Several lines of evidences suggest that macrophage apoptosis represents an innate defense against intracellular infection, analogous to the established role for apoptosis of virus-infected cells. Intracellular BCG and *M. tuberculosis* H37Ra are killed when host macrophages undergo apoptosis, while *M. tuberculosis* H37Rv grow inside host macrophages that remain viable. The virulence-associated trait of apoptosis suppression by *M. tuberculosis* involves several mechanisms including induction of the antiapoptotic bcl-2 family member mcl-1 and interference with TNF-α signaling at the level of TNFR1. Binding of TNF-α to TNFR1 also results in activation of signaling complexes, which signal inflammation and cell survival through IκB kinase (IKK) dependent activation of NF-κB. Several pathogens have been shown to activate NF-κB to secure their replication and survival within the host so this study was conducted to see the role of NF-κB in inhibition of apoptosis by *M. tuberculosis* H37Rv.

Preliminary experiments revealed that both *M. tuberculosis* H37Rv and *M. tuberculosis* H37Ra differ in their apoptotic potential where *M. tuberculosis* H37Ra infection lead more apoptosis of THP-1 cells than *M. tuberculosis* H37Rv infection. To see the role of NF-κB in inhibition of apoptosis by *M. tuberculosis* H37Rv, stable THP-1 cell-line (THP-1-IκBαM dn cells) over expressing IκBα mutated form was generated to inactivate NF-κB. Inactivation of NF-κB in THP-1-IκBαM dn cells was checked by studying NF-κB-DNA binding activity using EMSA and IκBα degradation by western blotting. Annexin V staining clearly showed that the proportion of apoptotic macrophages in THP-1-IκBαM dn cells harboring *M. tuberculosis* H37Ra or *M. tuberculosis* H37Rv were more in comparison to *M. tuberculosis* H37Ra or *M. tuberculosis* H37Rv infected THP-1 cells respectively. This result was further confirmed by TUNEL, DAPI staining and DNA fragmentation where comparable fractions of *M. tuberculosis* H37Ra or *M. tuberculosis* H37Rv infected THP-1-IκBαM dn cells showed chromatin condensation and nuclear fragmentation suggestive of apoptosis than infected THP-1 cells. Further to deduce the mechanism of apoptosis and taking into account the earlier published report suggesting central role of mitochondria in TNF-α mediated apoptosis of macrophages, Ψ_m of infected THP-1-IκBαM dn and THP-1 cells was examined by flow cytometry as well as by confocal microscopy where significant change in Ψ_m of infected THP-1-IκBαM dn cells was observed than infected THP-1 cells followed by

significant release of cyt. c from mitochondria. Effect of cyt. c release was also studied on downstream caspases like caspase-3 where *M. tuberculosis* H37Rv or *M. tuberculosis* H37Ra infected THP-1-IκBαM dn cells showed more caspase-3 activity than infected THP-1 cells.

RNase protection assay further gave insight into the mechanism of apoptosis where differential expression of bfl-1/A1 mRNA, an antiapoptotic member was found with *M. tuberculosis* H37Rv or *M. tuberculosis* H37Ra infection in THP-1 cells at 48 h of post-infection. No significant difference in mRNA expression of other apoptosis associated molecules (Caspase-8, FASL, FAS, FADD, DR3, FAP, FAF, TRAIL, TNFRp55, TRADD, RIP, XIAP, TRAF1, TRAF2, TRAF4, NAIP, c-IAP-2, c-IAP-1, TRPM2, TRAF3) was observed at 48 h of post-infection in infected THP-1 cells. We did not find any significant difference in the expression of other Bcl-2 family members like bcl-2, mcl-1 and bcl-x etc. at 48 h of post-infection. Since, bfl-1/A1 mRNA expression is regulated by NF-κB, activation of NF-κB was also studied at 48 h of post-infection. *M. tuberculosis* H37Rv infection showed p65 translocation, NF-κB-dependent SEAP reporter activity and NF-κB-DNA binding activity than *M. tuberculosis* H37Ra infection in THP-1 cells, suggestive of NF-κB activation.

It has been reported that TNF-α plays very important role in apoptosis of mycobacteria infected macrophages. Before studying the release of TNF-α in mycobacteria infected THP-1 and THP-1-IκBαM dn cells TNFR1 expression was first measured in THP-1 and THP-1-IκBαM dn cells where THP-1-IκBαM dn cells showed higher TNFR1 expression than THP-1 cells. At 48 h post-infection, increased production of TNF-α was observed in *M. tuberculosis* H37Rv or *M. tuberculosis* H37Ra infected THP-1-IκBαM dn cells than THP-1 cells. Role of TNF-α was investigated using neutralizing anti-TNF-α antibody where marked decrease in apoptosis was observed in both types of cells harboring *M. tuberculosis* H37Ra with anti-TNF-α antibody after 72 h of post-infection. In contrast, addition of anti-TNF-α antibody failed to show any effect on *M. tuberculosis* H37Rv infected THP-1 as well as THP-1-IκBαM dn cells.

