Dying to Live: How the Death Modality of the Infected Macrophage Modulates Immunity to Tuberculosis

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Abstract Virulent *Mycobacterium tuberculosis* (*Mtb*) inhibits apoptosis and triggers necrosis of host macrophages to evade innate delay in the initiation of adaptive immunity. Necrosis is a mechanism used by bacteria to exit macrophage, evade the host defenses, and disseminate while apoptosis is associated with diminished pathogen viability. We have recently demonstrated that eicosanoids regulate cell death program of either human or murine macrophages infected with *Mtb*. We have defined prostaglandin E_2 (PGE_2) as a pro-apoptotic host lipid mediator which protects against necrosis. In contrast, lipoxin A_4 (LXA_4) is a pro-necrotic lipid mediator which suppresses PGE_2 synthesis, resulting in mitochondrial damage and inhibition of plasma membrane repair mechanisms; this ultimately leads to the induction of necrosis. Thus, the balance between PGE_2 and LXA_4 determines whether *Mtb*-infected macrophages undergo apoptosis or necrosis and this balance determines the outcome of infection.

Keywords *Mycobacterium tuberculosis* (*Mtb*) · Macrophages · Necrosis · Apoptosis · B-cell lymphoma 2 (Bcl-2) · Bcl-2 associated X Protein (BAX) · Bcl-2 homologous antagonist killer (BAK) · BH3 interacting domain (BID) ·

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Extrinsic pathway · FLICE-inhibitory protein (FLIPS) · Intrinsic pathway · Mitochondrial outer membrane permeabilization (MOMP) · Mitochondrial permeability transition (MPT) · Cell death program · Lipoxins (LX) · Prostaglandins (PG) · Eicosanoids · Plasma membrane microdisruptions · Mycobacterial antigens · BCG vaccine · T cell response

1 Introduction

Mycobacterium tuberculosis (*Mtb*) is an extremely successful bacterium that is transmitted person-to-person by the aerosol route. The World Health Organization (WHO) has estimated that more than 2 billion persons are latently infected with *Mtb*. From this large reservoir of asymptomatic infected people emerges 8–10 million cases of active TB each year resulting in the deaths of nearly 1.7 million people each year [1]. The increased incidence of TB has been attributed to three major factors: the HIV pandemic, the emergence of multidrug-resistant strains of *Mtb*, and the failure of the major vaccine, BCG, to prevent pulmonary tuberculosis [2–4].

The success of this pathogen is closely linked to its ability to alter the intracellular environment of the alveolar macrophage. When inhaled, *Mtb* enters the lower respiratory tract and reaches distal alveoli where initially infects alveolar macrophages. Although macrophages excel at phagocytizing and destroying biological particles including dead cells and bacteria, *Mtb* has adapted to the harsh intracellular environment, which allows it to survive and replicate within these phagocytic cells. By subverting or avoiding critical components of macrophage immunity including phagolysosomal fusion, microbicidal effectors, and as will be discussed in this chapter, cell death pathways, *Mtb* evades both innate and adaptive immune responses. Therefore, delineating how *Mtb* and macrophages interact is fundamental to understand immunity to *Mtb*.

Manipulation of macrophage death pathways is one mechanism that allows *Mtb* to evade host defenses. Three major outcomes are observed following productive *Mtb* infection of human or murine macrophages in vitro: (a) necrosis, a form of death characterized by plasma membrane disruption; (b) apoptosis, a form of death in which the plasma membrane integrity is preserved; and (c) survival of the infected macrophages. Characterization of these different phenotypes is challenging because of the asynchronous nature of intracellular infection and heterogeneity among the bacteria and macrophages. Other factors such as the percentage of infected macrophages and variation in the number of bacteria internalized by each macrophage can affect the kinetics of cell death when studied in vitro. Nevertheless, a spectrum of all three phenotypes can be observed following infection of normal macrophages with virulent *Mtb*. In general, highly virulent *Mtb* strains predominantly induce necrosis [5]. The concept that virulent *Mtb* induce necrosis in part by actively inhibiting macrophage apoptosis [6], has gained additional support by the identification of bacterial mutants that induce apoptosis

instead of necrosis [7, 8]. The different cellular fates of *Mtb* infected macrophages are of great interest as the death modality influences the outcome of infection. In particular, apoptotic death reduces the viability of different mycobacterial species [9, 10] including *Mtb* [11, 12, 13]. Here, we discuss the cellular mechanisms that regulate the death modality of *Mtb*-infected macrophages and lead to important functional consequences on immunity to *Mtb*.

2 Macrophage Apoptosis is a Host Defense Mechanism Against *Mtb*

The discovery that many attenuated strains of mycobacteria induce more apoptosis than their wild-type counterparts supports the hypothesis that virulent mycobacteria inhibit macrophage apoptosis. Indeed, there exists a reciprocal relationship between virulence and apoptosis. As such, *Mtb* infection predominantly results in necrosis, while attenuated mutant strains including BCG and H37Ra primarily induce apoptosis. Now, investigators are identifying single gene mutations in *Mtb* that shift the balance from necrosis to apoptosis [7, 8]. Although it is not yet clear whether virulent *Mtb* block the triggering of apoptosis or inhibit downstream events that give rise to the typical cellular changes associated with apoptosis, it can be argued that by inducing necrosis, *Mtb* evades host defenses and provides a pathway for its exit from the infected cell and its dissemination. Detailed analysis of "necrosis" reveals to be heterogeneous and certain subtypes have been defined that have unique cellular triggers and molecular mechanisms. For instance, pyroptosis and necroptosis are forms of necrosis that are dependent on caspase 1 and receptor-interacting proteins 1 and 3 (RIP-1/3), respectively [14–16]. Thus, the idea that necrosis is a passive, accidental, and unregulated form of cell death is an old dogma that needs to be revisited. In fact, how *Mtb* induces necrosis is a question that remains unanswered.

