Host Defense Mechanisms Against Mycobacterium Tuberculosis

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HOST DEFENSE MECHANISMS AGAINST *MYCOBACTERIUM TUBERCULOSIS*

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

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B.S., Tennessee State University, 2006

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ABSTRACT

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*Mycobacterium tuberculosis* is one of the world’s most infectious diseases. Approximately 2 million people die each year from *Mycobacterium tuberculosis* infection and one-third of the world’s population remains infected. For decades research has focused on uncovering the tactics used by *Mycobacterium tuberculosis* to evade host immune responses and defense mechanisms used to fight tuberculosis infection. The following review focuses on the host defense mechanisms used to combat *Mycobacterium tuberculosis* with an emphasis placed on the roles that Toll-like receptors (TLRs), T cells (γδ, CD4⁺, CD8⁺), and macrophages play in mounting the innate and adaptive immune responses necessary to eradicate this disease. The challenges of designing an effective vaccine to fight *Mycobacterium tuberculosis* infection and the two first-line anti-tuberculosis inhalant drugs, Isoniazid (H) and Rifampicin (R) are also discussed.
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SIGNIFICANCE

*Mycobacterium tuberculosis* is a devastating disease that affects a large portion of the world’s population. The fight against *Mycobacterium tuberculosis* infection is currently a battle that is lifelong given that at the moment there is no vaccine that is 100% effective or a cure. The fact that latent *Mycobacterium tuberculosis* infection continues to pose the threat of reactivation at any moment puts more individuals at risk of contracting *Mycobacterium tuberculosis* infection. With the increasing number of immigrants entering into the United States from countries where adequate healthcare is minimal to nonexistent, it is vital that all of the necessary precautions are taken to prevent the incidence of *Mycobacterium tuberculosis* infection.
INTRODUCTION

The likelihood of becoming infected with *Mycobacterium* tuberculosis is multifactoral (Raja, 2004). *Mycobacterium* tuberculosis is the second leading cause of death among all infectious diseases worldwide. Approximately 2 million people die each year from *Mycobacterium* tuberculosis (Davis et al., 2007) and one-third of the world’s population remains infected with latent disease (van Crevel et al., 2002).

Naturally there are individuals who are more susceptible to mycobacterial infections than others. Individuals reported as having an increased risk of contracting mycobacterial infections include individuals that have suffered chronic renal failure, individuals with insulin-dependent diabetes, individuals undergoing immunosuppressive therapy, residents of prisons and homeless shelters, intravenous-drug users, and individuals infected with HIV (Horsburgh, 2004). These specific individuals’ increased susceptibility to *Mycobacterium* tuberculosis may stem from a decrease in their immune system’s ability to prevent infection, progression to primary infection, and infection reactivation (Horsburgh, 2004).

The objective of the following review is to examine the host defense mechanisms used to combat *Mycobacterium tuberculosis* with an emphasis placed on the roles that Toll-like receptors (TLRs), T cells (γδ, CD4+, CD8+), and macrophages play in mounting the innate and adaptive immune responses necessary to eradicate this disease.
INITIAL MYCOBACTERIUM TUBERCULOSIS INFECTION

*Mycobacterium* tuberculosis can cause infection anywhere within the body, although it mainly takes hold and resides within the lungs of infected individuals (van Crevel et al., 2002). Consequently, the route of infection plays an important role in determining how signal transduction, immune activation, and the intracellular survival of *Mycobacterium* tuberculosis occur (van Crevel et al., 2002). *Mycobacterium* tuberculosis is the most virulent of mycobacteria (Pieters et al., 2002). Mycobacteria are slow growing organisms that limit themselves to mainly infecting macrophages of the host (Pieters et al., 2002), specifically macrophages of the lung (alveolar macrophages) (van Crevel et al., 2002). Mycobacteria reside in structures known as phagosomes. Normally bacteria ingested by alveolar macrophages are destroyed within lysosomes that fuse with phagosomes. In contrast, *Mycobacterium* tuberculosis prevents lysosomal fusion with phagosomes. The failed fusion of these two structures prevents the formation of a third structure known as the phagolysosome, consequently leading to the mycobacteria’s avoidance of degradation and destruction within the macrophage, contributing to the survival of *Mycobacterium* tuberculosis infection (Pieters et al., 2002). This ability to evade destruction by the alveolar macrophage limits the immune response of the host to containing the infection, not eliminating it (Flynn et al., 2001).

Initiation of *Mycobacterium* tuberculosis infection begins with the inhalation of tubercle bacilli. Following inhalation, the tubercle bacilli are ingested by alveolar macrophages, resulting in either their destruction or persistence. The tubercle bacilli that
are capable of escaping destruction then multiply and disrupt the function of the alveolar
macrophage. When the actions of the alveolar macrophage become disrupted, other
inflammatory cells programmed to prevent the spread of infection are then recruited to
the site of infection within the lungs. Following inhalation of the tubercle bacilli, the
mycobacteria are either destroyed, in which case no infection occurs, or the mycobacteria
remain viable. In the event that the mycobacteria are not destroyed, a primary complex
forms. The primary complex consists of *Mycobacterium* tuberculosis infiltrate and a
draining lymph node. The primary complex gives rise to the positive skin test that marks
the *Mycobacterium* tuberculosis-specific T cell response, uncovering the existence of
infection. Once *Mycobacterium* tuberculosis infection has been established, the infection
is usually stabilized and goes into latency. In some cases however, the infection is not
stabilized and active disease develops (primary tuberculosis infection). Active
tuberculosis infection in turn uses one of two methods to remain active within the host.
Active tuberculosis disease either remains localized, keeping infection limited to the
lungs, or it disseminates and travels to other areas of the body. In the event of
*Mycobacterium* tuberculosis dissemination, infection stabilization and latency are still
possible however, large amounts of mycobacteria traveling throughout the body (miliary
tuberculosis) and meningitis remain risks. Although establishing a state of latency in a
*Mycobacterium* tuberculosis-infected individual is one means of controlling tuberculosis
infection, there always remains the risk of latent infection becoming reactivated in the
event of immune surveillance failure, thereby establishing post-primary *Mycobacterium*
tuberculosis infection (Fig. 1) (van Crevel et al., 2002).
FIG. 1 Chronological events after inhalation of *Mycobacterium tuberculosis*. Adapted from van Crevel et al., 2002
CONTROLLING DISSEMINATION WITHIN THE INFECTED HOST

Granuloma Formation

Within 2 to 6 weeks following Mycobacterium tuberculosis infection, cell-mediated immunity leads to granuloma formation (Raja, 2004; van Crevel et al., 2002). Granulomas consist of lymphocytes and macrophages and are protective structures that prevent the spread of infection to other parts of the body by localizing the inflammation and damage. Granulomas surround the bacteria-infected cells and starve them from nutrients that are essential for their survival. In the case of Mycobacterium tuberculosis infection, granulomas function to keep the infection localized within the lungs (Salgame, 2005). Tuberculous granulomas consist of epitheliod macrophages, T cells (CD4+ and CD8+), B cells, and fibroblasts (Fig. 2) (Co et al., 2004 as reviewed by Salgame, 2005). Lymphocytes and multinucleated Langerhans giant cells may also be found in a central location, surrounded by macrophages within the granuloma (Peters et al., 2003).

Formation of Three Granuloma Types

Three types of granulomas are capable of forming during a granulomatous response to Mycobacterium tuberculosis: the noncaseating granuloma, the caseating granuloma, and the reactivating granuloma (as reviewed by Salgame, 2005). Although there is no direct evidence, the caseating and reactivating granulomas may arise from noncaseating granulomas (Salgame, 2005). The noncaseating granuloma consists of CD4+ and CD8+ T cells, B cells, and macrophages harboring few tubercle bacilli. This specific granuloma is also surrounded by a layer of fibroblasts. Phagocytes carrying
tubercle bacilli from secondary infection sites migrate to these types of granulomas. Caseating granulomas are the classic type of granulomas found within tuberculosis patients. Caseating granulomas contain a central necrotic area that contains extracellular bacteria surrounded by macrophages and phagocytes. This specific granuloma also contains lymphoid follicle-like structures that are rich in T and B cells, and macrophages that contain tubercle bacilli. A reactivating granuloma is a very disorganized granuloma type that exhibits enhanced *Mycobacterium* tuberculosis replication. The reactivating granuloma is the least effective in fighting against *Mycobacterium* tuberculosis infection (Fig. 2) (Salgame, 2005).

**FIG. 2** The three types of granulomas capable of forming during the granulomatous response to *Mycobacterium tuberculosis*. Adapted from Salgame, 2005

**Cytokines Involved in the Host Defense Response Against Mycobacterium tuberculosis**

**Interleukin 1 Beta (IL-1β)**

IL-1β is a proinflammatory cytokine produced mainly by monocytes, macrophages, and dendritic cells during an immune response to *Mycobacterium*
tuberculosis (as reviewed by van Crevel et al., 2002). IL-1β is a key cytokine that plays an important role in the acute phase response to mycobacterial infection by inducing fever and cachexia (Raja, 2004). IL-1β is found in excess in tuberculosis patients (Schauf et al., 1993 as reviewed by van Crevel et al., 2002). IL-1R type-1-deficient mice displayed an increased susceptibility to mycobacterial outgrowth as well as defective granuloma formation following infection with *Mycobacterium tuberculosis* (Juffermans et al., 2000). This finding supports the importance of IL-1β in battling *Mycobacterium tuberculosis* infection.

**Interleukin 6 (IL-6)**

IL-6 is a cytokine that possesses both pro- and anti-inflammatory properties (VanHeyningen et al., 1997 as reviewed by van Crevel et al., 2002). IL-6 plays multiple roles during an immune response, including inflammation, hematopoiesis, and differentiation of T cells (Flynn et al., 2001). IL-6 is produced early in mycobacterial infections but also has the potential to be harmful in these infections by inhibiting the production of both TNF-α and IL-1β (Sallusto et al., 2000 as reviewed by van Crevel et al., 2002). IL-6 knockout mice administered a low dose of aerosolized tubercle bacilli maintained a higher bacterial load in the lungs and had decreased IFN-γ production (Saunders et al., 2000). This decrease in IFN-γ production may contribute to the increased susceptibility of IL-6-deficient mice to *Mycobacterium tuberculosis* infection.

**Interleukin 12 (IL-12)**

IL-12 is a very important cytokine with a key role in host defenses against *Mycobacterium tuberculosis*. IL-12 is produced by activated macrophages following phagocytosis of tubercle bacilli, triggering a TH1 response and IFN-γ production. Mice
deficient in the IL-12p40 gene were highly susceptible to intravenous infection with *Mycobacterium tuberculosis* and were unable to control bacterial growth in the lung. The mice also had a decreased survival time, reflecting the decrease in IFN-γ production (Cooper et al., 1997). Humans with mutations in either the IL-12p40 gene or IL-12R gene also showed a reduction in IFN-γ production, however, they were susceptible to disseminated Bacillus Calmette-Guerin (BCG) and *Mycobacterium avium* infections, not *Mycobacterium tuberculosis* (Ottenhoff et al., 1998). In a study with BABL/c mice, a mouse strain highly susceptible to *Mycobacterium tuberculosis* infection, administration of IL-12 early during infection significantly decreased the bacterial burden within their lungs; however, the mice eventually succumbed to infection and died (Flynn et al., 1995). In contrast, IL-12 had only marginal effects on the bacterial numbers in the lungs of C57BL/6 mice (Cooper et al., 1995). The difference in the results presented by these two sets of mice possibly result from the naturally resistant phenotype of the C57BL/6 mouse to tubercle bacilli.

**Interleukin 10 (IL-10)**

IL-10 is an anti-inflammatory cytokine produced by macrophages and T cells during *Mycobacterium tuberculosis* infection and antagonizes proinflammatory responses by downregulating IFN-γ, TNF-α, and IL-12 production (Flynn et al., 2001). Downregulation of proinflammatory cytokines by IL-10 inhibits CD4+ T cell responses and the function of mycobacteria-infected antigen presenting cells (APCs) (Rojas et al., 1999 as reviewed by Flynn et al., 2001). Intravenously BCG-infected IL-10 transgenic mice developed a large bacterial burden in the spleen and liver (Murray et al., 1997), contrasting from intravenously BCG-infected IL-10-deficient mice that displayed a
bacterial burden comparable to the control (Murray et al., 1999). In human tuberculosis, IL-10 production was shown to be higher in peripheral blood monocytes (PBMCs) of anergic tuberculosis patients both before and following stimulation with purified protein derivative (PPD). This finding suggests that *Mycobacterium* tuberculosis-induced IL-10 production is responsible for suppressing an effective immune response (Boussriotis et al., 2000).

**Transforming Growth Factor Beta (TGF-β)**

TGF-β is an anti-inflammatory cytokine produced by monocytes following the detection of mycobacterial products (Toossi et al., 1995). TGF-β is produced in excess and is expressed at sites of active *Mycobacterium* tuberculosis infection (Toossi et al., 1998). TGF-β acts by suppressing cell-mediated immunity. Deactivation of macrophages by TGF-β downregulates IFN-γ and TNF-α production. The generation of reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs) is also inhibited by TGF-β (Toossi et al., 1998). TGF-β was present in the cellular infiltrate of granulomas from patients with active *Mycobacterium tuberculosis* infection, suggesting its involvement in the tissue damage and fibrosis that occurs in cases of tuberculosis infection (Toossi et al., 1995).

**Tumor Necrosis Factor Alpha (TNF-α)**

TNF-α is an important mediator of inflammation during *Mycobacterium* tuberculosis and is believed to play multiple roles in the immune and pathologic responses to tubercle bacilli (Flynn et al., 2001). TNF-α is a proinflammatory cytokine produced by macrophages, monocytes, and T cells upon stimulation with mycobacteria or mycobacterial products (as reviewed by Flynn et al., 2001). TNF-α plays a key role in
granuloma formation, macrophage activation, and possesses immunoregulatory properties (as reviewed by van Crevel et al., 2002).

