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Determinants of Phagocytosis, Phagosome Biogenesis and Autophagy for *Mycobacterium tuberculosis*

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1.1
Introduction

Mycobacterium tuberculosis is an intracellular pathogen of mononuclear phagocytes and is highly adapted to the human host. This bacterium enters macrophages by the phagocytic process using a defined subset of receptors and subsequently multiplies within a unique phagosomal compartment. *M. tuberculosis* has developed multiple strategies to circumvent the normal fate of ingested pathogens both during and following phagocytosis. Evidence is emerging that surface mannosylation of *M. tuberculosis* bacilli is an important host adaptive mechanism for directing the phagocytic and post-phagocytic processes, especially within the unique microenvironment of lung alveoli. Key biochemical pathways and mycobacterial determinants in the development and maintenance of the mycobacterial phagosome are beginning to be identified. In addition, recently it has been shown that the induction of autophagy in the macrophage plays an important role in the innate immune response to *M. tuberculosis*. Major developments in these areas are the focus of this chapter.

1.2
Mycobacterial Determinants in Host Recognition: Example of Host Adaptation

The interaction between the *M. tuberculosis* cell wall components and host cell surface receptors is of major importance in the pathogenesis of infection. About 60% of the cell wall of *M. tuberculosis* is composed of lipids including mycolic acids, trehalose-containing lipids and several lipoglycoconjugates [1]. In particular, the surface of *M. tuberculosis* is dominated by a group of biosynthetically related mannosylated lipoglycoconjugates [2], which mediate recognition and entry of the bacillus into host cells through C-type lectin pattern recognition receptors (PRRs) [3]. These closely related mannose-containing lipoglycoconjugates include phosphatidyl-*myo*-inositol

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mannosides (PIMs), lipomannan (LM) and mannose-capped lipoarabinomannan (ManLAM) [2].

Evidence is increasing that mannosylation of the *M. tuberculosis* surface aids in host cell recognition and response. The terminal mannose caps of ManLAM have been shown to bind to the macrophage C-type lectin mannose receptor (MR) [4,5] – a prototypic PRR that links innate and adaptive immunity [6]. ManLAMs from different *M. tuberculosis* strains vary in the degree to which they bind to the MR pointing to a potential relationship between the length and/or presentation of the mannose caps and their avidity for the MR [7]. ManLAM caps also bind to dendritic cell (DC)-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) on DCs [8,9]. Thus, terminal components of ManLAM are very important in host cell recognition and response. In contrast, the phosphatidyl-*myo*-inositol caps of LAM from *M. smegmatis* do not bind to the MR or DC-SIGN [4,9].

PIMs are the major phospholipid components of the mycobacterial cell wall. They are defined as families by their number of mannose units (1–6) and as species by their number of fatty acids (2–4) [10]. It has recently been determined that higher-order PIMs (PIM_5f and PIM_6f) associate with the MR and that this association is directly influenced by their degree of acylation, where triacylated forms of higher-order PIMs preferentially bind to this receptor [11]. In contrast, lower-order PIMs associate poorly with macrophages and do not bind to the MR [11]. Conversely, lower-order PIMs and LM are recognized by DC-SIGN in comparable fashion to higher-order PIMs and ManLAM independent of their degree of acylation. Thus, recognition of *M. tuberculosis* PIMs by host cell C-type lectins is dependent on both the nature of their terminal carbohydrates and degree of acylation. These data further support the idea that the spatial orientation of the MR carbohydrate recognition domains (CRDs) limits their sugar recognition repertoire, whereas the clustered CRD conformation of the tetrameric DC-SIGN enables a broader range of mannose glycoconjugate recognition. The importance of PIMs in host cell recognition by the MR is also supported by a recent study where an *M. smegmatis* strain overexpressing higher-order PIMs was found to markedly increase its association with the human macrophage MR when compared to the *M. smegmatis* wild-type strain [12].

In addition to DC-SIGN, there is evidence that lower-order PIMs associate with complement receptor 3 (CR3) [13]. Thus, it seems that the lower-order PIMs preferentially associate with CR3 and DC-SIGN, both receptors being expressed on DCs. Since most macrophage subsets express low or negligible DC-SIGN [14–17], it remains unclear whether this is an important PRR for PIMs, LM and ManLAM on macrophages. Recent evidence also indicates that *M. tuberculosis* interacts with Toll-like receptors (TLRs) on murine bone marrow-derived DCs through their lower-order PIMs [18].

