Discussion
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*Mycobacterium tuberculosis* is known to modulate the activation and consequent functioning of the cells of immune system for its own benefit. It has devised numerous ways to evade protective immune responses by the modulation of host factors. These include downmodulation of surface MHC class II expression on macrophages and changes in the profile of cytokines and chemokines in DCs that individually and collectively affect the priming of T cells (Flynn and Chan 2001). Other modulations include downregulation of reactive nitrogen and reactive oxygen species generation, inhibition of IFN-γ receptor expression and activation on macrophages (Kincaid and Ernst 2003; Singhal *et al.*, 2007).

Till date, many *M. tb* antigens have been found to be promising vaccine as well as diagnostics candidates (Anderson 1994; Roberts *et al.*, 1995; Roche *et al.*, 1994, 1996; Brandt *et al.*, 1996; Elhay *et al.*, 1998; Tanghe *et al.*, 2001). However, despite the large volume of data available on these antigens, their physiological role(s) at sites of infection are not yet fully deciphered. Many *M. tb* protein and non-protein antigens have been demonstrated to play roles in immune evasion. For example, mycobacteria interact with DC-SIGN to modulate TLR4-mediated immune responses by DCs (Geijtenbeek *et al.*, 2003). The ligand responsible for this DC-SIGN-mediated immune modulation is Mannosylated lipoarabinomannan (ManLAM), a cell-wall component abundantly expressed by *M. tb*. ManLAM expressed on the surface and later secreted by virulent *Mycobacterium* species such as *M. tb* and *M. avium* (Wieland *et al.*, 2007; Noss *et al.*, 2001) has been demonstrated to bind DC-SIGN homologs such as DC-SIGNR1 (CD209b) and L-SIGN on both mouse and human cells (Wieland *et al.*, 2007). Binding of ManLAM to DC-SIGN impairs lipopolysaccharide (LPS)-induced maturation of DCs and increases the production of the immunosuppressive cytokine, interleukin-10 (IL-10).
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(Geijtenbeek et al., 2003). The intracellular signaling triggered upon DC-SIGN engagement (ERK and PI3K activation, transient rise in intracellular calcium concentration) impairs IL-12 and enhances IL-10 release. These could either individually or collectively contribute to DC-SIGN ligation favoring pro-Th2/protolerogenic dendritic cell maturation (Caparrós et al., 2006). The fact that DCs do not support the growth of mycobacteria due to IL-10 induced reversion of DC maturation indicates that pathogen recognition by DC-SIGN might modulate DC-induced immune responses, shifting the balance from immune activation towards impairment of immune responses, which would be beneficial to pathogen survival.

Also, mice lacking DC-SIGNR1 induce stronger T cell responses to M. tb (Noss et al., 2001) indicating an inhibitory role for ManLAM in T cell priming. Our lab has also recently demonstrated that one of the potent mechanisms by which ManLAM plays an inhibitory role via DC-SIGN homologs is its ability to inhibit IL-12 secretion from DCs. This was due to increased expression and recruitment of SOCS1 to DC-SIGN homologs (Srivastava et al., 2009). The study has shown that increased expression of SOCS1 could be one of the signalling mechanisms that contribute towards the induction of suppressive responses by ManLam via regulation of IL-12 and IL-12 receptor levels. In addition, many M. tb antigens modulate TLR signaling. These include LAM (Means et al., 1999), a 19-kDa lipoprotein (Noss et al., 2001), the CpG repeat of nonmethylated DNA (Hemmi et al., 2000) and peptidoglycan. These components interact with different TLRs, e.g., LAM and a 19-kDa lipoprotein act on TLR2, CpG DNA acts on TLR9, and peptidoglycan acts on TLR2 and TLR4. In particular, 19-kDa antigen has been well characterized and is involved in compromising many antibacterial functions of macrophages in TLR2 dependent manner (Pennini et al., 2006).