Increased apoptosis in infected THP-1-IκBα M dn cells also showed its effect on the intracellular survival of *M. tuberculosis* H37Ra by 3.1 fold in THP-1-IκBαM dn cells in

comparison to normal THP-1 cells and of *M. tuberculosis* H37Rv by 3.5 fold in THP-1-IκBαM dn cells in comparison to normal THP-1 cells.

Differential upregulation of bfl-1/A1 by *M. tuberculosis* H37Rv or *M. tuberculosis* H37Ra in THP-1 cells at 4 and 48 h of post-infection was studied using –1374/+83 Bfl-1 promoter-luciferase reporter construct where luciferase gene expression is under the control of bfl-1/A1 promoter, which contains NF-κB binding site. At 4 h of post-infection, both *M. tuberculosis* H37Rv or *M. tuberculosis* H37Ra infected THP-1 cells showed significant high luciferase activity whereas at 48 h post-infection only *M. tuberculosis* H37Rv infected cells showed luciferase activity. Differential activation of bfl-1/A1 in THP-1 cells with *M. tuberculosis* H37Rv or *M. tuberculosis* H37Ra infection was further confirmed at mRNA level using RT-PCR and at protein level by western blotting. RT-PCR and western blot analysis clearly showed that bfl-1/A1 expression was induced by *M. tuberculosis* H37Rv or *M. tuberculosis* H37Ra at 4 h of post-infection in THP-1 cells. On the contrary at 48 h post-infection only *M. tuberculosis* H37Rv infection induced the expression of bfl-1/A1.

In order to elucidate the role of bfl-1/A1 in apoptosis of *M. tuberculosis* H37Rv infected THP-1 cells, siRNA approach was undertaken. Silencing was confirmed at 48 h of post-infection in *M. tuberculosis* H37Rv infected THP-1 cells after siRNA transfection by western blotting where *M. tuberculosis* H37Rv induced expression of bfl-1/A1 was knocked down by bfl-1/A1 siRNA. Further, effect of bfl-1/A1 inhibition on apoptosis of *M. tuberculosis* H37Rv infected THP-1 cells was studied using annexin V staining at 72 h of post-infection. Annexin V staining clearly showed a greater proportion of apoptotic THP-1 cells transfected with bfl-1/A1 siRNA and harboring *M. tuberculosis* H37Rv as compared to infected THP-1 cells. We did not find any significant apoptosis in mock-transfected THP-1 cells.

In order to investigate whether increased apoptosis due to bfl-1/A1 inhibition has any effect on the intracellular survival of mycobacteria, growth of *M. tuberculosis* H37Rv was evaluated in siRNA transfected THP-1 cells at 72 h of post-infection. Interestingly, growth of intracellular mycobacteria was decreased by 2 fold in transfected THP-1 cells in comparison to infected THP-1 cells. We did not find reduction of mycobacterial growth in mock-transfected THP-1 cells.

Further to deduce the mechanism of decrease in intracellular survival of *M. tuberculosis* H37Rv or *M. tuberculosis* H37Ra in THP-1-IκBαM dn cells, role of ROI and RNI was studied. The intracellular generation of ROI with mycobacterial infection in THP-1 and THP-1-IκBαM dn cells was quantified by flow-cytometric analysis. In the presence of reactive oxygen species, non-fluorescent dihydrorhodamine 123 is oxidized to fluorescent compound rhodamine 123, which can be easily measured by flow cytometry. *M. tuberculosis* H37Ra or *M. tuberculosis* H37Rv infection did not induce any significant ROI production in THP-1-IκBαM dn cells than THP-1 cells at 4 and 48 h of post-infection and ROI inhibition by antioxidants like catalase and superoxide dismutase also did not show any significant effect on the intracellular viability of *M. tuberculosis* H37Rv in THP-1-IκBαM dn cells than infected THP-1 cells. Even mycobacteria infected THP-1 and THP-1-IκBαM dn cells did not produce significant amount of nitric oxide at 48 and 72 h of post-infection.

Further phagosome-lysosome fusion studies were also undertaken to see its role in intracellular killing of mycobacteria. Our results showed that GFP labeled mycobacteria in THP-1 cells appeared green, indicating that they were residing in non-acidic vesicles separate from the red stained lysosomes. However in bfl-1/A1 siRNA transfected cells, an increasing percentage of *M. tuberculosis* appeared yellow (due to colocalization with red LysoTracker), indicating that they were residing in acidic organelles that had fused with lysosomes.

This result was further verified in THP-1-IκBαM dn cells, which have already been shown to express low level of bfl-1/A1 in *M. tuberculosis* infection. P/L fusion was detected at 48 h post-infection in GFP-*M. tuberculosis* H37Rv infected THP-1 and THP-1-IκBαM dn cells. Interestingly, infected THP-1-IκBαM dn cells resulted in marked increase in colocalization of lysosomes with phagosomes than infected THP-1 cells, which exhibited negligible colocalization. These results clearly indicate that bfl-1/A1 downregulation enhances P/L fusion with mycobacterial infection.

So, our results for the first time showed in mycobacterial infection the role of NF-κB dependent bfl-1/A1 in controlling phagosome-lysosome fusion, which ultimately control the intracellular survival of mycobacteria.