Results
RESULTS

Cloning, expression and purification of recombinant *Mycobacterium tuberculosis* (Mtb) 6-kDa Early Secreted Antigenic Target (ESAT-6) protein

The open reading frame *Rv3875*, encoding ESAT-6 protein of *M. tuberculosis*, was amplified by PCR from the genomic DNA of a local clinical isolate, by using the following primers:

Forward, 5'-GGAATTCCATATGACAGCAGCAGTGGAA TTTCG -3',
Reverse, 5'- CCGCTCGAGTGCGAACATCCCAGTGACGTTGC-3' (*NdeI* and *XhoI* sites, respectively, are underlined). The PCR product obtained was of correct size (305bp) (Fig.1). The PCR product was gel-purified and directly cloned into an intermediate vector, pGEM-T-Easy® and the nucleotide sequence of the gene was validated and deposited with GenBank (Accession number, AF420491). Full-length authentic gene was then sub-cloned into bacterial expression vector pET23b+; this vector yielded satisfactory levels of polyhistidine-tagged recombinant ESAT-6 protein expressed as an insoluble protein in *E. coli* BL21(DE3)pLysS as inclusion bodies. The inclusion bodies were solubilized in 8M Urea pH 8.0. Recombinant ESAT-6 was purified by nickel-nitrilotriacetic acid (Ni^{2+}-NTA, Qiagen, Valencia, CA, USA) metal affinity chromatography according to the manufacturer's recommendations for purification of proteins under denaturing conditions. The SDS-PAGE analysis (Fig.2A) of the purified protein revealed it to be essentially (>90% pure) homogenous preparation. In an
immunoblot (Fig.2B), the purified ESAT-6 was recognized specifically by a mouse antipolyhistidine monoclonal antibody. The protein preparation was dialyzed against several changes of 10mM Na₂HPO₄ pH 8.0 to remove the urea and then against phosphate-buffered saline and stored in small aliquots at -20°C. In order to rule out the possibility of endotoxin contamination in the recombinant protein preparation, the endotoxin levels in each batch of ESAT-6 were determined using E-Toxate kit (Sigma). The endotoxin level did not exceed 0.03 endotoxin units in any batch of the purified recombinant ESAT-6 protein.

M 1 2 3 4

Figure.1

Figure.1 The ORF encoding Rv3875 was amplified from the genomic DNA of a local clinical isolate by PCR. M: 100bp DNA ladder (Promega), Lanes 1-4: PCR products.
Figure 2 – (A) The gel represents silver staining of a 12% SDS-PAGE; (B) Western blot using polyclonal anti-polyhistidine antibody (Sigma). M=Molecular weight marker, 1=Uninduced lysate, 2=Induced Lysate, 3=Flow through, 4=Wash 1 (8M Urea, pH 6.5), 5=Wash 2 (8M Urea, pH 6.5), 6=Wash 3 (8M Urea pH 5.9), 7=Elution 1 (8M Urea, pH 4.5), 8=Elution 2 (8M Urea, pH 4.5).

**ESAT-6 bound to the surface of RAW264.7 cells**

To determine whether ESAT-6 binds to the surface of RAW264.7 cells, 1x10^6 RAW264.7 cells were incubated with 5μg of FITC-conjugated ESAT-6 for 45 minutes in dark at 4°C, then cells were washed with FACS buffer and centrifuged at 2500 rpm for 5 minutes at 4°C. The supernatant was discarded and the cell pellet was resuspended in 200μl of 4% paraformaldehyde (PFA). The cells were then acquired and analysed in FACSCalibur (Becton-Dickinson). There was a 4-fold shift in mean fluorescence intensity (MFI) compared to the unstained cells (Fig.3) showing that ESAT-6 binds to the surface of RAW264.7 cells. To show the specificity of binding, RAW264.7 cells were
incubated with either 10-fold or 50-fold excess of unlabeled ESAT-6, then washed with FACS buffer and then stained with 5μg of FITC-conjugated ESAT-6 as before and then cells were washed and fixed. Addition of 10-fold excess of unlabeled ESAT-6 caused 50% decrease in MFI compared to the set without any unlabeled ESAT-6 while with 50-fold excess ESAT-6 there was complete reversal of Mean Fluorescence Intensity (MFI) to the level of unstained cells.

