Expression, production and release of the Eis protein by *Mycobacterium tuberculosis* during infection of macrophages and its effect on cytokine secretion

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The *eis* gene of *Mycobacterium tuberculosis* has been shown to play a role in the survival of the avirulent *Mycobacterium smegmatis* within the macrophage. *In vitro* and *in vivo* analysis of Δeis deletion mutants and complemented strains showed no effect on survival of *M. tuberculosis* in U-937 macrophages or in a mouse aerosol infection model, respectively. Further studies were done in an attempt to determine the role of *eis* in *M. tuberculosis* intracellular survival and to define a phenotypic difference between wild-type and the Δeis deletion mutant. Bioinformatic analysis indicated that Eis is an acetyltransferase of the GCN5-related family of *N*-acetyltransferases. Immunofluorescence microscopy and Western blot analysis studies demonstrated that Eis is released into the cytoplasm of *M. tuberculosis*-infected U-937 macrophages. Eis was also found in the extravesicular fraction and culture supernatant of *M. tuberculosis*-infected macrophages. The effect of Eis on human macrophage cytokine secretion was also examined. Eis modulated the secretion of IL-10 and TNF- α by primary human monocytes in response both to infection with *M. tuberculosis* and to stimulation with recombinant Eis protein. These results suggest that Eis is a mycobacterial effector that is released into the host cell to modulate inflammatory responses, possibly via transcriptional or post-translational means.

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INTRODUCTION

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), is one of the oldest pathogens known to man and is responsible for the deaths of over 1.8 million people every year (Corbett *et al.*, 2003). *M. tuberculosis* survives within the host macrophage by arresting the normal development of the phagosome (Chan *et al.*, 1991; Fratti *et al.*, 2003).

Phagosome–lysosome fusion is inhibited in part by effectors that are secreted by *M. tuberculosis*, such as mannose-capped lipoarabinomannan (ManLAM) (Chan *et al.*, 1991; Fratti *et al.*, 2003). In this manner, the bacterium is able to avoid the toxic effects of lysosomal enzymes and the normal acidified environment of a mature phagosome.

However, mycobacterial survival also hinges on its ability to modulate host cytokine secretion. *M. tuberculosis* infection or mycobacterial proteins alone induce the secretion of a large number of cytokines including interleukin (IL)-1, IL-2, IL-10, IL-12 and tumour necrosis factor alpha (TNF- α) by monocytes/macrophages (Barnes *et al.*, 1992; Fulton *et al.*, 1996; Lee *et al.*, 2003b; Wallis *et al.*, 1990). The important proinflammatory cytokine TNF- α plays a key role in the host immune defence against TB (Flynn *et al.*, 1995; Keane *et al.*, 2001). In contrast, IL-10 downregulates cytokine production and accessory cell function of mononuclear phagocytes

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Abbreviations: 3D-PSSM, three-dimensional position-specific scoring matrix; FCS, fetal calf serum; GNAT, GCN5-related *N*-acetyltransferase; IF, immunofluorescence microscopy; IL, interleukin; PPD, purified protein derivative; TB, tuberculosis; TNF- α , tumour necrosis factor alpha.

(D’Andrea *et al.*, 1993; de Waal Malefyt *et al.*, 1991) and its expression is also induced by various mycobacterial products (Barnes *et al.*, 1992).

A wide variety of mycobacterial proteins and lipids are secreted into the cytoplasm of *M. tuberculosis*-infected macrophages (Beatty & Russell, 2000; Beatty *et al.*, 2001, 2000; Xu *et al.*, 1994). The trafficking of these mycobacterial components within the endocytic network of the host cell may play a role in modulating macrophage function to the advantage of the bacterium. These mycobacterial components are also released from the macrophage into the extracellular milieu in vesicular compartments (Beatty *et al.*, 2001; Rhoades *et al.*, 2003). Furthermore, these vesicles are taken up by uninfected bystander cells (Beatty *et al.*, 2001, 2000). The propensity of *M. tuberculosis* to extend its influence beyond the constraints of the infected host cell is in keeping with its ability to subvert the immune system of the host to enhance its survival.

The *eis* (enhanced intracellular survival) gene of *M. tuberculosis* is only found in members of the *M. tuberculosis* complex and has been shown to confer enhanced survival on *Mycobacterium smegmatis* in U-937 macrophages and human macrophages (Wei *et al.*, 2000). The Eis protein is a 42 kDa protein that is secreted when *M. tuberculosis* is grown in liquid culture (Dahl *et al.*, 2001). In the present study, we constructed and utilized an *eis* mutant (Δeis) and complemented strains of *M. tuberculosis* H37Ra and H37Rv to study the role the gene plays in the microbe’s interactions with macrophages. We discovered that the Eis protein is produced and released by *M. tuberculosis* into the cytoplasm of the phagocyte during intracellular infection. We also found that Eis is able to modulate the secretion by human monocytes of the cytokines TNF- α and IL-10, which play a major role in the regulation of the inflammatory response to infection. Bioinformatic studies show that Eis is a putative

acetyltransferase of the GCN5-related family of *N*-acetyltransferases (GNAT) that may play a role in deregulating macrophage cytokine production.

METHODS

Strains and growth media. *M. tuberculosis* H37Rv and H37Ra were grown in Middlebrook 7H9 (Difco) broth. For growth on solid culture media, Middlebrook 7H10 (Difco) with 10% OADC was used. Mycobacterial inocula for infection of U-937 cells with MtbRa/*gfp* and MtbRa Δeis /*gfp* were prepared as previously described (Wei *et al.*, 2000). For growing *Escherichia coli* transformants, Luria–Bertani (LB) broth or agar with 200 μ g hygromycin B ml⁻¹ or 100 μ g ampicillin ml⁻¹ was used.

