Molecular basis of mycobacterial survival in macrophages

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Abstract

Macrophages play an essential role in the immune system by ingesting and degrading invading pathogens, initiating an inflammatory response and instructing adaptive immune cells, and resolving inflammation to restore homeostasis. More interesting is the fact that some bacteria have evolved to use macrophages as a natural habitat and tools of spread in the host, e.g., *Mycobacterium tuberculosis* (Mtb) and some non-tuberculous mycobacteria (NTM). Mtb is considered one of humanity's most successful pathogens and is the causal agent of tuberculosis, while NTMs cause opportunistic infections all of which are of significant public health concern. Here, we describe mechanisms by which intracellular pathogens, with an emphasis on mycobacteria, manipulate macrophage functions to circumvent killing and live inside these cells even under considerable immunological pressure. Such macrophage functions include the selective evasion or engagement of pattern recognition receptors, production of cytokines, reactive oxygen and nitrogen species, phagosome maturation, as well as other killing mechanisms like

autophagy and cell death. A clear understanding of host responses elicited by a specific pathogen and strategies employed by the microbe to evade or exploit these is of significant importance for the development of effective vaccines and targeted immunotherapy against persistent intracellular infections like tuberculosis.

AQ1

AQ2

Keywords

Intracellular pathogens
Phagocytosis
Tuberculosis
Immune evasion
Phagosome maturation
Inflammatory signaling

Introduction

The ability of pathogens to cause disease is what is referred to as virulence, and for some pathogens, this may depend on the ability to reside within host cells. Intracellular microorganisms cause severe diseases, including malaria, HIV/AIDS, tuberculosis, typhoid fever, and listeriosis, resulting in significant morbidity and mortality worldwide [1, 2, 3]. Common to these pathogens is the perturbation of host cell function, thus hampering or altering conventional combat strategies to promote an intracellular life. All viruses, certain bacteria (e.g., *Mycobacterium tuberculosis* (Mtb), *Listeria monocytogenes,* and *Salmonella typhi*), and protozoa can survive inside mononuclear phagocytes and, sometimes, within other cell types [4]. Within cells, bacteria are protected from humoral attack mechanisms like antibodies but are exposed to efficient cell-mediated immune responses. Depending on the invading microbe, the contribution of the different cell types in the immune system and molecules that the cells secrete to yield a desired response will vary widely. The innate immune response is effectuated by a diverse repertoire of conserved molecules and mechanisms of elimination of intruders. Microbes are first recognized by pathogen-recognition receptors (PRRs) at the plasma membrane of immune

cells like macrophages, followed by phagocytosis and the gradual maturation of the phagosome into a phagolysosome, where microbes are digested [5]. Different pathogen-associated molecular patterns (PAMPs) are displayed and activate distinct PRRs present in the various cellular compartments, thus initiating inflammatory signaling and antimicrobial pathways [6, 7]. Defense mechanisms must act together in perfect concert in space and time to ensure a rapid and potent response to invading pathogens. The majority of microbes are recognized and eliminated through degradation by these first-line innate defense mechanisms, usually without any signs or symptoms of disease.

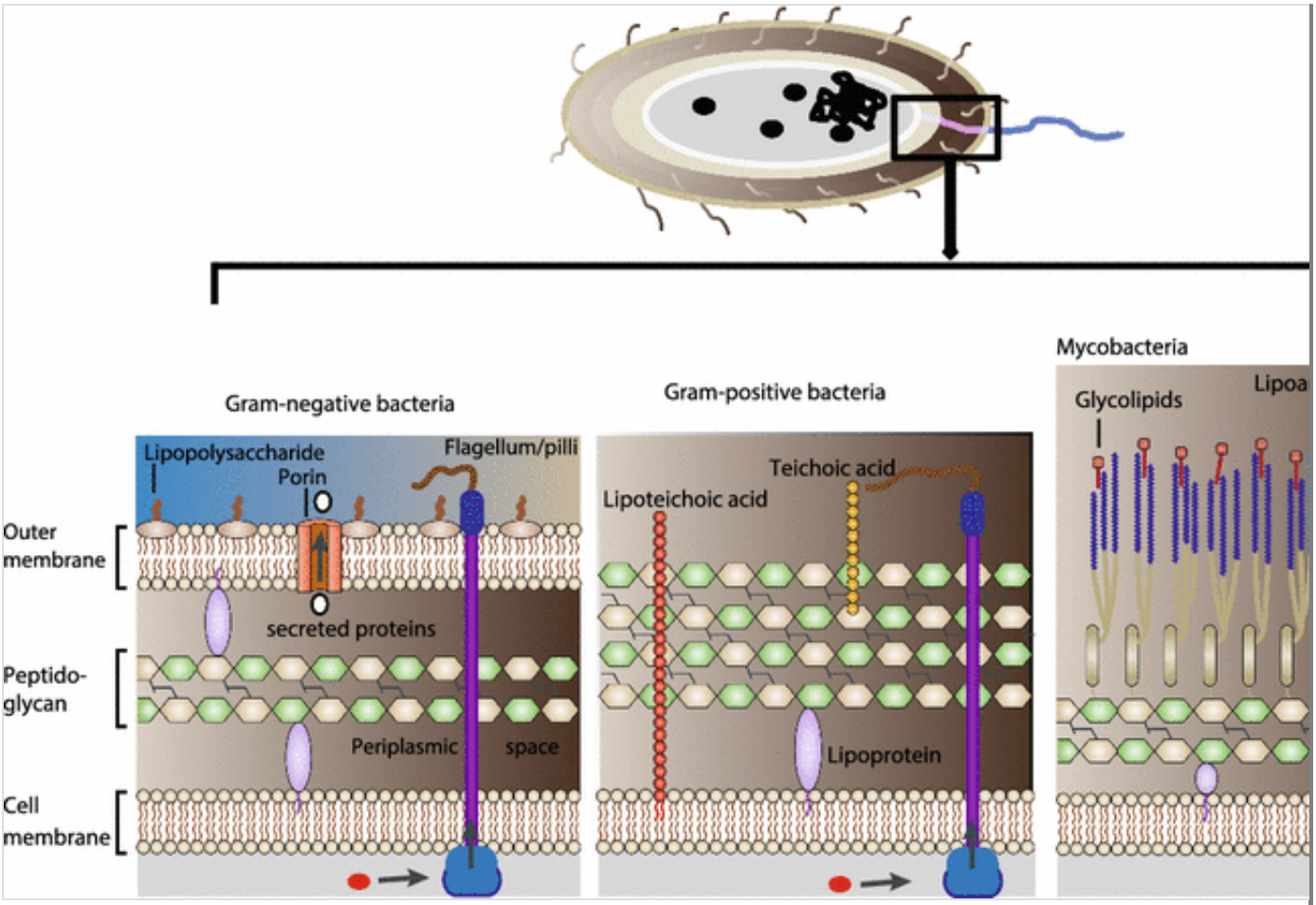
PRRs are found in different combinations in the various cell types of the immune system but also in non-immune cells like epithelial cells lining the body's mucosal surfaces [7, 8, 9, 10]. This means that such non-professional immune cells to some degree if infected might themselves be able to restrict growth and survival or eliminate intracellular pathogens. This model of infection control is now referred to as cell-autonomous immunity, commissioned to guard both individual immune and non-immune cells against the immediate threat of infection [9]. However, intracellular bacteria have evolved efficient specialized mechanisms to protect themselves from detection and the harsh environment of the degradative enzymes and antimicrobial molecules encountered within the cells and, when needed, purposely elicit host responses favoring their survival or spread. We review such concepts and effector mechanisms in bacteria, with an emphasis on mycobacterial infections believed to represent one of the most successful intracellular pathogens known to date. Tuberculosis (TB) is caused by Mtb and kills more than 1.5 million people worldwide each year, and estimated two billion people carry latent Mtb infection [11]. The prevalence of non-tuberculous mycobacterial infections caused by *Mycobacterium avium* is increasing in immunocompromised individuals due to underlying disease or immunosuppressant drugs [12, 13]. *M. avium* lacks several of the key virulence factors of Mtb, but nonetheless can establish chronic infections. Inefficient treatments and emerging antimicrobial resistance [11] create a need for novel treatment strategies; however, their development requires an improved understanding of host–pathogen relations.

All bacteria are composed of and secrete PAMPs, including cell wall components and nucleic acids that are potent stimulators of the immune response (Fig. 1) [14]. Mycobacteria possess a complex cell wall with a thin

peptidoglycan layer that acts as a protective barrier on the cell membrane and a scaffold for the attachment of polymers and proteins. An arabinogalactan layer associates with a dense layer of long-chain beta-hydroxy fatty acids called mycolic acids, making mycobacteria highly resistant to chemical damage, antibiotics, and dehydration. Mycobacterial infections are largely difficult to treat, because most antibiotics cannot breach the densely mycolylated cell walls [15]. Phosphatidyl inositol (PI) forms the backbone for the majority of the cell wall components, including lipomannan (LM), lipoarabinomannan (LAM), and mannosylated LAM (manLAM). Other components include hydrophobic lipids, such as cardiolipin (CL), mycobacterial cord factor, or trehalose dimycolate (TDM), and trehalose monomycolate (TMM), and hydrophilic PI-mannosides (PIMs), phthiocerol dimycocerosate (PDIM), glycolipids, and phospholipids, all important in bacterial virulence [15, 16].

