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IL-6 inhibits IFN-γ induced autophagy in *Mycobacterium tuberculosis* H37Rv infected macrophages

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ABSTRACT

The significance of IL-6 production in tuberculosis is yet to be fully elucidated, although it is known for quite some time that IL-6 interferes with IFN-γ induced signal. In order to know which cellular process induced by IFN-γ is actually counteracted by IL-6, we studied the role of IL-6 on IFN-γ induced autophagy formation in virulent *Mycobacterium tuberculosis* infection in THP-1 cells, since it is well characterized that induction of autophagy by IFN-γ eliminates intracellular mycobacterium by overcoming the phagosome maturation block imposed by bacilli. We report here that IL-6 inhibits both IFN-γ and starvation induced autophagy in *M. tuberculosis* H37Rv infected cells. *M. tuberculosis* H37Rv infection results in time dependent production of IL-6 in THP-1 cells and neutralization of this endogenous IL-6 by anti-IL-6 antibody significantly enhances the IFN-γ mediated killing of the intracellular bacteria. IL-6 time dependently lowers Atg12–Atg5 complex and therefore inhibits autophagosome biogenesis rather than autophagolysosome formation. IL-6 also affects IFN-γ mediated stimulation of mTOR, p-38 and JNK pathways. These results clearly indicate that virulent mycobacteria strategically upregulate IL-6 production to combat innate immunity.

1. Introduction

Tuberculosis is one of the oldest infectious disease that remain a threat for public health around the world. *Mycobacterium tuberculosis*, the etiologic agent for this disease infect one third of the world population and is responsible for 2–3 million deaths every year (WHO, 2006). This bacterium is known to induce strong immune responses, yet successfully survive within its host macrophages. Although, macrophages are equipped to phagocytose and then kill most of the pathogens they encounter, but virulent *M. tuberculosis* is only phagocytosed but not killed by these cells. Invading microorganisms after ingestion by macrophages remain within phagosomes, which then mature into the phagolysosome. This process involves sequential interactions of phagosome with components of the endocytic pathway and end in fusion with lysosome to form a phagolysosome. The phagolysosome is an organelle with acidic pH and high content of hydrolytic enzymes involved in routine elimination of ingested microorganisms (Kaufmann, 2001; Koul et al., 2004). However, *M. tuberculosis* is known to arrest phagosomal maturation and thus prevent killing (van Crevel et al., 2002). Recent studies have demonstrated that induction of autophagy leads to the delivery of mycobacteria into lysosomes thereby killing of the intracellular bacteria (Munz, 2009; Deretic, 2008).

Autophagy is a catabolic process for the degradation of a cell's own components through the lysosomal machinery. It is a highly regulated process that plays a normal part in cell growth, development and homeostasis, helping to maintain a balance between the synthesis, degradation, and subsequent recycling of cellular products (Mizushima, 2007; Mizushima et al., 2002). Recently autophagy has been characterized as an innate defense mechanism for elimination of intracellular pathogens (Harris et al., 2009). During this process regions of the cytoplasm as well as organelles are first engulfed by double or multiple membrane structures called autophagosomes and subsequently the trapped material is delivered to autolysosomes which are formed by the fusion of autophagosomes with lysosomes (Levine and Klionsky, 2004). The trapped cytoplasmic material is eventually degraded by lysosomal hydrolytic enzymes. Three main steps are involved in autophagy formation: initiation, elongation and maturation. The initiation step is characterized by the formation of an isolation membrane (phagophore) which is controlled by various autophagy related factors (Atgs). Elongation step is distinguished by membrane bending and increase in the size of the phagophore. Maturation step involves the fusion of autophagosomes with late endosomes and lysosomes. Lumenal acidification followed by lysosomal degradation of the ingested components completes the process. Autophagy formation

Abbreviations: mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; LC3, microtubule-associated protein 1 light chain 3.

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involves the action of two Atg5 dependent ubiquitin like conjugation systems. First Atg12 is activated by Atg7, then transferred to Atg10 and finally covalently linked to Atg5. The Atg12–Atg5 conjugate localizes to autophagosome precursors. The second conjugation system modifies the C-terminal glycine of Atg8/LC3 (LC3-I) with phosphatidylethanolamine, generating the lipid conjugated LC3-II which is specifically targeted to elongated autophagosomes (Levine and Klionsky, 2004; Meijer and Codogno, 2004)

IFN-γ, a predominant activator of the microbicidal function of macrophages, has been demonstrated to induce autophagy in mycobacteria infected cells. Induction of autophagy by IFN-γ is associated with protective immunity against tuberculosis. The mechanism by which IFN-γ induces autophagy in macrophages is not yet fully understood. However, it is clear that Irgm1 is involved in this process since transfection of murine macrophages with Irgm1 induces the formation of autophagosomes, while siRNA knockdown of Irgm1 inhibits IFN-γ-induced autophagosome formation (Gutierrez et al., 2004; Singh et al., 2006; Harris et al., 2009). The human ortholog of Irgm1, IRGM is also important in autophagy.

