Discussion:

The present study showed that MPT 70, a secretory antigen of *M. tuberculosis*, bound to the surface of macrophages and induced pro-inflammatory cytokines such as TNFα, IL-12, IL-1β and IL-6 but not the anti-inflammatory cytokine IL-10. MPT 70 also enhanced/stimulated LPS-induced NO production and synergized with IFNγ for the same suggesting that MPT 70 may have a pro-inflammatory function. MPT 70 was found to mediate these effects through ERK1/2 and p38 MAPK signaling pathways. The study suggests that MPT 70 might play a protective role against *M. tb* infection by tilting the macrophage effector functions towards pro-inflammatory response. This hypothesis seems to hold true in the light of previous findings that the virulent *M. tb* has downregulated the expression levels of this highly immunogenic protein, and that might protect the mycobacterium from being recognized by immune surveillance mechanism of the host.

Our data show that MPT 70 induced macrophages to produce significant levels of TNFα (Fig. 7a). TNFα is a pro-inflammatory cytokine but its role in tuberculosis infection is complex as it contributes to the host’s protective response as well as to the tissue damage (Bekker *et al.*, 2000; Tsenova *et. al*, 1999; Rook *et al.*, 1990; Rook, 1988). TNFα is required globally as well as locally at the intrapulmonary sites for an effective protective response against infection (Smith *et al*., 2002). TNFα plays a major role in the recruitment of immune cells for granuloma formation (Kindler *et al*., 1989; Mohan *et al*., 2001). In the presence of only Th1 responses, TNFα acts as a macrophage activating factor while in Th1 and Th2 mixed responses or Th0 responses, it causes tissue damage (Hernandez-Pando and Rook, 1994). However, experimental data on animals have shown that TNFα deprivation results in increased bacterial load and susceptibility to infection with shortened survival time, damaged histopathology and abnormal granuloma formation (Adams *et al*., 1995; Bean *et al*., 1999; Bekker *et al*., 2000; Flynn *et al*., 1995; Kindler *et al*., 1989). Also, patients with inflammatory bowel disease or rheumatoid arthritis receiving recombinant anti-
TNF antibodies have increased rates of tuberculosis (Keane et al., 2001). These reports suggest that stimulation of TNFα by MPT 70 elicited macrophages, observed in the present study might contribute to the host’s protective response to tuberculosis. Similar studies with other *M. tb* antigens such as MTSA 10 (aka CFP-10) and Ag 85B have earlier suggested their role in TNFα secretion (Phillips et al., 1996; Trajkovic et al., 2002). MPT 70 facilitating a protective immune response is justified by the fact that, in contrast to virulent *M. bovis*, *M. tb* secretes very low amounts of MPT 70, raising the possibility that *M. tb* has modified itself to deliberately decrease the production of highly immunogenic antigens such as MPT 70 so that it may evade host immune surveillance and hence better persist in the host. On the contrary, other reports have shown that TNFα secretion by human macrophages promotes intracellular replication of *M. tuberculosis* (Byrd, 1997; Engele et al., 2002), indicating that MPT 70 along with other *M. tb* secreted antigens might help mycobacteria to persist in infected macrophages by sustained release of TNFα leading to self-tissue destruction and hence, disease progression. In fact, a report has shown that administration of mycobacterial antigens in *M. tb* infected mice exacerbates disease manifestations (Moreira et al., 2002). In the light of these studies, the TNFα release by the MPT 70-induced macrophages seems to be an important factor in the pathogenesis as well as protective immunity to tuberculosis.

Since IL-12 has been implicated in controlling *M. tuberculosis* infection, we surmised that it would be important to investigate the effect of MPT 70 on IL-12 production. As evident from the results presented in Figure 7a, we observed that MPT 70 significantly increased the IL-12p40 production by macrophages in a dose-dependent manner, further supporting the notion of its possible role in aiding putatively protective host immune response (Fig. 7a). IL-12 is secreted by macrophages and dendritic cells upon phagocytosis of *Mycobacterium tuberculosis* and it drives the Th1 response by inducing the IFNγ production, the key cytokine in controlling *M. tuberculosis* infection (Ladel et al., 1997; Henderson et al., 1997). Previous studies have shown that administration of IL-
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12 in BALB/c mice induced resistance to *M. tuberculosis* infection whereas no significant change was observed in C57BL/6 mice, perhaps underlining the naturally resistant phenotype of C57BL/6 mice (Flynn et al., 1995; Cooper et al., 1995). Studies with the *IL-12p40* gene deficient mice have demonstrated its importance in *M. tuberculosis* resistance. In comparison to control mice, *IL-12p40* gene deficient mice showed high susceptibility to infection, increased bacterial burden and decreased survival time due to impaired IFNγ production (Cooper et al., 1997). Also, introduction of the IL-12 DNA in mice that had chronic *M. tuberculosis* infection significantly reduced the bacterial burden indicating its role in controlling the *M.tb* infection (Cooper et al., 1997). IL-12p70, the second subunit of IL-12 is required for the optimal interferon-γ (IFNγ) T-cell response, which is critical for control of *M.tb* growth (Cooper and Khader, 2008). However, the effect of MPT 70 on the production of IL-12p70, the second subunit of IL-12, is not yet studied.