**Influence of TNF-α on Granuloma Formation and Cytokine Production**

Mice treated with a TNF-α-neutralizing antibody displayed an increase in IFN-γ and IL-12 production and inducible nitric oxide synthase gene 2 (NOS2) expression within lung granulomas (Chakravarty et al., 2008). The production of the pro-inflammatory cytokines IFN-γ and IL-12 increases inflammation within the granulomas, defining a possible anti-inflammatory role for TNF-α. An increased production of the anti-inflammatory cytokine IL-10 was also present within the granulomas. The presence of IL-10 was hypothesized to decrease inflammation within the lungs of the TNF-α-neutralized mice (Chakravarty et al., 2008). To demonstrate the importance of TNF-α in mounting a successful protective immune response against *Mycobacterium tuberculosis* infection, BCG- immunized C57BL/6 mice were treated with TNF-α-neutralizing hamster monoclonal antibody one week prior to intravenous infection with *Mycobacterium tuberculosis*. Results from the experiment revealed that neutralization of TNF-α impaired the ability of the mice to control the infection. Mice with a disruption in the gene encoding for the 55kDa TNF receptor (TNFRp55⁻/⁻) were also unable to control *Mycobacterium tuberculosis* infection (Flynn et al., 1995). Granulomas were present in the lungs of both sets of mice; however they were necrotizing and contained an overwhelming number of tubercle bacilli (Flynn et al., 1995). The findings from both experiments define the importance of TNF-α in controlling *Mycobacterium tuberculosis* infection.
TNF-α-Induced Production of Nitric Oxide (NO)

It is hypothesized that TNF-α stimulates the production of nitric oxide (NO') by Mycobacterium tuberculosis-infected alveolar macrophages (Lyons, 1995 as reviewed by Rich et al., 1997). To study the effect of TNF-α on Mycobacterium tuberculosis-infected alveolar macrophages, human alveolar macrophages were infected with Mycobacterium tuberculosis and incubated with TNF-α or pentoxifylline, a non-specific inhibitor of TNF-α. The results showed that TNF-α alone increased Mycobacterium tuberculosis-induced NO' production by the alveolar macrophages. Pentoxifylline inhibited NO' production by the alveolar macrophages as expected (Rich et al., 1997). The results from this experiment show that TNF-α is required for NO' production by human alveolar macrophages.

Role of TNF-α in Macrophage Apoptosis

TNF-α is hypothesized to contribute to the apoptosis of Mycobacterium tuberculosis-infected macrophages. To investigate the role that TNF-α has in inducing the apoptosis of Mycobacterium tuberculosis-infected macrophages, alveolar macrophages obtained from bronchoalveolar lavage (BAL) of healthy subjects were infected with either the attenuated (H37Ra) or virulent (H37Rv) Mycobacterium tuberculosis strains. Following ELISA analysis of TNF-α levels, apoptosis of the alveolar macrophages was measured using the TUNEL assay. Results from the experiment showed significant TNF-α-induced apoptosis in both Mycobacterium tuberculosis strains compared to the control. H37Ra however, displayed a higher level of TNF-α-induced apoptosis than H37Rv (Balcewicz-Sablinska et al., 1998).
Use of TNF-α Blockers

Systemic spillover of TNF-α can lead to unwanted inflammatory effects such as fever, weight loss, tissue necrosis, and systemic shock (as reviewed by Bekker et al., 1998). In tuberculosis patients receiving treatment, an increase of TNF-α was observed in their plasma and it was accompanied by weight loss and clinical deterioration (Bekker et al., 1998). In order to prevent or limit the deleterious effects of TNF-α, it is necessary to downregulate systemic production of TNF-α and increase soluble TNF-α receptors that block TNF-α activity (as reviewed by van Crevel et al., 2002).

To determine a cause for reduced apoptosis in H37Rv-infected alveolar macrophages (Balcewicz-Sablinska et al., 1998), soluble tumor necrosis factor receptor (sTNFR), a natural inhibitor of TNF-α, was analyzed for its release during Mycobacterium tuberculosis infection. Results uncovered that there was a six-fold increase in the amount of sTNFR present in the supernatant of H37Rv-infected alveolar macrophages than the supernatant of H37Ra-infected alveolar macrophages (Fig. 3) (Balcewicz-Sablinska et al., 1998).
FIG. 3 Higher amount of sTNFR present in the supernatant of H37Rv-infected alveolar macrophages. Adapted from Balcewicz-Sablinska et al., 1998

The presence of sTNFR in the supernatant of H37Rv-infected alveolar macrophages was responsible for the decrease in TNF-α-induced apoptosis. Further analysis showed that increased IL-10 production by the H37Rv-infected macrophages was responsible for the accumulation of sTNFR in the supernatant. The increase in IL-10 inactivated TNF-α. When anti-IL-10 antibody was added to the supernatant, sTNFR release was significantly reduced and apoptosis was increased (Balcewicz-Sablinska et al., 1998). Taken together, the findings from this experiment suggest a role for IL-10 in regulating the release of sTNFR, ultimately controlling TNF-α-induced apoptosis of H37Rv-infected alveolar macrophages.

Individuals treated with TNF-α blockers contract Mycobacterium tuberculosis infection at an increased rate (Salvi et al., 2006). Three TNF-α-inhibiting drugs that are currently being used for the treatment of autoimmune diseases such as rheumatoid arthritis are being studied to determine their effect on the macrophage response to
mycobacterial infection (Harris et al., 2008). The three TNF-α-inhibiting drugs, also known as TNF-α blockers, currently being used are adalimumab (Humira), infliximab (Remicade), and etanercept (Enbrel). In whole blood samples stimulated with Mycobacterium tuberculosis, Humira and Remicade inhibited T cell activation and IFN-γ production whereas Enbrel did not (Table 1) (Saliu et al., 2006).

**Tumor-necrosis-factor blockade: effects on T cell activation (in terms of CD69 expression).**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Cell type</th>
<th>No treatment</th>
<th>Etanercept at 2 μg/mL</th>
<th>Adalimumab at 5 μg/mL</th>
<th>Infliximab at 80 μg/mL</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB-BCF</td>
<td>CD4</td>
<td>0.73</td>
<td>0.60</td>
<td>0.37*</td>
<td>0.22*</td>
<td>.003</td>
</tr>
<tr>
<td>PHA</td>
<td>CD4</td>
<td>14.4</td>
<td>15.1</td>
<td>10.5</td>
<td>10.1*</td>
<td>.015</td>
</tr>
<tr>
<td>PHA</td>
<td>CD8</td>
<td>11.1</td>
<td>9.1</td>
<td>7.8</td>
<td>5.9</td>
<td>.064</td>
</tr>
</tbody>
</table>

**NOTE.** Data are median values for 10 subjects. An asterisk (*) indicates significant difference, compared with values in untreated control cultures, as determined by repeated-measures analysis of variance on ranks followed by post-hoc testing by Duni’s method. Adalimumab and infliximab both significantly inhibited antigen-induced CD4 activation, and similar effects of smaller magnitude were also observed in mitogen-stimulated cells; etanercept had no significant effect. PHA, phytohemagglutinin; MB-BCF, Mycobacterium tuberculosis culture filtrate.

**TABLE 1** Tumor-necrosis-factor blockade: effects on T cell activation (in terms of CD69 expression). Adapted from Saliu et al., 2006

The difference in the effect that Enbrel has on T cell activation and IFN-γ production suggest that it uses a different mechanism than the one used by Humira and Remicade, possibly making it a better drug for the treatment of Mycobacterium tuberculosis infection.

To determine the effect of Humira, Remicade, and Enbrel on phagosome-lysosome fusion, human monocytes were infected GFP-BCG or PKH67 labeled H37Ra and analyzed using confocal microscopy. In the absence of IFN-γ stimulation none of the drugs had an effect on phagosome acidification in BCG-infected monocytes. However, in H37Ra- and H37Rv-infected monocytes pretreated with IFN-γ, Humira, Remicade,
and Enbrel all inhibited IFN-γ phagosome acidification (Harris et al., 2008). In macrophages derived from PBMCs, Humira and Remicade inhibited phagosome acidification in both untreated and IFN-γ-stimulated cells whereas Enbrel did not (Harris et al., 2008). The difference in the macrophage types may have contributed to the differences seen in the actions of the drugs.

**Interferon Gamma (IFN-γ)**

IFN-γ is a cytokine of great importance in controlling *Mycobacterium* tuberculosis infection. IFN-γ is produced by T cells and NK cells and is required for the protective response against *Mycobacterium* tuberculosis. The role of IFN-γ in opposing survival of tubercle bacilli is to promote phagosome acidification and phagolysosome fusion (as reviewed by Salgame, 2005).

**Defective genes for IFN-γ in Mice and Humans**

Humans defective in genes for IFN-γ or the IFN-γ receptor are highly susceptible to mycobacterial infections, including *Mycobacterium tuberculosis* (Jouanguy et al., 1996 as reviewed by Raja, 2004). A lack of IFN-γ also poses a threat to the immune response of mice. IFN-γ gene knockout mice infected with the virulent *Mycobacterium* tuberculosis Erdman strain either intravenously or with aerosol were unable to control infection, making them highly susceptible to *Mycobacterium* tuberculosis infection (Cooper et al., 1993).

**Implication of IFN-γ in NO Production**

IFN-γ is hypothesized to be involved in NO production by activated monocytes. In an experiment where *Mycobacterium* tuberculosis-infected PBMCs were stimulated
with IFN-γ, NO⁻ production was significantly increased (Sharma et al., 2004). The finding from this experiment supports the hypothesis that IFN-γ induces NO⁻ production.
THE INNATE IMMUNE RESPONSE AGAINST MYCOBACTERIUM TUBERCULOSIS

Apoptosis

Apoptosis, also referred to as programmed cell death, is a process that is important to the development and maintenance of the immune system (Kelly et al., 2008). In the host defense against Mycobacterium tuberculosis, apoptosis promotes microbicidal activity in alveolar macrophages in an effort to remove the intracellular macrophage niche (as reviewed by Gan et al., 2008). Macrophage apoptosis may contribute to the innate immune system response against Mycobacterium tuberculosis by containing and limiting the growth of tubercle bacilli (as reviewed by Kelly et al., 2008) by keeping the membrane barrier intact (Gan et al., 2008). Macrophage necrosis, on the other hand, releases viable tubercle bacilli from macrophages for the extracellular spread of infection (as reviewed by Gan et al., 2008). Macrophage necrosis causes cell lysis and death, leading to the inflammation that promotes tissue damage. The act of macrophage necrosis is responsible for the recruitment of naïve macrophages that ultimately become infected, thus prolonging Mycobacterium tuberculosis infection (Gan et al., 2008). The attenuated H37Ra Mycobacterium tuberculosis strain induces macrophage apoptosis while the virulent H37Rv Mycobacterium tuberculosis strain induces macrophage necrosis (Frataazzi et al., 1997 as reviewed by Gan et al., 2008).
Role of Bystander Apoptosis in Reducing the Spread of Mycobacterium tuberculosis

In an attempt to uncover how the process of apoptosis occurs in macrophages of Mycobacterium tuberculosis-infected patients, Kelly et al. (2008) conducted a series of three experiments. Based on findings from previous studies involving the attenuated Mycobacterium tuberculosis strain H37Ra and human macrophage apoptosis, Kelly et al. (2008) conducted an experiment that investigated if apoptosis also occurred in uninfected bystander macrophages. Using the TUNEL staining technique, infected and uninfected macrophages were co-cultured and observed for apoptosis for 24, 48, and 96h. Apoptosis of infected macrophages was observed 24h postinfection with significant increases in apoptosis seen at 48 and 96h postinfection. The results also showed bystander apoptosis in the uninfected macrophages occurring at 48 and 96h postinfection (Fig. 4) (Kelly et al., 2008).

FIG. 4 Mycobacterium tuberculosis strain H37Ra induces bystander apoptosis in uninfected human macrophages. Adapted from Kelly et al., 2008

To determine if bystander apoptosis of the uninfected macrophages was due to the release of soluble factors, supernatant from infected apoptotic macrophages was collected and
filtered to remove any mycobacteria. The supernatant was then added to the uninfected macrophages and the level of apoptosis was measured using ELISA. The results revealed that the supernatant did not induce apoptosis in the uninfected macrophages at any timepoint (Fig. 5) (Kelly et al., 2008).

![Bar graph showing apoptosis relative to uninfected control at various timepoints.](image)

**FIG. 5** Soluble factors released from infected apoptotic human macrophages do not induce apoptosis in uninfected bystander macrophages. *Adapted from Kelly et al., 2008*

In a final attempt to determine the cause of bystander apoptosis, the uninfected macrophages were labeled with PKH67, a green fluorescent dye that gets incorporated into the lipid regions of the plasma membrane, and added to infected macrophages. The cells were co-incubated for 24 and 48h then stained using TUNEL TMR (red) to determine if cell-cell contact was required for bystander apoptosis to occur. Bystander macrophages undergoing apoptosis were labeled with red-green stains, showing an increase in the level of bystander apoptosis in uninfected macrophages (Fig. 6).
FIG. 6 Cell-cell contact between infected and uninfected macrophages induces bystander apoptosis. Adapted from Kelly et al., 2008

The results from the experiment showed that cell-cell contact between the infected and uninfected macrophages induced significant bystander apoptosis. Bystander apoptosis may be a mechanism used by the innate immune response to mediate the killing of uninfected potential niche cells, an occurrence that may serve as a way to control and limit the progression of *Mycobacterium tuberculosis* infection (Kelly et al., 2008).

**Inducible Nitric Oxide Synthase (iNOS) and Nitric Oxide (NO)**

Reactive nitrogen intermediates (RNIs) are antimicrobial agents formed by
macrophages (as reviewed by Miller et al., 2004). RNIs are produced by inducible nitric oxide synthase (iNOS) and gives rise to NO\(^{\cdot}\) (Miller et al., 2004). NO\(^{\cdot}\) is one of the few bactericidal agents produced in vivo that is capable of successfully killing Mycobacterium tuberculosis. NO\(^{\cdot}\) is a highly active free radical that is both lipid and water soluble and has the ability to react with oxygen and oxygen intermediates to yield mycobactericidal products such as NO\(_2\), NO\(_2^{-}\), NO\(_3^{-}\), N\(_2\)O\(_3\), and ONOO\(^{\cdot}\) (as reviewed by Davis et al., 2007).