Fig. 1

Schematic illustration of the different bacterial cell walls and their associated PAMPs. *Left* Gram-negative bacterial cell wall consists of a thin layer of peptidoglycan in the periplasmic space between the inner and outer lipid membranes. The outer membrane contains lipopolysaccharides on its outer leaflet and transport channels, such as porins. *Centre* Gram-positive bacteria lack an outer membrane but have a single lipid membrane surrounded by a cell wall composed of a thick layer of peptidoglycan and lipoteichoic acid, which is anchored to the cell membrane by diacylglycerol. *Right Mycobacteria* spp cell wall components surround a lipid membrane and include thin peptidoglycan and arabinogalactan layers, and a thick layer of mycolic acids. Glycolipids, porins and lipoarabinomannan and its variants, which are anchored to the cell membrane by diacylglycerol, are also important components. In addition, these bacteria secrete proteins and nucleic acids are also recognized by host PRRs



Surface recognition and phagocytosis of mycobacteria

Phagocytic receptors

Extracellular PRRs are the first receptors to encounter, recognize, and facilitate internalization of microbes through phagocytosis in macrophages, dendritic cells (DCs), and neutrophilic granulocytes. Actin- and receptor-mediated opsonic or non-opsonic phagocytosis may occur depending on whether or not the microbe is coated by soluble PRRs like complement and antibodies. Opsonic receptors include Fc receptors, integrins, and complement receptors (CR), although CR3 can also mediate recognition without opsonins. Non-opsonic receptors important in microbial recognition include CD169 and CD33, C-type lectins (Dectin-1 and -2, mannose receptor (MR), MCL, Mincle, and DNGR-1) that recognize carbohydrate moieties, and scavenger receptors (SR-A, AIM, MARCO, and CD36) that recognize lipoteichoic acids, lipoglycans, and TDMs [16, 17]. Together with toll-like receptors (TLR)2/6, Dectin-1 can mediate both particle uptake and modulate cytokine production. Dectin-1

dimerizes via a cytoplasmic ITAM motif, recruits Src, and then Syk and CARD9, which have been implicated in the uptake of fungi, including *Candida albicans,* and mycobacteria [18, 19, 20]. Recently, Dectin-2 was identified as the direct receptor for mycobacterial manLAM in bone-marrow-derived dendritic cells (BMDCs), leading to production of cytokines and promotion of antigen presentation. In addition, upon mycobacterial infection, Dectin-2 knockout mice showed augmented lung pathology [21]. Mincle is a receptor for the mycolic acid, TDM [22]. The immunogenic properties of TDM have been linked to specific genes, including *pcaA*, *cmaA2,* and *mmA4*, which upon deletion led to different modifications of mycolate structure and altered host immune responses [23, 24]. However, Mincle-deficient mice revealed no significant differences compared with wild type in organ pathology or bacterial burden [25] and preliminary studies in our lab have not yielded any differences between wild-type and Mincle-deficient macrophages. Engagement of MR by LAM and manLAM can also mediate uptake of Mtb and modulate cytokine production by human macrophages [16]. However, MR-deficient mice show similar bacterial growth and pathology to wild-type mice following aerosol infections [26]. Thus, a considerable redundancy in the use of phagocytic receptors is obvious for mycobacterial uptake [26]. Other intracellular pathogens may actively induce or block their uptake by different cell types. *Shigella*, *Salmonella*, enteropathogenic *E. coli*, and *Yersinia* species use the type III secretion systems (T3SS) to deliver virulence proteins directly into the cell cytosol [27]. *Salmonella* and *Shigella* secreted effectors promote the uptake of these bacteria into non-phagocytic cells, whereas those from *Yersinia* inhibit phagocytosis by macrophages [27]. Some of these proteins induce membrane ruffling and uptake via micropinocytosis [28].

Surface Toll-like receptors

TLRs are the most widely studied PRRs thought to be key sensors of invading pathogens and cooperate with phagocytic receptors to promote uptake and signaling [17]. In humans, 10 TLRs have been identified and 12 in mice; TLR1 to 9 are conserved in both humans and mice, while TLR10 is expressed in humans but not in mice, and TLR11, 12 and 13 are expressed only in mice. TLRs are distributed to different cellular compartments to provide optimal access to their ligands [7]. Human TLR1, 2, 5 and 6 are found on the plasma membrane where they respond to external PAMPs like peptidoglycans,

lipopolysaccharide (LPS), lipoteichoic acids (LTAs), flagellin, arabinogalactan, and LAM [7]. Our group has contributed extensively to the understanding of how TLRs function in host defense reactions against pathogens [29, 30, 31, 32]. TLR homo- or heterodimers activate many signaling pathways mediated via the recruitment of different Toll-IL-1 receptor (TIR) domain containing adaptor molecules, such as MyD88, TRIF, TIRAP, and TRAM, to the TIR domains of the different TLRs [33]. MyD88 recruitment activates IL-1R1-associated protein kinases (IRAKs) to form a large complex, the 'myddosome'. This complex interacts with TNF receptor-associated factor 6 (TRAF6) that activates the TAK1 complex that leads to NF-κB activation and MAP kinase activation. TAK1 phosphorylates IκB kinase (IKK) β in the canonical IKK complex comprised of IKKα-IKKβ-NEMO which, in turn, phosphorylates NF-κB inhibitory protein, IκBα, targeting it for proteasomal degradation and release of NF-κB. Activated NF-κB and MAPKs, in turn, drive transcription of inflammatory cytokines and antimicrobial programs. TLR signaling is regulated by various factors, including; CD14, CD36, Nrdp1, A20, SHP-1, TMED7, CD11b, NLRP4, NLRX1, Keap1/Cul3/Rbx1 complex, and multiple miRNAs [7, 34].

In general, with TLR1 or TLR6, TLR2 recognizes di-acyl or triacyl lipopeptides, respectively [7, 16, 31]. In mycobacteria, TLR2 recognizes PIMs, PDIM, LM, glycoproteins, and LAM (Table 1). Through the less activating mannosylated LAM (manLAM), virulent mycobacteria may avoid the TLR2 pathway to modify the host environment for an intracellular life. In addition, although contradictory, some studies have found an association of TLR2 polymorphisms in humans with enhanced susceptibility to pulmonary TB [35, 36]. LPS is detected by TLR4 as a homodimeric complex with myeloid differentiation 2 (MD2) protein [37]. In Mtb, TLR4 recognizes cell wall lipids, glycoproteins, and secreted proteins, although tetra-acylated LM is considered the most specific TLR4 ligand [16]. The role for TLR4 in the innate response to Mtb in vivo has been shown in mouse models [38], although it is not entirely clear how the signaling occurs. TLR2 responses to mycobacteria appear to be more prominent than TLR4 responses and may be largely strain dependent. TLR mutations appear to be rarely fatal, but four primary immunodeficiencies involving adaptors, MyD88, IRAK4, NEMO, and IκBα mutations, are associated with susceptibility to Mtb. Mice deficient of MyD88, TNF, or IL-1R

are extremely susceptible to infections with either Mtb or *M. avium* [39, 40]. However, this susceptibility appears to be mainly due to the role of MyD88 in IL-1R signaling, which also shares this adaptor with TLRs [41, 42]. The importance of this MyD88-dependent pathway in Mtb immunity in humans remains to be established, as the few individuals with natural deficiencies in MyD88 or IRAK4 do not show enhanced susceptibility to mycobacterial infections as opposed to other deficiencies as in IL-12 and interferon (IFN)-γ [43, 44].

Table 1

Relevant pattern recognition receptors (PRRs), cellular localization, and associated pathogen-associated molecular patterns (PAMPs) for mycobacteria

PRRs	Localization	PAMPs	References
TLRs			
TLR2	Plasma membrane	LAM, LM, 38- and 19-kDa (LpqH) mycobacterial glycoproteins, PIM, triacylated (TLR2/TLR1), or di-acylated (TLR2/TLR6) lipoproteins, chaperon proteins	[31, 35, 36, 38, 274, 275]
TLR4	Plasma/endosomal membrane	Tetra-acylated LM, HSP65, 50S ribosomal protein	[16, 38, 276, 277]
TLR9	Endosomal membrane	CpG DNA	[143, 144, 278]
NLRs			
NOD2	Cytosol	MDP	[165, 166, 168]
NLRP3	Cytosol	Undefined but ESAT-6 is implicated	[177, 178, 190]
DNA sensors			
AIM2	Cytosol	dsDNA	[183]
cGAS	Cytosol	dsDNA	[194]
CLRs			

MR	Plasma membrane	Mannose (LAM and manLAM)	[16]
DC-SIGN (human)/SIGNR3 (mouse)	Plasma membrane	LAM, manLAM, LM and LpqH	[279, 280]
Dectin-1	Plasma membrane	Uncharacterized	[281]
Dectin-2	Plasma membrane	High mannose, α-mannans, lipoproteins	[21]
Mincle and MCL	Plasma membrane	α-Mannans, glycolipids, mycolic acids (TDM)	[22]
Scavenger receptors			
CD36	Plasma membrane	ManLAM and LM	[282]
MARCO (with TLR2/CD14)	Plasma membrane	TDM	[283]
SR-A	Plasma membrane	TDM	[283]
CD5L (AIM)	soluble	Unspecific	[284]
Other receptors			
CD14	Plasma membrane	LAM and chaperonin 60.1	[285, 286]
CR3	Plasma membrane	Mycobacterial antigen 85C, LAM	[287]
Innate immune cells like macrophages, dendritic cells (DCs), neutrophils, and other non-professional immune cells recognize and engage germline-encoded PRRs that recognize conserved microbial structures of pathogens known as pathogen-associated molecular patterns (PAMPs) and activate the expression of inflammatory mediators			
LAM lipoarabinomannan, *LM* lipomannan, *manLAM* mannosylated LAM, *PIM* phosphatidylinositol mannosides, *TDM* trehalose 6,6′-dimycolate, *ESAT* early secreted antigenic target-6, *SR-A* scavenger receptor A, *TLR* toll-like receptor, *NLR* NOD-like receptor, *NLRP3* NACHT, LRR and PYD domains-containing protein 3, *MR* mannose receptor, *CR3* complement receptor 3, *DC-SIGN* dendritic cell-specific intercellular adhesion molecule-3-Grabbing non-integrin, *AIM2* absent in melanoma 2, *CLR* C-type lectin receptor			

Intracellular pathogens attempt to skew the balance of inflammatory cytokines, towards anti-inflammatory conditions to improve their survival chances within macrophages. Mtb proteins, such as hsp60 and PPE18, induce the anti-inflammatory cytokine IL-10 in human THP-1 cells in a TLR2-dependent

