

## *Mycobacterium tuberculosis* secreted antigen (MTSA-10) inhibits macrophage response to lipopolysaccharide by redox regulation of phosphatases

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The present study was undertaken to investigate the possible role of a 10-kDa, secretory antigenic protein of Mtb (MTSA-10) in regulating macrophage response to lipopolysaccharide (LPS). MTSA-10 inhibited the lipopolysaccharide (LPS)-induced oxidant species generation in the macrophage. Treatment of macrophages with MTSA-10 activated their protein tyrosine phosphatases (PTPs) in a redox-regulated fashion. These activated phosphatases then interfered with the early events of LPS signaling and lower the strength and magnitude of the signal generated, thereby preventing macrophages from making an effective immune response. *Mycobacterium tuberculosis* Region of Deletion-1 (RD-1)-specific secretory antigen MTSA-10 (encoded by ORF *Rv3874* of Mtb genome) modulated the macrophage signaling machinery and prevented it from responding to further activation by LPS.

**Keywords:** Antigenic (MTSA-10), Lipopolysaccharide, Macrophage, *Mycobacterium tuberculosis*, Phosphatase, Redox regulation

Tuberculosis, caused by *Mycobacterium tuberculosis* (Mtb), is one of the most prevalent microbial diseases of humans, claiming millions of lives every year. It is estimated that one-third of the world population is infected with Mtb and the number of deaths due to tuberculosis is the highest for any single bacterial pathogen<sup>1</sup>. Emergence of multi-drug resistant strains (MDR) of Mtb and the failure of *M. bovis* bacille Calmette-Guerin (BCG) vaccine (the only vaccine against tuberculosis) have further worsened the situation.

Mtb, a facultative intracellular pathogen, invades the host macrophages that constitute the first line of host defense to any invading pathogen. In response to Mtb invasion, the macrophage becomes severely compromised in its anti-bacterial functions<sup>2-3</sup>. Several studies have revealed that Mtb has evolved multiple strategies to survive in the harsh environment of the

macrophage<sup>4-6</sup>, with the activation status of the macrophage determining whether Mtb proliferates or lies dormant within it<sup>7</sup>. Thus, the interplay between mycobacteria and host response determines the fate of an infection. Having co-evolved with their hosts, microbial pathogens have acquired subtle molecular strategies to regulate the host activation response in order to establish a favourable niche for themselves. Thus, several bacterial pathogens are known to secrete a variety of virulence factors to modulate the host anti-bacterial response<sup>8-10</sup>.

Many secretory proteins of Mtb are known to be prominent targets of host immune response. The antigen 85 (Ag85) complex, and 6-kDa, early secretory antigenic target (ESAT-6) proteins are being extensively studied as candidate vaccines against tuberculosis<sup>11</sup>. However, few detailed studies have been done to elucidate the putative role of Mtb secretory proteins in regulation of the macrophage immune functions. We have been studying the immunomodulatory role of a 10-kDa, culture filtrate protein (CFP-10) of Mtb, also known as *Mycobacterium tuberculosis*-specific antigen-10 (MTSA-10)<sup>12</sup>. MTSA-10 and ESAT-6 are two major secreted antigens of Mtb. MTSA-10 and ESAT-6 are co-transcribed from *Rv3874* and *Rv3875* genes, respectively<sup>13</sup> located in the "region of deletion-1"

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(RD-1) locus of Mtb genome<sup>14</sup>. Since RD-1 locus has been lost in all BCG vaccine strains of *M. bovis*<sup>15</sup>, and has not been detected in a majority of environmental non-pathogenic mycobacteria, MTSA-10 and ESAT-6 are considered to be the antigens specifically secreted by pathogenic mycobacteria. Recent studies have documented the importance of RD-1 locus for mycobacterial virulence. For example, targeted deletion of RD-1 from Mtb attenuated the organism<sup>16</sup>. Conversely, incorporation of RD-1 locus of Mtb into BCG<sup>17</sup> or *M. microti*, a natural RD-1 deletion mutant, imparted them with enhanced virulence and immunogenicity<sup>18</sup>. Recently, RD-1 region has been characterized as a specialized secretion system of Mtb meant to transport MTSA-10 and ESAT-6 out of the cell. It was also found that mutations in this system profoundly attenuated Mtb virulence in the mouse model<sup>19</sup>. The reduced virulence of the mutants during *in vivo* infection may be attributed partly to their inability to limit the macrophage activation response, which makes MTSA-10 and ESAT-6 the two important microbial determinants in the modulation of host immune function.

Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria is one of the most potent activators of macrophages<sup>20</sup>. Recognition of LPS and release of inflammatory mediators and anti-bacterial products are the hallmarks of the involvement of the cells in the pathophysiology of inflammatory conditions<sup>21</sup>. In early immune response to LPS, macrophages play a central role in host defense with physical and immune response against bacterial infections<sup>20</sup>. In a previous study, we have shown that MTSA-10 can render macrophage tolerant to the subsequent challenge with LPS<sup>22</sup>. This has been found to differ from the phenomenon of "LPS tolerance" as it is confined to disabling the primed macrophages from generating nitric oxide (NO) response without affecting TNF- $\alpha$  or IL-10 production, and thus seem to suggest a complex interplay of different signaling pathways<sup>22</sup>.

In the present study, we have examined the possible role of MTSA-10 in regulating macrophage response to LPS. We have provided evidence to suggest that MTSA-10 interferes with the initial events of LPS signaling by down regulation of reactive oxygen species (ROS) generation by macrophages in response to LPS triggering. This in turn prevents the macrophage from responding to the

activation stimulus, which is essential for making an effective anti-microbial response.

## Materials and Methods

*Cell lines and reagents*—A mouse tumor derived macrophage-monocyte cell line J774A.1, originally obtained from the American Type Culture Collection (ATCC; Manassas, VA), was procured from the National Centre for Cell Science, Pune, (India), and maintained in DMEM (Life Technologies, Carlsbad, CA) supplemented with 10% heat-inactivated Fetal Bovine Serum (Life Technologies) at 37°C in 5% CO<sub>2</sub>.

Affinity-purified, lipopolysaccharides (LPS)-free, recombinant 6xHis-tagged MTSA-10 protein was cloned in bacterial expression vector pQE-31 (Qiagen, Valencia, CA) and purified using Ni-NTA-Agarose matrix. The QIAexpressionist® protein expression and purification kit including Ni-NTA-Agarose, gel purification kit and all primers used for amplification of c-DNA and RNAiFect were from Qiagen (Valencia, CA). *E. coli* lipopolysaccharide (LPS), 5 - bromo - 4 - chloro - 3 - indolyl - beta - D - galactopyranoside (X-gal), protein A-agarose, isopropyl-thio-D-galactopyranoside (IPTG), ampicillin, kanamycin, para-nitrophenyl phosphate (pNPP), N-acetylcysteine (NAC), dephosphorylated casein, sodium orthovanadate (Na<sub>2</sub>VO<sub>3</sub>), sodium fluoride, aprotinin, pepstatin, leupeptin, iodoacetamide were purchased from Sigma (St. Louis, MO). Bacto tryptone, yeast extract and Bacto agar were from Difco (San Diego, CA). The immobilized pH gradient (IPG) dry-strips, dithiothreitol (DTT) and other chemicals used for two-dimensional gel electrophoresis (2-DE) were from Amersham Biosciences, (Uppsala, Sweden). Dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) was from Molecular Probes (Eugene, OR). The SMARTpool® siRNA for mouse HePTP gene was from Dharmacon, Inc., (Lafayette, CO). The IL-12p40 detection ELISA kit was from BD Pharmingen (San Diego, CA). All antibodies used for immunoprecipitation, western blotting, and the enhanced chemiluminescence (ECL) kit was from Santa Cruz Biotechnology (Santa Cruz, CA). [<sup>32</sup>P]-orthophosphoric acid was from Perkin-Elmer (Boston, MA).

