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Breaching the phagosome, the case of the tuberculosis agent

(Phagosome/invasion dynamics)

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Abstract

The interactions between microbes and their hosts are among the most complex biological phenomena known today. The interaction may reach from overall beneficial interaction, as observed for most microbiome/microbiota related interactions to interaction with virulent pathogens, against which host cells have evolved sophisticated defense strategies. Amongst the latter, the confinement of invading pathogens in a phagosome plays a key role, which often results in the destruction of the invader, whereas some pathogens may counteract phagosomal arrest and survive by gaining access to the cytosol of the host cell. In the current review we will discuss recent insights into this dynamic process of host-pathogen interaction, using *Mycobacterium tuberculosis* and related pathogenic mycobacteria as main examples.

Take Aways

- Phagosomal rupture is a key event in mycobacterial host-pathogen interaction
- Combined action of protein and lipid virulence factors induce phagsomal rupture
- Mycobacteria-induced phagosomal rupture leads to enhanced intracellular signalling
- Egress of mycobacteria or their products to the cytosol is linked to host cell death

Introduction

In this review dedicated to recent research on bacteria-induced phagosomal rupture, our discussion will mainly focus on *Mycobacterium tuberculosis (Mtb)*, the causative agent of human tuberculosis (TB). This key human pathogen was only relatively recently counted among the pathogens that have the ability to rupture the phagosome and egress to the cytosol of host phagocytes. A short comparative analysis of *Mtb* with selected classical cytosolic pathogens of other bacterial phyla will complete the presented perspectives.

Factors of *Mtb* that enable phagosomal rupture:

Mtb represents a bacterial pathogen that has caused billions of deaths in the history of mankind, and tuberculosis remains a leading cause of death in many countries today. Despite numerous studies that have aimed during many years to elucidate the actions that *Mtb* is using to circumvent host defenses, our knowledge on these features is still limited. Thus, it is not surprising that novel aspects linked to the infection mechanisms of *Mtb* are identified more or less regularly, even more than 130 years after the discovery of the tubercle bacillus. One such recently revealed aspect that has changed our perception of mycobacterial infection biology

was the discovery of a concerted action between well-known virulence lipids and equally well-known virulence proteins of *Mtb*, acting together to induce phagosomal rupture in phagocytes (J Augenstreich et al., 2017; J. Augenstreich et al., 2020; Barczak et al., 2017).

Indeed, *Mtb* produces a large variety of unique and complex lipids (Daffe & Marrakchi, 2019), which contribute to the physical resistance of *Mtb* and its interaction with the host. Briefly, *Mtb's* envelope has 4 parts: (1) a plasma membrane, which is mainly composed of phospholipids; (2) a layer of peptidoglycan covalently linked to a layer of arabinogalactan; (3) a mycomembrane, also called mycobacterial outer membrane, which has an inner and an outer leaflet, whereby the inner leaflet is composed of mycolic acids that are esterified with the arabinogalactan layer and the outer leaflet is composed of a variety of non-covalently linked lipids, such as phthiocerol dimycocerosates (abbreviated as DIM or PDIM), trehalose dimycolates (TDMs) and sulfolipids (SL) that are involved in the interaction between *Mtb* and host cells; (4) at the outermost part, a capsule layer is present, which is formed by a matrix of glucan, proteins, (lipo)polysaccharides and small amounts of lipids. The example of DIM/PDIM being involved in phagosomal rupture is thus one new aspect of the previously defined functions of mycobacterial lipids that contribute to the virulence of *Mtb* (Camacho, Ensergueix, Perez, Gicquel, & Guilhot, 1999; Cox, Chen, McNeil, & Jacobs, 1999).

Apart from various lipids, the cell envelope of *Mtb* also contains numerous protein secretion systems, which are usually embedded within the plasma membrane, and play important roles in host pathogen interaction. Among them are the Sec-dependent general secretory pathway (Feltcher, Sullivan, & Braunstein, 2010), the Twin Arginine Translocation (TAT) system (Palmer & Berks, 2012; Solans et al., 2014) and the ESX/Type VII Secretion Systems (T7SS) (Abdallah et al., 2007; Chirakos, Balaram, Conrad, & Champion, 2020; Groschel, Sayes, Simeone, Majlessi, & Brosch, 2016).