Figure.3

Figure.3 – Binding of ESAT-6 on the surface of RAW264.7 were assessed by flow cytometer. Unstained RAW264.7 cells are represented as normal line, the bold line represents cells incubated with 5μg of FITC-conjugated ESAT-6, the dotted line represents cells preincubated with 10-fold excess (50μg) of unlabeled ESAT-6 and then stained with 5μg of FITC-conjugated ESAT-6 and the broken line indicates cells preincubated with 50-fold excess (250μg) of unlabeled ESAT-6 and then stained with
5μg of FITC-conjugated ESAT-6. After staining cells were washed and fixed and acquired in FACSCalibur.

**ESAT-6 triggered phosphorylation of extracellular signal regulated kinases 1/2 mitogen -activated protein kinase (ERK1/2) in the cytoplasm but not in the nucleus**

To identify the signaling pathways triggered by ESAT-6, the extracellular signal regulated kinases 1/2 of the MAP kinase family was studied. The mitogen activated protein (MAP) kinases play diverse roles in the cell, ranging from apoptosis, cell differentiation, cell proliferation, stress response, production of proinflammatory cytokines etc., (van der Bruggen et al, 1999). The virulent strains of mycobacteria cause greater inhibition of MAP kinases, particularly ERK1/2 pathway, as compared to avirulent strains (Blumenthal et al, 2002; Roach and Schorey, 2002).

In our studies RAW264.7 cells were stimulated for different time intervals of 0, 15, 30, 60 and 120 minutes with 5μg/ml of ESAT-6 protein. The cells were harvested after the indicated time points and lysed as described in Materials and Methods. The cytoplasmic and nuclear extracts were then run on 10% SDS-PAGE and probed for phospho-ERK1/2 after western blotting. It was found that ESAT-6 caused phosphorylation of ERK1/2 in the cytoplasm in a time-dependent manner (Fig.4A); phosphorylation of ERK1/2 increased by 1.5 fold at 15 minutes and by 2.5 fold at 120 minutes compared to unstimulated cells. Normally ERK1/2, after phosphorylation, would translocate to the nucleus but in this case no phospho-ERK1/2 could be detected in the nucleus (Fig.4C). The total ERK1/2 levels were found to remain unchanged in cytoplasm (Fig.4B) and in nucleus (Fig.4D) over the experimental time period. We checked for the
phosphorylation of another MAP kinase p38, ESAT-6 did not have any effect on phosphorylation of p38 in cytoplasm (Fig.4E) and nucleus (Fig.4G). Total p38 levels remained constant in cytoplasm (Fig.4F) and nucleus (Fig.4H).

Figure.4

Figure.4 – (A) and (C) Phosphorylation of ERK1/2 in cytoplasm and nucleus respectively at 0, 15, 30, 60 and 120 minutes upon stimulation with 5μg/ml of ESAT-6. (B) and (D) Total ERK1/2 levels in cytoplasm and nucleus respectively over the same time period. (E) and (G) Phosphorylation of p38 in cytoplasm and nucleus respectively at 0, 15, 30, 60 and 120 minutes upon stimulation with 5μg/ml of ESAT-6. (F) and (H) Total p38 levels in cytoplasm and nucleus respectively over the same time period.
LPS triggered phosphorylation of ERK1/2 in both cytoplasm and nucleus

The absence of phospho-ERK1/2 from the nucleus of ESAT-6 stimulated RAW264.7 cells was not a cell type specific activity; rather, it was specific for ESAT-6. To prove this, RAW264.7 cells were stimulated with 0.1µg/ml of Lipopolysaccharide (LPS) for the same time points as before. LPS, which is a general activator of macrophages (van der Bruggen et al, 1999; Yang et al, 1999; Hopkins et al, 1995) triggered time-dependent phosphorylation of ERK1/2 in both cytoplasm (Fig.5A) and nucleus (Fig.5C) indicating that absence of phospho-ERK1/2 from the nucleus was specific for ESAT-6.