*Treatment of cells with MTSA-10 and resolution of phosphorylated proteins by two-dimensional gel electrophoresis (2-DE)*—J774A.1 cells (1×10<sup>7</sup>) were placed in phosphate-free DMEM supplemented with FCS(1%) for 2 hr before [<sup>32</sup>P]-orthophosphoric acid

(0.5 mCi/mL) was added and the incubation continued for an additional 4-5 h. These cultures were then treated with affinity purified, LPS-free, recombinant MTSA-10 protein at a final concentration of 10 µg/mL for indicated time. In a similar fashion, LPS (Sigma) was used for stimulation at a final concentration of 100 ng/mL. The cytoplasmic fractions of cell lysates were prepared and resolved by 2-DE, using IPG strips (Amersham Biosciences) as per manufacturer's protocol. Briefly, J774.1 cells from the MTSA-10- or mock-treated cultures were harvested and washed once with culture medium by centrifugation, disrupted in the lysis buffer [(8M, urea; 4%, CHAPS; and 2% (v/v) IPG buffer (pI 4-7)], and their cytoplasmic fractions obtained by ultracentrifugation. The cell extracts corresponding to different time points were diluted in rehydration buffer [(8M, urea; 2% (v/v), CHAPS; 0.28% (w/v), DTT; 0.5% (v/v), IPG buffer (pI 4-7)], and applied to IPG strips (pI 4-7) using the "in-gel rehydration" method. The isoelectrofocusing (IEF) was performed in the IPGphor IEF system (Amersham Biosciences), with the following voltage program: gradient from 0-500 V in 2 h, 500 V constant for 5 h, gradient from 500-4000 V in 2 h, step up to 4500 V and hold for 1 h, and finally step up to 5000 V constant until 22-24 kVh. Later, IPG strips were equilibrated in solutions A [50 mM Tris/HCl (pH 8.8) containing 6M, urea; 30%, glycerol; 2%, SDS; 1%, DTT) and B (solution A without DTT, but with 2.5%, iodoacetamide; and 0.005%, bromophenol blue). Each strip was then loaded on to the top of a SDS-polyacrylamide gel slab (12% gel, 1mm thick) and electrophoresis was performed at a constant current of 20 mA per gel, to resolve the focused proteins in the second dimension.

The resultant gels were silver stained to ascertain that comparable quantities of proteins from experimental and control groups were loaded for analysis. Dried gels were then exposed to X-ray films. Phosphoproteins were visualized by autoradiography and digitized on a Molecular Dynamics computing densitometer using Image Quant software, version 5.2, and were analyzed using Image Master 2D software version 4.01 (Amersham). Only spots with an area greater than 75 pixels were considered, and the minimum intensity surrounding the spot on the film was taken as its background and subtracted to give the true intensity. Relative quantification was achieved by normalizing against three distinct spots

that were apparently unaffected upon stimulation of cells. Calibration for Mw and pI was done on the basis of standard markers that were run on parallel gels.

*Cellular fractionation and phosphatase assay*—Following incubation of J774.1 cells with MTSA-10 or LPS for indicated time, cells were fractionated into membrane and cytoplasmic fractions. Briefly, the cells were collected in hypotonic fractionation buffer [10 mM, HEPES (pH 7.4); 4.5 mM, EGTA; 2.5 mM, EDTA; 1.0 mM, Na<sub>2</sub>VO<sub>3</sub>; 1.0 mM, phenylmethylsulfonyl fluoride; 10 µg/mL, each of aprotinin, leupeptin, and pepstatin], and lysed for 20 min at 4°C with constant rotation. Cell lysates were ultracentrifuged at 100,000 × g for 30 min, yielded cytosolic fraction as the supernatant. The particulate pellet was resuspended in extraction buffer containing 1% of Triton X-100 for 20 min at 4°C, and spun at 16,000×g for 20 min at 4°C, and the detergent-soluble supernatant was collected as the membrane fraction. Protein contents in either fraction were estimated using standard Bradford assay. Twenty microgram of each protein fraction were then assayed for phosphatase activity. In addition, two phosphatases, *viz.*, hematopoietic protein tyrosine phosphatase (HePTP), and Src homology domain-2 (SH2) containing phosphatase-2 (SHP-2) were immunoprecipitated from the cytoplasmic fractions by incubating 1.0 mg of either fraction with 1 µg of respective antibody for 2 h at 4°C, followed by addition of 40 µL of protein A-Sepharose for another 2 h. The resultant immunoprecipitates were assayed for phosphatase activity using *para*-nitrophenyl phosphate (pNPP) as the substrate.

*Isolation of RNA and RT-PCR*—RNA was isolated from the treated cells (1×10<sup>6</sup>) at indicated time points using Trizol reagent according to manufacturer's recommendations. Two microgram of RNA from each group was subjected to reverse transcription (RT) using oligo(dT)<sub>20</sub> and Superscript II Reverse Transcriptase (Invitrogen). cDNAs were amplified by PCR with appropriate primers. Sequence of primers used for PCR amplification of different genes is given in Table 1.

*Lyn kinase assay*—For kinase assay, Lyn was immunoprecipitated from various groups of cells (1×10<sup>7</sup>). The immunoprecipitates were washed and then incubated in the kinase reaction buffer [25 mM, Tris (pH 7.5); 0.5 mM, DTT; 0.1mM, orthovanadate; 50 µM, unlabelled ATP; 10 mM, MgCl<sub>2</sub>; and

Table 1—Sequence of the primers used in RT-PCR

Gene	Primer Sequence
IL-6	Forward: 5'-ATA ACA AGA AAG ACA AAG CCA GAG-3' Reverse: 5'-TGA TTT CAA GAT GAA TTG GAT GGT-3'
IL-1 $\beta$	Forward: 5'-TGG CAA CTG TTC CTG AAC TCA A-3' Reverse: 5'-TCC ACG GGA AAG ACA CAG GTA-3'
Egr-1	Forward: 5'-GAG CGA ACA ACC CTA TGA GC-3' Reverse: 5'-AGG TCT CCC TGT TGT TGT GG-3'
iNOS	Forward: 5'-AAG AGT TCC CTT CCT TGC ATG T-3' Reverse: 5'-CAC TGA CAC TTC GCA CAA AGC-3'
c-Myc	Forward: 5'-TCC TGT ACC TCG TCC GAT TC-3' Reverse: 5'-AAT TCA GGG ATC TGG TCA CG-3'
$\beta$ -actin	Forward: 5'-CTA TGC TCT CCC TCA CGC CA-3' Reverse: 5'-CCG CTC GTT GCC AAT AGT GAT-3'
MIP-1 $\alpha$	Forward: 5'-ATG AAG GTC TCC ACC ACT GC-3' Reverse: 5'-ACC AAC TGG GAG GGA GAT G-3'
MIP-1 $\beta$	Forward: 5'-GAA GCT CTG CGT GTC TGC C-3' Reverse: 5'-GAA GAG GGG CAG GAA ATC TG-3'
ICAM-1	Forward: 5'-TCT CGG AAG GGA GCC AAG TAA-3' Reverse: 5'-CTC TTG CCA GGT CCA GTT CC-3'
IL-12p40	Forward: 5'-AAG TAT TCA GTG TCC TGC CAG G-3' Reverse: 5'-CGA ACA AAG AAC TTG AGC GAG AAG-3'
IFN- $\gamma$ receptor-2	Forward: 5'-GAT TCT AAC TTG GGA GCC GTC A-3' Reverse: 5'-CAC CCA CTT GGA AGT GAG GTT-3'
TNF- $\alpha$ receptor-1a	Forward: 5'-CCC CAC CTC TGT TCA GAA ATG G-3' Reverse: 5'-TAC TTC CAG CGT GTC CTC GT-3'

10  $\mu$ Ci/tube of ( $^{32}$ P)-ATP]. The reaction was initiated by addition of 5  $\mu$ g/tube of de-phosphorylated casein at 30°C for 10 min. The reaction was terminated by addition of 6 $\times$ SDS loading buffer followed by boiling for 5 min. The reaction mixtures were subjected to SDS polyacrylamide gel electrophoresis. Comparable amounts of Lyn in different groups were ascertained by silver staining of the resultant gels. Dried gels were then exposed to X-ray films and the amount of [ $^{32}$ P]-ATP incorporation in the substrate was established by autoradiography followed by densitometric analysis.

