MicroRNA 17-5p regulates autophagy in Mycobacterium tuberculosis-infected macrophages by targeting Mcl-1 and STAT3

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Summary

Autophagy plays a crucial role in the control of bacterial burden during Mycobacterium tuberculosis infection. MicroRNAs (miRNAs) are small non-coding RNAs that regulate immune signalling and inflammation in response to challenge by pathogens. Appreciating the potential of host-directed therapies designed to control autophagy during mycobacterial infection, we focused on the role of miRNAs in regulating M. tuberculosis-induced autophagy in macrophages. Here, we demonstrate that M. tuberculosis infection leads to downregulation of miR-17 and concomitant upregulation of its targets Mcl-1 and STAT3, a transcriptional activator of Mcl-1. Forced expression of miR-17 reduces expression of Mcl-1 and STAT3 and also the interaction between Mcl-1 and Beclin-1. This is directly linked to enhanced autophagy, because Mcl-1 overexpression attenuates the effects of miR-17. At the same time, transfection with a kinaseinactive mutant of protein kinase C δ (PKCδ) (an activator of STAT3) augments M. tuberculosis-induced autophagy, and miR-17 overexpression diminishes phosphorylation of PKCδ, suggesting that an miR-17/PKC δ/STAT3 axis regulates autophagy during M. tuberculosis infection.

Autophagy is widely recognized as a process in which organelles or long-lived cellular proteins are encapsulated in a vesicle with a double membrane and delivered to lysosomes for degradation (to remove damaged organelles) or recycling (to provide amino acids during starvation). Macrautophagy is the major pathway of autophagy. It involves the formation of macromolecular complexes at different steps involving a group of proteins conserved from yeast to mammals. In the canonical pathway of autophagy, autophagosome formation is initiated by the nucleation complex involving class 3 phosphoinositide-3-kinase (Vps34), Atg6 (or Beclin-1) and ubiquitin-like conjugation reactions. The elongation complex requires Atg7, Atg5 and Atg12. There is covalent conjugation of Atg8 homologues (or LC3) to phosphatidylethanolamine and expansion of the autophagic membrane. Finally, the autophagosome travels to the lysosome where its contents are degraded via acidic lysosomal hydrolases (He and Klionsky, 2009). It is now recognized that autophagy plays a crucial role in pathogen capture and degradation (Pareja and Colombo, 2013; Huang and Brumell, 2014).

MicroRNAs (miRNAs) are widely recognized as regulators of the host immune response to bacterial pathogens. It also appears that the differential regulation of miRNAs often provides a survival advantage to bacteria. MiRNAs have been profiled, and their role in influencing cellular signalling pathways have been reported in infections by pathogens such as Salmonella, Helicobacter pylori, Francisella tularensis and Listeria monocytogenes (reviewed by Maudet et al., 2014).
Tuberculosis is a cause of global concern, particularly in view of the rising incidence of mortality associated with AIDS and multi-drug resistant tuberculosis (World Health Organization, 2014). Host-directed therapies are being widely explored in an effort to develop adjunctive therapies for controlling the disease. In this scenario, it is essential to understand the immune regulatory mechanisms that are critical in the control of the disease. Autophagy is recognized to play a role in restricting bacteria within the host in a number of settings (reviewed by Deretic et al., 2013). It facilitates lysosomal killing of mycobacteria (Gutierrez et al., 2004; Alonso et al., 2007) and is associated with the control of the disease (Castillo et al., 2012; Bradfute et al., 2013). Small molecule inhibitors that restrict intracellular Mycobacterium tuberculosis growth have been reported to activate autophagy (Stanley et al., 2014). Recent studies using zebra fish and human macrophage models of mycobacterial infection show that DNA damage-regulated autophagy modulator (DRAM) I functions in the TLR-MyD88 pathway to mediate autophagic defense against mycobacteria (van der Vaart et al., 2014). DRAMI-dependent targeting of mycobacteria to autophagosomes depends on the STING pathway. Watson et al. (2012) have independently shown that after bacteria permeabilize the phagosomal membrane, the STING pathway ubiquitinates and targets the bacteria for lysosomal destruction.