**Determination of iNOS Recruitment to Phagosomes with the Use of Latex Beads**

Mycobacterium tuberculosis has the ability to disrupt iNOS recruitment to phagosomes (Davis et al., 2007; Miller et al., 2004). In testing the recruitment of iNOS to phagosomes, it is important that the alveolar macrophages are stimulated and ready to battle infection. In studies conducted by Davis et al. (2007) and Miller et al. (2004) alveolar macrophages were stimulated with IFN-\(\gamma\) and lipopolysaccharide (LPS) prior to infection with mycobacteria. In the study conducted by Miller et al. (2004) mouse macrophages were infected with IgG coated latex beads and incubated with polyclonal rabbit anti-iNOS antibody. Using immunofluorescence microscopy, the intracellular recruitment of iNOS to phagosomes infected with mycobacteria was examined. The results revealed that the latex bead phagosomes recruited iNOS, while iNOS recruitment to mycobacterial phagosomes did not occur because of the mycobacteria (Fig. 7) (Miller et al., 2004).
FIG. 7 Latex bead phagosomes recruit iNOS but phagosomes containing mycobacteria do not. J774 macrophages (A and B). Bone marrow-derived macrophages (C and D). Adapted from Miller et al., 2004

Using immunofluorescence laser scanning confocal microscopy, Davis et al. (2007) compared iNOS recruitment of phagosomes containing live mycobacteria to that of phagosomes containing heat-killed mycobacteria. The results revealed that phagosomes containing live mycobacteria had a reduction in iNOS recruitment when compared to latex bead phagosomes, while phagosomes containing heat-killed mycobacteria had no effect on iNOS recruitment to phagosomes (Fig. 8). Taken together, these results suggest that recruitment of iNOS to mycobacteria containing phagosomes is only inhibited in alveolar macrophages harboring live mycobacteria (Davis et al., 2007).
FIG. 8 Live mycobacteria reduces iNOS recruitment to phagosomes. (A) Localization of iNOS (green/red) to the phagosome for latex beads (red) and dead mycobacterial phagosomes (red), but not in live mycobacterial phagosomes (green). (B) iNOS localization to phagosomes in macrophages infected with latex beads, live, or dead mycobacteria. Adapted from Davis et al., 2007

The Actin Cytoskeleton and iNOS Recruitment

It was once believed that iNOS was diffusely distributed in the cytosol of phagosomes but recently it has been recognized that iNOS recruitment to phagosomes within alveolar macrophages requires a functional actin cytoskeleton. Ezrin-radixin-moesin (ERM)-binding phopshoprotein 50 (EBP50) is a protein that belongs to a family of membrane-cytoskeleton linking proteins. EBP50 interacts with the CD44 glycoprotein located at the actin filament-plasma membrane interaction site. The interaction of EBP50 with CD44 allows EBP50 to anchor different proteins to the actin cytoskeleton. Activated mouse alveolar macrophages transfected with GFP-labeled EBP50 were infected with Texas-Red-labeled latex beads and stained for iNOS. Using fluorescence microscopy, the localization of iNOS to the latex bead phagosomes was analyzed. The results from the experiment showed an increase in iNOS recruitment to the latex bead phagosomes.
However, when the mouse macrophages were transfected with a short interfering RNA (siRNA) sequence directed against EBP50, iNOS recruitment to the latex bead phagosomes decreased. These findings show that EBP50 is in fact required for iNOS recruitment to phagosomes (Fig. 9) (Davis et al., 2007).

![Graph A](image1)

**FIG. 9** EBP50 is required for iNOS recruitment to latex bead phagosomes. (B) iNOS recruitment using EBP50. (D) iNOS recruitment using EBP50 siRNA. Adapted from Davis et al., 2007

Coronin-1 is an actin-associated protein that functions in the rearrangement of actin. Coronin-1 is important for cell motility and phagocytosis. Mouse macrophages transfected with coronin-1 siRNA and incubated with Alexa 568-labeled latex beads showed a significant increase in iNOS recruitment to the LB phagosomes (Fig. 10) (Davis et al., 2007).

![Graph B](image2)
Miller et al. (2004) utilized the actin microfilament disrupter cytochalasin D and the actin microfilament stabilizer, jasplakinolide, to show that they cause a decrease in iNOS recruitment to latex bead phagosomes. Cytochalasin D is a membrane permeable peptide that has the ability to bind to actin filaments and prevent their polymerization and elongation. Jasplakinolide is a membrane permeable peptide that participates in actin polymerization and actin filament stabilization. When cytochalasin D treatment was followed by jasplakinolide, iNOS recruitment to latex bead phagosomes was comparable to the control (Fig. 11) (Miller et al., 2004).

Taken together, these findings clearly suggest that interfering with actin microfilament motility can disrupt the recruitment of iNOS to latex bead phagosomes and that it is necessary to have a functional actin cytoskeleton in order to ensure iNOS recruitment to phagosomes (Miller et al., 2004).

Taking their experiment a step further, Davis et al. (2007) performed immunofluorescence laser scanning confocal microscopy to test iNOS recruitment to
mycobacterial phagosomes using EBP50 and coronin-1. Macrophages transfected with siRNA against EBP50 followed by phagocytosis of Alexa-568 labeled dead mycobacteria showed a decrease in iNOS recruitment, demonstrating that a mutation in EBP50 alters iNOS recruitment to mycobacteria-infected alveolar macrophages (Fig. 12).

![Graph showing iNOS recruitment to dead BCG](image)

**FIG. 12** Mutations in EBP50 alters iNOS recruitment to mycobacteria-infected phagosomes. *Adapted from Davis et al., 2007*

When macrophages were transfected with a siRNA against coronin-1 and incubated with Alexa-568 labeled live mycobacteria, no effect on iNOS recruitment to phagosomescontaining live mycobacteria was observed (Fig. 13).

![Graph showing iNOS recruitment to live BCG](image)
FIG. 13 Mutations in coronin-1 does not increase iNOS recruitment to mycobacteria-infected phagosomes. Adapted from Davis et al., 2007

This finding shows that coronin-1 has no role in mycobacteria-induced inhibition of iNOS recruitment to mycobacteria-infected phagosomes (Davis et al., 2007).

Mouse Model of iNOS Recruitment

Variability in the sensitivity of different Mycobacterium tuberculosis strains is hypothesized to affect the ability of RNIs to limit the growth and replication of Mycobacterium tuberculosis (Scanga et al., 2001). To test this possibility, three anonymous clinical isolates of Mycobacterium tuberculosis from patients harboring active infection and the laboratory Mycobacterium tuberculosis Erdman strain were used to test the importance of iNOS-mediated effector functions. C57BL/6 mice and iNOS gene-disrupted mice (NOS2<sup>−/−</sup>) were infected either intravenously or with aerosol containing the different Mycobacterium tuberculosis strains. The results showed the three clinical strains and Erdman strain growing at a higher rate in the NOS2<sup>−/−</sup> mice following intravenous infection than in the C57BL/6 wild-type mice (Fig. 14) (Scanga et al., 2001).
FIG. 14 NOS2<sup>−/−</sup> mice have a higher bacterial burden in the lungs following intravenous infection. NOS2<sup>−/−</sup> mice (closed circles). Wild-type C57BL/6 mice (open circles) mice. *Mycobacterium tuberculosis* strains: (A) Erdman (B-D) Anonymouse clinical isolates. Adapted from Scanga et al., 2001

The NOS2<sup>−/−</sup> mice also had a higher bacterial burden in their lungs following aerosol infection with the clinical and Erdman *Mycobacterium tuberculosis* strains (Fig. 15) (Scanga et al., 2001).
Without the ability to produce iNOS, the mice quickly succumbed to infection regardless of the route of administration. The results from these experiments display the importance of iNOS in controlling *Mycobacterium tuberculosis* infection.

To investigate the role of the adaptive immune response in host resistance to *Mycobacterium tuberculosis*, C57BL/6 mice and NOS2−/− were infected with H37Rv or H37Ra in aerosol and observed for their CD4+ and CD8+ T cell responses. The number of infiltrating leukocytes into the lungs of both sets of mice was similar to one another at day 14 and day 30 postinfection (Fig. 16) (Beisiegel et al., 2009).

Of the infiltrating leukocytes, CD4+ T cell numbers were higher than CD8+ T cell numbers in both sets of mice (Fig. 17).
Further investigation revealed that the CD4⁺ and CD8⁺ T cells predominately produced IFN-γ and TNF-α with negligible IL-17 production (Fig. 18) (Beisiegel et al., 2009).
FIG. 18 CD4⁺ and CD8⁺ T cells of NOS2⁻/⁻ and wild-type C57BL/6 mice predominately produce IFN-γ and TNF-α with negligible IL-17 production. *Adapted from Beisiegel et al., 2009*

This study shows that NOS2⁻/⁻ mice are capable of mounting an adaptive immune response comparable to wild-type control mice upon challenge with *Mycobacterium tuberculosis*.

**Human Study of iNOS Expression**

The production of NO⁺ by *Mycobacterium tuberculosis*-infected mouse alveolar macrophages has led to the hypothesis that human *Mycobacterium tuberculosis*-infected alveolar macrophages are also capable of producing NO⁺. Supernatant from *Mycobacterium tuberculosis*-infected human alveolar macrophages showed an accumulation of NO⁺ that could be inhibited in a dose-dependent manner when an iNOS inhibitor was used (Fig. 19).

![Graph showing the inhibition of NO production by an iNOS inhibitor](image)

**FIG. 19** iNOS inhibitor can inhibit NO⁺ production in human alveolar macrophages in a dose-dependent manner. *Adapted from Rich et al., 1997*

The use of the iNOS inhibitor increased the bacterial burden in the *Mycobacterium tuberculosis*-infected alveolar macrophages (Rich et al., 1997). The results from this specific experiment demonstrate that human *Mycobacterium tuberculosis*-infected...
alveolar macrophages are capable of producing NO and that this production of NO is important for inhibiting the growth of *Mycobacterium tuberculosis*.

iNOS expression and generation of RNIs by alveolar macrophages of patients harboring active *Mycobacterium tuberculosis* infection was hypothesized to be detectable through an increased level of exhaled NO. Wang et al. (1998) conducted a study that measured the level of NO exhaled by patients with active infection. Patients with active *Mycobacterium tuberculosis* infection exhaled significantly higher levels of NO than healthy control subjects. iNOS expression by the alveolar macrophages of the tuberculosis patients was significantly higher than the control subjects and correlated with the level of NO that was exhaled (Fig. 20) (Wang et al., 1998).
FIG. 20 Increased iNOS expression by alveolar macrophages of tuberculosis patients correlates with increased NO exhalation. Top figure: Level of iNOS expression. Bottom figure: Concentration of exhaled NO; TB patients (open circles); Control subjects (closed circles). Adapted from Wang et al., 1998

Confirming these findings, BAL supernatant from the tuberculosis patients contained levels of nitrite that was significantly higher than the nitrite levels observed in the control subjects. Upon further investigation, cultured alveolar macrophages from tuberculosis patients showed a spontaneous release of nitrite in culture medium at a greater extent than that released by control subjects (Fig. 21) (Wang et al., 1998).

FIG. 21 Alveolar macrophages from tuberculosis patients show a higher release of nitrite than control subjects. Adapted from Wang et al., 1998

Overall, the finding from this study supports the hypothesis that an increase in exhaled NO results from increased expression of iNOS by alveolar macrophages.

Toll-Like Receptors (TLRs)

The first step in mounting an effective host response against Mycobacterium tuberculosis begins with innate recognition of mycobacterial products. The purpose of the innate immune system is to mount a rapid protective response during the early phase of infection while instructing adaptive T cell-mediated immune responses during the later phase of infection (Medzhitov et al., 2000 as reviewed by Branger et al., 2004). TLRs are activated immediately upon or shortly after the uptake of Mycobacterium tuberculosis.
by macrophages (Heldwein et al., 2002) which make their successful activation one of vital importance.

The Toll-like receptor was first studied in the fruit fly, *Drosophila melanogaster*. Through the study of *Drosophila melanogaster* TLR proteins, mammalian homologues were discovered. The human homologue of the *Drosophila melanogaster* Toll protein is Toll-like receptor 4 (TLR4). There are three features of the Toll protein that link it with innate immunity. The first feature is its participation in antifungal responses. The second feature is its participation in signal transduction pathways. The similarity between the *Drosophila melanogaster* Toll protein and mammalian homologues is that the *Drosophila melanogaster* Toll protein leads to the activation of the transcription factor Dorsal, the fly homologue of NF-κB in mammals that signals cytokine production. The final feature refers to the similar intracellular domains that the *Drosophila* Toll protein and mammalian TLRs share (as reviewed by Means et al., 1999).

**Mycobacterial Antigens Recognized by TLRs**

To mount a successful response against mycobacterial infection TLRs must be able to recognize and process mycobacterial antigens. Three common antigens known to trigger a response in the event of *Mycobacterium tuberculosis* infection are 19kDa lipoprotein, lipoarabinomannan (LAM), and soluble tuberculosis factor (STF).

### 19kDa Lipoprotein

19kDa lipoprotein is a bacterial lipoprotein that belongs to a group of molecules possessing pathogen-associated molecular patterns. The pathogen-associated molecular patterns present in this group of molecules contain molecular motifs that are found in microorganisms, not mammalian hosts (Stenger et al., 2002). 19kDa lipoprotein is an
antigen secreted by *Mycobacterium tuberculosis* and is a potent inducer of T\(_H\)1 and cytolysis T cell responses through the induction of IL-12 (Brightbill et al., 1999). 19kDa lipoprotein induced IL-12 release in both mouse and human macrophages. The interaction of 19kDa lipoprotein with TLR2 shows that it is capable of inducing NO production in mouse macrophages (Brightbill et al., 1999).

*Lipoarabinomannan (LAM)*

LAM is a glycolipid found in the walls of mycobacteria and plays a key role in triggering cytokine secretion by macrophages. LAM is a potent inducer of TNF-\(\alpha\) in both mouse and human macrophages (Moreno et al., 1989). LAM activates mouse macrophages by interacting with the cell surface molecule CD14 (Means et al., 1999). Through the interaction of LAM with CD14, an intracellular signal is generated by TLR2 to activate macrophages (Means et al., 1999).

*Soluble Tuberculosis Factor (STF)*

STF is a short-term-culture filtrate of *Mycobacterium tuberculosis* that contains the mycobacterial glycolipid phosphatidylinositolmannan (PIM). PIM induces TNF-\(\alpha\) secretion by mouse macrophages. STF is similar to both 19kDa lipoprotein and LAM in that it only interacts with TLR2, not TLR4 (Jones et al., 2001).

*Role of Myeloid Differentiation Protein 88 (MyD88) in TLR Signal Transduction*

Signal transduction by TLRs is necessary for carrying out an effective innate immune response. Proper signal transduction in most TLRs require myeloid differentiation protein 88 (MyD88) (as reviewed by Tjarnlund et al., 2006). MyD88 is an adaptor molecule that links TLR recognition to the activation of interleukin (IL)-1/IL-1 receptor-associated kinase (IRAK), a factor essential for *Mycobacterium tuberculosis*-
induced activation of macrophages (as reviewed by van Crevel et al., 2002). Multiple studies reviewed by Tjarnlund et al. (2006) showed that mice deficient in MyD88 failed to generate any proinflammatory responses when stimulated through their TLRs, leaving them highly susceptible to mycobacterial infections.