**Measurement of nitric oxide and IL-12p40**—For measurement of secreted nitric oxide and IL-12p40,  $1 \times 10^5$  cells/well were treated with MTSA-10 and LPS as mentioned in the text. After 18 hr of incubation, the supernatant was used for the assays. Nitrite accumulation, an indicator of nitric oxide production, was measured in the supernatant using the Griess reagent. An IL-12p40 ELISA kit from BD Biosciences was used to determine the amount of

secreted IL-12p40 according to manufacturer's protocol.

**Measurement of reactive oxygen species (ROS)**—For flowcytometry analysis, the cells ( $5 \times 10^6$  cells in each case) were suspended in 1 mL of medium and labeled with 1  $\mu$ M of dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) for 15 min at 37°C. Cells were washed twice with medium and re-suspended in 1 mL of medium. Treatment was done as required and cells were used for FACS analysis after 10 min of stimulation.

**Isolation of peritoneal macrophages**—Mouse peritoneal macrophages were obtained from resident peritoneal cells of BALB/c mice. The animals were anesthetized and the resident peritoneal cells were harvested, pooled, checked for viability by trypan blue dye exclusion, and counted with a haemocytometer. This cell suspension was adjusted to  $1 \times 10^6$  viable cells/mL in DMEM medium and incubated for 2 h at 37°C. The adherent cells were collected and used for further experiments.

**siRNA**—SMARTpool siRNA specific to cDNA sequence of mouse HePTP gene from Dharmacon (USA) was used to knockdown HePTP expression. siRNA was used at 25  $\mu$ g/ $5 \times 10^6$  cells and transfection was achieved using RNAiFect kit (Qiagen) strictly following the protocol recommended by the manufacturer. Our preliminary experiments suggested that maximum silencing was obtained after 36 h of transfection. Hence after 40 h in culture, cells were harvested and stimulated as mentioned in the text.

## Results

**MTSA-10 prevents LPS-induced phosphorylation of macrophage proteins**—Exposure of macrophages to bacterial LPS triggers a series of biochemical and functional changes including secretion of inflammatory mediators such as IL-1 and TNF- $\alpha$  and stimulation of bactericidal activity<sup>20,23</sup>. Several studies have shown that the early events of LPS response involve rapid tyrosine phosphorylation of macrophage proteins and activation of protein tyrosine kinases<sup>24,25</sup>. To study the overall effect of MTSA-10 on LPS-induced phosphorylation of the macrophage proteins, we monitored the intracellular phosphorylation events in J774.1 cells. The cells were pre-equilibrated with [ $^{32}$ P]-orthophosphoric acid and stimulated with LPS, in the presence or absence of MTSA-10. The cell lysates prepared at different time points of stimulation were resolved by two-dimensional gel electrophoresis

(2-DE) and subsequently analyzed by autoradiography.

The results obtained from one such experiment (out of three reproducible ones) are shown in Figure 1A. Stimulation of cells with LPS led to an increase in the number of phosphoproteins that peaked by 15 min of stimulation. Concomitant presence of MTSA-10 for 15 min in the cultures resulted in a massive decrease in the number of phosphoprotein spots observed (Fig. 1A). After normalizing the two autoradiograms for background intensity, and analyzing them using the Image Master 2D Elite software (Amersham

Bioscience), the number of phosphoproteins detectable after 15 min of stimulation is presented in Figure 1B and C. Among 165 phosphorylated proteins detectable in the LPS-stimulated cells, 27 were common with the 54 phosphoproteins present in the profile of unstimulated cells, indicating that at least 138 proteins got newly phosphorylated upon LPS stimulation (Fig. 1B).

Interestingly, when cells were stimulated with LPS in the presence of MTSA-10, only 48 proteins got phosphorylated as compared to the 138 proteins obtained after stimulation with LPS alone (Fig. 1C).

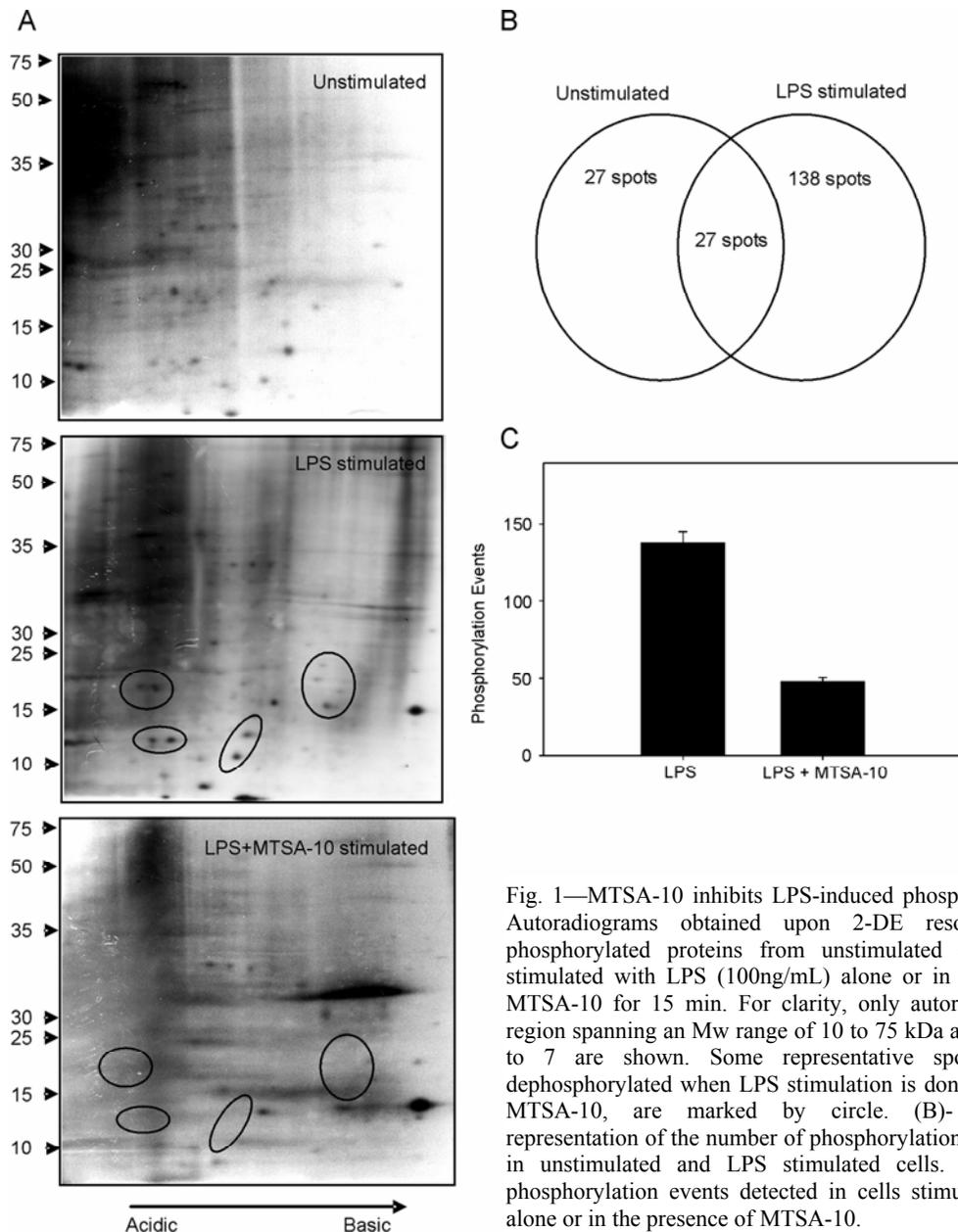


Fig. 1—MTSA-10 inhibits LPS-induced phosphorylation. (A)- Autoradiograms obtained upon 2-DE resolution of the phosphorylated proteins from unstimulated cells and cells stimulated with LPS (100ng/mL) alone or in the presence of MTSA-10 for 15 min. For clarity, only autoradiograms from region spanning an Mw range of 10 to 75 kDa and pI range of 4 to 7 are shown. Some representative spots, which get dephosphorylated when LPS stimulation is done in presence of MTSA-10, are marked by circle. (B)- Diagrammatic representation of the number of phosphorylation events detected in unstimulated and LPS stimulated cells. (C)-Number of phosphorylation events detected in cells stimulated with LPS alone or in the presence of MTSA-10.