Next we wanted to know whether LPS can overcome the ESAT-6 induced inhibition of phosphorylation of ERK1/2 in nucleus; for this RAW264.7 cells were co-stimulated for the same time intervals with LPS (0.1µg/ml) and ESAT-6 (5µg/ml). Co-stimulation with LPS and ESAT-6 resulted in diminished phosphorylation of ERK1/2 in the nucleus (Fig.6C) as compared to stimulation by LPS alone. In the cytoplasm (Fig.6A), however, there was no significant change. Thus ESAT-6 had an opposing effect on LPS-induced phosphorylation of ERK1/2, thereby dampening the MAP kinase signaling in the nucleus. The levels of total ERK1/2 were constant in both cytoplasm (Fig.6B) and the nucleus (Fig.6D) over the experimental time points.
Figure.5 – (A) Phosphorylation of ERK1/2 in the cytoplasm upon stimulation with LPS (0.1μg/ml) for 0, 15, 30, 60 and 120 minutes. (C) Phosphorylation of ERK1/2 in the nucleus. (B) and (D) represents total ERK1/2 levels in cytoplasm and the nucleus respectively over the same time points.

Figure.6 – (A) Phosphorylation of ERK1/2 in the cytoplasm upon stimulation with LPS (0.1μg/ml) and ESAT-6 (5μg/ml) for 0, 15, 30, 60 and 120 minutes. (C) Phosphorylation of ERK1/2 in the nucleus. (B) and (D) represents total ERK1/2 levels in cytoplasm and the nucleus respectively over the experimental time points.
Diminished ERK1/2 activation in the nucleus by ESAT-6 was due to some tyrosine phosphatase

To determine whether the absence of phospho-ERK1/2 from the nucleus of ESAT-6-treated cells was due to some putative phosphatase(s) present in the nucleus, RAW264.7 cells was stimulated with ESAT-6 (5μg/ml) in presence of 1mM sodium orthovanadate (Na3VO4), a tyrosine phosphatase inhibitor (Sugano et al, 2004) for 0, 15, 30, 60 and 120 minutes. Stimulation of cells with ESAT-6 along with sodium orthovanadate causes appearance of phospho-ERK1/2 in the nucleus (Fig.7C) with a 4-fold increase in phosphorylation at 15 minutes and upto 10-fold at 60 minutes thus corroborating the involvement of some putative phosphatase(s) in the dephosphorylation of ERK1/2 in the nucleus. The phosphorylation pattern of ERK1/2 in the cytoplasm (Fig.7A) was same as before. The total ERK1/2 levels in cytoplasm (Fig.7B) and nucleus (Fig.7D) were constant over the time period.

To confirm whether the appearance of phospho-ERK1/2 upon stimulation by ESAT-6 in the presence of sodium orthovanadate was not due to the effect of the latter alone, RAW264.7 cells were treated with 1mM sodium orthovanadate for the same time points. Sodium orthovanadate alone weakly induced phosphorylation of ERK1/2 in the cytoplasm (Fig.8A) and almost none in the nucleus (Fig.8C). As before the total ERK1/2 levels in the cytoplasm and the nucleus remained constant over the time points.
Figure 7 – (A) Phosphorylation of ERK1/2 in the cytoplasm after stimulation with ESAT-6 (5μg/ml) in presence of 1mM sodium orthovanadate(Na₃VO₄) for 0, 15, 30, 60 and 120 minutes. (C) Phosphorylation of ERK1/2 in the nucleus. (B) and (D) represents total ERK1/2 levels in the cytoplasm and the nucleus respectively over the experimental time period.

