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Cell Death and Autophagy in TB

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Abstract

Mycobacterium tuberculosis has succeeded in infecting one third of the human race though inhibition or evasion of innate and adaptive immunity. The pathogen is a facultative intracellular parasite that uses the niche provided by mononuclear phagocytes for its advantage. Complex interactions determine whether the bacillus will or will not be delivered to acidified lysosomes, whether the host phagocyte will survive infection or die, and whether the timing and mode of cell death works to the advantage of the host or the pathogen. Here we discuss cell death and autophagy in TB. These fundamental processes of cell biology feature in all aspects of TB pathogenesis and may be exploited to the treatment or prevention of TB disease.

Keywords

Tuberculosis; Phagocyte; Apoptosis; Necrosis; Autophagy

1. Introduction

Programmed cell death and autophagy are fundamental processes of cell biology intimately involved in the interaction between *Mycobacterium tuberculosis* (*Mtb*) and the phagocytes it infects, including macrophages, dendritic cells (DC) and neutrophils. The remarkable success of *Mtb* as a human pathogen results from its capacity to evade the innate antimicrobial effector mechanisms of mononuclear phagocytes (MPs) and leverage the intracellular environment as a replication niche. Infected MPs are faced with a pathogen surviving in phagosomes that fail to incorporate the molecular machinery needed to reduce vacuolar pH and generate free radicals of oxygen or nitrogen, and that fail to fuse with lysosomes to expose bacilli to damaging hydrolases [1]. Plan B for the infected MP is to undergo programmed cell death, which eliminates the intracellular sanctuary and exerts other potentially host-protective effects described in section 3.1.2. Alternatively, a variety of extracellular signals may activate the autophagic machinery of infected MP to drive *Mtb* into lethal autolysosomes as described in section 5. These responses set the stage for what are now recognized as a very complex series of measures and countermeasures culminating in

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the survival or death of the infecting pathogen or its host cell, the progression or resolution of immune pathology, and outcome of tuberculosis (TB) disease.

2. Overview of programmed cell death

A requirement for regulated cell death to support tissue development and homeostasis was conceived by Karl Vogt in 1842 but the term apoptosis to describe a morphologically distinct form of non-traumatic cell death and the understanding of its biochemical mechanisms did not emerge until the late 20th century [2]. Apoptosis is a tightly regulated process of cellular deconstruction. It minimizes inflammation and bystander injury by containing the dismembered nuclear and cytoplasmic contents of dying cells within membrane-bound vesicles called apoptotic bodies that are engulfed by other phagocytes in a process called efferocytosis (section 3.1.2). Binding of apoptotic bodies to specific receptors on MPs responding to “find me” and “eat me” signals induces the expression of anti-inflammatory cytokines including transforming growth factor-β and interleukin (IL)-10 to further insure the silent elimination of cellular corpses [3, 4]. The ultrastructural morphology of apoptosis is characterized by cell shrinkage and chromatin condensation (pyknosis), nuclear fragmentation (karyorrhexis), and blebbing of the outer cell membrane that culminates in apoptotic body formation. Chromosomal DNA is cleaved at inter-nucleosomal boundaries, demonstrated by laddering of DNA bands on gel electrophoresis. Phosphatidylserine (PtdSer), a membrane component that in viable cells is held facing the cytosolic side of the plasma membrane by the enzyme flippase, translocates to the outward-facing surface in apoptotic cells. Exposure of PtdSer on the cell surface plays an important role in membrane stability and clearance of apoptotic bodies (section 3.1.2).

Necrosis is a much different death, defined by the loss of outer cell membrane integrity with release of cytoplasmic and nuclear contents to the extracellular space. Necrosis was originally thought to result only from accidental events (e.g. freezing or crushing) but regulated mechanisms of necrosis were later identified (section 2.2) [5]. The ultrastructural morphology of necrosis is characterized by cytoplasmic swelling (onicosis), cytoplasmic vacuolization and swelling of organelles including mitochondria and cell nuclei [6]. These changes result from ATP depletion and the failure of plasma membrane ion pumps to maintain a stable osmotic gradient. Necrosis can also result from direct plasma membrane damage, which disrupts the cells without onicosis. Rupture of the plasma membrane provokes inflammation by releasing damage-associated molecular patterns (DAMPs) such as heat shock proteins, high-mobility group box 1, S100 proteins, extracellular genomic and mitochondrial DNA, ATP, monosodium urate, and heparin sulfate [7, 8, 8-12]. Binding of DAMPs to their cognate receptors activates an innate inflammatory response and sends “endogenous adjuvant” signals that can stimulate DC to promote T cell activation [9]. The diversity of protein and non-protein DAMPs ensures redundancy in immune stimulation but most converge on common pathways involving inflammasomes, IL-1 and leukotriene (LT)B4 [9, 13].

2.1 Apoptosis signaling and execution

Three major pathways of apoptosis initiation (extrinsic, intrinsic and perforin/granzyme) converge on a common execution mechanism with degradation of chromosomal DNA and

nuclear and cytoskeletal proteins. Both steps in this process involve caspases; a family of cysteine-dependent aspartate-directed proteases constitutively expressed as zymogens. Caspases operate in a cascade for the rapid induction of apoptosis, which is energy-dependent but independent of transcription [14]. Initiator caspases-8, -9, and -10 are activated by dimerization following recruitment to signaling complexes (sections 2.1.1 and 2.1.2) [15]. Activated initiator caspases then cleave and activate the pre-formed dimeric zymogens of the executioner caspases-3 and -7. Activation of executioner caspases is necessarily a tightly regulated event but can in some circumstances be mediated by proteases other than initiator caspases.

2.1.1 Extrinsic apoptosis—The extrinsic pathway begins with ligand binding to tumor necrosis factor receptor (TNFR) family proteins containing a death domain in their cytoplasmic tail which serves as the site for signal complex formation [16]. The receptor/ligand pairs most relevant to TB are TNF-α/TNFR1 and Fas ligand/Fas [17, 18]. TNF-α binding trimerizes TNFR1 allowing recruitment of TNFR1-associated death domain (TRADD), receptor interacting protein kinase (RIPK)1, TNFR-associated factor (TRAF)2, TRAF5, cellular inhibitor of apoptosis protein (cIAP) 1, and cIAP2 to form membrane-associated complex I. Signals from complex I activate NFκB that upregulates pro-survival genes [19]. Apoptosis is initiated from TNFR1 following dissociation of complex I constituents to form cytoplasmic complexes. Complex IIA contains TRADD, Fas-associated death domain (FADD) and caspase-8. Its formation is opposed by cellular FLICE-like inhibitory protein (cFLIP) that is induced by NFκB [20]. The TRADD-independent complex IIB (also called the ripoptosome) forms when TNFR1 is activated but cIAP1 is inhibited by mimetics of second mitochondria-derived activator of caspases (Smac). Formation of complex IIB also requires deubiquitination of RIPK1 by cylindromatosis. FasL binding to Fas recruits FADD which in turn recruits procaspase-8 and/or cFLIP via death-effector domain interactions. This forms a membrane-associated death inducing signal complex but a secondary cytosolic complex of FADD, cFLIP, and caspase-8 can be released to further amplify apoptosis initiation [19].

2.1.2 Intrinsic apoptosis—The intrinsic apoptosis pathway is induced by diverse intracellular stresses such as DNA damage, starvation, and oxidative stress that lead to outer mitochondrial membrane (OMM) permeabilization. Cytochrome *c* released from the mitochondrial inter-membrane space binds the cytosolic protein apoptotic protease activating factor (Apaf-1) to form a multimeric signaling complex called the apoptosome. The apoptosome recruits and activates procaspase-9, which in turn activates executioner caspases [21]. Smac and the serine protease HtrA2/OMI are also released from the inter-membrane space; they amplify intrinsic apoptosis by relieving the constitutive caspase repression mediated by cIAP family members. Mitochondrial permeability is controlled by Bcl-2 family proteins that either promote or inhibit apoptosis [22, 23]. Pro-apoptotic Bax and Bak form pores in the OMM to release cytochrome *c*. This is opposed by anti-apoptotic family members (e.g. Bcl-2, Bclx-L, Mcl-1) but further promoted by other pro-apoptotic Bcl-2 proteins. Cell fate is determined by the integrated activities of pro- and anti-apoptotic Bcl-2 family proteins. Activated caspase-9 cleaves pro-apoptotic Bid into an enzymatically active truncated form (tBid), which orchestrates the activities of Bax to accelerate

cytochrome *c* release. Caspase-8 can also cleave Bid, providing a means for crosstalk between the extrinsic and intrinsic apoptosis pathways [24].

2.1.3 Perforin/granzyme mediated apoptosis—A third apoptosis induction pathway is mediated by serine proteases of the granzyme family contained, along with perforin, in granules of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells [25]. Perforin creates pores in the plasma membrane of target cells through which the granzymes are introduced. Granzyme B cleaves the initiator caspases -8 and -10 and the executioner caspases -3 and -7, and it has other substrates relevant to apoptosis induction including inhibitor of caspase-activated DNase and Bid [26, 27]. The former mediates DNA fragmentation while the latter links perforin/granzyme to the mitochondrial pathway. Granzymes A and C are also implicated in apoptosis although their roles are less well characterized [28]. While perforin/granzymes is the primary mechanism for killing, CTL can also cause apoptosis by engaging Fas on target cells with FasL to trigger the extrinsic pathway [29].

