Microreview

Mycobacterium tuberculosis inhibition of phagolysosome biogenesis and autophagy as a host defence mechanism

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Summary

A marquee feature of the powerful human pathogen Mycobacterium tuberculosis is its macrophage parasitism. The intracellular survival of this microorganism rests upon its ability to arrest phagolysosome biogenesis, avoid direct cidal mechanisms in macrophages, and block efficient antigen processing and presentation. Mycobacteria prevent Rab conversion on their phagosomes and elaborate glycolipid and protein trafficking toxins that interfere with Rab effectors and regulation of specific organelar biogenesis in mammalian cells. One of the major Rab effectors affected in this process is the type III phosphatidylinositol 3-kinase hVPS34 and its enzymatic product phosphatidylinositol 3-phosphate (PI3P), a regulatory lipid earmarking organelar membranes for specific trafficking events. PI3P is also critical for the process of autophagy, recently recognized as an effector of innate and adaptive immunity. Induction of autophagy by physiological, pharmacological or immunological signals, including the major antituberculosis Th1 cytokine IFN-γ and its downstream effector p47 GTPase LRG-47, can overcome mycobacterial phagosomal maturation block and inhibit intracellular M. tuberculosis survival. This review summarizes the findings centred around the PI3P-nexus where the mycobacterial phagosome maturation block and execution stages of autophagy intersect.

Received 17 December, 2005; accepted 30 January, 2006.
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Mycobacterial entry into the host macrophage – a striking resemblance to HIV

Mycobacterium tuberculosis macrophage parasitism is central to tuberculosis infection, latency, disease activation and transmission (Russell, 2001). M. tuberculosis enters the host macrophages where it resides in a phagosome that does not mature into the phagolysosome (Armstrong and Hart, 1971; Vergne et al., 2004a). M. tuberculosis interactions with the macrophage are dominated by the ability of the pathogen to prevent phagolysosome biogenesis (Vergne et al., 2004a), referred to in older texts as the phagosome–lysosome fusion block.

A number of studies focusing on macrophage surface receptors and M. tuberculosis entry (Ernst, 1998) have emphasized complement receptors and complement opsonization of mycobacteria (Ferguson et al., 2004) as well as C-type (calcium-dependent) lectins such as mannose receptor (Kang et al., 2005). The engagement of multiple receptors during entry is in keeping with the findings that blocking individual receptors does not significantly alter M. tuberculosis intracellular trafficking (Ernst, 1998). Nevertheless, individual receptor roles may be difficult to detect when using whole organisms. This was recently substantiated when using purified M. tuberculosis ligands showed that mannose receptor contributed to the modulation of phagosomal maturation by mycobacteria (Kang et al., 2005). Mannose receptor is only one of the many members of the large and heterogeneous family of C-type lectins (Cambi et al., 2005), which include several receptors known to interact with mycobacteria, such as dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) (Geijtenbeek et al., 2003). A report (Gatfield and Pieters, 2000) showing potential engagement during mycobacterial entry of cholesterol-rich lipid domains, referred to as rafts, may be related to the utilization of C-type lectins, as C-type lectins seem to be affected by lipid rafts (Cambi et al., 2005). Incidentally, lipid rafts and C-type lectins (Cambi et al., 2005), including DC-SIGN (Geijtenbeek et al., 2000), whose expression is debatable in lymph node dendritic
cells but is clearly detected on macrophages (Granelli-Piperno et al., 2005) and in human foreskin dendritic cells (Soilleux and Coleman, 2004), are also targeted by HIV envelope protein gp120 containing highly conserved mannoseylated oligosaccharides. The entry of HIV appears to be redundantly supported by C-type lectins other than DC-SIGN, and HIV also gets opsonized with complement degradation products. This adds to the previously noted repertoire of overlaps between M. tuberculosis and HIV in their interactions with the macrophage (Deretic et al., 2004).