Figure 8 – (A) Phosphorylation of ERK1/2 in the cytoplasm of RAW264.7 cells after treatment of cells with 1mM sodium orthovanadate(Na₃VO₄) for 0, 15, 30, 60 and 120 minutes. (C) Phosphorylation of ERK1/2 in the nucleus. (B) and (D) represents total ERK1/2 levels in cytoplasm and nucleus respectively.
ESAT-6 antagonized LPS-induced ERK kinase activity

We have earlier observed that ESAT-6 dampened phosphorylation of ERK1/2 in the nucleus and also LPS-induced ERK1/2 phosphorylation in the nucleus. Therefore, to further confirm the levels of activation of ERK1/2 in cytoplasm and in the nucleus, RAW264.7 cells were stimulated with LPS (0.1μg/ml), ESAT-6 (5μg/ml) and/or sodium orthovanadate, Na3VO4 (1mM) for 60 minutes, then from the cytoplasmic and nuclear extracts ERK is immunoprecipitated and the kinase assay was done using myelin basic protein (MBP) as substrate at 30°C. The reaction was stopped using 2X-SDS-loading dye, boiled and reaction products were run on 12% SDS-PAGE, the gel was dried and exposed to X-ray film. The kinase assay confirmed the western blot results. ESAT-6 antagonized LPS-induced ERK1/2 phosphorylation in the nucleus (Fig.9C).

1 2 3 4 5 6

Cytoplasm

Figure.9A

Fold Change

0 0.5 1 1.5 2 2.5 3

1 2 3 4 5 6

Figure.9B
Figure 9 – (A) Kinase assay for ERK1/2 in the cytoplasm using myelin basic protein (MBP) as substrate, (C) ERK1/2 kinase activity assay in the nucleus. Lane.1. Unstimulated cells, Lane.2. Cells stimulated with 5μg/ml ESAT-6, Lane.3. Cells stimulated with 0.1μg/ml of LPS, Lane.4. Cells stimulated with LPS and ESAT-6, Lane.5. Cells stimulated with 1mM Na3VO4 and 5μg/ml ESAT-6, Lane.6. Cells stimulated with 1mM Na3VO4. (B) and (D) is the graph showing fold change of the densitometric values obtained from the densitometric studies of the autoradiogram of (A) and (C) respectively. Unstimulated cells were given a value 1.00.
ESAT-6 stimulated phosphatase activity

Whether the absence of phospho-ERK1/2 in nucleus was really due to some phosphatase, we determined phosphatase activity associated with ERK1/2 for the same time points in the nucleus after stimulation with ESAT-6 (5μg/ml). For this, total ERK was immunoprecipitated from the nuclear extract obtained after stimulation for different time intervals of 0, 15, 30, 60 and 120 minutes and in the immunoprecipitate, phosphatase activity was determined using p-nitrophenyl phosphate as the substrate. The reaction was stopped with 10N NaOH and the absorbance was read at 405nm. Determination of phosphatase activity showed that upon stimulation with ESAT-6 there was 1.5 fold increase in the phosphatase activity at 15 minutes, and 2.5 fold at 120 minutes over the unstimulated cells (Fig.10A), the antibody control in which cells were stimulated with ESAT-6 (5μg/ml) for 120 minutes but to the nuclear extract to which no anti-ERK-1 antibody was added, showed less than 1.5 fold increase in phosphatase activity over the unstimulated cells. This showed that the phosphatase activity associated with ERK1/2 was responsible for dephosphorylating the ERK1/2 as it translocated from cytoplasm to the nucleus.

