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IMMUNE MODULATION OF THE MYCOBACTERIUM TUBERCULOSIS GRANULOMATOUS RESPONSE

Kerry J. Welsh

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**IMMUNE MODULATION OF THE *MYCOBACTERIUM TUBERCULOSIS*
GRANULOMATOUS RESPONSE**

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A

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The University of Texas

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in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

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Houston, Texas

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Tuberculosis (TB) remains a major public health burden. The immunocompetent host responds to *Mycobacterium tuberculosis* (MTB) infection by the formation of granulomas, which initially prevent uncontrolled bacterial proliferation and dissemination. However, increasing evidence suggests that granuloma formation promotes persistence of the organism by physically separating infected cells from effector lymphocytes and by inducing a state of non-replicating persistence in the bacilli, making them resistant to the action of antibiotics. Additionally, immune-mediated tissue destruction likely facilitates disease transmission. The granulomatous response is in part due to mycobacterial glycolipid antigens. Therefore, studies were first undertaken to determine the innate mechanisms of mycobacterial cord factor trehalose-6'6-dimycolate (TDM) on granuloma formation. Investigations using knock-out mice suggest that TNF- α is involved in the initiation of the granulomatous response, complement factor C5a generates granuloma cohesiveness, and IL-6 is necessary for maintenance of an established granulomatous responses. Studies were next performed to determine the ability of lactoferrin to modulate the immune response and pathology to mycobacterial cord factor. Lactoferrin is an iron-binding glycoprotein with immunomodulatory properties that decrease tissue damage and promote Th1 responses. Mice challenged with TDM and treated with lactoferrin had decreased size and numbers of granulomas at the peak of the granulomatous response, accompanied by increased IL-10 and TGF- β production. Finally, the ability of lactoferrin to serve as a novel therapeutic for the

treatment of TB was performed by aerosol challenging mice with MTB and treating them with lactoferrin added to the drinking water. Mice given tap water had lung \log_{10} CFUs of 7.5 ± 0.3 at week 3 post-infection. Lung CFUs were significantly decreased in mice given lactoferrin starting the day of infection (6.4 ± 0.7) and mice started therapeutically on lactoferrin at day 7 after established infection (6.5 ± 0.4). Total lung inflammation in lactoferrin treated mice was significantly decreased, with fewer areas of macrophages, increased total lymphocytes, and increased numbers of CD4⁺ and CD8⁺ cells. The lungs of lactoferrin treated mice had increased CD4⁺ IFN- γ ⁺ cells and IL-17 producing cells on ELISpot analysis. It is hypothesized that lactoferrin decreases bacterial burden during MTB infection by early induction of Th1 responses.

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List of abbreviations

BMM	bone marrow-derived macrophages
BSA	bovine serum albumin
DMEM	Dulbecco's modified eagle medium
DC	dendritic cell
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FM	foamy macrophage
H&E	hematoxylin & eosin
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
LWI	lung weight index
MDR	multi-drug resistant
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MTB	<i>Mycobacterium tuberculosis</i>
PBS	phosphate buffered saline
SD	standard deviation
TDM	trehalose dimycolate
TNF	tumor necrosis factor

Chapter One

General Introduction

Epidemiology of tuberculosis

Tuberculosis (TB) is a major cause of infectious morbidity and mortality. TB causes approximately 1.8 million deaths annually, making it the leading cause of death due to bacterial infections worldwide [1]. Additionally, nearly one-third of the world's population is latently infected with *Mycobacterium tuberculosis* (MTB) [1]. Over 95% of drug-susceptible MTB can be cured with a four drug regimen of isoniazid, rifampin, pyrazinamide, and ethambutol for two months followed by four additional months of isoniazid and rifampin [2]. However, it is estimated that only half of TB patients are effectively treated worldwide [1]. Treatment failure occurs because of noncompliance to the lengthy drug regimen, individual variation in response to antimicrobials against TB, and drug resistance [2]. With the exception of the potential role of the fluoroquinolones, there have been no new anti-TB drugs created since rifampin was introduced in the 1960s [3]. Thus, agents with anti-mycobacterial activity or a beneficial effect when used in combination with the current anti-TB drugs are desperately needed.

TB infection is transmitted by aerosolization of MTB containing droplet nuclei produced from an infected person by coughing, sneezing, or forced respiratory activities [4]. Only about 30% of people exposed to TB bacilli get infected. The risk factors for infection with TB in the United States include residing in the same household as a person infected with TB, living in an institutionalized setting with infectious TB cases, older age, immigration from a country with a high prevalence of TB, and living in an area with a high incidence of TB such as inner cities. The initial infection is asymptomatic in most individuals. However, a small percentage of infected persons (2 – 23 %) will develop active TB disease [5]. The risk factors for progression to active TB

disease are malnutrition, certain gastrointestinal surgeries, young and old age, kidney disease, HIV infection, cancer, immunosuppressive therapies, and intravenous drug use [4].

TB remains a public health burden despite the availability of antimicrobial chemotherapy. The incidence of TB significantly decreased in the United States in the 1800s before the advent of TB antibiotics [6]. The decline in TB incidence is possibly attributed to better housing and ventilation, improved nutrition, milk pasteurization, and use of sanatoriums to isolate infectious TB cases. The development of streptomycin, para-aminosalicylic acid, and isoniazid combination therapy in the mid-20th century resulted in TB becoming a curable disease [7]. Anti-TB chemotherapy was significantly improved with the introduction of rifampin in a multi-drug regimen, which resulted in an even more rapid decline in TB incidence and mortality. TB case number decreased approximately 6% annually from 1953 to 1985 [8]. However, TB incidence in the United States and other industrialized countries increased 20% from 1985 to 1992. The resurgence of TB in developed countries is attributed to the HIV/AIDS epidemic, transmission of TB in institutionalized settings, a rise in immigration from areas with a high prevalence of TB, and a lack of funding and infrastructure for TB control programs [6]. The increase in TB incidence was reversed in 1993, with the number of TB cases in the United States decreasing by approximately 5-7% per year from 1993-1997.

Multidrug resistant (MDR)-TB emerged with the increase of TB incidence in the United States [8]. Poor patient adherence to treatment regimens and incorrect or erratic treatment caused by a number of clinical and social factors contributed to the development of drug-resistant TB [9]. Thus, new anti-TB agents are needed for

treatment of MDR-TB and to improve current treatment options to allow for shorter and more intermittent therapy.

***M. tuberculosis* induced pathology**

The hallmark of MTB pathology in the immunocompetent adult is the caseating granuloma, which consists of central infected macrophages that may differentiate into foamy macrophages, epithelioid cells, and Langhans' giant cells (Figure 1.1A) [10]. The majority of cells in the granuloma are positive for CD68 and CD4 [11]. A layer of fibrous surrounds the infected macrophages, which isolates the organism and prevents bacterial dissemination. The periphery of the granuloma consists of lymphocytes that are a mixture of CD4+, CD8+, and CD20+ cells [11]. Occasional T-regulatory cells are observed in the lymphocytic portion of the granuloma [11, 12]. The fibrotic portion of the granuloma causes physical partitioning of infected cells from effector lymphocytes that are able to kill infected cells and inhibits the penetration of antibiotics [10].

The majority of granulomas persist for the life of the individual without causing active TB disease. However, approximately 2 - 23% of infected individuals develop reactivation of infection [5]. These post-primary (secondary) TB cases cause the majority of TB disease and nearly all transmission of the bacilli [13]. Post-primary TB is characterized histologically as a lipid pneumonia, with foamy macrophages in alveoli and lymphocytes in the alveolar walls and alveoli (Figure 1.1B) [11, 14]. The majority of lymphocytes in lipid pneumonia are positive for CD8, with relatively fewer CD4+ lymphocytes compared to other tissue manifestations of TB. It is hypothesized that there is an accumulation of mycobacterial antigens, possibly glycolipids, that

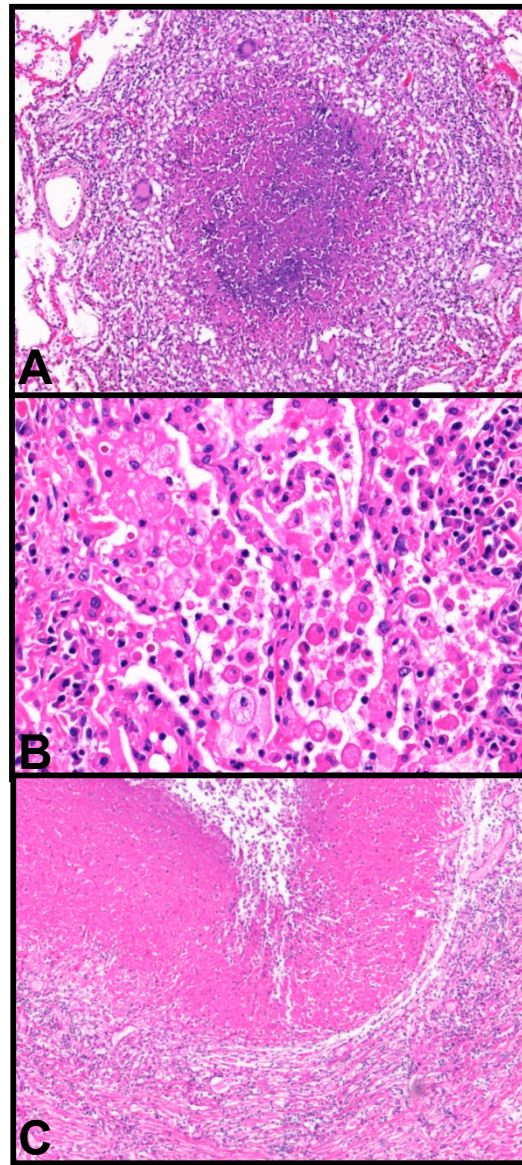


Figure 1.1. Tuberculosis induced pathology. **A.** Typical caseating granuloma of primary TB consists of central necrosis flanked by foamy and epithelioid macrophages. Occasional Langhan's giant cells are noted. The periphery of the granuloma consists of lymphocytes. **B.** The lipid pneumonia of early post-primary TB is characterized by alveoli filled with foamy macrophages. Lymphocytes are found in the alveoli and alveolar walls. **C.** Cavitory lesions consist of a wall of macrophages and necrotic debris. Abundant fibrosis and lymphocytes are noted surrounding the cavity wall. Welsh KJ, et al. Clin Devel Immuno. 2011 [11].

initiates tissue necrosis and the formation of cavities.

Cavitary lesions, the hallmark of active TB disease, likely result from abrupt necrosis of the lipid pneumonia [11, 14]. The wall of the cavity consists of necrotic tissue and foamy macrophages (Figure 1.1C). Numerous lymphocytes line the wall of the cavity and consist of CD4+, CD8+, CD20+, as well as numerous T-regulatory cells [11]. The damage to the lung resulting from TB-induced immunopathology causes a productive cough, producing infectious aerosols that may infect a new host. Modulation of the inflammatory pathology will likely facilitate improved response to antimicrobials, limit transmission of the disease, and decrease morbidity [15].

The immune response to *M. tuberculosis*

MTB infection is initiated by inhalation of TB bacilli that are deposited in the alveoli, which become phagocytosed by alveolar macrophages [10]. The macrophages produce tumor necrosis factor-alpha (TNF- α) and chemokines that recruit systemic monocytes, forming the nascent granuloma (Figure 1.2). The acquired immune response develops after migration of dendritic cells to the draining lymph nodes. Dendritic cell production of IL-12 initiates the development of interferon-gamma (IFN- γ) producing Th1 cells, while the synthesis of interleukin (IL)-6 and transforming growth factor-beta (TGF- β) promotes the development of Th17 cells [16]. Th1 and Th17 cell populations migrate to the lung in response to chemokine gradients. The induction of the specific immune response correlates with control of MTB infection. This section reviews the cell types and cytokines essential for immunity to MTB.

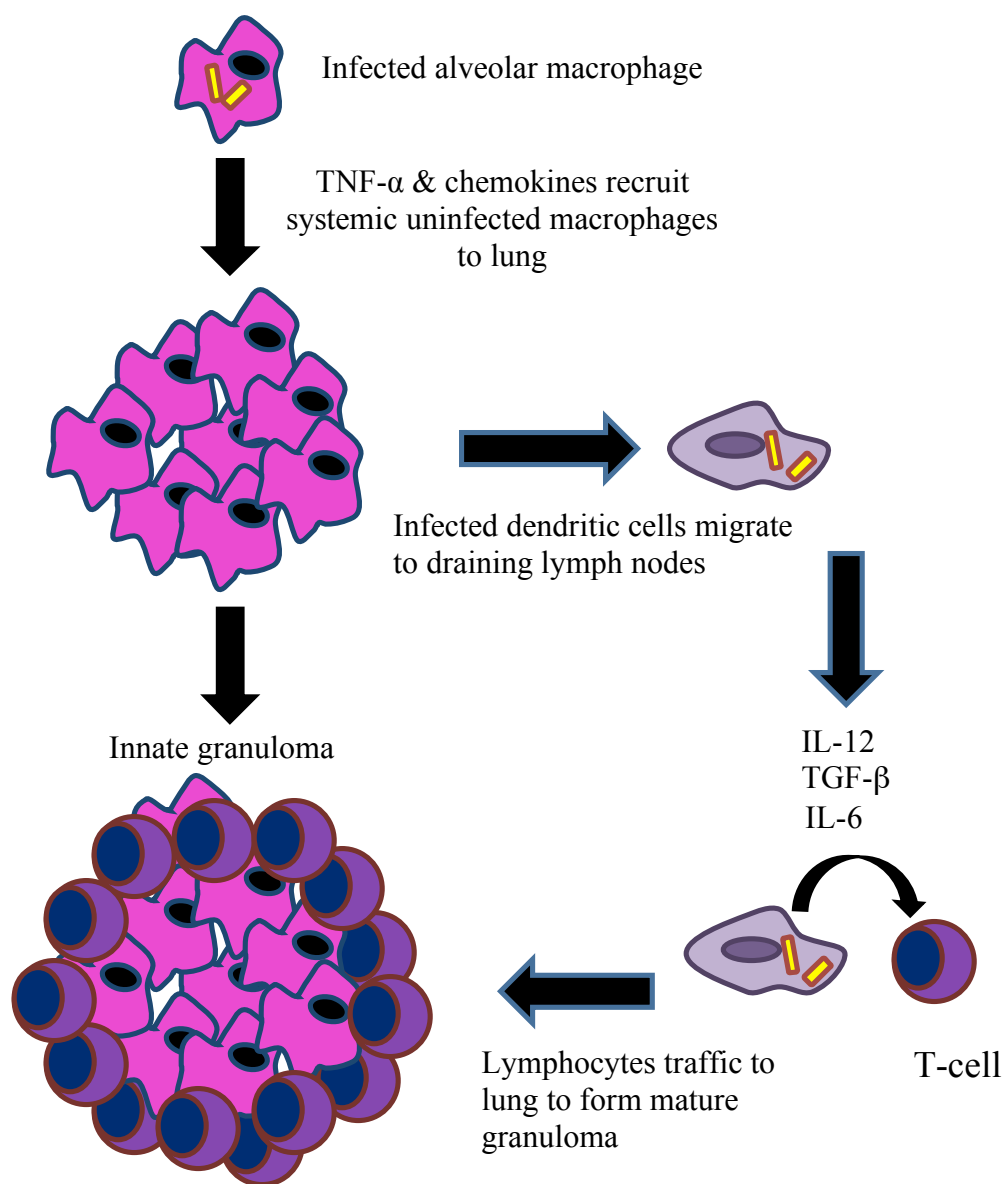


Figure 1.2. Schematic depicting the major immunologic events leading to granuloma formation. Infection with MTB initiates with the deposition of bacilli into the alveoli. MTB are phagocytosed by alveolar macrophages that secrete cytokines such as TNF- α and chemokines that recruit systemic monocytes to form the nascent granuloma. The adaptive immune response begins when infected dendritic cells migrate to the draining lymph nodes of the lung. Dendritic cell production of IL-12 polarizes naive T-cells to the Th1 phenotype, while TGF- β and IL-6 promote Th17 cells. Lymphocytes migrate to the lung to form the mature granuloma.

Macrophages

The first cells to encounter TB bacilli are likely alveolar macrophages [17]. Macrophages phagocytose mycobacteria using mannose receptors, complement receptors, and scavenger receptors [18-20]. The phagosome in most infections undergoes maturation processes that result in fusion with lysosomes, inducing organelle acidification and the activation of lytic enzymes that result in bacterial death. However, MTB is able to prevent phagolysosomal fusion, giving the organism the ability to survive within macrophages [21]. The mechanism of MTB inhibition of phagolysosomal fusion may be due to mycobacterial cell wall products, such as sulfatides and glycolipids [22, 23].

Macrophages have mechanisms to control proliferation of MTB. Macrophage activation by IFN- γ , and to a lesser extent by TNF- α , causes inducible nitric oxide synthetase (iNOS) to produce NO and reactive nitrogen intermediates using arginine as the substrate [21]. Numerous studies have demonstrated the critical importance of nitrogen oxides in protection against MTB, both *in vitro* and *in vivo* [24, 25]. Furthermore, macrophages play a role in antigen presentation and production of IL-12 that are critical for the generation of the adaptive immune response [26, 27].

While macrophages possess the ability to kill MTB, there is some evidence that macrophages contribute to the chronic nature of MTB infection. Macrophages in TB infection may become foam cells, possibly by dysregulation of host lipid metabolism [17]. It was recently reported that foamy macrophages (FM) positive for the dendritic cell (DC) marker DEC-205 collect in the alveoli along with CD8⁺ lymphocytes in both the alveolar walls and alveoli in humans with early post-primary TB [11]. Ordway et al.

[28] demonstrated that the FM in mouse granulomas stained positive for DEC-205. The DEC-205 positive FM in mice had additional markers of DCs such as CD40, CD11c, and major histocompatibility complex II (MHC II) in addition to high levels of antiapoptotic markers. The importance of FM expressing DC markers has not been elucidated. However, DCs infected with MTB can harbor the bacilli for long periods of time and lack efficient mechanisms for eliminating MTB [29-31]. FM with DC characteristics may thus provide a favorable environment for the growth of MTB. Furthermore, FM may suppress the cell-mediated immunity that is necessary to eliminate MTB. For example, FM produce high amounts of TGF- β that can induce apoptosis of immune cells [32]. Additionally, FM synthesize high levels of iNOS, which may cause suppression of T-cells [33]. Therefore, FM may cause local immune suppression that contributes to inability of the immune system to clear post-primary TB.

Dendritic cells

DCs are another innate immune system cell essential for protection against MTB. DCs are considered permissive for MTB growth because they harbor the bacilli for extended periods of time and do not appear to be capable of killing MTB, despite activation with IFN- γ [29-31]. However, DCs are essential for host defense against MTB because mice lacking CD11c cells were less able to control MTB growth, possibly by delayed CD4⁺ T-cell activation [34].

The major function of DCs is to promote T-cell responses following infection with MTB [35]. DCs that become infected with MTB or have phagocytosed MTB antigen become activated and produce IL-12p40, enabling the DC to respond to

chemokines such as CCL19 and CCL21 that allow the DC to migrate to the draining lymph nodes [36]. DC production of IL-12 to naive T-cells polarizes T-cells to the Th1 phenotype, which are essential for bacterial control. DCs also synthesize IL-6 and TGF- β that generate Th17 cells that play a number of important roles in the immune response against MTB [36].

T-cells

Cell mediated immunity is essential for host defense against MTB. This is due to the fact that MTB is an intracellular organism and thus effector T-cells are needed to control bacterial growth. Both CD4⁺ and CD8⁺ T-cells become activated in the draining lymph nodes of the lung during week one of infection in the mouse model of MTB; these cells migrate to the lung between two and four weeks post-challenge [37, 38]. A significant portion of these cells are positive for CD69, suggesting that activated T-cells are migrating to active sites of infection [21].

CD4⁺ T-cells are critical to the outcome of MTB infection. CD4⁺ T-cells respond to antigen presented on MHC II, which present exogenous antigen that was processed in phagolysosomes [39]. Studies in mice using knock-out mice, adoptive transfer, and antibody neutralization of CD4⁺ cells all demonstrate that CD4⁺ T-cells are an absolute requirement for infection control [40-44]. Another example of the importance of the CD4⁺ T-cell response can be found in humans with advanced HIV, who develop disseminated mycobacterial infections when CD4⁺ T-cells reach low levels [45]. CD4⁺ T-cells also promote the development of CD8⁺ cytotoxic T-cells, which are also important for control of disease pathology [46]. There are four subtypes of CD4⁺

T-cells that play various roles during MTB infection; these are T-cell helper type 1 (Th1), Th2, Th17, and T-regulatory (Treg) cells.

1. Th1 phenotype. Control of MTB infection is strongly dependent on the induction of a Th1 immune response. The Th1 response is characterized by populations of CD4⁺ T-cells that produce IFN- γ upon activation [47]. Th1 responses are initiated by innate immune cell production of IL-12 during antigen presentation to naive T-cells. The importance of IL-12 is demonstrated by IL-12p40 deficient mice that have markedly increased bacterial burdens and decreased survival time when infected with MTB [48]. The IL-12 induced generation of CD4⁺ IFN- γ producing cells are critical for the host defense against MTB. IFN- γ production by Th1 CD4⁺ cells causes activation of macrophages, resulting in phagosome acidification and production of reactive nitrogen species capable of killing MTB [46]. IFN- γ additionally promotes upregulation of MHC II expression that further induces antigen presentation and T-cell stimulation [39, 49]. The importance of IFN- γ production is highlighted by IFN- γ knock-out mice that are extremely susceptible to MTB [50, 51]. Additionally, humans with mutations in IFN- γ or its receptor are likely to have serious mycobacterial infections [52]. Therefore, it is critical that immunomodulatory agents useful in control of MTB infection produce a strong Th1 response.

2. Th2 phenotype. Th2 immune responses are characterized by the production of cytokines such as IL-4 and IL-10. The role of Th2 responses in TB infection is unclear. The increase in bacterial burden that occurs after 3 weeks in mice challenged with a high dose of MTB is accompanied by increased IL-4 [53]. Additionally, IL-4 knock-out mice were more resistant to MTB infection [21]. However, IL-10 deficient mice do not have

increased resistance in comparison to wild-type mice [54]. Detection of Th2 responses in humans is difficult [21]. It has been reported in humans that decreased Th1 responses, but not increased Th2 responses, are found in PBMCs from TB patients [55-57]. Thus, elevated Th2 responses are not likely associated with TB in humans.

3. Th17 phenotype. While Th1 responses are clearly necessary for host defense against MTB, this immune response alone is not sufficient [21]. Th17 cells are a recently discovered T-cell subset with a number of regulatory functions. Th17 responses are initiated by TGF- β in combination with IL-6, and further expanded by IL-23 [58]. Th17 cells synthesize characteristic cytokines IL-17A, IL-17F, IL-21, and IL-22 that promote upregulation of other proinflammatory mediators including chemokines, IL-6, and GM-CSF [59-63]. A number of investigations have demonstrated that Th17 cells may play an important role in the immune response against MTB [64-66]. Enhancement of IL-17 responses early in MTB infection by use of an IL-23 synthesizing adenovirus resulted in reduced lung pathology and decreased bacterial burden [67]. Additionally, Th17 cells from humans recently infected with TB had the phenotypic markers of long-lived central memory cells and were distinct from Th1 effector memory cells [68], suggesting that Th17 cells may give long-lasting immunity to TB.

4. Treg cells. Treg cells, identified by Foxp3 expression, are considered essential for preventing autoimmunity [69]. However, the function of Tregs in infectious diseases including MTB is incompletely understood. Tregs synthesize suppressive cytokines such as IL-10 and TGF- β that limit excess inflammation, but may prevent clearance of pathogens [70]. Treg cells in the peripheral blood decrease in newly infected contacts of patients with active TB disease, possibly due to their migration to the infected lung [71].

Tregs accumulate in sites of disease and in blood as the infection progresses [12, 72-74]. Differential Foxp3 expression in PBMCs differentiated active TB from latent TB infection [75]. Foxp3+ cells were found to decrease T-cell responses to mycobacterial antigens in human patients [76]. Neutralization of Treg cells during mouse models of infection enhanced elimination of MTB [77, 78], providing further support that Treg cells have a negative impact on MTB infection. Another investigation showed that small numbers of Tregs specific for MTB prevent the aggregation of CD4+ and CD8+ T-cells in infected mouse lungs [79]. Finally, it was recently shown that human cavitory lesions, lesions that rarely spontaneously heal, contained a higher percentage of Tregs compared to other pathological manifestations of TB [11].

CD8 T-cells. CD8+ T-cells also potentially play a role in MTB infection. These cells recognize antigen presented on MHC I molecules, and may produce IFN- γ when activated. MTB antigens are thought to gain access to the MHC I molecules by pores in the vesicular membrane of the phagosome or an alternate processing pathway for MHC I [80]. CD8+ lymphocytes can be found in granulomas at approximately the same frequency as CD4+ cells [11]. CD8+ T-cells may directly lyse MTB-infected cells, and have been reported to potentially overcome the absence of CD4+ T-cells [43, 81, 82]. The mechanisms of CD8+ cell dependent lysis are perforin and granulysin [83-85]. However, mice lacking CD8+ T-cells have higher bacterial burdens, but generate normal granulomas and ultimately control the infection [86].

Soluble factors

A number of protein mediators such as the complement system and cytokines play essential roles in during bacterial infections. Cytokines function as signaling mediators between cells and are essential regulators of all aspects of immunity. The cytokines central to the adaptive immune response have been discussed elsewhere in this review. This section will discuss the functions of the complement system and the cytokines TNF- α and IL-6.