Features of the M. tuberculosis phagosome

The most prominent characteristics of the M. tuberculosis phagosome are its incomplete luminal acidification (Sturgill-Koszycki et al., 1994) and absence of mature lysosomal hydrolases. Accessibility to transferrin-bound iron (Sturgill-Koszycki et al., 1996; Schaible et al., 2002; Kelley and Schorey, 2003), and trafficking of transferrin receptors through mycobacterial phagosome (Clemens and Horwitz, 1996) indicate that the mycobacterial phagosome is not a static organelle despite a block in the acquisition of lysosomal compartments and is accessible to early endosomal contents. A specific mycobacterial lipid product, PIM, stimulates fusion of early endosomes with mycobacterial phagosomes (Vergne et al., 2004b). Stimulating macrophages with the macrophage-activating cytokine IFN-γ overcomes the maturation block (Via et al., 1998). This process is not secondary to killing of mycobacteria by a trafficking-independent mechanism, but involves an active membrane sorting mechanism dependent on LRG-47, a member of the family of 47 kDa GTPases inducible by interferon-γ (MacMicking et al., 2003) (LRG-47 mode of action will be covered in the section on Rab conversion and M. tuberculosis phagosome maturation arrest). An important aspect of the mycobacterial phagosome is its inefficient antigen processing capacity (Pancholi et al., 1993; Ramachandra et al., 2001). It has been shown using purified phagosomes that the majority of peptide–MHC-II complexes are formed within phagosomes, by loading MHC-II molecules without prior export of bacterial antigens from phagosomes to conventional antigen processing compartments (Ramachandra et al., 2001). In these assays, heat-killed mycobacteria were processed more readily than live M. tuberculosis (Ramachandra et al., 2001). This has been recently revisited, suggesting participation of two separable processes, depending on the type of inactivation process used to kill mycobacteria (Ramachandra et al., 2005). Incidentally, at least two mycobacterial products have been identified as affecting phagosome maturation (Vergne et al., 2005), one a protein and the other a lipid, with differential sensitivity to inactivating agents.

Inhibition of Rab conversion and M. tuberculosis phagosome maturation arrest

An early study has uncovered that the phagolysosome biogenesis block occurs between the maturation stages controlled by the small GTP binding proteins Rab5 (early endocytosis) and Rab7 (late endosome) (Via et al., 1997), known to direct endosomal sorting. Rab5 was detected on mycobacterial phagosomes, while Rab7 was absent at times expected for its recruitment (Via et al., 1997). Several studies on Rab5 and Rab7 in mycobacterial phagosome maturation (Clemens et al., 2000a,b; Kelley and Schorey, 2003) have led to an observation that Rab5 facilitates mycobacterial acquisition of iron by ensuring proper endocytic sorting and delivery to mycobacterial phagosomes (Kelley and Schorey, 2003).

Recently, a fundamental breakthrough (Rink et al., 2005) in understanding the role of the Rab5 and Rab7 switch on endocytic organelles, referred to as Rab conversion (Rink et al., 2005), has helped place into a new perspective the initial observations with Rab5 and Rab7 on mycobacterial phagosomes (Via et al., 1997) (Fig. 1). Rabs are small GTPases (the human genome encodes 70 Rabs) that control the identity of intracellular organelles and direct membrane trafficking and protein sorting in all eukaryotic cells (Pereira-Leal and Seabra, 2001; Pfeffer, 2005). Rabs, like their close relative Ras, operate on the basis of a molecular ON–OFF switch depending on GTP or GDP bound states. Rab5 and Rab7 are central players within the endosomal pathway. Rab5 participates in receptor-mediated endocytosis and formation of clathrin-coated vesicles containing the endocytosed cargo, and controls fusion of such vesicles into an early (sorting) endosome, where the endocytosed cargo and receptors are sorted for degradation or recycling. Several Rabs, e.g. Rab4, Rab5, Rab11 (de Renzis et al., 2002) within the early endosomal compartments and Rab7 and Rab9 in late endosomes (Barbero et al., 2002), help segregate recycling components by sequential passage through a membrane continuum on the same sacculotubular organelle divided into domains marked by appropriate Rabs, until physically fully separated by fission (Fig. 1A). However, very little was known about how Rabs control anterograde processes of cargo transport to the degradative compartments, i.e. the maturation of the leftover sorting endosome and its conversion into a late endosomal organelle. It was thought that this process could occur via transport intermediates with small vesicles carrying cargo between Rab5 and Rab7 organelles, which retained their original identity along with their marquee Rabs. However, the work by Rink et al. (Rink et al., 2005) has now shown that this process occurs via a bulk change of the whole Rab5 organelle into a Rab7 late endosome, once the sorting for recycling is completed. The abrupt change of
a Rab5 organelle into a Rab7 organelle occurs by a synchronous and wholesale removal of Rab5 and its nearly instantaneous replacement by Rab7, called Rab conversion. Thus, the initial observation of Rab5 presence and Rab7 absence on mycobacterial phagosomes (Via et al., 1997) identifies a critical block in phagosomal Rab conversion (Fig. 1B). As the signals and molecular machinery controlling Rab conversion are presently not known (Deretic, 2005a; Rink et al., 2005), this is an area of expected experimental growth. Mycobacterial action may provide clues regarding Rab conversion process, and vice versa, delineating the exact molecular processes governing Rab conversion will shed new light on the mycobacterial phagolysosome biogenesis block.

