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1 Breaching the phagosome, the case of the tuberculosis agent

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3 (Phagosome/invasion dynamics)

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14

15 **Abstract**

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

26 **Take Aways**

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

32 **Introduction**

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

40 **Factors of *Mtb* that enable phagosomal rupture:**

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

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

51 Indeed, *Mtb* produces a large variety of unique and complex lipids (Daffe &
52 Marrakchi, 2019), which contribute to the physical resistance of *Mtb* and its interaction with
53 the host. Briefly, *Mtb*'s envelope has 4 parts: (1) a plasma membrane, which is mainly
54 composed of phospholipids; (2) a layer of peptidoglycan covalently linked to a layer of
55 arabinogalactan; (3) a mycomembrane, also called mycobacterial outer membrane, which has
56 an inner and an outer leaflet, whereby the inner leaflet is composed of mycolic acids that are
57 esterified with the arabinogalactan layer and the outer leaflet is composed of a variety of non-
58 covalently linked lipids, such as phthiocerol dimycocerosates (abbreviated as DIM or PDIM),
59 trehalose dimycolates (TDMs) and sulfolipids (SL) that are involved in the interaction
60 between *Mtb* and host cells; (4) at the outermost part, a capsule layer is present, which is
61 formed by a matrix of glucan, proteins, (lipo)polysaccharides and small amounts of lipids.
62 The example of DIM/PDIM being involved in phagosomal rupture is thus one new aspect of
63 the previously defined functions of mycobacterial lipids that contribute to the virulence of
64 *Mtb* (Camacho, Ensergueix, Perez, Gicquel, & Guilhot, 1999; Cox, Chen, McNeil, & Jacobs,
65 1999).

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

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

82 2016) and (Chirakos et al., 2020), respectively). Moreover, a study on clinical isolates of *Mtb*
83 identified one strain harboring a frameshift mutation that inhibited EsxA secretion, which led
84 to an attenuation of virulence in both macrophage and mouse infection models and also
85 caused a decrease of pro-inflammatory cytokine release (Clemmensen et al., 2017).

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

113 The interaction between *Mtb* and host cells is a combination of strategic steps from both sides
114 that have been selected during long-lasting coevolution (Bottai et al., 2020; Ngabonziza et al.,
115 2020; Queval, Brosch, & Simeone, 2017). *Mtb* is phagocytosed by macrophages and interferes

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

149 However, as briefly mentioned above, a functional ESX-1 system is not the only
150 requirement for *Mtb* to rupture the phagosome. Recent studies from independent groups,
151 established that the ability of *Mtb* to gain access to the cytosol also required the production
152 and export of the virulence lipids DIM/PDIM (J Augenstreich et al., 2017; Lerner et al., 2017;
153 Lerner et al., 2020; Quigley et al., 2017). Indeed, these extractable lipids have been shown to
154 play important roles in host-pathogen interaction, such as the arrest of phagosomal
155 acidification (Astarie-Dequeker et al., 2009) and they are also involved in inducing host cell
156 death (Passemar et al., 2014). Most recently, employment of different techniques in parallel,
157 including the labelling of galectin-3 and ubiquitinated proteins for the identification of
158 damaged phagosomal membranes in infected macrophages and a FRET-based
159 cytofluorometric approach for the detection of the induction of phagosomal rupture, first
160 revealed that (i) DIM/PDIM are involved in the induction of the phagosomal rupture and (ii)
161 that both EsxA/ESX-1 and DIM/PDIMs were required to induce phagosomal rupture and
162 membrane damage in *Mtb*-infected phagocytes (J Augenstreich et al., 2017). Moreover, it was
163 found that complementation of BCG strains with the ESX-1 system from *Mtb* only enabled
164 DIM/PDIM-producing BCG substrains to induce changes in the FRET signals, whereas ESX-
165 1 complementation of BCG strains that had lost the ability to produce and/or secrete
166 DIM/PDIMs, (e.g. BCG Japan or BCG Moreau (Chen, Islam, Ren, & Liu, 2007)), did not
167 induce changes in the FRET signals (J Augenstreich et al., 2017). The significance of
168 DIM/PDIM in the process of phagosomal rupture was independently confirmed by a study
169 that used an *Mtb* strain bearing a transposon insertion in the *mmpL7* gene, which encodes a
170 transporter of DIM/PDIM across the bacterial plasma-membrane to the mycomembrane
171 (Camacho et al., 1999). It was found that infection of macrophages with the *MmpL7*-ko *Mtb*
172 strain showed much less induction of phagosomal damage compared to cells infected the *Mtb*
173 *MmpL7* WT strain (Quigley et al., 2017). Moreover, in a study that used a non-redundant *Mtb*
174 transposon mutant library, high-content imaging and a multiparametric analysis for evaluating
175 pathogen and host phenotypes, it was observed that mutants in the DIM/PDIM and ESX-1
176 pathways both showed similar profiles of impaired intracellular survival and reduced
177 induction of type I interferon responses, suggesting a concerted impact of both pathways on
178 phagosomal damage and cytosolic signaling (Barczak et al., 2017). Finally, DIM/PDIM have
179 also been shown to facilitate cytosolic access of *Mtb* in human lymphatic endothelial cells
180 (hLEC), as evaluated by electron microscopy (Lerner et al., 2018). Taken together,
181 convergent data from experiments done by different research groups and by using different