The complement system. The complement system plays a central role in innate immunity and the initiation of adaptive immunity [87]. The three major pathways of complement are the classical, alternative, and lectin pathways. The classical pathway begins when certain subclasses of IgG or IgM bind to antigen. C1q binding to Fc sites induces C1r to activate C1s. C1s cleaves C4 and C2, which forms the classical pathway C3 convertase (C4b2a). The classical C3 convertase cleaves C3, generating the C5 convertase (C4b2a3b) that cleaves C5. The result may be lysis of cells by the deposition of C5b-9 on the surface of cells, inflammation by C3a and C5a-induced chemotaxis of leukocytes, or opsonization and phagocytosis of cells mediated by C3b. The lectin pathway is similar to the classical pathway, except that it starts with the binding of mannose-binding lectin to microbial surfaces that induces proteases to cleave C4 and C2. The alternate pathway is initiated when C3 undergoes spontaneous auto-cleavage to C3b. C3b binds to factor B and properdin to form the C3 convertase (C3bBb). The C3 convertase enzymatically cleaves additional C3 to form the C5 convertase (C3bBbC3b), which cleaves C5 [87].

New evidence suggests that complement plays a major role in the induction of T-cell responses in addition to its clear role in innate and humoral immunity [88]. The

binding of complement factors to the C3a and C5a receptors promotes the migration of antigen presenting cells to areas of infection, where they present antigen to T-cells and modulate IL-12 production [89-91]. Binding of immune complexes to the C1q receptor on T-cells promotes T-cell activation with production of TNF- α and IFN- γ [92]. C5a binding to the C5a receptor on T-cells directs their migration to sites of inflammation [93]. Both CD4⁺ and CD8⁺ T-cells responses in C3 deficient mice are decreased in viral infection models [94-96]. The precise role of complement factors in immunity and pathology during MTB infection is unclear. It was reported that mice deficient in complement C5 have decreased survival, increased inflammation, and decreased macrophage cytokine production [97, 98]. The studies presented here examine the role of C5a in the early initiation and maintenance of the granulomatous response.

TNF- α . TNF- α is a proinflammatory cytokine synthesized by dendritic cells, macrophages, and T-cells that plays a critical role in the regulation of acute and chronic granulomas [99]. Neutralization of TNF- α or mice with a deficiency of TNF- α or its receptor rapidly succumb to MTB infection, have markedly increased bacterial loads, and a dysregulated granulomatous response [100-102]. The mechanism of TNF- α in host resistance to MTB is possibly due to its ability to partially activate macrophages, increasing their cytotoxicity, and facilitating the interactions between macrophages and T-cells that are necessary to generate granulomas [103].

However, there is increasing evidence that the granulomatous response promotes a survival advantage to MTB in the host and that the organism possesses specific mechanisms to drive TNF- α production [15]. Mycobacterial cell walls contain glycolipids, such as trehalose dimycolate, that induce TNF- α and drive granuloma

formation [99]. MTB may also promote TNF- α production by direct interference with intracellular signaling. A MTB mutant deficient in the adenylate cyclase Rv0386 was characterized by reduced cAMP after infection, accompanied by decreased protein kinase A and CREB activation, and ultimately decreased TNF- α synthesis by infected macrophages [104]. The mutant MTB had poor survival in a mouse infection model and decreased lung pathology. It has thus been proposed that the induction of excessive TNF- α production by MTB is a component of the virulence of the organism [15].

IL-6. The role of IL-6 in the host defense against MTB is unclear. This cytokine is produced by fibroblasts, macrophages, B-cells, and T-cells. IL-6 possibly interferes with IFN- γ signaling and increases IL-4 production, both of which may inhibit protective Th1 responses [105]. Production of IL-6 by BCG infected macrophages inhibited T-cell responses [106]. However, IL-6 $-/-$ mice infected with MTB have produced mixed results. Intravenous challenge MTB was lethal for IL-6 knock-out mice [107]; however, aerosol challenge of the IL-6 $-/-$ mice had later production of IFN- γ but were ultimately able to control the infection [105]. Thus, IL-6 may play a role in the immune response against MTB, but its precise function remains to be determined.

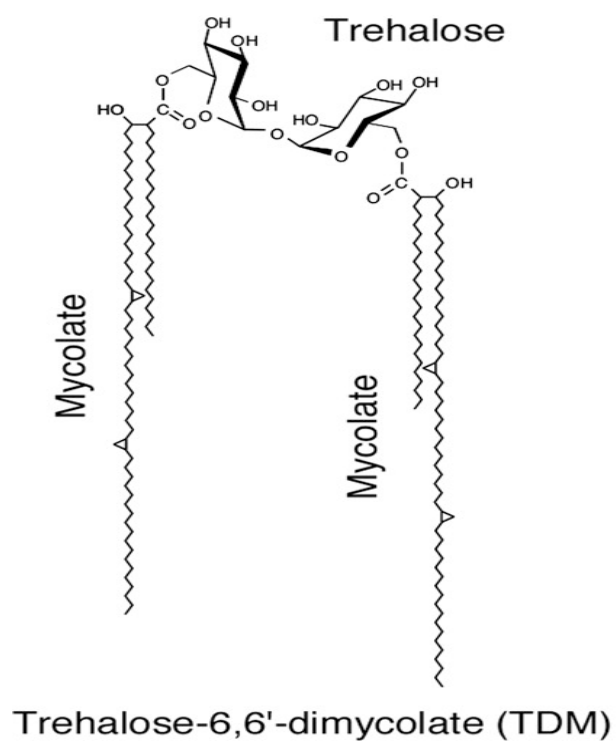
The role of trehalose 6,6'-dimycolate on MTB pathogenesis

Mycobacterial cell walls possess abundant lipid, accounting for approximately half of the dry weight of MTB [108]. The lipid content of MTB includes mycolic acids, which consist of long chains of fatty acids with 78 – 90 carbons [109]. Trehalose 6,6'-dimycolate (TDM) is the most abundant glycolipid on the surface of MTB [13]. TDM, also referred to as mycobacterial cord factor, was identified due to the ability of MTB to

form serpentine cords. TDM structurally consists of two trehalose molecules, each with a mycolic acid chain with 60 – 90 carbons (Figure 1.3) [13]. The biologic activity of TDM depends on its presentation [110]. TDM mixed in an aqueous solution forms micelles that are non-toxic; TDM on the surface of MTB is hypothesized to exist in the micelle form. In contrast, TDM monolayers formed on hydrophobic surfaces, such as oil-water interfaces, are toxic in microgram quantities [110]. TDM is thought to play a number of important roles in MTB pathogenesis, including virulence and granuloma formation.

TDM is hypothesized to contribute to the virulence of MTB. Higher levels of TDM are found on the surface of virulent MTB than avirulent organisms [111]. Extraction of TDM from mycobacteria decreases the organism's ability to survive in both macrophages and mice; reconstituting purified TDM largely restores the organisms' survival in macrophages [112-114]. Decreased MTB ability to produce excess TDM is accompanied by a decrease in virulence [115, 116]. Furthermore, alterations in the structure of TDM such as absence of the cyclopropane rings results in decreased bacterial growth in macrophages and mice [117]. Removal of TDM from the surface of MTB resulted in enhanced macrophage ability to undergo phagosome acidification and fusion with lysosomes [112]. Additionally, surface TDM decreases macrophage expression of MHC II, CD40, CD80, CD86, and CD1d, markers that are essential for antigen presentation and stimulation of T-cells [23].

TDM is likely involved in induction of the granulomatous response during MTB infection. Administration of a single dose of TDM in emulsion form induces foreign



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Figure 1.3. Structure of trehalose-6,6'-dimycolate (TDM). TDM is composed to two molecules of trehalose each with a mycolate attached. The structure contains double bonds and cyclopropane molecules that are necessary for its activity [123].

body lung granulomas; these granulomas peak in size and number at one week and completely resolve two-weeks post-challenge [80]. This granulomatous response is accompanied by pro-inflammatory cytokine production including TNF- α , IL-6, IL-1 β , IFN- γ , IL-12, as well as chemokines [118, 119]. TDM can additionally promote hypersensitive granulomatous response by TDM challenging mice that have been immunized with TDM and protein [80, 120, 121]. Hypersensitive granulomas in response to TDM are characterized by large, destructive granulomas that have a significant lymphocyte composition. The hypersensitive response is accompanied by synthesis of Th1 cytokines such as IFN- γ and IL-2. The hypersensitive granulomatous response was demonstrated to be transferable by CD3⁺ TDM immunized cells; CD4⁺ cells were later determined to be responsible [120, 121]. TDM has also been implicated in the induction of caseating granulomas [110].

It has long been established that the proinflammatory responses to TDM are mediated by macrophages [122]. However, receptors for binding and signaling for TDM were only recently discovered. Bowdish et al [122] reported that the macrophage scavenger receptor MARCO forms a complex with TLR2 and CD14 that recognizes TDM-coated microspheres. Other investigators showed that macrophage inducible C-type lectin (Mincle) bound TDM coated onto plates [123, 124]. Mincle response to TDM requires the Fc receptor γ chain (FcR γ) and Sky-Card9-Bcl10-Malt1 signaling pathway [125].

Glycolipid-specific T-cells are induced in mycobacterial infected humans and guinea pigs [126]. However, antigen presentation occurs via CD1 rather than MHC I or II. CD1 molecules present hydrophobic ligands [80]. Humans possess five CD1 family

members, divided into group 1 (CD1a, CD1b, CD1c) and group 2 (CD1d, CD1e). Mice, however, have only two CD1 genes (CD1d1 and CD1d2) [80]. Therefore, it is difficult to compare studies of humans and mice on CD1 presentation of glycolipids. Murine macrophages upregulate CD1d1 after stimulation with TDM [127], but TDM-specific CD1d T-cells have not been identified in mice. However, it was demonstrated that mice lacking CD1d (CD1d $-/-$) have dysregulated acute granulomatous response to TDM [128]. In the hypersensitive granuloma model, CD1d $-/-$ mice had less destructive lung lesions, loosely organized CD3⁺ lymphocytes, and increased proinflammatory cytokines [129]. Thus, the precise role of CD1 in the presentation of TDM is still being delineated.

It is well established that TDM induces a number of immunological mediators; however, the specific cytokines that necessary for granuloma formation are unknown. Studies from our laboratory demonstrated that regulation of corticosterone in the lung by 11 β -hydroxysteroid dehydrogenases modulates the progression TDM-induced granuloma formation [130]. The work in this thesis explores the role of complement factor C5a, TNF- α , and IL-6 in the initiation and maintenance of TDM-induced granuloma, and the ability of lactoferrin to modulate this granulomatous response.

Modulation of TB-induced immunopathology as an adjuvant to antimicrobial chemotherapy

Granuloma formation is considered a favorable response that prevents bacterial replication and dissemination to other organ systems. However, MTB localized within granulomas are protected from sterilizing antimicrobials and immune-mediated killing [15]. MTB within granulomas undergo conversion to a state of non-replicating

persistence that includes changes in biosynthetic pathways and decreased replication [131, 132]. The action of antibiotics requires bacilli that are actively replicating for effectiveness and thus are unable to eliminate the dormant MTB within granulomas. Additionally, reduction of lung inflammatory pathology may facilitate antibiotic penetration into infected tissue, thereby inducing a faster response to antibiotics [15, 133]. Indeed, MTB mutants that lack the ability to induce granulomas have significantly reduced survival in mice and less lung pathology [104]. Additionally, it is hypothesized that the inflammatory response to MTB is responsible for disease transmission; modulation of the immune response may limit dissemination of the bacilli to new hosts [15, 17, 134].

Immunomodulators combined with TB antibiotics has generated favorable results in human clinical studies. One clinical trial combined etanercept, a soluble TNF- α receptor, with standard TB chemotherapy in HIV patients with TB and reported significantly increased bacterial clearance and improved chest x-ray results in the patients treated with the combination therapy [135]. Another study explored high-dose corticosteroids with anti-TB drugs in patients co-infected with HIV and TB [136]. Sputum culture conversion was significantly increased in the patients given prednisolone compared to patients given standard TB chemotherapy alone. The studies in this thesis use lactoferrin as an immunomodulator during MTB infection; lactoferrin has numerous advantages over other available immunomodulators because it does not suppress the immune system and has a proven safety record in a number of animal studies and human clinical trials [137-139].

Lactoferrin as an immune modulator

New evidence identifies lactoferrin as an immune response modulator to a number of infectious and inflammatory stimuli [140, 141]. Lactoferrin is member of the transferrin family and is found in epithelial secretions as well as in the granules of neutrophils [140]. The serum concentration of lactoferrin is normally low, at less than 1 $\mu\text{g/ml}$, but markedly increases during injury and inflammation [142, 143]. Lactoferrin receptors are found on many different immune cells, such as macrophages, dendritic cells, and T-cells [144-146]. Reported immunomodulatory properties of lactoferrin include macrophage activation, promoting polymorphonuclear cell phagocytosis, enhancement of T- and B-cell maturation, and augmentation of antigen specific immune responses [147-149].

Lactoferrin modulation of innate immunity

Lactoferrin modulates the activity of innate antigen presenting cells (APCs), which in turn have the potential to alter T-cell responses. APCs, including dendritic cells, process and present antigen to naive CD4^+ T-cells using MHC II along with co-stimulatory molecules such as CD40, CD80, and CD86 [39, 150]. APC production of IL-12 promotes the development of naive CD4^+ T-cells to a Th1 phenotype [26, 151]. Thus, alteration of APC cytokine production and surface molecule expression has the potential to enhance the development of the acquired immune response.

Lactoferrin enhances dendritic cell and macrophage function as APCs to activate CD4^+ T-cells. Macrophages stimulated with IFN- γ and infected with mycobacteria have decreased MHC II expression [152-154]. Lactoferrin added to activated macrophages

infected with BCG significantly increased expression of MHC II [155, 156]. Lactoferrin increased the CD86:CD80 ratio in dendritic cells and macrophages infected with BCG [155, 157], suggesting that APCs treated with lactoferrin are better at promoting activation of T-cells during antigen presentation [158, 159]. Indeed, dendritic cells and macrophages infected with BCG and incubated with lactoferrin had significantly enhanced IFN- γ production from both CD3⁺ and CD4⁺ cells in comparison to APCs cultured in the absence of lactoferrin [155, 157]. Additionally, lactoferrin increased expression of CD40 on the mouse macrophage cell line RAW 264.7 as well as peritoneal macrophages [160]. Immature human dendritic cells cultured with recombinant human lactoferrin increased human leukocyte antigen II, CD80, CD86, CD83, in addition to chemokine receptors necessary for migration to draining lymph nodes [161].

IL-12 is essential for the promotion of the Th1 cytokine IFN- γ , both in the maturation of naive T-cells to the Th1 phenotype and in maximizing production of IFN- γ from established Th1 effector and memory cells [162, 163]. Injection of lactoferrin intraperitoneally into mice increased production of IL-12 from peritoneal macrophages recovered from the mice [147]. Other investigators have shown that lactoferrin administered orally increases IL-12p40 expression, accompanied by decreased expression of IL-10 [164]. Indeed, macrophages infected with BCG and treated with lactoferrin demonstrated a significantly increased ratio of IL-12 to IL-10, a cytokine that inhibits the action of IL-12 [147]. Furthermore, lactoferrin increased TGF- β production from dendritic cells and macrophages infected with BCG [155, 157]. TGF- β in combination with IL-6 promotes the generation of Th17 responses [59], which may play an important role in protection against MTB [64, 68]. In summary, these studies indicate

that lactoferrin strongly modulates APC function. The impact of lactoferrin on innate cell function involved in the initial interaction with microbes gives it potential to enhance the generation of the adaptive immune response, with clear mechanisms to support its use as an adjunct therapeutic for the treatment of TB.

Lactoferrin modulation of the acquired immune response

Lactoferrin has a number of direct effects on T-cell responses in addition to modulation of innate responses. CD4⁺ and CD8⁺ T-cells possess receptors for lactoferrin [165]. Lactoferrin affects the levels of co-stimulatory surface molecules that impact T-cell function, suggesting that lactoferrin modulates T-cell response to antigen. The maturation of double negative mouse T-cells preferentially towards CD4⁺ T-cells is enhanced by human lactoferrin, possibly through Erk2 and p56^{lck} to activate the MAP kinase pathway [166, 167]. Administration of oral lactoferrin to mice increased CD4⁺ and $\gamma\delta$ T-cells as well as total circulating granulocytes [164]. Lactoferrin increased expression of leukocyte function associated antigen, an adhesion molecule involved in cell-to-cell contact on T-cells [168]. Expression of the human T-cell ζ -chain, a component of the CD3 T-cell receptor complex involved in signaling, was enhanced by lactoferrin [169].

Lactoferrin can polarize naive T-cells to either Th1 or Th2 phenotypes, depending on the cytokine environment and antigen. Studies demonstrate that lactoferrin can augment production of cytokines needed for the development of a Th1 response, with increase of the delayed-type hypersensitivity (DTH) response to BCG, sheep red blood cells, and ovalbumin [147, 170, 171]. Transgenic mice expressing human lactoferrin and infected

with *Staphylococcus aureus* had increased IFN- γ and TNF- α , along with decreased IL-10 and IL-5 [172]. Oral administration of lactoferrin increased Th1 T-cell responses, indicated by enhanced levels of IFN- γ , in both naive and tumor-harboring mice [173, 174]. The IL-12:IL-10 ratio was increased by lactoferrin in LPS stimulated splenocytes [175]. Furthermore, chronic hepatitis C virus elimination was augmented by the combination of lactoferrin and interferon therapy, possibly by increasing Th1 responses [176]. In contrast, lactoferrin increased IL-10 and decreased IFN- γ in an infection model of *Toxoplasma gondii*, indicating the enhancement of Th2 responses [177]. The effects of lactoferrin on Th17 cells are currently unknown.

Lactoferrin additionally alters B-cell responses. Immature B-cell incubation with lactoferrin increased their proliferation in response to antigen-specific T-cells [149]. Lactoferrin augments maturation of immature mouse B-cells, indicated by increased expression of the complement 3 receptor and IgD [149]. Lactoferrin increases mouse Peyer's patches production of IgG and IgA [178]. Mice treated with lactoferrin had increased levels of IgG in both the serum and intestine [179]. Additionally, lactoferrin increases antibody-forming cells and humoral responses to sheep red blood cells in mice treated with methotrexate and cyclophosphamide [180-182]. Taken together, these investigations suggest that lactoferrin directly affects B-cells, and enhances their function as APCs to generate T-cell responses.

Essential for the studies performed here, lactoferrin decreases immune-mediated tissue damage. For example, mice administered lactoferrin had decreased mortality and reduced destruction of gut tissue following LPS injection [183]. Lactoferrin also

decreased tissue damage in mouse models of rheumatoid arthritis and inflammatory bowel disease [184-186].

Summary of Thesis

This thesis will explore the cytokine mechanisms of TDM-induced granulomas and the ability of lactoferrin to modulate TB-induced immunopathology. Initial studies examined the role of TNF- α , complement factor C5a, and IL-6 in the initiation and maintenance of TDM-induced granulomas (Chapter 2). Studies were next undertaken to explore the ability of lactoferrin to modulate immune-induced pathology to mycobacterial antigen (Chapter 3). Finally, the ability of lactoferrin to serve as an adjuvant therapeutic for TB was evaluated by treating MTB infected mice orally with lactoferrin (Chapter 4). The mechanisms of lactoferrin-mediated immune modulation during MTB infection are discussed.

Chapter Two

The role of TNF- α , complement C5a, and IL-6 in the induction and maintenance of mycobacterial cord factor trehalose 6,6'-dimycolate induced granulomas

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Introduction

Mycobacteria are responsible for a number of infectious pathologies that are a significant global health problem. Disease caused by *Mycobacterium tuberculosis* (MTB) results in approximately 2 million deaths annually [1]. Infection usually presents as pulmonary pathology, characterized by the generation of granulomas that prevent bacterial dissemination. However, extrapulmonary disease also occurs in which any organ can become infected including the bones, kidneys, central nervous system, and gastrointestinal tract [187]. The cytokine mechanisms that induce granuloma formation have not been fully elucidated. Due to the increasing number of drugs developed to target cytokines in the therapy of immune-mediated disorders, it is essential to determine the role these mediators play in the formation and maintenance of mycobacterial induced granulomas.

Tumor necrosis factor- α (TNF- α) is the major cytokine necessary for the generation and maintenance of MTB induced granulomas [188]. TNF- α has a number of immunologic functions that include activation of endothelial cells, initiation of apoptosis, and proliferation of thymocytes [189]. Antibody neutralization of TNF prevented control of infection with bacillus Calmette-Guérin (BCG) and granuloma formation in mice [190]. Mice deficient in the TNF receptor and wild-type mice administered a TNF- α neutralizing antibody rapidly succumb to infection with MTB [101]. Additionally, mice lacking TNF do not generate granulomas during mycobacterial infection, accompanied by delayed recruitment of CD11b⁺ cells and production of C-C and C-X-C chemokines [191]. TNF is also essential for long term

control of persistent infections, with elimination resulting in inability to maintain granulomas [192].

Complement C5 is another potentially important mediator in the granulomatous response. Its split product, C5a, recruits cells to sites of inflammation and promotes the synthesis of cytokine subsets, such as TNF- α , IL-6, and IL-12 [193]. A/J mice, which lack complement C5, have increased mortality accompanied by markedly increased inflammatory responses and do not form granulomas in a mouse model of MTB infection [97]. The complement deficient A/J mice had significantly earlier reactivation of latent infection, exacerbated responses in an induced chronic infection, and reduced macrophages synthesis of cytokines and chemokines in comparison to complement-sufficient mice [98]. A study by Borders and colleagues [194] showed that mice deficient in the C5a receptor had exacerbated inflammatory responses with absence of granulomas. Thus, complement C5 is likely involved in the early initiation of the granulomatous response, possibly due to recruitment of cells that produce regulating proinflammatory cytokines to the site of inflammation.

Interleukin-6 (IL-6) is a proinflammatory mediator synthesized by fibroblasts, macrophages, B-cells, and T-cells that possibly plays a role in mycobacterial infections by its involvement in acute phase protein and immunoglobulin production, and adaptive cell activation [195]. *In vitro* studies possibly indicate a pathologic role for IL-6 in MTB infection, but these experiments need further clarification. IL-6 synthesis by macrophages infected with mycobacteria decreases T-cell responses [106] in addition to decreasing transcriptional activation of interferon- γ (IFN- γ) in uninfected, nearby macrophages [196]. *In vivo* studies have generated mixed results. One study found that

MTB infection was lethal for mice lacking IL-6 [107]; however, another investigation using IL-6 $-/-$ mice reported that the deficient mice were still able to control the infection despite delayed synthesis of IFN- γ [105]. Thus, IL-6 likely plays a role in the immune response to MTB, but its precise function is unclear.

Trehalose-6,6'-dimycolate (TDM) is a glycolipid found on the mycobacterial cell wall. Purified TDM causes lung pathology in mice that mimics some aspects of MTB infection, such as granuloma formation and induction of proinflammatory cytokines [197, 198]. TDM plays a number of important roles in the pathogenesis of MTB infection, including the induction of caseating granulomas [13, 110, 199]. The TDM model system may thus be a useful tool to explore the factors necessary for the early granulomatous response. The precise roles for TNF- α , C5a, and IL-6 in initiation and maintenance of the granulomatous response to isolated TDM are unknown; thus, experiments were conducted to explore the responses of mice lacking these critical immune factors.

Materials and Methods

Animals. Three-to-five week-old, wild-type, female C57BL/6 mice and mice lacking TNF- α (B6.129S6-Tnftm1Gkl/J), C5a (B10.D2-Hc0H2dH2-T18c/oSnJ), or IL-6 (B6.129S2-Il6tm1Kopf/J) were purchased from the Jackson Laboratories (Bar Harbor, ME). Animal experiments were performed under the approval of the UTHSC animal ethics committee (AWP 04-065). Four to six mice per group per indicated time points were used.

Preparation of bone marrow-derived macrophages, stimulation with TDM-coated microspheres, and MTB infection. Bone marrow-derived macrophages from wild-type C57BL/6 and complement factor C5a deficient (A/J) mice were generated by previously described methods [200]. Briefly, femurs were flushed with McCoy's media (Sigma, St. Louis, MO), and 2×10^6 cells were added to 24 well tissue culture plates (Corning Incorporated, Corning, NY). Cells were cultured in McCoy's media (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma), $100 \mu\text{g ml}^{-1}$ gentamycin (Sigma), 100 U ml^{-1} penicillin (Sigma), and 10 ng ml^{-1} recombinant murine granulocyte/macrophage colony stimulating factor (GM-CSF; Chemicon, Billerica, MA). The cells were cultured at 37°C in 5% CO_2 for seven days, with two media changes containing GM-CSF. Finally, cells were washed and suspended in Dulbecco's modified eagle's medium (DMEM; Sigma) supplemented with 10% FBS.

TDM-coated and BSA-coated microspheres were generated by previously described methods [112], and were a generous gift from C. Kan. The microspheres were added to the bone marrow-derived macrophages at a ratio of 10 beads per cell. IL-6 and TNF- α protein production was determined in the cell supernatants after a 72 hour incubation using an ELISA (see below).

MTB Erdman (ATCC 35801) was grown to log phase in Dubos broth (Difco, Franklin Lakes, NJ) with 7.5% dextrose and 5% BSA as supplements. Petroleum ether (Sigma) was used to extract mycobacterial surface lipids to generate delipidated MTB, as previously detailed [112, 114]. The bacteria were vortexed after adding petroleum ether for 2 minutes, after which they were incubated for 5 minutes at room temperature. The bacteria were then centrifuged for 10 minutes at 500 g. After removing the supernatants,

the extraction process was repeated two additional times followed by suspending the culture in phosphate buffered saline (PBS). Lipid extraction by petroleum ether does not alter the acid fastness of organisms or viability [112, 114]. Delipidated MTB were reconstituted by adding 0.01% (50 $\mu\text{g ml}^{-1}$) purified TDM (Sigma) in petroleum ether [112, 114]. The bacteria were resuspended in PBS after evaporation of the ether.

