Specific T Cells Restore the Autophagic Flux Inhibited by *Mycobacterium tuberculosis* in Human Primary Macrophages

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**Background.** Autophagy inhibits survival of intracellular *Mycobacterium tuberculosis* when induced by rapamycin or interferon γ (IFN-γ), but it remains unclear whether *M. tuberculosis* itself can induce autophagy and whether T cells play a role in *M. tuberculosis*–mediated autophagy. The aim of this study was to evaluate the impact of *M. tuberculosis* on autophagy in human primary macrophages and the role of specific T cells in this process.

**Methods.** *M. tuberculosis* (H37Rv)–infected macrophages were incubated with naive or *M. tuberculosis*–specific T cells. Autophagy was evaluated at 4 hours and 8 hours after infection by analyzing the levels of LC3-II (a hallmark of autophagy) and p62 (a protein degraded by autophagy). *M. tuberculosis* survival was evaluated by counting the colony-forming units.

**Results.** *M. tuberculosis* infection of macrophages inhibited the autophagic process at 8 hours after infection. Naive T cells could not rescue this block, whereas *M. tuberculosis*–specific T cells restored autophagy degradation, accompanied by enhanced bacterial killing. Notably, the effect of *M. tuberculosis*–specific T cells was not affected by neutralization of endogenous IFN-γ and tumor necrosis factor α and was blocked by preventing contact between macrophages and T cells, suggesting that cell-cell interaction is crucial.

**Conclusions.** *M. tuberculosis* inhibits autophagy in human primary macrophages, and specific T cells can restore functional autophagic flux through cell-cell contact.

Among humans infected with *Mycobacterium tuberculosis*, only 10% at most will eventually progress to active tuberculosis [1]. *M. tuberculosis* has evolved elaborate survival mechanisms that allow the bacterium to persist in absence of clinical manifestations, although it constantly engages with the immune system. In this state, the immune response prevents active replication but fails to eradicate the bacteria. The mechanisms for these differential outcomes to *M. tuberculosis* exposure remain unclear, although the fundamental role of the T-cell response is well established [2, 3]. *M. tuberculosis* has the capacity to prevent eradication by the host by persisting within macrophages. This is accomplished by means of a variety of immune evasion strategies that interfere with the recognition of infected macrophages by T cells and inhibition of phagosome maturation [4].

It is well-known that the degradation of intracellular mycobacteria occurs by autophagy [5], a primordial function preserved in all eukaryotic organisms. During autophagy, an isolation membrane wraps around portions of the cytoplasm to form a double-membrane organelle known as the autophagosome. The cytoplasmic material engulfed in an autophagosome is degraded after fusion with late endosomes or lysosomes. LC3 is the hallmark of autophagic activation, and the cytoplasmic form (LC3-I) is processed by site-specific
proteolysis and lipidation, which allows for its recruitment to the autophagosome membranes [6]. The autophagic activity is biochemically measured by analyzing both the increase in LC3-II level and the rate of LC3-II degradation within the lysosome [7]. Autophagy plays many physiological roles in mammals [8, 9]. Among them, autophagy functions in diverse aspects of innate and adaptive immunity that mainly involve defense against intracellular pathogens [10, 11]. Direct autophagic capture of intracellular microbes occurs with the help of a new class of innate immunity receptors in the cytoplasm, termed sequestosome (p62/SQSTM1)–like receptors (SLRs). SLRs are autophagic receptors/adapters that recognize targets that are earmarked for autophagy by ubiquitination tags and by the presence of an LC3-interacting region (LIR) [12–16]. SLRs play a role in autophagy of intracellular Salmonella species [13, 15], Shigella species [14], streptococci [13], Listeria species [16], and Sindbis virus [12]. Regarding M. tuberculosis, it has been shown that p62 also plays an indirect role in modulating autophagy [17] by harvesting seemingly innocuous cytoplasmic proteins, such as ubiquitin or ribosomal precursor proteins, and delivering them to autophagosomes, where they are digested into peptides with new biologic activities. Several studies have highlighted the role of acquired-immunity cyto- kines in the regulation of the autophagic process by showing that interferon γ (IFN-γ) produced by T-helper 1 cells induces autophagy, whereas interleukin 4 and interleukin 13, specific for the T-helper 2 cell response, inhibit the process [18].

