IMMUNOGENICITY, SUBCELLULAR LOCALIZATION AND FUNCTION OF THE EIS PROTEIN OF *MYCOBACTERIUM TUBERCULOSIS*

by

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DEDICATION

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ABSTRACT

The *eis* gene of *M. tuberculosis* is believed to play a role in the intracellular survival of this pathogen. Significantly higher levels of antibodies to Eis were detected by ELISA in the sera of patients with tuberculosis as compared to healthy controls. PBMCs from recovered TB donors were also found to demonstrate significantly higher levels of proliferation in response to stimulation with the Eis protein than PBMCs from either active TB cases or healthy controls. Neither the active TB population nor the healthy controls showed significant levels of IFN-γ or IL-4 secretion in response to stimulation of PBMC with Eis or ESAT-6. Far Western analysis determined that Eis interacts with a ~65 kDa protein that localizes to the cytoplasmic fraction of *M. tuberculosis* lysate. Real-time PCR analysis of *M. tuberculosis* infected U-937 macrophages showed that the *eis* gene is constitutively expressed during infection. Using immunofluorescence microscopy (IF), the Eis protein was detected within the cytoplasm of *M. tuberculosis* infected macrophages indicating that the protein was being released/secreted from the mycobacterium containing phagosomes. Western blot analysis of the cytoplasm of macrophages infected with *M. tuberculosis* expressing green fluorescent protein confirmed these results. Western blot analysis also detected the presence of native Eis both in the culture supernatant of infected macrophages and vesicles released from the macrophages. IF also detected the presence of Eis in uninfected macrophages. The Eis protein in the cytoplasm of *M. tuberculosis* infected macrophages was also found to colocalize with EEA1, an endosomal marker, indicating a possible association of the protein with early endosomes. Eis was also shown to elicit higher levels of IL-10

secretion than PPD in human monocytes. Infection of monocytes from healthy tuberculin reactors with *M. tuberculosis* wild type and Δ*eis* mutant demonstrated that *eis* plays a role in modulation of IL-10/TNF-α secretion in response to infection. Bioinformatic analysis of the amino acid sequence of Eis indicates that Eis is an acetyltransferase of the GCN5 related family of N-acetyltransferases. Further work is required to determine the role Eis plays in the survival of *M. tuberculosis* within the macrophage.

INTRODUCTION

History

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB) is one of the oldest pathogens known to man. Recent reports indicate that the disease was prevalent in ancient Egypt as far back as 3500 BC (172). Over the centuries, this bacterium has proved to be a formidable foe. Assyrian clay tablets from the 7th century BC describe patients coughing blood. In the 5th century BC, Hippocrates accurately described the symptoms of patients suffering from tuberculosis, an affliction that he termed "Consumption" due to the drastic weight loss seen in victims. In the 16th and 17th centuries, the disease ravaged the populations of Europe where it was known as the "White Plague" and is estimated to have killed over one quarter of all Europeans (110). The arrival of the industrial revolution, which was marked by poor living conditions for the masses and high levels of pollution did nothing to stem the spread of this killer. It was not until the 1950s that the advent of antibiotics, the use of the *M. bovis* BCG vaccine, and improved living standards led to a decline in the incidence of tuberculosis. This fall in the number of cases led to the mistaken belief that the disease was close to being contained and possibly eradicated. In 1972, the U.S. Congress responded by eliminating all funding for tuberculosis control programs and a similar trend was seen all over the world (http://www.hopkins-tb.org/news/5-1-2000.shtml). The resulting collapse of TB health systems was followed by an explosion of tuberculosis cases in the 1980s. The acquired immune deficiency syndrome (AIDS) epidemic (42) and the emergence of

multi-drug resistant (MDR) strains of *M. tuberculosis* further exacerbated the situation (122).

In 1993, the World Health Organization (WHO) declared the spread of tuberculosis a global emergency and advocated the implementation of the Directly Observed Therapy Strategy (DOTS) (58). In spite of the success achieved by this program in containing the spread of tuberculosis, the statistics remain staggering. Tuberculosis kills more adults worldwide than any other single infectious agent (119). Over one-third of the world population is infected with *M. tuberculosis*. An estimated 5-10% of the people who are infected will develop the disease at some point in their life. In 2002 alone, nearly 9 million new cases of tuberculosis were detected and almost 2 million deaths were recorded (59). In 2000, half a million deaths were attributed to co-infection with *M. tuberculosis* and the human immunodeficiency virus (HIV). Tuberculosis is the leading cause of death among people who are HIV positive (http://www.who.int/hiv/ topics/tb/tuberculosis/en/) (42). Further complications have arisen from the emergence of MDR strains of *M. tuberculosis*. MDR strains are resistant to both isoniazid and rifampicin, which are the most powerful anti-tubercular drugs. In many countries, over 15% of the cases of tuberculosis are caused by MDR strains and worldwide it is estimated that 50 million people are infected with MDR strains of *M. tuberculosis* (122). Treatment of MDR tuberculosis requires up to 2 years of drug therapy, is more toxic to the patient and is over 100 times more expensive than regular treatments (http://www.who.int/mediacentre /factsheets/fs104/en/). The spread of MDR strains has slowed the progress in containment of tuberculosis (TB). In the US, the

average year to year drop in tuberculosis cases was 6.8% between 1993 and 2002, however in 2003, the decrease was only 1.9%. Current worldwide rates of TB incidence are the highest in recorded history (http://www.who.int/mediacentre /factsheets/ fs104/en/).

Pathogenesis of Tuberculosis

M. tuberculosis is usually transmitted by aerosolized droplets originating from people with active pulmonary disease. It enters the body via the alveolar passages and is ingested by the macrophages, although this may also occur with type II alveolar epithelial pneumocytes, which are more commonly found in the lungs (21). Additionally, the bacterium may also infect dendritic cells (79). Dendritic cells are more migratory and better antigen presenting cells than macrophages, but unlike activated macrophages, are unable to kill engulfed *M. tuberculosis* and hence may be responsible for the dissemination of the bacterium (151). Walgren divided the progression of the disease after infection into 4 stages (164). In the first stage, which lasts 3-8 weeks after infection, the bacteria are disseminated to the regional lymph nodes where they form the primary or Ghon lesions. Conversion to tuberculin reactivity i.e. skin test positive for the Mantoux test occurs at this stage. The second stage lasts about 3 months and is characterized by the hematogenous spread of the bacterium to other parts of the lung and various organs. In some cases, this can lead to acute and sometimes fatal cases of miliary (disseminated) tuberculosis. The third stage may last 3-7 months and involves inflammation of the pleura (pleurisy) caused by release of bacteria from the lung. Resolution of the disease occurs in the last stage. One-third of the people who are exposed to *M. tuberculosis* become

infected with the bacterium. Of this number, 3-5% develop the disease in the first year while another 3-5% develop the disease later in life through reactivation of latent bacteria (144). In comparison, 50% of *M. tuberculosis* infected HIV-positive patients will develop tuberculosis by reactivation (42, 144).

Once inside the human host, initial uptake of the bacterium occurs by the interaction with macrophage mannose and complement receptors (139). Various other receptors including human toll-like receptor (TLR-2) are also believed to play a role in this process (118). *M. tuberculosis* survives within the macrophage by arresting the normal development of the phagosome. This is partly achieved by the exclusion of the proton ATPases from phagosomes containing the bacterium (149). *M. tuberculosis* containing phagosomes acquire the early endosomal marker Rab5 but not the late endosomal markers Rab7 or lysosome associated membrane glycoprotein 1 [LAMP1] (37, 160). *M. tuberculosis* has also been found to enhance retention of coronin-1 (TACO-tryptophan aspartate-containing coat) on the surface of the phagosome, which in turn prevents phagolysosome fusion (60). Phagosome-lysosome fusion is also inhibited, in part by effectors such as mannose capped lipoarabinomannan (ManLAM) that are secreted by *M. tuberculosis* (67). Among other things, ManLAM causes depressed TNF-α and IL-12 secretion by human mononuclear phagocytes resulting in poor macrophage responses to infection (98). In this way, the bacterium is able to avoid the toxic effects of lysosomal enzymes and the normal acidified environment of a mature phagosome.

Latent Tuberculosis

The Th1 immune response is primarily responsible for resistance to intracellular pathogens such as *M. tuberculosis*. In a normal healthy individual, an *M. tuberculosis* infection is contained by formation of a granuloma (48). More specifically, the local innate immunity characterized primarily by the involvement of alveolar macrophages controls but fails to eradicate the *M. tuberculosis* infection. Granuloma formation is characterized by the aggregation of monocytes, lymphocytes and neutrophils around infected macrophages (156). This leads to the formation of focal lesions composed of lymphocytes and macrophage-derived giant cells. The destruction of infected macrophages within the granuloma results in the formation of a caseous center within which *M. tuberculosis* bacilli are believed to survive (48). This stage is referred to as latent tuberculosis. In healthy individuals, the granulomas heal to form small calcified lesions. In cases of immune suppression however, the granuloma may serve as the source material for a renewed infection possibly resulting in active tuberculosis infection (48). This commonly occurs in cases of AIDS and when immunocompetence of the host wanes as seen in the elderly and cancer patients (42, 50).

CHAPTER I: IMMUNOGENICITY OF THE EIS PROTEIN OF *MYCOBACTERIUM TUBERCULOSIS*

Host Immune Responses and TB Countermeasures

CD4+ and CD8+ T cells

As mentioned earlier, resistance to an *M. tuberculosis* infection is mediated by a Th1 type immune response. Macrophages are potent effectors of this resistance. They kill ingested bacteria by a variety of mechanisms, including generation of reactive oxygen intermediates and reactive nitrogen intermediates (2), as well as phagolysosome fusion which normally results in acidified vacuoles containing potent hydrolytic enzymes. Along with dendritic cells, macrophages also act as antigen presenting cells (6). It has been firmly established that CD4+ T cells play a central role in protection against tuberculosis. CD4+ T cells secrete IFN-γ and TNF-α which activate macrophages for better mycobacterial killing (25). Activated CD4+ T cells also play a role in the destruction of *M. tuberculosis* infected macrophages via the expression of granzymes, Fas-L, and perforin (31). Furthermore, they also participate in the activation of B cells, CD8+ and γδ T cells by the secretion of IL-2 (91).

However, chronic infection of macrophages with *M. tuberculosis* has been shown to inhibit expression of MHC class II molecules on the surface of these cells (86). While baseline levels of MHC class II were not affected, IFN-γ mediated upregulation of MHC class II molecules was inhibited in *M. tuberculosis* infected macrophages (117). This in turn would interfere with the presentation of MHC class II restricted antigens required for activation of CD4+ T cells. (86).

CD8+ T cells also play a major role in the control of *M. tuberculosis* infection. They are cytolytic T-lymphocytes that bring about the lysis of *M. tuberculosis* infected macrophages via the same mechanisms as CD4+ T cells (31). Most *M. tuberculosis* reactive CD8+ T cells recognize mycobacterial antigens presented in concert with MHC class I molecules. *M. tuberculosis* antigens can also enter the MHC class I processing pathway through an alternate route that does not require the classic ER-golgi pathway (32). Particulate antigens such as those from *M. tuberculosis* can be processed by this pathway which does not require penetration into cytosol and thus provides an additional means for activation of CD8+ T cells (32).

Th1/Th2 Immune Response, Cytokines and Tuberculosis

M. tuberculosis strongly induces secretion of various cytokines during infection and these play a major role in the inflammatory response, chronic nature, and ultimately control of the infection. The Th1 cell mediated immune response is primarily induced by IL-12 while the Th2 humoral immune response is induced by IL-4 (1). In an *M. tuberculosis* infection, Th1 cytokines are required for an appropriate immune response. Th2 cytokines on the other hand may inhibit production of Th1 cytokines and weaken the host response to a mycobacterial infection (52). There are conflicting data showing that a shift in immune response from Th1 to Th2 type may be brought about by *M. tuberculosis* infection and correlates to a decline in the clinical status of the patient (22, 54).

M. tuberculosis is a strong inducer of IL-12 production following infection of dendritic cells and macrophages in the human host (79, 100). This in turn drives the Th1 response along with IFN-γ production. IL-12 knockout mice have been shown to be

highly susceptible to mycobacterial infection (41). Patients with mutations in the genes encoding IL-12p40 (an IL-12 subunit) and IL-12R (an IL-12 receptor), suffer from recurrent mycobacterial infections and have been shown to display a reduced ability to produce IFN-γ (125). IFN-γ is produced by CD4+ T cells, CD8+ T cells and NK cells (14, 101, 107, 120, 121, 140). This cytokine plays a major role in macrophage activation leading to enhanced mycobacterial killing and antigen presentation via upregulated expression of MHC class II molecules. However *M. tuberculosis* has been shown to inhibit IFN-γ mediated activation of macrophages by interfering with the association of transcriptional activator STAT1 with the transcriptional coactivators CREB binding protein and p300 (152).

The data regarding the role of IL-4 in a mycobacterial infection are conflicting but its importance stems from the fact that it plays a role in inhibiton of IFN-γ production (105). It may be required for the control of the inflammatory response. Whether IL-4 plays a role in protection against *M. tuberculosis* however is unclear. TNF-α on the other hand is essential for the control of an *M. tuberculosis* infection. This cytokine is secreted by macrophages, dendritic cells, and T cells (100). TNF-α plays an important role in granuloma formation, macrophage activation, and cell migration to sites of infection via upregulation of adhesion molecules and chemokine expression (97, 105).

Th2 mediated humoral immunity to *M. tuberculosis* has long been considered to be insignificant (73). However, this may not be completely true. Serum antibodies to lipoarabinomannan (LAM), a major *M. tuberculosis* virulence factor, have been shown to be involved in classical complement activation (81). Specific antibody to trehalose-6,6-

dimycolate (cord factor) has also been shown to neutralize the important immuno-regulatory activity of this *M. tuberculosis* virulence factor (81). TNF-α secretion in response to purified protein derivative (PPD) in humans has been found to directly correlate to plasma concentration of anti-PPD IgG_1 (89). On the other hand, a major Th2 mediator, IL-10, is an anti-inflammatory cytokine produced by macrophages and T cells (157). IL-10 is known to be capable of macrophage deactivation and downregulation of IL-12 production which in turn leads to decreased IFN-γ production (127). Macrophages from TB patients are known to suppress *in vitro* T cell proliferation and this was reversed by inhibition of IL-10 activity (74), indicating that the suppression was IL-10 mediated.

In addition to induction of the secretion of anti-inflammatory cytokines such as IL-10 and TGF-β, *M. tuberculosis* has other means to inhibit T cell responses. *M. tuberculosis* disease is also associated with increased apoptosis of mycobacterium-specific T cells (85). Stimulation of peripheral blood mononuclear cells (PBMC) from TB patients with heat-killed *M. tuberculosis* resulted in a significant reduction of the numbers of IFN-γ+ CD4+ T cells. This in turn resulted in the dominance of IL-4+ CD4+ T cells (22). This selective depletion of T cells can only have a detrimental effect on the host response to *M. tuberculosis* infection. T cell responses to mycobacterial antigens have been shown to be considerably diminished in TB patients even during and after completion of treatment, while responses to control antigens such as tetanus toxoid remain normal (5). Peripheral blood mononuclear cells (PBMCs) from patients with active cases of tuberculosis stimulated by *M. tuberculosis* demonstrate diminished production of the Th1 cytokines IL-2 and IFN-γ but no reduction in secretion of Th2

cytokine such as IL-4, IL-10 and IL-13 (171). This combination of factors may result in the shift of the immune response to *M. tuberculosis* from Th1 to Th2 type immune response to the detriment of the host.

Other T Cell Subsets and Tuberculosis

γδ T cells are characterized by a unique T cell antigen receptor composed of γ and δ chains. In adults, the majority of γδ T cells express Vδ9 and Vδ2 elements (78). These T cells are involved in IFN-γ secretion and can lyse infected macrophages thus helping to contain a mycobacterial infection. In healthy individuals sensitized to mycobacterial antigens, there is a greater ability to activate these cells in response to mycobacterial infection as compared to non-sensitized individuals (16). Patients with active cases of *M. tuberculosis* infection however, show a diminished ability to activate these cells. There is in fact, a selective depletion of Vδ9 and Vδ2 T cells in tuberculin negative patients with cases of tuberculosis (150).

CD1-restricted αβ TCR+ T cells are the least common T cells in the peripheral blood. These cells are referred to as double negative because they express neither CD4 or CD8 (28). They do however play an important role in immunity to *M. tuberculosis* infection because of their ability to bind the polar lipid antigens of *M. tuberculosis* such as mycolic acids and phophatidyl-inositol-mannosides (PIMs) (64). Like the other T cells, these CD1 restricted T cells also secrete IFN-γ and lyse infected macrophages (109). Here too however, *M. tuberculosis* being the consummate pathogen, has found a way to sabotage the immune system. Infection of CD1+ antigen presenting cells (APCs) with *M. tuberculosis* is found to downregulate expression of the CD1 molecule on the

surface of the cell resulting in the complete loss of the ability to present mycobacterial antigens to CD1 restricted T cells (147).

Diagnosis of Tuberculosis

In spite of the fact that tuberculosis has been a threat to mankind for thousands of years, we still lack the ability to quickly and accurately diagnose this disease. There is no rapid, simple, specific and sensitive test to differentiate patients with active disease from those who may be latently infected, vaccinated or have simply been exposed to the bacterium at some point in their lifetime (29). Errors in diagnosis using X-rays, Mantoux skin tests and Ziehl-Neelsen stain are quite common (70). This leads to errors in treatment with healthy patients unnecessarily being given drug therapy while at the same time patients with active but undiagnosed tuberculosis continue to unwittingly spread the disease.

The Mantoux/tuberculin skin test is the traditional method for diagnosis of *M. tuberculosis*. This involves the use of the PPD to detect a delayed type hypersentivity (DTH) response to *M. tuberculosis*. PPD is a crude mixture of*M. tuberculosis* secreted and somatic antigens. However, this method is fraught with problems. A positive result does not confirm active disease and a negative result does not exclude it. Positive results may be due to active infection, vaccination, past infection or exposure to environmental mycobacteria (70, 155).

The most commonly used diagnostic method is the Ziehl-Neelsen stain which involves detection of acid-fast bacilli in sputum samples by microscopy. This method however is plagued by poor sensitivity with about 40-60% of patients with pulmonary

tuberculosis and about 75% of patients with extrapulmonary tuberculosis escaping diagnosis (49, 70). This method also fails to distinguish between *M. tuberculosis* and other mycobacteria since all of them are acid-fast.

Culture methods for *M. tuberculosis* are extremely slow and clinical samples may require 4-8 weeks to produce a positive result. The need to perform antibiotic sensitvity tests is also hindered because of this fact. Even with the latest culture methods such as the BACTEC 460 radiometric system, a positive result still requires 1-3 weeks (145). These methods are also not usually cost effective (70). More recently, polymerase chain reaction (PCR) techniques have been used with some success on clinical samples to confirm presence of *M. tuberculosis* (99).

Research has now begun to focus on the use of Enzyme linked immunosorbent assay (ELISA) and T cell proliferation assays for the diagnosis of *M. tuberculosis* infection. The identification of novel *M. tuberculosis* antigens and their availability in purified recombinant form have enhanced the specificity of these assays. Several *M. tuberculosis* antigens have been identified for this purpose. Most prominent among these are Early Secretory Antigen Target-6 (ESAT-6) and Culture Filtrate Protein [CFP-10] (55, 128, 158). ESAT 6 is a 6 kDa protein that has been shown to be a strong inducer of Th1 type CD4+ T cells early during infection with *M. tuberculosis* (128). The combination of these antigens was found to have high sensitivity (73%) and higher specificity (93%) as compared to PPD (82% sensitivity and 7% specificity, respectively). This was measured in terms of *in vitro* IFN-γ production of human PBMC (peripheral blood mononuclear cells) in response to stimulation with mycobacterial antigens (158).

Andersen *et al.* showed that when testing a panel of recombinant mycobacterial antigens for the T cell proliferative response of PBMC from TB patients, ESAT-6 was found to show higher sensitivity than any other single antigen and performed comparably to complex mycobacterial antigens such as PPD (114). PPD along with other complex mycobacterial antigens is characterized by poor specificity in similar experiments. Converse *et al.*showed that 33% of healthy skin test negative subjects showed positive *in vitro* IFN-γ response to PPD (40). Similarly Fiavey and Frankenburg demonstrated that using PPD as the antigen for a T cell proliferation assay, they were only able to get sensitivity of 63.6% and specificity of 59.5% (61). Using ESAT-6 alone, Ravn *et al.* was able to show that T cells of 59% of tuberculosis patients responded to stimulation, with no positive responses from any of the PPD negative healthy controls (128). Combinations of recombinant antigens may therefore enable us to devise a test for tuberculosis with sufficiently high specificity and sensitivity.

The use of enzyme linked immunosorbent assay (ELISA) and enzyme linked immunospot assays (ELISPOT) as diagnostic tools for the detection of *M. tuberculosis* infection has been touted as a possible answer to the difficulties faced by clinicians in properly diagnosing cases of active disease in the early stages as compared to healthy PPD skin test positive subjects. Using a combination of two *M. tuberculosis* antigens, ESAT-6 and CFP-10, Hill *et al.* were able to develop an ELISPOT assay that offered increased specificity for the diagnosis of *M. tuberculosis* infection in tropical settings at the cost of some sensitivity (82).

Protection Against Tuberculosis

The only vaccine currently available for use against *M. tuberculosis* is *Mycobacterium bovis* BCG (Bacillus Calmette Guerin). Camille Guerin and Albert Calmette developed this strain from *M. bovis* by subculturing a virulent bovine strain 230 times over a period of 13 years (39). For 70 years now, this vaccine has been widely used as the only form of protection against *M. tuberculosis* infection. Analysis of recent clinical trials has concluded however that there is a wide range (0%> to <80%) in the efficacy of this vaccine (38, 39). BCG is believed to provide some protection against *Mycobacterium leprae* as well as against childhood forms of TB (miliary and meningeal) (62, 135). A 15 year trial involving 360,000 adults in Chingleput, India, concluded that BCG provided no protection against *M. tuberculosis* in adults (154).

Brandt *et al*. (27) were able to demonstrate that sensitization of mice with environmental mycobacteria inhibited the initial multiplication of BCG and resulted in poor protection against *M. tuberculosis* infection. It has been hypothesized that in tropical regions, exposure of local populations to environmental mycobacteria can explain the poor efficacy of the BCG vaccine in these areas. Sensitization of the local population by environmental mycobacteria may inhibit multiplication of the *M. bovis* BCG vaccine and hence diminish the immune response and subsequent protection *to M. tuberculosis* infection (23). These data are supported by studies showing that IFN-γ responses to BCG in individuals in Malawi who were sensitized to environmental mycobacteria were repressed as compared to responses in non-sensitized individuals in the United Kingdom (23, 24).

For several years now there has been a major push to develop alternative vaccine candidates. There are four basic vaccine types being examined (72):

1. **Subunit vaccines:** These consist of combinations of one or more mycobacterial proteins, lipids or even carbohydrates. Adjuvants are sometimes used to enhance the immune response.

2. **DNA vaccines:** Eukaryotic plasmid vectors expressing mycobacterial antigens.

3. **Vaccines using live attenuated mycobacteria:** These include attenuated forms of *M. tuberculosis*, non-pathogenic mycobacteria or BCG expressing recombinant antigens or cytokines.

4. **Non-mycobacterial vectors:** The fourth type are vaccines comprised of live attenuated non-mycobacterial vectors such as *Salmonella spp*. or vaccinia virus expressing mycobacterial antigens (72).