Preparation of TDM, tissue processing, and histopathology. A TDM oil-in-water emulsion was prepared by previously detailed methods [118], with minor modifications discussed below. Mice were intravenously injected with 50 μL of emulsion, generated by dissolving 25 μg of purified TDM (Sigma) in 9:1 hexane/ethanol, after which the solvent was evaporated. One μL of Drakeol (Penreco, Dickinson, TX) was homogenized into the TDM. Finally, 48 μL of 0.2% Tween 80 (Mallinckrodt, Hazelwood, MO) in PBS was admixed. Mice were sacrificed by cervical dislocation at days 0, 4, 7, and 14 after TDM challenge. Lung tissue was aseptically removed, weighed, and prepared for analysis of cytokines or fixed in 10% formalin for histology. The lung weight index (LWI) was calculated as an indicator of inflammation intensity [129, 194, 201] using the following formula:

$$\text{LWI} = \sqrt{\frac{\text{Lung weight (mg)}}{\text{Mouse weight (g)}/10}} / 10$$

Lung tissue (approximately 30 mg) was homogenized and put into 2 mL DMEM (Sigma) supplemented with 10% FBS, 0.01% L-Arginine (Sigma), 0.01% HEPES (Sigma), 50 $\mu\text{g/mL}$ gentamycin (Sigma) and 100 $\mu\text{g/mL}$ penicillin (Sigma). Samples were cultured for 4 hours at 37 °C with 5% CO_2 . The supernatants were kept at -20 °C until analysis by enzyme-linked immunoassay (ELISA). For histopathologic studies, the

left lung was placed in 10% formalin, sectioned (5 μ m thick), and stained with hematoxylin and eosin (H & E) per standard methodologies. The remainder of the lung was frozen in RNA-Bee (TelTest, Inc., Friendswood, TX) and stored at -80 °C for RNA studies. No significant differences were observed in histopathology or lung weight in mice given emulsion alone, as previously described [129].

ELISA analysis for lung cytokine and chemokine protein. Levels of interleukin-1 β (IL-1 β), IL-6, interleukin-10 (IL-10), TNF- α , interleukin-12p40 (IL-12p40), IFN- γ , and macrophage inflammatory protein-1 α (MIP-1 α) in lung homogenates were analyzed by a sandwich ELISA as per the manufacturer's instructions (R&D Systems, Minneapolis, MN). Briefly, capture antibody was coated on to Costar 96-well plates followed by washing (0.5% Tween-20 in PBS) and blocking (1% BSA, 5% sucrose, 0.05% NaN₃ in PBS). Lung homogenates were incubated at room temperature for 2 hours, after which biotin-conjugated secondary antibodies were used for detection with visualization by streptavidin-horseradish peroxidase (R&D) and TMB Microwell Peroxidase Substrate (KPL, Gaithersburg, MD). The reactions were stopped with 2 N H₂SO₄. The absorbance was read at 570 nm and 450 nm using an ELISA plate reader (Molecular Devices, Menlo Park, CA). The average of duplicate wells was generated using on a standard curve made for each assay with recombinant molecules supplied by the manufacturer (R&D). The lower limit of detection was 15 - 32 pg/mL.

Extraction of mRNA, reverse transcription, and real-time PCR. Sectioned lung tissue was minced in 1 ml of RNA-Bee, and RNA extracted using previously detailed methods [120]. cDNA was generated from 2 μ g RNA in First Strand buffer (250 mM Tris-HCL, pH 8.3, 375 mM KCl, 15 mM MgCl₂) (Invitrogen, Carlsbad, CA), with 2.5

mM deoxynucleotides-triphosphate (dNTPs) (Invitrogen), 0.1 M dithiothreitol (DTT) (Invitrogen), 20 U RNase inhibitor (Promega, Madison, WI), and 80 U random hexamer oligonucleotides (Roche Diagnostics GmbH, Mannheim, Germany). 200 U of SuperScript II reverse transcriptase (Invitrogen) was admixed after heating the reaction mixture to 70 °C for 5 minutes, followed by incubation at 42 °C for 50 minutes; the reactions were halted by heating to 70 °C for 15 minutes. The samples were diluted 1:8 with distilled water.

cDNA was quantified with the Taqman assay using fluorogenic probes with FAM (6-carboxyfluorescein) reporter and BHQ-1 (black hole quencher) quencher (Biosearch, Novato, CA). The primer and probe sequences are shown in Table 2.1. The reaction mixture contained 1X PCR Buffer (20 mM Tris-HCL pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 0.5% Tween 20, 0.5% Igepal CA-630) (5 Prime, Gaithersburg, MD), 200 nM dNTPs (Invitrogen), 1X ROX Reference Dye (Invitrogen), 400 nM of each primer (Integrated DNA Technologies, Coralville, IA), 100 nM probe, and 1 U/45 µL Taq DNA Polymerase (5 Prime). The ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) was used for amplification. The reaction cycle consisted of 95 °C for one minute, followed by 40 cycles of a 12 second step at 95 °C and a one minute step at 60 °C. Data were analyzed by the $-2^{\Delta\Delta C_T}$ method using β -actin message as the calibrator [202].

Statistics. The data are shown as the mean \pm one standard deviation. Two way ANOVA was used to determine differences between groups. A p-value <0.05 was considered significant.

<i>Protein</i>		<i>Primers</i>	<i>Probes</i>
β -actin	S ^a	5' TCTGGCTCCTAGCACCATGA	5' ATCAAGATCATTGCTCCTCCTGAGCGC
	AS	5' CCACCGATCCACACAGAGTACT	
IL-1 β	S	5' CTCATTGTGGCTGTGGAGAA	5' TGGCAGCTACCTGTGTCTTTCCCG
	AS	5' GGTGCTCATGTCCTCATCCT	
IL-6	S	5' CCCAATTTCCAATGCTCTC	5' TAGCCACTCCTTCTGTGACTCCAGCT
	AS	5' TGAATTGGATGGTCTTGGTC	
TNF- α	S	5' CCGATGGGTTGTACCTTGTCT	5' TCTTCAAGGGACAAGGCTGCCCC
	AS	5' TGGGTGAGGAGCACGTAGTC	
MIP-1 α	S	5' ACTAAGAGAAACCGGCAGAT	5' TGCCTGACTCCAAAGAGACC
	AS	5' TTCAGTTCCAGGTCAGTGAT	
IFN- γ	S	5' AGCAACAGCAAGGCGAAA	5' TCAAACCTGGCAATACTCATGAATGCATCCT
	AS	5' CTGGACCTGTGGGTTGTTGA	
IL-12p40	S	5' AAGTGTGAAGCACCAAATTACTC	5' ACGGTTACCGTGCTCATGGCT
	AS	5' TTCAAGTCCATGTTTCTTTGC	
IL-4	S	5' ATCGGCATTTTGAAACGAGGT	5' CAGGAGAAGGGACGCCATGCAC
	AS	5' GACGTTTGGCACATCCATCT	
IL-10	S	5' CAGCCGGGAAGACAATAACTG	5' CCCACTTCCCAGTCGGCCAG
	AS	5' CCGCAGCTCTAGGAGCATG	
TGF- β	S	5' CACTGGAGTTGTACGGCAGTG	5' TGAACCAAGGAGACGGAATACAGGGC
	AS	5' AGAGCAGTGAGCGCTGAATC	
IL-17	S	5' GCTCCAGAAGGCCCTCAGA	5' ACCTCAACCGTTCCACGTCAC
	AS	5' CTTTCCCTCCGCATTGACA	

^aS, sense; AS, antisense

Table 2.1. Oligonucleotide primers and probes.

Results

TDM induced inflammatory response in wild-type and C5a deficient bone marrow derived macrophages.

TDM is a major generator of proinflammatory cytokines from monocytic cells [118]. The amounts of IL-6 and TNF- α were significantly increased in the supernatants from both wild-type C57BL/6 and C5a-deficient A/J bone marrow derived macrophages stimulated with TDM coated microspheres (Table 2.2). Prior studies showed that removal of surface TDM from MTB resulted in a significantly diminished proinflammatory response [200]. A number of recent studies suggest that complement factors play an important role in this response [97, 194]; thus, IL-6 and TNF- α were also evaluated in bone marrow derived macrophages lacking complement C5a and infected with MTB, or organisms from which surface TDM (delipidated; d*Mtb*) was removed. C5a-sufficient derived cells were used as a comparison. Delipidation of both groups resulted in a significantly decreased cytokine response. Of importance, the C5a-deficient derived cell response was always significantly decreased compared to C5a-sufficient derived cells. The cytokine responses were almost completely restored when the cells were stimulated with organisms that had been reconstituted with purified TDM (r-d*Mtb*), suggesting the importance of TDM in generating proinflammatory responses to MTB.

Dysregulated TDM-induced responses in TNF- α , C5a, and IL-6 knock-out mice.

Lung weight indices (LWI) were determined as an indicator of general inflammation for wild-type and deficient mice after intravenous challenge with TDM

TNF-α	BSA beads	TDM beads	MTB	dMtb	r-dMtb	uninfected
C5a-sufficient	24 (2)	1294 (173)*	541 (70)	253 (39)**	461 (44)	19 (5)
C5a-deficient	14 (1)	322 (19)*	303 (50)	166 (32)**	339 (50)	10 (1)
IL-6						
C5a-sufficient	7 (1)	245 (4)*	335 (75)	102 (6)**	227 (14)	5 (1)
C5a-deficient	< 10	24 (4)	30 (1)	< 10	< 10	< 10

Table 2.2. TDM induced cytokine production in C5a-sufficient and C5a-deficient bone marrow derived macrophages. Production of TNF- α and IL-6 by bone marrow derived macrophages to TDM-coated beads, native MTB, delipidated Mtb (dMtb), delipidated Mtb reconstituted with TDM (r-dMtb), or untreated cells. Values were measured by ELISA and expressed as average pg protein/ 10^6 macrophages (\pm SD), three replicates per time point. * p <0.05 between treatment groups (TDM-coated vs. uncoated) as analyzed by Student's t test; ** p <0.05 between groups compared against dMtb or against TDM reconstituted organisms; all responses from C5a-deficient (A/J) derived BMMs were significantly lower (p <0.05) than the C5a-sufficient (C57BL/6) BMM treated in the identical manner.

(Table 2.3). The wild-type, C5a $-/-$, and IL-6 had significantly ($p < 0.05$) increased LWI at both 4 and 7 days post-TDM challenge compared to untreated controls, or in comparison to emulsion controls (data not shown, [97]). In comparison, the LWI of the TNF- α $-/-$ mice were unchanged after TDM-challenge, indicating that the TNF- α deficient mice did not generate a significant inflammatory reaction to TDM.

Histopathology is altered in TNF- α , C5a, and IL-6 deficient mice in response to TDM.

C57BL/6 mice form cohesive granulomas after intravenous challenge with TDM (Figure 2.1). Small, focal, monocytic clusters of cells were observed 4 days post-TDM challenge. The granulomas increased in both size and number by day 7. The granulomas were found in the lung parenchyma, without occlusion of lymphatics or blood vessels. The granulomatous response completely resolved by day 14.

The TNF- α $-/-$ mice did not produce a histopathological response to TDM, in contrast to the wild-type mice. Inflammation was not observed by day 4 after TDM challenge. On day 7, examination revealed a few cellular infiltrates that did not form obvious granulomas; infiltrating cells were primarily monocytic. Occlusion of vasculature, edema, and pneumonitis were not observed.

C5a deficient mice also did not generate granulomas to isolated TDM. However, the histopathology was markedly different from the TNF- α $-/-$ mice. The complement knock-out mice had a marked inflammatory response that was non-focal at day 4. They did not form cohesive cellular aggregates at day 7, and had a considerable amount of monocytic and lymphocytic infiltrates. The lung parenchyma demonstrated occluded

	<i>Day 0</i>	<i>Day 4</i>	<i>Day 7</i>	<i>Day 14</i>
wild-type	1.00 ± 0.033	1.31 ± 0.073	1.63 ± 0.232	1.06 ± 0.154
TNF- α -/-	0.98 ± 0.026	0.99 ± 0.012*	1.01 ± 0.010*	0.96 ± 0.035
C5a -/-	0.93 ± 0.144	1.32 ± 0.156	1.53 ± 0.157	1.04 ± 0.196
IL-6 -/-	1.02 ± 0.048	1.30 ± 0.051	1.55 ± 0.082	1.19 ± 0.216

Table 2.3. Lung Weight Indices Following TDM Administration. Lung weight

indices were calculated at days 0, 4, 7, and 14 post-TDM administration. Individual values are average responses (\pm SD) and compared to wild-type C57BL/6 mice (* $p < 0.05$) at each indicated time point.

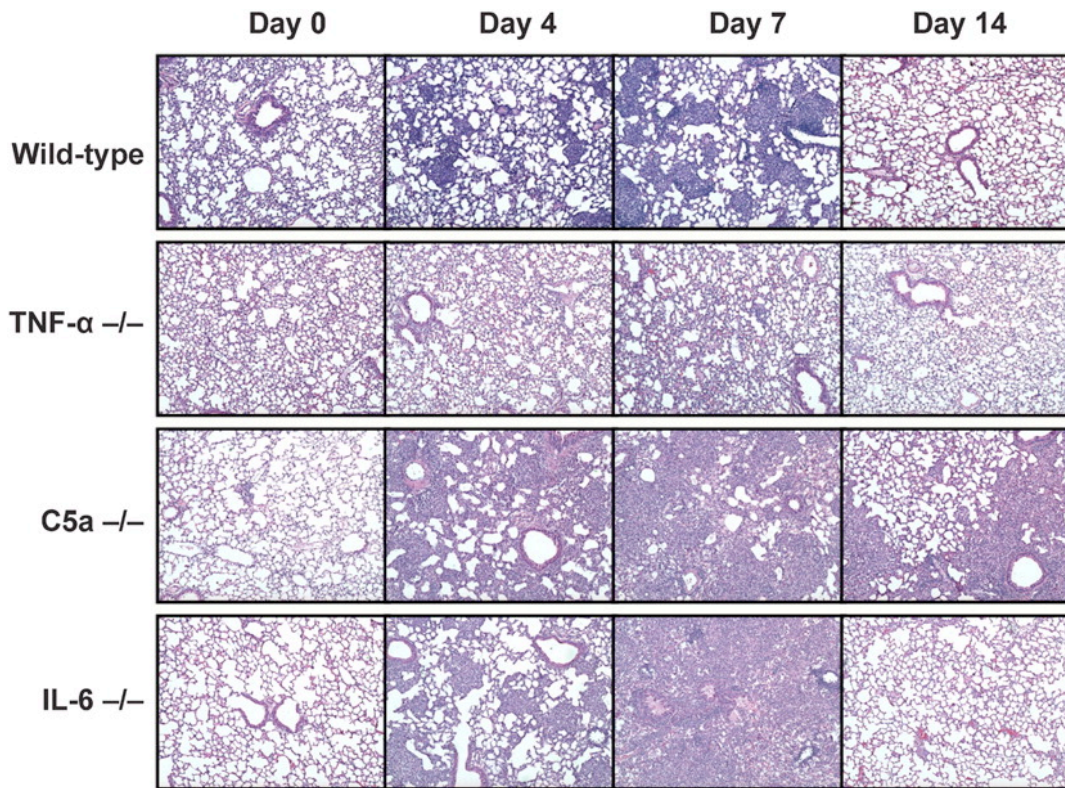


Figure 2.1. Granulomatous response to TDM in mice deficient in TNF- α , C5a, or IL-6. Wild-type mice challenged with mycobacterial TDM demonstrate small focal pulmonary granulomas by 4 days post-TDM administration, which peak at day 7 and resolve by day 14. In comparison, challenge of TNF- α -deficient mice did not elicit significant histopathology, with failure to mount significant inflammation in the lung. Mice with deficiency in C5a demonstrated a non-focal inflammatory response by day 4, and with no cellular aggregation of accompanying lymphocytic and monocytic infiltration or true resolution by 14 days post-challenge. The IL-6-deficient mice initiated granuloma histopathology, but by day 7 exhibited marked monocytic infiltration, lymphocytic cuffing around occluded vesicles, and edema with accompanied alveolar cell wall thickening. Sections representative of 4–6 mice per group. Hematoxylin and eosin staining; magnification $\times 40$.

vessels and areas of pulmonary edema. Incomplete resolution of the response occurred, with some degree of inflammation evident on day 14.

The IL-6 $-/-$ mice appeared to initially generate granulomas after TDM challenge, with monocytic, focal clusters visible at day 4. However, these mice did not maintain this response. Acute inflammation was increased with considerable monocytic infiltration apparent at day 7. Lymphocytic cuffing of occluded blood vessels was observed, along with alveolar cell wall thickening and edema. This response appeared to be transient, with resolution occurring by day 14.

Dysregulated cytokine and chemokine protein and mRNA expression in deficient mice following TDM challenge

Production of cytokine and chemokine proteins were evaluated in the lungs of mice administered TDM (Figures 2.2 and 2.3). Wild-type mice produced significantly higher ($p < 0.05$) IL-6, TNF- α , IL-1 β , IL-12p40, IL-10, and MIP-1 α on both days 4 and 7 in compared to naive mice. Additionally, there was a transient, significant, increase in IFN- γ synthesis on day 4. Cytokine protein levels returned to near normal levels by day 14 after TDM administration.

Lung cytokine protein production in control and deficient mice was markedly different 4 days post TDM challenge. The TNF- α knock-out mice failed to elevate levels of cytokines and chemokines after TDM challenge. The amount of IL-6, IL-1 β , IFN- γ , IL-12p40, IL-10, and MIP-1 α were not different compared to naive mice, and were significantly below values generated by wild-type mice at all time points. TNF- α was not produced by the TNF- α $-/-$ mice (data not shown).

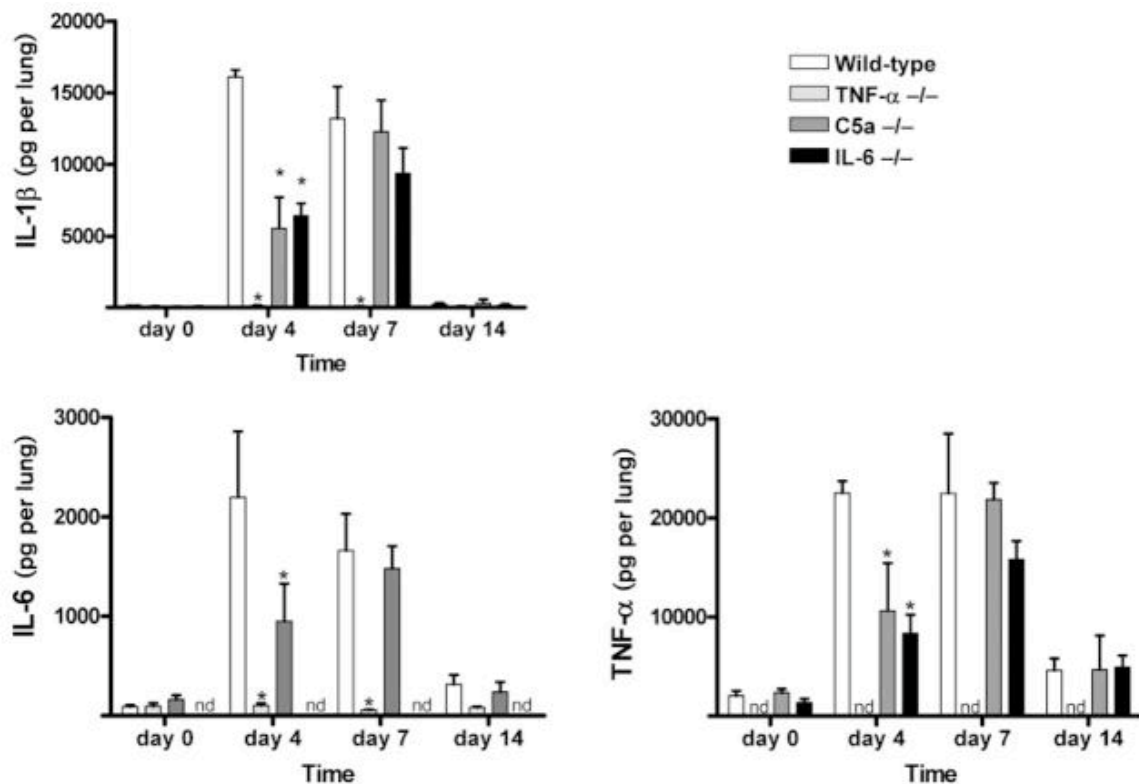


Figure 2.2. TDM-elicited proinflammatory mediator production from lungs of deficient and wild-type mice. Levels of pulmonary proinflammatory mediators IL-1 β , IL-6 and TNF- α were quantified post-challenge in the lungs of mice administered TDM. Mean values per lung are shown for mice deficient in TNF- α , C5a or IL-6 prior to challenge (day 0) and at 4, 7 and 14 days post-TDM challenge. Comparisons are made to the wild-type control animals administered TDM. Data are represented as the mean \pm SD for duplicate wells per mouse ($n = 4-6$ mice per group per time point). * $P < 0.05$; nd, none detected.

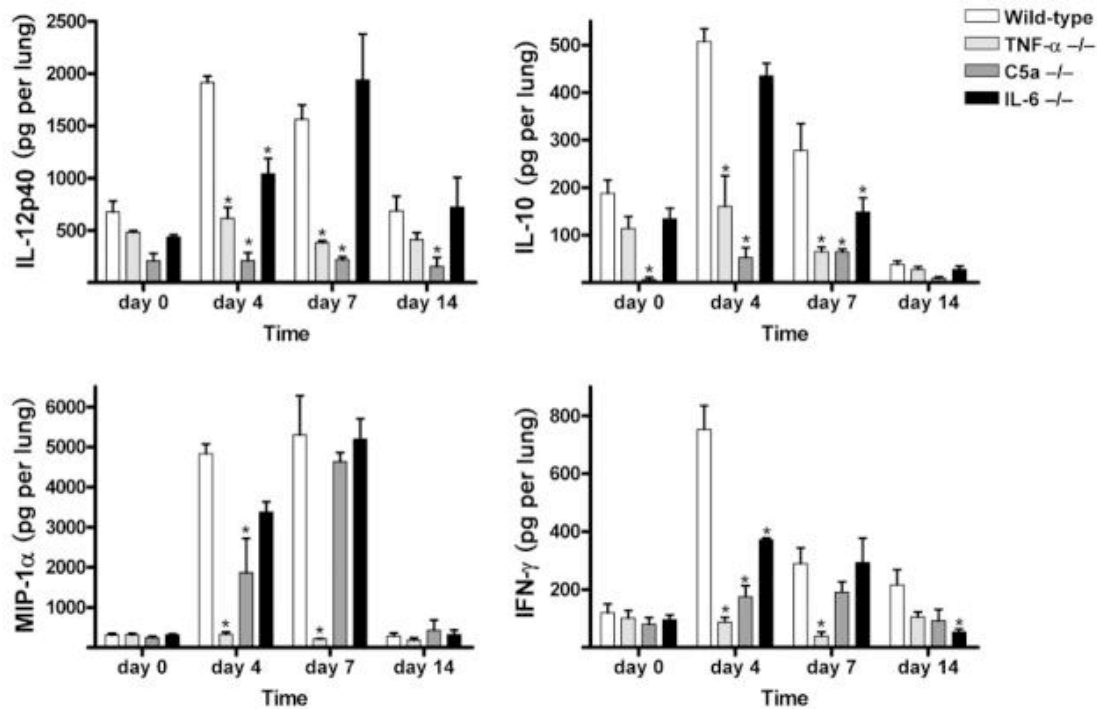


Figure 2.3. TDM-elicited cytokine production from lungs of deficient and wild-type mice. Levels of pulmonary cytokines IL-12p40, IL-10, MIP-1 α and IFN-1 γ were quantified post-challenge in the lungs of mice administered TDM. Mean values per lung are shown for mice deficient in TNF- α , C5a or IL-6 prior to challenge (day 0) and at 4, 7 and 14 days post-TDM challenge. Comparisons are made to the wild-type control animals administered TDM. Data are represented as the mean \pm SD for duplicate wells per mouse ($n = 4-6$ mice per group per time point). * $P < 0.05$.

The complement C5a knock-out strain had significantly decreased lung synthesis of IL-1 β , IL-6, TNF- α , IFN- γ , and MIP-1 α on day 4, in comparison to the wild-type mice. However, levels of protein became comparable to wild-type mice by day 7. MIP-1 α , a chemokine that directs migration of a variety of effector cells, was significantly decreased in the C5a deficient mice at day 4. Additionally, IL-12p40 was also significantly reduced on all time points compared to complement-sufficient mice.

The IL-6 $-/-$ mice also had decreased synthesis of IL-1 β , TNF- α , IL-12p40 and IFN- γ in the lungs 4 days after TDM administration. The levels of these cytokines were similar to the wild-type mice by day 7. IL-10 levels were significantly reduced at day 7 in the IL-6 knock-out mice. MIP-1 α levels were not significantly different from the wild-type mice on any time points tested. Production of IL-6 protein in the IL-6 deficient mice was below the limit of detection (data not shown).

Examination of mRNA in wild-type and deficient mice given TDM generated similar results to lung protein levels (Table 2.4). The wild-type C57BL/6 mice produced a significant increase (20-fold or greater) in proinflammatory message of IL-1 β , IL-6, TNF- α , and MIP-1 α , in comparison to naive or emulsion treated controls (not shown). A modest increase in IL-12p40 was noted on both days 4 and 7. There was additionally an early rise in expression of IL-10 and IFN- γ that decreased on day 7. The deficient mice had reduced expression of all cytokines examined, with the exception of IL-12p40, in comparison to wild-type mice. Significant differences in message levels in the knockout strains compared to wild-type control mice ($p < 0.05$) are shown in Table 2.4. Expression of IL-6 or TNF- α in the IL-6 $-/-$ mice or the TNF- α $-/-$ mice, respectively, was not observed.