Recent studies have clearly demonstrated that miRNAs regulate the immune response to Mycobacterium tuberculosis infection (reviewed by Maudet et al., 2014). The production of inflammatory signalling molecules may be regulated by virulent mycobacteria through differential regulation of miRNAs (Rajaram et al., 2011; Kumar et al., 2012). In addition, miR-155 has been reported to induce autophagy through repression of the negative regulator Rheb (Wang et al., 2013). In view of the role of autophagy in controlling Mycobacterium tuberculosis infection and the role of miRNAs in regulating autophagy, we sought to further explore how the differential expression of miRNAs during Mycobacterium tuberculosis infection regulates autophagy. Here we show that miR-17-5p (or miR-17) is downregulated during Mycobacterium tuberculosis infection of macrophages, as well as in mice infected with Mycobacterium tuberculosis. Other members of this family of miRNAs, namely, miR-20a, miR-20b, miR-93 and miR-106a, are also downregulated in infected macrophages. Our results show that the levels of Mcl-1 and its transcriptional activator STAT3 are regulated by miR-17. Mcl-1 suppresses autophagy by virtue of its ability to sequester Beclin-1. In addition, we also show that (a) protein kinase C δ (PKC δ) acts as a negative regulator of Mycobacterium tuberculosis (Mtb)-induced autophagy by virtue of its ability to activate STAT3 and (b) miR-17 overexpression inhibits the phosphorylation of PKC δ. The miR-17-PKCδ-STAT3-Mcl-1 pathway emerges as a key regulatory axis of autophagy during Mycobacterium tuberculosis infection suggesting that downregulation of miR-17 during infection likely suppresses autophagy. Our results uncover an additional layer of regulation of autophagy during mycobacterial infection, dependent on the downregulation of miR-17 and possibly other members of this family of miRNAs.

Results

Mycobacterium tuberculosis infection is associated with differential regulation of miR-17-5p

Our earlier report of global miRNA profiling of macrophages infected with Mycobacterium tuberculosis showed downregulation of several miRNAs including miR-17 (Kumar et al., 2015). In order to validate the differential expression of miR-17, RAW264.7 cells were infected with Mycobacterium tuberculosis at an MOI of 10 for different periods of time. Quantitative reverse transcription polymerase chain reaction showed a gradual decrease in miR-17 expression in a time-dependent manner (Fig. 1A). We found that an MOI of 10 was suitable for achieving optimal infectivity without loss of cell viability under the present conditions. Therefore, infections were carried out at an MOI of 10 in this study as in our previous studies (Kumar et al., 2012, Kumar et al., 2015). miR-17 was also downregulated in bone marrow-derived macrophages (BMDMs) infected with Mycobacterium tuberculosis (Fig. 1B). In order to understand the mechanism of regulation of miR-17, we assessed the levels of its precursor. Pre-miR-17 levels were lower in infected BMDMs compared with uninfected BMDMs (Fig. 1C), suggesting that the regulation occurs at the transcriptional level. We also checked the levels of miR-17 in various organs of infected mice. Downregulation of miR-17 levels was consistently observed 12 weeks post-infection in the lungs, spleen and lymph nodes of infected mice compared with uninfected mice (Fig. 1D–F). The decrease in miR-17 with progression of disease is likely to be of physiological relevance. In addition to the expression of miR-17, we also tested the expression of some of the other members of the miR-17 family, which share a similar seed sequence. miR-106a, miR-20a, miR-20b and miR-93 were downregulated when RAW264.7 was infected with Mycobacterium tuberculosis at an MOI of 10 (Fig. S1).

miR-17 regulates autophagy in Mycobacterium tuberculosis-infected macrophages

miR-17 or members of its family target autophagy-associated genes such as ULK1 (Wu et al., 2012), Beclin 1 (Chatterjee et al., 2014), ATG7 (Comincini et al., 2013) ATG16L1 (Zhai et al., 2013) and p62 (Meenhuis et al., 2011). We therefore hypothesized that miR-17 could be a possible regulator of autophagy in Mycobacterium tuberculosis-infected macrophages. In order to test this, we transfected cells with an miR-17 mimic prior to infection. The Atg3/Atg7-
mediated conversion of LC3-I to its C-terminally lipidated form, LC3-II, is used as a marker of autophagosome formation (Klionsky et al., 2012). Using this assay, we observed a time-dependent increase in LC3-II formation following \( \text{M. tuberculosis} \) infection, in cells transfected with a miR-17 mimic, suggesting that miR-17 enhances rather than inhibits autophagy in the present scenario (Fig. 2A). The increased conversion of LC3-I to LC3-II in infected macrophages, which had been transfected with miR-17 mimic, was also observed in BMDMs (Fig. 2B). At the same time, transfection with the miR17 mimic did not alter the expression of the autophagy-associated proteins ULK1, Beclin1 or ATG7 in infected macrophages (Fig. S2). Increased LC3-II levels could also indicate a block in autophagy flux. In order to rule out this possibility, we tested the effect of miR-17 in bafilomycin-treated RAW264.7 infected with Mtb. Conversion of LC3I to LC3II was further augmented when phagosomal acidification was inhibited by bafilomycin A (Fig. 2C). The role of miR-17 in regulating autophagy was confirmed by immunostaining Mtb-infected RAW264.7 with LC3B antibody and enumerating punctae. A significant increase in the number of punctae was observed when cells were transfected with miR-17 mimic prior to infection (Fig. 2D,E). Tandem fluorescent-tagged LC3 (mRFP-EGFP-LC3) is a convenient tool for measuring autophagic flux (Ni et al., 2011). Autophagic flux is associated with a selective loss of green fluorescence but retention of red fluorescence in acidic compartments. We observed a significantly increased proportion of red fluorescence compared with yellow (merging of green and red) when cells were treated with miR-17 mimic prior to infection (Fig. 2F,G). This confirmed that miR-17 facilitates autophagic flux in \( \text{M. tuberculosis} \)-infected cells. Taken together, our observations suggest that miR-17 is a positive regulator of autophagy in \( \text{M. tuberculosis} \)-infected macrophages.