In contrast to necrosis, the past decades have made tremendous progress in unraveling the signaling pathways that lead to initiation of apoptosis. Hallmarks of apoptosis include the segmentation of DNA [17], exposure of phosphatidylserine on the outer leaflet of the plasma membrane, and finally, packaging of cellular components into membrane-bound blebs [18, 19]. During apoptosis the dying cell produces 'find me' and 'eat me' signals that aid its rapid clearance by phagocytes through the process of efferocytosis [13, 20].

Apoptosis is initiated by two major pathways

(1) *The extrinsic pathway:* The induction of apoptosis by attenuated *Mtb* in human monocyte-derived macrophages is mediated by the executioner caspases 3 and 7 and requires two distinct signals: one is a lipid and the other is a protein [21, 22] the protein-dependent signal can be reconstituted by TNF. Indeed, the induction of apoptosis in macrophages by *Mtb* requires the action of TNF to

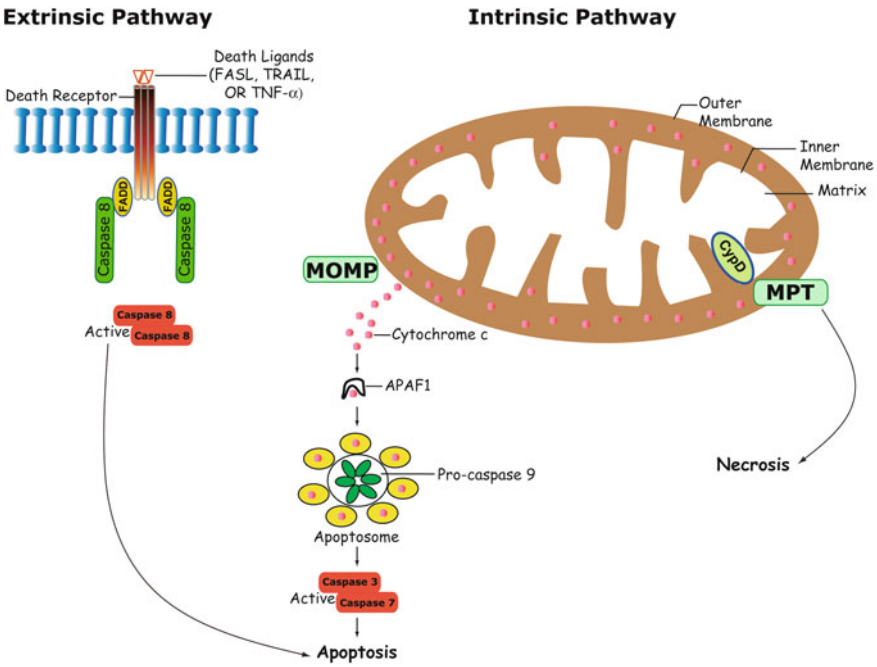

Fig. 1 Two major pathways leading to macrophage death following *M. tuberculosis* infection. Some macrophages death programs are triggered by the extrinsic pathway (surface receptor mediated): Ligation of death receptor (e.g., Fas or TNF-α receptors) is followed by activation of caspase 8 which leads to apoptosis. Alternatively, the intrinsic pathway (mitochondria-mediated) is activated: Permeabilization of mitochondria outer membrane potential (MOMP) leads to the release of apoptotic mediators such as cytochrome c from the mitochondrial intermembrane space into the cytosol leading to formation of apoptosome complex and activation of caspases (9, 3, and 7) which in turn induce apoptosis. However, during necrosis mitochondrial permeability transition (MPT) causes mitochondrial inner membrane perturbation (MIMP), collapse of the membrane potential, uncoupling of the respiratory chain, and overproduction of reactive oxygen species (ROS). Cyclophilin D (CypD) is a mitochondrial protein which is involved in MPT and necrosis

activate the extrinsic death receptor-dependent pathway (Fig. 1). Although both bacterial strains produce comparable amounts of TNF [23], the avirulent strain H37Ra much more potently induces apoptosis than the virulent H37Rv strain. One potential explanation for this observation is that soluble TNFR-2 is shed by macrophages infected with virulent *Mtb*, and neutralizes TNF, resulting in a "TNF-poor" microenvironment [24]. This model is also consistent with data that caspase 8 activation, which is an essential and early step in the induction of apoptosis by the extrinsic pathway, is inhibited in H37Rv-infected macrophages (Remold, unpublished observation). Recently, some of the early components of the extrinsic apoptotic pathway activated by *Mtb* in the murine macrophage cell line RAW 264 have been identified [25]. In *Mtb*-infected cells, TNF production induces reactive oxygen species

(ROS)-dependent activation of apoptosis signal-regulating kinase (ASK1; A002816), a member of the mitogen-activated protein kinase family causing $FLIP_s$ phosphorylation. Phosphorylated $FLIP_s$ interacts with the E3 ubiquitin ligase c-CYBL facilitating proteasomal $FLIP_s$ degradation involving the tyrosine kinase c-Abl. $FLIP_s$ degradation then enables activation of caspase 8 leading to caspase 3/7 activation and apoptosis.