M. tuberculosis H37Ra wild-type and Δeis were transformed with the vector pBEN, which contains *gfp* driven by *phsp60*, a strong heat-shock promoter from *Mycobacterium bovis* BCG (Table 1) (Saviola *et al.*, 2003) to generate the *gfp*-expressing strains MtbRa/*gfp* and MtbRa Δeis /*gfp*, respectively. The promoterless *gfp* vector pFPV27 was used as the negative control for these studies. Both vectors were generously supplied by Stanley Falkow, Stanford University School of Medicine. Bacteria were grown on Middlebrook 7H10 medium containing 25 μ g kanamycin ml⁻¹. Fluorescence of transformants was confirmed by fluorescence microscopy, as described below.

Human macrophage-like U-937 cells (ATCC; 1593.2 CRL) were grown in RPMI 1640 medium (Sigma) containing 10% fetal calf serum (FCS) (Atlanta Biologicals) under 5% CO₂ at 37 °C (Nilsson & Sundstrom, 1974). U-937 macrophage monolayers were prepared by transformation of cells in T75 flasks (Corning) using phorbol myristic acetate (PMA), as previously described (Wei *et al.*, 2000).

General DNA methods. For DNA manipulations, standard protocols were followed (Sambrook *et al.*, 1989). Agarose was purchased from Promega. Restriction enzymes, Klenow fragment of T4 DNA polymerase, T4 DNA ligase and calf intestinal alkaline phosphatase (CIAP) were from New England BioLabs or Gibco-BRL Life Technologies. All reagents were used according to the manufacturer’s instructions. Plasmids were constructed in *E. coli* HB101 or DH5 α cells and prepared using a Qiaprep spin miniprep kit (Qiagen).

Table 1. Plasmids used in this study

Plasmid	Relevant characteristics	Reference or source
pMV306	Mycobacterial integrating vector, integrates into the <i>attB</i> site, Kan ^r	Stover <i>et al.</i> (1991)
pSP72	<i>E. coli</i> cloning vector, Amp ^r	Promega
pRL498	pUC-based vector, containing 1.3 kb hygromycin resistance cassette, Hyg ^r Kan ^r	C. E. Barry, III*
pMJ10	<i>ts oriM</i> ; <i>sacB</i> counterselection, Kan ^r Gent ^r	Pelacic <i>et al.</i> (1997)
p69	pOLYG with 2.99 kb <i>M. tuberculosis</i> genomic DNA fragment containing the <i>eis</i> gene	Wei <i>et al.</i> (2000)
p6237	1.3 kb hygromycin gene released by <i>Xba</i> I from pRL498 replacing a 367 bp internal <i>Nhe</i> I fragment from p6297	This study
p6297	pSP72 with 1.6 kb <i>Apo</i> I– <i>Pvu</i> II <i>eis</i> -containing fragment from pOLYG	This study
p6301	pMV306 with 1.6 kb <i>Cl</i> aI– <i>Hind</i> III <i>eis</i> -containing fragment from p6297	This study
p6308	pMJ10 with 2.5 kb <i>Xba</i> I– <i>Bam</i> HI fragment from p6237 containing <i>eis</i> disrupted by the <i>hyg</i> gene	This study
pBEN	<i>gfp</i> driven by <i>phsp60</i> from <i>M. bovis</i> BCG	Saviola <i>et al.</i> (2003)
pFPV27	Promoterless <i>gfp</i> reporter vector	Barker <i>et al.</i> (1998)
pET-15b	Carries N-terminal His tag	Novagen
pET-15b- <i>eis</i>	pET vector with <i>eis</i> fragment	This study

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Recombinant Eis. In order to obtain purified Eis protein in sufficient quantities for use in our studies, we constructed a recombinant His-tagged Eis protein. The 1.3 kb *eis* gene was PCR amplified and cloned into the pET-15b vector (Novagen) so as to obtain a recombinant protein with an N-terminal His-tag. The plasmid bearing the insert (pET15b-*eis*) was transformed into the *E. coli* expression strain BL-21 DE-3 pLysS (Novagen) (Table 2). Recombinant protein was then induced, harvested and purified as per the manufacturer's protocol (Novagen). The one step Ni-NTA purification process resulted in a single protein band, of molecular mass similar to that predicted for Eis (42 kDa) (Wei *et al.*, 2000). Purity of the recombinant Eis was greater than 99%, as determined by scanning densitometry analysis of SDS-PAGE gels, and the His-tagged Eis protein also reacted with the anti-Eis antibody (data not shown). Limulus lysate amoebocyte assay was utilized to test for endotoxin contamination.

Infection of mice. Five- to eight-week-old female C57/BL6 mice were purchased from Taconic and aerosol infected as described previously (Boshoff *et al.*, 2003). Each mouse was infected with approximately 50 c.f.u. *M. tuberculosis* and bacterial burdens were monitored by plating of lung and spleen homogenates from four or five mice at different times during the infection. In addition, mice lungs and spleens were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained for histology with either haematoxylin/eosin or Ziehl–Neelsen stain.