In vitro studies involving immortalized cell lines or animal primary cells have shown that autophagy induced by drugs or starvation may play a fundamental role in clearance of mycobacteria (mainly bacille Calmette-Guérin strains) [5, 10]. However, because of the complexity of the immune response involved, the relevance of these findings to M. tuberculosis infection in humans is still unknown.

The objective of this study was to evaluate the role of autophagy in the immune response against M. tuberculosis in a cell system involving human primary macrophages infected in vitro, without use of drugs or starvation to manipulate autophagy. We also investigated the impact of naive or M. tuberculosis–specific T cells on the autophagic process.

METHODS

Macrophages

Buffy coats and peripheral blood specimens were obtained from healthy donors, who provided written informed consent. The study was approved by the ethical committee at the National Institute for Infectious Diseases (protocol n.4/2009). Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation [19]. Monocytes were purified from PBMCs by positive sorting, using anti-CD14–conjugated magnetic microbeads (Miltenyi Biotec [Auburn, CA]). By flow cytometry (FACS Canto II flow cytometer; Becton Dickinson [Milan, Italy]), >99% of selected cells were CD14+, and <1% were CD3+. Macrophage-derived monocytes were obtained by cultivating adherent monocytes for 5 days in X-Vivo 15 medium (Lonza [Walkersville, MD]), 2% human serum (Euroclone [Paignton, United Kingdom]). Cells were seeded in 24-well flat-bottom tissue culture plates (1 × 10⁶ cells/well, 1 mL/well; Corning [New York, NY]) and maintained at 37°C in 5% CO₂.

IFN-γ (at 200 IU/mL; R&D [Minneapolis, MN]) was added to the macrophages 2 hours after infection. Specific lysosomal enzyme inhibitors, E64d/PepStatinA (5 µg/mL; Sigma-Aldrich [St. Louis, MO]), were added 4 hours before protein extraction, and autophagy was evaluated 8 hours after infection in all M. tuberculosis–infected and -uninfected cells. Soluble receptors for IFN-γ and tumor necrosis factor α (TNF-α) (3 µg/mL and 0.09 µg/mL, respectively; R&D) were added to the M. tuberculosis–infected macrophages 2 hours after infection. Neutralization of endogenous cytokines was confirmed by the subsequent evaluation of cytokines in the supernatants.

Bacteria

M. tuberculosis H37Rv was grown in 7H9 medium (Difco BD, NY) supplemented with ADC (bovine albumin fraction V, dextrose, and catalase; Microbiol [Cagliari, Italy]), glycerol (0.5% vol/vol), and 0.05% Tween 80 until the late log phase. One-milliliter aliquots of culture supplemented with 10% glycerol were stored at −80°C until use. M. tuberculosis viability in these stocks was determined by plating serial dilutions in 7H11 agar (Difco, Becton Dickinson [Franklin Lakes, NJ]) supplemented with OADC (oleic acid, bovine albumin fraction V, dextrose, catalase powder, and sodium chloride; Microbiol). All mycobacteria preparations were analyzed for lipopolysaccharide contamination by the Limulus amoebocyte lysate assay (Lonza) and contained <10 pg/mL of lipopolysaccharide.

M. tuberculosis Infection of Macrophages and Colony Forming Unit

Macrophages were infected with M. tuberculosis at a multiplicity of infection (MOI) of 1:1. Two hours after infection, macrophages were washed once with phosphate-buffered saline (PBS) and then either fresh medium (see above), naive T cells, or specific T cells (described below) were added. To determine the intracellular bacterial load, colony-forming units (CFU) of M. tuberculosis–infected macrophages were measured in triplicate 8 hours after infection. Briefly, M. tuberculosis–infected cell cultures were lysed in PBS 0.1% Triton X (Sigma-Aldrich), and the serial dilution was prepared in PBS 0.05% Tween 80 (Sigma-Aldrich). Fifty-microliter aliquots of each M. tuberculosis dilution were plated on 7H11/OADC agar plates. Plates were incubated for 3 weeks.