Over the last few years, our lab has also been involved in the characterization of these events that, in turn, mediates priming and long-term survival of M. tb. Till now, these studies have been focused on elucidating the interactions of M. tb secretory antigens with dendritic cells (DCs) and the outcome of these interactions on host mediated immune responses. Our lab has demonstrated the role of M. tb antigens such as CFP-10 in
modulating DC functions. CFP-10 induces the differentiation of DCs (Latchumanan et al., 2002). However, these CFP10 differentiated DCs induce suppressor responses as opposed to the conventional DCs (GM-CSF differentiated DCs). These suppressor responses include decreased levels of IFN-γ and increased IL-10 levels from T cells (Natarajan et al., 2003; Balkhi et al., 2004). In addition, CFP10-DCs were found to modulate ROS levels leading to the increased survival of mycobacteria within DCs (Sinha et al., 2006). This indicated that secretion of antigens (like CFP-10) at sites of infection could be a strategy employed by M. tb towards downregulation of proinflammatory and protective immune responses via differentiation of DCs. It was also seen that conditioning DCs with appropriate cytokines and chemokines result in the mounting of protective immune responses and clearance of an established M. tb infection in mice (Salam et al., 2008).

Similarly, many more antigens leaching out of infected macrophages at different phase of infection dictate the immune responses mounted against M. tb. These include various proteins, cell wall components such as LAM, mycolic acid and other lipids. M. tb proteins that are secreted from infected macrophages are the early antigens captured by professional antigen presenting cells such as the dendritic cells (DCs). Consequently, the nature of the immediate and long-term immune responses mounted against pathogen is largely determined by the quality and quantum of responses initiated by M. tb and its antigens. In view of the above reports, we argued that M. tb would express many more antigens like CFP-10 and 19-kDa etc., as a function of time inside macrophages that would result in the continued suppression of macrophage and DC activation. This could further serve as an immune evasion mechanism and result in the creation of a niche for long-term survival of M. tb.

The first step towards proving this hypothesis was to enrich M. tb antigens that were expressed inside infected macrophages as a function of time. Macrophages were infected with M. tb for two different time points (selected on the basis of infectivity in macrophages and viability of intracellular bacteria). Using the series of procedures, we could enrich 10 antigens from the M. tb infected macrophages at different time points of
infection - 5 antigens from day 1 (24 h) of infection and 5 antigens from day 5 (120 h) of infection, hereafter, called day 1 and day 5 antigens respectively. Results indicated that \textit{M. tb} expresses different antigens at different times post-infection. While antigens expressed immediately following infection (day 1 antigens) are required for the survival of pathogen in infected cells as revealed by their presence in the Rubin list (Rengarajan \textit{et al.}, 2005); antigens expressed at later times post-infection (day 5 antigens) work towards the establishment of persistent infection (latency). All the day 5 antigens have been previously shown to play a major role in promoting latent infection under \textit{in vitro} culture conditions (Singh \textit{et al.}, 2009; Manganelli \textit{et al.}, 2004; Mack \textit{et al.}, 2009). Significant among these were Rv3416 (\textit{WhiB3}), Rv3911 (\textit{SigM}) and Rv2391 (\textit{SirA}). Interestingly, antigens expressed at day 1 of infection were not expressed at day 5 of infection and similarly, antigens expressed at day 5 of infection were not detected early in the infection process. Also, none of the day 5 antigens matched with the Rubin list. These findings were further confirmed \textit{in vivo} wherein most day 1 antigens were expressed within 3 days of infection, while the expression of day 5 antigens was observed at day 15 of infection and not at early times. These results indicated that expression of \textit{M. tb} antigens during infection is finely tuned and temporally regulated.

