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Targeted depletion of BTF3a in macrophages activates autophagic pathway to eliminate *Mycobacterium tuberculosis*

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ABSTRACT

Aims: β casein fragment peptide (54–59) downregulates Basic Transcription factor 3a (BTF3a) in macrophages and exhibits enhanced clearance of *M. bovis* BCG and several other intracellular pathogens. However, the direct effect of BTF3a downregulation on *Mycobacterium tuberculosis* (*Mtb*) survival and the probable pathways involved have not yet been studied. Therefore, the present study was undertaken to deduce the antimycobacterial significance of BTF3a in human macrophages.

Main methods: CRISPR/Cas 9 gRNA was designed to downregulate BTF3a in THP1 derived macrophages. Fold change in BTF3a, p62 and Lamp 1 expression was evaluated through immune blot analysis. CFU assay was done to enumerate the intracellular burden of *Mtb* H37Rv. LC3B-II turnover and Lamp 1 expression was checked through immunoblotting and also visualized through confocal microscopy. Colocalization of *Mtb* H37Rv with LC3B, Lysotracker and Rab 7 was visualized through confocal microscopy.

Key findings: The current study identifies BTF3a as a critical host factor assisting intracellular survival of *Mtb*. In THP1 derived macrophages, infection with *Mtb* H37Rv resulted in upregulation of BTF3a and targeted depletion of BTF3a resulted in augmented *Mtb* clearance. Furthermore, BTF3a knockdown demonstrated increased autophagy flux and ameliorated the lysosomal targeting of *Mtb* containing autophagosomes for lysosomal degradation.

Significance: Deep understanding of macrophage-*Mtb* interactions and their roles in the pathogenesis can offer exciting new therapeutic targets for alternative host-specific adjunct therapies in tuberculosis treatment. The present study highlights a novel and significant role of BTF3a in curbing the intracellular survival of *Mtb* through modulation of autophagy and lysosome biogenesis.

1. Introduction

Mycobacterium tuberculosis (*Mtb*), the etiological agent of human tuberculosis (TB), continues to be a leading cause of morbidity and mortality in humans worldwide [1–3]. Currently, the AIDS-pandemic, failure of BCG vaccine, limited number of effective antimicrobial drugs, and the ongoing emergence of antibiotics resistance are the main challenges in current global TB control efforts [1,4,5]. Hence, there is increasing demand of innovative therapeutic interventions, which can improve the treatment outcomes. In this scenario, attaining a parallel approach to target microbe as well as host intrinsic factors may escalate discovery of novel adjunct host-directed therapies (HDTs) that will boost current treatment protocols. For that, obtaining a better understanding of the host milieu contributing towards disease progression is paramount ([6–8].

Basic Transcription Factor 3 (BTF3) was originally identified as a general transcription factor from HeLa cell lysates [9]. In humans, gene encoding *BTF3* has two splicing variants: BTF3a (27 kDa), which has

Abbreviations: BTF3, basic transcription factor 3; CFU, colony forming unit; Lamp 1, lysosomal-associated membrane protein 1; MAPLC3B II/LC3B-II, microtubule-associated protein 1 light chain 3 B II; PFA, paraformaldehyde; SD, standard deviation; PMA, phorbol 12-myristate; RPMI, roswell park memorial institute; Baf A1, bafilomycin A1; V-ATPase, vacuolar-type H+ ATPase; Icd, inhibitor of cell death; sgRNA, single guide RNA; gRNA, guide RNA; HDT, host directed therapy; MOI, multiplicity of infection; GFP, green fluorescent protein; m, minute; h, hour

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the transcription characteristics of BTF3, and BTF3b (22 kDa), which lacks the first 44 amino acids of BTF3a and is transcriptionally inactive [10,11]. Few studies have also reported that neither BTF3a nor BTF3b are essential for the transcription initiation [12]. Due to lack of *in vivo* evidence, now the view that BTF3a is actually a general transcription factor is questionable and debated. Nevertheless, investigations are still being carried out on BTF3/BTF3a mediated transcriptional regulation of tumor-associated genes in various forms of cancer [13–17]. The biological importance of BTF3 was carried out later in mice, where an insertional mutation between exon 3 and 4 targeting both BTF3a and BTF3b cause early post-implantation lethality around day 6 [18]. The study however does not highlight the influence of the individual isoforms on the early post-implantation development of mice. BTF3b (β_1NAC) is also known as β subunit of nascent polypeptide associated complex (βNAC), which along with the alpha subunit (NACA) functions to prevent the targeting of the nascent polypeptide chains that lacks a signal sequence to the endoplasmic reticulum [19,20]. Although BTF3a (β_2NAC) and NACA complex is not yet fully characterized *in vitro*, but the properties of the complex are assumed to be similar to BTF3b - NACA complex [19–22]. Despite of all the evidences presented, the distinct function of BTF3a in humans is still unclear. We are the first to report that BTF3a is the target of β casein fragment peptide, in human macrophages [23]. β casein fragment peptide, is an immunostimulatory peptide isolated from the tryptic digest of human milk protein, casein and is known to clear several intracellular pathogens from macrophages ([23–28]. Furthermore, the downregulation of BTF3a mediated by the peptide is assumed to be responsible for augmenting intracellular clearance of *M. bovis* BCG from the macrophages [23]. Unfortunately, nothing has been reported about the antimycobacterial role of BTF3a in absence of the peptide treatment. In this work, we have demonstrated the impact of BTF3a knock down on intracellular survival of *Mtb* H37Rv. The study illustrates that BTF3a may have enough potential as an immunotherapeutic target against *Mtb* from the host's perspective.

2. Materials and method

2.1. Reagents

px330-U6-Chimeric_BB-CBh-hSpCas9 was a gift from Feng Zhang (Addgene plasmid # 42230). HPLC grade guide RNA (gRNA) oligonucleotides were synthesized from IDT. Cell culture reagents were purchased from Gibco, Life Technologies, unless stated otherwise. Lysotracker DND-99 (L7528) was purchased from Life technologies Invitrogen, phorbol 12-myristate 13-acetate (PMA) and Bafilomycin A1 (B1793) from Sigma-Aldrich. Anti-BTF3 (ab66940), anti-LC3B (ab51520), anti-rabbit alexa fluor (ab181474) and anti-mouse alexa fluor 555 (ab150114) antibodies were from Abcam, U.K. Anti-p62 (AP2183B) and anti-Lamp 1 (AP1823a-ev) antibodies were from abjent, anti-β actin antibody (sc-1616-R) was from Santa Cruz Biotechnology and anti- Rab 7 (#9367) and anti – Cleaved Caspase 3 (#9664) antibodies were purchased from Cell Signaling Technology. Restriction enzymes *Age*I and *Bbs*I were purchased from Thermo Scientific.

2.2. Cell culture and maintenance

Human leukemia monocyte cell line,THP-1 cells (ATCC TIB-202) were maintained in Gibco RPMI-1640 medium supplemented with fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin in a 5% CO2 incubator at 37 °C. THP-1 cells were differentiated to macrophages using 30 nM of Phorbol 12-myristate 13-acetate (PMA) as mentioned elsewhere [29].