The importance of higher-order PIMs along with ManLAM in *M. tuberculosis* recognition by the MR and their potential involvement in *M. tuberculosis* pathogenesis is suggested further by the results of a direct comparison of the amounts of purified PIMs from the virulent *M. tuberculosis* Erdman and H37Rv strains, the attenuated *M. tuberculosis* H37Ra strain and the avirulent *M. smegmatis* mc^2155 strain [11]. *M. tuberculosis* strains have significantly more higher-order PIMs and

much less lower-order PIMs when compared to *M. smegmatis*. In addition, virulent *M. tuberculosis* strains have quantitatively more higher-order PIMs (especially Ac_1PIM_6) than the attenuated *M. tuberculosis* H37Ra strain. This result along with previous studies with ManLAM indicates that virulent *M. tuberculosis* has evolved to more heavily construct its cell wall lipoglycoconjugates with α(1–2) mannosylated termini. These termini resemble those present in high mannose *N*-linked oligosaccharides of newly produced glycoproteins in eukaryotic cells [19]. Such heavily α(1–2) mannosylated *N*-glycoproteins released into circulation are scavenged by the macrophage MR through pinocytosis to maintain homeostasis [20]. This leads to the speculation that increased α(1–2) mannosylation on the surface of *M. tuberculosis* allows for preferential engagement of the MR in a form of exploitation to enhance its survival in macrophages (also see below).

Other *M. tuberculosis* surface mannosylated molecules accessible to the MR are arabinomannans, mannans and mannoproteins [21,22]. The potential involvement of the MR in human mycobacterial diseases including tuberculosis (TB) and leprosy has been highlighted by human genetic studies [23].

1.3 Hierarchy of Host Receptors: Importance of the Lung Environment

Alveolar macrophages (AMs) are the first line of cellular defense against inhaled environmental particles and infectious microorganisms that enter the lungs. These cells express a broad range of immune receptors, including Fcγ receptors (FcγRs), CRs and TLRs, and particularly high levels of PRRs such as the MR [24], dectin-1 (β-glucan receptor) [25] and scavenger receptors (SRs) [26]. Despite constant stimulation by inhaled particles and pathogens, AMs display an anti-inflammatory phenotype described as an 'alternative activation' state, which includes altered cytokine responses [27], reduced oxidant production in response to stimuli [28] and reduced microbicidal activity [29]. Thus, AMs seem best adapted for removal of small airborne particulates with minimal induction of inflammatory immune responses. While alternatively activated macrophages may be adequate for the efficient clearance of routinely inhaled extracellular pathogens, they may be inadequate for a host-adapted intracellular pathogen such as *M. tuberculosis*. This point is highlighted in a recent report that alternatively activated (type 2) macrophages do not produce interleukin (IL)-23 or -12, but instead predominantly secrete IL-10, downregulate antigen-presenting and costimulatory molecules, poorly support type 1 helper function, and enhance mycobacterial growth [30]. The importance of alternatively activated macrophages such as AMs in the pathogenesis of *M. tuberculosis* has recently been demonstrated in other studies [31,32].

There has been considerable interest in the discovery of the molecules such as IL-4, IL-13, transforming growth factor-β and glucocorticoids, which induce alternative activation states in macrophages [29]. Data are emerging which indicate that components of pulmonary surfactant also contribute to this process, especially surfactant protein (SP)-A and -D, which play important roles in innate immunity,

serving as innate defense proteins and modulators of cellular immune activities [33,34]. SP-A has been shown to have multiple effects on macrophage biology *in vitro*, including increased PRR activity, increased phagocytosis, altered production of pro-inflammatory cytokines, and decreased production of nitric oxide (NO) and reactive oxygen intermediates (ROIs) in response to stimuli [35,36].

SP-A and -D regulate the early interaction between *M. tuberculosis* and macrophages. In the case of SP-A, this has been shown to increase the phagocytosis of *M. tuberculosis* through a direct interaction of the protein with macrophages [37], which upregulates MR activity [38]. A study using bronchoalveolar lavage fluid from human immunodeficiency virus patients, which contains high amounts of SP-A, shows that upon opsonization of *M. tuberculosis* by SP-A, bacilli increase their attachment to murine AMs [39]. In addition, it is reported that SP-A binds to the attenuated *M. bovis* bacillus Calmette-Guérin (BCG) strain, increasing uptake of the bacilli by murine macrophages [40] by engagement of SPR210, a receptor for SP-A [41], and mediating NO-dependent inhibition of mycobacterial growth [42]. In contrast, SP-A has been found to inhibit *M. tuberculosis*-stimulated NO production from interferon-γ-primed murine macrophages [43]. In opposition to SP-A, SP-D has been shown to decrease *M. tuberculosis* phagocytosis by macrophages by binding with high avidity to the mannose caps of ManLAM on the bacilli, and thereby reducing the interaction of *M. tuberculosis* with the macrophage MR [44]. SP-D-opsonized *M. tuberculosis* bacilli that are phagocytosed undergo increased phagosome–lysosome fusion and have reduced intracellular growth [44,45].

1.4 Other Macrophage Receptors for *M. tuberculosis*

There are abundant data to support the role of C3 opsonization and the CRs (CR1, CR3 and CR4) in the phagocytosis of *M. tuberculosis* [3]. The expression of CRs (particularly CR4) and the MR increases during the differentiation of monocytes into macrophages, with CR4 and the MR being highly expressed on AMs.