Fig. 1. \( \text{Mycobacterium tuberculosis} \) downregulates the expression of miR-17 in macrophages. RAW264.7 cells or BMDMs were infected with \( \text{M. tuberculosis} \) for different periods of time as indicated in the figure. RNA was isolated and expression of miR-17 was assessed by qRT-PCR in RAW264.7 cells using snoR-142 expression for normalization (A) or by Northern blotting in BMDMs (B) using U6 for normalization. The blot in (B) is representative of two different experiments. (C) The expression of pri-miR-17 in BMDM was assessed by RT-PCR using gapdh for normalization. (D–F) RNA was isolated from different organs of mice after infection with \( \text{M. tuberculosis} \) for 12 weeks. Expression of miR-17 was analysed by Northern blotting in lungs (D), spleen (E) or lymph nodes (F). Intensities of bands were measured by densitometric scanning. Each data point is represented as ratio of intensities of miR-17 and U6. Symbols represent individual mice (panels D-F) [three in the uninfected and four (D,E) or five (F) in the infected groups]. For (A) results are means ± SD, \( n = 3 \). * = \( p < 0.05 \); ** = \( p < 0.01 \), *** = \( p < 0.001 \).
Mcl-1 expression is regulated by miR-17 in Mycobacterium tuberculosis-infected macrophages

Considering that miR-17 augments autophagy, we searched for negative regulators of autophagy likely to be targeted by miR-17 (or members of its family). Mcl-1 emerged as one likely target. Mcl-1 is a target of the miR-17 family member miR-106a (Rao et al., 2013). We tested whether miR-106a regulates autophagy in the present scenario. We observed that like miR-17, miR-106a overexpression is also associated with increased conversion of LC3-I to LC3-II (Fig. S3). We argued that because miR-17 and miR-106a share the same seed sequence, Mcl-1 could be a target of miR-17 also. In order to test this, we cloned the 3'-UTR of Mcl-1 downstream of a constitutively expressing luciferase gene (Fig. 3A) and tested its expression in HEK293 cells in the absence or in the presence of a miR-17 mimic. Forced expression of miR-17 repressed luciferase activity (Fig. 3B). This effect was not observed when the putative miR-17 seed sequence was mutated. Mcl-1 levels increased over time during the early periods of infection, decreasing thereafter (Fig. 4A). This time-dependent increase in expression of Mcl-1 in infected macrophages was suppressed in the presence of the miR-17 mimic in both RAW264.7 (Fig. 4B) and BMDMs (Fig. 4C). On the other hand, transfection with miR-17 inhibitor prior to infection augmented Mcl-1 expression (Fig. 4D) confirming that miR-17 downregulates levels of Mcl-1 in infected macrophages. To test our hypothesis that Mcl-1 is a negative regulator of autophagy, we silenced Mcl-1 (Fig. 5A) and checked the conversion of LC3-I to LC3-II in infected cells. Silencing of Mcl-1 augmented Mtb-induced autophagy in RAW264.7 (Fig. 5B), confirming that Mcl-1 dampens autophagy induced by M. tuberculosis. Next, we cotransfected cells with Mcl-1 expressing plasmid along with miR-17 mimic prior to infection. The autophagy-enhancing effect of miR-17 was blocked by overexpression of Mcl-1 (Fig. 5C), supporting the view that miR-17 enhances M. tuberculosis-mediated autophagy in an Mcl-1-dependent manner.
Mcl-1 interacts with Beclin-1 and regulates autophagy in Mycobacterium tuberculosis-infected macrophages in an miR-17-dependent manner

Mcl-1 is known to interact with the autophagy inducing protein Beclin-1, leading to the abrogation of autophagy (Germain et al., 2011). In order to test whether miR-17 augments autophagy by interfering with the interaction of Mcl-1 with Beclin-1, RAW264.7 cells were transfected with FLAG-Beclin-1 and either control mimic or miR-17 mimic, prior to infection. Post-infection, cells were lysed and immunoprecipitated with FLAG-Beclin-1 expressing plasmid and either control mimic or miR-17 mimic, along with miR-17 mimic (or control mimic). After 24 h of transfection, luciferase assays were performed and luciferase counts were normalized to β-galactosidase readings. The data is representative of at least three independent experiments. *** = p < 0.001.