2.2 Regulated necrosis

The field of programmed cell death has become increasingly complex since the discovery of apoptosis. Much recent interest has focused on pathways of regulated necrosis, which currently comprise necroptosis, pyroptosis, pyronecrosis, ETosis, cyclophilin D (CYPD)-dependent necrosis, parthanatos, and autophagic cell death [5]. Necroptosis is the best characterized pathway while pyroptosis, pyronecrosis and ETosis have been most closely identified in the context of infection. Necroptosis occurs when TNFR1 signaling is activated but caspase-8 is inhibited by drugs or virus-encoded anti-apoptotic proteins. This results in formation of a complex called the necrosome, comprising RIPK1, RIPK3, FADD, and caspase-8 [30, 31]. The necrosome recruits and activates mixed lineage kinase domain like which then translocates to the plasma membrane where it mediates TNF- and Ca^{2+}-dependent necrotic cell death (necroptosis) through several terminal mechanisms including energy depletion, reactive oxygen species (ROS) production, lysosomal membrane permeabilization (LMP), and lipid peroxidation [5].

2.2.1 Pyroptosis and pyronecrosis—Pyroptosis and pyronecrosis were discovered in the context of intracellular bacterial infection (e.g. *Francisella tularensis*, *Listeria monocytogenes*, and *Shigella flexneri* [32-34]) although non-infectious triggers have also been identified [35]. These death subroutines share dependence on inflammasome constituents and terminal plasma membrane pore formation resulting onicosis. A common trigger for pyroptosis and pyronecrosis is NOD-like receptor (NLR) activation by pathogen-associated molecular patterns (PAMPs) from intracellular microbes. Distinguishing characteristics are the dependence of pyroptosis on caspase-1 or caspase-11 (human caspase-4) while pyronecrosis is caspase-independent and requires the inflammasome component apoptosis-associated speck-like protein containing a CARD (ASC) and the lysosomal protease cathepsin B. Pyroptosis and pyronecrosis share rapid kinetics of lethality and considerable overlap in triggers. As an example, *S. flexneri* at low multiplicity of infection (MOI) induces macrophage pyroptosis dependent on the inflammasome constituent NLRC4 but higher MOI triggers pyronecrosis via NLRP3 [36]. These death modes are

highly inflammatory, with abundant production of IL-1β and IL-18 that is perpetuated by the release of aggregated ASC multimers (ASC specks) which continue processing pro-IL-1β in the extracellular space and even following phagocytosis by naïve macrophages [37, 38].

2.2.2 CYPD-dependent necrosis—Mitochondrial permeability transition (MPT) occurs when the inner membrane (IMM) becomes permeable to solutes <1500 Da [39]. This is a sudden event caused by opening of a channel in IMM called the permeability transition pore (PTP) [40]. The mitochondrial matrix protein CYPD is a key regulator of PTP formation although not an essential component of the pore. Binding of cyclosporin A to CYPD inhibits MPT [23]. Transient MPT is survivable but sustained PTP opening results in Ca^{2+} efflux, depletion of pyridine nucleotides from the mitochondrial matrix, and matrix swelling that culminates in OMM rupture with release of inter-membrane space components including cytochrome *c*, Smac, and apoptosis-inducing factor (AIF). The intrinsic apoptosis pathway is activated by cytochrome *c* and Smac, (another example of crosstalk between death pathways), while AIF mediates caspase-independent DNA fragmentation and chromatin condensation [41]. AIF also plays a role in parthanatos, a regulated necrosis pathway mediated by poly ADP ribose polymerase hyper activation that is reviewed elsewhere [42]. The CYPD pathway is tied to necrosis as result of catastrophic mitochondrial dysfunction. The inducers of CYPD-dependent necrosis are not fully understood but Ca^{2+}, ROS, adenine nucleotides, and ubiquinones have been implicated [5].

2.2.3 ETosis—This mode of programmed necrosis was first identified in neutrophils and features chromatin disgorgement to the extracellular space, forming extracellular traps (NETs) [43]. In contrast to apoptosis, chromatin decondensation is an early step in ETosis and PtdSer does not flip to the outer cell surface. Unfolded genomic DNA presents a web-like anionic polymer that binds bacteria and fungi by electrostatic interactions. NETs also bind proteins including neutrophil elastase, histones, and antimicrobial peptides that kill bound pathogens [44]. Release of extracellular traps has been identified in eosinophils, mast cells, and macrophages, hence the term ETosis [45, 46].

Triggers for ETosis include lipopolysaccharide, interferon (IFN)-γ, bacteria, fungi, and phorbol myristate acetate [47]. NET release in most instances requires NADPH oxidase (NOX) although a NOX-independent pathway stimulated by CXCL2 was reported [48]. The ETosis pathway, including the requirement NOX, is incompletely understood. Upstream signaling via Raf-MEK-ERK activates NOX and ETosis, as does activation of the mammalian target of rapamycin (mTOR) pathway. Inhibitors of ETosis include serine protease inhibitor B1 and the eicosanoid 5-hydroxyeicosatetraenoic acid, a product of 5-lipoxygenase [48, 49]. ETosis is fundamentally a mode of necrotic cell death but there is remarkable evidence for the persistence of post-ETotic, anuclear neutrophil zombies capable of directed migration and phagocytosis [50].

2.3 Detection of apoptosis and necrosis

Many assays are available to discern whether dead and dying cells are undergoing apoptosis or necrosis. The most common of these test the integrity of the outer cell membrane (e.g. propidium iodide [PI] or 7-aminoactinomycin D), presentation of PtdSer on the outer surface

of the plasma membrane (e.g annexin V binding), nuclear pyknosis and fragmentation (e.g. 4',6-diamidino-2-phenylindole staining), DNA fragmentation (e.g. terminal deoxynucleotidyl transferase dUTP nick end labeling [TUNEL] assay), caspase activation, and the state of mitochondrial transmembrane potential ($\Delta\Psi_m$). Transmission electron microscopy (TEM) is perhaps the most accurate method to distinguish between apoptotic and necrotic cell death based on distinct ultrastructural features. Each method has strengths and weaknesses depending on cells and experimental conditions being investigated. TUNEL is highly sensitive but has poor specificity while TEM is highly specific but lacks sensitivity since it is difficult to interrogate large numbers of cells, particularly in vivo.

To critically evaluate the literature on cell death in TB it is important to understand the limitations of these assays and the fundamental problem that none can unambiguously define cell fate. The Cell Death Nomenclature Committee has published guidelines for the use and interpretation of cell death assays [51]. The heterogeneity of cell death and unique characteristics of linked to specific cell types and conditions means that only general guidelines can be suggested. These include determining whether the cells in question are truly dead and then interrogating the responsible cell death subroutine using at least two methodologically unrelated assays. The challenge of defining the specific nature and subroutine of cell death is made greater by the extensive crosstalk between pathways so that different death subroutines may be simultaneously activated in a single cell with one or another appearing predominant based on experimental conditions [5, 51, 52]. Common examples of such ambiguities include: caspase activation in viable cells; caspase activation as a secondary effect of proteolysis in necrotic cells; crosstalk between extrinsic and intrinsic apoptosis pathways; secondary necrosis of apoptotic bodies; and the convergent triggers and execution mechanisms of the different programmed necrosis subroutines.

3. Cell death in TB

Apoptosis can serve as a defense mechanism for cells confronted with intracellular pathogens that consume host resources for survival and replication. This paradigm was discovered the context of virus infection (reviewed in [53]) but has been extended to a wide spectrum of pathogenic microbes. Apoptosis offers several potential benefits for the host including elimination of a replication niche, exposure of pathogens to humoral immunity, and forcing intracellular pathogens to reestablish residence in naïve host cells. Efferocytosis provides a means to defeat the virulence mechanisms of intracellular pathogens that inhibit vesicular trafficking and maturation. It also delivers pathogens or their antigenic components to DC for efficient priming and cross presentation (section 3.1.2) [54]. The rapid, immunostimulatory demise of cells by programmed necrosis might benefit the host by accelerating neutrophil recruitment to control fast-replicating bacteria [55]. Conversely, apoptosis could be disease-promoting if it eliminates key host defense cells, facilitates penetration of epithelial barriers, or spreads infection through efferocytosis [56, 57].

Macrophage-pathogen interactions play a central role in TB pathogenesis. The bacillus is a facultative intracellular parasite that cannot gain a foothold in new hosts without this replication sanctuary. Once *Mtb* has established infection in the lung, transmission to new hosts by infectious aerosols requires its transition to the extracellular space. It is therefore

unsurprising that cell death in its many forms has been identified in the context of *Mtb* infection in vitro and in vivo. A recent review on this topic cited 48 manuscripts reporting a spectrum of death modes and subroutines linked to TB including extrinsic, intrinsic and perforin/granzyme-mediated apoptosis, and several type of necrosis [58]. In this review we focus primarily on macrophage cell death resulting directly from *Mtb* infection but the fates of infected DC and neutrophils will also be discussed (section 3.3). While beyond the scope of this review, CTL-mediated death, activation-induced cell death, bystander death of T cells, and *Mtb*-induced death of epithelial cells all participate in TB pathogenesis.

