Mycobacterium tuberculosis Genes Involved in Regulation of Host Cell Death

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Abstract The topic of host cell death response upon *Mycobacterium tuberculosis* (Mtb) infection has been a controversial one [1]. Recent findings demonstrate that one of the important confounding factors was most likely the fact that while Mtb inhibits host cell apoptosis induction early during the infection it clearly induces a necrotic form of cell death during later infection stages [2, 3]. This bi-phasic intracellular lifestyle in regard to host cell death manipulation is emerging as a common theme shared with other facultative and obligate intracellular bacterial pathogens such as *Chlamydia* and *Legionella* [4–6]. Accordingly, the list of discovered bacterial proteins involved in host cell apoptosis inhibition is growing [7, 8]. At the same time it is clearly beneficial for the resistance of the host to overcome the bacterial apoptosis block during the early stage of the infection [9–11]. Hence, host cell components have evolved to recognize intracellular pathogens and mediate host cell apoptosis induction if necessary [12]. There have been several reviews on the various aspects of the host cell death response upon Mtb infection [1, 3, 13–15]. Thus in this chapter I will focus on the pathogen side of the equation and describe the tremendous progress that has been made in the identification and characterization of Mtb genes involved in manipulation of host cell death pathways.

Keywords Apoptosis · Pyroptosis · Necrosis · Mycobacterium · Tuberculosis · Cell death

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1 Introduction

The topic of host cell death response upon *Mycobacterium tuberculosis* (Mtb) infection has been a controversial one [1]. Recent findings demonstrate that one of the important confounding factors was most likely the fact that while Mtb inhibits host cell apoptosis induction early during the infection it clearly induces a necrotic form of cell death during later infection stages [2, 3]. This bi-phasic intracellular lifestyle in regard to host cell death manipulation is emerging as a common theme shared with other facultative and obligate intracellular bacterial pathogens such as *Chlamydia* and *Legionella* [4–6]. Accordingly, the list of discovered bacterial proteins involved in host cell apoptosis inhibition is growing [7, 8]. At the same time it is clearly beneficial for the resistance of the host to overcome the bacterial apoptosis block during the early stage of the infection [9–11]. Hence, host cell components have evolved to recognize intracellular pathogens and mediate host cell apoptosis induction if necessary [12]. There have been several reviews on the various aspects of the host cell death response upon Mtb infection [1, 3, 13–15]. Thus in this chapter I will focus on the pathogen side of the equation and describe the tremendous progress that has been made in the identification and characterization of Mtb genes involved in manipulation of host cell death pathways.

2 Genes Involved in Inhibition of Host Cell Death

2.1 Superoxide Dismutase A

The first evidence for a gene of Mtb with anti-apoptotic function was provided by the analysis of lungs of mice infected with either Mtb or a strain with reduced superoxide dismutase A (SodA) expression using an anti-sense approach [16]. The SodA-knock-down strain was attenuated in mice and induced more apoptosis in lung cells [16]. These findings could be confirmed and extended in a more recent study which used an Mtb mutant deficient in the SecA2 secretion system that induced increased caspase-dependent apoptosis in macrophages. The pro-apoptotic phenotype of the *secA2* mutant was caused by deficient export of SodA since episomal expression of SodA with a signal peptide, targeting it to the SecA1 secretion system for export, could rescue the *secA2* mutant phenotype [17]. Interestingly, the overexpression of SodA in the vaccine strain BCG resulted in a reduction of macrophage apoptosis induction and a reduced protective capacity of the vaccine against challenge with Mtb in the mouse and guinea pig models [18].

