Mycobacterium tuberculosis and the Four-Minute Phagosome

By arresting the maturation of phagosomes, M. tuberculosis avoids being delivered to lysosomes

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Macrophages kill many household microbes with minimal fuss, efficiently producing reactive oxygen and nitrogen intermediates, and also delivering internalized microbes to the low-pH, hydrolytically active environment of the lysosome. However, Mycobacterium tuberculosis parasitizes host macrophages, subverting phagosome maturation and thus evading this ordinarily effective host defense mechanism.

By arresting maturation of phagosomes, M. tuberculosis avoids being delivered to lysosomes. Despite this block, however, the vacuole that this bacillus inhabits remains fusion competent and behaves like an early endosome (Fig. 1). Its pH is 6.4, it derives key nutrients from the rapidly recycling endosomal system, and it retains the membrane-fusion machinery associated with early endosomes. Two big questions are associated with this phenomenon. How does the microbe do this, and what are the consequences with respect to the environment within the phagosome?

Markers of Phagosome Maturation

The differentiation of endosomes and phagosomes is documented extensively. Early endosomes and phagosomes acquire the small GTPase rab5, which helps to recruit the PI3-kinase, Vps34. As PI3-P increases on the membrane, it binds EEA1. EEA1 concentration increases concomitantly with a decrease in rab5 concentration and the appearance of rab7, another small GTPase that is abundant in late endosomes. The vacuole also acquires lysosome-associated membrane proteins, such as LAMPs 1 and 2, as well as lysosomal enzymes that are delivered through fusion with other lysosomes and also from the trans-Golgi network via the mannose 6-phosphate receptor.

To document the degree of maturation of microbe-containing phagosomes, researchers typically use antibodies that recognize some of these protein markers. Despite their value and wide use, however, these antibody-based methods provide only static measurements of a dynamic process, and they are often employed subjectively, and provide limited information about the environment within phagosomes.

Phagosome Modulation by Mycobacterium: a Two-Step Process?

Since at least the 1970s, researchers suspected that mycobacterial cell wall lipids
modulate membrane fusions inside host cells. Recent studies, notably by Vojo Deretic of the University of New Mexico in Albuquerque and his collaborators, suggest that the lipid lipoarabinomannan inhibits Vps34 activity, limiting production of PI3-P and arresting maturation of the phagosome prior to its accumulation of EEA1. Pharmacologic studies further implicate calcium, calmodulin, calmodulin-dependent kinase II, and sphingosine kinase as regulating maturation of phagosomes. For instance, David Kusner of the University of Iowa has shown that treating infected macrophages with a calcium ionophore recruits calmodulin, activates calmodulin-dependent kinase II, and enhances maturation of bacterium-containing vacuoles. Although ordinary phagosomes induce calcium flux during maturation, *M. tuberculosis* appears to inhibit this signaling by suppressing sphingosine kinase activity.

Lipoarabinomannan and other lipids appear capable of blocking phagosome maturation, which seems contrary to the widely held belief that dead bacilli fail to arrest phagosome/lysosome fusion. However, directly measuring vacuolar pH sheds light on this disparity (Fig. 2). Phagosomes containing inert particles such as immunoglobulin G (IgG) beads are processed rapidly, acidifying to pH 4.5 within 15 minutes of uptake, while phagosomes that contain live *Mycobacterium bovis* BCG equilibrate slowly to pH 6.4. Meanwhile, phagosomes containing heat-killed *M. bovis* BCG stabilize at a pH of about 5.8, suggesting that cell wall lipids influence phagosome maturation but that other effectors are also required to modulate this process.

This interpretation is supported by independent data generated through analysis of transposon-mutagenized *M. tuberculosis* mutants that were isolated through a genetic screen that enriches for bacteria defective in arresting phagosome/lysosome fusion. Pools of transposon-mutagenized bacteria were fed to macrophages whose lysosomes were loaded with iron dextran. Shortly after infection, the macrophages were lysed, and the lysosomes isolated by magnetic selection. Mutants that traffic preferentially to these lysosomes were selected through several rounds of enrichment. The majority of these mutants are attenuated for intracellular survival and show enhanced fusion with late endosomes and lysosomes containing colloidal gold. Of particular significance, the most-attenuated mutants sequester in vacuoles at about pH 5.7. The overlap in phagosome conditions between dead bacteria and these mutants reinforces the idea that cell wall lipids go so far, but require further help to attain full modulation.

**What Other Effectors Might Modulate Maturation?**

What molecules other than lipids modulate phagosomes? One possibility comes from study-
ing mutants defective in producing the serine/threonine kinase PknG; these mutants cannot arrest maturation of their phagosomes and show reduced survival in macrophages, according to Jean Pieters of the University of Basel in Switzerland and his collaborators. They report that the kinase gains access to the cytosol of the host cell and interferes with the signaling cascade that drives maturation. How the kinase PknG moves from the lumen of the phagosome across the vacuolar membrane and into the cell cytosol is a mystery, because Mycobacterium spp. do not have type III secretion systems.