**Control of Mycobacterium tuberculosis Infection by TLR2 and TLR4**

TLR2 and TLR4 have both demonstrated the ability to mediate *Mycobacterium tuberculosis*-induced intracellular signaling in vitro and are therefore the TLRs of interest (Branger et al., 2004). The mouse types used for the TLR studies were the C3H/HeJ (TLR4-defective) mouse and its control C3H/HeN (TLR4-competent) mouse as well as the C57BL/6 (TLR2-competent) and BALB/c (CD14-competent) mouse. In a study conducted by Reiling et al. (2002) mice deficient in CD14, TLR2, and TLR4 were administered 100 CFU of *Mycobacterium tuberculosis* aerosol. All three sets of mice contained bacterial loads in their lungs similar to those of their control mice at 105 days postinfection (Fig. 22) (Reiling et al., 2002).

![FIG. 22](image)

**FIG. 22** TLR2 and TLR4 are not effective alone, they require aid from other innate immune components. Control (closed circle) and deficient mice (open circle). *Adapted from Reiling et al., 2002*

The results showed that neither TLR2 nor TLR4 were effective in providing a significant innate immune response by themselves but that they require aid from other innate
immune components for a successful immune response when challenged with *Mycobacterium tuberculosis*. In a study conducted by Tjarnlund et al. (2006), TLR2 and TLR4 knockout mice and C57BL/6 wild-type mice were also infected with *Mycobacterium tuberculosis* aerosol. The results showed the bacterial growth in the lungs of both the TLR2 and TLR4 knockout mice to be significantly higher during the early phase, 3 weeks postinfection, in comparison to the C57BL/6 wild-type mice. However, during the later phase, 8 weeks postinfection, there were no differences between the three groups of mice (Fig. 23) (Tjarnlund et al., 2006).

![FIG. 23](image)

**FIG. 23** TLR2/4-deficient mice display increased susceptibility to aerosol infection with *Mycobacterium tuberculosis* H37Rv. *Adapted from Tjarnlund et al., 2006*

To test the importance of TLR4 in preventing the advancement of tuberculosis infection, C3H/HeJ mice were administered 100 CFU aerosol of *Mycobacterium tuberculosis* and observed for 250 days. All of the C3H/HeJ mice died between 160 and 200 days post-infection (Fig. 24) (Abel et al., 2002).
FIG. 24 Reduced survival of the TLR4 mutant C3H/HeJ mice after *Mycobacterium tuberculosis* H37Rv infection. Wild-type C3H/HeN mice (open circles). TLR4 mutant C3H/HeJ mice (filled circles). Adapted from Abel et al., 2002

A similar fifteen week study conducted by Branger et al. (2004) showed similar results when seven of twelve C3H/HeJ mice administered a 100 CFU aerosol of *Mycobacterium tuberculosis* died and all of the C3H/HeN wild-type mice survived (Fig. 25). The findings from both experiments suggest an important role for TLR4 during the later phase of *Mycobacterium tuberculosis* infection.

FIG. 25 Decreased survival time of TLR4 mutant C3H/HeJ mice following aerosol infection with *Mycobacterium tuberculosis* H37Rv. Adapted from Branger et al., 2004

An experiment performed by Drennan et al. (2004) spanning a 6 month period tested the ability of TLR2-deficient mice to control *Mycobacterium tuberculosis* infection. The mice were administered 100 CFU of *Mycobacterium tuberculosis* aerosol and survived
the entire 6 month experimental period. This finding indicates that TLR2 has a different way of combating *Mycobacterium tuberculosis* infection than the one used by TLR4.

In separate experiments, higher doses of *Mycobacterium tuberculosis* were administered by aerosol to the mice to determine if an increased dose of aerosol could further affect the function of TLRs. In a second experiment performed by Reiling et al. (2002), a higher dose of *Mycobacterium tuberculosis* aerosol was administered to both C3H/HeJ and TLR2-deficient mice. Both sets of mice were administered a 2000 CFU inoculum of *Mycobacterium tuberculosis* aerosol and were monitored for 210 days. The C3H/HeJ mice were as resistant to the high-dose *Mycobacterium tuberculosis* infection as their C3H/HeN control mice while the TLR2-deficient were significantly more susceptible to the high-dose aerosol infection than the C57BL/6 mice (Fig. 26).

![FIG. 26 TLR2-deficient mice are significantly more susceptible to high dose aerosol infection than TLR4 mutant C3H/HeJ mice. Adapted from Reiling et al., 2002](image)

When TLR2-deficient mice were administered a 500 CFU inoculum of *Mycobacterium tuberculosis* aerosol in an experiment conducted by Drennan et al. (2004), the mice began
to die between 7 and 11 weeks postinfection and were all dead by week 22. C57BL/6 mice administered the same infectious dose remained healthy and survived the entire 10 month experimental period. Data collected from the experiment showed the number of viable mycobacteria in the lungs of the TLR2-deficient mice to be highest between week 8 and 13 postinfection. In a 23 week study conducted by Branger et al. (2004) the same 500 CFU aerosol dose was administered to C3H/HeJ mice and all of the mice died (Fig. 27).

Because of the differences in the survival rates between the C3H/HeJ and C3H/HeN mice Branger et al. (2004) went a step further and conducted an experiment to determine whether bacterial loads in the mice differed during the early phase of infection. The lungs of both the C3H/HeJ and C3H/HeN mice were observed at 2 and 5 weeks postinfection. At 2 weeks postinfection, the lungs of the C3H/HeJ mice contained more viable mycobacteria than the C3H/HeN mice and at 5 weeks postinfection the lungs of
the C3H/HeJ mice displayed a 2.8-fold increase of mycobacteria in their lungs (Fig. 28) (Branger et al., 2004).

**FIG. 28** The lungs of TLR4 mutant C3H/HeJ mice contain more viable bacteria at both 2 and 5 weeks postinfection. Adapted from Branger et al., 2004

**Histological Analyses of Mice Lacking TLR4**

To gain a visual understanding of the damage done to the lungs of mice lacking TLR4 during *Mycobacterium tuberculosis* infection, Branger et al. (2004) performed a histopathological analysis of the lungs of C3H/HeJ and C3H/HeN mice at 2 and 5 weeks following aerosol infection. At 2 weeks postinfection, the lungs of the C3H/HeN mice displayed a slight cell infiltration consisting of macrophages, lymphocytes and neutrophils (Fig. 29a) while the C3H/HeJ mice displayed larger areas of inflammation (Fig. 29b) that was attributed to the higher number of leukocytes (Table 2). At 5 weeks postinfection, inflammation was more pronounced in both the C3H/HeN and C3H/HeJ mice. The inflammatory infiltrate of the C3H/HeN mice consumed between 50 and 60% of their lung surface (Fig. 29c) (Table 2) and the infiltrate of the C3H/HeJ mice
consumed between 70 and 80% of their lung surface (Fig. 29d) (Table 2) (Branger et al., 2004).

FIG. 29 Histopathological analysis of the lungs of C3H/HeJ and C3H/HeN mice at 2 and 5 weeks postinfection. (A) Wild-type lung tissue 2 weeks postinfection. (B) TLR4 mutant lung tissue 2 weeks postinfection. (C) Wild-type mouse lungs 5 weeks postinfection. (D) TLR4 mutant lungs 5 weeks postinfection. Adapted from Branger et al., 2004

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Cell subsets in the left lung in TLR4 mutant and wild-type mice during pulmonary tuberculosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>TLR4 mutant (×10⁶)</td>
</tr>
<tr>
<td>Leukocytes (%)</td>
<td>19.1 ± 0.9</td>
</tr>
<tr>
<td>Macrophages (%)</td>
<td>16.5 ± 1.0</td>
</tr>
<tr>
<td>Granulocytes (%)</td>
<td>39.2 ± 1.5</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>44.3 ± 1.7</td>
</tr>
<tr>
<td>CD4⁺</td>
<td>60.4 ± 0.9</td>
</tr>
<tr>
<td>CD8⁺</td>
<td>26.5 ± 3.1</td>
</tr>
<tr>
<td>CD4⁺/CD8⁺</td>
<td>77.7 ± 1.3⁺</td>
</tr>
<tr>
<td>CD4⁺/CD25⁺</td>
<td>82.1 ± 2.1</td>
</tr>
<tr>
<td>CD8⁺/CD4⁺</td>
<td>27.2 ± 1.5</td>
</tr>
<tr>
<td>CD8⁺/CD25⁺</td>
<td>12.0 ± 0.2</td>
</tr>
</tbody>
</table>

Cell subsets in the lungs of TLR4 mutant and wild-type mice infected with M. tuberculosis 2 and 5 weeks post-infection. Total leukocyte counts per left lung. Differential counts as percentage of total leukocytes. Flow cytometry results are expressed as the percentage of CD4⁺, CD8⁺, CD4⁺/CD25⁺ and CD8⁺/CD4⁺ T cells within the CD3⁺ population in the left lung. Data are mean ± SEM of six to nine mice per group. *P < 0.05 versus wild-type at the same time point.  

TABLE 2 Cell subsets in the left lung in TLR4 mutant and wild-type mice during pulmonary tuberculosis. Adapted from Branger et al., 2004

Abel et al. (2002) also studied the inflammatory responses in the lungs of C3H/HeJ and C3H/HeN mice exposed to Mycobacterium tuberculosis aerosol. Microscopy of the
lungs of both the C3H/HeJ and C3H/HeN mice 28 days postinfection showed the formation of small granulomas (Fig. 30a; Fig. 30b). At 140 days postinfection the C3H/HeJ mice had massive inflammation in their lungs with large numbers of macrophages and neutrophils consuming majority of the airspace (Fig. 30d) while the C3H/HeN mice did not (Fig. 30c) (Abel et al., 2002).

FIG. 30 The inflammatory responses in the lungs of both C3H/HeJ and C3H/HeN mice exposed to Mycobacterium tuberculosis aerosol. (A) C3H/HeN and (B) C3H/HeJ mice 28 days following aerosol infection. (C) C3H/HeN and (D) C3H/HeJ mice 140 days following aerosol infection. Adapted from Abel et al., 2002

To further investigate the inflammatory cells recruited to the lungs of the mice during the inflammatory response, BAL was performed 4 weeks following aerosol infection with Mycobacterium tuberculosis. The results showed comparable total cell counts for the two groups of mice, however the C3H/HeN mice had a significantly larger proportion of macrophages present than the C3H/HeJ mice (Fig. 31a). Besides the number of macrophages present in the two groups of mice, the lymphocyte population was also comparable (Fig. 31b). BAL analysis showed a significant number of neutrophils being recruited to the lungs of the C3H/HeJ mice but not the C3H/HeN mice (Fig. 31c). The
data obtained from this experiment support the histological findings of reduced macrophage but significant neutrophil recruitment to the lungs of C3H/HeJ mice harboring *Mycobacterium tuberculosis* infection (Abel et al., 2002).

**FIG. 31** Decreased macrophage and increased neutrophil recruitment into the lungs of TLR4 mutant C3H/HeJ mice 4 weeks postinfection. *Adapted from Abel et al., 2002*

**TLR2- and TLR4-Mediated Cytokine Production**

After observing how a lack of TLR2 and/or TLR4 could affect cell recruitment to the lungs of *Mycobacterium tuberculosis*-infected mice, Reiling et al. (2002) compared the production of IL-12 in TLR2-deficient and C3H/HeJ mice in a 10 day study to determine if a lack in cell recruitment placed a damper on important cytokine production. There were no significant differences observed in IL-12 production between the C3H/HeJ and C3H/HeN mice at day 0 or day 10 postinfection (Fig. 32a). The TLR2-deficient mice however showed a significant decrease in the IL-12 production in comparison to C57BL/6 mice at day 10 (Fig. 32b). This difference in IL-12 production at day 10 is hypothesized to be responsible for the TLR2-deficient mice’s higher susceptibility to *Mycobacterium tuberculosis* infection and decreased proinflammatory response (Reiling et al., 2002).
Abel et al. (2002) also conducted an experiment to observe cytokine production in the lungs of C3H/HeJ mice following *Mycobacterium tuberculosis* infection. At 4 weeks postinfection, BAL fluid of C3H/HeJ mice was assessed using ELISA. The results showed a significant reduction in IL-12 production (Fig. 33a) while TNF-α and IFN-γ production remained steady (Fig. 33b; Fig. 33c).

**FIG. 32** IL-12 production in C3H/HeJ and TLR2-deficient mice following high-dose aerosol infection with *Mycobacterium tuberculosis*. Adapted from Reiling et al., 2002

**FIG. 33** Decreased IL-12 production in BAL fluid of TLR4 mutant C3H/NeJ mice following *Mycobacterium tuberculosis* infection. (A) IL-12p40, (B) IFN-γ, (C) TNF-α. Adapted from Abel et al., 2002
To further assess cytokine production in the C3H/HeJ mice, the infected lungs were homogenized and the supernatants were analyzed. The results once again showed a significant reduction in both IL-12 and TNF-α (Fig. 34a; Fig. 34c) while the level of IFN-γ was only slightly lower than the C3H/HeN mice (Fig. 34b). The results from this study suggest that the reduction in proinflammatory cytokines upon *Mycobacterium tuberculosis* infection is associated with an increased susceptibility to *Mycobacterium tuberculosis* infection (Abel et al., 2002).

**FIG. 34** Decreased cytokine levels in the lungs of TLR4 mutant C3H/HeJ mice. (A) IL-12, (B) IFN-γ, (C) TNF-α. Adapted from Abel et al., 2002

IFN-γ production in C3H/HeJ mice following aerosol infection with *Mycobacterium tuberculosis* displayed similar IFN-γ levels to C3H/HeN mice at 2 weeks postinfection.

At 5 weeks postinfection, however, IFN-γ levels in the C3H/HeJ mice were elevated (Fig. 35), coinciding with the increased mycobacterial load observed in their lungs (Branger et al., 2004).
FIG. 35 IFN-γ concentrations in lungs of TLR4 mutant C3H/HeJ and wild-type mice following aerosol infection with *Mycobacterium tuberculosis*. Adapted from Branger et al., 2004

In the case of TLR2 and TLR4 knockout mice, mRNA expression of both IFN-γ and TNF-α were the same as the wild-type mice at both 3 and 8 weeks postinfection (Tjarnlund et al., 2006).