After the phosphatase activity was determined, the immunoprecipitate was mixed with 2X-SDS-loading dye and boiled for 5 minutes, the samples were then run on 10% SDS-PAGE, western blotted and probed for total ERK1/2. The total ERK levels were constant in all the samples showing equal immunoprecipitation (Fig.10B).
Figure 10A

Figure 10B

Figure 10 – (A) The phosphatase activity in the nucleus of cells stimulated with ESAT-6 (5μg/ml) 0, 15, 30, 60 and 120 minutes respectively. The data represents the mean of three independent experiments. (B) The total ERK1/2 in all the immunoprecipitates to confirm equal pull down.
ESAT-6 downregulated LPS induced c-myc expression

To determine whether the effect of ESAT-6 on activation of ERK1/2 group of MAP kinases can affect LPS-induced expression of certain proto-oncogenes like c-myc (Cheng et al, 1999) encoding a transcription factor c-Myc which plays a role in cell proliferation and programmed cell death (Lemaitre et al, 1996), cells were stimulated with different stimulus for 120 minutes, then the total RNA was isolated and RT-PCR was done for c-myc. ESAT-6 (5μg/ml) did not change the c-myc expression over the basal level, LPS (0.1μg/ml) upregulated c-myc expression that was antagonized by ESAT-6 (Fig.11A). Again treatment with sodium orthovanadate (1mM) alongwith ESAT-6 enhanced the c-myc levels while sodium orthovanadate (1mM) alone did not have any effect on the c-myc expression over the basal level.

To investigate the role of ERK1/2 MAP kinase in LPS-induced c-myc expression, the cells were treated with ESAT-6 and Na3VO4 along with MEK-1 inhibitor PD98059 (10μM) and also p38 MAP kinase inhibitor SB203580 (10μM). As observed before, treatment with Na3VO4 and ESAT-6 enhanced c-myc expression over ESAT-6 stimulation; interestingly treatment with MEK-1 inhibitor PD98059 downregulated c-myc expression to the level of ESAT-6 stimulation while the p38 inhibitor SB203580 had no effect on c-myc expression levels (Fig.11B). This indicates that ESAT-6+Na3VO4 treatment causes phospho-ERK1/2 to appear in the nucleus, which was again inhibited by PD98059, and therefore the level of c-myc expression falls to the level of ESAT-6 stimulation; the ERK1/2 activation caused by treatment with Na3VO4 is offset by
PD98059. The p38 MAP kinase pathway was not involved in c-myc expression, as the addition of SB203580 did not have any effect on c-myc levels.

![Figure 11](image1.png)

![Figure 12](image2.png)
Figure 11 – (A) RT-PCR for c-myc gene expression, Lane 1. Unstimulated cells, Lane 2. Cells stimulated with 5μg/ml ESAT-6, Lane 3. Cells stimulated with 0.1μg/ml of LPS, Lane 4. Cells stimulated with LPS and ESAT-6, Lane 5. Cells stimulated with 1mM Na₃VO₄ and 5μg/ml ESAT-6, Lane 6. Cells stimulated with 1mM Na₃VO₄. Stimulation time is 120 minutes. (B) β-actin control.

Figure 12 – (A) RT-PCR for c-myc gene expression, Lane 1. Unstimulated cells, Lane 2. Cells stimulated with 5μg/ml ESAT-6, Lane 3. Cells stimulated with 1mM Na₃VO₄ and 5μg/ml ESAT-6, Lane 4. Cells stimulated with Na₃VO₄ and ESAT-6 and 10μM of MEK-1 inhibitor PD98059, Lane 5. Cells stimulated with Na₃VO₄ and ESAT-6 and 10μM of p38 inhibitor SB203580, Lane 6. Cells stimulated with 1mM Na₃VO₄. (B) β-actin control.

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Figure 13
Summarizing the above results, ESAT-6 limited the activation of extracellular signal-regulated kinases 1/2 (ERK1/2) MAP kinase in the nucleus of RAW264.7 cells. This effect of ESAT-6 was specific for the ERK1/2 pathway as another MAP kinase p38 was not affected. ESAT-6 also opposed the LPS-induced ERK1/2 activation in the nucleus. The effect of ESAT-6 on ERK1/2 pathway could be countered by addition of sodium orthovanadate (a tyrosine phosphatase inhibitor) suggesting the role of some putative phosphatase(s). Measurement of phosphatase activity in the nucleus associated with ERK1/2 showed a time-dependent increase in activity. To see whether the effect of ESAT-6 could be exerted at the level of LPS-induced gene expression, ESAT-6 was found to downregulate LPS-induced c-myc gene expression which could be reverted by addition of sodium orthovanadate. Treatment with MEK-1 inhibitor PD98059 again led to the decrease in c-myc levels while addition of p38 inhibitor SB203580 did not had any effect on c-myc levels.

