Summary

Tuberculosis disease of ancient time annually engulfs the life of millions of people worldwide. *M. tuberculosis*, the causative agent of tuberculosis has the capacity to subvert host immune responses and survive in the intracellular niche of the host macrophages. Despite the strong host mediated immune responses *M. tuberculosis* survives and disseminates further by subverting phagosomal acidification induced after the bacterium gets internalized by the macrophages (Philips and Ernst, 2012). Autophagy induction by various stimuli including, amino acid starvation, rapamycin treatment or activation by IFN-γ results in the fusion of bacteria containing phagosomes with autophagosomes and leading to the destruction of *M. tuberculosis* and suppresses its intracellular survival (Gutierrez et al., 2004).

Autophagy is an important catabolic process which regulates the degradation of old cellular components. Autophagy induction results in the generation of double membrane autophagosomes around the material to be degraded and then deliver the cargo material to lysosome by autophagosome-lysosome fusion process. This degraded matter is then reutilized by the cell and that is how the recycling goes on. Recently, role of autophagy has been also demonstrated in the elimination of intracellular pathogens know as xenophagy. Autophagy sequesters the bacteria which has gained access to the host cytoplasm. Cytosolic bacteria are coated with ubiquitin which get enclosed in double membrane autophagosomes and then degraded by fusion with lysosomes. Autophagy is regulated at various points. mTOR is one of the major regulators of autophagy. mTOR regulates autophagy by directly phosphorylating ULK1/ATG13 and by regulating the signaling pathways associated with the proteins regulating autophagy (Alers et al., 2012). Majority of signaling pathways controlling autophagy converge at mTOR, like AMPK, which activates autophagy by inhibiting the activity of mTOR, PI3K/Akt which down-regulates autophagy by activating mTOR (Alers et al., 2012). Autophagy is also regulated by various Bcl-2 members, Mcl-1 belongs to the same family and by directly interacting with Beclin 1 it negatively regulates autophagy (Germain et al., 2011). Mcl-1 expression is regulated by PI3K pathway and is reported to be induced by multiple growth factors (Thomas et al., 2010). Autophagy is induced by various stimuli including amino acid starvation, TLR ligands, ER stress and cytokines including TNF-α, IL-1 and IFN-γ (Harris, 2011).

Although the immunological nexus during the disease has been poorly understood but the major role of cytokines has been emerging gradually. IFN-γ an essential pleotropic cytokine

provides protection against *M. tuberculosis*. IFN-γ activates macrophages to promote the killing of intracellular *M. tuberculosis* by various mechanisms. Autophagy induction is one of the major effectors mechanism associated with IFN-γ mediated mycobactericidal effects. Treatment of murine macrophages with IFN-γ eliminates significant fraction of intracellular *M. tuberculosis* by producing reactive nitrogen intermediates (RNI) (Chan et al., 1992). IFN-γ also promotes phagosome maturation in immunity related GTPases (IRGM) dependent manner (Singh et al., 2006). IFN-γ also induces ER stress mediated autophagy in ATF6 dependent manner (Gade et al., 2012). Despite the local production of IFN-γ, the immune responses generated are not enough to completely eradicate *M. tuberculosis* infection in human (Jo et al., 2003; Raja, 2004). It is well reported that *M. tuberculosis* modulates macrophages responses towards IFN-γ. It has been reported recently that IL-27 promotes IL-10 production and antagonizes IFN-γ action (Cooper et al., 2011) but the role of IL-27 in the regulation of IFN-γ induced autophagy has not been reported yet. Neutralization of IL-27 during mycobacterial infection has been documented to enhance the elimination of *M. tuberculosis* (Jung and Robinson, 2014). Therefore, the present study was designed to investigate the effects of IL-27 on IFN-γ induced autophagy in mycobacterial infection.

First of all we were interested to elucidate the effect of IL-27 on various autophagy inducing stimuli including IFN-γ and amino acid starvation condition in THP-1 cells (taken as human macrophage model system). Here, we have found that IL-27 significantly inhibited IFN-γ and starvation induced autophagy, as confirmed by acridine orange staining. These results were further confirmed by investigating changes in well-known autophagy marker LC3 tagged with GFP as GFP-LC3 puncta formation in GFP-LC3 over-expressing THP-1 cells. We observed that IL-27 inhibited the generation of GFP-LC3 puncta formation induced by IFN-γ and starvation. IFN-γ and starvation induced autophagy inhibition by IL-27 was further confirmed from LC3-I to LC3-II conversion by western blotting. Here also IL-27 significantly lowered the IFN-γ and starvation induced conversion of LC3-I to II. Observation in autophagy inhibition by GFP-LC3 puncta formation itself does not give conclusive picture about the inhibitory stage of autophagy, as decrease in GFP-LC3 puncta formation can be due to two reasons either autophagy is inhibited at the initial stage of autophagosome formation or enhancement in the degradation of LC3. IL-27 inhibited autophagosome formation as evident by reduction of IFN-γ induced GFP-LC3 puncta formation even in the presence of lysosomal protease inhibitors E64D and pepstatin-A.