In the next series of experiments, we characterized the role played by these antigens in the modulation of host immune responses and the mechanisms employed thereof. Results indicated that antigens expressed by \textit{M. tb} at different times post infection differentially modulate the activation of DCs and macrophages. Although day 1 and day 5 antigens mediate immune suppression, they employ different strategies that are commensurate with the functioning of the cells of immune system. It is already established that interaction of human DCs with \textit{M. tb} or BCG results in increased surface densities of a number of molecules such as MHC II, CD40, CD54, CD58, CD80, CD86 and CD83 and IL-12 production that are involved in interactions with T cells (Hickman \textit{et al.}, 2002; Giacomini \textit{et al.}, 2001; Henderson \textit{et al.}, 1997; Kim \textit{et al.}, 1999), suggesting that \textit{M. tb} infection directly induces DCs to mature. Our results also showed that day 1 antigens enhanced \textit{M. tb} mediated activation of DCs as evident by the increased surface densities of key activation markers such as MHC molecules, costimulatory molecules and
receptors for IL-12 and IFN-γ. But, on the other hand, contrary to day 1 antigens, day 5 antigens were found to prevent any such activation indicating suppression of DC functions at later times post infection. Further, all the antigens were found to curtail pro-inflammatory cytokine secretion from DCs. These results indicated that early activation of DCs could be beneficiary to the pathogen but at later times post-infection, inhibition of DC activation is needed to establish a safe niche for the bacteria.

Similar experiments in macrophages, however, showed that both day 1 and day 5 antigens inhibited the activation of these cells. M. tb is known to downmodulate the expression of MHC class II molecules on infected macrophages early during the infection process (Wang et al., 2005). TLR2-dependent inhibition of MHC class II transactivator expression, MHC class II molecule expression and antigen presentation leads to immune evasion by M. tb to establish persistent or latent infection in macrophages. This reduction of antigen presentation might reflect a general mechanism of negative-feedback regulation that prevents excessive T cell-mediated inflammation and that M. tb has subverted to create a niche for the survival in infected macrophages and evasion of recognition by CD4+ T cells (Harding and Bloom 2010).

In the present study, we have shown that antigens expressed early during the infection contribute towards this downmodulation, and for the first time, present data that this modulation would continue at later times during the infection with the expression of day 5 antigens. These results have an important bearing on the extent of early and late T cell responses mediated by these antigens.

An important function of DCs is to prime T cells (Banchereau et al., 1998) that then mediate effector functions, e.g. activation of infected macrophages in case of M. tb infection. The above results indicated that by the time day 1 antigen-activated DCs would prime T cells, the infected macrophages would have already expressed day 1 and later day 5 antigens which would result in the downmodulation of MHC class I and class II molecules on the macrophage cell surface. Therefore, in effect, the T cells primed by antigen activated DCs would be blind to the infected macrophages and as a result, both
the effector functions (CD4⁺ T cell mediated activation or CD8⁺ T cell mediated cytotoxicity) would be impaired. Thus, even if day 1 antigens mediated activation of DCs, indicating the possibility of a productive T cell response, it would not ensue. In addition, should a possible T cell recognition is mediated by infected macrophages, the low levels of pro-inflammatory cytokines, IL-12, IL-6 and IL-17 (that serves as a third signal during T-cell-APC interaction), secreted by antigen activated DCs would ensure that these T cells mediate suppressor responses. To validate this hypothesis and to give the functional relevance to the above results, we investigated the cytokine profiles secreted during a cognate DC - T cell interaction. The cytokine profile displayed a phenotype indicative of suppressor responses with high levels of IL-10 and low levels of IL-17 and IFN-γ. These results indicated that all the antigens essentially abrogated the pro-inflammatory T-cell responses irrespective of the time at which they were expressed using different strategies.

The next step in study dealt with the understanding of mechanisms employed by these antigens in the downmodulation of immune responses from DCs and macrophages. Since essentially similar responses were observed when the comparisons were made between the results obtained from all day 1 antigens and all day 5 antigens, we initially restricted our experiments to a single day 1 and a single day 5 antigen. Later when recombinant proteins were used, we extended the study to four antigens. Also, the two antigens that were selected for the further study i.e. Rv2463 and Rv3416, were most potent of all the other antigens in downmodulating pro-inflammatory responses (Table 6.4). Using these antigens as models, we analyzed their ability to modulate key functions of DCs and macrophages that are indicative of protective responses (i.e. oxidative and nitrosative bursts, SOCS1 levels and induction of IL-12 levels).