3.1 Apoptosis of Mtb-infected macrophages

Before the discovery of an intrinsic apoptosis response to *Mtb* infection, Molloy et al. [59] reported that treating BCG-infected monocytes with exogenous ATP induced apoptosis and killed bacilli while BCG viability was preserved after H_2O_2-induced monocyte necrosis. In retrospect, the antimicrobial activity of ATP might have been mediated by autophagy (section 5.2) [60] but this publication introduced the concept of apoptosis has host-protective in TB. Keane et al. [18] were the first to describe apoptosis as an autonomous response of primary human alveolar macrophages infected with live but not heat-killed *Mtb*. Apoptosis was verified by TEM, internucleosomal laddering of genomic DNA, and TUNEL assay (the latter including human TB lung sections). The extrinsic pathway was triggered TNF-α in an autocrine/paracrine manner and required infection-induced priming for TNFR death signals since uninfected cells were resistant to exogenous TNF-α. The attenuated *Mtb* strain H37Ra was a much stronger apoptosis inducer than virulent H37Rv. Oddo et al. [17] subsequently reported that exogenous FasL or TNF-α induced apoptosis of *Mtb*-infected macrophages (defined by annexin V/PI staining and by TUNEL) was accompanied by reduced bacillary viability. No antimicrobial effect occurred with complement-induced necrosis of infected macrophages.

A simple model of apoptosis as a host-protective response in TB emerged from those early reports. Subsequent in vitro studies confirmed the association of apoptotic cell death with reduced *Mtb* viability [61-65]. The concept of apoptosis evasion as a virulence-associated trait of *Mtb* was substantiated and several mechanisms of apoptosis evasion were described including downregulation of Fas on infected macrophages, interference with death signals downstream of TNFR1, and shedding of soluble TNFR2 that neutralized TNF-α [17, 66, 67].

Recent findings have underscored the complexity of macrophage apoptosis in TB and its role in host defense. Data from the zebrafish/*M. marinum* model show that apoptosis can facilitate spread of infection to naïve macrophages in vivo [57]. Accelerated dissemination was also demonstrated in mice infected with a pro-apoptotic mutant of virulent *Mtb* (section 3.1.1) in experiments that also failed to support a direct antimicrobial effect of macrophage apoptosis in vivo [68].

3.1.1 Mtb genes linked to apoptosis evasion and induction—A major advance in this field was provided by the discovery in 2007 of two *Mtb* genes (*secA2* and *nuoG*) linked to the suppression macrophage apoptosis, both acting reduce levels of ROS in the

mycobacterial vacuole [69, 70]. This jibes with the models of extrinsic apoptosis in *Mtb*-infected macrophages where ROS play an intermediary role in TNF-α signaling for apoptosis (and necrosis), and intrinsic apoptosis that can also be triggered by ROS [71, 72]. Mutation of *secA2* in impairs secretion of bacterial superoxide dismutase (SodA) and confers an apoptosis-inducing phenotype on H37Rv demonstrated by TUNEL and caspase activation in THP-1 cells and mouse bone marrow-derived macrophages (BMM] [70]. This result was anticipated by an earlier study of H37Rv mutants where SodA expression was knocked down with anti-sense RNA, resulting in vivo attenuation with less inflammation and more MP apoptosis after high dose intravenous infection in C57BL/6 mice [73]. The Δ*secA2* mutant strongly induced antigen-specific $CD8^+$ T cell priming in vivo, which was attributed to its pro-apoptotic phenotype (section 3.1.2).

The anti-apoptotic activity of *nuoG* was revealed in a gain-of-function screen in *M. smegmatis*. Deletion of *nuoG* in *Mtb* H37Rv conferred a pro-apoptotic phenotype on infection of THP-1 cells and BMM in vitro [69]. The *nuoG* gene encodes a subunit of a type I NADH dehydrogenase that neutralizes ROS generated by host NOX2, thereby inhibiting TNF-α-stimulated apoptosis as well as TNF-α secretion [74]. The mutant RvΔ*nuoG* strain grows normally in broth but it is attenuated in SCID and wild-type BALB/c mice after high dose intravenous challenge [69]. Growth of RvΔ*nuoG* in the lung during the first 3 weeks post-infection matched that of wild-type H37Rv and a complemented mutant strain; its attenuation in terms of bacterial burden was only evident at later times points with a 0.8 log reduction compared to wild-type H37Rv at 20 weeks. Bacterial loads in liver and spleen did not differ at any time point. These data suggest that the host-protective role of macrophage apoptosis in TB may be restricted in time and tissue compartment, following the induction of adaptive immunity and in the period when bacterial burden in the lung is normally held at a plateau level in wild-type mice infected with wild-type *Mtb*. That notion is supported by data from aerosol infection of protein kinase R $(PKR)^{-/-}$ mice [75]. These mice exhibit increased macrophage apoptosis during TB and have lower lung CFU than wild-type mice at 70 days post-infection but not at 21 days.

The enhanced intracellular survival (*eis*) gene of *Mtb* is a more recently described pro-survival factor that acts through an ROS-dependent pathway reminiscent of *secA2* and *nuoG* [76]. The phenotype of RvΔ*eis* differs, however, by increasing a caspase-independent cell death that is not clearly apoptotic in nature. This death is not accompanied by a strong TUNEL signature or caspase activation and it is only partially blocked by the pan-caspase inhibitor z-VAD-fmk. The Eis protein also modulates autophagy, and the autophagy inhibitor 3-methyladeneine enhances the survival of macrophages infected with RvΔ*eis*. Following low dose aerosol challenge of wild-type C57BL/6 mice, lung and spleen bacterial burden was no different between RvΔ*eis*, wild-type H37Rv or a complemented mutant strain but PI-positive cells and inflammation (IL-6 and TNF-α levels) were higher in mice infected with RvΔ*eis*. Despite the common theme of ROS reduction, the data suggest that the Eis protein inhibits a predominantly necrotic rather than apoptotic macrophage death mode. This may be explained by its mechanism of action, based on acetylation of DUSP/MKP-7 and inhibition of JNK-dependent autophagy and ROS generation (section 5.4)[77].

An anti-apoptotic function was proposed for the *Mtb* protein MPT64 acting in a pathway involving NF-κB, miR21, and Bcl-2 [78]. The *Mtb* Rv3654c and Rv3655c genes were shown to participate in suppressing extrinsic apoptosis by reducing the availability of caspase-8 which has the effect of promoting regulated necrosis (section 2.2) [79]. Finally, protein kinase E of *Mtb* was reported to suppress macrophage apoptosis, specifically in the context of nitric oxide stress [80].

Far less is known about *Mtb* genes required for apoptosis induction. The *Mtb* 6 kDa early secretory antigenic target (ESAT6) is a pore-forming, virulence-associated gene product exported by the Esx-1 secretion system in a complex with 10-kDa culture filtrate protein [81]. The pore-forming function of ESAT6 is a leading candidate for *Mtb*-mediated LMP that precedes several forms of infection-induced cell death. There is considerable data linking ESAT6 and the Esx-1 secretion system to regulated necrosis of *Mtb*-infected macrophages (section 3.2) but ESAT6-mediated caspase upregulation and induction of THP-1 apoptosis (defined by annexin-V binding and sensitivity to pan-caspase inhibition) has been described [82]. It is uncertain whether these data obtained with immortalized monocytic cells reflect death mechanisms in primary macrophages infected *Mtb*. Others found that *M. bovis* BCG, which lacks ESAT6/CFP10 and the Esx-1 secretion system, is a potent inducer of apoptosis [61, 83, 84].

3.1.2 Efferocytosis in TB defense—Cells undergoing apoptosis release “find me” signals (e.g. CXC3CL1, lysophosphatidylcholine, sphingosine-1-phosphate, ATP, and UTP) that attract phagocytic cells for the clearance of apoptotic bodies [85]. Which of these or other possible signals operate in TB is presently conjectural but available data hint at a role for CX3CL1 and its receptor CX3CR1 [86]. While ATP/P2X7R interactions are implicated in TB defense [87], P2Y2 is the sensor for the small amounts of nucleotides released from apoptotic cells and so far this receptor has not been linked to TB. Neutrophils could respond to “find me” signals but the non-inflammatory clearance of apoptotic corpses is maintained by lactoferrin released from apoptotic cells. Lactoferrin serves as a “stay away” signal specifically inhibiting neutrophil but not MP migration to the vicinity of apoptotic cells [88].

Caspase-dependent exposure of PtdSer on the outer leaflet of plasma membranes is the best studied “eat me” signal although multiple signals may be required to promote efferocytosis [85]. Apoptotic corpses are recognized by a variety of tethering, signaling and/or phagocytic receptors on responding MPs (e.g. CD36, integrins, scavenger receptors, TAM receptors, TIM4, lectins, and RAGE), some of which require bridging molecules (e.g MFG-E8 and thrombospondin). These interactions initiate efferocytosis primarily by activating the Rho family GTPase Rac via CrkII-Dock180-ELMO or LPR1/MEGF10-GULP-ABCA1/ABCA7 pathways. The internalization process of efferocytosis resembles macropinocytosis more than receptor-mediated phagocytosis and delivers apoptotic bodies to acidified phagolysosomes [89]. Specificity is further ensured by the expression of “don't eat-me” signals on viable sells (e.g. CD47 which binds to SIRPα to inhibit efferocytosis) [90].