2.2 nuoG *(NDH-1) and* eis

The first conclusive report that the inhibition of host cell apoptosis is important for the virulence of Mtb was provided by the analysis of the pro-apoptotic mutant of Mtb deficient in the *nuoG* gene [19]. In this study a "gain-of-function" genetic screen was used to identify three genomic regions in Mtb that contain anti-apoptotic genes and *nuoG* was identified as an anti-apoptotic gene in one of these regions [19]. The *nuoG* gene is part of a 14 gene operon coding for the type I NADH dehydrogenase, NDH-1. Subsequent investigations discovered that a functional NDH-1 is important for neutralizing reactive oxygen species (ROS) created by the macrophage phagocyte oxidase (NOX2) [20]. In *nuoG* mutant infected macrophages ROS accumulated in the phagosome as late as 24 h after infection but not in NOX2-deficient macrophages. The nuoG mutant did not induce apoptosis in the NOX2 knockout macrophages demonstrating the causal effect of increased ROS on host cell apoptosis. Phagosomal ROS mediated host cell apoptosis induction via the extrinsic apoptosis pathway was dependent upon TNF receptor signaling and caspase-8 activation [20]. The enhanced intracellular survival (*eis*) gene of Mtb is also important for suppression of ROS increases in the host cell but in this case the *eis* mutant induced ROS generation not via NOX2 but also via host cell mitochondria [21]. Another important difference of this mutant with the *nuoG* mutant phenotype is that the *eis* mutant induces caspase-independent cell death and autophagy. Eis is a member of the GCN5-related family of N-acetyltransferases and the acetyltransferase activity of Eis is necessary for its anti-apoptotic activity [21]. Eis is a secreted protein and thus it is conceivable that it reaches the host cell cytosol to acetylate and thus inactivate target proteins in a manner similar to the *Yersinia* spp. secreted effector YopJ [21, 22].

2.3 *Protein Kinase E*

It is of interest to note that neither the *nuoG* nor the *eis* mutant-induced apoptosis depends on the phagosomal generation of reactive nitrate intermediates (RNI) that are derived from nitric oxide produced by the iNOS enzyme. Instead, nitric oxide induces transcription of protein kinase E (*pknE*) and in the absence of PknE, Mtb causes more host cell apoptosis early during infections and less necrosis during the later stage of the infection [23]. A detailed analysis of the host transcriptome upon infection with the *pknE* mutant demonstrated an increased expression of pro-apoptotic BCL-2 family members *bax* and *bid* genes and suppressed expression of anti-apoptotic *mcl-1* among a list of genes involved in apoptosis regulation, co-stimulation, pro- and anti-inflammatory cytokines [24]. It is still unclear which of these host genes are of functional importance for the phenotype of the *pknE* mutant, neither is the target of pknE known. Nevertheless, a model emerges in which Mtb has evolved to sense the important host phagosomal defense responses

in ROS and RNI and developed multiple pathways to evade their detrimental effects. Furthermore, besides their involvement in direct bactericidal activity, the prolonged production and accumulation of ROS and RNI leads to host cell apoptosis induction. This could be regarded as a last resort of the macrophage defending against intracellular pathogens that have adapted to the hostile macrophage environment.

2.4 Rv3364c *and* Rv3654c

Several anti-apoptotic Mtb genes have recently been discovered via a "loss-of-function" genetic screen [25, 26]. The first report describes the importance of a 7-gene operon (*Rv3654c-Rv3660c*) for Mtb-mediate apoptosis inhibition. The operon contains four genes with homology to type IV pili. It could be determined that Rv3654c is secreted into the host cell cytosol and binds to protein-associated splicing factor (PSF) which inhibits splicing of caspase-8 pre-mRNA and thus downregulates caspase-8 protein levels in macrophages [26]. Consistently, knockdown of host cell PSF via siRNA abrogates the pro-apoptosis phenotype of the *Rv3654c* mutant [26]. The second finding reported from this genetic screen describes another operon (*Rv3361c-Rv3365c*) but this operon was important for inhibition of host cell pyroptosis [25]. Pyroptosis is defined as a form of apoptosis that depends upon inflammasome activation and in particular the activation of the caspase-1 [12]. Interestingly, the Rv3364c protein can enter the host cell cytosol, bind to, and inhibit the protease cathepsin G which leads to less apoptosis induction. Furthermore, a reduction of cathepsin G activity also reduces activation of caspase-1 which further

