THE MYCOBACTERIUM TUBERCULOSIS EARLY SECRETED ANTIGENIC TARGET OF 6 kDa INHIBITS T CELL INTERFERON-\(\gamma\) PRODUCTION THROUGH THE p38 MITOGEN-ACTIVATED PROTEIN KINASE PATHWAY

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Running head: Protein kinases and ESAT-6 inhibition of T cell IFN-\(\gamma\) production

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Summary

We reported previously that the early secreted antigenic target of 6 kDa (ESAT-6) from Mycobacterium tuberculosis directly inhibits human T cell IFN-\(\gamma\) production and proliferation in response to stimulation with anti-CD3 and anti-CD28. To determine the mechanism of this effect, we treated T-cells with kinase inhibitors before stimulation with ESAT-6. Only the p38 MAPK inhibitor, SB203580, abrogated ESAT-6-mediated inhibition of IFN-\(\gamma\) production in a dose-dependent manner. SB203580 did not reverse ESAT-6-mediated inhibition of IL-17 and IL-10 production, suggesting a specific effect of SB203580 on IFN-\(\gamma\) production. SB203580 did not act through inhibition of AKT (PKB), as an AKT inhibitor did not affect ESAT-6 inhibition of T cell IFN-\(\gamma\) production and proliferation. ESAT-6 did not reduce IFN-\(\gamma\) production by expanding FoxP3+ T regulatory cells. Incubation of T cells with ESAT-6 induced phosphorylation and increased functional p38 MAPK activity, but not activation of ERK or JNK. Incubation of peripheral blood mononuclear cells (PBMC) with ESAT-6 induced activation of p38 MAPK and inhibition of p38MAPK with SB203580 reversed ESAT-6 inhibition of M. tuberculosis-stimulated IFN-\(\gamma\) production by PBMC from subjects with latent tuberculosis infection. Silencing of p38\(\alpha\) MAPK with siRNA rendered T-cells resistant to ESAT-6 inhibition of IFN-\(\gamma\) production. Taken together, our results demonstrate that ESAT-6 inhibits T cell IFN-\(\gamma\) production in a p38 MAPK-dependent manner.

Introduction

Mycobacterium tuberculosis is a Gram-positive bacterium with a distinctive lipid-rich cell wall that enters the body through the airways, resides in alveolar macrophages and causes chronic granulomatous inflammation. Despite the strong cellular and humoral immune responses in infected persons (1) and experimental animals (2), M. tuberculosis causes persistent latent infection in an estimated 2 billion people worldwide, and disease in 5-10% of infected immunocompetent persons. Tuberculosis remains the leading cause of death globally from a single pathogen, and this problem is exacerbated by the epidemic of HIV infection, which has created a large population of susceptible hosts. In addition, the spread of multidrug-resistant and extensively drug-resistant strains of M. tuberculosis, combined with the absence of an effective vaccine, make tuberculosis an urgent threat to public health (3,4). Therefore, it is imperative to develop improved antituberculosis vaccines, and vaccine development depends on a detailed understanding of the host-pathogen interactions during M. tuberculosis infection.

Early secreted antigenic target of 6-kDa...
(ESAT-6)$^3$ is a *M. tuberculosis* protein that elicits strong T cell recall immune responses in mouse models of infection, and is considered a promising vaccine candidate (5,6). However, accumulating evidence suggest that ESAT-6 and its molecular partner, culture filtrate protein of 10 kDa (CFP10), mediate virulence and pathogenicity. These proteins lack classical signal peptides, but are secreted through the ESAT-6 (ESX-1) or Type VII secretion system (7,8). ESAT-6 and CFP10 and related proteins associated with this secretion system are encoded by a cluster of genes which are located within the region of difference (RD)1 (9) of *M. tuberculosis*. RD1, which resembles the pathogenicity islands of several other human bacterial pathogens (10,11), is deleted in all the attenuated *M. bovis* bacillus Calmette-Guerin (BCG) strains (12) and the naturally attenuated strain of the *M. tuberculosis* complex, *M. microti* (12). Introduction of RD1 into BCG increased virulence in animal models of infection (12,13). These findings suggest that secretion of ESAT-6 and CFP10 is associated with virulence.

We have previously demonstrated that ESAT-6 directly binds to human T cells and inhibits IFN-γ production and proliferation without affecting cell viability and apoptosis (14). Since T cells and IFN-γ production are pivotal in protection against tuberculosis (15), identifying the underlining mechanisms by which ESAT-6 inhibits T cell IFN-γ production is critical to understand the pathogenesis of tuberculosis and design improved ESAT-6-based vaccines against tuberculosis infection.

In this study, we found that ESAT-6 activated p38 MAPK in T cells without inducing Ca$^{2+}$ influx, unlike the case for most bacterial pore-forming toxins that activate p38 MAPK. Neutralizing p38 MAPK with a specific chemical inhibitor or with siRNA rendered T cells resistant to ESAT-6-induced inhibition of IFN-γ production. ESAT-6 did not expand T regulatory cells (Tregs). We conclude that ESAT-6 inhibits human T cell IFN-γ production in a p38 MAPK-dependent manner.

**Materials and Methods**

**Human subjects**

Heparinized venous blood was obtained from 8 healthy donors with latent tuberculosis infection and 21 without latent tuberculosis infection, based on results of the QuantiFERON-TB GOLD test. All donors were employees or students at the University of Health Science Center at Tyler. Blood samples were collected after obtaining informed consent, as approved by the Institutional Review Board.