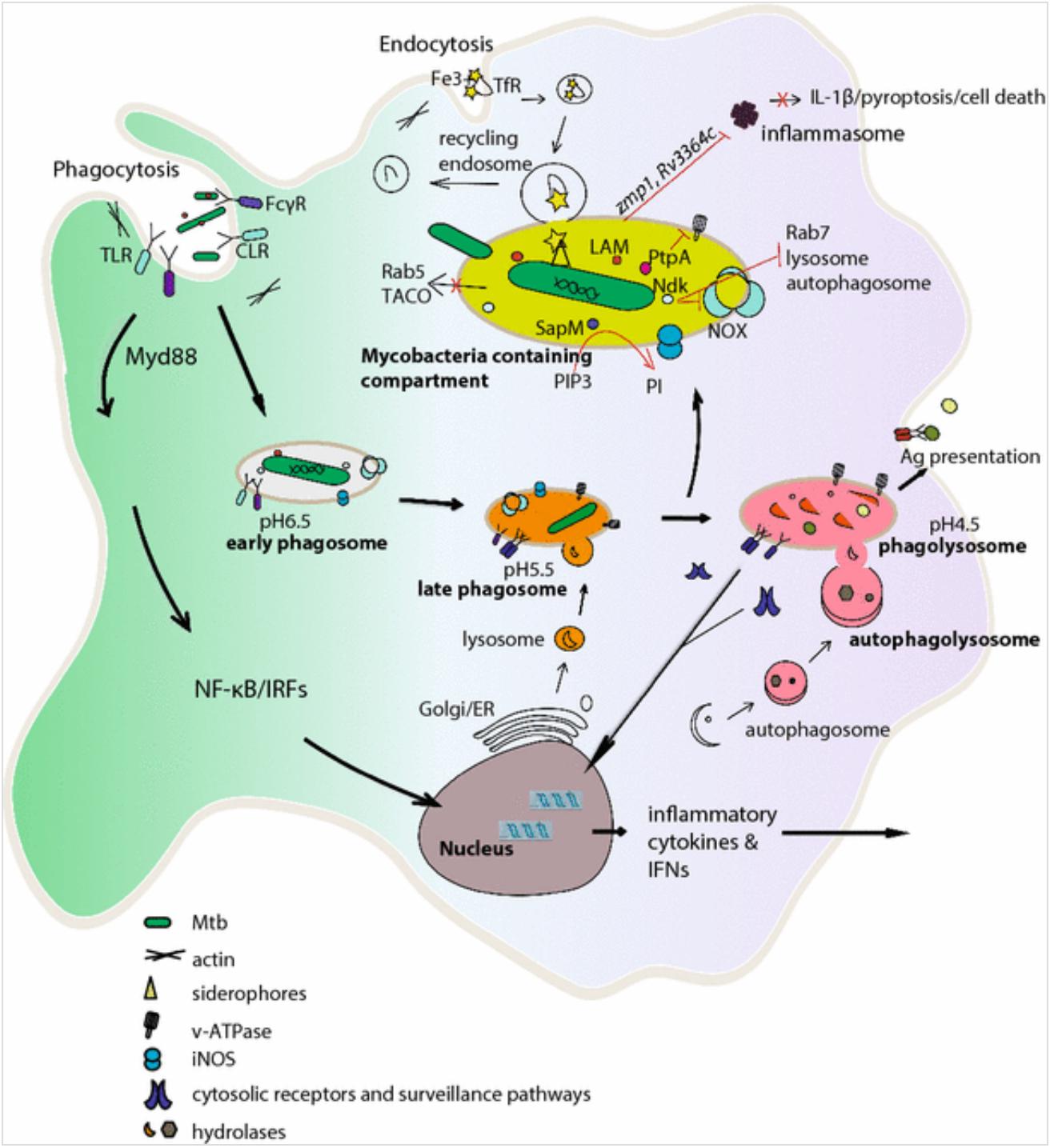
manner [45, 46]. The mycobacterial early secreted antigenic target (ESAT-6) also binds TLR2 and inhibits IL-12 production in RAW macrophages [47]. Furthermore, Mtb LpqH, LprA, and LprG proteins may interact with TLR2 to inhibit IFNγ-induced MHC-II antigen presentation in THP-1 cells and murine macrophages [31, 48]. These inhibitions are lost when cells are pre-treated with an anti-TLR2 antibody or siRNA, making TLR2 a valuable target for Mtb. Another interesting turn is that Mtb-infected macrophages may release chemokines that attract Mtb-permissive cells [49]. PIM and a di-acylated LM can inhibit MyD88- and TRIF-dependent signaling, likely downstream of TLR4, in response to LPS [50]. PDIM is another lipid that may be used by Mtb to evade TLR signaling, as shown in a zebrafish model of *M. marinum* infection. These data suggest that the absence of PDIM leads to increased TLR activation and thus increased bacterial clearance [51].

Receptor-mediated internalization of microbes occurs via actin-dependent zippering of the plasma membrane around the microbes (Fig. 2). Actin polymerization is controlled by Rho GTPase proteins and involves the small GTPases, Rac and Cdc42, and the Arp2/3 complex that nucleates actin filaments [17, 52]. Yet another mechanism of uptake is through lipid raft aggregation on target host cells in response to interactions with microbes [53]. Some bacterial proteins induce vital changes in lipid raft formation and organization [54, 55, 56] in a caveolin-dependent manner [55]. During Mtb and *M. avium* infections [54, 57], translocation of TLR2 to lipid rafts was shown in BMDMs treated with the Mtb lipoprotein LpqH [58], and cholesterol clustering around *M. bovis* BCG during infection has also been shown. Subsequent depletion of cholesterol inhibited uptake of the bacteria in murine macrophage cell line [57]. Furthermore, cell wall phenolic glycolipids have been shown to promote the recruitment of macrophages that are tolerant to Mtb. This seemed to occur via upregulation of host chemokine receptor 2 expression and inhibition of the microbicidal effects of macrophages that responded to TLR ligands and produce reactive radicals [51].

Fig. 2

Mycobacterial phagocytosis and interference with phagosomal maturation and autophagy. Mycobacteria enter the cell through passive phagocytosis and there is a great deal of redundancy in receptor usage, once inside the macrophage phagosomes normally fuse with lysosomes for degradation of the content.

Pathogenic mycobacteria are known to interfere with phagosome maturation and take residence within a macrophage compartment. Our research indicates that the compartment may be in the endosomal recycling pathway. Within macrophages mycobacteria experience iron starvation, but can access iron through interaction with endosomes providing them with transferrin-iron. Mycobacteria can be detected by various factors on membranes and cytosol, and autophagy, a mechanism whereby the bacterium or the compartment is wrapped by a double membrane into an autophagosome that will fuse with lysosomes for degradation. Recent studies have shown that phagosomal escape of Mtb or at least permeabilisation of the phagosome triggers autophagy. Some mycobacteria, e.g., *M. avium,* may escape phagosomal degradation and signaling even when fused with late endosomes or lysosomes, although the mechanisms around this remain elusive. Mycobacterial evasion and survival strategies, in particular, mycobacterial iron acquisition, and also mycobacterial proteins that may interfere with phagosomal maturation and autophagy are illustrated in *red lines*



Host–pathogen interactions during phagosomal maturation

Reactive oxygen and nitrogen species (ROS and RNS) production

Full activation of murine macrophages depends on IFNγ, PRR activation, and/or TNF, while human primary macrophages need vitamin D2 in addition as a cofactor, which enhances the expression of antimicrobial peptides/proteins

(AMPs), such as cathelicidin and other antimicrobial capacities, including ROS and RNS generation [6]. RNS/ROS production is a rapid process occurring within minutes in response to phagocytosis of microbes via the enzymatic activity of inducible nitric oxide synthase 2 (NOS2) and NADPH oxidase (NOX2), respectively [59]. NOS2 is expressed primarily in murine macrophages and is induced by cytokines and microbial products, notably IFNγ and LPS [60]. Similarly, activated macrophages produce ROS via activation of NOX2. The NOX2 complex is made up of two associated transmembrane proteins; $gp91^{phox}$ and $gp22^{phox}$, four cytosolic subunits, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac1, and a role in innate immunity is well established [61]. In the phagosome, NO and ROS can spontaneously react to generate highly reactive intermediates that destroy microbial membrane lipids, DNA, and thiol- and tyrosine residues by oxidation, and NO can directly target the iron sulfur clusters of bacterial enzymes. In humans, polymorphisms in the *NOS2* or *CYBB* (coding for gp91phox) genes have been associated with an increased susceptibility to TB [62, 63]. However, while ROS is important for cell signaling, sustained ROS production can be detrimental to the cells and tissues. The Keap1-Nrf2 system is a well-established system that regulates the expression of cyto-protective genes in response to electrophilic and oxidative stress. The function of Keap1 as a stress sensor and an adaptor component of the Cullin 3 (Cul3)-based ubiquitin E3 ligase complex has been quite well studied [64, 65]. Apart from its role as a ROS sensor, there is mounting evidence that Keap1 and its Cul3-Rbx1 E3 ligase complex are involved in the regulation of inflammatory responses in cancers [66, 67], and we have shown a role in *M. avium* infection in primary human macrophages [34].

Mycobacteria have evolved to also dampen the antimicrobial activity of ROS. The Mtb protein, enhanced intracellular survival (Eis), may enhance survival of intracellular mycobacteria by ROS-dependent modulation of autophagy, inflammatory responses, and cell death. Macrophages infected with an Mtb Eis-deficient mutant H37Rv displayed elevated TNF, IL-6, and ROS levels, and increased accumulation of large autophagic vacuoles compared with wild-type or complemented strains [68]. The Mtb nucleoside diphosphate kinase (Ndk) is shown to interact with and inactivate Rac1, important in recruiting NOX2 to the phagosome, thus preventing NOX2 assembly. Consistent with these data, Ndk-deficient Mtb showed impaired survival both in vitro and in vivo [69].

Similarly, Mtb protein, *nuoG*, is a potential virulence factor that seems to inhibit NOX2, although the mechanism behind this is still undefined [70]. Mycobacteria and other intracellular bacteria, such as *Salmonella*, *Yersinia,* and *Staphylococcus,* employ a battery of protective enzymes, including superoxide dismutase, catalase, alkyl hydroperoxidase, and peroxiredoxins, to neutralize the free radicals generated by the host cell well discussed by Trivedi and colleagues [71]. To release this arsenal of protective enzymes, Mtb must sense changes in its environment. Oxygen, NO, and CO levels in the environment are sensed via proteins, DosS, and DosT, while WhiB3 and anti-sigma factor RsrA monitor changes within the cell. Mtb has thus evolved to survive in nutrient-deficient, acidic, oxidative, nitrosative, and hypoxic environments, such as in granulomas [71].