Cocultivation of T Cells and M. tuberculosis-Infected Macrophages

In the PBMCs, the CD4/CD8 T-cell ratio evaluated by cytometry was 1.8:2.2. To induce an antigen-specific response, autologous

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PBMCs were seeded in 6-well flat-bottom tissue culture plates (3 × 10^6 cells in 3 mL of medium; Corning) and stimulated with or without purified protein derivative (PPD; at 10 µg/mL; Staten Serum Institute [Copenhagen, Denmark]). Interleukin 2 (at 5 IU/mL; R&D) was added to the PBMC culture after 72 hours to expand the antigen-specific T cells [19]. After a 5-day culture, a fraction of PBMCs was taken, washed, and then stimulated again with PPD in vitro. Median cell mortality was 30%. IFN-γ-specific response was evaluated by ELISPOT (Oxford Immunotec [Abingdon, United Kingdom]) after 20 hours of PPD-specific restimulation. PBMCs were defined as from PPD responders if the IFN-γ-specific restimulation. PBMCs were thawed 1 day before M. tuberculosis infection, washed, counted, and seeded (as described above). Median cell mortality after thawing was 25%. Naive T cells were added to the infected macrophages 2 hours after infection, in a 1:1 proportion of lymphocytes to macrophages. Two hours after infection, the specific T cells were seeded to or without purified protein derivative (PPD; at 10 µg/mL; Staten Serum Institute [Copenhagen, Denmark]). Interleukin 2 (at 5 IU/mL; R&D) was added to the PBMC culture after 72 hours to expand the antigen-specific T cells [19]. After a 5-day culture, a fraction of PBMCs was taken, washed, and then stimulated again with PPD in vitro. Median cell mortality was 30%. IFN-γ-specific response was evaluated by ELISPOT (Oxford Immunotec [Abingdon, United Kingdom]) after 20 hours of PPD-specific restimulation. PBMCs were defined as from PPD responders if the IFN-γ response to PPD was ≥10-fold higher than that in unstimulated controls. PBMCs from the PPD nonresponders were frozen after isolation from blood, whereas PBMCs from the PPD responders were stimulated as above.

PBMCs from PPD nonresponders, defined as naive T cells, were thawed 1 day before M. tuberculosis infection, washed, counted, and seeded (as described above). Median cell mortality after thawing was 25%. Naive T cells were added to the infected macrophages 2 hours after infection, in a 1:1 proportion of lymphocytes to macrophages. M. tuberculosis-specific T cells after a 5-day culture (described above) were washed twice, counted, and added to the infected-macrophages 2 hours after infection, in a 1:1 proportion of lymphocytes to macrophages.

Transwell supports (Corning, NY) were used to inhibit the contact between specific T cells and macrophages. Two hours after M. tuberculosis infection, the specific T cells were seeded inside the supports.

**Western Blot Assays**

Cells were lysed in CellLytic buffer (Sigma-Aldrich) plus protease and phosphatase inhibitors (protease inhibitor cocktail: 1 mM sodium fluoride, 1 mM sodium orthovanadate, and 1 mM sodium molybdate; 1 mM phenylmethylsulfonyl fluoride; and 1 mM phosphoinositidase C [Sigma-Aldrich]). Proteins were separated on NuPAGE Bis-Tris gel (Invitrogen [Carlsbad, CA]) and electroblotted onto PVDF membranes (Millipore [Billerica, MA]). Blots were incubated with primary antibodies in 5% nonfat dry milk in PBS plus 0.1% Tween20 (Sigma-Aldrich) overnight at 4°C. Proteins were detected using horseradish peroxidase-conjugated secondary antibody (Jackson Laboratory; [Bar Harbor, ME]) and visualized with ECL plus (GE Healthcare, [Little Chalfont, UK]).