Of these 48 phosphoproteins, 32 were common in both the profiles, while 16 represented new phosphorylation events. Thus, in the J774.1 cells, MTSA-10 blocked/prevented phosphorylation of as many as 106 out of 138 proteins that got phosphorylated as a result of stimulation with LPS. Furthermore, among the 32 proteins that were common to both profiles, 12 underwent partial dephosphorylation while 14 showed a gain in their phosphorylation level upon stimulation with LPS plus MTSA-10 as compared to that obtained with LPS alone. Phosphorylation status of the remaining six proteins was not affected by MTSA-10 (data not shown). Furthermore, we found that the effect of MTSA-10 on the LPS-induced phosphorylation was not restricted to any particular subset of proteins; rather it seemed to be a “global phenomenon” in which a significant proportion of macrophage proteins were affected (data not shown).

*MTSA-10 regulates macrophage phosphatase activity*—Failure of the macrophage proteins to get phosphorylated upon LPS stimulation in the presence of MTSA-10 was surprising, especially since we found no intrinsic phosphatase activity in MTSA-10. Several studies have shown that cellular response to activating stimuli leads to activation of receptor tyrosine kinases<sup>26-27</sup>. Subsequent phosphorylation of downstream signaling molecules by these kinases results in signal amplification and progression, leading to an activation response. On the other hand, the pathogens and/or their products, like *Leishmania donovani* or Mtb lipoarabinomannan (LAM), are known to activate macrophage cellular phosphatases, thereby rendering the host cell unresponsive to further activation<sup>28-29</sup>. We wondered whether MTSA-10 was also playing a similar role and checked the phosphatase activity of macrophages upon stimulation.

The results of such experiments are shown in Figure 2. Stimulation of macrophage cells with LPS induced rapid inactivation of the membrane-associated phosphatases. Maximum inhibition (~40%) was achieved by 10 min post-stimulation. However, the activity was restored to normal level by 30 min of treatment (Fig. 2A). Interestingly, MTSA-10 tended to block or antagonize the LPS-mediated inactivation of membrane-associated phosphatases as evident from the net increase in their phosphatase activity in cells stimulated with LPS in the presence of MTSA-10 (Fig. 2A). LPS treatment led to inactivation of

cytoplasmic phosphatases as well. When we probed for the cytoplasmic phosphatase activity in cells stimulated with LPS for different time intervals, we found that LPS caused maximum inhibition of cytoplasmic phosphatases by 15 min of treatment. However, the phosphatase activity was gradually restored by 30 min (Fig. 2B). Thus, MTSA-10 seemed to prevent LPS from inhibiting phosphatases, as evident from a moderate increase in the net cellular phosphatase activity observed in the cells stimulated with LPS in the presence of MTSA-10. This indicated that MTSA-10 was intercepting with LPS signaling by activating the cellular phosphatases, and thereby preventing the macrophage proteins from getting phosphorylated in response to LPS stimulation. It is also worthwhile to mention that this effect of MTSA-10 on LPS-stimulated macrophage was not restricted to any individual signaling pathway, but was a global effect as observed from the activity of the individual phosphatases (Fig. 3), where all of them showed a similar pattern of activity when stimulated by LPS in the presence or absence of MTSA-10.

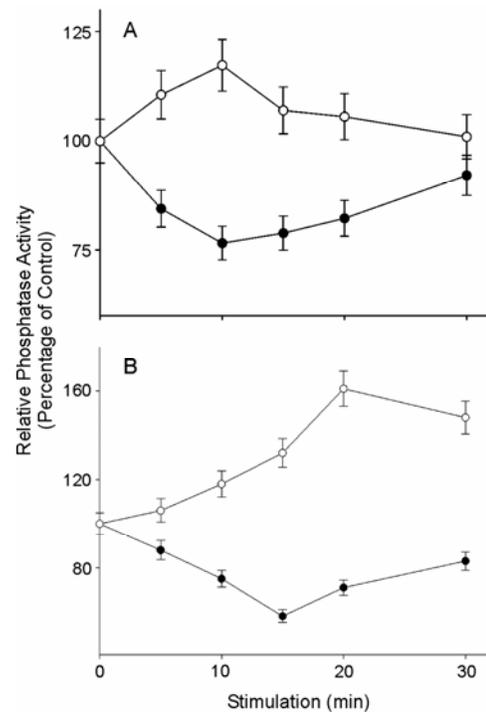


Fig. 2—MTSA-10 reverts the effect of LPS-induced phosphatase activity. Phosphatase activity measured in J774.1 cells ( $2 \times 10^7$ /aliquot) stimulated with LPS in presence (○) or absence (●) of MTSA-10 for indicated time. (A)—Membrane fractions were isolated and equal amount of membrane protein fraction from each time point were subjected to an assay for detecting phosphatase activity. (B)—Phosphatase activity was measured from

the cytoplasmic fractions of cells stimulated with LPS in presence (○) or absence (●) of MTSA-10 for indicated time. [Values are mean ± SD of 4 separate experiments]

*MTSA-10 prevents LPS-induced ROS generation in the macrophage*—Increasingly, a second messenger function of ROS has been implicated in diverse receptor mediated signal transduction systems including EGF, PDGF, insulin, BCR and TCR<sup>30-31</sup>. Signal activation by ROS has been proposed to occur through H<sub>2</sub>O<sub>2</sub>-mediated inhibition of protein tyrosine phosphatases (PTPs), which have a redox-regulated cysteine in their active site, thus, shifting the equilibrium in favor of kinase activation<sup>32-34</sup>. Phago-

cytes such as macrophages and neutrophils are specially endowed to produce ROS through activation of NADPH oxidase<sup>35</sup>. LPS is also known to induce ROS generation in macrophages that in turn helps in their activation<sup>36</sup>. We have observed earlier that MTSA-10 can activate cellular phosphatases by reducing macrophage ROS generation<sup>37</sup>. So we monitored the ROS levels in LPS stimulated J774.1 cells that had been pre-loaded with a ROS-sensitive dye, dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA). The results of such experiments are shown in Fig. 4. Stimulation of cells with LPS led to robust increase in