	<i>IL-1α</i>	<i>IL-6</i>	<i>TNF-α</i>	<i>MIP-1α</i>	<i>IFN-α</i>	<i>IL-12p40</i>	<i>IL-10</i>
Day 4							
wild-type	23.89 \pm 9.37	32.01 \pm 7.71	20.71 \pm 3.67	29.57 \pm 10.59	23.09 \pm 14.60	2.99 \pm 0.32	29.17 \pm 17.71
TNF- α -/-	3.79 \pm 2.24*	5.45 \pm 3.41*	---	4.61 \pm 1.87*	4.12 \pm 1.76*	3.21 \pm 1.74	7.10 \pm 4.02*
C5a -/-	15.25 \pm 1.94*	16.57 \pm 6.76	31.34 \pm 13.99	19.59 \pm 20.04	12.43 \pm 2.10*	1.35 \pm 0.98	11.45 \pm 7.86*
IL-6 -/-	5.02 \pm 1.42*	---	5.72 \pm 1.50*	5.54 \pm 2.00*	7.02 \pm 2.48*	2.04 \pm 1.02	7.02 \pm 3.22*
Day 7							
wild-type	32.96 \pm 10.66	27.87 \pm 26.62	43.59 \pm 13.60	42.51 \pm 16.84	9.63 \pm 5.95	2.19 \pm 0.72	11.84 \pm 3.34
TNF- α -/-	2.54 \pm 1.73*	1.08 \pm 0.88*	---	2.32 \pm 1.77*	2.31 \pm 1.36	0.89 \pm 0.68	2.49 \pm 1.64
C5a -/-	15.94 \pm 4.17*	3.28 \pm 0.97 *	18.44 \pm 10.55*	16.36 \pm 10.88*	10.57 \pm 5.37	1.79 \pm 0.45	5.73 \pm 1.20
IL-6 -/-	5.04 \pm 1.77*	---	5.97 \pm 2.21*	7.04 \pm 1.89*	2.18 \pm 0.87	0.95 \pm 0.55	2.07 \pm 0.81
Day 14							
wild-type	0.61 \pm 0.20	0.53 \pm 0.05	10.29 \pm 15.73	0.72 \pm 0.56	1.75 \pm 1.04	1.09 \pm 0.29	0.67 \pm 0.30
TNF- α -/-	2.30 \pm 1.25	1.50 \pm 1.53	---	2.14 \pm 1.14	3.42 \pm 1.97	1.13 \pm 0.69	2.16 \pm 1.39
C5a -/-	0.68 \pm 0.55	0.44 \pm 0.22	0.63 \pm 0.60	0.94 \pm 0.55	1.37 \pm 1.48	1.58 \pm 1.19	1.79 \pm 2.24
IL-6 -/-	2.01 \pm 1.32	---	3.29 \pm 2.72	2.65 \pm 1.05	2.56 \pm 1.71	1.35 \pm 0.60	3.41 \pm 2.29

Table 2.4. Relative Change in mRNA following TDM administration.

Proinflammatory mediator mRNAs in lungs of mice deficient in TNF- α , C5a, or IL-6 were evaluated by RT-PCR and compared to wild-type controls, after challenge with TDM. Results shown are normalized to β -actin, and represented as fold-change from non-injected mice. Data are expressed as mean values with standard deviations. * p<0.05.

Discussion

Approximately one-third of the world's population is considered latently infected with MTB [203]. Granuloma formation is an essential host response for containment of the bacilli and requires concerted regulation of immunological mediators. An increasing number of chemotherapeutics that target cytokines for the treatment of immune-mediated diseases makes it essential to explore how cytokines are involved in the initiation and maintenance of MTB-induced granulomas. For example, use of the TNF- α neutralizing antibody infliximab resulted in modulated inflammatory patterns that caused reactivation of latent pulmonary TB and greater occurrences of extrapulmonary disease [204]. Furthermore, cytokines are being considered as adjunct therapeutics to enhance antibiotic efficacy for TB treatment in cases of multidrug-resistant strains and immunodeficient patients [205, 206]. An understanding the implications of cytokine dysregulation on granulomatous responses is essential when evaluating treatment for these individuals.

TNF- α is a central mediator in initiation of granuloma formation. TNF- α deficient mice did not generate granulomas following challenge with MTB, instead forming necrotic lesions lacking epithelioid cells [207]. The TNF- α deficient mice in the results reported in this study did not generate a histological response following challenge with TDM, accompanied by markedly reduced cytokine protein and message synthesis. Of importance, these deficient mice also had decreased amounts of the Th1 cytokines IL-12p40 and INF- γ . A study by Kindler and colleagues [190] noted that antibody neutralization of TNF inhibited granuloma formation following infection with BCG and dissolved chronic granulomas. Transgenic mice producing high levels of human soluble

TNF receptor 1 also did not form granulomas to BCG, accompanied by delayed synthesis of both IL-12p40 and IFN- γ [208]. The absence of granuloma generation in TNF- α $-/-$ mice is possibly a result of an inability to synthesize a cytokine cascade that includes chemokines that recruit both monocytes and T-cells to lung tissue. TNF neutralization in macrophage cultures infected with MTB resulted in a decrease in synthesis of CCL5, CXCL9, and CXCL10 [209]. The same investigation reported that CD11b $^{+}$ cells recovered from mice deficient in the 55 kDa TNF receptor and infected with MTB had delayed synthesis of chemokines. Additionally, TNF- α is an essential regulator of Th1 immune responses necessary for control of mycobacterial infections. Treatment of macrophages with a TNF-neutralizing antibody or macrophages deficient in the TNF receptor 1 did not synthesize IL-12 following BCG infection [210]. TNF- α was necessary for the initiation of IL-12-induced Th1 responses in BALB/c mice [211]. Furthermore, Ahlers et al. [212] reported that a synergism between TNF- α and IL-12 was essential to promote the IL-12R β 2 chain and IFN- γ to induce the development of Th1 cells. The essential role of T cells in TDM-induced granulomas has been explored [120, 213], with T helper cells of central importance for the generation of hypersensitive pathology [120, 214].

The studies reported here suggest that complement C5a is necessary for development of cohesive granulomas following challenge with mycobacterial glycolipid TDM, once the response is initiated. C5a deficiency resulted in development of pneumonitis pathology accompanied by edema and monocytic infiltration with incomplete resolution of inflammatory responses, suggesting a role for C5a in granuloma maintenance and resolution. Furthermore, the complement knock-out mice

had reduced early synthesis of proinflammatory cytokines accompanied by consistently decreased production of IL-12p40. A/J mice, which are deficient in the fifth component of complement along with other defects, failed to generate granulomas and had increased mortality to MTB infection [98]. A study using the same C5a deficient mice used in this investigation reported increased MTB growth in lung tissue in addition to failing to form granulomas [97]. Both investigations reported early decreases in cytokines and chemokines, similar to results presented here for the chemokine MIP-1 α . Complement C5 is necessary for variety of additional processes that are possibly involved in granuloma formation or maintenance. C5a promotes NF κ B, a regulator of expression of a number of inflammatory mediators, including TNF- α and IL-6 [215]. Both C5a and C5b induce adhesion molecule expression on neutrophils, endothelial cells, and macrophages [216, 217]. Finally, C5 is necessary for IL-12 generated cell-mediated immunity [218].

The investigations presented here additionally suggest that IL-6 plays a role in maintenance of established granulomas, but not in their initiation. The IL-6 $-/-$ mice generated similar granulomas to wild-type mice after TDM challenge at day 4. Of significance, this occurred in the presence of decreased levels of proinflammatory cytokines. However, the granulomatous response was not maintained through day 7, despite levels of cytokines similar to those of wild-type mice. The IL-6 deficient mice had severe, edematous pathology; a recent investigation demonstrated that IL-6 is necessary for regulating local concentrations of glucocorticoids that modulate pathological responses [219]. Investigations on the role of IL-6 in control of mycobacterial infections have yielded conflicting results. Even less is known of the role

IL-6 plays in granuloma formation during MTB infection. Ladel and coworkers [107] reported that IL-6 deficient mice had significantly higher bacterial loads and ultimately succumbed to infection, while Saunders and colleagues [105] demonstrated that mice lacking IL-6 were able to contain the infection in the presence of higher bacterial burdens and decreased early IFN- γ production. The conflicting results between these two studies may be due to the number of organisms and routes used for the infectious challenge. Further complicating interpretation, *M. avium*-infected mice treated with IL-6 neutralizing antibodies had increased bacterial loads; however, no differences in granuloma size or number were observed [220]. Infection of IL-6 knock-out mice with *M. avium* resulted in fewer necrotic lesions in comparison to wild-type mice [221]. IL-6 $-/-$ mice infected with *Rhodococcus aurantiacus*, an organism that produces short chain TDM and induces Th1 granulomas that are similar to those induced by mycobacterial organisms, reported increased inflammatory infiltrates after challenge and granulomas that were increased in size and had central necrosis in comparison to the wild-type mice [222]. IL-6 is generally thought of as a Th2 cytokine [195, 223]. However, this may not be a stand-fast rule in all models of intracellular infections [224]. IL-6 has been reported to play a role in the development of T-cell responses against *M. avium* [220]. It is well-established that maintenance of the granulomatous response requires antigen-specific T-cells [225]. Thus, IL-6 is possibly a regulator of T-cell responses that are essential for maintenance of established granulomas, especially when persistent antigen is present.

The TDM model of granuloma formation mimics in part certain aspects of mycobacterial histopathology seen during aerosol challenges of mice. Thus, this model may be useful for investigations of the roles of complement and certain cytokine

mediators involved in the early response to mycobacterial glycolipid antigens. The investigations reported here suggest that TNF- α is an essential mediator required in initiation of TDM-induced granulomas (Figure 2.4). Once the granulomas have been initiated, mediators such as complement component C5a and IL-6 generate secondary responses. C5a is most likely is an important regulator of responses that generate cohesive granuloma pathology, while IL-6 is involved in the maintenance of established granulomas. Deficiency of any of these mediators results in an inability to effectively generate and maintain a granulomatous response, and would thus also likely be important in the control of mycobacterial infections.

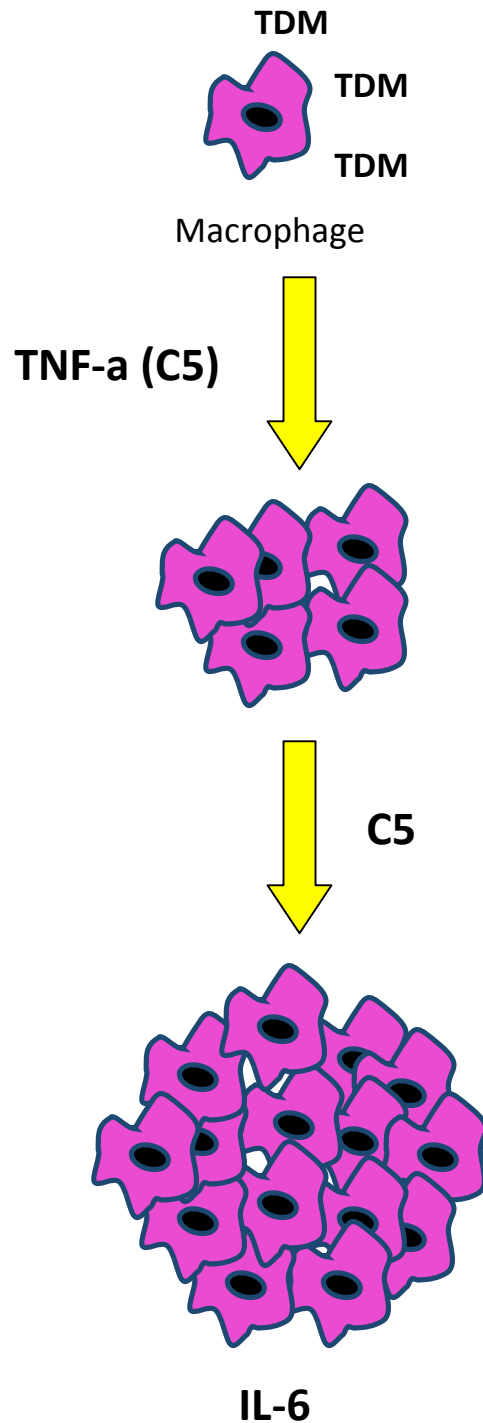


Figure 2.4. Proposed mechanism for TDM-induced granulomas. TNF- α is essential for a histological response to TDM. Once a response has been initiated, complement C5a and IL-6 recruit cells that produce regulating cytokines and upregulate integrin that allow cells to form organized, cohesive structures.

Chapter Three

Lactoferrin decreases mycobacterial antigen induced lung immunopathology

The studies presented in this chapter were published in the journal Translational Research, 2010 156: 207 – 15 [226]

Introduction

Tuberculosis (TB) causes approximately 1.8 million deaths annually, making it the leading cause of death worldwide due to bacterial infections [203]. Additionally, roughly one-third of the world's population is considered latently infected with *Mycobacterium tuberculosis* (MTB) [203]. Effective chemotherapy for TB is available; however, treatment requires at least six months of a complex multi-drug regimen for which effectiveness is threatened due to the emergence of multi-drug resistant organisms. Unfortunately, there have been no new commercial antibiotics against TB created since rifampin was invented in the 1960s [3]. Therefore, new agents with activity against MTB or a favorable effect when used in combination with the standard anti-TB drugs are desperately needed.

The normal, immune competent host responds to infection with MTB by forming granulomas, which structurally consist of central infected macrophages that can differentiate into foamy macrophages, epithelioid cells, and Langhan's giant cells [17]. A layer of fibrosis develops and surrounds the infected macrophages, which prevents bacterial dissemination. However, granulomas also result in physical separation of infected cells from effector lymphocytes that are capable of eliminating infected cells [17]. Additionally, the TB bacilli enter a state of non-replicating persistence within granulomas; these organisms are resistant to sterilizing antimicrobials [131, 132]. Most granulomas heal, but viable MTB persist. Individuals at this stage of infection are

known as latently infected because they do not have clinical symptoms of disease, sputums do not contain acid-fast bacilli, and chest X-rays are stable [227]. However, a small percentage of patients experience reactivation of latent disease that histologically is characterized as a lipid pneumonia that may undergo necrosis to generate cavitory lesions [11]. The resulting lung damage causes a productive cough that generates infectious aerosols, which may infect a new individual. Therefore, modulation of TB-induced inflammatory pathology will likely decrease morbidity, facilitate improved response to antibiotics, and limit disease transmission.

Increasing evidence identifies lactoferrin as a modulator of the immune response to a number of inflammatory and injurious agents. Lactoferrin is a component of the transferrin family of glycoproteins and is produced in neutrophil granules and mucosal secretions [140]. Lactoferrin receptors are found on numerous cells of the immune system, such as macrophages, dendritic cells, and T-cells [144-146]. Lactoferrin effects include macrophage activation, enhancement of polymorphonuclear cell phagocytosis, increasing killer cell activity, and maturation of B- and T-cells [148, 149, 228-230]. Of significance, lactoferrin alters macrophage cytokine production depending on the environmental context [142, 156, 231]. For example, addition of lactoferrin to macrophages stimulated with low levels of LPS produced increased levels of proinflammatory cytokines [175]. However, macrophages fully stimulated with LPS and given lactoferrin had decreased cytokine production. Of importance to the studies proposed here, lactoferrin protects against immune-mediated tissue damage in a number of model systems. Mice injected with LPS and treated with lactoferrin had decreased gut tissue destruction and increased survival [140, 183]. Additionally, lactoferrin as an

adjuvant to the BCG vaccine resulted in decreased lung damage and increased protection following an aerosol TB challenge [232].

Trehalose-6'6-dimycolate (TDM) is the most abundant glycolipid produced by MTB. TDM has multiple hypothesized roles in MTB pathogenesis [13], including involvement in the formation of lung caseation [110]. TDM prepared in emulsion form and injected into mice intravenously induces lung pathology that mimics certain aspects of MTB infection, such as production of proinflammatory cytokines and granuloma formation. The TDM granuloma model has been used to explore the immunological mediators involved in initiation of the granulomatous response [119, 130]. TDM induces foreign body granulomas in naïve mice [118, 233], and hypersensitive granulomas in previously sensitized mice [69, 110, 121, 213]. Thus, this model system may be used to explore the potential of immunomodulators to modulate granuloma structure.

Materials and Methods

Animals. Four-week old, female C57BL/6 mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Animal studies were conducted under approval by the UTHSC Institutional Review Board (protocol number HSC-AWP 07-093). Eight mice were used per group, for the time points indicated.

Lactoferrin and MTB. Bovine-derived lactoferrin (>95% purity, <20% iron saturated, <0.2 endotoxin units/mg) was purchased from PharmaReview Corporation (Houston, TX). Lactoferrin was dissolved in phosphate buffered saline (PBS), and stored at -80°C until use.

MTB Erdman (American Type Cell Culture 35801) was grown in 7H9 broth (Remel, Lenexa, KS) with 10% supplement at 37°C to logarithmic phase. The MTB were diluted in 1X PBS using McFarland standards (Sigma, St. Louis, MO) to 3×10^8 bacteria per ml. The final bacterial concentration was confirmed by plating serial dilutions onto 7H11 plates (Remel), and counted after incubating at 37°C for 3-4 weeks.

Generation of bone marrow-derived macrophages (BMM), stimulation with TDM-coated beads, and MTB infection. BMMs from C57BL/6 mice were generated by previously detailed procedures [155]. McCoy's medium (Sigma) was used to flush femurs, followed by adding 5×10^5 of the recovered cells to 24-well tissue culture plates (Corning, Corning, NY). The cells were cultured in McCoy's medium supplemented with 10% fetal bovine serum (FBS) (Sigma), antibiotics (100 µg/ml penicillin G and 50 µg/ml gentamycin) (Sigma), and 10 ng/ml recombinant murine granulocyte/macrophage colony stimulating factor (GM-CSF) (Cell Sciences, Canton, MA). Cells were cultured at 37°C with 5% CO₂ for one week, with two additional media changes containing GM-CSF. The cells were washed one time with 1X PBS and the media changed to Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 0.01% HEPES (Sigma), and 0.01% L-arginine (Sigma). The cells were rested overnight and then stimulated with TDM-coated microspheres or infected with MTB.

TDM- and BSA-coated beads were generated as per previously described methods [23], and were a generous gift from C. Kan. The beads were briefly sonicated and then added to the BMMs at a ratio of 10 beads per one cell with or without 100 µg/ml lactoferrin that was added simultaneously with bead stimulation or three hours later. Alternatively, the BMM were infected with MTB using a multiplicity of infection

(MOI) of 1:1 in the presence or absence of lactoferrin (100 $\mu\text{g/ml}$) added at the same time as infection or three hours after. For both bead stimulation and infection, the supernatants were collected after 24 and 72 hours of incubation and analyzed for cytokine protein production by ELISA (see below).

In vivo challenge with TDM, tissue processing, and lung immunopathology. An oil-in-water emulsion of TDM was generated by previously described methods [118, 119]. Briefly, mice were intravenously injected in the tail vein with 50 μl of the emulsion. The TDM emulsion was generated by dissolving 25 μg of purified TDM (Sigma) in 9:1 hexane/ethanol, after which the solvent was evaporated. Two percent Drakeol (Penreco, Dickinson, TX) was homogenized into the TDM. Finally, 0.2% Tween 80 (Mallinckrodt, Hazelwood, MO) in PBS was admixed. A control emulsion lacking TDM was also generated. Subsets of mice were given either 1 mg of lactoferrin or 1 mg of bovine apo-transferrin (Sigma) in 100 μl of PBS that was administered intravenously 24 hours after TDM challenge.

Mice were sacrificed at 0, 4, and 7 days after challenge with TDM. The lungs were immediately removed, weighed, and sectioned for cytokine analysis or histological studies. Approximately 30 mg of lung tissue was minced and put into 2 ml of DMEM and cultured at 37 °C for 4 hours. The supernatants were collected and stored at -20 °C until ELISA analysis for cytokine protein production. For histological studies, the left lung was fixed in 10% buffered formalin, sectioned (5 μm), and stained with hematoxylin and eosin per standard methods. The entire 5 μm lung section for at least 6 mice per group was photographed with a 2x objective. A quantitative analysis was performed with the NIH Image J program (U.S. National Institutes of Health, available

at <http://rsb.info.nih.gov/nih-image/>). The total number of granulomas was counted over an entire section of the lung. The average granuloma size was calculated using a random selection of 10 granulomas in 3 different microscopic fields for each mouse.

The lung production of IL-1 β , IL-6, TNF- α , IL-12p40, IL-10, IFN- γ , and TGF- β was determined using a sandwich ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The average of duplicate wells was determined by a standard curve created for each assay by use of manufacturer supplied recombinant molecules. The limit of detection sensitivity was 15-32 pg/ml, according to the manufacturer.

Statistics. The data are shown as the mean \pm 1 SD. Two-way ANOVA was used to evaluate the differences between groups using GraphPad Prism software (GraphPad Software, La Jolla, CA). Statistical significance was defined as a p-value of < 0.05 .

Results

Lactoferrin modulates bone marrow-derived macrophage inflammatory response to isolated TDM.

TDM induces proinflammatory cytokine production by macrophages [17, 118, 200]. TNF- α , IL-6, and IL-12p40 production were significantly increased in BMMs stimulated with TDM-coated microspheres, in comparison to macrophages given BSA-coated beads (Figure 3.1). In contrast, TGF- β production significantly decreased with TDM-bead challenge. Treating the BMM with 100 μ g/ml bovine lactoferrin simultaneously with TDM bead stimulation or three hours later resulted in markedly decreased synthesis of TNF- α , IL-6, and IL-12p40 that was accompanied by increased

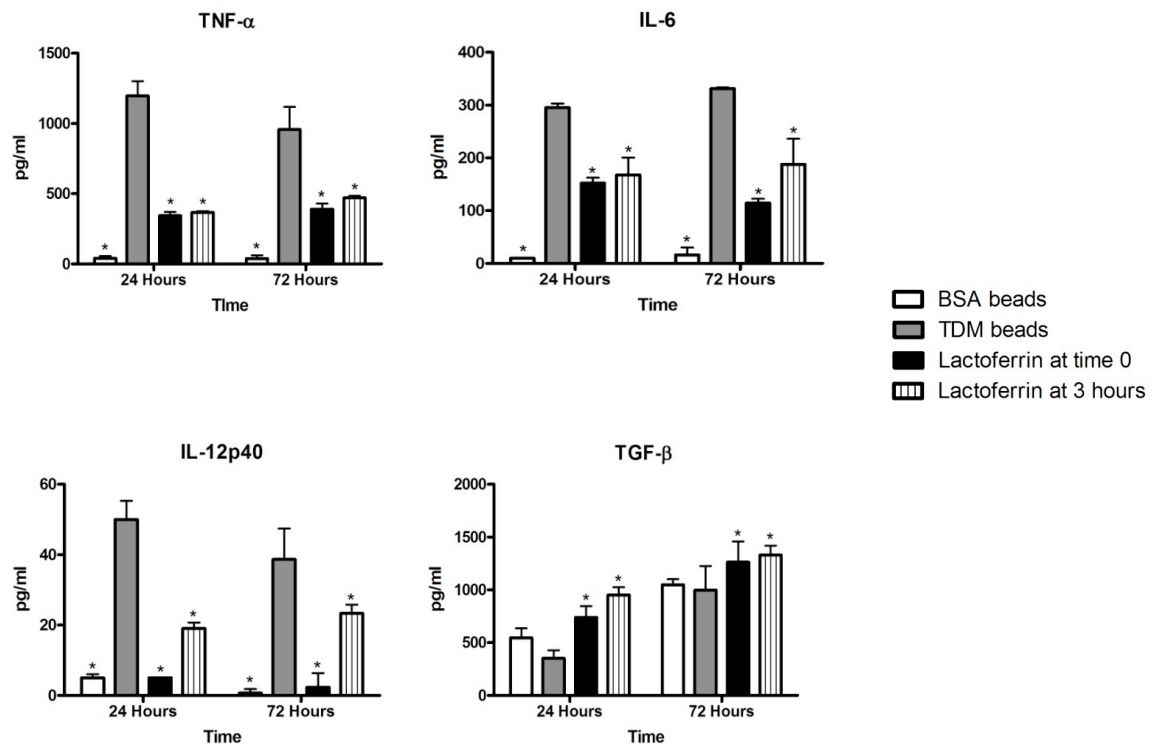


Figure 3.1. Lactoferrin modulation of TNF- α , IL-6, IL-12p40, and TGF- β production by BMM in response to TDM-coated beads. Cells were treated with 3 μ m BSA- or TDM-coated beads alone at a ratio of 10:1, 100 μ g/ml lactoferrin at the same time as beads stimulation, or 100 μ g/ml lactoferrin 3 hours after bead addition. Values were measured by ELISA and expressed as mean pg/ml with SD. * $p < 0.05$ compared to treatment with TDM-coated beads alone.

production of TGF- β . TGF- β was elevated when the cells were stimulated with control BSA-coated beads and given lactoferrin, as has been previously described [157].

Lactoferrin decreases TDM-induced immunopathology.

C57BL/6 mice challenged with TDM generated lung granulomas following a single intravenous dose. Focal, small, monocytic clusters were visible by 4 days after challenge with TDM (Figure 3.2A). Granuloma size and number increased by day 7 (Figure 3.2B) and was accompanied by other pathological manifestations, such as perivascular cuffing of lymphocytes. Mice given an emulsion control without TDM did not generate granulomas and had normal lung histology. Mice administered TDM and treated with 1 mg of intravenous bovine lactoferrin 24 hours after challenge had smaller and fewer granulomas, with the granulomatous response significantly decreased at 7 days. This response is not likely attributed to lactoferrin's iron binding capacity because mice given transferrin, an iron-binding control, did not have altered granuloma size or number.