CR3 is the major integrin of phagocytic cells (mononuclear phagocytes and neutrophils), and is also expressed on natural killer cells and a small set of lymphocytes [46]. Evidence exists for C3-mediated recognition of *M. tuberculosis* by human and murine macrophages, as well as for a direct interaction between *M. tuberculosis* and CR3 [47,48]. Mycobacterial polysaccharides [49], lower-order PIMs (see above) and glycopeptidolipids (GPLs) [13] have been shown to interact with CR3, presumably through binding to the lectin site of the receptor. Binding sites on CR3 for the bacterium (I-domain, which recognizes C3bi versus lectin sites) are predicted to impact the cellular response [50]. In this respect, the host cell response to mycobacterial adherence may also be influenced by the relative involvement of other macrophage receptors, such as the MR, or other CR3-associated receptors such as CD14 [51].

Although CR3 mediates a substantial amount of both opsonic and non-opsonic phagocytosis of *M. tuberculosis* by macrophages, its role in pathogenesis remains unclear. When bone marrow-derived macrophages from CR3-knockout mice were

used, results showed a reduced binding and phagocytosis of *M. tuberculosis* without affecting the production of ROIs/NO radicals and bacterial survival [52]. An *in vivo* study using intravenous inoculation of *M. tuberculosis* [53] did not show a difference in bacterial burden or pathology between CR3-knockout and wild-type mice. As the unique compartmentalization of the immune response in the lung is well established, the relative role of the C3–CR3 pathway in *M. tuberculosis* pathogenesis during airborne infection remains unresolved.

Apart from the MR and CRs, other macrophage receptors may also participate in *M. tuberculosis* phagocytosis either alone or in conjunction with CRs and/or the MR (Figure 1.1). SR-A participates in the uptake of non-opsonized *M. tuberculosis* by macrophages [54]. CD14 has been found to mediate uptake of non-opsonized *M. tuberculosis* by human microglia, the resident macrophage in the brain [51], and uptake of *M. bovis* by porcine AMs [55]. Dectin-1 is primarily a PRR for fungal β-glucan [56]. Since mycobacteria lack β-glucan on their surface, dectin-1 may not be considered as a potential adhesion receptor. However, there is recent evidence that dectin-1 in cooperation with TLR-2 mediates the production of tumor necrosis factor-α by mouse bone marrow-derived macrophages infected with avirulent or attenuated mycobacteria strains, but not virulent strains [57]. Since TLRs are known as 'signaling receptors' rather than phagocytic receptors, several phagocytic receptors cooperate ('cross-talk') with TLRs for downstream signaling processes in order to induce the production of immune modulators in the host cells. The TLR agonists derived from mycobacteria and the TLR coreceptors on host cells involved during mycobacterial infections have been described elsewhere [3]. It is noteworthy that FcγRs do not play a role in the phagocytosis of *M. tuberculosis* in the absence of specific antibody [58], whereas opsonization with immunoglobulin in the immune host can lead to phagolysosomal fusion presumably via entry through FcγRs [59].

1.5
Relationship Between Route of Entry and Survival for *M. tuberculosis*

For professional phagocytes such as macrophages, the time frame from the phagocytosis of a microbe to the maturation of its phagosome is relatively short [60]. Thus, it is critical to explore the phagocytic process itself in initiating the development of the unique phagosome of *M. tuberculosis* as well as the nature of the early inflammatory response. Macrophage PRRs are known to recognize a variety of microbial surface determinants, and bridge innate and adaptive immune responses by regulating endosomal and phagosomal traffic as well as pro- and anti-inflammatory cytokine production. Although much attention has focused on the unique nature of the *M. tuberculosis* phagosome, only recently has there been evidence for the role of specific macrophage PRRs in directing this process (Figure 1.1). As noted above, human macrophages primarily use the MR and CR3 for the phagocytosis of *M. tuberculosis* [47]. These receptors are distinguished by the fact that they mediate the internalization of microbes without necessarily triggering a pro-inflammatory response [60] and thus are postulated to enhance the early intracellular survival of host-adapted intracellular pathogens.