**Effect of CFP-10, ESAT-6 and the 1:1 CFP10:ESAT6 complex on macrophage signaling**

The next part of our studies focussed on the effect of CFP-10, ESAT-6 and CFP10:ESAT6 complex in modulation of macrophage signaling. It has been reported that the genes esxA and esxB which encode CFP-10 and ESAT-6 respectively are
cotranscribed and both the proteins form a tight 1:1 complex (Renshaw et al, 2002 and Renshaw et al, 2005). We compared the effect of the CFP10:ESAT6 complex along with the individual proteins on macrophages.

We looked at the production of reactive oxygen species (ROS) in the macrophages as the measure of macrophage activation. The generation of ROS has been connected to stress response, apoptosis, ageing and cell death (Adler et al, 1999; Buttke and Sandstrom, 1995). ROS plays a role in host defense mechanism against bacteria by oxidizing the -SH group of cysteine in the proteins. Several studies have suggested that ROS can regulate the production of cytokines in macrophages through mechanisms that are dependent on NF-κB. LPS, which stimulates the production of TNF-α, induced the production of ROS via a pathway dependent on Rac1 and the activation of NF-κB through IκB kinase in murine macrophage cells (Sanlioglu et al, 2001). NF-κB plays a role in containment of infection by inducing the expression of several proinflammatory cytokines like TNF-α, IL-12, IFN-γ and nitric oxide synthase-2 (Lee and Schorey, 2005; Yue et al, 2005; Kim et al, 2006).

Structural characterization of CFP-10, ESAT-6 and CFP10:ESAT6 complex

Circular Dichroism Spectroscopy

The structural states of CFP-10, ESAT-6 and CFP10:ESAT6 complex were confirmed under the experimental conditions by far UV CD and fluorescence spectroscopy. The far UV CD spectra shown in Fig.14 are representative of those obtained for ESAT-6, CFP-10 and CFP10:ESAT6 complex. The spectra for ESAT-6 and
the CFP10:ESAT6 complex are similar to proteins which have significant amount of helical content whereas significantly different spectrum observed for CFP-10 indicates unstructured, random coil polypeptide.

Figure 14 - The Circular Dichroism spectra of CFP-10 (A), ESAT-6 (B) and the 1:1 CFP10:ESAT6 complex (C) between wavelength range of 190-250nm.
**Fluorescence spectroscopy**

The fluorescence spectra for ESAT-6, CFP-10 and CFP10:ESAT6 complex are shown in Fig.15. The spectra of both ESAT-6 and CFP-10 are characterized by a fluorescence maximum of 355.5 and 358nm respectively at 18°C which can be expected for proteins where all tryptophans are fully exposed to aqueous environment, whereas in the CFP10:ESAT6 complex, the fluorescence maxima has shifted to around 350nm towards blue region which indicates that one or more tryptophans which was earlier exposed outside has moved to less polar region upon complex formation.

![Fluorescence spectra](image)

**Figure.15**

Figure.15 - The Fluorescence spectra of (A) CFP-10, (C) ESAT-6 and the (B) 1:1 CFP10:ESAT6 complex between wavelength range 300-450nm
Inhibition of ROS production by CFP-10, ESAT-6 and CFP10:ESAT6 complex