Gamma Delta (γδ) T Cells

γδ and CD4+ cells are the two dominant T cell subsets found in the human immune response to *Mycobacterium tuberculosis* that contribute to the proinflammatory responses involved in protective immunity (Tsukaguchi et al., 1999). In normal healthy individuals, there are more *Mycobacterium tuberculosis*-reactive γδ T cells than αβ T cells. All γδ T cells express a TCR encoded by a single Vγ9 and Vδ2 gene (as reviewed by Li et al., 1998) and studies have shown that γδ T cells are the main source of IFN-γ production (Tsukaguchi et al., 1999). Li et al. (1998) showed that patients harboring active tuberculosis infection have a significantly reduced number of *Mycobacterium tuberculosis*-reactive γδ T cells in their blood and BAL in comparison to normal healthy individuals. This finding may explain the progression and severity of *Mycobacterium tuberculosis* disease. The loss or absence of γδ T cells in tuberculosis patients may
reflect anergy, inactivation or dysfunction of immune cell populations necessary for γδ T cell activation and growth, or activation-induced cell death (AICD) (as reviewed by Li et al., 1998).

**Effect of Drug Chemotherapy on γδ T Cell Numbers**

As drug treatment for patients harboring active Mycobacterium tuberculosis infection evolves, a question of whether or not drug chemotherapy is responsible for the decreased numbers of Mycobacterium tuberculosis-reactive γδ T cells in tuberculosis patients remains unclear. In an attempt to determine if drug chemotherapy adversely affects γδ T cells or has a beneficial effect on γδ T cell numbers, Li et al. (1998) conducted an experiment to analyze the number of peripheral γδ T cells during and after drug chemotherapy. The results from the study showed a significant reduction in the γδ T cell numbers of non-treated active disease patients in comparison to the γδ T cell numbers of non-treated normal patients. Non-treated patients with inactive (latent) disease showed a slight decrease in their γδ T cell numbers in comparison to non-treated normal patients but showed a significant increase in their γδ T cell numbers in comparison to non-treated patients with active disease (Fig. 36) (Li et al., 1998).
FIG. 36 Tuberculosis patients with active disease have reduced numbers of *Mycobacterium tuberculosis*-reactive $\gamma\delta$ T cells. Adapted from Li et al., 1998

All of the active and inactive disease patients and normal patients undergoing drug treatment were followed for a period of time spanning from 2 to 16 months. The treated active disease patients had a decrease in their $\gamma\delta$ T cell numbers despite the length of treatment. $\gamma\delta$ T cell numbers were recovered in the treated inactive (latent) disease patients (Fig. 36). Based on the results received from the treated groups of patients, it was concluded that drug chemotherapy treatment for *Mycobacterium tuberculosis* was not responsible for the loss of $\gamma\delta$ T cell numbers in patients harboring active *Mycobacterium tuberculosis* infection (Li et al., 1998).

$\gamma\delta$ T Cells vs. CD4$^+$ T Cells: IFN-$\gamma$ Production

$\gamma\delta$ T cells and CD4$^+$ T cells produce IFN-$\gamma$ and serve as cytotoxic effector cells in response to *Mycobacterium tuberculosis*-infected macrophages. To test IFN-$\gamma$ production by $\gamma\delta$ T cells and CD4$^+$ T cells in response to *Mycobacterium tuberculosis* infection,
supernatant from human PMBCs were stimulated with either Mycobacterium tuberculosis or PPD and analyzed using ELISA and ELISPOT. CD4\(^+\) T cells produced IFN-\(\gamma\) in response to PPD and Mycobacterium tuberculosis, while \(\gamma\delta\) T cells produced IFN-\(\gamma\) in response to Mycobacterium tuberculosis only (Fig. 37) (Tsukaguchi et al., 1999). The findings from this experiment suggest that CD4\(^+\) T cells and \(\gamma\delta\) T cells differ in the mycobacterial antigens that they recognize. Findings from the experiment also suggest that \(\gamma\delta\) T cells are capable of producing more IFN-\(\gamma\) on a per cell basis than CD4\(^+\) T cells in response to tubercle bacilli (Fig. 37) (Tsukaguchi et al., 1999).

**FIG. 37** Comparison of IFN-\(\gamma\) production by CD4\(^+\) and \(\gamma\delta\) T cells in response to *Mycobacterium tuberculosis*. Adapted from Tsukaguchi et al., 1999

TNF-\(\alpha\) plays a role in protective immunity against mycobacterial infections in both mice and humans (as reviewed by Tsukaguchi et al., 1999). To determine the role of \(\gamma\delta\) T cells and CD4\(^+\) T cells in TNF-\(\alpha\) production, the supernatant from Mycobacterium tuberculosis-infected human monocytes in both the presence and absence of \(\gamma\delta\) T cells and CD4\(^+\) T cells was analyzed using ELISA. Results from the experiment revealed that
TNF-α is increased in the presence of γδ T cells and CD4+ T cells (Tsukaguchi et al., 1999).

**Influence of NK Cells on γδ T Cell Numbers**

NK cells are effective regulators of the innate and adaptive immune responses. The participation of NK cells in the early immune response to intracellular pathogens has been recognized. NK cells are capable of direct cell lysis, IFN-γ production, and initiation of apoptosis (as reviewed by Brill et al., 2001). Isolated human NK cells added to Mycobacterium tuberculosis-infected monocytes mediated the killing of intracellular tubercle bacilli within 24h of coculture (Brill et al., 2001), demonstrating the effector function of this cell type. Because the relationship between NK and γδ T cell numbers in active tuberculosis infection has not been studied, Zhang et al. (2006) hypothesized that NK cells might be involved in the immune regulation of γδ T cells in response to Mycobacterium tuberculosis and that the loss of γδ T cells may be due to a decrease in NK cell activity. To investigate the role of NK cells in the immune response of γδ T cells to Mycobacterium tuberculosis infection, heat-treated antigens from Mycobacterium tuberculosis were used to expand γδ T cells in vitro. Results from the experiment showed that γδ T cells proliferate faster in a high NK cell-percentage population than in a low NK cell-percentage population (Fig. 38) (Zhang et al., 2006).
FIG. 38 γδ T cells proliferate faster in a high NK cell-percentage population. Adapted from Zhang et al., 2006. This finding suggests that NK cell numbers affect the proliferation response of γδ T cells and that in the event of NK cell depletion, γδ T cell proliferation would decrease (Zhang et al., 2006).
Interleukin 17 (IL-17)

IL-17 is a potent inflammatory cytokine secreted by T lymphocytes such as Th17 and CD4⁺ T cells (as reviewed by Peng et al., 2008). IL-17 induces chemokine production and augments neutrophil accumulation (as reviewed by Lockhart et al., 2006). The production of IL-17 by Th17 cells is regulated by IFN-γ. It is hypothesized that IFN-γ and IL-17 have the ability to counterregulate each other during chronic mycobacterial infections (Cruz et al., 2006).

**IL-17-Producing Cells**

In a study to investigate the source of IL-17 in tuberculosis patients, the proportion of IL-17-producing cells in their peripheral blood was measured. γδ T cells were the main source of IL-17, with other IL-17-producing cells making up a small proportion of the peripheral blood lymphocytes. The overall percentage of IL-17-producing cells in the peripheral blood of the tuberculosis patients and healthy donors was very similar (Fig. 39b), however the percentage of IL-17-producing γδ T cells was significantly higher in tuberculosis patients (Fig. 39c) (Peng et al., 2008).
FIG. 39 The percentage of IL-17–producing γδ T cells is significantly higher in tuberculosis patients. (B) The percentages for IL-17+ cells of lymphocytes. (C) The percentages for CD3+ cells and γδ T cells among IL-17+ cells. Adapted from Peng et al., 2008

Purified cells from the lungs of Mycobacterium tuberculosis-infected mice at 2, 4, and 52 weeks postinfection were studied. The purified cells were fractioned into three groups. Group 1 consisted of CD4+ and CD8+ T cells, B cells, and macrophages (“CD4 pool”). Group 2 was composed of γδ T cells. Group 3 consisted of NK cells, NK T cells, and other cell types (“flow through”). Each cell group was stimulated with supernatant from Mycobacterium tuberculosis-infected dendritic cells for 48h and tested for IL-17 production using ELISPOT. The results showed that the γδ T cells and “flow through” fractions were capable of producing IL-17 at all three time points at a higher level than the “CD4 pool” (Fig. 40) (Lockhart et al., 2006).
FIG. 40 γδ and other non-CD4 T cells contribute to IL-17 production in Mycobacterium tuberculosis-infected mice. (B) Lungs at 2 weeks postinfection, (C) Lungs at 4 weeks postinfection, (D) Lungs at 52 weeks postinfection. Adapted from Lockhart et al., 2006

The results obtained from this particular experiment show that γδ T cells and other non-CD4⁺ or CD8⁺ T cells are capable of producing IL-17 during Mycobacterium tuberculosis infection and that γδ T cells produce more IL-17 than CD4⁺ T cells (Lockhart et al., 2006).

**Counterregulation of IL-17 and IFN-γ**

IFN-γ regulates IL-17 production both in vitro and in vivo (as reviewed by Cruz et al., 2006). In an experiment using C57BL/6 and IFN-γ-deficient mice, IFN-γ production was tested to see if it could limit IL-17 T cell responses to BCG. The C57BL/6 and IFN-γ-deficient mice were intravenously infected with BCG and the expansion of IL-17-producing CD4⁺ T cells was observed. The IFN-γ-deficient mice produced more IL-17 than the C57BL/6 mice at both 15 and 21 days postinfection (Fig. 41). This finding supports the hypothesis that IFN-γ regulates the IL-17 response (Cruz et al., 2006).
FIG. 41 IFN-γ-deficient mice produce more IL-17 than wild-type C57BL/6 mice following BCG infection. C57BL/6 mice (shaded bar). IFN-γ-deficient mice (striped bar). Adapted from Cruz et al., 2006

In an experiment conducted by Peng et al. (2008) the percentage of IFN-γ-producing γδ T cells in the peripheral blood of tuberculosis patients was significantly lower than healthy donors while the percentage of IL-17-producing γδ T cells was significantly higher in the tuberculosis patients (Fig. 42b). Further testing showed the ratio of IFN-γ-producing γδ T cells to IL-17-producing γδ T cells in the peripheral blood of tuberculosis patients to be significantly lower than those of the healthy donors (Fig. 42c). This finding confirms that IL-17-producing γδ T cells are increased in tuberculosis patients (Peng et al., 2008).

FIG. 42 IL-17-producing γδ T cells are increased in the peripheral blood of tuberculosis patients. Adapted from Peng et al., 2008

The findings from these experiments support the hypothesis that an inability of tuberculosis patients to produce enough IFN-γ to dampen IL-17-mediated inflammation during chronic Mycobacterium tuberculosis infection predisposes them to inappropriate tissue-damaging responses (Cruz et al., 2006).

CD4+ and CD8+ T Cells

The protective immune response against Mycobacterium tuberculosis relies on cell mediated immunity. Mycobacterium tuberculosis is a pathogen that primarily resides within cells, usually macrophages. Because of the survival method used by
Mycobacterium tuberculosis, T cell effector mechanisms rather than antibodies are required to control and/or eliminate the bacteria (Flynn et al., 2001). It is the interaction between infected macrophages and T cells that defines the successful elimination of Mycobacterium tuberculosis from an infected individual (van Crevel et al., 2002). A proper protective immune response mounted against Mycobacterium tuberculosis occurs in a series of steps. First, the bacteria are delivered by resident and/or recruited APCs to secondary lymphoid tissues. Second, the APCs migrate to regions of the secondary lymphoid tissues that are enriched in naïve CD4+ and CD8+ T cells that become activated. Third, the activated CD4+ and CD8+ T cells migrate via the bloodstream to the site/s of infection. Lastly, the CD4+ and CD8+ T cells emigrate from the intravascular space into the tissue where they either deliver macrophage-activating cytokines or kill the infected cells by direct contact (Peters et al., 2003).

**CD8+ T Cell Response to Mycobacterium tuberculosis Infection**

At one time research focused primarily on CD4+ T cell responses to Mycobacterium tuberculosis infection. Studies have shown CD4+ T cells to be two-fold more abundant than CD8+ T cells at sites of tuberculosis infection in both mice and humans (as reviewed by Peters et al., 2003). Recently, more interest has been placed into the role that CD8+ T cells have in mounting an immune response against Mycobacterium tuberculosis infection. Serbina et al. (2001) reported that the percentage of CD8+ T cells in Mycobacterium tuberculosis-infected lungs of CD4+ T cell-deficient mice was two-fold higher than in the lungs of wild-type mice following aerosol infection (Fig. 44).
FIG. 43 CD4$^+$ T cell-deficient mice have more CD8$^+$ T cells in their lungs than wild-type mice following *Mycobacterium tuberculosis* infection. Wild-type mice (open square). CD4$^+$ T cell-deficient mice (closed square). *Adapted from Serbina et al., 2001*

Direct lysis and IFN-γ production are two effector functions used by CD8$^+$ T cells (Flynn et al., 2001) and CD4$^+$ T cells are important for the maintenance of CD8$^+$ T cell effector functions (Serbina et al., 2001). So despite the increase in the number of CD8$^+$ T cells, further investigation revealed significant reductions in the specific lysis mediated by the cytotoxic CD8$^+$ T cells in the lungs of CD4$^+$ T cell-deficient mice (Fig. 45).

FIG. 44 Lysis mediated by cytotoxic CD8$^+$ T cells is reduced in the lungs of CD4$^+$ T cell-deficient mice. Wild-type mice (squares). CD4$^+$ T cell-deficient mice (circles). *Adapted from Serbina et al., 2001*

(Serbina et al., 2001). The results from the two experiments conducted by Serbina et al. (2001) suggest that the cytotoxic activity of *Mycobacterium tuberculosis*-specific cytotoxic CD8$^+$ T cells is compromised in the absence of CD4$^+$ T cells.
Based on the newfound information pertaining to the role that CD8\(^+\) T cells have in combating *Mycobacterium tuberculosis* infection in the mouse model, Carranza et al. (2006) analyzed the role of this specific T cell subset in individuals harboring active *Mycobacterium tuberculosis* disease. To test if exposure to *Mycobacterium tuberculosis* aerosol induced bactericidal activity mediated by T cells, autologous blood CD8\(^+\) and CD4\(^+\) T cells from healthy individuals were added to attenuated H\(_{37}\)Ra and virulent H\(_{37}\)Rv *Mycobacterium tuberculosis*-infected alveolar macrophages. The H\(_{37}\)Ra and H\(_{37}\)Rv *Mycobacterium tuberculosis*-infected cultures both controlled the growth of the tubercle bacilli when the alveolar macrophages were cocultured with CD8\(^+\) T cells (Fig. 46) (Carranza et al., 2006).