2.3. gRNA designing and cloning

Two 20-bp guide sequence GCCCCGAGAGTCAGCCTGAG-3′ (BTF3a sgRNA1) and 5'-GCCAGGGGCGGCTGCCCTGG-3′ (BTF3a sgRNA2) targeting within BTF3 Exon 2 positive and negative strands were identified using the 'CRISPR Design Tool' (http://crispr.mit.edu/). The sgRNA were selected on the basis of binding to Exon 2 of the gene with no off targets. These sgRNA sequences were further validated by NCBI BLASTn tool. The sgRNA nucleotides (IDT) were annealed, phosphorylated and cloned in px330 Cas9 vector using *Bbs*I Enzyme and the construct was validated through *Bbs*I and *Age*I double restriction digestion.

2.4. BTF3a knockdown in THP1 derived macrophages

For transfection, the pre-differentiated macrophages are detached enzymatically by Accutase I (A6964 Sigma Aldrich) treatment. 5 μg of gRNA construct (2.5 μg of each sgRNA) or 5 μg Vehicle control (px330) was used to transfect 4 million cells. The transfection is performed using Y-001 program of Nucleofector 2b device for electroporation of the THP1 derived macrophages [30]. After transfection, differentiation is continued in appropriate media as described before for another 24 h. After 72 h, whole lysate of the cells was checked for BTF3a expression through western blotting. For experiments with serial transfections, cells were transfected with gRNA as described above on day one and with GFP-LC3 plasmid using the same protocol on day 2.

2.5. Analysis of Mtb intracellular growth

The *Mtb* H37Rv strain (ATCC-27294) was cultured in Middlebrook 7H9 broth (BD Difco Laboratories, 271310), supplemented with ADC, 0.2% glycerol (Sigma Aldrich, G2025) and 0.05% tyloxapol at 37 °C. Middlebrook 7H11 agar plates supplemented with 0.2% glycerol, 0.05% Tween 80 (Sigma Aldrich, P4780) and 10% Middlebrook oleic acid albumin dextrose catalase (OADC; BD Difco Laboratories, 211886) to log phase. PMA differentiated macrophages were transfected with BTF3a gRNA and vehicle control as described above. After 24 h of differentiation, the transfected macrophages were detached and seeded in 6 well plates (0.3×10^6 cells/well). The macrophages were infected with *Mtb* H37Rv at multiplicity of infection 1 at 37 °C for 4 h, washed thrice with medium to remove unphagocytosed bacteria. The media for these cells were replaced on alternate days with fresh RPMI 1640 with 10% FBS and gentamicin. On days 0 (4 h) and 5, the infected macrophage monolayers (three wells per treatment) were washed twice with fresh RPMI 1640 media with 10% FBS and then lysed with 0.1 ml of 1% Triton X-100 (Sigma Aldrich) to release intracellular mycobacteria, which were then enumerated in the form of Colony forming units (CFU) by plating serial dilutions on Middlebrook 7H11 agar plates incubated at 37 °C for ~25 days for estimating the cfu. All the experiments were performed independently thrice in triplicate and mean values were plotted log CFU/ml.

2.6. Immunoblotting

These cells were then harvested, washed, and total protein was extracted using 1× Protein lysis buffer (Promega) with phosphatase and protease inhibitor cocktail. Later, protein was estimated in the lysate using Bradford assay reagent (Sigma Aldrich, B6916) as per manufacturer's protocol and SDS-PAGE was performed (For LC3B and BTF3, a 15% SDS-PAGE gel was used). After transfer to PVDF membranes in a semi dry transfer unit (Amersham Biosciences) and subsequent blocking, the membranes were immunoblotted with Antibodies (Abs) against BTF3, LC3B, p62, Lamp 1, Cleaved-caspase 3 and loading control β actin. Blots were developed using a Luminata chemiluminescence kit (Milipore) in a Chemidoc gel imaging system (Syngene Bioimaging). Image analysis was performed with ImageJ and My image analysis softwares.

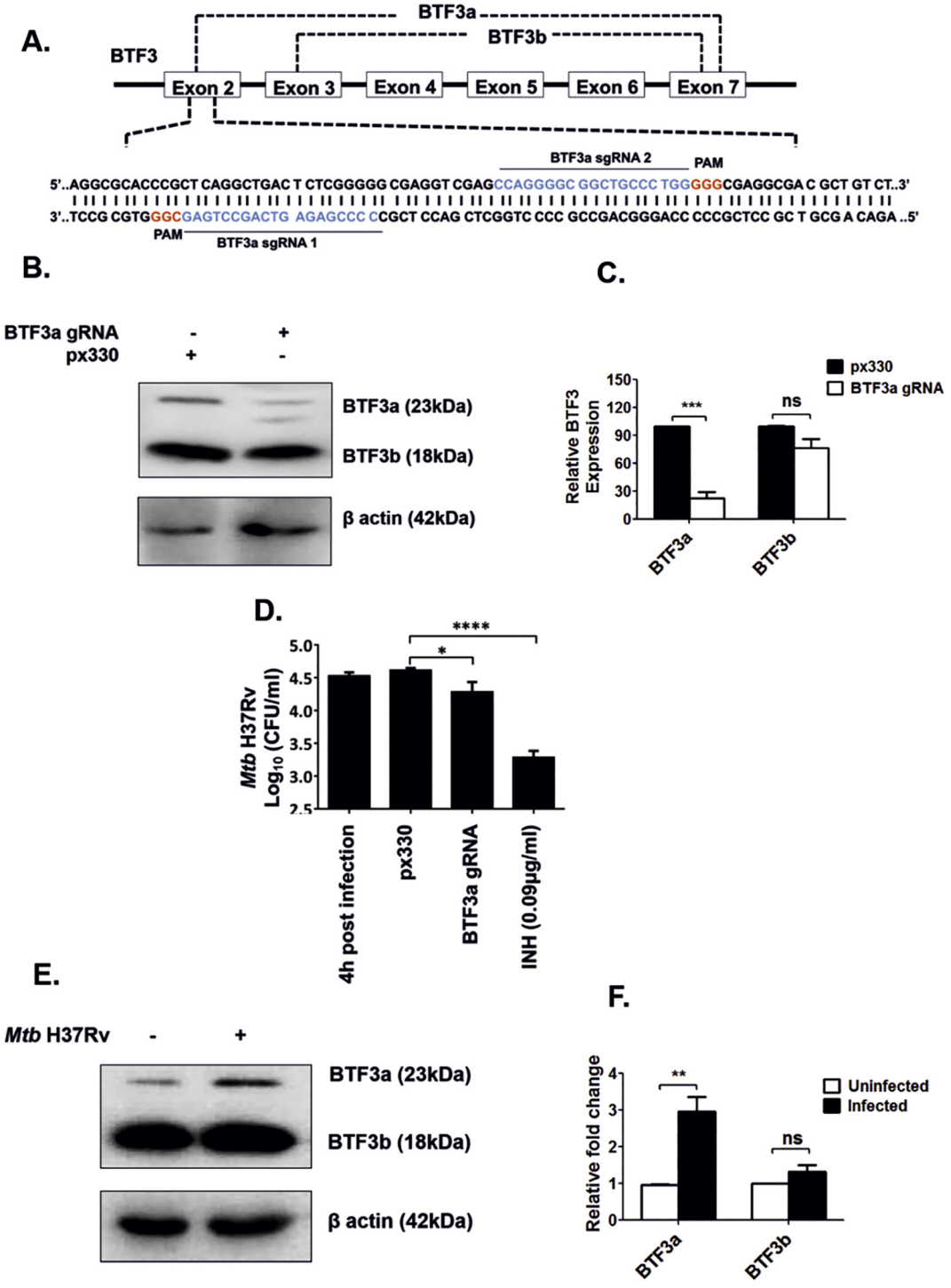

Fig. 1. Loss of BTF3a expression reduces intracellular survival of *Mtb* H37Rv in THP1 derived macrophages.