The potential for efferocytosis to enhance the innate antimycobacterial properties of MPs was first proposed by Fratazzi et al. [91] in experiments using an apoptosis-inducing strain of *M. avium*. Adding naïve macrophages to cultures of infected, apoptotic macrophages

reduced mycobacterial viability in a contact-dependent manner that was not seen if the infected macrophages were made necrotic. Definitive evidence for efferocytosis-dependent killing of *Mtb* was provided by Martin et al. [92]. Primary mouse macrophages infected with mCherry-expressing H37Rv in vitro were shown to undergo apoptosis via the intrinsic pathway with subsequent delivery of apoptotic bodies to naïve macrophages. Transfer of *Mtb* from necrotic macrophages was observed in parallel and both phenomena were demonstrated in vivo after transfer of Rv/mCherry-infected DiO-labeled $CD45.2^+$ macrophages into $CD45.1^+$ recipients. Efferocytosis-dependent killing of H37Rv was demonstrated in vitro by blocking this pathway with anti-TIM4 mAb or by pre-treating naïve macrophages with forskolin or prostaglandin (PG)E2 that inhibit efferocytosis through a mechanism involving increased intracellular cAMP. Bacilli ingested by efferocytosis were delivered to capacious, acidified phagolysosomes that co-localized with LAMP1 and vacuolar H^+-ATPase. An antimicrobial effect of efferocytosis in vivo was demonstrated by intraperitoneal transfer of H37Rv-infected arachidonate 5-lipoxyengase (*Alox5*)$^{-/-}$ macrophages into $Rag^{-/-}$ recipients. *Alox5*$^{-/-}$ macrophages are more prone to apoptosis following *Mtb* infection (section 3.2). In this infection/transfer model, recipients treated with anti-TIM4 had roughly 2-fold higher bacterial load in spleen and lung than recipients treated with isotype control mAb. The data clearly demonstrate the potential for efferocytosis-dependent killing of *Mtb* in TB although results from in vivo challenge with RvΔ*nuoG* [68] suggest that this operates mainly at later stages of TB disease (section 3.1.1).

The potential for efferocytosis to promote cross-presentation of mycobacterial antigens for MHC class-I- and CD1-dependent T cell activation was initially reported by Schaible et al. [54]. Restimulation of $CD8^+$ T cells from donors with latent TB infection co-cultured with *Mtb*-infected macrophages required the intermediary function of naïve DCs that acquired antigen through efferocytosis. Similar results were achieved when naïve DCs were pulsed with sterile, purified vesicles derived from *Mtb*-infected MPs. These investigators subsequently demonstrated that immunization with *Mtb* antigen-containing vesicles can cross-prime naïve $CD8^+$ T cells in vivo, which requires DC homing to lymph nodes [93]. They further showed that apoptotic bodies possess adjuvant activity mediated by Toll-like receptor (TLR)-2, and that $CD4^+$ T cells are also stimulated in vivo. Evidence of efferocytotic antigen transfer to DC and cross-priming occur during TB disease was reported by Divangahi et al. [94] in experiments using intratracheal transfer of *Mtb*-infected *Alox5*$^{-/-}$ macrophages. Transfer of these apoptosis-prone macrophages accelerated the activation of TB10.4-specific $CD8^+$ T cells and depended on apoptosis (suppressed by inhibition of caspase-8 and caspase-9) as well as DC. Altogether, the data suggest that the capacity of virulent *Mtb* to suppress the apoptotic death of infected macrophages could contribute to the delayed priming of adaptive immunity that characterizes the early host response in TB.

3.2 Necrosis of Mtb-infected macrophages

The capacity of virulent *Mtb* to suppress apoptosis implies an alternative exit strategy for the bacillus to reach the extracellular environment. The first evidence of programmed necrosis in *Mtb* was described by Duan et al. [95] using in vitro infection of human monocyte-derived macrophages with attenuated H37Ra. They found that some infected macrophages died by apoptosis while others died by necrosis, which correlated with MPT. The induction

of MPT and CYPD-dependent necrosis by *Mtb* was subsequently shown to be inhibited by cyclosporin A [96] and to be more potently induced by H37Rv than H37Ra [97]. It was later shown that PGE2 induced by H37Ra protects against mitochondrial PTP formation and necrosis while lipoxin (LX)A4 induced by H37Rv and Erdman suppresses PGE2 production and promotes necrosis [98]. The direct antimicrobial effect of apoptosis against *Mtb* observed under in vitro culture conditions was not seen when infected cells died by necrosis. These data supported the concept of programmed necrosis as means for replication-competent bacilli to exit host macrophages for spreading infection.

The apoptotic death of infected macrophages confines *Mtb* in membrane-bound vesicles but this potentially host-protective outcome is lost if the apoptotic bodies decompose before they are cleared by efferocytosis. Plasma membrane stability is another determinant of the fate of *Mtb*-infected macrophages. This was first revealed by experiments showing that virulent *Mtb* H37Rv inhibits the cross-linking of annexin-I bound to PtdSer, which serves to stabilize apoptotic membranes [99]. In a series of carefully conducted, mechanistic studies the capacity of *Mtb*-infected macrophages to repair membrane damage was shown to be a major determinant of an apoptotic versus necrotic demise [94, 100]. Cell fate in this model hinges on eicosanoid regulation of the lysosome-mediated membrane repair machinery, which is induced by PGE2 via the E2 receptor. Virulent H37Rv promotes elevated LXA4 levels in macrophages, which in turn downregulates cyclooxygenase (COX)2 mRNA thereby reducing PGE2 and its protective effects. Infection of *Alox5*$^{-/-}$ macrophages that cannot synthesize LXA4 results in increased apoptosis even with H37Rv. Conversely, prostaglandin E synthase (*Pges*)$^{-/-}$ macrophages cannot produce PGE2 and die by necrosis even with H37Ra infection. These data suggest that virulent and attenuated *Mtb* strains trigger a common pathway that can terminate in apoptosis if mitochondrial and plasma membrane integrity is protected by PGE2, or progresses to necrosis if LXA4 predominates. How virulent *Mtb* strains induce LXA4 production and what causes the membrane injury counteracted by repair remains to be determined. The pore forming activity of ESAT6 is one suggested mechanism for LMP as well as plasma membrane microdisruptions [101]. Another candidate is the product of the *Mtb* Rv3903c gene (CpnT) whose C-terminal fragment induces necrotic cell death with plasma membrane disruption that is not blocked by a pan-caspase inhibitor (therefore not caspase-3 mediated apoptosis or caspase-1 dependent pyroptosis) and not blocked by the RIPK1 inhibitor necrostatin-1 (therefore not necroptosis) [5;102].

The in vivo relevance of excess lipoxins as detrimental to TB defense is supported by genetic data from the mouse and zebrafish models, and in human TB patients. *Alox5*$^{-/-}$ mice are more resistant to aerosol TB than wild-type controls and make a more robust Th1 biased adaptive immune response [103]. Reminiscent of kinetics of RvΔ*nuoG* growth in vivo, lung bacterial load in *Alox5*$^{-/-}$ mice is only lower than wild-type ≥21 days post-infection, suggesting that its protective effect is not manifest during the primarily innate phase of TB defense. A zebrafish mutant hypersusceptible to *M. marinum* mapped to the *lta4h* locus encoding leukotriene A4 hydrolase, which catalyzes the last step in LTB4 synthesis [104]. Zebrafish with this mutation are deficient in pro-inflammatory LTB4 and over-produce anti-inflammatory lipoxins. Human resistance to TB and multibacillary leprosy is associated with

heterozygosity of *LTA4H* polymorphisms that correlate with differential production of LTB4 [104, 105]. These data suggest that an optimal balance of pro- and anti-inflammatory eicosanoids protects against the adverse effects of poorly controlled bacillary replication on one hand and against damaging immune pathology on the other. Whether these in vivo phenotypes reflect differential regulation of MP apoptosis and necrosis, or some other effects on immunity of these pleiotropic eicosanoids remains to be determined

Inflammasome activation and the induction of pyroptosis is postulated as a defense mechanism that deprives rapidly growing intracellular bacterial pathogens of a growth niche. Many pathogenic bacteria have mechanisms to subvert this response [55]. Slow growing *Mtb* requires the intracellular niche of MPs, so its replication would be restricted the rapid induction of programmed necrosis of its host cells. In this regard, the *Mtb* Rv3364c gene product was reported to suppress caspase-1 and pyroptosis in macrophages [106].