Lung cytokine protein levels in mice challenged with TDM and treated with lactoferrin.

The levels of lung cytokines were examined in mice administered TDM (Figures 3.3 and 3.4). Mice challenged with the TDM emulsion had significantly increased levels of IL-1 β , IL-6, TNF- α , IL-12p40, and IFN- γ at 4 and 7 days post-challenge in comparison to mice given an emulsion control without TDM (Figure 3.3). Mice given 1 mg of bovine lactoferrin intravenously 24 hours post-TDM administration produced

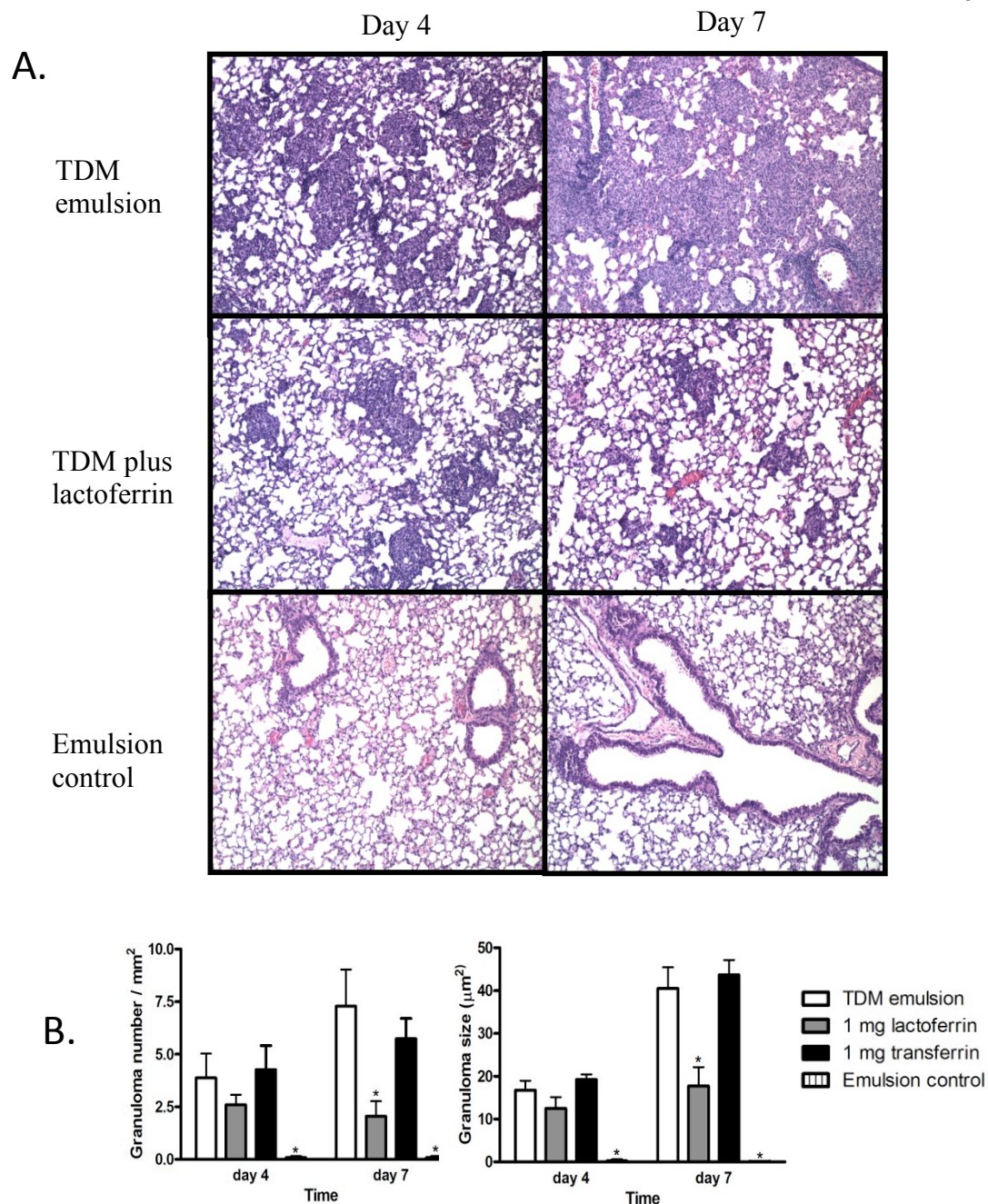


Figure 3.2. Granulomatous response to TDM in lactoferrin treated mice. A) Mice were challenged with 25 μg TDM prepared in a water-in-oil emulsion. One mg of lactoferrin or transferrin (not shown) was administered 24 hours post-TDM challenge. Hematoxylin and eosin staining, magnification 100X. **B)** Number of lung granulomas per mm² and the size of the granulomas (μm²). Granulomas were enumerated and measured as described in the materials and methods section. Data are expressed as the mean ± SD. * p<0.05 with comparisons made to the mice administered the TDM emulsion.

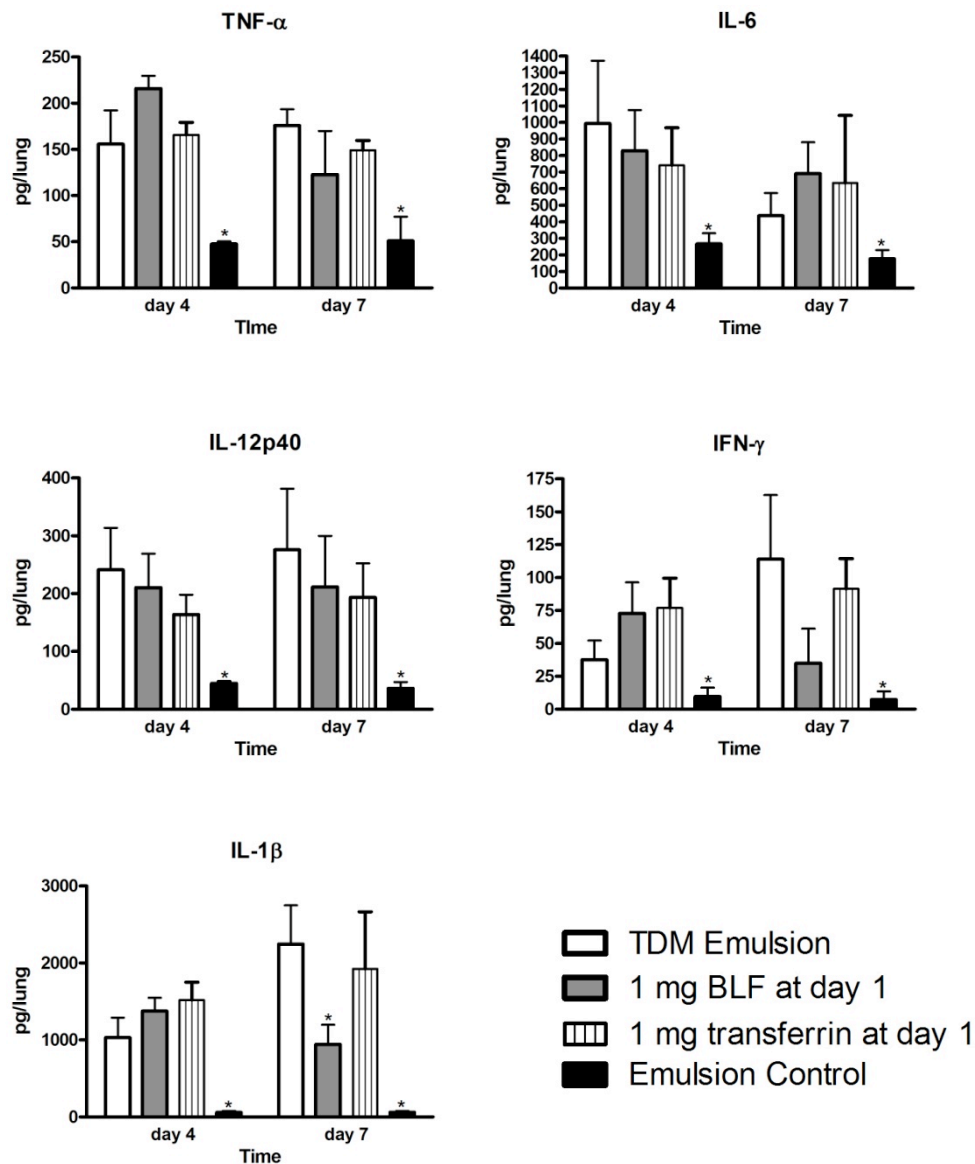


Figure 3.3. Production of pro-inflammatory mediators in mice challenged with TDM alone or with lactoferrin. The levels of TNF- α , IL-1 β , IL-6, IL-12p40, and IFN- γ in lung tissue were determined by ELISA. Data are shown as the mean with standard deviation. N = 8 mice per group, per time point. * $p < 0.05$ with comparison to the TDM emulsion treated mice.

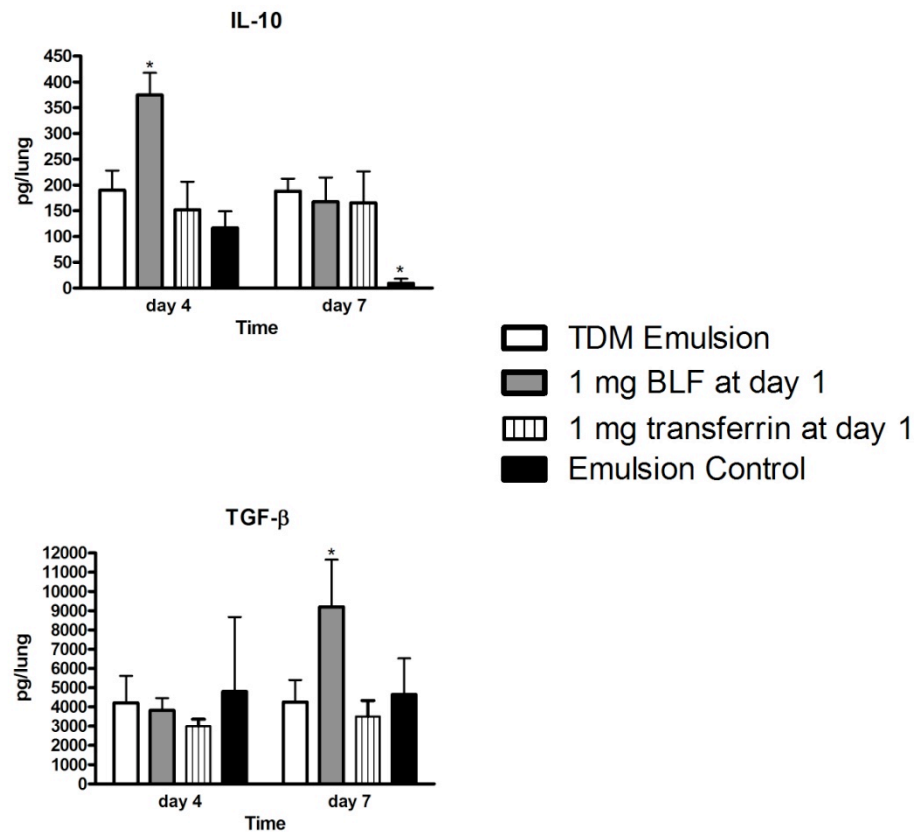


Figure 3.4. Lactoferrin induced production of anti-inflammatory cytokines in TDM challenged mice. The levels of IL-10 and TGF- β were quantified in lung tissue homogenates by ELISA. Data are expressed as the mean with standard deviation. N = 8 mice per group, per time points indicated. * $p < 0.05$, comparisons made to the TDM-treated mice.

similar levels of most pro-inflammatory cytokines, except for decreased IL-1 β at day 7. Of potential significance, the levels of TNF- α and IFN- γ were not significantly altered in the mice treated with lactoferrin. In comparison, the amounts of IL-10 and TGF- β , cytokines that can have anti-inflammatory functions, were elevated at day 4 and day 7, respectively, in the mice treated with lactoferrin (Figure 3.4). Mice given transferrin 24 hours after the mice were challenged with TDM produced similar levels of cytokines in comparison to the mice administered a TDM emulsion alone.

Lactoferrin modulation of BMM cytokine production in response to mycobacterial infection

Certain proinflammatory cytokines such as TNF- α are of critical importance for host defense during MTB infection [190, 207, 234]. Thus, there was concern that the decrease in TNF- α production from BMMs stimulated with TDM-coated beads and treated with lactoferrin may produce favorable conditions for MTB proliferation within host cells. Thus, C57BL/6 BMMs were infected with MTB in the presence or absence of lactoferrin. Production of cytokines in response to MTB infection is presented in Figure 3.5. TNF- α levels were significantly increased in BMMs treated with lactoferrin either simultaneously with bacterial seeding or three hours after. IL-6 was significantly decreased, and IL-12p40 production was not altered. Additionally, TGF- β was significantly elevated at 72 hours post-infection. These data suggest a potential for lactoferrin to decrease the cytokines responsible for tissue destruction while maintaining production of those essential for the generation of responses necessary for protection

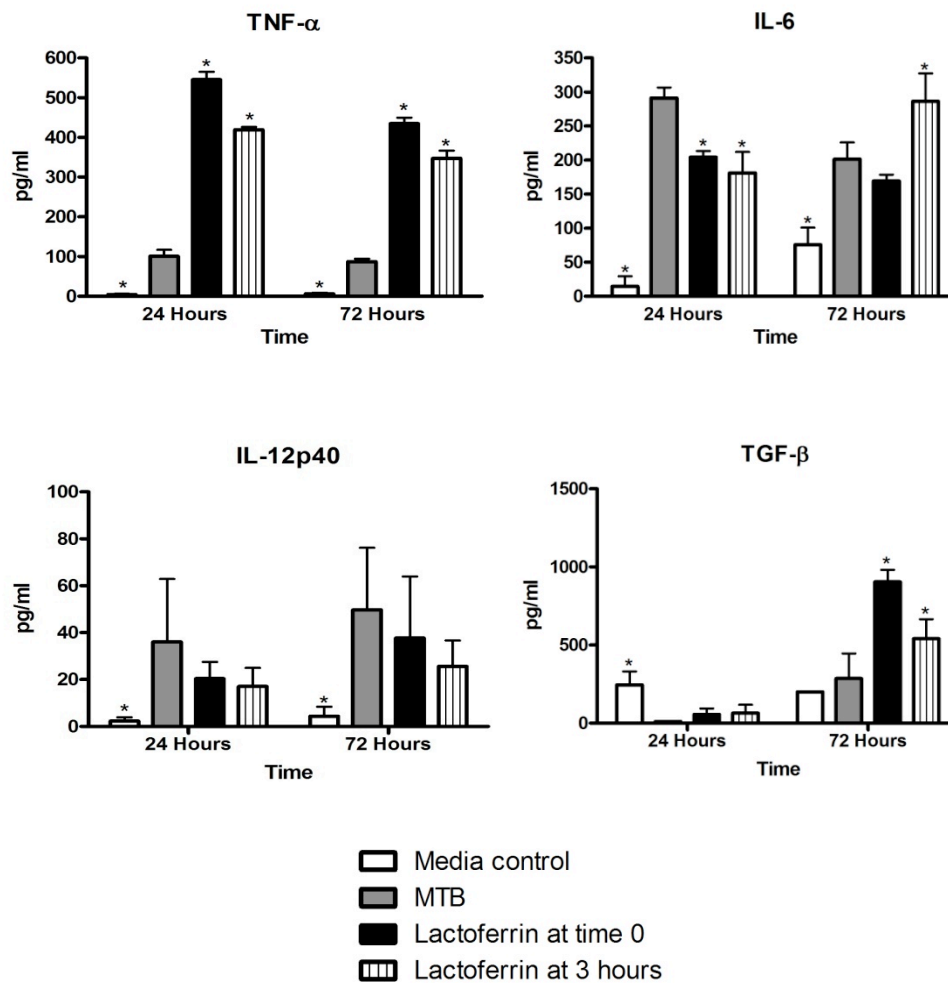


Figure 3.5. Lactoferrin modulation of BMM cytokine production in response to MTB infection. BMM were infected with MTB Erdman using a MOI of 1:1. 100 $\mu\text{g/ml}$ of bovine lactoferrin was added at the same time as infection or 3 hours after infection. The levels of TNF- α , IL-6, IL-12p40, and TGF- β were measured in the filtered supernatants 24 and 72 hours after infection by ELISA. Data are shown as the mean and SD. * $p < 0.05$, comparisons are to cells infected with MTB alone.

against mycobacteria. Lactoferrin alone does not alter the proliferation of MTB within macrophages (Chapter 4).

Discussion

MTB causes approximately 1.8 million deaths annually and infects nearly one-third of the world's population [203]. The number of cases of this disease continues to rise, possibly due to the emergence of multi-drug resistant organisms and HIV co-infection [133]. The therapy for TB is complex, lengthy, and requires antibiotics that have significant side-effects, often resulting in poor compliance to treatment. The World Health Organization's directly observed therapy short-course (DOTS) program is effective, but puts a significant financial burden on the resource poor countries that are disproportionately impacted by TB. Therefore, new agents that can shorten TB treatment are needed. Agents with immunomodulatory functions, such as lactoferrin, are a novel approach to TB treatment in that they may be able to circumvent drug resistance. Additionally, lactoferrin's ability to decrease immune-related pathology may enhance antibiotic penetration into tissue and augment the generation of immune responses that can kill latent bacilli [131, 133].

TDM is a highly immunostimulatory glycolipid synthesized by MTB. Challenge with a single dose of TDM results in recruitment of immune cells to lung tissue accompanied by production of cytokines that are similar to those synthesized during natural TB infection [17, 110]. The data presented here demonstrate that mice treated with lactoferrin following challenge with TDM had decreased lung immunopathology at the height of the granulomatous response. Alteration of granuloma structure has a

number of potential implications for the therapy of both active and latent TB. MTB within granulomas convert to a state of non-replicating persistence that is characterized by changes in biochemical pathways and decreased replication [15, 131, 132].

Antimicrobial agents require actively replicating bacilli for effectiveness and are therefore unable to kill the dormant MTB isolated within granulomas. Additionally, it is hypothesized that decreasing lung immunopathology may enhance the penetration of antibiotics within infected tissue, thereby enabling a faster response to TB chemotherapy [15, 133]. Use of TB antibiotics in combination with immunomodulators has generated promising results in human clinical studies. One clinical trial evaluated addition of the soluble TNF- α receptor etanercept to antibiotic therapy in HIV patients with TB; there was significantly lessened time to sputum culture conversion accompanied by improved chest x-rays in the patients given the combination of anti-TB agents and etanercept [135]. Another trial explored combining high-dose prednisolone with anti-TB agents in patients co-infected with HIV and TB [136]. Sputum culture conversion rate was significantly increased in patients treated with prednisolone, in comparison to antibiotics alone. Use of lactoferrin in combination with TB therapy has numerous advantages over other immunomodulators in use because it does not suppress the immune system and has a proven safety record in a number of animal models and human studies [134, 138, 139, 235, 236].

A hypothesized mechanism for the reduction in lung pathology in lactoferrin treated mice is the increased synthesis of TGF- β and IL-10, cytokines that have essential roles in decreasing pathology due to the immune system [58]. It is possible that T_H2 cytokines may decrease protective immune responses to TB [131]. However, the

cytokines considered critical for host defense against TB, IFN- γ and TNF- α [46], were not significantly altered by treatment with lactoferrin. IFN- γ is necessary for control of mycobacterial burden; an example of the importance of this cytokine is the severe mycobacterial infections that occur in patients deficient in IFN- γ signaling factors [237]. TNF- α is necessary for initiation and maintenance of granulomas; deficiency of TNF- α results in reactivation of latent disease and severe necrotic pathology [15, 46, 238]. Lactoferrin's ability to generate an anti-inflammatory response simultaneously with pro-inflammatory cytokine synthesis has been described in other studies [140]. For example, lactoferrin administered orally to rats in a model of colitis increased the Th2 cytokines IL-4 and IL-10 [186], in addition to decreased immune-mediated pathology in mouse LPS sepsis models [183, 239]. In comparison, lactoferrin increased the delayed-type hypersensitivity response to ovalbumin, BCG, and sheep red blood cells [170, 171, 228]. Lactoferrin's ability to produce pro-inflammatory and anti-inflammatory responses makes it an ideal immunomodulator for a number of inflammatory and infectious diseases.

We report that macrophages treated with lactoferrin and infected with MTB produced higher levels of TNF- α . This is in marked contrast to the overall reduction in proinflammatory cytokines when the BMM were stimulated with TDM-coated microspheres and given lactoferrin. This is possibly attributed to the fact that MTB produces a variety of immunostimulatory moieties in addition to TDM, such as ligands for the toll-like receptors, NOD-like receptors, and C-type lectins [123]. Of significance, these experiments found that lactoferrin alone did not alter MTB growth in BMMs. However, lactoferrin does have an impact on the growth of MTB in

macrophages, but only if the macrophages are activated with IFN- γ in addition to lactoferrin treatment (Chapter 4). The data presented here on infected macrophages indicate the necessity to determine the impact of lactoferrin in MTB infection models.

The TDM granuloma model mimics certain aspects of the immune responses observed in murine aerosol infections. This model system is thus ideal for screening immunomodulatory agents that may alter TB-induced lung pathology. These studies suggest that lactoferrin may decrease lung pathology in a TB granuloma model while maintaining protective immune responses. Lactoferrin may thus have potential advantages as an adjuvant therapeutic in the treatment of TB.

Chapter Four

Lactoferrin as a therapeutic to modulate *Mycobacterium tuberculosis* induced immunopathology

Introduction

Tuberculosis (TB) remains a significant global public health burden. There are approximately 9.27 million new cases of this disease and nearly two million deaths each year [1]. Over 95% of drug-susceptible *Mycobacterium tuberculosis* (MTB) can be cured with a four-drug regimen of isoniazid, rifampin, pyrazinamide, and ethambutol for two months followed by four additional months of isoniazid and rifampin [2]. However, the lengthy, complex drug regimen required to treat TB is difficult to administer in the resource poor countries that TB disproportionately affects. Furthermore, the efficacy of the available drug regimen is threatened by the emergence of multi-drug resistant strains [1]. The last major advancement in TB therapeutics was made when rifampin was introduced in the 1960s [7]. Thus, there is considerable need to develop novel agents for the treatment of TB.

Th1 responses are critical for host defense against MTB [46]. MTB infection begins with uncontrolled growth of the TB bacilli within macrophages. An innate granuloma forms due to the accumulation alveolar and recruited systemic macrophages. Dendritic cell presentation of TB antigens to naive CD4⁺ T-cells in the presence of IL-12 generates a Th1 immune response [240]. Interferon-gamma (IFN- γ) production by Th1 cells activates macrophages, resulting in phagosome acidification, phagolysosome fusion, and synthesis of reactive nitrogen species that kill MTB [46]. CD4⁺ T-cells are also essential for the generation of CD8⁺ cytotoxic T-cells that may play a role in control of disease progression [46]. An example of the importance of the CD4⁺ T-cell response can be found in humans with advanced HIV, who develop disseminated mycobacterial infections when CD4⁺ T-cells reach low levels [45]. Additional immune

system responses are also likely important. For example, a number of studies have highlighted the role of IL-17 in the immune response against MTB [64, 65, 241]. Specifically, early enhancement of IL-17 responses by use of an IL-23 producing adenovirus during MTB infection resulted in decreased bacterial burden and reduced lung pathology [67]. Therefore, immunomodulatory agents useful in control of MTB infection are expected to produce a strong Th1 response, and perhaps IL-17 mediated responses.

Modulation of immune-mediated pathology while preserving essential immune responses may represent a novel therapeutic strategy for the treatment of TB. The immunocompetent host responds to MTB infection by the formation of granulomas, which may prevent dissemination of the bacilli [17]. However, MTB within granulomas are protected from immune-mediated killing due to the sequestration of infected macrophages from immune effector cells [15]. MTB within granulomas adapt by conversion to a dormancy phenotype that includes decreased replication and changes in biochemical pathways, making the bacilli relatively resistant to the action of sterilizing antimicrobials [131, 132]. Furthermore, decreasing lung immunopathology may enhance antibiotic penetration in infected tissue, thus promoting a faster response to antimycobacterials [15, 133].

A number of studies suggest that lactoferrin has a number of immune modulating properties that may be favorable to the host during MTB infection [140]. Lactoferrin is an iron-binding glycoprotein member of the transferrin family that is found in mucosal secretions and neutrophil granules. Specific effects of lactoferrin include macrophage activation, enhancement of phagocytosis, and enhancement of the delayed type

hypersensitivity response to a number of antigens [140]. Importantly, lactoferrin enhances Th1 immune responses in a number of model systems, a response essential for host defense against MTB [134, 140, 155, 157, 242, 243]. Critical to the studies proposed here, lactoferrin has been shown to protect against immune-mediated tissue damage. For example, mice treated with lactoferrin had increased survival and decreased gut tissue destruction after LPS injection [183]. Additionally, lactoferrin added to the BCG vaccine resulted in increased protection against an aerosol TB challenge, with evidence of decreased lung damage [232].

These studies explored the ability of lactoferrin to modulate lung pathology during a mouse of MTB infection using a rapidly proliferating variant of MTB Erdman. We found that lactoferrin added to the drinking water during MTB infection decreased organ bacterial burden and lung immunopathology. Lung immune responses were explored to determine the ability of lactoferrin to promote protective Th1 and IL-17 mediated responses.

Materials and methods

Animals. Four week-old, female C57BL/6 mice were purchased from Jackson Laboratories. All studies were conducted under the approval from the animal ethics committee at the UTHSC, protocol AWC-08-050. Four to six mice were used per group, per time points indicated. All MTB infections occurred in biosafety level 3 facilities.

