**Mycobacterium tuberculosis** infection of host cells in space and time

Claudio Bussi and Maximiliano G. Gutierrez*

Host-pathogen interactions in tuberculosis laboratory, The Francis Crick Institute, 1 Midland Road, London, NW1 1AT, United Kingdom

*Corresponding author: Host-pathogen interactions in tuberculosis laboratory, The Francis Crick Institute, 1 Midland Road, London, NW1 1AT, United Kingdom. Tel: +44(0)2037961460; E-mail: max.g@crick.ac.uk

One sentence summary: The review describes the complex dynamics underlying the interactions between **Mycobacterium tuberculosis** and the human host cell with an emphasis on the cell biology approaches and potential challenges to study these processes.

**ABSTRACT**

Tuberculosis (TB) caused by the bacterial pathogen **Mycobacterium tuberculosis** (Mtb) remains one of the deadliest infectious diseases with over a billion deaths in the past 200 years (Paulson 2013). TB causes more deaths worldwide than any other single infectious agent, with 10.4 million new cases and close to 1.7 million deaths in 2017. The obstacles that make TB hard to treat and eradicate are intrinsically linked to the intracellular lifestyle of Mtb. Mtb needs to replicate within human cells to disseminate to other individuals and cause disease. However, we still do not completely understand how Mtb manages to survive within eukaryotic cells and why some cells are able to eradicate this lethal pathogen. Here, we summarise the current knowledge of the complex host cell-pathogen interactions in TB and review the cellular mechanisms operating at the interface between Mtb and the human host cell, highlighting the technical and methodological challenges to investigating the cell biology of human host cell-Mtb interactions.

**Keywords:** **Mycobacterium tuberculosis**; macrophage; phagosome; autophagy; Tuberculosis

**FROM TISSUES TO PHAGOSOMES: THE ENVIRONMENTS MTB FACES IN THE HOST**

The tubercle bacillus spreads from person-to-person almost exclusively by aerosolized particles. The size of infectious droplets from Mtb infected patients ranges from 0.65 (small) to > 7.0 μm (medium–large) (Fennelly and Jones-Lopez 2015). While small Mtb aerosol particles are expected to transit the nasopharyngeal or tracheobronchial region to be deposited in the distal airways, larger particles can be trapped in the upper airway or oropharynx where they can potentially lead to Tuberculosis (TB) of the oropharynx or cervical lymph nodes (Laal 2012; Fennelly and Jones-Lopez 2015).

Once in the lower respiratory tract, Mtb is primarily phagocytosed by macrophages and dendritic cells. In addition to macrophages and dendritic cells, studies analysing the sputum of TB patients identified neutrophils as the predominant phagocytic cell infected with Mtb (Eum et al. 2010). There is also evidence that Mtb is also found in non-myelocytic cells of TB patients (Lerner, Borel and Gutierrez 2015; Randall et al. 2015; Gabban et al. 2016) (Fig. 1). The bacilli that manage to pass through the upper airways will be delivered to the alveoli. The alveoli consist of type I and II epithelial cells with a number of other immune cells such as alveolar macrophages, dendritic cells and neutrophils (Lerner, Borel and Gutierrez 2015). Infection of type II alveolar epithelial cells by Mtb has been extensively studied in vitro and DNA from Mtb has been detected within these cells in post-mortem studies (Hernández-Pando et al. 2000). Among the specialised epithelial cells of the upper airway, Mtb invades the cells known as a microfold cell (M cell) in the lungs of mice to initiate infection (Nair et al. 2016). Infected cells will trigger a local inflammatory response that will attract...
immune cells into the site of infection. These cellular aggregates, that contain many cell types, form the granulomas, the pathological hallmarks of TB. These granulomatous structures represent a complex environment for Mtb, very different from the one associated with healthy tissue. Although the mechanism is not entirely clear, these single lesion environments are likely very permissive for bacterial growth as well as impairing dissemination (Rubin 2009).

Outside the lungs, Mtb can virtually disseminate to any organ, with the lymphatics and lymph nodes being the main sites of extrapulmonary TB (Sehr and Waters 2014). In this context, aerosol infection of mice lacking M cells have significantly delayed dissemination to lymph nodes and reduced mortality, suggesting that M cells contribute to dissemination and disease progression (Nair et al. 2016). In the lymph nodes of patients with extrapulmonary TB, Mtb was found to infect lymphatic endothelial cells (LEC) (Lerner et al. 2016; Lerner et al. 2017). LEC are cells that line the lymphatic vessels and therefore the first ones to encounter bacteria that disseminate through lymphatics. Because these cells are non-motile, they could provide a niche for Mtb that facilitates persistent infection in lymph nodes (Lerner et al. 2016). Moreover, the adipose tissue (Beigier-Bompade et al. 2017) and bone marrow (Mayito et al. 2019) have been proposed as extrapulmonary niches where the tubercle bacillus could persist for long periods of time modulating the local tissue environment.

Collectively, in vitro and in vivo evidence show that Mtb infects different cell types during infection, encountering distinct environments within these cells (Fig. 1). The impact of these environments for Mtb on TB dissemination or disease progression remain unclear. One of the main arguments against a role for non-myelocytic cells during TB is based on the idea that only a minor proportion of these cells are potentially infected. However, it is still unclear whether a minor proportion of infected non-myelocytic cells could affect the outcome of active TB. The evidence arguing for several cell types playing a role during TB needs to be carefully considered and not necessarily seen as a potential bystander effect. How different specific cell-type environments affect Mtb localisation and survival (Fig. 1) is poorly characterised and these differences could have a profound impact during disease dissemination, progression and resolution. For example, Mtb must face very different intracellular niches in macrophages when compared to neutrophils or endothelial cells (Fig. 1).

**THE SPACE**

**In a membrane-bound compartment: tight and spacious phagosomes**

Mtb is internalised by phagocytosis in phagocytic cells such as macrophages (Fig. 2), dendritic cells and neutrophils. This actin-dependent process is regulated by many receptors and depends on the cell type. Phagocytosis of Mtb by macrophages is mediated via an array of different receptor molecules, including dectin-1, the complement receptor 3, Toll-like receptors, mannose receptor, the dendritic cell-specific intercellular adhesion molecule (ICAM)-3-grabbing nonintegrin (DC-SIGN), Fc receptors, scavenger receptors and CD14 (Pieters 2008; Schafer et al. 2009).

Although the relevance during human TB is unclear, numerous studies performed mostly in vitro, showed the internalization of Mtb by non-phagocytic cells such as type II alveolar epithelial cells, fibroblasts and stem cells. This process involves heparin-binding hemagglutinin, TLRs, surfactant proteins and complement and scavenger receptors (Lerner, Borel and Gutierrez 2015). In human tissue from TB patients, mycobacteria were also found in adipocytes, LEC and stem cells, suggesting these cellular environments are permissive for Mtb survival and subsequent reactivation (Neyrolles et al. 2006; Das et al. 2013; Lerner et al. 2016; Tornack et al. 2017).

While in vitro studies have demonstrated clear roles for particular receptor(s), it is more than likely that in vivo, Mtb is not internalized by macrophages using a single receptor-mediated pathway. Mycobacteria display numerous and diverse ligands on their surface which might either engage multiple receptors of multiple types simultaneously, or might bind to those receptors that are available on a particular cell. Moreover, mycobacterial surface molecules might also function to impair the formation of adequate receptor clustering (Schafer et al. 2009; Liu, Liu and Ge 2017). Importantly, the type of receptor engaged in phagocytosis can influence downstream signalling and influence phagosomal fate at the single phagosome level,
After Mtb is internalised in a phagosome, these phagosomes will subsequently interact with early and late endocytic organelles as well as other intracellular organelles (see below) in a process referred to ‘phagosome maturation’ (Fig. 2). These interactions are very dynamic and rapidly change the membrane and luminal composition of the phagosome, with the primary aim of restricting the growth of the internalised pathogen.

Mtb has been considered to be a classical bacterial pathogen that primarily resides within phagosomes of macrophages (reviewed in Russell 2001; Vergne et al. 2004a; Pieters 2008). Pioneering studies using electron microscopy (EM) from D’Arcy Hart and collaborators localised Mtb in phagosomes (Armstrong and Hart 1971). Other EM studies performed in the 80’s led to conclusions that differed from D’Arcy Hart’s original observations and localised Mtb outside the phagosome in the cytosol of macrophages (see ‘in the cytosol’ section below). Notably, in the studies from D’Arcy Hart the possibility that Mtb phagosomes were fusing with late endocytic organelles (herein referred as lysosomes for simplicity) was observed (Armstrong and Hart 1975). Phagosomes containing Mtb or a related pathogen, M.
avium in human macrophages were non-acidic suggesting a defect in phagosomal acidification (Crowle et al. 1991). In human monocytes, Mtb was mostly localised in phagosomes that do not fuse with late endosomes/lysosomes (Clemens and Horwitz 1995) but display some interactions with early endosomes and remained accessible to transferrin (Clemens and Horwitz 1996). More recent EM studies confirmed that a significant proportion of mycobacteria localise in membrane bound compartments (Jordao et al. 2008). Strikingly, EM localisation in alveolar macrophages isolated from a patient with active TB in Malawi showed membrane-bound bacteria which localised sometimes in large vacuoles containing multiple bacilli (Russell, Mwandumba and Rhoades 2002).

Because of the observed deficient luminal acidification, efforts focused mostly on finding the mechanisms by which Mtb remained in a non-acidic compartment and ‘arrested’ phagosome maturation. Research in this area contributed to the idea that Mtb interferes with the maturation of phagosomes to survive within macrophages and a striking feature of the Mtb phagosome was its defective recruitment of the vacuolar ATPase (vATPase) and reduced levels of mature lysosomal hydrolases (Sturgill-Koszycki et al. 1994). The mechanism of the vATPase exclusion by Mtb phagosomes is associated to the STAT-5-mediated expression of cytokine-inducible SH2-containing protein, which selectively targets the vATPase for ubiquitination and degradation (Queval et al. 2017).

As our understanding of the molecular machinery controlling phagosome and endosome maturation gradually emerged, this knowledge was applied to the study of the Mtb phagosome. Mechanistic reports of the process leading to the mycobacterial phagosome maturation arrest began with the study of endocytic Rab GTPases, which regulate intracellular trafficking and maintain the identity of cellular organelles (Vergne et al. 2004a). Early studies indicated that a functional block occurred between the maturation stages controlled by the small GTPase Rab5 (early endosomal) and Rab7 (late endosomal/lysosomal) (Vas et al. 1997). Rab5 accumulated on mycobacterial phagosomes, whereas Rab7 was not recruited to the phagosomes (Vas et al. 1997). A complementary study found that while Rab5 effectors are recruited to the mycobacterial phagosome, the Rab5 effector early endosomal autoantigen 1 (EEA1) associated with the process of membrane fusion was excluded (Fratti et al. 2001). EEA1 directly interacts with Rab5 and its recruitment and association with endosomal membranes are strengthened by the binding of its FYVE domain to phosphatidylinositol 3-phosphate (PI3P) (Simonsen et al. 1998). PI3P is generated on endosomal membranes by the action of the class III phosphatidylinositol 3-kinase (PI3K) and Rab5 effector, hVPS34 (Christoforidis et al. 1999). Thus, the impaired recruitment of EEA1 observed in mycobacterial phagosomes provided a mechanistic link between PI3K/PI3P and the mycobacterial phagosome maturation arrest (Fratti et al. 2001).

Experiments showing that mycobacteria-containing compartments access transferrin-bound iron (Sturgill-Koszycki, Schaible and Russell 1996), accumulation of transferrin receptor on mycobacterial phagosomes (Clemens and Horwitz 1996) and accessibility to glycosphingolipids (Russell, Dant and Sturgill-Koszycki 1996) supported the idea that the mycobacterial phagosome is not a static organelle. For instance, the survival of Mtb is associated with Coronin 1 (also known as TACO), which transiently accumulates on phagosomal membranes (Ferrari et al. 1999; Mori, Mode and Pieters 2018) and is actively retained by living mycobacteria residing inside phagosomes. Coronin 1 is responsible for the activation of the Ca\(^{2+}\)-dependent phosphatase calcineurin, which in turn impairs the delivery of mycobacteria to phagolysosomes (Jayachandran et al. 2007). On the other hand, a number of reports suggested that mycobacteria inhibit actin polymerization (Guerin and de Chaufflard 2000; Anes et al. 2003).