Lytic viruses induce host cell necrosis after an optimal period of replication to a “burst size” intracellular load. Similar dynamics were suggested for *Mtb*, which triggers an atypical necrosis subroutine in macrophages at intracellular loads in the range of 20-40 bacilli [106,108]. Dying cells exhibit early apoptotic features of PtdSer externalization and nuclear pyknosis, but progress within hours to necrosis without nuclear fragmentation or apoptotic vesicle formation. This death is caspase-independent and does not require pro-apoptotic Bcl2 family proteins. This death differs from the necrosis of bioenergetic collapse by the absence of osmotic swelling, and it differs from pyroptosis and pyronecrosis, being independent of caspase-1 and cathepsin B [108]. It is not inhibited by cyclosporin A and is therefore distinct from CYDP-dependent necrosis [107]. Death is initiated upon LMP, followed by lipolytic attack on mitochondrial, nuclear and outer cell membranes. It could therefore be classified as a form of lysosomal cell death but is atypical in its dependence on lipase more than protease activities. The bacterial determinants of this death mechanism remain undefined, but it requires one or more genes of the PhoPR regulon since a Δ*phoPR* mutant of H37Rv fails to cause LMP or kill macrophages at MOI 25 [108]. In contrast, BCG and a defined RvΔRD1Δ*espA* mutant lacking the ESX-1 secretion system are fully capable of provoking necrosis if introduced at high MOI. Both of those strains are capable of only limited intracellular replication, so this pro-necrotic phenotype is not evident when they are introduced at low MOI. The dynamics of *Mtb* burden per cell in MPs after aerosol challenge of mice fits a burst size model of necrosis [109]. Neutrophils are the predominant phagocytes harboring *Mtb* cell during periods of unrestricted bacillary replication [109, 110]. This is consistent with the inflammatory nature of necrotic cell death which recruits neutrophils and absence of the “stay away” signal lactoferrin that is associated with apoptosis [13, 88].

3.3 Programmed death of other myeloid cells in TB

Macrophages have been the focus of research on cell death in TB but data from the mouse model and human TB indicate that DC and neutrophils are important and in some circumstances the predominant phagocytes harboring *Mtb* in vivo [109-111]. Neutrophils are readily identified by common laboratory methods but distinguishing between tissue-resident macrophages, recruited macrophages, monocytes, and DC is more challenging and some

have questioned whether these cell types represent distinct lineages or simply alternative and plastic phenotypes a common monocytic progenitor [112-114]. Recognizing this controversy, we will refer to DC as defined by the authors of cited publications who used generally accepted methods to isolate and phenotype these cells.

The limited literature on the fate of *Mtb*-infected DC reflects many of the same challenges to interpreting the often contradictory literature on macrophage cell death. An early study compared the activation and survival of C56BL/6 mouse bone marrow derived DC challenged with Erdman at MOI 10 [115]. Immature DC matured in response to infection and survived >48 hours but (unlike macrophages) were unable to kill intracellular bacilli even with IFN-γ activation. The authors noted that high rates of cell death (trypan staining) in DC and macrophages at later time points. More recently, Ryan et al. [116] reported that human peripheral blood monocyte-derived DC undergo necrosis (PI staining) that was caspase-independent (not rescued by Q-VD-Oph) and lacked nuclear fragmentation but did feature DNA cleavage (nucleosomal particle ELISA) despite no activation of the executioner caspases 3 and 7. The induction of DC death required live bacilli and was triggered by H37Rv and H37a. In contrast, a study using bone marrow-derived DC from C57BL/6 mice and H37Rv (MOI 10) described activation of inflammasome and executioner caspases 3/7 with along with apoptosis (defined by TUNEL) but no necrosis (defined by adenylate kinase release) [117]. Based on these and other data the authors concluded that *Mtb* induces DC apoptosis dependent on Esx-1 but does not cause pyroptosis or pyronecrosis. We are left to conclude that *Mtb* can infect and activate DC and that apoptotic or necrotic death might ensue depending on variables likely to include the origin, subtype and activation state of the DCs prior to infection. Necrotic death of DC could plausibly delay immune priming while an apoptotic death might facilitate priming. These are important considerations since there is substantial evidence that DC are the major phagocytes that convey viable bacilli to lung-draining lymph nodes for adaptive immune priming [110, 118]. The kinetics of these events has a major impact on the subsequent outcome of disease at least in mice [119].

3.3.1 Neutrophil cell death in TB—Much recent interest has focused on the roles that neutrophils play in TB [120]. A fascinating dynamic has been proposed for the early events following inhalation of *Mtb*, where neutrophils accelerate immune priming by undergoing infection-induced apoptosis and passing bacilli to migratory DC in a manner that facilitates DC trafficking to the lymph node [68, 121]. Evidence that virulent *Mtb* strains inhibit neutrophil apoptosis and thereby delay immune priming was obtained using the pro-apoptotic Rv/Δ*nuoG* mutant [68]. In contrast, *Mtb* H37Rv was reported to trigger apoptosis of human peripheral blood neutrophils that was dependent on TLR2 and independent of TNF-α [122]. Adding complexity to this interaction, comparison of two different clinical *Mtb* isolates identified one strain that strongly induced human neutrophil apoptosis while the other strain did not induce apoptosis [123].

While neutrophils may exert a host protective function as an early event in TB, they also appear to be important mediators of tissue injury at later stages of disease if they are present in excess as occurs with poorly controlled *Mtb* infection in susceptible hosts [65, 124, 125]. Neutrophil lifespan is prolonged in I/St mice with TB, which may be a factor in the

increased neutrophil accumulation and lung damage seen in that highly susceptible mouse strain [126]. NETs may contribute to lung tissue damage [47] and ETosis is another fate that has been described for human neutrophils and macrophages infected with *Mtb* in vitro [46, 127], in the mice with poorly controlled TB in vivo [109]. While NETs exert antimicrobial activity against a range of bacteria, this activity does not extend to *Mtb* [127]. Release of traps by human macrophages is accelerated by IFN-γ [46] but NETs were identified in lung lavage of IFN-$\gamma^{-/-}$ mice with TB [109]. The diverse fates of *Mtb*-infected neutrophils in TB feature in the host-protective and damage-inducing roles that these cells play in TB pathogenesis.

3.4 Translation opportunities

Manipulation of apoptosis in the context of vaccination holds the most promise compared other therapeutic goals for early translation of knowledge about cell death in TB. The pro-apoptotic recombinant strain BCGΔureC::hly+ expresses the pore-forming listeriolysin of *L. monocytogenes* and lacks urease C to ensure an optimal intraphagosomal pH for listeriolysin activity. This strain is more immunogenic than the parental BCG for type 1 responses and uniquely induces type 17 as well [128, 129]. These results are attributable to exploitation of the the efferocytotic pathway discussed in section 3.1.2. The human vaccine candidate based on this technology, VPM1002, demonstrated safety in a phase I clinical trial [130] and a phase II trial is currently underway.

Opportunities for adjunctive TB therapies that directly enhance apoptosis are less obvious and could risk killing necessary cells (e.g T cells) and/or generating an excess of apoptotic bodies at risk for secondary necrosis. Nonetheless, a more comprehensive understanding of how different fates for MPs and neutrophils influence the effectiveness of host defense and the quantity and quality of immune pathology in TB is vital to understanding TB pathogenesis and new targets for treatment and diagnosis.

3.5 Summary of cell death in TB

The diverse outcomes and conclusions of published studies on cell death in TB (Table 1) reflect differences in host species and strains, mycobacterial species and strains, cell types and their activation state, conditions of infection, and the likelihood that multiple cell death programs can be simultaneously activated in cells stressed by *Mtb* infection. These variables determine which fate predominates in particular experimental systems. Results from many studies support the concept that apoptosis of macrophages and neutrophils contributes mainly host-protective effects, while macrophage necrosis is mainly linked to adverse outcomes. However, these are clearly not absolute paradigms since apoptosis can promote disseminated infection and accelerated necrosis might limit intracellular bacillary replication.

Given the complex interplay of multiple host and bacterial mechanisms to promote or prevent cell death, more research with in vivo approaches is needed to identify what truly matters in TB disease. A comprehensive model must also integrate a multitude of common factors that are often excluded in reductionist experimental systems. Examples include the effects of metabolic disorders like diabetes and hyperlipidemia where methylglyoxal,

oxidized LDL cholesterol, or an excess of intracellular free cholesterol that promote apoptosis or necrosis [131, 132]; cytokines, such IFN-γ that promotes survival of macrophages at low MOI but accelerates necrosis with high bacillary loads [133, 134]; and apoptosis induced by CTL. The best studies will confirm unequivocally that cells thought be to dying are indeed dying and ideally use multiple unrelated assays if making a case for one particular fate.

4. Overview of Autophagy

Autophagy ("self-eating") is an evolutionarily conserved pathway in cell biology that serves to control cytoplasmic content including organelles, to recycle chemical resources in bulk, and to regulate cellular functions under basal conditions and in response to variety of stresses. Macroautophagy refers to the isolation of cytoplasmic content by the formation of lipid bilayer vacuoles called autophagosomes which fuse with lysosomes to degrade their cargoes. Macroautophagy is the autophagic process most relevant to TB and will be simply called "autophagy" in this review. Other autophagic processes such as microautophagy (direct sequestration of cytoplasm within lysosomes) and chaperone-mediated autophagy that targets proteins with a specific signal sequence for degradation are reviewed elsewhere [135].