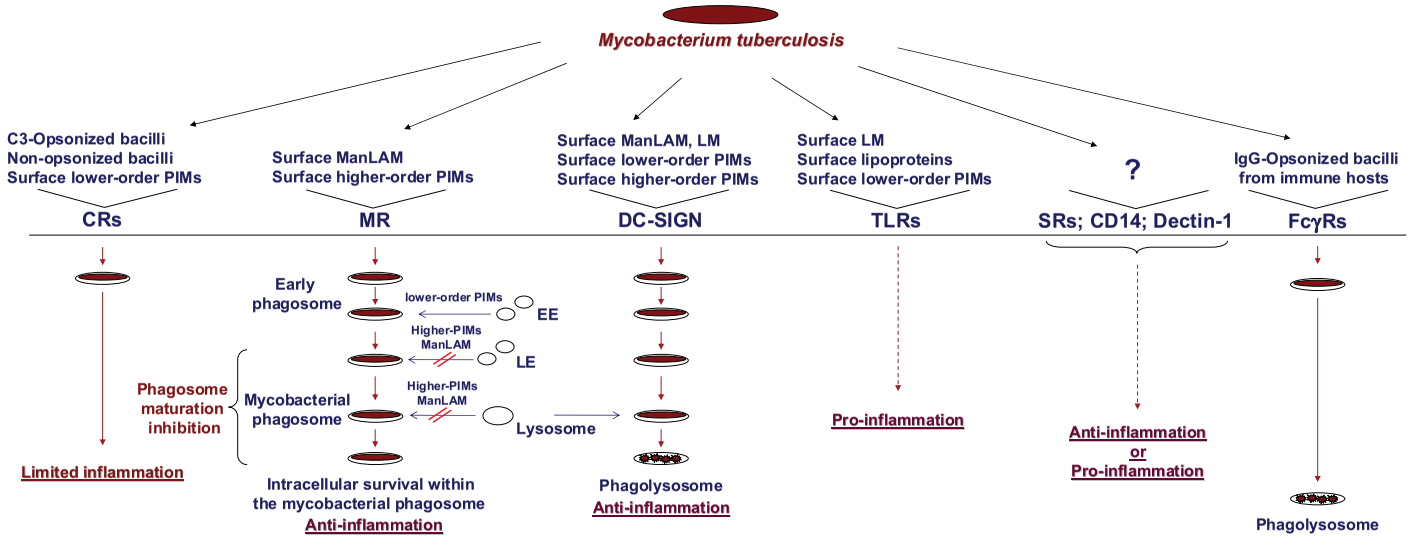

Figure 1.1 Host cell recognition and response to *M. tuberculosis*. Mycobacterial cell wall components and opsonized bacilli associate with a subset of immune receptors and PRRs to initiate phagocytosis and the development of specific host cell responses. Mannosylated lipoglycoconjugates on the surface of *M. tuberculosis* engage the MR on macrophages resulting in the development of a mycobacterial phagosome that has limited fusion with lysosomes; whereas engagement of DC-SIGN on DCs by these lipoglycoconjugates leads to the development of a phago-lysosome. Interaction with specific receptors also leads to the activation of pro- and/or anti-inflammatory pathways in the host cell that influences intracellular survival of the bacterium. EE, early endosome; LE, late endosome.

M. tuberculosis surface mannosylated lipoglycoconjugates also play an important role in regulating phagocytosis and the host response. *M. tuberculosis* ManLAM inhibits phagosome–lysosome fusion in macrophages [61,62] (see below). The MR has recently been shown to regulate this process of reduced phagosome–lysosome fusion in human macrophages following phagocytosis of ManLAM-coated beads and live *M. tuberculosis* bacilli [63]. Higher-order PIMs from *M. tuberculosis* which bind to the MR also participate with ManLAM in limiting phagosome–lysosome fusion [11], consequently aiding the survival of the bacillus in human macrophages. A similar role of the MR in inhibiting phagosome–lysosome fusion in the human macrophage-like cell line THP-1 has been implicated with *M. avium* GPLs [64]. Thus, engagement of specific receptors on the macrophage surface can initiate and influence the development of the unique mycobacterial phagosome (Figures 1.1 and 1.2). The recently published work [63] promotes a two-step model for *M. tuberculosis* phagosome biogenesis. Step 1 involves the recognition of *M. tuberculosis* surface determinants by specific receptors leading to receptor-mediated signaling, actin cytoskeleton reorganization, and directed entry into the nascent phagosome. In step 2, the unique *M. tuberculosis* phagosome is maintained over hours to days. Here *M. tuberculosis* viability is particularly critical and a number of bacterial- and host-derived factors are active in blocking phagosome maturation (see below).

Recent studies show that the MR and DC-SIGN, both C-type lectins that recognize *M. tuberculosis* ManLAM, regulate phagosome trafficking differently. DC-SIGN and its homologues L-SIGN and SIGNR1 target ManLAM and/or mycobacteria to lysosomes in DCs [8,65,66]. This observation is supported by the fact that the cytoplasmic tail of DC-SIGN possesses two signaling motifs, the tri-acidic cluster and di-leucine motif, which target endocytosed molecules to lysosomes and to major histocompatibility complex class II-positive late endosomes [67]. The MR lacks such motifs and has only one tyrosine-based cytoplasmic motif containing Tyr18 (a member of a di-aromatic amino acid sequence), which is involved in efficient phagocytosis and endocytosis [68,69] and endosomal sorting [69]. Thus, these studies raise the possibility that the trafficking fate of *M. tuberculosis* bacilli and ManLAM in a given cell following phagocytosis will depend on the relative amount and activity of the MR and DC-SIGN on the cell surface. Since macrophages express high MR and DCs high DC-SIGN on their surface, it is speculated that this difference may provide one explanation for why macrophages serve as the major intracellular niche for *M. tuberculosis* rather than DCs, which are more specialized for processing endocytosed products in lysosomes.

Apart from its role in directing phagosomal development, MR-dependent phagocytosis is associated with an anti-inflammatory program and not associated with activation of the NADPH oxidase [70,71]. These findings along with the fact that ManLAM inhibits IL-12 production via the MR by generating a negative signal in the cell [72], provide further support that involvement of mannosylated lipoglycoconjugates of *M. tuberculosis* and the MR pathway are beneficial for the early intracellular survival of *M. tuberculosis* in macrophages. The use of this pathway is likely to be particularly important in the lung environment where macrophages express high MR activity.