Table 1 Mtb genes important for apoptosis inhibition

Gene	Protein function	How it inhibits apoptosis	Ref.
sodA	Superoxide dismutase	Neutralizes phagosomal ROS?	[16–18]
nuoG	Electron acceptor; part of type I NADH dehydrogenase	Neutralizes and/or inhibits production of phagosomal ROS	[19, 20]
pknE	Serine/threonine protein kinase	Suppresses nitric oxide stress induced apoptosis	[23, 24]
Rv3364c	Roadblock/LC7 family like protein	Binds to host cell cathepsin G which leads to suppression of pyroptosis	[25]
eis	GCN5-related family of N-acetyltransferases	Mediates ROS suppression via N-acetyltransferase domain; inhibits autophagy and caspase-independent cell death	[21]
Rv3654c	Part of 7-gene operon (type IV pili like)	Binds and cleaves host cell protein-associated splicing factor (PSF) leading to less caspase-8 in host cell	[26]

cements the importance of cathepsin G in pyroptosis induction and suggests a role for cathepsin G in caspase-1 activation [25] (Table 1).

3 Genes Involved in Induction of Host Cell Death

3.1 esxA (esat-6)

After replication and inhibition of host cell apoptosis Mtb escapes the phagosome [27, 28] and induces a form of necrotic cell death [2, 3]. It is unclear which genes of Mtb are important for triggering this necrotic death response but it seems likely that active bacterial manipulation is required [3, 29]. Mycobacteria contain five type VII secretion systems (ESX1-ESX5) [30, 31]. The ESX-1 secretion system is clearly important for escape of Mtb and *M. marinum* (Mm) out of the phagosome into the host cell cytosol [27, 28, 32, 33]. It is also well established that Mtb and Mm mutants deficient in functional ESX-1 induce less host cell necrosis and reduced inflammasome activation [32–36]. In general, the problem with the analysis of the ESX-1 system is that the deletion of any of its known components inhibits the whole system and hence it is difficult to demonstrate which proteins of the system are the secreted effectors and which are part of the secretion machinery. The esxA (ESAT-6) component of ESX-1 is able to form dimers which can insert into lipid bilayers and form pores [32, 34]. This could explain the importance of ESX-1 for phagosomal escape and it also makes the ESX-1 system a candidate for the induction of necrosis-type cell death induction during the later stage of the infection [37, 38]. Alternatively, the recognition of ESX-1 effectors by host cell the NLRP3 inflammasome induces pyronecrosis in human macrophages [39]. It remains to be seen if these findings can be linked to the previously described exit pathway involving host cell eicosanoide manipulation [3]. It will be experimentally challenging to prove the role of ESX-1 in host cell exit since ESX-1 mutants do not escape from the phagosome and hence do not enter the normal later stage of the replication cycle. Nevertheless, the dual role of the ESX-1 system for two important functions in the intracellular life cycle of Mtb would also explain the severe attenuation of mycobacteria without this secretion system [34, 40–42].

Interestingly, the *esxA* mutant induces less host cell apoptosis compared to wild-type Mtb early during the infection when Mtb inhibits phagosome maturation and replicates in the phagosome [38, 43]. This suggests that host cell proteins are able to recognize ESX-1 secreted effectors to induce apoptosis. Interestingly, purified EsxA can bind to and induce signaling of the TLR-2 which leads to inhibition of signaling response of TLR-4, TLR-7, and TLR-9 [44]. These studies were performed using purified ligands and hence it remains to be seen how all of these signals integrate during an infection with live Mtb. It would not be surprising to find that human macrophages have evolved to target one or more of the components of Mtb essential for intracellular survival during the early stage of the

infection to induce host cell apoptosis. To that effect, genetic variability in the capacity to mediate an apoptotic response is associated with differences in host susceptibility to mycobacterial infections [9–11].

