SUMMARY

Tuberculosis still remains as one of the major health challenges to human mankind. There are other diseases like smallpox and plague that have killed millions in the past but in the twenty first century their threat has receded. As recently as 2013 around 9 million new cases and 1.5 million human fatalities were documented involving *Mycobacterium tuberculosis*.

Iron is an essential nutrient element from all organisms ranging from bacteria to mammals. Like other prokaryotes, iron has an important role in mycobacterial physiology. Iron, being part of the iron-sulfur cluster plays a cardinal role in its metabolism. During infection, there is an intense struggle between host and pathogen to withhold or acquire iron respectively. It is known that M.tb acquires iron from host transferrin and lactoferrin. It has been showed that transferrin provided extracellularly is trafficked into inside phagosomes of infected macrophages. The host attempts to withhold iron by downregulating the expression of transferrin receptors and by initiating the synthesis of siderocalins that sequester bacterial siderophores to minimize the supply of iron within the phagosome. In order to survive bacilli utilize multiple iron acquisition mechanisms including the use of siderophores, hemophores and transferrin receptors. Siderophores withdraw iron from transferrin and delivers it to mycobacteria. However, the utility of siderophores can only be manifested when iron is delivered to those compartments of the host macrophage to which the siderophores have access. Till date siderophores have been shown to be limited to be present only within the infected macrophage. In addition, there are several published reports have suggested that even mycobacteria that do not express siderophores continue to acquire iron while resident within the host. Together these observations imply the existence of additional unknown pathways for transferrin iron delivery to the intracellular pathogen. Recently our group demonstrated that transferrin is internalized by M.tb. via its surface receptor GAPDH (Rv1436). However in the case of bacilli within cells for this process to play a significant role the prior trafficking of extracellular transferrin into the phagosome (where it can encounter mycobacterial surface GAPDH) is a prerequisite and the down regulation of macrophage cell surface transferrin receptors presents a paradox.

Earlier our laboratory had also established the role of mammalian GAPDH in transferrin acquisition in mammalian tissues. We demonstrated that mammalian surface GAPDH binds and internalizes transferrin and lactoferrin into various primary cells and cancer cell lines. GAPDH is known to be secreted out of the cells and constitutes a normal component of serum. Our research has also proved that secreted GAPDH acts as a soluble receptor for

effecting transferrin delivery into the cells in autocrine or paracrine manner. In combination with transferrin secreted GAPDH traffics into cells via multiple endocytosis pathways including lipid raft-mediated endocytosis and macropinocytosis and is dependent upon surface uPAR (Urokinase-type plasminogen activator receptor) expression. Interestingly, mammalian cell GAPDH secretion is observed to increase upon iron starvation which results in delivery of transferrin much more efficiently than via surface transferrin receptors. Since secreted GAPDH is involved in transferrin iron acquisition by macrophages we considered it plausible that secreted GAPDH may function as a means of transferrin delivery into the infected macrophages and bacilli thereby contributing to the establishment of a successful infection. The current research was focused primarily on the role of secreted GAPDH in M.tb transferrin iron acquisition.

In the current study, we first investigated the status of surface receptors (TfR1 and GAPDH) on infected macrophages. We found that TfR1 and surface GAPDH either remained unchanged or decreased both in M.tb H37Ra and M.tb H37Rv infected THP1 and murine macrophages (*in vitro* as well as *in vivo*). These results were in agreement with the conclusions of earlier workers regarding the host macrophages interest being in limiting the supplies of iron to invading mycobacteria. Decrease in macrophage receptors for iron carrier proteins was also reflected in the labile iron pool of the cells and we observed that there was a decrease in the labile iron pool of peritoneal macrophages (unchanged in THP1 cells infected with M.tb H37Ra). Next, we checked the soluble transferrin receptor *i.e.* secreted GAPDH in the culture of infected THP1 and murine macrophages infected with M.tb H37Rv. Significantly, we found that GAPDH secretion was enhanced upon infection our observation on the increase in secretion of GAPDH by infected cells corresponds with recent reports that upon infection there is an increase in GAPDH in lungs granuloma tissues formed by M.tb infection in mice.

In a previous study from our laboratory, we have shown that secreted GAPDH enhances transferrin delivery into the numerous mammalian cells. We checked the same in the case of infected macrophages and found that here too it increases transferrin delivery into the infected cells (THP1 and peritoneal macrophages).

After establishing that GAPDH is able to deliver more transferrin into the infected cell as a whole, we looked at the subcellular delivery of transferrin into the phagosome containing mycobacteria. We found that both exogenously added GAPDH and transferrin not only traffic into phagosomes but also continue their interaction with each other as analyzed by

immuno electron microscopy and co-immunoprecipitation studies. Taking these investigations further we concluded that GAPDH mediated enhanced transferrin delivery into the phagosomal compartment of infected cells results in a net increased transferrin delivery inside the mycobacterial cells resident within phagosomes. The essential role of macrophage uPAR in this route of transferrin delivery into infected macrophages and resident infecting mycobacteria was also confirmed using M.tb H37Ra GFP infected uPAR knockdown THP1 cells.

Dissemination of bacilli from the site of infection to other organs of the body involves the release of mycobacteria from macrophage into the extracellular milieu. During this intervening period, prior to their entry into another cell, they are dependent upon direct uptake of transferrin from the extracellular fluid to meet their iron requirements. Using M.tb H37Ra/H37Rv bacilli in culture we were again able to demonstrate an enhanced uptake of Tf in the presence of GAPDH by both strains. Finally, TEM confirmed co-internalization of Tf and GAPDH in M.tb H37Rv bacilli.

The current belief is that mycobacteria in host primarily acquire transferrin iron via siderophores. The significance of the GAPDH mediated enhanced transferrin delivery to mycobacteria can only be fully assessed in the absence of siderophores. We, therefore proceeded to evaluate if GAPDH was able to enhance delivery of transferrin into a siderophore knockout *mbtB* mutant strain of mycobacteria M.tb H37Rv and found that to be the case. GAPDH significantly enhanced delivery of transferrin into both; the *mbtB* mutant of M.tb H37Rv in culture as well as when the mutant M.tb was resident inside phagosomes of THP1 macrophages. Finally, we imaged the presence of both transferrin and sGAPDH inside the mutant bacteria by Super Resolution Microscopy.

The ultimate role of acquiring transferrin by any cell is to obtain iron. After confirming the GAPDH mediated transferrin trafficking into the bacteria, we looked for enhanced Tf-bound iron transport into bacteria. In presence of GAPDH, the transport of Tf-Fe^{55} into phagosomes of M.tb H37Ra infected THP1 cells was significantly enhanced in a dose-dependent manner while control protein BSA failed to have any effect on Tf-iron delivery. Not only was the delivery of iron into phagosomes increased, GAPDH was also able to enhance the delivery of Tf-bound iron into the intraphagosomal M.tb H37Ra infecting THP1 cells as well as into isolated M.tb H37Ra cells grown in culture.

In the current study, we have established that M.tb can acquire transferrin using a host molecular delivery pathway for its own need of iron. It is a siderophore-independent

pathway for the acquisition of iron by mycobacteria both intracellularly and extracellularly which can contribute to its pathogenesis. It provides a new dimension in the hijacking of host carrier proteins for iron metabolism by the invading M.tb.