Phagosome maturation

Early phagosomes mature into late phagosomes, and then into phagolysosomes via sequential fusion and fission with intracellular vesicles, referred to as phagosome maturation. Antimicrobial mechanisms of the mature macrophage phagosome include acidification, production of AMPs, activation of NADPH oxidase and NOS2, and degradative enzymes, such as cathepsins. Various Rab GTPases, phosphoinositides, and kinases control-specific stages of this process, and, therefore, can be used as markers to identify intracellular compartments and follow trafficking [5]. The proton pump v-ATPase is recruited to phagosomes to facilitate luminal acidification and to activate lysosomal hydrolases and cathepsins that degrade phagolysosomal content. Different PAMPs become exposed and activate distinct PRRs present in the various cellular compartments and the cytosol, thus initiating inflammatory signaling and antimicrobial pathways [6, 7] (Fig. 2). However, intracellular bacteria, such as Mtb*, S. aureus, L. pneumophila,* and some fungi [72, 73, 74, 75], can escape or block phagosomal maturation, reside, and replicate in compartments that are uniquely adapted to each pathogen. Mtb has been proposed to manipulate the macrophage by modifying the phagosomal environment to make it more conducive, or escape to the cytosol. MR interaction with the cell wall lipids, manLAM, or PIMs may contribute to the generation of the mycobacterial compartment [76] and then some sort of maturation block or delay thereafter. This is thought to happen at an early endosomal stage, because first, the Mtb and *M. avium* phagosomal compartments (MtbC and MavC, respectively)

maintain their pH at 6–6.5 by excluding v-ATPases and preventing fusion with lysosomes while still accessing essential nutrients like iron and fats [39, 77, 78]. Second, MtbC and MavC retain the early endosomal marker Rab5, but not the late endosomal marker Rab7, thus suggesting arrest at an early stage. We have shown that the MavC interacts with the Rab11+ recycling endocytic pathway, from which it can access iron loaded on transferrin, and thus avoid the antibacterial protein, lipocalin 2 that traffics to lysosomes [79, 80]. A few electron microscopy studies in mouse macrophages suggest that pathogenic mycobacteria do not block phagosomal maturation at an early endosomal stage, but instead temporarily reside in phagolysosomes before re-establishing a new compartment [81, 82]. Our preliminary data suggest that it is true for *M. avium* in human primary macrophages; *M. avium* traffics to phagolysosomes from where a fraction is sorted to LAMP-1-negative compartments that support replication and long-term survival (Gidon and Flo et al., unpublished). To determine definitively when phagosomal maturation arrest occurs, careful analysis of the spatiotemporal localization of mycobacteria within cells is needed.

Mycobacterial phagosomes thus need to be constantly maintained through virulence factors and secretion machineries that allow the pathogens to deliver effector proteins into the cytosol to manipulate host cell signaling and avoid lysosomal fusion. Mycobacteria in non-degrading phagosomes require an all-around tight apposition between the mycobacterial surface lipids and the phagosome membrane. Interference with this close apposition leads to rapid maturation of phagosomes [83]. Other stress factors like low pH, oxidative stress, and nutrient deficiency in the maturing phagosome can act as important cues to the bacteria enabling them to mount counteractive mechanisms. Several studies have analyzed the transcriptome and proteome of Mtb and *M. avium* grown under stress conditions like pH and oxidative stress in culture or isolated from infected macrophages [39, 78, 84, 85, 86]. The induction of pH-responsive genes and those involved in utilization of alternative carbon sources, such as host-derived fatty acids and cholesterol, have been shown to be important [84, 85, 86]. Host cholesterol is essential for persistence of Mtb in the lungs of chronically infected mice and for growth within IFNγ-activated macrophages that mainly occur in chronic infection. Mtb thus have an unusual capacity to catabolize sterols and circumvent nutrient deprivation, and thereby sustain a

persistent infection [87]. Cholesterol mediates the phagosomal recruitment of TACO/coronin-1, an actin-binding host protein that is normally released prior to phagosome fusion with lysosomes [57]. Recruitment and retention of TACO to mycobacterial phagosomes prevent phagosome maturation and allow the mycobacteria to escape the bactericidal action of lysosomes [88]. In addition, in a TLR2/4-dependent manner, lipid requirement was also recently validated by a demonstrated decrease in phagosomal lipolysis and an increased retention of lipids by Mtb-infected macrophages [89]. Several studies have provided insight into the regulation mechanisms of nitrogen metabolism in Mtb, showing in particular the key role of the glutamine synthetase GlnA1 and its regulator GlnE [90], and the asparagine transporter AnsP2 and the secreted asparaginase AnsA to assimilate nitrogen and resist acid stress through asparagine hydrolysis and ammonia release. AnsA seems to be crucial in both phagosome acidification arrest and intracellular replication, as an Mtb asparaginase mutant was severely restricted in macrophages and mice [91].

Other factors implicated in mycobacterial modulation of phagosome maturation include: cell wall lipids, lipoproteins, secretory systems, enzymes, etc., as well as host-associated factors, and the list seems to continuously grow. At least partial phagosome maturation arrest by Mtb is dependent on the cell wall component, LAM [92, 93]. LAM incorporates into human macrophage membrane rafts, blocks calcium signaling, and inhibits the type III phosphatidylinositol 3 kinase (PI3 K) necessary for the fusion and fission of vesicles involved in phagosome maturation in murine macrophage cell lines [93, 94]. The lipoprotein LprG, which binds to lipoglycans, such as LAM, and mediate Mtb immune evasion, facilitates the transfer of LAM from the plasma membrane into the cell envelope, increasing surface-exposed LAM, allowing inhibition of phagosome–lysosome fusion, and enhancing Mtb survival in macrophages. *lprG*$^{-/-}$ mutants had lower levels of surface-exposed LAM and failed to inhibit phagosome–lysosome fusion in murine macrophages and were promptly cleared in the lungs of infected mice [95, 96]. Another strategy used by Mtb and *M. avium* to overcome phagosomal maturation is the secretion of phosphatases which dephosphorylate kinases required for phagosome maturation. The secreted 28-kDa acid phosphatase, SapM and protein- and lipid-phosphatases, protein-tyrosine phosphatase A (PtpA), and PtpB are among these. SapM inhibits the generation of phosphatidylinositol 3-phosphate (PI3P),

which is essential for phagosome biogenesis [97]. PtpA blocks v-ATPase trafficking to the phagosome and phagosome–lysosome fusion [98, 99]. Mtb Ndk blocks murine macrophage Rab5 and Rab7 GTPases, and inhibits recruitment of their effector, RILP, leading to reduced phagolysosome fusion [100]. We have preliminary data suggesting that inflammatory signaling is initiated from *M. avium* phagolysosomes but not from MavCs, suggesting that *M. avium* not only escape lysosomal degradation but also PRR recognition and inflammatory signaling (Fig. 2) (Gidon and Flo, unpublished). Clarifying how *M. avium* manages to establish this safe haven, e.g., if PRRs are excluded from the mycobacterial compartments, if ligands are not proteolytically processed for recognition due to elevated pH, or if downstream signaling is prevented is essential for a complete picture of phagosome maturation block, with implications for other similarly pathogenic bacteria.

Mycobacteria may also exit the phagosomal location or communicate with the extra-phagosomal environment via the secretion of virulence factors through a specialized ESX secretion system also known as type VII secretion system. The ESX-1 secretion system is encoded by a genomic region called the region of difference 1 (RD1), and secretes ESAT-6 and its associated protein, the 10-kDa culture filtrate protein (CFP-10) [101]. A part of the RD1 region is deleted from the genome of other NTMs and *M. bovis* BCG, the only licensed vaccine strain against tuberculosis [102]. Genomic integration of the extended RD1 locus from Mtb into the genomes of *M. bovis* BCG and *M. microti* restored ESAT-6 secretion and increased the virulence of these strains [103, 104], and deletion of this region from Mtb resulted in attenuation of Mtb, underlining the role of the ESX-1 secretion system in Mtb virulence [105]. An important effect of the ESX-1 secretion system is its contribution to Mtb translocation from the phagosome to the cytosol of macrophages and DCs, observed after about 2–4 days. In the cytosol, bacteria undergo replication and culminate in the death of infected cells and bacterial spread [104, 106]. In fact, some Mtb strains were shown to be poorly adapted for subsistence within endocytic vesicles of infected macrophages. Instead, through a mechanism involving activation of host cytosolic phospholipase A2, they could escape and multiply in the cytosol [107]. ESAT-6 seems to be the culprit here where it inserts into membranes forming a membrane-spanning pore that disrupts the phagosomal membrane providing access of the bacteria and bacterial products to the cytosol [104, 108].

Mtb nucleic acids and a necrotizing toxin are believed to enter the cytosol via the ESX-1 secretion system and trigger cytosolic PRRs and autophagy, or necrosis by hydrolyzing NAD, respectively [108, 109, 110]. *M. avium,* on the other hand, that lacks the ESX-1 secretion system does not seem to translocate into the cytosol. There are four other ESX clusters in the Mtb genome, ESX-2–ESX-5 well covered by Simeone and colleagues [101]. There is an emerging role for ESX-3 of Mtb and *M. smegmatis* in iron and zinc acquisition [111, 112]. ESX-5 of Mtb and *M. marinum* are involved in the maintenance of cell wall integrity and the secretion of a group of proline–proline–glutamate (PPE) motif containing proteins thought to be recognized by host cells and implicated in antigenic variation of mycobacterial strains [113]. In Mtb, the accessory SecA2 system, which is conserved in bacteria [114], has also been implicated in phagosome maturation arrest. Sullivan and colleagues showed that shortly, after infection of primary murine BMDMs, phagosomes containing a ΔsecA2 mutant of Mtb were more acidified and showed greater association with markers of late endosomes than phagosomes containing wild-type Mtb. This study also suggests that effectors of phagosome maturation are exported into the cytosol by the accessory SecA2 system [115]. ~~If the SecA2 system translates for *M. avium* remains to be determined. However, an *M. avium*-specific~~In *M. avium* the gene MAV_2941 encodes a PI3 K mimic protein that is exported by the oligopeptide transporter OppA in the ATP-binding cassette (ABC) transport system to the macrophage cytoplasm. MAV_2941 protein interacts with host proteins and interferes with normal phagosome maturation. Mutations in MAV_2941 were associated with impairment of growth in THP-1 macrophages [116].