While the genome of *Mtb* (Cole et al., 1998) encodes five ESX T7SS (ESX-1 to ESX-5), in the current review, we will mainly focus on the ESX-1 system, which is absent from live attenuated vaccine strains *Mycobacterium bovis* BCG (BCG) (Hsu et al., 2003; Pym, Brodin, Brosch, Huerre, & Cole, 2002) and *Mycobacterium microti* M.P. Prague (Orgeur et al., 2021), and its role in processes leading to induction of phagosomal rupture. The most well-known ESX-1 substrate in this context is EsxA (also known as Early Secretory Antigenic Target of 6 kDa, ESAT-6) (Andersen, Andersen, Sorensen, & Nagai, 1995; Sorensen, Nagai, Houen, Andersen, & Andersen, 1995), which is required for full virulence of *Mtb* and the related fish pathogen *Mycobacterium marinum* (reviewed in (Groschel et al.,

2016) and (Chirakos et al., 2020), respectively). Moreover, a study on clinical isolates of *Mtb* identified one strain harboring a frameshift mutation that inhibited EsxA secretion, which led to an attenuation of virulence in both macrophage and mouse infection models and also caused a decrease of pro-inflammatory cytokine release (Clemmensen et al., 2017).

Concerning the predicted functional implications of EsxA in virulence, the first membranolytic activity of EsxA was described by the use of purified EsxA protein that caused lysis of artificial lipids bilayers (Hsu et al., 2003). These studies were followed by demonstration of membranolytic activity of EsxA on liposome preparations, representing phospholipid compositions typical for eukaryotic membranes (de Jonge et al., 2007). Notably, this study also tested an EsxA preparation that was extracted directly from *Mtb*. Several other studies have focused on the EsxA membrane lysis activity by comparing the activities of EsxA proteins from pathogenic and non-pathogenic mycobacteria (De Leon et al., 2012; Peng & Sun, 2016) or by generating specific point mutations in order to disrupt the membrane lytic activity of EsxA (Ma, Keil, & Sun, 2015). However, the membranolytic activity of EsxA, produced in *Escherichia coli*, has been reconsidered based on recent *in vitro* studies, which found that traces of detergent ASB-14 remained attached to purified ESAT-6 and were responsible for the lytic activity of these preparations, even if proteinase K treatment removed EsxA from the sample (Conrad et al., 2017; Refai et al., 2015). While the ASB-14-mediated lytic activity of EsxA preparations that used this detergent in the purification protocol, were recently confirmed by independent experiments (J. Augenstreich et al., 2020), such studies also showed that EsxA produced without the use of ASB-14 still showed membranolytic activity (Aguilera et al., 2020; J. Augenstreich et al., 2020). These results are in good agreement with results obtained from lytic assays using native EsxA preparations from *Mtb* that did not use ASB-14 in the purification protocol (J. Augenstreich et al., 2020; de Jonge et al., 2007). Taken together, it seems that some confusion on the lytic activity of certain EsxA preparations were caused by particular purification protocols, which, however, have been resolved in recent studies by the use of EsxA preparations that were prepared without ASB-14 and had retained membranolytic activity. From these latter experiments it seems clear that EsxA itself does show intrinsic membranolytic activity, which is in good agreement with results from *in vivo* experiments that show loss of biological activity in case of certain attenuating mutations or truncation of EsxA (Brodin et al., 2005).

The interaction between *Mtb* and host cells is a combination of strategic steps from both sides that have been selected during long-lasting coevolution (Bottai et al., 2020; Ngabonziza et al., 2020; Queval, Brosch, & Simeone, 2017). *Mtb* is phagocyted by macrophages and interferes

