The present study reveals that glyceraldehyde 3-phosphate dehydrogenase primarily known as a glycolytic enzyme, moonlights as a novel lactoferrin receptor in mammalian cells and especially macrophages. Till date no other molecule has been definitively proved to function as a lactoferrin receptor in macrophages. The presence of receptors for lactoferrin is vital due to their involvement in several crucial physiological functions including; iron homeostasis (Ward and Conneely, 2004), cell signaling (He and Furmanski, 1995), host defence against a broad range of microbial infections (Lonnerdal and Iyer, 1995), anti-inflammatory activity (Conneely, 2001), regulation of cellular growth and differentiation (Bi et al., 1997). Lactoferrin has also been implicated in protection against cancer development and metastasis (Artym, 2006). Because of the involvement of lactoferrin in such a vast range of functions, studies on, the cellular machinery that can deliver requisite amounts of lactoferrin into cells on demand, continue to be an area of intense interest.

Mammalian LfRs were first reported to be present in macrophages over three decades ago (Van Snick and Masson, 1976). However the study did not characterize any specific receptor. Our laboratory, has now identified GAPDH as a novel lactoferrin receptor present on the surface of macrophages and also on other mammalian cells. Though several other molecules have been reported to function as LfRs (on cell other than macrophages) we have now been able to demonstrate that GAPDH is the preferred lactoferrin receptor for the cells upon iron depletion.

Earlier our laboratory has reported that iron depletion causes a significant increase in mammalian cell surface GAPDH (Kumar et al., 2011) which facilitates an increased uptake of iron. Solid phase assay demonstrated that GAPDH and lactoferrin interact *in-vitro*. This was further confirmed using *in-vitro* homologus inhibiton assay. Homologus inhibiton assay also demonstrated the specificity of binding between the two proteins. Affinity analysis was performed using surface plasmon resonance (also a solid phase assay), which demonstrated a weak interaction between chip immobilized lactoferrin and GAPDH with average

dissociation constant in μM range (4.32 μM). However isothermal titration calorimetry (a solution phase assay) revealed an affinity constant value in the nanomolar range (43.8 ± 8.26 nM). The reason for the relatively low affinity value obtained using Biacore may be the result of conformational changes in the protein which are probably associated with protein is immobilization on a surface. Treatment with pronase, a non-specific protease, resulted in complete abolishment of lactoferrin binding to cell surface thereby demonstrating the involvement of a protein receptor in binding. Treatment of cells with 0.5 M sodium chloride, which removes any loosely bound peripheral surface proteins, resulted in no alteration in lactoferrin binding to the cell surface thus indicating the involvement of a non-peripheral protein receptor.

To confirm the interaction *ex-vivo*, membrane co-immunoprecipitation experiments were performed. Lactoferrin pulled down membrane fraction demonstrated the presence of GAPDH confirming that lactoferrin interacts with cell surface GAPDH. The interaction was visualized by confocal microscopy based co-localization study which showed that the two proteins are co-localized on the surface of CHO-TRVb and J774 cells. To demonstrate the close proximity associated with molecular interaction, foster resonance energy transfer (FRET) experiments were performed using GAPDH-mCherry transfected CHO-TRVb cells incubated with lactoferrin-FITC. Photobleaching of the GAPDH-mCherry signal resulted in a simultaneous increase of the lactoferrin-FITC signal thus confirming that the two proteins are present at close proximity (<100Å) to each other as required for protein-protein interaction. *Ex-vivo* binding assay also demonstrated the saturable nature of lactoferrin binding to J774 and CHO-TRVb cell surface. This indicates that binding of lactoferrin to the cell surface receptor is specific. B max value was 2.374 and Kd value was calculated to be 99.39nM for J774 cells. For CHO-TRVb cells, B max value was 0.1487 and Kd value was calculated to be 370.2 nM. To confirm the specificity of lactoferrin binding to cell surface, homologus inhibition assays were performed. Three different concentrations (150nM, 200nM & 300nM) were used for homologus inhibiton and

calculated Kd values were 44.17nM, 51.8nM & 92.14nM respectively. Average Kd value was calculated to be 62.7 ± 25.8 nM (mean ± SD). Similar results were obtained with THP1 cells using two different concentrations (200nM and 400nM) and Kd values were calculated to be 129.7nM and 139.4nM respectively. Nanomolar range of dissociation constant values demonstrates the relatively high affinity interaction of lactoferrin with its mammalian cell surface receptor. Characterization of the membrane binding domains for lactoferrin revealed that lactoferrin interacts with both detergent resistant membrane (DRM, also known as raft domain), and with detergent soluble membrane (DSM) domains. This was done by purifying DRM and DSM fractions from the cell membranes and performing co-IP from both the fractions. Raft associated GADPH was also shown to mediate trafficking of lactoferrin into cells as visualized by the co-localization of GAPDH, lactoferrin and raft marker using confocal microscopy. To identify the mode of trafficking, transferrin was used as an early endosomal marker and it was demonstrated that incubation of THP1 cells with both lactoferrin and transferrin results in the internalization of both the proteins. Co-localization of lactoferrin and transferrin demonstrates that the mode of lactoferrin trafficking is via early endosomal pathway. Co-IP study of purified endosomes from J774 and CHO-TRVb cells revealed that lactoferrin not only binds to GAPDH at the cell surface but the complex is also internalized to the endosomal compartment of cells. The association in the endosomes was confirmed by performing double immunogold transmission electron microscopy. Macrophages are the scavenging cells of the body and possess scavenger receptors on their surface. These are non-specific receptors which can bind to many ligands. The involvement of scavenger receptors in lactoferrin binding was ruled out by, the absence of any inhibition in lactoferrin binding to J774 cells, when Poly I:C (a specific ligand of scavenger receptor) was incorporated into the incubation media.