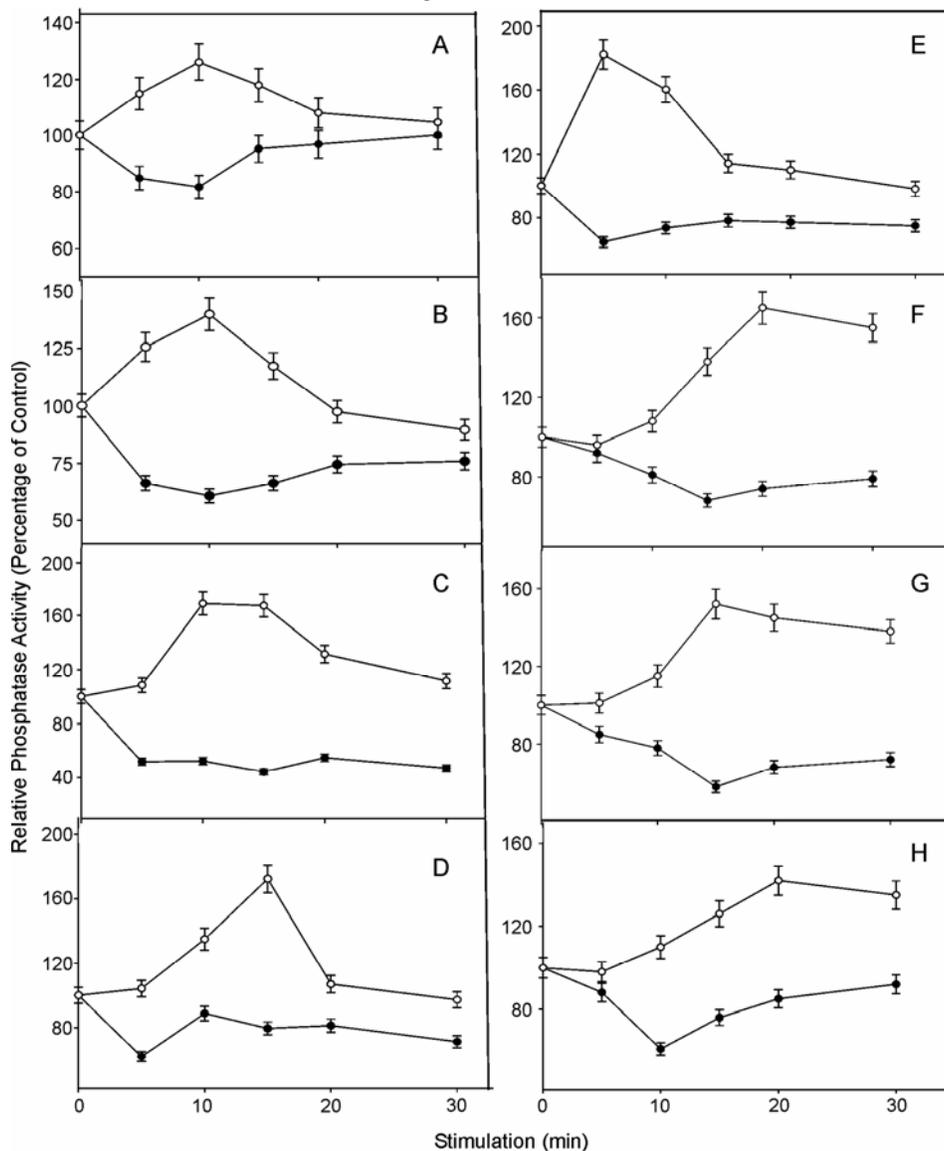


Fig. 3—MTSA-10 influence activity of phosphatases in LPS stimulated macrophage—Cells were stimulated with LPS in absence (●) or in presence (○) of MTSA-10 for the indicated times. (A)–SHP-2; (B)–HePTP; (C)–PTP1B; (D)–MKP-1; (E)–MKP-2; (F)–PP1; (G)–PP2A; and (H)–PP2B were immunoprecipitated from the cytoplasmic fractions of the stimulated cells and were employed in an assay to detect the phosphatase activity. The data represents the percentage of activity from stimulated cells as compared to that of unstimulated cells.

ROS generation, however, this increase was severely curtailed when stimulation was done in the presence of MTSA-10 (Fig. 4A). Interestingly, we found that though Mtb secretory protein ESAT-6 had no effect on LPS-induced ROS generation (Fig. 4B), addition of MTSA-10 along with ESAT-6 reduced the ROS levels in a similar fashion as observed with MTSA-10 alone (Fig. 4C). It is also worthwhile to mention that 1:1 complex of MTSA-10: ESAT-6 also reduced the LPS-induced ROS generation in J774.1 cells similar to that observed with MTSA-10 alone (Fig. 4D). A similar effect of MTSA-10 on LPS-induced ROS generation was observed in the freshly isolated mouse peritoneal macrophages (Fig. 5A-D).

Role of ROS in LPS-induced inhibition of phosphatases was also evident from our observation that in the presence of an ROS scavenger, N-acetylcysteine (NAC), LPS was unable to inhibit the macrophage phosphatases. Interestingly, presence of MTSA-10 further aggravated the reduction in ROS levels leading to additional increase in the macrophage phosphatase activity (Fig. 6).

The above findings indicated that MTSA-10 inhibited LPS-induced ROS generation and activated

phosphatases. We also found that this effect on macrophages was specifically due to MTSA-10 as opposed to ESAT-6 in the 1:1 complex of MTSA-10:ESAT-6.

*MTSA-10 intercepts the early events of LPS signaling in a ROS dependent manner*—One of the initial steps of host response to bacterial endotoxin is the binding of LPS to its cell surface receptor, CD14, expressed on the surface of monocytes and macrophages<sup>38</sup>. Co-immunoprecipitation of Src kinase Lyn with CD14 and its activation by LPS has identified Lyn to be one of the early kinases that initiate the downstream LPS signaling<sup>39</sup>. In order to understand how MTSA-10 might interfere with LPS signaling, we looked at the tyrosine phosphorylation of Lyn, immunoprecipitated from the LPS-stimulated cells. Whereas LPS induced maximal phosphorylation of Lyn within 5 min, the presence of MTSA-10 delayed this maximal phosphorylation to 10 min, followed by quick dephosphorylation (Fig. 7A). We also observed an overall decrease in the magnitude of Lyn phosphorylation in cells stimulated with LPS in the presence of MTSA-10. Thus, MTSA-10 not only delayed the kinetics of Lyn phosphorylation by LPS,

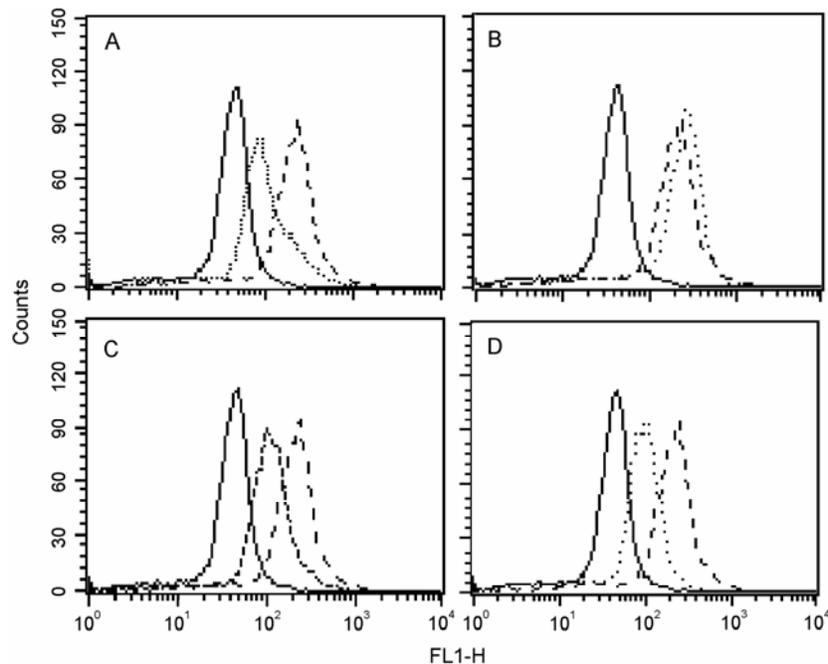


Fig. 4—Effect of MTSA-10 on LPS-induced ROS generation in macrophages. Figures A-D show the FACS profile of ROS generation as measured by DCFDA oxidation. All treatments were for 10 mins. (A)-FACS profiles of untreated J774.1 cells (solid line), and the cells treated with LPS in the absence (broken line), or presence of MTSA-10 (dotted line). (B)- Profiles of untreated cells (solid line), and LPS-stimulated cells in the absence (broken line) or presence of 10  $\mu$ g/ml of ESAT-6 (dotted line). (C)-Profiles of untreated cells (solid line), and the cells stimulated with LPS in the absence (broken line) or presence of both MTSA-10 and ESAT-6 (dotted line). (D)-Profiles of untreated cells (solid line), and LPS-stimulated cells in absence (broken line) or presence (dotted line) of MTSA-10: ESAT-6 (1:1) complex (10  $\mu$ g/mL). All the figures are representative of three different experiments.

but also reduced the magnitude of the initial signal generated.