A. Schematic diagram of sgRNA targeting the BTF3 Exon 2 locus. The sgRNA targeting site on the sense strand is labeled as sgRNA2 while those on the antisense are labeled as sgRNA1, Both the sgRNAs are highlighted in blue whereas the PAM sequences are highlighted in red.

B. Immunoblot represents the activity of BTF3a gRNA (sgRNA1 + sgRNA2) and vehicle control (px330) transfection on the expression of BTF3a in THP1 derived macrophages. The expression was checked 72h post transfection.

C. Densitometry quantification of panel (B).

D. CFU from vehicle control, BTF3a knock down and Isoniazid (INH) treated THP1 derived macrophages after 5 days post infection (dpi) with *Mtb* H37Rv at MOI of 1. Control CFU was also checked after 4h of infection with *Mtb* H37Rv. Isoniazid at a concentration of 0.09µg/ml treatment is taken as positive control.

E. Immunoblot represents the changes in expression levels of BTF3 isoforms upon infection with *Mtb* H37Rv. THP1 derived macrophages were infected with *Mtb* H37Rv at MOI = 1 and whole cell lysates of infected and uninfected control were analyzed by Western blotting for BTF3 expression levels.

F. Quantification of panel (E).

Data are shown as the mean ± SD of at least three independent experiments with similar results. Asterisks denote significant differences assessed by Student's *t*-test (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$, ns: not significant, *Mtb*, *Mycobacterium tuberculosis*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.7. Quantification of LC3B and Lamp 1 puncta by confocal microscopy

Following the transfection process in THP1 derived macrophages as described above; cells were seeded on sterilized coverslips (0.1 × 106/well) in 12 well cell culture plates. Two different sets of cells were used in the study, one infected with *Mtb* H37Rv (MOI = 1) as mentioned in analysis of *Mtb* intracellular growth segment and other the uninfected one. After 24 h, cells were washed and fixed with 4% paraformaldehyde for 20 min at 37 °C followed by washing with thrice with 1XPBS. After permeabilization with 0.5% Triton X-100 for 10 min at 37 °C, cells were blocked with BSA (3%) for 1 h and incubated individually with rabbit polyclonal antibody against LC3B (1/100), an autophagosoml marker and rabbit polyclonal antibody against LAMP1 (1/100), late endosome-lysosome marker overnight at 4 °C. Washing is repeated and cells were incubated with alexa fluor 647 conjugated goat anti-rabbit Ab for 2 h at 37 °C. Thereafter, the coverslips (with cells) were washed and mounted on slides and visualized in LSM510 META confocal laser scanning microscope (Carl Zeiss) with 63× 1.4 oil emersion. Number of puncta formation was counted manually from 40 different cells. LC3B punctate per cell body were presented in bar diagram.

2.8. Colocalization experiments with Lysotracker or LC3B and Rab7

Macrophages were infected with *Mtb* H37Rv-GFP at a MOI of 1 for 4 h in RPMI 1640 at 37 °C. Cells were then thoroughly washed and incubated with fresh RPMI media at 37 °C under an atmosphere containing 5% CO_2. Further, the cells were incubated with Lysotracker red diluted 1:10,000 for 1 h. Cells were then rinsed, fixed with 4% paraformaldehyde for 20 min.

For LC3B and *Mtb* H37Rv-GFP colocalization analysis, macrophages were fixed as described above, permeabilized by incubation with 0.5% Triton X-100 for 10 min at room temperature (RT), blocked by incubation with 3% BSA for 20 min and incubated with rabbit polyclonal antibody against LC3B (1/100) overnight at 4 °C, revealed with alexa fluor 647 conjugated goat anti-rabbit Ab.

For *Mtb* H37Rv-GFP, LC3B and Rab 7 colocalization experiment, the cells are processed as described above, however the cells were also incubated with anti-Rab7 (1/100) overnight alongwith LC3B (1/100) antibody at 4 °C. The LC3B expression was revealed using alexa fluor 647 conjugated anti-rabbit Ab and Rab 7 expression was checked using alexa fluor 555 anti- mouse Ab.

For all the experiments, thereafter the coverslips (with cells) were washed, mounted and viewed with visualized in LSM510 META confocal laser scanning microscope (Carl Zeiss) with 63× 1.4 oil emersion. The colocalization of LC3B, Lysotracker and GFP- *Mtb* H37Rv was quantified by manually counting colocalization in 40 cells.

2.9. Statistics and data analysis

All the densitometric analyses were performed using ImageJ 1.47v. Results are expressed as the mean ± SD, unless otherwise mentioned. Graphs were plotted using GraphPad Prism 5.0 software and statistical significance was defined by one-way ANOVA and Student paired 2-tailed test.

3. Results

3.1. BTF3a knock down augments intracellular killing of Mtb H37Rv in human macrophages

Because our previous study has illustrated indirect evidence towards the antimycobacterial role of BTF3a macrophages, present work was undertaken as an endeavor to identify the role of BTF3a on intracellular survival of *Mtb* H37Rv. For this, we performed knock down of BTF3a in THP1 derived macrophages (PMA treated) employing CRISPR/Cas 9 gRNA approach by targeting Exon 2 of BTF3 (Fig. 1A). Western blotting analysis demonstrated > 70% reduction in BTF3a expression after 72 h of BTF3a gRNA transfection in THP1 derived macrophages (Fig. 1B & C). We also evaluated the specificity of BTF3a gRNA by targeting exon 3 which is common between BTF3a and BTF3b. Targeting exon 3 resulted in downregulation of both BTF3a and BTF3b isoforms (Fig. S1A, B & C).

We next evaluated the antimycobacterial role of BTF3a knock down in macrophages through CFU assay. After 5 days of infection, a significant 0.33 $\log_{10}$CFU/ml reduction in the intracellular load of *Mtb* H37Rv was observed upon targeted depletion of BTF3a in THP1 derived macrophages when compared with vehicle control macrophages (Fig. 1D). Previously, the expression level of BTF3a was found modulated upon proteome analysis of J774A.1 macrophages infected with *Mtb* LPS [31]. Therefore, we next determined the effect of *Mtb* H37Rv infection on intracellular expression of BTF3a in human macrophages. Strikingly, infection with *Mtb* H37Rv for 24 h significantly upregulated the expression levels of BTF3a in THP1 derived macrophages confirmed through western blotting (1E & F). Collectively, these data suggest that the modulation in BTF3a expression could be a strategy amongst many, applied by *Mtb* for its survival or replication inside host macrophages.