with phagolysosome maturation by blocking the phagosomal acidification in order to survive inside host cells (Lugo-Villarino & Neyrolles, 2014; C.J. Queval et al., 2017). Since the seminal work of Armstrong and Hart in 1971, the intracellular localization of *Mtb* inside host cells was thought to be exclusively within the phagosome (Armstrong & Hart, 1971). Although this view was challenged by selected electron microscopy (EM)-based studies (Leake, Myrvik, & Wright, 1984; McDonough, Kress, & Bloom, 1993; Myrvik, Leake, & Wright, 1984), it was mainly the use of sophisticated cryo-immunogold EM, which finally questioned the previously widely-accepted hypothesis of the exclusive intracellular localization of *Mtb* (Houben et al., 2012; van der Wel et al., 2007). These results suggested that *Mtb* is able to gain access to the cytosol three days post-infection and that this phenomenon was ESX-1-dependent (Houben et al., 2012; van der Wel et al., 2007). Indeed, several research groups using independent techniques confirmed and extended these initial findings, and established that a functional ESX-1 system was fundamental for *Mtb* to rupture the phagosome and gain access the cytosol of phagocytes. Wong and Jacobs used monoclonal antibodies against Galectin-3 and ubiquitinated proteins for identification of damaged phagosomal-membranes in *Mtb*-infected THP-1 cells (Wong & Jacobs, 2011). As initially shown in the context of *Shigella* and *Listeria* infections, intracellular galectin-3 accumulates in structures in vicinity of bacteria that lyse the phagocytic vacuole (Paz et al., 2010). Similarly, an assay that was based on β-lactamase-activated interruption of a Fluorescence Resonance Energy Transfer (FRET) signal has been successfully adapted for identification of phagosomal rupture and contact of *Mtb* with the cytosol of host cells (Simeone et al., 2012; Simeone et al., 2015). In such a FRET assay, after phagocytosis, *Mtb*-infected phagocytes are loaded with a FRET-inducing probe that is sensitive to cleavage by β-lactamase activity, which stems from bacteria that have established cytosolic contact (Simeone, Majlessi, Enninga, & Brosch, 2016). As an initial readout for this approach, fluorescent microscopy was employed (Simeone et al., 2012), and then the assay was adapted to flowcytometry as readout (Simeone et al., 2015). Both methods confirmed that interruption of the FRET signal, visible as a change from green to blue fluorescence, was only obtained when phagocytes were infected with *Mtb* strains harboring an intact ESX-1 system, while infections with the naturally ESX-1-deficient BCG vaccine strains did not induce changes in the fluorescence type, thereby indicating that BCG strains are unable to rupture the phagosome and to establish cytosolic contact, unless they are complemented with a functional ESX-1 system from *Mtb* (Simeone et al., 2012), or *M. marinum* (Groschel et al., 2017).

However, as briefly mentioned above, a functional ESX-1 system is not the only requirement for *Mtb* to rupture the phagosome. Recent studies from independent groups, established that the ability of *Mtb* to gain access to the cytosol also required the production and export of the virulence lipids DIM/PDIM (J Augenstreich et al., 2017; Lerner et al., 2017; Lerner et al., 2020; Quigley et al., 2017). Indeed, these extractable lipids have been shown to play important roles in host-pathogen interaction, such as the arrest of phagosomal acidification (Astarie-Dequeker et al., 2009) and they are also involved in inducing host cell death (Passemar et al., 2014). Most recently, employment of different techniques in parallel, including the labelling of galectin-3 and ubiquitinated proteins for the identification of damaged phagosomal membranes in infected macrophages and a FRET-based cytofluorometric approach for the detection of the induction of phagosomal rupture, first revealed that (i) DIM/PDIM are involved in the induction of the phagosomal rupture and (ii) that both EsxA/ESX-1 and DIM/PDIMs were required to induce phagosomal rupture and membrane damage in *Mtb*-infected phagocytes (J Augenstreich et al., 2017). Moreover, it was found that complementation of BCG strains with the ESX-1 system from *Mtb* only enabled DIM/PDIM-producing BCG substrains to induce changes in the FRET signals, whereas ESX-1 complementation of BCG strains that had lost the ability to produce and/or secrete DIM/PDIMs, (e.g. BCG Japan or BCG Moreau (Chen, Islam, Ren, & Liu, 2007)), did not induce changes in the FRET signals (J Augenstreich et al., 2017). The significance of DIM/PDIM in the process of phagosomal rupture was independently confirmed by a study that used an *Mtb* strain bearing a transposon insertion in the *mmpL7* gene, which encodes a transporter of DIM/PDIM across the bacterial plasma-membrane to the mycomembrane (Camacho et al., 1999). It was found that infection of macrophages with the MmpL7-ko *Mtb* strain showed much less induction of phagosomal damage compared to cells infected the *Mtb* MmpL7 WT strain (Quigley et al., 2017). Moreover, in a study that used a non-redundant *Mtb* transposon mutant library, high-content imaging and a multiparametric analysis for evaluating pathogen and host phenotypes, it was observed that mutants in the DIM/PDIM and ESX-1 pathways both showed similar profiles of impaired intracellular survival and reduced induction of type I interferon responses, suggesting a concerted impact of both pathways on phagosomal damage and cytosolic signaling (Barczak et al., 2017). Finally, DIM/PDIM have also been shown to facilitate cytosolic access of *Mtb* in human lymphatic endothelial cells (hLEC), as evaluated by electron microscopy (Lerner et al., 2018). Taken together, convergent data from experiments done by different research groups and by using different

methods indicate that phagosomal rupture induced by *Mtb* is an ESX-1 and DIM/PIDM-dependent process, which can be observed in various cellular infection models.