Vojo Deretic and his collaborators identified a secreted phosphatase, SapM, that is capable of dephosphorylating PI3-P, the phosphoinositol species required for recruiting EEA1 in maturing phagosomes. Their studies indicate that this enzyme can augment the activity of lipoarabinomannan, maintaining low PI3-P levels in the Mycobacterium-containing phagosome and therefore avoiding accumulation of EEA1. Because the substrate of SapM is a membrane component, the enzyme could certainly work on the lipid within the phagosome. Whether PI3-P is mobile within the lipid bilayer and whether the phosphorylated form of the phosphatidylinositol flips to the inner face of the membrane are unaddressed questions.

Our transposon mutants include several genotypes yet to be characterized. Although the explanation for some of these phenotypes may be fitness and not mechanism dependent, at least one is involved in synthesizing a geranylgeranyl pyrophosphate-based isoprenoid, which could act as an effector molecule. This possibility remains to be explored.

New Assays Help To Explore the Environment within Phagosomes

Despite knowing the pH of phagosomes containing pathogenic Mycobacterium spp., we have few other direct physical measurements to describe changes in the lumen of such phagosomes as they mature. In general, cell biologists have studied how this process is regulated, focusing almost exclusively on cytosolic and membrane-associated proteins that mediate the membrane-fusion events required for passage to the lysosomes.

In recognition of the dynamic nature of phagosome maturation, we developed several assays to quantify functional characteristics that are key to the biology of these compartments (Fig. 3). For instance, we use fluorescence energy resonance transfer (FRET) microscopy to measure phagosome/lysosome fusion.

This is achieved by allowing macrophages to pinocytose an acceptor fluorochrome that is concentrated in lysosomes. Later, we add particles labeled with a donor fluorochrome that the macrophages phagocytose, then we use the FRET signal to measure the degree of mixing of the phagosomes with lysosomal contents—exciting the fluorochrome at the donor wavelength and measuring the signal at the acceptor wavelength. This assay provides a dynamic readout over a period of 90 minutes before mixing, or phagolysosomal fusion, reaches steady state. Compounds that inhibit phagosome/lysosome
fusion, such as concanamycin A (V-ATPase), W7 (calmodulin), and SB 203580 (MAP kinase), depress the FRET signal. We also developed an assay to measure directly the hydrolytic capacity of phagosomes that take up particles. To visualize protease activity, we load ligand-bearing particles with the fluorogenic substrate (biotin-Phe-Arg)2-rhodamine 110, which is recognized by the cysteine proteinase cathepsin L. Alternatively, to detect lipase activity we load particles with the lipase substrate 1-trinitrophenyl-amino-dodecanoyl-2-pyrenedecanoyl-3-O-hexadecyl-sn-glycerol. In both cases, the fluorescent moiety, rhodamine 110 and pyrene, respectively, remains quenched until hydrolyzed by an appropriate enzyme. The cysteine proteinase substrate is processed more rapidly than the lipase substrate, and the assays provide dynamic readouts over 15 and 60 minutes, respectively.

While inhibitors of the V-ATPase complex block acidification and suppress both phagosome/lysosome fusion and cysteine proteinase activity, they increase the lipolysis rate. Whether this is due to an increased delivery of lipases or to a higher pH optimum for this enzyme remains to be determined. In any case, these assays demonstrate the rates by which phagosomes acquire hydrolytic activities, which indicates how quickly the compartment becomes hostile to intracellular pathogens.

**M. tuberculosis and the Concept of the Four-Minute Phagosome**

Now that we have the tools to measure the hydrolytic capacity of the M. tuberculosis-containing vacuole, it is interesting to extrapolate from the above studies to predict what this environment might be. For instance, an IgG-bead phagosome reaches a pH of about 6.5, the same pH as the M. tuberculosis-containing vacuole, within four minutes of internalization. At four minutes, the FRET profile indicates that phagosome/lysosome fusion is just entering a phase of exponential increase, meaning that there is still very little mixing with lysosomal contents. The hydrolytic capacity of a phagosome depends on several factors, including the concentrations, activation status, and pH optimum of its hydrolytic enzymes. In four-minute phagosomes, both the lipase and cysteine proteinase substrates register minimal activity, predicting that this environment will not be particularly hostile to M. tuberculosis.

The reporter fluorochromes and fluorogenic substrates can be linked to the surface of M. tuberculosis, with no impact on viability, paving the way for direct experimental analysis of the phagosomal environment. Moreover, we can also test the compartment to which dead bacteria and transposon-mutagenized bacteria gain access. Finally, our longer-term goal is to use pharmacological agents and the maturation arrest-defective mutants to manipulate the phago-
somtes containing *M. tuberculosis* and to perform microarray transcriptional analysis to build a picture of the differential responses induced by the environments experienced by *Mycobacterium* during intramacrophage infection. So, while it is important to identify the mechanism(s) responsible for arresting the maturation of the phagosome, we feel that the information gained from those studies can be exploited further to assemble a much broader understanding of the intravacuolar environment and how the bacterium detects and responds to its host.

SUGGESTED READING


