Summary
We have studied the effect of 6-kDa Early Secreted Antigenic Target (ESAT-6) protein in modulation of macrophage function. The open reading frame Rv3875 encoding the ESAT-6 protein was amplified from the genomic DNA of a local clinical isolate and cloned into pET23b(+) vector and expressed as a hexahistidine-tagged protein which was then purified by Ni²⁺-NTA column; it was found to be pure and of correct size as confirmed by silver staining and western blotting respectively.

Next we looked at the effect of ESAT-6 on macrophage signaling pathways. We looked at the status of extracellular signal-regulated kinases 1/2 (ERK1/2). ESAT-6 induced phosphorylation of ERK1/2 in cytoplasm, however no phosphorylated ERK1/2 could be detected in the nucleus. The macrophage activator Lipopolysaccharide (LPS) induced phosphorylation of ERK1/2 in both cytoplasm and the nucleus proving that the effect of ESAT-6 was not a property of the RAW264.7 cell line. ESAT-6 was found to dampen the LPS-induced phosphorylation of ERK1/2 in the nucleus thereby exerting an antagonistic effect. Treatment of cells with a tyrosine phosphatase inhibitor, sodium orthovanadate (Na₃VO₄) alongwith ESAT-6 caused phospho-ERK1/2 to appear in the nucleus while Na₃VO₄ alone did not induce ERK1/2 phosphorylation in the nucleus. This indicates there were some putative phosphatase(s) in the nucleus responsible for dephosphorylating ERK1/2. The activation of ERK1/2 was further confirmed by doing kinase assay after immunoprecipitating ERK1/2 from the cytoplasmic and nuclear extracts using Myelin Basic Protein (MBP) as the substrate. The measurement of kinase activity confirmed
the results obtained from western blotting on ERK1/2 phosphorylation. Measurement of ERK1/2 associated-phosphatase activity in the nucleus showed a time-dependent increase in the phosphatase activity.

To check the effect of ESAT-6 on LPS-induced gene expression, the expression of an early response gene \textit{c-myc} was studied. The \textit{c-myc} expression was known to be regulated by the MEK-ERK pathway. ESAT-6 itself did not altered the \textit{c-myc} expression over the basal level. However the LPS-induced \textit{c-myc} expression was found to be downregulated by ESAT-6 compared to the \textit{c-myc} expression by LPS stimulation alone. Again stimulation with ESAT-6 alongwith 1mM sodium orthovanadate (Na$_3$VO$_4$) increased the level of \textit{c-myc} compared to ESAT-6 stimulation alone while Na$_3$VO$_4$ alone did not have any effect on \textit{c-myc} levels over the basal level. To confirm the role of ERK1/2 pathway in \textit{c-myc} expression, we determined \textit{c-myc} expression in presence of MEK-1 inhibitor PD98059 and p38 MAP kinase inhibitor SB203580 alongwith Na$_3$VO$_4$ and ESAT-6. Treatment with PD98059 along with ESAT-6 and Na$_3$VO$_4$ downregulated \textit{c-myc} levels while SB203580 did not have any effect on \textit{c-myc} levels. Since p38 MAP kinase pathway was not involved, hence treatment with SB203580 did not have any effect on \textit{c-myc} expression. The effect of ESAT-6 on LPS-induction of some other genes like \textit{Icam-1, Bax, IL-1\beta} and \textit{Tnf-r1a} were also studied and ESAT-6 was found to downregulate the LPS-induced the expression of these genes.

We compared the effect of CFP-10, ESAT-6 and CFP10:ESAT6 complex on macrophage signal transduction. The complex formation between CFP-10 and ESAT-6 proteins was confirmed by Circular Dichroism and Fluorescence Spectroscopy. From our studies with the CFP-10, ESAT-6 and 1:1 CFP10:ESAT6 complex, it was
observed that they downregulated ROS production in RAW264.7 macrophages. 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. Lipopolysaccharide is known to induce the production of ROS. These antigens *i.e.* CFP-10, ESAT-6 and CFP10:ESAT6 complex were found to antagonize LPS-induced ROS production in these cells. The ROS affects intracellular signal transduction and is known to activate the transcription factor NF-κB. We first looked at the binding of NF-κB p65 subunit to the NF-κB consensus binding sequence in the nucleus as a measure of its activation; LPS was found to enhance NF-κB p65 DNA binding which was downregulated by CFP-10, ESAT-6 and the CFP10:ESAT6 complex as well as by ROS scavenger N-Acetyl cysteine. Next we determined whether LPS-induced ROS production resulted in NF-κB-dependent gene transcription in a reporter assay system. The promoter of the reporter gene is the promoter for *IL-2* and contains binding sites for NF-κB p65 subunits. LPS was found to induce the expression of NF-κB-dependent reporter gene (Chloramphenicol acetyl transferase, *cat*) over the basal level. This increase in reporter gene expression was downregulated by CFP-10, ESAT-6 and the 1:1 complex. Addition of N-Acetyl cysteine, which is a ROS scavenger along with LPS stimulation led to significant reduction in reporter gene expression even below the basal level. This indicates that ROS produced upon LPS stimulation were completely scavenged by N-Acetyl cysteine and no ROS species were left to activate NF-κB and hence reporter gene expression falls.

Therefore from our studies with *M. tuberculosis* secretory protein ESAT-6 we have found that it modulates the ERK1/2 signaling cascade without affecting the p38 MAP kinase pathway. ESAT-6 also antagonized LPS-induced ERK1/2 activation and
subsequent gene expression. These effect of ESAT-6 on gene expression might help the tubercle bacilli to modulate the host response against it by altering the profile of gene expression. Furthermore our studies on ROS indicated a toning down of ROS production in macrophages. ROS is known for its role as a defense mechanism employed by the host cell to eliminate the pathogen. The role of ROS in the context of mycobacterial infection has not been studied in detail. Here we show for the first time that *M. tuberculosis* antigens CFP-10, ESAT-6 and CFP10:ESAT6 complex downregulated LPS-induced ROS production. This downregulation of ROS production inhibits NF-κB transactivation capacity. Since NF-κB plays a very important role in orchestrating inflammatory response, cytokine production, cell proliferation and apoptosis; therefore down regulation of NF-κB transactivation property could be a vital strategy employed by the bacilli to subvert the host response. Further studies will in this context will give a detailed insight into the mechanisms operating inside the macrophage during Mtb infection.