**Chemicals and bacterial preparations**

We used inhibitors for PI3K (LY294002), ERK (PD98059, both from InvivoGen), JNK (SP600125), PKC (Ro318220), p38 MAPK (SB203580) and AKT/PKB (124005, all from Calbiochem/EMD Biosciences). Heat-killed *M. tuberculosis* Erdman (provided by Dr. Patrick Brennan, Colorado State University, Fort Collins, CO) was used as antigen to stimulate peripheral blood mononuclear cells (PBMC) from persons with latent tuberculosis infection.

**Preparation of recombinant ESAT-6**

The original recombinant plasmid (pET23b) containing Rv3875 was obtained from Colorado State University at Fort Collins through the TB Vaccine Testing and Research Materials Contract. ESAT-6 was cloned from this plasmid and inserted into the same expression plasmid. After confirming the correct sequence of the inserted *esat-6* gene by commercial PCR sequencing, the plasmid was transformed into *Escherichia coli* BL21 (DE3) cells. The cells were cultured in LB medium and ESAT-6 expression was induced by addition of isopropyl -D-1-thiogalactopyranoside. Recombinant ESAT-6 was purified, LPS was removed, and its purity was >95%, based on SDS-PAGE, followed by Coomassie staining and Western blotting with anti-ESAT-6 mAb (HYB 76-8), as previously described (14). Recombinant ESAT-6
contained <50 pg of LPS per mg protein, by the QCL-1000 Limulus amebocyte assay, and was resuspended in HBSS (2 mg/ml), aliquoted, and stored at -70°C until use.

Cell preparation, culture, and detection of cytokines in culture supernatants

PBMC were isolated by Ficoll–Hypaque density gradient centrifugation of heparinized blood, and untouched CD3+ T cells were purified by immunomagnetic negative selection (Human Pan T cell isolation kit, Miltenyi Biotec). The purity of CD3+ cells was ≥95-98%, as measured by staining with FITC anti-CD3, followed by flow cytometry analysis with a FACSCalibur (BD Biosciences). PBMC or CD3+ cells from QuantiFERON-negative subjects were suspended at 2 × 10^6 cells/ml in RPMI 1640 medium (Invitrogen), supplemented with 10% heat-inactivated pooled human serum (Atlanta Biologicals), 100 units or 100 μg/ml penicillin and streptomycin, 1 mM sodium pyruvate and 0.1 mM MEM nonessential amino acids (all from Invitrogen).

PBMC or CD3+ cells were cultured with different concentrations of PK inhibitors or in media alone for 1 h at 37°C, 5% CO2, followed by incubation with 3.3 μM (20 μg/ml) recombinant ESAT-6. After 1 h, PBMC were stimulated with heat killed M. tuberculosis Erdman (2 μg/ml), and CD3+ cells were cultured in a 96-well plate precoated with 5 μg/ml anti-CD3 (OKT3, eBiosciences) plus 1 μg/ml anti-CD28 (CD28.2, BD Biosciences) in PBS at 37°C for 2 h. After 48-96 h, cell-free supernatants were collected and IFN-γ, IL-10 (both from BD Biosciences) and IL-17 (R&D Systems) levels were measured by ELISA.

Flow cytometry

For intracellular IFN-γ staining, purified CD3+ cells were treated with SB203580 for 1 h and ESAT-6 for 1 h, followed by stimulation with anti-CD3 and anti-CD28 for 48 h, as outlined above. GolgiStop™ (BD Biosciences, Palo Alto, CA) was added during the last 6 h of stimulation. The cells were collected, washed and stained with PE-anti-CD4 (eBiosciences). After 30 min surface staining, the cells were washed, fixed and permeabilized, then incubated with FITC-anti-IFN-γ (BD PharMingen) for 30 min at room temperature, according to the manufacturer’s protocols. The cells were washed and IFN-γ-positive cells were identified by flow cytometry with a FACS Calibur.

To quantify T regulatory cells (Tregs), purified CD3+ cells were cultured with anti-CD3 and anti-CD28, as outlined above. After 72 h, the cells were stained with PE-anti-CD4 (eBiosciences) and FITC-anti-CD25 (BD PharMingen), prior to intracellular staining with APC-anti-Foxp3 (eBiosciences). The stained cells were then analyzed by flow cytometry.

Real-time PCR

Total RNA was extracted from 8 × 10^5 CD3+ cells with TRIzol reagent (Invitrogen), cDNA was synthesized and IFN-γ cDNA was quantified by real-time PCR, with 18S ribosomal RNA as an endogenous control, as previously described (14). The relative quantity of IFN-γ mRNA was calculated by the ΔCt method (16).

Western blot

Total cell protein extracts were prepared from purified CD3+ cells and PBMC, and Western blotting was performed by standard methods, as previously described (14). Each nitrocellulose membrane was blotted, stripped, and rebotted with a series of primary Abs (all from Santa Cruz Biotechnology) to p38α MAPK, activating transcription factor (ATF)-2 (c-19) and GAPDH (FL-335). To detect phosphorylated MAPK, we used...
anti-phospho-ERK (Thr202/Tyr204), anti-phospho-JNK (Thr183/Tyr 185) and phosphor-p38 MAPK (Thr180/Tyr182) rabbit polyclonal antibodies, all from Cell Signaling. Anti-vinculin mAb (Sigma) was used to verify equal protein loading.