Measurement ROS production in RAW264.7 cells show that all the three components CFP-10, ESAT-6 and CFP10:ESAT6 complex downregulated ROS over 5 minutes. The downregulation observed with CFP-10:ESAT6 complex was more than observed with CFP-10 and ESAT-6 alone. The complex at 5μg/ml decreased ROS by 60% compared to 40% reduction with ESAT-6 and 10% reduction observed with CFP-10 compared to normal unstimulated cells (Fig.16A). To check for the specificity for the effects we used two non-ESAT-6 family proteins CFP-21 and Antigen 85b at the same doses and measured ROS production (Fig.16B). CFP-21 at 20μg/ml reduced ROS by only 10% while Antigen 85b at the same dose increased ROS production by 10% over the basal level.
Figure.16B

Figure.16 – (A) The production of reactive oxygen species (ROS) by stimulation of RAW264.7 cells with 5μg/ml, 10μg/ml and 20μg/ml of CFP-10 (MTSA-10), ESAT-6 and the CFP10:ESAT6 complex. The graph shows the fold change in ROS over the unstimulated cells which are given a value of 1.00.

(B) The production of ROS upon stimulation with 5μg/ml, 10μg/ml and 20μg/ml of CFP-21 and Antigen-85b. As before unstimulated cells were given a value 1.00. The data represented as fold change in ROS.
Next we wanted to see the effect of CFP-10, ESAT-6 and CFP10:ESAT6 1:1 complex on Lipopolysaccharide-induced ROS production. LPS is known to induce ROS production in macrophages (Hsu and Wen, 2002; Woo et al, 2004), therefore we used LPS as a positive control. CFP-10 reduced LPS-induced ROS production in cells by 60% (Fig.17), ESAT-6 reduced LPS-induced ROS production by 50% (Fig.18) and the CFP10:ESAT6 complex reduced LPS-induced ROS production by 70% (Fig.19).

Figure.17

Figure.17 – (1) Unstimulated cells, (2) Cells+LPS (0.1μg/ml), (3) Cells+CFP-10 (5μg/ml), (4) Cells+LPS (0.1μg/ml)+CFP-10 (5μg/ml). The data represented as fold change over the basal level, unstimulated cells were given a value of 1.00.
Figure. 18

Figure. 18 – (1) Unstimulated cells, (2) Cells+LPS(0.1μg/ml), (3) Cells+ESAT-6 (5μg/ml), (4) Cells+LPS(0.1μg/ml)+ESAT-6(5μg/ml). The data represented as fold change over the basal level, unstimulated cells were given a value of 1.00.

Figure. 19

Figure. 19 – (1) Unstimulated cells, (2) Cells+LPS (0.1μg/ml), (3) Cells+CFP10:ESAT6 complex (5μg/ml), (4) Cells+LPS (0.1μg/ml)+CFP10:ESAT6 complex (5μg/ml). The
data represented as fold change over the basal level, unstimulated cells were given a value of 1.00.

**Inhibition of LPS-induced NF-κB DNA binding activity by CFP-10, ESAT-6 and the CFP10:ESAT6 complex**

LPS is a known inducer of ROS production and the latter activates transcription factor NF-κB leading to gene expression (Lu and Wahl, 2005; Yoo et al, 2002; Li and Engelhardt, 2006; Gloire et al, 2006). Since CFP-10, ESAT-6 and CFP10:ESAT6 complex antagonized ROS production in macrophages, we determined the effect of ROS production on the NF-κB p65 DNA binding activity in the nucleus as a measure of its activation. For this RAW264.7 cells were treated with LPS (0.1μg/ml) and/or CFP-10 (5 μg/ml), ESAT-6 (5μg/ml), complex (5μg/ml) and N-Acetyl cysteine (10mM) for 30 minutes. Then cells were harvested and nuclear extracts were prepared as described in Materials and Methods. Then 10μg of nuclear extract was used for EMSA binding reaction with 32P-labelled NF-κB consensus binding sequence as probe. A control was set up where 100-fold excess of non-radioactive (cold) probe was added along with labelled probe. In another set, anti-p65 antibody (SantaCruz) was added in the binding reaction. LPS induced p65 DNA binding 2-fold over basal level while CFP-10, ESAT-6 antagonized the effect of LPS down to basal level; the CFP10:ESAT6 complex downregulated the effect of LPS by 40% compared unstimulated cells (Fig.20A). Addition of N-Acetyl cysteine (10mM), a ROS scavenger reduced LPS induction of p65 DNA binding activity by 60% over unstimulated cells. This indicates the role of ROS in the activation of NF-κB p65 and its subsequent DNA binding activity; addition of N-
Acetyl cysteine removes the ROS species and therefore this effects counters the positive effect of LPS on p65 and hence binding of p65 subunit falls below the basal level. The control where 100-fold excess of unlabelled probe was added showed no binding of p65. The control where anti-p65 antibody was added showed inhibition of binding of p65 to DNA; it is reported that inhibition of DNA-protein complexation by antibody also indicates specificity (George et al, 2006).