**Fig. 3.** miR-17 inhibits Mcl-1 expression by interacting with its 3’UTR.
A. Schematic representation of Mcl-1 mRNA with relative positions of coding sequence and 3’-UTR regions. Depiction is not to scale.
B. HEK293 cells were co-transfected with plasmids expressing Mcl-1 3’UTR (wild type, WT or mutant, MUT) and β-galactosidase, along with miR-17 mimic (or control mimic). After 24 h of transfection, luciferase assays were performed and luciferase counts were normalized to β-galactosidase readings. The data is representative of at least three independent experiments. *** = p < 0.001.

**Fig. 4.** miR-17 regulates Mcl-1 expression in M. tuberculosis infected macrophages. RAW264.7 cells (A, B, D) or BMDMs (C) were left untransfected (A) or were transfected with either control or miR-17 mimic (B, C) or miR-17 inhibitor and its appropriate control (D) followed by infection with M. tuberculosis at an MOI 10 for different periods of time as indicated. Cell lysates were immunoblotted with Mcl-1 (A-D) or STAT3 antibody (C) followed by reprobing with actin antibody. Fold changes were calculated from densitometric scanning of the blots shown in panel B and C. The data shown are representative of two independent experiments.
antibody. Immunoprecipitates were tested for the presence of Mcl-1 by Western blotting. Association of Mcl-1 with FLAG-Beclin-1 was diminished in the presence of the miR-17 mimic (Fig. 5D), suggesting that miR-17 overexpression interferes with the ability of Mcl-1 to sequester Beclin-1, thereby facilitating autophagy.

**Protein kinase C δ regulates Mycobacterium tuberculosis-induced autophagy**

Based on reports that protein kinase C (PKC) δ activates STAT3 during keratinocyte proliferation (Gartsbein et al., 2006), we tested whether PKCδ regulates Mtb-induced autophagy by functioning as an upstream regulator of STAT3. Transfection of RAW264.7 with a dominant-negative (kinase-inactive, K376R) mutant of PKCδ (PKCδ-DN) augmented Mtb-induced conversion of LC3-II (Fig. 7B), suggesting that PKCδ serves as a negative regulator of autophagy in this setting. Treatment of cells with the PKCδ inhibitor rottlerin, prior to infection, showed a similar effect (Fig. 7C). Formation of LC3 punctae after infection was also increased in rottlerin-treated cells (Fig. S5A and B). STAT3 underwent time-dependent tyrosine phosphorylation in *M. tuberculosis*-infected cells (Fig. S5C). Transfection of cells with PKCδ-DN attenuated the tyrosine phosphorylation of STAT3 (Fig. 7D). Considering that STAT3 is a negative regulator of Mtb-induced autophagy, this suggested that PKCδ also serves as a negative regulator of autophagy by virtue of its ability to phosphorylate STAT3. Transfection of cells with PKCδ-DN (Fig. 7E) also diminished Mtb-induced Mcl-1 expression. Taken together, these results suggest that Mtb-induced autophagy in macrophages is negatively regulated by the PKCδ-STAT3-Mcl-1 axis.

**miR-17 regulates phosphorylation of protein kinase C δ**

It was of obvious interest to test the link between miR-17 and PKCδ considering the effects of both regulators on autophagy. We therefore analysed the regulation of phosphorylation of PKCδ on Y311 by miR-17. Transfec-
tion of cells with miR-17 inhibitor augmented the phos-
phorylation of PKCδ following M. tuberculosis infection; whereas, overexpression of miR-17 had the opposite effect (Fig. 7F,G). This suggested a link between miR-17 and PKCδ, although the mechanistic details of this link remain to be worked out. miR-17 therefore appears to regulate M. tuberculosis-mediated autophagy through multiple pathways.

