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Autophagy, an immunologic magic bullet: *Mycobacterium tuberculosis* phagosome maturation block and how to bypass it

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Abstract

Mycobacterium tuberculosis is a facultative intracellular pathogen that parasitizes host macrophages where it persists in immature phagosomes by avoiding their maturation into phagolysosomes. The mechanisms of how *M. tuberculosis* inhibits phagolysosome biogenesis have been researched in detail and the maturation block at least partially depends on the manipulation of host phosphoinositide interconversions, with phosphatidylinositol 3-phosphate (PI3P) being a central target since it has been shown to be required for phagolysosome biogenesis. PI3P earmarks intracellular organelles for binding and assembly of effector molecules that interact with PI3P or its derivatives, including Class E Vps proteins such as Hrs and ESCRT components, early endosome antigen 1, which are required for sequential protein and membrane sorting within the endosomal and, by extension, phagosomal systems. In a search of a cellular mechanism that can bypass the tubercule bacillus-imposed PI3P block, researchers have uncovered a new general bactericidal process, autophagy, which can eliminate intracellular pathogens. This is a new, rapidly growing field replete with possibilities for novel, previously untried immunologic and pharmacologic interventions applicable not only to TB but to other stubborn bacterial, parasitic and viral diseases.

Keywords

autophagy; macrophage; phagosome; phosphoinositide; Rab; tuberculosis

Fundamental principles of phagosomal organelle maturation

To understand the *Mycobacterium tuberculosis* phagosome maturation block, one needs an advanced knowledge of mammalian cell biology, basic principles of organelle biogenesis and the control of membrane and protein trafficking in the host cells. *M. tuberculosis* interferes with small GTPases, termed Rabs, which control nearly every aspect of intracellular trafficking in mammalian cells. Mycobacterial manipulation of Rab recruitment and function has a cascade of downstream effects, involving Rab-interacting partners and effectors, such as phosphatidylinositol-3-kinase (PI3K), which modifies membrane-associated lipids, specifically phosphatidylinositol (PI), by phosphorylating PI into phosphatidylinositol-3-phosphate (PI3P) **(Figure 1)**, with PI3P serving as a tag for the

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recruitment of factors that execute conversion of phagosomal organelles into the phagolysosome.

The Rab family of GTPases consists of nearly 70 members in humans [1]. Individual Rabs, or sets of Rabs, specialize in regulating intracellular trafficking in, out and within a given intracellular compartment. Rabs and their effectors accomplish this by organizing other proteins and lipids to either maintain a given state of an organelle, or lead to its transformation into a different compartment. Rabs and Rab-interacting proteins, including organelle-bridging/membrane-tethering molecules, such as early endosome antigen (EEA)1, molecular motors that move organelles and trafficking intermediates along the cytoskeletal elements and lipid modifying enzymes (kinases and phosphatases), all play a role in maintaining or changing organellar identity and control the progression of cargo along an intracellular pathway. As examples of these activities, Rab4, -11 and -22a control recycling of components (e.g., receptors and membrane) back from early endosomes to the plasma membrane. A wholesale replacement of one Rab (e.g., early endosomal Rab5) with another (late endosomal Rab7) on an entire organelle, through a process termed Rab conversion, is observed in both endosomal [2] and phagosomal systems [3,4] and leads to transformation of an early endosomal or phagosomal organelle to a lysosomal, degradative compartment – often referred to as organellar maturation. Rabs primarily function as molecular switches and are considered to be active while loaded with GTP, becoming inactive when GTP becomes hydrolyzed into GDP, which follows stimulation of Rab GTPase activity by a cognate GTPase-activating protein. Consequently, a pivotal regulatory event in membrane trafficking is the loading of Rab with GTP, and this is catalyzed by a guanine nucleotide exchange factor, with an event termination mediated by GTP hydrolysis. Another essential feature of Rabs is their recruitment to membranes, where they anchor themselves by insertion of their doubly-prenylated lipid tails into the membrane bilayer [1]. Thus, localization to a specific organelle and the GTP–GDP-bound states of a Rab are its defining characteristics.