IL-10 being a major anti-inflammatory cytokine is implicated in macrophage deactivation. The data obtained in the present study indicated that macrophages stimulated with MPT 70 did not produce IL-10 in detectable levels (Fig. 7a). IL-10 is produced by macrophages, causing macrophage deactivation and decreased IL-12 and hence low IFNγ production by T cells. An earlier study has shown that IL-10 inhibition partially uplifted the suppression of macrophages for T cell proliferation in tuberculosis patients (Gong et al., 1996). Also, IL-10 has been shown to inhibit the antigen processing and presentation functions of infected cells and the CD4 T cell responses directly, suggesting that IL-10 acts as a key cytokine to counter macrophage activation (Rojas et al., 1999a, Rojas et al., 1999). However, IL-10−/− mice were not resistant to acute tuberculosis, compared to the wild type mice (North et al., 1998). Failure of MPT 70 to produce IL-10 suggests that perhaps, MPT 70 selectively stimulates production of proinflammatory cytokines and not anti-inflammatory cytokine IL-10, thereby resulting in an efficient macrophage activation, and shifting the balance towards Th1 responses, and hence better containment of the tuberculosis.
In the present study, we also found that MPT 70 stimulated IL-1β secretion by macrophages (Fig. 7a). This again highlights the proinflammatory nature of MPT 70 as IL-1 is a pro-inflammatory cytokine implicated in containment of intracellular pathogens. The IL-1β expression is seen in the broncho-alveolar lavage from the infected lungs of TB patients (Shimokata et al., 1991; Law et al., 1996). Studies with the IL-1 type 1 receptor deficient (IL-1R^-) mice have shown that IL-1R^- mice were unable to respond to IL-1 and upon M.tb infection, were more susceptible to pulmonary TB with increased mortality, enhanced mycobacterial load in the lungs, defective granuloma formation and migration of the cells at the infection site and also decreased production of IFNγ by the splenocytes (Juffermans et al., 2000). Another study has shown that absence of IL-1R signals leads to defects in early control of M.tb infections, and that IL-1 along with IL-1-induced innate responses is important for MyD88-dependent host response to acute M.tb infections (Cecile et al., 2007). These studies suggest that IL-1 plays an important role in the host defense against active pulmonary TB. However, it would be interesting to study the effect of MPT 70 on secretion of IL-1 receptor antagonist (IL-1ra) and soluble IL-1 receptor type II (sIL 1RII) also. It has been shown that IL-1 receptor antagonist (IL-1ra) regulates IL-1 activity by competitively blocking IL-1R type I and, soluble IL-1 receptor type II (sIL-1RII), the shedded ligand-binding part of the corresponding cellular receptor and functions as a competitive inhibitor of the binding of IL-1 to surface IL-1 receptors (Dinarello, 1996), thus regulating IL-1 signalling and affecting the pathogenesis of the infection.