3.2. BTF3a knock down stimulates autophagy induction and modulates lysosome formation in THP1 derived macrophages

Macrophages represent the forefront of innate immune defense against different bacterial invaders. At the same time, macrophages are also primary targets of intracellular bacteria to be abused as host cells [32]. Amongst many, *Mtb* is the most successful intracellular bacterium which survives within macrophages. *Mtb* has learned during evolution to counter-balance the host's immune defense strategies for its survival or multiplication within this otherwise hostile environment. Primarily, *Mtb* inhibits i) the apoptosis of infected macrophages to secure a perfect niche for its survival and multiplication, ii) the lysosomal degradation of the autophagosomes, where it resides. Therefore, next we investigated BTF3a regulated probable pathways leading to *Mtb* clearance in macrophages. We also checked the impact of BTF3a knock down on macrophages without infection.

Given that BTF3 downregulation in different cancer forms has been associated with the modulation of apoptosis [13,15], we first monitored the impact of BTF3a depletion on apoptosis in THP1 derived macrophages through cleaved-caspase 3 based immunoblotting (Fig. 2A) and annexin V staining (Fig. S4). Interestingly, we did not find any significant changes in apoptosis in macrophages upon debilitating the activity of BTF3a in both presence and absence of *Mtb* H37Rv infection. This may be due to cell specific functioning of BTF3a. Additionally, it was also confirmed that BTF3a knock down does not eliminate intracellular *Mtb* infection through cell apoptosis. We further aimed to determine the modulation in autophagy through studying expression patterns of different protein markers involved in the process. Monitoring levels of processed microtubule-associated protein light chain 3 (LC3B-II) is used as a central readout for autophagosome formation [33]. Sequestosome 1 or p62 is an adaptor protein which interacts with polyubiquitinated protein aggregates and LC3B-II, thereby targets these aggregates for degradation at the autolysosome. Changes in p62 protein levels are used to indicate a defect in the turnover of poly-ubiquitinated protein aggregates [33,34]. Lysosomal-associated membrane protein 1 (Lamp 1) is a late endosomal marker involved in lysosome biogenesis [35]. Our results clearly demonstrated that BTF3a downregulation in *Mtb* H37Rv infected macrophages led to significantly increased turnover of LC3B-II expression, reduced expression of p62, enhanced Lamp 1 expression when compared to infected vehicle control (Fig. 2A). Enhanced LC3B-II turnover and p62 downregulation signify a positive regulation of autophagy, whereas increased Lamp 1 expression indicates some regulatory role of BTF3a in lysosome biogenesis. However, in uninfected macrophages BTF3a knock down does modulated LC3B-II turnover and Lamp 1 expression but was lesser in magnitude when compared with BTF3a knock down macrophages in presence of *Mtb*

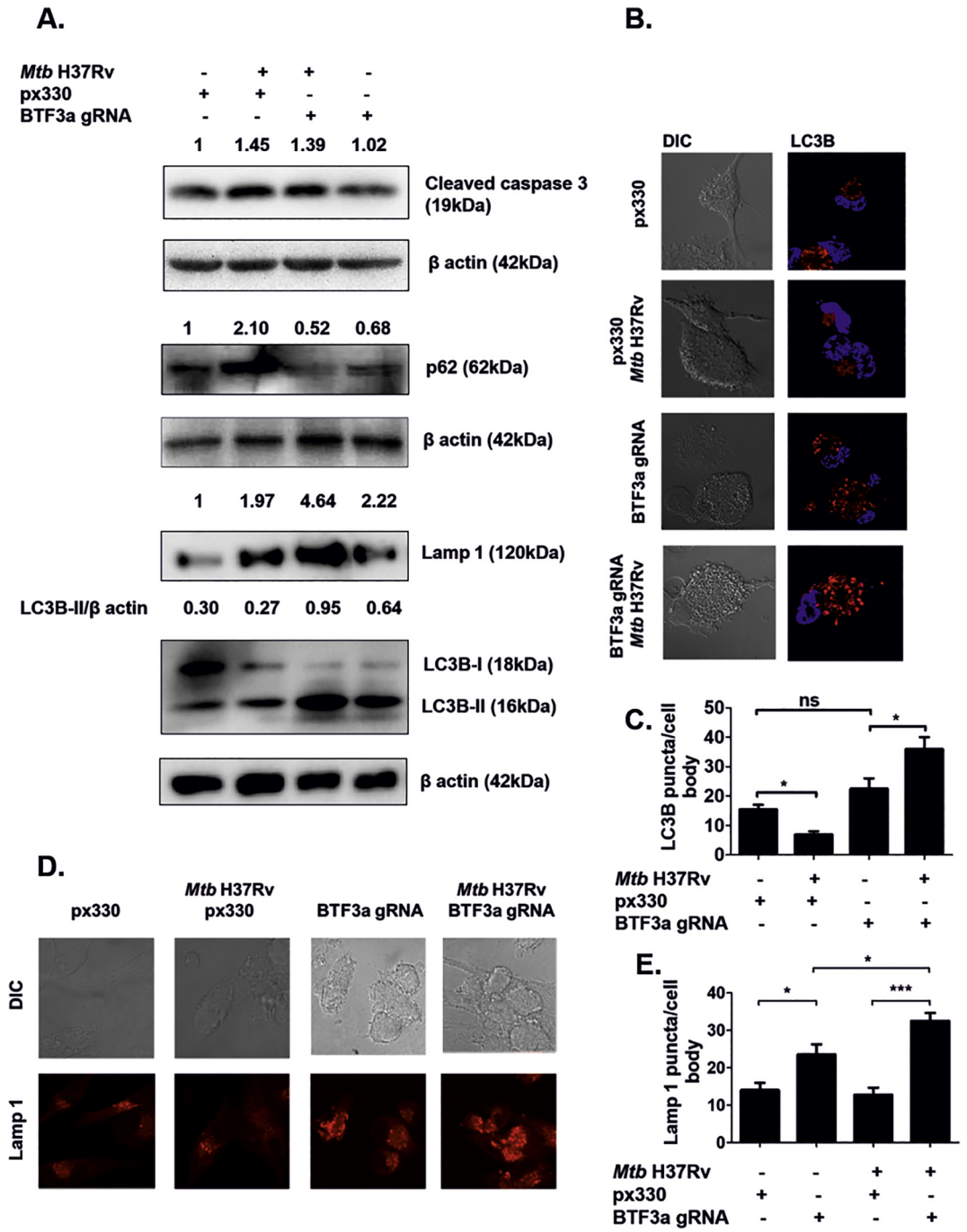

Fig. 2. BTF3a knock down induces autophagy and lysosome biogenesis in THP1 derived macrophages.

A. Vehicle control and BTF3a silenced THP1 derived macrophages were infected with *Mtb* H37Rv at MOI of 1 for 24 h and whole cell lysates of infected and uninfected sets were analyzed by Western blotting for LC3B-II, p62, Caspase 3, Lamp 1 and β actin levels respectively. The original gel image for LC3B-II and p62 is provided in supplementary data (S3).

B. Induction of autophagosomes was assessed through endogenous LC3B staining in vehicle control and BTF3a silenced THP1 derived macrophages in presence and absence of *Mtb* H37Rv infection at MOI of 1 for 24 h. Scale bar, 1 μm.