**Discussion**

Given that miRNA families defined by a common seed sequence can regulate an overlapping set of targets and that a single target can be regulated by a number of miRNAs, there has been growing appreciation of the regulatory potential of miRNAs in diverse processes. Expectedly, there is now a body of research documenting the role of miRNAs in inflammation and the interaction between host and pathogen (Eulalio et al., 2012; O’Connell et al., 2012). There has been considerable interest in understanding the miRNA response of mammalian cells to infection. Included among the pathogens studied are intracellular bacterial pathogens such as Salmonella typhimurium and M. tuberculosis. One of the first analyses of the global miRNA response to infection focused on S. typhimurium and underscored the involvement of a trinity of miRNAs, miR-155, miR146a/b and miR-21 in the innate immune response (Schulte et al., 2011). Also noteworthy was the downregulation of the let-7 family. Since then, we and other laboratories have explored the global miRNA response of host cells to pathogenic mycobacteria. In mouse macrophages, miR-155 is highly upregulated (Kumar et al., 2012). On the one hand, it downregulates BTB and CNC homology 1 (BACH1) and SHIP1, thereby regulating activation of AKT and expression of heme oxygenase-1. On the other hand, it targets Rheb, a negative regulator of autophagy (Wang et al., 2013). miR-125a inhibits autophagy during mycobacterial infection by targeting UVRAG (Kim et al., 2015); whereas, miR-125b targets the 3’UTR of TNF to restrict TNF expression in M. tuberculosis-infected human macrophages (Rajaram et al., 2011). miR-99b is upregulated in M. tuberculosis-infected dendritic cells and macrophages (Singh et al., 2013) to regulate proinflammatory cytokines. We have demonstrated that let-7 is downregulated in infected macrophages, and its levels decline during the course of infection in mice (Kumar et al., 2015). Because let-7 targets A20, a negative regulator of NF-κB signalling, it regulates a range of proinflammatory cytokines and inducible nitric oxide synthase. These studies consolidate the view that miRNAs are important modulators of the immune response to M. tuberculosis infection and that the differential regulation of miRNAs could, under certain conditions, benefit the pathogen. Autophagy enhances targeting of M. tuberculosis to acidic compartments and helps in controlling bacterial burden. Proteolysis of ubiquitin and ribosomal proteins by the autophagy pathway generates antimicrobial peptides, which eliminate mycobacteria (Alonso et al., 2007; Ponpuak et al., 2010; Pilli et al., 2012). Autophagy helps the frontline drugs isoniazid and pyrazinamide in exerting their bactericidal effects (Kim et al., 2012). We attempted
to gain a better understanding of the role of miRNAs as regulators of autophagy during *M. tuberculosis* infection. Among the miRNAs transcribed from a single polycistronic transcript are those of the miR17-92 cluster (He et al., 2005) as well as its paralogues miR106a-363 and miR106b-25 (Khuu et al., 2014). We observed downregulation of representative members of each cluster, namely, miR-17, miR-106a, miR-20b and miR-93. These clusters have been associated with deregulated developmental programmes, oncogenic properties and autophagy (Hayashita et al., 2005). Based on the reports that miR-17 targets multiple players in the autophagic pathway, we focused on miR-17. The downregulation of miR-17 in *M. tuberculosis*-infected macrophages as well as in infected mice was striking. Surprisingly, miR-17 or miR-106a overexpression augmented Mtb-induced autophagy, prompting us to search for negative regulators of autophagy, which are likely miR-106a and miR-17 targets.

The Bcl-2 family members control programmed cell death or apoptosis by regulating mitochondrial outer membrane permeability (Green and Kroemer, 2014). The proapoptotic homologues include Bax and Bak. The activity of Bax and Bak is inhibited by the antiapoptotic Bcl-2, Bcl-XL, A1 and Mcl-1 (Youle and Strasser, 2008).
addition to regulating apoptosis, Bcl-2 homologues also regulate autophagy. The antiapoptotic Bcl-2 family members, Bcl-2, Bcl-XL and Mcl-1, can bind to Beclin-1 and inhibit autophagy (Pattingre et al., 2005; Maiuri et al., 2007; Germain et al., 2011). In addition, Mcl-1 has been shown to be upregulated in M. tuberculosis-infected THP-1 cells and to facilitate survival of the bacterium in macrophages (Sly et al., 2003). Based on reports that Mcl-1 is targeted by miR-106a, we tested and confirmed that it is also an miR-17 target. We observed that the time-dependent increase in Mcl-1 levels in infected macrophages was attenuated upon overexpression of miR-17. This strongly suggested a link between miR-17, Mcl-1 and autophagy. We have further confirmed this link by demonstrating that overexpression of Mcl-1 dampens the ability of miR-17 to enhance Mtb-induced autophagy. Mcl-1 inhibits autophagy in infected macrophages likely through its ability to sequester Beclin-1. The second miR-17 target that emerged as a negative regulator of autophagy in this study is STAT3, a transcription factor required for Mcl-1 expression. The role of STAT3 in regulating T cell-driven cytokine networks is documented. STAT3 regulates IL-23 receptor expression and IL-17 production by CD4+ cells during tuberculosis infection in humans (Bandaru et al., 2014). STAT3 also plays a role in driving fibroblast-dependent MPM-1 production that may be crucial in tissue destruction in tuberculosis patients (O’Kane et al., 2010). Less is known about its role in the macrophage response to M. tuberculosis infection. STAT3 is an inhibitor of HIV-1-mediated autophagy in bystander monocytc cells (Van Grol et al., 2010). Here, we show that STAT3 is a regulator of macrophage autophagy. While there are several reports showing that miR-17 targets important players in the canonical autophagy pathway, this study is unique in the sense that it demonstrates that miR-17 can also target negative regulators of autophagy. We further demonstrate that PKCδ is a likely regulator of STAT3 phosphorylation and nuclear translocation. In harmony with this, rottlerin, an inhibitor of PKCδ, augments LC3-II levels and the formation of LC3 puncta in infected cells. The phosphorylation of PKCδ in infected macrophages is attenuated by miR-17 overexpression. The pathway linking miR-17 to PKCδ phosphorylation is yet to be uncovered. However, this provides an additional layer of complexity to the role of miR-17 in regulating autophagy. The diverse mechanisms through which miR-17 regulates Mtb-induced autophagy are summarized in Fig. 7H.