(2) *The intrinsic pathway:* Induction of apoptosis in vertebrate cells most commonly proceeds through the intrinsic apoptotic pathway, which is functionally defined by mitochondrial outer membrane permeabilization (MOMP) [26]. MOMP is a central event that can lead to apoptosis as it results in release of apoptotic mediators, including cytochrome c, Smac-DIABLO, AIF, and other factors from the mitochondrial inter-membrane space and ultimately results in the activation of caspases 9, 3, and 7 (Fig. 1). Although these events usually occur independently of other changes in the mitochondria, they can also be associated with the opening of the mitochondrial inner membrane pore (PT pore), which leads to mitochondrial permeability transition (MPT), loss of the mitochondrial inter-membrane potential ($\Delta\psi_m$), and necrosis. We found that *Mtb* infection, whether virulent or avirulent, induces such changes in the mitochondrial membranes and that these changes are the key events that determine the death modality of infected macrophages.

3 Mitochondrial Damage and Macrophage Death

Considering mitochondria as a key player in regulation of cell death program, the different combinations of MOMP and mitochondrial permeability transition (MPT, the opening of a pore in the inner mitochondrial membrane) in model experimental systems, and their effect on the cellular outcome are reviewed below. Thus the changes in MOMP and MPT induced by either virulent or avirulent *Mtb* infection will be discussed in the context of these scenarios.

In Scenario I, MPT causes the mitochondria to become leaky to water, which results in swelling, dysfunction, and eventually necrosis [26]. Irreversible MPT can lead to outer mitochondrial membrane damage, which manifests itself as MOMP, in this case a by-product of MPT. This scenario emerges when hepatocytes under oxidative stress or due to other toxic treatment undergo both necrosis and apoptosis [27].

However, in Scenario II, MOMP and apoptosis can occur independently of MPT. This is the case when MOMP is induced by members of the Bcl-2 family of apoptosis-inducing proteins, which do not affect the mitochondrial inner membrane [28]. Specifically, processing of the Bcl-2 protein by BID leads to activation of the pro-apoptotic Bcl-2 family proteins, BAX and BAK, causing MOMP and translocation of pro-apoptotic factors including cytochrome c into the cytosol, activation of caspase 9, and eventually caspase 3. This process neither induces nor requires MPT [29].

In Scenario III, effector molecules capable of damaging the mitochondrial inner membrane gain access to the mitochondrial inter-membrane space if the mitochondrial outer membrane is permeable. This seems to be the mechanism by which Ndufs1, the 30 kDa subunit of mitochondrial complex 1 of the electron transport chain, is damaged by caspase 3 [30]. Caspase 3 is thought to access the mitochondrial inter-membrane space via pores generated in the mitochondrial outer membrane, which allow pro-apoptotic factors including cytochrome c to escape into the cytosol [29]. Damage of Ndufs1 disrupts the electron transport chain in the inner membrane leading to ROS accumulation and necrosis.

Finally, MPT can also occur independently of MOMP (Scenario IV). This is thought to be how granzyme A damages components of the mitochondrial inner membrane [31]. Hsp70 and Hsp90 are candidate molecules that serve as cytosolic chaperones for granzyme A and allow the protease to enter the mitochondrial inter-membrane space without damaging the mitochondrial outer membrane leading to cleavage of Ndufs3 [31].

In macrophages infected with attenuated *Mtb*, apoptosis is associated with MOMP yet MPT is not induced, as described in Scenario II [29]. Inhibition of MOMP diminishes only apoptosis, but does not affect MPT [5]. Silencing of the gene for the pro-apoptotic Bcl-2 protein BAX, which is required for the release of cytochrome c and AIF from the mitochondrial inter-membrane space, abrogates *Mtb*-induced apoptosis, but does not affect MPT or necrosis [5]. In contrast, virulent *Mtb* induce both MOMP and MPT leading to irreversible mitochondrial swelling and necrosis [5]. MPT can be inhibited by cyclosporin A (which selectively blocks the function of cyclophilin D in the mitochondrial inner membrane), has a requirement for mitochondrial Ca^{++} loading and is independent of Bcl-2 family member-induced apoptosis [32]. Inhibition of MPT vis Cyclophilin D, downregulates only necrosis, but does not affect the degree of MOMP or apoptosis [33, 34]. It is not clear at present whether in *Mtb* infected macrophages MPT is dependent on opening of pores in the mitochondrial outer membrane (MOMP—Scenario III) or whether toxic molecular species enter the mitochondrial inter membrane space via chaperones (Scenario IV). The different mechanisms induced by virulent and avirulent *Mtb* indicate that in *Mtb*-infected macrophages MOMP and MPT are independent phenomena; virulent *Mtb* are unique in their induction of MPT that leads to the destruction of the mitochondrial outer membrane causing secondary cytochrome c release (see Scenario I) [5]. In summary, induction of apoptosis or necrosis in *Mtb*-infected macrophages depends on highly specific mechanisms leading to different types of mitochondrial membrane perturbation. Attenuated and virulent *Mtb* alike cause transient MOMP characterized by cytochrome c release from the mitochondrial inter-membrane space, which requires BAX. In contrast, only virulent H37Rv causes MPT.