The ability to gain access to the cytosol by phagosomal rupture is a key process for *Mtb* that likely has been selected during evolution in order to circumvent host defenses and get access to nutriments during infection (Simeone et al., 2016). However, it is also clear that cellular mechanisms exist that try to prevent or repair such phagosomal damage. Earlier studies showed that the Endosomal Sorting Complex Required for Transport (ESCRT) machinery is involved in repair of phagosomal membranes (Jimenez et al., 2014). Indeed, the ESCRT machinery has been reported to be recruited to *Mtb*-containing phagosomes in an ESX1-dependent manner (Mittal et al., 2018), thereby regulating inflammation and cell viability (Beckwith et al., 2020) (Figure 1). These findings suggest a dynamic interaction between bacterial factors that induce phagosomal rupture and host factors that may repair or prevent this process. Similar observations have also been made for *M. marinum* during infection of the social amoeba *Dictyostelium discoideum* (López-Jiménez et al., 2018).

Most interestingly, *Mtb*-induced phagosomal rupture has also been observed under *in vivo* conditions, using a mouse model that included an enrichment of infected phagocytes and a screen for tracking these rare cells, which were characterized by carrying a CD45.1 hematopoietic allelic marker (Simeone et al., 2015). In the same study, it was also shown that inhibition of phagosomal acidification intensified phagosomal rupture in *Mtb*-infected phagocytes, which pointed to the importance of the immune system and certain host resistance factors, such as the natural resistance-associated macrophage protein (Nramp)-1, in the control of *Mtb*-induced phagosomal damage (Simeone et al., 2015). Strong impact of immune control on the frequency of phagosomal rupture during *in vivo* infections with *Mtb* was also reported in a recent pre-print showing results from a collaborative project led by the team of N. van der Wel (van der Niet et al., 2020). In this study, which was focused on the analysis by electron microscopy of samples from mycobacteria-infected human- and animal-tissues, only low numbers of cytosolic bacilli were found in mouse-, zebrafish-, armadillo- and patient tissues infected with *Mtb, M. marinum* or *Mycobacterium leprae*, respectively. In contrast, when innate or adaptive immunity was compromised, as in SCID or IL-1R1-deficient mice, a larger amount of cytosolic *Mtb* bacilli were detected in lungs of infected mice, suggesting that the cytosolic localization of mycobacteria *in vivo* is controlled by adaptive immune responses and selected host resistance factors (van der Niet et al., 2020).

Indeed, phagosomal rupture has also a strong impact on host cell signaling and host immune responses, which will be discussed in the following section of the review.

Impact of phagosomal rupture on host signaling

As discussed briefly above, mycobacteria-induced phagosomal membrane damage is an ESX-1 and DIM/PDIM-dependent mechanism, which triggers various host intracellular signaling pathways, leading to cytokine production, inflammasome activation and autophagy that profoundly influence cell fate and the outcome of infection (Bussi & Gutierrez, 2019; Chai, Wang, Liu, & Ge, 2020; Groschel et al., 2016; Kroesen, Madacki, Frigui, Sayes, & Brosch, 2019).