In addition, the composition, the movement and the role of metals and trace elements have an impact on the biology of the microbial phagosome (Soldati and Neyrolles 2012). For instance, the mycobacterial phagosome is readily emptied of phosphorus, sulphur and chlorine upon inflammatory stimulation (Wagner et al. 2005). However, if phosphate depletion from the vacuole is associated to the induction of a dormancy program previously reported in mycobacteria cultured in phosphate-limiting conditions (Rifat, Bishai and Karakousis 2009), remains to be determined.

Mtb also directly alters host signalling through the secretion of phosphatases, thereby shutting down critical cellular processes and promoting its survival within macrophages (Saleh and Belisle 2000; Bach et al. 2008; Wong et al. 2011; Wong et al. 2013). Mtb-secreted protein- and lipid-phosphatases protein-tyrosine phosphatase A and B (PtpA and PtpB), and secreted acid phosphatase M have been shown to contribute to Mtb pathogenicity by disrupting the endocytic pathway and promoting phagosome maturation arrest (reviewed in (Wong, Chao and Av-Gay 2013)). These secreted enzymes represent a good strategy to target Mtb and avoiding the development of drug resistance. For example, selective inhibitors of MptPb alter Mtb phagosomal PI3P dynamics that correlates with increased bacterial control in macrophages and guinea pigs (Vickers et al. 2018).

The above mentioned mechanistic studies of phagosome maturation ‘arrest’ are important for our understanding of the phagosomal stage of mycobacteria. Conclusions from these studies were generally based on limited quantitative imaging data and performed in host cells from different sources and species, making comparisons difficult. The postulated mechanisms were mostly based on data generated using avirulent mycobacteria such as the vaccine strain Bacillus Calmette-Guerin (BCG) and in many cases using Western blot analysis of mycobacteria-containing phagosomes, a method that is prone to significant contamination with other organelles. In consequence, and mostly due to the lack of appropriate technologies to address these questions, the current paradigm states that the majority of membrane-bound Mtb resides in an early ‘arrested’ phagosome in macrophages.

With the advent of new technologies, data using live cell imaging show that in macrophages, the majority of individual Mtb phagosomes follow a conventional maturation pathway interacting with lysosomes (Schnettger et al. 2017). These results are in agreement with previous observations showing that Mtb can eventually survive upon trafficking into late endosomal compartments, either through Fc receptor-mediated phagocytosis or by co-infection with the pathogen Coxiella burnetti (Armstrong and Hart 1975; Gomes et al. 1999).

A kinetic study showed that Rab7 is transiently recruited to Mtb phagosomes, suggesting that dissociation of Rab7 from the Mtb phagosome is important for altered phagolysosome biogenesis (Seto et al. 2009; Seto, Tsujimura and Koida 2011). The presence of late endosomal markers in Mtb phagosomes was confirmed by using a novel method of phagosome purification using magnetic particles (Steinhauser et al. 2013).

Mtb can also be localised in spurious phagosomes, as originally described in EM studies (De Chaufflard and Thilo 1997).
The occurrence of tight and spacious Mtb phagosomes are conserved across host species and likely to be relevant in vivo since these spacious phagosomes have been observed in monocytes of TB patients (Russell, Mwandumba and Rhoades 2002), as well as in infected mice (Moreira et al. 1997), zebrafish (Hosseini et al. 2014) and Dicyostelium (Barisch et al. 2015). Interferon-gamma (IFN-γ) is a key mediator of macrophage activation and resistance to intracellular pathogens. This cytokine increases interactions of Mtb phagosomes with the endosomal network, targeting mycobacteria to spacious, proteolytic compartments that reduce Mtb replication. Furthermore, this targeting of Mtb to spacious phagosomes also reduced phagosomal damage and access of mycobacteria to the cytosol. Without IFN-γ activation, fully virulent Mtb is able to bypass this targeting to spacious compartments, suggesting a complex interplay between immune activation and Mtb effectors (Schnettger et al. 2017).

In a damaged compartment: causes and consequences

In addition to the bacterial Sec and Tat secretion pathways, Mtb possesses the ESX secretion system, a Type VII secretion system (T7SS) which is found to be non-essential for growth in vitro, but is required for bacterial virulence in vivo (Hau et al. 2003). The first and most studied member of the five ESX secretion systems, ESX-1 is encoded by the region of difference (RD) 1 and its deletion results in attenuation of Mtb. Several Mtb proteins are secreted but the ESX-1 system and the 6-kDa early secreted antigenic target (ESAT-6; EsxA) and Culture filtrate protein 10 (CFP-10; EsxB) are the most abundant proteins found in supernatants from Mtb cultures.

Studies using a Mtb mutant lacking the RD1 region suggested that the ESX-1 system is implicated in the Mtb access to the cytosol, arguing for a role of ESX-1 in phagosomal membrane damage (Fig. 2) (van der Wel et al. 2007). There is also compelling evidence that specific cell wall lipids can contribute to membrane damage and the virulence-associated Phthiocerol dimycocerosates were shown in several systems to facilitate cytosolic localisation of Mtb in host cells (Augusteich et al. 2017; Barczak et al. 2017; Quigley et al. 2017; Lerner et al. 2018). It is still not clear precisely how Mtb damages membranes; for example, we do not completely understand the complete structure of the assembled T7SS ESX-1 and how stable damaged phagosomes are, since damaged membranes are very unstable in a cytosolic (hydrophilic) environment. Moreover, the role of EsxA as pore toxin that was implicated in membrane damage is still unclear. The ability of EsxA to lyse membranes could be due to the way the protein is purified in the laboratory, which could explain the differences in results between groups (Refai et al. 2015). The purification method used is very important to the conformational state of EsxA which affects its ability to create pores. In agreement with this notion, in M. marinum some of the EsxA lytic activity was found to be due to detergent contamination (Conrad et al. 2017).

The fate of damaged Mtb phagosomes is relatively well characterised. Damaged phagosomes are now known to be rapidly recognised by the selective autophagy machinery (see below). Autophagy is a major intracellular pathway for the degradation and recycling of long-lived proteins and cytoplasmic organelles. Autophagy of foreign entities such as bacteria, viruses and parasites is termed xenophagy, an evolutionarily conserved mechanism classically observed to target and remove pathogens after host cellular invasion (reviewed in Kimme and Stallings 2016). After damage of Mtb phagosomes, Mtb components (e.g. DNA/RNA) access the cytosol, triggering signalling important for the innate immune response. During Mtb infection of macrophages, activation of cytosolic surveillance pathways is dependent on ESX-1, leading to type I interferon (IFN) and interleukin-1β (IL-1β) production. The inflammasome regulates IL-1β secretion and the nucleotideylltransferase Cyclic GMP-AMP synthase (cGAS), that induces synthesis of type I IFN via the cyclic di-nucleotide cGAMP in response to cytosolic DNA derived from viruses or retroviruses such as the human immunodeficiency virus (Cai, Chiu and Chen 2014). In this context, three independent reports showed the association of cGAS with antimycobacterial immunity (Collins et al. 2015; Wassermann et al. 2015; Watson et al. 2015). Altogether, these studies support a model whereby subsequent to Mtb infection, cGAS and mycobacterial DNA co-localize in the macrophage cytoplasm and form aggregates. This interaction initiates the synthesis of cGAMP by cGAS and consequent activation of the STING-TBK-1-IRF-3 signalling axis that drives transcription of IFN-β (Majlessi and Brosch 2015). STING is a signalling molecule essential for controlling the transcription of numerous host defence genes, including type I IFNs and pro-inflammatory cytokines, following the recognition of aberrant DNA species or cyclic dinucleotides in the cytosol of the cell (Barber 2015). These studies revealed the detection of cytosolic mycobacterial DNA by cGAS, emphasizing the concept of cytosolic access by Mtb. However, the in vivo relevance remains to be defined, since mice lacking cGAS are only moderately more susceptible to Mtb infection than their wild type littermates. Mycobacterial RNA accesses the cytosol through a SecA2- and ESX-1-dependent mechanism and activates the retinoic acid-inducible gene (RIG-I)/mitochondrial antiviral signalling protein (MAVS)/tank-binding kinase 1 (TBK1)/IRF7 signalling pathway. Activation of this RNA sensing pathway requires prior STING activation and works synergistically with the DNA sensing pathway to stimulate IFN-β production in host cells during Mtb infection (Cheng and Schoepe 2018).

If cytosolic access occurs after complete disassembly of the phagosomal membrane, as observed in previous EM studies (Leake, Myrvik and Wright 1984; Myrvik, Leake and Wright 1984; McDonough, Kress and Bloom 1993) or it represents a dynamic process associated with membrane rupture and subsequent membrane repair, remains poorly characterised. Such conclusions require time-resolved data at ultrastructural resolution that are not readily obtainable (Simeone et al. 2016). Studies using a Fluorescent Resonance Energy Transfer (FRET)-based assay, were able to confirm that Mtb indeed accesses the cytosol in an RD1 dependent manner. (Simeone et al. 2012; Simeone et al. 2015; Wang et al. 2015) These reports showed that during infection of THP-1 macrophages, Mtb and BCG expressing ESX-1 displayed phagosomal rupture 3 to 4 days post-infection, which was followed by host cell death (Simeone et al. 2012). A later adaptation of the method allowed to observe a phagosome-to-cytosol connection in vivo in Mtb-infected phagocyte populations inside the lung parenchyma, granuloma and spleen of mice at the chronic phase of infection (Simeone et al. 2015; Simeone et al. 2016). This latter assay has been useful to define access of luminal components to the cytosol; however, it does not allow one to rigorously define if the bacteria are free in the cytosol (Fig. 2).

An important consequence of Mtb phagosome damage is the leakage of molecules from the phagosomal lumen. For example, biochemical properties that rely on an intact membrane such as pH will not be retained in a damaged phagosome. If damaged phagosomes are stable organelles, as shown for lysosomes (Skowyra et al. 2018), and displaying limited damage with pores of around 100 nm, remain to be investigated. The
situation is similar for ions, cations and many amino acids that are contained within the lumen of a phagosome. It is likely that phagosomes that have been considered in several studies ‘lysotracker negative’, and therefore non-acidic, in fact are leaky phagosomes unable to maintain a gradient through the phagosomal membrane (Schnettger et al. 2017). It is becoming clearer that the host cell attempts to repair damaged Mtb phagosomes. One of the mechanisms is mediated by fusion of endosomal membranes with Mtb phagosomes that restore phagosomal membrane integrity (Schnettger et al. 2017). This pathway repairs damaged phagosomes, leading to the retention of Mtb in intact phagosomes that restrict Mtb replication in vitro (Schnettger et al. 2017). Interestingly, the endolysosomal repair machinery ESCRT-III is recruited into Mtb phagosomes in an EsxA dependent manner, suggesting a dynamic balance between bacterial damage and host repair mechanisms (Mittal et al. 2018). During infection of Dictyostelium discoideum with M. marinum, the ESCRT-I component Tsg101, the ESCRT-III protein Snf7/Chmp4/Vps32 and the AAA-ATPase Vps4 are recruited after mycobacteria phagosome damage. While membrane damage induced by the ESX-1 secretion system of M. marinum are targeted by both ESCRT and autophagy, in absence of Tsg101, M. marinum accesses prematurely the cytosol, where the autophagy machinery restricts its growth (Lopez-Jimenez et al. 2018).

Notably, not all bacteria are able to induce damage and only a minor fraction is found to be ubiquitinated or positive for galectins, beta-galactoside binding lectins containing homologous carbohydrate recognition domains that recognise damaged membranes. These subpopulations of Mtb positive for galectins could result from dynamic changes between membrane-bound and cytosolic states at specific time points and dynamic studies (e.g. by using live-cell imaging) may reveal that a greater proportion become positive for galectins at a specific time point (Fig. 2). These dynamic cycles of damage and repair could depend on the timing and levels of the production of Mtb damaging factors. One possibility is that phenotypic variation within the bacterial population drives the heterogeneous response (Stanley and Cox 2013). For instance, stochastic fluctuations in ESX-1 activity may give rise to cell-to-cell variations in the flux of EsxA and thus create variations in membrane permeability from phagosomal to phagosome (Ohol et al. 2010).