The primary antibodies used in this study were rabbit anti-LC3 (Cell Signaling [Danvers, MA], mouse anti-p62 (Santa Cruz Biotech [Santa Cruz, CA]), and goat anti-GAPDH (Santa Cruz Biotech).

**Confocal Microscopy Analysis**

Lentivirus encoding GFP-RFP LC3 [20] were prepared as described [21]. Macrophages were infected with the GFP-RFP LC3 lentivirus 48 hours before M. tuberculosis infection. Cells were fixed with 4% paraformaldehyde in PBS for 1 hour at room temperature, deposited on a glass slide, mounted in SlowFade-Anti-Fade (Invitrogen), and examined under a confocal microscope (TCS SP2; Leica Microsystems [Wetzlar, Germany]). Digital images were acquired with Leica software. A minimum of 50 cells/sample were counted for triplicate samples per condition.

**Real-Time Polymerase Chain Reaction (PCR)**

RNA was prepared as described [21]. Real-time PCR reactions were performed with the LightCycler (Roche, [Basel, Switzerland]) and were prepared as described elsewhere [21]. p62 primer sets for amplicons were designed using the Primer Express software system (version 1.0; Applied Biosystems) and were as follows: forward: 5′-ACAGATGCCAGAATCCGAAG-3′; reverse: 5′-TG-GAGAGGGACTCAATCAG-3′. Levels of p62 messenger RNA (mRNA) were normalized to those of the ribosomal gene L34; the values are means ± SD of triplicate determinations.

**Cytokine Detection**

Supernatant from M. tuberculosis–infected macrophages cultures were collected at different time points after infection, and cytokines were evaluated by a cytometric bead array (BD Biosciences [San Jose, CA]).

**Statistical Analysis**

Data were analyzed by the GraphPad Prism software, version 4.00 for Windows (GraphPad Software [San Diego, CA]). Means and SDs were calculated. The statistical significance of the differences between 2 groups was determined using the t test. Differences were considered significant if P values were ≤.05.

**RESULTS**

M. tuberculosis Induces Autophagy in Human Macrophages at Early Time Points After In Vitro Infection and Subsequently Blocks It

Autophagy levels in human primary macrophages infected in vitro with M. tuberculosis were analyzed at different time points after infection by means of immunoblotting and confocal microscopy. M. tuberculosis–infected macrophages showed higher levels of LC3-II, the lipoidated isoform of LC3 that specifically associates with autophagosome membranes [7], compared with uninfected cells, at both 4 hours and 8 hours after infection (Figure 1A). An increased number of autophagosomes was confirmed by analyzing the number of LC3-positive vesicles in M. tuberculosis–infected macrophages expressing a fluorescent LC3 reporter (Figure 1B and 1C).

To evaluate the occurrence of lysosome-dependent autophagic degradation (autophagic flux), we studied p62 level modulation in parallel. p62 was downregulated at 4 hours after infection, indicating effective stimulation of autophagy, whereas
it dramatically increased at 8 hours after infection, suggesting impairment of autophagy-dependent degradative activity (Figure 1A).

Autophagic flux in M. tuberculosis–infected macrophages was also monitored by taking advantage of the double-tagged GFP/RFP-LC3 reporter (Figure 1B), which allows for discrimination between autophagosomes (GFP-positive and RFP-positive dots) and autophagolysosomes (only RFP-positive dots) owing to the inactivation of GFP signals in acidic compartments. While a higher number of autophagolysosomes were detected at 4 hours after infection, compared with uninfected controls, their number significantly decreased at 8 hours ($P = .0006$), indicating impairment of autophagosome maturation by M. tuberculosis (Figure 1C).