Figure 20A
Figure 20B

Figure 20 (A) – Autoradiogram of the gel after doing electromobility shift assay. Lane 1. Unstimulated cells, Lane 2. LPS (0.1μg/ml), Lane 3. LPS+CFP10 (5μg/ml), Lane 4. LPS+ESAT6 (5μg/ml), Lane 5. LPS+CFP10:ESAT6 complex (5μg/ml), Lane 6. CFP-10 (5μg/ml), Lane 7. ESAT-6 (5μg/ml), Lane 8. CFP10:ESAT6 complex (5μg/ml), Lane 9. LPS+N-Acetyl cysteine (10mM), Lane 10. 100-fold excess (5 picomoles) of unlabelled probe compared to labelled probe (50 femtomoles), Lane 11. Antibody control having 1μg of anti-p65 antibody. (B) The graph showing the plot of densitometric values obtained from the densitometric studies of the autoradiogram. The graph shows fold change over the basal level; unstimulated cells were given a value of 1.00.
Inhibition of LPS-induced NF-κB-dependent reporter gene expression by CFP-10, ESAT-6 and CFP10:ESAT6 complex

Next we wanted to know whether inhibition of LPS-induced ROS production by the proteins have any effect on NF-κB dependent gene expression. RAW264.7 cells were transfected with a reporter plasmid containing chloramphenicol acetyl transferase (cat) gene under IL-2 promoter (which has binding sites for NF-κB p65 subunit) for 6 hours. After transfection is over cells were stimulated with different stimuli for 2 hours. LPS (0.1μg/ml) upregulated cat expression by 90% over the basal level as shown in Fig.21B. The LPS-induced increase was downregulated by CFP-10, ESAT-6 and CFP10:ESAT6 complex by 66%, 56% and 54% respectively (Fig.21B). Stimulation with LPS alongwith N-acetyl cysteine (10mM) which is a scavenger of ROS (Oh and Lim, 2005) led to significant reduction (64%) in cat expression. These results indicate inhibition of LPS-induced ROS production by these proteins affect NF-κB transcriptional activity.
Figure 2.1A

Figure 2.1B

Fold change in reporter gene expression

0 0.5 1 1.5 2 2.5

1 2 3 4 5 6 7 8 9
Figure 21 – (A) The products of the CAT assay were run on a thin-layer chromatography plate using methanol:chloroform (95:5) as a solvent. The autoradiogram shows di-acetylated chloramphenicol on the upper row, the monoacteylated chloramphenicol in the middle row and unacetylated chloramphenicol in the lower row. Lane 1. Unstimulated cells, Lane 2. LPS (0.1 μg/ml), Lane 3. LPS+ESAT-6 (5 μg/ml), Lane 4. LPS+CFP-10 (5 μg/ml), Lane 5. LPS+CFP10:ESAT6 complex (5 μg/ml), Lane 6. ESAT-6 (5 μg/ml), Lane 7. CFP-10 (5 μg/ml), Lane 8. CFP10:ESAT6 (5 μg/ml), Lane 9. LPS+N-Acetyl cysteine (10 mM). Stimulation was done for 2 hours after transfection. (B) The graph shows the fold change in densitometric values obtained after densitometric studies of the upper row (di-acetylated chloramphenicol) of the autoradiogram.