![FIG. 45](image)

FIG. 45 Both H\(_{37}\)Ra- and H\(_{37}\)Rv-infected alveolar macrophage cultures control the growth of tubercle bacilli when cocultured with CD8\(^+\) T cells. Alveolar macrophages alone (closed circles). Alveolar macrophage/CD8 coculture (open circles). Adapted from Carranza et al., 2006

This finding further supports the participation of CD8\(^+\) T cells in the immune response against *Mycobacterium tuberculosis* infection.
**IFN-γ Production by CD8⁺ T Cells**

To test the influence that CD8⁺ T cells had on the production of IFN-γ, alveolar macrophage cultures and alveolar macrophage/CD8⁺ T cell cocultures of healthy individuals were infected with the H₃₇Ra or H₃₇Rv *Mycobacterium tuberculosis* strains for one week. Both strains caused an increase in IFN-γ concentration from day 0 to day 7. The H₃₇Rv-infected alveolar macrophage cultures and alveolar macrophage/CD8⁺ T cell cocultures displayed IFN-γ concentrations that were two-three times higher than the H₃₇Ra strain. However, IFN-γ concentrations of the alveolar macrophage/CD8⁺ T cell cocultures were one- to four times higher than the alveolar macrophage cultures of both strains (Fig. 47) (Carranza et al., 2006).

![IFN-γ production in alveolar macrophage/CD8⁺ T cell cocultures](image)

**FIG. 46** IFN-γ production in alveolar macrophage/CD8⁺ T cell cocultures is significantly higher than alveolar macrophage cultures alone. Alveolar macrophages alone (black bars). Alveolar macrophage/CD8⁺ coculture (white bars). **Adapted from Carranza et al., 2006**

The results show that CD8⁺ T cells are capable of inhibiting *Mycobacterium tuberculosis* growth and producing IFN-γ, two elements necessary in combating tuberculosis. To compare the amount of IFN-γ produced by CD8⁺ T cells to that produced by CD4⁺ T
cells, the same experiment was performed except that alveolar macrophage/CD4⁺ T cell cocultures were used in place of alveolar macrophage/CD8⁺ T cell cocultures. Similar to the results given when studying CD8⁺ T cells, the IFN-γ concentrations were higher in the alveolar macrophage/CD4⁺ T cell cocultures than in the alveolar macrophage cultures alone (Fig. 48) (Carranza et al., 2006).

FIG. 47 IFN-γ concentrations are higher in alveolar macrophage/CD4⁺ T cell cocultures than alveolar macrophages alone. Alveolar macrophages alone (black circles). Alveolar macrophage/CD4 coculture (white circles). IFN-γ production in alveolar macrophages alone (black bars). IFN-γ production in alveolar macrophages/CD4 coculture (gray bars). Adapted from Carranza et al., 2006

Overall, it was shown that IFN-γ levels of the alveolar macrophage/CD4⁺ T cell cocultures were higher than those of the alveolar macrophage/CD8⁺ T cell cocultures regardless of the *Mycobacterium tuberculosis* strain used. The findings from these two experiments suggest that *Mycobacterium tuberculosis* antigen-specific human CD4⁺ T cells are the primary source of IFN-γ and that CD8⁺ T cells although less than CD4⁺ T cells, do contribute to IFN-γ production in the event of *Mycobacterium tuberculosis* infection (Carranza et al., 2006).

**IFN-γ Production by CD4⁺ T Cells**

To take a closer look at the role of CD4⁺ T cells in *Mycobacterium tuberculosis*, Caruso et al. (1999) performed a study involving wild-type, MHC II-deficient, and CD4⁺ T cell-deficient mice. The results from their experiment showed all three groups of mice
producing low levels of IFN-γ one week postinfection while at two weeks postinfection
IFN-γ production in the MHC II-deficient and CD4⁺ T cell-deficient mice was only 44% of that in the wild-type mice. It was not until four weeks postinfection that the total amount of IFN-γ within the lungs of the wild-type and CD4⁺ T cell-deficient mice was similar (Fig. 49) (Caruso et al., 1999).

![Graph showing IFN-γ production over time](A)

FIG. 48 IFN-γ production in lung cells from *Mycobacterium tuberculosis* -infected mice. Wild-type mice (open square). MHC II-deficient mice (closed circle). CD4⁺ T cell-deficient mice (closed triangle). Adapted from Caruso et al., 1999

Further analysis uncovered that IFN-γ production by CD8⁺ T cells in the CD4⁺ T cell-deficient and wild-type mice was the same at two weeks postinfection but that the contribution of CD4⁺ T cells to IFN-γ production was absent in the MHC II-deficient and CD4⁺ T cell-deficient mice (Caruso et al., 1999). It is hypothesized that the absence of CD4⁺ T cells in MHC II-deficient and CD4⁺ T cell-deficient mice may be responsible for preventing CD8⁺ T cells from becoming cytotoxic, an occurrence that may contribute to MHC II-deficient and CD4⁺ T cell-deficient mice being more susceptible to *Mycobacterium tuberculosis* infection. The findings from this experiment suggest that IFN-γ production by CD4⁺ T cells is needed to have an effective immune response early
in *Mycobacterium tuberculosis* infection and that IFN-γ production by CD8\(^+\) T cells is insufficient to control infection on its own (Caruso et al., 1999).
DRUG TREATMENT FOR *MYCOBACTERIUM TUBERCULOSIS*

As more knowledge and understanding about how *Mycobacterium tuberculosis* is recognized and controlled by the immune system surfaces, the development of effective vaccines remains an important goal in controlling tuberculosis infection. Vaccination is still the best hope for ultimately eradicating this disease (Schluger, 2001), however, there are challenges in attempts to design and develop successful vaccines to fight against *Mycobacterium tuberculosis* infection. The pathogenesis of tuberculosis infection is studied in animal models and there is no completely satisfactory animal model of human tuberculosis available (Schluger, 2001). This presents a challenge because animal models permit the study of the risk of actual tuberculosis infection whereas human studies focus on the progression of tuberculosis infection to active disease in patients harboring latent infection (Schluger, 2001).

Although there is no perfect animal model for the study of *Mycobacterium tuberculosis* in humans, animal studies have the potential to provide useful information about *Mycobacterium tuberculosis* infection. Various animal hosts (Table 3) have been used for tuberculosis studies but the expense of some of the animals and the cost of keeping them under level III biosafety conditions has made the mouse the model of choice (Orme, 2005).
Vaccine testing in the mouse model occurs in a series of steps. First the mice are vaccinated against *Mycobacterium tuberculosis* then infected with virulent *Mycobacterium tuberculosis* a few weeks later. Following infection with *Mycobacterium tuberculosis* the mice are screened at various time points and at 30 days postinfection are tested for the bacterial load in their lungs and other organs such as the spleen and liver. The bacterial loads of the infected mice are then compared to those of the uninfected control mice (Orme, 2005). The results produced in these studies allow for identification and evaluation of vaccine longevity and effectiveness as well as the degree to which the mice are able to generate both a T\(_{11}\)1-type CD4\(^+\) and CD8\(^+\) T cell response (Orme, 2005).

**Vaccines**

In designing an effective vaccine against *Mycobacterium tuberculosis* infection it is important for the vaccine to possess certain qualities. An effective vaccine should be able to induce a good immune response. The vaccine should consist of molecules that are foreign to the host and complex, demonstrating stability and degradability so that the antigenic material gets processed and presented to lymphocytes (Sable et al., 2007). In searching for good vaccine candidates it is ideal for the vaccine to be safe and

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**TABLE 3** Overview of animal models for vaccine testing. *Adapted from Orme, 2005*

Vaccine testing in the mouse model occurs in a series of steps. First the mice are vaccinated against *Mycobacterium tuberculosis* then infected with virulent

*Mycobacterium tuberculosis* a few weeks later. Following infection with *Mycobacterium tuberculosis* the mice are screened at various time points and at 30 days postinfection are tested for the bacterial load in their lungs and other organs such as the spleen and liver. The bacterial loads of the infected mice are then compared to those of the uninfected control mice (Orme, 2005). The results produced in these studies allow for identification and evaluation of vaccine longevity and effectiveness as well as the degree to which the mice are able to generate both a T\(_{11}\)1-type CD4\(^+\) and CD8\(^+\) T cell response (Orme, 2005).

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inexpensive, provide full protection after a single administration, provide worldwide protection against disease and infection, induce lifelong immunological memory, and not compromise the tuberculin skin tests (TSTs) (Andersen, 2001).

Recombinant Bacillus Calmette-Guerin (BCG)

The recombinant Bacillus Calmette-Guerin (BCG) vaccine, originally developed from *Mycobacterium bovis*, was designed nearly a century ago. BCG is considered to be a safe cost effective option for vaccinating against *Mycobacterium tuberculosis* infection (Orme, 2005). However, multiple BCG vaccine trials carried out in different countries have shown this vaccine to be variable in its degree of protection against *Mycobacterium tuberculosis* infection. BCG possesses an effectiveness ranging from 0-80% and its efficacy is believed to wane with time, remaining protective for only 10-20 years (as reviewed by Sable et al., 2007). Studies have shown that tuberculin reactivity following BCG vaccination depends on the age of administration. Administration of the BCG vaccine during infancy has been shown to be highly effective in protecting against severe cases of *Mycobacterium tuberculosis* due to the rapid waning of tuberculin reactions (as reviewed by Menzies, 2000). BCG vaccination of individuals after the first year of life, however, has been shown to result in persistent tuberculin reactions (as reviewed by Menzies, 2000). It is argued that this loss of protection from childhood through young adult life may be the reason for the increased incidence of tuberculosis infection (Sable et al., 2007). Several reasons have been suggested for variation in the protective efficacy an overall failure of the BCG vaccine. Variations in the effectiveness of the BCG vaccine are believed to be either directly or indirectly related to genetic differences in vaccinated populations, differences in BCG strains, dose and vaccination protocols, over-attenuated
parent strains, administration of the vaccine to already sensitized or infected individuals, and the interaction between BCG and environmental mycobacteria which can mask or block its protective effect (as reviewed by Sable et al., 2007).

The 0-80% variable efficacy of BCG and the difficulty of improving its protection have lead to the need for more effective tuberculosis vaccines (as reviewed by Sable et al., 2007). The United States and other industrialized countries with low incidences of tuberculosis infection have discontinued the use of the BCG vaccine. BCG vaccination has been shown to affect tuberculin skin tests (TSTs) which makes it difficult to interpret the results (Cohn, 2001). Because there is no reliable method to distinguish between an individual displaying a true-positive TST because of actual Mycobacterium tuberculosis infection from an individual with a false-positive TST resulting from prior vaccination with BCG, creating a more effective vaccine to aid in the prevention of Mycobacterium tuberculosis is one of extreme importance (Cohn, 2001).

_Culture Filtrate Protein (CFP)_

In early attempts to develop effective vaccines against Mycobacterium tuberculosis infection, heat-killed bacilli were the preparations of choice. However, vaccines containing heat-killed tubercle bacilli only gave rise to non-specific inflammatory responses as opposed to the long-term protection that was desired (Orme, 1988). Only mice that were immunized with live Mycobacterium tuberculosis generated a protective T cell response over a 30 day experimental period (Orme, 1988). It is hypothesized that the secreted proteins of Mycobacterium tuberculosis present in the secretory or total culture filtrate protein (CFP) pools are the key protective antigens of the tubercle bacillus responsible for the rise of the T cell-mediated host immune responses.
necessary for the control and containment of *Mycobacterium tuberculosis* infection (Roberts et al., 1995). Today, the use of CFP vaccines is being explored because of their ability to impart significant protection against tuberculosis infection in the mouse model. Mice immunized with purified CFP antigens from the *Mycobacterium tuberculosis* Erdman strain and challenged with a low-dose aerosol of *Mycobacterium tuberculosis* were resistant to tuberculosis infection. Immunization with the CFP antigens generated CD4+ T cells that secreted IFN-γ (Roberts et al., 1995), presenting the possibility for the use CFP vaccines. However, despite findings from CFP experiments, there are two limitations preventing CFP from being an effective vaccine. The first limitation is that CFP provides initial protection upon infection with *Mycobacterium tuberculosis*, but like BCG, the effectiveness wanes with time. The second limitation of using CFP as vaccine is that it has to be delivered in adjuvant (Roberts et al., 1995). Another setback for the use of CFP vaccines reside in the differences that arise with each culture condition and the risk of the vaccine possibly possessing both protective and deleterious molecules because of improper purification (Sable et al., 2007).

*Early Secretory Antigenic Target of 6 kDa (ESAT-6)*

Live vaccines can cause life-threatening disease in immunocompromised individuals, a fact that has lead to the development of subunit vaccines. Mycobacterial components such as proteins, lipids, and carbohydrates are screened from both secretory and subcellular compartments to develop subunit vaccines (Sable et al., 2007). The early secretory antigenic target of 6 kDa (ESAT-6), a secreted antigen of *Mycobacterium tuberculosis*, has been shown to give a strong protective response against tuberculosis infection (Orme, 2005). Low molecular weight polypeptides such as ESAT-6 make ideal
vaccine candidates because of their ability to induce dominant $T_{H1}$ responses with negligible $T_{H2}$ responses (Sable et al., 2007). In mouse macrophages ESAT-6 demonstrated the ability to enhance IFN-$\gamma$-induced accumulation of iNOS, suggesting its participation in NO release via macrophage stimulation (Singh et al., 2005). The strong antigenicity of ESAT-6 is hypothesized to result from its small size, possibly rendering it more susceptible to presentation to and processing by CD4$^+$ T lymphocytes following its release in the phagosome. It is also hypothesized that ESAT-6 gains access to the cytoplasm and class-I processing machinery contained in the proteosome, leading to strong cytotoxic T cell responses (as reviewed by Sable et al., 2007). As with other vaccines however, vaccination with ESAT-6 has presented a problem. The problem is that ESAT-6 has a low inherent immunogenicity and requires being complexed with multiple strong adjuvants to mount a sufficient immune response. Mice immunized with ESAT-6 complexed with the hydrophobic adjuvant monophosphoryl lipid A (MPL) did not give rise to IFN-$\gamma$-producing T cells and mice immunized with ESAT-6 complexed with dimethyl dioctadecylammonium bromide (DDA) give rise to a low number IFN-$\gamma$-producing T cells. It was not until ESAT-6 was emulsified in DDA-MPL that a very high number of IFN-$\gamma$-secreting CD4$^+$ T cells was observed (Brandt et al., 2000). The results from this experiment emphasizes how the use and choice of adjuvant influences the efficacy of ESAT-6 in mounting a sufficient IFN-$\gamma$-producing T cell response.