C. Quantitative analysis of panel (B). The frequency of puncta formation was enumerated by manual counting of 40 cells.

D. Modulation in lysosome biogenesis was confirmed through confocal microscopy based on Lamp 1 staining in vehicle control and BTFa knock down THP1 derived macrophages in presence and absence of *Mtb* H37Rv infection at MOI of 1 (24 h). Scale bar, 10 μm

E. Quantitative analysis of panel (D). The frequency of puncta formation was enumerated using 4–5 different microscopic fields and counting 40 cells.

All data show mean ± SD of at least two to four independent experiments with similar results assessed by student *t*-test and significant difference were identified on the basis of $*p \leq 0.05$ and $***p \leq 0.001$, ns: not significant. DIC: differential interference contrast.

H37Rv. The results also explained the possible role of BTF3a in maintaining the autophagic flux [36]. These findings were also verified through LC3B based immunostaining experiment, which confirm the results in the form of enhanced LC3B puncta reflecting increased number of autophagosomes in infected BTF3a knock down cells when compared to infected vehicle control cells (Fig. 2B & C). We also performed LAMP 1 based confocal microscopy to enumerate acidic vacuoles. Intriguingly, we observed a slight increase in puncta formation

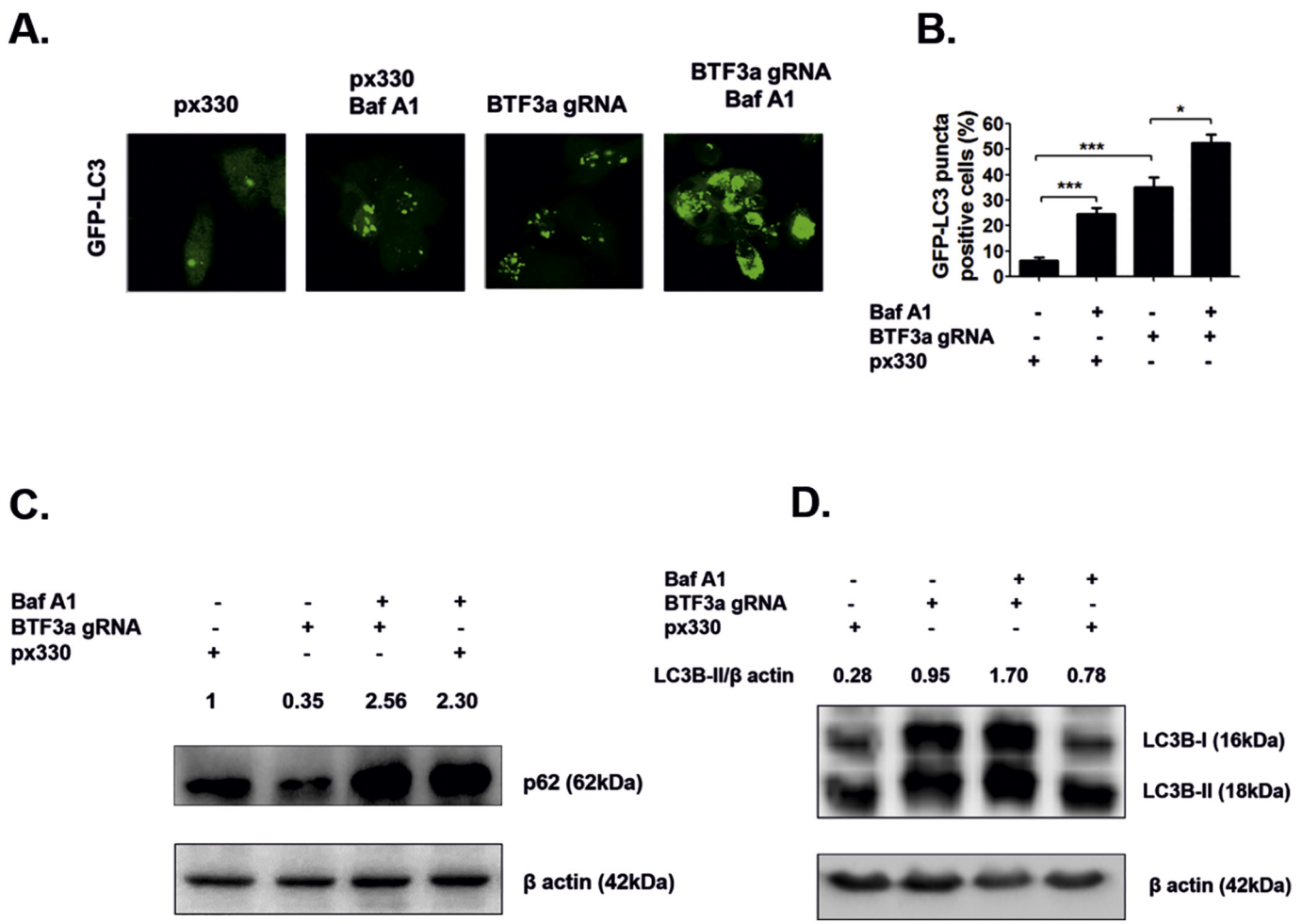

Fig. 3. BTF3a knock down induced autophagic flux in *Mtb* H37Rv infected macrophages

A. PMA-differentiated THP-1 cells were transfected with vehicle control or BTF3a gRNA for 24 h followed by transfection with GFP-LC3 and infection with *Mtb* H37Rv (MOI = 1). The cells were then treated with or without (Bafilomycin A1) Baf A1 (100nM) for 4h and GFP-LC3 puncta formation was observed by confocal microscopy 24 h later. Scale bar-10μM.

B. Quantitative analysis of panel (A). The number of GFP-LC3 puncta in each cell was counted for 40 cells in each group.

C. Vehicle and BTF3a knock down macrophages (THP1) were treated and not with 100 nM Baf A1 for 4 h followed by *Mtb* H37Rv infection (MOI = 1, 24 h). Whole cell lysates of Baf A1 treated and untreated were analyzed by immunoblotting for p62 and β actin expression levels respectively.

D. Vehicle and BTF3a knock down macrophages (THP1) were treated and not with 100 nM Baf A1 for 4 h followed by *Mtb* H37Rv infection (MOI = 1, 24 h). Whole cell lysates of Baf A1 treated and untreated were analyzed by immunoblotting forLC3B-II and β actin expression levels respectively.

Data is represented in the form of mean ± S.D. from 3 independent experiments. $*p \leq 0.05$ and $***p \leq 0.001$. DIC: differential interference contrast.

upon BTF3a knock down in infected macrophages when compared to the infected vehicle control and BTF3a knock down uninfected macrophages indicating that BTF3a has its activating effect in lysosomal formation also (Fig. 2D & E). Altogether, these results indicate positive regulation of autophagy and lysosome biogenesis in *Mtb* infected macrophages upon BTF3a knock down.