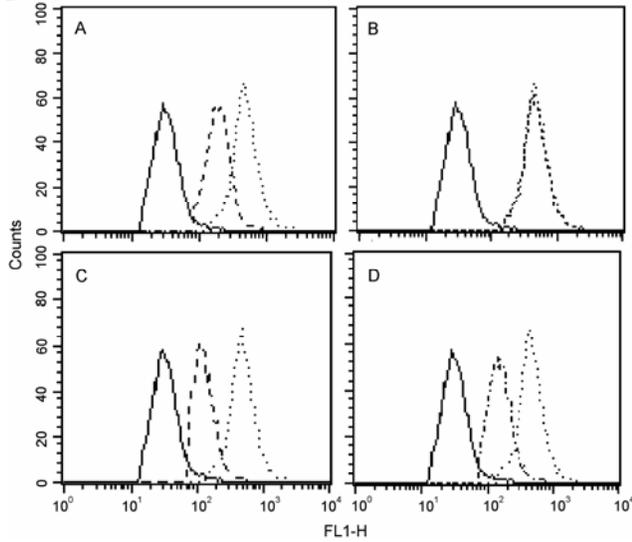


Fig. 5—M TSA-10 exerts similar effects on primary macrophages—Figures (A-D) show FACS profile of ROS generation by mouse peritoneal macrophages. All the treatments shown are of 10 mins. (A)—Profiles of untreated macrophage cells (solid line), LPS-stimulated cells in absence (dotted line) and in presence of M TSA-10 (broken line). (B)—Profiles of untreated cells (solid line), along with LPS-stimulated cells in absence (dotted line) and in presence of 10  $\mu$ g/ml of ESAT-6 (broken line). (C)—Profiles of the untreated cells (solid line), and of cells stimulated with LPS in absence (dotted line) and in presence of both M TSA-10 and ESAT-6 (broken line). (D)—Profiles of untreated cells (solid line), and LPS-stimulated cells in absence (dotted line) and in presence of 1:1 complex (10  $\mu$ g/ml) of M TSA-10: ESAT-6 (broken line). [All the figures are representative of three different experiments each]

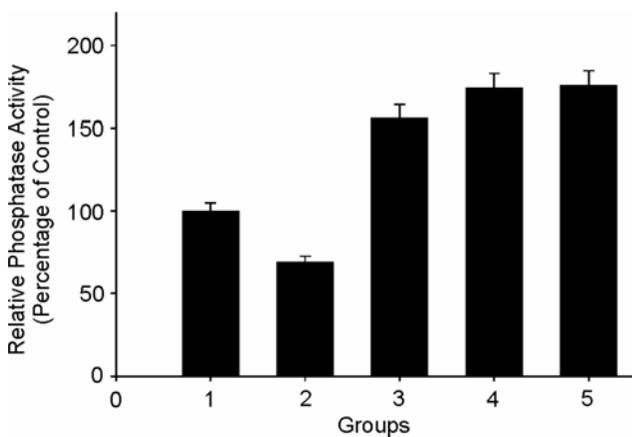


Fig. 6—LPS regulates macrophage phosphatase in a ROS dependent manner. Cells were stimulated for 10 min with LPS in absence (Group 2) or in presence of M TSA-10 (Groups 3, 4 and 5) where cells were pretreated with 50mM of NAC (Groups 4 and 5) before stimulation. Equal amount of cytoplasmic proteins were

subjected to phosphatase assay. [Phosphatase activities are expressed as percentage of that obtained in unstimulated cells (Group 1)]

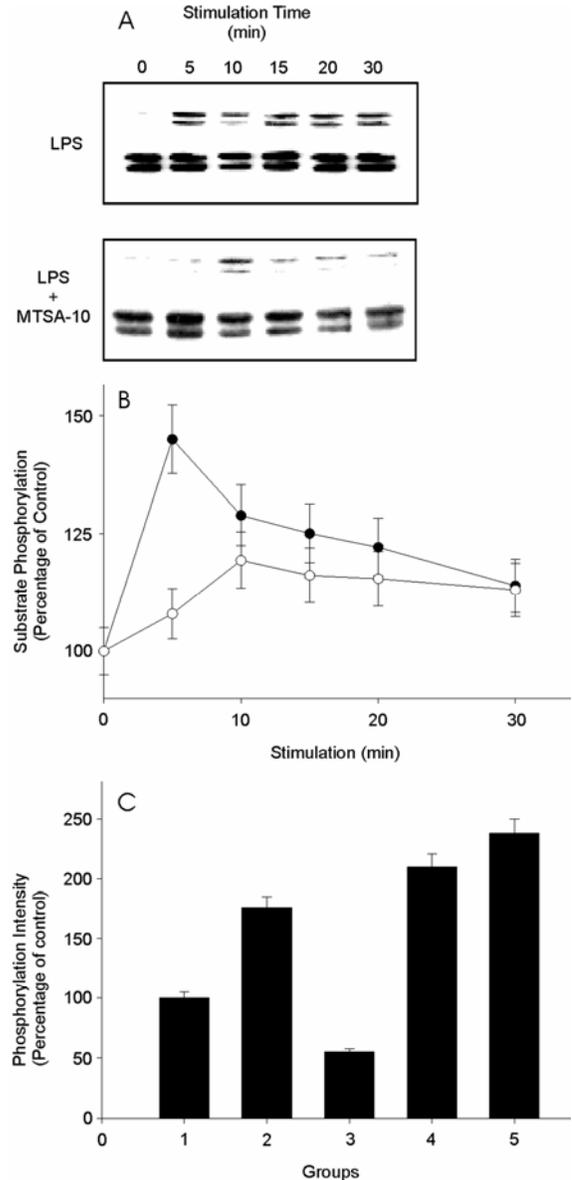


Fig. 7—M TSA-10 influence Lyn activity in LPS stimulated macrophage. (A)—Lyn was immunoprecipitated from the cells stimulated by LPS in presence or in absence of M TSA-10 for the indicated times. The immunoprecipitates were probed with anti-phosphotyrosine antibodies (pP) and the blots were stripped and reprobbed with anti-Lyn antibodies (P). (B)—Lyn was immunoprecipitated from cells stimulated by LPS alone (●) or in the presence of M TSA-10 (○) for the indicated times. The immunoprecipitates were used in a phosphorylation assay of de-phosphorylated casein. Results indicate net incorporation of radioactive phosphate in casein after background subtraction. (C)—Lyn was immunoprecipitated from unstimulated cells (Group 1), cells stimulated for 5 mins by LPS alone (Group 2) or in the presence of M TSA-10 (Groups 3, 4 and 5) where cells were pretreated with H<sub>2</sub>O<sub>2</sub> (Group 4) and sodium orthovanadate (group

5) before stimulation. The immunoprecipitates were probed with anti-phosphotyrosine antibodies. [Intensity of Lyn phosphorylation is expressed as percentage of that seen in unstimulated cells]

Further, we studied the effect of MTSA-10 on activation of Lyn kinase by LPS. Lyn was immunoprecipitated from stimulated cells and its kinase activity was measured using de-phosphorylated casein as a substrate (Fig. 7B). Stimulation with LPS led to rapid activation of Lyn kinase that peaked at 5 min. This activation was severely compromised in cells stimulated in the presence of MTSA-10. Again, a delay in kinetics of activation was observed as evident from the shift in the activation peak to 10 min of stimulation (Fig. 7B). Thus, MTSA-10 exerted a profound effect on LPS signaling, as both the initial intensity and the strength of signal were compromised. Interference by MTSA-10 with LPS-induced Lyn activity seemed to be mediated through reduction in ROS generation. We observed that inhibition of LPS-mediated Lyn phosphorylation by MTSA-10 could be reversed by external supplementation of ROS in the form of  $H_2O_2$ , or by inhibiting the cellular phosphatases with sodium orthovanadate (Fig. 7C). Thus, it seems that inhibition of ROS generation by MTSA-10 was the key event responsible for modulation of macrophage response to LPS.