3.2 ESX-5 Locus and PE_PGRS33

The ESX-5 type VII secretion system is a major pathway for the export of PE and PPE domain containing family of proteins [45, 46]. The ESX-5 deficient Mtb and Mm induce less inflammasome activation and host cell necrosis [36]. The host cell necrosis induction is mediated via lysosomal rupture and depends upon the cathepsin B protease. Interestingly, the ESX-5 system is not required for phagosomal escape and thus a model was proposed where ESX-1 is important for phagosomal escape and ESX-5 for necrosis induction and consequently the exit of Mtb out of the host macrophage [36]. The PE family protein PE_PGRS33 has pro-apoptotic activity via interaction with Toll-like receptor 2 when purified protein was added to various cell types [47]. It remains to be shown if this protein has any activity when analyzed in the context of live mycobacteria and if it could be an ESX-5 secreted effector involved in host cell death induction.

3.3 Heparin-Binding Hemagglutinin

The Heparin-binding hemagglutinin (HBHA) is a 28 kDA protein found in the cell wall and culture filtrate of Mtb. It facilitates binding of Mtb to nonphagocytic epithelial cells and is important for extrapulmonary dissemination of Mtb [48, 49]. Interestingly, in macrophages HBHA is targeted to host cell mitochondria after infection to induce apoptotic cell death [50]. In this study, BMDMs were infected with either Mtb deficient in HBHA expression which led to less apoptosis induction or with Msme ectopically expressing Mtb-HBHA which led to an increase in apoptosis. The apoptosis induction is dependent on the activation of pro-apoptotic host cell protein Bax and on an increase in mitochondrial ROS. A specific role of this apoptosis induction for Mtb pathogenesis remains to be determined but it seems unlikely that this mechanism is involved in the exit of Mtb from macrophages because it does not induce cell membrane rupture [50].

3.4 OppABCD

Methylglyoxal (MG) and advanced glycation end products (AGE) may cause apoptosis and IL-1β, IL-6 and TNF cytokine secretion [51–53]. Interestingly, both of these products accumulate in host cells upon Mtb infection [54]. The oligopeptide transporter OppABCD of Mtb is able to bind glutathione and import it into

Table 2 Mtb genes important for apoptosis/necrosis induction

Gene	Proposed function	How it induces apoptosis/necrosis	Ref.
esxA (esat-6)	Component of ESX-1 type VII secretion system	Forms homodimers to induce pores in host cell membranes	[32, 34, 43]
hbha	Adhesion to lung epithelial cells	Localizes to host cell mitochondria to induce Bax activation, loss of MOMP and cytochrome C release	[50]
ESX-5 locus	Type VII secretion system	Necrotic cell death dependent upon cathepsin B	[36]
oppA-D	Oligopeptide transporter	OppA binds to host cell glutathione, leads to increase in host cell TNF production	[53]

the bacterial cytosol [53]. The decreased glutathione levels in the host cell lead to an accumulation of MG and AGE in cells infected with wild-type Mtb but not in cells infected with an *oppD* mutant [53]. These findings thus provide a molecular mechanism for the Mtb-mediated manipulation of host cell glutathione levels and its effect on cell apoptosis and cytokine response. The role for this regulation in pathogenicity of Mtb during in vivo infections remains to be determined. In view of the in vitro data, one could speculate that an *oppA-D* deficient mutant will be hypervirulent since it induces less apoptosis and cytokine secretion (Table 2).

4 Conclusion

The manipulation of host cell death pathways by Mtb is complex but can be divided into at least two phases: an early anti-apoptotic and a later pro-necrotic phase. The analysis and characterization of the genes involved in the pro-necrotic phase will be complicated by the fact that the host cell also aims to undergo apoptosis and hence recognizes mycobacterial components for cell death induction. Consequently, in future studies it will be important to differentiate between mycobacterial genes important for the regulated exit and genes encoded for components that are recognized by the host cell for immune defense purposes. The execution of "loss-"and "gain-of-function" genetic screens has been successful in identifying Mtb genes dedicated to host cell apoptosis inhibition during the early phase of infection. These approaches revealed that the Mtb genome encodes multiple effectors dedicated to inhibiting the extrinsic pathway of caspase-dependent apoptosis, the caspase-1-dependent pathway of pyroptosis, and caspase-independent, autophagy-associated apoptosis.

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