Immunofluorescence microscopy of infected U-937 macrophages. One-millilitre aliquots of RPMI containing 2×10^5 U-937 cells were plated into each well of a 24-well tissue culture plate (Falcon) containing sterile glass coverslips. Cells were infected the next day as described previously (Wei *et al.*, 2000) with MtbRa/*gfp* and MtbRaΔ*eis*/*gfp* at an m.o.i. of 20. At appropriate time points, coverslips were washed three times with PBS and cells fixed by addition of 1 ml 3% formaldehyde in PBS to each well. After 15 min, coverslips were washed three times with PBS and cells were permeabilized by addition of 0.1% Triton X-100 for 5 min. Coverslips were again washed, and incubated with a 1:500 dilution of anti-Eis antibody for 1 h at room temperature on an orbital shaker. Anti-Eis antibody is an affinity-purified antibody to Eis generated in rabbits as described previously (Dahl *et al.*, 2001). Alexafluor 594 goat anti-rabbit IgG linked to a fluorescent red dye (Molecular Probes;

1:10 000) in PBS containing 10% FCS was then added and incubated with the coverslips. Wells were washed and coverslips were removed to a paper towel to air dry in the dark. Two microlitres of *p*-phenylenediamine mounting medium was added to each coverslip, which was then inverted onto a glass slide, sealed with clear nail polish and viewed using a Nikon Eclipse 2000-S fluorescent/phase-contrast microscope. Images were obtained as TIFF files using Metacam software and adjusted for consistent contrast using Photoshop 7.0 (Adobe).

Infection, harvesting and fractionation of infected U-937 macrophage monolayers for Western blot analysis of macrophage cytoplasm.

Infection of U-937 monolayers was carried out as described above (Wei *et al.*, 2000). An m.o.i. of 15 was used and the infection was allowed to proceed for 4 h. Monolayers were then washed and 15 ml RPMI + 5% human AB sera was added to each flask and incubated for 48 h. Monolayers were then washed with Hanks' balanced salts solution (HBSS) and harvested into 15 ml HBSS by agitation with 3 mm glass beads. Cells were washed and resuspended in cold homogenization buffer (250 mM sucrose, 0.5 mM EDTA, 0.5 mM EGTA, 20 mM HEPES) and then lysed by repeated passage through a 25 gauge needle. Lysate was centrifuged at 300 *g* for 10 min followed by two subsequent spins at 100 *g* for 5 min each. The supernatant was carefully layered over a 12% sucrose cushion and centrifuged at 800 *g* for 45 min at 4 °C. The cytoplasmic fraction remained in a distinct upper layer, separate from mycobacteria and phagosomes containing mycobacteria in the lower layer and pellet, respectively.

Isolation of exocytic vesicles from culture supernatant of infected U-937 macrophages.

Exocytic vesicles were isolated from tissue culture supernatants as described previously (Beatty *et al.*, 2001). Briefly, tissue culture supernatants from U-937 macrophages infected with *M. tuberculosis* H37Ra were harvested and subjected to a series of centrifugation steps to remove whole cells and bacteria. The supernatant was then centrifuged for 60 min at 100 000 *g*. The resulting pellet contained the vesicles released from the macrophages which were analysed by Western blotting for the presence of the Eis protein. Culture supernatant obtained after

Table 2. Bacterial strains used in this study

Strain	Description	Reference or source
<i>E. coli</i>		
DH5α	<i>supE44 ΔlacU169 (φ80lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Gibco-BRL
BL-21 DE-3 pLysS	<i>ompT hsdS (r_B⁻ m_B⁻) dcm⁺ Tet^r gal endA Hte</i>	Stratagene
<i>M. tuberculosis</i>		
H37Ra	Attenuated H37Rv	D. Young*
H37RaΔ <i>eis</i>	Double-crossover recombinant of H37Ra with p6308, Δ <i>eis</i> :: <i>hyg</i> , Hgr ^r	This study
H37Ra <i>eis</i> complemented	H37Ra containing integrated copy of p6308 and p6301 Δ <i>eis</i> :: <i>hyg</i> , <i>eis</i> :: <i>attB</i> , Hgr ^r Kan ^r	This study
H37Rv	Virulent laboratory strain (ATCC 27294)	CSU†
H37RvΔ <i>eis</i>	Double-crossover recombinant of H37Rv with p6308, Δ <i>eis</i> :: <i>hyg</i> , Hgr ^r	This study
H37Rv <i>eis</i> complemented	H37Rv containing integrated copy of p6308 and p6301 Δ <i>eis</i> :: <i>hyg</i> , <i>eis</i> :: <i>attB</i> , Hgr ^r , Kan ^r	This study
MtbRa/ <i>gfp</i>	H37Ra transformed with pBEN so as to express <i>gfp</i> , Kan ^r	This study
MtbRaΔ <i>eis</i> / <i>gfp</i>	H37RaΔ <i>eis</i> transformed with pBEN so as to express <i>gfp</i> , Kan ^r	This study

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processing as described above was concentrated threefold using YM-10 Centriprep filters (Millipore) and analysed by Western blot for the presence of Eis protein.

Western blot analysis. Sample protein concentrations were determined by BCA protein assay (Pierce). Samples were separated on 10% SDS-PAGE gels along with prestained molecular mass standards (Gibco-BRL). Electrophoresis was performed at 200 V and 80 mA for 3–4 h. Proteins were transblotted for 1 h using MiniProtein 3 cells (Bio-Rad) onto PVDF membranes (Millipore) which were then blocked with 5% skim milk in PBS containing 0.02% sodium azide. Membranes were incubated with primary antibody in PBS containing 0.25% powdered skim milk for 1 h, washed three times with PBS and then probed with secondary antibody for 1 h. Secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) diluted 1:20 000 and used in blots. Membranes were then washed three times with PBS and incubated with chemiluminescent substrate for 5 min followed by exposure to X-ray film for appropriate periods of time. All reagents for development of the chemiluminescent Western blots were obtained from Pierce.

Isolation and cultivation of human monocytes for analysis of cytokine secretion in response to antigenic stimulation.