Some progress has already been made in the search for novel TB vaccines. BCG expressing recombinant Antigen-85 (Ag85- a major *M. tuberculosis* antigen) was found to provide better protection than BCG alone in a mouse model (87). Brandt *et al.* also showed that vaccination using ESAT-6 in a formulation of adjuvants [dimethyldioctadecylammonium bromide (DDA) and monophosphoryl lipid A (MPL)] resulted in an immune response comparable to that achieved with BCG (26). Mice immunized with a plasmid encoding Ag85 also showed resistance to subsequent infection with *M. tuberculosis* although it was not as effective as BCG (104). A study by Kamath *et al.* (92) compared the protection conferred on mice against *M. tuberculosis* infection by 3 different plasmids expressing Ag85B, ESAT-6 and MPT64 respectively. The

plasmid expressing Ag85B was found to confer the highest level of protection followed by the plasmids expressing ESAT-6 and MPT-64 respectively, although none performed better than BCG. More interestingly, co-immunization with all three DNA vectors induced better protection than with any single vector. Olsen *et al.*designed a fusion protein comprised of Ag85B and ESAT-6 and tested its efficacy as a vaccine in combination with the adjuvants DDA and MPL (168). The fusion protein provided a level of protection in mice similar to BCG and better than either of the individual proteins alone or even as a mixture.

Another factor in the failure of BCG as a vaccine is the lack of its ability to prime CD8+ T cells. It has been reported that *M. tuberculosis* antigens have easier access to the host cytosolic compartment and are hence able to better induce an MHC I mediated CD8+ T cell response (3, 80). The use of DNA/viral vaccine delivery systems utilizing combinations of antigens may enable us to devise a better vaccine candidate that would circumvent this problem.

Eis: Introduction

The *eis* gene of *M. tuberculosis* is believed to play a role in intracellular survival of this bacterium (167). The Eis protein is a 42-kDa protein that is predominantly hydrophilic with a potential hydrophobic amino terminus (45). A carboxy terminal polypeptide sequence was used to generate affinity purified rabbit polyclonal antibodies that reacted with the 42-kDa Eis protein in Western blot analysis. Fractionation of *M. tuberculosis* lysates by differential centrifugation revealed that Eis was localized mainly in the cytoplasmic fraction but was also found in the membrane, cell wall and culture

supernatant fractions (45). Dahl *et al*. also showed that forty percent of patients with pulmonary tuberculosis tested positive for the presence of anti-Eis antibody in their sera using Western blot analysis. Initial studies seem to indicate that Eis elicits a significant immune response in *M. tuberculosis* patients (45). This has led us to believe that Eis may be a candidate to be studied further to determine its potential for use as a diagnostic tool or as a vaccine candidate.

Based on current research, it seems likely that the future of the development of new diagnostic techniques and vaccines for *M. tuberculosis* infection lies in the use of combinations of mycobacterial antigens. The work presented here aims to demonstrate that Eis is an immunodominant antigen of *M. tuberculosis* that shows promise as a vaccine candidate and diagnostic tool in the same vein as ESAT-6 and Ag85. Studies were done using ELISA to compare the levels of antibodies to Eis in healthy controls and TB patients. Experiments were also done to demonstrate whether Eis is capable of eliciting a T cell proliferative response from *M. tuberculosis* infected subjects. Finally, the cytokine response in healthy donors and *M. tuberculosis* infected subjects in response to stimulation with Eis was compared to determine the nature of the immune (Th1 vs Th2) response to Eis.

Materials and Methods

Study Population

Patients with active pulmonary disease (n=8) were recruited at Maricopa County Health Department, Phoenix, Arizona. *M. tuberculosis* infection was confirmed by acid-fast stain, culture and PCR of sputum samples. Prospective donors were screened for HIV infection. Blood samples (45 ml) were drawn after start of treatment. Heparinized venous blood was collected and shipped overnight in ExaktPak containers. Recovered donors (n=4) were subjects who had undergone treatment and remained free of disease for over a year. Healthy controls (n=6) were recruited from PPD skin test negative volunteers at the University of Arizona, Tucson. Separate samples without heparin were also collected from donors for sera for ELISA.

Generation and Purification of Recombinant Eis Protein

In order to obtain purified Eis protein in sufficient quantities for use in our assays, it was necessary to generate a recombinant His-tagged Eis protein. The 1.3 kb *eis* gene was PCR amplified using the forward primer 5'-3' CGA CTG GCC CAT ATG TTC CTA CTG G and the reverse primer 5'-3' CGC GGC GGA TCC CCA TCC. The *eis* PCR product was cloned into the pET-15b vector (Novagen) using the *Bam*H1 and *Nde*1 restriction sites, so as to obtain a recombinant protein with an N-terminal His tag. This vector construct was transformed into *Escherichia coli* strain DH5α. The plasmid bearing the *eis* gene insert (pET15b-*eis*) was purified and transformed into the *E. coli* expression strain BL-21 DE-3 pLysS [Novagen] (Table 1 and 2). Single colonies were isolated and used to initiate overnight 3 ml starter cultures in LB (Luria Bertani) [Difco] which were

used to inoculate 1 liter cultures of LB media containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Cultures were incubated at 37°C with agitation at 200 rpm until an OD600 of 0.8-0.9 was reached. The *E. coli*cultures were then cooled to 4 °C for 1 h. Induction was carried out by addition of 0.5 mM isopropyl-thio-D-galactopyranoside (IPTG) and cultures were then incubated at room temperature with agitation for 36 h. The cells were harvested by centrifugation (10,000 x *g* for 20 minutes at 4°C), and the pellets were stored at –70°C until further use.

Pellets were then exposed to two freeze thaw cycles and resuspended in 10 ml lysis buffer (20 mM Tris base, 1.25 M NaCl, 45 mM imidazole, 10% glycerol, pH 7.5). Twenty microliters of protease inhibitor cocktail (Sigma Diagnostics) was also added. After 30 min incubation on ice, the viscosity of the solution was decreased by addition of DNase (20 μg/ml)(Sigma). The cells were then sonicated (Branson Sonifier 450, VWR) on ice for 5 x 1 minutes (cycle 20, power setting 7) with 1 min intervals cooling on ice between bursts. The suspension was then centrifuged (10,000 x *g* for 30 min) to remove cell debris. The supernatant was then filtered with a 0.2 μm syringe filter. The filtered supernatant was then applied twice to a pre-equilibrated 1.5 ml Ni-NTA agarose column (Qiagen). The column was then washed 3 x with 3 ml volumes of wash buffer (20 mM Tris base, 1.25 M NaCl, 50 mM imidazole, 10% glycerol, pH 7.5). His-tagged bound protein was eluted by applying 4 volumes of 750 μl of elution buffer (20 mM Tris base, 1.25 M NaCl, 200 mM imidazole, 10% glycerol, pH 7.5). Fractions were collected and examined for purity by electrophoresis at 200v and 80 mA for 3 h on a 10% SDS-PAGE gel which was subsequently stained with Coomassie blue stain (Pierce) for visualization

of protein bands (Figure 1). One liter of culture yielded 4 mg of purified Eis protein. Western blot analysis of purified protein using rabbit anti-Eis antibodies confirmed that the purified protein was indeed Eis (data not shown). Eluted protein was then dialyzed into storage buffer Phosphate Buffered Saline (PBS-modified with 0.5 M NaCl and 10% glycerol to prevent precipitation of protein) and stored at -80^{o}C. Flow through, cell lysate pellets and wash fractions from the purification process were analyzed to confirm that no inclusion body formation or large scale loss of Eis occurred during the purification procedure.

Antigens

Recombinant Eis protein with an N-terminal His tag was purified from *E. coli* expression strain BL21 DE3 pLysS (Novagen) using a Ni-NTA agarose column (Qiagen) as described previously. ESAT-6 was obtained from Colorado State University through the repository of tuberculosis research materials, NIAID, NIH contract number A1-75320 entitled "Tuberculosis Research Materials and Vaccine Testing". Tissue culture tested Concanavalin A (ConA) [Sigma Diagnostics] was used as positive control for T cell proliferation assays. PPD was obtained from Statens Serum Institut, Denmark.

ELISA for the Detection of Antibodies to Eis in Donor Sera

Purified recombinant Eis protein was diluted in Superblock buffer (Pierce) and used to coat the wells of NUNC Maxisorp flat bottom plate (Nunc) at a concentration of 2.5 μg/ well. Plates were incubated overnight at 4^{o}C and then washed 3 times with PBS/2% Tween using an EL 404 Microplate Autowasher (Bio-Tek Instruments). Two hundred microliter of Superblock was then added to each well. Plates were then

incubated at 37^{o}C for 1 h and washed again 3 x with PBS/2% Tween. One hundred microliters of serial dilutions (in Superblock) of the sera to be tested were added to each well. Sera dilutions used ranged from 1:100 to 1:12,800. Each sample was tested in duplicate at each dilution. Controls for each sera tested included wells that had not been coated with Eis protein. Plates were incubated at room temperature for 1 h and washed again 3 x with PBS/2% Tween. One hundred microliters/well of secondary antibody (goat anti-rabbit IgG conjugated to HRP) [Pierce] diluted 1:10,000 in Superblock was added and plates incubated at room temperature. Plates were then washed 3 x with PBS/2% Tween. One hundred µl/well of TMB substrate was then added and plates incubated at room temperature till color developed. The reaction was stopped with the addition of 50 µl 1N hydrochloric acid and absorbance read at 450 nm using a Biorad Microplate Reader (Biorad). Mean values were calculated for each dilution. Statistical significance was calculated using the Mann-Whitney U test.

T Cell Proliferation Assays for Determination of Donor Response to Stimulation of PBMC with Mycobacterial Antigens

T cell proliferation assays were performed according to standard methods (114). PBMC were separated from whole blood by density gradient centrifugation using Histopaque (Sigma Diagnostics). Briefly, whole blood was mixed in a 1:1 proportion with PBS and 7 ml of this was layered over 5 ml Histopaque in 15 ml conical tubes (Corning). Samples were then centrifuged at 300 × *g* for 45 minutes at room temperature. The buffy coat containing PBMC was aspirated and washed 3 times in PBS. PBMC were resuspended in X-VIVO-15 tissue culture medium (Cambrex) which contained

gentamicin and phenol red. PBMC were counted and 2 x 10^5 cells in 100 µl of medium were seeded into 96 well round bottom Falcon tissue culture plates (Becton Dickinson) in triplicate. Antigens in optimal concentrations were added in another 100 µl of medium to each well. Eis, ESAT-6, PPD (5 µg/ml each), and Concanavalin A (ConA, 1 µg/ml) were used. The final volume in the culture wells was 200 µl. The plates were incubated at 37^{o}C in a humidified atmosphere of 5% CO_2 and 95 % air. On day 5, 50 µl of supernatant was harvested from each well and stored at –80^{o}C for later analysis of cytokine content. The cultures were then pulsed with 1 µCi [^{3}H]-thymidine (PerkinElmer), harvested on filter mats using a Filtermate 196 harvester (Packard) and the radioactivity incorporated measured by a Topcount Microplate scintillation counter (Packard). Mean values were calculated for each antigen.

$$\text{Stimulation Index (SI)} = \frac{\text{(counts per minute) cpm of cells stimulated with antigen}}{\text{cpm of unstimulated cells}}$$

Proliferative responses were considered positive if the SI>3. Statistical significance was calculated using the Mann-Whitney U test.

ELISA for Determination of IFN-γ and IL-4 Levels in Culture Supernatant of PBMC Stimulated with Mycobacterial Antigens

ELISA for the detection of IFN-γ and IL-4 in the culture supernatant of PBMCs obtained from healthy controls and TB patients and stimulated with mycobacterial antigens was performed using the Human IFN-γ ELISA Kit and the Human IL-4 ELISA Kit (Pierce). Anti-human IFN-γ and IL-4 pre-coated 96-well strip plates were used. All samples and reagents were thawed to room temperature before use. Standards were

reconstituted in ultrapure water and prepared in serial dilutions with one for each point of the standard curve-1000, 400, 160, 64, 25.6 and 0 pg/ml for IFN-γ and 400, 160, 64, 25.6, 10.24 and 0 pg/ml for IL-4. Fifty microliter of reconstituted samples or test reagents were added to each well followed by 50 µl of biotinylated antibody reagent. Plates were sealed with adhesive covers and incubated at room temperature for 2 h. Subsequently, plates were unsealed and washed 3 x with wash buffer using a squirt bottle. Plates were then blotted onto paper towels. Streptavidin-HRP solution was prepared from concentrate and 100 µl of this was added to each well. Plates were tightly sealed once more and incubated at room temperature for another 30 min. At the end of this time, plates were unsealed and washed as described previously. One hundred microliter of TMB substrate solution was pipetted into each well. Enzymatic color was allowed to develop in the dark at room temperature for 30 min followed by addition of 100 µl of the stop solution to each well. Absorbance was read at 450 nm using a Biorad Microplate Reader. Standard curves were generated using readings obtained by testing of serially diluted cytokine standards and this was used to calculate IFN-γ and IL-4 concentrations in test samples.

Statistical Analysis

Statistical analysis for comparison of differences between various data sets was carried out using Mann-Whitney U test (77). Differences were considered significant for $p<0.05$.

Table 1: Plasmids used in this study

Plasmids	Relevant characteristics	Reference or Source
pBEN	*gfp* driven by p*hsp60* from *M. bovis BCG*	(136)
pFPV27	promoterless *gfp* reporter vector	(12)
pET-15b	carries N-terminal His tag	Novagen
pET-15b-*eis*	pET vector with *Bam*HI-*Nde*I *eis* fragment	This study

Table 2. Bacterial strains used in this study

Strains	Description	Reference or source
E. coli		
DH5α	*supE44 ΔlacU169 (Δ80lacZ ΔM15) hsdR17recA1 endA1 gyrA96 thi-1 relA1*	Gibco BRL
BL-21 DE-3 pLysS	*ompT hsdS(rB mB –) dcm+ Tetr gal endA Hte*	Stratagene
M. tuberculosis		
H37Ra	attenuated H37Rv	D. Young[a]
H37Ra Δ*eis*	double-crossover recombinant of H37Ra with p6308, Δ*eis::hyg*, Hgr^r	(166)
H37Ra *eis* complemented	H37Ra containing integrated copy of p6308 and p6301 Δ*eis::hyg*, *eis::attB*, Hgr^r, Kan^r	(166)
H37Rv	Virulent laboratory strain (ATCC 27294)	CSU[b]
H37Rv Δ*eis*	double-crossover recombinant of H37Rv with p6308, Δ*eis::hyg*, Hgr^r	(166)
H37Rv *eis* complemented	H37Rv containing integrated copy of p6308 and p6301 Δ*eis::hyg*, *eis ::attB*, Hgr^r, Kan^r	(166)
MtbRa/*gfp*	H37Ra transformed with pBEN so as to express *gfp*, Kan^r	This study
MtbΔ*eis/gfp*	H37Ra Δ*eis* mutant transformed with pBEN so as to express *gfp*, Kan^r	This study

[a] Imperial College School of Medicine at St. Mary's, London, England
[b] Colorado State University.

Results

Purification of Recombinant His-tagged Eis Protein

As described above, the *eis* gene was cloned into a pET expression vector under the control of a T7 promoter and transformed into an *E. coli* expression strain. *E. coli* cultures were grown and production of recombinant protein was induced by the addition of IPTG as described in materials and methods. Bacteria were then pelleted, lysed and passaged over a Ni-NTA agarose column to purify the His-tagged recombinant protein. Multiple washes were carried out to remove any contaminants due to non-specific

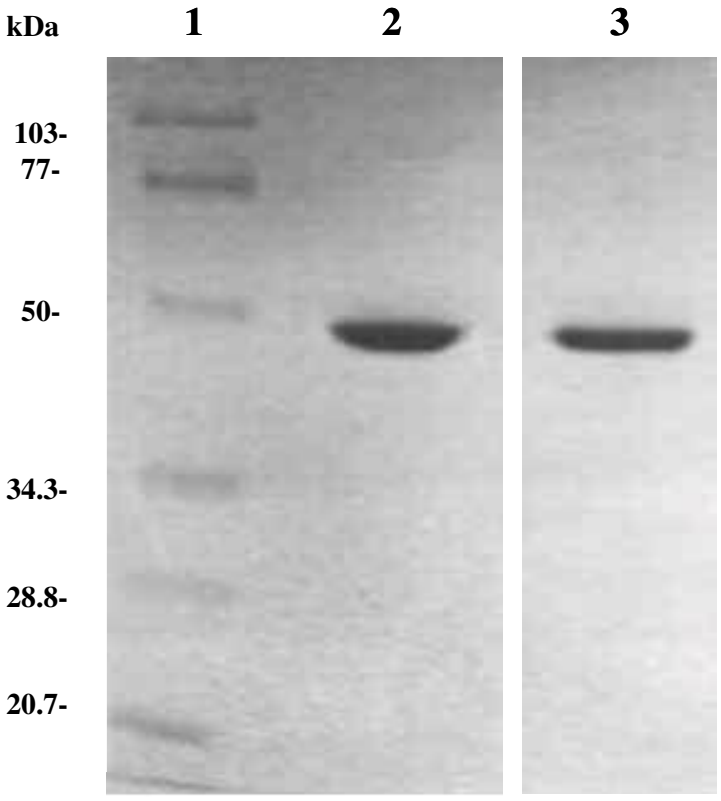


Figure 1: SDS-PAGE analysis showing purified recombinant His-tagged Eis protein. Recombinant His-tagged Eis was purified on a Ni-NTA agarose column and 5 μg loaded onto a 10% SDS-PAGE gel for analysis. The gel was stained with Coomassie blue. (**Lane 1**) Molecular weight markers, (**lane 2**) Purified Eis protein, (**lane 3**) recombinant Eis probed with rabbit anit-Eis antibody.

interactions. Initially, the resulting purified protein precipitated out of solution within a few hours after purification. Attempts to resolve this problem by using established protein denaturation and renaturation protocols failed. Ultimately, this problem was solved by increasing the concentration of NaCl in the lysis, wash and elution buffers from 0.5 M to 1.25 M and by the addition of 10% glycerol to all the buffers. This reduced the chance of non-specific protein-protein interactions and kept the final purified recombinant Eis product in solution. The recombinant His-tagged Eis protein preparation was >99% pure as evinced by scanning densitometry analysis of the protein on SDS-PAGE (Figure 1, lane 2). The recombinant protein also reacted with the anti-Eis antibody (45) (Figure 1, lane 3) that was generated in rabbits to the N-terminus of the protein. The recombinant protein ran at the same size as native protein when run on a 10% SDS-PAGE gel (Figure 1).

Presence of Antibodies to Eis in Sera of Patientsw ith Active Cases of Tuberculosis

Dahl *et al.*had previously demonstrated the presence of antibodies to the Eis protein in the sera of patients infected with *M. tuberculosis* (45). Since antibodies are markers of antigens expressed *in vivo*, this lends credence to the argument that the Eis protein is produced during human infection. To further characterize the immune response to Eis, sera from patients with active cases of tuberculosis (n=13) and healthy controls (n=7) were analyzed for levels of antibody to recombinant Eis using ELISA. ELISA plates were incubated with recombinant Eis in blocking buffer as described above. The wells were then washed and diluted serum samples were added. Serial dilutions of patient sera were

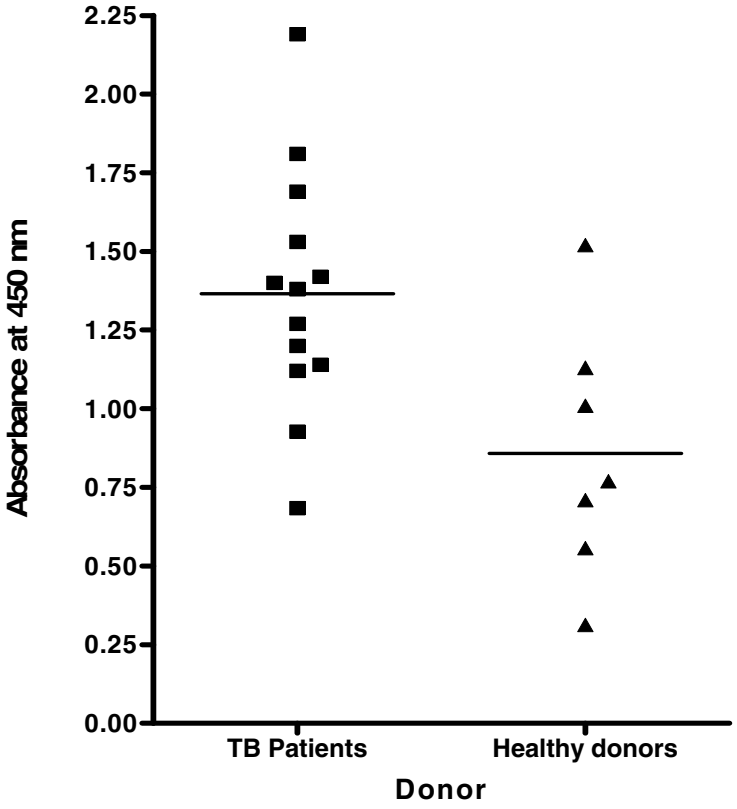


Figure 2: Sera from TB patients and healthy controls tested for the presence of antibodies to Eis. ELISA data show significantly higher levels of antibody to Eis in sera of TB patients than healthy controls (dilution = 1:200, patient sera in Superblock). $p<0.05$, statistical significance by Mann-Whitney U test when levels of antibody to Eis in healthy and TB donors was compared. n=13 for TB patients and n=7 for healthy controls. Horizontal bars represent mean values for each data set.

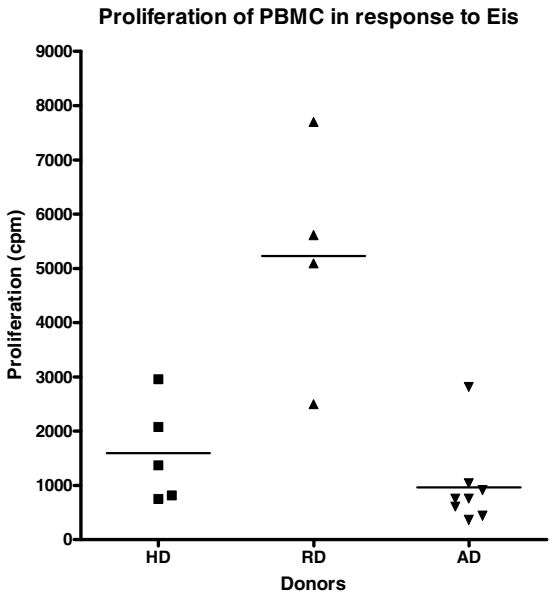
tested in duplicate for this purpose and the data presented (Figure 2) is from the 1:200 dilution data set. Significantly higher levels of antibodies ($p<0.05$) were detected in sera of patients with active cases of tuberculosis as compared to healthy controls (Figure 2). The role of antibodies in protection against *M. tuberculosis* infection is questionable, however the presence of antibodies to the Eis protein in the sera of patients undergoing

treatment for active cases of tuberculosis is a strong indicator that the protein is produced and released by the bacterium during infection, as it is in culture. These findings also indicate that antibodies to Eis in patient sera may be of some use for diagnostic purposes.

T Cell Proliferation of PBMC in Response to Stimulation with Eis

Several *M. tuberculosis* proteins have been demonstrated to be immunodominant T cell antigens. Prime examples of these are Antigen 85 and ESAT-6 (88, 128). They have been shown to elicit T cell proliferative responses from subjects infected with *M. tuberculosis* and have shown promise as diagnostic tools and vaccine candidates. Studies were therefore done to determine whether Eis could induce a similar response. Peripheral blood mononuclear cells (PBMC) were isolated from healthy controls, donors with active cases of tuberculosis, and recovered TB donors. Cells were purified from whole blood as described in Materials and Methods section and treated with 5 μg of recombinant Eis or ESAT-6. PBMC from recovered TB donors showed significantly higher proliferation in response to stimulation with the Eis protein as compared to PBMC from healthy controls and patients with active disease ($p<0.05$ in both cases) (Figure 3A). Similar results were obtained when PBMC were stimulated with ESAT-6 ($p<0.05$ in both cases) (Figure 3B). When the data were viewed in terms of stimulation index (SI), it was noted that while 4/4 of the recovered TB patients demonstrated a positive response to Eis (SI >3), only 2/8 of the donors with active disease responded positively to Eis (Table 3). None of the healthy controls (0/6) showed positive responses to stimulation with the Eis protein (SI<3) (Table 3).

