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## **Mycobacteria and Macrophage Apoptosis:**

### **Complex Struggle for Survival**

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### **Abstract**

*M. avium*-infected macrophages undergo apoptosis, but *M. tuberculosis* suppresses apoptosis and triggers necrosis

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Although distinct, *Mycobacterium tuberculosis* and *Mycobacterium avium* both infect and survive in host macrophages. *M. tuberculosis* is more virulent, specifically adapted to humans, and grows poorly outside of the host. In contrast, *M. avium* can survive in the environment and also can infect several different host species in addition to humans, including birds, pigs, dogs, and monkeys.

*M. tuberculosis* is primarily a pathogen of the respiratory tract, whereas *M. avium*, which can infect the lungs, can also gain access to hosts by crossing the intestinal barrier. Although *M. avium* infections in healthy individuals are rarely disseminated, this bacterium can persist within macrophages in mesenteric lymph nodes. Both types of bacteria survive within host mononuclear phagocytes, inhibiting vacuole acidification and phagosome-lysosome fusion. However, while the reactive nitrogen intermediates (RNIs) in activated macrophages kill *M. tuberculosis*, *M. avium* is resistant to RNIs.

Following infection, virulent mycobacteria replicate within macrophages of susceptible hosts and subsequently infect surrounding macrophages. To spread the infection, mycobacteria escape one macrophage, probably once it becomes dysfunctional, to infect another. In the case of *M. avium*, infected macrophages undergo apoptosis, which triggers the escape of the bacterium, enabling it to spread to other cells. Meanwhile, *M. tuberculosis* inhibits apoptosis and exits cells by inducing necrosis.

### **Apoptosis Amounts to an Innate Immune Response against *M. tuberculosis***

A number of pathogens interfere with host cell apoptosis, either promoting it or suppressing it. For example, some pathogens, such as *Shigella*, *Salmonella*, and *Legionella*, activate pro-

caspase-1, which is a principal agent of the apoptosis cascade. However, other pathogens, including *Mycobacterium tuberculosis*, inhibit apoptosis.

Apoptosis is a form of innate immunity against some pathogens. Both extrinsic factors, such as cytokines induced by phagocytized bacteria, or intrinsic stimuli, such as host proteins that, once activated, increase the permeability of the mitochondrial membrane, can trigger apoptosis. In some cases, intracellular pathogens disable cells, such as mononuclear phagocytes, as a means of establishing an infection. However, the disabling of host cells sometimes backfires when those cells no longer provide nutrients or other environmental conditions that the pathogen needs to replicate and survive.

Recent studies demonstrate that macrophages and lymphocytes of patients with tuberculosis undergo apoptosis. Moreover, experiments *in vitro* establish that macrophages infected with viable virulent strains of *M. tuberculosis* undergo apoptosis with less frequency than macrophages infected with attenuated strains of *M. tuberculosis*. Strain virulence in mice correlates directly to the extent to which apoptosis is inhibited. Thus, virulent strains of *M. tuberculosis* apparently suppress apoptosis in host cells. Conversely, macrophage apoptosis appears to provide host cells a means for killing other types of intracellular microorganisms, with host cells likely initiating apoptosis following infection. Secreted bacterial proteins likely interact with proteins in the host-cell cytoplasm, members of the Nod-leucine-rich repeats (LRR-CARD) family, to trigger this process in macrophages.

### ***M. tuberculosis* and Macrophage Apoptosis**

Hardy Kornfeld of the University of Massachusetts Medical School in Worcester once suggested that *M. tuberculosis* inhibits macrophage apoptosis by decreasing the number of tumor necrosis factor-receptor-1 (TNF-R1) molecules on the phagocytic cell surface and lowering its secretion of interleukin-10. After *M. tuberculosis* infects macrophages, TNF- $\alpha$  secretion is suppressed, with the speed and intensity of suppression being strain-dependent. Some other *M. tuberculosis* strains are thought to induce TNF- $\alpha$  production. In addition, the response of *M. tuberculosis*-infected macrophage to TNF- $\alpha$  stimulation is impaired.

We propose that TNF- $\alpha$  is secreted upon bacterial uptake, and its synthesis and secretion then decrease progressively, while TNFR1 is down-regulated. However, at least in infected macrophage monolayers, *M. tuberculosis* does not fully inhibit apoptosis, which starts by day 3 and peaks at day 4 or 5. By day 5, secretion of TNF- $\alpha$  is probably down-regulated in some, but not all, macrophage cells.

Both *M. avium* and *M. tuberculosis* can down-regulate interleukin-12 (IL-12), a cytokine that macrophages produce, according to reports from ours as well other labs. However, when *M. tuberculosis* infects macrophages, it suppresses apoptosis triggered by other extracellular stimuli, such as recombinant TNF- $\alpha$ . This finding suggests that the TNFR1 mechanism, as well as the decreased secretion of TNF- $\alpha$ , do not completely explain bacterium-associated suppression.