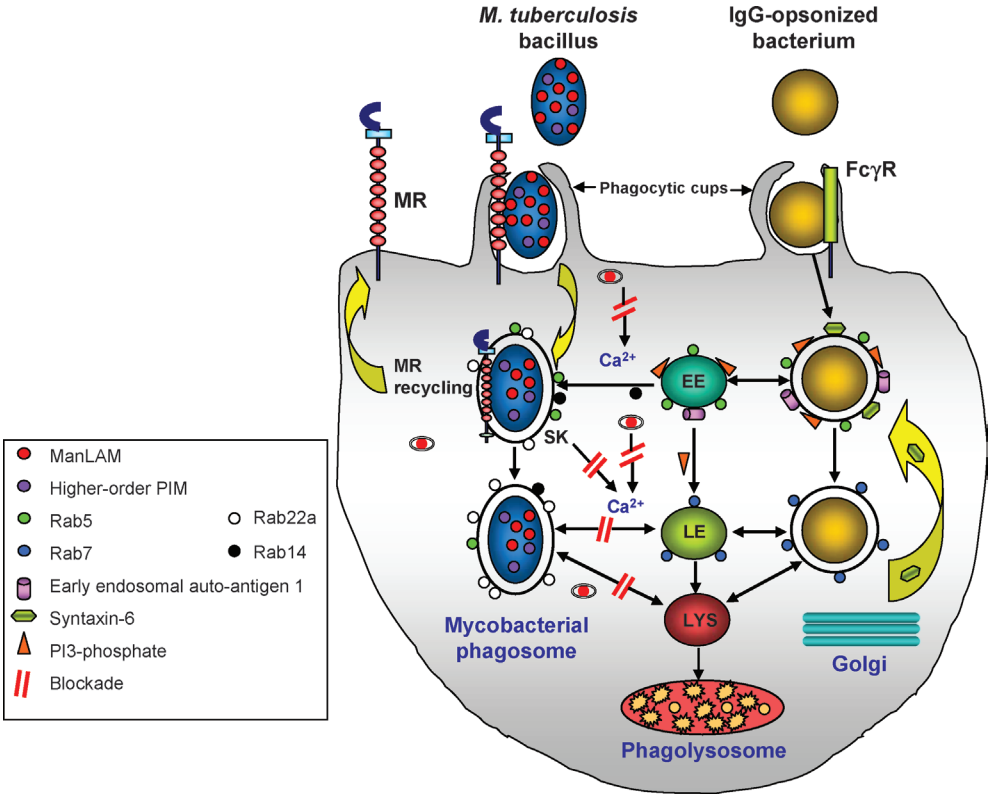

Figure 1.2 Receptor-mediated phagocytic pathways affect the trafficking fate of *M. tuberculosis*. *M. tuberculosis* ManLAM engages the MR during phagocytosis and directs the bacterium to a phagosome with limited phagosome–lysosome fusion. The MR lacks a lysosomal targeting motif and recycles from the early endosomal compartment. *M. tuberculosis* inhibits macrophage sphingosine kinase, and both bacilli and ManLAM inhibit the Ca^{2+}/CaM- and Rab5-dependent recruitment of PI3K. The result is reduced formation of PI3P on the phagosomal membrane. Decreased Rab5 activity on the phagosomal membrane inhibits recruitment of the Rab5 effectors EEA1 and syntaxin-6, which normally allow for full maturation of the phagosome to a phagolysosome. In addition to Rab5, there is recruitment of Rab22a and Rab14 to the mycobacterial phagosome which also contributes to the phagosome maturation block (see in the text). The mycobacterial phagosome excludes Rab7 (thus precluding Rab5 to Rab7 conversion) which is normally present on late endosomes and maturing phagosomes leading to phagosome–lysosome fusion. On the other hand, opsonization of bacteria with IgG in the immune host leads to their uptake by FcγRs on macrophages. FcγR-mediated phagocytosed bacteria follow the normal trafficking pathway culminating in phagosome–lysosome fusion. EE, early endosome; LE, late endosome; LYS, lysosome; SK:, sphingosine kinase.

Studies show that engagement of DC-SIGN by ManLAM leads to suppression of DC maturation through TLR signaling [65]. Both *M. tuberculosis* and *M. bovis* BCG strains can infect DCs by association with DC-SIGN through their surface ManLAM and downregulate host innate immune responses by suppressing IL-12 and inducing IL-10 production [65]. However, in the case of *M. bovis* BCG, the exclusive role of DC-SIGN in such events is controversial [73]. In a recent study [17], DC-SIGN expression was shown to be induced in AMs from TB patients (not expressed in uninfected individuals) by an as yet unidentified factor, therefore indicating a role for DC-SIGN in *M. tuberculosis* colonization of the lung.

