Discussion
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The success of mycobacteria as pathogens for the humankind is largely dependent on their capacity to avoid destruction by host cells, particularly the macrophages. Mycobacteria are internalized into the macrophage phagosome by various mechanisms. However, in contrast to the normal course of events in which the phagocytosed cargo is shuttled to lysosomes where it is efficiently destroyed; mycobacteria are able to block the delivery to lysosomes. In doing so, pathogens manage to circumvent immediate destruction enabling them to establish a niche inside the macrophage where they can survive and even replicate. A good number of studies have led us to understand intricacies of the macrophage-mycobacterium interaction. However, several important questions are still unaddressed.

One third of the world’s total population is considered to be infected with *M. tuberculosis*. However, amongst the infected people only about 10% develop overt disease in their lifetime. The remaining 90% people are only latently infected and are able to successfully contain the bacteria. Though earlier researchers have related this phenomena to the socio-economic status, natural resistance associated membrane protein (NRAMP), immunological make of person, etc.; no attempts were made to understand the factors operational at the macrophage level which could possibly delineate these two populations. This study was designed to address this question.

The study subjects were from a TB endemic region (north India) and were also vaccinated with BCG, hence they were expected to be exposed as well as immunologically reactive to *M. tuberculosis*. To ascertain that the donors were indeed pre-exposed to mycobacteria, T cell proliferative response as well as IFN-γ production against mycobacterial antigens derived from cytosol and membrane were determined. All donors showed a positive T cell stimulation by these criteria. Earlier also, our lab had shown that subcellular proteins of *M. tuberculosis* were strong T stimulants for healthy donors of this region (Mehrotra et al., 1999; Sinha et al., 2005).

In addition to the determination of T cell responses, we also determined the anti-mycobacterium antibodies in sera of the donors. A positively high antibody level against *M. tuberculosis* membrane antigens provided yet another evidence for exposure of the donor population.
With the classical observation which gave birth to the concept that pathogenic mycobacteria avoid phagosome-lysosome fusion, and reversal of this can happen upon serum pretreatment of bacteria (Armstrong and Hart, 1971; Armstrong and Hart, 1975); lot of work has been done to understand the cell biology of macrophage-mycobacterium interaction. These studies (cited in the literature review section), most of which were done in mice, have led us to understand many facets of molecular mechanisms of this interaction.

The reported enhanced microbicidal capacity of macrophages upon phagocytosis of opsonized mycobacteria was an interesting point for us and we explored this phenomenon further through this study. Opsonization was done with anti-mycobacterium antibodies present in heat inactivated sera, a process which depletes the serum of complement components. The mycobacterium uptake happens through several receptors, important among them are complement, mannose, scavenger and Fc-γ receptors. Since the complement system is universal and non-specific in nature, we were interested in looking more specifically at the influence of anti-mycobacterial antibodies on phagocytosis which most likely occurs through Fc-γ receptors. Therefore, we heat-inactivated the complement from test sera. We found that the antibodies were still active after heat treatment of sera at 56 °C.

In order to test our hypothesis that factors operating at the level of macrophage-mycobacterium interaction determine the fate of the latent infection in healthy persons, it was necessary to delineate our test population into two subgroups- those who had an inherent capability to clear or contain the infection (‘high’ responders), and those who were unable to do so (‘low’ responders). We decided that the rate of clearance of the pathogen by the infected macrophages could serve as a robust criterion to differentiate the two groups. Intracellular survival and growth of bacteria was thus determined by enumerating colony forming units (CFUs). After infection with *M. tuberculosis*, the mean survival time of macrophage was 9 to 10 days. Therefore CFU counting was done on days 0 (immediately after infection), 4 and 8. Just after entry into the macrophage, the mycobacterium has to face an arsenal of defense mechanisms. However once the infection sets in, the bacilli start multiplying intracellularly.
On the basis of multiplication of live (unopsonized) bacilli from day 4 to day 8, it was possible to segregate low responders (fold multiplication >3) and high responders (fold multiplication <2). Both groups had 5 donors each. This ratio (50:50) was different from the reported 10:90, for high and low responders, respectively. There could be two reasons for this difference. First, our donors were limited in number (n=10), hence the proportion existing in population at large was not reflected. Second, the ratio could indeed be higher than 10:90 in the studied population as no such surveys have been made on Indian subjects. It was difficult for us to recruit more donors since each one was required to donate blood on 3-4 occasions in order to complete various components of the study.