Autophagy is controlled primarily by Atg proteins, a family with more than 30 members that were revealed in screens for yeast mutants defective for autophagy [136]. The Atg family and other proteins operate in a complex conjugation cascade; many of the interactions and multiple functions of these players are incompletely understood, particularly in mammalian cell systems. A simplified scheme for autophagy includes four subgroups of core proteins involved in three major steps of vesicle formation. Autophagy is initiated by the Atg1/ULK1 complex comprising Unc-51 like autophagy activating kinases 1 and 2 (ULK1/2), Atg13, FIP2000, and Atg101 [137]. Initiation is under negative and positive regulation by mTOR and AMP kinase (AMPK), respectively, as well as mTOR-independent pathways. The Vps34 complex comprised of the class III phosphatidylinositol-3 kinase Vps34, Beclin 1 (homolog of yeast Atg6), p150 (Vps15 in yeast) and Atg14, also participates in autophagy induction. Together, these two complexes promote vesicle nucleation with formation of the "omegasome", a cup-shaped protrusion from the endoplasmic reticulum (ER). Although other vesicle nucleation sources have been described, ER is the best understood. An ensuing conjugation cascade directs formation of a "phagophore" from repurposed ER, building a double-layered "isolation membrane" on the ring-like base of omegasome. Elongation of this membrane requires two ubiquitination-like reactions and two protein complexes. The first involves conjugation of the ubiquitin-like protein Atg12 to Atg5, which then form a complex with Atg16L. The second reaction follows cleavage of microtubule associated protein-1 light chain-3 (LC3), yielding cytosolic LC3-I (Atg8) that is converted to LC3-II through conjugation to phosphatidylethanolamine, tethering LC3-II to the isolation membrane. Through mechanisms not yet fully defined, these complexes facilitate expansion and bending of the isolation membrane. Autophagosome formation is completed upon closure of the isolation membrane, sequestering the targeted cytosolic constituents. In the final step, mediated by SNARE proteins, the autophagosome fuses with a lysosome to form an "autolysosome" where digestion of the sequestered vacuolar contents proceeds [138].

The ratio of LC3-II to LC3-I on immunoblots and a punctate as opposed to cytosolic distribution of LC3 identified by fluorescence microscopy are commonly used as assays to quantify autophagy. They are useful but can be misleading; alternative measures of autophagic flux may be required to distinguish between accelerated autophagosome biogenesis versus reduced turnover [139]. In the related but distinct pathway called LC3-associated phagocytosis (LAP), LC3-II is recruited to conventional, single-membrane phagosomes and promotes their fusion with lysosomes. LAP has been implicated in the clearance of apoptotic and necrotic corpses among other settings [140].

Autophagy proceeds at basal levels to maintain the homeostasis of unstressed cells and is further activated by a variety of factors including starvation, hypoxia, extremes of pH or temperature, growth factor withdrawal, oxidative stress, ER stress, and infection [141-143]. Targeting signals (e.g. externalization of cardiolipin to the limiting membrane of damaged mitochondria [144]) initiate autophagosome formation, which is subject to a wide range of regulatory influences, some dependent and some independent of mTOR and AMPK [145]. Among its many functions, autophagy provides a mechanism to counteract the various strategies employed by intracellular pathogens to use host cells to their advantage. The detection and autophagic destruction of intracellular pathogens, also called xenophagy, has been linked to host defense against certain viruses (e.g. HIV and influenza A virus), bacteria (e.g. Shigella, Salmonella, Group A Streptococcus, and Listeria), fungi (e.g. Candida and Cryptococcus), and protozoa (e.g. Toxoplasma) [146-148]. Conversely, certain intracellular pathogens subvert autophagy to promote infection [149]. Xenophagy plays an integral role in innate and adaptive immunity to infection with *Mtb* that is the focus of this review.

5. Autophagy in TB

As noted in section 1, the capacity of *Mtb* to inhibit phagosome biogenesis and survive inside macrophages that eliminate most other phagocytosed bacteria is an essential virulence mechanism. Despite this countermeasure, a proportion of internalized bacilli are directed to acidified compartments and this is accelerated by macrophage activating factors such as IFN-γ. The role of autophagy in this successful outcome was first identified by Gutierrez et al. in 2004 [150]. They reported that induction of autophagy in *Mtb*-infected macrophages by starvation or by treatment with the mTOR inhibitor rapamycin delivered bacilli to phagolysosomes and increased co-localization of *Mtb* with LC3 and Beclin 1. They also found that autophagy was induced by treatment with IFN-γ or transfection with the IFN-γ effector LRG-47. Since that initial report, a substantial body of evidence has established the major role that autophagy plays in TB defense.

5.1 Targeting Mtb for autophagic elimination

Following receptor-mediated phagocytosis, most *Mtb* bacilli reside in early endosome-like vacuoles that resist acidification or fusion with lysosomes. This niche is permissive for some movement of macromolecules to and from the cytoplasm, to other vesicular compartments, and even the extracellular environment [151-153]. Such bidirectional transfer is required for *Mtb* to acquire nutrients and manipulate host cell functions but also confers vulnerability to host defense [154, 155]. The LMP generated at least in part by ESAT6 (section 3.1.1) enables detection of PAMPs by innate cytosolic sensors. Genomic DNA of *Mtb* activates the

STING-dependent cytosolic pathway, leading to ubiquitination of bacilli and their delivery to autophagosomes in a process dependent on the ubiquitin-autophagy receptors p62 and NDP52, and the serine/threonine protein kinase TBK1 [154]. Data from the zebrafish model of *M. marinum* infection corroborated the involvement of RD1 and STING in autophagic defense against mycobacteria, and further identified the participation of the autophagy modulator DRAM1 in this response [156]. The contribution of autophagy to TB defense in mammals was demonstrated by the increased susceptibility of Lyz-Cre-*Atg5*$^{fl/fl}$ mice with targeted deletion of *Atg5* in myeloid cells [154, 157].

Redundant pathways for innate recognition and autophagic targeting of intracellular *Mtb* have been identified. The ubiquitin ligase Parkin, which mediates the autophagic elimination of dysfunctional mitochondria (mitophagy), was shown to play a critical role in the colocalization of ubiquitin and *Mtb* in macrophages [158]. The dual role of Parkin in mitophagy and xenophagy for intracellular bacteria is unsurprising since mitochondria evolved from intracellular prokaryotes. Once tagged with ubiquitin, bacteria may be targeted by cytoplasmic sequestasome (p62/SQSTM1)-like receptors (SLRs). By virtue of its ubiquitin-associated domain and LC3 interaction region, p62 serves as an adaptor bridging ubiquitinated bacilli and LC3 on the preautophagosome [146]. A role of p62 in recruiting an E3 ligase to ubiquitinate *Mtb* has also been proposed but the precise targets for ubiquitination on bacterial cells and the full spectrum of mediators for this conjugation reactions are incompletely defined [159].

Innate cytosolic receptors bind PAMPS, providING surveillance for intracellular infection. The NLR subfamily receptor NOD2 binds bacterial peptidoglycans like muramyl dipeptide and is activated in macrophages infected with *Mtb* [160]. Signaling downstream of NOD2 in *Mtb*-infected macrophages induces nuclear translocation of NF-κβ, resulting in increased expression of the autophagy-related genes IRGM, LC3, and ATG16L1, the antimicrobial peptide human cathelicidin (hCAP-18/LL-37), and the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α [161].

The capture of *Mtb* in autophagosomes that subsequently fuse with lysosomes delivers bacilli to a hostile, acidified compartment replete with hydrolytic enzymes and free radicals of oxygen and nitrogen. Microbicidal activity against *Mtb* in autolysomes was recently shown involve another role for p62, namely the delivery of ribosomal precursor and other ubiquitinated proteins from the cytosol to autolysosomes where they are cleaved to generate “neo-antimicrobial peptides” [162]. Whether the cytosolic precursor proteins are delivered directly to autolysosomes containing *Mtb* or if killing depends on subsequent fusion of peptide-containing vesicles with *Mtb*-containing phagosomes remains to be determined.

5.2 Regulation of autophagy in TB

Following inhalation of *Mtb* by naïve mice, lung bacterial burden rises in a log linear manner for ~3 weeks until bacterial replication is curtailed by adaptive immunity and particularly IFN-γ produced mainly by Th1 cells [163]. Inducible nitric oxide synthase was considered the major host-protective macrophage response to IFN-γ activation but it is now clear that accelerated autophagic flux is another important effect of IFN-γ [150]. Host-protective effects of IFN-γ are mediated in part by members of the immunity-related GTPase

family (IRG proteins, also called p47 GTPases). Two pathways for autophagy induction by IFN-γ have been reported. One involves Irgm1, a STAT1-dependent IRG previously called Lrg47 (IRGM in humans) [164]. The alternative IFN-γ autophagy pathway is independent of STAT1 but requires JAK 1/2, PI3K, and p38 mitogen-activated protein kinase (MAPK) [165]. The Th1 cytokine TNF-α also induces autophagy and there is at least indirect evidence for this effect in *Mtb*-infected macrophages [166]. In contrast, the Th2 cytokines IL-4 and IL-13 inhibit autophagy at least in in mice [167]. There is evidence for and against autophagy induction by *Mtb* in the absence of exogenous activators like IFN-γ, and different mycobacterial strains may be high or low inducers [168]. Indeed, the suppression of autophagy may be a virulence mechanism of the bacillus (section 5.4). Infection is associated with increased mTOR activity, indicating that innate activation autophagy by mycobacteria is independent of mTOR [168].