While cell death is a tightly regulated process, the host–pathogen interaction adds several layers of complexity. How the death modality of infected cells affects the outcome of infection, particularly during different clinical states in people (e.g., latency versus disease), remains a pertinent question. Here, other investigators working on the genetics of susceptibility to *Mtb* provide an important

perspective. Gene expression profiling finds that several genes related to apoptosis are expressed less in active TB patients than in latently infected people, suggesting that decreased apoptotic activity is associated with the reactivation of latent infection [35]. Using a more targeted approach, Abebe et al. found that patients with active TB in Ethiopia had elevated expression of genes associated with the extrinsic apoptosis pathway including TNF, Fas, FasL, and caspase 8. However, the expression of FLIP, an intrinsic inhibitor of caspase 8 was also significantly elevated [36]. Although the upregulation of TNF, Fas/FasL, and caspase 8 may be the signature of an immune response capable of inducing apoptosis in infected cells, the authors propose a model in which *Mtb* inhibits the extrinsic apoptosis pathway by upregulating FLIP to evade an apoptotic death. Finally, the eicosanoid biosynthetic pathways, which regulate the death modality of infected human and murine macrophages, have now been identified as important genetic loci that regulate susceptibility to tuberculosis and leprosy in people [37–39]. While the genetic and functional data require greater scrutiny and functional correlation, they independently provide scientific motivation to better understand how death is regulated in *Mtb*-infected macrophages.

4 Host Lipid Mediators Modulate *Mtb*-Infected Macrophage Death Modality

As bacterial factors can affect the death modality, host factors also determine whether an infected cell undergoes apoptosis or necrosis. In particular, the eicosanoids appear to be critical regulators of apoptosis following *Mtb* infection [11, 12, 40]. *Mtb* induces apoptosis and triggers concomitant antimycobacterial activity of human macrophages based on the activity of cytosolic phospholipase A_2-γ (cPLA$_2$-γ), a group IV cytosolic PLA_2, which catalyzes the release of arachidonic acid from the *sn-2* position of membrane phospholipids [22]. Arachidonic acid and its diverse products regulate death in several cell types [41]. For example, arachidonic acid products are second messengers in TNF-induced apoptosis [42], and oxygen radicals, which are produced during lipoxygenation of arachidonic acid, induce ROS production, which can induce cell death [43]. Arachidonic acid also activates sphingomyelinase leading to ceramide production and apoptosis [44]. Which of these mechanisms are important in vivo is not clear [45].

An interesting area of research focuses on the role of the eicosanoids prostaglandin E_2 (PGE_2) and lipoxin A_4 (LXA_4) in regulating programmed cell death of macrophages [11, 12, 40]. The cyclooxygenases COX1 and COX2 convert arachidonic acid into the central intermediate PGH_2 [46], which is converted by specific synthases into diverse prostanoids [47]. Interaction of these prostanoid species, which includes the prostaglandins PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and thromboxane, with an array of specific prostanoid receptors affects many cellular pathways. In the case of PGE_2, interaction with one of four receptors, EP1, EP2,

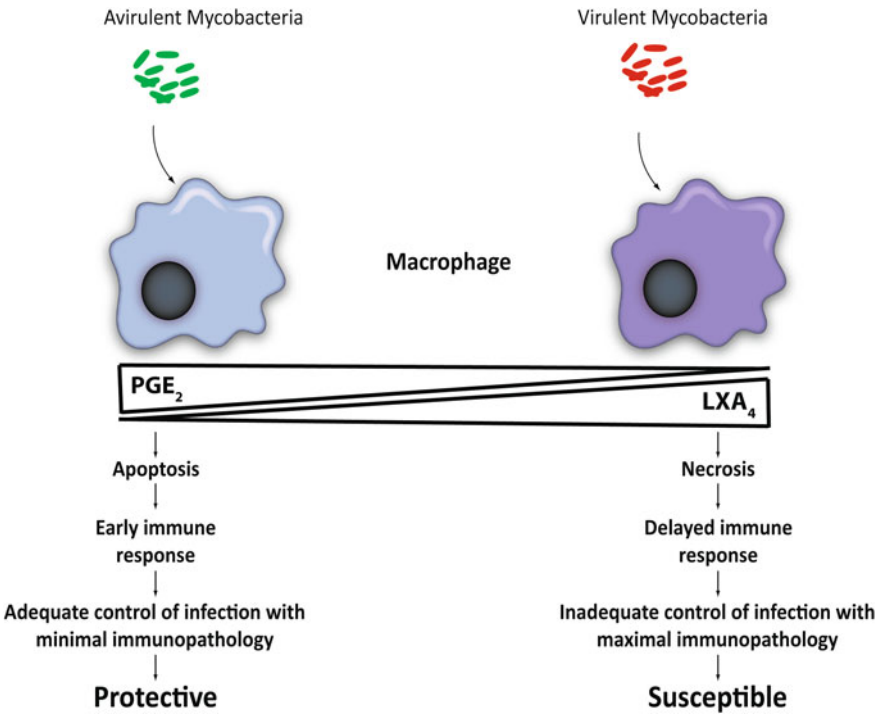

Fig. 2 Virulent mycobacteria tip the balance between PGE_2 and LXA_4 production in macrophages. Infection with virulent *Mtb* induces LXA_4, which inhibits the production of COX-2 dependent PGE_2. In the absence of PGE_2 mitochondria are damaged and membrane microdisruptions remain unrepaired triggering macrophage necrosis. We hypothesize that bacterial inhibition of prostaglandin production is an immune evasion strategy that allows *Mtb* to avoid the consequences of apoptosis, which leads to early immune response