An important mechanism by which Rabs prepare membranes for fusion is their interactions with PI kinases (such as hVPS34 PI3K) and phosphatases. For example, as Rab5 recruits hVPS34, this lipid kinase converts PI into PI3P on the cytosol-facing leaflet of the endosomal membrane. The membrane areas earmarked by PI3P recruit proteins through their PI3P-binding domains, which act as organellar tethering factors (e.g., EEA1) or regulators of protein and membrane sorting (e.g., Hrs). It turns out that Rabs, their interacting partners and phosphoinositide interconversions – in particular PI3P generation – are targets of mycobacterial factors that block *M. tuberculosis* phagosomal maturation [5].

M. tuberculosis interferes with Rab organization & function on phagosomal organelles

The *M. tuberculosis* phagosome does not acidify properly [6] because it does not acquire the late endosomal Rab, Rab7 **(Figure 1)** [3], which brings about recruitment of the vacuolar proton ATPase, which is responsible for phagosomal acidification. It has been shown that Rab7 acquisition by phagosomes is similar to a critical point in the endosomal pathway, recently termed ‘Rab conversion’ [7]. In the process of Rab conversion (in our case, specifically Rab5 to Rab7) the key early endosomal Rab, Rab5, is simultaneously substituted by Rab7 on an entire early endosomal organelle. The Rab5 to Rab7 conversion ushers the transformation of an early endosomal or early phagosomal organelle into a late endosome or phagolysosome, with degradative properties. A recent study demonstrated that Rab22a plays a pivotal role in this process [4]. *M. tuberculosis* recruits copious amounts of Rab22a to the phagosome. Rab22a controls the terminal stages of endosomal recycling and, upon recycling of the necessary reusable components, its change in activation status sends

an 'all clear' signal to the trafficking machinery, allowing Rab5 to Rab7 conversion to proceed. The recruitment to, and maintenance of, Rab22a on *M. tuberculosis* phagosomes blocks Rab conversion, thus prohibiting acquisition of Rab7 and blocking subsequent acidification and proteolytic competency of a phagosome. The altered Rab complement on mycobacterial phagosomes is reflected in aberrant acquisition of Rab effectors, including membrane tethering molecules such as EEA1 [8] and the multivesicular body endosome sorting regulator Hrs [9]. These changes have been shown to play a functional role in mycobacterial phagosome maturation block.

A block in Rab conversion may result in an erroneous, static view of the *M. tuberculosis* phagosome. In fact, the *M. tuberculosis* phagosome receives nutrients such as iron-loaded transferrin and membrane from early endosomal organelles [10]. This is an important feature of *M. tuberculosis* phagosomes, as Rab5, with a defining role in endocytosis and early endosomal trafficking, supports intracellular mycobacterial survival [11]. Recently, another Rab, Rab14, has been shown to promote *M. tuberculosis* phagosome fusion with early endosomes [12], and thus acts in tandem with Rab5. Rab5 is involved during the early stages, while Rab14 is important for long-term interactions of the mycobacterial phagosome with early endosomal organelles. Rab14 is necessary for proper viability of intraphagosomal mycobacteria. Interestingly, it also causes phagosome–phagosome homotypic fusion [12], a previously unappreciated phenomenon that may play a role in the intracellular growth of *M. tuberculosis*.

M. tuberculosis interference with PI3P production

PI3P is important for maturation into a late endosomal compartment, and is required for phagosome maturation [8,13]. Mycobacteria suppress PI3P production on their phagosomes [8,14,15]. Normally, PI3P is generated on endomembranes in mammalian cells by an endocytic Rab effector, the type III PI3K hVPS34. Mycobacteria interfere with PI3P production **(Figure 1)** as follows:

- *M. tuberculosis* inhibits Ca^{2+} flux in infected macrophages [16,17];
- Lack of an increase in cytosolic Ca^{2+} inhibits calmodulin (CaM) and CaM kinase II recruitment to mycobacetrial phagosomes [18];
- Since CaM complexes with hVPS34 [19], interruption of Ca^{2+}–CaM–hVPS34 assembly, coupled with the inhibition of Rab conversion and exclusion of Rab7 – also an hVPS34-interacting Rab [20] – reduces PI3P production on *M. tuberculosis* phagosomes.