Vitamin D receptor (VDR) signaling complements IFN-γ for *Mtb* clearance by a pathway that may be highly significant in humans but is absent in mice. Shin et al. [169] reported that stimulation of TLR2/1 by the *Mtb* lipoprotein LpqH induces autophagy in human monocyte cultures supplemented with the pro-vitamin D hormone calcifediol (25-dihydroxyvitamin D3) [169]. Their data support a model where signaling by TLR2/1 and CD14 induces Ca^{2+} influx through PLC-γ activation. Calcium flux results in AMPK phosphorylation and activation of p38 MAPK that in turn upregulates VD3 1A hydroxylase, which catalyzes the hydroxylation of calcifediol to biologically active calcitriol (1,25-dihydroxyvitamin D3). Calcitriol binding to VDR induces expression of cathelicidin that increases the expression Beclin-1 and Atg5 and promotes autophagic flux [170,171]. The antimicrobial activity of IFN-γ activated human macrophages requires a sufficient level of calcifediol, linking the VDR-stimulated autophagic pathway the protective effect of IFN-γ in human TB [172]. The VDR pathway is not required for autophagy induction by rapamycin or starvation [172]. Discovery of the VDR pathway and its role in TB defense stimulated renewed interest in the therapeutic potential of vitamin D in human TB (section 5.6).

A number of other mediators and pathways have been linked to autophagy induction in *Mtb*-infected macrophages. Given the links between intracellular and cell surface innate receptors and autophagy, it is unsurprising that the TLR4 ligand LPS and IL-1β (via Myd88 and TBK1) have been linked to the autophagic elimination of *Mtb* [173,174]. The growing list autophagy activators in *Mtb*-infected MPs includes the scavenger protein Apoptosis Inhibitor of Macrophages (a target of liver X receptor activation) that was also shown to inhibit apoptosis of THP1 cells challenged with *Mtb* at low MOI [175]. Extracellular ATP (a DAMP discussed in section 2) promotes autophagy via $P2X_7$ receptor, linking autophagy induction and the host response to necrosis [60]. MicroRNA-155 has been linked to TB defense in several reports including evidence that its induction is dependent on ESAT6, that it regulates apoptosis of *M. bovis* BCG-infected macrophages, and that it accelerates autophagy and the killing of intracellular *Mtb* by suppressing the Ras homolog Rheb [176-178]. It is likely that a wider array of pathways and mediators regulating xenophagy in *Mtb*-infected MPs will be identified in the future. Prioritizing these pathways in terms of their protective effects against TB disease in vivo will help direct translational research on harnessing autophagy for TB treatment (section 5.6).

5.3 Autophagy-mediated regulation of immunity in TB

Autophagy participates in TB defense beyond its effector function to kill intracellular bacilli. Since *Mtb* inhibits conventional phagosome biogenesis, its capture and degradation in autolysosomes provides an opportunity to generate peptides for loading onto MHC class II molecules. This outcome was demonstrated by Seto et al. [159] in murine bone marrow derived DC and DC2.4 cells infected with *Mtb* Erdman. The recruitment of MHC II followed the capture of ubiquitinated bacilli in autophagosomes and fusion with lysosomes. In contrast to Erdman, BCG was not ubiquitinated in DC. This is consistent with a requirement for ESAT6 (which is deleted from BCG) to cause LMP. Treating BCG-infected DC with rapamycin, however, increases colocalization of bacilli with autophagosomes and enhances the immunogenicity and protective efficacy of BCG [179].

Autophagy also functions to modulate inflammation through targeted degradation of cytokines such and TNF-α and IL-1β, and by preventing the accumulation of damaged mitochondria that activate the NLRP3 inflammasome via ROS and mtDNA [180-184]. The effect of this regulation to limit inflammation in TB was reported by the Castillo et al. [157]. Pulmonary TB in autophagy-deficient *Atg5*$^{fl/fl}$ LysM-Cre^{+} mice challenged with a low aerosol dose of *Mtb* H37Rv featured increased bacterial burden compared to wild-type controls but also increased neutrophilic inflammation with tissue necrosis, Th17 skewing, and elevated levels of IL-1α, IL-12, IL-17, and CXCL1. The authors concluded that the host-protective effects of autophagy in TB include modulation of the inflammatory response to infection. Bonilla et al. [185] similarly reported increased inflammation and bacillary load in autophagy-deficient *Atg7*$^{-/-}$ LysM-Cre^{+} mice challenged with a high intranasal dose of BCG. Cytokine levels were not reported and the authors attributed susceptibility to accelerated phagocytosis of mycobacteria due to increased surface expression of the scavenger (and *Mtb*) receptors MARCO and MSR1. An excess of p62 in the *Atg7*$^{-/-}$ macrophages bound KEAP1, a suppressor of the transcription factor Nrf2, resulting in increased expression of Nrf2 regulated genes including scavenger receptors. Parenthetically, these authors observed no difference in autophagy induction between BCG (lacking ESAT6 and the Esx-1 secretion system) and *Mtb* H37Rv.

5.4 Evasion of autophagy by Mtb

Evasion of the vesicular trafficking systems that deliver most phagocytosed bacilli to acidified phagolysosomes is a key to *Mtb* virulence and is mediated through inhibition of Ca^{2+} flux and Rab family GTPases [1]. Similarly, the bacillus has evolved countermeasures to evade xenophagy. Zhang et al. [186] reported that ectopic expression of ESAT6/CFP10 inhibited autophagosome formation in with H37Rv-infected RAW264.7 cells, along with reduced expression of Atg8 and other autophagy-related genes. Romangoli et al. [187] compared autophagic flux in human monocyte-derived DC infected with H37Rv, H37Ra or BGG, finding inhibition of autophagosome-lysosome fusion only in DC infected with H37Rv. The capacity to inhibit autophagic flux was restored by complementation of BCG with the Esx-1 region from *Mtb* and by complementation of H37Ra with the PhoP gene. They also showed that the Esx-1 dependent autophagic block exerted by H37Rv could be overcome with rapamycin.

The *Mtb* Eis protein, which inhibits infection-induced macrophage cell death through acetylation of DUSP16/MKP-7 and downregulation of JNK-induced ROS generation (section 3.1.1) inhibits autophagy as well [76]. Macrophage cytolysis triggered by RvΔ*eis* was attributed to autophagic cell death but the relationship between autophagy and cytotoxicity is controversial since autophagy is commonly activated as a prosurvival response in stressed cells that may succumb to one or another subroutine of programmed death [51]. The *Mtb* cell wall component lipoarabinomannan is another virulence factor associated with autophagy inhibition. Phagocytosis of lipoarabinomannan-coated latex meads by RAW264.7 cells inhibited the accumulation of autophagosomes while beads coated with *E. coli* LPS or phophatidyl-myo-inositol of *M. smegmatis* had no suppressive effect [188]. Further research will likely reveal more host and pathogen countermeasures regulating the predominantly host-protective effects of autophagy in TB.

5.5 Genetic associations of autophagy and TB

A body of genetic evidence supports the relevance of autophagy as a biologically significant host defense mechanism in TB. Mice with targeted mutations in the autophagy-related genes Atg5, Atg7, and Irgm1 all demonstrate increased susceptibility to *Mtb* [154,157,185,189]. In a genome-wide siRNA screen in human (THP-1) cells infected with *Mtb* H37Rv or a several clinical isolates of different clades, Kumar et al. [190] identified 275 genes functionally involved in control of infection. Seventy-four of these genes defined essential components active against the spectrum of different *Mtb* isolates and within that subset, more than half were associated with the regulation of autophagy.

Several studies identified association of polymorphic alleles in autophagy-related genes with TB susceptibility or resistance. The complexity of these interactions is highlighted by several studies investigating polymorphisms in *IRGM*. The -261TT allele of *IRGM* was negatively associated with TB in a cohort from the west African country of Ghana but this applied only to disease caused by *Mtb* strains of the Euro-American lineage [191]. The -261TT allele (rs9637876) is predicted to eliminate binding sites for inhibitory transcription factors and therefore increase IGRM expression. In the same Ghanaian population, this allele was not associated with protection from TB caused by *M. africanum* or *M. bovis*. Several other studies variously identified associations of polymorphic *IRGM* alleles with protection or susceptibility to TB in African-American, Iranian, Chinese and Korean populations [191-194]. In some cases opposing effects on TB susceptibility were associated with the same allele in different populations. Analysis of 22 polymorphisms in 14 autophagy genes in an Indonesian population found an association of TB disease with polymorphisms in LAMP1 and MTOR but statistical significance was lost after correction for multiple comparisons [195]. These divergent associations may reflect differences in host genetic background, locally prevalent *Mtb* strains and/or environmental influences but regardless of the effect the data clearly link the *IRGM* gene to TB defense.

5.6 Therapeutic opportunities

The goal of TB elimination is hampered by challenges of compliance with prolonged multi-drug antibiotic regimens and the emergence of drug-resistant *Mtb* strains. This has stimulated interest in the potential for host-directed therapies (HDTs) to amplify endogenous

effector mechanisms and accelerate the response to antituberculous chemotherapy [196]. Among the pathways that may be amenable to this approach, autophagy is an attractive candidate based on its anti-mycobacterial and anti-inflammatory effects in TB. Interestingly, the first line anti-TB drugs isoniazid and pyrazinamide were reported to induce autophagy selectively in *Mtb*-infected but not uninfected macrophages [196]. Activation of autophagy was dependent on ROS and attributed to bacteria-derived hydroxyl radicals produced in response to these drugs. In the fly model of *M. marinum* infection, these authors showed that that the anti-mycobacterial activity of the antibiotics depended at least in part on autophagy. The anti-protozoal drug nitazoxanide and its metabolite tizoxanide inhibit mammalian target of rapamycin complex 1 (mTORC1) signaling and stimulate autophagy by a mechanism attributed to suppression of the quinone oxidoreductase NQO1 [197]. This drug also kills replicating and non-replicating *Mtb* in broth culture but its antimicrobial activity was greatest in *Mtb*-infected macrophages and it was more potent than rapamycin. Drugs with dual antibiotic and autophagy-stimulating effects may particularly attractive candidates for TB treatment.