In case of low responders, the opsonization of mycobacteria with heat inactivated serum reduced intracellular CFU counts significantly. This was in concordance with reports showing enhanced phagosome-lysosome fusion and intracellular killing of mycobacteria upon opsonization (Armstrong and Hart, 1971; Armstrong and Hart, 1975; Oh and Straubinger, 1996). However, in case of high responder group, the macrophages did not allow growth of either live or opsonized mycobacteria more than two fold. This suggests that these macrophages were already activated to their optimal levels so as to take protective action against the invading bacteria.

As a corollary to the ‘protective’ influence of opsonization with antibodies, we looked for a possible correlation between circulating anti-mycobacterial antibodies and high or low responses of the donor macrophages. Serum antibody levels were determined against both cytosol and membrane antigens of *M. tuberculosis* and the antibody level against cytosol was found to be lower than that against membrane. More importantly, the high responder group had significantly higher level of antibodies compared to low responders. It is generally accepted that cellular, rather than humoral, immunity plays a crucial role in defense against intracellular pathogens. However, accumulating data indicate the importance of humoral immunity in the defense against a number of intracellular bacteria. Recently it was shown that polymeric IgR (pIgR) knock out mice were more susceptible to mycobacterial infections of the respiratory tract compared to the wild type mice. It also caused reduced IFN-γ and TNF-α production in lungs. The secreted antibodies (IgA type) have been shown to play an important
role in protection against mycobacterial infection by blocking bacilli in the lung, in addition to modulation the mycobacterium induced pro-inflammatory responses (Tjarnlund et al., 2006). When peritoneal macrophages of IFN-γ activated mouse were infected with bacilli opsonized with IgA, it resulted in synergistic increase in nitric oxide and TNF-α production and 2-3 fold decrease in the bacterial counts (Reljic et al., 2006). Monocytes have high affinity receptors for IgG1 and IgG3 classes of antibodies that mediate endocytosis. In advanced tuberculosis, selective up-regulation of IgG1 (which bind to Fc-γ receptors) occurs and it has been shown that PPD specific IgG1 antibodies have capacity to augment expression of TNF-α in PPD stimulated monocytes (Hussain et al., 2001). Mycobacterial lipoarabinomannan (LAM) specific monoclonal antibodies proved to be protective against M. tuberculosis infection in terms of dose dependant reduction in bacterial load in spleens and lungs, reduced weight loss and increased long term survival (Hamasur et al., 2004). Monoclonal antibodies bound to surface of mycobacterium increased survival time of mice and changed morphology of granuloma and distribution of acid fast bacilli in the lungs (Chambers et al., 2004). Protective role of immune sera against reactivation of M. tuberculosis infection in SCID mice has been studied and it was found that passive immunization with sera obtained from mice treated with detoxified M. tuberculosis extracts (delivered in liposome in a composition known as RUTI) exerted significant protection (Guirado et al., 2006).

Following infection with M. tuberculosis, differences in cytokine expression are likely to determine whether the infection progresses, resolves, or remains latent. In particular, the balance between T helper type-1 (Th-1) and Th-2 cytokines has been shown to influence the expression of disease in individuals with TB. In addition, the protection also depends on certain monokines (monocyte derived cytokines). In view of this background information, the levels of TNF-α, IL-12, IL-1, IL-4, and IL-6 were estimated in culture supernatants of the infected macrophages.