AQ3

Other classic examples of pathogens known to escape phagosome maturation include *Salmonella*, group A *Streptococcus* (GAS), *Listeria*, *Shigella*, and *Rickettsia* species [117, 118, 119]. *L. monocytogenes* produces listeriolysin and two phospholipase C enzymes (PlcA and PlcB) [119] and GAS makes streptolysin O [120], that make pores and ruptures phagosomes allowing escape of bacteria into the cytosol. *Shigella*, *Salmonella*, enteropathogenic *E. coli*, and *Yersinia* species use the T3SS to deliver virulence proteins directly into the cell cytosol [27]. The *Shigella* T3SS apparatus was shown to be directly involved in mediating cytosolic translocation in epithelial cells [121], and the secreted

protein, IpgD, promotes the efficiency of *Shigella* escape [118]. The bacterial factors known to affect *Salmonella* containing vacuole (SCV) integrity depend on the stage of the SCV. Escape from nascent SCVs seems to involve the T3SS translocon, although the mechanism by which T3SS-mediated pore formation facilitates that vacuole membrane destabilization is not clear and possibly involves additional factors as is the case in *Listeria* [122]. A homologue of the *Shigella* effector IpgD, SopB can dephosphorylate soluble inositol polyphosphates and inositol phospholipids, and hence regulate SCV maturation [123]. In late SCVs, a number of T3SS effectors contribute to stability: SifA, PipB2, SopD2, and SseJ [122]. ActA is a critical virulence protein in *Listeria* that triggers actin polymerization around bacteria, and forms actin comet tails facilitating bacterial motility in the cytosol, the formation of host cell surface protrusions, and cell-to-cell spread [124]. *M. marinum* escapes from its phagosome and also develops actin tails that allow it to move freely in the cell cytosol as seen in primary mouse and fish macrophage cells [125, 126].

Phagosomal PRRs

Microbial nucleic acids are the major components recognized by endosomal TLRs. TLR3, 7, 8, and 9 are primarily expressed in endosomes and sense nucleic acid PAMPs [7, 127, 128] and host nucleic acids [129]. These TLRs, except for TLR3, recruit MyD88 and initiate MyD88-dependent signaling, but also activate the interferon (IFN) regulatory factors (IRFs) to induce IFNs in macrophages and DCs. TLR3 is abundantly expressed in most innate immune cells and recognizes viral double-stranded RNA in endosomes in a TRIF-dependent mechanism [7, 33]. TRIF-dependent pathways in complex with the adaptor TRAM on the endosome activate IRFs that drive the expression of type I IFNs (IFNα/β) important in the host defense against infection and particularly viral infections. TRIF-dependent signaling of TLR4 occurs from endosomes in response to LPS. This initiates the signaling required for IRF3 activation [130, 131]. While MyD88, TIRAP, TRIF, and TRAM form complexes with TLRs to promote TLR signaling, the most recent TLR adaptor to be found, sterile α, and armadillo motif containing protein (SARM), negatively regulates TLR3 and TLR4 signaling through direct binding to MyD88 and TRIF [132].

TLR7 and TLR8 recognize viral ssRNA as well as bacterial RNA from, e.g., *S. pyogenes* and *S. aureus* in macrophages and conventional DCs in a species-

specific manner [133, 134, 135, 136]. TLR8 recognizes uridine together with short oligoribonucleotides [137, 138], whereas TLR7 senses guanosides or modified guanosides together with oligoribonucleotides [139]. Recent data identify human TLR8 as functional murine TLR13 equivalent that promiscuously senses ssRNA [140]. Its ligand consensus motif was broader than the 'UGG' motif described earlier [137], including both 'UAA' and 'UGA' [138]. This implies that human TLR8 can bind to a variety of RNA ligands, including Sa19 mitochondrial (mt) 16S rRNA sequence-derived oligoribonucleotides, *S. aureus*-, *E. coli*-, and mitochondrial RNA in peripheral blood mononuclear cells, and THP-1 cells [138]. We have shown that TLR2 may negatively regulate TLR8-induced IFNβ in response to *S. aureus* RNA [134]. TLR8 seems important in the control of viral infections and some bacterial infection, and interestingly, two studies point to an association of *tlr7* and *tlr8* polymorphisms with pulmonary TB [141, 142]. The mechanism through which TLR8 recognizes Mtb and signals intracellularly remains unknown. TLR9 is expressed by pDCs, macrophages, and B cells, and recognizes bacterial and viral DNA that is rich in unmethylated CpG DNA motifs, as well as synthetic CpG oligonucleotides [7, 143]. The exact signaling compartments used, and if different endolysosomal compartments show different signaling capacities are still controversial and require more detailed evaluation. In fact, the manner in which the ligands become available to receptors may also direct the signaling outcome. Both murine and human studies have provided some more insight in to the role of TLR9 in Mtb infections. TLR9-deficient mice are more susceptible to a low dose of Mtb aerosol infection compared with wild types, although there is no significant difference in bacterial load [144]. Similarly, in humans, TLR9 polymorphisms have been associated with susceptibility to Mtb [145, 146], suggesting that TLR9 may play a role in primary human infections.

Type I IFNs are central in viral defenses, whereas the role in bacterial infections is less clear and may vary with the pathogen. Perhaps, the best-described examples of a harmful role for IFNα/β are in infections with *L. monocytogenes* and Mtb. Three research groups independently found that *Ifnar1*$^{-/-}$ mice are resistant to *L. monocytogenes* infection, with a longer survival than wild-type mice [147, 148, 149], attributed to reduced apoptotic cell death, particularly of lymphocytes, with IFNα/β sensitizing these cells to the *L. monocytogenes* LLO

and resultant cell death in wild-type mice. In later studies, *L. monocytogenes*-induced IFNα/β could potently inhibit antimicrobial pathways during infection for example by blocking the responsiveness of macrophages to IFNγ through downregulation of IFNγR expression [150, 151]. In Mtb infections, similar to *Listeria*, some studies performed in patients and mouse models have reported a decreased bacterial burden and/or improved host survival in the absence of IFNα/β-mediated signaling [152, 153, 154]. Berry and colleagues first showed that patients with active tuberculosis had a prominent whole blood IFNα/β-inducible transcriptional profile that correlated with disease stage, corroborated by others [155, 156, 157]. In mice, infection with hyper-virulent Mtb strains showed a correlation between increased levels of IFNα/β and increased virulence [152, 154]. The exact bacterial effectors involved in this IFNα/β-mediated virulence are still unclear, but recent studies point to induction of immunosuppressive IL-10 and IL-1Ra, downregulation of IFNγR, and inhibition of protective IL-1 and IL-12 [158, 159].

Cytosolic detection of bacteria

The mechanisms governing the fate of bacterial pathogens that enter or, from an intracellular compartment communicate with the cytosol of mammalian cells continue to be of profound interest. Various mechanisms aid macrophages to sense the presence of invaders within the cytosol. Cytosolic PAMPs include nucleic acids, peptidoglycans, polysaccharides, proteins, and lipids. Nucleic acid sensors exist with a seemingly ever expanding list extensively covered by Pandey and colleagues [7]. Cytosolic receptors include leucine-rich repeat (LRR)-containing nucleotide-binding and oligomerization domain (NOD) proteins, NOD-like receptors (NLRs), retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs), and other DNA and RNA sensors. Engagement of these receptors results in pro-inflammatory cytokine production, autophagy activation, and different forms of cell death [16]. Type I IFNs are also induced through the stimulator of interferon genes (STING) and the adaptor TBK1, which are central in some of these pathways [108, 160]. Three distinct subfamilies exist within the human NLR family; the NODs (NOD1, NOD2, NOD3/NLRC3, NOD4/NLRC5, NOD5/NLRX1, and CIITA), the NLRPs (NLRP1-14, or NALPs), and the IPAFs (IPAF/NLRC4, NAIP) [161]. NLRs sense a wide range of cytoplasmic ligands. Upon engagement, they activate NF-κB or MAP kinases to induce inflammatory cytokines, type I IFNs or activate

the assembly of a multiprotein complex, the inflammasome [162]. NOD1 and NOD2 are mainly expressed in the cytosol and recognize D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), respectively [163]. PAMP recognition initiates oligomerization of these sensors, which subsequently recruit a CARD-containing adaptor protein, RIP1 via CARD–CARD interactions, and activate NF-κB and MAP kinases [164]. $NOD2^{-/-}$ murine macrophages and DCs produce low levels of TNF, IL-12, RANTES, and NO following Mtb infection, although $NOD2^{-/-}$ mice were no more susceptible than their wild-type counterparts [165]. Similarly, knockdown of NOD2 in human macrophages led to lower TNF and IL-1β following Mtb infection. In contrast to mouse macrophages, NOD2 knockdown in human macrophages led to increased intracellular bacterial growth [166]. Finally, treatment of Mtb-infected human alveolar macrophages with MDP appeared to improve their ability to control Mtb infection possibly by upregulating TNF and IL-6 production and autophagy [167]. An important outcome of cytosolic recognition is the induction of type I IFNs and as earlier noted, Mtb benefits from these type I IFNs. Pandey and colleagues showed in mouse models that intraphagosomal Mtb and MDP stimulate NOD2 in the cytosol via membrane damage by ESX-1-associated mechanisms. Interestingly, this recognition triggered the expression of type I IFN in a TBK1- and IRF5-dependent manner [168], differing fundamentally from stimulation by bacterial DNA, as shown for *L. monocytogenes*, which depends entirely on IRF3 [169, 170]. Thus, the NOD2 system is specialized to recognize bacteria that actively perturb host membranes and remarkably so, mycobacteria. However, the contribution of NOD2 directly to human immunity to Mtb remains unknown.