Rab effectors and mycobacterial phagosome

The recognition of the mycobacterial phagosome maturation arrest as what is now referred to as a Rab conversion block, has led to a search for Rab5 interacting components in an effort to pinpoint the potential host target(s) (Fratti et al., 2001). The majority of Rab5 effectors that have been examined appear to be recruited to both the model (latex bead) and mycobacterial phagosomes (Fratti et al., 2001), with the notable exceptions of a Rab5 effector EEA1 (early endosomal autoantigen 1) (Fratti et al., 2001). In addition to its interactions with Rab5, EEA1 associates with phosphatidylinositol 3-phosphate (PI3P) on organellar membranes. The latter is also true for Hrs, another endosomal regulatory PI3P-binding protein (Vieira et al., 2004). PI3P is generated on organellar membranes by the action of a critical Rab5 effector, the type III phosphatidylinositol 3-kinase (PI3K) hVPS34 (Christofidis et al., 1999). Hence, the reduced or altered recruitment of EEA1 and Hrs to mycobacterial phagosomes (Fratti et al., 2001; Vieira et al., 2004) have implicated hVPS34 and PI3P in the mycobacterial phagosome maturation block (Fratti et al., 2003). Consequently, phosphatidylinositol phosphates have become the focus of studies aimed at pinpointing the exact molecular events causing M. tuberculosis phagosome maturation arrest (Fratti et al., 2001; 2003; Vergne et al., 2003a,b; 2004a; 2005; Kelley and Schorey, 2004; Vieira et al., 2004; Purdy et al., 2005).

Inhibition of PI3P generation by M. tuberculosis products plays a central role in mycobacterial phagolysosome biogenesis block

Figure 2 summarizes how mycobacterial factors converge upon PI3P as a trafficking regulatory lipid within the cytoplasmic leaflet of organellar membranes. PI3P, generated by the host cell enzyme hVPS34, is essential for proper membrane trafficking and sorting events within the endosomal system, leading to the formation of organelles such as late endosomal multivesicular bodies (Piper and Luzio, 2001; Katzmann et al., 2002; Gruenberg and Stenmark, 2004) and phagolysosomes (Fratti et al., 2001; Vieira et al., 2001). Two products generated by M. tuberculosis, one a lipid (liparabinomannan, LAM) (Fratti et al., 2001; 2003; Hmama et al., 2004; Kang et al., 2005), and the other an enzyme (a PI3P phosphatase, SapM) (Saleh and Belisle, 2000; Vergne et al., 2005), ensure phagosome maturation block. Both products act upon PI3P, with LAM preventing its generation and SapM removing whatever PI3P may escape the LAM block.
Ca\(^{2+}\) and phagosomal maturation