The best studied non-antibiotic effector of autophagy in the context of TB is vitamin D (section 5.2). Low serum levels of calcifediol are associated with increased TB susceptibility and adverse treatment outcomes in different geographic locations [198-203]. None of these studies established causation, and a cautionary note comes from a case-control study in Greenland where serum calcifediol levels below and above 75-140 nmol/l was associated with increased odds for TB [202]. Two trials have tested vitamin D supplementation in TB. The first of these was a randomized, double-blind, placebo-controlled trial conducted in Guinea-Bissau [204]. Adult patients starting TB treatment received 100,000 IU of cholecalciferol on enrollment and after 5 and 8 months of inclusion. Vitamin D treatment did not reduce the clinical TB severity score (primary outcome) or 12-month mortality. The second study enrolled, new smear-positive adult pulmonary TB patients in the United Kingdom who had corrected serum calcium concentration >2.65 mmol/l for randomization to receive four oral doses of 2.5 mg cholecalciferol or placebo given at baseline and then 14, 28 and 42 days after starting TB treatment. Supplementation was shown to increase serum calcifediol concentrations in the patients receiving intensive-phase TB treatment but did not significantly influence the time to sputum culture conversion (primary endpoint) across the whole cohort. Sputum culture conversion was, however, accelerated by vitamin D supplementation in the subgroup of participants having the *tt* genotype of the *TaqI* VDR polymorphism. Data from that trial were re-analyzed a separate publication that included only patients who fulfilled per-protocol analysis criteria [205]. In this patient group, vitamin D supplementation accelerated sputum smear conversion and resolution of lymphopenia and monocytosis, and it suppressed pro-inflammatory cytokines in the circulation and in cultures of *Mtb* antigen-stimulated whole blood.

Some drugs already in clinical use for other indications can stimulate autophagy and therefore might be considered for adjunctive therapy in TB. Rapamycin is used for immunosuppression in organ transplantation and to inhibit endothelial proliferation on coronary stents [206,207]. It potently suppresses mTOR via inhibition of mTORC1 [208]. Systemic immunosuppression is clearly an unacceptable effect for TB patients but this might

be mitigated by delivery of rapamycin in inhalable particles [209]. Alternative drugs with less troubling side effects include statins that where shown to have activity against *Mtb* in vitro and in vivo [201,211]. A high-throughput screen for small molecules that restrict *Mtb* growth in macrophages identified activity for gefitinib and fluoxetine, both of which also stimulate autophagy [166]. Of these, gefitinib was tested in mice and restricted *Mtb* growth following aerosol infection of BALB/c mice.

Many other approved drugs are known to induce autophagy although their application to TB has not yet been reported. Among these, the oral anti-diabetic biguanide metformin is an attractive candidate. Metformin activates AMPK and stimulates autophagy as well as cell death in malignant cells and has attracted considerable interest in for cancer treatment [212]. Metformin also appears to stimulate autophagy by an AMPK-independent mechanism linked to increased expression of REDD1, which is a negative regulator of mTOR [213]. Zhang et al. [214] identified eight small molecule regulators of autophagy using an image-based high-throughput screen. Three of the compounds are U.S. Food and Drug Administration-approved anti-psychotic drugs, three are Ca^{2+} current inhibitors approved for cardiovascular indications (niguldipine, nicardipine, amiodarone), and one (loperamide) is approved for treatment of diarrhea. None of these compounds modulated mTOR phosphorylation, indicating that they induce autophagy through pathways distinct from rapamycin. Minoxidil and clonidine were identified in another screen for autophagy enhancers restricted to drugs previously used in humans without major side effects [215]. As more autophagy-promoting drugs are evaluated against *Mtb* it will be interesting to learn if particular pathways offer better efficacy advantages as HDTs for TB. At the same time it will be necessary and informative to test whether candidate HDTs adversely influence the balance of protective versus damaging immunity through mechanisms like mTOR inhibition. A cautionary, if unrelated, note is provided by the experience with glyburide in patients infected with *Burkholderia pseudomallei* (melioidosis). Koh et al. [216] reported that glyburide improved survival in diabetic melioidosis patients independent of glycemic control; an effect attributed to inhibition of inflammasome assembly. A contrasting effect of glyburide was later reported by Liu et al. [217] who found higher rates of septic complications in melioidosis patients treated with (unspecified) sulfonylureas and suppressive effects of glyburide on inflammatory cytokine production by peripheral blood mononuclear cells treated with *B. pseudomallei* antigen.

5.6 Conclusions

Autophagy provides a means for infected MPs to overcome the key virulence mechanism of *Mtb*; inhibition of phagosome biogenesis. Its contributions to TB defense include killing of intracellular bacilli, promoting MHC class II restricted antigen presentation, and modulating the inflammatory responses that cause tissue damage and the clinical manifestations of TB disease. Phagocytes use several cytosolic sensors recognize intracellular bacilli and tag them for destruction. Further research will likely identify additional cytosolic and cell surface receptors involved in the detection of *Mtb* and stimulation of autophagic flux. Autophagy is managed by a complex regulatory network with evidence of mTOR-dependent and independent pathways linked to TB defense. The relative contribution of these different pathways to positive outcomes in TB remains to be determined. Such knowledge might be

leveraged for the optimal design of host-directed therapies that stimulate autophagy. The exciting translational opportunities for autophagy in TB will also be advanced by better understanding of the countermeasures that have evolved in the bacillus to escape detection and inhibit autolysosome biogenesis.

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Abbreviations

AIF	apoptosis-inducing factor
AMPK	AMP kinase
Apaf-1	apoptotic protease activating factor
BMM	bone marrow-derived macrophages
cFLIP	cellular FLICE-like inhibitory protein
cIAP	cellular inhibitor of apoptosis protein
COX	cyclooxygenase
CTL	cytotoxic T lymphocytes
CYPD	cyclophilin D
DAMP	damage-associated molecular pattern
DC	dendritic cell
FADD	Fas-associated death domain
HrtA2/Ommi	high temperature requirement
IL	interleukin
IMM	inner mitochondrial membrane
LT	leukotriene
LX	lipoxin
MAPK	mitogen-activated protein kinase
MPT	mitochondrial permeability transition
Mtb	Mycobacterium tuberculosis
mTOR	mammalian target of rapamycin
NET	neutrophil extracellular trap
NK	natural killer
OMM	outer mitochondrial membrane
PG	prostaglandin

PI	propidium iodide
PtdSer	phosphatidylserine
PTP	permeability transition pore
RIPK	receptor interacting serine/threonine protein kinase
Smac	second mitochondria-derived activator of caspases
SLR	SodA, superoxide dismutase
SLR	sequestasome-like receptor
TEM	transmission electron microscopy
TB	tuberculosis
TLR	Toll-like receptor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRADD	TNFR-associated death domain
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end-labeling
VDR	vitamin D receptor

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HIGHLIGHTS

Cell Death and Autophagy in TB (Moraco and Kornfeld)

- Phagocytes infected with *M. tuberculosis* may undergo apoptosis or necrosis
- Apoptosis promotes immune priming and kills bacilli but can spread infection
- Apoptosis can also facilitate spread of *M. tuberculosis* infection
- Autophagy sends *M. tuberculosis* to acidified lysosomes and regulates inflammation
- Apoptosis and autophagy may be leveraged for TB prevention and treatment

Table 1

Myeloid cell death and host defense in TB

Cell death type	Effects on host-pathogen interaction	Consequences for host defense	References
Extrinsic and intrinsic MΦ apoptosis	Eliminates replication niche Induces anti-inflammatory cytokines Lactoferrin inhibits neutrophil recruitment Packages Mtb and antigens in apoptotic vesicles	Lower bacillary load Less immune pathology Less immune pathology Efferocytosis by DC promotes immune priming and cross presentation Efferocytosis by MO kills Mtb *Efferocytosis promotes spreading infection*	17;18;59; 61-65 3;4 88 54;93;94 91;92 57;68
CYPD-dependent necrosis of MΦ MΦ necrosis due to inhibition of membrane repair MΦ necrosis with high Mtb burden CpnT-mediated MΦ necrosis	Releases bacteria to extracellular space Induces pro-inflammatory cytokines	*Promotes spreading infection and transmission* *More immune pathology*	95;96 94;100 107-109 102
MΦ ETosis	Unknown	Unknown	46
DC apoptosis	Packages Mtb and antigens in apoptotic vesicles	Unknown	117
DC necrosis	Unknown	Unknown	116
Neutrophil apoptosis NETosis	Antigen transfer to DC Binds Mtb to extruded DNA but no antimicrobial effect in vitro	Accelerated immune priming Unknown -restricted spread of Mtb? *-increased tissue injury?*	68 109;127

MΦ, macrophage.