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Living on the edge: Inhibition of Host Cell Apoptosis by *Mycobacterium tuberculosis*

Volker Briken[1,2,3] and **Jessica L. Miller**[1,2]

1*Department of Cell Biology and Molecular Genetics, University of Maryland College Park, MD, 20742, USA*

2*Maryland Pathogen Research Institute University of Maryland College Park, MD, 20742, USA*

Summary

Tuberculosis is a human disease of global importance caused by infection with *Mycobacterium tuberculosis*. Thus, an estimated one-third of the world's population is latently infected; there are 2-3 million annual deaths and an increasing amount of multi-drug resistant and extreme-drug resistant tuberculosis cases. *M. tuberculosis* is a highly adapted human pathogen that has evolved to employ multiple strategies in its attempt to avoid an efficient host immune response. The induction of host cell death is an ancient immune defense strategy that is conserved throughout the animal and plant kingdoms. Here we review the current status of the research involving interaction of mycobacteria with host cells regarding the induction of host cell death by apoptosis. We conclude that virulent strains of *M. tuberculosis* employ several strategies to avoid the induction of macrophage cell death and success in this process is clearly important for bacterial virulence. The molecular mechanisms of host cell apoptosis inhibition are little understood, but the recent identification of anti-apoptosis genes in the genome of *M. tuberculosis* has provided the tools necessary to investigate the details of this host-pathogen interaction. The results of these future studies may prove useful for the development of new drug targets and/or vaccine candidates.

Keywords

Apoptosis; Mycobacterium; Infection; NADH dehydrogenase; vaccine development; drug target; virulence; cell death; signal transduction; TNF

The capacity of *M. tuberculosis* (Mtb) to manipulate infected host cells to its own advantage is well documented, although the underlying molecular mechanisms are often only poorly understood [1-4]. One of the most ancient defense mechanism of multicellular organisms is the sacrifice of infected cells for the benefit of the remaining cells. In fact, the induction of programmed cell death or apoptosis after the encounter of pathogenic microorganisms is one aspect of host innate immune response that is conserved among the animal and plant kingdoms [5,6].

The capacity of some viral pathogens to inhibit host cell apoptosis is well studied, and protozoan and bacterial pathogens are described as having anti-apoptotic capacities [7-10]. The conclusions of publications on the interaction of Mtb with macrophages in regarding the fate of the infected cells are somewhat contradictory. In some reports virulent Mtb is described to induce apoptotic host cell death, whereas in others the pathogen is thought to inhibit this process. Some of these differences might be explained by the complexity of the experimental

[3]corresponding author: vbriken@umd.edu

system which depends on the interaction of viable bacteria and host cells. Thus, there is variation among the publications in regards to the strains/isolates of mycobacteria used, differences in the infection procedures and multiplicity of infection, and finally, the nature of the host cell. In this first section of the review we will present a survey of the literature and conclude that indeed virulent Mtb induces host cell apoptosis, while at the same time clearly employing multiple strategies to inhibit apoptosis. The net result is that virulent isolates of Mtb induce very little host cell death when compared to non-pathogenic or facultative-pathogenic strains of mycobacteria which lack the anti-apoptosis mechanism of the virulent Mtb.

Thesis: *M. tuberculosis* induces host cell death

What results support the argument that Mtb induces host cell apoptosis? Several publications explicitly conclude that virulent Mtb induces apoptosis. For example, in two studies the level of macrophage apoptosis was demonstrated to be elevated in primary human alveolar macrophage obtained after bronchoalveolar lavage of tuberculosis patients when compared to healthy subjects [11,12]. Consistently, Mtb-H37Rv induced apoptosis in *in vitro* infection assays using primary alveolar macrophages [13]. Additional evidence was presented by infecting monocyte derived macrophages obtained from human blood with virulent *M. tuberculosis* and documenting an increase in host cell apoptosis after infection of the cells [14-16]. Similar results were obtained in the human macrophage-like cells using U937 and THP-1 cell lines [14,16]. Finally, bone-marrow derived murine macrophages (BMDM) were infected with Mtb and apoptosis levels increased [17]. Thus, in a variety of systems, virulent Mtb induces an increase in host cell apoptosis.