**Figure 1.** Mycobacterium tuberculosis induces autophagy in human macrophages at early time points after in vitro infection and subsequently blocks it. **A,** Representative example of 3 Western blot analyses of the levels of the autophagic markers LC3-II and p62 in human macrophage cell cultures performed 4 hours or 8 hours after M. tuberculosis infection. GAPDH was used as a loading control. In vitro infection of macrophages with M. tuberculosis increases the LC3-II level, compared with uninfected control, 4 hours and 8 hours after infection. In contrast, p62 was downregulated 4 hours after in vitro infection, indicating a positive autophagic process, whereas it was dramatically increased 8 hours after infection. **B,** Macrophages were transduced with a lentivirus encoding GFP-RFP-LC3 48 hours before M. tuberculosis infection. The number of autophagosomes and autophagolysosomes was examined by analyzing GFP-RFP-LC3 fluorescence in human macrophage cell cultures 4 hours or 8 hours after M. tuberculosis infection. Autophagosomes show both GFP and RFP signals. Autophagosome-lysosome fusion results in RFP-positive, GFP-negative dots because of the inhibition of GFP fluorescence in the acidic compartment. Scale bar: 10 μm. **C,** Quantification of results described in panel B. D, Results of the real-time polymerase chain reaction from 3 different experiments to determine p62 RNA level, whose representative example is panel A. E, Densitometric analysis of 3 Western blotting experiments, whose representative examples are presented in panel A. The band density ratio of p62 levels relative to GAPDH levels is reported on the graph. The ratio of p62 to GAPDH in uninfected and infected cells was set to 1. Data are reported as means ± SD. A t test was performed to compare means. Abbreviations: Ctr, control; mRNA, messenger RNA.
To better evaluate the modulation of p62, mRNA and its protein levels were evaluated over time. We observed a transcriptional modulation of p62 mRNA after in vitro *M. tuberculosis* infection, both 4 hours and 8 hours after infection (Figure 1D); in contrast, the levels of p62 protein decreased 4 hours after infection, while it increased 8 hours after infection, with a fold-induction significantly higher than that observed at the RNA level (Figure 1E). The different modulation of p62 mRNA, compared with protein, suggests that autophagy is an active process 4 hours after infections, while there is flux impairment 8 hours after infection.

Moreover, we confirmed that the increase of LC3-II in *M. tuberculosis*-infected macrophages 8 hours after infection (Figure 1A) was due to blockage of the autophagosome-lysosome fusion rather than to induction of the autophagic process, since p62 in these cells was not degraded. Lysosomal enzyme inhibitors increased LC3-II and p62 levels in uninfected controls, indicating that both proteins are degraded within the lysosomes by autophagy (Figure 2A–C). Conversely, in *M. tuberculosis*-infected macrophages, inhibition of lysosomal activity did not lead to increased LC3 and p62 levels, compared with untreated cells, indicating that the autophagic flux was already inhibited by *M. tuberculosis* (Figure 2A–C).

Together, these results suggest that, in human macrophages, *M. tuberculosis* induces the autophagic process immediately after infection but that, at later time points, this process is blocked by inhibiting the autophagosome-lysosome fusion.

### M. tuberculosis-Specific T Cells Positively Modulate the Autophagic Process in M. tuberculosis–Infected Macrophages

Autophagy is important in the innate and adaptive immune responses mainly involved in defending from intracellular pathogens [11, 22]. These findings and the well-characterized role of IFN-γ in the protective immunity against tuberculosis [23] led us to investigate the ability of *M. tuberculosis*-specific T cells to modulate the autophagic process in *M. tuberculosis*-infected human macrophages. As shown in Figure 3A and 3E, the addition of naive T cells to infected macrophages did not modulate LC3-II and p62 levels, compared with infected macrophages. Conversely, the exogenous addition of IFN-γ to the *M. tuberculosis*-infected macrophages, which were used as positive controls for the modulation of autophagy [5], resulted in higher levels of LC3-II and reduced levels of p62, compared with controls (Figure 3B and 3E). Interestingly, the addition of *M. tuberculosis*-specific T cells to the *M. tuberculosis*-infected macrophages (Figure 3C and 3E) resulted in a significant reduction of p62 levels (*P* = .04) but did not impact LC3-II levels, indicating enhancement of the autophagic flux. To confirm that specific T cells promote the autophagic flux, lysosomal protease inhibitors were added to the coculture of specific T cell and *M. tuberculosis*-infected macrophages (Figure 3D and 3F), and indeed p62 levels were restored, indicating that specific T-cell degradation of p62 was mediated by the induction of autophagy. These results highlight the role of *M. tuberculosis*-specific T cells in positively modulating the autophagic process and, consequently, preventing *M. tuberculosis* from blocking it.
Endogenous IFN-γ and TNF-α Are Not Involved in Modulating the Autophagic Process by Specific T Cells in M. tuberculosis–Infected Macrophages