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

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

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

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

216

217 **Impact of phagosomal rupture on host signaling**

218 As discussed briefly above, mycobacteria-induced phagosomal membrane damage is an ESX-
219 1 and DIM/PDIM-dependent mechanism, which triggers various host intracellular signaling
220 pathways, leading to cytokine production, inflammasome activation and autophagy that
221 profoundly influence cell fate and the outcome of infection (Bussi & Gutierrez, 2019; Chai,
222 Wang, Liu, & Ge, 2020; Groschel et al., 2016; Kroesen, Madacki, Frigui, Sayes, & Brosch,
223 2019).

224 It has been shown that cytosolic release of dsDNA inside infected host macrophages is
225 recognized as pathogen-associated molecular pattern (PAMP) by the host innate cytosolic
226 sensor AIM2 (Absent in Melanoma 2). This event likely represents the first crucial step in the
227 activation of the NLRP3 (nucleotide-binding oligomerization domain (NOD)-like receptor
228 pyrin domain-containing-3)-dependent inflammasome activation cascade that then leads to
229 caspase-1 activity, which cleaves the inactive pro-IL-1 β and pro-IL-18 cytokine precursors
230 into the active cytokines IL-1 β and IL-18, respectively (Dorhoi et al., 2012; Mishra et al.,
231 2010). IL-1 β is a key innate pro-inflammatory cytokine that plays a central protective role
232 during infection with mycobacteria (Bussi & Gutierrez, 2019; Fremond et al., 2007).
233 Moreover, ESX-1-dependent NLRP3 inflammasome activation and subsequent IL-18
234 secretion in CD11c⁺ cells were also found to stimulate noncognate IFN- γ production by *Mtb*
235 antigen-independent memory CD8⁺ T cells and NK cells, conferring an additional level of
236 early protective immune responses against TB in mice (Kupz et al., 2016). This process does
237 not seem to be restricted to infection with *Mtb*, but was also reported for infection with the
238 emerging mycobacterial pathogen *Mycobacterium abscessus* (Kim, Kim, Kook, & Kim,
239 2020), whereby bacteria with rough colony morphology type increased mitochondrial ROS
240 and increased release of oxidized mitochondrial DNA into the cytosol of murine
241 macrophages, resulting in enhanced NLRP3 inflammasome-mediated IL-1 β and cGAS-
242 STING-dependent IFN-I production (Kim et al., 2020). This finding is in agreement with
243 results from a study using a high-density transposon screen, which found the involvement of
244 the *M. abscessus* ESX-4 type VII secretion system in intracellular growth and cytosolic
245 access of *M. abscessus* (Laencina et al., 2018). In this regard it is particularly noteworthy that
246 the fast-growing *M. abscessus* does not harbor an ESX-1 secretion system, but encodes an
247 EccE4 protein, which is missing from most other mycobacterial ESX-4 secretion systems
248 (Dumas et al., 2016). Finally, it also should be emphasized that IL-1R1-deficient mice show
249 more cytosolic *Mtb* than fully immunocompetent mice in their lungs (van der Niet et al.,

250 2020), suggesting that ESX-1-induced phagosomal rupture and cytosolic localization of *Mtb*
251 is counteracted by immune responses of the IL-1 cytokine / receptor families.

252 In parallel to the NLRP3-mediated signaling pathway, ESX-1 mediated cytosolic
253 release of dsDNA also plays an important role for the cGAS–STING pathway. Several
254 independent studies showed that transfer of dsDNA to the host cytosol induced the synthesis
255 of cyclic GMP-AMP (cGAMP), a second messenger that interacts with STING (stimulator of
256 IFN genes) (Collins et al., 2015; Wassermann et al., 2015; Watson et al., 2015). This process
257 activates TBK-1 (tank-binding kinase 1) and triggers the STING/TBK-1/IRF3 signaling
258 pathway, leading to IFN- β production and transcription of a subset of interferon-stimulated
259 genes, including IP-10 and IL-10 (Ablasser & Chen, 2019; Dey et al., 2015; Kroesen et al.,
260 2019; Majlessi & Brosch, 2015; Manzanillo, Shiloh, Portnoy, & Cox, 2012) (Figure 1).