Kusner and colleagues have demonstrated in a series of articles the importance of Ca\(^{2+}\) and Ca\(^{2+}\)/calmodulin/calmodulin kinase II in phagolysosome biogenesis (Malik et al., 2000; 2003; Thompson et al., 2005) and antigen presentation (Herrmann et al., 2005). The Ca\(^{2+}\)-binding protein calmodulin, which acts as a sensor for increased cytosolic Ca\(^{2+}\) and transduces the Ca\(^{2+}\) signal upon binding to effector proteins, is critical for phagosomal maturation (Malik et al., 2000). Ca\(^{2+}\) turned out (Vergne et al., 2003b; 2004a) to be critical for the PI3P-dependent pathway of delivery of lysosomal components (lysosomal hydrolases and V\(_{5}\), H\(^{+}\)ATPase) to the phagosome (Fratti et al., 2003). Of particular significance are inhibitory effects of LAM on Ca\(^{2+}\) fluxes (Vergne et al., 2003b). As Ca\(^{2+}\) and calmodulin influence the recruitment of the PI3K hVPS34 to phagosomes and other endosomes in macrophages, interference with Ca\(^{2+}\) fluxes by LAM inhibits this pathway (Vergne et al., 2003b) (Fig. 2).

Glycosylated phosphatidylinositol LAM

The molecular mechanism of LAM action has been partially established (Vergne et al., 2003b; 2004a). LAM functions as a preformed trafficking toxin, acting on contact with macrophages (Vergne et al., 2003b; Kang et al., 2005). LAM acts by inhibiting phosphatidylinositol phosphorylation into PI3P (Vergne et al., 2003b; 2004a). LAM is itself a glycosylated phosphatidylinositol, made in copious amounts by fresh clinical isolates (Torrelles et al., 2004). LAM has a variety of modifications on its carbohydrate antennae including terminal mannose residues, succinyl groups and phosphatidylinositol end groups (Torrelles et al., 2004). Both the carbohydrate and the lipid portions of LAM are important for its action (Kang et al., 2005). At least one aspect of LAM function is related to its carbohydrate interaction with the mannose receptor during mycobacterial entry into macrophages, albeit by a presently unknown mechanism (Kang et al., 2005). Mannose receptor eventually carries LAM to late endosomal compartments for antigen presentation by CD1b (Prigozy et al., 1997). CD1b is a member of the CD1 family of non-polymorphic, β2m-associated lipid antigen presenting molecules that are evolutionarily distant relatives of MHC I (Brigl and Brenner, 2004). Recently, the role of the LAM lipid moiety has been reassessed in the context of the sustained action of this glycolipid (Kang et al., 2005). It has been established that LAM inserts via its phosphatidylinositol portion into the host cell membrane (Ilangumaran et al., 1995; Shabaana et al., 2005) and affects host cell signalling. How LAM affects Ca\(^{2+}\) signalling remains to be delineated, but it is likely to be related to the mycobacterial inhibition of sphingosine kinase and sphingosine 1-phosphate generation, recently demonstrated by Kusner and colleagues (Thompson et al., 2005).

Mycobacterial secreted lipid phosphatase SapM

In addition to LAM, Mycobacterium tuberculosis makes and secretes a second factor influencing PI3P levels on mycobacterial phagosomes. The search for a factor other than LAM was initiated in a follow-up to the observation that live and dead mycobacteria differ in their ability to maintain a PI3P-free phagosome (Vergne et al., 2005). While LAM alone can inhibit initial Ca\(^{2+}\) fluxes, mycobacterial viability and continual protein synthesis are needed to maintain a PI3P-less phagosome over prolonged periods of time (Vergne et al., 2005). Among the potential candidates for a PI3P phosphatase encoded by the Mycobacterium tuberculosis genome, only SapM (Saleh and Belisle, 2000) proven to correspond to the PI3P phosphatase activity initially detected in culture filtrate proteins (Vergne et al., 2005). SapM is secreted into the culture filtrate by the virtue of its leader peptide (Saleh and Belisle, 2000), and is also present in phagosomes in infected macrophages (Vergne et al., 2005). Pharmacological inhibition of SapM (Vergne et al., 2005). Pharmacological inhibition of SapM (Vergne et al., 2005) renders mycobacteria susceptible to phagosomal uptake by a double-latch mechanism minimizing the risk of undergoing a PI3P-dependent maturation into the phagolysosome (Fig. 2).