We have recently shown that stimulation of mouse and human DC-SIGN homologs induce higher expression of SOCS1, while stimulation of TLR2 results in lower SOCS1 expression (Srivastava et al., 2009). High SOCS1 expression results in lower levels of IL-12 in the context of M. tb infection. Inhibiting SOCS1 by RNAi enhanced IL-12 levels and reduced intracellular bacterial loads. SOCS1 is found to negatively regulate LPS and
IL-4-induced DC maturation (Yu et al., 2004) and is also involved in the regulation of DC subset distribution (Tsukada et al., 2005). Further, SOCS proteins are also involved in the regulation of early Th1- and Th2-cell differentiation.

It was, therefore, interesting to study whether these day 1 and day 5 antigens also employ the modulation of SOCS1 expression as one of the strategies to modulate the host immune responses. Results indicated that both day 1 and day 5 antigens quantitatively as well as kinetically increased TLR-2 mediated SOCS1 expression in DCs, also suggesting that the downmodulatory effects of SOCS1 could be initiated early during the course of infection. Further, higher expression of SOCS1 translated into subversion of IL-12 production thereby influencing the induction of suppressor responses.

*M. tb* employs different mechanisms to withstand various stresses mounted by the host immune cells such as macrophages and DCs that eventually help it to establish its own niche. DCs are known to produce reactive oxygen species (ROS), with both signaling and anti-pathogenic functions. It has been reported that oxidative stress, induced following exposure to H$_2$O$_2$, influenced DC maturation and function by regulating surface expression of MHC molecules, chemokine and cytokine expression including TNF-α (Kantengwa et al., 2003). Bovine DCs have been shown to produce ROS in response to Toll-like Receptor (TLR) agonists (Werling et al., 2004; Elsen et al., 2004). On the similar lines, activated macrophages express two enzymes, phagocyte oxidase (NOX2/gp91phox) and inducible nitric oxide synthase (iNOS), which generate reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) respectively. Both ROI as well as RNI are bactericidal as they react with a wide range of molecules, including nucleic acids, proteins, lipids and carbohydrates. To counteract these stresses, *M. tb* uses a variety of molecules to either detoxify ROI and RNI before they can cause harm or to repair the damage they caused. One such resistance mechanism against ROI is mediated by the *katG* product catalase-peroxidase, which decomposes H$_2$O$_2$ into water and oxygen. *M. tb* lacking *katG* (Mt$b\Delta katG$) exhibited no catalase activity and was hypersusceptible to H$_2$O$_2$ in culture (Ng et al., 2004). On similar lines, our lab has also demonstrated the role of secretory antigens in modulation of generation of reactive
oxygen species. It was found that CFP-10 differentiated DCs serve as depots for the survival of mycobacteria by downregulating oxidative burst following infection with mycobacteria (Sinha et al., 2006). This was achieved at two levels. The first was the increase in the levels of a well-known quencher, namely, superoxide dismutase 1 (SOD1) and the second was the poor induction of calcium influx upon infection. SOD1 serves to absorb reactive oxygen species and converts them into hydrogen peroxide. H$_2$O$_2$ in turn is scavenged by peroxiredoxins and converted into water and harmless divalent oxygen. In fact, M. $tb$ encodes the mammalian homolog of SOD that are active inside infected macrophages (Flynn and Chan 2001). Addition of H$_2$O$_2$ or increasing Ca$^{2+}$ inside CFP10-DCs resulted in better clearance of bacteria (Sinha et al., 2006). Furthermore, the Cu,Zn superoxide dismutase of M. $tb$ has been demonstrated to play a role in enhancing its survival such that deletion of the same results in increased killing of intracellular mycobacteria in infected macrophages (Piddington et al., 2001).