Lactoferrin and MTB. Bovine-derived lactoferrin (15 – 20% iron saturated, <0.2 endotoxin units/mg) was supplied by PharmaReview Corporation (Houston, TX). A rapidly-proliferating variant of MTB Erdman (TMC 107, American Type Cell Culture)

was cultured in Middlebrook 7H9 broth with 10% supplement (5% bovine serum albumin, 2% dextrose, and 0.5% Tween 20 in distilled water) to log phase. Pelleted bacteria were resuspended in phosphate buffered saline (PBS) and diluted to 3×10^8 colony forming units (CFU) per ml using McFarland standards. Bacteria were sonicated to disperse aggregates. The bacterial CFUs were confirmed by plating serial dilutions on Middlebrook 7H11 agar plates (Remel, Lenexa, KS), which were incubated at 37 °C for 3 – 4 weeks.

Acute tuberculosis infection of mice. MTB strain Erdman was cultured to log phase as described above. C57BL/6 mice were infected using an aerosol inhalation exposure system (GLAS-COL Model #A4212 099c) to achieve an aerosol implantation of 100 CFUs. The inoculation dose was confirmed by sacrificing a subset of mice at day one post-challenge, and the lung bacterial load determined by plating lung homogenates onto 7H11 agar plates. The mice were randomized to either untreated controls or mice treated with lactoferrin at the start of infection to determine the effect of lactoferrin prior to development of histopathology, or beginning 7 days later to evaluate the impact of lactoferrin on established infection. Lactoferrin was administered in the drinking water at 5 mg/ml to give a dose of approximately 20-25 mg per mouse, assuming mice drink 4-5 ml of water per day [244], as was done in prior studies of oral lactoferrin-mediated immune modulation [180, 245]. Mice were sacrificed at 1, 2, and 3 weeks post-infection. The lung, spleen, and liver were aseptically removed, placed into 5 ml of PBS, and homogenized. Serial dilutions of organ homogenates were plated onto Middlebrook 7H11 agar plates and incubated for 3 – 4 weeks at 37 °C.

Lung histopathology analysis. Lung tissue was fixed in 10% formalin and embedded in paraffin. Five μm thick sections were stained with hematoxylin and eosin by standard methods. Acid-fast staining was performed by the Zhiel-Neelsen method. Twelve lungs from each group pooled from two different experiments at three weeks post-challenge were randomly selected for immunohistochemistry (IHC) for CD4 and CD8. Rat anti-mouse monoclonal antibodies (R&D Systems, Minneapolis, MN) were used to detect CD4⁺ and CD8⁺ cells, with detection using an anti-rat horseradish peroxidase 3,3'-diaminobenzidine cell and tissue staining kit according to the manufacturer's instructions (R&D Systems).

All images of histopathology were obtained with the Nuance multispectral imaging system (CRI, Woburn, MA). This technology allows quantification of cell types in defined regions of pathology. The entire region of the immunostained lungs was captured using the 10x objective. Enumeration of normal lung, area percentages of macrophages and lymphocytes, and total number of CD4⁺ and CD8⁺ cells was done using the tissue and cell segmenting abilities of Inform software (CRI) as detailed by the manufacturer's instructions.

Lung cytokine expression. Total lung RNA was isolated by homogenizing lung tissue in 1 ml of RNA-Bee (Tel-Test, Friendswood, TX), followed by addition of 0.1 ml of chloroform (Sigma). The samples were mixed, incubated on ice for 15 min, and centrifuged for 15 min at 13,000 rpm. The supernatants were removed and placed into an equal volume of isopropanol for overnight precipitation. The samples were centrifuged at 13,000 rpm for 15 min. The resulting RNA pellets were washed with 75% ethanol and resuspended in water with 1 mM EDTA. cDNA was generated by mixing 2

μg of RNA into first strand buffer (Invitrogen), 2.5 mM dNTPs (Invitrogen), 0.1 M DTT (Invitrogen), 80 U random hexamer (Roche Diagnostics, Mannheim, Germany), and 20 U RNasin (Promega, Madison, WI). The reaction mixture was heated to 70°C for 5 min, followed by addition of 200 U SuperScript II RT (Invitrogen). The mixture was heated to 42°C for 50 min, and then 70°C for 15 min to terminate the reaction.

Analysis of cDNA was performed using the Taqman assay. The sequences of primers and probes (FAM reporter and BHQ-1 quencher) for the cytokines are shown in Table 2.1. The reaction mixture contained 5 μl cDNA, 1X PCR buffer (5Prime, Fisher Scientifics, Pittsburg, PA), 200 nM dNTPs (Invitrogen, Carlsbad, CA), 400 nM each for the forward and reverse primer (Integrated DNA Technologies, San Diego, CA), 100 nM probe (Biosearch Technologies, Novato, CA), 1x ROX reference dye (Invitrogen), and 1 U/50 μl Taq DNA polymerase (Fisher). The reaction was performed using the ABI Sequence Detection System (Applied Biosciences, Carlsbad, CA) by heating for 1 min at 95 °C, and then 40 cycles of 95 °C for 12 seconds and 60 °C for 1 min. Data are expressed as fold change expression relative to naive controls after normalization to β-actin [202].

Preparation of lung digests and ELISpot analysis. Lung tissue from infected mice was removed, minced, and incubated at 37°C with 30 μg/ml DNase (Roche Diagnostics, Mannheim, Germany) and 1 mg/ml type I collagenase (Worthington Biochemical Corporation, Lakewood, NJ) for 60 min on a rotating shaker. The lung digests were passed through a 40 μm filter (Fisher) followed by centrifugation at 1500 rpm. Red blood cells were lysed with ACK lysing buffer (Lonza, Walkersville, MD). The cells were washed 2 times with PBS and counted using a hemacytometer. One hundred μl of

lung digests or splenocytes (2×10^5 cells) (see above) were examined for IFN- γ and IL-17 producing cells (eBioscience, San Diego, CA) and CD4⁺ IFN- γ cells (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Recall response to MTB antigens. Recall responses by splenocytes to heat-killed MTB was performed to evaluate lactoferrin modulation of the systemic immune response to MTB. Spleens were harvested from infected mice at the times points designated above. The tissues were minced using a glass homogenizer. Red blood cells were lysed with ACK lysing buffer (Lonza), followed by washing twice with PBS. After counting with a hemacytometer, cells were resuspended in Dulbecco's modified eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% FBS, 0.01% HEPES (Sigma), and 0.01% L-arginine (Sigma). Cells were plated into 24-well tissue culture plates at a concentration of 2×10^6 cells/ml. Heat-killed MTB was added at a MOI of 10:1, generated by autoclaving MTB suspended in 1xPBS at 121°C for 10 mins. Subsets of cells were stimulated with 2 μ g/ml conA or 10 ng/ml LPS as controls. Supernatants were collected at 72 hours post-stimulation, filtered with a 2 μ m filter, and analyzed by ELISA for IL-17, IFN- γ , IL-10, IL-12p40, TNF- α , IL-6, and TGF- β (R&D Systems).

Proliferation of MTB in broth and macrophage culture in the presence of lactoferrin. MTB were grown in 7H9 broth alone, with 100 μ g/ml lactoferrin, or with 1 mg/ml of lactoferrin. Bacteria were sonicated every 4 hours, the OD₆₀₀ obtained, and serial dilutions plated on 7H11 agar plates. The plates were incubated at 37 °C for 3 – 4 weeks.

For macrophage proliferation studies, the J774A.1 (ATCC TIB-67) were cultured

in antibiotic-free DMEM with 2% FBS in 24-well tissue culture plates at a concentration of 5×10^5 cells/ml. MTB Erdman were added at a MOI of 1:1. Subsets of cells were given combinations of 10 ng/ml recombinant IFN- γ (Cell Sciences, Canton, MA), 100 μ g/ml lactoferrin, and 1 mM of the NO synthetase inhibitor N-mono-methyl-arginine (Sigma). The cells were lysed every 48 hours with 0.05% SDS, neutralized with 15% bovine serum albumin (Sigma), and the lysates plated onto 7H11 agar plates. The cell supernatants were also removed, filtered with a 0.2 μ m filter and examined for IL-6, TNF- α , IL-12p40, and TGF- β production by ELISA (R&D Systems). Production of NO was assessed by the Griess reaction as previously described [246]. Briefly, 50 μ l of the culture supernatants were mixed with 50 μ l of Griess reagent (1% sulfanilamide and 0.1% naphthyl-ethylenediamine dihydrochloride in 1 N HCL). The optical density at 550 nm was determined. NaNO₂ was used to produce a standard curve.

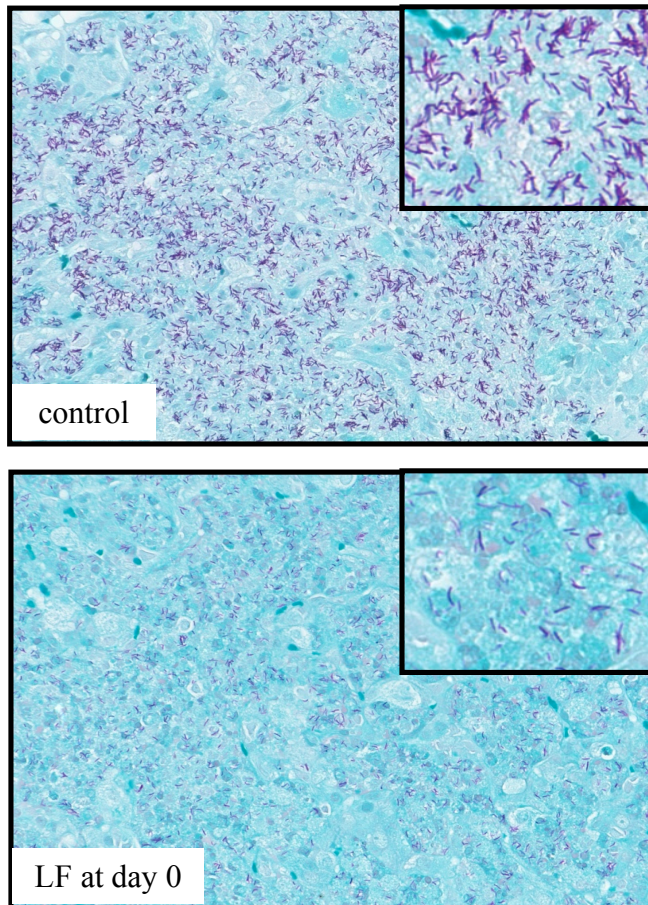
Statistics. The data are shown as the mean \pm SD. Two-way ANOVA was used to determine the differences between groups by use of GraphPad Prism software. A p-value of less than 0.05 was defined as statistically significant.

Results

Lactoferrin decreases bacterial burden in a mouse model of MTB infection

Acid-fast staining of lung tissue was performed by the Zhiel-Neelsen method (Figure 4.1A). Control mice administered tap water had numerous acid-fast bacilli in the lung at three weeks post-challenge with a highly virulent variant of MTB Erdman. However, mice treated with lactoferrin had markedly fewer organisms visible on acid-

A.



B.

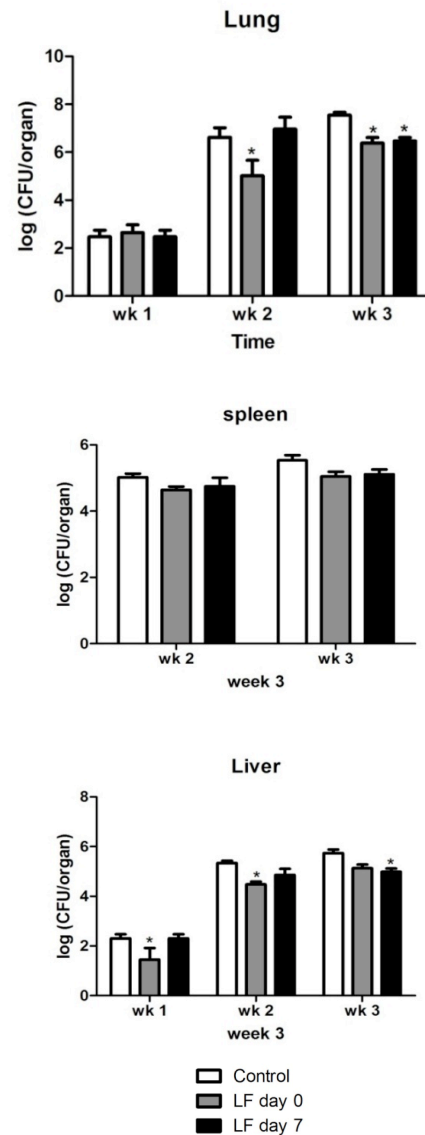


Figure 4.1. Decreased organ bacterial burden in lactoferrin treated mice. A.

Acid-fast staining in control mice three weeks post-infection demonstrates numerous acid-fast bacilli. A similar region of histopathology in mice given lactoferrin at day 0 shows markedly fewer bacteria. **B.** Mice treated with lactoferrin at day 0 had significantly reduced bacterial burden in the lung at weeks 2 and 3 post-challenge, accompanied by decreased CFUs in the liver compared to mice given tap water. Mice started on lactoferrin at day 7 had significantly decreased lung and liver CFUs at week 3. Data are presented as the mean and SEM, $n = 6$ mice per group, per time point. $*p < 0.05$ with comparisons to control mice.

fast staining in regions with comparable histopathology. Bacterial CFUs in the lung, liver, and spleen are shown in Figure 4.1B. Specifically, control mice administered tap water had lung \log_{10} CFUs of 2.5 ± 0.7 at week 1, 6.6 ± 0.9 at week 2, and 7.5 ± 0.3 at week 3. In comparison, the mice treated with lactoferrin at the start of infection had significantly fewer lung CFUs at week 2 (5.0 ± 1.4) and week 3 (6.4 ± 0.7). Mice treated with lactoferrin beginning at day 7 after established infection at day 7 had significantly reduced lung CFUs at week 3 (6.5 ± 0.4). Bacterial growth in the spleen was not observed until week 2 and was not significantly different between the two groups. Mice treated with lactoferrin at day 0 had significantly reduced liver CFUs at weeks 1 and 3 compared to control mice. Mice given lactoferrin at day 7 had significantly decreased liver bacterial loads only at week 3.

Lactoferrin reduces MTB induced lung immunopathology

Representative images of lung histopathology at three weeks post-challenge using hematoxylin and eosin staining are shown in Figure 4.2. Control mice given tap water have severe inflammation with nearly complete lung occlusion and large areas of pulmonary edema (Table 4.1 and Figure 4.2A). High power images demonstrate a predominance of macrophages, cellular debris, and tissue beginning to undergo necrosis (Figure 4.2B). Few areas of lymphocytes were observed, with relatively few CD4⁺ and CD8⁺ cells visualized with IHC (Table 4.1).

In contrast, mice treated with lactoferrin had significantly reduced lung occlusion compared to control mice (Figure 4.2C and Table 4.1). There was significantly

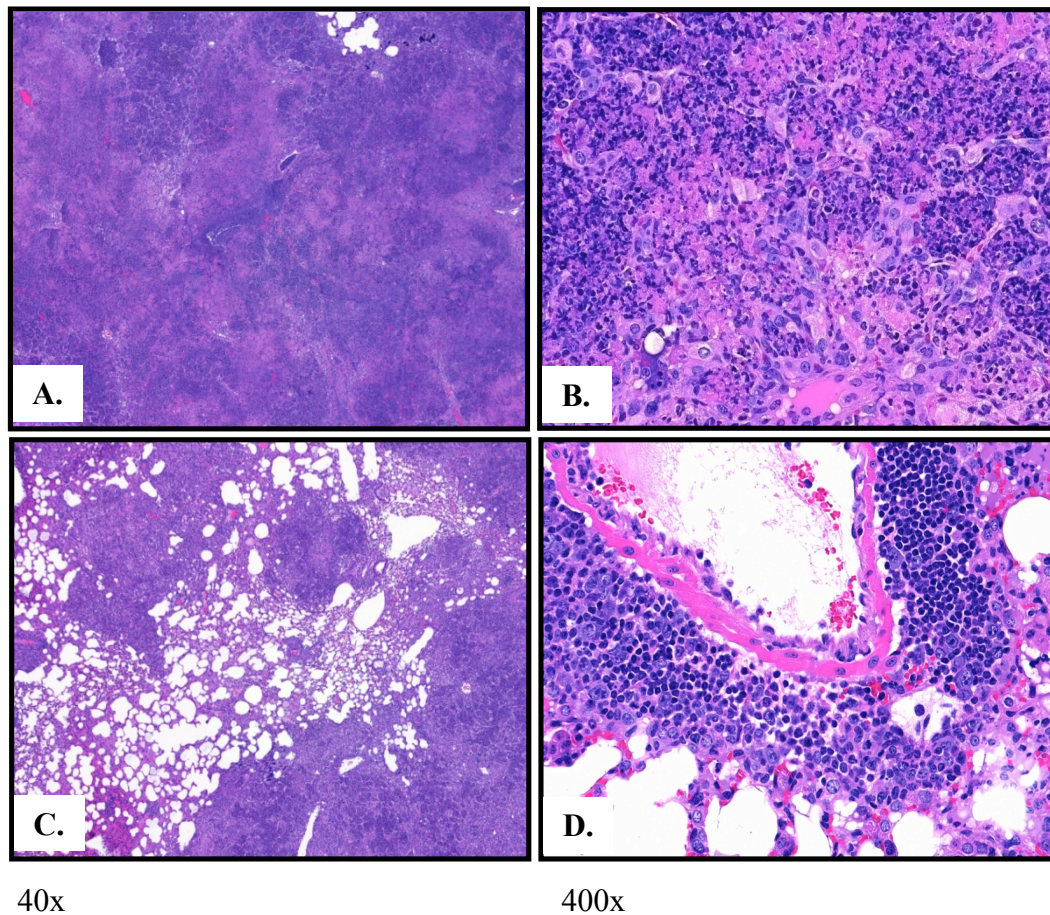


Figure 4.2. Histopathology in control (A & B) and lactoferrin treated mice beginning at day 0 (C & D) at three weeks post-infection. A. Control mice demonstrate nearly complete lung occlusion, 40X. **B.** High power image from control mice shows large numbers of macrophages, edema, and cellular debris, 400X. **C.** Mice treated with lactoferrin have significantly less areas of inflammation and occlusion, 40X. **D.** Mice treated with lactoferrin have clusters of lymphocytes, fewer areas of foamy macrophages, and less edema, 400X. n = 6 mice per group.

Table 4.1. Lactoferrin decreases lung immunopathology during MTB infection

	Control	LF at day 0	LF at day 7
% lung occlusion	89.4 ± 4.8	*71.6 ± 6.0	*79.0 ± 1.8
% macrophages	79.5 ± 11.5	*33.0 ± 16.8	*52.3 ± 8.3
% lymphocytes	4.1 ± 5.6	*22.6 ± 7.4	*25.9 ± 8.1
CD4+ lymphocytes	175.3 ± 292.6	*1425.0 ± 589.0	*1843 ± 511.1
CD8+ lymphocytes	30.0 ± 27.3	*379.2 ± 237.3	*598.8 ± 488.3

* p< 0.05 compared to control mice

decreased overall lung inflammation, less pulmonary edema and occluded vasculature, and reduced areas of macrophages in mice started on lactoferrin at day 0 and day 7 post-infection. Clusters of lymphocytes were frequently observed (Figure 4.2D). The majority of the lymphocytes were CD4⁺ cells; abundant CD8⁺ cells were observed as well.

Lactoferrin enhances IFN- γ mediated killing of MTB and modulates cytokine production in macrophage culture

Studies were undertaken to determine if lactoferrin has a direct effect on MTB proliferation. The results of lactoferrin's effect on MTB growth in broth culture are shown in Figure 4.3A. A physiologic concentration of lactoferrin (100 μ g/ml) did not alter the growth of MTB in 7H9 broth using either the OD₆₀₀ or CFUs. Only a very high, non-physiologic concentration (1mg/ml) had a slight inhibitory effect on MTB growth, possibly due to iron sequestration.

MTB proliferation with or without lactoferrin in macrophage culture was also assessed (Figure 4.3B). Lactoferrin alone did not affect MTB growth in naive J774 cells. However, there was enhanced early killing when lactoferrin was used in combination with IFN- γ . This effect is possibly mediated by NO, as macrophages given the combination of lactoferrin and IFN- γ produced significantly more NO compared to control and activated macrophages (Figure 4.4A). The addition of the NO synthetase inhibitor N-mono-methyl-arginine abolished the enhanced MTB killing effect of lactoferrin and IFN- γ (Figure 4.4B).

Lactoferrin modulated cytokine production from infected macrophages

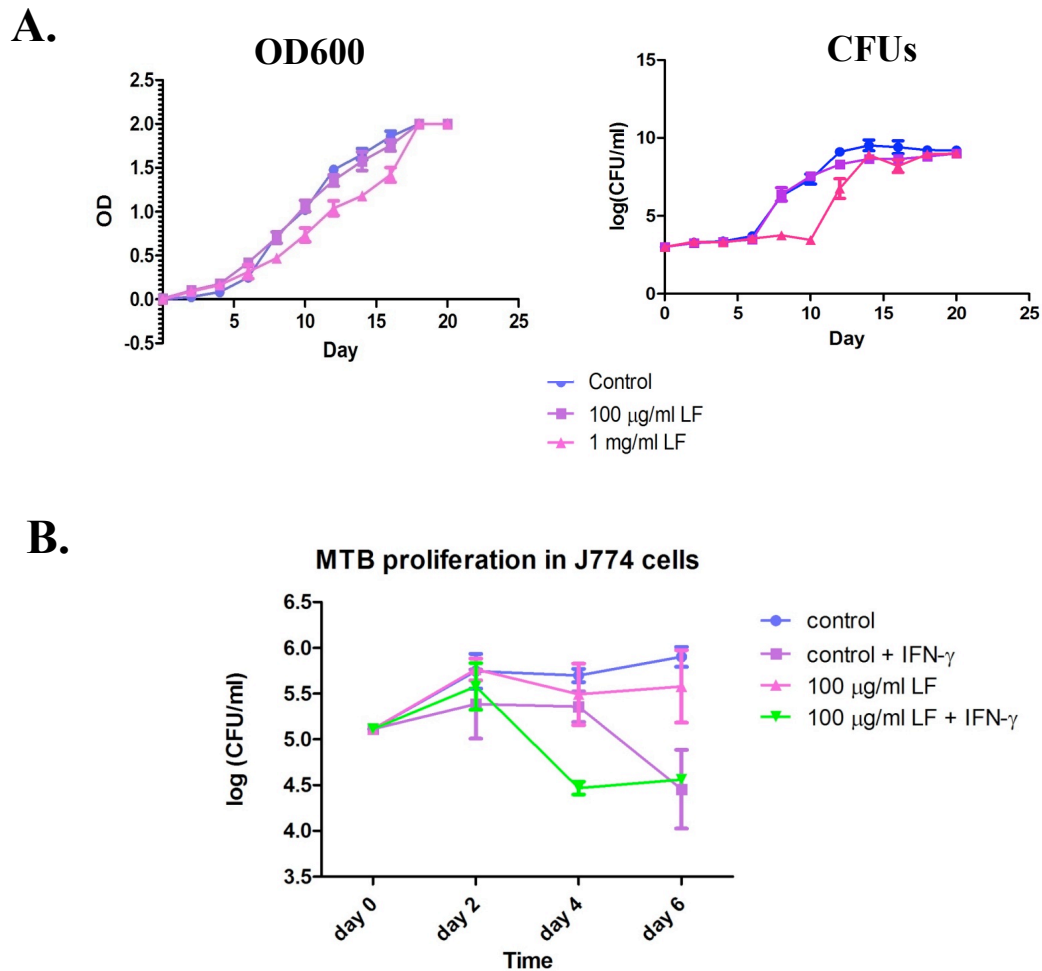


Figure 4.3. MTB proliferation in broth and macrophage culture in the presence of lactoferrin. **A.** MTB were grown in 7H9 broth alone, with 100 µg/ml lactoferrin, or 1 mg/ml lactoferrin. The OD600 values and bacterial CFUs both show that physiologic concentrations of lactoferrin (100 µg/ml) do not alter MTB growth. Only very high, non-physiologic concentrations of lactoferrin have a slight impact of MTB growth. **B.** J774 macrophages were cultured in DMEM, infected with MTB at a MOI of 1:1, and treated with various combinations of lactoferrin and IFN- γ . Lactoferrin alone did not impact MTB proliferation in macrophages. However, there was enhanced early killing by the combination of lactoferrin and IFN- γ .

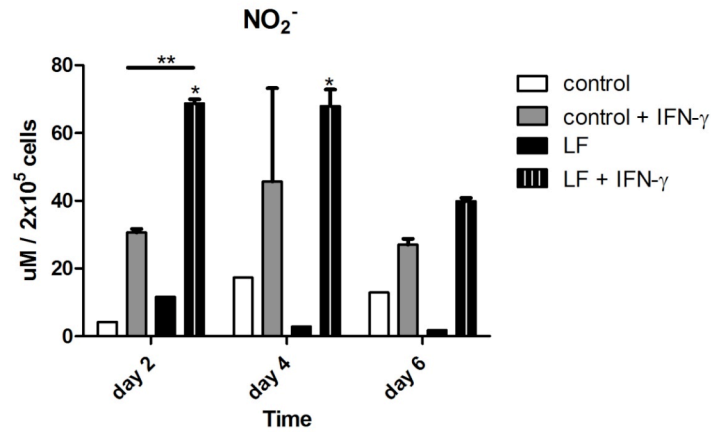
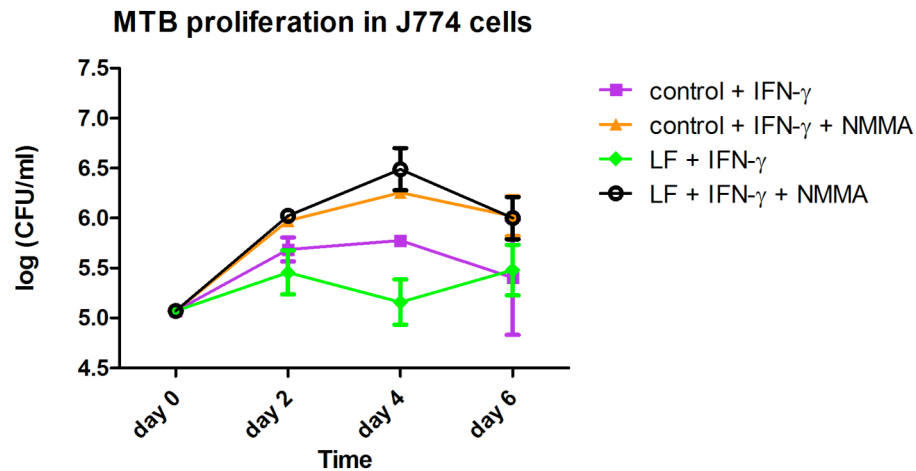
A.**B.**

Figure 4.4. Lactoferrin enhances IFN- γ mediated NO production.