A



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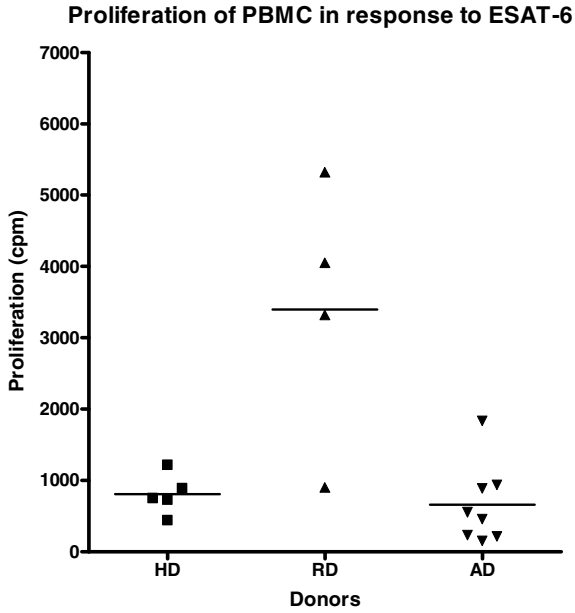


Figure 3: Comparison of proliferative responses of PBMC to Eis and ESAT-6 in different donor populations. PBMCs were isolated from healthy donors (HD, n=5), recovered TB donors (RD, n=4) and active cases of tuberculosis (AD, n=8) and stimulated with 5 μg/ml Eis and ESAT-6. (**A**) Recovered TB donors show significantly

higher proliferation in response to stimulation with Eis as compared to active cases and healthy donors ($p<0.05$ in both cases). (**B)** Recovered TB donors show significantly higher proliferation in response to stimulation with ESAT-6 as compared to active cases and healthy donors ($p<0.05$ in both cases). Horizontal bars represent mean values for each data set.

In the case of PBMC stimulated with ESAT-6, 2/4 of the recovered TB donors exhibited positive responses while all of the healthy controls and patients with active cases of tuberculosis demonstrated negative responses (Table 4). This correlates with

PBMC proliferation in response to Eis expressed as stimulation index (SI)		
Healthy Donors	**Recovered Donors**	**Active Disease Donors**
2.03	**4.56**	**3.08**
0.75	**3.49**	**4.08**
2.35	**4.98**	2.70
0.77	**6.50**	1.91
1.25		0.96
1.25		0.69
		2.04
		2.06

Table 3: PBMC proliferation in response to Eis expressed as stimulation index (SI) . 4/4 recovered donors show positive responses while only 2/8 active cases and 0/6 healthy controls respond positively to Eis. Positive responses are shown in bold. SI>3 is considered positive.

PBMC proliferation in response to ESAT-6 expressed as stimulation index (SI)		
Healthy Donors	**Recovered Donors**	**Active Disease Donors**
1.82	**4.76**	0.94
0.44	1.26	2.05
0.09	2.62	0.85
0.68	**3.84**	2.92
1.99		1.20
0.45		1.22
		0.72
		0.71

Table 4: PBMC proliferation in response to ESAT-6 expressed as stimulation index (SI). 2/4 recovered donors show positive responses while none of the active cases or healthy controls respond positively to ESAT-6. Positive responses are shown in bold**.** SI>3 is considered positive.

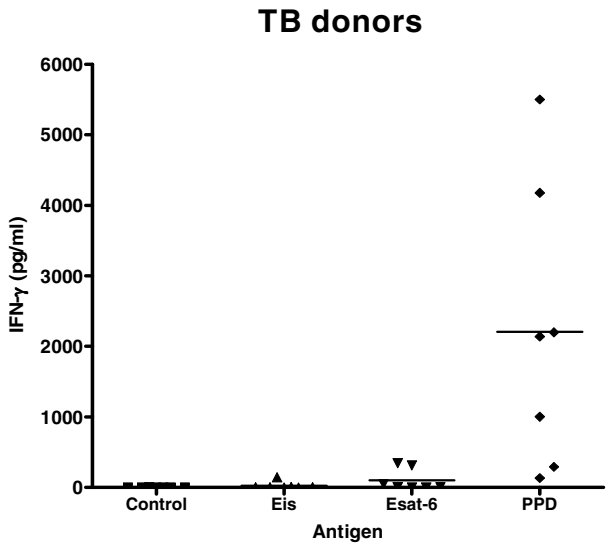
reports in the literature which have documented response levels to ESAT-6 in TB donors ranging from 30-60% (114, 128). The negative responses to Eis and ESAT-6 seen in patients with active cases of *M. tuberculosis* infection are not surprising given the ability of the bacterium to specifically suppress T cell proliferative responses to mycobacterial antigens while not affecting T cell responses to other antigens (5, 114). These findings further strengthen the argument that the *eis* gene is expressed during infection by *M. tuberculosis* and also provide the impetus for further study into the immunogenicity of the Eis protein.

Cytokine Secretion by PBMC in Response to Stimulation with Eis

The cytokine response of PBMC in response to stimulation with an antigen is an important indicator of the type of response elicited by that antigen. IFN-γ secretion is characteristic of a Th1 type cell mediated immune response that is normally responsible for controlling intracellular pathogens such as *M. tuberculosis*. IL-4 secretion is characteristic of a Th2 type immune response that is humoral in nature. In order to determine the nature of the response elicited by Eis, studies were done to analyze the levels of these cytokines secreted into the culture supernatant of PBMC in response to stimulation with Eis and other mycobacterial antigens. Culture supernatants from PBMC of healthy controls and patients with active disease were stimulated with Eis, ESAT-6 and PPD and harvested for analysis of cytokine secretion in response to antigenic stimulation. Samples were stored at –70^{o}C until ready for use. Once a sufficient number of samples had been obtained, they were analyzed by ELISA for the detection and quantification of IFN-γ and IL-4 levels.

High levels of IFN-γ were seen in response to stimulation of PBMC from patients with active disease with PPD. However, almost no IFN-γ secretion was observed in response to stimulation with Eis or ESAT-6 (Figure 4A). PBMC from healthy donors showed lower levels of IFN-γ secretion in response to PPD and also showed very low or negligible levels of IFN-γ secretion in response to stimulation with Eis and ESAT-6 (Figure 4B). This seemed in keeping with the T cell proliferation data from cases of active TB showing very poor or no response to stimulation with the Eis protein (Figure 3).

A



B

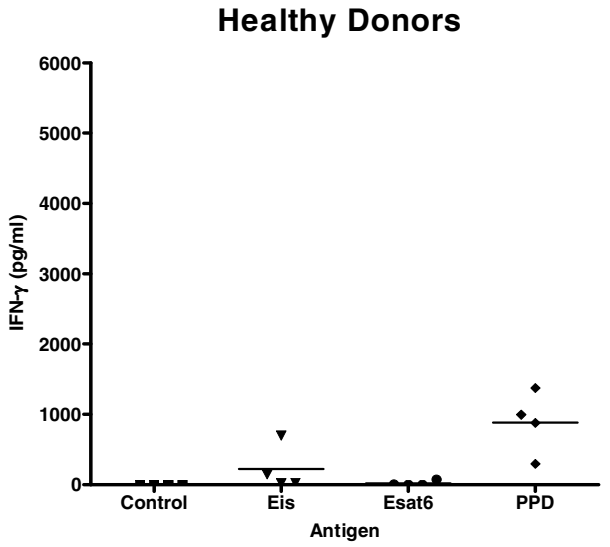
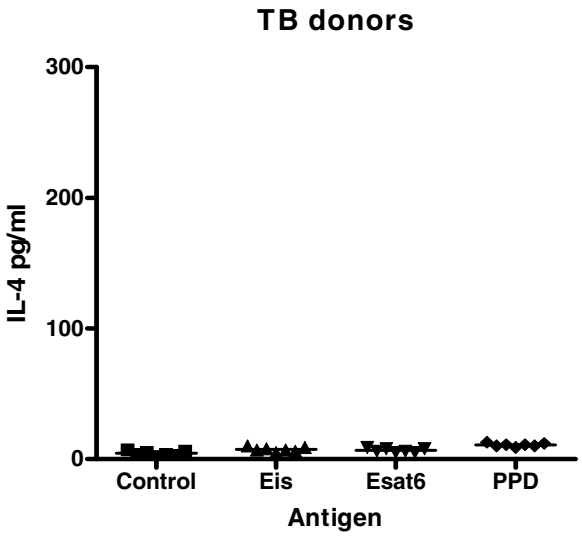


Figure 4: Interferon-γ response of PBMC to antigenic stimulation in healthy donors and patients with active disease. Culture supernatant from PBMC proliferation reactions were harvested after 5 days and analyzed for IFN-γ levels by ELISA. Stimulation with 5 μg/ml Eis or ESAT-6 does not elicit IFN-γ secretion in either (**A**) active TB cases or (**B**) healthy donors. High levels are seen in response to stimulation with PPD in active cases but not in healthy controls. n=7 for active cases and n=4 for healthy controls. Horizontal bars represent mean values for each data set.

A



B

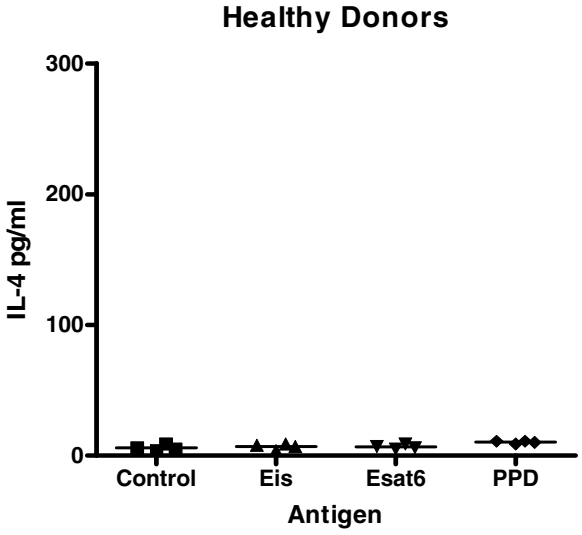


Figure 5: IL-4 response of PBMC to antigenic stimulation in healthy donors and patients with active TB. Culture supernatant from PBMC proliferation reactions were harvested after 5 days and analyzed for IL-4 levels by ELISA. Stimulation with 5 μg/ml Eis, ESAT-6 and PPD does not elicit IL-4 secretion in either (**A**) active TB cases or (**B**) healthy donors. n=7 for active cases and n=4 for healthy controls. Horizontal bars represent mean values for each data set.

Unfortunately samples from recovered cases of TB could not be obtained for this experiment.

Similar results were obtained on analysis of IL-4 levels in the culture supernatant of PBMC stimulated with Eis, ESAT-6 and PPD. No IL-4 secretion was detected by PBMC from either healthy controls or active cases of TB in response to stimulation with Eis, ESAT-6 or PPD (Figure 5A, B). This is not surprising given that the immune response induced by mycobacteria is generally a Th1 type cell mediated immune response. Here too, samples from recovered TB donors were unavailable for analysis.

Discussion

The need for development of alternative diagnostic tools for tuberculosis has made it essential to identify immunodominant antigens of *M. tuberculosis*. Even more crucial is the need to develop an alternative to the BCG vaccine given its failings. Whether searching for diagnostic tools or vaccine candidates, current research indicates that the most successful option will likely be a cocktail of immunodominant T cell antigens of *M. tuberculosis*. Important steps have been made in this direction with the identification of ESAT-6 and Antigen-85, among others, as major T cell antigens of *M. tuberculosis* (88, 128). Our task here is to determine whether Eis could join this pantheon of proteins as a major antigen of *M. tuberculosis*.

The presence of IgG antibodies to the Eis protein in the sera of patients with active cases of *M. tuberculosis* (Figure 2) is a clear indication that Eis is expressed during human infection with *M. tuberculosis* and that the protein is taken up by B cells. T cell proliferative responses are seen in response to stimulation with the Eis protein in

recovered TB donors but not in active cases of tuberculosis or healthy controls (Figure 3). This is in keeping with our knowledge of the ability of *M. tuberculosis* to suppress the various facets of the immune response that are required to protect the host. This includes suppression of Th1 cytokine (IL-12, IL-2 and IFN-γ) production (68, 84, 102) and the subsequent upregulation of anti-inflammatory cytokines (IL-10 and IL-13) which are of little use in protecting against *M. tuberculosis* infection and only serve to hinder the host immune response (46, 51, 113, 171). T cell proliferative responses to mycobacterial antigens have been shown to be suppressed in patients undergoing treatment for *M. tuberculosis* and subsequently, responses have been shown to improve on completion of treatment (5). As mentioned earlier, patients with active cases of tuberculosis also show a depletion of CD4+ IFN-γ+ T cells in their peripheral blood (22). This in combination with the selective loss of the relevant γ/δ T cells severely restricts the ability of TB patients to fight the disease (150). In addition, it has been reported that *M. tuberculosis* inhibits the ability of dendritic cells to present antigens to activate CD4+ and CD8+ T cells (117, 171). The ripple effect of this defect will literally cripple the immune system in its attempts to fight the disease.

It is therefore not surprising that our results show a significant T cell response in recovered TB donors but not in active cases of TB (Figure 3). Also, 100% (4/4) of the recovered TB donors responded positively to Eis while only 50% (2/4) responded to stimulation with ESAT-6, a protein that has been established as an immunodominant T cell antigen of *M. tuberculosis* (Tables 3 and 4).

Analysis of the cytokine response of PBMC in response to stimulation with our panel of mycobacterial antigens is incomplete due to the absence of the recovered TB donor population from this experiment. The data however does seem to reflect what is observed with the T cell proliferation experiments. Healthy controls and active cases of TB fail to show significant IFN-γ secretion in response to stimulation with either Eis or ESAT-6 (Figure 4A, B). This could be attributed to the immunosuppressive effects of *M. tuberculosis* infection in active cases of TB infection. Given that their T cell responses were suppressed, it is not surprising that IFN-γ secretion was suppressed as well in patients with active disease since CD4+ T cells play a major role in driving the Th1 response. It is therefore safe to conjecture that since the recovered TB donors showed significant T cell proliferation when stimulated with Eis and ESAT-6, they would probably have shown significantly higher levels of IFN-γ secretion on stimulation with the same antigens. The low levels of IL-4 secretion seen in all donor groups with all antigens indicates that none of them stimulate a Th2 type response (Figure 5).

In summary, it is safe to say that Eis shows promise as an immunodominant T cell antigen of *M. tuberculosis*. Further work needs to be done before progressing to the stage where one can conclusively state that it is a candidate for diagnostic and vaccine development purposes. The data however looks promising and warrants further study of the antigenicity of the Eis protein of *M. tuberculosis*.

CHAPTER II: SUBCELLULAR LOCALIZATION AND FUNCTION OF THE EIS PROTEIN

Introduction: Mycobacterial Constituents Released in Macrophage Cytoplasm

A wide variety of mycobacterial proteins and lipids have been shown to be secreted into the cytoplasm of infected macrophages (17-19, 116, 130, 170). The trafficking of these mycobacterial components within the endocytic network of the host cell may provide source material for antigen processing and presentation. It is however postulated that these components may also play a role in modulation of macrophage function to the advantage of the bacterium. These mycobacterial components are not only released into the macrophage cytoplasm but are also released from the macrophage into the extracellular milieu in vesicular form (19, 130). Furthermore, these vesicles are taken up by uninfected bystander cells which leads to the phenomenon whereby these cells are found to contain mycobacterial proteins/lipids in the absence of infecting bacteria (17, 19). Antigen 85 is a mycolyltransferase involved in cell wall metabolism of *M. tuberculosis* (20) that has been shown to be released into the cytoplasm of infected macrophages (18). Xu *et al.* demonstrated the presence of lipoarabinomannan (LAM), a mycobacterial membrane glycolipid, in vacuoles discrete from the bacterium within infected macrophages (170). Similarly, the 19-kDa lipoprotein antigen and the fibronectin attachment protein (FAP) along with proteins of the antigen 85 complex have been shown to traffic within the cytoplasm of infected macrophages (18, 116). Mycobacterial glycolipids such as LAM and phosphatidylinositol mannosides have been found in uninfected cells, probably exiting infected cells via late endosomes/lysosomes (137).

Mycobacterial cell wall constituents tagged with fluorescent markers have also been demonstrated in the microvesicular fraction of the culture supernatant of infected macrophages (18). Subsequently these bacterial constituents were found to make their way into uninfected macrophages (17). In addition, human monocytes isolated from peripheral blood and infected with *M. tuberculosis* have been shown to release phosphatidylethanolamine and phosphatidylinositol of mycobacterial origin which subsequently activates suppressor T cells (161). Other mycobacterial proteins/lipids secreted into the macrophage cytoplasm have also been shown to be effectors of pathogenesis. Protein kinase G inhibits phagosome-lysosome fusion and hence enables intracellular survival of *M. tuberculosis* (163), while nucleoside diphosphate kinase acts as a cytotoxic factor for macrophages by enhancing ATP–induced cell death (36, 163). Most prominent however is LAM which has been implicated in a myriad of activities including inhibition of T cell proliferation and modulation of cytokine secretion (35, 98, 112, 131, 133). Mycobacterial proteins also induce the secretion of a large number of cytokines including IL-10, TNF-α, IL-1, and IL-12 by monocytes/macrophages (15, 69, 102, 103, 165).

The mode of release of these mycobacterial components into the macrophage cytoplasm has not yet been characterized. It is possible that *M. tuberculosis* has an yet undescribed mechanism by which it secretes its effectors out of the phagosome. It is more likely however that this release occurs during the exchange of materials between the phagosomes and the various organelles that interact with it. This phenomenon is described as the “kiss and run” hypothesis by Desjardins (53). Phagosomes along the

course of their maturation interact with early and late endosomes and lysosomes. Desjardins *et al.*argue that what occurs between these organelles is not fusion but rather a microtubule mediated transient interaction with the concurrent exchange of luminal contents and membrane markers (53). It is possible therefore that mycobacterial proteins/lipids exit the phagosome via this route.

The propensity of *M. tuberculosis* to extend its influence beyond the constraints of the host cell it resides in, and is in keeping with its ability to subvert the immune system of the host to suit its purposes. Patients with active cases of tuberculosis have been shown to have depressed immune responses to stimulation with *M. tuberculosis* antigens while their responses to antigens from other pathogens remain normal (84, 85, 102, 103, 114).

Eis

Fractionation of *M. tuberculosis* lysates by differential centrifugation revealed that Eis was predominantly localized in the cytoplasmic fraction of the bacillus (45). The stringent response gene, Rel_{Mtb}, of *M. tuberculosis* which plays a major role in survival during nutrient deprivation and persistence in the mammalian host and has been shown to negatively regulate the expression of several immunodominant mycobacterial proteins including ESAT-6 and Antigen 85 (44). This gene also appears to have the same effect on the expression of *eis* (43). During induction of stringent response by nutrient starvation conditions, as one would expect to see during infection of macrophages, levels of *eis* expression remained constant in wild type *M. tuberculosis* but were upregulated 6.3-fold in *M. tuberculosis* Δrel_{Mtb} (43, 44).

The immune response to the Eis protein in TB patients indicates that the protein is produced during human infection. While *eis* has been shown to enhance intracellular survival of *M. smegmatis* (167), its role in the virulence of *M. tuberculosis* is unclear. Intracellular survival assays failed to detect a phenotype using the *M. tuberculosis* wild type and *eis* mutant strains (166). In order to characterize the role of *eis* in the pathogenesis of *M. tuberculosis*, studies were conducted to examine the production and release of Eis during the infection of macrophages. Protein-protein interaction assays were utilized in an attempt to identify proteins interacting with Eis, thus providing clues as to the function of Eis. The role of Eis in the modulation of cytokine secretion by infected macrophages was also examined. Finally, bioinformatic analysis was utilized to try to determine the function of the Eis protein.

Materials and Methods

Strains and Culture Media

M. tuberculosis strains were grown in Middlebrook 7H9 media (Difco) supplemented with 10% OADC (Becton Dickinson) at 37^{o}C with constant agitation till the required ODs were achieved. For growth on solid culture media, Middlebrook 7H10 (Difco) with 10% OADC was used. *M. tuberculosis* H37Ra Δ*eis* (Ra610, Table 2) and H37Rv Δ*eis* mutants (Rv6, Table 2) were contructed by allelic replacement using the suicide vector pMJ10 (166). The *eis* complemented strains (Ra610-16 and Rv8-1, Table 2) were constructed using the integrative single copy *eis*-bearing plasmid p6301 and

grown as described previously (166). The deletion and complementation of the *eis* gene was confirmed by both Southern and Western blot analysis in all strains (166).

E. coli DH5α was used as the host for all plasmid constructions. It was grown on Luri-Bertani medium (Difco) supplemented with the appropriate antibiotics.

U-937 macrophage cell line was obtained from the American Type Culture Collection, (ATCC, Rockville, MD, U.S.A.) and cultured in RPMI 1640 culture media (Sigma) supplemented with 10 % heat inactivated fetal bovine serum (Atlanta Biologicals). This will subsequently be referred to as RPMI unless stated otherwise. J774 macrophage cell line was obtained from the American Type Culture Collection. (ATCC, Rockville, MD, U.S.A.). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum.

Western Blots

Western blot analysis was carried out using the chemiluminescent Supersignal West Pico detection kits (Pierce). Sample protein concentrations were determined by the BCA assay (Pierce). Samples were separated by 10% SDS-PAGE gels along with prestained MW standards (Gibco BRL). Electrophoresis was carried out at 200V and 80mA for 3-4 h. Proteins from the gels were transblotted for 1 h using MiniProtean 3 cells (Biorad) onto PVDF membranes (Millipore) which were then blocked with 5% skim milk in PBS for 1 h. All antibodies used were diluted into PBS containing 0.25% powdered skim milk with 0.02% sodium azide. Membranes were incubated with primary antibody for 1 h, washed 3 x with PBS and then probed with secondary antibody for

another hour. Secondary antibodies conjugated to horseradish peroxidase [HRP] (Pierce) were used at 1:20,000 dilution. Membranes were then washed 3 x with PBS and treated with HRP substrate (Pierce) for 5 minutes followed by exposure to X-ray film (Pierce) for appropriate periods of time.

Preparation of Mycobacterial Subcellular Fractions by Differential Centrifugation Partitioning

Subcellular fractionation was performed essentially as described previously (123), with minor modifications. Briefly, 1 liter of *M. tuberculosis* H37Ra was grown to mid-log phase (3 weeks) in Middlebrook 7H9 medium (Difco) at 37°C with agitation before harvesting. Cells were collected by a 30 min centrifugation at 10,000 × *g*. Pelleted cells were washed, resuspended in PBS with protease inhibitor cocktail, and lysed using silica beads in a Fastprep beadbeater (ThermoSavant) using maximum settings 3 times for 45 sec each cycle with 5 min intervals for cooling on ice. Lysates were centrifuged twice at 11,000 × *g* for 5 min at room temperature to remove unbroken cells and insoluble material. Lysate supernatants were centrifuged at 27,000 × *g* for 1 h at 4°C to pellet cell walls. The supernatant was collected and ultracentrifuged at 100,000 × *g* for 4 h at 4°C to separate cell membranes (pellet) from the cytoplasmic fraction (supernatant). The cell wall and cell membrane fractions were each washed three times with PBS and resuspended in 500 µl of PBS. The cytoplasmic fraction was ultracentrifuged three more times for 4 h at 100,000 × *g* to ensure that all residual membranes were removed. The final volume of the cytoplasmic fraction was 3.5 ml, seven times the volumes of the cell wall and cell membrane fractions.