Infection with M. tuberculosis triggers secretion of proinflammatory cytokines such as TNF-α and IFN-γ [24, 25], which are 2 important modulators of autophagy [5, 26]. Indeed, both TNF-α and IFN-γ were induced following infection with M. tuberculosis in our model, and this effect was enhanced by the addition of T cells (Figure 4). To analyze whether the positive modulation of the autophagic process in M. tuberculosis–infected macrophages was dependent on the endogenous production of cytokines, we measured autophagy levels following neutralization of IFN-γ and TNF-α (Figure 5A and 5B). While the addition of specific T cells to M. tuberculosis–infected macrophages increased the autophagic flux, as indicated by the reduction of p62 level, neutralization of IFN-γ and TNF-α did not prevent autophagy induction. These results suggest that specific T cells positively modulate autophagy independent of IFN-γ- and TNF-α.

Figure 4. Interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α) are induced by Mycobacterium tuberculosis in macrophages and T-cell cultures. IFN-γ and TNF-α levels were measured in macrophages and T-cell cultures 1 and 3 days after in vitro M. tuberculosis infection. Data are reported as means ± SD, and the experiments were repeated 3 times.
Cell Interaction Is Required for Positive Modulation of the Autophagic Flux by Specific T Cells in \textit{M. tuberculosis}–Infected Macrophages

Several studies have shown that cell contact is important in triggering the autophagic process and, consequently, inducing clearance of pathogens, such as \textit{Toxoplasma} species, in macrophages [27]. This prompted us to investigate whether cell interaction was involved in the induction of autophagy. As shown in Figure 6A and 6B, prevention of cell contact between T cells and \textit{M. tuberculosis}–infected macrophages by means of a transwell system abolished p62 degradation, clearly indicating that cell interaction is required to positively modulate autophagy.

T-Cell–Induced Autophagy Enhances the Antimycobacterial Activity in Macrophages

Finally, we investigated whether there was an association between autophagy modulation and \textit{M. tuberculosis} survival. In all experiments, the intracellular amount of CFU at time zero was assessed. No modulation of intracellular survival at time zero and 8 hours after infection was observed, suggesting that all wells were infected with the same bacterial concentration (data not shown). The results are reported as CFU per million cells (Figure 7A) and normalized and expressed as the percentage of \textit{M. tuberculosis} that survived in macrophages (Figure 7B). Addition of \textit{M. tuberculosis}–specific T cells significantly limited intracellular survival of \textit{M. tuberculosis} (\(P = .02\)), similar to the extent observed following the addition of IFN-\(\gamma\) (\(P = .01\)). Conversely, we did not observe any reduction of intracellular \textit{M. tuberculosis} when naive T cells were added. Interestingly, the effect of specific T cells on mycobacterial...