Mycobacterial products that interfere with phagolysosome biogenesis

A number of random genetic screens have yielded nonoverlapping sets of genes **(Figure 1)** [21–23]. Their further characterization will be necessary to assign putative functions. Complementary to these shotgun approaches, a rational search for lipid and protein factors has yielded several lipids [24,25] and proteins [15,26] that have been shown or have been proposed to play a role in mycobacterial phagosome maturation arrest.

Mycobacteria produce copious amounts of complex lipids, accounting for 60% of the cell wall biomass [27]. Many of them, including the glycoconjugates PI mannoside (PIM) and lipoarabinomannan (LAM) **(Figure 1)**, are both anchored in the lipid bilayer of the bacterial plasma membrane and are also shed. PIM and LAM appear to be mimics of host-cell phosphoinositides, and have been investigated for their interference potential in the context of host cell phosphoinositide interconversions (e.g., PI3P generation). LAM interacts with the mannose receptor and this plays a role in phagolysosome maturation block [25].

Following gathering and local concentration by mannose receptors, LAM intercalates into the membrane [28] and traffics within the cellular endomembranes [29]. The intercalated LAM inhibits intracellular Ca^{2+} flux preventing Ca^{2+}-dependent recruitment of hVPS34, thus blocking initial PI3P production on mycobacterial phagosomes [19] and interfering with key processes in phagolysosome biogenesis [5]. The less extensively glycosylated LAM precursor, PIM, plays a different role in phagosome maturation arrest. It promotes fusion between mycobace-trial phagosomes and early endosomes [30]. A secreted *M. tuberculosis* protein, SapM, which has been originally described as an acid phosphatase [31], has strong PI3P phosphatase activity, and plays a role in keeping the mycobacterial phagosome PI3P-free and thus plays a role in long-term phagosomal maturation block [15].

It should be noted that an eventual delivery of mycobacteria to the phagolysosome does not ensure their elimination; classical experiments performed by D'Arcy Hart and colleagues demonstrated that antibody-opsonized myco-bacteria end up in phagolysosomes, but survive this environment in resting macrophages [32]. In the same study, Armstrong and Hart commented on the possibility that phagolysosomal contents may be different in activated macrophages, which brings us to the phenomenon of autolysosomes described below. Prior to that, however, it is important to mention two additional phenomena – recently brought to the fore – related to the *M. tuberculosis* phagosome, which have identified mycobacterial factors as causing or contributing to the effects.

In addition to mycobacteria surviving in phagosomes (or ineffective phagolysosomes, as described above), a recent report demonstrates that after long incubation times in tissue culture, *M. tuberculosis* (but not the vaccine strain BCG) escapes into the cytosol in dendritic cells (and possibly in macrophages) where it can apparently replicate robustly [33]. Furthermore, van der Wel *et al.*, have shown that this process requires ESAT6 and CFP10 [33], a mysterious set of proteins that are not produced by BCG owing to a genomic deletion of the RD1 locus. Of particular importance is the so-called 'escape' of mycobacteria into the cytosol, which has been a controversial topic, with such claims stemming all the way back to the early work by Myrvik *et al.*, who reported disruption of phagosomal membranes in rabbit alveolar macrophages infected with *M. tuberculosis* H37Rv [34]. In patients with active disease, *M. tuberculosis* escapes from cells within necrotic lesions, and thus escapes not only from the phagosome but also from the macrophage. It is perhaps this stage of infection progression that is modeled in the studies by van der Wel and others, as tissue culture cells grown for prolonged periods may lose their viability and undergo cell death, possibly in a necrotic fashion.

Recruitment of coronin 1 to mycobacterial phagosomes, prominently seen with large myco-bacterial clumps (a natural tendency with *M. tuberculosis* and thus of likely pathophysiological importance) [35], appears to be exacted by the mycobacterial lipoamide dehydrogenase possibly by partitioning into cholesterol-rich domains of the membrane [36]. The status of mycobacterial clumps, explicitly whether they do [37] or do not [35] mature into phagolysosomes, is controversial and remains to be settled. Nevertheless, the recent progress in identifying the mycobacterial component that interacts with coronin 1, and the demonstration that it may be a target for processes including autophagy, induced by the p47 GTPase, specifically LRG-47 or Irgm1 in murine cells (with IRGM being the equivalent in human cells; see subsequent sections), lends interest to these observations [36].