It has been shown that cytosolic release of dsDNA inside infected host macrophages is recognized as pathogen-associated molecular pattern (PAMP) by the host innate cytosolic sensor AIM2 (Absent in Melanoma 2). This event likely represents the first crucial step in the activation of the NLRP3 (nucleotide-binding oligomerization domain (NOD)-like receptor pyrin domain-containing-3)-dependent inflammasome activation cascade that then leads to caspase-1 activity, which cleaves the inactive pro-IL-1β and pro-IL-18 cytokine precursors into the active cytokines IL-1β and IL-18, respectively (Dorhoi et al., 2012; Mishra et al., 2010). IL-1β is a key innate pro-inflammatory cytokine that plays a central protective role during infection with mycobacteria (Bussi & Gutierrez, 2019; Fremond et al., 2007). Moreover, ESX-1-dependent NLRP3 inflammasome activation and subsequent IL-18 secretion in $CD11c^+$ cells were also found to stimulate noncognate IFN-γ production by *Mtb* antigen-independent memory $CD8^+$ T cells and NK cells, conferring an additional level of early protective immune responses against TB in mice (Kupz et al., 2016). This process does not seem to be restricted to infection with *Mtb*, but was also reported for infection with the emerging mycobacterial pathogen *Mycobacterium abscessus* (Kim, Kim, Kook, & Kim, 2020), whereby bacteria with rough colony morphology type increased mitochondrial ROS and increased release of oxidized mitochondrial DNA into the cytosol of murine macrophages, resulting in enhanced NLRP3 inflammasome-mediated IL-1β and cGAS-STING-dependent IFN-I production (Kim et al., 2020). This finding is in agreement with results from a study using a high-density transposon screen, which found the involvement of the *M. abscessus* ESX-4 type VII secretion system in intracellular growth and cytosolic access of *M. abscessus* (Laencina et al., 2018). In this regard it is particularly noteworthy that the fast-growing *M. abscessus* does not harbor an ESX-1 secretion system, but encodes an EccE4 protein, which is missing from most other mycobacterial ESX-4 secretion systems (Dumas et al., 2016). Finally, it also should be emphasized that IL-1R1-deficient mice show more cytosolic *Mtb* than fully immunocompetent mice in their lungs (van der Niet et al.,

2020), suggesting that ESX-1-induced phagosomal rupture and cytosolic localization of *Mtb* is counteracted by immune responses of the IL-1 cytokine / receptor families.

In parallel to the NLRP3-mediated signaling pathway, ESX-1 mediated cytosolic release of dsDNA also plays an important role for the cGAS–STING pathway. Several independent studies showed that transfer of dsDNA to the host cytosol induced the synthesis of cyclic GMP-AMP (cGAMP), a second messenger that interacts with STING (stimulator of IFN genes) (Collins et al., 2015; Wassermann et al., 2015; Watson et al., 2015). This process activates TBK-1 (tank-binding kinase 1) and triggers the STING/TBK-1/IRF3 signaling pathway, leading to IFN-β production and transcription of a subset of interferon-stimulated genes, including IP-10 and IL-10 (Ablasser & Chen, 2019; Dey et al., 2015; Kroesen et al., 2019; Majlessi & Brosch, 2015; Manzanillo, Shiloh, Portnoy, & Cox, 2012) (Figure 1).

Several studies that used murine models or human cellular models highlighted immune modulating and anti-inflammatory properties of IFN-β, or other type I IFNs (Cooper & Khader, 2008; Moreira-Teixeira et al., 2020; Novikov et al., 2011). However, the contributing role of type I IFN in the protection against virulent *Mtb* during the early and late phases of infection is not fully understood, as type I IFN release might under some circumstances also counteract protective IL-1β responses (Desvignes, Wolf, & Ernst, 2012; Manca et al., 2001). From a practical perspective, the integration of a virulence-neutral ESX-1 secretion system, originating from *M. marinum*, into the *M. bovis* BCG genetic background, which induced IFN-β release and higher IL-1β responses, also significantly increased the protective efficacy of this recombinant BCG::Esx-1^{Mmar} vaccine candidate in different murine models, as compared to the BCG parental strain (Groschel et al., 2017).

Apart from DNA-induced signaling pathways, it was recently also reported that mycobacterial RNA is released into the infected macrophage cytosol in SecA2- and ESX-1-dependent manner (Cheng & Schorey, 2018), thereby triggering IFN-β production through a RIG-1 (retinoic acid-inducible gene-1) mediated pathway and a cross-talk between DNA and RNA sensor pathways (Cheng & Schorey, 2018) (Figure 1). Taken together, it is clear that gaining access to the cytosol during mycobacterial infection is a key process that determines a cascade of innate and adaptive immune responses that decide whether the pathogen or the host will "win" the control of the infection process.