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

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

280

281 **Phagosomal rupture and autophagy induction**

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

284 pathway, is linked to the detection of phagosome-derived cytosolic DNA, involving the
285 cGAS/STING/TBK-1 signaling cascade. This process is a powerful host defense mechanism
286 to control intracellular pathogens and usually plays a beneficial role in anti-mycobacterial
287 innate and adaptive immunity (Krakauer, 2019; Xiao & Cai, 2020). Knockdown of cGAS in
288 human or mouse macrophages blocks cytokine production and induction of *Mtb*-induced
289 selective autophagy pathway (Collins et al., 2015; Watson et al., 2015), whereas TBK-1 acts
290 as a pivotal regulator of host innate immune control of mycobacterial growth (Pilli et al.,
291 2012). Recognition of cytosolic dsDNA by the STING cytosolic pathway was also required
292 for targeting bacteria with LC3, p62 and NDP52 ubiquitin adaptors, leading to their delivery
293 to autophagosomes and restricted mycobacterial replication (Watson et al., 2015; Watson,
294 Manzanillo, & Cox, 2012). In the earlier study, it was shown that mouse cells missing the
295 autophagy protein 5 (Atg5) were highly susceptible to infection with *Mtb*, which led to the
296 assumption that Atg5-mediated autophagy was an important mechanism to help control
297 mycobacterial infections, similar to findings by Castillo and colleagues (Castillo et al., 2012).
298 In a parallel study, cytosolic access of mycobacteria was found essential for the accumulation
299 of the lipidated autophagosome-associated isoform of LC3 (LC3-II) in dendritic cells
300 (Romagnoli et al., 2012). Moreover, for IFN- γ -activated macrophages, it was demonstrated
301 that the host protein ubiquilin 1 promoted IFN- γ -mediated autophagic clearance of *Mtb*
302 (Sakowski et al., 2015). Similarly, another study reported that ubiquitin binds to an *Mtb* PE-
303 PGRS surface protein, Rv1468c, and directly recruits autophagy receptor p62 to deliver
304 mycobacteria into LC3-associated autophagosomes, thereby contributing to mycobacterial
305 clearance (Chai et al., 2019). A study using *Mtb*-infected primary murine macrophages
306 revealed that a particular autophagy receptor, TAX1BP1, mediates clearance of
307 ubiquitinated *Mtb* and targets bacteria to LC3-positive phagophores (Budzik et al., 2020).
308 By using mass spectrometry and bio-informatics analyses, the authors identified hundreds of
309 dynamically regulated phosphorylation and ubiquitylation sites, suggesting that dramatic
310 remodeling of multiple host pathways occurred during infection with *Mtb* (Budzik et al.,
311 2020). In a parallel study which used human induced pluripotent stem cell-derived
312 macrophages (iPSDMs) it was found that upon ESX-1-mediated phagosomal rupture, *Mtb*
313 induced the formation of LC3B-positive tubulovesicular autophagosomes (Bernard et al.,
314 2020).

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

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

322

323 **Impact of phagosomal rupture on cell death**

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

337

338 **How can this knowledge be used for vaccine development ?**

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

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

351 1^{Mmar} provided superior protection in murine aerosol challenge models, compared to parental
352 BCG, which used various highly virulent *Mtb* strains (Groschel et al., 2017).

353 The *Mtb* Δ pe25-pe19 strain (Bottai et al., 2012) is another live-attenuated anti-TB
354 vaccine candidate that elicits ESX-1-mediated cytosolic immune signaling, due to its
355 functional ESX-1 type VII secretion system (Bottai et al., 2012; Sayes et al., 2016; Sayes et
356 al., 2012). This strain efficiently activates innate and adaptive immune responses comparable
357 to virulent *Mtb*, including mycobacteria-specific effector memory CD4⁺ T cells, while
358 showing strong attenuation in cellular and *in vivo* infection models (Bottai et al., 2012; Sayes
359 et al., 2012). Its use as an attenuated live vaccine candidate displayed significant improved
360 TB protection efficacy in selected mouse models relative to BCG (Sayes et al., 2016; Sayes et
361 al., 2012).

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

368

369 **Comparison of mycobacterial phagosomal rupture to other bacteria**

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

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

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

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

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

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

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

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

434

435 **Conclusions**

436 From recent years' research it has become more and more evident that mycobacteria-induced
437 phagosomal rupture represents a key event in pathogen-host interaction. While for many years
438 it was thought that *Mtb* exclusively replicated inside vacuoles, convergent immunological and
439 cell biological data point to periods during infection when *Mtb* gains access to the cytosol of
440 host phagocytes. Despite intense research in the last years this process still holds many
441 secrets, but which step by step are being elucidated, as was for example recently shown for
442 the infection process inside selected *in vivo* models (Simeone et al., 2015; van der Niet et al.,
443 2020). Given the novel tools that are progressively becoming available for detailed
444 observations of the nanomachines involved in host-pathogen interaction (Lawarée &
445 Custódio, 2019), deeper insights into mechanistic details of the interaction between
446 mycobacterial pathogens and host cells shall be obtained in the near future. Research linked to
447 phagosomal rupture induced by pathogenic mycobacteria will thus remain an exciting subject,
448 whose new insights might also help to find new intervention strategies against a pathogen that
449 continues to threaten the health and lives of millions of people.

450

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457

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863 **Figure legends:**

864
865 **Figure 1.** Schematic view of an *Mtb*-infected phagocyte, highlighting the various signaling
866 events induced, as described in different sections of the review. Note that the red structures
867 drawn inside the mycobacterial cell envelope are meant to represent the mycobacterial ESX-1
868 secretion system.

869

870 **Data Availability Statement**

871

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

874

875 **Conflict of interest statement**

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

877

878 **Graphical Abstract text:**

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