Considering the above studies and to further our understanding on the physiological roles played by the M. $tb$ antigens expressed inside macrophages during the course of infection, it was investigated if DCs, in the presence of the day 1 and day 5 antigens, could modulate the generation of reactive oxygen species. In agreement to the results obtained with CFP-10 DCs, it was found that that both day 1 and day 5 antigens prevent the generation of oxidative burst in DCs that might lead to better survival of the pathogen in these cells.

Results, thereby, indicated that all the key functions of DCs that are indicative of protective responses were modulated by one antigen or the other but with different kinetics. Both day 1 and day 5 antigens subvert DC functions by similar mechanisms (involving upregulation of SOCS1 levels and thereby lowering IL-12 production), thereby ensuring that the priming of T cells is constantly subverted as the infection progresses.

On similar lines, the mechanisms by which day 1 and day 5 antigens modulates protective responses in macrophages were also looked upon. Importantly, M. $tb$ has been
shown to interact differently with DCs as compared to macrophages. The most potent antimicrobial molecules produced by macrophages and probably the most effective antimicrobial molecules produced by any effector cell are reactive nitrogen intermediates (RNIs). High output nitric oxide (NO) production by immunologically activated macrophages is a major antimicrobial mechanism of these cells (Fang 1997; MacMicking et al., 1997; Chan and Flynn 1999). Macrophage activation with IFN-γ and TNF-α leads to enhanced production of reactive nitrogen intermediates in the murine system. High level expression of iNOS and RNI production were also detected in human alveolar macrophages lavaged from the lungs of tuberculosis patients (Choi et al., 2002) accompanied with increased level of exhaled NO in such patients (Wang et al., 1998). It has been also argued that intracellular survival of mycobacteria within macrophages is better regulated by reactive nitrogen intermediates as compared to ROS (Nathan et al., 2000; Gomes et al., 2002). As compared to DCs, the roles of both day 1 and day 5 antigens in modulating macrophage functions matches with the kinetics of different protective responses that is generally mounted by the infected macrophage. This is evident from the results obtained on iNOS2, IL-12 and SOCS1 levels, wherein day 1 antigens downregulated the expression of iNOS2 levels while day 5 antigens upregulated the SOCS1 levels. Also, day 1 antigens downregulated the levels of NO produced as monitored by the nitrite levels. This points to the fact that day 1 antigens help the bacteria to overcome the stress induced by toxic nitrogen intermediates, which constitute a major microbicidal mechanism of macrophages, and day 5 antigens modulated the immune response by increasing SOCS1 levels and promoted the survival of *M. tb* inside macrophages. Since macrophages rely more on the reactive nitrosative pathways for mounting protective responses immediately following infection (Flynn and Chan 2001), subversion of these reactive species *via* downmodulation of iNOS2 by day 1 antigens would be strategically useful for *M. tb* in early adaptation to stress and for survival inside macrophages. Further, the results obtained for the modulations of ROS levels in macrophages were in agreement to the previous reports that macrophages are the poor generators of ROS as compared to DCs.
Quenching and sequestration of ROS and RNIs are some of the many strategies employed by various pathogens to evade host-mediated killing and elimination (Flynn et al., 2001). This is more true with respect to the ‘danger hypothesis’ in the context of peripheral activation of DCs. ROS forms an integral component of defense with respect to invading pathogens (Lenaz 2001; McPhail et al., 1992; Hingley-Wilson et al., 2003). Therefore, reduced ROS levels in DCs in the presence of \textit{M. tb} antigens might modulate danger signals seen by the host, which again would favor the pathogen.

Thus, depending upon the time of induction of the effector molecules in the host cells, the antigens adjust and complement the functions of each other such that protective immune responses are essentially thwarted.