Autophagy: a process that can bypass *M. tuberculosis* phagosome maturation block & eliminate intracellular mycobacteria

Another host cell membrane-trafficking process dependent on PI3P is autophagy **(Figure 2)**. Autophagy is a homeostatic cellular maintenance mechanism [38,39], whereby cells digest

parts of their own cytoplasm for the turnover of stable macromolecules or, more importantly for the topic of this review, for the removal of unwanted organelles and macromolecular aggregates in the cytoplasm. In every cell of the body, autophagosomal membranes can corral portions of the cytoplasm and sequester it into autophagosomes, creating morphologically distinct organelles that briefly exist as closed double membranes that wrap around the intended cytoplasmic target. An autophagosome delivers the captured cytoplasmic content to lysosomes for degradation, reuse or elimination. Physiologically, when under starvation conditions, which can occur either by withdrawal of nutrients or by absence of growth factors even when the nutrients are present, autophagy is primarily aimed at recycling long-lived cytosolic macromolecules, such as stable proteins, to support cellular anabolic demands; at other times, autophagy is used to simply trim surplus organelles (e.g., overproliferated endoplasmic reticulum), thus ensuring cellular viability [40]. Autophagy can remove spuriously damaged organelles (including mitochondria), which have accidentally compromised membranes, thus protecting cells from unscheduled apoptosis. Accordingly, autophagy, when induced in moderation, is primarily a cell-survival mechanism, however, chronic activation at high levels can also lead to apoptosis [41] referred to as programmed cell death type II. When autophagy causes cell death, a link between autophagy and apoptosis also becomes apparent [42]. With such a disparate role in controlling cell health, biomass and intracellular organellar integrity, it is not surprising that autophagy has been implicated in cancer, development, aging/longevity, and neurodegenerative diseases (e.g., Huntington's, Parkinson's and Alzheimer's disease) and myodegenerative disorders [38,43]. Of particular significance, autophagy has been previously overlooked or unappreciated as an immune effector against intracellular bacteria and viruses [44–47]. The capture and distruction of intracytoplasmic contents by autophagy appears to be ‘just what the doctor ordered’, and is ideally suited for the removal and destruction of intracellular pathogens. Not just any autophagic organelle would do, however, as the formation of an effective autolysosome is necessary, including appropriately delivered bactericidal ingredients [48]. This may explain why, in a report of a cholesterol-depletion-driven *Myco-bacterium avium* delivery to an organelle, which morphologically appeared as autophagic, the authors could not observe killing of this micro-organism [49]. Furthermore, a handful of highly adapted pathogens have specialized systems to escape autophagic elimination [50] and, thus, the pathways necessary for the assembly of effective autophagolysosomes need to be dissected to allow for targeted drug design.

The execution stages of autophagy **(Figure 2)** are controlled by upstream signal transduction systems feeding various nutritional and other signals to the central gatekeeper, target of rapamycin (Tor). A classical physiological inducer of autophagy via Tor is amino acid starvation, in particular the withdrawal of branched aliphatic side-chain amino acids or absence of growth factors that normally regulate the uptake of nutrients [51]. A pharmacologic induction of autophagy can be exacted using rapamycin, which targets the Ser/Thr kinase Tor [52]. An active Tor suppresses autophagy, but when Tor is inhibited (e.g., in cells treated with rapamycin) this activates the execution stages of autophagy. Importantly, PI3P hVPS34 and the PI3K involved in phagosome maturation complexed with an autophagy-specific subunit termed Beclin 1, are essential for autophagy [53]. Owing to the PI3P and hVPS34 connection, researchers have tested whether induction of autophagy can overcome *M. tuberculosis* phagosome maturation arrest. This has now been proven to be the case [46,54]; physiologic, pharmacologic or immunologic stimulants of autophagy can activate mycobacterial phagosome maturation, and they can also kill *M. tuberculosis* and diminish its viability and colony counts within the course of several hours. The most recent finding in this area is that the immunity-related GTPases (IRGs; also known as p47 GTPases), which have been implicated in the control of intracellular pathogens but whose mechanism of action remained elusive, are now known to induce autophagy [54,55]. The majority of the initial studies with IRGs have focused on work in mice, where there are over