In the cytosol

The emerging concept is that after Mtb-induced membrane damage occurs, there is communication or access to the cytosol and eventually the host cell will try to repair the damage via different mechanisms (Fig. 2). If this process is not properly contained, phagosome membrane disruption allows Mtb to freely localise in the cytosol. Crucially, the access of Mtb to the cytosol is critical for downstream signalling and modulation of the immune response. However, exactly how Mtb enters the cytosol, for how long it remains there and how the host cell recognises cytosolic Mtb remains to be fully characterised.

The cytosolic localisation of Mtb was reported in the early 80’s and subsequent reports using EM contributed to the idea that some Mtb could be free in the cytosol (Leake, Myrvik and Wright 1984; Myrvik, Leake and Wright 1984; McDonough, Kress and Bloom 1993). However, it was not clear to what extent this phenomenon was occurring. The main challenge to interpreting these data is related to the fact these studies were performed with conventional chemically fixed and plastic embedded cells, a method that is prone to artefacts. Methods and reagents available (e.g. antibodies) to investigate the phagosomal stage of Mtb were much better established and most of the research focused on this intracellular aspect of Mtb. However, studies using M. marinum (Mm) revealed that this close related of Mtb was free in the cytosol of macrophages. The cytosolic localisation of Mm was rapidly accepted because of the presence of actin tails in EM studies clearly confirmed Mm was free in the cytosol and propelled by actin tails (Stamm et al. 2003).

More than 20 years after the first descriptions of Mtb free in the cytosol, one study investigated again this aspect of the Mtb biology using a different approach. The use of thawed cryosections combined with immunogold EM allowed for good preservation of cellular membranes and to define cytosolic vs. phagosomal Mtb (van der Wel et al. 2007). However, as with earlier studies, this method used chemical fixation and the lack of a marker could not always mean absence of a surrounding membrane. By using both the absence of a membrane and a late endosomal marker, it was shown that the ESX-1 T7SS is one of the main Mtb factors responsible for the localisation of bacteria in the cytosol (van der Wel et al. 2007).

Despite several studies showing evidence that Mtb accesses the cytosol of host cells, the cytosolic localisation of Mtb is still debated and not widely accepted. For example, if the subcellular localisation of Mtb within cells and the physiological relevance of the cytosolic localisation is relevant for the disease is still disputed (Harriff, Purdy and Lewinsohn 2012). It is now clear that these original observations were, at least in part, correct based on more recent and compelling data from several independent groups using alternative methods. Moreover, the in vivo evidence of Mtb cytosolic access remains to be defined. A previous study carried out in mice using the FRET assay described above combined with flow-cytometry, showed that Mtb induces phagosomal rupture in the mouse model of infection. The assay is, as the one described for in vitro studies above, based on FRET changes depending on the β-lactamase activity present on the surface of bacteria. Although, the authors showed that this response is abrogated when mice are infected with Mtb strains lacking the ESX-1 secretion system, the possibility that some of these effects may have been caused by bacterial products translocating through permeable phagosomal membranes cannot be excluded (Simeone et al. 2015).

The current evidence argues strongly for a scenario whereby Mtb can be localised in functionally different intracellular locations, namely: (1) membrane-bound compartments, (2) transiently in damaged phagosomes or (3) free in the cytosol (Fig. 2). This mixed localisation has important implications for future interpretation of experimental data and also in drug development. It is clear that bacteria that is in a membrane bound compartment will be more affected by innate immune pathways that operate in the lumen of phagosomes (Reactive nitrogen species, ROS, hydrolytic enzymes of any class). The dynamics, functions and relevance of these sub-populations of Mtb, and in particular 2 and 3, in human macrophages remain unknown. In this context, if cytosolic Mtb constitutes a major target from a therapeutic standpoint remains to be defined (Russell 2016).

In time

The recently formed Mtb phagosome (e.g. after phagosomal membrane closure) will dynamically interact with numerous organelles derived from the endocytic, secretory and autophagic pathways (Fig. 3). Mtb phagosomes also interact with other membrane-less organelles such as lipid droplets (LD), double membrane organelles such as mitochondria and the multi-membrane network of the endoplasmic reticulum, although
Figure 3. The time: spatiotemporal dynamics of Mtb interactions with host cell organelles. At least four different populations of intracellular Mtb (early phagosome, late phagosome, damaged phagosome and free in the cytosol) are localised in different environments and will interact dynamically with host cell organelles such as vesicles from the endocytic pathway (e.g. early, recycling and late endosomes); the autophagy pathway, endoplasmic reticulum, post-Golgi vesicles, the extensive network of mitochondria, lipid droplets and peroxisomes. Because of the dynamic nature of these Mtb populations, their interactions with host organelles will be different and likely regulated in a spatiotemporal manner by the bacteria and the host cell. Distinct molecular players involved in these interactions are shown. Known host factors are shown in black and Mtb factors in green.

these interactions are poorly understood (Fig. 3). Crucially, these highly dynamic interactions between the endocytic and phagocytic pathway endorse the phagosome with antibacterial properties as part of the innate immune response.

Interactions with the endocytic pathways

Pathogenic mycobacteria have evolved mechanisms to interfere with both (glyco)lipid and protein-mediated mechanisms that regulate the trafficking of bacteria to lysosomal organelles for destruction (Pieters 2008). Mtb glycolipids can interfere with phagosome-lysosome fusion through blocking a normal host trafficking event that is regulated by PI3P. PI3P is a host membrane component that is essential for phagolysosome biosynthesis (Roth 2004). Locally generated by PI3K on early endosomal and phagosomal membranes, PI3P represents a docking site for several regulatory proteins involved in the maturation of phagosomes into lysosomes, such as the hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) and EEA1 (Birkeland and Stenmark 2004; Pizarro-Cerda and Cossart 2004). The generation of PI3P regulates the delivery of phagocytosed cargo to lysosomes and Mtb interferes with this trafficking event by actively preventing PI3P accumulation on phagosomal membranes (Fratti et al. 2003).

Mannose-capped lipoarabinomannan (ManLAM), a macroamphiphilic lipoglycan exposed at the surface of Mtb cell envelope (Pitarque et al. 2008), is a key lipid that modulates phagocyte functions (Briken et al. 2004; Fukuda et al. 2013; Vergne, Gilleron and Nigou 2014). ManLAM interferes with the endocytic pathway by modulating calcium signalling and recruitment to the phagosome of EEA1, a tethering protein and Rab5 effector (Vergne et al. 2004a). EEA1 recruitment is necessary for delivering hydrolases such as Cathepsin D and vATPase from the Trans-Golgi-Network to the phagosome (Fratti et al. 2003). The impairment of phagosome maturation by ManLAM has been shown in different systems by several groups (Hmama et al. 2004; Kang et al. 2005; Welin et al. 2008).

Phagocytosis of dead but not live Mtb triggers an increase of cytosolic Ca²⁺ that results in activation of calmodulin-dependent kinase II (CaMKII) (Malik, Denning and Kusner 2000; Malik, Iyer and Kusner 2001). Inhibition of Ca²⁺, Calmodulin (CaM) and CaMKII prevents phagosomes containing dead Mtb from fusing with lysosomes. ManLAM prevents cytosolic Ca²⁺ concentration increase and through p38 MAPK activation, it
contributes to privation of PI3K on the Mtb containing phagosomes and reduction of Rab5 levels in early endosomes, thus providing a rationale for its effect on limiting EE1A recruitment and phagosome maturation (Fratti et al. 2003; Vergne, Chua and Deretic 2003; Vergne et al. 2004b).

There is also evidence that Mtb phagosomes interacts with Golgi-derived vesicles that contain enzymes implicated in innate immunity (Fig. 3). Sorting of luminal and membrane proteins into phagosomes is critical for the immune function of this organelle. Sortilin, also known as neurotensin receptor 3 is a transmembrane receptor that transports lysosomal proteins from the trans-Golgi network into lysosomes, as an alternative route to mannose-6-phosphate receptors (Braulke and Bonifacino 2009). The phagosomal association of sortilin is critical for the delivery of acid sphingomyelinase and required for efficient phagosome maturation. Furthermore, in vitro and in vivo studies showed that sortilin is implicated in a Golgi-to-phagosome pathway that is required for controlling intracellular mycobacteria and lung inflammation (Vazquez et al. 2016).

**Interactions with the autophagic pathway**

Whereas there is a large body of literature linking xenophagy as an anti-mycobacterial pathway (Deretic; Gutierrez et al. 2004), how and when Mtb is targeted to autophagic compartments, and eventually autophagylysosomes in human macrophages is still unknown. There are at least four possible alternative mechanisms: (1) direct sequestering of intact Mtb phagosomes by phagophores, the membranes that initiate autophagy; (2) targeting via selective autophagy of cytosolic bacteria and/or damaged phagosomes; (3) direct fusion of autophagosomes with intact phagosomes and (4) direct recruitment of autophagic proteins (e.g. LC3B) into Mtb phagosomes, as in non-canonical autophagy (Fig. 3). This information is critical, given the data showing that Mtb can eventually block the fusion of autophagosomes with lysosomes (Romagnoli et al. 2012; Lerner et al. 2016) and data showing that Mtb can evade the autophagic responses in the mouse model of TB (Kimmey and Stallings 2016). Induction of autophagy promotes maturation and acidification of Mtb phagosomes and their conversion into mycobactericidal organelles (Gutierrez et al. 2004; Harris et al. 2007; Fabri et al. 2011) through the canonical autophagy machinery or alternative pathways such as non-canonical autophagy (Cemma and Brumell 2012).

Compelling evidence shows that Mtb is targeted to autophagosomes via ubiquitination. However, it is not yet clear if the substrates for ubiquitination in infected cells are intact mycobacteria that escaped into the cytosol, mycobacterial components inserted on host membranes, secreted bacterial molecules, damaged host membranes or simply host cell proteins found on Mtb phagosomes. Independently of the substrate identity, a small fraction of damaged Mtb phagosomes are tagged with ubiquitin, and ubiquitin moieties are recognised by autophagic adaptors and the selective autophagic machinery that will target Mtb into autophagosomes, and presumably into the lysosomal degradation pathway (Watson, Manzanillo and Cox 2012). Evidence argues that different Mtb clinical strains may be differentially recognised by the selective autophagy pathway, suggesting different behaviours of different Mtb isolates in their ability to damage host membranes (Kumar et al. 2010; Haque et al. 2015).

A more detailed analysis of autophagy factors such as Sequestosome 1/p62 has established that the entire pathway is important for antimycobacterial action of autophagy (Ponpuak et al. 2010). A specific manifestation of this is initiation of autophagy generates and delivers a mixture of antimicrobial peptides, known as cryptides, into Mtb phagosomes (Ponpuak and Deretic 2011). These peptides are produced through autophagic proteolysis of otherwise innocuous cytosolic proteins such as ribosomal proteins (Ponpuak et al. 2010) and ubiquitin (Alonso et al. 2007). As already mentioned, a fraction of intracellular Mtb bacilli escape from phagosomes into the cytoplasm or are in contact with the cytosol (van der Wel et al. 2007). The bacilli that are in the cytosol represent a minor proportion of the total intracellular Mtb, but are nevertheless subject to targeting through selective autophagy (Watson, Manzanillo and Cox 2012).

The kinase TBK-1 also plays a critical role in the regulation of mycobacterial control by autophagy (Pilli et al. 2012). TBK-1 is required for IL-1β-induced clearance of Mtb by autophagy since the TBK-1 inhibitor BX795 or TBK-1 depletion reduces mycobacterial killing when autophagy is induced by IL-1β (Pilli et al. 2012).

The elimination of Mtb via a ubiquitin-dependent mechanism where phagosomes enclosing bacteria are tagged with ubiquitin chains is partially regulated by the E3 ligase Parkin that triggers K63-ubiquitination of mycobacteria. Macrophages lacking Park2 show a significant reduction in ubiquitin-positive mycobacteria compared with normal cells (Manzanillo et al. 2013). Infected Park2−/− macrophages also revealed decreased recruitment of the ubiquitin adaptors, P62, NDP52 and NBR1, and the autophagy proteins, LC3 and ATG12, to mycobacterial cells, compromising their efficient elimination (Manzanillo et al. 2013). Ubiquitin tagging of Mtb is likely more complex since other E3 Ubiquitin ligases have been implicated in Xenophagy of Mtb (Franco et al. 2017; Pei et al. 2017).