There have been a multitude of reports suggesting that virulent Mtb inhibits host cell apoptosis. However, a careful analysis of these publications reveals that in several cases, cells infected with virulent Mtb actually induced more apoptosis than seen in uninfected cells. For example, although Mtb-H37Rv clearly induces less apoptosis than the attenuated Mtb-H37Ra, it still induces a twofold increase in apoptosis compared to apoptosis levels in uninfected primary human alveolar macrophages [18]. In our own recent study we demonstrated a strong increase of apoptosis induction in differentiated human macrophage-like cells (THP-1) infected with the Mtb *nuoG* deletion mutant when compared to wild-type Mtb [19]. Nevertheless, we could always detect a small but significant increase of apoptosis in infected cells when compared to uninfected THP-1 cells [19]. Furthermore, the analysis of the host cell transcriptional response to infection with virulent Mtb demonstrated an increase in both pro- and anti-apoptotic genes [14,20]. Finally, recent reports described the induction of host cell death by virulent Mtb via a novel caspase-independent pathway that shares features of apoptotic and necrotic cell death [21,22]. This cell death pathway may help intracellular bacteria to escape infected macrophages of a permissive host and promote extracellular spread of the infection. In conclusion, it is most likely that even virulent strains of *M. tuberculosis* induce an apoptotic response in the host cell, but the degree of apoptosis induction depends on the multiplicity of infection and the genetic background of the host cell [17,21,23].

Anti-Thesis: *M. tuberculosis* inhibits host cell death

However, there is an increasing amount of evidence from three independent lines of investigation establishing that virulent Mtb does indeed inhibit host cell apoptosis. First of all, Keane *et al.* showed that virulent species of mycobacteria (Mtb-H37Rv, Mtb- Erdman, *M. bovis*) induced considerably less apoptosis in primary human alveolar macrophages than non virulent mycobacterial species (BCG, Mtb-H37Ra and *M. kansasii*) [24]. This is a feature also observed in THP-1 [25,26] and J774 cell lines [27] and supports the hypothesis that virulent mycobacterial species can actively inhibit or down regulate infection induced apoptosis of host macrophages.

Secondly, several recent studies have identified mycobacterial genes that mediate inhibition of host cell apoptosis. For instance, a "gain-of-function" genetic screen was used in our laboratory to identify Mtb genes that can actively inhibit host cell apoptosis induced by non-virulent mycobacterial species. Thus, three independent regions of Mtb genomic DNA were identified because they reduced apoptosis induction of the non-virulent mycobacterial species *M. smegmatis* and *M. kansasii*. These results provide strong genetic evidence that the genome of virulent Mtb strains encodes anti-apoptotic mechanisms. Characterization of one of these genetic loci led to the identification of the mycobacterial gene *nuoG*, which encodes one subunit of the type I NADH dehydrogenase, NDH-1, in Mtb. Interestingly, the overexpression of Mtb-*nuoG* in the apoptosis-inducing species *M. kansasii*, conferred the ability to inhibit apoptosis of infected human and murine macrophages. Conversely, the deletion of *nuoG* from Mtb ablated its ability to inhibit macrophage apoptosis [19]. In addition to nuoG, two other Mtb proteins, SecA2 and protein kinase E (PknE), have been implicated in the inhibition of host cell apoptosis [28,29]. *SecA2* codes for a mycobacterial secretion system that, among other proteins, mediates secretion of superoxide dismutase A (SodA), an enzyme that catalyzes conversion of superoxide anions to hydrogen peroxide [30]. Interestingly, the *secA2* deletion mutants of Mtb induce more apoptosis upon macrophage infection than wild-type Mtb. However this phenotype is lost when SecA2 deficient bacteria are complemented with a SodA fusion protein that is secreted independently of the SecA2 system, insinuating that it is the extracellular SodA that is involved in inhibiting apoptosis [28]. PknE is a serine threonine kinase whose promoter is induced during nitric oxide (NO) stress. The deletion of *pknE* resulted in a mutant that was more susceptible to NO exposure, and also capable of inducing a higher level of apoptosis in human macrophages compared to wild-type Mtb [29].