20 genes encoding distinct IRG products. The sole human *IRG* gene (*IRGM*) has only recently been shown to play a role in defense against *M. tuberculosis* via autophagy [54]. Within months, it was also revealed [56] that *IRGM*, along with another autophagy gene *ATG16L1* [57,58], are susceptibility loci for Crohn's disease, a form of inflammatory bowel disease potentially linked to mycobacterial infection. Thus, the IRGs, orphaned in terms of function for a long time, finally have a role – in autophagy.

Conclusion

A steady stream of independent publications have confirmed the role of autophagy in innate immunity [44–46,49,59–61]. Autophagy eliminates intracellular microbes in a similar manner to autophagic capture and results in the digestion of unwanted or damaged intracellular organelles. It is now well established that autophagy represents a previously unappreciated mechanism that is efficient in the removal of intracellular bacteria and viruses [62,63], in keeping with its primary function as a cytoplasmic housekeeping service. Now, researchers must learn how to manipulate this built-in system for the removal of unwanted guests that invade host cells. It is almost certain that there will be many pharmacologic and immunologic advances in this area; holding strong promise for the discovery of methods to control and eliminate the hardiest of microbes.

Future perspective

The future perspective on utilizing autophagy against *M. tuberculosis* and other intracellular pathogens including bacteria, viruses and protozoans, is exciting and clear. The discovery of autophagy as an immune mechanism for intracellular pathogen elimination opens completely new vistas and opportunities for the development of antimicrobials and for dealing with the most recalcitrant pathogens. It is likely that the pharmaceutical industry will start to invest more heavily in this area and we can expect to see the development of a completely new class of antimicrobials. New drug development programs aimed at autophagy are underway [64], while classical activators or inhibitors of autophagy are already in clinical studies to treat cancer and neurodegenerative diseases – where autophagy also plays a role. Drugs that induce autophagy to treat hardy intracellular microbes and modulate inflammatory responses may be next.

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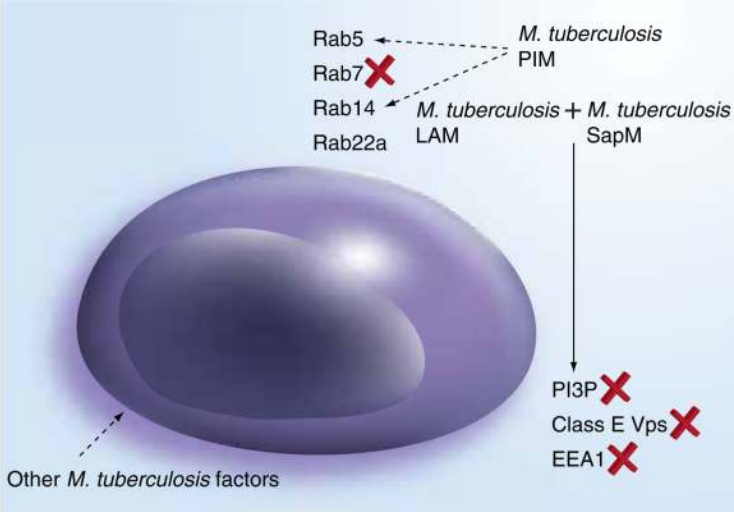
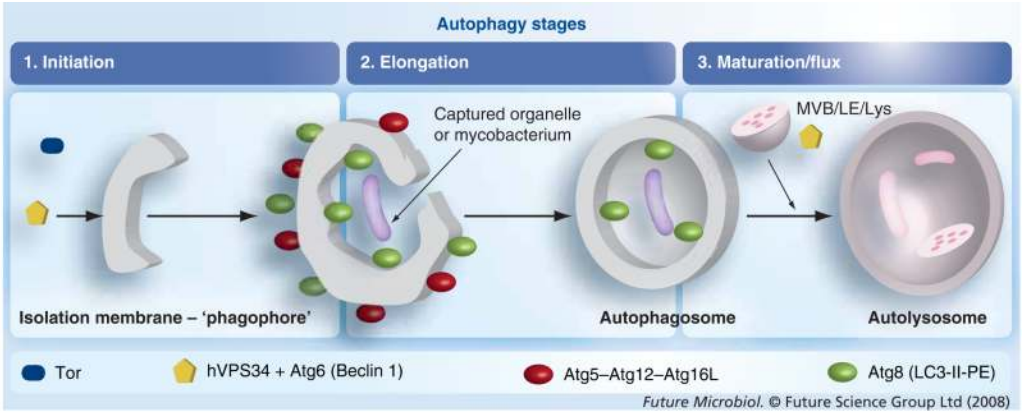
Executive summary