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\caption{Interferon \(\gamma\) (IFN-\(\gamma\)) and tumor necrosis factor \(\alpha\) (TNF-\(\alpha\)) are not involved in positive modulation of the autophagic process in \textit{Mycobacterium tuberculosis}–infected macrophages. \textbf{A}, Representative example of 4 Western blot analyses of LC3-II and p62 in human macrophage cell cultures 8 hours after \textit{M. tuberculosis} infection, in the presence or absence of neutralizing soluble receptors (sRec) for IFN-\(\gamma\) and TNF-\(\alpha\). GAPDH was used as loading control. \textbf{B}, Densitometric analysis of the Western blotting experiments whose representative examples are presented in panels \textbf{A} and \textbf{B}, and the density ratios of LC3-II (black bar) and p62 (white bar) relative to GAPDH are reported on the graphs. LC3II/GAPDH and p62/GAPDH ratio in infected cells was set to 1. A \(t\) test was performed for pairwise comparisons of means. Data are reported as means \(\pm\) SD.}
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\caption{Cell interaction is involved in positive modulation of the autophagic flux in \textit{Mycobacterium tuberculosis}–infected macrophages. \textbf{A}, Representative example of 4 Western blot analyses of LC3-II and p62 was performed in human macrophage cell cultures 8 hours after \textit{M. tuberculosis} infection, in the presence or absence of a transwell membrane that prevented cell contact between macrophages and T cells. GAPDH was used as loading control. \textbf{B}, Densitometric analysis of 3 Western blotting experiments whose representative experiment is reported in \(\textbf{A}\). The band density ratio of LC3-II (black bar) and of p62 (white bar) relative to GAPDH is reported on the graph. LC3II/GAPDH and p62/GAPDH ratio in infected cells was set to 1. A \(t\) test was performed to compare means for pairwise comparisons. Data are reported as means \(\pm\) SD.}
\end{figure}
Figure 7. Modulation of specific T cells on the intracellular survival of Mycobacterium tuberculosis is associated to the autophagic process. Enumerations of colony-forming unites (CFU) was performed 8 hours after in vitro infection of human macrophages with M. tuberculosis. Infected macrophages were incubated with interferon γ (IFN-γ), naive T cells, or specific T cells 2 hours after M. tuberculosis infection. CFU were also analyzed in human macrophage cultures cocultivated with specific T cells in the presence of transwell membrane that prevented cell contact between macrophages and T cells, or in the presence of E64d/Pep 5 μg/mL, added 4 hours before the CFU assay, to block lysosomal degradation. A, Triplicates of each condition for at least 3 independent experiments are reported and expressed as CFU/million cells. B, The results are normalized and expressed as percentage of M. tuberculosis surviving in macrophages. In all experiments, the intracellular amount of CFU at time zero was assessed to make sure that all wells were
survival was prevented when inhibitors of lysosomal activity were added or when cell-to-cell contact was inhibited. These results suggest that there is a direct association between modulation of *M. tuberculosis*–specific T cells on the intracellular survival of *M. tuberculosis* and autophagic flux.

**DISCUSSION**

In this study, we implemented an in vitro system involving primary human macrophages to investigate the role of autophagy during infection with fully virulent *M. tuberculosis*. To our knowledge, this is the first study to demonstrate that *M. tuberculosis* induces early postinfection autophagy in human primary macrophages and blocks the autophagic flux later on. Moreover, we showed that autologous *M. tuberculosis*–specific T cells restore a functional autophagic process through cell contact, independent of endogenous cytokine secretion. Finally, we showed that autophagy induction by *M. tuberculosis*–specific T cells inversely correlates with bacterial survival. Together, these data help elucidate the potential mechanisms underlining the interaction among macrophages, *M. tuberculosis*, and T cells in the human cell system. Moreover, we developed an in vitro experimental model involving multiple immunological players directly isolated from human subjects that, at least in part, mimics the pathophysiology of tuberculosis, which could be very useful for addressing scientific questions regarding *M. tuberculosis*–induced autophagy.

*M. tuberculosis* can persist in the host mainly as a result of its ability to parasitize host macrophages [4], where it resides in phagosomes that do not acquire phagolysosomal properties, such as luminal acidification and lysosomal hydrolases [28]. The autophagic process may induce degradation of intracellular mycobacteria by promoting their transfer into degradative autolysosomal organelles [5]. The engulfed cytoplasmic material present in an autophagosome is degraded after fusion with late endosomes or lysosomes. Here, we showed that *M. tuberculosis* is able to induce autophagy early after infection, although *M. tuberculosis* subsequently hampers the autophagic process by blocking autophagosome maturation. These results are of relevance because they suggest that *M. tuberculosis* senses the harsh macrophage–associated intracellular environment immediately after infection and regulates the expression of a set of genes to actively interfere with the autophagic process and evade macrophage killing.