The presence of cholesterol within lipid rafts on the cell membrane plays an important role in receptor-mediated phagocytosis and in regulating phagosome maturation. Non-opsonic phagocytosis of *M. kansasi* by CR3 on human neutrophils was found to be cholesterol-dependent [74]. In a recent study, depletion of cholesterol in *M. avium*-infected mouse bone marrow-derived macrophages resulted in phagosome maturation and fusion with lysosomes, and led to the assembly of live bacilli in phago-lysosome-derived autophagic vacuoles [75]. The lack of cholesterol in micro-domains of the membrane abolishes the sequestration of many signaling molecules and is likely to impair the anchorage and trafficking of relevant host receptors. Thus, the composition of the cell membrane and its associated receptor complexes are also important in regulating the entry of mycobacteria into host cells and the nature of the host response.

1.6
Host Intracellular Trafficking Regulators and Phago-Lysosome Biogenesis

An understanding of the block in *M. tuberculosis* phagosome maturation requires in depth knowledge of the fundamental aspects of mammalian cell biology, membrane trafficking and protein sorting, the specifics of phago-lysosome biogenesis, as well as identification of mycobacterial factors affecting such processes. The *M. tuberculosis* maturation block involves interference with the recruitment and function of the small Rab GTPases, Rab-interacting effectors, for example, phosphatidylinositol 3-kinases (PI3Ks) and membrane tethering molecules, membrane fusion proteins SNAREs (for soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) molecules, and membrane-associated lipids that serve as tags recruiting and guiding membrane trafficking machinery.

There are close to 70 members of the Rab family of GTP-binding proteins in the human genome [76], with members specializing in regulating intracellular trafficking in various organellar compartments and pathways. Rabs and Rab-interacting partners [effectors such as the long-range membrane tethering molecule early endosome antigen 1 (EEA1) and short-range catalyzers of membrane fusion called SNAREs], including lipid-modifying enzymes (kinases and phosphatases), play a role in maintaining organellar identity and progression along a given pathway. For example:(1)Rab11 and its effectors define the pericentriolar recycling endosome and(2)Rab5 to Rab7 conversion (i.e. a coordinated replacement of one Rab with the other on an entire organelle) in the endosomal [77] or phagosomal systems [78,79]

controls the transition of an endosome or a phagosome from an early, non-degradative organelle to a lysosomal, degradative compartment. Rabs and their effectors accomplish this by organizing other proteins and lipids to either maintain a given state of an organelle or promote its change, in the endosomal and phagosomal systems, often referred to as organellar maturation. Rabs function as molecular switches, and are considered to be in the ON state when bound to GTP and switched OFF when bound to GDP. Consequently, a major regulatory point in membrane trafficking is whether a Rab is loaded with GTP, which happens with a help of a GTP nucleotide exchange factor (GEF), or whether it is induced by GTPase activating factors to undergo GTP hydrolysis, thus acting as a GTPase, and becomes GDP loaded. Another essential feature of Rabs is that they are recruited to membranes, often via GEFs, and insert their doubly prenylated lipid tails in the membrane that they either regulate or use as a transit station to a point further downstream of the organellar trafficking pathway where they finally act [76]. Thus, specific organellar association and GTP/GDP bound states are the major determinants of Rab function.

An important aspect of how Rabs earmark membranes for fusion is their interactions with phosphatidylinositol kinases (such as hVPS34 PI3K) and phosphatases. For example, as Rab5 recruits hVPS34, this lipid kinase converts phosphatidylinositol into phosphatidylinositol 3-phosphate (PI3P) on the cytofacial leaflet of an endosomal membrane. The membrane patches of PI3P recruit proteins that serve as organellar tethering factors (e.g. EEA1) or regulators of protein and membrane sorting (e.g. Hrs) via their PI3P-binding domains. Rabs also interact with bifunctional effectors, which bridge and transfer the function between different Rabs that act in succession within a pathway. This is how Rabs control organellar identity and remodeling as organelles transit through different stages of a pathway. It turns out that Rabs, their effectors and lipid product are targeted by mycobacteria to modulate *M. tuberculosis* phagosomal characteristics [80].

1.7 Rabs and Rab Effectors Affected by *M. tuberculosis*

The close relationship of phago-lysosome biogenesis to the endosomal pathway has facilitated work on and understanding of the *M. tuberculosis* phago-lysosome biogenesis block. It was established in the 1990s that the *M. tuberculosis* phagosome, which is not acidified to the extent seen in phagosomes harboring inanimate objects or dead bacteria [81], do not acquire the key late endosomal Rab, Rab7 [78]. It turned out recently that the process of Rab7 acquisition by phagosomes is akin to a critical point in the endosomal pathway, referred to as Rab conversion [82], where the marquee early endosomal Rab, Rab5, is abruptly and simultaneously replaced on an entire early endosomal organelle by Rab7. The departure of Rab5 and concomitant replacement by Rab7 is the key event in changing an endosomal organelle: the repertoire of interactions between various trafficking pathways in the cell and an endosome transforms it from a sorting organelle into a degradative, lysosomal compartment. A recent study [79] has shown that another Rab, Rab22a, controls this process.

M. tuberculosis recruits to its phagosome copious amounts of Rab22a. Rab22a controls aspects of endosomal recycling and appears to signal to the trafficking machinery that not all recycling is completed, precluding a Rab5 to Rab7 conversion. The recruitment and maintenance of Rab22a contributes to the block in acquisition of Rab7, precluding subsequent steps of acidification and activation of hydrolases.