EP3, and EP4 triggers intracellular pathways that either promote or inhibit inflammation [48]. Importantly, the functional outcome of PGE_2 signaling is largely determined by its interaction with its specific receptors [48]. For example, EP1 mediates the elevation of intracellular Ca^{++}. By contrast, EP2, which is involved in joint inflammation and neutrophil recruitment, and EP4, which induces cell migration in tumor invasion, both lead to an increase in intracellular cAMP levels. EP2 signaling results in PKA activation and triggering EP4 activates adenylate cyclase and phosphatidylinositol 3 kinase. Triggering EP3 decreases cAMP concentrations and is known to mediate fever and angiogenesis [48].

D'Avila et al. find that lipid bodies form at distinct cytoplasmic sites following infection of murine macrophages with the attenuated *M. bovis* strain BCG. These lipid bodies are the site of COX2 activity and PGE_2 generation [49]. Indeed, PGE_2 production has been a consistent finding following BCG infection of mice and macrophages [50]. We find that macrophages infected with attenuated *Mtb* also activate the PGE_2 production, which prevents necrosis and leads instead to an

apoptotic death (Fig. 2) [12]. In contrast, virulent *Mtb* strains, such as H37Rv or Erdman, only minimally induce the production of PGE_2 by macrophages [12]. This raises the possibility that virulent *Mtb* actively inhibits PGE_2 production. Thus, an important strategy that *Mtb* exploits to avoid death by apoptosis is the subversion of host eicosanoid biosynthetic pathways [11, 12].

Lipoxins are also generated from arachidonic acid but require the action of different enzymes including 5- and 15-lipoxygenases [51]. Lipoxins are anti-inflammatory and modulate chemokine and cytokine expression, monocyte trafficking and efferocytosis (phagocytosis of apoptotic cells) [52]. In contrast to attenuated strains, virulent *Mtb* induces LXA_4 production, which inhibits cyclo-oxygenase-2 production effectively shutting down PGE_2 biosynthesis, and provides an explanation for how *Mtb* inhibits PGE_2 production [11, 12]. In a PGE_2-poor microenvironment, the macrophage cannot prevent mitochondrial damage nor enable repair of plasma membrane disruptions effectively [5, 11, 12]. Both processes are required to prevent macrophage necrosis and induce apoptotic cell death [11, 12]. Virulent *Mtb* in pre-necrotic macrophages continues to replicate and once the cells are lysed, propagate the infection by spreading to uninfected macrophages. Thus, the balance of PGE_2 and LXA_4 production by the infected macrophage regulates the relative amount of apoptosis and necrosis following *Mtb* infection and has important functional consequences for innate control of intracellular *Mtb* infection.

Induction of LXA_4 by virulent *Mtb* inhibits PGE_2 production and triggers mitochondrial permeability transition (MPT) leading to irreversible mitochondrial damage [12]. By triggering LXA_4 production in the host macrophage virulent *Mtb* inhibits prostanoid production by blocking COX2 mRNA accumulation. By contrast, attenuated *Mtb* induce only minimal amounts of LXA_4 and cause instead production of substantial amounts of PGE_2. We found that when macrophage are infected with attenuated *Mtb*, PGE_2 actively suppresses mitochondrial inner membrane perturbation, which is the outcome in an infection with virulent *Mtb* [12]. Therefore, infection with virulent H37Rv, a PGE_2 non-inducer, causes MPT, which is suppressed by reconstitution with PGE_2.

Our model that lipoxin production by *Mtb*-infected macrophages is associated with increased bacterial replication and greater virulence is strengthened by the recent genetic analysis of zebrafish susceptibility to *M. marinum* [53]. Multiple mutant classes with different innate susceptibilities to *M. marinum* were identified by Tobin et al. [53]. A hypersusceptible zebrafish mutant was found to map to the LTA4H locus, which encodes leukotriene A_4 hydrolase (LTA4H), an enzyme that is required for the final step of leukotriene B_4 (LTB_4) synthesis. While LTA4H deficiency results in the loss of LTB_4 production, addition of LTB_4 did not complement the genetic defect nor increase host resistance. In the absence of LTA4H, its substrate, LTA_4, accumulates and can lead to redirected eicosanoid synthesis and increase lipoxin synthesis. Therefore, Tobin et al. hypothesize that the increased susceptibility of the zebrafish LTA4H mutant is due to an increase in lipoxin production. The same study presents human genetic data that polymorphisms in the

LTA4H gene are associated with susceptibility to pulmonary and meningeal tuberculosis [39, 53]. Thus, from fish to man, eicosanoids appear to play an unexpected role in susceptibility to tuberculosis.