When the cytokine levels in high and low responders were compared, the high responder group was found to produce a significantly higher level of TNF-α than the low responders. Role of TNF-α in protection against TB has been very well described in literature. It is associated with immunology and pathophysiology tuberculosis. Blocking of TNF-α allows the disease to emerge from latent infection and hence it has
tremendous importance in protective immunity against TB (Gomez-Reino et al., 2003; Mohan et al., 2004). TNF-α antagonists Infliximab, Etanercept and Adalimumab used in therapy of rheumatoid arthritis were found to be associated with development of tuberculosis in such patients, as remarked by Food and Drugs Administration of USA and in a multi-centric active surveillance document (Gomez-Reino et al., 2003). Many mycobacterial proteins viz. 38-kDa glycoprotein, PPD, matrix metallo-protease -9 (MMP-9) cause increase in secreted TNF levels which directly correlates with protective immunity (Barry et al., 2003; Hussain et al., 2000; Hussain et al., 2001; Jung et al., 2006; Price et al., 2003). PPD and M. tuberculosis have been shown to induce apoptosis in monocytes from PPD-positive healthy subjects. On the other hand, cells from TB patients showed both apoptosis and necrosis. The monocytes from control subjects produced mainly TNF-α, whereas those from TB patients produced mainly IL-10 (Gil et al., 2004). M. tuberculosis induces high production of nitric oxide in coordination with production of TNF-α in patients with fresh active TB, but not in multi-drug resistant (MDR) cases of TB (Sharma et al., 2004). TNF-α was also significantly increased in tuberculous lungs and was principally localized to the necrotic, and to a lesser extent, the inflammatory and fibrotic areas of the granuloma (Choi et al., 2002). Cachexia is one of the prominent features of advanced tuberculosis seen in association with increased expression of TNF-α. In another study, when intracellular growth of M. tuberculosis was correlated with production of various cytokines, none of cytokine combination was as effective as TNF-α in killing the intracellular bacilli (Denis et al., 1990).

Interleukin-12 is a potent inducer of IFN-γ production and it has been used as immune adjuvant for activation of macrophages through enhancement of T-cell function (Russo et al., 2000). IL-12 also activates NK cells and gamma/delta T cells, both of which secrete various macrophage-activating factors to kill M. tuberculosis. Thus it is considered as one of the potent immunotherapeutic agent. In single nucleotide polymorphism (SNP) analysis done in Mexico, it was observed that macrophage chemotactattractant protein (MCP)-1 genotype GG produces higher concentrations of MCP-1, which inhibits production of IL-12p40 in response to M. tuberculosis and increases the likelihood that infection will progress to active tuberculosis (Flores-Villanueva et al., 2005). Taking all above literature into consideration, it was interesting to study the status of IL-12 in infected macrophages. Our results show that
the high responder group had a higher capacity to produce IL-12 (though not statistically significant) than the low-responders.

Monocyte-derived macrophages are known to secrete pro-inflammatory cytokines TNF-α, IL-1 and IL-6 upon infection with *M. tuberculosis*. At the same time, they produce IL-10 which is a potent inhibitor of IL-12 secretion. Neutralization of IL-10 was found to restore IL-12 secretion by *M. tuberculosis* infected macrophages (Giacomini et al., 2001). With respect to the levels of IL-1 and IL-10 in infected macrophages, we found a variable response in donors and it was difficult to find any correlation with intracellular survival or killing of the mycobacteria. In case of IL-4 and IL-6, no response was observed.