On the other hand, Mtb has been shown to subvert the function of some intrinsic host mechanisms favouring their own survival in host cells. For instance, Coronin 1a (CORO1A) is a host F-actin-binding protein that is activated by Mtb and impairs autophagosome formation around the bacillus-containing phagosomes. This observed reduction on autophagosome formation is likely the result of impaired activation of the p38 MAPK necessary for autophagy induction through TLR signalling (Seto, Tsujimura and Koide 2012). In addition, the Mtb protein Eis has been shown to acetylate Lys-55 in a JNK-specific phosphatase (Kim et al. 2012). JNK action is important for activation of the autophagy regulator Beclin-1, and thus Eis may modulate autophagy (Shin et al. 2010; Ganaie et al. 2011; Kim et al. 2012). A mycobacterial glycolipid lipoolarabinomannan has also been reported as a strong inhibitor of autophagy (Shui et al. 2011). The Mtb effector protein EssA is secreted via the ESX-1 secretion system and blocks Mtb phagosomal maturation into degradative autolysosomal organelles (Romagnoli et al. 2012; Zhang et al. 2012). Nonetheless, the precise mechanisms and pathways used by Mtb to evade autophagy are poorly understood (Deretic 2014).

One of the non-canonical pathways of autophagy is LC3-associated phagocytosis (LAP) where LC3 is rapidly conjugated to a single-membrane phagosome in a process that is independent of the autophagy initiation complex Ulk-1 (Martinez et al. 2015). LAP is initiated by signalling through pathogen recognition receptors such as TLR2 and CLRs, resulting in the recruitment of the nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) to the phagosome. The NADPH oxidase generates Reactive Oxygen Species, which are essential for LAP. In addition, Rubicon, a negative regulator of the PI3K complex, which is also required for LAP, stabilizes the NADPH oxidase. Although little is known about the role of LAP during
Mtb infection, there are data showing that, similar to canonical autophagy, Mtb inhibits LAP through CpsA, a LytR-CpsA-Psr (LCP) domain-containing protein which disrupts the NADPH oxidase. However, how CpsA impairs NADPH oxidase recruitment and eventually LAP remains to be investigated (Köster et al. 2017).

**Interactions with other organelles**

It has been postulated that interactions between Mtb and LD are important for the infection. Foamy macrophages accumulate in granulomas during mycobacterial infections, a process that Mtb mycolic acids can induce (Peyron et al. 2008). Moreover, these investigators found that mycobacteria were ultimately delivered into the LD, where bacilli accumulate lipids (Peyron et al. 2008). A later study showed that accumulation of lipids by Mtb within foamy macrophages mainly results from the incorporation of fatty acids derived from host TAG in a process largely mediated by mycobacterial triacylglycerol synthase 1 (Daniel et al. 2011). These observations indicate that Mtb can use host LD as a source of nutrients. However, there are limited dynamic studies on how LD associate with Mtb during intracellular infection, particularly in human host cells, in real time. In addition, most of the original observations lack quantitative analysis at the single cell level (Fig. 3). In contrast, there is another study suggesting that LD formation is not a Mtb-driven process, but rather occurs as a result of IFN-γ activation of macrophages and as part of a host defense mechanism (Knight et al. 2018).

Interaction of mycobacteria with the host macrophage also results in plasma membrane microdisruptions (Roy et al. 2004; Divangahi et al. 2009). Resealing of these lesions, a process crucial for preventing necrosis and promoting apoptosis, required translocation of lysosomal and Golgi apparatus-derived vesicles to the plasma membrane. Plasma membrane repair depended on prostaglandin E2, which regulates synaptotagmin 7 (Syt-7), a calcium sensor involved in the lysosome-mediated repair mechanism (Divangahi et al. 2009). Although there is insufficient evidence supporting a role of the ER during Mtb infection, previous reports have shown an association between mycobacterial secreted proteins and the activation of the ER stress response, which could mediate apoptosis of macrophages and epithelial cells during infection (Choi et al. 2010; Grover et al. 2018).

Bacterial effectors can target mitochondria and manipulate their function and dynamics (Jain, Luo and Blanke 2011; Lum and Morona 2014; Lobet, Letesson and Arnould 2015; Chowdhury et al. 2017; Escoll et al. 2017) (Fig. 3). The effects
Figure 5. Different fluorescent-based approaches to study the intracellular localisation of Mtb. A. One of the most widely used strategies to study bacteria localisation is based on a biased judgement to determine if a particular intracellular marker (shown in red) co-localise with bacteria (shown in green). B. Alternatively, in cases where the cellular markers co-localise almost completely with bacteria, a more quantitative and unbiased method can be employed using a masking strategy to select bacteria and applying this selection to the cellular marker channel/image to determine the pixel number and intensity associated with bacteria. C. A variant of this unbiased method can also be applied when the intracellular marker is associated with the bacteria membrane but it does not cover all the surface. In this case, it is necessary to dilate the original bacteria mask. The basic effect of this operator (on a binary image) is to gradually enlarge the boundaries of regions of foreground pixels. Then, the bacteria pixels will be subtracted and the resulting mask will be used to quantify the corresponding pixels in the cellular marker image/channel. D. Examples where unbiased methods are not suitable for quantification and positive or negative co-localisation is difficult to determine since the distribution of the intracellular marker is asymmetric or it only partially covers the bacteria surface. In these cases, artificial intelligence represents a promising methodological advance.

of mycobacterial proteins and the interaction of Mtb with the mitochondrial network remain largely unexplored. Recently, the host Immune-Responsive Gene 1 (Irg1; also called Acod1), a mitochondrial enzyme induced under inflammatory conditions that produces the metabolite itaconate, has been shown to be regulated Mtb. By using Irg1−/− and Irg1fl/fl conditional gene-deleted mice this study showed that complete absence of Irg1 during Mtb infection resulted in severe pulmonary disease and ultimately death (Nair et al. 2018). Although macrophage necroptosis, a programmed form of necrosis that is dependent on activation of receptor-interacting kinase 3 (RIPK3 and the mixed lineage kinase domain-like pseudokinase), is associated with depolarized mitochondria and impaired ATP synthesis, known hallmarks of Mtb-induced cell death, the effects of mycobacterial proteins and the interaction of Mtb with the mitochondrial network remain largely unexplored.
Challenges to study human macrophage-Mtb interactions in vitro

Human macrophages

Although Mtb is a human pathogen, the majority of the in vitro host-pathogen studies in TB have been carried out in mouse cells. Detailed cell biology studies of Mtb infected human macrophages in the literature are limited since, until recently the available in vitro systems were not ideal. The most commonly used human macrophage models are cancer cell lines such as the human monocyte-like cell line THP-1 (Tschiya et al. 1980) or U937 (Sundstrom and Nilsson 1976) (Fig. 4). However, these cells need to be chemically activated with phorbol esters before use and are karyotypically abnormal. Moreover, by definition, they are not terminally differentiated macrophages, complicating the interpretation of results. Many signalling pathways are partially active (or completely absent) in these cells, such as the inflammasome pathways (Gaidt et al. 2016). Immunity in these proliferating cells might be different from physiological conditions and this has to be considered when selecting these cell lines as models to study immune responses. Another macrophage cell line model is the BLaER1 cells that uses lineage conversion by the inducible nuclear translocation of a C/EBPα transgene from malignant B-lineage cells to monocytes/macrophages. Using this model, it has been possible to characterize a novel species-specific NLRP3 inflammasome pathway that existed in human and porcine peripheral blood mononuclear cells but was absent from murine peripheral blood mononuclear cells and THP-1 cells (Gaidt et al. 2018). A great advantage of the cell line-based macrophage systems is the possibility to perform genetic-based studies using CRISPR/Cas9 (Fig. 4).

It is thought that after infection in humans Mtb travels into the lungs and once there, alveolar macrophages will phagocytose the bacilli. There is little evidence in humans about this critical event, there are data in mouse models of TB indicating that alveolar macrophages are important for disease progression and dissemination (Cohen et al. 2018). Alveolar macrophages are therefore considered the niche for Mtb and to carry out experiments with primary alveolar macrophages clearly represents a more physiological setting. One of the caveats of this model are the cell numbers since these cells are obtained from bronchoscopy and bronchoalveolar lavage fluid or post-mortem lung tissue. Therefore, these cells are difficult to culture in sufficient numbers for extensive in vitro experiments (Fig. 4). There are however available protocols that are suitable for experiments (O’Leary et al. 2014) to investigate macrophage metabolism (Gleeson et al. 2018) and autophagy (Coleman et al. 2018).

A widely used cellular system of human macrophages is peripheral blood-derived mononuclear cell macrophages (MDM) that are differentiated in vitro using a wide range of cytokines. Human primary macrophages are physiologically relevant but highly variable across donors and are not amenable to genetic manipulation using CRISPR/Cas9 or other gene editing systems. In contrast to mouse macrophages, one important physiological feature of MDM is that despite the expression of inducible nitric oxide synthase (iNOS), these cells produce low (or absent) levels of nitric oxide after activation. It is important to consider that independently of the method used to isolate and differentiate the MDM, these cells have a limited lifetime that is normally about 2 weeks. Careful studies that compared oxygen levels, source of serum, and cytokines were able to significantly extend the survival of MDM for longer than 2 weeks (Vogt and Nathan 2011). Therefore, in well-defined systems MDMs represent a good source of physiologically relevant macrophages.

The fast-moving field of stem cell research has significantly increased the options to work with human macrophages (Lee, Kozaki and Ginhoux 2018). In particular, human induced pluripotent stem cell- (hiPSC) derived macrophages (iPSDM) represent a very attractive system to study the biology the human macrophages (van Wilgenburg et al. 2013) (Fig. 4) since gene expression studies showed that iPSDM resembles human monocyte-derived macrophages (Hale et al. 2015). One of the most useful advantages is the possibility to use CRISPR/Cas9-based genome editing. Moreover, there are continuous efforts in multiple laboratories to differentiate hiPSC into different physiological human alveolar-like macrophages, a system that would be extremely useful to study Mtb infection. Although how closely these macrophages represent physiologically relevant macrophages remains to be defined (Lee, Kozaki and Ginhoux 2018). These cells are derived from single donors (either individual donors or from a stem cell bank) and experiments need to be repeated with several donors. The possibility to use iPSDM from patients with specific mutations to investigate biological functions is also very attractive. However, in addition to the costs associated with this method, an important aspect to consider is that these cells contain a specific genetic background with natural mutations. So, if mutations affecting the pathway under study are present, this should be analysed before starting with any particular clone in cases where the genome of that particular clone is available. Overall, iPSDM represent a powerful and genetically amenable tool to investigate the cell biology of human macrophages during infection and it will certainly be more used in future studies.

Another aspect to consider is that biology is in three dimensions. The relevance of 3-dimensional organisation in the context of Mtb infection and granuloma formation is key to our understanding of TB pathogenesis and 3-dimensional cellular systems have been developed for studying TB treatment (Bielecka and Elkington 2018). In these models, infected primary human cells are co-cultured with collagen matrix gels, agarose beads or agarose-coated plates and it could also include epithelial cells and fibroblasts (Fonseca et al. 2017). A microsphere system, has also been used to test efficacy of anti-TB drugs. In this system, microspheres are generated within a cell encapsulator, incorporating Mtb-infected primary human cells including monocytes and T cells within extracellular matrix (Tezera et al. 2017). Interestingly, lung organoids and lung-on-a-chip models have been investigated in the context of infection (Sachs et al. 2019), however, further development and validation is required, for example by incorporating different cell types relevant to the infection, before applying these experimental systems to the study of TB disease.