Our studies provide insight into the role of miRNAs in regulating autophagy during M. tuberculosis infection. The miR-17 family and its paralogues could likely play a crucial role in the fate of the bacterium in macrophages. During infection, autophagy has been reported to limit IL-1β production, thereby linking autophagy to inflammasome activation (Shi et al., 2012). Nitazoxanide-stimulated autophagy inhibits intracellular proliferation of M. tuberculosis (Lam et al., 2012). It therefore appears that downregulation of miR-17 associated with suppression of autophagy could possibly help in the establishment of infection. The role of miR-17 in vivo remains open to further investigation using appropriate animal models.

**Experimental procedures**

**Reagents**

Antibodies for Mcl-1 and tubulin were from Santa Cruz Biotechnology; STAT3, p-STAT3 (Y705), Phospho-PKC δ (Y311) and LC3 were from Cell Signaling Technology; beta-actin and FLAG antibodies were from Sigma Chemical Co. The HRP-tagged secondary antibodies used were either from Santa Cruz Biotechnology or from Cell Signaling Technology. For immunostaining, the antibodies used were LC3 (MBL) and Alexa-546 conjugated secondary antibody (Molecular Probes). For Northern blotting, digoxigenin (DIG)-labelled probes for miR-17 and U6 were purchased from Eurogentec and Exiqon respectively. Transfection reagents Lipofectamine 2000 and Dharmafect2 were purchased from Invitrogen and Thermo Scientific respectively. Macrophage colony stimulating factor (M-CSF) was purchased from Prolspec or PeproTech. MiR-17 mimic, control mimic, miR-17 inhibitor and control inhibitor were obtained from Ambion. siRNAs against STAT3 and Mcl-1 were purchased from Eurogentec, while control siRNAs were from Santa Cruz Biotechnology. Bafilomycin A1 and rottlerin were purchased from Sigma and Calbiochem respectively.

**Cell culture and preparation of bone marrow-derived macrophages**

The murine macrophage cell line RAW264.7 and HEK293 cells were obtained from the National Centre for Cell Science, Pune. RAW264.7 and HEK293 were maintained in Dulbecco’s modified Eagle’s medium or minimal essential medium respectively, supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO₂. For preparation of BMDMs, monocytes were isolated from the bone-marrow of Balb/C mice and cultured in the presence of M-CSF for 6–8 days for their maturation (Boone et al., 2004; Gómez-Muñoz et al., 2004).

**Transfections**

RAW264.7 cells or BMDMs were plated on 12-well plates at a seeding density of 1.5 x 10⁵ in an antibiotic free medium a day before transfection. Cells were transfected with miR-17 or control mimic (20 nM) [or miR-17 or control inhibitor (150 nM)] using Lipofectamine 2000 following the manufacturer’s instructions. After 24 h of transfection, cells were infected as indicated. siRNAs against Mcl-1 or STAT3 were transfected at 50 nM concentration using Dharmafect2 transfection reagent. Cells

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were grown for 48 h followed by infection. Silencing was confirmed by immunoblotting.

Cells were transfected with plasmids expressing PKCδ-DN or STAT3 (Y705F) or mRFP-EGFP-LC3 (ptLC3) using Lipofectamine 2000 following the manufacturer’s instructions. Co-transfections of RAW264.7 cells with plasmids pTOPO-MCL-1 and miR-17 mimic or Flag–Beclin-1 and miR-17 mimic were carried out similarly.