In the present study, MPT 70 was also found to stimulate IL-6 production by macrophages (Fig. 7a). IL-6 is regarded as a proinflammatory cytokine which induces the production of acute phase proteins in response to infection either alone (Geiger et al., 1988), or in synergy with IL-1, resulting in pyrexia and leukocytosis (Helle et al., 1988). IL-6 is produced by a variety of cells, including macrophages, T cells, endothelial cells and fibroblasts (Van Snick et al., 1987;
Hirano et al., 1985; Corbel et al., 1984; Weissenbach et al., 1980), and exerts its effects on multiple cell types, and hence, known as a pleiotropic cytokine. IL-6 is also a B cell growth and differentiation factor (Kopf et al., 1998) and it has also been shown to play a role in the initiation of T cell activation during tuberculosis subunit vaccination (Leal et al., 1999). Studies have shown that, regulatory T cells were suppressed by IL-6 that was induced by microbial components supporting in vivo effector T cell proliferation (Pasare and Medzhitov, 2003). However, contradictory studies have shown IL-6 to inhibit T cell responses and also macrophage responsiveness to IFNγ, thereby inhibiting Th1 differentiation in naive CD4 T cells (VanHeyningen et al., 1997; Diehl et al., 2000; Nagabushanan et al., 2003). Although the role of IL-6 as a proinflammatory cytokine is controversial, a recent report has shown its role in inducing protective T cell memory against M.tb infection (Singh et al., 2011). In the above mentioned study, IL-6, IL-1 and TNFα when co-administered along with M.tb infected macrophages (which were used as a candidate vaccine), induced better T cell memory response than BCG. Taking these observations into account, MPT 70 again seemed to have a protective role as it stimulated secretion of IL-6 along with IL-1β, TNFα and IL-12p40, promoting Th1 response through IFNγ secretion by T cells.

Our study has also demonstrated the effect of MPT 70 on the nitric oxide production by macrophages. MPT 70, though unable to induce NO release on its own, enhanced LPS-induced NO production upon pre-sensitization of macrophages with MPT 70 (Fig. 8). Nitric oxide generated by macrophages via iNOS has been implicated in clearance of mycobacterium in mice. This has been supported by the studies showing that the inhibition of NO synthesis by iNOS inhibitors or by iNOS gene knockout resulted in increased susceptibility of mice to mycobacterial infection (Chan et al., 2001; Chan et al., 1995; MacMicking et al., 1997). The protective role of NO in humans remains somewhat controversial, although the in vitro studies with M.tb infected monocytes and alveolar macrophages have supported its implication in controlling M.tb infection in
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humans (Jagannath et al., 1998; Rockett et al., 1998; Wang et al., 2001). In the present study, MPT 70 could indeed prime murine macrophages for NO release and hence increased the production of NO triggered by subsequent exposure to M.t.b whole cell lysate or LPS. If this phenomenon is also mimicked in vivo, then MPT 70-primed macrophages might be better equipped for NO-mediated killing and therefore, more efficient in controlling M.t.b infection. This is in contrast to a study with another M.t.b antigen, CFP 10, which was reported to desensitize the macrophages and hence reduce LPS-induced NO (Trajkovic et al., 2002). In fact, M.t.b proteins CFP 10 and ESAT 6 have been implicated in dampening the host cell responses by downregulating NO and ROS generation and MHC II expression (Sinha et al., 2006; Ganguly et al., 2007; Kumar et al., 2011). Also, MPT 70 is expressed in very small amounts by M.t.b than M. bovis, the virulent mycobacterium causing bovine TB in cattle, whereas CFP 10 and ESAT 6 are secreted by M.t.b in high abundance. Thus, M.t.b being a pathogen of humans may have evolved a mechanism to downregulate or decrease the synthesis of proteins like MPT 70 which might render them susceptible to host’s inflammatory immune surveillance, and increase the synthesis of proteins like CFP 10 and ESAT 6 which modulate host responses to benefit the pathogen survival and propagation. Therefore, whether administration of MPT 70 and similar proteins along with BCG or any other vaccine will or will not confer better protection against M.t.b remains an issue to be investigated. Also, in all likelihood, MPT 70 seems to be associated with the dormant phase of TB and therefore, it perhaps may also provide protection against latent TB infection, though its efficacy as a vaccine candidate has to be explored in this respect. It may be pertinent to note that in a recent study, Tyagi and co-workers have shown that antigens expressed at the latent stage of infection, when administered as vaccine, provide better protection against TB (Dey et al., 2011).