Far Western Analysis of *M. tuberculosis* H37Ra Lysates for Detection of Eis -Protein Interactions

Far Western analysis is a method utilized to detect protein-protein interactions. *M. tuberculosis* H37Ra and *Mycobacterium smegmatis* cultures were allowed to grow in 7H9 media to mid-log phase, bacteria were pelleted and subsequently bacterial lysates were prepared as described previously. Samples were loaded onto 10% SDS-PAGE at 50-100 μg protein/lane and allowed to run for 3 h at 200v. Subsequently, samples were transferred onto PVDF membrane (Immobilon) as described previously in the Western blot protocol. The membrane was then blocked for 2 h in Blocking buffer I (0.05% Tween 20 in PBS) with gentle agitation followed by blocking for another 2 h in Blocking buffer II (1 gm BSA in 100 ml PBS). This extended blocking process allows the protein to renature on the membrane to a certain extent and thus enhances the ability of the process to detect protein-protein interactions. The membrane was rinsed briefly in PBS and then probed with 5 μg/ml recombinant Eis protein in probe dilution buffer (3 gm BSA, 10 ml bovine sera, 100 ml PBS) for 2 h with gentle agitation. It was then washed 4 x with 200 ml PBS each time. The blot was then incubated with 200 ml of Blocking buffer III (5% non-fat milk in 1 x Tris buffered saline (TBS) with 0.05% Tween) for 1 h at room temperature with gentle agitation. The primary antibody (rabbit anti-Eis antibody) (45) was then diluted in Blocking buffer III at a concentration of 1 μg/ml and incubated with the membrane for 1 h with gentle agitation at room temperature. The membrane was then washed 3 x, 10 min each time, with 200 ml TBS with 0.05% Tween (TBST) and then probed goat anti-rabbit IgG conjugated to horseradish peroxidase

(Pierce) diluted to 1:20,000 in Blocking buffer III. The membrane was then washed 6 x with 200 ml TBST, 5 min each, incubated in TMB substrate (Pierce) for 5 min and then exposed to X-ray film (Pierce) for appropriate periods of time.

Purification of Protein Binding to Eis Using a Ni-NTA Agarose Column

Ni-NTA agarose column (1.5 ml) was prepared and stored at 4^{o}C as described previously. *M. tuberculosis* H37Ra lysate was prepared in PBS as described above. The column was loaded with 250 µg recombinant His-tagged Eis. Five milligrams of *M. tuberculosis* H37Ra lysate was passaged multiple times over the column by gravity flow. The column was then washed 3 x with 3 ml wash buffer (20 mM Tris base, 0.5 M NaCl, 50 mM imidazole, pH 7.5). Finally bound proteins were eluted from the column using 3 washes of 750µl elution buffer (20 mM Tris, 0.5 M NaCl, 200 mM imidazole, pH 7.5). Each fraction was collected and stored separately. Samples were not pooled but were loaded separately onto 10% SDS-PAGE for analysis as described previously. Each eluate fraction was thus loaded onto the gel separately giving us 3 fractions each for the test (*M. tuberculosis* H37Ra +Eis) and control (*M. tuberculosis* H37Ra –Eis) samples.

Coimmunoprecipitation for Detection of Eis-Protein Interactions

Protein A-Sepharose CL-4B (Sigma) was obtained as a lyophilized powder and 250 mg was rehydrated in 5 ml of 50 mM Tris-HCl (Sigma), pH 7.5 for 1 to 2 h on ice. Protein A-Sepharose CL-4B was used because it has been treated with cyanogen bromide so as to generate a sepharose matrix that it is cross-linked to withstand centrifugation. The binding capacity of this matrix is 20 mg/ml of IgG. Protein A-Sepharose was washed 4 x in coimmunoprecipitation buffer (CoIP) (50 mM Tris-HCl pH7.5, 15 mM EGTA, 100

mM NaCl, 0.1% Triton X-100, 1 mM sodium azide, added fresh 1 mM DTT, 1 mM PMSF and 20 µl protease inhibitor cocktail (Sigma) added before use) with gentle centrifugation at 1000 rpm for 1 min. Matrix was prepared and stored in 2.5 ml CoIP buffer at a concentration of 100 mg/ml at 4^0C. It can be stored for several months in this manner.

M. tuberculosis H37Ra wild type and Δ*eis* mutant strain lysates were prepared as described previously. One microgram of anti-Eis antibody was added to 500 µg of each lysate in 500 µl of CoIP buffer and incubated on ice for 90 min with occasional agitation. The mixture was then microcentrifuged for 10 min at 13,000 rpm to pellet non-specific aggregates. Then 50 µl of Protein A-Sepharose was added and the preparation rotated at 4^oC for 1 h. The sample was then centrifuged for 30 sec at 1000 rpm on a table top centrifuge. The pellet thus obtained was washed 3 x with 1 ml each time of CoIP buffer followed by gentle centrifugation for 30 sec at 1000 rpm. Finally, supernantant was removed by aspiration with a needle. To the pelleted Protein-A Sepharose beads was added 25 µl of SDS sample buffer and boiled for 5 min, vortexed and microcentrifuged to pellet beads. The supernatant was then loaded onto 10 % SDS-PAGE gel for analysis. The gel was run at 200 v and 80 mA for 3 h and protein bands visualized by staining with Coomassie blue (Pierce).

Culture and Preparation of Mycobacterial Inocula for Macrophage Infection

M. tuberculosis strains were cultured in Middlebrook 7H9 culture medium supplemented with 10% OADC (Becton Dickinson), 1% glycerol and 0.05% Tween 80. Mid-log phase cultures (OD 0.6-0.7) were then plated onto Middlebrook 7H10 solid

media for preparation of inocula for infections. On day of infection, bacterial growth was swabbed from the plate into 5 ml of 7H9 media, vortexed 10 sec at full power and spun down at 3000 rpm for 10 min. The bacterial pellet was resuspended in another 5 ml of 7H9 with the addition of 50-3 mm glass beads and vortexed for 30 sec at full power. Inocula was then centrifuged for 5 min at 1000 rpm with the brake off in a Beckman Model T J-6R centrifuge. OD650 readings were utilized to dilute the inocula to an MOI of 15 for the assay.

Infection of Macrophage Cell Lines

U-937 macrophage monolayers were prepared by transformation of U-937 cells in T75 flasks (Corning). Ten microliters of a 10 mg/ml solution of phorbol myristic acetate (PMA) (Sigma) in dH_2O was added to each flask containing 2.5 x 10^7 cells. The next day, the cells were washed 3 x with HBSS and harvested using 3 mm glass beads. Cells were then plated into fresh T75 flasks for infection. Infection of U-937 monolayers was carried out as described previously (167). Briefly, concentrated *M. tuberculosis* H37Ra wild type and Δ*eis* mutant inocula were diluted in RPMI supplemented with 10% human AB serum (Omega Scientific) and allowed to stand at room temperature for 20 min to allow for opsonization. Macrophage monolayers were then washed with 25 ml Hbss and 15 ml of diluted inocula was then added to each flask. An MOI of 15:1 was used for these experiments. The flasks were incubated at 37^{o}C and 5% CO_2 and the infection was allowed to proceed for 4 h. The inoculum was aspirated and monolayers washed 3 x with RPMI + 1% human AB sera. Finally, 15 ml of RPMI + 5% human AB sera was added to each flask and incubated for 48 h at 37^{o}C and 5% CO_2.

J774 macrophages are adherent in nature and were cultured in T75 flasks at 37 ^{0}C and 5% CO_2. For immunofluorescence assays, J774 cells were harvested using glass beads and seeded to 24 well plates (Falcon) containing sterile glass coverslips at 2 x 10^5 cells/well and infected as described previously.

Real-time PCR Analysis of *eis* Transcripts Using *sigA* as an Internal Standard

PMA-treated U-937 human macrophage monolayers in 24 well plates were infected with *M. tuberculosis* H37Ra at an MOI of 10 as described above. For each time point, 16 wells of infected macrophages were lysed using 1 ml of TRIZOL reagent (Invitrogen). Four wells of mock-infected U-937 cells were also lysed using 1 ml TRIZOL. Samples were transferred to a 2 ml screwcap tube, vortexed for 10 sec at maximum settings to improve lysis, and snap-frozen in liquid nitrogen. Samples were stored at -70°C until ready for processing. On the day of processing, infection samples in TRIZOL were thawed on ice for 20 min and ~0.5 ml of 0.1 mm RNase-free zirconium beads were added to each tube. Mycobacteria were then lysed in a FastPrep FP120 reciprocating angular shaker three times at maximum settings (45 sec at 6.5 m/s) with 5 min incubations on ice between runs. RNA was then extracted according to the manufacturer's (RNeasy, Qiagen) protocol including the initial centrifugation step for cells rich in lipids. RNA was re-suspended in 20 µl of diethylpyrocarbonate treated water and quantified by spectrophotometry at $OD_{260/280}$. For RNA isolated from mycobacteria grown in 7H9 or exposed to RPMI-10% FCS + human sera, ~ 1 µg RNA was treated with Amplification grade RNase-free DNaseI (Invitrogen) in a 50 µl total reaction

according to the manufacturer's protocol. For RNA isolated from infections or mock infections, ~ 5 μg of RNA was treated with DNaseI as described above. Eight microliters of DNaseI treated RNA was incubated with a 1:1:1 mixture (16.67 ng each) of *sigA*.rt and *eis*2.rt primers (Table 5) to avoid sample to sample variability in reverse transcription for *M. tuberculosis* H37Ra samples.

The parameters for *sigA* real-time PCR are as follows: an initial denaturation at 94°C/10 min; (15 cycles with a decrease of 0.5°C/cycle at the annealing step): 94°C/30 sec, 65°C/30 sec, 72°C/30 sec; (25 cycles): 94°C/30 sec, 57°C/1 min, 72°C/30 sec; followed by a final elongation at 72°C/5 min. The cycling parameters for *eis* are as follows: an initial denaturation at 94°C/10 min; (20 cycles with a decrease of 1°C/cycle at the annealing step): 94°C/30 sec, 75°C/30 sec, 72°C/30 sec; (25 cycles): 94°C/30 sec, 60°C/30 sec, 72°C/30 sec; followed by a final elongation at 72°C/5 min.

Table 5: Primers used in Chapter II of this study:

Primers	Sequence[a]	Ref[b]
sigA.rt	CGGACGAGACCATGGTGCGGC	(56)
sigA.up	GGCCAGCCGCGCACCCTTGAC	(56)
sigA.down	GTCCAGGTAGTCGCGCAGGACC	(56)
*eis*2.rt	GGTCCTGTGGATGGGTGATG	(This study)
*eis*2-nf	TCGTTCGCTTACTGCATCC	(This study)
*eis*2-r	ACAATGCGCATCTGCGGTTACCGCC	(This study)

[a] Primers are listed 5' to 3'.
[b] Unless indicated otherwise, primers used are from this work.

Gene specific cycling parameters described above were used, except that the final elongation step was not included. SYBR green was used to detect double-stranded,

fluorescent amplicons using 10 ρmol of each gene specific primer (Table 5) according to the manufacturer's protocol (Invitrogen) during the elongation step of the second set of cycles in the PCR. An ABI Prism 7000 (Applied Biosystems) was used for real-time quantitation. *sigA* real-time PCR was performed independently due to differences in cycling parameters. Fluorescence from mock RT reactions was subtracted from true RT reactions for all samples to account for background amplification from contaminating DNA. The results are expressed as the ratios of the numbers of RNA copies detected in RNA samples taken at various times from infected samples to the numbers of RNA copies detected in samples obtained from bacteria growing in 7H9 medium. The values were normalized to the *sigA* RNA value. Each measurement was obtained at least twice using independent RNA preparations.

Construction of *M. tuberculosis* H37Ra Wild Type and Δ*eis* Mutant Expressing Green Fluorescent Protein

M. tuberculosis H37Ra wild type and Δ*eis* mutant were tranformed with the vector pBEN, which contains *gfp* driven by by p*hsp60*, a strong heat shock promoter from *Mycobacterium bovis BCG* [Table 1](13, 136). The promoterless *gfp* vector pFPV27 was used as the negative control for these studies. Bacteria were grown on Middlebrook 7H10 media containing 25 μg/ml Kanamycin. Fluorescence of transformants was confirmed by fluorescence microscopy (data not shown).

Immunofluorescence Microscopy (IF) of Macrophages Infected With *M. tuberculosis* H37Ra Wild Type and Δ*eis* Mutant

One ml of RPMI containing 2 x 10^5 PMA-activated U-937 cells were plated into each well of a 24-well tissue culture plate (Falcon) containing sterile glass coverslips (Falcon). Cells were infected, as described previously, with *M. tuberculosis* H37Ra wild type and H37Ra Δ*eis* expressing *gfp* at an MOI of 20. At appropriate time points, coverslips were washed 3 x with PBS and 1 ml of 3% formaldehyde in PBS was added as fixative to each well. After 15 min, coverslips were washed 3 x with PBS and cells permeabilized by addition of 0.1% Triton 100X for 1 min. The coverslips were then washed 3 x with PBS and 0.5 ml 1:500 dilution of anti-Eis antibody (45) was added per well, followed by incubation for 1 h at room temperature on an orbital shaker. The wells were then washed 3 x with PBS followed by addition of 1:10,000 dilution of the secondary antibody - Alexafluor 594 goat anti-rabbit IgG (Molecular Probes) linked to a fluorescent red dye in PBS + 10% FBS. Plates were again incubated for 1 h at room temperature on an orbital shaker. Coverslips were washed 3 x with PBS and removed to a paper towel to air dry in the dark. Finally, 2 μl p-phenylenediamine (ppd) mounting media was added to each coverslip, which was then inverted onto a glass slide, allowed to air dry and then visualized using a Nikon Eclipse 2000-S microscope. The same procedure was used for IF analysis of both infected J774 murine macrophages and primary human monocytes.

Immunofluorescence Microscopy of Macrophages to Detect Uptake of Recombinant Eis

For demonstration of recombinant Eis uptake by macrophages from the culture supernatant, the same protocol as described above was followed with the obvious exception that the U-937 macrophages were not infected. Instead, 5 μg/ml recombinant Eis was added to the culture supernatant of the macrophages. Macrophage nuclei were stained red with propidium iodide (1:10,000 dilution in PBS for 1 min). Cells were washed, fixed, permeabilized and probed with rabbit anti-Eis antibody (45) followed by Alexafluor 488 goat anti-rabbit IgG (Molecular probes) [1:10,000 dilution] linked to a fluorescent green dye.

Harvesting and Lysis of Infected U-937 Macrophage Monolayers for Isolation of Macrophage Cytoplasmic Fraction

M. tuberculosis infected macrophage monolayers in 20 T75 flasks, 48 h after infection at an MOI of 15, were washed with 20 ml HBSS each and harvested into 20 ml HBSS using 3 mm glass beads. Lysis was carried out as described previously (34). Briefly, cells were washed and resuspended in cold homogenization buffer (250 mM sucrose, 0.5 mM EDTA, 0.5 mM EGTA, 20 mM Hepes). Macrophages were then lysed by repeated passage through a 25 G needle. Macrophage lysate was then centrifuged at 300 x *g* for 10 min followed by 2 subsequent spins at 100 x *g* for 5 min each to remove whole macrophages, cellular debris, and nuclei. The post-nuclear supernatant was carefully layered over a 12% sucrose solution in 15 ml conical tubes (Corning) and centrifuged at 800 x *g* for 45 min at 4^{o}C. Subcellular organelles remained in a distinct

upper layer, separate from bacteria and mycobacteria containing phagosomes in a lower layer and pellet. The macrophage cytoplasmic fraction was then harvested from the surface of the sucrose solution, quantified by BCA and 200 μg total protein was loaded onto a 10% SDS-PAGE gel for subsequent analysis by Western blot as described previously. The blot was probed for the presence of the Eis protein in the macrophage cytoplasm.

Isolation of Exocytic Vesicles From the Culture Supernatant of Infected U-937 Macrophages

Exocytic vesicles were isolated from culture supernatants as described previously (130). Briefly, culture supernatant from U-937 macrophages infected with *M. tuberculosis* H37Ra as described previously were harvested 48 h after infection. A total of 300 ml of tissue culture media was harvested from 20 T75 flasks. This was then centrifuged twice at 300 x *g* for 10 min to remove whole macrophages. Supernatant was further centrifuged twice for 10 min at 2000 x *g* to remove any bacilli. The supernatant was recovered and subjected to a series of centrifugation steps- 30 min at 25,000 x *g*, supernatant was recovered and centrifuged for 30 min at 70,000 x *g*, supernatant was recovered again and centrifuged for 60 min at 100,000 x *g*. The resulting pellet contained the extravesicular fraction from the culture supernatant of infected U-937 macrophages. This pellet was resuspended in 100 μl PBS and the vesicles were lysed by addition of 0.1% SDS. Protein concentration was determined by BCA assay and 200 μg of sample was loaded onto a 10% SDS PAGE gel and analyzed by Western blot for the presence of the Eis protein.

Culture supernatant obtained after multiple centrifugation steps described above was concentrated 3-fold using a YM-10 Centriprep filter (Millipore) and analyzed by Western blot for the presence of native Eis protein.

Measurement of Lactate Dehydrogenase Release from Infected Macrophages

A Cytotox 96 non-radioactive cytotoxicity assay (Promega) kit was used to measure levels of lactate dehydrogenase (LDH) in the macrophage culture supernatant to determine viability during infection with *M. tuberculosis*. This assay quantitatively measures LDH levels using a 30 min coupled enzymatic assay. Samples included culture supernatant from U-937 macrophages infected with *M. tuberculosis*, uninfected U-937 macrophages in culture medium and culture medium alone. All three samples were incubated for the same time period as previous infections and harvested. Uninfected U-937 macrophages were lysed in the culture media by freeze thawing. Fifty microliter of each sample was loaded, in quadruplicate into 96 well ELISA plates (Falcon) and 50 µl of substrate was added. Plates were incubated at room temperature for 30 min in the dark. Fifty microliter of stop solution was added and absorbance recorded at 490 nm using a Biorad Plate Reader (Biorad). Background values were subtracted from sample readings. Percentage of cell death was determined using the formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental LDH release (OD490)}}{\text{Maximum LDH release (OD490)}}$$

Density Gradient Separation of Macrophage Organelle Fractions to Determine Localization of Eis

The macrophage cytoplasmic fraction obtained by centrifugation of the lysate of U-937 macrophages infected with *M. tuberculosis* H37Ra wild type and Δ*eis* mutant strains over a 12% sucrose solution was harvested. Protease inhibitor cocktail (Sigma) (20 μl) was added and 1.2 ml of 62% sucrose solution was added per ml of sample to bring the sucrose concentration of the sample to 40%.

A sucrose step gradient was prepared using varying concentrations of sucrose in PBS- 60%, 40%, 35%, 25% and 10% sucrose concentrations were used as described previously (106). In brief, this was done by carefully layering the sucrose (~2ml for each fraction) in order of decreasing concentration in a 12 ml volume Ultraclear centrifugation tube (Beckman). The macrophage cytoplasmic fraction (4.5 mg) was incorporated into this gradient as the 40% fraction, and layered over the 60% fraction. This gradient was then centrifuged at 100,000 x *g* for 1 h at 4^{o}C using a SW 41 rotor in a Beckman L8-80 Ultracentrifuge (Beckman). The different fractions were harvested from the tube by puncturing the side of the tube using syringes tipped with 18 G needles. Samples were harvested from the 10%, 25%, 35% and 60% fractions. Samples were dialyzed against water overnight to remove sucrose. Protein concentrations were determined by the BCA assay and entire fractions were then loaded onto a 10% SDS-PAGE gel for Western blot analysis. In order to determine the localization of Eis with particular macrophage organelles, the blot was probed with antibodies to Eis as well as multiple macrophage organelle markers. Mouse anti-calreticulin IgG_1 (BD Biosciences), mouse anti-early

endosome antigen-1 (BD Biosciences) and mouse anti-LAMP-1 IgG1 (Developmental Studies Hybridoma Bank, University of Iowa) were used. The secondary antibody used here was goat anti-mouse IgG conjugated to horse radish peroxidase (1:20,000 dilution).

Calreticulin is a 60-kDa luminal endoplasmic reticulum protein. Early endosome antigen-1 (EEA1) is a 180-kDa hydrophilic membrane protein that colocalizes to early endosomes. Lysosome associated membrane protein-1 (LAMP-1) is a 110-kDa protein associated with lysosomes.

Preparation of Human Monocytes for IF

Peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy donors by centrifugation on Ficoll-hypaque (Pharmacia Fine Chemicals) as described previously (42, 128). Briefly, 50 ml of blood was diluted 1:1 with warm HBSS buffer, layered on Ficoll-hypaque, and centrifuged at 2,000 rpm for 40 min in a Beckman Model T J-6R centrifuge. After the centrifugation, the total leukocyte fraction was collected and washed with 10 ml of Hbss buffer at 1,500 rpm for 5 min. The cells were resuspended in RPMI 1640 (Sigma) containing 15 % autologous human serum and adjusted to 10^6 cells per ml. Appropriate volumes of mononuclear cells (to give 2 x 10^5 monocytes per well) were incubated in wells of a 24-well tissue culture plate (Costar) containing sterile glass coverslips. After 2 h incubation at 37^{o}C in a CO_2 incubator, cells were washed three times with warm RPMI-1 % autologous serum to remove nonadherent cells. Adherent cells were 90 to 95% monocytes, as verified by Giemsa stain. Adherent cells were cultured in 1 ml of RPMI-15 % autologous serum at 37 ^{o}C with 5 % CO_2 overnight to allow maturing into macrophages prior to the infection with *M. tuberculosis*

for immunofluorescence microscopy. Normally, macrophages and serum from the same donor were used. The number of adherent cells per well was determined by counting nuclei after lysis of the monolayer (213). At this point, each well contained approximately 2 to 4 × 10^5 cells.

Isolation and Cultivation of Human Monocytes for Analysis of Cytokine Secretion in Response to Antigenic Stimulation

Venous blood was drawn from subjects into sterile blood collection tubes, and PBMCs were isolated by density sedimentation over Histopaque-1077 (Sigma). They were suspended at a density of 2 x 10^6 viable cells/ml in a complete medium (RPMI 1640 [Gibco BRL]) with 10% fetal bovine serum [Gibco BRL], sodium pyruvate, nonessential amino acids, penicillin G [100 IU/ml], and streptomycin [100 µg/ml]). Cells were incubated for 1 h at 37°C, and nonadherent cells were removed by pipetting off the supernatant. Adherent monocytes were collected as previously described (146). The recovered cells were >95% CD14+ cells, as determined by flow cytometry using an anti-CD14 antibody. The cells were then stimulated with PPD (Statens Serum Institut), Eis, or lipopolysaccharide (LPS, 10 ng/ml; Sigma) and incubated at 37°C in a 5% CO_2 humidified air atmosphere. The supernatants from 18 h (for TNF-α) and 48 h (for IL-10) cultures were frozen at -80°C until used in ELISA.

Preparation of Inocula and Infection of Human Monocytes with *M. tuberculosis* for Analysis of Cytokine Secretion

M. tuberculosis H37Rv wild type, Δ*eis* mutant, and complemented strains were grown to late log phase in Middlebrook 7H10 agar medium supplemented with 10%

OADC (Becton & Dickinson Immunocytometry) and 0.05% Tween 80 (Sigma). Batch cultures were aliquoted and stored at -70°C. Representative vials were thawed and enumerated for viable CFU on Middlebrook 7H10 agar. Single-cell suspensions of mycobacteria were obtained by a modification of the standard methods. Briefly, aliquots of frozen *M. tuberculosis* were cultured in 7H9 broth with 0.5% glycerol at 37°C in 5% CO_2 for 7 to 10 d so that cultures reached mid-exponential growth phase. Bacterial cultures were pelleted at 3,000 × *g* for 10 min and resuspended in 7H9. Clumped mycobacteria were dispersed with an ultrasonic cell disrupter (3 to 5 min, 35 kHz; Bandelin). Bacteria were then resuspended in 1 ml of RPMI 1640 medium and clumps were disrupted by multiple passages through a 25 G needle. Mycobacterial viability was assessed by the number of CFUs and was found to be 60 to 70%.