To further characterize the antigens, these proteins were recombinantly expressed and purified. Henceforth, the functional characterization was done with the exogenous stimulation of these antigens. Results from these studies indicated that irrespective of whether the antigens were expressed inside the cells or were provided as an exogenous stimulation, they modulated major DC and macrophage functions like down modulation of pro-inflammatory cytokine secretion, down regulation of Th1 responses, increased expression of TLR2 induced SOCS1 levels in DCs and downmodulation of iNOS2 levels in macrophages.

It has been argued that a balance between activation of TLR2 and Dendritic Cell-Specific ICAM-3 Grabbing Non-integrin (DC-SIGN) during \textit{M. tb} infection governs the generation of protective versus suppressor responses (Kooyk and Geijtenbeek 2003). TLR2 mediated activation results in higher IL-12 expression \textit{via} increased activation of NF-κB, while stimulation of DC-SIGN blocks NF-κB activation resulting in lower IL-12 secretion. Based on this, it has been proposed that at initial stages of infection when the pathogen load is low, TLR2 triggering induces protective immunity and prevents the development of active TB disease. Following increased bacterial burden as a result of HIV infection or other factors and the development of active disease (Flynn and Chan 2001), soluble ManLAM is secreted from the infected macrophages at later times post
infection that triggers DC-SIGN to induce suppressor responses, thereby favoring the pathogen. Therefore, in context of the above data, an increase in TLR2 mediated SOCS1 expression by day 1 and day 5 antigens in DCs would inhibit Th1 cell priming and by day 5 antigens (at later times post infection) in macrophages would inhibit TLR2 signals and possibly amplify signals by DC-SIGN. This once again emphasizes the complementary roles played by the antigens that are expressed at different times post-infection and collectively works towards immune suppression.

Importantly, M. tb has been shown to interact differently with DCs as compared to macrophages. For example, infection of DCs with M. tb induces their activation by upregulating MHC and costimulatory molecules (Henderson et al., 1997; Tsuji et al., 2000). This also results in secretion of IL-12 from infected DCs. On the other hand, infection of macrophages with M. tb results in downregulation of MHC class I and class II molecules, IFN-γ responsiveness and IL-12 production (Flynn and Chan 2001; Kincaid and Ernst 2003; Singhal et al., 2007; Hisert et al., 2004; Sendide et al., 2005). The upregulation of these surface markers in infected DCs underlines the capacity of DCs to mature following M tb infection, which correlates with the acquired ability to present antigens to T lymphocytes, indicating that while M. tb infection results in the direct activation and maturation of DCs followed by enhanced presentation of antigen and capacity to stimulate T cells, it impairs the ability of macrophages to process and/or present soluble antigen and in turn, to serve as accessory cells in T cell activation (Giacomini et al., 2001). Our results also suggest that M. tb antigens expressed inside macrophages at different times post infection interact differentially with DCs and macrophages but the cumulative outcome of these effects essentially result in compromising the ability of the infected cell to mount effector responses to the pathogen. Nevertheless, the use of either mechanism leads to a better survival of the pathogen in infected cells.

Collectively, above results point towards a unique survival strategy employed by mycobacteria towards immune evasion and increased intracellular survival in DCs and macrophages. This includes the strategically expressed different antigens at different
times post infection by \textit{M. tb}. These antigens differentially modulate key functions of DCs and macrophages, as the infection progresses, resulting in the generation of suppressor responses and better survival of the pathogen inside host. Put together, these results exemplify the role of diverse antigens in regulating the immune responses by various cells of the immune system.

All the above observation reveals that since DCs and macrophages are at the forefront of induction of immunity, it is pertinent that some of their functions will be modulated by \textit{M. tb} or other pathogens to shift the balance of immune response where it is beneficial for the pathogen. Interfering with or modulating effector functions of T cells is probably among the highest priorities for \textit{M. tb}, given the central role of T cells in resistance and control of infection with this organism.