Finally, further evidence that virulent strains of Mtb can actively inhibit apoptosis stems from the observations that Mtb infection confers resistance in host cells to apoptotic stimuli, such as Fas ligand (FasL) and TNFα. TNFα can induce apoptosis via TNFα receptor-1 (TNFR1) signaling. Keane *et al*, showed that Mtb-H37Rv infected cells can inhibit TNFα mediated cell death, while those infected with Mtb-H37Ra cannot. When macrophages infected with non-virulent Mtb-H37Ra were exposed to TNFα, they exhibited significantly higher levels of apoptosis (from 28.6% to 47.9%). However, exposure of cells infected with Mtb-H37Rv to TNFα led to only a slight increase in apoptosis (12.6% to 17.4%) [13]. These data suggest that virulent Mtb is able to inhibit the extrinsic apoptosis pathway induced by the addition of TNFα. Virulent Mtb infection can also protect host cells against Fas mediated apoptosis. Macrophages infected with Mtb-H37Rv were less susceptible to FasL mediated cell death than untreated, or those infected with Mtb-H37Ra [31].

Synthesis: Virulent *M. tuberculosis* induces and inhibits host cell death

The recent identification of three genes in the Mtb genome that mediate a reduction in infection-induced apoptosis [19,28,29] provides the strongest evidence to date that Mtb is able to inhibit apoptosis. These results are corroborated by the finding that the expression of these anti-apoptosis genes in *M. smegmatis* reduces the strong pro-apoptosis phenotype [19]. Nevertheless, Mtb clearly induces some level of apoptosis and is therefore not as potent an inhibitor of host cell apoptosis as other intracellular pathogens, such as *Chlamydia trachomatis*, which confers resistance to infected cell against most known apoptosis stimuli [32]. In conclusion, Mtb infection results in pro- and anti-apoptotic responses of the host cell. The pro-apoptotic response is of clear benefit to the host since Mtb bacteria need to survive and persist in macrophages. Nevertheless, at a later stage of the infection, after the successful intracellular replication of Mtb, it may actually be advantageous for the bacteria to induce necrotic cell death in order to escape from the macrophage and infect new cells [21,22,33]. This process would be similar to the pathology of *Legionella* and *Chlamydia* in macrophages, which both go through an anti-apoptotic phase early during host cell infection, and then change

into a pro-necrotic phase at later stages [34-36]. Furthermore, it might be of benefit for Mtb to induce necrotic cell death of lung epithelial cells during the initial phase of the infection in order to effectively colonize the lung [14,37,38]. In summary, the complex interaction of Mtb with the host cell illustrates a constant evolutionary battle between the host trying to sense the intracellular pathogen and inducing apoptosis and the pathogen trying to hide within the host cell and thus inhibiting apoptosis.

Mechanisms of Mtb mediated host cell apoptosis inhibition

In general, apoptosis can be induced via two pathways: the extrinsic pathway, which involves death receptors like Fas/CD95 or the TNF-α receptor 1 (TNFR1) that activate Caspase-8/10 upon ligation, and the intrinsic pathway, which is triggered upon intracellular stress sensed by the mitochondria and initiated by activation of Caspase-9. Both pathways converge at the level of the Caspase-3/6/7 activation, which then triggers the subsequent events associated with apoptosis, e.g. fragmentation of genomic DNA (for a detailed review see [39,40]).