Venous blood was drawn from tuberculin-positive healthy subjects into sterile blood collection tubes, and peripheral blood mononuclear cells (PBMCs) were isolated by density sedimentation over Histopaque-1077 (Sigma). PBMCs were suspended at a density of 2×10^6 cells ml^{-1} in complete RPMI medium (Gibco-BRL) with 10% fetal bovine serum (Gibco-BRL), sodium pyruvate, nonessential amino acids, penicillin G (100 IU ml^{-1}) and streptomycin (100 $\mu\text{g ml}^{-1}$). Cells were incubated for 1 h at 37 °C and non-adherent cells were removed by pipetting off the supernatant. Adherent monocytes were collected as previously described (Song *et al.*, 2003). The recovered cells were >95% CD14⁺ cells, as determined by flow cytometry using an anti-CD14 antibody. The cells were then stimulated with either purified protein derivative (PPD) (Statens Serum Institut), Eis, or lipopolysaccharide (LPS, 10 ng ml^{-1} ; Sigma) and incubated at 37 °C in a 5% CO₂ 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 was grown to late exponential phase in Middlebrook 7H9 as described previously, aliquoted and stored at -70 °C. Representative vials were thawed and c.f.u. enumerated on Middlebrook 7H10 agar. Single-cell suspensions of mycobacteria were obtained by a modification of standard methods. Briefly, aliquots of frozen *M. tuberculosis* were cultured in 7H9 broth with 0.5% (v/v) glycerol at 37 °C in 5% CO₂ for 7–10 days so that cultures reached mid-exponential growth phase. Bacterial cultures were pelleted at 3000 g for 10 min and resuspended in 7H9 broth. Clumped mycobacteria were dispersed with an ultrasonic cell disrupter (3–5 min, 35 kHz; Bandelin). Bacteria were then resuspended in 1 ml RPMI 1640 medium and clumps were disrupted by multiple passages through a 25 gauge needle. Mycobacterial viability, as assessed by the number of c.f.u., was 60–70%.

Adherent human monocytes were washed three times with Ca²⁺- and Mg²⁺-free PBS, and adherent monolayers were replenished with complete medium without antibiotics. The cells were incubated overnight without stimulation at 37 °C in 5% CO₂. After overnight incubation, monocytes ($2 \times 10^5 \text{ ml}^{-1}$) were infected with mycobacteria using an m.o.i. of 1 for either 18 h or 48 h, and the supernatants were then recovered for ELISA determination of cytokine levels of TNF- α and IL-10, respectively.

ELISA. ELISA was used to detect TNF- α and IL-10 levels (BD Biosciences) in monocyte culture supernatants, as previously described (Lee *et al.*, 2003a). 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 millilitre. The difference between triplicate wells was consistently less than 10% of the mean.

Bioinformatic analysis. Protein sequences for analysis were obtained in FASTA format and input into respective programs at the interface locations described below. Reverse position-specific (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 (Marchler-Bauer & Bryant, 2004). 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. The 3D-PSSM (three-dimensional position-specific scoring matrix) server is designed to take a protein sequence of interest and attempt to predict its 3D structure and its probable function. This program is able to confidently predict structural and functional relationships better than standard local alignment search tools such as PSI-BLAST (Kelley *et al.*, 2000). 3D-PSSM is administered by Lawrence Kelley and is available for use at <http://www.sbg.bio.ic.ac.uk/servers/3dpssm/>.

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

RESULTS

Survival of *M. tuberculosis* wild-type and *eis* mutant *in vitro* and *in vivo*

Having demonstrated previously that the *eis* gene enhanced intracellular survival of *M. smegmatis* (Wei *et al.*, 2000), it was only natural to look for a similar phenotype in *M. tuberculosis*. The first step towards answering this question was to develop a Δeis and complemented strain in both *M. tuberculosis* H37Ra and H37Rv. Deletion and complementation of the *eis* gene was confirmed by both Southern and Western blot analysis (data not shown). Survival of the H37Rv wild-type, Δeis mutant and complemented strains in U-937 macrophage monolayers was then compared. There was no significant difference between the three strains in terms of intracellular survival in U-937 macrophages for the duration of the 7 day experiment (data not shown).

Survival of these three strains was then compared in an *in vivo* mouse aerosol model (Dahl *et al.*, 2003). Six- to eight-week-old female C57BL/6 mice were infected via the aerosol route with *M. tuberculosis* H37Rv wild-type, Δeis mutant and complemented strains. At various time points over a 17 week period, the mice were sacrificed and lung and spleen homogenates were plated for c.f.u. determinations and tissue samples were also taken for histological evaluation. There appeared to be no significant difference between survival of the wild-type, Δeis mutant and complemented strains in the *in vivo* model as determined by viable plate counts (Fig. 1). Also, no histological

Fig. 1. Growth of *M. tuberculosis* H37Rv, Δeis and complemented strains in the lungs (a) and spleens (b) of aerosol-infected mice. Female C57/BL6 mice were infected via the aerosol route and monitored by c.f.u. determinations of lung and spleen homogenates via viable plate counts. These data are representative of three independent experiments using four or five mice per time point after initial infection. $\square$, Wild-type; $\triangle$, Δeis ; $\blacktriangle$, complemented Δeis .

differences were observed between infected lung or spleens from wild-type or Δeis mutants. This included severity of granuloma formation and inflammation, granuloma numbers and numbers of acid-fast bacteria per tissue section (data not shown).