In addition to its effect on the LPS-induced NO release, MPT 70 was also found to synergize with IFN\(\gamma\) (Fig.9). IFN\(\gamma\) is a major activator of macrophages and is central to effective immune response against Mycobacterium tuberculosis by
activating macrophages to produce mycobactericidal NO via iNOS (Dalton et al., 1993; Flynn et al., 1993). The present study has shown that MPT 70 synergizes with IFNγ in a dose dependent manner to induce NO synthesis in J774 or murine peritoneal macrophages when applied before, simultaneously or after MPT 70 (Fig. 9). This is in contrast to that reported for another M. tb secreted protein, namely CFP 10 which failed to stimulate macrophage NO synthesis when applied before IFNγ. The effect of CFP 10 on macrophage NO release seemed to be directed by IFNγ, in the absence of which CFP 10 would inhibit LPS-induced NO but in the presence, it overcame the inhibitory effect and synergized with IFNγ to produce NO (Trajkovic et al., 2002). This suggests the modulatory role for CFP-10 and its ability to subvert the host cell immune response in the absence of adequate IFNγ response. Whereas results with MPT 70 in the present study show that it induced macrophages to produce NO in the presence of LPS as well as IFNγ which suggests that MPT 70 would induce macrophage to release NO even during inadequate IFNγ responses, further strengthening the view about its role as a protective molecule. Though MPT 70 and IFNγ synergize to induce macrophage NO release, they seem to trigger distinct intracellular events in macrophages as the former induced TNFα but no NO synthesis, while latter induced NO but no detectable TNFα production (Fig. 7a, 9). As mentioned in the results, the synergistic induction of NO by IFNγ and MPT 70 primed macrophages was through iNOS as the NO release was sensitive to aminoguanidine which is a well-known specific inhibitor of iNOS (McDaniel et al., 1993). In contrast to the microbicidal role of NO, it has been known to induce T cell hypo- responsiveness in M. tb infected mice (Nabeshima et al., 1999). NO is also known to induce apoptosis in M. tb infected murine macrophages (Rojas et al., 1997). Since MPT 70 is a secretory protein, it might induce iNOS and apoptosis of uninfected macrophages, thereby reducing the protective capability of the host. Therefore, further studies with MPT 70 in M. tb infected mice need to be carried out to ascertain its role as a protective molecule.
Since Mitogen-activated protein kinases (MAPK) play important role in signal transduction pathways involved in production of the above mentioned pro-inflammatory molecules, we also investigated the effect of MPT 70 on ERK1/2 and p38 MAPK activation. Previous studies have shown that intracellular pathogens target the MAPK pathways to survive inside the macrophages (Ruckdeschel et al., 1997), prompting us to study the influence of MPT 70 on the MAP kinases. ERK1/2 and p38 MAPK are serine/threonine kinases implicated in NO and TNFα release by macrophages upon activation by LPS. Our study has shown that MPT 70 and MPT 70- IFNγ induced TNFα release and NO induced by pre-MPT 70- LPS and MPT 70- IFNγ are dependent on both p38 and ERK1/2 pathways (Fig. 10). Although, cells were pre-treated with IFNγ and then stimulated with MPT 70 for NO induction to avoid any interference with IFNγ signals, one cannot completely rule out the possibility that IFNγ induced signals were also interrupted by the kinase inhibitors. Nevertheless, our results suggest that both TNFα and NO release are induced by the same ERK1/2 and p38 pathways. Studies have shown that virulent strains of mycobacteria cause greater inhibition of MAPKs, particularly ERK1/2 pathway, than avirulent strains (Blumenthal et al., 2002). To further examine the ERK1/2 and p38 activation, the expression of p-ERK1/2 and p-p38, the activated form of these molecules, was examined in MPT 70 treated macrophages. Expression of both these MAPKs increased upon MPT 70 treatment (Fig. 11, 12, 13, 14). The activation was similar to that of LPS and stimulation of MPT 70 pre-treated macrophages was further increased after LPS stimulation suggesting that MPT 70 heightens LPS-induced ERK1/2 and p38 activation and thus magnifies LPS effects (Fig 11, 12, 13, 14). This might help in eliciting better immune responses against M.tb and may help in driving T cell responses towards Th1 type and further aid in better recognition and subsequent elimination of the pathogen by the host immune system.
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*M. tuberculosis* regulates the macrophage cytokines and NO production during infection through its secreted proteins and cell wall components, adding to the complexity of the immune responses against *M. tb*. However, proteins like MPT 70 have shown to have pro-inflammatory functions, which may provide better immune recognition and hence clearance of *M. tb*. However, further studies need to be carried out to establish the role of MPT 70 as a protective molecule. The current study will help in understanding the immune-pathogenesis of *M. tb*, paving way for development of MPT 70 based candidate vaccines. MPT 70, thought to be expressed as a dormant phase protein, may also serve as a promising marker for diagnosis of latent tuberculosis infection. Studies need to be carried out to explore its expression and immune effects in *M. tb* infected humans.