**Western blotting and co-immunoprecipitation**

Cells lysates were denatured, separated by SDS-PAGE and electrophoretically transferred to PVDF membranes. Membranes were blocked in 5% (w/v) non-fat dry milk in tris-buffered saline with Tween 20 (TBST) (20 mM Tris–HCL, pH 7.5, 150 mM NaCl, 0.1% Tween-20) and incubated in primary antibodies at 4°C overnight, followed by washing with TBST thrice. The immunoprecipitated complexes were further incubated with secondary antibodies in 5% non-fat dry milk (w/v) in TBST and developed using HRP substrate following manufacturer’s instructions. For co-immunoprecipitation assays, lysates were incubated in lysis buffer with anti-Flag antibodies at 4°C overnight with gentle rotation followed by incubation with protein A/G agarose (Santa Cruz Biotechnology) at 4°C overnight, followed by washing with TBST thrice. The immunoprecipitated complexes were centrifuged at 3000 x g at 4°C for 5 min following by washing in lysis buffer at 4°C. The immunoprecipitated complexes were analysed by western blotting using antibodies against Mcl-1 and Flag as described previously.

**RNA extraction and Northern blotting**

Total RNA was extracted using the mirVana miRNA isolation kit (Ambion) following the manufacturer’s instructions. Northern blotting was carried out using DIG-labelled LNA probes as described by Kim et al. (2010). Briefly, RNA was separated using 8 M urea-15% polyacrylamide gels and electrophoretically transferred to positively charged nylon membrane (Roche Applied Science) followed by cross-linking of RNA using EDC crosslinking solution for 1 h at 60°C. The membranes were pre-hybridized in UltraHyb hybridisation buffer (Ambion) at 37°C for 1 h followed by hybridization with 1 nM DIG-labelled probe for miR-17 (Eurogentec) or 0.25 nM for U6 (Exiqon) at 37°C for 16 h with slow rotation. After incubation, membranes were washed twice for 5 min with low stringent buffer (2X SSC, 0.1% w/v SDS) and twice with high stringent buffer (0.1X SSC, 0.1% w/v SDS) at 37°C for 5 min. Then the membranes were blocked for 3 h at 25°C in blocking solution (DIG Block and Wash buffer set, Roche), followed by incubation with anti-DIG-Ap-Fab fragment in blocking buffer at 25°C for 30 min. The membranes were washed four times with wash-buffer and incubated in detection buffer (DIG Block and Wash buffer set, Roche) containing CSPD (Roche) at a dilution of 1:100 for 15 min at 37°C. The membranes were exposed to X-ray film and developed. For reprobing, membranes were kept in boiling buffer (5 mM EDTA, 0.1% SDS) for 10 min.

**Generation of Mcl-1 3′UTR reporter constructs and luciferase assay**

The Mcl-1 3′UTR containing the seed region for miR-17 was amplified using mouse genomic DNA as template and the sense and antisense primers 5′ TCACTAAGTACCT CAGAAATGTGACCTTTA 3′ (a) and 5′AACAAAGCTT CAGTGGTCTCAACCTTTA 3′ (b) respectively. The PCR product was cloned in pMIR-Report between the asymmetric Spel and HindIII sites. Site directed mutagenesis of the seed region in the Mcl-1 3′UTR was performed by overlap extension PCR using the primer pairs a (sense) and 5′ GTTTATTCACACTCATCTGAAGTCGGAAGA3′ (b, antisense); and 5′ TCTTCAGGACTCTAGGTAGTTCAATAAC3′ (sense, c) and d (antisense). The final PCR product was generated using the aforementioned products as templates and primers a and d. This product was cloned in pMIR-Report and sequenced.

For testing whether the Mcl-1 3′UTR is targeted by miR-17, the Mcl-1 3′UTR construct was cotransfected with miR-17 mimic (or control mimic) and β-galactosidase expressing construct in HEK 293 cells using Lipofectamine 2000. Cells were plated at a seeding density of 10^5 cells/well in a 24 well plate a day before transfection. After 24 h of transfection, cells were lysed and luciferase activity was measured using the Luciferase Reporter Assay System (Promega) in a microplate reader (Perkin Elmer). Readings for luciferase activity were normalised for transfection efficiency by assaying β-galactosidase activity.