Live and thriving bacteria are better than dead bacteria at blocking apoptosis. However, treating *M. tuberculosis*-infected macrophages with rifampin at levels that inhibit protein

synthesis without affecting bacterial viability significantly increases apoptosis, suggesting that newly synthesized *M. tuberculosis* proteins are required to inhibit extrinsic apoptosis. In sum, *M. tuberculosis* requires ongoing protein synthesis to inhibit macrophage apoptosis in vitro and in vivo.

### ***M. avium* Does Not Suppress Macrophage Apoptosis**

Unlike *M. tuberculosis*, *M. avium* apparently does not suppress macrophage apoptosis. Indeed, we and others showed that, when infected with *M. avium*, both human and mouse macrophages undergo apoptosis.

Many but not all of the infecting *M. avium* organisms appear to be killed following macrophage apoptosis, and thus some *M. avium* bacteria survive to infect other macrophages, thereby disseminating. In fact, *M. avium* can exit the phagocyte vacuole, and subsequently the cell, after apoptosis. Other bacteria, such as *Listeria monocytogenes*, also escape the vacuole, but in this case by a listeriolysin-dependent mechanism. Although *Mycobacterium marinum* similarly acidifies the phagocytic vacuole, its escape is not known to be associated with secretion of a lytic enzyme.

Using video microscopy, we found that some *M. avium* cells leave the phagocytic cell, while others remain within the apoptotic body and soon are ingested by another macrophage. Whether the failure of some bacteria to escape apoptotic cells is due to the lack of an efficient propulsion mechanism or a failure to come into contact with the cytoplasmic membrane is being investigated. Although *M. tuberculosis* and *M. avium* were considered to behave fairly similarly when occupying macrophages, evidence of their differences is accumulating.

### **Genes Associated with the Spreading of Mycobacteria among Host Cells**

*M. tuberculosis* and *M. avium* spread from one host cell to another by different mechanisms. *M. avium* escape from macrophages following apoptosis, whereas *M. tuberculosis* partially inhibits macrophage apoptosis.

We recently identified an operon of *M. tuberculosis* that can inhibit apoptosis. The operon is composed of seven genes: four of them resemble a pilus operon, the fifth gene encodes a transmembrane protein, and the other two genes encode secreted proteins. When a construct carrying the two genes encoding those two secreted *M. tuberculosis* proteins transfects macrophages, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and staurosporine-mediated apoptosis are inhibited. The mechanism(s) of action of the two secreted proteins is under investigation.

Based on the homology between four genes in this *M. tuberculosis* operon and type IV pili genes, we hypothesized that the *M. tuberculosis* genes might encode a secretion apparatus for the apoptosis-inhibiting proteins. In attempting to visualize such an apparatus, freeze-fraction transmission electron microscopy showed that both the bacterial and vacuole membranes have pores. The *M. tuberculosis* pores show two different morphologies; one type is similar to porins that are found in *Mycobacterium smegmatis*, while the other pores contain a protruding edge.

Other *M. tuberculosis* genes appear to be involved in inhibiting macrophage apoptosis. For instance, several encode enzymes that appear to be involved in synthesizing and degrading lipids. Another encodes mmpL8, a membrane protein that is involved in transporting fatty acids. All of these genes are associated with inactivating the extrinsic pathway of apoptosis. In fact, *M. tuberculosis* infections of macrophages do not inhibit the intrinsic pathway, raising the possibility of a link between this pathway and escape of *M. tuberculosis* from the host cell, which occurs secondary to necrosis. Many studies indicate that the pathogenesis-related region RD-1 and, more specifically, the genes encoding proteins ESAT-6 and CPF-10, are directly involved in macrophage cytotoxicity and spread of this bacterial pathogen. The mechanisms remain unknown.

DNA array studies of macrophages infected with *M. tuberculosis* mutants that do not inhibit apoptosis show that the wild-type bacterium inhibits expression of TNF- $\alpha$  and induces expression of TNF-RI. When macrophages are infected with one of these mutants, there is a 25-fold increase in expression of the pro-apoptotic gene *bim-L*. Apparently, one of the secreted proteins blocks expression of *bim-L*.

We found four *M. avium* mutants that do not escape macrophages upon apoptosis. Although these mutants escape the phagosome, they cannot exit the cell. We do not know whether the vacuole membrane disintegrates upon apoptosis or whether the virulent bacteria respond to changes in the vacuoles by triggering the disintegration of the vacuole membrane.