Adherent human monocytes were washed 3 x with Ca^{2+}- and Mg^{2+}-free PBS, and adherent monolayers were replenished with complete medium without antibiotics. The cells were incubated overnight without stimulation at 37°C in a 5% CO_2 atmosphere. After the overnight incubation, monocytes (2 x 10^5/ml) were infected with mycobacteria using bacteria-to-cell ratios of 1:1 for 18 h to obtain the supernatants for cytokine analysis.

Enzyme-linked Immunosorbent Assay for TNF-α and IL-10

ELISA was used for detecting TNF-α and IL-10 levels (BD Biosciences) in monocyte culture supernatants, as described (103). Assays were performed as recommended by the manufacturers. Cytokine concentrations in the samples were

calculated using standard curves generated from recombinant cytokines, and the results were expressed in picograms per milliliter. The difference between triplicate wells was consistently less than 10% of the mean.

Statistical Analysis

For statistical analysis, data obtained from independent experiments are presented as the mean ± SD and were compared by Student's *t* test or for multiple comparisons by ANOVA. Differences were considered significant for $p < 0.05$.

Bioinformatics

Protein sequences for analysis were obtained in FASTA format and input into respective programs at the interface locations described below. RPS-BLAST is a variation of PSI-BLAST which utilizes the Conserved Domain Database (CDD) to compare a single sequence against a database of PSSM (Position-Specific Score Matrix) models (5). This is available for use at http://www.ncbi.nlm.nih. gov/ Structure/ cdd/ wrpsb.cgi. Pfam is a comprehensive database of protein domain families based on seed alignments using hidden Markov model profiles, while CDD is a collection of multiple sequence alignments representing protein domains conserved in molecular evolution (4, 5). The 3D-PSSM server is a designed to take a protein sequence of interest and attempt to predict its 3-dimensional structure and its probable function (96). 3D-PSSM utilizes a library of known protein structures onto which each query sequence is "threaded" and scored for compatibility. It uses a variety of scoring components: 1D-PSSMs (sequence profiles built from relatively close homologues), 3D-PSSMs (more general profiles containing more remote homologues) matching of secondary structure elements, and

propensities of the residues in the query sequence to occupy varying levels of solvent accessibility (96). Initially, the entire amino acid sequence of Eis was used as input for the program but subsequently, shorter sequences that match up to known structures were used as input. 3D-PSSM is administered by Lawrence Kelley and is available for use at http://www.sbg.bio.ic.ac.uk/servers/3dpssm/.

Multiple Expectation Maximization for Motif Elicitation (MEME) uses statistical modeling techniques to automatically choose the best width, number of occurrences, and description for each motif (10, 75, 76). It represents motifs as position-dependent letter-probability matrices which describe the probability of each possible letter at each position in the pattern. MEME can be found at http://meme.sdsc.edu and is administered by the University of California, San Diego.

Results

Far Western Analysis of *M. tuberculosis* H37Ra Lysates for Detection of Eis -Protein Interactions

In an attempt to identify a possible function for the Eis protein, experiments were done utilizing Far Western blot analysis as a method of detecting protein-protein interactions that may occur between Eis and other proteins in *M. tuberculosis*. It was thought that assuming that Eis possesses some enzymatic function, this would give clues as to possible substrate(s) and therefore the role of Eis in the pathogenesis of *M. tuberculosis*. *M. smegmatis* and *M. tuberculosis* lysates (50-100 µg each) were loaded onto 10% SDS-PAGE gel, separated by electrophoresis and transferred onto PVDF membranes as described previously in the Materials and Methods section. The membrane

was then blocked for several hours as described in materials and methods to enable renaturation of the denatured protein on the membrane. This would enable the proteins to regain their original three dimensional structures to some extent and thus make it more likely to detect the protein-protein interactions that occur during the normal course of the life cycle of the bacterium. The blocking process would also reduce the incidence of non-specific protein-protein interactions.

The membranes loaded with *M. tuberculosis* H37Ra wild type and *M. smegmatis* lysates were probed with 5 ml of 5 μg/ml recombinant Eis in probe dilution buffer and then washed rigorously as described in the Materials and Methods section so that only Eis bound to proteins in the membrane blots was retained. Thus, when the membrane was probed with anti-Eis antibody, the Eis protein was detected in the *M. tuberculosis* lysate as well as a 65-kDa protein that bound recombinant Eis on the membrane (Figure 6, lane 2). The protein which wasnamed , eX, was not detected in the lysates of *M. smegmatis* (Figure 6, lane 1) or lysates of *M. tuberculosis* Δ*eis* mutant (data not shown). Protein eX was also not detected in *M. tuberculosis* lysate that was not probed with recombinant Eis protein (Figure 6, Western Blot, lane 2). It therefore seemed likely that identification and characterization of protein eX would give us some clue as to the function of the Eis protein.

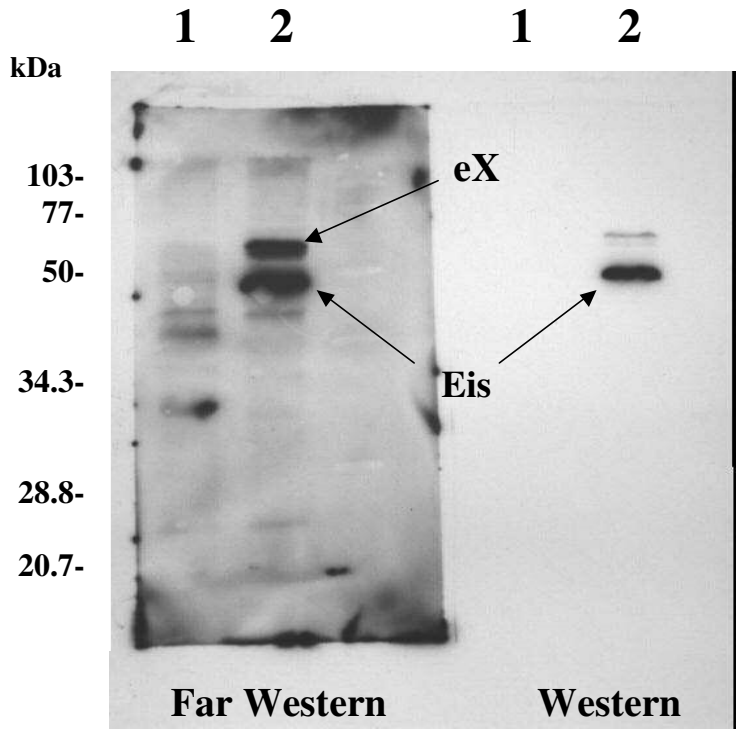


Figure 6: Far Western and Western blot analysis *M. tuberculosis* and *M. smegmatis* lysates. *M. tuberculosis* and *M. smegmatis* lysates were loaded onto 10% SDS-PAGE gels, separated by electrophoresis and transblotted onto PVDF membranes. After blocking, membranes were probed with 5 μg/ml Eis for Far Western analysis followed by anti-Eis antibody for both Far Western and Western blots. Recombinant Eis bound to protein eX was detected in *M. tuberculosis* lysate (**Lane 2**) but not in *M. smegmatis* lysate (**Lane 1**) on the Far Western blot. Western blot analysis of the same lysates reveals the presence of Eis alone in *M. tuberculosis* lysate (**Lane 2**). Each lane contained 100 μg total protein.

To this end, attempts were made to determine the localization of protein eX in *M. tuberculosis*. *M. tuberculosis* H37Ra lysate was separated by differential centrifugation into cytoplasmic, cell wall, and cell membrane fractions as described previously (45). Equal amounts of each fraction (150 μg/lane total protein) were loaded onto 10% SDS-PAGE gels, separated by electrophoresis, transblotted onto membranes, and subjected to Far Western analysis to determine the localization of protein eX. It has been previously

demonstrated that Eis localizes mainly in the cytoplasmic and cell membrane fractions of *M. tuberculosis* (45) and this was confirmed in this study. Interestingly, protein eX was also found to localize to the cytoplasmic fraction of *M. tuberculosis* (Figure 7, lane 1). Attempts to identify protein eX by excision of the band from the membrane followed by N-terminal sequencing have failed as the sequence was found to be blocked at the N-terminus (data not shown).

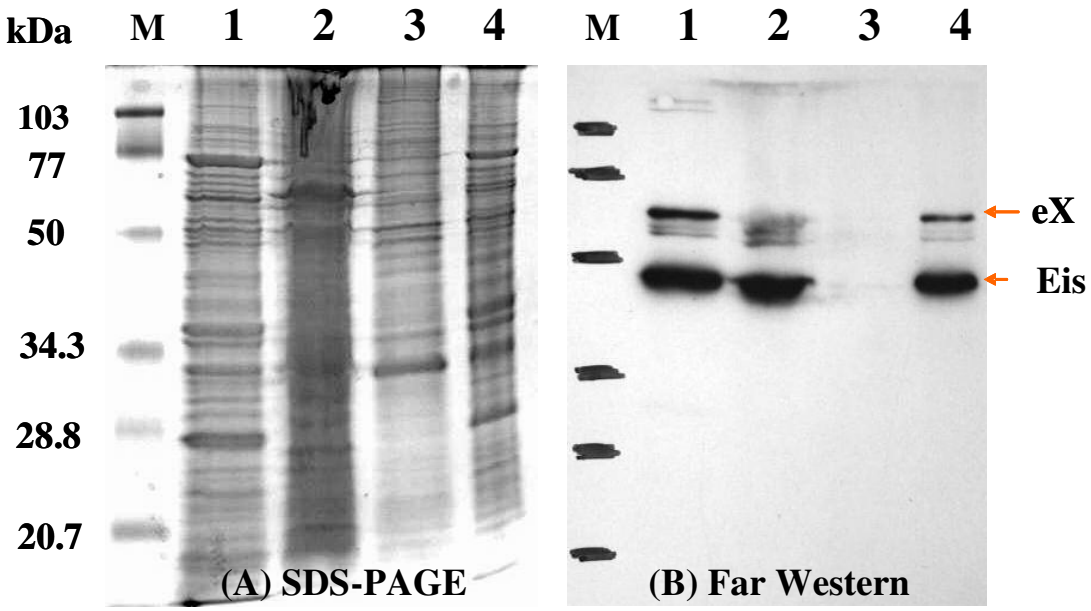


Figure 7: Far Western analysis of *M. tuberculosis* lysate fractions separated by differential centrifugation to determine localization of eX. *M. tuberculosis* H37Ra lysate was separated into cytoplasmic, cell membrane and cell wall fractions by differential centrifugation. These were then loaded onto 10% SDS-PAGE gel and (**A**), stained with Coomassie blue or (**B**), subjected to Far Western analysis using recombinant Eis. Protein eX was found to localize to the cytoplasmic fraction of *M. tuberculosis* lysate (B, lane 1) while Eis localized to the cytoplasmic and cell membrane fractions (B, lanes 1 and 2). (M) Molecular weight markers, (1) Cytoplasmic fraction, (2) Cell membrane fraction (3) Cell wall fraction (4) Total lysate. Each lane contained 150 µg total protein.

Attempted Isolation of eX using Recombinant His-tagged Eis Bound to a Ni-Agarose Column

Given the apparent interaction between Eis and eX as demonstrated by Far Western analysis (Figure 6 and 7), it was only logical to try and utilize a Ni-NTA agarose column with recombinant His-tagged Eis immobilized on it as bait to try and purify eX from *M. tuberculosis* lysate. This would simplify the task of isolating and identifying eX. Accordingly, 250 μg recombinant His-tagged Eis was loaded onto the Ni-NTA agarose column (1.5 ml). *M. tuberculosis* H37Ra wild type lysate was prepared as described previously and passaged over the column several times. The expectation was that Eis would bind and retain eX on the Ni column whereas all other proteins would be washed off using the wash buffer. Analysis of the eluted fractions however failed to reveal the presence of either the 65-kDa protein eX or any other bands that were unique to the *M. tuberculosis* H37Ra lysate (Figure 8). The control sample was *M. tuberculosis* H37Ra lysate passaged over the Ni-NTA agarose column without recombinant Eis loaded onto it. It is likely that the buffers utilized during this procedure may not have been conducive to the replication of the Eis-protein eX interaction seen with Far Western blot analysis.

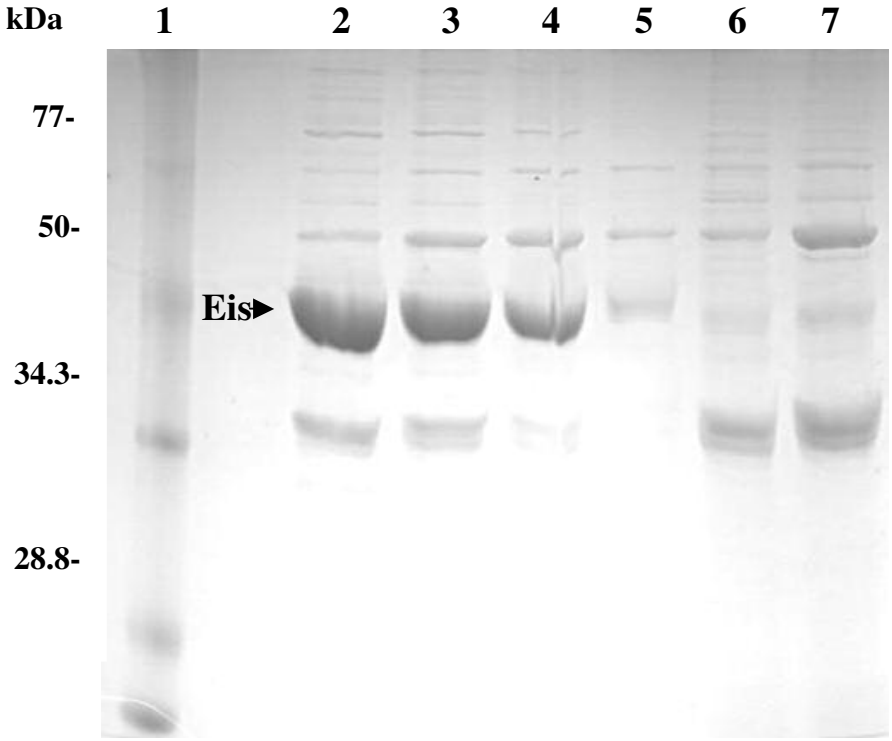


Figure 8: Attempted isolation of protein eX using His-tagged recombinant Eis as bait on a Ni-NTA agarose column. *M. tuberculosis* H37Ra lysate (5 mg) was passaged over a Ni-NTA agarose column (1.5 ml) loaded with recombinant His-tagged Eis (250 μg). A control column was not loaded with recombinant Eis prior to passage of *M. tuberculosis* lysate. Columns were washed and eluted 3 x with 750 μl elution buffer each. Each sample was collected separately and loaded onto 10% SDS-PAGE gel and stained with Coomassie blue for analysis. (**Lane 1**) Molecular weight markers, (**Lane 2-4**) Eluates 1-3 from columns loaded with Eis, (**Lane 5-7**) Eluates 1-3 from control columns not loaded with Eis. No unique bands representing protein eX or any other proteins that may be interacting with Eis are visible in lanes 2-4 as compared to lanes 5-7. Fifty micrograms of total protein was loaded per lane.

Analysis of Eis-Protein Interaction by Coimmunoprecipitation

Considering the failure of repeated attempts to identify protein eX, attempts were made to utilize anti-Eis antibody (45) to isolate both protein eX and Eis from *M. tuberculosis* lysate using coimmunoprecipitation. This would allow the use of other methods such as mass spectrometry to identify protein eX. Coimmunoprecipitation

involves using Sepharose beads linked to Protein A which binds IgG. Initially *M. tuberculosis* H37Ra wild type and Δ*eis* mutant lysates were incubated with anti-Eis antibody with occasional agitation. The expectation was that Eis would bind to protein eX and to the anti-Eis antibody. Subsequently, this complex would bind to Protein A bound to Sepharose. The cross-linking of Sepharose beads to form a matrix enables it to resist low speed centrifugation without being crushed.

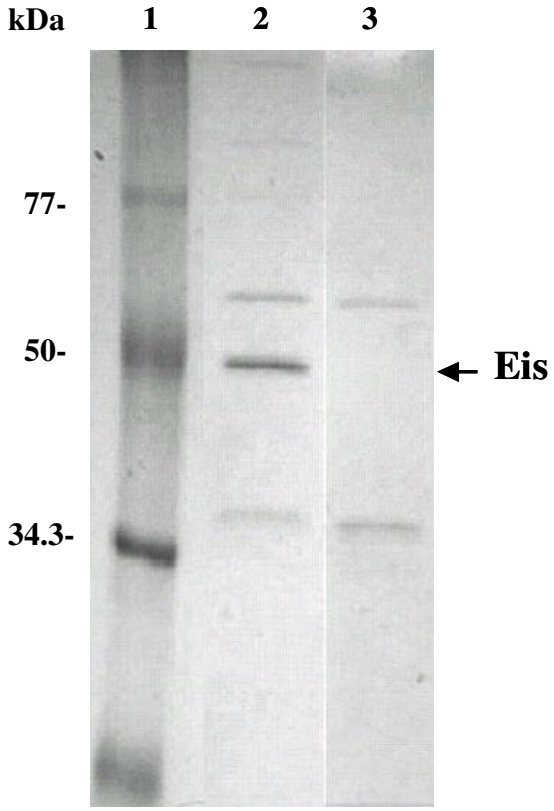


Figure 9: Attempted coimmunoprecipitation of protein eX from *M. tuberculosis* lysate using anti-Eis antibody bound to a Protein A-Sepharose matrix. *M. tuberculosis* H37Ra lysate (0.5 mg total protein) was incubated with 1 µg anti-Eis antibody and mixed with 50 µl Protein A-Sepharose in 0.5 ml CoIP buffer. The complex thus formed was then washed, boiled in SDS buffer and loaded onto 10% SDS-PAGE gel and stained with Coomassie blue for analysis. (**Lane 1**) Molecular weight markers, (**Lane 2**) *M. tuberculosis* H37Ra lysate with added anti-Eis antibody and Protein A-Sepharose, (**Lane 3**) *M. tuberculosis* H37Ra Δ*eis* mutant with anti-Eis antibody and Protein A-

Sepharose as control. Eis is coimmunoprecipitated from the mixture (**Lane 2**) but not protein eX.

The binding of the Protein A-Sepharose complex with the Eis/protein eX/anti-Eis antibody complex should in theory have isolated protein eX out of the *M. tuberculosis* lysate. Boiling the samples in SDS buffer would then dissociate the complex from the Protein A-Sepharose. The samples were then loaded onto 10% SDS-PAGE gel, separated by electrophoresis and stained with Coomassie blue for visual confirmation of the isolation of protein eX. Unfortunately, repeated attempts to isolate protein eX by coimmunoprecipitation failed, although we were able to isolate Eis from the *M. tuberculosis* lysate (Figure 9, lane 2). Addition of recombinant Eis to enhance the chances of coimmunoprecipitating protein eX also failed (data not shown).

Real-time PCR of *eis* From *M. tuberculosis* H37Ra Infection of U-937 Macrophages

With all indicators pointing towards the production of Eis by *M. tuberculosis* during human infection, experiments were done to define the expression pattern of *eis* during the infection of U-937 macrophages by *M. tuberculosis* H37Ra using quantitative real-time PCR analysis. *sigA* was used as a reference gene for these studies because its level of expression is relatively unaffected by changes in *M. tuberculosis* environment (108). Real-time PCR analysis of *eis* expression in *M. tuberculosis* H37Ra infecting U 937 macrophages showed that *eis* was constitutively expressed throughout the duration of the infection up to 48 h with no significant differences in expression between time points tested (Figure 10). In addition, *eis* expression does not change during incubation in the

RPMI media used for preparing the infection inoculum (Figure 10). This work was carried out by Dr. Esteban Roberts.

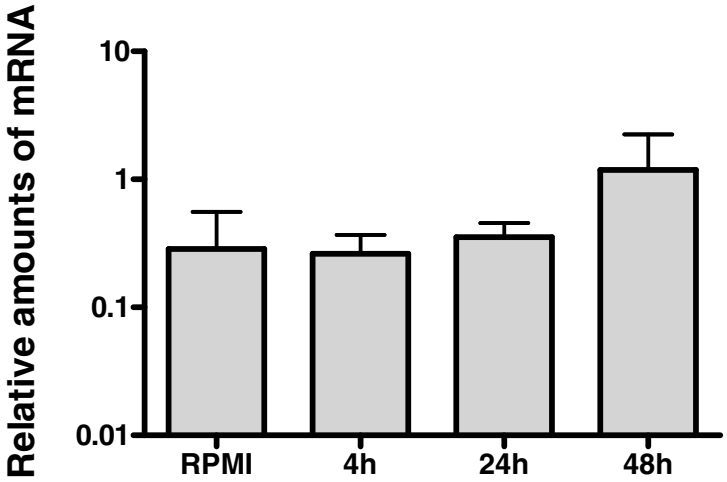


Figure 10: Expression of *eis* mRNA by *M. tuberculosis* H37Ra during infection of U-937 cells. The results are expressed as the ratios of the numbers of RNA copies detected in RNA samples taken at various times (shown on the *x* axis and expressed in h) from cultures of *M. tuberculosis* H37Ra infected U-937 cells to the numbers of RNA copies detected in samples obtained from bacteria growing in 7H9 medium. The values were normalized to the *sigA* RNA value. Each measurement was obtained at least twice using independent RNA preparations. *eis* appears to be constitutively expressed during the infection of macrophages.

Immunofluorescence Microscopy (IF) of Macrophages Infected with *M. tuberculosis* H37Ra Wild Type and Δ*eis* Mutant

Numerous mycobacterial proteins and lipids have been reported to be released into the cytoplasm of infected macrophages (18, 116, 162, 163). Many of them have been shown to act as effectors for the modulation of the immune response to mycobacterial infection to the advantage of the bacterium. Having determined that *eis* is expressed

during the infection of macrophages and armed with the knowledge that the Eis protein is secreted into the culture medium by *M. tuberculosis* (45), studies were done to examine infected macrophages for the presence of the Eis protein in the host cell cytoplasm. *M. tuberculosis* H37Ra wild type and Δ*eis* mutant were transformed with the pBEN plasmid which contains the green fluorescent protein (*gfp*) gene driven by p*hsp60* from *Mycobacterium bovis BCG* (136). The expression of *gfp* by the bacteria during infection renders the bacillus bright fluorescent green and enables one to visualize the microbe within the macrophage during the course of infection by IF. *M. tuberculosis* H37Ra wild type and Δ*eis* mutant expressing *gfp* were named MtbRa/*gfp* and MtbΔ*eis*/*gfp,* respectively.

U-937 macrophage monolayers on glass coverslips in 24-well plates were infected at an MOI of 20 with either MtbRa/*gfp* or MtbΔ*eis*/*gfp*. At various time points, the macrophage monolayers were fixed, permeabilized and probed with antibodies for IF as described in the Materials and Methods section. Macrophages infected with MtbΔ*eis*/*gfp* showed no red fluorescence (staining of the Eis protein) at any time point due to the absence of Eis in the *Δeis* mutant (Figure 11A and B). Figure 11A shows a typical macrophage infected with fluorescent green MtbΔ*eis*/*gfp* at 24 h after infection. Figure 11B shows the same field viewed by phase contrast microscopy. Note the presence of fluorescent green bacteria but the absence of red stained Eis (Figure 11A and B).