**Kinase Assay for p38 MAPK activity**

Purified human CD3⁺ cells (6-8 × 10⁶ cells per reaction) were incubated with ESAT-6 (3.3 µM) or media alone for different periods, and functional p38 MAPK activity was measured, using the p38 MAPK assay kit (Cell Signaling). Briefly, cellular protein extracts were prepared in ice-cold cell lysis buffer with phosphatase inhibitors and 1 mM PMSF, and activated p38 MAPK was immunoprecipitated from the cell extracts by incubating with bead-immobilized anti-phospho-p38 MAPK (Thr180/Tyr182) mAb at 4°C with gentle rocking. After incubation for 16 h, the beads were washed twice with cell lysis buffer, then twice with kinase buffer. The beads were then resuspended in 50 µl of kinase buffer, supplemented with 200 µM ATP, and incubated at 30°C with recombinant ATF-2 (encompassing amino acids 19-96) as substrate. After 30 min incubation, the kinase reaction was terminated by adding 3 × SDS-PAGE loading buffer. The samples were boiled for 5 min, resolved by SDS-PAGE, electroblotted to a nitrocellulose membrane and blotted with anti-phospho-ATF-2 (Thr76).

**Evaluation of calcium influx**

Freshly purified CD3⁺ cells from healthy donors were resuspended at 8 × 10⁶/ml in loading buffer (Hanks solution with 1 mM CaCl₂, 1 mM MgCl₂ and 1% FBS) and incubated with the Ca²⁺-indicator fluorescent dye, fluo-4 AM (Invitrogen), at 4 µg/ml at 37°C in the dark. After 30 min, the cells were washed once with loading buffer and resuspended at 2 × 10⁶/ml in loading buffer, supplemented with 10% FBS. The loaded cells were divided into three Eppendorf tubes with 1 × 10⁶ cells per tube, and cultured either in loading buffer alone as a negative control, with ESAT-6, or with OKT3 as a positive control. Some cells were incubated with 3.3 µM ESAT-6 in loading buffer at room temperature for 1 h. Other cells were incubated with OKT3 at 1 µg/ml on ice for 15 min, followed by incubation on ice with goat anti-mouse IgG at 20 µg/ml to crosslink cell-bound OKT3, as described previously (14). The cells with loading buffer alone were kept at room temperature. At the end of treatment, the cells in all three tubes were resuspended in 100 µl of ice cold loading buffer with 10% FBS after removing the free ESAT-6 and antibodies. All the tubes were incubated in a 37°C water bath for 5 min before recording cellular Ca²⁺ influx by flow cytometry for 250 sec.

**Transfection of siRNA**

Transfection of siRNA was performed, by a modification of previously described methods (17). Briefly, purified CD3⁺ cells were stimulated with PHA-L (Sigma) at 1 µg/ml for 20 h. The cells were collected, washed and resuspended at 8-10 × 10⁶/ml in a Dharmacon Accell siRNA delivery medium (Thermo Scientific), supplemented with 5% heat-inactivated pooled human serum, and plated in a 24-well plate at 400 µl per well. The cells were then transfected with scrambled siRNA or different concentrations of Smartpool siRNA for p38 MAPKα (MAPK14 on target plus smart pool) in 3 µl of DharmaFECT1 transfection reagent per well, based on pilot experiments (all reagents from Thermo Scientific). Fresh Dharmacon Accell siRNA delivery medium (2 ml per well) was then added with 10% human serum. Forty-eight h post-transfection, the cells were collected, washed with RPMI-1640 without serum and resuspended at 2 × 10⁶/ml in RPMI-1640 medium with 10% human serum. The cells were treated with ESAT-6 at 3.3 µM for 1 h at 37°C and 5% CO₂. The cells were then plated in a 96-well plate precoated with α-CD3 (5 µg/ml) and α-CD28 (1 µg/ml) and incubated at 37°C with 5% CO₂. After 72 h, the culture
supernatants were collected and IFN-γ levels were measured by ELISA. To determine the effect of siRNA on p38 MAPK expression, some cells were collected 48 and 72 h post-transfection, and lysed in 1 x SDS-PAGE loading buffer. After boiling for 8 min, the proteins were resolved on a 10% SDS-PAGE gel, electroblotted to a nitrocellulose membrane, and Western blotting was performed with anti-p38α MAPK (Santa Cruz Biotechnology). To control for protein loading, the blot was stripped and rebotted with anti-GAPDH.

Results

Inhibition of p38 MAPK reverses ESAT-6-mediated reduction of IFN-γ production by T cells

We have shown previously that ESAT-6 of M. tuberculosis binds to human T cells and inhibits T cell IFN-γ production and proliferation in response to stimulation with anti-CD3 and anti-CD28, without affecting cell viability or TCR proximal signaling pathways that activate Zap70 (14). We hypothesized that ESAT-6 inhibits IFN-γ production through effects on the major PKs, which control T cell cytokine production and proliferation, and are affected by toxins of several bacterial pathogens, including Helicobacter pylori (18,19), Bacillus anthracis (20-22) and Bordetella pertussis (23). To test this, we pretreated T cells from five healthy donors with different PK inhibitors, then stimulated T cells in the absence or presence of ESAT-6. The concentrations of inhibitors used were based on the literature and on our pilot experiments. Stimulation of T cells with anti-CD3 and anti-CD28 induced high levels of IFN-γ (14.207 ± 2.739 pg/ml), which were significantly reduced by ESAT-6 (4.147 ± 726 pg/ml), as we reported (14). Pretreatment with the p38 MAPK inhibitor, SB203580, at both concentrations tested, restored IFN-γ production by T cells in the presence of ESAT-6 (11.020 ± 1,469 pg/ml and 12,710 ± 1,444 pg/ml with SB203580, versus 4.147 ± 726 pg/ml with ESAT-6, p=0.007 for both comparisons, Fig. 1A). The other PK inhibitors did not affect ESAT-6 inhibition of IFN-γ production, and inhibitors of PI3K and PKC further reduced IFN-γ levels, perhaps because these kinases are required for T cell activation (24) and IFN-γ production (25).