**Inhalable Microparticles**

The manner in which drugs are delivered to targeted alveolar macrophages can affect their activation (Sharma et al., 2007). Sharma et al. (2001) showed that drug concentrations found in the serum or macrophages of mice can vary based on the route of
administration. They observed that high serum drug concentrations did not necessarily lead to high intracellular drug concentrations and that high intracellular drug concentrations achieved by administering microparticles did not necessarily lead to high serum drug concentrations (Fig. 50) (Sharma et al., 2001).

A proposed reason for treatment failure in the event of *Mycobacterium tuberculosis* infection is that drug concentrations achieved in the cytosol of targeted cells through oral administration are not sufficient enough to kill the bacteria residing within the macrophages (Sharma et al., 2001).

Macrophages infected with *Mycobacterium tuberculosis* retain their ability to phagocytose and process additional material (Oh et al., 1996 as reviewed by Sharma et al., 2007). Because the role of alveolar macrophages is to uptake bacteria found in the lungs and transport it to secondary lymphoid organs, it is possible that the bacteria could
be exposed to, taken up by the bloodstream, and delivered to sites of macrophage trafficking. Sharma et al. (2001) proposed that loading resident alveolar macrophages of the lung with biodegradable drug-containing microparticles ranging in size from 0.5-3 microns would lead to transport of the drug to sites where alveolar macrophages migrate, thus preventing the spread of *Mycobacterium tuberculosis* which would otherwise go undetected. Inhaled microparticles phagocyted by alveolar macrophages may deliver larger amounts of drug to the cytosol than using the oral route or by dissolving the drugs in body fluids (Sharma et al., 2001). Goals of using inhalable microparticles as opposed to oral drug administration to battle *Mycobacterium tuberculosis* infection include reducing the drug dose, reducing the dose frequency and toxicity, improving patient compliance, and most importantly targeting specific macrophages that harbor *Mycobacterium tuberculosis* (Sharma et al., 2001). The overall objective of using inhalant microparticles to treat tuberculosis infection is to achieve high intracellular drug concentrations in the infected macrophages (Sharma et al., 2007).

*Isoniazid (H) and Rifampicin (R)*

In developing an effective aerosol drug to treat *Mycobacterium tuberculosis* infection, it is important for the drug to be potent and long-lived. In an attempt to prevent drug resistance while treating *Mycobacterium tuberculosis*, single-drug therapy aerosols are not used (as reviewed by Sharma et al., 2001) and inhalable microparticles containing multiple drugs in a dry powder are preferred (Sharma et al., 2001). In treating *Mycobacterium tuberculosis* infection Isoniazid (H) and Rifampicin (R) are the two first-line anti-tuberculosis inhalant drugs of choice (Sharma et al., 2001). To test the potency of H and R, mouse macrophages were exposed to equivalent amounts of H and R in a
time spanning from 0 to 30 minutes. The macrophages were exposed to H and R dissolved in culture medium and in microparticle form. Both H and R maintained higher concentrations within the macrophages after 30 minutes in microparticle form as opposed to the soluble form that presented a lower concentration within the macrophages (Fig. 51) (Sharma et al., 2001).

![Graph showing concentration of H and R in microparticles and soluble form over time](image)

**FIG. 50** Both H and R maintain higher concentrations within alveolar macrophages in microparticle form. Adapted from Sharma et al., 2001

In a three day study of mice infected with *Mycobacterium tuberculosis*, administration of H and R aerosol resulted in the microparticles targeting alveolar macrophages of both the *Mycobacterium tuberculosis*-infected mice and the uninfected control mice. Following the administration of H and R, the macrophages of the mice were up to 50µm in diameter and contained 10 or more particles. The alveolar macrophages also displayed no signs of
intact mycobacteria, a finding that presents promise for the use of aerosols to treat

*Mycobacterium tuberculosis* infection (Fig. 52) (Sharma et al., 2007).

**FIG. 51** Alveolar macrophages display no signs of intact mycobacteria following the administration H and R aerosol. The arrow indicates electron-opaque debris.  *Adapted from Sharma et al., 2007*

*Effect of H and R on NO Production*

Intracellular RNIs are direct in their anti-mycobacterial function (Sharma et al., 2007). An increase in NO production indicates that alveolar macrophages have become activated. Sharma et al. (2007) exposed uninfected alveolar macrophages and *Mycobacterium tuberculosis*-infected alveolar macrophages to H and R drug containing microparticles over a set period of 3, 9, and 24h. The results showed larger amounts of NO being produced 9h after infection with *Mycobacterium tuberculosis* when compared to the untreated control. When equivalent amounts of H and R were used in solution, no significant increases in NO production were observed (Fig. 53) (Sharma et al., 2007).
FIG. 52 H and R activate alveolar macrophages better in microparticle form than in solution at 3 (filled bars), 9 (hatched bars) and 24 (white bars) h after treatment. ‘U+MP’ refers to uninfected cells treated with microparticles, ‘Inf’ refers to cells receiving 25 moi of M. tuberculosis but no further treatment, ‘Inf+ D’ refers to infected cells exposed to drugs in solution and ‘Inf+ MP’ refers to infected cells treated with an equivalent dose of microparticles.  Adapted from Sharma et al., 2007

These findings support the observation that inhaled microparticles have a greater effect on alveolar macrophage activation than drugs in solution.

Effect H and R on Cytokine Production

To understand the effect that drug treatment has on *Mycobacterium tuberculosis* infection, a three day experiment comparing TNF-α, IFN-γ, IL-10, and IL-12 cytokine production by cells recovered from different mouse subjects was conducted. The results showed an up-regulation of all four cytokines in untreated *Mycobacterium tuberculosis*-infected mice; however, there was no significant increase in IL-12 production. Oral drug treatment with H and R down-regulated the production of all four cytokines while the combination of oral and inhalant drug administration had no significant effect on any of the cytokines. Inhalation drug treatment alone induced significant TNF-α production and downregulated IFN-γ production (Fig. 54) (Sharma et al., 2007).
Of the four cytokines tested, TNF-α and IL-12 were the most significantly induced following treatment with H and R microparticles (Fig. 55) (Sharma et al., 2007).
or blank, drug-free microparticles (falling hatching). Uninfected cells administered drug-containing microparticles (vertical lines) served as an additional control. Adapted from Sharma et al., 2007

These findings continue to support the important roles that TNF-α and IL-12 have in fighting *Mycobacterium tuberculosis* infection.
DISCUSSION

*Mycobacterium tuberculosis* is a devastating disease that strategically evades destruction by the host immune response. The innate and adaptive immune responses triggered by this mycobacterium create a cascade of events that work to limit and contain the growth of infection. The host defense mechanisms mounted against *Mycobacterium tuberculosis* provides a glance into how the body naturally attempts to fight this disease and are summarized in Table 4 and Figure 55.

Macrophage apoptosis is hypothesized to contribute to the host innate immune response by containing and limiting the growth of tubercle bacilli. The study conducted by Kelly et al. (2008) addressed the possibility of bystander apoptosis where uninfected bystander cells undergo apoptosis as a way to prevent the dissemination of infection. It was concluded that bystander apoptosis was not due to the release of soluble factors by apoptotic *Mycobacterium tuberculosis*-infected macrophages but rather the result of cell-cell contact between the infected apoptotic cells and the uninfected bystander cells. Cell-cell contact between the infected and uninfected cells may have been required for apoptosis to occur, but Kelly et al. (2008) failed to investigate the mechanism responsible for the cell-cell contact-induced apoptosis of the uninfected bystander cells. It would have benefited them to take the experiment a step further to investigate the reason for this occurrence by considering the participation of gap junctions or the interaction of cell surface molecules with one another. Because no further experimentation was conducted to explain the mechanism behind the cell-cell contact-induced apoptosis, their findings
have to be interpreted with great caution and perhaps a follow-up study designed to address how cell-cell contact-induced apoptosis occurred should be conducted.

Nitric oxide (NO) is one of the few bactericidal agents produced by infected macrophages that is capable of killing \textit{Mycobacterium tuberculosis}. Miller et al. (2004) showed that iNOS is recruited to phagosomes not containing mycobacteria and Davis et al. (2007) showed that iNOS is only recruited to phagosomes containing dead mycobacteria. The combined findings from both experiments show that live mycobacteria have a way of disrupting the recruitment of iNOS to infected phagosomes and it encourages the progression of \textit{Mycobacterium tuberculosis} infection. Further experimentation by Miller et al. (2004) and Davis et al. (2007) showed that alterations or deletions in one or more of the elements contributing to the action of the actin cytoskeleton can interfere with microfilament motility and disrupt the recruitment of iNOS to phagosomes.

iNOS gene-disrupted mice (NOS2\textsuperscript{-/-}) displayed a higher bacterial burden in their lungs following \textit{Mycobacterium tuberculosis} infection, however they were able to mount an adaptive immune response comparable to that of wild-type mice (Beisiegel et al., 2009). The fact that NOS2\textsuperscript{-/-} mice had an infiltration of IFN-\(\gamma\)- and TNF-\(\alpha\)-producing CD4\(^+\) and CD8\(^+\) T cells similar to that of wild-type mice but still succumbed to infection strongly supports the notion that NO production is vital for fighting \textit{Mycobacterium tuberculosis} infection. The results from their experiment also suggest that proinflammatory cytokines alone are not enough to fight \textit{Mycobacterium tuberculosis} infection in NOS2\textsuperscript{-/-} mice.

The hypothesis that human alveolar macrophages are capable of producing NO in the event of \textit{Mycobacterium tuberculosis} infection was confirmed in a studies by Rich et
al. (1997) and Wang et al. (1998). Rich et al. (1997) showed that supernatant from human *Mycobacterium tuberculosis*-infected alveolar macrophages contained NO that could be inhibited through the use of a iNOS inhibitor. Wang et al. (1998) conducted a clinical study that fell in line with the results given by Rich et al. (1997). Wang et al. (1998) showed that patients with active *Mycobacterium tuberculosis* infection had significantly higher levels of NO exhaled in their breath than healthy control subjects. Further experimentation revealed that iNOS expression by the alveolar macrophages of the tuberculosis patients was significantly higher than the control subjects and that the increase in iNOS expression correlated with the level of exhaled NO. The findings from this clinical study have the potential to be used as a screening tool for people suspected of harboring *Mycobacterium tuberculosis* infection. Since the level NO exhaled in the breath of the tuberculosis patients correlated with the amount of NO being produced, perhaps breathalyzer screenings could be used to determine the existence of a mycobacterial infection and its seriousness.

TLR2 and TLR4 have displayed their importance in limiting and controlling the advancement of *Mycobacterium tuberculosis*. In the absence of TLR2 and/or TLR4, infection with *Mycobacterium tuberculosis* was shown to be fatal in mutant mice. Following infection with *Mycobacterium tuberculosis*, TLR2- and TLR4-deficient mice displayed bacterial burdens in their lungs that were significantly higher than the bacterial burdens present in the wild-type control mice. The mice were ultimately shown to have a large decrease in IL-12 production (Reiling et al., 2002) which is hypothesized to be responsible for their increased susceptibility to infection with *Mycobacterium tuberculosis*. This finding is of great importance because IL-12 is a proinflammatory
cytokine produced upon TLR activation and in essence bridges the innate and adaptive immune responses through CD4⁺ T cell recruitment and NK cell activation. Without adequate IL-12 production, receiving aid from other innate immune components such as γδ T cells or the proinflammatory cytokines IFN-γ and TNF-α is inhibited and results in uncontrollable growth of tubercle bacilli.

γδ T cells contribute to the proinflammatory responses triggered upon infection with *Mycobacterium tuberculosis*. In a study conducted by Li et al. (1998) patients with active tuberculosis infection were shown to have a significantly reduced number of *Mycobacterium tuberculosis*-reactive γδ T cells in their blood and bronchoalveolar lavage (BAL) both before and after receiving drug chemotherapy. The decrease in the number of *Mycobacterium tuberculosis*-reactive γδ T cells was attributed to the progression and severity of infection in the patients. The problem with this conclusion is that the type of drug treatment chosen to treat the *Mycobacterium tuberculosis* infection is one that is non-specific and kills infected cells as well as healthy cells. Active disease patients and patients with latent disease were administered chemotherapy for a time span of 2 to 16 months. At the 2 month mark only the active disease patients’ γδ T cell numbers were counted. Beyond 2 months, both the active and latent disease patients’ γδ T cell numbers were counted. This presents a problem because the counting of the γδ T cells for the 2 patient groups was not consistent throughout the experiment. Since the γδ T cell numbers of patients with latent *Mycobacterium tuberculosis* was not measured 2 months after drug chemotherapy, how can it be concluded that they regained their γδ T cell numbers after 16 months of treatment? I understand that healthy individuals cannot be administered chemotherapy treatments for experimental purposes; however, Li et al. (1998) concluded
that the γδ T cell numbers of the active tuberculosis patients was significantly decreased despite the experimental length. Without the use of a healthy control to undergo the same treatment regimen to see exactly how their γδ T cell numbers are affected following drug chemotherapy, how can it be concluded that the decrease seen in the γδ T cell numbers of the tuberculosis patients is really as significant as the experiment claims? Perhaps to address this question future studies should be performed using mice so that the extent of the effect that drug chemotherapy places on γδ T cell numbers in healthy subjects, active, and latent *Mycobacterium tuberculosis*-infected subjects can be observed.

Lockhart et al. (2006) conducted a study to show that γδ T cells and other non-CD4⁺ or CD8⁺ T cells are capable of producing IL-17 during *Mycobacterium tuberculosis* infection. The findings from their study show that γδ T cells produce more IL-17 than CD4⁺ T cells however, the way that they present their conclusion is misleading. Purified cells from *Mycobacterium tuberculosis*-infected mice were fractioned into three groups but the group referred to as the “CD4 pool” consisted of more than CD4⁺ T cells. The “CD4 pool” also included CD8⁺ T cells and B cells. The results from their experiment are misleading because if it is concluded that other non-CD4⁺ cells are capable of producing IL-17, then it only makes sense to have a group only consisting of CD4⁺ cells to serve a control. It would have possibly been more accurate to refer to the “CD4 pool” as the “adaptive immune cell pool” considering that all of the cells in the group are involved in adaptive immunity.