The NLRs (NLRP1, NLRP3, NLRC4/IPAF, and NAIP) and non-NLRs could assemble upon sensing different bacterial components except nucleic acids to form the inflammasome complex that is important in the processing of pro-inflammatory cytokines, IL-1β and IL-18, and pyroptotic cell death [171]. A central adaptor of inflammasome assembly is the PYRIN domain (PYD)-caspase activation and recruitment domain (CARD) adaptor (ASC) required by all the receptors [161]. Inflammatory caspases (caspase-1, -4, -5, and -11) are important innate defense mediators associated with inflammasomes. Caspase-1 is activated by ligands of various canonical inflammasomes, and caspase-4/5 and -11 directly recognize bacterial LPS, and can trigger pyroptosis, an

inflammatory cell death process [171, 172, 173, 174]. Upon stimulation, NLRs, ASC, and pro-caspase-1 assemble promoting the proteolytic cleavage and activation of pro-caspase-1 to yield caspase-1. Caspase-1, in turn, promotes cleavage of pro-IL-1 and pro-IL-18 to IL-1 and IL-18, respectively [161, 171]. NLRP1 inflammasome assembly is stimulated by bacterial components like MDP and toxins like anthrax toxin from *Bacillus anthracis,* while IPAF/NAIPs/NLRC4 inflammasomes are induced by needle and rod proteins from bacterial T3SS and flagellin in both human and murine macrophages [161, 175]. The NLRP3 inflammasome is the most widely studied and diverse PAMPs from viruses, bacteria, and fungi, and host DAMPs like ROS that disrupt mitochondrial membrane polarization and dynamics have been reported to activate the NLRP3 inflammasome, in addition to non-microbial substances, such as uric acid and cholesterol crystals [161, 176]. NLRP3 is activated in Mtb infection of both human and mouse macrophages, and human macrophages with an NLRP3-CARD8 gain-of-function mutation are less susceptible to infection [177, 178]. Yet, a specific Mtb ligand that binds directly to NLRP3 remains elusive.

Human caspase-4/5 and the murine homolog caspase-11 are gaining attention and found to be differentially activated by cytosolic and vacuolar bacteria, and by cytosolic bacterial components like LPS which potentiates NLRP3-dependent caspase-1 activation [161, 179, 180]. Caspase-11 function in detecting Gram-negative bacteria has led to the classification of canonical and non-canonical inflammasomes activated by caspase-1 and caspase-11, respectively. Caspase-11 was shown to protect specifically against cytosolic bacteria and not vacuolar bacteria in a mechanism that did not involve the classical inflammasomes [172, 174]. Absent in melanoma 2 (AIM2) inflammasome recognizes cytosolic DNA from bacteria [181]. Mtb DNA is proposed to enter the cytosol via ESX-1 [182] and co-localized with AIM2 in infected macrophages [183], but activation of the AIM2-inflammasome pathway only seems to occur following infection with avirulent Mtb or other avirulent mycobacteria like *M. smegmatis* [184]. However, AIM2-inflammasome activation may be triggered in vivo in the response to virulent Mtb as $AIM2^{-/-}$ mice show impaired IL-1β and IL-18 production and increased bacterial burden compared with wild-type mice [183], although this effect might be due to excess accumulation of DNA in the cytosol leading to a pathologic

production of type I IFNs [182]. Mycobacteria also activate a non-canonical caspase-8-dependent inflammasome and processing of pro-IL-1β through engagement of Dectin-1, although with varying importance for Mtb, *M. leprae* and *M. bovis* BCG [185]. Guanylate-binding proteins (GBPs) are increasingly being implicated in regulating inflammasome complex formation [186, 187, 188]. In mice, GBPs promote innate immune recognition of the vacuolar *S. typhimurium* by destabilizing its vacuole, leading to the translocation of bacteria into the cytosol and subsequent detection of LPS by the caspase-11 inflammasome [188]. Furthermore, GBP1 and GBP7 restrict *M. bovis* BCG and *L. monocytogenes* by recruiting antimicrobial effectors to the pathogen-containing vacuoles [189].

Bacteria can inhibit inflammasome activation by limiting levels of inflammasome triggers or by active inhibition of inflammasome assembly. It has been suggested that virulent mycobacteria can inhibit AIM2 activation in vitro [184]. While infection with *M. smegmatis* could induce both NLRP3 and AIM2 inflammasomes, co-infection with virulent Mtb, resulted in lower AIM2 inflammasome activation. This inhibition of AIM2 required the Mtb ESX-1 secretion system, suggesting that a yet to be defined ESX-1-secreted effector protein(s) modulates AIM2 inflammasome activation in response to Mtb [184]. However, ESX-1 is also inflammasome inducer and activation of cytosolic detection mechanisms can benefit intracellular pathogens. Mtb has been implicated in activation of inflammasomes in an ESAT-6-dependent manner in mouse DCs and macrophages and occasionally without cell death [178, 190]. Inflammasome activation may thus be deliberately triggered or inhibited by virulent mycobacteria to promote intracellular survival, although the mechanisms behind this remain unclear.

Cytosolic DNA may also be sensed via other sensors in the cytosolic surveillance pathway (CSP), centered on the activation of STING. Cyclic GMP-AMP (cGAMP) synthase (cGAS), a member of the nucleotidyltransferase family, has been shown to act as a sensor for cytosolic dsDNA via the endogenous second messenger cGAMP for STING activation in multiple cell types, including macrophages by numerous studies [191]. cGAS interaction with DNA precedes the synthesis of cyclic-di-GMP-AMP (c-di-GAMP) from ATP and GTP, which then binds to STING that dimerizes, translocates to the Golgi from the ER, and facilitates TBK1 activation leading to the induction of

IRF3-mediated IFNβ production [191, 192]. Multiple cell types from *cGAS* knockout mice showed impaired cytokine production in response to transfected immunostimulatory DNA or DNA virus (HSV-1, VACV) infection, while the responses to poly I:C, poly dAdT, and RNA virus infection remained intact, and these knockout mice were more susceptible to lethal HSV-1 infection than wild-type mice [192]. In vitro, cGAS also plays a role in IFNβ production in response to the intracellular pathogens like *L. monocytogenes* [193]. In response to Mtb, the CSP pathway is also activated and was recently show to be needed to induce IFNβ in macrophages [194].

Other bacterial survival strategies

Bacterial inhibition of the lysosomal degradative machinery

Lysosomal enzymes include nucleases, proteases, among others, and ubiquitin-derived peptides required for degradation of microbes. Amphipathic cationic AMPs also play a role in controlling infections, such as the cathelicidin-derived peptide LL-37, that co-localized in MtbC upon TLR2 activation, and this co-localization was associated with decreased bacterial viability [195]. Vitamin D-mediated induction of LL-37 is known to enhance control of pathogens, such as Mtb [195, 196]. AMPs can insert into membranes to form pores by specific mechanisms and several observations suggest that translocated peptides can alter cytoplasmic membrane septum formation, and inhibit cell wall, nucleic acid, and protein synthesis or enzymatic activity [197, 198]. Mechanisms of resistance to these peptides have been well covered in a recent special issue entitled: Bacterial Resistance to Antimicrobial Peptides, in *Biochimica et Biophysica Acta* (*BBA*)—*Biomembranes* [197]. In a screen to determine genes encoding proteins that are associated with resistance to AMPs, a transposon library of *M. avium* strain 104 for susceptibility to polymyxin B, an antimicrobial peptide surrogate was generated. The majority of the genes identified were related to cell wall synthesis and permeability, with impaired ability to enter macrophages and to survive macrophage killing. The mutants were also shown to be susceptible to LL-37, indicating that the *M. avium* envelope is the primary defense against host antimicrobial peptides [199]. Furthermore, pathogenic NTMs appear to resist and inactivate LL-37. LL-37 exposed to mycobacteria had reduced antimicrobial activity likely due to inactivation of LL-37 by mycobacterial lipid component(s) of the cell envelope,

although the mechanisms remain to be clarified [200].

Ubiquitin-derived peptides are antimicrobial molecules generated by proteolysis of ubiquitinated proteins by hydrolytic enzymes. Solubilized lysosomes from resting BMDMs were shown to be bactericidal towards both Mtb and *M. smegmatis* [201]. This bactericidal activity was associated with ubiquitin. Full-length ubiquitin lacks bactericidal activity, but ubiquitin-derived peptides obtained from a cathepsin digest of purified ubiquitin or a synthesized peptide Ub2 were bactericidal against mycobacteria. Like cationic peptides, Ub2 likely polymerizes and inserts into bacterial membranes compromising their integrity [202]. To counter these antimicrobial molecules, the reduced membrane permeability of mycobacteria provides intrinsic resistance against antimicrobial compounds, including bactericidal ubiquitin-derived peptides [203]. In addition, the *M. smegmatis AhpD* transposon mutant was shown to be Ub-peptide hyper-susceptible. Overexpression of OxyS in *M. smegmatis* reduced transcription of the *AhpCD* genes, which encode a peroxide detoxification system. Thus, RoxY, OxyS, and AhpD play a role in the mycobacterial oxidative stress response, and are important for resistance to host antimicrobial peptides [204].

Manipulation of autophagy

One prominent mechanism that effectively eliminates intracellular bacteria is autophagy, originally thought to be a bulk degradation process important for nutrient turnover in cells. Engulfment and degradation of microbes via autophagy are referred to as xenophagy [205]. Several studies have shown xenophagy as a downstream effector of PRR signaling with roles in immunity and inflammation [206, 207]. One of the first reports that showed autophagy as an autonomous innate immunity mechanism capable of killing and removing intracellular bacteria was on Mtb [208]. Since then, several relationships between known immune regulators and autophagy have been shown in different systems. Xu et al. first showed that TLRs trigger an autophagic response by showing the formation of autophagosomes in response to LPS stimulation in the murine macrophage RAW264.7 cell line. They further proposed that by recruiting both the MyD88 and TRIF signaling cascades, TLR4 could promote both a fast phagocytic response and a slower autophagic response, respectively [209]. Other studies have proposed a mechanism by which TLRs might regulate autophagy. Shi and Kehri showed that TRIF and MyD88 target Beclin 1 and

reduced its binding to Bcl-2 (an anti-apoptotic protein that inhibits Beclin 1-dependent autophagy), upon stimulation with different TLR ligands [210]. Other TLR family members have been implicated in the control of autophagy in RAW264.7 macrophages that were stimulated with different TLR ligands: CpG DNA (TLR9), poly (I:C) (TLR3), and ssRNA (TLR7) [207]. However, it is still unclear why only some PAMPs induce autophagy and how infected cells specifically direct the isolation membrane to intracellular bacteria, and not to other cellular components and organelles.