To confirm the effects of p38 MAPK inhibition, we evaluated the effect of different concentrations of SB203580 on anti-CD3 plus anti-CD28-stimulated IFN-γ production by T cells from a larger group of 13 healthy donors (Fig. 1B). SB203580 did not affect IFN-γ production by anti-CD3 plus anti-CD28-stimulated cells, but negated the capacity of ESAT-6 to inhibit anti-CD3 plus anti-CD28-induced IFN-γ production in a dose-dependent manner. We next determined if the effect of SB203580 was mediated at the transcriptional level (Fig. 1C). ESAT-6 inhibited IFN-γ transcription in T cells, as we previously demonstrated (14). SB203580 did not affect anti-CD3 plus anti-CD28-induced IFN-γ mRNA expression by T cells, but completely abrogated the capacity of ESAT-6 to reduce anti-CD3 plus anti-CD28-stimulated IFN-γ transcription. Thus, the results from this study suggest that inhibition of p38 MAPK abrogates ESAT-6 inhibition of T cell IFN-γ production at the transcriptional level.

p38 MAPK inhibition does not affect the capacity of ESAT-6 to reduce production of IL-10 or IL-17

We previously showed that ESAT-6 inhibits T cell production of IL-10 and IL-17, as well as IFN-γ (14). We treated the T cells with different concentrations of SB203580 and ESAT-6, before stimulation with anti-CD3 and anti-CD28. In the absence of ESAT-6, addition of increasing concentrations of SB203580 reduced levels of IL-10 induced by anti-CD3 plus anti-CD28 stimulated in a dose-dependent manner with significant reduction at 20 µM concentration, (Figs. 1D). This confirms a previous report that IL-10 production by T cells in response to TCR stimulation depends in part on p38 MAPK (26).
Anti-CD3 and anti-CD28 stimulation induced increased IL-17 production by T cells, and presence of increased concentrations of SB203580 did not significantly reduce IL-17 production (Fig. 1E), suggesting that T-cell production of IL-17 is p38 MAPK independent. ESAT-6 reduced anti-CD3-stimulated IL-10 (Fig. 1D) and IL-17 levels (Fig. 1E) significantly, but these levels were not restored by preincubation with SC203580. Therefore, inhibition of p38 MAPK abrogated the inhibitory effect of ESAT-6 on T cell IFN-γ production, but did not affect ESAT-6 inhibition of IL-10 and IL-17 production.

ESAT-6 inhibits IFN-γ production by both CD4+ and CD8+ T cells through p38 MAPK

To determine if p38 MAPK mediates the effects of ESAT-6 on IFN-γ production and the number of IFN-γ producing CD4+ and CD8+ T cells, we used intracellular staining and flow cytometry analysis to evaluate IFN-γ-producing cells. Stimulation of T cells with anti-CD3 and anti-CD28 markedly increased the percentage of IFN-γ-producing CD4+ and CD8+ cells (Fig. 2A). ESAT-6 reduced the frequency of IFN-γ+ cells by 50-80%, with similar degrees of reduction for CD4+ and CD8+ cells (Figs. 2A and B). Treatment of cells with SB203580 before ESAT-6 largely restored the percentages of IFN-γ+CD4+ and CD8+ cells (Figs. 2A and B). ESAT-6 inhibited anti-CD3 plus anti-CD28-induced T cell proliferation and IFN-γ production (14). To determine if ESAT-6 inhibition of T cell proliferation is mediated through the p38 MAPK pathway, we used CFSE stained T cells and flow cytometry to analyze T cell proliferation (Fig. 3). ESAT-6 markedly inhibited proliferation of both CD4+ and CD8+ cells, and pretreatment with SB203580 enhanced proliferation of ESAT-6-treated T cells. However, this effect was not statistically significant, and was much less than the effect of SB203580 on IFN-γ production (compare Figs. 3B and 1B). Therefore, ESAT-6-mediated inhibition of T cell proliferation is not mediated primarily through p38 MAPK, and the increase in IFN-γ-producing cells by p38 MAPK inhibition (Fig. 2) is not merely due to increased T cell proliferation.

ESAT-6 inhibition of T cell IFN-γ production is AKT kinase-independent

High concentrations of the p38 MAPK inhibitor, SB203580, can also inhibit AKT in T cells as an off-target effect to reduce IL-2-dependent proliferation of a murine T cell line (27). To determine if the same mechanism (AKT) contributed to the effects of SB203580 observed above, we used an AKT-specific inhibitor. The results (Fig. 4) demonstrated that inhibition of AKT did not affect ESAT-6-mediated suppression of IFN-γ production and proliferation (not shown). This suggests that ESAT-6 inhibits IFN-γ production through p38 MAPK but not through AKT.

ESAT-6 inhibition of T cell IFN-γ production is not mediated by Tregs

p38 MAPK is critical for generation of induced Tregs (28) and for Tregs to exhibit suppressor function (29,30). To determine if the effects of p38MAPK inhibition observed above were mediated through reducing the capacity of ESAT-6 to enhance Treg function, we studied the effect of ESAT-6 on generation of Tregs by staining for FoxP3 and CD25. ESAT-6 reduced the percentage of anti-CD3 plus anti-CD28-stimulated FoxP3+CD25+ cells (Figs. 5A and B), indicating that inhibition of IFN-γ production by ESAT-6 was not due to increased numbers of Tregs. Addition of SB203580 to ESAT-6-treated cells increased the number of Tregs, perhaps because Tregs typically expand when the number of activated T cells increases.
These findings indicate that the effects of SB203580 and ESAT-6 on IFN-γ production in our experimental system were unlikely to be mediated by Tregs.