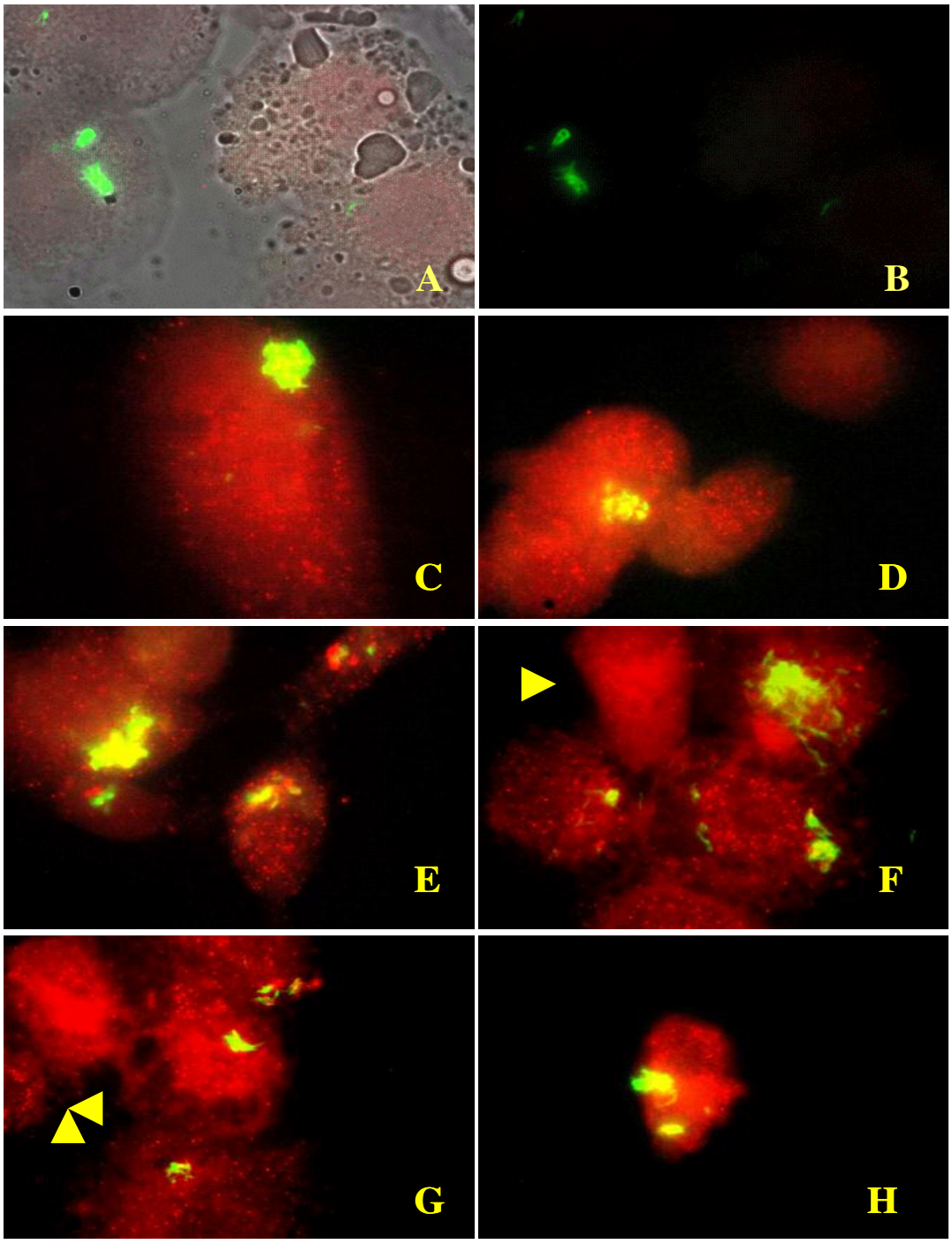


Figure 11: Immunofluorescence microscopy demonstrating the presence of Eis in the cytoplasm of U-937 macrophages infected with MtbRa/*gfp*. U-937 macrophages were infected at an MOI of 20 and at various time points, cells were permeabilized, fixed and probed with anti-Eis antibody followed by fluorescent red dye labeled secondary antibody and viewed by IF. Mycobacteria appear as fluorescent green clumps while the

Eis protein appears as fluorescent red dispersed throughout the cytoplasm of MtbRa/*gfp* infected macrophages. (**A**) Phase contrast microscopy image merged with the IF microscopy image showing fluorescent green MtbΔ*eis*/*gfp* in U-937 macrophages. (**B**) IF microscopy of the same field seen in A. No Eis was seen in the cytoplasm of these macrophages as observed by the absence of red fluorescence. (**C**) U-937 cells infected with MtbRa/*gfp* demonstrating red fluorescence throughout the cytoplasm due to the release of Eis protein from mycobacterial phagosomes at 4 h. (**D**) 12 h (**E**) 24 h (**F**) 48 h (**G**) 72 h (**H**) Eis is present in the cytoplasm of MtbRa/*gfp* infected macrophages even at 96 h after infection. In both (**F**) and (**G**) uninfected macrophages (indicated by arrowheads) demonstrate the presence of Eis protein in cytoplasm in the absence of infecting bacteria.

Starting at 4 h, U-937 cells infected with MtbRa/*gfp* demonstrated the presence of Eis protein in the cytoplasm as evidenced by red fluorescence when viewed by IF (Figure 11C to H). The red fluorescence, indicating the presence of Eis protein, was visibly dispersed throughout the cytoplasm of infected macrophages with no apparent localization. Mycobacteria were visible as bright green due to the presence of GFP while the Eis protein was visible in the cytoplasm of the macrophages as dispersed red color due to the labeling with the fluorescent red secondary antibody (Alexafluor 594 goat anti-rabbit antibody). This was typical of what was observed with infected macrophages. Eis was visible in the cytoplasm of infected macrophages even up to 96 h after infection (Figure 11H). This correlated with the data from real time PCR indicating that *eis* is constitutively expressed by *M. tuberculosis* during infection of macrophages (Figure 10). One interesting observation was that in many instances, macrophages appeared to contain Eis protein in the absence of visible infecting bacteria (Figure 11F and G, arrowheads denote uninfected macrophages). Mycobacterial proteins have been shown to be released

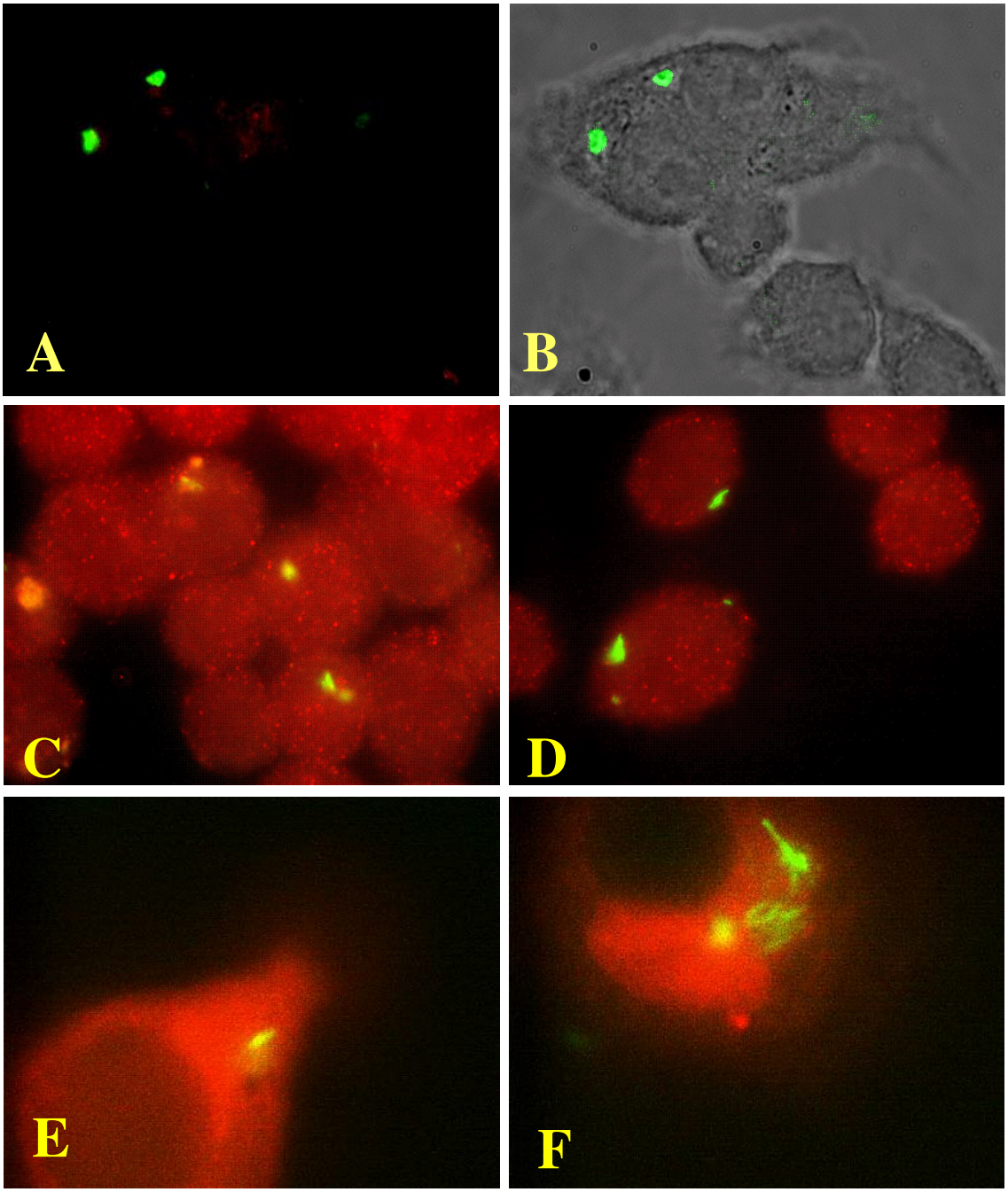


Figure 12: Immunofluorescence microscopy demonstrating the presence of Eis in the cytoplasm of J774 murine macrophages and human monocytes infected with MtbRa/*gfp*. Cells were infected with MtbRa/*gfp* and MtbΔ*eis*/*gfp* at an MOI of 20 and processed as described with U-937 cells. (**A**) J774 macrophages infected with MtbΔ*eis*/*gfp* at 24 h after infection. Fluorescent green mycobacteria are seen within the cytoplasm of the macrophages but no red fluorescence is visible due to the absence of Eis, (**B**) Phase contrast microscopy image merged with IF image of the same field as seen

in A, (**C**) J774 macrophages infected with MtbRa/*gfp* at 24 h and, (**D**) 48 h, demonstrating red fluorescence dispersed throughout the cytoplasm due to presence of Eis, (**E**) Human monocytes infected with MtbRa/*gfp* at 24 h and, (**F**) 48 h also demonstrating presence of Eis in cytoplasm of infected macrophages.

by infected macrophages in vesicle form and taken up by uninfected macrophages (17). This could explain the presence of Eis in the cytoplasm of uninfected macrophages.

Similar results were achieved using both J774 murine macrophages and primary human monocytes. J774 cells infected with MtbΔ*eis*/*gfp* did not show any red fluorescence that would indicate the presence of Eis in the macrophage cytoplasm (Figure 12A). Figure 12B shows the same field as viewed by phase contrast microscopy. J774 macrophages infected with MtbRa/*gfp* demonstrated the presence of the fluorescent green bacteria and fluorescent red labeled Eis in their cytoplasm by IF (Figure 12C and D). Primary human monocytes demonstrated the same results (Figure 12E and F). Fluorescent red color was not seen in cells that were not permeabilized (data not shown).

Western Blot Analysis of the Cytoplasm and Culture Supernatant of Macrophages Infected with *M. tuberculosis*H37Ra

Studies were then done to confirm the presence of the Eis protein in the cytoplasm of infected macrophages by Western blot analysis of infected macrophage lysates as described previously (34). Infected U-937 macrophage monolayers were harvested, lysed and separated over a 12% sucrose solution to remove mycobacteria containing phagosomes (34). The cytoplasmic fraction of the lysed macrophages remained in a discrete layer over the 12% sucrose solution while the phagosomal fraction containing infecting mycobacteria formed a pellet/layer at the bottom of the tube. The cytoplasmic fraction was harvested and 200 μg total protein was loaded onto a 10% SDS-PAGE gel

for Western blot analysis as described in materials and methods. The Eis protein was detected in the cytoplasm of infected macrophages independent of the presence of infecting bacteria (Figure 13, lane 2). This correlated with the IF data indicating that Eis is secreted into the cytoplasm of infected macrophages (Figure 11C to H). The cytoplasmic fraction obtained from U-937 macrophages infected with *M. tuberculosis* H37Ra Δ*eis* mutant failed to demonstrate the presence of Eis protein by Western blot analysis as expected (data not shown). To discount the possibility that the presence of Eis in the macrophage cytoplasm may be due to lysis of phagocytosed bacterium, the cytoplasmic fraction was probed by Western blot for the presence of *hspx*, a mycobacterial protein that is upregulated in *M. tuberculosis* during infection of macrophages (56). *Hspx* was not detected in the cytoplasmic fraction of macrophages infected with *M. tuberculosis* (data not shown).

Having observed the presence of Eis protein in macrophages that appeared to be visibly uninfected (Figure 11F and G), this phenomenon was further investigated. Tissue culture supernatant from U-937 cells infected with *M. tuberculosis* H37Ra was harvested and centrifuged multiple times to remove both contaminating bacteria and macrophages. The resulting fraction was then subjected to a series of centrifugation steps as described previously in the Materials and Methods section (17) to remove any contaminating macrophage organelles and to separate out the microvesicular fraction. A final spin at 100,000 x *g* for 1 h resulted in a pellet that contained vesicles released from macrophages into the tissue culture supernatant. Analysis of this pellet by Western blot detected the

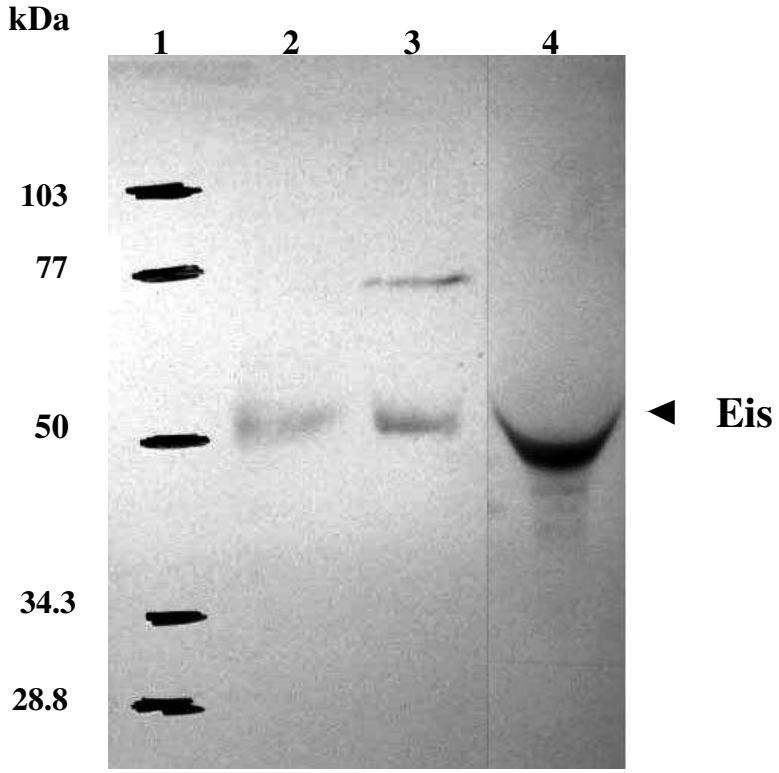


Figure 13: Western blot detection of Eis in the cytoplasm and tissue culture supernatant of U-937 macrophages infected with *M. tuberculosis* H37Ra. Infected U-937 macrophages were harvested, disrupted by syringe lysis, layered over 12% sucrose, and centrifuged to separate out mycobacterium containing phagosomes which pelleted to the bottom of the tube. **(Lane 1)** Molecular weight markers, (**Lane 2**) Macrophage cytoplasmic fraction free of infecting mycobacteria was harvested from the surface of the 12% sucrose solution and subjected to Western blot analysis for the presence of Eis using anti-Eis antibody. The Eis protein was detected in the cytoplasm of infected macrophages, (**Lane 3**) Tissue culture supernatant of *M. tuberculosis* infected U-937 macrophages was harvested and centrifuged to remove any macrophages or bacteria. The final pellet obtained after centrifugation at 100,000 x *g* for 1 h contained the extravesicular fraction and was probed by Western blot with anti-Eis antibody. Eis was discovered to be associated with vesicles released from infected U-937 macrophages, (**Lane 4**) Presence of native Eis protein in the tissue culture supernatant of U-937 macrophages infected with *M. tuberculosis* H37Ra after removal of all vesicular material followed by concentration using Centriprep filters. In all cases, samples were loaded onto 10% SDS-PAGE gel, separated by electrophoresis, and transblotted onto PVDF membranes for Western blot analysis. Two hundred micrograms of total protein were loaded per lane.

presence of the Eis protein (Figure 13, lane 3). These findings are consistent with the detection of other mycobacterial proteins/lipids in vesicles released into the culture supernatant by *M. tuberculosis* infected macrophages as described by Beatty *et al.* (17, 19, 130). The uptake of these vesicles by uninfected macrophages may explain the presence of Eis in these macrophages.

The steps utilized to obtain the vesicular pellet left us with a supernatant fraction devoid of any host cellular structural material. An aliquot of this sample was concentrated three-fold using Centriprep filters and analyzed by Western blot. The Eis protein was detected in this concentrated culture supernatant fraction (Figure 13, lane 4). The source of this Eis protein can only speculated on, but may be due to the degradation of vesicles containing mycobacterial proteins in the tissue culture supernatant or possibly due to the direct release of the protein by the infected macrophage.

Viability of infected macrophages was determined using LDH release assay to ensure that release of Eis and vesicles containing Eis into the culture supernatant was not due to lysis of infected macrophages. Based on LDH release, it was determined that macrophage viablity was >90% at time of harvest (48 h) (data not shown).

Localization of Eis in Sucrose Gradient Separated Fractions of Infected U-937 Macrophages

Having demonstrated that Eis is released into the cytoplasm of infected macrophages, studies were carried out to determine its association, if any, with macrophage organelles. The *M. tuberculosis* infected U-937 macrophage cytoplasmic

fraction obtained after removal of mycobacterial phagososmes was separated over a sucrose step gradient. The gradient was prepared as described in materials and methods.

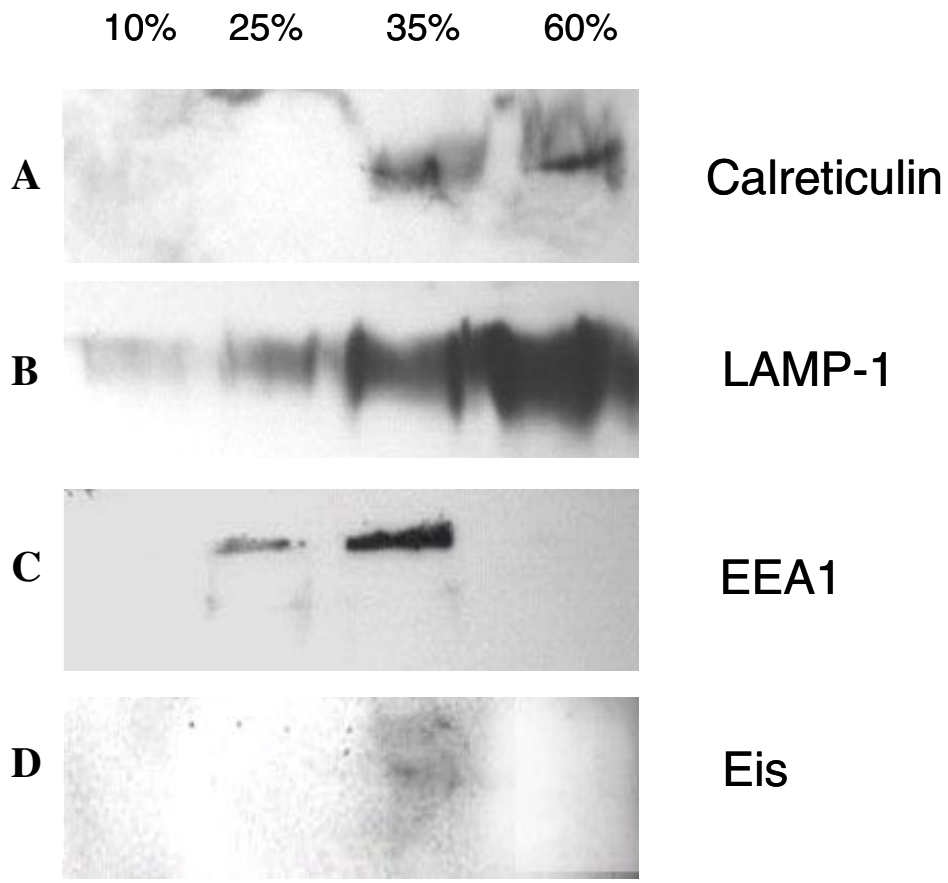


Figure 14: Western blot analysis of fractions obtained by sucrose step gradient separation of lysate of *M. tuberculosis* infected U-937 macrophages. After separation of the lysate over 12% sucrose to remove the phagosomal fraction, the cytoplasmic fraction free of bacteria thus obtained was fractionated by centrifugation over a sucrose step gradient. The 10%, 25%, 35% and 60% fractions were harvested, dialyzed against distilled, loaded onto 10% SDS-PAGE gel, transblotted onto a PVDF membrane, and probed with antibodies to calreticulin (**A**), LAMP-1 (**B**), EEA1 (**C**), and Eis (**D**). LAMP-1 and calreticulin localize mainly in the 35% and 60% fractions while EEA1 and Eis localize mainly in the 35% fraction. The same blot was stripped and reprobed for each marker.

The resulting fractions were then probed with antibodies to Eis as well as markers for various macrophage organelles. Eis was found to localize specifically in the 35% fraction (Figure 14D). Calreticulin, which is a marker for the endoplasmic reticulum (ER) was found to concentrate in the 35% and 60% fractions (Figure 14A). LAMP-1, which is found in the membranes of late lysosomes, was found in all the fractions but was concentrated mainly in the 35% and 60% fractions (Figure 14B). EEA1, a membrane protein of early endosomal compartments was detected mainly in the 35% but was also found in the 25% fraction (Figure 14C). From this data, it can be speculated that given the absence of Eis in the 60% fraction where LAMP-1 and calreticulin are found, it is likely that Eis colocalizes with EEA1 and hence with the early endosomes.