Xenophagy may occur via; (1) direct pathogen removal mediated by ubiquitination, sequestosome 1-like receptors (SLRs) and DAMPs; (2) regulation of effector functions of PRRs, involving NOD 1/2; (3) galectin-dependent mechanisms; and (4) di-acylglycerol-associated pathway. Specific autophagic recognition of intracellular microbes is facilitated by autophagic adaptors or cargo receptors, referred to as SLRs, and these microbes targeted for degradation must be tagged with ubiquitin to be recognized. SLRs are characterized by LC3 interacting regions and cargo recognition domains like ubiquitin domains, and are regulated by protein kinases. Known examples include p62, Neighbor of BRCA1 gene 1 (Nbr1), nuclear domain 10 protein (NDP52), and optineurin, well-reviewed by Rogov and colleagues [211]. Microbial factors may contribute to autophagy activation, e.g., GAS is targeted into autophagosomes following escape from its early endosomal compartment into the cytosol. GAS lacking the streptolysin O remain within endosomes and avoid autophagic degradation indicating a role for streptolysin O in induction of autophagy [120]. Similarly, *Salmonella, Listeria and Shigella* that escape from phagosomes are ubiquitinated and recognized by specific SLRs for autophagic degradation [212, 213]. E3 ubiquitin ligases have also joined the ranks of the regulators of xenophagy. LRR-containing RING E3 ligase, LRSAM1, was identified as an important E3 ligase in *Salmonella* infections [214] and, more recently, Parkin in Mtb infections [215]. NOX2 and mitochondria are the major sources of ROS, which may contribute to the induction of autophagy and killing of intracellular pathogens by macrophages [68, 216]. IFNγ and its effector, the IFN-inducible immunity-related GTPase family M member 1 (Irgm1), induce autophagy in macrophages and may inhibit the survival of intracellular pathogens like mycobacteria [217]. IFNγ-mediated induction of autophagy was shown to inhibit survival of virulent Mtb H37Rv in human and murine

macrophage cell lines. IFNγ increased proteolysis of long-lived proteins, translocation of LC3, and subsequent formation of autophagosomes [208]. Autophagy apparently plays a key role in the clearance of mycobacteria, although much of how this happens remains unexplained. A few studies present possible mechanisms of ubiquitin-dependent autophagic clearance. An earlier study implicated TBK1 as key regulator of autophagy maturation [218], and the ubiquitin ligase, Parkin, as central in ubiquitin mediated delivery of Mtb to autophagosomes [215], while a very recent study identifies a Rab7-dependent autophagy pathway of mycobacterial killing in THP-1 macrophages [219]. Galectins are cytosolic glycan-binding lectins that detect damage to endosomes or lysosomes as luminal glycans become exposed to the cytosol. Galectins thus can detect membrane damage caused by several intracellular pathogens, including *Salmonella*, *Shigella*, *Legionella*, *Listeria,* and even viruses [220, 221, 222, 223]. Recruitment of galectin-8/3/9 to damaged SCV and ubiquitination of the membrane remnants of vacuolar rupture in *Shigella*-infected cells suggest that compromised membranes and cytosolic bacteria could trigger xenophagy [188, 221]. DAG also accumulates around SCV and inhibition of DAG formation results in inhibition of xenophagy, although the exact role or mechanisms of DAG remain largely unclear [224].

Notwithstanding, pathogens have evolved mechanisms to inhibit, modulate, or exploit the autophagy response of the host. Autophagy has been shown to be induced in *M. avium* infected macrophages by few studies, including our preliminary observations, but the significance of this is not clear as the bacteria seem to be able to avoid killing [225]. Mtb may inhibit autophagy initiation upstream of autophagosome formation [68], and autophagy activation stimuli, like oxidative stress, starvation, and treatment with the drug rapamycin restrict Mtb in infected macrophages in vitro [208]. Mtb also inhibits Rab7 recruitment to phagosomes, thus selectively modulating autophagy flux in macrophages [219]. The ability of Mtb to survive in macrophages may, therefore, depend in part on its ability to modulate autophagy at least in macrophages in vitro [208]. The significance of these in vitro observations remains unclear as in vivo mouse models of Mtb infection in mice with myeloid cells deficient of the *Atg5* gene show a loss of bacterial control [108, 226], suggesting that Atg5 and the autophagy pathway are essential for Mtb control in vivo. However, Kimmey et al. recently showed that loss of autophagy related genes: *Atg3*, *Atg7*, *Atg12*,

Atg14 l, and *Atg16l1* did not show similar phenotypes as for *Atg5* implying that Atg5 may regulate an autophagy pathway that is independent of the other *Atg* genes tested or Atg5 may function in a non-autophagic processes like LC3-associated phagocytosis (LAP), that contributes to Mtb restriction [227, 228]. More studies to determine how Atg5 influences mycobacterial infections are necessary to enhance the quest for strategies to control mycobacterial infections, in general. In LAP, LC3 is recruited directly to the single membrane of bacterial phagosomes or other particles for rapid degradation in lysosomes [206]. The first report of this recruitment showed the appearance of LC3 on phagosomes within 5–10 min of internalization of *E. coli* and yeast by RAW macrophage cells expressing GFP-LC3 [206]. Similarly, endogenous LC3 was seen to decorate *M. marinum* phagosomes shortly after infection of RAW264.7 cells in an ESX-1-dependent mechanism. The $LC3^{+}$ compartment showed features of late endosomes, but did not acquire hydrolytic enzymes or properties. This indicates that *M. marinum* is capable of blocking autophagosomal maturation even if autophagy was already triggered [229].

L. monocytogenes cleverly recruits a complex of host proteins; GTPases, actin, the Arp2/3 complex, and Ena/VASP, via the bacterial ActA protein, to its surface thus disguising the bacteria from autophagic recognition [230]. Other bacteria induce autophagy and rather exploit the mechanism [231]. This is always a rather interesting phenomenon as to why a pathogen would enhance a host defense mechanism. However, if we recall that autophagy is actually a source of nutrition for host cells, evolution of bacterial mechanisms to hijack autophagosomes and redirect the by-products of degradation to enhance their own replication is rather resourceful as is the case with *F. tularensis* [232]. Autophagosomes may also provide a source of membrane, along with late endosomes, for the expansion of the *Yersinia*-containing vacuole (YCV) into a spacious compartment [233]. A non-canonical autophagy may be thus triggered by some intracellular bacteria for supplying nutrients or membranes needed for growth.

Manipulation of host cell death programs

If intracellular microbes escape killing, they can escape from the cells altogether and, perhaps, infect neighboring cells. Cell-to-cell spread can occur either directly or through the induction of cell death processes. Hence,

pathogens can target cell death pathways as a virulence strategy. If all else fails, as a final push macrophages can induce suicide programmes to prevent further microbial intracellular replication and increase immune exposure. Apoptotic cell death may be triggered by activating pro-death signals which results in activation of caspases [234], usually without activation of inflammatory cytokines. Apoptotic host cells and the pathogens they may contain are subsequently cleared by uninfected macrophages, typically in a cell-autonomous manner [234]. Apoptotic cells emit ‘find me’ signals, such as ATP or expose ‘eat me’ signals like phosphatidylserine (PS) that attract circulating phagocytes predominantly macrophages to engulf the apoptotic cells and contents. This engulfment is called efferocytosis, distinct from phagocytosis by engaging a different set of signals; lysoPS, cardiolipin, calreticulin, and CD31, and crucial for the resolution of inflammation [6]. Efferocytic Mtb phagosomes readily fuse with lysosomes and lead to bacterial killing both in vitro and in vivo [234]. A plausible speculation is that engulfed Mtb within an apoptotic cell further compartmentalizes the bacterium and prevents its virulence factors from interfering with phagosome maturation. On the contrary, bacteria may use this process for spread exemplified by *M. avium* which was shown to survive autophagic killing and induces apoptotic escape [225]. *M. avium* subsp. *hominissuis* (MAH) infects macrophages, and after several days, the infection triggers apoptosis leading to infection of neighboring macrophages. Through transposon mutations, some genes were identified that abrogated bacterial exit from macrophages upon apoptosis. Although the mechanism associated with bacterial escape from apoptotic macrophages is unknown, the identification of macrophage proteins targeting the MAH proteins suggests an interference with protein degradation or post-translational mechanisms. TatC, a bacterial protein, that transports large folded proteins across membranes was identified to be involved in the ability of MAH to leave macrophages, and suggests that secreted effector(s) are involved in the process [235]. Several genes and gene products have been implicated in the modulation of apoptosis in Mtb reviewed by others. Depending on the environmental cues, Mtb will either induce (e.g. *nuoG*) or inhibit (e.g., *pknE*) apoptosis to survive [236].

Necrosis is cell death that depends on the phosphorylation of RIPK1/3 and the activation of the pseudokinase MLKL accompanied by highly lytic and inflammatory responses [237]. Pyroptosis is a form of necrosis that involves the

activation of caspase-1 and caspase-4/5 or -11 that leads to a rapid and lytic form of macrophage death, accompanied by strong pro-inflammatory cytokine secretion, and or release of alarmins, which activate neutrophils for the clearance of released intracellular pathogens [173, 174]. Caspase-1 and caspase-11 both trigger pyroptosis by cleaving the newly identified gasdermin D protein [238, 239]. Gasdermin D makes pores in the plasma membrane releasing cytosolic cellular components which is recognized and removed in a PS- and T-cell immunoglobulin and mucin 4 (TIM4)-dependent manner [240]. An early pyroptotic response during infection may reveal and compromise the intracellular replicative niches that pathogens try to exploit and lead to clearance of the infection. Pathogens thus avoid triggering this response by various mechanisms that restrict expression of inflammasome ligands, modify the structure of the ligands, or directly inhibit inflammasome function [173], to maintain their intracellular niche. Such microbes include *S. typhimurium*, *L. monocytogenes,* and *L. pneumophila* that avoid inflammasome detection by the NLRC4 and AIM2 inflammasomes and caspase-11 [174, 175, 241]. Pyroptosis has not been reported in Mtb infections in DCs although, Lerm and colleagues showed that, in human primary macrophages, some necrotic cell death induced by Mtb independent of caspase-1 or cathepsin B but dependent on mycobacterial ESAT-6 [178, 242]. In addition, Niederweis' group identified a necrotizing toxin secreted by Mtb that cause host cell necrosis, possibly pyroptosis, by hydrolyzing NAD, although the exact mechanism of killing was not elucidated [109, 110]. Mtb genes that are so far important for the inhibition of pyroptosis include *zmp*1 and *Rv3364c* by inhibiting inflammasome formation required for IL-1β secretion and cathepsin G in both human and mouse macrophages, respectively [243, 244]. In general, cell death by mycobacteria seems to be highly strain dependent and the mechanisms behind this are still elusive as apoptosis, pyroptosis, necrosis, and necroptosis, and even Mtb-specific cell death are terms often used to describe the cell death.