Multiple groups have shown that Mtb can inhibit the intrinsic pathway of host cell apoptosis via the regulation of pro- and anti-apoptotic proteins. For instance, upon infection, Mtb induces the upregulation of the anti-apoptosis genes *mcl-1* [41] and *A1* [42,43], both of which encode for Bcl-2-like proteins residing in the mitochondria. These results were corroborated by functional data using anti-sense oligonucleotides to knock-down mcl-1 and A1, as well as A1 knock-out mice, to demonstrate the importance of these genes for Mtb mediated host cell apoptosis inhibition [41,43,44]. Importantly, upregulation of A1 was not observed upon infection with Mtb-H37Ra, a non-virulent, high apoptosis inducing strain of Mtb [26]. Other studies have also shown that the anti-apoptotic protein Bcl-w is upregulated in Mtb-H37Rv, but not in Mtb-H37Ra infected cells [45], while the pro-apoptotic protein, Bad, is inactivated following Mtb-H37Rv infection [46].

Mtb infected macrophages have also been shown to inhibit the extrinsic apoptosis pathway by modifying the expression of death receptors such as Fas (CD95) and the soluble TNF receptor 2 (sTNFR2). Cell surface levels of Fas were shown to be decreased in host cells upon infection by Mtb [31], which may help to protect infected cells from Fas-ligand induced apoptosis. Furthermore, Mtb lipoglycan can stimulate the activation of NF-κB, a pro-survival factor, via TLR-2. The NF-κB activation leads to the subsequent upregulation of the cellular anti-apoptotic protein FLIP, which in part mediates the inhibition of FasL-mediated apoptosis [47]. In order to inhibit TNFα induced apoptosis Mtb infected macrophages have been reported to exhibit increased secretion of soluble TNFR2 (sTNFR2). The sTNFR2 binds to TNFα in the extracellular milieu and thus inhibiting its binding to the TNFR1 [18,48].

Why does Mtb inhibit host cell apoptosis?

Virulent but not avirulent strains of mycobacteria inhibit apoptosis of primary human alveolar macrophages. Thus, the capacity of Mtb to inhibit apoptosis was proposed to be a virulence factor [24]. The importance of apoptosis in the host's innate immune response was underlined by a report that apoptotic cell death reduced mycobacterial viability, whereas necrotic cell death had no effect on bacterial viability [49-51]. In line with these findings is a report demonstrating that the susceptibility of different mouse strains to mycobacterial infections could be linked to the capacity of infected macrophages to either undergo necrotic or apoptotic cell death upon infection, with the former imparting a susceptible phenotype and the latter a resistant phenotype [23]. The importance of host cell apoptosis for the acquired immune response against Mtb was suggested by the demonstration that phagocytosis of apoptotic bodies derived from Mtb-infected macrophages by dendritic cells (DCs) could lead to the presentation of mycobacterial lipid and peptide antigens and subsequent activation of specific T-cells [52]. Phagocytosis of apoptotic bodies seems to be an important mechanism in DCs by which

extracellular antigens gain access to MHC I molecules for priming of cytolytic T-cells, a process defined as “crosspriming” [53]. Remarkably, apoptotic bodies containing mycobacterial antigens have the capacity to protect mice from challenge by virulent Mtb [54]. All of these results support the importance of the inhibition of apoptosis in the virulence of *M. tuberculosis* and vice versa, the necessity of the host cells to undergo apoptosis in order to induce a protective immune response.

However, only the identification of pro-apoptotic Mtb mutants allowed performing the *in vivo* studies necessary to establish a causal link between the capacities of the bacteria to inhibit host cell apoptosis and their virulence [19,28,55]. Furthermore, the study by Hinchey et al. provided strong evidence that one mechanism of attenuation of the pro-apoptosis mutants is due to the increased cross-priming of $CD8^+$-Tcells [28].

