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SUMMARY OF THE THESIS

Autophagy is an intracellular degradative process for the delivery of cytoplasmic constituents to lysosome. Recent progress has demonstrated that autophagy plays a wide variety of physiological and pathological roles which are sometimes complex. Autophagy has been linked to innate and adaptive immune response to numerous intracellular pathogens, including mycobacteria. It is tightly regulated biological process that plays a central role in tissue homeostasis, development and disease. Antiapoptotic Bcl-2 homologs downregulate autophagy through interactions with the essential autophagy effector protein, Beclin 1. Beclin 1 contains a BH3 domain, similar to that of Bcl-2 proteins, which is necessary and sufficient for binding to antiapoptotic Bcl-2 homologs and required for Bcl-2 mediated inhibition of autophagy. Bfl-1/A1 is a NF-κB dependent Bcl-2 family antiapoptotic protein which also contain BH3 domain but so far its role in autophagy has not been studied. Present study has identified Bfl-1/A1 as a negative regulator of autophagy. Moreover, this study helped to understand the significance of differential expression of Bfl-1/A1 in virulent and avirulent *M. tuberculosis* infected macrophages.

To see the role of Bfl-1/A1 in autophagy, stable THP-1 cell line (HABfl-1-THP-1) overexpressing HA-tagged human Bfl-1 was constructed and used for further studies. Overexpression of Bfl-1 clearly inhibited starvation, rapamycin and IFN-γ induced autophagosome formation as revealed by acridine orange as well as MDC staining. Less autophagosome formation was further confirmed by GFP-LC3 puncta formation where HABfl-1-THP-1 cells showed very less LC3 puncta formation after starvation as compared to starved THP-1 cells. The inhibitory effect of Bfl1/A1 is not cell type specific as HABfl-1-HEK293 cells also showed less GFP-LC3 puncta formation after starvation as compared to control HEK293 cells. Maturation of autophagosome was also studied by co-localization of GFP-LC3 with lysosomes stained with LysoTracker. HABfl-1-THP-1 cells showed decreased LC3 puncta under starvation and which did not co-localize with LysoTracker, whereas control THP-1 cells showed increased LC3 puncta formation and most of them fused with lysosomes indicating that overexpression of Bfl-1 inhibits maturation of autophagosomes. In contrast, enhanced autophagosome formation was obtained in THP-1-IκBαM dn cells which stably overexpress the mutated form of IκBα

and consequently less NF-κB and Bfl-1/A1. These results, taken together, demonstrate that Bfl-1/A1 negatively regulate autophagy.

Next, we tried to understand the significance of preferential expression of Bfl-1/A1 in *M. tuberculosis* infected macrophages. Previous study from our laboratory has found that virulent but not avirulent strain of mycobacteria upregulate and thereafter maintain Bfl-1/A1 level for longer period in infected cells. Inhibition of Bfl-1/A1 by siRNA enhanced P/L fusion of mycobacteria loaded phagosomes as well as increased the rate of apoptosis in virulent mycobacteria infected macrophages. As Bfl-1/A1 enhances P/L fusion, we studied the role of Bfl-1/A1 in autophagosome formation in mycobacterial infection.

First, the effect of NF-κB inhibition on autophagosome formation in mycobacteria infected macrophages was studied. Enhanced co-localization of mycobacteria loaded phagosomes was seen with both LC3 and LysoTracker at 48 h of infection or after 4 h of cytokine (IFN-γ) treatment in THP-1-IκBαM dn cells compared to that in THP-1 cells. The effect of EGCG, a NF-κB inhibitor, was also studied on autophagosome formation in mycobacteria infected THP-1 cells. We found that EGCG treatment inhibited the mycobacteria induced NF-κB activation and Bfl-1/A1 expression as well as phosphorylation of Akt and mTOR. In addition, EGCG treatment enhanced the conversion of LC3-I to LC3-II in infected THP-1 cells. We also observed the enhanced LC3 co-localization and acidification of mycobacteria loaded phagosomes in EGCG treated THP-1 cells. Moreover, inhibition of Bfl-1/A1 by siRNA enhanced the acidification as well as autophagosome formation in mycobacteria infected macrophages and decreased mycobacterial survival in macrophages. These results taken together showed that NF-κB plays an important role in autophagosome formation in mycobacterial infection and inhibition of NF-κB and Bfl-1/A1 enhances autophagosome formation in mycobacteria infected THP-1 cells.

Effect of overexpression of Bfl-1 on autophagosome formation in mycobacterial infection was also studied. Our results revealed that overexpression of Bfl-1 inhibited both P/L fusion and autophagosome formation of avirulent mycobacteria loaded phagosome after 48 h of infection. Interestingly, Bfl-1 overexpression helps the intracellular bacteria *M. tuberculosis* H37Ra to survive in its host cells as revealed by the increased number of CFU in HABfl-1-THP-1 cells as compared to that in THP-1 cells.

Further, depletion of Beclin 1 effectively blocked LC3 translocation to mycobacteria loaded phagosomes in THP-1 cells which clearly indicated the involvement of functional autophagic pathway in phagosome maturation.

Bcl-2 family proteins have been known to physically interact with BH3 domain of Beclin 1, therefore we performed co-immunoprecipitation experiments to see the interaction between Bfl-1 and Beclin 1. Interestingly, our co-immunoprecipitation results showed that Bfl-1 co-precipitated with Beclin 1. Further, endogenous interaction of Bfl-1/A1 with Beclin 1 was found only in virulent mycobacteria infected THP-1 cells.

It is known that autophagy can be induced in mycobacteria infected macrophages by rapamycin. Rapamycin inhibits mTOR activity and by some unknown mechanism induces autophagy. We also found that rapamycin treatment inhibits mTOR activity in infected THP-1 as well as in HABfl-1-THP-1 cells. Rapamycin also lowered Bfl-1/A1 level in *M. tuberculosis* H37Rv infected THP-1 cells. When autophagy formation was compared in both the cells, lesser co-localization of LC3 as well as Beclin 1 with mycobacterial phagosomes were detected in HABfl1-THP-1 than those in THP-1 cells. In addition, conversion of LC3-I to LC3-II was found to be less in Bfl-1 overexpressing cells with rapamycin treatment. Significant difference in survival percentage of *M. tuberculosis* H37Rv was observed in THP-1 and HABfl-1-THP-1 cells treated with rapamycin. These results, taken together further establish the negative regulatory role of Bfl-1 in autophagy formation in mycobacterial infection.

It has been previously shown by our laboratory that virulent mycobacteria induce less apoptosis compared to avirulent one. Hence, the effect of overexpression of Bfl-1 on avirulent mycobacteria induced apoptosis was studied. Overexpression of Bfl-1 inhibited the apoptosis in avirulent mycobacteria infected macrophages. There was comparatively lesser change in MMP in HABfl-1-THP-1 cells infected with avirulent mycobacteria compared to infected THP-1 cells. These results supported the role of Bfl-1/A1 in protecting cells from loss of MMP and apoptosis.

So, taken together our results for the first time showed that Bfl-1/A1 directly interacts with Beclin 1 and inhibits autophagosome formation in mycobacterial infection, thereby helping mycobacterial survival inside macrophages. We also found that inhibition of NF-κB and Bfl-1/A1 can enhance autophagosome formation in mycobacterial infection and plays an important role in autophagy regulation. Therefore, Bfl-1/A1 acts as a negative regulator of autophagy in mycobacteria infected macrophages.