To investigate further as to how ROS regulated phosphorylation of Lyn, we immunoprecipitated Lyn from LPS-stimulated cells and probed for any associated phosphatase activity. We found that Lyn immunoprecipitated from unstimulated cells showed a high degree of associated phosphatase activity that decreased with increasing time of LPS stimulation. But when the cells were stimulated with LPS in the presence of MTSA-10, the phosphatase activity increased up to 10 min of stimulation before it gradually declined (Fig. 8A). Since there is no report of any phosphatase activity in Lyn *per se*, we tend to speculate that some tyrosine phosphatase may be associated with Lyn, keeping Lyn in an un-phosphorylated state; upon stimulation with LPS, the phosphatase activity associated with Lyn decreases facilitating phosphorylation of Lyn. The presence of MTSA-10 seemed to block the decrease in phosphatase activity, and this in turn prevented Lyn from getting phosphorylated. Immunoprecipitation of Lyn followed by western blot analysis revealed the tyrosine phosphatase, HePTP, to be associated with Lyn. Interestingly, neither the amount of HePTP association, nor its phosphorylation status changed

upon stimulation. So its activity was probably regulated by ROS generated by the macrophage (Fig. 8B).

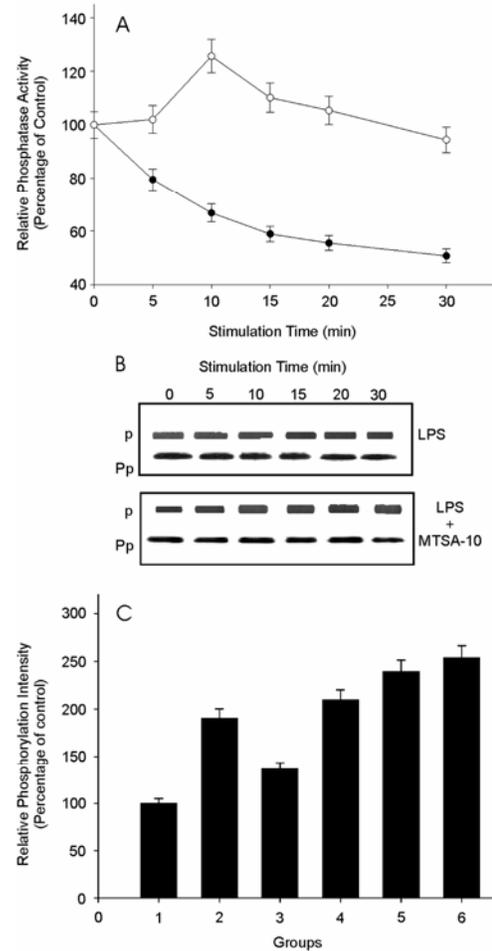


Fig. 8—MTSA-10 influence LPS-stimulated Lyn phosphorylation by activating phosphatase in macrophage. (A)—Lyn was immunoprecipitated from the cells stimulated by LPS in presence (○) or in absence (●) of MTSA-10 for the indicated times. The immunoprecipitates were employed in an assay for detecting the phosphatase activity. (B)—Lyn was again immunoprecipitated from the cells stimulated by LPS in presence or in absence of MTSA-10 for the indicated times and were subjected to western blotting followed by probing with anti-HePTP antibodies (P), the blots were stripped and re-probed with anti-phosphotyrosine antibodies (pP). (C)—Lyn was immunoprecipitated from unstimulated cells (Groups 1 and 4), cells stimulated for 5 min by LPS alone (Groups 2 and 5) or in the presence of MTSA-10 (Groups 3 and 6) where cells used in Groups 4, 5 and 6 were pre-transfected with siRNA targeted for mouse HePTP gene. The immunoprecipitates were probed with anti-phosphotyrosine antibodies. [Intensity of Lyn phosphorylation is expressed as percentage of corresponding intensity observed in unstimulated cells]

To confirm further the role of HePTP in Lyn phosphorylation, we silenced HePTP gene expression using siRNA and then checked the level of Lyn

phosphorylation. We found Lyn to be hyperphosphorylated in HePTP-deficient cells as compared to normal cells in unstimulated condition, indicating that HePTP was necessary to keep a check upon Lyn phosphorylation in untreated cells (Fig. 8C). We also found that MTSA-10 failed to down regulate LPS-induced Lyn phosphorylation in HePTP-deficient cells indicating that MTSA-10 affected Lyn phosphorylation by activating the phosphatase HePTP (Fig. 8C).

*MTSA-10 inhibits LPS-induced gene expression in macrophages*—Cellular signaling has direct implication for transcriptional changes in the nucleus in eukaryotic cells. The cell-specific gene expression involves a cascade of controls over transcription factors and the signals that activate these factors<sup>40</sup>. Mtb is known to reprogram macrophage transcriptome for its own benefit and to influence transcription of a large number of macrophage genes considered essential for the host cell activation response<sup>41,42</sup>. Impact of MTSA-10 on cellular signaling was reflected in the way it modulated the macrophage gene expression in response to LPS

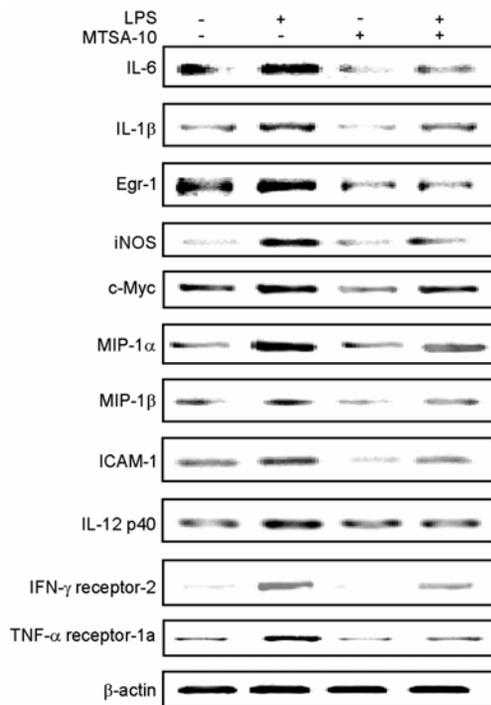


Fig. 9—MTSA-10 regulates gene expression in LPS-activated macrophage. The figure shows the mRNA levels of some common LPS-induced genes. Total RNA was isolated from unstimulated cells and cells stimulated for 6 h by LPS in presence or absence of MTSA-10 and subjected to RT-PCR. Levels of mRNA of the genes from cells treated with MTSA-10 for 6 h are also shown.

stimulation. A variety of known LPS-inducible genes in the macrophage were probed for their expression by RT-PCR amplification analysis on LPS-stimulated cells in the presence or absence of MTSA-10. This experiment revealed that the signaling interception caused by MTSA-10 extended down to transcription of genes encoding co-stimulatory molecules (ICAM-1), transcription factors (Erg-1), cytokines (IL-6), and cell surface receptors (IFN- $\gamma$  receptor 2) among others (Fig. 9). Thus, MTSA-10 blocked the macrophage from responding to LPS activation in multiple ways.