3.3. BTF3a knockdown augments autophagic flux in Mtb H37Rv infected macrophages

Enhanced LC3B-II turnover in cells not necessarily reflects increased autophagic flux and may be a reflection of blocked autophagosome fusion or degradation, such autophagosomes do not colocalize with lysosome [34,37]. To assess, the role of BTF3a in autophagic flux, vehicle control and BTF3a knock down cells were transfected with GFP-LC3, followed by *Mtb* H37Rv infection. Autophagosome formation was observed in presence and absence of Bafilomycin (Baf) A1. Baf A1 prevents maturation of autophagic vacuoles by inhibiting vacuolar type H ATPase (V-ATPase), thereby preventing degradation of autophagosomes [38]. We found significant increase in GFP-LC3 punta in BTF3a knock down cells treated with Baf A1 (Fig. 3A & B). Also, through western blotting analysis we observed increased accumulation of LC3B-II and p62 markers in BTF3a knock down *Mtb* infected macrophages after Baf A1 treatment, indicating that BTF3a knock down was able to overcome the *Mtb* induced block in autophagic flux in macrophages (Fig. 3C & D).

3.4. Loss of BTF3a expression enhances Mtb targeting to phagolysosomes through recruitment of Rab7

The observation of increased autophagy and lysosome formation upon BTF3 knock down in the present study led us to examine the lysosomal targeting of *Mtb* containing autophagosomes. For that, we first analyzed the localization of *Mtb* to LC3B-positive compartments. Through confocal microscopy we found significant enhanced colocalization of GFP tagged *Mtb* H37Rv with the autophagosomes (LC3B tagged) in macrophages upon BTF3a knock down indicating enhanced targeting of *Mtb* to autophagosomes (Fig. 4A & B). Activation of autophagy initiates a bactericidal process that involves the maturation of mycobacterial autophagosomes [39]. Following uptake, inhibition of

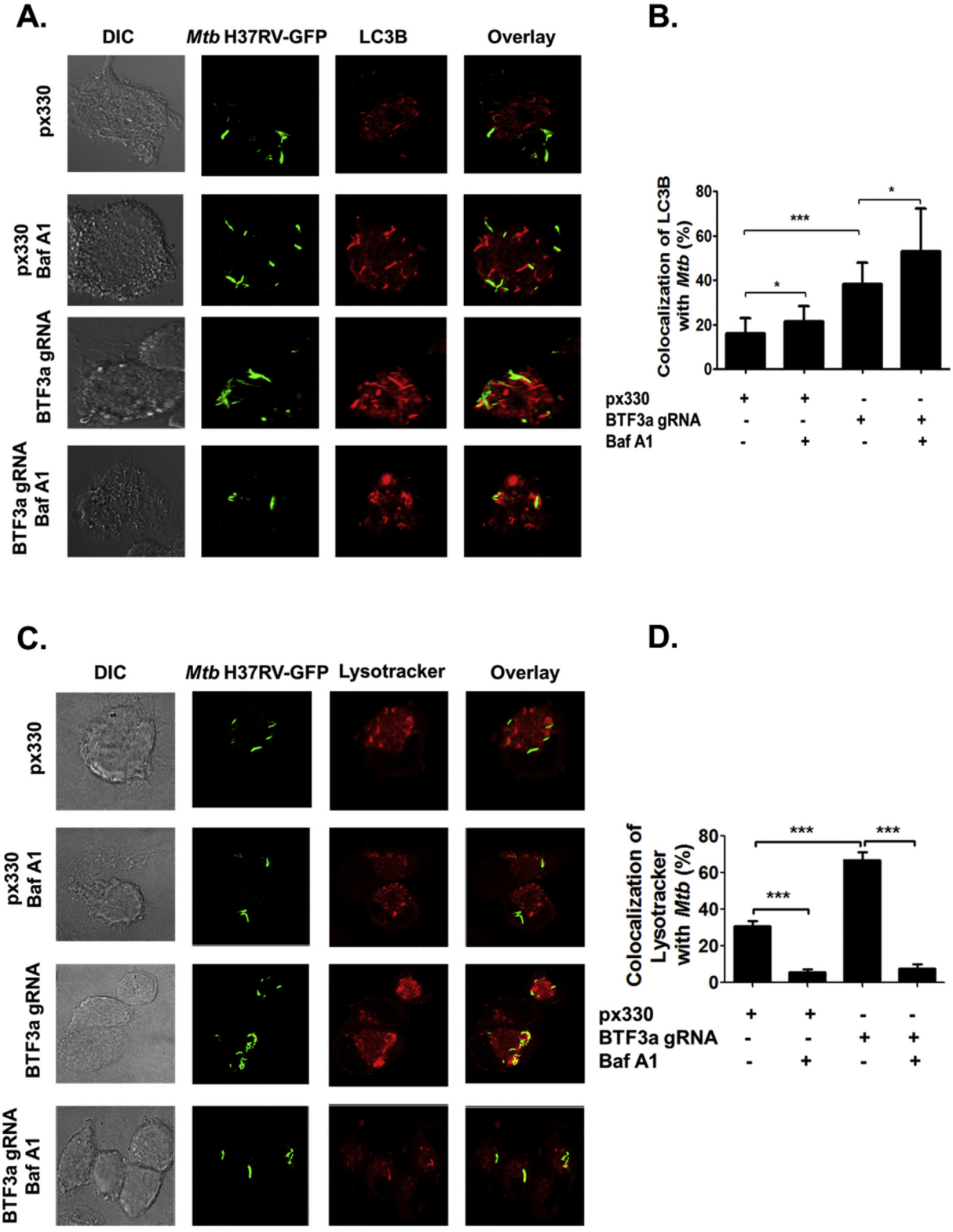

Fig. 4. BTF3a knockdown promotes lysosomal targeting of *Mtb* H37Rv carrying autophagosomes.

A. Vector control and BTF3 knock down THP1 macrophages were infected with *Mtb* H37Rv-GFP at MOI of 1 for 24 h, Colocalization of LC3B with *Mtb* was analyzed in presence and absence of Baf A1 treatment by immunofluorescence. Scale bar, 10 μm.

B. Quantitative analysis of the percentage of colocalization of LC3B and *Mtb* H37Rv- GFP in panel (A).

C. Colocalization of *Mtb* H37Rv-GFP (MOI = 1) with acidified lysosomes (stained with LysoTracker Red) was determined in THP1 derived macrophages (Vector control, BTF3a knock down and BTF3a knock down in presence of Baf A1) by confocal microscopy after 24h of infection. Scale bar, 10μM.