Conclusion

M. tuberculosis and humans have co-evolved over thousands of years. This resulted in a selection for bacteria that are best suited to exploit the host cells. For instance, *M. tuberculosis* has adapted to human host cells by developing anti-apoptosis mechanisms and concomitantly reducing exposure of apoptosis-inducing components at its cell surface when compared to nonpathogenic mycobacteria [56,57]. Nevertheless, human macrophages have evolved to sense persisting intracellular pathogens. It is in this context that one has to consider the evidence that Mtb inhibits intrinsic and extrinsic apoptosis pathways, while at the same time induces pro-apoptosis signals. This leads to a delicate equilibrium between cell survival or cell death signaling that depends very much on the host cell type, its resistant or susceptible genetic background, and on the genetic composition of the pathogen. The molecular mechanisms of these complex interactions are little understood and provide a treasure trove for scientific discovery that may prove to be of importance beyond the field of tuberculosis research since some of these mechanisms are most likely shared with other persisting intracellular pathogens.

Future Perspective

The recent identification of several anti-apoptosis genes in Mtb and the subsequent demonstration of their role in bacterial virulence underlined the importance of apoptosis in Mtb-host cell interaction. The potential implications for clinical aspects of tuberculosis research are that the anti-apoptosis gene products of Mtb constitute a new group of drug targets [58] and secondly, anti-apoptosis genes are promising targets for improving the existing and developing new attenuated live vaccine strains [28,59]. From the viewpoint of the scientist interested in basic research, these mutants and the potential for the discovery of additional anti-apoptosis genes in Mtb provide a phenomenal opportunity to investigate the molecular mechanisms of this particular host-pathogen interaction. The host cell signal transduction pathways for the induction or inhibition of apoptosis are very complex and highly interconnected, as they should be, since the decision to die is not one to be taken lightly. Therefore, Mtb may have adapted to interfere with many of these signaling pathways as suggested by the identification of multiple anti-apoptotic genes. Once again, the interrogation of a highly adapted human pathogen may help us to learn something about ourselves, as the investigation of viral pathogens has done so many times before.

Executive Summary

M. tuberculosis induces host cell death

- Careful analysis of the literature demonstrates that there is a good amount of evidence suggesting that virulent Mtb induces host cell apoptosis albeit the extent of which

varies widely due to differences in host cells, Mtb isolates and multiplicities of infection used

M. tuberculosis inhibits host cell death

- The comparison of virulent and nonvirulent mycobacterial species demonstrates that the latter induce a much stronger apoptotic response in primary human alveolar macrophages
- Three recent publications identify anti-apoptosis genes (*nuoG*, *sodA* and *PknE*) in Mtb
- Expression of the anti-apoptotic gene, *nuoG*, in the apoptosis-inducing strain *M. kansasii* confers inhibition of apoptosis
- Mtb-infected macrophages are more resistant to apoptosis induction via Fas and TNF-α receptors

M. tuberculosis inhibits intrinsic and extrinsic apoptosis host cell pathways

- Mtb can increase host cell anti-apoptosis gene expression (e.g. *mcl-1*, *bcl-w*) and downregulated the pro-apoptosis gene, *bad*
- Mtb inhibits FasL and TNF-α induced apoptosis by either downregulating FasL-receptor or increasing secretion of soluble TNF-receptor

Inhibition of host cell apoptosis is a virulence factor of M. tuberculosis

- Mutants of Mtb that induce increased apoptosis are less virulent in mice

Conclusion

- Mtb clearly has pro- and anti-apoptosis mechanisms and the final outcome may depend on the nature and activation status of the host cell
- Inhibition of host cell apoptosis is important for virulence of Mtb

Future perspective

- Anti-apoptotic genes may be used for discovery of new anti-TB drugs and/or improvement of live vaccines
- The discovery of anti-apoptotic gene deletion mutants allows the analysis of the molecular mechanism of this host-pathogen interaction

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