Other mycobacterial products affecting phagosome maturation and additional host mechanisms potentially involved

At least two genetic screens (Pethe et al., 2004; Stewart et al., 2005) using comprehensive mutant libraries of

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**Fig. 2.** Phosphatidylinositol 3-phosphate (PI3P) is a regulatory trafficking lipid targeted by M. tuberculosis factors. ManLAM, mannosecapped lipoarabinomannan prevents PI3P generation. SapM, an M. tuberculosis-secreted PI3P phosphatase maintains the mycobacterial phagosome PI3P-free. TGN, trans-Golgi network. For details, see text.
M. tuberculosis and BCG suggest that additional mycobacterial products directly or indirectly (by affecting the fitness of the organism) influence trafficking processes and undermine intracellular survival. No overlaps among the identified candidates were seen between the genes reported in the two screens (Pethe et al., 2004; Stewart et al., 2005) Neither screen showed potential overlaps with LAM biosynthetic or processing pathways or SapM. Nevertheless, it is likely that some of the mutants in these libraries or additional factors will turn out to play a role in intracellular survival processes pending further detailed studies. On the host side, additional studies suggest that there is an involvement of actin in the modulation of mycobacterial intracellular survival, possibly by affecting phagosomal maturation (Anes, 2003) or by exclusion of iNOS from the vicinity of mycobacterial phagosome (Miller, 2004).

Autoptahgy is a PI3P-dependent process mechanism overcoming mycobacterial phagosome maturation block

The recognition of PI3P as a centrepiece of phagosomal maturation and mycobacterial phagolysosome biogenesis block has opened a new area of investigation (Deretic, 2005b) that was not anticipated before PI3P was identified as a critical nexus of regulation. In a search of a mechanism that could boost cellular ability to generate PI3P and to override the mycobacterial inhibition of PI3P production, we have found that another PI3P-dependent process, autophagy, can bypass the mycobacterial phagolysosome biogenesis block (Gutierrez et al., 2004; Deretic, 2005b). Initially, these studies have been motivated by the possibility that activation/mobilization of hVPS34 PI3K during autophagy might enhance PI3P production and thus override M. tuberculosis-imposed block via lower PI3P levels.

Autophagy is a fundamental biological process defined as a cytoplasmic homeostasis pathway (Shintani and Klionsky, 2004) whereby discrete portions of the cytoplasm are sequestered into a specialized double membrane vacuole, termed the autophagosome, and delivered to lysosomes for degradation (Fig. 3). This process removes damaged or surplus organelles, including leaky mitochondria and excess peroxisomes. In addition, by turning over long-lived cytosolic macromolecules, such as stable proteins, autophagy supports viability under starvation conditions (Shintani and Klionsky, 2004). Autophagy is a cell survival mechanism, but under certain conditions excessive autophagy can cause non-apoptotic programmed cell death, and the two processes are balanced at least in part via Bcl-2 interactions with Beclin, a subunit of the hVPS34 complex (Pattingre et al., 2005). Autophagy has been implicated in both health-promoting and disease-associated states in cancer, neurodegeneration, development and ageing (Shintani and Klionsky, 2004). Recently, autophagy has been demonstrated to represent a previously unrecognized immune effector against infectious diseases (Gutierrez et al., 2004; Nakagawa et al., 2004; Ogawa et al., 2005; Paludan et al., 2005). It has become evident that autophagy serves as a mechanism for removal of intracellular bacteria and viruses, in keeping with cytoplasmic maintenance as a primary function of autophagy (Deretic, 2005b). Autophagy is more than just an innate defence mechanism and its role in adaptive immunity enabling antigen processing for MHC II presen-