D. Quantitative analysis of Panel (C).

Data represent mean ± SD from at least three experiments. $*p \leq 0.05$, $***p \leq 0.001$ two-tailed Student *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

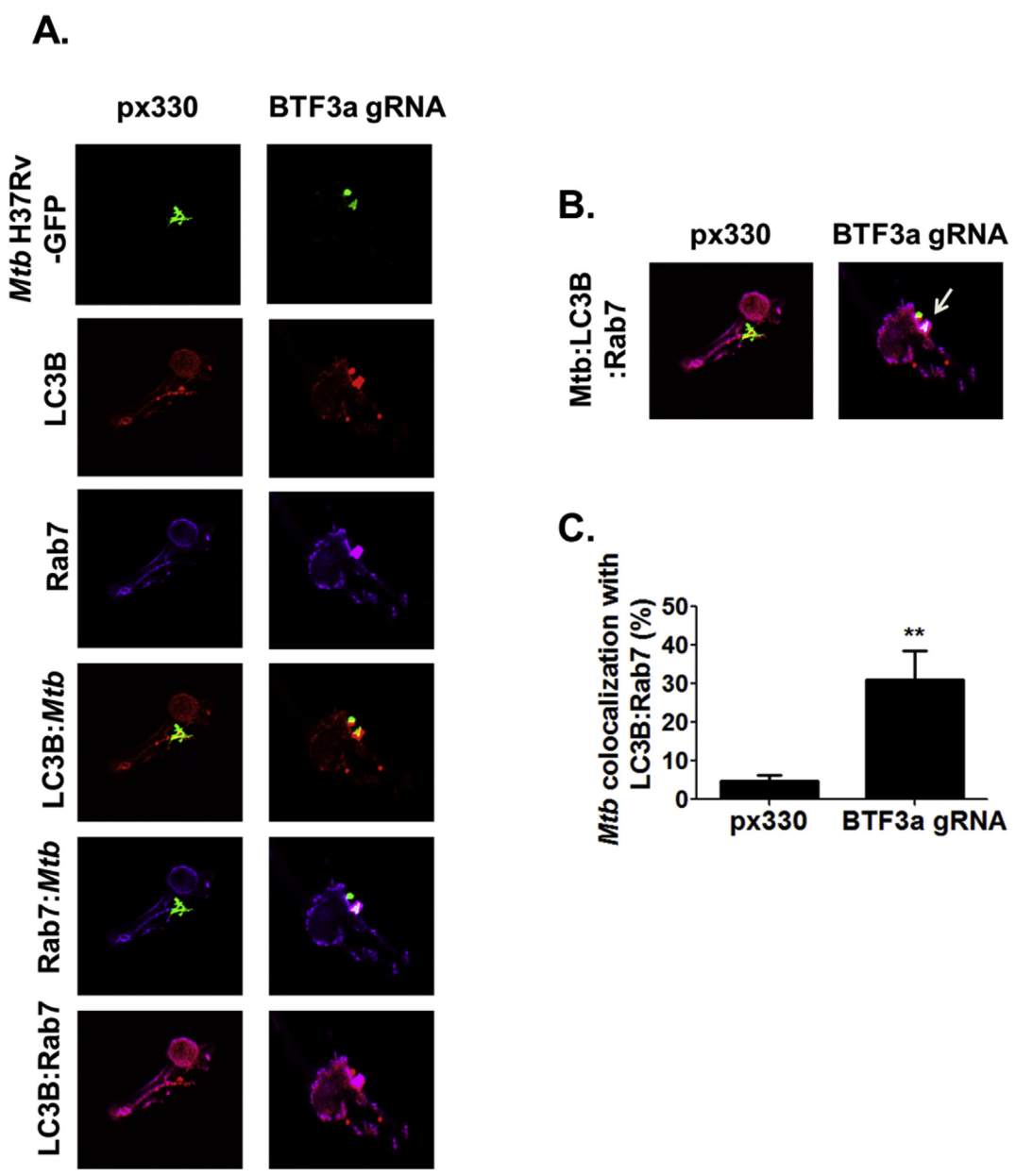

Fig. 5. BTF3a knock down promotes Rab7 recruitment on *Mtb* occupied autophagosomes.
(A and B) THP1 derived macrophages were transfected with gRNA against BTF3a or vehicle control (px330), followed by infection with *Mtb* H37Rv-GFP (MOI = 1). Recruitment of Rab7 (Purple) with *Mtb* H37Rv (GFP) containing autophagosomes (LC3B, Red) was analyzed by immunofluorescence. Scale bar, 5 μM.
C. Quantitative analysis of *Mtb* (white arrow) in LC3B-RAB7 amphisomes from panel (B).
Data is represented in mean ± SD, $^{**}p \leq 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

phagosome-lysosome fusion is critical for the survival of *Mtb* within human macrophages. This is mediated by pathogen-dependent manipulation of host signaling pathways, which ensures that the bacteria remain in early endosome [40]. It has been reported that autophagosomes that contain the virulent strains of *Mtb* show selective resilience to the maturation phase of autophagy [41]. Literatures suggest that *Mtb* somehow blocks the selective acquisition of Rab7, a late endosomal marker on the autophagosomes carrying the bacilli itself and thereby hinders the phagolysosome fusion to avoid the lysosomal degradation [41–43]. Lysotracker red is an acidotropic acid dye that serves as an indicator of lysosomal pH. Lysotracker Red freely permeates cell membranes and remains trapped in acidic compartments upon protonation. Therefore, we monitored the colocalization of GFP-tagged *Mtb* H37Rv with lysotracker Red and observed enhanced colocalization of *Mtb* H37Rv with lysotracker dye upon BTF3a knock down, which was found, reversed upon Baf A1 treatment confirmed through confocal microscopy (Fig. 4C & D). Also, confocal microscopy-based experiment revealed the enhanced recruitment of Rab7 in *Mtb* H37Rv containing autophagosomes (LC3B) in BTF3a knock down macrophages (Fig. 5A–C). On the basis of the results, we concluded that BTF3a knock down promotes auto-phagosomal maturation of *Mtb* containing autophagosomes through the recruitment of Rab7.

4. Discussion

Rapid increase in drug-resistant TB and comorbid conditions such as HIV infection has imposed great challenges in the field of TB therapeutics. Current strategy for managing *Mtb* pathogenesis emphasizes