As lactoferrin is a glycosylated protein, we checked for the possibility of any involvement of sugar residues in lactoferrin binding to GAPDH. This was done by performing *in-vitro* sugar inhibiton assay. We found that the binding of

lactoferrin to GAPDH remained unaltered in presence of high concentrations of glucose, mannose, galactose and α-methylmannose. However D-glucosamine in the incubation media caused an inhibition in lactoferrin binding. This suggests the involvement of amine sugars in interaction. Expression analysis using reverse transcription PCR indicated the absence of intelectin and AGPR (previously reported intestinal and hepatic lactoferrin receptors respectively) in CHO-TRVb & J774 cells. On the other hand, LRP was expressed in both cell lines while CD14, which is present in macrophages, was not expressed in CHO-TRVb cells. Studies on alteration of surface lactoferrin receptor expression and subsequently lactoferrin binding & uptake was performed using iron depleted cells. It was observed that binding of lactoferrin is enhanced in CHO-TRVb cells upon iron depletion. This observation is in parallel to the observed increase in the expression of cell surface GAPDH upon iron depletion (Kumar et. al, 2011). No change in the expression of LRP was observed at mRNA as well as protein level. However, a decrease in lactoferrin binding was observed in case of J774 cells. LRP expression was also found to decrease in J774 which might be the reason for the observed decrease in lactoferrin binding to these cells inspite of a simultaneous increase in cell surface GAPDH expression. Previously studies from our laboratory have shown that GAPDH is secreted in cell culture media. Therefore we checked if the soluble GAPDH (sGAPDH) is able to mediate delivery of lactoferrin into cells, thereby functioning as a soluble receptor in addition to a membrane localized one. Uptake studies revealed a significant increase in lactoferrin uptake by cells when sGAPDH was present in the incubation media. The uptake was also demonstrated to be concentration dependent and was found to be maximum at 100 μg/ml GAPDH in case of THP1 cells and 500 μg/ml in case of J774 cells. Co-trafficking studies were performed in J774, THP1, peritoneal and spleen macrophages which revealed that both the proteins are trafficked together. FACS based quadrant analysis revealed that GAPDH positive cells show enhanced uptake of lactoferrin in comparison to control cells. Co-trafficking was also confirmed by co-localization study using confocal microscopy. To check if the mechanism of soluble GAPDH mediated lactoferrin trafficking is physiologically significant, iron

uptake studies were performed using ^{55}Fe radiolabeled lactoferrin. We were able to demonstrate that addition of sGAPDH caused an enhanced delivery of lactoferrin bound iron into differentiated THP1 cells. While, in the course of the time period studied, cells demonstrated more incorporation of iron into their labile iron pool, however presence of soluble GAPDH also resulted in increased iron delivery to the protein bound iron pool of macrophages. Lactoferrin has long been a molecule of active research due to its multifunctional role. Role of lactoferrin in iron metabolism & transport is already well known. This function is very important in case of macrophages as these cells are involved in maintaining iron homeostasis. Many vital functions in cells such as; oxygen transport, mitochondrial energy metabolism, electron transport, DNA & RNA synthesis and detoxication of oxidants are crucially dependent on the presence of iron. Therefore all the living organisms need to have a constant iron supply for their survival. To explore if intracellular pathogens can exploit this host mechanism for their own iron requirements while residing in mammalian host, Mtb infected macrophages were utilized. A phagosomal co-IP study of infected macrophages revealed the presence of both lactoferrin and sGAPDH in the phagosomes. The result was further confirmed by doing co-localization study using confocal microscopy. The data demonstrated that the host mechanism might be hijacked by the microbe for its own advantage.

In conclusion, the present study characterizes a novel receptor for lactoferrin on macrophage cell surface and also describes one more novel function for a multifunctional protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The study also reveals the physiological significance of the mechanism and demonstrates the utilization of this mechanism by macrophages and other mammalian cells for enhanced delivery of iron, therefore catering to their requirements for iron and maintaining iron homeostasis. In addition, the study revealed the possibility of appropriating this host mechanism by resident intracellular microbes for their own survival inside host.