Detection of Immunofluorescence Due to Uptake of Exogenously Added Eis Protein by U-937 Macrophages

Our observations regarding the presence of Eis in culture supernatant of infected macrophages and its appearance in the cytoplasm of uninfected macrophages (Figure 11F and G) led us to examine by IF the ability of macrophages to endocytose and retain exogenously added Eis. Recombinant His-tagged Eis protein (5 μg/ml) was added to the culture supernatant of U-937 macrophages. At various time points, the cells were washed, permeabilized and probed with the rabbit anti-Eis antibody. The secondary antibody used here was Alexafluor 488 goat anti-rabbit antibody conjugated to a fluorescent green dye. The macrophage nuclei were stained red with propidium iodide. When viewed by IF,

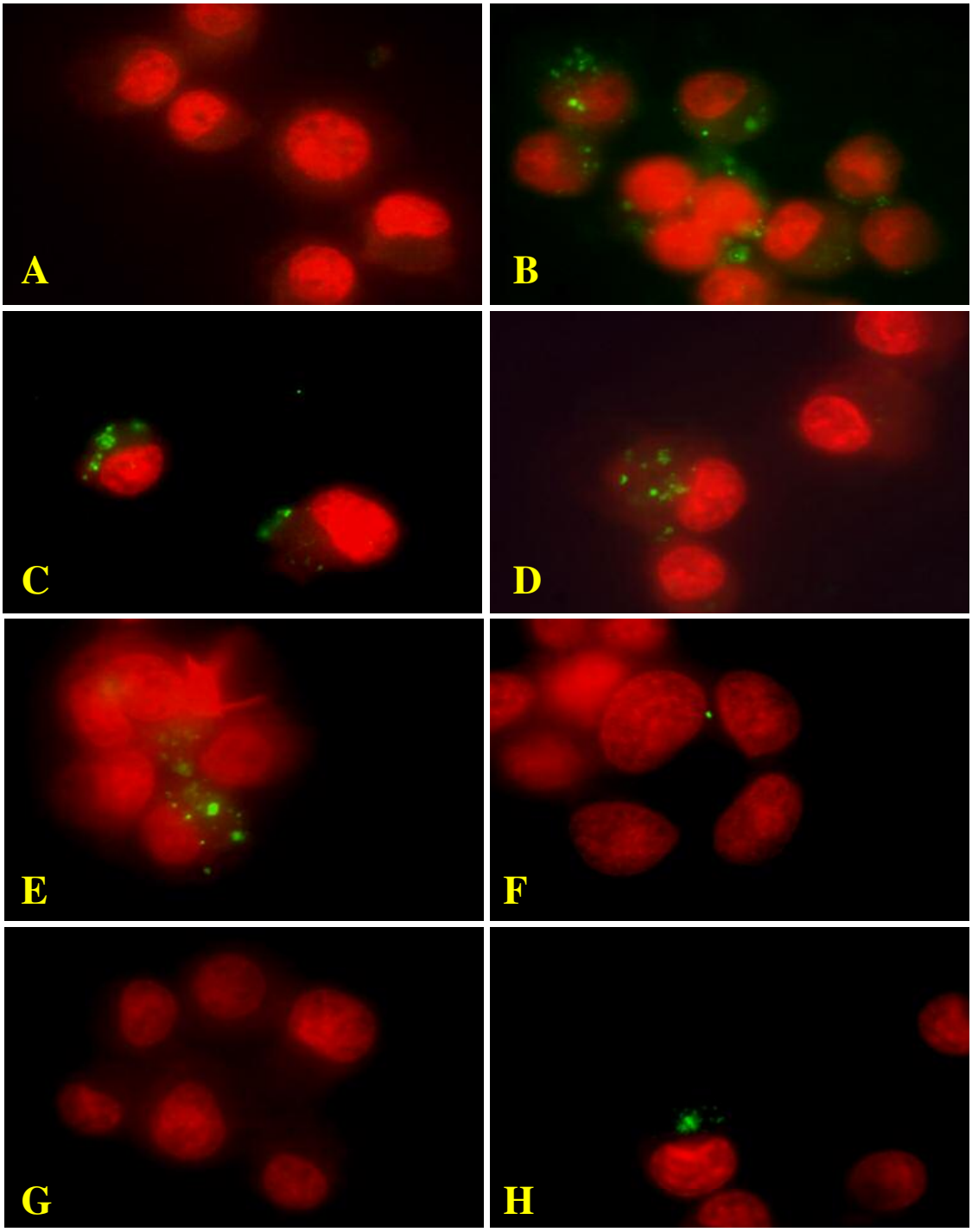


Figure 15: Immunofluorescence microscopy showing the uptake and retention of exogenously added recombinant Eis protein by U-937 macrophages from culture supernatant. Five microgram per ml of recombinant His-tagged Eis was added to culture supernatant of U-937 macrophages which were then washed 3 x with PBS, fixed, permeabilized, and probed with rabbit anti-Eis antibody at different time points. The cells

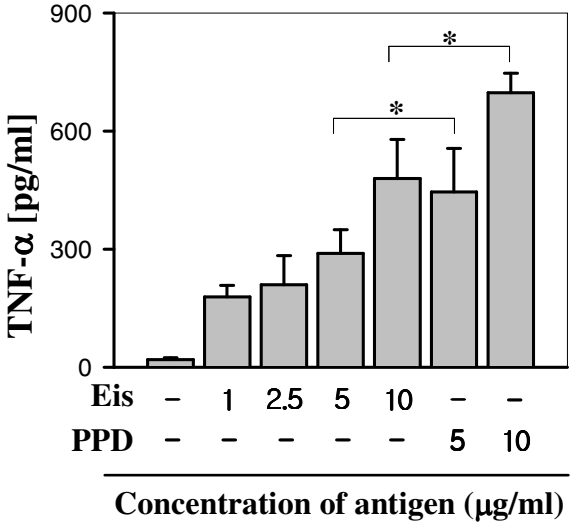
were then probed with a goat anti-rabbit secondary antibody conjugated to a fluorescent green dye. The nuclei of the macrophages were stained with propidium iodide (PI) and appear red while the Eis protein appears green. (**A**) U-937 macrophages without added Eis protein showing fluorescent red nuclei due to PI staining but no green fluorescence, (**B**) U-937 macrophages 30 min after addition of Eis showing green fluorescence within the macrophage due to the uptake of Eis, (**C**) after 2 h, (**D**) 4 h, (**E**) 8 h, (**F**) 12 h, (**G**) 24 h. The Eis protein appeared to be retained in the cytoplasm of macrophages up to 8 h after addition of protein although occasional fields showing green fluorescence due to Eis could be observed even at 24 h (**H**).

control U-937 macrophages with no added Eis protein showed no green fluorescence (Figure 15A). Eis protein was clearly visible within the cytoplasm of the macrophages labeled with fluorescent green dye from 30 min up to 8 hr after addition of the protein (Figure 15B to E). After 8 h of incubation, Eis was not easily visible in the macrophages by immunofluorescence although occasional fields showing green fluorescence within macrophages due to presence of Eis could be observed up to 24 h after addition of Eis (Figure 15H). No green fluorescence was observed in cells that were not permeabilized (data not shown).

Production of TNF-α and IL-10 in Human Primary Monocytes After *in vitro* Stimulation With Eis Protein

To evaluate TNF-α and IL-10 production in primary monocytes in response to Eis or PPD antigen, assays were performed to detect these cytokines by ELISA. Experiments using monocytes from three healthy tuberculin reactors showed that TNF-α and IL-10 levels were not produced in freshly isolated cells but detectable 3 h after and peaked from 18-24 h (for TNF-α) and 48 h (for IL-10) after stimulation with Eis or PPD antigen (Dr. Eun Jo, personal communication). Therefore, the 18 h and 48 h time points were used for studying the levels of TNF-α and IL-10 production respectively, after

A



B

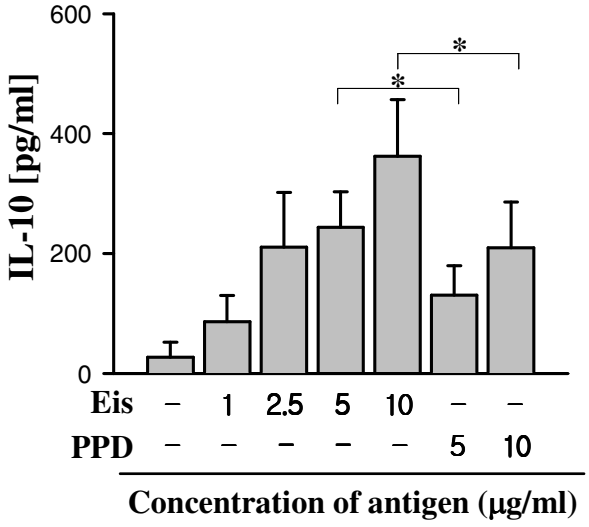


Figure 16: Cytokine secretion by human monocytes treated with mycobacterial antigens. The cells were isolated from tuberculin reactive healthy donors and either left untreated or stimulated with increasing amounts of PPD and Eis antigen. (A) TNF-α was measured by ELISA in the cell culture supernatants that were harvested at 18 h following stimulation with each antigen and expressed as the mean ± SE of three independent

experiments (B) IL-10 was measured by ELISA in the cell culture supernatants were harvested at 48 h following stimulation with each antigen and expressed as the mean ± SE of three independent experiments. n=3 for each experiment. *p<0.05

stimulation with the Eis or PPD antigens of *M. tuberculosis.* All experiments were performed at least three times times using cells from different donors, and the quantitative effects described here were reproduced in all individuals.

As shown in Figure 16A, Eis induced TNF-α production was increased in human monocytes in a dose-dependent manner. However, the Eis protein at 5 and 10 μg/ml concentration elicited significantly decreased production of TNF-α, when compared with the same concentrations of the PPD antigen (P < 0.01; Figure 16A). Stimulation of human monocytes with LPS (positive control) resulted in the secretion of TNF-α (1,000 to 2,500 pg/ml). In addition, IL-10 production was evaluated using the 48 h supernatants (Figure 16B). Significant dose-dependent IL-10 production was observed in human monocytes after *in vitro* stimulation with the Eis protein. In contrast to the findings for TNF-α, we observed that the IL-10 production induced by Eis protein at 5 and 10 μg/ml concentration was significantly higher (p<0.05), as compared to that induced by the PPD antigen at the same concentrations (Figure 16B). Stimulation of human monocytes with LPS (positive control) resulted in the secretion of IL-10 (2,000 to 4,000 pg/ml).

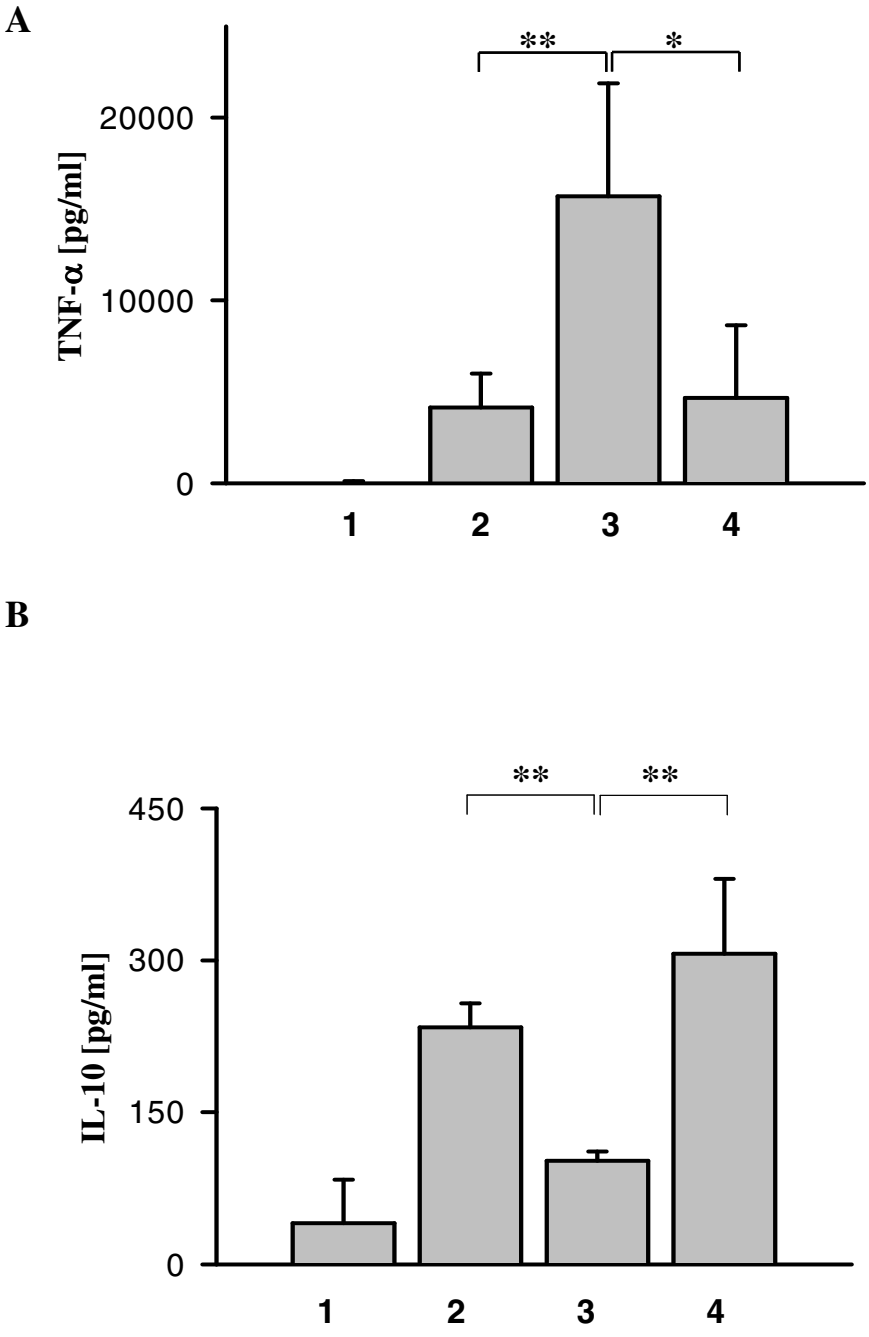


Figure 17: Cytokine release from human monocytes infected with *M. tuberculosis* H37Ra wild type, Δ*eis* mutant and *eis* complemented strains. Monocytes from healthy tuberculin reactive donors were harvested and infected with *M. tuberculosis* H37Ra wild type, Δ*eis* mutant and complemented strains at an MOI of 1. Culture supernatant from

infected human monocytes was harvested 18 h after infection to determine levels of cytokine secretion in (**1**) Uninfected monocytes, (**2**) monocytes infected with *M. tuberculosis* H37Rv, (**3**) monocytes infected with *M. tuberculosis* H37Ra Δ*eis* mutant, (**4**) monocytes infected with *M. tuberculosis eis* complemented strain. (**A**) TNF-α secretion by monocytes from healthy donors in response to infection. TNF-α levels in the culture supernatants were monitored by ELISA. Representative experiment, n=3. Significantly higher levels of TNF-α secretion were observed by monocytes in response to infection with the Δ*eis* mutant as compared to monocytes infected with wild type and complemented strains, (**B**) IL-10 secretion by infected monocytes from healthy donors. IL-10 levels in the supernatants were monitored by ELISA. Representative experiment, n=3. Significantly higher levels of IL-10 secretion were observed by monocytes in response to infection with wild type and complemented strains as compared to Δ*eis* mutant infected monocytes. *p<0.05; **p<0.01.

Production of TNF-α and IL-10 in Human Primary Monocytes After *in vitro* Infection With Wild Type, Δ*eis* Mutant or Complemented Strains of *M. tuberculosis* H37Rv

Next, TNF-α and IL-10 secretion by human monocytes infected with wild type *M. tuberculosis* H37Rv, Δ*eis* mutant and *eis* complemented strains at an MOI of 1 was evaluated. As shown in Figure 17A, TNF-α induction by human monocytes infected with the H37Rv Δ*eis* mutant was significantly greater than those infected with wild type H37Rv ($P < 0.01$) or complemented strain ($P < 0.05$). Although the mean TNF-α production by *M. tuberculosis* infected monocytes increased slightly with bacterial load at MOIs of 10, H37Rv wild type infected monocytes still secreted significantly less TNF-α than those infected with Δ*eis* at an MOI of 10 (Dr. Eun Jo, personal communication).

In contrast to TNF-α, IL-10 secretion by the H37Rv Δ*eis* mutant infected monocytes was significantly lower than induction by H37Rv wild type ($P < 0.01$) or *eis*

complemented strains ($P < 0.01$) (Figure 17B) at an MOI of 1. At MOIs of 10, the Δ*eis* mutant infected monocytes still secreted significantly less IL-10 than those infected with wild type or complemented strains (Dr. Eun Jo, personal communication). Therefore, this data shows that the H37Rv Δ*eis* mutant induced more TNF-α, but less IL-10, production in human primary monocytes. All experiments were performed at least three times using cells from different donors, and the quantitative effects described here were reproduced in all individuals.

The work on cytokine release by monocytes in response to stimulation with mycobacterial antigens and infection with *M. tuberculosis* was carried out in a collaborative effort with Dr. Chang-Hwa Song and Dr. Eun-Kyeong Jo at Chungnam University, South Korea.

Bioinformatic Analysis

Given the apparent ability of Eis to modulate cytokine secretion of macrophages, yet having no indication as to the mechanism whereby Eis was able to modulate these changes, bioinformatic analysis was used to try and determine the possible function of Eis. BLAST analysis of the Eis protein using the protein-protein blastp program (7) yielded a large number of homologous proteins most of which have not been characterized (data not shown). The presence of several acetyltransferases of the GCN5 related N-acetyltransferase (GNAT) superfamily of proteins in those results however led us to examine the possibility that Eis may exhibit remote homology to proteins of that family. Bioinformatic analysis of the amino acid sequence of Eis using RPS-BLAST (Reverse Position Specific BLAST) revealed the presence of an acetyltransferase domain

of the pfam00583 family of GNAT acetyltransferases (Figure 18). The acetyltransferase domain extends from amino acid 61 to 137 of the 402 amino acid long Eis protein sequence and contains the conserved residues that are characteristic of this domain (Figure 18).

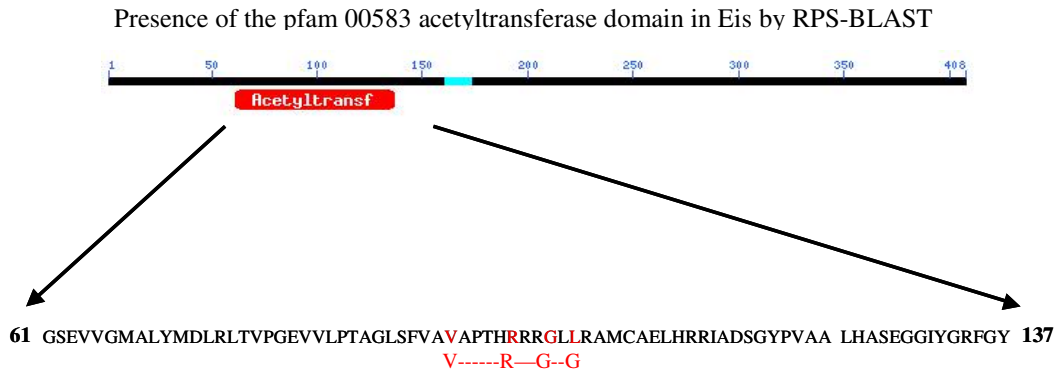


Figure 18: RPS-BLAST analysis of the Eis protein showing the presence of the acetyltransferase domain of the GCN5 related family of N-acetyltransferases. RPS-BLAST analysis of the amino acid sequence of the Eis protein detected the presence of the acetyltransferase domain of the pfam 00583 GNAT family. The domain extends from amino acid 61 to 137 and includes the conserved residues commonly seen among members of this family VxxxxRxxGxG. The substitution seen in the case of the last conserved residue is deemed acceptable as it is seen among several members of this family (data not shown).

GNAT acetyltransferases can acetylate the N-terminal α-amino group of proteins, the ε-amino group of lysine residues, aminoglycoside antibiotics, spermine and arylalkylamines such as serotonin (8). This family of proteins are involved in a wide variety of activities ranging from transcriptional activation to antibiotic resistance. Proteins of the GNAT superfamily are found in all the kingdoms of life and include over a dozen protein families involved in a broad range of functions and phenotypes (57, 115). The lack of sequence homology among members of this family makes classification of

possible new members difficult unless three dimensional structural data is available (115).

The GNAT superfamily is characterized by the presence of four conserved motifs A-D only one of which, motif A, the acetyltransferase domain (Figure 18), is universally present in all members of this family (115). This domain contains the residues that are conserved across the GNAT family: V/I-x-x-x-x-Q/R-x-x-G-x-G/A (Figure 18). In the case of the last conserved residue, a substitution of glycine or alanine with lysine as seen here may be acceptable as it is seen in other members of this family as well (115). Furthermore, Eis has also been allocated its own COG (Cluster of Orthologous Groups) by NCBI. The COG 4552.1 has been named Eis and contains proteins from many diverse bacteria sharing multiple domains including the acetyltransferase domain. The COGs were constructed by comparing protein sequences encoded in 43 complete genomes, representing 30 major phylogenetic lineages. Each COG consists of individual proteins or groups of paralogs from at least 3 lineages and thus corresponds to an ancient conserved domain.

We then proceeded to use the MEME program (Multiple Expectation Maximization for Motif Elicitation) to analyze Eis along with other members of COG 4552.1. MEME is used as a tool to discover conserved motifs in groups of related protein or DNA sequences (9). The analysis demonstrated the presence of multiple motifs shared by Eis and the other acetyltransferases including the GNAT acetyltransferase motif which is the only motif common to all members of the GNAT superfamily (Figure 19).

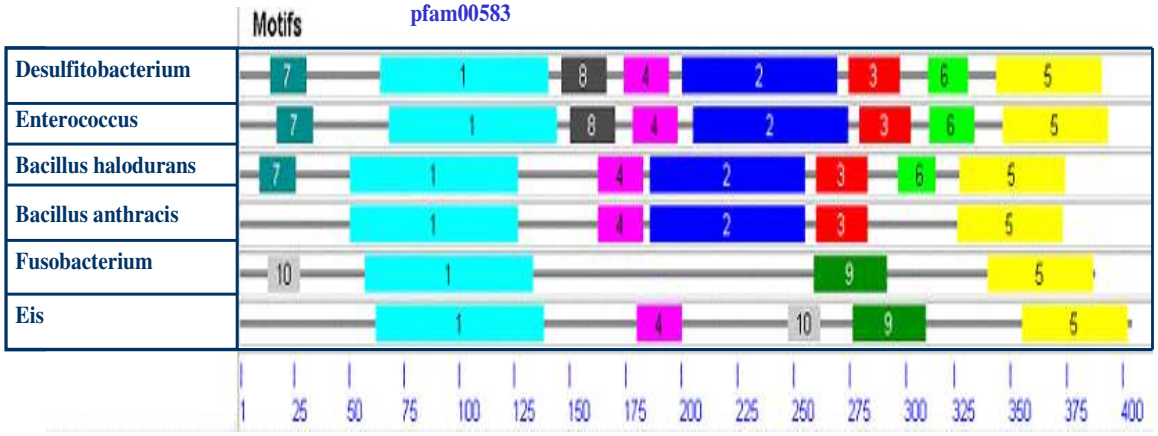


Figure 19: MEME analysis of members of the COG 4552.1 (Eis) family of acetyltransferases. Given the poor sequence homology of members of the GNAT acetyltransferase superfamily, the amino acid sequences of members of the COG 4552.1 (Eis) family of acetyltransferases were compared by MEME analysis to detect the presence of common motifs within the family. Members of COG 4552.1 shared multiple common motifs. Motif 1 is the GNAT acetyltransferase domain that is common to all members of the family. Motifs 2-8 have not yet been characterized and are of unknown function.