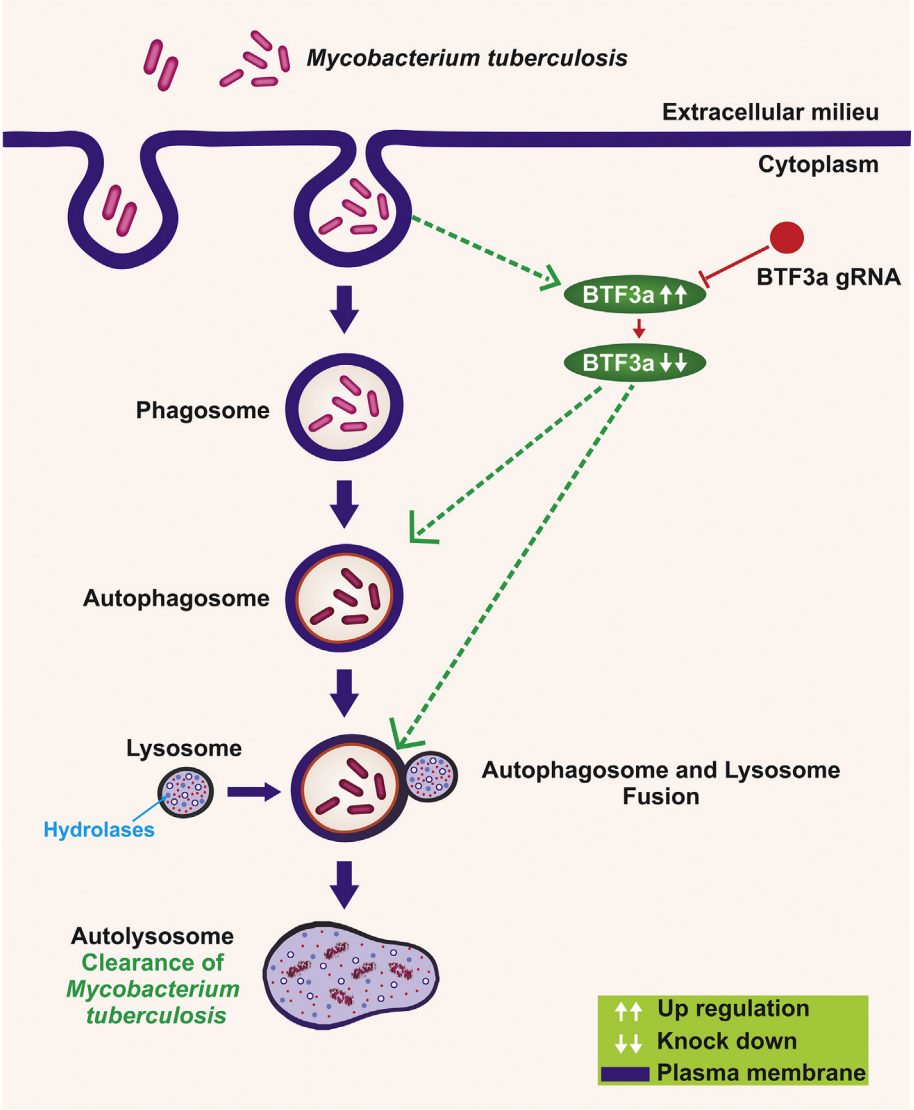

Fig. 6. Schematic representation of BTF3a knock down mediated clearance of *Mycobacterium tuberculosis* through autophagy. Infection with *Mtb* H37Rv (MOI = 1) upregulates the expression of BTF3a in THP1 derived Human macrophages. Specific gRNA mediated targeted depletion of BTF3a promotes autophagosome formation and phagolysosomal fusion of *Mtb* H37Rv containing autophagosomes leading to enhanced intracellular clearance of *Mtb* H37Rv.

mainly on targeting pathogens with specific drugs. However, despite success stories, a serious downside of this pathogen-directed strategy has been the development of drug resistance forms of the infection. Hence, there is an urgent need to identify alternative therapeutic interventions that can potentiate the host antimicrobial response or overcome host subversion by *Mtb*, which are less likely to induce drug resistance [44]. Host-directed therapy (HDT) is such an emerging approach in the field of anti-infectives that target clinically pertinent biological pathways in the host to inflect and rectify pathological immune responses, thus, making the host cell non-permissive[45].

In the past three decades, several functions of BTF3a have been studied, ranging from transcription initiation to translational regulation and apoptosis in cancer cells [9,11,13,14,21]. Currently, the role of BTF3/BTF3a in infectious diseases has been studied only till expression levels. For instance, BTF3 protein downregulation has been reported in human bronchial epithelial cell line (BEAS-2B) in presence of *Bordetella pertussis* infection [46], BTF3a upregulation was found in J774A.1 macrophages upon infection with *Mtb* LPS [31] and upregulation of BTF3 was also found in human lung A549 cells infected with human influenza virus A/PR/8/34 (H1N1) for 24 h [47]. Although these findings suggests a role for BTF3a/BTF3 in host-pathogen interactions but further validation is required. We are the first to demonstrate the role of BTF3a in regulating intracellular survival of *Mtb*. Because we had previously reported that treatment of immunostimulatory peptide, β casein fragment (54–59) exhibit significant downregulation of BTF3a and enhanced *M.bovis* BCG clearance in THP1 derived macrophages [23]. We hypothesized that the inhibition of mycobacterial growth inside macrophages is due to downregulation of BTF3a. Specific CRISPR/Cas 9 gRNA mediated knockdown of BTF3a in THP1 derived macrophages augmented the intracellular clearance of *Mtb* H37Rv (Fig. 1B). Furthermore, *Mtb* H37Rv infection resulted in upregulation of BTF3a expression in macrophages (Fig. 1C & D). On the basis of the results described above, our study demonstrates the novel regulatory role BTF3a in mycobacterial survival within macrophages, where depletion of BTF3a results in reduced survival of *Mtb* in macrophages.

The remarkable success of *Mtb* as a human pathogen results from its potentiality to circumvent the innate antimicrobial effector defense mechanisms of macrophages, its primary host and exploit the intracellular environment as a replication niche [48]. *Mtb* is an obligate intracellular pathogen that parasitizes host macrophages where it persists in immature phagosomes and arrests phago-lysosomal biogenesis [49]. Several reports suggest that *Mtb* does not impose any impact on

basal cellular autophagy, however, it only avoids the maturation of the autophagosomes occupied by the bacteria [41]. To avoid stereotypical phagosome maturation, *Mtb* selectively modulates the intracellular trafficking signaling. Evidences suggest that, the bacteria efficiently excludes sequestration of the late endosomal marker Rab 7, thereby blocking subsequent acidification and proteolytic competency of the phagosome ([49,50] and, thus, the autophagosomes containing bacteria do not accomplish the late endosomal fusion with lysosomes. The study presented here demonstrate that BTF3a knock down enhanced the autophagic flux and lysosome biogenesis in macrophages infected with *Mtb* H37Rv and leading to its intracellular clearance (Fig. S2). Elevated expression levels of LC3B-II and reduced expression of p62 indicates the positive regulation of autophagy upon BTF3a knock down (Fig. 2A), The autophagy flux was also verified in presence of Bafilomycin A1 (Fig. 3A–D). While analyzing candidate regulators of autophagy, we found that debilitating the function of BTF3a by gRNA results in a marked increase in Lamp 1 expression, a marker of late endosomes-lysosome (Fig. 2A & D). We also demonstrated that BTF3a knock down enhanced the targeting of *Mtb* H37Rv to lysosomes (Fig. 4C & D) mediated by the recruitment of Rab7 on autophagosomes containing the bacilli (Fig. 5B & C). Our results conclusively illustrate that BTF3a knock down mediated enhanced autophagic flux in conjugation with a positive regulation on lysosome biogenesis accounts for augmented *Mtb* clearance in macrophages. A schematic representation is drawn that depicts the BTF3a knock down mediated clearance of *Mycobacterium tuberculosis* clearance through modulation of autophagy and lysosome biogenesis (Fig. 6). In essence, this study highlights the anti-mycobacterial activity of BTF3a and further understanding of the role of BTF3a in microbial pathogenesis might make it a possible target for a host directed therapeutic application against TB. Since BTF3a is a host target of immunostimulatory β casein fragment peptide it may be further exploited in case of intra-macrophagic infections also.

5. Conclusion

The current study delineated a novel function of host BTF3a protein with respect to intracellular survival of *Mtb*. We demonstrated that the knockdown of BTF3a in presence of *Mtb* infection not only resulted in modulation of autophagic flux but also promoted lysosome biogenesis. Further, the contribution of BTF3a to autophagy is found to facilitate the removal of intracellular *Mtb* within macrophages. Identification of BTF3a as a novel regulatory factor in autophagy may implicate its therapeutic potential for control of *Mtb* infection.

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Conflict of interest

The author declares no conflict of interest.

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