Along with cytokines, many researchers have shown a role of reactive nitrogen- and oxygen- intermediates (RNI and ROI) in intracellular killing of bacteria. In healthy household contacts of tuberculosis patients, when alveolar macrophages were co-cultured with mycobacterium along with TNF-α, nitric oxide levels increased on day 4 and day 7 but the same was not observed in case of subjects from the unexposed community (Carranza et al., 2006). This observation should also be applicable to people from a TB endemic region such as India. Single nucleotide polymorphism (SNP) in nitric oxide synthase 2A gene leads to production of higher amount of macrophage chemo-attractant protein-1 (MCP-1), which in turn inhibits production of IL-12p40 in response to *M. tuberculosis* infection (Flores-Villanueva et al., 2005). Nitric oxide synthase knock out mice were much more susceptible than the wild type mice to clinical isolates as well as laboratory strains of *M. tuberculosis*, which prove the role of RNI in *M. tuberculosis* infection control (Scanga et al., 2001). In one study, the release of nitrite, IL-1β and TNF-α was found to be much greater from alveolar macrophages of tuberculosis patients than normal subjects. In the presence of NO inhibitor NG-monomethyl-L-arginine (L-NMMA), the production of nitrite as well as IL-1β and TNF-α was inhibited (Wang and Kuo, 2001). It has been observed that when human blood derived monocytes were infected with *M. tuberculosis*, detectable nitrite level in the supernatant started appearing after 72 hours after infection, increased progressively up to day 10 (Jagannath et al., 1998). Another report suggests that with a multiplicity of infection (MOI) 1:5 (monocytes: mycobacterium), monocytes stimulated very little or no nitric oxide, while alveolar macrophages
produced nitric on days 4 and 7 after infection (Rich et al., 1997). In our experimental setting, we wanted to study whether nitric oxide is induced or not in human monocyte derived macrophages upon infection with mycobacteria. Also, whether opsonization of mycobacteria with heat inactivated serum has any influence over NO release. Our results show that the macrophages infected with heat-killed mycobacteria release very low (<4 μM) or no nitric oxide, but the live and opsonized mycobacteria induced very high levels (>20 μM). Thus NO release was primarily induced by live mycobacteria and opsonization apparently had no effect on these levels. Moreover, the levels of NO in low and high-responders were similar. In our view, NO release is live mycobacterium specific phenomenon, but its role in protection is doubtful. Earlier researchers have shown that human monocytes do not release nitric oxide (Arias et al., 1997), but in our experimental set up where monocytes were differentiated into macrophages, NO production was induced.

ROIs produced by macrophages have also been shown to have antimicrobial activity, mainly in the mouse model of TB (Scanga et al., 2001). To circumvent their onslaught, M. tuberculosis expresses two genes encoding superoxide dismutase proteins- sodA and sodC (Piddington et al., 2001). In one study, the production of ROIs in granulocytes and macrophages from healthy volunteers infected in vitro with live Bacille Calmette-Guerin (BCG) was estimated and significant differences in the biochemical reactions in granulocytes and monocytes were observed (Paziak-Domanska et al., 2000). With the available limited information in the literature, there is no direct evidence about role of ROIs in killing of M. tuberculosis within human macrophages. We estimated H$_2$O$_2$ concentrations in the supernatants of infected macrophages in all the donor samples. However, the levels were variable and did not show any correlation with killing/survival of the phagocytosed bacilli.

To differentiate the high and low responders groups more precisely on the basis of handling of mycobacteria by their macrophages, it was necessary to study intracellular trafficking of the bacilli. Though ample literature and a number of markers are available for studying intracellular trafficking of mycobacteria, we had chosen only some of them, representing different classes of markers involved in trafficking. This study was done qualitatively. To analyze time bound events, the bacteria must enter macrophages at the same time, though such synchronous uptake/ infection is
practically difficult to attain. Some authors have used protocols wherein the cells were kept at 4 °C for some time assuming that the mycobacteria will settle down on macrophage and when transferred to 37°C, phagocytosis will commence. The use of single cell bacterial suspension in our experiment showed that the bacilli did not settle down even after centrifugation at 480 x g 10 min (twice the time mentioned by other authors). In order to circumvent this problem, we preferred to increase the MOI for this set of experiments to 1:200 (macrophage: mycobacteria). We assumed that increase in MOI will make the phagocytosis process synchronous, though we did not have any methods to prove so. Trafficking within the macrophage was studied using Syto-9 (green fluorescent dye) labeled *M. tuberculosis*. The infection was given for period of one hour, cells were fixed immediately and immuno-cytochemical staining for different markers was performed for confocal microscopy. The snapshots at this time point were used for data analysis.