**ESAT-6 directly activates p38 MAPK in T cells**

Because our data demonstrated that ESAT-6 inhibits IFN-γ production through p38 MAPK, we hypothesized that ESAT-6 directly activates p38 MAPK in T cells. To test this, we incubated freshly isolated T cells with 3.3 μM of ESAT-6 for different time points and evaluated the activation of p38 MAPK. Treatment of T cells with ESAT-6 for 30-60 mins induced phosphorylation of p38 MAPK, based on Western blotting with anti-phospho-p38 MAPK (Thr180/Tyr182), which detects the active form of the kinase (Fig. 6A). ESAT-6 did not phosphorylate ERK or JNK. We next immunoprecipitated the ESAT-6-induced phosphorylated p38 MAPK from T cell extracts, and measured its functional kinase activity, based on phosphorylation of its specific substrate, ATF-2. Incubation of T cells with ESAT-6 for as little as 15 min induced p38 MAPK activity and this increased further until 60 min post-incubation (Fig. 6B). Western blotting also demonstrated increased ATF-2 phosphorylation in ESAT-6-treated T cells, compared with untreated cells (not shown), confirming that ESAT-6 induced p38 MAPK activity in T cells. These results strongly suggest that ESAT-6 directly activates p38 MAPK in T cells.

**ESAT-6 does not induce Ca^{2+} influx in T cells**

Many bacterial virulence factors are pore-forming toxins that induce extracellular calcium influx (31), which in turn activates p38 MAPK as a cellular protective mechanism (31-33). ESAT-6 can induce cytolysis of alveolar epithelial cells and macrophages (34-36) and cause pore formation in mammalian cells (37). To determine if ESAT-6 activates p38 MAPK through pore formation and increased calcium influx in our experimental system, we loaded T cells with a Ca^{2+}-sensitive fluorescent dye, then treated the cells with ESAT-6 and evaluated the changes in intracellular calcium by flow cytometry. ESAT-6 did not affect intracellular calcium levels (Fig. 7). In contrast, α-CD3 cross-linking significantly increased cellular calcium, compared to that in control cells incubated in medium alone, suggesting that ESAT-6-mediated activation of p38 MAPK is not due to membrane pore formation and increased cellular calcium levels.

**Inhibition of p38 MAPK by siRNA reverses ESAT-6-mediated reduction of IFN-γ production by T cells**

Our results above show that a chemical inhibitor of p38 MAPK reverses ESAT-6-mediated reduction of IFN-γ production by T cells (Fig. 1). To confirm that this was due to inhibition of p38 MAPK, we silenced p38α MAPK in T cells with siRNA, then incubated the cells with ESAT-6 and measured anti-CD3-induced IFN-γ production. In four donors, p38α MAPK siRNA reduced p38α expression in a dose-dependent manner, with 40 nM lowering expression by > 85%, compared to scrambled siRNA at the same concentration (Fig. 8A). p38 MAPK siRNA also abrogated the capacity of ESAT-6 to inhibit IFN-γ production in response to anti-CD3 plus anti-CD28 (Fig. 8B), indicating that p38α MAPK is required for this effect. We conclude that ESAT-6 inhibits IFN-γ production by directly activating p38 MAPK in T cells.

**ESAT-6 inhibits M. tuberculosis-induced IFN-γ production through p38 MAPK**

Our results above show that ESAT-6 inhibits IFN-γ production in response to T cell receptor stimulation in a p38 MAPK-dependent manner. To determine if ESAT-6 also inhibits IFN-γ production in response to bacterial antigens through this mechanism, we evaluated the response of SB203580-treated PBMC to stimulation with heat-killed *M. tuberculosis*. Pretreatment of PBMC with SB203580
abrogated the capacity of ESAT-6 to inhibit antigen-induced IFN-\(\gamma\) production (16,216 ± 3,210 pg/ml vs 6,806 ± 2,557 pg/ml, p<0.05, Fig. 9A), and SB203580 did not affect M. tuberculosis-stimulated IFN-\(\gamma\) production by PBMC in the absence of ESAT-6. When PBMC were incubated with ESAT-6, Western blotting showed marked accumulation of phosphorylated p38 MAPK over 60 mins (Fig. 9B). ERK was activated to a lesser extent, and JNK MAPK was not affected. These findings suggest that ESAT-6 inhibits antigen-induced and T cell receptor-elicited IFN-\(\gamma\) production through p38 MAPK activation.

Discussion

ESAT-6 is a secreted protein of M. tuberculosis that contributes to virulence through multiple mechanisms, including direct suppression of T cell production of IFN-\(\gamma\), which is central to immune defenses against mycobacteria. In the current report, we demonstrated that inhibition of p38 MAPK with a chemical inhibitor or with siRNA abrogated the ESAT-6-mediated reduction of IFN-\(\gamma\) production by anti-CD3-stimulated T-cells and M. tuberculosis-stimulated PBMC, affecting both CD4+ and CD8+ T cells. Inhibition of p38 MAPK reversed the effects of ESAT-6 on IFN-\(\gamma\) production at the IFN-\(\gamma\) mRNA expression, and these effects are neither through Tregs nor AKT. These effects were specific for IFN-\(\gamma\), as inhibition of p38 MAPK did not affect the capacity of ESAT-6 to reduce production of IL-10 and IL-17. Stimulation of T cells with ESAT-6 induced phosphorylation and increased functional kinase activity of p38 MAPK, but not ERK or JNK. Our results demonstrate that ESAT-6 inhibits T-cell IFN-\(\gamma\) production by inducing functional p38 MAPK activity in T cells, providing a new mechanism by which M. tuberculosis subverts host immune responses.