Phagosomal rupture and autophagy induction

In addition to the above described effects of phagosomal rupture on innate and adaptive immunity processes, another important cell biological process, known as selective autophagy

pathway, is linked to the detection of phagosome-derived cytosolic DNA, involving the cGAS/STING/TBK-1 signaling cascade. This process is a powerful host defense mechanism to control intracellular pathogens and usually plays a beneficial role in anti-mycobacterial innate and adaptive immunity (Krakauer, 2019; Xiao & Cai, 2020). Knockdown of cGAS in human or mouse macrophages blocks cytokine production and induction of *Mtb*-induced selective autophagy pathway (Collins et al., 2015; Watson et al., 2015), whereas TBK-1 acts as a pivotal regulator of host innate immune control of mycobacterial growth (Pilli et al., 2012). Recognition of cytosolic dsDNA by the STING cytosolic pathway was also required for targeting bacteria with LC3, p62 and NDP52 ubiquitin adaptors, leading to their delivery to autophagosomes and restricted mycobacterial replication (Watson et al., 2015; Watson, Manzanillo, & Cox, 2012). In the earlier study, it was shown that mouse cells missing the autophagy protein 5 (Atg5) were highly susceptible to infection with *Mtb,* which led to the assumption that Atg5-mediated autophagy was an important mechanism to help control mycobacterial infections, similar to findings by Castillo and colleagues (Castillo et al., 2012). In a parallel study, cytosolic access of mycobacteria was found essential for the accumulation of the lipidated autophagosome-associated isoform of LC3 (LC3-II) in dendritic cells (Romagnoli et al., 2012). Moreover, for IFN-γ-activated macrophages, it was demonstrated that the host protein ubiquilin 1 promoted IFN-γ-mediated autophagic clearance of *Mtb* (Sakowski et al., 2015). Similarly, another study reported that ubiquitin binds to an *Mtb* PE-PGRS surface protein, Rv1468c, and directly recruits autophagy receptor p62 to deliver mycobacteria into LC3-associated autophagosomes, thereby contributing to mycobacterial clearance (Chai et al., 2019). A study using *Mtb*-infected primary murine macrophages revealed that a particular autophagy receptor, TAX1BP1, mediates clearance of ubiquitinylated *Mtb* and targets bacteria to LC3-positive phagophores (Budzik et al., 2020). By using mass spectrometry and bio-informatics analyses, the authors identified hundreds of dynamically regulated phosphorylation and ubiquitylation sites, suggesting that dramatic remodeling of multiple host pathways occurred during infection with *Mtb* (Budzik et al., 2020). In a parallel study which used human induced pluripotent stem cell-derived macrophages (iPSDMs) it was found that upon ESX-1-mediated phagosomal rupture, *Mtb* induced the formation of LC3B-positive tubulovesicular autophagosomes (Bernard et al., 2020).

Taken together, all these studies suggest that autophagy is an important innate immune mechanism against pathogenic mycobacteria. Nevertheless, it should be mentioned that the role of certain proteins, such as Atg5, which are often considered as being mainly involved in

the autophagy pathway, might also have other cellular functions. For example, a study using Atg5-deficient mice as model has suggested that the increased susceptibility of Atg5-deficient mice to infection with *Mtb* was due to altered neutrophil recruitment and associated immune system pathologies, rather than the impairment of autophagy (Kimmey et al., 2015).

Impact of phagosomal rupture on cell death

Phagosomal rupture represents an important molecular event in mycobacteria-infected macrophages, which can have severe consequences for the infecting bacterium and/or the host macrophage. Apart from the damage of the vacuolar compartment caused by ESX-1 proficient *Mtb*, a subsequent damage of the host cell plasma membrane has also been observed, which caused K^+ efflux and activation of NLRP3-dependent IL-1β release and pyroptosis, facilitating the spread of *Mtb* to neighbouring cells (Beckwith et al., 2020). These observations are in agreement with previous data, that showed substantially increased cell death caused by ESX-1 proficient *Mtb* strains, compared to *Mtb* strains secreting truncated ESX-1 protein(s) (Simeone et al., 2012). Finally, it should also be mentioned that a recent paper reported that type I interferon signaling mediates *Mtb*-induced macrophage death (Zhang, Jiang, Pfau, Ling, & Nathan, 2021), which adds additional possibilities to other reported virulence-enhancing consequences of type I interferon release (Moreira-Teixeira, Mayer-Barber, Sher, & O'Garra, 2018; Moreira-Teixeira et al., 2020).

How can this knowledge be used for vaccine development ?

What can we learn from mycobacteria-induced phagosomal rupture and the involved cytosolic sensing pathways for developing new generations of more protective anti-TB vaccines, able to activate host cytosolic surveillance pathway and trigger optimal innate and adaptive immune responses ?