5 Blocking Plasma Membrane Repair

The ESAT-6 secretion system 1 (ESX-1), a specialized Type VII secretion system, is required for the secretion of certain virulence factors including the immunodominant antigens early secreted antigen 6 kilodaltons (ESAT6) and culture filtrate protein 10 (CFP10). Although known to contribute to bacterial virulence, why ESX-1 is required for bacterial survival in the host is unknown. Some data indicate that ESAT6 damages host cell membranes [54, 55]. We hypothesized that disruption of the plasma membrane by *Mtb* is one mechanism that induces necrosis of the macrophage. Interaction of mycobacteria with the host macrophage results in plasma membrane microdisruptions. Microdisruptions induced by attenuated *Mtb* are rapidly resealed by plasma membrane repair mechanisms that include recruitment of lysosomal and Golgi apparatus-derived vesicles to the macrophage surface lesions [11, 56, 57]. Lysosomal or Golgi membrane recruitment to the plasma membrane can be assessed by measuring LAMP1 or mannosidase II translocation to the macrophage surface [58, 59]. Active membrane repair prevents necrosis and is required for induction of apoptosis. By contrast, if resealing of the plasma membrane microdisruptions inflicted by the bacteria is inhibited, as is the case with virulent *Mtb* infection and necrosis ensues.

Ca^{++} sensors are of crucial importance for the recruitment of both lysosomal and Golgi vesicles to the membrane lesions. Gene silencing of the lysosomal Ca^{++} sensor synaptotagmin 7 (SYT7) impairs the recruitment of lysosomal, but not Golgi membranes to the cell surface [11, 60]. The recruitment of Golgi-derived vesicles to the cell surface, which occurs independently of lysosomal vesicle recruitment, requires the expression of neuronal calcium sensor 1 (NCS-1), a Ca^{++} sensor that is particularly abundant in the Golgi [11, 61]. Silencing NCS-1 gene expression, or the use of brefeldin A, a Golgi-specific transport inhibitor, both inhibit translocation of Golgi membranes. These data show that both lysosomal and Golgi membranes are involved in plasma membrane repair and are recruited independently to plasma membrane lesions of infected macrophage.

Plasma membrane resealing is cAMP dependent [62], and addition of forskolin, an activator of adenylate cyclase, results in greater translocation of lysosomal membranes to the cell surface [11]. The protective effect of PGE_2 on mitochondrial stability is mediated through the PGE_2 receptor EP2 [12] and binding of PGE_2 to either EP2 or EP4 causes increased cAMP accumulation [63]. Consistent with this, PGE_2 treatment of human macrophages infected with virulent H37Rv reconstitutes repair mediated by lysosomal membranes. By contrast, PGE_2 does not affect Golgi mediated repair [11]. Although the protective effects of PGE_2 on mitochondria require the EP2 receptor, PGE_2-dependent lysosomal membrane

translocation requires PI3 K activation, which indicates that signaling through EP4 is involved [11]. These findings have important functional consequences for control of intracellular mycobacterial replication. First, *Alox-5*$^{-/-}$ mice (unable to produce LXA_4 and other *Alox-5*-dependent products) survive longer than wild-type (WT) control mice after low dose aerosol infection with virulent *Mtb* [64]. Conversely, *Ptges*$^{-/-}$ (unable to produce PGE_2) mice succumb earlier than WT mice (unpublished observation: Divangahi, Behar, and Remold). However, as many cell types produce eicosanoids, these results do not provide information about the role of eicosanoids during innate immunity. In experiments using macrophages from *Ptges*$^{-/-}$ and *Alox-5*$^{-/-}$ mice, we found that *Ptges*$^{-/-}$ macrophages were unable to control intracellular *Mtb* infection, while *Alox5*$^{-/-}$ macrophages limited *Mtb* replication better than WT macrophages [11]. This phenotype is replicated in vivo when *Mtb* infected *Ptges*$^{-/-}$, *Alox5*$^{-/-}$ and WT macrophages were adoptively transferred into the lungs of V(D)J recombination-activating protein 1-deficient (*Rag*$^{-/-}$) recipient mice. Recipient mice that received infected *Alox5*$^{-/-}$ macrophages had a substantially lower mycobacterial lung burden than recipients that received infected *Ptges*$^{-/-}$ or WT macrophages. Since *Rag1*$^{-/-}$ mice lack B and T cells, the greater capacity of *Rag1*$^{-/-}$ mice to control pulmonary infection following transfer of *Mtb* infected *Alox5*$^{-/-}$ macrophages must be attributed to an intrinsic property of *Alox5*$^{-/-}$ macrophages or a unique interaction between *Alox5*$^{-/-}$ macrophages and the innate immune system [11].

One conceivable explanation for the role of PGE_2 in fostering membrane repair is that PGE_2 is required for the generation of SYT7, the lysosomal Ca^{++} sensor essential for plasma membrane repair. Virulent *Mtb* stimulate LXA_4 production in macrophages, which inhibits PGE_2 production by down regulation of COX2 mRNA accumulation [12]. Indeed we find that in contrast to LAMP1 expression, SYT7 transcription is specifically induced by PGE_2. Likewise, *Alox5*$^{-/-}$ macrophages infected with virulent *Mtb* express more SYT7 than WT or *Ptges*$^{-/-}$ macrophages [11]. Although it is not known how PGE_2 modulates SYT7 expression, collectively these data indicate that PGE_2 is an essential mediator of SYT7 expression and is therefore of critical importance for the prevention of necrosis and induction of apoptosis. Cumulatively, these studies show that the balance of PGE_2 and LXA_4 production by infected macrophages affects the outcome of infection in the microenvironment of the lung (Fig. 2).