*M. avium* does not seem to accumulate actin at its poles. In *Mycobacterium marinum*, however, polar actin appears responsible for propagating these bacteria in macrophage cytoplasm, according to Luisa Stamm of the University of California, San Francisco and her collaborators. Assuming *M. avium* lacks actin propulsion implies that these bacteria encounter host-cell cytoplasmic membranes with low efficiency. However, once the host cell dies, the volume of the cytoplasm decreases, thus bringing these bacteria into closer contact with the cell membrane. We have identified several genes that apparently enable *M. avium* to escape the cell membrane. These phospholipases or other enzymes with lytic activity might need the cytoplasmic environment to be activated before *M. avium* can be released.

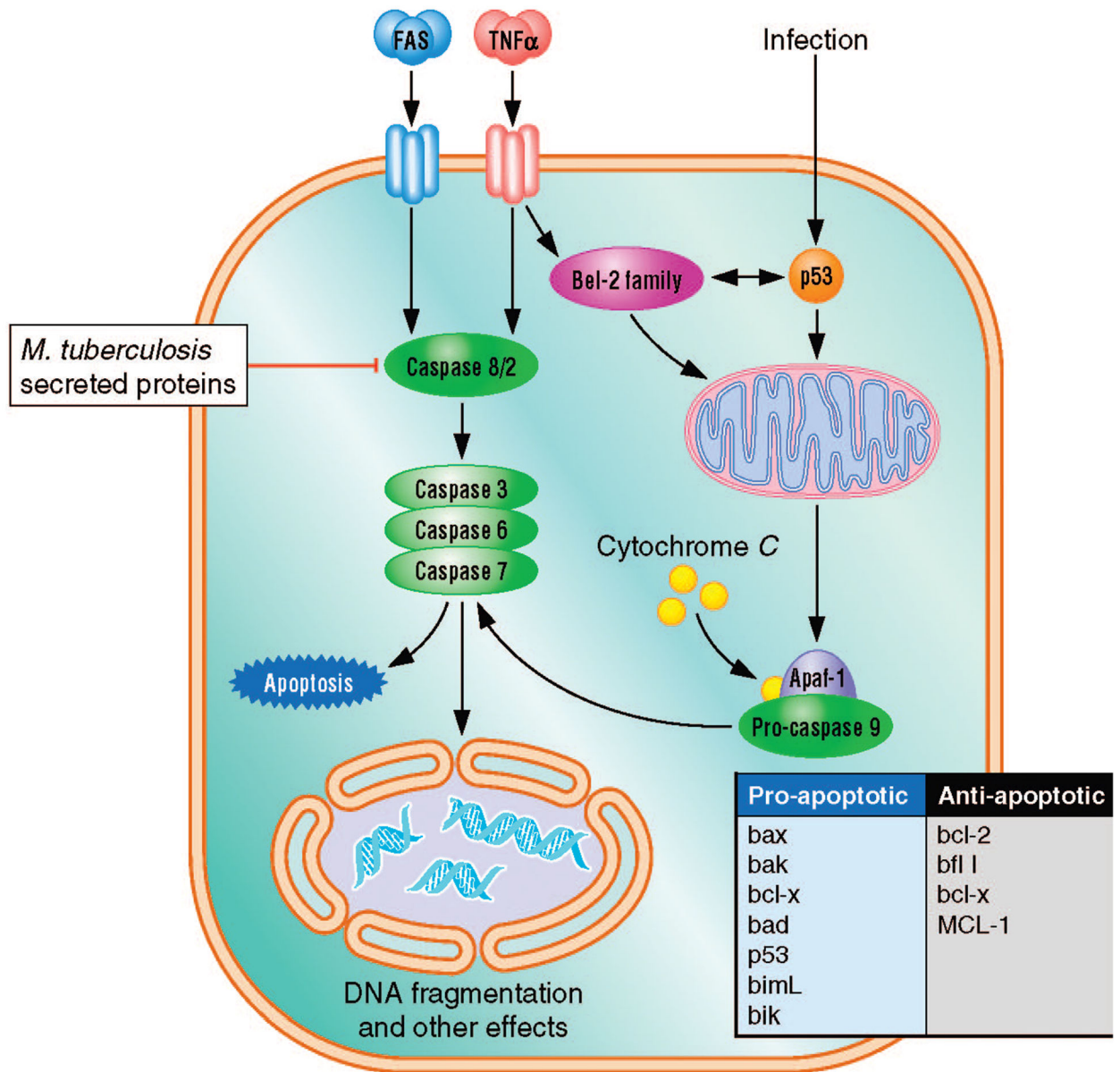
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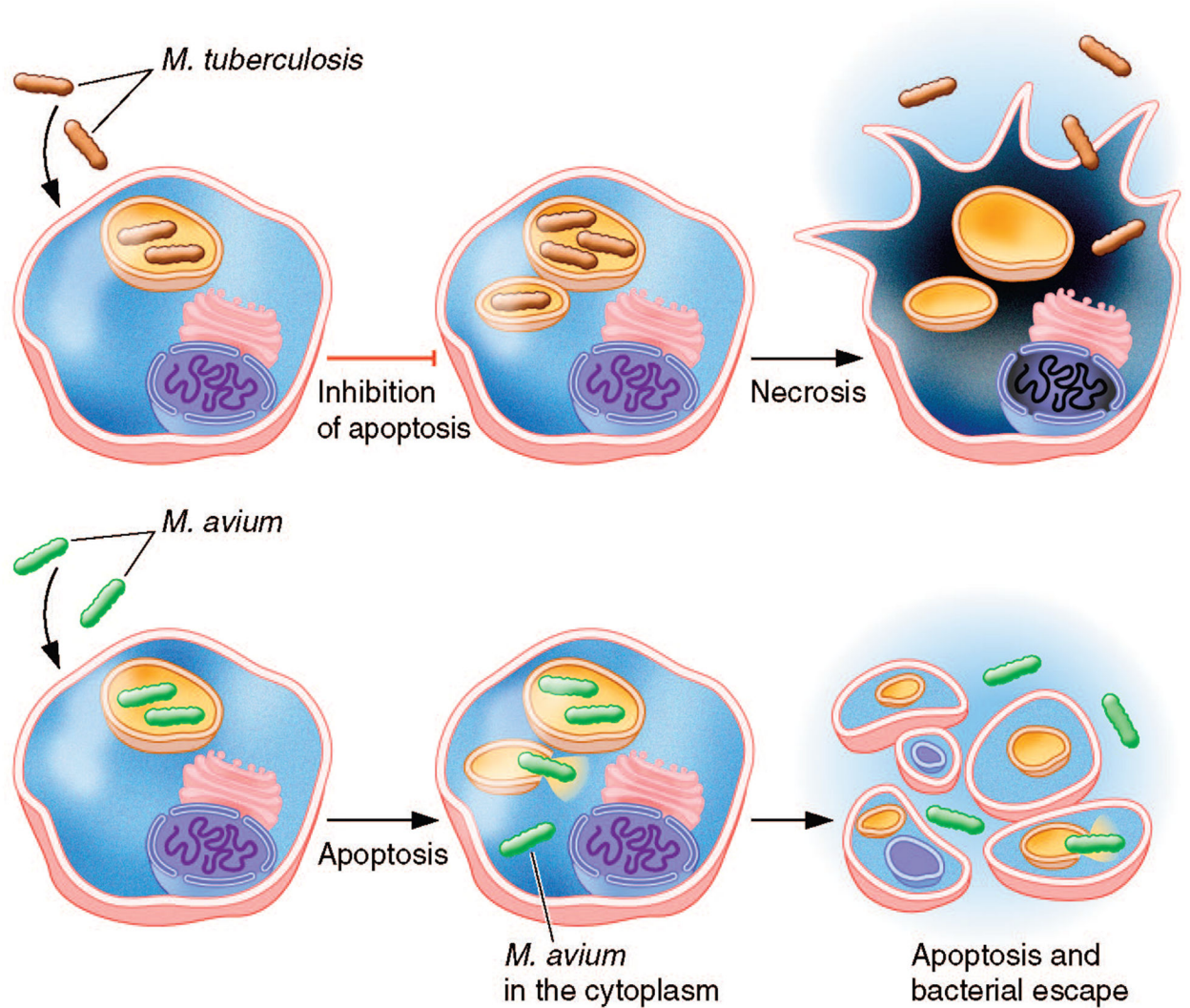
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### Summary