We have further extended our research in mycobacterial infection. We studied the effect of IL-27 on IFN-γ and starvation induced autophagy in *M. tuberculosis* infected macrophages. We observed that IL-27 significantly inhibited IFN-γ and starvation induced autophagy and phagosomal acidification in *M. tuberculosis* H37Rv infected macrophages. IL-27 treatment resulted in decreased IFN-γ and starvation induced co-localization of GFP-H37Rv containing phagosomes with LC3 and Beclin 1 positive phagosomes. IL-27 also lowered the IFN-γ induced co-localization of GFP-H37Rv with LysoTracker and CD63 in THP-1 cells, clearly indicating the inhibitory effect of IL-27 on IFN-γ induced phagosomal acidification in mycobacterial infection. We have further investigated the inhibitory effect of IL-27 on IFN-γ induced autophagy at ultra-structural level by transmission electron microscopy in *M. tuberculosis* infected THP-1 cells. IFN-γ induces the double membrane structure around *M. tuberculosis*, which was prevented by IL-27 treatment and can be seen as single membrane phagosomes, clearly indicating that IL-27 inhibits the generation of double membrane autophagosome induced by IFN-γ. These results were also confirmed by LC3-I to LC3-II conversion in infected THP-1 cells. Here also IL-27 reduced IFN-γ induced LC3-I to LC3-II conversion. Next, we have studied the effect of IL-27 on intracellular survival of *M. tuberculosis* H37Rv. Here, we have found that exogenous addition of IL-27 inhibited the mycobactericidal effect of IFN-γ and results in the promotion of intracellular survival of mycobacteria.

After establishing the role of IL-27 in autophagy, we have extended our investigation to find out the molecular mechanism behind the IL-27 mediated autophagy inhibition. We have observed that IL-27 activates JAK/STAT pathway which was evident by the phosphorylation of STAT-1 and STAT-3. We have found the role of JAK pathway in IL-27 mediated inhibition of autophagy, in the cells where JAK pathway was blocked by JAK inhibitor. In condition where JAK pathway was blocked IL-27 could not inhibit IFN-γ induced GFP-LC3 puncta formation, LC3-I to LC3-II conversion in uninfected THP-1 cells and co-localization of GFP-H37Rv containing phagosomes with Beclin 1 positive autophagosomes and LysoTracker positive auto-lysosomes in THP-1 cells as well as human MDMs. mTOR is a master regulator of autophagy. Activation of mTOR via PI3K class I and Akt inhibits the induction of autophagy by phosphorylating ULK-1 and ATG13 (Jung et al., 2010). We have found that IFN-γ inhibits whereas IL-27 activates mTOR and counteract the action of IFN-γ. mTOR activation by IL-27 was found to be JAK pathway dependent and interestingly IFN-γ mediated inhibition of mTOR was JAK independent. IL-27 mediated activation of mTOR

was observed to be PI3K class I and Akt pathway dependent. Role of mTOR activation by IL-27 in the inhibition of autophagy was further confirmed by mTOR inhibitor rapamycin or knock down of mTOR, where IL-27 could no longer inhibit IFN-γ induced autophagy in *M. tuberculosis* infected macrophages. These results clearly suggested the role of JAK/PI3K/Akt/mTOR pathway in IL-27 mediated inhibition of IFN-γ induced autophagy in *M. tuberculosis* infected macrophages. Mcl-1, another negative regulator of autophagy as reported in cortical neurons of mice is also regulated by various growth factors and interacts with the BH3 domain of Beclin 1 and results in the inhibition of autophagy (Germain et al., 2011). We have also investigated the effect of IL-27 on the expression on Mcl-1 in THP-1 cells. We have found that IFN-γ down-regulates whereas IL-27 up-regulates the expression of Mcl-1. IL-27 mediated increase in Mcl-1 was at protein level where as IFN-γ mediated down-regulation of Mcl-1 was also evident at transcriptional level. IL-27 enhanced Mcl-1 via JAK and PI3K pathway but was independent of mTOR as proved by JAK inhibitor, PI3K class I inhibitor and rapamycin respectively. Crucial role of Mcl-1 in IL-27 mediated inhibition of IFN-γ induced autophagy was also proved by knock down and over-expression of Mcl-1in THP-1 cells. In Mcl-1 silenced cells IL-27 could no longer inhibit IFN-γ induced autophagy as well as phagosomal acidification in *M. tuberculosis* infected macrophages. Mcl-1 over-expressing THP-1 cells where IL-27 was not provided exogenously also showed reduction in IFN-γ and starvation induced autophagy as well as phagosomal acidification in mycobacterial infection. Mcl-1 over-expression also abrogated IFN-γ mediated killing of *M. tuberculosis* and promotes intracellular survival of the bug.

Further we wanted to establish the role of IL-27 on IFN-γ mediated signaling cascade including ER stress pathway and associated autophagy induction in mycobacterial infection. We have found that IFN-γ treated cells showed increase in the expression of ER stress markers CHOP, XBP-1 and GRP-78 as well as induces the expression of both the subunits of IL-27 (EBI3 and p28). We have found similar results with TNF-α. IFN-γ and TNF-α treated cells also showed increase phosphorylation of one of the important ER stress marker known as eIF2α, which is involved in the translational attenuation, induces autophagy and support the survival of the cells during ER stress. Further we have found that *M. tuberculosis* infection also induces the expression of IL-27 (EBI3 and p28) which was further enhanced by IFN-γ treatment. We observed that inhibition of JAK pathway or neutralization of IL-27 during *M. tuberculosis* infection leads to enhanced elimination of *M. tuberculosis* due to

enhanced ER stress signaling and autophagy induction associated with it, as evident by ncreased eIF2α, JNK1/2 phosphorylation and LC3-I to LC3-II conversion.

Conclusively our results indicate that *M. tuberculosis* modulates the expression of IL-27 to subvert host protective mechanism for its survival. Despite the generation of various immune therapeutic approaches, this bacterium claims millions of life worldwide. To combat this deadly disease it is necessary to understand the complete nexus of innate immune responses and their subversion by mycobacteria and augmentation of the protective immune responses against *M. tuberculosis* especially in respect to the development of anti-TB immuno-therapeutics and vaccines.