Furthermore, LPS-activated macrophages are known to kill intracellular bacteria primarily by nitric oxide (NO), and by activation of T cells via cytokine IL-12<sup>43</sup>. Therefore, we measured the secretion levels of these two molecules in LPS-stimulated macrophages. We found that the secretion levels of both IL-12p40 and NO were significantly lowered in the cells stimulated with LPS in the presence of MTSA-10 as compared to those obtained upon stimulation with LPS alone (Fig. 10 A,B). The fact that MTSA-10 had no effect on the secretion of either of the molecules indicated that it had no effect on their induction, but its interference with the LPS signaling

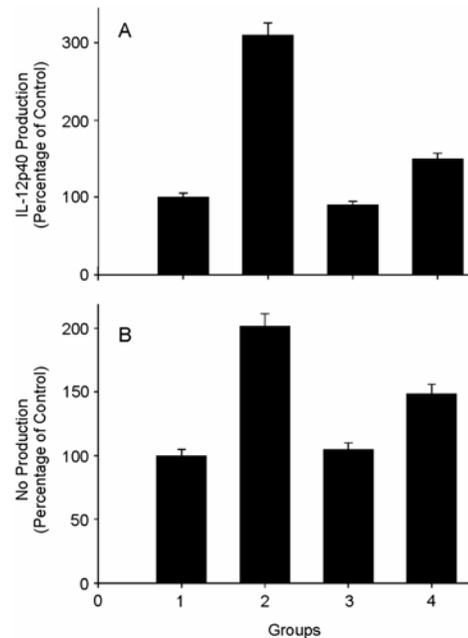


Fig. 10—MTSA-10 modulates the extent of LPS induced macrophage activation—The figure shows (A)—Levels of IL-12p40 secretion; and (B)—NO production by macrophage cells. In both the cases, the values obtained from cells stimulated for 18 h by LPS (Group 2), MTSA-10 (Group 3) and both LPS and MTSA-10 (Group 4). [Values are expressed as percentage of unstimulated cells (Group 1)]

down-regulated the expression and secretion of these two molecules essential to antibacterial responses of macrophages.

### Discussion

Macrophages respond to LPS by undertaking phosphorylation of a number of its intracellular signaling molecules<sup>24,25,44-46</sup>. This initiates a cascade of signaling events that lead to secretion of inflammatory cytokines like IL-1, IL-6 and TNF- $\alpha$  and anti-microbial immune responses<sup>47</sup>. While bacterial pathogens are known to disrupt host cell signaling<sup>10</sup>, the global dampening of macrophage signaling in the presence of MTSA-10 was unexpectedly strong enough to modulate LPS-mediated activation response. At least within the time frame of our experiments, the MTSA-10-treated macrophages failed to gain/maintain the LPS-induced phosphorylation status of majority of its proteins. This dephosphorylation was mainly achieved by activating the macrophage phosphatases, which would be otherwise inactivated by LPS. Mtb lipoarabinomannan (LAM)<sup>28</sup> and our earlier findings suggest MTSA-10 activates cellular phosphatases<sup>37</sup>. But, this is the first time when any Mtb secretory protein has been reported to exert such an effect on macrophage activation. Whereas activation of the membrane-associated phosphatases attenuated the initial events of LPS signaling, activation of cytoplasmic pool of phosphatases inhibited progression of the signal. Together these two events did not allow MTSA-10-treated macrophages to respond to activating stimuli.

LPS is known to induce oxidative burst in macrophages that helps to kill the invading pathogens, and it also plays an important role in signal transduction and the subsequent macrophage activation<sup>46,48</sup>. Our results indicate that MTSA-10, by inhibiting generation of ROS by LPS, blocked the shift of cellular equilibrium towards signal activation and brought about a global effect on the macrophage signaling. In a way, the inhibition of LPS-induced ROS by MTSA-10 functioned as a "control switch" that regulated the consequent events.

This was further supported by the effect of MTSA-10 on the phosphorylation status of Lyn, an Src kinase known to be involved in initial events of LPS signaling<sup>39</sup>. In the presence of MTSA-10, LPS-mediated phosphorylation of Lyn was attenuated in magnitude and delayed in kinetics as evident from the diminished ability of Lyn to phosphorylate the target substrate. Since the strength of initial signal generated

influences the extent to which it progresses, MTSA-10 exerted its control over signal progression in the case of LPS stimulation by simply lowering the strength of the initial signal. This attenuation of signal progression was evident on one hand from the level of protein dephosphorylation and on the other hand from the profile of gene expression. The effect of MTSA-10 on Lyn phosphorylation was most likely a consequence of phosphatase activation via ROS inhibition, as borne out by the fact that MTSA-10 failed to exert its effect when ROS was supplemented in the form of exogenous H<sub>2</sub>O<sub>2</sub> or phosphatases were pre-inactivated with sodium orthovanadate. Our results also indicated that LPS-induced ROS played a crucial role in generation of the initial signal. Although the precise intracellular source from which LPS induces ROS generation remains to be clarified, it has been reported that TLR4 directly interacts with NADPH oxidase 4, which is required for LPS-induced H<sub>2</sub>O<sub>2</sub> generation in the HEK-293 cells<sup>48</sup>. LPS might invoke a similar mechanism in the ROS-dependent activation of Lyn.

MTSA-10 has been reported to exist predominantly as 1:1 complex of MTSA-10:ESAT-6, however, it is pertinent to point out that Okkels and colleagues<sup>49</sup> employed 2-DE analysis to reveal the presence of eight species of ESAT-6 in the Mtb short-term culture filtrate; three of these species are acetylated and show preferential binding of MTSA-10. This observation raises the possibility that at least some proportion of MTSA-10 and ESAT-6 may indeed exist as independent moieties, potentially capable of exerting their individual influence on the host. Recently, it has been reported that in MTSA-10:ESAT-6 (1:1) complex, the C-terminal flexible arm of MTSA-10 actually binds to the macrophage surface, and that partial deletion of ESAT-6 has no effect on binding of the complex to the macrophage surface. Our results on LPS-induced ROS indicated that the effect was MTSA-10 specific, and at least at the level of ROS regulation, MTSA-10 exerted similar effect as the MTSA-10:ESAT-6 (1:1) complex. It seems plausible to suggest that MTSA-10 actually plays the role of the modulator of macrophage immune functions by MTSA-10:ESAT-6 (1:1) complex.

Microbial pathogens have evolved multiple strategies to disrupt host cell signaling, escape from host phagocyte responses, resist humoral defense mechanisms and inhibit T and B cell effector functions<sup>2,10</sup>. The first line of host defense against

invading mycobacteria is their killing by reactive nitrogen species (RNS) produced by activated macrophages. Subsequently, release of cytotoxic T-cell activators like IL-12 by macrophages further strengthens the host response. Mtb, like other microbial pathogens, has evolved several strategies to overcome this initial immune response, which enable it to establish a successful infection. We are now aware of some of these strategies and the responsible mycobacterial factors. Though possible role of Mtb secretory proteins in regulation of host immune response has been suggested<sup>50</sup>, there is very little information about host function modulation by any Mtb early-secretory antigens. In the present study, we have identified the role of one such early secretory protein MTSA-10, which exerted profound influence on macrophage response to activating stimuli. We found that by controlling the ROS generation by macrophage, MTSA-10 compromised its ability to respond to further stimulation. Our results further suggested that Mtb, by this secretory protein could control macrophage activation by virtually any activating agent, regulating the initial signal generation in a ROS dependent manner, and thereby influencing the consequent events. We also speculate that Mtb might utilize such a mechanism, with at least some of its secretory proteins, to attenuate the macrophage function. However, more work will be required to establish this speculation.

It must be mentioned, however, that the present results are from *in vitro* experiments using recombinant mycobacterial protein(s). Although the present study unravels certain interesting aspects of interaction between MTSA-10 and the host cell, further work with macrophages infected with MTSA-10-deleted Mtb mutants is required to understand the immunomodulatory role of this secretory protein.

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