Bioinformatic analysis of Eis

Bioinformatic analysis of the Eis protein sequence using the protein-protein BLAST (BLASTP) program (Altschul *et al.*, 1990) yielded a large number of homologous proteins, many of which were members of the GNAT superfamily of proteins. RPS-BLAST (Marchler-Bauer & Bryant, 2004) analysis of Eis revealed the presence of an acetyltransferase domain that is characteristic of the pfam00583 family of

GNAT acetyltransferases (Neuwald & Landsman, 1997). The acetyltransferase domain extends from residues 61 to 137 of the 402 aa Eis protein sequence and contains the conserved residues (V/I-x-x-x-x-Q/R-x-x-G-x-G/A) that are characteristic of this domain (Neuwald & Landsman, 1997). A substitution of glycine or alanine with lysine at the last conserved residue, as seen with Eis, is found in other members of this family (Neuwald & Landsman, 1997). Since members of the GNAT superfamily have poor sequence homology but share common 3D structural features (Dyda *et al.*, 2000), the 3D-PSSM program was used to search for proteins showing significant fold compatibility with Eis. Fold analysis of a 154 aa section of the Eis sequence, which included the acetyltransferase domain using 3D-PSSM, indicates that Eis shares strong similarities with several proteins belonging to the GNAT family of acetyltransferases including the conserved residues (Table 3). These bioinformatics analyses suggest that Eis may function as an acetyltransferase.

Presence of Eis in the cytoplasm of macrophages infected with *M. tuberculosis*

Because the Eis protein was shown to be secreted into the bacterial culture medium by *M. tuberculosis* *in vitro* (Dahl *et al.*, 2001), studies were done to examine infected macrophages for the presence of the Eis protein in the host cell cytoplasm. *M. tuberculosis* H37Ra and Δeis strains were transformed with the pBEN plasmid, which contains the green fluorescent protein (*gfp*) gene driven by *phsp60* from *Mycobacterium bovis* BCG (Saviola *et al.*, 2003). *M. tuberculosis* H37Ra wild-type and Δeis strains expressing *gfp* were named MtbRa/*gfp* and MtbRa Δeis /*gfp*, respectively. The MtbRa/*gfp*- and MtbRa Δeis /*gfp*-infected cells were washed, permeabilized, and probed with rabbit anti-Eis antibody (Dahl *et al.*, 2001) followed by goat anti-rabbit antibody labelled with a fluorescent red dye.

Macrophages infected with MtbRa Δeis /*gfp*, probed with both primary and secondary antibodies and visualized using immunofluorescence microscopy (IF) showed no red staining for the Eis protein at any time point due to the absence of Eis production in the MtbRa Δeis /*gfp* mutant

Table 3. 3D-PSSM search for structural homologues to the Eis protein

The predicted 3D structure of Eis shows significant homology to the known 3D structures of several members of the pfam00583 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.

Protein	Organism	Family	<i>E</i> -value
Aminoglycoside 2'- <i>N</i> -acetyltransferase	<i>M. tuberculosis</i>	Predicted GNAT	3.61×10^{-3}
Aminoglycoside 3'- <i>N</i> -acetyltransferase	<i>Serratia marcescens</i>	GNAT	9.56×10^{-3}
Histone acetyltransferase	<i>Saccharomyces cerevisiae</i>	GNAT	1.09×10^{-2}
Aminoglycoside 6'- <i>N</i> -acetyltransferase	<i>Enterococcus faecium</i>	GNAT	2.12×10^{-2}
Phosphinothricin <i>N</i> -acetyltransferase	<i>Bacillus subtilis</i>	Predicted GNAT	3.29×10^{-2}
Protein Yjcf	<i>B. subtilis</i>	GNAT	1.71×10^{-1}

(Fig. 2a, b). Starting at 4 h post-infection up to 96 h post-infection, 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 (Fig. 2c–h). No Eis protein was visible when the cells were examined 30 min post-infection (data not shown). In many instances, macrophages appeared to contain Eis protein in the absence of visible infecting bacteria (Fig. 2f, g; arrowheads denote uninfected macrophages). In control experiments, no IF was observed in uninfected U-937 cells that were probed in a similar manner. This was also true for cells that were not permeabilized before the addition of the primary and secondary antibodies (data not shown), indicating that the Eis protein being visualized was not tethered to the surface of the macrophage. This experiment was repeated three times and similar results were obtained each time. Similar results were also observed using both J774 murine macrophages and primary human monocytes (data not shown).

Western blot analysis of the cytoplasm of infected macrophages for the presence of Eis

Western blot analysis using the anti-Eis antibody of lysates prepared from infected macrophages as previously

described (Chakraborty *et al.*, 1994) was used to confirm the IF observation of the presence of Eis in the cytoplasm of these macrophages. The Eis protein was not detected in the cytoplasmic fraction of macrophages infected with *M. tuberculosis* H37Ra Δ *eis* (Fig. 3, lane 1) but was found in the cytoplasmic fraction of U-937 cells infected with *M. tuberculosis* H37Ra wild-type at 48 h after infection (Fig. 3, lane 2). The infecting bacteria had been removed from these fractions by centrifugation of the lysate over a 12 % sucrose solution. To discount the possibility that the presence of Eis in the macrophage cytoplasm may be due to intracellular lysis of phagocytosed bacteria, the cytoplasmic fraction was probed by Western blot analysis for the presence of the 16 kDa alpha crystallin protein (Acr) at 48 h after infection. Acr is a mycobacterial protein encoded by *hspX* that is upregulated in *M. tuberculosis* during infection of macrophages and is known not to be secreted from the bacteria (Dubnau *et al.*, 2002). Acr was not detected in the cytoplasmic fraction of macrophages infected with *M. tuberculosis* by Western blot analysis.