6 The Fate of *Mtb*-Infected Macrophages Determines Cross-Presentation of Mycobacterial Antigens

As elegantly discussed in Chap. 8 by Dr. Behar, an alternate possibility is that phagocytosis of dying infected macrophages leads to acquisition of bacterial antigens by DC, as has been shown for influenza and Listeria [65, 66]. The relevance of these processes to mycobacterial antigen presentation was first

investigated by Schaible et al. [67]. Extracellular vesicles derived from infected DC and macrophages were identified that were free of viable bacteria but contained mycobacterial lipids and proteins. The origin of these vesicles was not entirely clear, but they appear to be apoptotic blebs or possibly exosomes. While infected macrophages were not efficient to directly stimulate CD8+ T cells, their co-culture with uninfected DC led to the transfer of mycobacterial antigens to DC, which became competent to cross-present the antigens to CD8+ T cells. Presentation was TAP-1-dependent and required an intact class I MHC pathway. Thus, the antigenic cargo contained in these vesicles could be cross-presented by DC to CD8+ T cells. As these studies were done with previously activated T cells, the observed T cell activation was not true cross-priming but would be more accurately categorized as cross-presentation. Nevertheless, the uptake of antigen-containing vesicles by DC provides a mechanism by which uninfected DC can acquire *Mtb* antigens and prime naïve T cells.

Winau et al. used similar vesicles purified from BCG-infected murine macrophages to immunize mice [68]. Again, the purified apoptotic bodies contained bacterial antigens but no bacteria. CD8+ T cell priming was observed and required an intact class I MHC pathway. Successful T cell priming was associated with DC homing to the tissue sites where the purified vesicles were injected. Interestingly, initiation of the endosomal processing pathway abrogated CD8+ T cell priming—a feature that may be unique to cross-presentation of class I MHC-restricted peptides. The generation of CD8+ T cell responses in naïve mice indicates that true cross-priming occurred. Remarkably, not only did a CD8+ T cell response develop, but also vaccination with the vesicles generated immunity that protected mice against challenge with virulent *Mtb*.

The studies by Schaible and Winau provide the foundation for the "Detour Model" as proposed by Kaufmann [69]. They convincingly show that the mycobacterial antigens contained in purified vesicles are taken up by both human and murine DC and enter the class I MHC pathway. However, these studies fall short of demonstrating whether apoptosis of infected macrophage is required for the transfer of antigens to DC and whether this process occurs in vivo indicating physiological significance. Additionally, the apoptotic vesicles used in the studies by Winau et al. and Schaible et al. were derived from BCG-infected macrophages [68, 69] and it is not clear whether infection of macrophage with wild-type virulent *Mtb* would lead to apoptosis and enhanced T cell immunity. Finally, while immunization with purified vesicles cross-primes antigen-specific T cells, it is uncertain whether the generation of vesicles from infected macrophages is required for CD8+ T cell priming in vivo. The finding that *Mtb* infected DC traffic from the lung to the regional LN with kinetics mirroring T cell priming could be consistent with *Mtb*-infected DC directly priming *Mtb*-specific T cells and could indicate the existence of a priming pathway independent of the "Detour Pathway" [69]. Thus, the role of apoptosis and cross-priming in the generation of adaptive immunity during virulent *Mtb* infection remained an important unanswered

question. To confirm the existence of these pathways and to begin to elucidate their relevance, a better understanding of the host factors regulating cell death during *Mtb* infection was required.

7 The Role of Eicosanoids in Apoptosis-Mediated Cross-Presentation

Eicosanoids have been identified as important host lipid mediators that regulate inflammation and susceptibility following mycobacterial infection. One effect of eicosanoids is the regulation of cell death in both human and murine macrophages infected with *Mtb* [11, 12]. As discussed above, prostanoids such as the host lipid mediator PGE_2 induce plasma membrane repair and prevent mitochondrial damage; together these events protect infected macrophages against necrosis and instead promote apoptosis. Importantly, products of 5-lipoxygenase including LXA_4 are produced by macrophages after infection with virulent *Mtb*. LXA_4 inhibits COX-2 activity, which shuts down prostaglandin synthesis. As predicted, macrophages from mice that lack 5-lipoxygenase, produce prostaglandins even after infection with virulent *Mtb* and undergo more apoptosis than necrosis. Interestingly, *Alox5*$^{-/-}$ mice are more resistant to *Mtb*. Studies from Bafica et al. found that a more pronounced Th1 cytokine response is detected in the lungs of infected *Alox5*$^{-/-}$ mice compared to WT controls mice [64].