**Fig. 3.** Process of autophagy. Autophagy requires the action of hVPS34 and production of PI3P at the initiation and maturation stages along the execution stages of the autophagic pathway. Beclin (Atg6) is a subunit of the hVPS34 complex affecting autophagy. A nascent autophagosome termed isolation membrane (phagophore) forms around an organelle or section of the cytoplasm. It elongates and bends its membrane with the help of Atg factors forming two complexes: (i) Atg5 is conjugated to Atg12, and associates with Atg16; (ii) Atg8 is also known as LC3; its membrane associated form, known as LC3-II, is conjugated C-terminally to phosphatidylethanolamine (PE), MVB, multivesicular bodies; LE, late endosome; Lys, lysosome. Induction of autophagy by pharmacological, physiological, or immunological agonists results in control of intracellular M. tuberculosis. Although not shown, IFN-γ and one of its downstream effectors induce or modulate autophagy in macrophages. The details of how IFN-γ and LRG-47 affect autophagy are currently under investigation.
tation has only begun to be appreciated (Paludan et al., 2005).

Autophagy appears to be a particularly powerful mechanism clearing intracellular \textit{M. tuberculosis} (Gutierrez, 2004; No. 2741). This is in keeping with the function of autophagy to patrol the cytoplasm and sequester and degrade unwanted contents and organelles. Precisely how autophagy eliminates intracellular \textit{M. tuberculosis} remains to be established. Several non-mutually exclusive models can be envisioned including: (i) nascent autophagosomes envelop mycobacterial phagosomes in their maturation arrested state and proceed with the default pathway of autolysosome formation and content degradation; (ii) autophagosomes and mycobacterial phagosomes fuse and then undergo maturation and (iii) mycobacterial phagosome maturation is affected by a more robust late endosomal systems activated during autophagy induction. Questions related to these models include: (i) are there any special properties of the lytic mechanisms within autolysosomes that may be particularly robust against mycobacteria and other sturdy organisms? (ii) Are there any specific properties of the autophagosomes, including topological considerations, that render them less susceptible to mycobacterial factors? (iii) Are there any conditions under which mycobacteria can interfere with autophagy and what are the factors involved? The examination of these models and questions spells out an exciting area of future studies.

\textbf{IFN-γ, p47 GTPase LRG-47 and autophagy}

Recent studies have shown that one of the major protective Th1 cytokines, IFN-γ, induces autophagy in macrophages (Gutierrez et al., 2004) and in non-immune cells (Inbal et al., 2002; Pyo et al., 2005). Significantly, autophagy is induced or modulated by p47 GTPases (Gutierrez et al., 2004), which are in turn controlled by IFN-γ. The p47 family of GTPases are inducible by IFN-γ and have been implicated in defence against intracellular pathogens (MacMicking, 2004; Taylor et al., 2004), but their exact mode of action was not known prior to the connection with autophagy (Gutierrez et al., 2004). These and other aspects of autophagy as immune effectors of innate and adaptive immunity are sure to attract much attention in the field of immunology and infectious diseases, as well as in the areas of fundamental cell biology and cellular microbiology.

\textbf{Concluding remarks}

The \textit{M. tuberculosis} phagosome maturation arrest overlaps with fundamental immunological and cell biological processes. The finding that mycobacteria target host regulatory lipids and enzymes that modify them, as they remodel their phagosome, has led to identification of critical checkpoints in endosomal and phagosomal systems. The \textit{M. tuberculosis} genome is replete with lipid metabolism genes. Historically, phosphatidylinositolos have been first discovered in mycobacteria and only subsequently recognized in mammalian cells. Thus, we have come full circle in connecting the mycobacterial lipids and host lipids in a struggle to control the host cell. While many questions remain to be answered regarding details of how mycobacterial products act, the present knowledge has already permitted to make important progress. The specific host lipid involved, PI3P, has led to connections with autophagy, a fascinating new field of study regarding innate and adaptive immunity against intracellular pathogens. The latest link between IFN-γ, p47 GTPases and antimycobacterial action of autophagy is informative regarding the previously unappreciated mode of action of Th1 cytokines correlating with protective immunity against \textit{M. tuberculosis} and other intracellular pathogens. This particular aspect spells out an area where new progress and breakthroughs are expected to be made.

\textbf{Acknowledgements}

This work was supported by grants AI45148 and AI42999 from the National Institutes of Health.

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