Peng et al. (2008) showed that the percentage of IFN-γ-producing γδ T cells was lower than the percentage of IL-17-producing γδ T cells in tuberculosis patients. It is hypothesized that IFN-γ regulates the IL-17 response and that IL-17 can in return regulate
the IFN-γ response. The results from their study suggest that the inability of tuberculosis patients to produce enough IFN-γ to dampen IL-17-mediated inflammation during *Mycobacterium tuberculosis* infection predisposes them to inappropriate tissue-damaging responses. I on the other hand interpret their results a little differently. The way that I interpret their findings is that if IL-17 is placing a damper on the IFN-γ responses, then macrophage activation, CD8⁺ T cell recruitment, and most importantly NO production will be decreased. As I see it, this damper on IFN-γ production allows for the existence *Mycobacterium tuberculosis* infection, with IL-17 aiding in its survival.

The development of more effective drugs and the search for better treatment options for eradicating *Mycobacterium tuberculosis* remains ongoing. Despite the development of different vaccine options, each one presents its own set of problems. For instance, BCG is varies in its effectiveness, it has a waning efficacy, and is responsible for false-positive TSTs. The CFP vaccine also has a waning efficacy and it needs to be complexed with adjuvants. ESAT-6, like CFP, must be complexed with adjuvants. In light of this, Sharma et al. (2001) showed that the drug form and the route of administration can alter the effectiveness of the drug. In 2001, Sharma et al. proposed the use of the inhalant drugs Isoniazid (H) and Rifampicin (R) to treat *Mycobacterium tuberculosis* infection. Findings from their experiment showed that both H and R are most effective in microparticle form as opposed to being dissolved in solution (Sharma et al., 2001). Further studies in 2007 showed that the use of H and R in microparticle form can promote macrophage activation and NO production. In sync with the production of NO by activated macrophages, the proinflammatory cytokines IL-12 and TNF-α were produced the most upon treatment with H and R (Sharma et al., 2007). As it currently
stands, the use of inhalant microparticles is one of the most promising treatments in trying to eliminate *Mycobacterium tuberculosis* infection.

    TNF-α is a proinflammatory cytokine with a big responsibility in defending the host against *Mycobacterium tuberculosis*. TNF-α is shown to play a key role in granuloma formation and macrophage apoptosis. Mice that failed to produce adequate amounts of TNF-α or had a disruption in the gene encoding for the 55kDa TNF receptor (TNFRp55−/−) were unable to control infection and eventually died (Flynn et al., 1995). More importantly, in humans, Saliu et al. (2006) showed that individuals undergoing treatment with TNF-α blockers contract *Mycobacterium tuberculosis* infection at an increased rate. The three popular commercial TNF-α inhibitor drugs are Humira, Remicade, and Enbrel. Using these drugs, Saliu et al. (2006) showed that Humira and Remicade act by inhibiting T cell activation and IFN-γ production whereas Enbrel does not. The findings from their study is important because individuals being treated with TNF-α inhibitor drugs for autoimmune diseases such as rheumatoid arthritis have a higher risk of contracting *Mycobacterium tuberculosis*, specifically if they are using Humira or Remicade. At the moment, Enbrel sounds the most promising in the event that an individual with an autoimmune disease contracts *Mycobacterium tuberculosis* infection because it still allows for the activation of T cells and the production of IFN-γ, two elements absolutely necessary to successfully battle *Mycobacterium tuberculosis* infection.

    New strides are being made to advance the techniques used to study and develop a cure for *Mycobacterium tuberculosis*. In recent years there has been more interest placed into the role of vitamin D and its influence on *Mycobacterium tuberculosis* infection.
African-Americans have an increased susceptibility to *Mycobacterium tuberculosis* infection and usually have a more rapid and severe course of disease than Caucasian-Americans. Monocytes cultured in serum from African-Americans displayed vitamin D levels that were significantly lower than the vitamin D levels from cultures containing serum from Caucasian-Americans (Liu et al., 2006). This occurrence has been attributed to the increased skin melanin content of African-Americans that causes decreased serum levels of vitamin D (as reviewed by Liu et al., 2006).

Studies aimed at determining the role that vitamin D has in combating *Mycobacterium tuberculosis* have displayed conflicting results. Whole blood analyses from tuberculosis patients administered a single 2.5mg oral dose of vitamin D displayed enhanced immunity (Martineau et al., 2007) and when vitamin D was added to *Mycobacterium tuberculosis*-infected macrophages, the number of viable bacilli was reduced (Liu et al., 2006). Both of these studies support the notion that vitamin D promotes macrophage activation and helps to suppress the growth of *Mycobacterium tuberculosis*. On the other hand, vitamin D has been shown to elicit a suppressive effect on the production of T_{H1} cytokines, specifically IFN-γ and TNF-α. Prahbu et al. (2009) showed that vitamin D suppresses the production of both IFN-γ and TNF-α by CD4^{+} and CD8^{+} T cells. Another experiment analyzing the effects of vitamin D on cytokine production showed that tuberculosis patients had a significant decrease in the amount of IFN-γ and IL-12 produced by their PBMCs (Vidyarani et al., 2007). These findings however, suggest a role for vitamin D in reducing inflammation within the lungs of *Mycobacterium tuberculosis*-infected individuals.
The study of cholesterol is another avenue that is being explored to uncover its influence on the incidence of *Mycobacterium tuberculosis* infection. Cholesterol is a structural component of cellular membranes and it accumulates at the site of mycobacterial uptake in macrophages (Pieters et al., 2002). Infected mouse macrophages accumulated cholesterol at the site of bacterial entry while cholesterol of uninfected macrophages remained distributed within the plasma membrane (Gatfield et al., 2000). Mycobacteria bind to cholesterol with high affinity, so in an attempt to uncover the exact role of cholesterol in the phagocytosis of tubercle bacilli, cholesterol was depleted from the macrophages and the macrophages were observed for the uptake of the tubercle bacilli. The results showed an 85% reduction in the phagocytosis of tubercle bacilli (Gatfield et al., 2000). Gatfield et al. (2000) showed that the presence of cholesterol is required for the phagocytosis of tubercle bacilli. With this information future studies could focus on identifying the components of mycobacteria that bind to cholesterol. Such findings could lead to strategies that interfere with cholesterol-mediated phagocytosis of mycobacteria (Pieters et al., 2002). Gatfield et al. (2000) also showed that a depletion of cholesterol on the cell surface of macrophages could decrease phagocytosis of tubercle bacilli however; depleting cholesterol from the surface of macrophages may not serve as the best method to prevent *Mycobacterium tuberculosis* infection given that cholesterol has multiple roles in insuring the proper function of cells. In this case I think more research is needed to determine whether the act of reducing or completely depleting cholesterol from the surface of macrophages is more detrimental to the function of the macrophages in the long run despite the initial benefit that it offers in helping to prevent the uptake of tubercle bacilli.
Regardless of the direction that future studies decide to take I think that future animal studies should focus more on animals harboring latent *Mycobacterium tuberculosis* infection. By focusing on animals with latent infection, it could possibly lead to the design of new treatment options for humans harboring latent *Mycobacterium tuberculosis* infection. Finding a new way to battle and creating a cure for latent *Mycobacterium tuberculosis* infection will dramatically reduce the incidence of disease within the human population. Animal models should also focus on animals that have been previously vaccinated with the BCG vaccine. Focusing on these animals will allow for the study and possible development of a way to prolong the effectiveness and longevity of the BCG vaccine. The problem with current animal models is that most of them look at immune reactions in short-term assays and these type of assays do not allow time to access the vaccine’s longevity and the degree to which memory cells are or can be formed. Lastly, because *Mycobacterium tuberculosis* affects the production of the cytokines necessary for the successful containing and killing of infection, future drug treatments should consider adding cytokines such as TNF-α, IFN-γ or IL-12 to vaccines and aerosols as a means to help drive T cell recruitment and activation and NO production.
Table 4 Summary of Observations

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<th>Summary of Observations</th>
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<tr>
<td><strong>Mycobacterium tuberculosis</strong></td>
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<tr>
<td>- Second leading cause of death among all infectious diseases worldwide</td>
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<td>- Approximately 2 million people die each year from this disease</td>
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<td>- One-third of the world’s population remains infected</td>
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<tr>
<td><strong>Apoptosis</strong></td>
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<tr>
<td>- Cell-cell contact between <em>Mycobacterium tuberculosis</em> -infected and uninfected macrophages is required to induce bystander apoptosis</td>
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<tr>
<td><strong>Inducible Nitric Oxide Synthase (iNOS)</strong></td>
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<tr>
<td>- Mycobacteria-free phagosomes are capable of recruiting iNOS</td>
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<tr>
<td>- Phagosomes containing heat-killed mycobacteria have no effect on iNOS recruitment to phagosomes</td>
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<tr>
<td>- iNOS recruitment is disrupted in phagosomes containing live mycobacteria</td>
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<tr>
<td>- Alterations or deletions in one or more of the actin cytoskeleton elements can interfere with actin microfilament motility, disrupting the recruitment of iNOS to phagosomes</td>
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<td>- iNOS gene-disrupted mice (NOS2−/−) have a higher bacterial burden in their lungs following <em>Mycobacterium tuberculosis</em> infection</td>
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<tr>
<td>- NOS2−/− mice are capable of mounting an adaptive immune response comparable to wild-type control mice upon challenge with <em>Mycobacterium tuberculosis</em></td>
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<tr>
<td>- Human <em>Mycobacterium tuberculosis</em>-infected alveolar macrophages are capable of producing NO</td>
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<tr>
<td>- Patients with active <em>Mycobacterium tuberculosis</em> infection exhale significantly higher levels of NO than healthy control subjects</td>
</tr>
<tr>
<td>- iNOS expression by alveolar macrophages of tuberculosis patients is significantly higher than control subjects</td>
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**TLR2 and TLR4**
- Neither TLR2/TLR4 is effective in providing a significant innate immune response alone
- Mice deficient in TLR2/TLR4 have a significantly higher bacterial burden than wild-type mice

**γδ T cells**
- Patients with active tuberculosis infection have a significantly reduced number of *Mycobacterium tuberculosis*-reactive γδ T cells in their blood and BAL
- γδ T cells produce IFN-γ in response to *Mycobacterium tuberculosis* only
- γδ T cells are capable of producing more IFN-γ on a per cell basis than CD4⁺ T cells in response to tubercle bacilli
- γδ T cells proliferate faster in a high NK cell-percentage population than in a low NK cell-percentage population

**IL-17**
- The percentage of IL-17-producing γδ T cells is significantly higher in tuberculosis patients than in the healthy donors
- γδ T cells and other non-CD4⁺ or CD8⁺ T cells are capable of producing IL-17 during *Mycobacterium tuberculosis* infection
- γδ T cells produce more IL-17 than CD4⁺ T cells
- IFN-γ-deficient mice produced more IL-17 than wild-type mice

**CD4⁺ and CD8⁺ T cells**
- CD4⁺ T cells are two-fold more abundant than CD8⁺ T cells at sites of *Mycobacterium tuberculosis* infection in both mice and humans
- There is a significant reduction in the specific lysis mediated by cytotoxic CD8⁺ T cells in the lungs of CD4-deficient mice
- The cytotoxic activity of *Mycobacterium tuberculosis*-specific CD8⁺ T cells is compromised in the absence of CD4⁺ T cells
- In humans, CD4⁺ T cells are the primary source of IFN-γ but CD8⁺ T cells also contribute to IFN-γ production
**BCG Vaccine**
- Variable in its degree of protection against *Mycobacterium tuberculosis* infection
- Efficacy wanes with time
- Administration of the BCG vaccine during infancy is shown to be highly effective in protecting against severe cases of tuberculosis
- BCG vaccination of individuals after the first year of life results in persistent tuberculin reactions
- The United States and other industrialized countries with low incidences of *Mycobacterium tuberculosis* infection have discontinued the use of the BCG vaccine
- BCG vaccination affects tuberculin skin tests (TSTs)

**Culture filtrate protein (CFP) vaccines**
- Encourages competition for antigen presentation
- Different culture conditions and the risk of the vaccine possessing both protective and deleterious molecules makes it challenging to work with
- Immunization with CFP antigens generate CD4\(^+\) T cells that secrete IFN-\(\gamma\)
- Efficacy wanes with time
- CFP has to be delivered in adjuvant

**Early Secretory Antigenic Target of 6kDa (ESAT-6)**
- ESAT-6 enhances IFN-\(\gamma\)-induced accumulation of iNOS
- ESAT-6 has to be complexed with multiple strong adjuvants to mount a sufficient immune response

**Inhalant drugs: Isoniazid (H) and Rifampicin (R)**
- Microparticle form has a better effect on alveolar macrophage activation
- TNF-\(\alpha\) and IL-12 are the most significantly induced cytokines following treatment with H and R microparticles
### Important proinflammatory cytokines

**IL-12**
- Humans and mice with mutations in either the IL-12p40 gene or IL-12R gene show a reduction in IFN-γ production
- IL-12 links innate and adaptive immunity

**IFN-γ**
- Humans and mice with mutations in either the IFN-γ gene or IFN-γR gene are highly susceptible to mycobacterial infections
- NO\(^\text{•}\) production is significantly increased in *Mycobacterium tuberculosis*-infected PBMCs stimulated with IFN-γ

**TNF-α**
- Neutralization of TNF-α impairs the ability of the mice to control *Mycobacterium tuberculosis* infection
- Mice with a disruption in the gene encoding for the 55kDa TNF receptor (TNFRp55\(^{-/-}\)) are unable to control *Mycobacterium tuberculosis* infection
- TNF-α increases *Mycobacterium tuberculosis*-induced NO\(^\text{•}\) production by alveolar macrophages
  - The presence of sTNFR decreases TNF-α-induced apoptosis
  - Individuals treated with TNF-α blockers contract *Mycobacterium tuberculosis* infection at an increased rate
  - Humira and Remicade inhibited T cell activation and IFN-γ production whereas Enbrel does not
Inhalation of Mycobacterium tuberculosis

Phagocytosis of tubercle bacilli by the alveolar macrophage

Cytokine Production

Macrophage Apoptosis

Inducible Nitric Oxide Synthase (iNOS) & Reactive Nitrogen Intermediate (RNI) Production

IFN-γ

CD4+ T cell Recruitment

Granuloma Formation

CD8+ T cell Recruitment

Induces chemokine production & augments neutrophil accumulation

Nitric Oxide (NO) Production

Macrophage Activation

Activation of Toll-Like Receptors (TLRs)

IL-12

NK cell Activation

Macrophage Apoptosis

IFN-γ

CD4+ T cell Recruitment

γδ T cell Recruitment

Induces chemokine production & augments neutrophil accumulation

IFN-γ

CD8+ T cell Recruitment

Induces chemokine production & augments neutrophil accumulation

IFN-γ

Macrophage Activation

FIG. 55 Summary of Host Pro-Inflammatory Defense Mechanisms
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