1, providing further evidence that expression of the mce1 operon is critical for induction of a robust early innate response to infection with *Mycobacterium tuberculosis*. However, we also found that expression of the mce1 operon did not appear to be essential for inducing expression of CCR7 by dendritic cells, indicating that the migration potential of dendritic cells is not impaired in response to the mce1 operon mutant. As discussed, expression of CCR7 alone does not directly correlate with the migratory ability of dendritic cells, therefore, transwell migration assays need to be performed in order to conclusively determine whether Δmce1 infected dendritic cells can actively respond to chemokine gradients. In addition, the evidence in favor of Δmce1
being unable to activate dendritic cell maturation could be strengthened by investigating the ability of \( \Delta mce1 \) infected dendritic cells to induce a Th1 response.

**TLR2 Blocking**

The surface lipid extracts of \( \Delta mce1 \) were shown to contain an accumulation of free mycolic acids that were not present in the surface lipid extracts of wild-type *Mycobacterium tuberculosis*. Since it remains unclear whether these excess mycolic acids are within the cell envelope or on the surface of the bacteria, we hypothesized that they could be forming a barrier on the surface of the mutant bacteria that block mycobacterial TLR ligands and thus prevent the innate immune cells from mounting an immune response to infection with \( \Delta mce1 \). Due to the predominance of TLR2 ligands within the cell envelope of *Mycobacterium tuberculosis*, and their significance in activating an early innate immune response, we chose to focus on whether TLR2 signaling was impaired in response to infection with \( \Delta mce1 \). While we were able to show that preincubation of macrophages and dendritic cells with purified *Mycobacterium tuberculosis* mycolic acids was able to reduce the cytokine response to stimulation with TLR2 and TLR4 agonists, our data show that the aberrant immune response to \( \Delta mce1 \) is not solely due to an inability to activate TLR2 signaling.

**Direct Inhibition by Mycolic Acids**

Since we found that the accumulated mycolic acids in the surface lipid extracts of \( \Delta mce1 \) were not blocking TLR2 signaling, the final aim of this dissertation was to determine if the accumulated mycolic acids were directly inhibiting the innate immune
response. We compared the cytokine response of macrophages and dendritic cells to infection with native and delipidated bacteria as well as to stimulation with extracted bacterial surface lipids. Our results did not demonstrate a role for the Δmce1 surface lipids in directly inhibiting the immune response during infection. However, due to the difficulty of these experiments it is possible that this was due to experimental error, and thus the data remain inconclusive.

**Working Model**

Foreman-Wykert and Miller coined the term “anti-virulence genes” to describe genes that increase pathogen virulence when disrupted, such as seen in mice infected with the mce1 operon and mce1R mutants (Foreman-Wykert A. K. and J. F. Miller, 2003). They state that less virulent organisms may favor host survival and that a selective advantage in one environment could be balanced by a deficit in another environment. This coincides with the concept presented throughout this dissertation that establishment of a long term mycobacterial infection requires a balance of immune activation and immune inhibition. Thus, we propose a model in which expression of the mce1 operon modulates the host immune response for *Mycobacterium tuberculosis* persistence. In the intracellular environment expression of the mce1 operon is downregulated, which results in the accumulation of mycolic acids within the cell envelope. While we have shown that these excess lipids do not block TLR2 signaling nor do they appear to directly inhibit the immune response, it is possible that a buildup of these lipids within the cell envelope disrupts the cell envelope architecture in such a way as to block other innate immune signaling pathways, and thus protects the bacteria from the harsh intracellular
environment. This is balanced with upregulated expression of the mce1 operon extracellularly, which allows the extracellular bacteria to import lipids for energy metabolism. However, due to the loss of the protective lipid layer, the extracellular bacteria induce a robust early innate immune response for granuloma development. This allows the host to contain the bacteria, and thus favors host survival, but also induces migration of an ample supply of cells in which bacteria can replicate. This idea is supported by a study using transparent zebrafish embryos infected with Mycobacterium marinum as a model system for investigating granuloma formation in real time. Davis et al. demonstrated that within the granuloma there is continuous turnover of infected macrophages, which allows bacterial numbers to expand as they are released from dead macrophages and subsequently phagocytosed by newly recruited macrophages (Davis J. M. and L. Ramakrishnan, 2009). Therefore, over the course of a persistent infection expression of the mce1 operon fluctuates depending on its intra or extracellular location, respectively. It is the balance of these two bacterial populations inside granulomas that ultimately determine the clinical outcome. If the balance is tipped towards strains expressing the mce1 genes, the pro-inflammatory response is enhanced and disease progresses. If the balance is tipped towards strains not expressing the genes, the bacilli remain latent and there is no disease progression. This model could explain the two clinical manifestations of M. tuberculosis infection—active disease and latent TB infection.
REFERENCES