In order to determine whether apoptotic macrophages contribute to adaptive immunity, we established a novel adoptive transfer model in which macrophages from wild-type or knockout mice were infected in vitro with *Mtb* and then transferred by intra-tracheal instillation into normal recipient mice. This strategy was used to determine whether the macrophage genotype influences the T cell response and control of infection [40]. By using knockout macrophages that are prone to undergo either apoptosis (e.g., *Alox5*$^{-/-}$) or necrosis (e.g., *Ptges*$^{-/-}$) following infection, we determined how these two different cellular fates alter the course of infection in vivo. One advantage of this adoptive transfer infection model is that the development of tuberculosis occurs in a developmentally normal host with an intact immune system, which avoids the pitfalls of studying *Mtb* infection in knockout mice in which the genetic lesion affects multiple cell types and physiological processes.

We used the CD8+ T cell response to TB10.4, a mycobacterial antigen that elicits an immunodominant response following low dose aerosol *Mtb* infection, to track the CD8+ T cell response following intra-tracheal transfer of *Mtb*-infected macrophages [40]. An earlier TB10.4-specific CD8+ T cell response was detected both in the draining pulmonary LN and in the lung following transfer of pro-apoptotic macrophages compared to wild-type macrophages. Importantly, the cellular fate of the infected macrophages was crucial; pre-treatment of the pro-apoptotic macrophages with inhibitors of caspase 8 and caspase 9, which

prevented apoptosis of the infected macrophages, abrogated the enhancement of the CD8+ T cell response [40].

To determine how the *Mtb*-infected macrophages enhanced the CD8+ T cell response, the infected macrophage adoptive transfer model was adapted for use with OT-I TCR-transgenic mice (carry a transgenic CD8 T cell receptor (TCR) for the MHC class I-restricted $OVA_{257–264}$ peptide), so early events in T cell priming could be easily assessed. Similar to the intra-tracheal adoptive transfer of *Mtb*-infected macrophages, OT-I CD8+ T cell priming was detected earlier after the transfer of *Mtb*-infected OVA-pulsed *Alox5*$^{-/-}$ macrophages compared to wild-type macrophages. Importantly, the infected macrophages did not directly activate CD8+ T cells; instead, T cell priming required endogenous DC, since DC depletion abrogated OT-I CD8+ T cell expansion. Similar to the results of Winau et al. [68], CD8+ T cell priming required TAP-1 and an intact class I MHC pathway. These experiments show that CD8+ T cell priming requires cross-presentation of antigen acquired by DC from apoptotic *Mtb*-infected macrophage via the detour pathway. In addition, after the transfer of *Mtb*-infected pro-apoptotic *Alox5*$^{-/-}$ macrophages, not only was there an earlier and more robust *Mtb*-specific CD8+ T cell response, but the CD4+ T cell response to ESAT6 and Ag85B was also enhanced [40]. This may not be too surprising if DC phagocytosis of apoptotic vesicles transfers their cargo of *Mtb* antigens to the endocytic system, which intersects with the MHC II processing pathway. However, the mechanisms that govern this potential transfer have yet to be elucidated. Thus, while apoptosis has been directly linked to increased CD8+ T cell responses via cross-presentation, it also enhances class II MHC-restricted antigen presentation. This has important implications for the finding that vaccination with apoptosis-inducing bacterial vaccines or apoptotic vesicles induces protection against virulent *Mtb*: namely, the protective immunity elicited may be due to a combination of *Mtb*-specific CD4+ and CD8+ T cells.

Moreover, the pro-apoptotic mutants of *Mtb* prime a greater T cell response and enhance host control of infection [7]. This has generated considerable interest in whether pro-apoptotic mutants of *Mtb* could be used as a vaccine strategy. For example, vaccination with attenuated BCG or *Mtb* that induce greater macrophage apoptosis or with purified apoptotic bodies [68] may stimulate an enhanced T cell response. Collectively, these studies have provided important evidence that during pulmonary *Mtb* infection apoptosis of infected macrophages: (1) leads to innate control of early bacterial growth; and (2) acts as a reservoir of antigen that facilitates initiation of acquired T cell immunity via cross-priming by DC.

8 Conclusions

The finding that macrophages infected with virulent *Mtb* undergo necrosis while macrophages infected with attenuated mutant strains of *Mtb* undergo apoptosis, suggests that wild-type *Mtb* actively inhibits apoptosis. This forms the foundation

for the concept that apoptosis is an innate macrophage defense mechanism. Apoptosis is associated with a reduction in the viability of intracellular *Mtb* and provides an important link to the establishment of T cell immunity. Investigation of the interaction between *Mtb* and macrophages finds that three distinct mechanisms contribute to macrophages necrosis. First, *Mtb* inhibits plasma membrane repair. Second, virulent *Mtb* causes inner mitochondrial membrane damage. Third, *Mtb* inhibits generation of the apoptotic cellular envelope. These three effects predispose the infected macrophages to necrosis. In part, these events occur because virulent *Mtb* inhibits the production of PGE_2, a prostaglandin that is important for stimulation of membrane repair and protection of the mitochondrion. However, it is also important to note that some investigators have found that PGE_2 can impair immunity to other bacterial infections [70, 71] or Influenza viral infection (Divangahi, unpublished observation). Thus, how virulent *Mtb* subvert eicosanoid biosynthesis to alter the death modality of macrophages to foil both innate and adaptive immunity is an important area for future investigation. Given our capacity to manipulate eicosanoid-pathways, a better understanding of how their regulation is altered by mycobacteria may lead to novel approach to intervene therapeutically as well as to develop immunomodulatory strategies that can enhance vaccine efficacy.

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