We then attempted to explain the presence of Eis protein in macrophages that appeared to be visibly uninfected (Fig. 2f and g, arrowheads) by examining the vesicular fraction of the culture supernatant as described previously by Beatty

Fig. 2. 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 m.o.i. of 20 and at various time points cells were fixed, permeabilized, probed 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) IF showing fluorescent green *MtbRa/gfp* (white arrow) in U-937 macrophages. No Eis was detected in the cytoplasm of these macrophages as observed by the absence of red fluorescence. (b) Phase-contrast and fluorescent microscopy images of the field in (a) were merged, visualizing the macrophage and the fluorescent green bacteria (white arrow). (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 and (h) at 96 h after infection. In both (f) and (g) uninfected macrophages (indicated by white arrowheads) demonstrate the presence of the Eis protein in the cytoplasm in the absence of infecting bacteria. Bar, 20 μ m.

Fig. 3. Western blot detection of Eis in the cytoplasm and tissue culture supernatant of U-937 macrophages infected with *M. tuberculosis* H37Ra wild-type and Δeis mutant. Molecular mass markers and their sizes are represented at the left side of the blot. All lanes were probed with anti-Eis antibody. Lane 1, cytoplasmic fraction of U-937 macrophages infected with *M. tuberculosis* H37Ra Δeis . No Eis protein is detected. Lane 2, presence of Eis in the cytoplasmic fraction of U-937 macrophages infected with *M. tuberculosis* H37Ra wild-type. Lane 3, extravascular fraction from the tissue culture supernatant of *M. tuberculosis* H37Ra wild-type-infected U-937 macrophages. Eis was found 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. In all cases, samples were loaded onto 10% SDS-PAGE gels, separated by electrophoresis, and transblotted onto PVDF membranes for Western blot analysis. Two hundred micrograms of total protein was loaded per lane. The black arrowhead to the right of the blot denotes the position of the Eis protein.

et al. (2000). Tissue culture supernatant from U-937 cells infected with *M. tuberculosis* H37Ra was harvested at 48 h after infection and then subjected to a series of centrifugation steps to remove any contaminating bacteria and macrophage organelles and to separate out the microvesicular fraction. Analysis of this fraction containing the vesicles released by macrophages into the culture supernatant by Western blot detected the presence of the Eis protein (Fig. 3, lane 3). The 75 kDa band that is seen in this lane was not detected in either the cytoplasmic fraction (Fig. 3, lane 1) or the extravascular fraction of U-937 cells infected with *M. tuberculosis* H37Ra Δeis (data not shown). As the anti-Eis antibody used in these studies is affinity purified (Dahl *et al.*, 2001), it is unlikely to cross-react with host cell proteins. Eis protein has a tendency to aggregate in solution and this band is probably the result of aggregate formation. Nevertheless, the possibility that the 75 kDa band may represent Eis covalently bound to a mammalian protein needs to be further investigated.

These findings are consistent with the reports of detection of other mycobacterial proteins and lipids in vesicles released into the tissue culture supernatant by *M. tuberculosis*-infected macrophages (Beatty *et al.*, 2001, 2000; Rhoades *et al.*, 2003). The steps utilized to obtain the vesicular pellet resulted in a tissue culture supernatant fraction devoid of any host cellular structural material. Western blot analysis of a concentrated aliquot of this fraction detected the presence of the Eis protein (Fig. 3, lane 4), the source of which may be the degradation of vesicles containing mycobacterial proteins in the culture supernatant or possibly due to exocytic release of protein from *M. tuberculosis*-infected macrophages.

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

Cytokine release by human monocytes in response to stimulation with recombinant Eis

To evaluate cytokine production in primary human monocytes in response to recombinant Eis or PPD antigen (an *M. tuberculosis* antigen preparation commonly used in such immunological studies), assays were performed to detect TNF- α and IL-10 secretion by ELISA. These cytokines are known to play a significant role in the pro- and anti-inflammatory components respectively of the immune response to *M. tuberculosis* infection (Flynn *et al.*, 1995; Gong *et al.*, 1996). Mycobacterial proteins/lipids released from the phagosome of *M. tuberculosis*-infected macrophages have also been shown to modulate levels of cytokine secretion during infection, to the advantage of the pathogen (Gil *et al.*, 2004; Placido *et al.*, 1997; Rivera-Marrero *et al.*, 2004).

Experiments using human monocytes from three healthy tuberculin reactors showed that TNF- α and IL-10 were not produced in freshly isolated cells but were detectable 3 h after and peaked from 18–24 h (for TNF- α) and 48 h (for IL-10) after stimulation with recombinant Eis or PPD antigen (data not shown). Therefore, the 18 h and 48 h time points were used to study the levels of TNF- α and IL-10 production, respectively, after stimulation with the Eis or PPD antigens of *M. tuberculosis*. As shown in Fig. 4(a), Eis-induced TNF- α production was increased in human monocytes in a dose-dependent manner. However, Eis elicited significantly lower amounts of TNF- α at 5 and 10 $\mu\text{g ml}^{-1}$, when compared with the same concentrations of the PPD antigen ($P < 0.05$; Fig. 4a). Stimulation of human monocytes with 10 ng LPS (positive control) resulted in the secretion of TNF- α (1000–2500 pg ml^{-1}).