In this regard, as briefly mentioned above, we recently elaborated a low-virulent rBCG Pasteur::ESX-1^{Mmar} vaccine strain, which heterologously expresses the *esx-1* region of *M. marinum*, and thereby is able to induce phagosomal rupture and cGAS/STING/TBK-1/IRF3 cytosolic recognition in the host (Groschel et al., 2017). This strain induces IFN-β production and enhanced activation of AIM-2/NLRP3 inflammasome, representing innate immune signaling pathways that mimic the natural infection with virulent *Mtb*, resulting in higher initiation of mycobacteria-specific $CD8^+$ T cell immunity and a higher proportion of polyfunctional $CD4^+$ Th1 effectors specific to ESX-1 antigens. These features of rBCG::ESX-

1^{Mmar} provided superior protection in murine aerosol challenge models, compared to parental BCG, which used various highly virulent *Mtb* strains (Groschel et al., 2017).

The *Mtb* Δ*ppe25-pe19* strain (Bottai et al., 2012) is another live-attenuated anti-TB vaccine candidate that elicits ESX-1-mediated cytosolic immune signaling, due to its functional ESX-1 type VII secretion system (Bottai et al., 2012; Sayes et al., 2016; Sayes et al., 2012). This strain efficiently activates innate and adaptive immune responses comparable to virulent *Mtb*, including mycobacteria-specific effector memory $CD4^+$ T cells, while showing strong attenuation in cellular and *in vivo* infection models (Bottai et al., 2012; Sayes et al., 2012). Its use as an attenuated live vaccine candidate displayed significant improved TB protection efficacy in selected mouse models relative to BCG (Sayes et al., 2016; Sayes et al., 2012).

In conclusion, it seems well that breaching the phagosome represents an important feature for mycobacterial pathogens to evade destruction by the host cell, which can be exploited by specially engineered mycobacterial live vaccine strains to induce beneficial innate and adaptive immune responses that cannot be induced by standard BCG vaccination. The construction and use of ESX-1-proficient attenuated mycobacterial strains thus represent a promising strategy for the design of a new generations of anti-TB vaccine candidates.

Comparison of mycobacterial phagosomal rupture to other bacteria

Cytoplasmic access is a widely used strategy of bacterial pathogens that occurs in professional phagocytes and in invaded epithelial or endothelial cells. Phagosomal rupture and escape following secretion of bacterial effectors is not restricted to *Mtb*, as most cytosolic bacterial pathogens have adopted selected strategies of entry into the host cytosol (Pizarro-Cerdá, Charbit, Enninga, Lafont, & Cossart, 2016). In the following paragraph we will present a few selected examples, but we also would like to emphasize that there are many more cytosolic bacterial pathogens existing that would merit further description, which however is beyond the scope of the current review.

One of the best characterized examples of phagosomal rupture concerns the Gram-positive opportunistic pathogen *Listeria monocytogenes* that escapes from the vacuole *via* the action of listeriolysin O (LLO) and type C phospholipases (PLC), both secreted by the Sec pathway (Burg-Golani et al., 2013; Renier, Micheau, Talon, Hébraud, & Desvaux, 2012). LLO, a cholesterol-dependent cytolysin, binds cholesterol from the vacuolar membrane and forms large pores that prevent acidification of phagosomes in macrophages (Burg-Golani et al., 2013; Cossart, 2011). This process delays phagosome-lysosome fusion prior the

destruction of the vacuole membrane, which is finally achieved with the help of a PLC that directly hydrolyzes phospholipids from the membrane (Gilbert, 2010; Goldfine, Johnston, & Knob, 1993). Interestingly, *Mtb* also encodes up to four PLCs, which however, do not seem to be implicated in the phagosomal rupture process induced by *Mtb*. While a first study suggested that PLCs from *Mtb* might play a role in virulence in a mouse infection model (Raynaud et al., 2002), a latter study that used the same mutant strains and additional constructs did not find an attenuated virulence profile for PLC-deletion mutants of *Mtb* in a murine infection model (Le Chevalier et al., 2015). Moreover, the PLC-deletion mutants remained fully capable to induce phagosomal rupture in the widely used THP-1 cellular infection model, arguing that *Mtb* might use a different molecular mechanism to rupture phagosomal membranes than *L. monocytogenes* (Le Chevalier et al., 2015). Indeed, as described further above, *Mtb* requires an intact ESX-1 secretion system as well as export of PDIM virulence lipids for inducing phagosomal rupture and gaining access to the cytosol of the host macrophage (J Augenstreich et al., 2017). As for other Gram-positive bacteria, *Staphylococcus aureus* has been described to reach the host cytoplasm by escaping from phagosomes via the release of cytolytic peptides such as phenol-soluble modulins (Grosz et al., 2014).