Localisation of Mtb within host cells

Determining the localisation of Mtb within human macrophages (and other host cells) is critical to understand how cellular pathways contribute to bacterial control or replication. Defining whether Mtb is in an acidic phagolysosome, an autophagosome or remains in an early endosome is one of the cornerstones of the cellular microbiology of Mtb (and other intracellular pathogens). Understanding where Mtb is localised within host cells is important to understand signalling pathways triggered by Mtb. The majority of these studies of Mtb-infected
Figure 6. Measuring Mtb viability in host cells after infection. A. Currently, the conventional method to evaluate viability of Mtb relies on bacterial CFU enumeration on agar plates. This is a time-consuming approach due to the slow growth rate of Mtb that takes about 3–6 weeks to observe visible colonies on agar plates. One of its main disadvantages of this method is that clumps of bacteria cells can be miscounted as single colonies. B. The graph illustrates a genetic approach where Mtb constitutively express mCherry (red) and express GFP (green) using an inducible TetON promoter. After tetracycline induction, ‘live’ bacteria express both GFP (green) and mCherry (red) fluorescence, while ‘dead’ bacteria are only mCherry positive (red). C. A similar strategy to (B) where the Mtb reporter strains are constructed to constitutively express mCherry (smyc′::mCherry) that enables the visualization of all bacteria combined with the expression of GFP that is regulated by promoters that respond to environmental cues (for example, pH). D. Dual-targeting fluorogenic probes (CDG-DNBs) containing a BlaC-sensing unit, a caged fluorescent reporter, and a DprE1-binding unit for signal trapping. CDG-DNBs pass the Mtb cell wall through porins, BlaC and DprE1 enzymes located in the peptidoglycan layer and at the outer membrane will react with the probes. Then, BlaC will hydrolyze the lactam ring to activate the fluorophore, and DprE1 will covalently bind the anchor unit for fluorescence immobilization. The combined actions of BlaC and DprE1 would enable fluorescent labelling of single Mtb. Bacteria without any BlaC and/or DprE1 activity would not fluorescently label due to the lack of fluorescence activation (no BlaC) or signal retention (no DprE1) in cells. E. A FITC-trehalose probe that exploits the processing by Mtb Ag85 enzymes is specifically incorporated into Mtb growing in vitro and within macrophages.
cells are carried out by measuring the percentage of colocalisation of a selected cellular marker with Mtb. It is common practise to use a potentially biased quantification strategy that utilises human judgement to determine a positive or negative association of bacteria to the marker. This method, that consider events in the order of hundreds, is problematic because the localisation of Mtb is highly heterogeneous within a single cell, it shows a remarkable variability from cell to cell and it is difficult to define when an intracellular Mtb is positive or negative (Fig. 5). Despite its inherent problems, this method is commonly used and represents a useful choice in difficult cases, for example when bacteria are localised in an asymmetric and large compartment and semi or quantitative methods are imprecise (Fig. 5).

In the last decade, there has been a considerable advance in single cell quantitative image analysis. The use of unbiased quantitative image-based methods, which facilitates measuring large numbers of mycobacteria (in the order of thousands) and their association with specific markers circumvents this problem. This type of studies has considerably increased our knowledge of Mtb localisation and revealed a remarkable heterogeneity of intracellular populations. One of the most commonly used ways to do it is by masking bacteria and analysing the fluorescence of the cellular marker associated to single bacteria (Fig. 5). This approach is sometimes not useful when analysing membrane markers surrounding bacteria. For that, another approach that uses several steps of image processing (Fig. 5) can accurately measure a ring around single ‘masked’ bacteria. There are however cases where it is still difficult to use an image-based unbiased quantitative analysis. For example, in cases where bacteria are in a large and asymmetric compartment and a membrane marker needs to be measured. There are other situations where the marker is not clearly associated but overlaps with bacteria, this can be challenging when analysing markers of damaged membranes and/or phagosomes, that are partially associated with the bacteria (Fig. 5). Finally, there are conditions where a compartment positive for a specific marker contains either single or multiple bacteria. In that case, additional steps are required to distinctively quantify these events and evaluate their functional relevance. In the last few years, machine intelligence algorithms started to be used in the field of host-pathogen interactions and provide a promising approach to analyse these complex cases (Fisch et al. 2019).

Ideally, fluorescent studies using fluorescent probes and antibody-based labelling should be complemented and correlated with EM studies to observe membranes at the ultrastructural level. CLEM is useful because both fluorescence and EM-based information is combined but it is not suitable for high content studies. Tokuyasu thawed cryosectioning and immunolabelling is mostly the preferred method to visualise cellular markers and ultrastructure. This technique requires care and skill and in the case of Mtb, careful standardisation since most of the antibodies will cross-react with mycobacteria. EM stereology represents a very useful approach when analysing large populations of intracellular bacteria but in general, cellular markers are not considered. Given that the bacterial populations are very heterogeneous, correlation is one of the most desirable approaches to define localisation of intracellular Mtb.

**Intracellular Mtb viability**

At present, researchers mostly rely on rates of replication or killing of the mycobacteria at the population level e.g. by Colony Forming Unit (CFU) determination. The method of CFU to evaluate viability of Mtb in cellular models of infection is the most widely used but unfortunately the most problematic too (Fig. 6). CFU has several limitations and represents at best an approximation of the bacterial numbers in a defined system (e.g. macrophages infected with Mtb). The first point to consider is that bacteria recovered on agar plates of commercially available microbiological media are the ones able to grow under these defined conditions. This led to the concept of viable but non-culturable bacteria and this phenomenon could interfere with the interpretation of results. Another confounding factor is that the CFU method is based on the idea that one colony on the plate represents one bacteria, it is likely that is not the case in Mtb with its well-known ability to clump and form aggregates in solution (Fig. 6). Combined with the high variability associated to this technique, prone to high errors when working in Biosafety Level 3 lab with very high volume of samples and the fact that colony counts do not correlate to the dilution factor; this method needs to be carefully considered. Even more problematic is the fact that in cell biology studies that investigate the intracellular localization of Mtb, there is in general little information about the viability status of the bacteria. The main obstacle in elucidating the precise fate of Mtb has been the difficulty in unequivocally establishing if a single Mtb localised in a defined compartment (e.g. a late endosome) is able to replicate, or not, or is killed. Even under conditions where the majority of bacteria are killed, the ability of a minor pool to replicate, or to remain alive in a non-replicating state can have major implications for the future course of the infection.

In the last years, to overcome the problems associated to the CFU assay and trying to obtain information about the physiological state of Mtb within cells, image-based approaches, mostly based on fluorescence, have been developed.

A ‘live/dead’ Mtb H37Rv reporter that constitutively expresses mCherry and conditionally (following anhydrotracycline induction) expresses GFP in transcriptionally active bacteria has been developed (Martin et al. 2012). This system defines live bacteria as transcriptionally active and assumes the ‘inducer’, in this case tetracycline, is able to penetrate and reach all intracellular bacteria (Fig. 6). The ratio red/green (live/dead) pixels are averaged in untreated and antibiotic treated infected cell populations and plotted as % ‘live’ of control untreated infected cell populations. Live/Dead Mtb following treatment with the inducer. In this system where ‘live’ Mtb are mCherry + (red) and GFP + (green) while ‘dead’ Mtb are mCherry + the fluorescent ratio in cell populations is used to measure bacterial viability. Interestingly, using this approach it was found that after one day of rifampicin treatment, around 20% bacteria were still alive in Mtb-infected mouse macrophages (Martin et al. 2012).

A similar dual reporter strategy, that does not measure directly bacterial viability, has been used by combining promoters that respond to specific environmental cues in host cells and in vivo. In this system, one promoter drives constitutive expression of a fluorophore whereas a second promoter (that is regulated by environmental cues) controls the expression of a different fluorophore. Using this approach, it has been possible to define how Mtb respond to stress and nutrients in host cells and in vivo (Tan et al. 2013; Sukumar et al. 2014). Because it utilises highly stable GFP as a readout, with this system the spatiotemporal resolution is limited.

Another small molecular probe that uses a very elegant dual-targeting strategy has been developed to specifically label mycobacteria (Cheng et al. 2018). This probe discriminates
between live and dead *M. bovis* BCG. The probe is named CDG-DNB3 and fluoresces upon activation of the \(\beta\)-lactamase BlaC (a hydrolase expressed in Mtb). In a second step, the fluorescent product is retained within the bacilli through covalent modification of the Mtb enzyme decaprenylphosphoryl-\(\beta\)-d-ribose 2’-epimerase (DprE1). The probe works when bacteria are pre-treated and used to infect mouse macrophages and could potentially work to monitor viability of Mtb within host cells (Fig. 6). In long term experiments needed for monitoring Mtb replication, the system could be potentially diluted over time. Overall, it is not trivial to discriminate between live and dead bacilli when looking at infected cells and it depends largely on how Mtb viability is defined. Most of the systems have some difficulties with the interpretation of the results and ideally, a combination of more than one method is required.

Other probes exploited specific sugars that are only present in mycobacteria. The disaccharide sugar trehalose is absent in mammals and synthesized by Mtb via the enzymes Ag85A, Ag85B and Ag85C. These enzymes are sufficiently promiscuous to process a variety of trehalose analogs that are fluorescently labelled and trehalose-probe analogs are also efficiently anchored to Mtb. Trehalose probes are selectively taken up by live cells and label live Mtb in infected mammalian cells. These probes were able to differentially label relevant populations of intracellular Mtb that reflects intracellular localisation (Backus et al. 2011). These probes have not been extensively used to label replicating Mtb in macrophages. Some additional trehalose probes have been developed to detect Mtb in vitro and sputum and could represent another useful probe to monitor intracellular replication of Mtb (Kamariza et al. 2018).

### Intracellular Mtb replication

In the last few years, many groups have developed fluorescent reporters in Mtb to monitor bacterial replication in vitro. Macrophages are a niche for Mtb and resting macrophages in vitro provide very good conditions for bacterial replication. Understanding the location where Mtb replicates but also the conditions that restrict growth is critical to further delineate the cellular immune response to Mtb. This aspect however has been less investigated and establishing if a single Mtb localised in a defined compartment is able to replicate has been difficult. To measure bacterial replication quantitatively and at the single-cell level in host cells has been challenging due to the slow replication of mycobacteria and the limited availability of live cell imaging systems in Biosafety Level 3. One way to follow intracellular replication of Mtb is by monitoring active growth of fluorescent Mtb through time at single cell level in fixed cells (Fig. 7). With these methodologies it is however not possible to determine if bacteria are alive or dead as discussed above. Moreover, without spatiotemporal resolution, it is difficult to define whether Mtb was actually growing within the same cell due to cell death and efferocytosis.

Luciferase-luciferase systems are widely utilised in mycobacterial research (Andreu et al. 2010; Andreu et al. 2012). These reporters mainly exploits the firefly, *G. princeps* and Renilla Luciferase systems. In addition, bacterial luciferases have also been employed and the functional expression of the whole Lux operon in Mtb and *M. smegmatis* allowed the development of auto-luminescent mycobacteria (Andreu et al. 2010). Luciferase-based bacterial replication are useful for monitoring Mtb replication in bulk but do not allow for single cell studies in cell-based models of infection (Fig. 7).
red fluorescent protein DsRed2 (Manina, Dhar and McKinney 2015). Using this reporter strain, and quantitative time-lapse microscopy combined with a genetically encoded fluorescent reporter (rRNA-GFPdes), it is possible to investigate the single-cell dynamics of Mtb replication and rRNA expression in vitro and in vivo. These studies revealed a wide range of phenotypic heterogeneity even in bacteria grown under nutrient-rich conditions in vitro and increased heterogeneity in bacteria exposed to diverse stresses. For instance, stationary-phase populations showed a drop in rRNA-GFPdes expression followed by the appearance of a subset of non-replicating bacteria displaying high levels of fluorescence (Fig. 7). These observations confirmed that stationary-phase bacteria maintain a high level of de novo protein production, in agreement with studies in other bacteria (Rosenberg et al. 2012; Gefen, Fridman and Ronin 2014; Fridman et al. 2014). This reporter has been used mostly in vitro but one study also investigated mycobacteria in infected macrophages in drug screening studies looking for compounds that affect intracellular bacteria growth (Sorrentino et al. 2016). One of the caveats of this system is the low fluorescent signal from GFPdes and strategies using GFPdes in tandem could potentially increase signal to noise levels.

Another replication reporter has been developed and used to study the effect of vaccination on the Mtb replication status, at the single bacillus level. This reporter is based on an Mtb Erdman strain (ssb-GFP, smyc′::mCherry), consisting of a fusion of single stranded binding protein (SSB) to GFP, present on a replicating plasmid containing a constitutively expressed mCherry (Sukumar et al. 2014) (Fig. 7). Time-point-based experiments with the Mtb (ssb-GFP, smyc′::mCherry) reporter strain allow visualisation of the heterogeneity in replication status within the bacterial population at the level of the individual bacterium in mice.

**Spatiotemporal studies**

In most of the cases time courses are performed with multiple fixed-samples at pre-defined timepoints. However, it is clear that some of the interactions are transient and dynamic. In order to unambiguously define the subcellular sites of Mtb replication/restriction in human macrophages and define whether Mtb can replicate in multiple cellular compartments, including lysosomes, continuous live cell imaging is crucial. Nonetheless, there are important obstacles in the analysis of the spatiotemporal organelle dynamic interactions with Mtb and the development of reliable methods to measure bacterial replication: (1) macromolecule motility mass imaging problematic, (2) infection is rarely 100% synchronised making it difficult to capture statistically relevant numbers of events starting from zero time (time of bacterial entry), (3) macrophages die and aggregate at the site of bacterial replication and several processes of cell-to-cell transfer can dramatically interfere with the analysis and (4) the infection rates of macrophages are highly heterogeneous.