As a facultative intracellular pathogen that establishes chronic infection in humans, M. tuberculosis has evolved multiple mechanisms to affect intracellular signaling pathways to subvert host immunity(38). Most studies have focused on the effects of M. tuberculosis on mononuclear phagocytes, particularly on the signaling pathways that affect phagosomal maturation and apoptosis. M. tuberculosis activates p38 MAPK to reduce recruitment of early endosome autoantigen 1 to the phagosomal membranes and delay phagosomal maturation (39). In addition, M. tuberculosis activates p38 MAPK in monocytes through complement receptor 3 to reduce CD1 expression on monocyte-derived dendritic cells, limiting their capacity to present mycobacterial antigens to T cells (40). M. tuberculosis also activates p38 MAPK in neutrophils to induce apoptosis and inhibit expression of chemokine receptor, CXCR2 (41-43). Activation of p38 MAPK in macrophages and neutrophils is thought to be mediated by mannose-capped lipoarabinomannan, the major component of the M. tuberculosis cell wall (43). Thus, these studies suggest that M. tuberculosis activates p38 MAPK in neutrophils and monocytes to attenuate host immunity against M. tuberculosis. Our current results provide the first evidence that the secreted mycobacterial protein, ESAT-6, activates p38 MAPK in human T cells to inhibit production of T cell IFN-\(\gamma\), which is central for protective immunity against tuberculosis infection. This mechanism is reminiscent of that used by Herpes simplex virus, which activates p38 MAPK in infected T cells, inducing expansion of IL-10-producing T cells instead of protective IL-2- and IFN-\(\gamma\)-producing T cells to subvert host immune response (44).

MAP, composed of ERK, JNK and p38 MAPK, play crucial roles in regulating cellular activation, cytokine production, cell death and proliferation of T cells (45). Functional activity of MAPK requires phosphorylation of critical amino acids, and activation of p38 MAPK depends on dual phosphorylation at Thr180 and Tyr182 of its substrate recognition site, through
the MAPK activation cascade (46). Several bacterial toxins, including the *H. pylori* vacuolating toxin (18,19), *B. anthracis* lethal toxin (20-22) and *B. pertussis* adenylate cyclase (23), activate p38 MAPK by increasing Ca\(^{2+}\) influx through forming cell membrane pores in multiple cell types. However, ESAT-6 did not act through this mechanism as it did not affect cell viability (14) or Ca\(^{2+}\) influx (Fig. 7). p38 MAPKs are believed to be downstream signaling molecules that are mobilized after cellular activation by TLRs or cytokines. Although ESAT-6 was reported to act through TLR-2 in macrophages to inhibit TLR4-stimulated IL-12 production (47), this is unlikely to be the pathway for p38 MAPK activation in T cells, as ESAT-6 reduced anti-CD3 plus anti-CD28-stimulated TLR2 expression on T cells and anti-TLR-1/2 antibodies had no effect on ESAT-6-mediated inhibition of anti-CD3-induced IFN-\(\gamma\) production (B Samten, unpublished data). Furthermore, activation induces T cells to express TLR2, which acts as a costimulatory receptor to enhance T cell proliferation and IFN-\(\gamma\) production (48).

ESAT-6-mediated activation of p38 in T cells is unlikely to be the consequence of autocrine effects of ESAT-6-induced cytokines, because p38 MAPK activation occurred only 15 min after addition of ESAT-6. This period is too short for secretion of sufficient concentrations of inflammatory cytokines to activate p38 MAPK. ESAT-6 was shown to stimulate macrophages to produce NO and express surface molecules possibly through MAPK pathways (49). Thus, the mechanism by which ESAT-6 activates p38 MAPK in T cells remains uncertain, but is a fruitful topic for future investigation.

Because *M. tuberculosis* is an intracellular pathogen that resides in phagosomes surrounded by bilayer membranes, how would a secreted protein, such as ESAT-6, contact T cells to modulate p38 MAPK activity during infection *in vivo*? Recent studies have shown that exosomes, which are membrane-coated vesicles, are released by *M. tuberculosis*-infected macrophages, contain ESAT-6 and other mycobacterial proteins (50) and stimulate naïve T cells (51). These studies support the possibility that ESAT-6 may come in contact with T cells during infection.

The role of p38 MAPK in T cell proliferation and cytokine production depends on the mode of T cell stimulation. When T-cells are activated by cytokines such as IL-12 and IL-18, p38 MAPK enhances IFN-\(\gamma\) gene transcription, in part through activation of the IL-12-responsive transcription factor, STAT4 (52-55). In contrast, when T cells are activated through the T cell receptor, p38 MAPK inhibits proliferation and does not affect IFN-\(\gamma\) production (56,57). p38 MAPK activation can also inhibit cytokine production, as it reduces IL-2 and IFN-\(\gamma\) production by NKT cells in mice (58). We found that ESAT-6-mediated inhibition of anti-CD3-stimulated production of IFN-\(\gamma\), but not IL-10 or IL-17, depended on activation of p38 MAPK, suggesting a specific effect of p38 MAPK on ESAT-6 inhibition of IFN-\(\gamma\) production. p38 MAPK activation can dampen T cell responses by enhancing the expansion and functional activity of Tregs (28,29), but ESAT-6 does not increase the numbers of FoxP3\(^+\) Tregs (Fig. 5), suggesting that ESAT-6-mediated p38 MAPK activation does not inhibit IFN-\(\gamma\) production through this mechanism. We considered the possibility that ESAT-6 reduces the supply of p38 MAPK available to T-cells. However, this is unlikely because p38 MAPK is necessary for production of Th2 cytokines but dispensable for production of IFN-\(\gamma\) by T cell receptor-activated primary T-cells (56,57) or by T cells from mice with a p38\(\alpha\) MAPK gene deletion (59). Consistent with these reports, our data demonstrated that inhibition of p38 MAPK did not affect T cell IFN-\(\gamma\) production in response to either anti-CD3 or *M. tuberculosis* antigens, in the absence of ESAT-6 (Figs. 1B and
9. The molecular mechanisms for ESAT-6 inhibition of T cell IFN-\(\gamma\) production via activated p38 MAPK remain to be explored. In summary, our findings provide the first evidence that a microbial product can activate p38 MAPK to inhibit production of a cytokine that is central to protective immunity against \(M.\) \textit{tuberculosis} and other intracellular pathogens. Further studies to decipher the pathways by which ESAT-6 mediates these effects will provide insight into the mechanisms for pathogen-mediated inhibition of T cell responses and facilitate development of immunomodulatory therapy to reverse these effects.