Amongst the available markers, we had chosen to monitor mobilization of Rab5, LAMP-2, Cathepsin D and Fc-γ receptor. It is well established that phagosomes can fuse with lysosomes. The formation of phagolysosome is a dynamic process during which phagosome matures, modified by transient fusion and fission with endocytic organelles (Desjardins, 1995; Desjardins et al., 1994). Mycobacterial phagosomes retain Rab5, which plays a role in the interaction between early endocytic compartments and exclude Rab7, a GTPase that regulates late endosomal membrane trafficking. Earlier work has indicated that a block occurs between the maturation stages controlled by Rab5 and Rab7 (Via et al., 1998). In our experiments, Rab5 was found in close vicinity of mycobacteria following phagocytosis. Retention of Rab5 is an indication that the phagosome is not entering into maturation stages. Thus live *M. tuberculosis* has capability to modulate the fusion steps. This finding is in agreement with earlier studies.

The other well studied marker is lysosome associated membrane proteins (LAMPs). LAMP-1 is present in highest concentration within late endosomes and lysosomes. Its concentration has been shown to increase within the phagosomal membranes during the progression from early to late endosomes and phagolysosomal stages (Storrie and Desjardins, 1996). However, there is a disparity in reports regarding LAMP-1 levels within the phagosomes (Deretic et al., 1997; Kuehnel et al., 2001; Sturgill-Koszycki
et al., 1994; Xu et al., 1994). Some have described enrichment of LAMP-1 within phagosomes containing live mycobacteria, though LAMP-1 may be entering these compartments through alternate trafficking pathway rather than phagosome-lysosome fusion events. Specifically, one report describes equal levels of LAMP-1 colocalization with live and killed bacteria. These investigators felt that LAMP-2 may be a better marker for late endosomes/phagosomes (Kuehnel et al., 2001). Thus, in our study we preferred to use LAMP-2 (which is structurally similar to LAMP-1). When macrophages were infected with live mycobacteria, LAMP-2 was not colocalized with the bacilli. This was another indication that the phagosomes were not maturing as phagolysosomes. However, when the bacilli were opsonized with serum antibodies, they mostly got colocalized with LAMP-2 suggesting that the phagosome was entering into phagolysosome fusion; a step towards intracellular killing of the pathogen.

Cathepsin D was also studied as a marker for mycobacterial phagosome maturation. It is one of the lysosomal hydrolytic enzymes and is active only in an acidic environment. Although mycobacterial phagosome has been shown to acquire some lysosomal membrane protein and cathepsin-D, the staining for these markers remains less than that observed on the phagolysosome (Clemens and Horwitz, 1995). In our experiments, some of the mycobacteria got colocalized with cathepsin-D, while some others did not.

The Fc-γ receptor is one of the phagocytic receptors. We wanted to study change in surface expression as well as clustering of this receptor during uptake of live and live-opsonized bacteria. When mycobacteria are opsonized and come in contact with macrophages, the Fc-γ receptor clustering occurs in the form of pseudopodia so as to phagocytose the bacilli. Our analysis shows that Fc-γ receptor expression increased and its clustering occurred during the infection with live-opsonized bacteria. Further, most bacteria got colocalized with the receptor. Earlier researchers have shown that actin polymerization also occurs when uptake of the microbe is through downstream signaling of the Fc receptors and PI(3) kinase (Toker and Cantley, 1997). Our results also show colocalization of actin and Fc-γ receptors.