It has been known that the *M. tuberculosis* phagosome is not a static organelle and that it receives nutrients, such as iron-loaded transferrin, from early endosomal organelles [83]. It has been established that this is a vital feature of *M. tuberculosis* phagosomes, as Rab5, controlling endocytosis and early endosomal trafficking, was found to support intracellular mycobacterial survival [84]. Recently, Rab14 has been shown to promote *M. tuberculosis* phagosome fusion with early endosomes [85], thus acting in series with Rab5. Rab5 is involved in early stages, while Rab14 plays a role in long-term maintenance of mycobacterial phagosome interactions with early endosomal organelles.

Several studies have examined the role of Rab effectors in phagosomal maturation. It was found that the *M. tuberculosis* phagosome shows aberrant acquisition of tethering molecules, such as EEA1 [86], and the multivesicular body endosome sorting regulator Hrs [87]. It is likely that these changes on mycobacterial phagosomes reflect altered succession and aberrant composition of Rabs on the *M. tuberculosis* phagosome.

1.8
Host Lipids Affected by *M. tuberculosis*

It has been established that mycobacteria control levels of PI3P on their phagosomes [86,88,89]. Normally, PI3P is generated on endomembranes in mammalian cells by one of the Rab5 effectors, the type III PI3K hVPS34. The sequence of events is as follows.(1)*M. tuberculosis* inhibits Ca^{2+} fluxes in infected macrophages [90,91]. (2)This affects calmodulin (CaM) and CaM kinase II recruitment to mycobacterial phagosomes [92].(3)CaM is found in complexes with hVPS34 [93], and interruption of Ca^{2+}-CaM–hVPS34, coupled with the inhibition of Rab conversion and exclusion of Rab7 – a hVPS34-interacting Rab [94], leads to reduced PI3P production on *M. tuberculosis* phagosomes. PI3P is important for maturation into a late endosomal compartment and a requirement for PI3P has likewise been established in phagosome maturation [86,95].

1.9
Mycobacterial Products Affecting Phago-Lysosome Biogenesis

There have been several strategies undertaken to identify mycobacterial factors affecting phagosomes:(1)a random, genetic shotgun approach with mutant *M. tuberculosis* libraries in screens for mutants that are enriched in phagosomes progressing past the early, non-acidified phagosomal stage [96,97], and (2)rational testing of products

that interfere with trafficking processes in macrophages identified through cell biological studies [61,89]. The random screens have yielded non-overlapping sets of genes [96–98]. Their further characterization will be necessary to assign specific functions. The rational search for lipid and protein factors has identified several lipids [61,63] and proteins [89,99] shown or proposed to play a role in mycobacterial phagosome maturation arrest.

Mycobacterial PIMs and LAM represent nearly perfect mimics of host cell phosphatidylinositol and phosphoinositides, thus giving an incentive to investigate their potential interference with phosphoinositide interconversions (e.g. PI3P generation) on mycobacterial phagosomes. As noted earlier, ManLAM has been shown to interact with the MR and that this plays a role in the phago-lysosome maturation block [63] (Figure 1.2). ManLAM has been shown to intercalate into the membrane [100] and to traffic within the endomembranes of cells infected with mycobacteria [101]. ManLAM, by a yet to be fully delineated mechanism, affects intracellular Ca^{2+} fluxes leading to hVPS34 recruitment and PI3P production [93], thus interfering with a key process in phago-lysosome biogenesis [80]. Lower-order PIMs also play a role in phagosome maturation arrest, as they promote fusion between mycobacterial phagosomes and early endosomes [102]. Attesting to the key role of PI3P in mycobacterial phagosome maturation arrest is that a secreted protein, SapM, which has been originally described as an acid phosphatase [103], actually has a strong PI3P phosphatase activity, and plays a role in mycobacterial phagosome maturation block [89].

1.10
Induction of Autophagy Overcomes *M. tuberculosis* Phagosome Maturation Block and Kills Intracellular Mycobacteria

Another fundamental membrane trafficking process dependent on PI3P in host cells is macroautophagy (a major form of autophagy), herein referred to simply as autophagy (Figure 1.3). Autophagy is an important cellular maintenance mechanism [104,105], with cells digesting parts of their own cytoplasm for turnover of stable macromolecules or for removal of unwanted organelles and aggregates. During macroautophagy, portions of the cytoplasm are sequestered into autophagosomes, which are morphologically very distinct organelles delimited by a double membrane that wraps around the intended cytoplasmic target. An autophagosome ultimately delivers the trapped cytoplasmic content to lysosomes for degradation. Typically, under starvation conditions, autophagy is primarily aimed at turning over long-lived cytosolic macromolecules, such as long half-life proteins or overproliferated intracellular organelles, to support cellular anabolic needs and deplete surplus organelles thus ensuring cellular viability [106]. Autophagy furthermore removes damaged organelles including compromised mitochondria, thus protecting cells from unscheduled apoptosis. Although autophagy, when induced in moderation, is primarily a cell survival mechanism, its sustained activation at high levels can lead to programmed cell death [107] referred as type II. When autophagy leads to cell death, there is also a connection between autophagy and apoptosis [108]. Autophagy