Lymphocytes isolated from the lung of pulmonary tuberculosis patients typically have a Th1 phenotype secreting IFN-γ. However, despite the local production of IFN-γ, the immune response generated is not sufficient to eradicate tuberculosis infection in human (Jo et al., 2003; Raja, 2004). It is well documented now that *M. tuberculosis* prevents macrophages from responding to IFN-γ. Recently, it has been demonstrated that Th2 cytokines, IL-4 and IL-13 inhibit IFN-γ induced autophagy in both murine and human macrophages (Harris et al., 2007). Earlier, it was shown that IL-6 produced by mycobacteria infected macrophages selectively inhibits macrophage response to IFN-γ (Nagabhushanam et al., 2003) but no report exists to clarify whether IL-6 interferes with the autophagic process induced by IFN-γ.

Present study was designed to see the effect of IL-6 on IFN-γ induced autophagosome maturation process in virulent mycobacteria infected macrophages. We report here that IL-6 hinders autophagosome biogenesis stimulated by IFN-γ via counteracting with the expression of p38 and JNK pathways.

2. Materials and methods

2.1. Cell culture and reagents

The human promonocytic cell line, THP-1 was obtained from National Centre for Cell Science, (Pune, India) and processed for experiments as described earlier (Kathania et al., 2011). All cell culture reagents were purchased from Gibco Laboratories (Grand Island, NY, USA). Full Range Rainbow™ recombinant protein molecular weight marker and ECL Plus Western Blotting Detection System were purchased from GE Healthcare (USA). LysoTracker Red DND-99 was purchased from Molecular probes. p38 inhibitor, SB202190 and JNK inhibitor, SP600125 were purchased from Calbiochem (La Jolla, CA, USA). Human recombinant IFN-γ, IL-6, neutralizing human anti-IL-6 antibody, human IL-6 ELISA kit and antibodies against JNK1/JNK2, JNK/SAPK (pT183/pY185) were purchased from BD Pharmingen, USA. Beclin 1 siRNA (h), control siRNA, siRNA transfection reagent and antibodies against CD63, Beclin 1, p70 S6 kinase, p-p70 S6 kinase, p38, p-p38 were purchased from Santa Cruz (Santa Cruz Biotechnology, CA, USA). Antibodies against Atg5, Atg7, mTOR and phospho-(Ser 2448)-mTOR were purchased from Cell Signaling Technology, USA. Dehydrated mycobacteria growth media Middlebrook 7H9 and Middlebrook 7H10 agar, albumi-dextrose-catalase (ADC) and oleic acid-albumin-dextrose-catalase (OADC) supplements were purchased from Difco (Michigan, USA). Phorbol 12-myristate 13-acetate (PMA), p-formaldehyde, wortmannin, mouse anti-actin, anti-mouse HRP, anti-rabbit HRP and anti-IRGM antibodies and all other general chemicals were purchased from Sigma (St. Louis, MO, USA). The GFP-LC3 expression vector was kindly provided by Dr. Tamotsu Yoshimori (Tokyo Medical and Dental University, Tokyo, Japan). The RFP-LC3 plasmid was used as reported earlier (Kathania et al., 2011). All media and reagents used were endotoxin free.

2.2. Transfection and establishment of stable cell lines

THP-1 cells were transfected using Lipfectamine 2000™ according to manufacturer's protocol (Invitrogen, USA). Cells stably expressing GFP-LC3 or RFP-LC3 were selected and maintained in G418.

2.3. Bacteria and infection

M. tuberculosis H37Rv was maintained and used as described previously (Kathania et al., 2011). Briefly, PMA (25 ng/ml) differentiated THP-1 cells were plated into 6-well flat-bottom plates (Falcon) at 2.5×10^5 cells per well. Next day, cells were infected for 4 h with *M. tuberculosis* to give an infection ratio of 5–10 bacilli per macrophage as described previously (Kathania et al., 2011).

2.4. Infection of monocyte-derived macrophages (MDMs)

Human macrophages were derived from blood monocytes as described earlier (Kathania et al., 2011.) MDMs were infected with mycobacteria following the same method described for THP-1 cells as above.

2.5. Induction of autophagy in mycobacterial infection by IFN-γ in THP-1 and MDMs

Differentiated RFP-LC3-THP-1 cells were infected with GFP-*M. tuberculosis* H37Rv as described previously (Kathania et al., 2011) and then incubated in the presence or absence of IFN-γ (200 U/ml) or IL-6 (30 ng/ml) either alone or together for 4 h. After incubation, cells were fixed in 4% p-formaldehyde for 5 min and cells were analyzed under Carl Zeiss LSM 510 META confocal microscope and mycobacterial phagosomes were examined for colocalization with LC3 positive autophagosomes. Phagosome–lysosome fusion was seen by LysoTracker staining as described previously (Kathania et al., 2011).

For studying autophagy after mycobacterial infection in MDMs, cells were first transiently transfected with RFP-LC3 plasmid for 24 h using lipofectamine according to manufacturer's protocol (Invitrogen, USA). MDMs were then infected with GFP-*M. tuberculosis* H37Rv as described above and incubated in the presence or absence of IFN-γ (200 U/ml) or IL-6 (30 ng/ml) either alone or together for 4 h and examined for colocalization of LC3 positive autophagosomes with mycobacteria loaded phagosomes using confocal microscopy.

2.6. Induction of phagosome–lysosome fusion by starvation in mycobacterial infection

Differentiated THP-1 cells were first infected with GFP-*M. tuberculosis* H37Rv as described above and then incubated in amino acids and serum deprived starvation medium (Hank's balanced salt solution, HBSS) in the presence or absence of IL-6 (30 ng/ml). Phagosome–lysosome fusion was further studied under confocal microscope after LysoTracker staining.