Reference list
42. Aleman, M., Schierloh, P., de la Barrera, S. S., Musella, R. M., Saab, M. A., Baldini, M.,

Footnotes:
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Abbreviations used: ESAT-6, early secreted antigenic target protein of 6-kDa; CFP10, culture filtrate protein of 10 kDa; Tregs, T regulatory cells.

**Figure legends**

**Figure 1. Effects of PK inhibitors on ESAT-6 inhibition of T cell IFN-γ production.**

A. Purified T cells from 5 healthy individuals were incubated with medium or different PK inhibitors for 1 h, then cultured with ESAT-6 (3.3 μM) for 1 h. The cells were then plated in a 96-well plate coated with anti-CD3 and anti-CD28 for 48 h, and IFN-γ concentrations in the supernatants were measured by ELISA. IFN-γ levels were negligible in supernatants of unstimulated cells. Means and SEMs are shown. * p=0.007, compared to ESAT-6-treated cells without inhibitors.

B. Purified T cells from 13 healthy individuals were treated with medium or SB203580, then with or without ESAT-6, and incubated with anti-CD3 and anti-CD28 for 48 h, as in panel A. IFN-γ levels in the supernatants were measured by ELISA. Means and SEMs are shown. * p=0.03, ** p=0.001 compared to cells treated with ESAT-6 alone.

C. Purified T cells from four donors were treated as described in panel B, total cellular RNA was isolated and IFN-γ mRNA was measured by realtime PCR, using 18S rRNA as an internal control. Means and SEMs are shown. * p<0.05 compared to the cells with ESAT-6 without SB203580.

D. and E. Purified T cells from five donors were treated as described in panel B and the concentrations of IL-10 (D) and IL-17 (E) in the culture supernatants were measured by ELISA. Means and SEMs are shown. * p<0.05 compared to the cells stimulated with anti-CD3 and anti-CD28 with no SB203580 (D).

**Figure 2. Effect of inhibition of p38 MAPK on ESAT-6-mediated reduction of IFN-γ+ CD4+ and CD8+ T cells.**

Purified T cells from healthy subjects were treated with medium or different concentrations of SB203580 for 1 h, then with or without 3.3 μM ESAT-6 for 1 h. The cells were then cultured with plate-bound anti-CD3 and anti-CD28. After 48 h, the cells were collected and stained with anti-CD4 and anti-IFN-γ and analyzed by flow cytometry. A. A representative result is shown. B. Means and SEMs for results with five donors are shown. * p<0.05 compared to cells treated with ESAT-6 alone.

**Figure 3. Effect of inhibition of p38 MAPK on ESAT-6-mediated reduction of anti-CD3-induced T cell proliferation.**

Purified T cells from four donors were labeled with CFSE and treated with medium or different concentrations of SB203580 for 1 h, then with or without 3.3 μM ESAT-6 for 1 h. The cells were then cultured with plate-bound anti-CD3 and anti-CD28. After 96 h, the cells were stained with PE-anti-CD8, and cell proliferation was analyzed by CFSE dilution, using flow cytometry. A. A representative result is shown. B. Means and SEMs of four different experiments are shown for CD4+ and CD8+ cells.

**Figure 4. ESAT-6 inhibition of T cell IFN-γ production is independent of AKT.**

Purified T cells were treated with medium or different concentrations of AKT inhibitor for 1 h, then with or without 3.3 μM ESAT-6 for 1 h. The cells were then cultured with plate-bound anti-CD3 and anti-CD28 for 48 h. The supernatants were collected and IFN-γ concentrations were measured by ELISA. Means and SEMs for results with four donors are shown.

**Figure 5. The effects of ESAT-6 and SB203580 on IFN-γ production are not mediated by effects on Tregs.**

Purified human T cells were treated with medium or SB203580 for 1 h, then with or without 3.3 μM ESAT-6 for 1 h. The cells were then cultured with plate-bound anti-CD3 and anti-CD28. After 72 h, the cells were collected, washed and stained with anti-CD4 and anti-CD25,
followed by intracellular staining with anti-Foxp3. After gating on CD4+ cells, the frequency of CD25+ Foxp3+ cells (Tregs) was determined. A. A representative result is shown. B. Means and SEMs from experiments with four donors are shown. * p<0.05, compared to ESAT-6-treated cells without SB203580.

**Figure 6. ESAT-6 directly activates p38 MAPK in T cells.**

A. Purified T cells were incubated with 3.3 µM ESAT-6 for different periods, or stimulated with phorbol myristate acetate and ionomycin (PI) for 5 min as a positive control. The cells were collected and total protein extracts were prepared. Western blotting was performed with phosphospecific antibodies against p38, ERK and JNK. The same blot was stripped and blotted with vinculin as a protein loading control. A representative result of experiments performed on T cells from four donors is shown. B. T cells were treated with 3.3 µM ESAT-6 for different periods and total cell protein extracts were prepared. Phosphorylated p38 MAPK was immunoprecipitated with anti-phospho-p38 and the kinase activity in the immunoprecipitates was determined by Western blotting to measure the ability to phosphorylate the p38 MAPK substrate, recombinant ATF-2, as detailed in the methods. Total p38 MAPK levels in the protein extracts before immunoprecipitation, measured by Western blotting, were used as input controls. A representative result from experiments with three donors is shown.