Since members of the GNAT superfamily have poor sequence homology but share common three dimensional structural features, the 3D-PSSM program (three dimensional position-specific scoring matrix) was used to look for homologs to Eis. 3D-PSSM combines the power of multiple sequence profiles with the knowledge of protein structure to detect remote homologs (63, 96). This is accomplished by utilizing structural alignments of homologous proteins of similar 3D structure in the SCOP (structural classification of proteins) database to obtain a structural equivalence of residues. This is

Protein	Organism	Family	E-value
Aminoglycoside 2'-N-acetyltransferase	*M.. tuberculosis*	predicted GNAT	3.61×10^{-3}
Aminoglycoside-3-N-acetyltrasnferase	*S. marcescens*	GNAT	9.56×10^{-3}
Histone acetyltransferase	*S. cerevisiae*	GNAT	1.09×10^{-2}
Aminoglycoside 6'-N-acetyltransferase	*E. faecium*	GNAT	2.12×10^{-2}
Phosphinothricin N-acetyltransferase	*B. subtilis*	predicted GNAT	3.29×10^{-2}
Protein Yjcf	*B. subtilis*	GNAT	1.71×10^{-1}

Table 6: 3D PSSM search for structural homologs to the Eis protein. The predicted 3D structure of Eis shows significant homology to the known 3D structures of several members of the pfam 00583 family of GNAT acetyltransferases as demonstrated by their E-values. The 3D structures of the proteins with the lowest E-values demonstrate the highest levels of similarity to the predicted 3D structure of Eis.

then used to carry out multiple alignments of sequences obtained by standard sequence search methods. By converting this into a PSSM, this program is able to confidently predict structural and functional relationships better than standard local alignment search tools such as PSI-BLAST (96). Initially, the entire amino acid sequence of the protein was used as input, followed by segments of the protein that matched known 3D protein structures. Analysis of a 154 amino acid section of the Eis sequence, which included the acetyltransferase domain using 3D-PSSM, indicated that the predicted structure of Eis shared strong similarities with several proteins (Table 6). In short, the program predicted the 3D structure of Eis and compared it to a database of known 3D structures of proteins. The best matches which had the lowest E-values were all members of the GNAT family of acetyltransferases including a mycobacterial N-acetyltransferase that is involved in aminoglycoside resistance (4) (Table 6). It is unlikely however that Eis shares

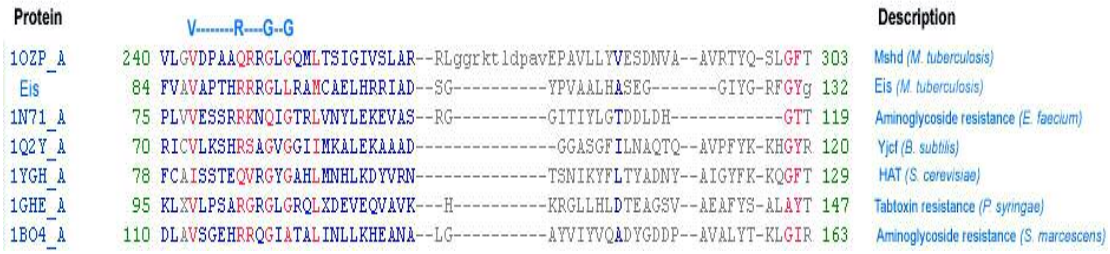


Figure 20: Alignment of the acetyltransferase domain of known members of the pfam 00583 family of GNAT acetyltransferases with the acetyltransferase domain of Eis. These proteins have been confirmed as GNAT acetyltransferases based on their three dimensional structures. The proteins analyzed here include those that were determined by 3D-PSSM to share significant structural homology with the predicted 3D structure of Eis (Table 6). The alignment shows the presence of conserved residues in the acetyltransferase domain that are characteristic of members of the GNAT superfamily and are shared by Eis.

this (antibiotic resistance) function since *M. smegmatis* strains expressing *eis* showed no resistance to amikacin (data not shown). Other matches included a histone acetyltransferases and a eukaryotic N-acetyltransferase. An alignment of the acetyltransferase domain of Eis with the acetyltransferase domains of known members of the GNAT superfamily of proteins, many of which show significant similarity to the predicted structure of Eis as shown by 3D-PSSM analysis, shows that Eis shares the conserved residues that are characteristic of this motif (Figure 20).

Discussion

The *eis* gene of *M. tuberculosis* was previously shown to enhance intracellular survival of *M. smegmatis* in U-937 macrophages (167). However, the mechanism whereby this was achieved was never elucidated. The *M. tuberculosis* H37Rv wild type, *eis* mutant and complemented strains showed no visible differences in terms of growth in culture media. Comparison of their survival in both U-937 macrophages and

the mouse aerosol infection model revealed no visible differences in terms of bacterial survival over the time period tested (166). Similarly, no difference was noted in bacterial load of lungs and spleen of mice infected with *M. tuberculosis* H37Rv wild type, Δ*eis* mutant and complemented strains over a period of 17 weeks (166). While these results were unexpected considering the significant impact of the *eis* gene on intracellular survival of *M.smegmatis*, it would be erroneous to assume that this negates the possibility for a role for *eis* in the pathogenesis of *M. tuberculosis* especially in the light of more recent data described here and elsewhere (33, 43).

The presence of higher titers of antibodies to Eis in the sera of TB patients as compared to healthy controls indicated initially that Eis is produced during human infection (Figure 2). Furthermore, the ability of Eis to induce a proliferative response from PBMC obtained from TB patients is further proof that this mycobacterial cytoplasmic protein is produced and secreted by the bacterium and enters the antigen presentation pathway (Figure 3).

Far Western blot analysis indicates that Eis interacts with a ~65-kDa *M. tuberculosis* protein which was subsequently named protein eX that localizes to the cytoplasmic fraction of *M. tuberculosis* lysate (Figure 6 and 7). Among the many possibilities, one must consider that eX may prove to be either a substrate or chaperone for Eis or perhaps is just a component in a pathway that involves Eis. The absence of eX in *M. tuberculosis* H37Ra Δ*eis* mutant lysate is also of interest. This would seem to indicate that there is perhaps a regulatory mechanism in place that shuts down production of eX in the absence of Eis or perhaps that the presence of Eis is necessary for the

production of eX. The localization of eX to the bacterial cytoplasm while Eis localizes to both the cytoplasmic and cell membrane fractions (Figure 7) makes it unlikely that eX was detected due to aggregation of the Eis protein itself. Identification of eX should go a long way towards clarifying some of these ideas and perhaps suggest a function for Eis. So far however, attempts to identify and isolate eX using techniques such as coimmunoprecipitation (Figure 9) have proved to be unsuccessful and other avenues need to be explored in order to resolve this pressing question. Initial attempts to utilize Far Western blot analysis and coimmunoprecipitation to detect any possible protein-protein interactions between Eis and U-937 macrophage lysate have so far proved unsuccessful (data not shown).

The discovery that *eis* is expressed during infection of human macrophages has been confirmed by others (33). The fact that real-time PCR indicated that *eis* is constitutively expressed by *M. tuberculosis* whether in culture media or within macrophages is not surprising given that *M. tuberculosis* is an intracellular pathogen that spends most of its time in one living host or the other (Figure 10). If*eis* is required for survival/virulence within the host then it would make sense to constitutively express the gene. In fact, this adds to the argument for a possible role for *eis* as an important constituent of the *M. tuberculosis* repertoire of weapons against host defenses. It is also corroborated by data mentioned previously as shown by Dahl *et al.*that*eis* is negatively regulated by the stringent response regulator Rel_{Mtb} so as to maintain constant levels of expression (44). Furthermore, recent studies have indicated that many of the genes

required for survival within macrophages by *M. tuberculosis* are constitutively expressed (129).

As mentioned earlier, many mycobacterial lipids and proteins are trafficked out of the mycobacterial phagosome. Multiple sources (18, 116, 162, 163) have also reported on the release of mycobacterial constituents from infected macrophages into the culture medium and then into uninfected bystander cells, in a sense expanding the sphere of influence of the bacterium beyond the infected cells. Immunofluorescence microscopy studies of *M. tuberculosis* infected U-937 macrophages showed the presence of Eis throughout the cytoplasm of the macrophage (Figure 11). The results obtained using primary human monocytes and J774 murine macrophages were consistent with those observed with U-937 macrophages (Figure 12). The Eis protein was observed in the cytoplasm of macrophages even up to 96 h after infection (Figure 11H) which would be expected given that real-time PCR data show that *eis* is constitutively expressed (Figure 10). This was further confirmed by Western blot analysis of macrophage lysate fractions free of infecting bacteria which demonstrated the presence of the Eis protein (Figure 13). The presence of Eis in the vesicles and tissue culture supernatant of infected macrophages puts it in the company of other established mycobacterial effectors of virulence such as LAM. The release of Eis into the tissue culture supernatant of infected macrophages, either in vesicles or free native form, makes it highly probable that the protein is taken up by uninfected macrophages.

The association of Eis with EEA1 (Figure 14) points to the possibility that the organelle with which this marker is associated (early endosomes) harbors the Eis protein

during infection of the macrophage. As mentioned earlier, it is possible that the protein is taken up from the mycobacterial phagosomes during the exchange of materials that is believed to take place during phagosome-endosome interactions as described in the "kiss and run" hypothesis (53). Early endosomes are also major sorting compartments for endocytosed material which are then transported to late endosomes and lysosomes or recycled to the plasma membrane (141). It is also possible that the Eis protein is contained within these endosomal compartments and is taken up from the tissue culture supernatant of the U-937 macrophages. This idea was given further impetus by immunofluorescence microscopy demonstrating the presence of Eis in uninfected macrophages (Figure 11F and G). U-937 macrophages are clearly capable of taking up and retaining recombinant Eis protein from the culture supernatant for significant periods of time (Figure 15).

The majority of mycobacterial proteins/lipids that have been characterized as being released into the subcellular fraction of infected macrophages appear to be associated with the mycobacterial cell wall (18). Dahl *et al.*showed previously that Eis is localized predominantly in the cytoplasmic fraction of *M. tuberculosis*. The findings reported here regarding the secretion of the Eis protein from the cytoplasm of *M. tuberculosis* into the cytoplasm of infected macrophages and its subsequent release into the culture media point to a possible role for Eis in modulation of macrophage response to infection.

The critical importance of TNF-α in antimycobacterial defense is well established in mice (66). TNF-α contributes to the prevention of reactivation of persistent TB, and

limits the pathological response of the host (111). In humans, the critical role of TNF-α was emphasized by the reactivation of TB in rheumatoid arthritis patients who were treated with anti-TNF-α antibodies (93). However, TNF-α may also be responsible for the toxic syndrome and tissue necrosis that accompany TB, since it has important proinflammatory activities (153). Thus, successful protective immunity to TB may require a balance between antimycobacterial cytokine and proinflammatory cytokine responses that may result in unwanted tissue damage (90).

The hypothesis that *M. tuberculosis* plays a role in modulation of the TNF-α/IL-10 axis is supported by the data showing that Eis modulates TNF-α and IL-10 secretion by human monocytes in response to infection by *M. tuberculosis* (Figure 17). The observation that stimulation of human monocytes with Eis elicits higher levels of IL-10 than TNF-α secretion as compared to PPD (Figure 16) led us to look at the differences in cytokine secretion in response to infection with *M. tuberculosis* H37Rv wild type, Δ*eis* mutant and *eis* complemented strains. Infection of human monocytes with H37Rv wild type induced moderate release of TNF-α and increased levels of IL-10, as compared to uninfected controls. In contrast, monocyte infection with the Δ*eis* mutant induced significantly higher levels of TNF-α ($p<0.05$) and significantly decreased levels of IL-10 as compared to infection with wild type H37Rv ($p<0.05$) and the complemented strain (Figure 17A and B). These results are powerful evidence that *M. tuberculosis* may utilize Eis either as an effector itself or as part of a pathway to alter the cytokine secretion profile of macrophages to its advantage.

Interleukin-10 is thought to play an important role in the regulation of microbial immunity. The induction of down-regulatory cytokines, such as IL-10 and transforming growth factor-β, can inhibit IFN-γ production by T cells and/or macrophage activation (65, 83, 124, 159). Antigen-presenting cell derived IL-10 can exert a major inhibitory effect on control of mycobacterial infection by a mechanism involving the suppression of macrophage effector function and apoptosis (132). Increased IL-10, but decreased TNF-α production induced by Eis may also contribute to the intracellular survival of the pathogen through alteration of host cell apoptotic response during mycobacterial infection.

It is widely known that in alveolar macrophages, apoptosis is a common defense mechanism against *M. tuberculosis* infection via a TNF-α mediated pathway (126). The ratio of TNF-α/IL-10 secretion in human monocytes has been shown to play a major role in determination of the route of cell death upon infection by *M. tuberculosis* (71). Studies have shown that the balance was tilted in favor of IL-10 in TB patients as compared to healthy controls (47, 71). This would suggest a role for Eis in regulating cell death to the advantage of the infecting bacterium. Subsequently, Jo *et al.*have also shown that Eis appears to play a role in the inhibition of induction of apoptosis in infected macrophages (personal communication). It is not uncommon for *M. tuberculosis* to utilize released mycobacterial proteins/lipids as effectors to subvert the immune system of the host to its advantage (35, 162).

Monocytes isolated from healthy donors have been shown to undergo apoptosis as a rule when exposed to PPD, whereas monocytes from TB patients treated similarly

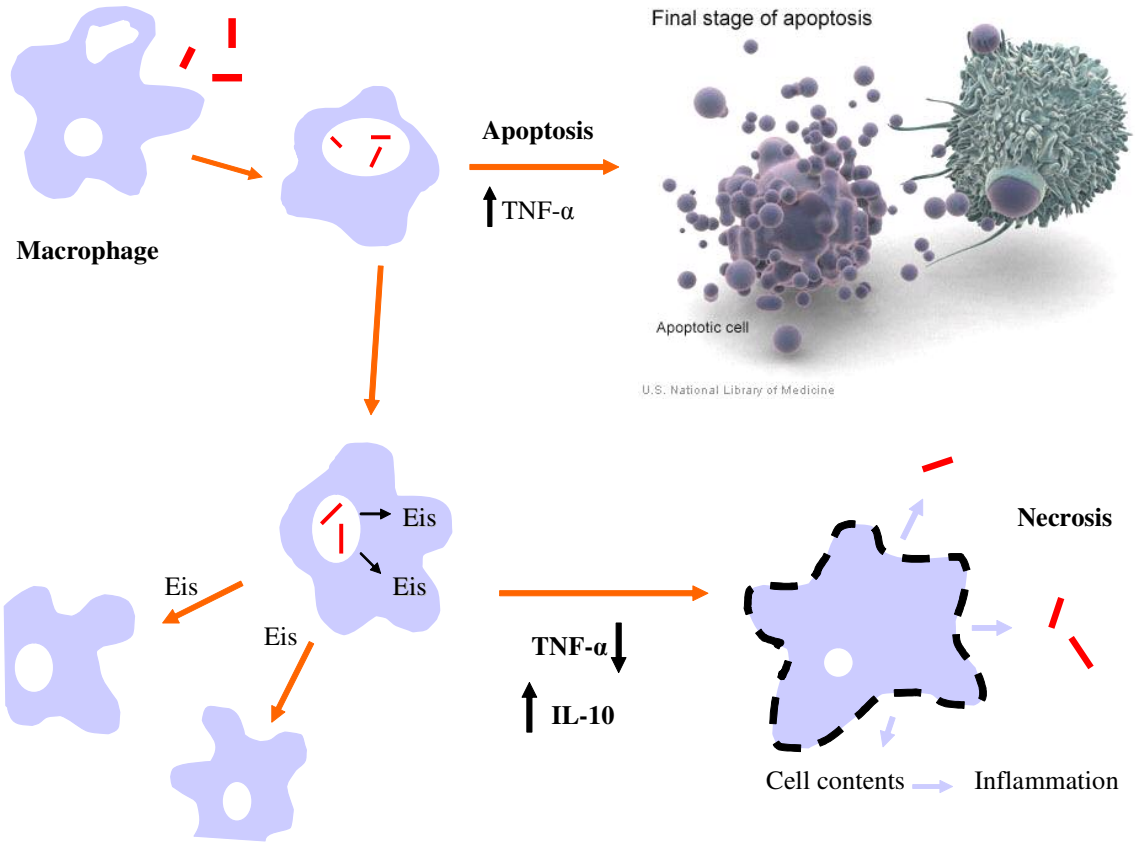


Figure 21: Hypothetical model demonstrating the possible role of Eis in altering the mode of cell death. In immune competent individuals, upregulated TNF-α secretion induces apoptotic cell death with the apoptotic blebs containing the bacteria being taken up and destroyed by activated macrophages. In susceptible individuals however, increased IL-10 and decreased TNF-α secretion mediated by Eis could result in necrotic cell death leading to release of bacteria and inflammatory mediators from the host cell.

underwent both apoptosis and necrosis in equal numbers (71), a difference that authors attributed to the altered balance of TNFα/IL-10 secretion by macrophages in these patients. It is conceivable that this difference is wrought by mycobacterial effectors released from infected cells. Apoptosis of infected macrophages would result in controlled cell death that would not only help to control the infection but also provide material for antigen presentation (138). Necrosis on the other hand would result in release

of the ingested bacteria and localized tissue damage due to release of lysosomal enzymes (Figure 21). Similar results regarding the differential induction of apoptosis and necrosis upon infection with *M. tuberculosis* were also noted in alveolar epithelial cells (47). Others have also shown that virulent strains of *M. tuberculosis* grow faster and induce less apoptosis than attenuated strains in alveolar macrophages (94). This phenomenon was found to be mediated in part by the differential release of the soluble TNFR2 which in turn was found to be IL-10 dependent (11). In addition, infection of THP-1 macophages with virulent strains of *M. tuberculosis* was found to upregulate expression of the antiapoptotic Bcl-2 family member Mcl-1 (143).

Roberts *et al.* (134) suggested that the *eis* gene is regulated by *sigA*, a finding that was subsequently corroborated by Barnes *et al*. (169). They also showed that expression of *eis* in the hyper virulent *M. tuberculosis* 210 Beijing strain was 12 times higher than in *M. tuberculosis* H37Rv during infection of a human monocyte cell line (169). These results seem to bolster the argument for a role for *eis* in pathogenesis of *M. tuberculosis* in the macrophage. The absence of a visible phenotype in the survival assays could be due to any number of reasons. In light of the findings regarding differences in production of Eis between H37Rv and the hypervirulent *M. tuberculosis* 210 Beijing strain (169), it would have been wiser to have picked a clinical strain for the survival assays as opposed to the laboratory strain H37Rv. The macrophage infection model utilizing U-937 human macrophage like cell line may not have been sensitive enough or capable of applying the selective pressure needed for demonstration of the phenotype. Knockouts of the *mptpB* gene in *M. tuberculosis* do not show a difference in survival as compared to wild type

during infection of macrophages unless grown in activated J774 murine macrophage cell line which have significantly higher killing ability than the U-937 cell line (142). The mouse aerosol infection model also did not detect any significant differences in survival of *M. tuberculosis* wild type and Δ*eis* mutant. However, comparison of the pathogenesis of *M. tuberculosis* H37Rv and CDC 1551 strains revealed no significant differences in pathogenesis or survival in the mouse model even though the CDC 1551 strain was significantly more virulent in humans, thus raising the possibility that the lack of a discernable phenotype could be due to shortcomings of the mouse model (95). It may also be possible taking into account the recent discoveries regarding the *eis* gene that we may have to reassess our expectations of what phenotype may be demonstrated by *M. tuberculosis* Δ*eis* mutant. The function of *eis* in intracellular survival and pathogenesis of *M. tuberculosis* appears to be far more complicated than previously believed.

Bioinformatic analysis suggests that Eis is an acetyltransferase of the family of GCN5 related N-acetyltransferases (Figure 18). 3D-PSSM analysis indicates that the predicted structure of Eis bears significant similarity to the structures of known members of the GNAT superfamily (Table 6). In addition, an alignment of the acetyltransferase domain of Eis as delineated by RPS-BLAST with the acetyltransferase domains of the proteins identified by 3D-PSSM shows the presence of conserved residues that are characteristic of the GNAT family (Figure 20). In the absence of candidate substrates, it is extremely difficult to demonstrate whether the Eis protein possesses functional acetyltransferase activity. Currently efforts are being directed towards resolution of the crystal structure of the Eis protein which may provide concrete evidence as to the

putative acetyltransferase activity of Eis and also provide direction as to the identity of its possible substrate(s). In the end, given the range of functions encompassed by members of the GNAT family, one can only speculate as to the possible roles of an acetyltransferase in the pathogenesis of *M. tuberculosis*. Acetylation of histones can result in reduced binding to DNA and ultimately enhance transcription and translation of protein products (30). Alternatively, acetyltransferases can act directly on transcription factors and thus alter the cytokine response of host cells to infection (148).

The release of Eis into the cytoplasm of infected macrophages and its apparent role in the modulation of cytokine secretion by macrophages raises many questions as to the mechanism whereby this is achieved. Further work needs to be done to determine whether Eis is indeed an acetyltransferase and if so, the nature of its role in the pathogenesis of *M. tuberculosis*.

APPENDIX A

DUE DATE:
1 OCTOBER 2004

HUMAN SUBJECTS COMMITTEE
PERIODIC REVIEW FORM

APPROVAL EXPIRES:
10/11/2004

Richard Friedman/A99.96/Micro/Immuno/M. Tuberculosis Survival in Macrophages
NAME OF INVESTIGATOR/PROJECT APPROVAL NUMBER/TITLE OF PROPOSAL

Human subjects approval for this activity expires on the date indicated above. Depending upon the activity status of the project, attachments may be required. Refer to IRB website (www.irb.arizona.edu) for detailed instructions. **Note:** If renewal is not granted before the expiration date, all study activities must stop at that time. If study procedures/treatment must be continued for subject safety, contact the IRB office immediately.

Activity Status – check one box only
Category A: attach items 1-13 listed on reverse
- ☒ Enrollment of new subjects in progress
- ☐ Enrollment not initiated, but still planned
- ☐ Enrollment closed to new subjects but current subjects are still undergoing study procedure or being entered into extensions and/or sub-studies

Category B: attach items 1-12 listed on reverse
- ☐ Enrollment closed, follow-up only (**non-sensitive** data collection via telephone contact, questionnaire and/or record review)
- ☐ Local data analysis only: no subject contact/no additional data collection (annual review required)

Category C: attach items 1-8 listed on reverse
- ☐ Concluded: enrollment and all participation/follow-up/local data analysis completed

Category D: no attachments required; complete and submit this form only
- ☐ Study not begun: permanent withdrawal of study

Subject Numbers (local enrollment)
If more than one study population is involved, report enrollment under number 2 of checklist (see reverse)

IRB NOTE: CORRECTIONS PER PJ E-MAIL OF 11/9/04

a) Number of new subjects enrolled (consented) since last reporting period: 19 ~~1~~ 23
b) Total number of subjects enrolled (consented) since start of project: 51 ~~2~~ 52
c) Male/female ratio of total enrolled since start of project: 25 ~~24~~ F/27 M

Conflict of Interest Statement (COI): see COI policies at http://vpr2.admin.arizona.edu/rie/confilict_of_interest.htm

a) Do any of the investigators serve as a speaker or consultant to the sponsor, the manufacturer, or the owner of the test article? ☐ Yes ☒ No
b) Do any of the investigators (or their family members) derive a direct or indirect benefit equity and/or royalty relationship with the sponsor, manufacturer, or owner of the test article? ☐ Yes ☒ No

If yes to either of the above, **attach** copy of U of A Conflict of Interest and Commitment Disclosure form.

I certify that this research will be conducted in accordance with the currently approved protocol/amendments and that no changes to procedures or study documents will be made without the knowledge/approval of the IRB.

Richard L. Friedman — 9/27/04
Signature of Principal Investigator (required for all projects) — **Date**

[signature] — 9/27/04
Signature of Departmental Review Chair (not required for concluded or not begun studies) — **Date**

FOR COMMITTEE USE ONLY

☒ Approve ☐ Disapprove — Period of Approval: NOV 16 2004 — OCT 11 2005

Subject to the following conditions: N/A. Revised Consent/Authorization Forms (updated to add page numbering/version dates only) approved concurrently. Lapse in approval due to delay in IRB processing.

David G. Johnson, MD — Date Reviewed: NOV 16 2004

David G. Johnson, M.D., Chair
Biomedical/Continuing Review Committee

☒ Expedited Review ☐ Full Committee Review

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