In recent years, several imaging approaches have been established to study the spatiotemporal detection of Mtb (Schnettger et al. 2017) and other mycobacteria (Delince et al. 2016) at the single cell level during in vitro infection. This represents an opportunity to study in more detail the precise roles of the different cellular pathways and their interplay with intracellular organelles for deciding the fate of Mtb in human macrophages.

**Concluding remarks: Mtb survival and replication in space and time**

The precise cellular mechanisms that control Mtb replication in human macrophages, and in particular the mechanisms by
which Mtb usurps the anti-bacterial host cell defence system, have not been completely elucidated. It is likely that the differentially localised populations of intracellular Mtb dynamically interact with cellular organelles during infection (Fig. 8). Spatiotemporal interactions between distinct Mtb populations and cellular organelles will dictate whether Mtb replicates, its growth restricted or eventually killed (Fig. 8). Given this background, there is a need to functionally define the precise locations where Mtb remains in host cells and their contribution to TB pathogenesis. In parallel, it will be important to identify the bacterial factors that allow Mtb to survive and eventually replicate in host cells and tissues.

The scarcity of knowledge in certain areas of host-cell-Mtb interactions is likely a consequence of a lack of technologies that allow spatiotemporal resolution at the single cell level. For example, although the long-standing dogma in the field states that the lysosomal environment is detrimental for Mtb replication, several studies with mycobacteria challenge the notion that lysosomes are the sites where bacterial killing occurs (Armstrong and Hart 1975; Jordao et al. 2008; Levitte et al. 2016). There is no clear correlation between Mtb delivery to phagolysosomes and control of bacterial replication. A previous study showed that during Mtb replication in human macrophages, the number of bacteria in membrane compartments positive for CD63 (which is a frequently used marker of fusion of phagosomes with late endosomes and lysosomes)- or LAMP-1-positive phagosomes increased, suggesting that Mtb was able to replicate within compartments that have acquired the markers expected for lysosomes (Welin et al. 2011). Additional reports also showed that a proportion of Mtb was positive for Rab7 and other late endocytic markers (Seto et al. 2009; Harriff et al. 2014). However, heterogeneity and lack of single cell studies made it difficult to define where Mtb replicates. For instance, it is not possible to exclude that Mtb replicated in a non-fusogenic phagosome and consequently had been taken up into an autophagosome that cert to cause phagosomal rupture and host cell apoptosis. Reversal of the usual nonfusion pattern and observations on bacterial survival. J Exp Med 1975;142:1–16. Armstrong JA, Hart PD. Phagosome-lysosome interactions in cultured macrophages infected with virulent tubercle bacilli. Cell Microbiol 2017;19:e12726. Bach H, Papavinasasundaram KG, Wong D et al. Mycobacterium tuberculosis virulence is mediated by PtpA dephosphorylation of human vacuolar protein sorting 33B. Cell Host Microbe 2008;3:316–22. Backus KM, Boshoff HI, Barry CS et al. Uptake of unnatural trehalose analogs as a reporter for Mycobacterium tuberculosis. Nat Chem Biol 2011;7:228–35. Barber GN. STING: infection, inflammation and cancer. Nat Rev Immunol 2015;15:760–70. Barczak AK, Avraham R, Singh S et al. Systematic, multiparametric analysis of Mycobacterium tuberculosis intracellular infection offers insight into coordinated virulence. PLoS Pathog 2017;13:e1006363. Barisch C, Paschke P, Hagedorn M et al. Lipid droplet dynamics at early stages of Mycobacterium marinum infection in Dictyostelium. Cell Microbiol 2015;17:1332–49. Behr MA, Waters WR. Is tuberculosis a lymphatic disease with a pulmonary portal? Lancet Infect Dis 2014;14:250–5. Beigier-Bompadre M, Montagna GN, Kuhl AA et al. Mycobacterium tuberculosis infection modulates adipose tissue biology. PLoS Pathog 2017;13:e1006676. Bielecka MK, Elkington P. Advanced cellular systems to study tuberculosis treatment. Curr Opin Pharmacol 2018;42:16–21. Birkeland HC, Stenmark H. Protein targeting to endosomes and phagosomes via FYVE and PX domains. Curr Top Microbiol Immunol 2004;282:89–115. Braulke T, Bonifacino JS. Sorting of lysosomal proteins. Biochim Biophys Acta 2009;1793:605–14.

From a therapeutic perspective, the intracellular lifestyle of Mtb represents a crucial stage in the disease and successful drug discovery programmes have to include in vitro studies using infected human host cells (Young et al. 2010; Small et al. 2013). This is consistent with the idea that Mtb experiences acid stress in vivo and that the pathogen possess factors to counteract an acidic environment (Vandal et al. 2008).

ACKNOWLEDGEMENTS

We thank Gareth Griffiths and members of the Host-Pathogen Interactions in TB Laboratory for critical comments on the manuscript. We are grateful to Joe Brock for preparing Figure 1 and our EM guru Tony Fearn for providing the EM images for Figure 2. MGG would like to dedicate this review to Maria Isabel Colombo, Michel Rabinhovitch, Vojo Deretic, Gareth Griffiths and Douglas Young for many years of great discussions on Mtb phagosomes and host-pathogen interactions.

REFERENCES

Alonso S, Pethe K, Russell DG et al. Lysosomal killing of Mycobacterium mediariumed by ubiquitin-derived peptides is enhanced by autophagy. Proc Natl Acad Sci USA 2007;104:6031–6. Andreu N, Fletcher T, Krishnan N et al. Rapid measurement of antituberculosis drug activity in vitro and in macrophages using bioluminescence. J Antimicrob Chemother 2012;67:404–14. Andreu N, Zelmer A, Fletcher T et al. Optimisation of bioluminescent reporters for use with mycobacteria. PLoS One 2010;5:e10777. Anes E, Kuhnel MP, Bos E et al. Selected lipids activate phagosome actin assembly and maturation resulting in killing of pathogenic mycobacteria. Nat Cell Biol 2003;5:793–802. Armstrong JA, Hart PD. Phagosome-lysosome interactions in cultured macrophages infected with virulent tubercle bacilli. Cell Microbiol 2017;19:e12726. Bach H, Papavinasasundaram KG, Wong D et al. Mycobacterium tuberculosis virulence is mediated by PtpA dephosphorylation of human vacuolar protein sorting 33B. Cell Host Microbe 2008;3:316–22. Backus KM, Boshoff HI, Barry CS et al. Uptake of unnatural trehalose analogs as a reporter for Mycobacterium tuberculosis. Nat Chem Biol 2011;7:228–35. Barber GN. STING: infection, inflammation and cancer. Nat Rev Immunol 2015;15:760–70. Barczak AK, Avraham R, Singh S et al. Systematic, multiparametric analysis of Mycobacterium tuberculosis intracellular infection offers insight into coordinated virulence. PLoS Pathog 2017;13:e1006363. Barisch C, Paschke P, Hagedorn M et al. Lipid droplet dynamics at early stages of Mycobacterium marinum infection in Dictyostelium. Cell Microbiol 2015;17:1332–49. Behr MA, Waters WR. Is tuberculosis a lymphatic disease with a pulmonary portal? Lancet Infect Dis 2014;14:250–5. Beigier-Bompadre M, Montagna GN, Kuhl AA et al. Mycobacterium tuberculosis infection modulates adipose tissue biology. PLoS Pathog 2017;13:e1006676. Bielecka MK, Elkington P. Advanced cellular systems to study tuberculosis treatment. Curr Opin Pharmacol 2018;42:16–21. Birkeland HC, Stenmark H. Protein targeting to endosomes and phagosomes via FYVE and PX domains. Curr Top Microbiol Immunol 2004;282:89–115. Braulke T, Bonifacino JS. Sorting of lysosomal proteins. Biochim Biophys Acta 2009;1793:605–14.
Briken V, Porcelli SA, Besra GS et al. Mycobacterial lipoarabinomannan and related lipoglycans: from biogenesis to modulation of the immune response. Mol Microbiol 2004;53:391–403.

Cai X, Chiu YH, Chen ZJ. The cGAS-cGAMP-STING pathway of cytosolic DNA sensing and signaling. Mol Cell 2014;54:289–96.

Cemma M, Brumell JH. Interactions of pathogenic bacteria with autophagy systems. Curr Biol 2012;22:R540–545.

Cheng Y, Scholey JS. Mycobacterium tuberculosis-induced IFN-beta production requires cytosolic DNA and RNA sensing pathways. J Exp Med 2018;215:2919–35.

Cheng Y, Xie J, Lee KH et al. Rapid and specific labeling of single live Mycobacterium tuberculosis with a dual-targeting fluorogenic probe. Sci Transl Med 2018;10:eaar4470.

Choi HH, Shin DM, Kang G et al. Endoplasmic reticulum stress response is involved in Mycobacterium tuberculosis protein ESAT-6-mediated apoptosis. FEBS Lett 2010;584:2445–54.

Chowdhury SR, Reimer A, Sharan M et al. Chlamydia preserves the mitochondrial network necessary for replication via microRNA-dependent inhibition of fission. J Cell Biol 2017;216:1071–89.

Christoforidis S, Miaczynska M, Ashman K et al. Phosphatidylinositol-3-OH kinases are Rab5 effectors. Nat Cell Biol 1999;1:249–52.

Clemens DL, Horwitz MA. Characterization of the Mycobacterium tuberculosis phagosome and evidence that phagosomal maturation is inhibited. J Exp Med 1995;181:257–70.

Clemens DL, Horwitz MA. The Mycobacterium tuberculosis phagosome interacts with early endosomes and is accessible to exogenously administered transferrin. J Exp Med 1996;184:1349–55.

Cohen SB, Gern BH, Delahaye JL et al. Alveolar macrophages provide an early mycobacterium tuberculosis niche and initiate dissemination. Cell Host Microbe 2018;24:439–446 e434.

Coleman MM, Basdeo SA, Coleman AM et al. All-trans Retinoic Acid Augments Autophagy during Intracellular Bacterial Infection. Am J Respir Cell Mol Biol 2018;59.

Collins AC, Cai H, Li T et al. Cyclop GMP-AMP synthase is an innate immune DNA sensor for mycobacterium tuberculosis. Cell Host Microbe 2015;17:820–8.

Conrad WH, Osman MM, Shanahan JK et al. Mycobacterial ESX-1 secretion system mediates host cell lysis through bacterium contact-dependent gross membrane disruptions. Proc Natl Acad Sci USA 2017;114:1371–6.

Crowle AJ, Dahl R, Ross E et al. Evidence that vesicles containing living, virulent Mycobacterium tuberculosis or Mycobacterium avium in cultured human macrophages are not acidic. Infect Immun 1991;59:1823–31.

Daniel J, Maamar H, Deb C et al. Mycobacterium tuberculosis uses host triacylglycerol to accumulate lipid droplets and acquires a dormancy-like phenotype in lipid-loaded macrophages. PLoS Pathog 2011;7:e1002093.

Das B, Kashino SS, Pulu I et al. CD271 (+) bone marrow mesenchymal stem cells may provide a niche for dormant Mycobacterium tuberculosis. Sci Transl Med 2013;5:176ra113.

De Chastellier C, Thilo L. Phagosome maturation and fusion with lysosomes in relation to surface property and size of the phagocytic particle. EUR J CELL BIOL 1997;74:49–62.

Delince MJ, Bureau JB, Lopez-Jimenez AT et al. A microfluidic cell-trapping device for single-cell tracking of host-microbe interactions. Lab Chip 2016;16:3276–85.

Deretic V. Autophagy in tuberculosis. Cold Spring Harb Perspect Med 2014;4:a018481.

Divangahi M, Chen M, Gan H et al. Mycobacterium tuberculosis evades macrophage defenses by inhibiting plasma membrane repair. Nat Immunol 2009;10:899–906.

Escoll P, Song OR, Viana F et al. Legionella pneumophila modulates mitochondrial dynamics to trigger metabolic repurposing of infected macrophages. Cell Host Microbe 2017;22:302–316e307.