A. NO production was measured in the supernatants from infected J774 cells using the Griess reaction. Macrophages treated with the combination of lactoferrin and IFN- γ had markedly enhanced NO production. * $p < 0.05$, with comparison to control macrophages. ** $p < 0.05$ with comparison to control macrophages activated with IFN- γ .

B. Macrophages were treated with 1mM of the nitric oxide synthase inhibitor n-monomethyl-L-arginine (NMMA) with various combinations of 100 μ g/ml lactoferrin (LF) and 10 ng/ml IFN- γ .

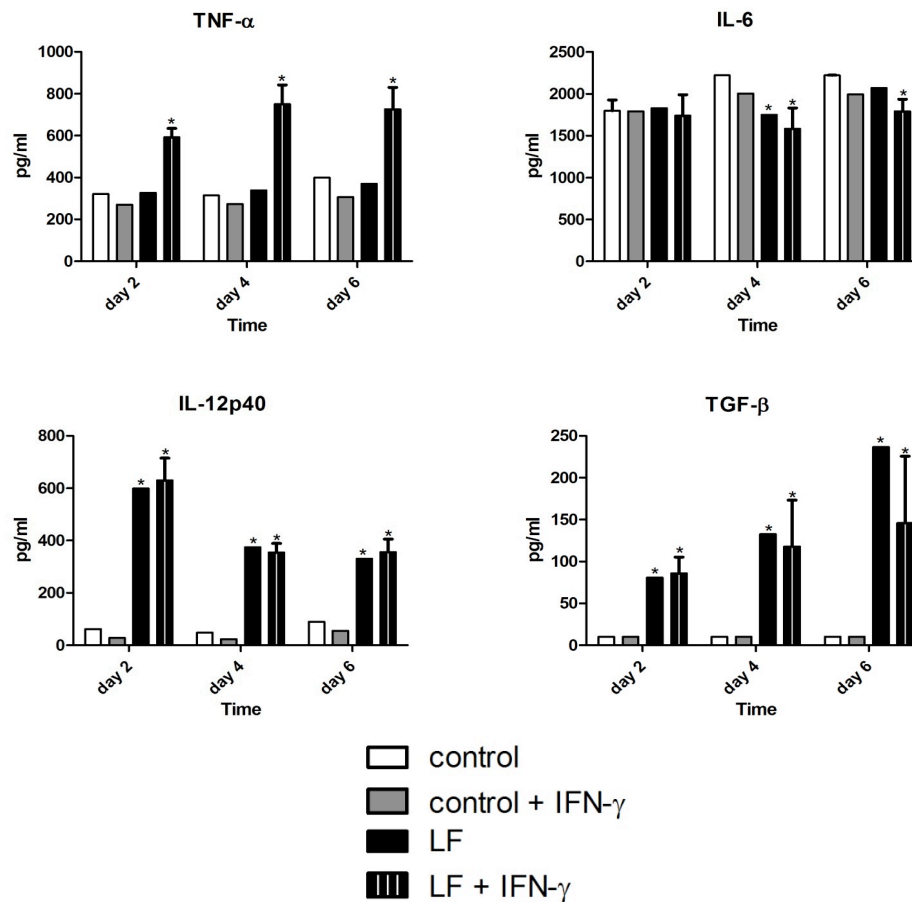


Figure 4.5. Lactoferrin modulates cytokine production by infected macrophages. J774 cells were cultured in DMEM, infected with MTB at a MOI of 1:1, and treated with various combinations of lactoferrin and IFN- γ . Supernatants were collected at the indicated time points, filtered with a 0.2 μ m filter, and analyzed for TNF- α , IL-6, IL-12p40, and TGF- β by ELISA. * $p < 0.05$ with comparisons to control macrophages.

(Figure 4.5). There was significantly increased TNF- α production when lactoferrin was added to macrophages activated with IFN- γ . Of potential importance, lactoferrin significantly increased IL-12p40 production, a cytokine of critical importance for the induction of Th1 responses [162, 163]. TGF- β synthesis was also significantly increased, a cytokine that has a number of regulatory functions [247].

Lactoferrin modulates lung cytokine expression in MTB infected mice

Lung cytokine expression of TNF- α , IL-6, IL-12p40, TGF- β , IL-10, IFN- γ , and IL-17 was evaluated by Taqman qPCR (Figures 4.6 and 4.7). Control mice increased expression of all cytokines over the three-week time course, with the exception of IL-12p40 expression that peaked at week 2. Lactoferrin administered both at day 0 and day 7 post-challenge significantly decreased expression of the proinflammatory cytokine IL-6. IL-10, a cytokine with inhibitory actions on cell-mediated immunity, was also decreased by lactoferrin treatment. There was a non-significant trend towards increased IFN- γ expression in the lactoferrin treated mice. IL-17 expression was significantly increased by lactoferrin treatment. There were no significant differences in lung expression of TNF- α , IL-12p40, or TGF- β between control and lactoferrin treated mice.

Increased Th1 and IL-17 producing cells in the lung and spleens of lactoferrin treated mice

Lung and spleen homogenates were examined for IFN- γ , and for CD4⁺ IFN- γ producing cells by ELISpot analysis (Figure 4.8). The levels of IFN- γ producing cells in

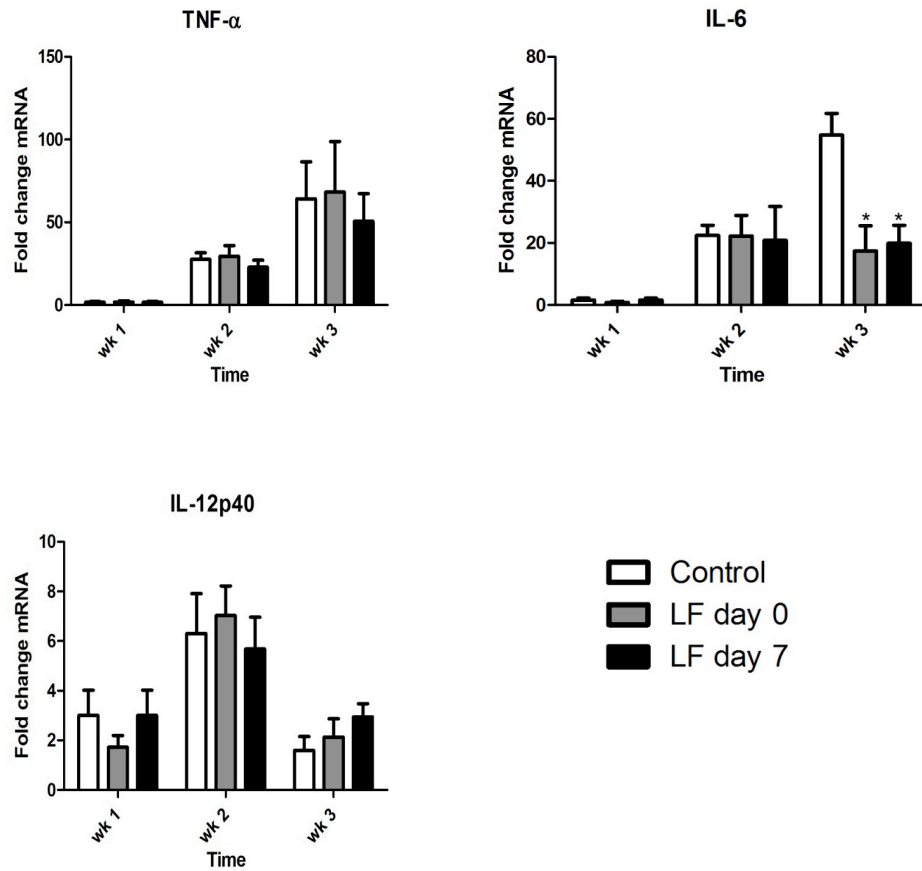


Figure 4.6. Lung expression of proinflammatory mediators in MTB-infected mice treated with lactoferrin. Expression of TNF- α , IL-6, and IL-12p40 mRNA was quantified in the lung in mice challenged with MTB and treated with or without lactoferrin. Data are expressed as fold change relative to naïve mice after normalization to β -actin. Data are presented as the mean with SD, $n = 6$ mice per group, per time point. * $p < 0.05$, comparisons are made to control mice.

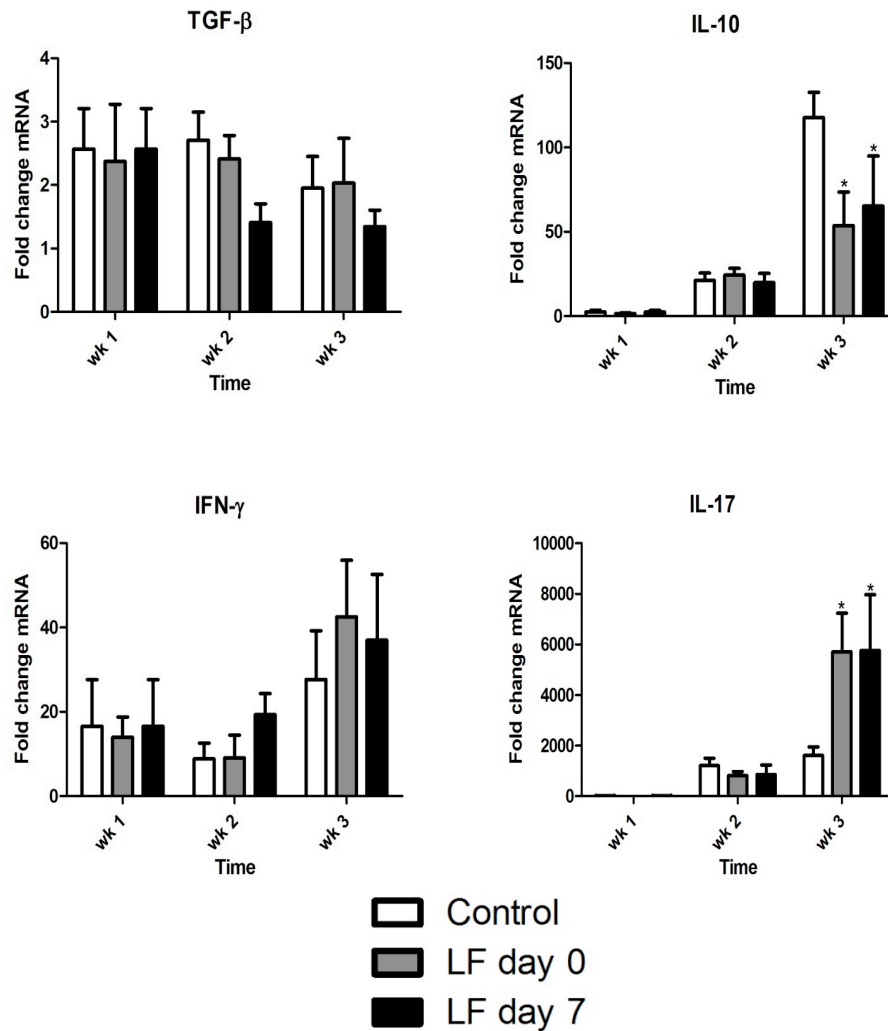


Figure 4.7. Lung expression of cytokines in MTB-infected mice treated with lactoferrin. Expression of TGF- β , IL-10, IFN- γ , and IL-17 was quantified in the lung in mice challenged with MTB and treated with lactoferrin. Data are expressed as fold change relative to naïve mice after normalization to β -actin. Data are presented as the mean with SD, n = 6 mice per group, per time point. * p < 0.05, with comparisons to control mice.

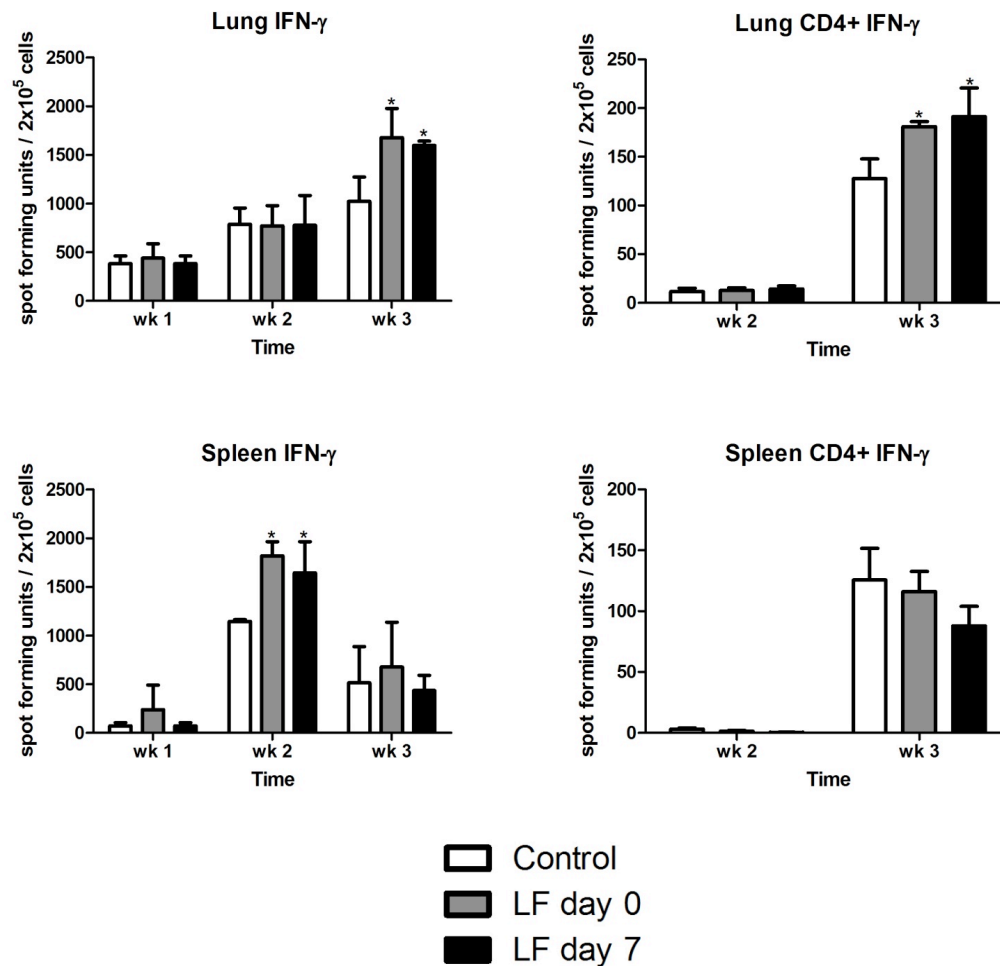


Figure 4.8. IFN- γ and CD4+ IFN- γ producing cells in the lung and spleen in control and lactoferrin treated mice. Lung and spleen homogenates were incubated with heat-killed MTB for 48 hours. Cells producing IFN- γ and CD4+ IFN- γ cells were enumerated by ELISpot analysis. Comparisons made to control mice, $n = 4$ mice per group per time point. * $p < 0.05$

the lung increased over the three-week time course in all groups examined. The number of lung IFN- γ synthesizing cells became significantly greater at week 3 in both lactoferrin treatment groups compared to control mice. The absolute numbers of IFN- γ producing cells in the spleen peaked at week 2 in all treatment groups. Splenocytes had a higher number of IFN- γ producing cells at week 2 post-MTB challenge in the lactoferrin treated mice compared to control mice. To determine if these IFN- γ synthesizing cells were Th1 cells, the lung and spleen digests were plated onto an ELISpot plate specific for CD4⁺ IFN- γ cells. The numbers of CD4⁺ IFN- γ ⁺ cells in the lungs were increased at week 3 in all groups examined, but significantly greater in the lactoferrin treatment groups. CD4⁺ IFN- γ ⁺ cells in the spleen were not significantly different between the groups.

IL-17 producing cells in the lung and spleen were also evaluated (Figure 4.9). The total number of IL-17⁺ cells increased in the lungs over the three-week observation period. There were significantly increased levels of IL-17 producing cells in the lung at week 2 in the lactoferrin treated mice. The spleen had relatively few IL-17 producing cells, with no significant differences between the groups.

MTB-specific responses by splenocytes are altered by lactoferrin treatment

Splenocytes from control and lactoferrin treated mice were isolated and stimulated with heat-killed MTB for 72 hours. Supernatant TNF- α , IL-6, IL-12p40, TGF- β , IL-10, IFN- γ , and IL-17 levels are shown in Figures 4.10 and 4.11. Splenocytes from both lactoferrin treatment groups had significantly elevated IL-12p40 at week 2 and IFN- γ at

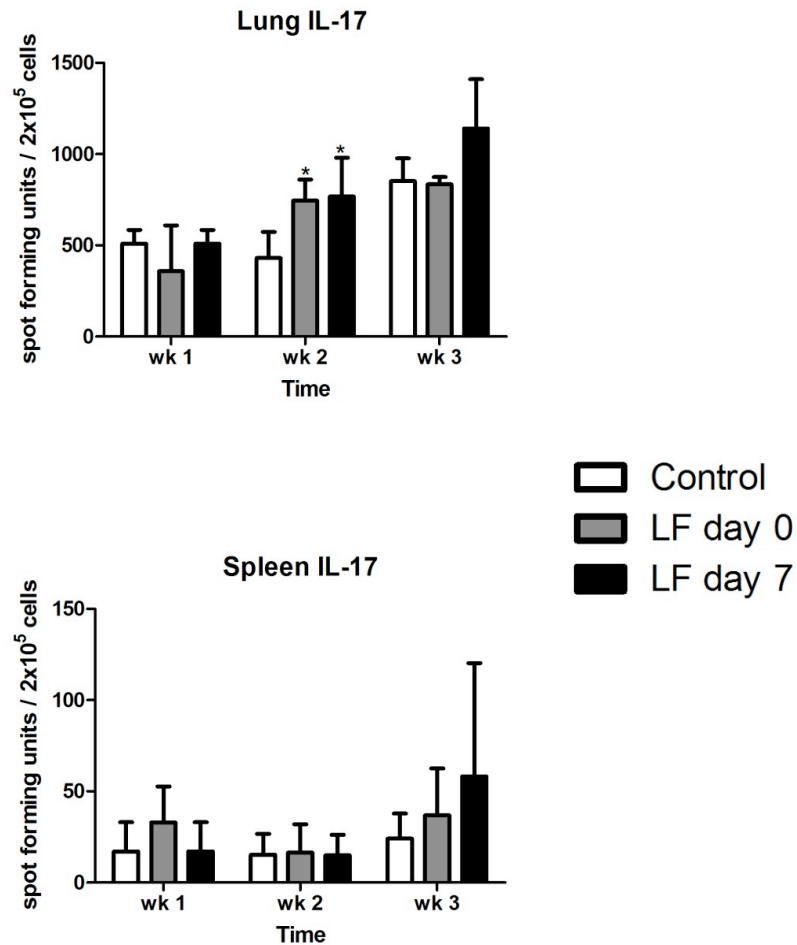


Figure 4.9. IL-17 producing cells in the lung and spleen in control and lactoferrin treated mice. Lung and spleen homogenates were incubated with heat-killed MTB for 48 hours. IL-17 producing cells were enumerated by ELISpot analysis. Comparisons are made to control mice, $n = 4$ mice per group per time point. * $p < 0.05$

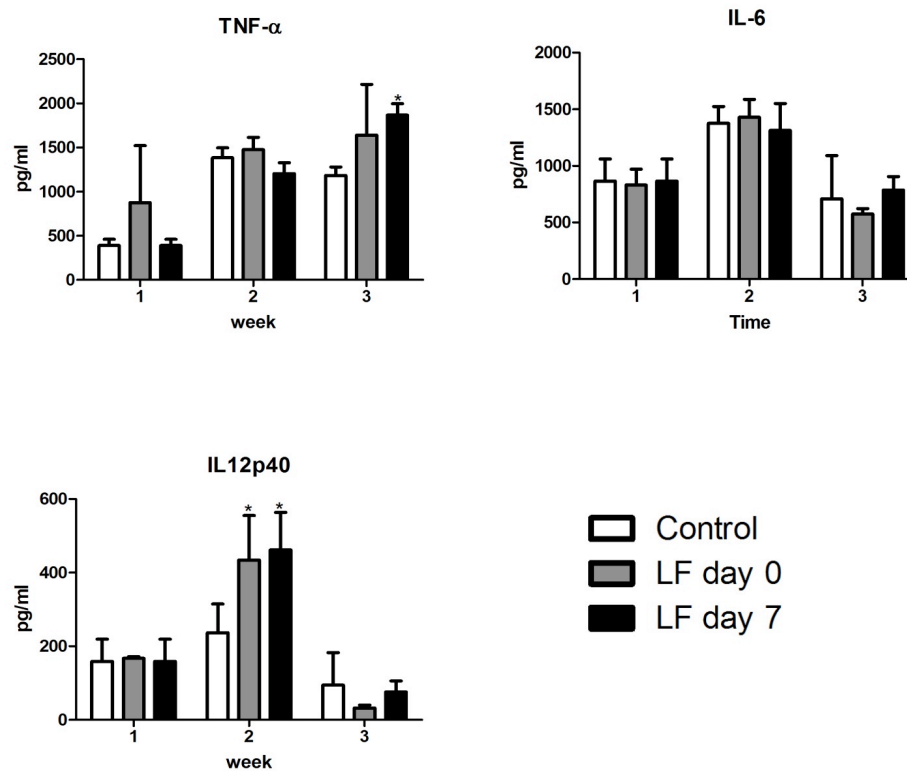


Figure 4.10. Specific protein production of proinflammatory mediators from control and lactoferrin treated mice. Splenocytes from control and lactoferrin treated mice were stimulated with heat-kill MTB for 72 hours. TNF- α , IL-6, and IL-12p40 were measured in the supernatants by ELISA. Comparisons are made to control mice. Data are presented as the mean with SD, $n = 4$ mice per group per time point. * $p < 0.05$

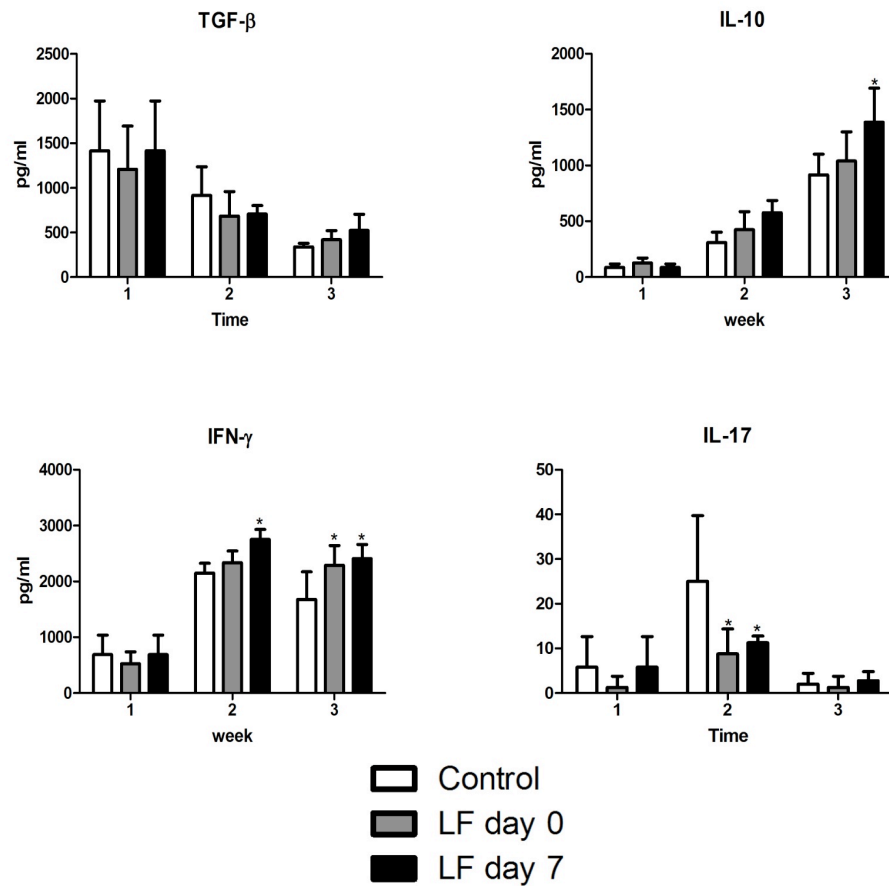


Figure 4.11. Specific protein production of cytokines from control and lactoferrin treated mice. Splenocytes from control and lactoferrin treated mice were stimulated with heat-kill MTB for 72 hours. TGF- β , IL-10, IFN- γ , and IL-17 were measured in the supernatants by ELISA. Comparisons are made to control mice. Data are presented as the mean with SD, n = 4 mice per group per time point. * p < 0.05

week 3. The mice given lactoferrin at day 7 after infection had increased TNF- α and IL-10 at week 3, and increased IFN- γ recall response at week 2. Both lactoferrin treatment groups had decreased IL-17 splenocyte synthesis at week 2. There were no differences in splenocyte synthesis of IL-6 or TGF- β in response to heat-killed MTB.