Among Gram-negative bacteria, *Shigella flexneri* is one of the most studied examples that gains access to the host cytosol. Indeed, upon internalization into the host cell, *S. flexneri* ruptures the vacuole membrane within 10 minutes (Paz et al., 2010), following the type III secretion system (T3SS)-dependent insertion of the effectors/translocators IpaB and IpaC into the vacuole membrane (Du et al., 2016; Picking et al., 2005; Russo, Duncan, Wiscovitch, Hachey, & Goldberg, 2019). In a similar way than LLO, IpaB displays haemolytic activity (High, Mounier, Prévost, & Sansonetti, 1992). Once inserted into the membrane, IpaB tetramers form a discrete ion channel that permits inflows and outflows of small molecules, leading to phagosomal membrane rupture and bacterial escape (Dickenson et al., 2013).

While mainly extracellular, a recent study has demonstrated that *Pseudomonas aeruginosa* also actively replicates inside macrophages (Kroken, Chen, Evans, Yahr, & Fleiszig, 2018), and escapes from the phagosome in a T3SS dependent manner (Garai, Berry, Moussouni, Bleves, & Blanc-Potard, 2019).

Phagosomal escape is also essential for *Francisella tularensis* intracellular replication within the host and depends entirely on secretion of the so-called pathogenicity determinant proteins PdpC and PdpD via the type VI secretion system (T6SS), known to deliver bacterial effectors across both bacterial and eukaryotic cells (Brodmann, Dreier, Broz, & Basler, 2017).

The exact molecular mechanism employed by PdpC and PdpD to rupture the phagosomal membrane remains to be elucidated. However, it is known that these two proteins are encoded within the *Francisella* Pathogenicity Island (FPI), but do not share similarity with known bacterial effectors or pore forming toxins. This finding is reminiscent to some extent of the low level of mechanistic insights that are available for the ESX-1- and PDIM-mediated phagosomal rupture processes induced by *Mtb* and related mycobacteria, which are still under discussion (J Augenstreich et al., 2017; J. Augenstreich et al., 2020; Conrad et al., 2017; de Jonge et al., 2007; De Leon et al., 2012).

Finally, it is interesting to underline that some intracellular bacteria do not trigger phagosomal escape and prevent phagosome-lysosome fusion by secreting dedicated bacterial effectors, which help them to create a replication-permissive vacuole within the infected host. Indeed, in both *Brucella* spp. and *Legionella* spp., the bacterial effectors are secreted by the type IV secretion system (T4SS) or the Dot/Icm T4SS, respectively (Atluri, Xavier, de Jong, den Hartigh, & Tsolis, 2011; Isberg, O'Connor, & Heidtman, 2009), further demonstrating the importance of specialized secretion systems for bacterial replication within the host.

Conclusions

From recent years' research it has become more and more evident that mycobacteria-induced phagosomal rupture represents a key event in pathogen-host interaction. While for many years it was thought that *Mtb* exclusively replicated inside vacuoles, convergent immunological and cell biological data point to periods during infection when *Mtb* gains access to the cytosol of host phagocytes. Despite intense research in the last years this process still holds many secrets, but which step by step are being elucidated, as was for example recently shown for the infection process inside selected *in vivo* models (Simeone et al., 2015; van der Niet et al., 2020). Given the novel tools that are progressively becoming available for detailed observations of the nanomachines involved in host-pathogen interaction (Lawarée & Custódio, 2019), deeper insights into mechanistic details of the interaction between mycobacterial pathogens and host cells shall be obtained in the near future. Research linked to phagosomal rupture induced by pathogenic mycobacteria will thus remain an exciting subject, whose new insights might also help to find new intervention strategies against a pathogen that continues to threaten the health and lives of millions of people.

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Figure legends:

Figure 1. Schematic view of an *Mtb*-infected phagocyte, highlighting the various signaling events induced, as described in different sections of the review. Note that the red structures drawn inside the mycobacterial cell envelope are meant to represent the mycobacterial ESX-1 secretion system.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study

Conflict of interest statement

The authors declare no conflict of interests linked to this work.

Graphical Abstract text:

The genome of *Mycobacterium tuberculosis*, the causative agent of human tuberculosis, harbors two loci (ESX-1 and DIM/PDIM), whose gene products are essential for the bacterium's ability to induce phagosomal rupture, when ingested by host phagocytes. The access to the host cytosol leads to important cellular signaling events and immune reactions.