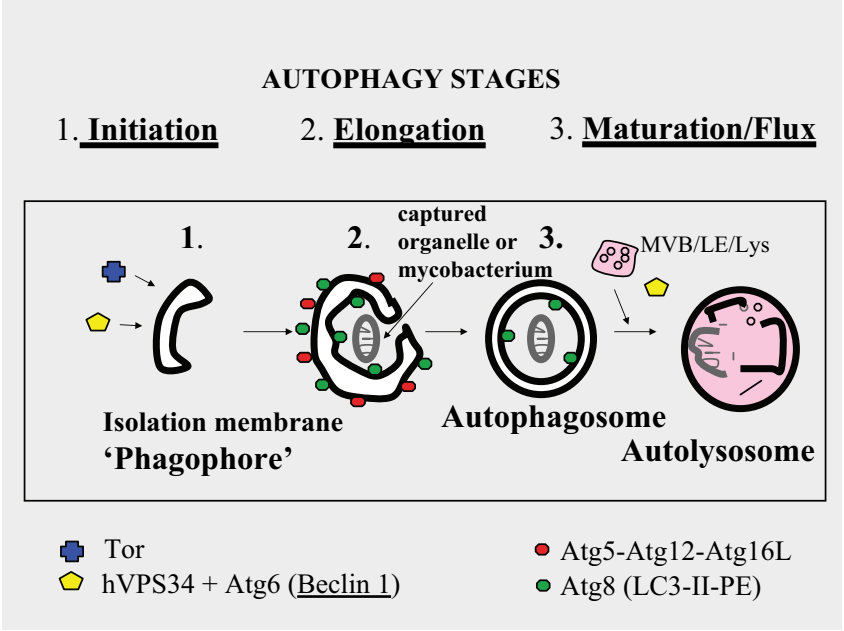

Figure 1.3 Autophagy as a homeostatic process captures and destroys cytoplasmic organelles or intracellular pathogens. Autophagy requires the action of hVPS34 and production of PI3P at the initiation and maturation stages along the execution stages of the autophagic pathway. Beclin 1(Atg6) is a subunit of the hVPS34 complex affecting autophagy. A nascent autophagosome, termed isolation membrane (phagophore), forms around and organelle, a section of the cytoplasm, or around a bacterium or a phagosome containing mycobacteria. It elongates and bends its membrane with the help of Atg factors forming two complexes: (1) Atg5 is conjugated to Atg12, and associates with Atg16. (2) Atg8 is also known as LC3; its membrane associated form, known as LC3-II, is conjugated C-terminally to phosphatidylethanolamine (PE) (3) MVB, multivesicular bodies; LE, late endosome; Lys, lysosome. Induction of autophagy by pharmacological, physiological, or immunological agonists results in control of intracellular *M. tuberculosis* (see text). Reproduced from [124] with permission.

has been implicated in development, aging, cancer and neurodegenerative disorders, such as Huntington's, Parkinson's and Alzheimer's diseases [104,109]. Of particular significance, autophagy is also an immunity effector against intracellular bacteria and viruses [110–113]. The capture and destruction of intracytoplasmic contents by autophagy appears custom made for cleaning up intracellular microbes.

The execution aspects of autophagy shown in Figure 1.3 are controlled by the upstream signal transduction systems. A classical physiological inducer of autophagy is amino acid starvation, in particular a withdrawal of branched aliphatic side-chain amino acids or absence of growth factors that normally regulate uptake of nutrients [114]. A pharmacological induction of autophagy can be exacted using rapamycin, which targets the Ser/Thr kinase Tor [115]. When Tor is active, this suppresses autophagy, but when Tor is inactivated (e.g. when the cells are treated with rapamycin) Tor promotes autophagy. Importantly, PI3P and the same PI3K involved in

phagosome maturation, hVPS34, only with a different, autophagy-specific subunit termed Beclin 1, are essential for autophagy [116]. This has served as an impetus for researchers to test whether induction of autophagy could overcome the phagosome maturation block imposed by *M. tuberculosis*. Indeed, this has proven to be the case [112,117]. It turned out that not only can physiological, pharmacological or immunological agonists of autophagy stimulate mycobacterial phagosome maturation, but they can also reduce viable *M. tuberculosis* counts in the course of hours. The most recent advance in this area is that the immunity-related GTPases (also known as p47 GTPases), which have been implicated in the control of intracellular pathogens, but whose mechanism of action remained elusive, induce autophagy [117,118].

The idea that autophagy may defend against pathogens hails back to a number of studies implicating autophagy in host–pathogen interactions [105]. A stream of recent publications has fully affirmed the role for autophagy in innate immunity [75,110–112,119–121]. Autophagy eliminates intracellular microbes similarly to autophagic capture and digestion of unwanted or damaged intracellular organelles. It is now evident that autophagy serves as a mechanism for the removal of intracellular bacteria and viruses [122,123], in keeping with its primary function as a cytoplasmic clean-up process.

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