Nutrient acquisition

Nutritional immunity is an aspect of the immune response to infection in which a host organism sequesters trace minerals in a bid to limit pathogenicity. Fatty acid catabolism, amino-acid synthesis, and acquisition of minerals like iron, among others are requirements for bacteria like Mtb to survive in macrophages. Reduced circulation of important minerals like iron, copper, manganese, and

zinc during infection starves invading pathogens of these essential minerals, thus limiting disease progression. Iron is mainly present as heme in the human body and free iron concentrations are maintained at even lower levels to avoid cytotoxicity [245]. Consequently, highly specialized proteins that maintain normal iron homeostasis and prevent deleterious effects have evolved. In plasma, iron is found almost exclusively bound to transferrin which is normally 30–40% saturated with iron, reviewed by Correnti and Strong [246]. Monocytes and macrophages may also regulate transporters, such as natural resistance-associated macrophage protein 1 (NRAMP1/SLC11A1), to deprive pathogens of minerals. *NRAMP1*$^{-/-}$ mice studies show reduced inflammatory responses to infection and iron recycling [247, 248] and meta-analysis data suggest that polymorphisms in the *NRAMP1* gene contribute to TB [249]. There is thus a strong functional link between metal sequestration and immunity. As a counter-measure, transcriptional studies have demonstrated that mycobacteria regulate several genes to secure a sufficient supply of iron within the cell [78]. Bacteria may acquire iron directly from heme, transferrin or lactoferrin; or through secreted ferric-specific chemical chelators called siderophores [246]. Using multiple or modifying siderophores with different chelation chemistries and backbone structures, iron can be acquired even in the presence of host siderophore-binding proteins like lipocalin 2 (Lcn2) [80, 250, 251]. Iron acquisition by Gram-positive bacteria like *S. aureus* involves siderophores, staphyloferrin A and B, which binds the lipoproteins HtsA and SirA, respectively, leading to uptake directly by their cognate membrane-spanning permeases [252]. In Gram-negative bacteria, a second cell membrane makes iron uptake considerably more complicated. Siderophores have to be bound by the outer membrane receptors, which transport the ferric siderophores into the periplasm. Specific periplasmic proteins then mediate transport across the inner membrane through their cognate permeases. In this group, pathogenic *E. coli* secretes both enterochelin and aerobactin [250]. Similar to Gram-negative bacteria, synthesis and transport of mycobacterial siderophores (mycobactins and carboxymycobactins) utilize the membrane proteins MmpS4 and MmpS5 associated with the corresponding transporters, MmpL4 and MmpL5 [253]. While ferric-carboxymycobactins cross the inner membrane using the IrtA/IrtB protein complex [254], it is not clear exactly though how they are taken up again by Mtb or how they cross the outer membrane. The ESX-3 secretion system of Mtb is required for iron acquisition, although it is still unclear how

this occurs [111, 112]. We have shown another clever mechanism via which mycobacteria may harness intracellular nutrient supply. Although Lcn2 was produced by murine macrophages, it did not impede growth in tissues and during long-term infections. Lcn2 trafficked to lysosomes but not *M. avium*, whereas transferrin was efficiently transported to the mycobacteria in a $Rab11^+$ compartment. Thus, mycobacteria seem to reside in the $Rab11^+$ endocytic recycling pathway, thereby retaining access to nutrition and avoiding antimicrobial proteins like Lcn2 [79]. Others have shown that this may be different in lung epithelial cells where Mtb encounters intracellular Lcn2 [255]. Zinc and copper are other minerals important in monocyte chemotaxis, phagocytosis, and cytokine production by macrophages [256, 257] that may be harnessed by bacteria, including Mtb, for their survival in a manner similar to iron [258].

Lipids are essential for mycobacteria survival; hence, macrophages try to curtail the supply of these nutrients. Mtb genes involved in fatty acid metabolism are upregulated during infection of macrophages and mice [259, 260]. Fatty acid metabolism is a preferred source of carbon in Mtb survival corroborated by the extensive duplication of genes involved in lipid metabolism in mycobacterial genomes [78]. Mycobacteria exploit lipids derived from macrophage-derived triacylglycerol and cholesterol. These lipids accumulate in bacterial phagosomes during infection to form foamy cell macrophages [261]. Through a mechanism involving ESAT-6, Mtb enhances ketone biosynthesis instead of the glycolytic pathway [262], whose intermediate products, like propionyl–CoA, could mount considerable metabolic stress to mycobacteria. Bacteria detoxify this intermediate for their own use by converting it to less toxic acetyl-CoA. An example of proteins involved in fatty acid metabolism is the glyoxylate shunt enzyme isocitrate lyase (ICL) of Mtb, earlier shown to be essential for metabolism of even chain fatty acids during infection in mice [78, 263]. Finally, amino-acid deprivation by NOS2 and indoleamine 2,3-dioxygenase (IDO), for instance, is also a strategy to limit and eliminate intracellular mycobacteria. However, IDO activation leads to breakdown of tryptophan that appears to be ineffective in controlling Mtb infection in mice as Mtb is able to synthesize tryptophan [264].

Macrophage-directed therapies for eradication of

persistent intracellular pathogens

Rising bacterial resistance to antibiotics and the need for new antibiotics only begs for exploration of all venues to control persistent infectious diseases. Traditionally, it is common to target pathogens and their components as is the case with the use of antibiotics. However, strategies to reinforce host strategies to remove invading pathogens represent an attractive alternative or supplement to antibiotic treatment known as host-directed therapy (HDT) [265]. Mononuclear phagocytes are central to the pathophysiology of inflammation and infectious diseases. The mechanisms of tumor cell killing by macrophages have been studied extensively and several clinical trials have already been performed or underway aiming at activating or enhancing macrophages or DCs [266]. The establishment of immunological memory in macrophages might also be a valuable asset in counteracting pathogens. HDTs range from re-evaluation of commonly used, affordable, and safe drugs, through immuno-modulators, biologics, and nutritional products, to cellular therapy using stem cells. The considerable decline in TB in the developed world occurred already early in the 19th century before the antibiotic era. This suggests that host factors have an important role to play in achieving anti-Mtb immunity. A concerted action of specific immune cells (T-lymphocytes and natural killer T cells) and cytokines is required to restrict Mtb survival [267]. The problem of resistance remains a high priority with a few new drugs in clinical trials, and resistance is still likely to develop against new TB drugs over time. Short treatment duration of TB (currently 6 months in patients with drug-sensitive tuberculosis and up to 2 years in patients with multidrug resistant TB), with improved patient compliance and outcome would be invaluable. Limiting inflammatory damage may also be an approach for better disease outcome using endogenous pro-resolving and anti-inflammatory mediators like protectins, lipoxins, and resolvins, depending on the circumstances [268]. Lipoxins were implicated as key chemical mediators in resistance to Mtb infection [269]. High levels of IL-12, IFNγ, and NOS2 mRNA levels were observed in murine tissues deficient of the lipoxin LXA4, accompanied by lower bacterial burdens compared with WT mice. This enhancement in the resistance of the LXA4-deficient animals to Mtb was completely lost by administration of a stable LXA4 analog. The authors concluded that lipoxins negatively regulate protective Th1 responses against mycobacterial infection in vivo and suggest that the inhibition of lipoxin

biosynthesis could serve as a strategy for enhancing host resistance to Mtb [269]. Activation and recruitment of antigen-presenting cells with the pro-inflammatory cytokines like IFNγ and GM-CSF could augment the antimicrobial immune response [265].

Other host-directed therapies for use as adjunct treatments to TB therapy are under development including using stem cells; redirecting commonly used drugs for diabetes, epilepsy, etc: micronutrients and other immune-modulators; antimicrobial peptide inducers, and checkpoint inhibitors; and therapeutic vaccines [265]. As discussed above, microbial virulence factors hinder intracellular processes that are required for successful eradication of the pathogen. HDTs could be directed to such impaired intracellular processes like activation of autophagy, induction of oxidative and nitrosative stress, and increased antigen processing and presentation. Autophagy induction with DAMPs like ATP, vitamin D3, or ROS has been shown to enhance elimination of Mtb in vitro, presenting autophagy with strong therapeutic potentials reviewed by Songane and colleagues [270]. The drugs isoniazid and pyrazinamide currently used for the treatment of TB are bactericidal and a recent study demonstrated autophagy as a mechanism of action of these drugs via activation of cellular and mitochondrial ROS [271]. Increased pressure on Mtb within the phagosome influences the outcome of macrophage infection [272], and Mtb mutants defective in the inhibition of phagosome maturation display reduced survival in murine macrophages [273]. Thus, phagosome maturation also presents a potential target for new drugs that enhance the innate immune response against Mtb and other intracellular pathogens.

Conclusion

We are now aware that intracellular pathogens are unarguably quite clever. However, continuous research efforts keep uncovering new host immune response mechanisms to counter these pathogens. A better understanding of such processes, including cell-autonomous immunity, autophagy, and efferocytosis or cell death, and nutritional immunity will spawn new knowledge and awareness on new therapeutic targets. Such insights will also strengthen the new approach of host-directed therapies that appear to be relevant for control of intracellular pathogens, notwithstanding the challenges that may be associated with the concept. Use of host-directed therapeutic strategies alongside standard

use of antibiotics might just be the way forward for the control of persistent and obligate intracellular infections to meet the challenges of increasing antibiotic resistance.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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