- *Mycobacterium tuberculosis* targets phosphatidylinositol-3-phosphate (PI3P) production due to its role as a main player in phagolysosome biogenesis.
- *M. tuberculosis* products, PI3P phosphatase SapM, glycosylated phosphatidylinositol-mimic lipoarabinomannan and phosphatidylinositol mannoside (PIM), play a role in suppressing PI3P production and cause arrest of mycobacterial phagosome maturation.
- *M. tuberculosis* prevents Rab5 to Rab7 conversion on its phagosome via Rab22a and Rab14 recruitment and retention, derailing the programmed succession of Rab GTPases on phagosomes as they mature into phagolysosomes.
- Other mycobacterial factors have been implicated as a result of random genetic screens.
- Autophagy is a previously untapped PI3P-dependent process that, when induced, can bypass mycobacterial phagosome maturation block and eliminate intracellular *M. tuberculosis.*
- Targeting autophagy is a general, very promising and previously unutilized approach to eliminating intracellular bacteria, viruses and parasites.
- P47 GTPases, also known as immunity-related GTPases, can induce autophagy, thus explaining their previously mysterious and unknown antimicrobial action.



LAM, PIM and SapM: *Mycobacterium tuberculosis* products implicated in inhibition of phagolysosome biogenesis. Rabs 5, 14 and 22a: early endosomal or recycling Rabs associated with phagosomes that do not mature in phagolysosomal organelles. X: points of inhibition imposed by mycobacterial factors. Note that *M. tuberculosis* PIM does not inhibit but activates processes involving Rab5 and Rab14, promoting fusion of mycobacterial phagosomes with early endosomes. See text for detailed explanations.
EEA: Early endosome antigen; LAM: Lipoarabinomannan;
PIM: Phosphatidylinositol mannoside; PI3P: Phosphatidylinositol 3-phosphate.

Figure 1.
Regulators of intracellular membrane trafficking and organelle identity in mammalian cells and *Mycobacterium tuberculosis* products causing phagosome maturation arrest.



Execution of autophagy requires the action of the PI3 kinase hVPS34 and production of PI3-phosphate at initiation and maturation. Beclin (Atg6) is a subunit of the hVPS34 complex that is required for the autophagy aspect of hVPS34 function. A nascent autophagosome is termed an isolation membrane (phagophore). It wraps around its cytoplasmic target (e.g., an organelle, a section of the cytoplasm or around a bacterium or a phagosome containing mycobacteria). The autophagosome elongates and bends its membrane with the help of Atg factors, generally folding into two complexes: Atg5 conjugated to Atg12, which associates with Atg16, and Atg8 (also known as LC3, its membrane-associated form, known as LC3-II) is C-terminally conjugated to PE. Induction of autophagy by pharmacologic, physiologic or immunologic agonists results in elimination of intracellular *Mycrobacterium tuberculosis* (described in the text).
LE: Late endosome; Lys: Lysosome; MVB: Multivesicular body; PE: Phosphatidylethanolamine; PI: Phosphatidylinositol; Tor: Target of rapamycin.

Figure 2.
Autophagy is an intracellular homeostatic process for the capture and destruction of intracellular pathogens.