**Bacterial culture and infection**

*Mycobacterium tuberculosis* strain H37Rv was grown in Middlebrook 7H9 supplemented with 10% ADC (Becton Dickinson) and 0.05% Tween 80 up to OD_{600} ≤ 0.2. The bacterial cells were washed thoroughly and passed through a 27 gauge needle thrice to remove clumps prior to infection. Infection was carried out for 4 h at an MOI of 10, followed by washing and incubating cells in medium containing gentamycin for 2 h in order to remove adhered bacterial cells. Cells were further incubated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

**Immunostaining and microscopy**

For immunostaining, cells were plated on cover slips in 24 well plates at a seeding density of 10^5 cells/well a day before the experiment. Cells were fixed by directly adding 10% neutral buffered formalin equal in amount to the medium present in the well. Cells were permeabilized with 0.05% Triton-X 100 for 5 min, blocked by adding 2% BSA in PBS for 30 min and incubated overnight with primary antibody at a dilution of 1:1000 at 4°C. Cells were subsequently washed and incubated with secondary antibody at dilution of 1:400. After washing, cells were mounted using slow fade anti-fade (Invitrogen). After drying for a few hours, cells were observed under a Zeiss AxioImager A1 fluorescence microscope (Carl Zeiss, Germany). Autophagic flux was analysed by confocal microscopy (TCS SP8, Leica Microsystems, Germany) to monitor mRFP-EGFP fluorescent signals.
Quantitative real time polymerase chain reaction for analysis of miRNAs

cDNAs for miRNAs were prepared using a Taqman cDNA synthesis kit and specific primers from Applied Biosystems following the manufacturer’s protocol. Real time PCR was carried out using TaqMan Universal Master Mix II and specific primers from Applied Biosystems in a 7500 Real-time PCR system (Applied Biosystems). Expression of snoR142 was used for normalization. The relative expression was calculated using the comparative ΔΔCT method, and the values were expressed as $2^{-\Delta\Delta CT}$.

Reverse transcription polymerase chain reaction for analysing mRNA

The RevertAid First Strand cDNA Synthesis Kit (Fermentas) was used for preparing cDNA. The sequences of primers used were: GAPDH (5′-gaa cgggaagcttgt cat caa-3′ and 5′-ctgagtctiegctgtcct-3′); pri-miR-17-92 (5′-tggacacctggcttcatatgc-3′ and 5′-caagttggagttcatctttgctc-3′). PCR was performed using ExPrime Taq DNA polymerase. PCR was carried out for 25 cycles.

Infection of mice

Animal experiments were approved by the institutional Animal Ethics Committees of the National Jaura Institute for Leprosy and other Mycobacterial Diseases (NJIL&OMD), Agra and the Bose Institute, Kolkata, India. Mice were purchased from the Central Drug Research Institute, Lucknow and were infected by aerosol inhalation of *M. tuberculosis* at the NJIL&OMD, Agra as described by Kumar et al. (2015). Effective dose of infection was confirmed by harvesting the lungs from mice 1 day after infection and determining colony-forming units. Infections were performed at a dose of about 100 cfu per mouse. Animals were euthanized 12 weeks post-infection. Lungs, spleen and lymph nodes were isolated, homogenized in Trizol and total RNA was isolated.

Statistical analysis

GRAPHPAD PRISM software was used for statistical analysis. Two groups were compared using 2-tailed Students t test. The data were presented as means ± SEM and p-value less than 0.05 were considered significant.

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**Supporting information**

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Fig. S1.** (A) Similarity in the seed sequence of mature miRNAs of the miR-106 family. (B,C) Expression of selected miRNAs of the miR-106 family analyzed by Taqman qRTPCR 4 (B) and 24 (C) hours after infection. B, C. Results represent means ± S.D., n=3. *, p < 0.05; ** p < 0.01

**Fig. S2.** RAW264.7 cells were transfected with either control or miR-17 mimic followed by infection with *M. tuberculosis*. Cell lysates were immunoblotted with the indicated antibodies and reprobed with tubulin antibody. The blot is representative of the results obtained in two separate experiments.

**Fig. S3.** RAW264.7 cells were transfected with either control or miR-106a mimic followed by infection with *M. tuberculosis* at an MOI of 10 for 4 or 6 h in the presence of 50 nM bafilomycin. Cell lysates were immunoblotted with LC3-II antibody and reprobed with actin antibody. The data represent at least two independent experiments.

**Fig. S4.** BMDMs were transfected with either control or STAT3 siRNA followed by infection with *M. tuberculosis*. Cell lysates were immunoblotted with Mcl-1 antibody and reprobed with actin antibody. The blot is representative of the results obtained in two separate experiments.

**Fig. S5.** (A,B) RAW264.7 cells were treated without or with Rottlerin for 30 min followed by infection with *M. tuberculosis* in the presence of bafilomycin. The cells were fixed and incubated with LC3 antibody, followed by staining with Alexa-546-conjugated secondary antibody. LC3 puncta (red) formation was detected by fluorescence microscopy (A) and puncta formation was quantitated (B). The experiment was done in triplicate and at least 100 cells were counted for each condition. The data represent three independent experiments. **p<0.01. (C) Time-dependent tyrosine phosphorylation of STAT3 was analyzed by Western blotting in *M. tuberculosis*-infected RAW264.7 cells. The blot is representative of the results obtained in two separate experiments.