As shown in Fig. 4(b), significant dose-dependent IL-10 production was observed by human monocytes after *in vitro* stimulation with the Eis protein. In contrast to the findings

Fig. 4. Cytokine secretion by human monocytes in response to Eis. Monocytes were isolated from tuberculin-positive healthy donors and either left untreated or stimulated with increasing amounts of PPD and Eis antigen. (a) TNF- α concentration was measured by ELISA in the cell culture supernatants harvested at 18 h following stimulation with each antigen and expressed as the mean $\pm$ SE of three independent experiments. (b) IL-10 concentration was measured by ELISA in the cell culture supernatants harvested at 48 h following stimulation with each antigen and expressed as the mean $\pm$ SD of three independent experiments. $n=3$ for each experiment. * $P<0.05$. (c) Monocytes from healthy tuberculin-positive donors were harvested and either not infected (1), or infected with *M. tuberculosis* H37Rv wild-type (2), Δeis (3) and complemented strains (4) at an m.o.i. of 1. Culture supernatants were harvested 18 h after infection to determine levels of cytokine production. TNF- α levels in the culture supernatants were monitored by ELISA. A representative experiment is shown, $n=3$. (d) IL-10 secretion by infected monocytes from healthy donors as described for (c). IL-10 levels in the supernatants were monitored by ELISA. A representative experiment is shown in (b), $n=3$. * $P<0.05$; ** $P<0.01$.

for TNF- α , the IL-10 production induced by Eis protein at 5 and 10 $\mu\text{g ml}^{-1}$ was higher ($P<0.05$) than that induced by the PPD antigen at the same concentrations (Fig. 4b). Stimulation of human monocytes with 10 ng LPS (positive control) resulted in the increased secretion of IL-10 (2000–4000 pg ml^{-1}). Recombinant protein preparations were tested to ensure absence of any contaminating endotoxins.

Cytokine release by human monocytes in response to infection with *M. tuberculosis* H37Rv wild-type, Δeis and complemented strains

Next, TNF- α and IL-10 secretion by human monocytes infected with wild-type *M. tuberculosis* H37Rv, Δeis and *eis* complemented strains at an m.o.i. of 1 were evaluated. As shown in Fig. 4(c), TNF- α production by human monocytes infected with the H37Rv Δeis was significantly greater than those infected with wild-type H37Rv ($P<0.01$) or the complemented strain ($P<0.05$). Although the mean TNF- α production by *M. tuberculosis*-infected monocytes increased

slightly with bacterial load, H37Rv wild-type infected monocytes still secreted significantly less TNF- α than those infected with the Δeis strain at an m.o.i. of 10 (data not shown).

In contrast to TNF- α , IL-10 secretion by H37Rv Δeis -infected monocytes was significantly lower than induction by H37Rv wild-type ($P<0.01$) or *eis*-complemented strains ($P<0.01$) (Fig. 4d). With increased bacterial load (m.o.i. of 10), the H37Rv Δeis -infected monocytes secreted significantly less IL-10 than those infected with wild-type or complemented strains (data not shown). These results show that strain H37Rv Δeis induced more TNF- α but less IL-10 production in primary human monocytes than either H37Rv wild-type or complemented strains.

DISCUSSION

The *eis* gene of *M. tuberculosis* enhances the intracellular survival of *M. smegmatis* (Wei *et al.*, 2000). However, its function in the virulence of *M. tuberculosis* has not yet been elucidated. No significant difference was noted in the

survival of *M. tuberculosis* H37Rv wild-type, Δeis mutant and complemented strains in either *in vitro* or *in vivo* models (Fig. 1). However, Barczak *et al.* (2005) demonstrated that there was no significant difference in the growth of the hypovirulent CDC 1551 and the hypervirulent HN878 strains of *M. tuberculosis* in prestimulated macrophages. They similarly noted no difference in the mycobacterial loads of the lungs of mice infected with either strain. Instead the difference between the two strains lay in the growth kinetics and mouse survival (Barczak *et al.*, 2005). Further studies are required to determine whether the same is true of strains deficient in *eis*. It is possible that the role of *eis* in the pathogenesis of *M. tuberculosis* may differ significantly from the phenotype observed in *M. smegmatis* (Wei *et al.*, 2000), a surrogate host for this *M. tuberculosis*-derived gene, thus explaining the lack of an intracellular survival phenotype in the current study.

The discovery that *eis* is expressed during infection of activated human macrophages by clinical strains of *M. tuberculosis* has been confirmed by others (Cappelli *et al.*, 2001). In these present studies, RT-PCR indicated that *eis* is constitutively expressed by the *M. tuberculosis* H37Ra strain whether in culture media or in macrophages (data not shown). This idea is corroborated by studies by Dahl *et al.* (2005) showing that *eis* is negatively regulated by the stringent response regulator Rel_{Mtb} so as to maintain constant levels of expression during starvation conditions. On the other hand, Wu *et al.* (2005) have demonstrated that the expression of *eis* was enhanced 12-fold in the clinical strain *M. tuberculosis* 210 as compared to the H37Rv strain when grown in a human monocyte cell line, whereas the expression was the same when the strains were grown *in vitro*. They also showed that SigA binds to the promoter region of *eis* and is responsible for the upregulation of *eis* expression in strain 210. They concluded that *eis* may contribute to the enhanced growth of the 210 strain in human macrophages. Morris *et al.* (2005) have also recently demonstrated that *eis* is a component of the *whiB7* regulon, whose expression is upregulated by exposure to sublethal concentrations of antibiotics or fatty acids such as would be encountered within the host macrophage during infection. These reports in conjunction with the data presented here point to a significant but not yet clearly defined role for *eis* in the pathogenesis of *M. tuberculosis*.