Discussion

TB continues to be a major cause of morbidity and mortality due to infectious diseases. Treatment of this disease requires 6 – 9 months of anti-TB chemotherapy that is difficult to administer in the regions of the world that have the highest burden of TB cases. Thus, there is a considerable need to develop novel agents for the treatment of TB both to shorten the treatment time and combat the emergence of drug resistant organisms. Increasing evidence suggests that immune modulating therapies that target granulomas and enhance protective immune responses may be useful as adjunct therapeutics for the treatment of TB [15].

Lactoferrin treated mice demonstrated a decrease in lung bacterial CFUs and a reduction in bacterial dissemination to the liver. These favorable effects were evident even when the mice were treated one week post-infection, indicating that lactoferrin has the potential as a novel agent for the treatment of TB. This decrease in bacterial burden is not likely due to a direct effect of lactoferrin on MTB because physiologic concentrations of lactoferrin did not alter MTB proliferation in either broth or macrophage culture. Thus, we hypothesize that the mechanism of bacterial reduction is due to immune modulation. Indeed, mice treated with lactoferrin had higher numbers of Th1 cells in the lung at three weeks after infection. Lactoferrin has been shown to

increase cell-mediated immune responses in a number of infectious disease models [140]. Enhancement of antigen presenting cell activity is one potential mechanism by which lactoferrin may promote Th1 responses. Lactoferrin increased expression of MHC II and the CD86:CD80 ratio in BCG-infected macrophages and dendritic cells, and resulted in increased production of IFN- γ from overlaid CD3⁺ and CD4⁺ cells compared to cells cultured without lactoferrin [155-157]. Lactoferrin may also enhance Th1 responses by modulation of antigen presenting cell cytokine production. Lactoferrin was demonstrated to increase production of IL-12 in a number of studies [155-157, 164, 228], including the data presented here. IL-12 is an essential cytokine for induction of IFN- γ from naive T-cells and enhancing production from mature Th1 cells [162, 163]. Furthermore, lactoferrin appeared to enhance IFN- γ mediated bacterial killing in macrophage culture; this effect is possibly due to enhancement of NO production. Other studies have reported increased NO production by lactoferrin, both in macrophage culture and *in vivo* [231, 248]. IFN- γ induced NO synthesis by macrophages is considered a crucial antimycobacterial activity [249]. Thus, we hypothesize that a major mechanism of lactoferrin's favorable effect during MTB infection is the generation of IFN- γ producing cells; IFN- γ in turn acts synergistically with lactoferrin to enhance macrophage killing of MTB, possibly through NO production.

Lactoferrin increased IL-17 producing cells in the lung in addition to enhancement of IFN- γ mediated responses. To our knowledge, this is the first report of lactoferrin modulation of IL-17 responses. Several studies suggest that IL-17 synthesizing cells may play an important role in MTB host defense [64, 65, 241]. Early enhancement of IL-17 responses by use of an IL-23 producing adenovirus during MTB

infection resulted in decreased bacterial burden and reduced lung pathology [67]. Thus, an early increase in IL-17 responses in the lactoferrin treated mice may have contributed to the reduction in bacterial CFUs and lung histopathology. The mechanisms by which IL-17 may be protective during MTB challenge include augmentation of bacterial killing by enhancement of IFN- γ responses that activate macrophages, direct stimulation of phagocytic cells, recruitment of neutrophils, and increasing expression of antimicrobial peptides that have activity against MTB [67, 250].

Mice treated with lactoferrin had a marked reduction in lung immunopathology in addition to a decrease in bacterial CFUs. Modulation of MTB-induced inflammatory pathology has been proposed as a mechanism to decrease the treatment time for TB [15], an approach successfully used in animal studies and human clinical trials. For example, the TNF- α reducer thalidomide used in combination with anti-TB antibiotics reduced mortality, brain pathology, and leukocytosis in a rabbit model of TB meningitis compared to anti-TB chemotherapy alone [251]. A clinical trial exploring the combination of etanercept, a soluble TNF- α receptor, with TB antibiotics in patients co-infected with HIV and TB reported increased bacterial clearance and improved chest-rays with the combination therapy [135]. A second clinical trial in individuals with HIV and TB demonstrated a higher rate of sputum culture conversion when high dose prednisolone was used as the immunomodulator in conjunction with TB antimicrobial chemotherapy [136]. In addition to an overall reduction in lung pathology, lactoferrin-treated mice had a decreased percentage of macrophages, an increased percentage of lymphocytes, and increased numbers of CD4⁺ and CD8⁺ cells, suggesting an increase in immune cells with protective effects during MTB infection.

In light of the increased emergence of drug resistant organisms and the increasing incidence of TB, it is essential to develop new agents for the treatment of TB. It is noteworthy that lactoferrin reduced bacterial burden, accompanied by an increase in certain proinflammatory responses while decreasing overall lung immunopathology. Lactoferrin has a number of advantages over the current immunomodulators in use because it does not suppress the immune system and has a proven safety record in a number of animal models and human clinical trials [138, 139, 235, 236]. These investigations indicate that lactoferrin has potential as a novel therapeutic for the treatment of TB.

Chapter Five

General Discussion

A major focus of this thesis centered on investigations into the molecular control of the granulomatous response to mycobacterial cord factor trehalose-6'6-dimycolate (TDM). The granuloma is the histopathologic hallmark of *Mycobacterium tuberculosis* (MTB) infection. The tuberculosis (TB) bacilli are likely recognized by alveolar macrophages following inhalation of the organism [17]. These macrophages secrete cytokines and chemokines that recruit monocytes from systemic circulation and form the innate granuloma. At some point after infection, a dendritic cell becomes infected or processes MTB antigen and migrates to the draining lymph node of the lung. Dendritic cell presentation of MTB antigen to naive T-cells generates adaptive immune responses. Antigen-specific T-cells migrate to the lung and surround infected macrophages, generating mature granulomas [17].

Granuloma formation is often considered a “double-edged sword” in the host defense against MTB [15]. Granuloma formation is clearly needed to prevent uncontrolled infection and bacterial dissemination, as demonstrated in both animal and studies where factors essential for granuloma generation and maintenance are dysregulated [190, 204, 207]. However, there is increasing evidence that MTB possess certain factors that specifically drive granuloma formation and tissue remodeling that results in physical sequestration of the bacilli from effector immune cells capable of killing the organism [15]. Specifically, MTB has been shown to directly interfere with intracellular signaling to drive granuloma formation. A MTB mutant lacking the adenylate cyclase Rv0386 was characterized by reduced cAMP after infection, accompanied by decreased protein kinase A and CREB activation, and ultimately decreased TNF- α synthesis by infected macrophages [104]. The mutant MTB had poor

survival in a mouse infection model and decreased lung pathology. MTB also possess mycolic acids, such as trehalose dimycolate, that have been shown to drive granuloma formation [13, 116, 118]. Thus, an understanding of the immunological factors that drive granuloma formation may lead to novel targets for the treatment of tuberculosis. A portion of this thesis specifically explores the role of TNF- α , complement factor C5a, and IL-6 in the generation and maintenance of TDM induced granulomas.

The TDM model of granuloma formation mimics certain aspects of MTB induced immunopathology and is ideal for studying the immunologic factors necessary for early granuloma formation [13, 116, 118, 119, 130]. The investigations presented in this thesis demonstrate that TNF- α is a key cytokine in mycobacterial TDM induced granulomas. TNF- α deficient mice challenged with intravenous TDM prepared in emulsion form failed to generate a histological response to TDM, accompanied by an absence of proinflammatory mediators and chemokines. Macrophages are the innate cell type that responds to TDM [122] and are likely the source of TNF- α during the initial immune response to MTB [252]. TNF- α possibly acts by inducing chemokines during early infection that facilitate recruitment of other immune system cells [191]. Studies performed on animal models of MTB infection indicate that TNF- α is clearly involved in the maintenance of established granulomas because treatment with a TNF- α neutralizing antibody disrupts chronic granulomas [190], but this phenomenon could not be evaluated in the TDM granuloma model. Use of TNF- α neutralizing therapy in patients latently infected with MTB resulted in reactivation of latent infection, often accompanied by extrapulmonary disease [204]. Inhibition of TNF- α during TB chemotherapy has been proposed as a novel therapeutic strategy because neutralization

of TNF- α has been shown to convert MTB from a state of non-replicating persistence to active replication, thus making the bacteria susceptible to antibiotics [15]. This approach was successful in both an animal model and a human clinical trial [135, 251], indicating that TNF- α may be a useful target employed during antimycobacterial chemotherapy.

The studies in this thesis also demonstrate that complement factor C5a plays a role in the generation of cohesive granulomas to TDM. C5a $-/-$ mice developed severe pathology accompanied by reduced early synthesis of proinflammatory mediators and an absence of IL-12. Complement C5a is a potent anaphylatoxin that recruits cells to the sites of active inflammation [87]. Thus, it is intriguing that deficiency of a chemotactic factor resulted in increased influx of cells that produced less inflammatory cytokines. C5a is necessary for NF κ B activation, an expression regulator for a variety of inflammatory mediators, thus possibly accounting for the delayed expression of proinflammatory mediators [215]. Furthermore, C5a induces macrophage adhesion molecule expression [216, 217], possibly accounting for the inability of C5a $-/-$ mice to form cohesive cellular aggregates. The inability of complement deficient animals to produce IL-12 has been documented in a number of other investigation, but a precise mechanism has yet to be defined [88]. A number of studies have indicated that the decreased IL-12 production by complement deficient animals results in defective cell-mediated immunity [92, 212, 253]. However, studies in humans have not demonstrated an increased susceptibility to TB in patients deficient in complement components; in fact, it has been suggested that such patients may be less susceptible to TB because of

reduced opsonization and phagocytosis [254]. Therefore, the role of the complement system in TB infection and as a therapeutic target remains to be determined.

IL-6 also appears to play a role in TDM induced granulomas. Mice deficient in this cytokine initially formed granulomas, but failed to maintain them and had severe, edematous inflammation. The IL-6 $-/-$ mice had decreased early production of proinflammatory mediators, similar to the complement deficient mice. An investigation by Clahsen and Schaper [255] demonstrated that IL-6 promoted activation of β 1-integrin and cellular migration across endothelial cells, suggesting that IL-6 has chemokine-like properties. It is hypothesized that IL-6 from macrophages may thus be needed to maintain a coordinated response to mycobacterial antigens. Additionally, a recent investigation demonstrated that IL-6 is necessary for regulating local concentrations of glucocorticoids that modulate pathological responses [219]. The TDM model used in these experiments explores early, innate immune responses. Thus, the role of IL-6 in chronic TB infection cannot be determined from these studies. Long-term studies of the role of IL-6 using MTB infection have generated mixed results. An intravenous challenge of 10^6 MTB was lethal for IL-6 $-/-$ mice in one study [107]; however, a low dose aerosol challenge of IL-6 deficient mice in a different investigation had reduced IFN- γ production but ultimately contained the infection [105]. The role of IL-6 in human TB infection is less clear. To date, there have been no studies that link IL-6 polymorphisms or disruption of IL-6 to TB susceptibility [256]. Therefore, the role of IL-6 as a therapeutic target for TB treatment remains to be clarified.

The overall hypothesis from our studies on the cytokine mechanisms of TDM-induced granuloma formation is that TNF- α is essential for the initiation of an

inflammatory response to TDM, possibly by inducing chemokines needed to recruit cells to the lungs. Once a response has been initiated, complement C5a and IL-6 recruit cells that produce regulating cytokines and upregulate integrin that allow cells to form organized, cohesive structures. Such studies may provide insight on the implications of using cytokine inhibitors to treat patients with chronic inflammatory disorders who may have latent TB disease as well as provide targets for the treatment of TB.

Granulomas are clearly essential in the prevention of uncontrolled bacterial proliferation and dissemination. However, it is hypothesized that modulation of granuloma structure may be a novel target for the treatment of TB [15]. MTB sequestered within granulomas enter a state of non-replicating persistence characterized by absent bacterial division and the use of altered biosynthetic pathways [131, 132], thus rendering the bacteria relatively resistant to the action of antibiotics. The physical structure of the TB granuloma is such that killer lymphocytes capable of lysing infected cells are physically separated from the infected cells by a layer of fibrosis [17]. Finally, reducing the inflammatory pathology induced by MTB may enhance antibiotic penetration into tissues. Reduction of pathology has led to favorable results in both animal and human studies [135, 136, 251]. Therefore, studies were undertaken to develop a therapeutic to reduce the pathological response to MTB and its antigens.

Lactoferrin was selected the candidate immune modulator for these studies because it has been shown to reduce immune mediated tissue destruction in a variety of inflammatory and infectious disease models. For example, lactoferrin added to the BCG vaccine as an adjuvant resulted in both increased protection following challenge with virulent MTB and decreased lung occlusion [232]. Additionally, mice injected with LPS

and treated with lactoferrin had decreased mortality and reduced gut tissue destruction [183]. Therefore, studies were conducted to determine if lactoferrin is able to modulate the immune response to mycobacterial antigens using TDM.

Macrophages were first stimulated with TDM-coated beads and lactoferrin added to the culture media; lactoferrin treatment of the macrophages resulted in globally reduced proinflammatory cytokine production. Mice challenged intravenously with TDM and treated with 24 hours later had significantly fewer and smaller granulomas at the peak of the granulomatous response. Overall, proinflammatory cytokine production in the lungs of TDM-challenged mice was not significantly altered by lactoferrin; however, TGF- β and IL-10, cytokines that can have anti-inflammatory functions, were increased in lactoferrin treated mice. When macrophages were infected with MTB and treated with lactoferrin, production of certain proinflammatory cytokines were increased. It is hypothesized that lactoferrin modulated macrophage cytokine production *in vivo* to reduce the histopathological response to TDM. However, it is intriguing that different cytokine responses were obtained when lactoferrin was added to macrophages stimulated with TDM-coated microspheres, mice challenged with TDM, and macrophages infected with MTB. Lactoferrin is known to have different effects depending on the model system, the inflammatory stimulus, and the immune status of the host [140]. For example, lactoferrin treated macrophages stimulated with a low dose of LPS had increased production of proinflammatory cytokines compared to untreated cells [175]. However, macrophages given higher doses of LPS had reduced proinflammatory cytokine production when lactoferrin was added to the culture media.

This unique property of lactoferrin makes it an ideal candidate immunomodulator for a number of inflammatory and infectious diseases.

The differing cytokine production when macrophages are given isolated TDM and live mycobacteria suggested that *in vivo* infection with MTB would likely give different results than those obtained from the TDM model. Additionally, the TDM model used in these studies only allows exploration of innate responses. Cell-mediated immunity is critical to the control of TB infection, which cannot be evaluated in the two-week TDM model. Furthermore, the alteration of granuloma structure and production of cytokines with anti-inflammatory properties may result in enhanced bacterial dissemination from the lung [131], a phenomenon that cannot be evaluated using the TDM model. Therefore, mice were aerosol challenged with a low dose of MTB strain Erdman and treated with lactoferrin by adding it to the drinking water. We found that mice treated with lactoferrin at the start of infection or one week after infection had decreased organ bacterial burden. Furthermore, lactoferrin treated mice had decreased lung histopathology, fewer areas of foamy macrophages, and increased numbers of lymphocytes. The fact that lactoferrin had a favorable effect even when given after established infection indicates that lactoferrin has the potential to be a novel therapeutic for the treatment of TB.

The hypothesized mechanism of lactoferrin-mediated reduction in bacterial burden is the early induction of Th1 immune responses. Th1 cells produce IFN- γ , which in turn acts synergistically with lactoferrin to augment nitric oxide mediated killing of MTB by macrophages (Figure 5.1). A number of other investigations have reported that lactoferrin increases Th1 responses. For example, lactoferrin administered orally

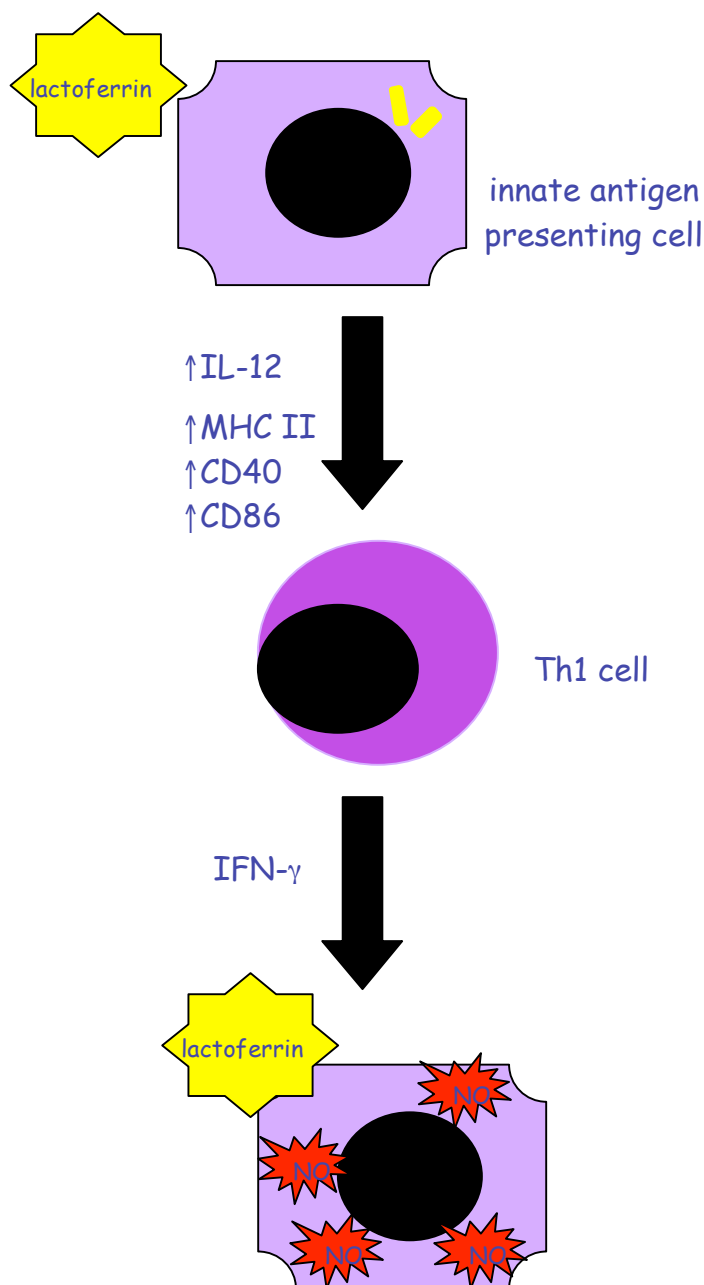


Figure 5.1. Hypothesized mechanism for the reduction in MTB bacterial burden by lactoferrin treatment. Innate antigen presenting cells present MTB antigen to naive T-cells on MHC II. Lactoferrin augments the induction of Th1 cells by increasing IL-12 production, as well as increasing expression of MHC II and the costimulatory molecules CD40 and CD86. IFN- γ production by Th1 cells acts synergistically with lactoferrin to promote MTB killing by macrophages in a nitric oxide dependent manner.

increased Th1 responses, indicated by increased IFN- γ production, in both naive and tumor-harboring mice [173, 174]. Transgenic mice expressing human lactoferrin and infected with *Staphylococcus aureus* had increased IFN- γ and TNF- α , along with decreased IL-10 and IL-5 [172]. The possible mechanism by which lactoferrin increases Th1 responses is enhancement of innate immune cell antigen presentation and increased production of IL-12 [140]. While lactoferrin increased Th1 responses in the studies presented here, others have reported that lactoferrin can increase Th2 responses depending on the model system. For example, lactoferrin increased IL-10 and decreased IFN- γ in an infection model of *Toxoplasma gondii*, indicating the enhancement of Th2 responses [177]. The precise mechanisms by which lactoferrin promotes Th1 versus Th2 responses is unknown; however, this ability of lactoferrin gives it the potential to be beneficial in a number of disease states.

Lactoferrin treatment also increased the numbers of IL-17 producing cells in the lungs of MTB infected mice. To our knowledge, this is the first report of lactoferrin affecting levels of IL-17+ cells. New evidence suggests that IL-17 producing cells play an important role in the host defense against MTB [64, 65, 241]. Of importance to these studies, early enhancement of IL-17 responses by administration of an IL-23 producing adenovirus during infection with MTB reduced lung bacterial burden and pathology [67]. Thus, the early appearance of IL-17 producing cells in the lactoferrin treated mice may have contributed to the reduced pathology and bacterial burden. Potential mechanisms by which IL-17 may be protective during MTB infection include enhancement of IFN- γ responses that activate macrophages, neutrophil recruitment,

stimulation of phagocytic cells, and increasing expression of antimicrobial peptides with activity against MTB [67, 250].

While a number of receptors for lactoferrin have been reported [141], the precise intracellular signaling mechanisms induced after activation by lactoferrin remain undefined. A 105-kDa lactoferrin receptor is found on platelets and lymphocytes and possibly initiates signaling cascades [166, 257]. Lactoferrin was shown to bind surface nucleolin, followed by endocytosis, and translocation of the lactoferrin/nucleolin complex to the nucleus [258]. Other lactoferrin receptors are the low-density lipoprotein receptor related proteins (LRPs) that are found on a number of cell types including macrophages [141]. Finally, an intestinal receptor that induces lactoferrin endocytosis is hypothesized to promote IL-18 synthesis and increase in systemic Th1 responses [259]. How lactoferrin promotes different immune responses is likely dependent on the model system under investigation.

The ability of lactoferrin to have a favorable effect during MTB infection when it is administered orally increases its appeal as a therapeutic, especially in drug-resistant infections that require the administration of injectable medications. However, it is not clear if lactoferrin's effects were mediated by modulation of systemic immune responses or local action at the lung. The decreased CD4+IFN- γ + and IL-17+ cells in the spleen compared to the lung suggest that lactoferrin had different local and systemic responses. Lactoferrin undergoes partial digestion in the gastrointestinal tract to produce peptides, such as lactoferricin, that have antimicrobial effects [260]. The effects of specific lactoferrin peptides on MTB growth and immune modulation during MTB infection are currently unknown. However, an investigation in humans reported that gastric survival

of lactoferrin is at least 60% [261]. Studies in mice demonstrate that intact lactoferrin accumulates in a number of organs shortly after oral administration [262]. A study using a peptide of human lactoferrin showed that it accumulates at sites of active inflammation in a mouse model of MRSA infection [263]. Thus, orally administered lactoferrin has the potential to modulate infection and inflammation in specific tissues.

Further increasing the appeal of lactoferrin is its favorable safety profile in a number of animal and clinical studies. A major concern over the use of novel agents in the treatment of TB is that the long use required for treatment may result in toxicity. F344/DuCrj rats given bovine lactoferrin in their diet for 40 weeks had no clinical signs of toxicity and had significantly lower liver transaminases, blood urea nitrogen, and triglyceride levels compared to control rats [138]. A phase 2 clinical trial was conducted in patients with disseminated renal cell carcinoma who were given a recombinant form of human lactoferrin for 12 weeks reported good tolerability and no renal, hematologic, or hepatic toxicities [137]. No major toxicities were reported in a 12 week trial evaluating the impact of bovine lactoferrin on patients with chronic hepatitis C [139]. Thus, lactoferrin represents a natural molecule that is well suited for clinical use.

Future Directions

TB remains a major public health problem, accounting for approximately 2 million deaths each year [1]. The studies presented in this thesis indicate that lactoferrin has the potential to be a novel therapeutic in the treatment of TB. However, TB treatment requires the use of a combination of at least four drugs [2]. It is essential to confirm that lactoferrin still has a favorable effect when used in a combination drug

regimen. It is theoretically possible that lactoferrin may antagonize the action of a specific antimycobacterial antibiotic. Furthermore, such studies will determine if lactoferrin can be used in combination with standard TB drugs to generate novel drug regimens that will allow for shortening the time needed to treat TB.

To our knowledge, these studies are the first to report that lactoferrin increases production of IL-17. Future studies can be performed to further characterize the importance of these responses. For example, IL-17 knock-out mice (currently not commercially available) can be infected with MTB and treated with lactoferrin to determine if lactoferrin still has a favorable effect in the absence of IL-17. Alternatively, wild-type mice can be treated with a neutralizing IL-17 antibody, but such experiments are extremely expensive.

Additionally, it is not known if lactoferrin acted systemically or locally in the lungs when either TDM-challenged or MTB-infected mice were treated with lactoferrin. Studies can be undertaken to label lactoferrin and determine its distribution in MTB infected mice. Additionally, it is possible that lactoferrin peptides generated in the digestive tract of treated animals may be responsible for some of the effects observed. Thus, peptides of lactoferrin can be generated and tested for immune modulating activity and therapeutic potential during MTB treatment.

Finally, these experiments explored the utility of lactoferrin in an acute model of MTB. Human patients are likely to present for treatment with chronic lesions. It is unclear at this time if administration of lactoferrin to individuals with on-going chronic disease will alter pathology. Experiments can be performed to determine if lactoferrin still has a favorable effect when administered to animals with established chronic

infection. However, humans with active TB often have cavitory lesions. Unfortunately, the animal models that adequately replicate the cavitory lesions that are found in human post-primary TB patients are not completely standardized [13]; it would be premature to investigate lactoferrin effects in these model systems until this obstacle is overcome.

In summary, these studies provide insights into the cytokine mechanisms by which mycobacterial antigens induce granulomas. The lung immunopathology induced by trehalose-6'6-dimycolate can be decreased by lactoferrin, possibly by modulation of macrophage cytokine production. Finally, lactoferrin has a number of favorable effects during MTB infection including reduction of organ bacterial burden and lung inflammation. The hypothesized mechanism by which lactoferrin decreases bacterial CFUs is by increasing Th1 responses and increased IFN- γ mediated killing by macrophages. Thus, lactoferrin has the potential as a novel therapeutic for TB treatment.

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