- Between *Mycobacterium tuberculosis* and *Mycobacterium avium*, the former is more virulent, specifically adapted to humans, and grows poorly on its own, whereas *M. avium* survives in the environment and readily infects several host species.
- *M. avium*-infected macrophages undergo apoptosis, whereas *M. tuberculosis* suppresses apoptosis but allows necrosis.
- A *M. tuberculosis* operon that inhibits apoptosis contains seven genes, including four that resemble a pilus operon, a fifth that encodes a transmembrane protein, and two genes that encode secreted proteins; still other genes appear to be involved in inhibiting this process.
- Several genes, encoding phospholipases or other enzymes with lytic activity, apparently enable *M. avium* to escape the cell membrane.



**FIGURE 1.** Main pathways of apoptosis. Infecting macrophage cells with a virulent strain of *M. tuberculosis* inhibits apoptosis by the extrinsic pathway. When *M. tuberculosis*-infected macrophages are exposed to TNF- $\alpha$ , caspase-8 cannot be activated. The existence of additional targets in the pathway is possible.



**FIGURE 2.**

*M. tuberculosis* and *M. avium* interact differently with macrophages. For example, *M. avium*-infected macrophages undergo apoptosis, which triggers the escape of *M. avium* from the cell, whereas *M. tuberculosis*-infected macrophages suppress apoptosis, and the bacterium ultimately escapes the macrophages by inducing necrosis.