Multiple sources have reported on the release of mycobacterial constituents from infected macrophages into the culture medium followed by their uptake into uninfected bystander cells (Beatty & Russell, 2000; Beatty *et al.*, 2001; Rhoades *et al.*, 2003). Our studies using IF of MtbRa/*gfp*-infected U-937 macrophages demonstrate the production and presence of Eis in the macrophage cytoplasm from 4 h to 96 h post-infection (Fig. 2c–h). Furthermore, macrophage lysate fractions, purged of infecting bacteria, demonstrated the presence of the Eis protein by Western blot analysis (Fig. 3, lane 2). The presence of Eis in the extravesicular fraction and the tissue culture supernatant of

infected macrophages (Fig. 3, lanes 3 and 4) places it in the company of other known mycobacterial effectors of virulence such as lipoarabinomannan (LAM) (Beatty *et al.*, 2000). These mycobacterial protein/lipid-containing vesicles appear to be taken up by uninfected macrophages, which accounts for the presence of mycobacterial components within host cells in the absence of bacteria (Beatty *et al.*, 2000). The release of Eis into the culture supernatant by infected macrophages, either in vesicles or in native form, followed by its uptake into uninfected macrophages (Fig. 2g, h), is the most likely explanation for its presence in these cells. Further work needs to be done to determine the localization of secreted Eis within the macrophage and examine its significance.

The critical importance of TNF- α in anti-mycobacterial defence is well established in mice and humans (Flynn *et al.*, 1995; Keane *et al.*, 2001). TNF- α helps prevent the reactivation of persistent TB and limits the pathological response of the host. The concept that *M. tuberculosis* plays a role in modulation of the TNF- α /IL-10 axis is supported by our data showing that Eis modulates TNF- α and IL-10 secretion by human monocytes in response to infection by *M. tuberculosis* (Fig. 4c, d). Because purified Eis elicits higher levels of IL-10 than TNF- α secretion, as compared to PPD (Fig. 4a, b), we examined the differences in cytokine secretion in response to infection with viable *M. tuberculosis* H37Rv wild-type, Δeis and *eis*-complemented strains. In contrast to H37Rv and the complemented strain, the Δeis mutant induced significantly higher levels of TNF- α and lower levels of IL-10 in human monocytes (Fig. 4c, d). This provides powerful evidence that *M. tuberculosis* utilizes Eis to manipulate the host TNF- α /IL-10 axis to its advantage by inducing a state of localized immunosuppression in the host.

IL-10 is thought to play an important role in the regulation of host antimicrobial immunity. The induction of down-regulatory cytokines, such as IL-10 and transforming growth factor- β , can inhibit interferon (IFN)- γ production by T cells and block or inhibit macrophage activation and apoptosis (Flesch *et al.*, 1994; Othieno *et al.*, 1999). It is widely known that in alveolar macrophages, apoptosis is a common defence mechanism against *M. tuberculosis* infection via a TNF- α -mediated pathway (Placido *et al.*, 1997). Monocytes from patients with active TB when compared to those from healthy donors are not only likely to secrete more IL-10 than TNF- α in response to stimulation with PPD, but they are also more likely to undergo necrosis than apoptosis (Gil *et al.*, 2004). Utilizing the slowly progressive primary tuberculosis (SPTB) mouse model, Abebe *et al.* (2006) have demonstrated a 20-fold increase in the expression levels of IL-10 in the lungs of mice with progressive tuberculosis as compared to mice with latent tuberculosis. Increased IL-10, but decreased TNF- α production driven by Eis, and possibly other *M. tuberculosis* antigens, may contribute to the intracellular survival of the pathogen by suppressing host cell apoptosis during mycobacterial infection. It is quite likely that the modulation of TNF- α and IL-10 secretion by

Eis will have a further effect on the levels of other cytokines due to their roles as prominent components of the pro- and anti-inflammatory immune response. Immunohistochemical analysis of *M. tuberculosis*-infected tissues for secreted Eis as well as altered cytokine expression may shed light on the role of *eis* in the pathogenesis of *M. tuberculosis*.

Bioinformatic analysis suggests that Eis is an acetyltransferase of the family of GCN5-related *N*-acetyltransferases. 3D-PSSM fold analysis shows that the predicted structure of Eis bears significant similarity to the structures of known members of the GNAT superfamily (Table 3) and also shares the conserved residues that are characteristic of this family. This family of proteins is involved in a wide variety of activities ranging from transcriptional activation by histone acetyltransferases to antibiotic resistance by aminoglycoside acetyltransferases. The lack of sequence homology among members of this family makes classification of possible new members difficult unless 3D structural data are available (Neuwald & Landsman, 1997). In the absence of candidate substrates, it is extremely difficult to demonstrate whether the Eis protein possesses functional acetyltransferase activity. Current efforts are being directed towards identification of possible substrates and resolution of the crystal structure of the Eis protein, which may provide concrete evidence as to the putative acetyltransferase activity of Eis and also offer direction as to the identity of possible substrates.

The absence of an observable intracellular survival phenotype for *eis*, while confounding, should not detract from the significant nature of the other findings presented here. Recent reports regarding the role of *eis* in clinical strains of *M. tuberculosis* indicate that further studies should be conducted using these strains. The release of Eis into the cytoplasm of infected macrophages and its apparent role in the modulation of cytokine secretion in these important host immune cells, essential for defence against tuberculosis infection, raises many interesting questions as to the mechanism(s) whereby this modulation is achieved. Perhaps Eis acetylates histones as other acetyltransferases do (Brownell & Allis, 1995) in the macrophage nucleus, resulting in reduced binding to DNA and enhanced transcription and translation of IL-10. Alternatively, Eis may act directly on mammalian cell transcription factors and alter the response of the host cell to infection as described for other acetyltransferases (Sternier & Berger, 2000). Eis, by one of these mechanisms, may directly affect macrophage cytokine production via activity in the host cell nucleus. Whether Eis may actually enter and localize in the macrophage nucleus is unknown and is presently under investigation. The data presented here point toward a significant and novel role for *eis* as yet another virulence factor in the pathogenesis of *M. tuberculosis* that warrants further study.

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