Eum SY, Kong JH, Hong MS et al. Neutrophils are the predominant infected phagocytic cells in the airways of patients with active pulmonary TB. Chest 2010;137:122–8.

Fabri M, Realegeno SE, Jo EK et al. Role of autophagy in the host response to microbial infection and potential for therapy. Curr Opin Immunol 2011;23:65–70.

Fennelly KP, Jones-Lopez EC. Quantity and quality of inhaled dose predicts immunopathology in tuberculosis. Front Immunol 2015;6:313.

Ferrari G, Langen H, Naito M et al. A coat protein on phagosomes involved in the intracellular survival of mycobacteria. Cell 1999;97:435–47.

Fisch D, Yakimovich A, Clough B et al. Defining host-pathogen interactions employing an artificial intelligence workflow. Elife 2019;8:e40560. DOI: 10.7554/eLife.40560.

Fonseca KL, Rodrigues PNS, Olsson IAS et al. Experimental study of tuberculosis: From animal models to complex cell systems and organoids. PLoS Pathog 2017;13:e1006421.

Franco LH, Nair VR, Scharn CR et al. The ubiquitin ligase smurf1 functions in selective autophagy of mycobacterium tuberculosis and anti-tuberculous host defense. Cell Host Microbe 2017;22:421–3.

Fratti RA, Backer JM, Gruenberg J et al. Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest. J Cell Biol 2001;154:631–44.

Fratti RA, Chua J, Vergne I et al. Mycobacterium tuberculosis glycosylated phosphatidylinositol causes phagosome maturation arrest. Proc Natl Acad Sci USA 2003;100:5437–42.

Fridman O, Goldberg A, Ronin I et al. Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. Nature 2014;513:418–21.

Fukuda T, Matsumura T, Ato M et al. Critical roles for lipo- mannan and lipoarabinomannan in cell wall integrity of mycobacteria and pathogenesis of tuberculosis. MBio 2013;4:e00472–00412.

Gaidt MM, Ebert TS, Chauhan D et al. Human monocytes engage an alternative inflammasome pathway. Immunity 2016;44:833–46.

Gaidt MM, Rapino F, Graf T et al. Modeling primary human monocytes with the trans-differentiation cell line BLaER1. Methods Mol Biol 2018;1714:57–66.

Ganaie AA, Lella RK, Solanki R et al. Thermostable hexameric form of Eis (Rv2416c) protein of M. tuberculosis plays an important role in the intracellular survival of mycobacteria. Eur J Cell Biol 2016;95:556–61.

Geeleson LE, O’Leary SM, Ryan D et al. Cigarette smoking impairs the bioenergetic immune response to mycobacterium tuberculosis infection. Am J Respir Cell Mol Biol 2018;59.

Gefen O, Fridman O, Ronin I. Direct observation of single stationary-phase bacteria reveals a surprisingly long period of constant protein production activity. Proc Natl Acad Sci USA 2014;111:556–61.

Gleeson LE, O’Leary SM, Ryan D et al. Cigarette smoking impairs the bioenergetic immune response to mycobacterium tuberculosis infection. Am J Respir Cell Mol Biol 2018;59.
Gomes MS, Paul S, Moreira AL et al. Survival of Mycobacterium avium and Mycobacterium tuberculosis in acidified vacuoles of murine macrophages. *Infect Immun* 1999;67:3199–206.

Grover S, Sharma T, Singh Y et al. The PGRS domain of mycobacterium tuberculosis PE_PGRS protein Rv0297 is involved in endoplasmic reticulum stress-mediated apoptosis through toll-like receptor 4. *MBio* 2018;9:e01017–18.

Guérin I, de Chastellier C. Pathogenic mycobacteria disrupt the macrophage actin filament network. *Infect Immun* 2000;68:2655–62.

Gutierrez MG, Master SS, Singh SB et al. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell 2004;119:753–66.

Hale C, Yeung A, Goulding D et al. Induced pluripotent stem cell derived macrophages as a cellular system to study salmonella and other pathogens. *PLoS* One 2015;10:e0124307.

Haque MF, Boonhok R, Prammanan T et al. Resistance to cellular autophagy by Mycobacterium tuberculosis Beijing strains. *Innate Immun* 2015;21:746–58.

Harriff MJ, Cansler ME, Toren KG et al. Immunity to induced host cell death. *PLoS Pathog* 2018;14:e1006874.

Kumar D, Nath L, Kamal MA et al. Genome-wide analysis of the host intracellular network that regulates survival of Mycobacterium tuberculosis. Cell 2010;140:731–43.

Köster S, Upadhyay S, Chandra P et al. Mycobacterium tuberculosis is protected from NADPH oxidase and LC3-associated phagocytosis by the LCP protein CpsA. *Proc Natl Acad Sci* 2017;114:E8711–20.

Lee CZW, Kozaki T, Ginhoux F. Studying tissue macrophages in vitro: are iPSC-derived cells the answer? *Nat Rev Immunol* 2018;18:716–25.

Lerner TR, Borel S, Greenwood DJ et al. Mycobacterium tuberculosis replicates within necrotic human macrophages. *J Cell Biol* 2017;216:583–94.

Lerner TR, Borel S, Gutierrez MG. The innate immune response in human tuberculosis. *Cell Microbiol* 2015;17:1277–85.

Lerner TR, de Souza Carvalho-Wodarz C, Repnik U et al. Lymphatic endothelial cells are a replicative niche for Mycobacterium tuberculosis. *J Clin Invest* 2016;126:1093–108.

Lerner TR, Queval CJ, Fearsn A et al. Phtioceroldimycocerosates promote access to the cytosol and intracellular burden of Mycobacterium tuberculosis in lymphatic endothelial cells. *MBMC Biol* 2018;161.

Levitte S, Adams KN, Berg RD et al. Mycobacterial acid tolerance enables phagolysosomal survival and establishment of tuberculous infection in vivo. *Cell Host Microbe* 2016;20:250–8.

Liu CH, Liu H, Ge B. Innate immunity in tuberculosis: host defense vs pathogen evasion. *Cell Mol Immunol* 2017;14:963–75.

Lobet E, Letesson JJ, Arnould T. Mitochondria: A target for bacteria. *Biochem Pharmacol* 2015;94:173–85.

Lopez-Jimenez AT, Cardenal-Munoz E, Leuba F et al. The ESCRT and autophagy machineries cooperate to repair ESX-1-dependent damage at the Mycobacterium-containing vacuole but have opposite impact on containing the infection. *PLoS Pathog* 2018;14:e1007501.

Lum M, Morona R. Dynamin-related protein Drp1 and mitochondria are important for Shigella flexneri infection. *Int J Med Microbiol* 2014;304:530–41.
Mahamed D, Boule M, Ganga Y et al. Intracellular growth of Mycobacterium tuberculosis after macrophage cell death leads to serial killing of host cells. Elife 2017;6:e22028 1-26. DOI: 10.7554/eLife.22028

Majlessi L, Brosch R. Mycobacterium tuberculosis meets the cytosol: The role of cGAS in Anti-mycobacterial Immunity. Cell Host Microbe 2015;17:733–5.

Malik ZA, Denning GM, Kusner DJ. Inhibition of Ca(2+) signaling by Mycobacterium tuberculosis is associated with reduced phagosome-lysosome fusion and increased survival within human macrophages. J Exp Med 2000;191:287–302.

Malik ZA, Iyer SS, Kusner DJ. Mycobacterium tuberculosis phagosomes exhibit altered calmodulin-dependent signal transduction: contribution to inhibition of phagosome-lysosome fusion and intracellular survival in human macrophages. J Immunol 2001;166:3392–401.

Manina G, Dhar N, McKinney JD. Stress and host immunity amplify Mycobacterium tuberculosis phenotypic heterogeneity and induce nongrowing metabolically active forms. Cell Host Microbe 2015;17:32–46.

Manzanillo PS, Ayres JS, Watson RO et al. The ubiquitin ligase parkin mediates resistance to intracellular pathogens. Nature 2013;501:512–6.

Martin CJ, Booty MG, Rosebrock TR et al. Effecrotysin is an innate antibacterial mechanism. Cell Host Microbe 2012;12:289–300.

Martinez J, Malireddi RKS, Lu Q et al. Molecular characterization of LC3-associated phagocytosis reveals distinct roles for Rubicon, NOX2 and autophagy proteins. Nat Cell Biol 2015;17:893.

Mayito J, Andia I, Belay M et al. Anatomic and cellular niches for mycobacterium tuberculosis in latent tuberculosis infection. J Infect Dis 2019;219:685–94.

McDonough KA, Kress Y, Bloom BR. Pathogenesis of tuberculosis: interaction of Mycobacterium tuberculosis with macrophages. Infect Immun 1993;61:2763–73.

Mittal E, Skowrya ML, Uwase G et al. Mycobacterium tuberculosis Type VII secretion system effectors differentially impact the ESCRT Endomembrane Damage Response. MBio 2018;9:1-21 e01765-18.

Moreira AL, Wang J, Tsenova-Berkova L et al. Sequestration of Mycobacterium tuberculosis in tight vacuoles in vivo in lung macrophages of mice infected by the respiratory route. Infect Immun 1997;65:305–8.

Mori M, Mode R, Pieters J. From phagocytes to immune defense: Roles for corinon proteins in dictyostelium and mammalian immunity. Front Cell Infect Microbiol 2018;8:77.

Myrvik QN, Leake ES, Wright MJ. Disruption of phagosomal membranes of normal alveolar macrophages by the H37Rv strain of Mycobacterium tuberculosis. A correlate of virulence. Am Rev Respir Dis 1984;129:322–8.

Nair S, Huynh JP, Lampropoulou V et al. Irg1 expression in myeloid cells prevents immunopathology during M. tuberculosis infection. J Exp Med 2018;215:1035–45.

Nair VR, Franco LH, Zacharia VM et al. Microfold cells actively translocate mycobacterium tuberculosis to initiate infection. Cell Rep 2016;16:1253–8.

Neyrolles O, Hernandez-Pando R, Pietri-Rouxel F et al. Is adipose tissue a place for Mycobacterium tuberculosis persistence? PLoS One 2006;1:e43.

Ó’Leary SM, Coleman MM, Chew WM et al. Cigarette smoking impairs human pulmonary immunity to Mycobacterium tuberculosis. Am J Respir Crit Care Med 2014;190:1430–6.

O’Hoi YM, Goetz DH, Chan K et al. Mycobacterium tuberculosis MyCP1 protease plays a dual role in regulation of ESX-1 secretion and virulence. Cell Host Microbe 2010;7:210–20.

Paulson T. Epidemiology: A mortal foe. Nature 2013;502:52–3.

Pei G, Buijze H, Liu H et al. The E3 ubiquitin ligase NEDD4 enhances killing of membrane-perturbing intracellular bacteria by promoting autophagy. Autophagy 2017;13:2041–55.

Peyron P, Vaubourgeix J, Poquet Y et al. Foamy macrophages from tuberculous patients’ granulomas constitute a nutrient-rich reservoir for M. tuberculosis persistence. PLoS Pathog 2008;4:e1000204.

Pieters J. Mycobacterium tuberculosis and the macrophage: Maintaining a balance. Cell Host Microbe 2008;3:399–407.

Pilli M, Arko-Mensah J, Ponpuak M et al. TBK-1 promotes autophagy-mediated antimicrobial defense by controlling autophagosome maturation. Immunity 2012;37:223–34.

Pitarque S, Larrouy-Maumus G, Payne B et al. The immunomodulatory lipoglycans, lipoarabinomannan and lipomannan, are exposed at the mycobacterial cell surface. Tuberculosis (Edinb) 2008;88:560–5.

Pizarro-Cerda J, Cossart P. Subversion of phosphoinositide metabolism by intracellular bacterial pathogens. Nat Cell Biol 2006;6:1026–33.

Ponpuak M, Davis AS, Roberts EA et al. Delivery of cytotoxic components by autophagic adaptor protein p62 endows autophagosomes with unique antimicrobial properties. Immunity 2010;32:329–41.

Ponpuak M, Deretic V. Autophagy and p62/sequestosome 1 generate neo-antimicrobial peptides (cryptides) from cytosolic proteins. Autophagy 2011;7:336–7.

Queval CJ, Song OR, Carralot JP et al. Mycobacterium tuberculosis controls phagosomal acidification by targeting CISH-Mediated Signaling. Cell Rep 2017;20:3188–98.