An inhibitory effect of *M. tuberculosis* in late stages of autophagy was not previously reported in studies involving in vitro–infected macrophages. We think that the specific settings of our experimental model may explain the differences we observed. First, we used human primary cells and the pathogenic strain of *M. tuberculosis* in the same cell system, while most studies were performed using bacille Calmette-Guérin strains [5, 10, 17, 29] and/or immortalized macrophage cell lines [5, 30] or mouse primary cells [10]. Second, we evaluated autophagy levels by introducing autologous T cells as potential physiological modulators of this process, rather than by inducing it by exogenous manipulation via pharmacological compounds or starvation [5, 10, 17, 29]. Third, we evaluated autophagy by concurrently analyzing 2 parameters, LC3-II and p62 levels, which allowed us to evaluate autophagy as a dynamic flux. Together, these experimental conditions may have helped to uncover antiautophagic activities of *M. tuberculosis* that like occur during actual infection of humans.

Notably, autophagy inhibition by *M. tuberculosis* has been recently proposed by Kumar et al. [30] on the basis of their high-throughput screening aimed at identifying host genes able to modulate *M. tuberculosis* replication.

With regard to the impact of T cells on autophagy, we showed that specific T cells may overcome the ability of *M. tuberculosis* to block autophagosome maturation in macrophages by enhancing autophagic flux. Lymphocyte-dependent inhibition of *M. tuberculosis* growth was previously reported in a study showing that CD4+ T cells from PPD responders are required to inhibit intracellular bacterial replication in human monocytes 4 and 7 days after infection [31]. In contrast to our study, Silver and collaborators [31] did not observe any effect of these T cells on intracellular *M. tuberculosis* replication a few hours after infection, although they used monocytes rather than fully differentiated human macrophages.

Notably, we also found that, although IFN-γ and TNF-α were induced by *M. tuberculosis*, neutralization of their activity by soluble receptors or neutralizing antibodies (data not shown) did not overcome the proautophagic effect induced by the *M. tuberculosis*–specific T cells and the related antimycobacterial activity associated with it. These results are in agreement with recent findings showing that there are pathways independent of IFN-γ and TNF-α that efficiently control *M. tuberculosis* replication. It has been postulated that identification of these alternative effector functions may provide new therapeutic avenues to combat *M. tuberculosis* through vaccination [32].

Moreover, it has recently been shown that the mutation rate of *M. tuberculosis* during latency is not less than the rate during active tuberculosis [2, 3]. Therefore, on the basis of this assumption, control of *M. tuberculosis* may be mediated by engagement of specific T cells in cell-cell contact with
M. tuberculosis–infected macrophages and by induction of the autophagic process. We also demonstrated that interaction between specific T cells and macrophages is required for positive autophagy modulation and decreasing bacterial survival. Interestingly, in other experimental infections, such as Toxoplasma gondii infection [27, 33], the role of plasma membrane-associated CD40/CD40L factors and decreasing bacterial survival. Interestingly, in other experimental infections, such as Toxoplasma gondii infection [27, 33], the role of plasma membrane-associated CD40/CD40L factors and decreasing bacterial survival. Interestingly, in other experimental infections, such as Toxoplasma gondii infection [27, 33], the role of plasma membrane-associated CD40/CD40L factors and decreasing bacterial survival.

In conclusion, our results underline the importance of studying the relationship between the autophagic process and M. tuberculosis in a more “physiological” experimental system. By analyzing primary human macrophages, we demonstrated that M. tuberculosis per se inhibits the autophagic flux and that autologous specific T cells may restore functional autophagic flux in M. tuberculosis–infected cells, where cell–cell contact is essential. These findings may help improve understanding about the pathogenesis of M. tuberculosis infection and lead to development of efficacious vaccines.

Notes
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