**Figure 7. Effect of ESAT-6 on calcium influx in T cells.** Purified T cells from healthy donors were loaded with a Ca2+-sensitive fluorescent dye, then cultured in medium alone, with ESAT-6 or with anti-CD3 and anti-CD28 for 1 h at room temperature. The cells were then incubated at 37°C for 5 min, after which Ca2+ influx was measured for 250 secs (x-axis) by flow cytometry as the mean fluorescence intensity for fluo-4 (y axis). The basal Ca2+ influx in untreated cells is shown as the gray area. A representative result from four experiments is shown.

**Figure 8. The effect of silencing p38 MAPK on ESAT-6-mediated inhibition of T cell IFN-γ production.** Purified human T cells were transfected with scrambled or p38 MAPK siRNA for 48 h. A. The silencing efficiency of siRNA was evaluated by Western blotting of total T cell protein extracts with anti-p38α MAPK. The membranes were stripped and blotted with anti-GAPDH to control for protein loading. A representative result of four different experiments is shown. B. siRNA-transfected cells were incubated, with or without 3.3 µM of ESAT-6, for 1 h before further treatment with plate-bound anti-CD3 and anti-CD28. After 72 h, the supernatants were collected and IFN-γ levels were measured by ELISA. Means and SEMs from experiments with five donors are shown. * p<0.05, compared to ESAT-6-treated cells with scrambled siRNA.

**Figure 9. ESAT-6 inhibits IFN-γ production by M. tuberculosis-stimulated PBMC through activation of p38 MAPK.** A. PBMC from seven donors with latent tuberculosis infection were treated with medium or SB203580 (20 µM) for 1 h, then with or without 3.3 µM ESAT-6 for 1 h. The cells were then incubated with heat-killed *M. tuberculosis*. After 48 h, supernatants were collected and IFN-γ concentrations were measured by ELISA. Means and SEMs are shown. * p=0.04, compared to ESAT-6-treated cells. B. PBMC from three donors with latent tuberculosis infection were incubated with 3.3 µM ESAT-6 for different time periods, total cell protein extracts were prepared and activation of p38, ERK and JNK MAPK were evaluated by Western blotting with phosphor-specific antibodies. Vinculin was used as a protein loading control. PBMC were stimulated with phorbol myristate acetate and ionomycin (P/I) as a positive control. A representative result is shown.
Figure 2

A

B

The graphs illustrate the percentage of IFN-γ+ cells in CD4+ and CD8+ cell populations treated with different conditions.

- **A:** Scatter plots showing the distribution of IFN-γ+ cells with various treatments.
  - Medium
  - α-CD3/CD28
  - α-CD3/CD28/ESAT-6
  - α-CD3/CD28/ESAT-6/SB (10 μM)
  - α-CD3/CD28/ESAT-6/SB (20 μM)

- **B:** Bar graphs comparing the percentage of IFN-γ+ cells in CD4+ and CD8+ cell populations.
  - CD4+ cells:
    - Medium
    - α-CD3/CD28
    - α-CD3/CD28/ESAT-6
    - α-CD3/CD28/ESAT-6/SB (10 μM)
    - α-CD3/CD28/ESAT-6/SB (20 μM)
  - CD8+ cells:
    - Medium
    - α-CD3/CD28
    - α-CD3/CD28/ESAT-6
    - α-CD3/CD28/ESAT-6/SB (10 μM)
    - α-CD3/CD28/ESAT-6/SB (20 μM)

Statistical significance is indicated by asterisks (*) for certain conditions.
Figure 3

A

SB203580

None  5 μM  10 μM  20 μM

α-CD3/CD28

α-CD3/CD28 + ESAT-6

B

% proliferation

CD4+ cells  CD8+ cells

No ESAT-6  ESAT-6  No ESAT-6  ESAT-6

Medium  No SB  SB203580 (5 μM)  SB203580 (10 μM)  SB203580 (20 μM)

N.S.

N.S.
Figure 4

![Graph showing IFN-γ levels with different conditions.]

- Medium
- α-CD3/CD28
- α-CD3/CD28/AKT I (5 μM)
- α-CD3/CD28/AKT I (10 μM)
- α-CD3/CD28/AKT I (20 μM)
Figure 5

A

No SB203580

SB203580

α-CD3/CD28

α-CD3/CD28 + ESAT-6

B

% CD25^+Foxp3^+ cells

No ESAT-6

ESAT-6

Medium

α-CD3/CD28

α-CD3/CD28/SB203580

*
Figure 6

A

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Figure 7
**Figure 8**

A. Immunoblot analysis showing the expression of p38 and GAPDH under different conditions.

B. Quantitative analysis of IFN-γ levels with and without ESAT-6, treatment with scrambled and p38 (20 nM and 40 nM) conditions. The graph shows a significant increase in IFN-γ levels with ESAT-6 treatment compared to other conditions, indicated by the asterisk (*).
Figure 9

A

B

**M. tb**

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- p-p38
- p-Erk
- p-JNK
- Vinculin
The mycobacterium tuberculosis early secreted antigenic target of 6 kDa inhibits T cell interferon-\(\gamma\) production through the p38 mitogen-activated protein kinase pathway

Hui Peng, Xisheng Wang, Peter F. Barnes, Hua Tang, James C. Townsend and Buka Samten

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