Quigley J, H Hughitt VK, Velikovsky CA et al. The cell wall lipid PDIM contributes to phagosomal escape and host cell exit of Mycobacterium tuberculosis. MBio 2017;8:e00148-17.

Randall PJ, Hsu NJ, Quesniaux V et al. Mycobacterium tuberculosis infection of the ‘non-classical immune cell’. Immunol Cell Biol 2015;93:789–95.

Refai A, Haoues M, Othman H et al. Two distinct conformational states of Mycobacterium tuberculosis virulent factor early secreted antigenic target 6 kDa are behind the discrepancy among its biological functions. FEBS J 2015;282:4114–29.

Rifat D, Bishai WR, Karakousis PC. Phosphate depletion: a novel trigger for Mycobacterium tuberculosis persistence. J Infect Dis 2009;200:1126–35.

Romagnoli A, Etna MP, Giacomini E et al. ESX-1 dependent impairment of autophagic flux by Mycobacterium tuberculosis in human dendritic cells. Autophagy 2012;8:1357–70.

Rosenberg A, Sinai L, Smith Y et al. Dynamic expression of the translational machinery during Bacillus subtilis life cycle at a single cell level. PLoS One 2012;7:e41921.

Roth MG. Phosphoinositides in constitutive membrane traffic. Physiol Rev 2004;84:699–730.

Roy D, Liston DR, Idone VJ et al. A process for controlling intracellular bacterial infections induced by membrane injury. Science 2004;304:1515–8.

Rubin EJ. The granuloma in tuberculosis-friend or foe? N Engl J Med 2009;360:2471–3.

Russell DG, Dant J, Sturgill-Koszycki S. Mycobacterium avium and Mycobacterium tuberculosis-containing vacuoles are dynamic, fusion-competent vesicles that are accessible to glycosphingolipids from the host cell plasmalemma. J Immunol 1996;156:4764–73.
Russell DG, Mwandumba HC, Rhoades EE. Mycobacterium and the coat of many lipids. J Cell Biol 2002;158:421–6.

Russell DG. Mycobacterium tuberculosis: Here today, and here tomorrow. Nat Rev Mol Cell Biol 2001;2:569–77.

Russell DG. The ins and outs of the Mycobacterium tuberculosis-containing vacuole. Cell Microbiol 2016;18:1065–9.

Sachs N, Papasyproupolou A, Zomer-van Ommen DD et al. Long-term expanding human airway organoids for disease modeling. EMBO J 2019;38:e100300 1–20.

Saleh MT, Belisle JT. Secretion of an acid phosphatase (SapM) by Mycobacterium tuberculosis that is similar to eukaryotic acid phosphatases. J Bacteriol 2000;182:6850–3.

Schafer G, Jacobs M, Wilkinson RJ et al. Non-opsonic recognition of Mycobacterium tuberculosis by phagocytes. J Innate Immun 2009;1:231–43.

Schnettger L, Rodgers A, Repnik U et al. A Rab20-dependent membrane trafficking pathway controls M. tuberculosis replication by regulating phagosome spaciousness and integrity. Cell Host Microbe 2017;21:619–628 e615.

Seto S, Matsumoto S, Ohta I et al. Dissection of Rab7 localization on Mycobacterium tuberculosis phagosome. Biochem Biophys Res Commun 2009;378:272–7.

Seto S, Tsujimura K, Koide Y. Coronin-1a inhibits autophagosome formation around Mycobacterium tuberculosis-containing phagosomes and assists mycobacterial survival in macrophages. Cell Microbiol 2012;14:710–27.

Seto S, Tsujimura K, Koide Y. Rab GTPases regulating phagosome maturation are differentially recruited to mycobacterial phagosomes. Traffic 2011;12:407–20.

Shin DM, Jeon BY, Lee HM et al. Mycobacterium tuberculosis eis regulates autophagy, inflammation, and cell death through redox-dependent signaling. PLoS Pathog 2010;6:e1001230.

Shui W, Petzold CJ, Redding A et al. Organelle membrane proteomics reveals differential influence of mycobacterial lipoglycans on macrophage phagosome maturation and autophagosome accumulation. J Proteome Res 2011;10:339–48.

Simeone R, Bobard A, Lippmann J et al. Phagosomal rupture by Mycobacterium tuberculosis results in toxicity and host cell death. PLoS Pathog 2012;8:e1002507.

Simeone R, Majessi L, Enninga J et al. Perspectives on mycobacterial vacuole-to-cytosol translocation: the importance of cytosolic access. Cell Microbiol 2016;18:1070–7.

Simeone R, Sayes F, Song O et al. Cytosolic access of Mycobacterium tuberculosis: critical impact of phagosomal acidification control and demonstration of occurrence in vivo. PLoS Pathog 2015;11:e1004650.

Simonsen A, Lippe R, Christoforidis S et al. EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. Nature 1998;394:494–8.

Skowyra ML, Schlesinger PH, Naismith TV et al. Triggered recruitment of ESCRT machinery promotes endolysosomal repair. Science 2018;360:eaar5078.

Small JL, O’Donoghue AJ, Boritsch EC et al. Substrate specificity of MarP, a periplasmic protease required for resistance to acid and oxidative stress in Mycobacterium tuberculosis. J Biol Chem 2013;288:12489–99.

Soldati T, Neyrolles O. Mycobacteria and the intraphagosomal environment: take it with a pinch of salt(s)! Traffic 2012;13:1042–52.

Sorrentino F, Gonzalez del Rio R, Zheng X et al. Development of an intracellular screen for new compounds able to inhibit mycobacterium tuberculosis growth in human macrophages. Antimicrob Agents Chemother 2016;60:640–5.

Stamm LM, Morisaki JH, Gao LY et al. Mycobacterium marinum escapes from phagosomes and is propelled by actin-based motility. J Exp Med 2003;198:1361–8.

Stanley SA, Cox JS. Host-pathogen interactions during Mycobacterium tuberculosis infections. Curr Top Microbiol Immunol 2013;374:211–41.

Steinhauser C, Heigl U, Tchikov V et al. Lipid-labeling facilitates a novel magnetic isolation procedure to characterize pathogen-containing phagosomes. Traffic 2013;14:321–36.

Sturgill-Koszycki S, Schaible UE, Russell DG. Mycobacterium-containing phagosomes are accessible to early endosomes and reflect a transitional state in normal phagosome biogenesis. EMBO J 1996;15:6960–8.

Sturgill-Koszycki S, Schlesinger PH, Chakraborty P et al. Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science 1994;263:678–81.

Sukumar N, Tan S, Aldridge BB et al. Exploitation of Mycobacterium tuberculosis reporter strains to probe the impact of vaccination at sites of infection. PLoS Pathog 2014;10:e1004394.

Sundstrom C, Nilsson K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int J Cancer 1976;17:565–77.

Tan S, Sukumar N, Abramovitch RB et al. Mycobacterium tuberculosis responds to chloride and pH as synergistic cues to the immune status of its host cell. PLoS Pathog 2013;9:e1003282.

Tezera LB, Bielecka MK, Chancellor A et al. Dissection of the host-pathogen interaction in human tuberculosis using a bioengineered 3-dimensional model. Elife 2017;6:e21283. DOI: 10.7554/eLife.21283.

Tornack J, Reece ST, Bauer WM et al. Human and mouse hematopoietic stem cells are a depot for dormant mycobacterium tuberculosis. PLoS One 2017;12:e0169119.

Tsuchiya S, Yamabe M, Yamaguchi Y et al. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int J Cancer 1980;26:171–6.

Vandahl OH, Pierini LM, Schnappinger D et al. A membrane protein preserves intrabacterial pH in intraphagosomal Mycobacterium tuberculosis. Nat Med 2008;14:849–54.

VanderVen BC, Huang L, Rohde KH et al. The minimal unit of infection: Mycobacterium tuberculosis in the macrophage. Microbiol Spectr 2016;4. doi: 10.1128/microbiolspec.TBTB2-0025-2016.

van der Wel N, Hava D, Houben D et al. Tuberculosis and M. leprae translocate from the phagosomal to the cytosol in myeloid cells. Cell 2007;129:1287–98.

van WiJlgenburg B, Browne C, Vowles J et al. Efficient, long term production of monocye-derived macrophages from human pluripotent stem cells under partly-defined and fully-defined conditions. PLoS One 2013;8:e71098.

Vazquez CL, Rodgers A, Herbst S et al. The proneurotrophin receptor sortilin is required for Mycobacterium tuberculosis control by macrophages. Sci Rep 2016;6:29332.

Vergne I, Chua J, Deretic V. Tuberculosis toxin blocking phagosome maturation inhibits a novel Ca2+/calmodulin-Pi3K hVPS34 cascade. J Exp Med 2003;198:653–9.

Vergne I, Chua J, Singh SB et al. Cell biology of mycobacterium tuberculosis phagosome. Annu Rev Cell Dev Biol 2004a;20:367–94.
Bussi and Gutierrez

Vergne I, Fratti RA, Hill PJ et al. Mycobacterium tuberculosis phagosome maturation arrest: Mycobacterial phosphatidylinositol analog phosphatidylinositol mannoside stimulates early endosomal fusion. Mol Biol Cell 2004b;15:751–60.

Vergne I, Gilleron M, Nigou J. Manipulation of the endocytic pathway and phagocyte functions by Mycobacterium tuberculosis lipoarabinomannan. Front Cell Infect Microbiol 2014;4:187.

Via LE, Deretic D, Ulmer RJ et al. Arrest of mycobacterial phagosome maturation is caused by a block in vesicle fusion between stages controlled by rab5 and rab7. J Biol Chem 1997;272:13326–31.

Vickers CF, Silva APG, Chakraborty A et al. Structure-based design of MptpB inhibitors that reduce multidrug-resistant mycobacterium tuberculosis survival and infection burden in Vivo. J Med Chem 2018;61:8337–52.

Vogt G, Nathan C. In vitro differentiation of human macrophages with enhanced antimycobacterial activity. J Clin Invest 2011;121:3889–901.

Wagner D, Maser J, Lai B et al. Elemental analysis of Mycobacterium avium-, Mycobacterium tuberculosis-, and Mycobacterium smegmatis-containing phagosomes indicates pathogen-induced microenvironments within the host cell's endosomal system. J Immunol 2005;174:1491–500.

Wang J, McIntosh F, Radomski N et al. Insights on the emergence of Mycobacterium tuberculosis from the analysis of Mycobacterium kansasii. Genome Biol Evol 2015;7:856–70.

Wassermann R, Gulen MF, Sala C et al. Mycobacterium tuberculosis Differentially Activates cGAS- and Inflammasome-Dependent Intracellular Immune Responses through ESX-1. Cell Host Microbe 2015;17:799–810.

Watson RO, Bell SL, MacDuff DA et al. The Cytosolic Sensor cGAS Detects Mycobacterium tuberculosis DNA to Induce Type I Interferons and Activate Autophagy. Cell Host Microbe 2015;17:811–9.

Watson RO, Manzanillo PS, Cox JS. Extracellular M. tuberculosis DNA targets bacteria for autophagy by activating the host DNA-sensing pathway. Cell 2012;150:803–15.

Welin A, Raffetseder J, Eklund D et al. Importance of phagosomal functionality for growth restriction of Mycobacterium tuberculosis in primary human macrophages. J Innate Immun 2011;3:508–18.

Welin A, Winberg ME, Abdalla H et al. Incorporation of Mycobacterium tuberculosis lipoarabinomannan into macrophage membrane rafts is a prerequisite for the phagosomal maturation block. Infect Immun 2008;76:2882–7.

Wong D, Bach H, Sun J et al. Mycobacterium tuberculosis protein tyrosine phosphatase (PtpA) excludes host vacuolar-H+-ATPase to inhibit phagosome acidification. Proc Natl Acad Sci USA 2011;108:19371–6.

Wong D, Chao JD, Av-Gay Y. Mycobacterium tuberculosis-secreted phosphatases: from pathogenesis to targets for TB drug development. Trends Microbiol 2013;21:100–9.

Young DB, Perkins MD, Duncan K et al. Confronting the scientific obstacles to global control of tuberculosis. J Clin Invest 2008;118:1255–65.

Zhang L, Zhang H, Zhao Y et al. Effects of Mycobacterium tuberculosis ESAT-6/CFP-10 fusion protein on the autophagy function of mouse macrophages. DNA Cell Biol 2012;31:171–9.