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Apoptosis inhibition by intracellular bacteria and its consequence on host immunity

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Regulated cell death via apoptosis not only is important for organismal homeostasis but also serves as an innate defense mechanism. The engulfment of apoptotic infected cells, a process known as efferocytosis, is a common pathway for the destruction of many intracellular bacteria. Some pathogens take advantage of efferocytosis to prevent activation of macrophages and thereby facilitate their dissemination. Conversely, many obligate intracellular bacterial pathogens and some facultative-intracellular bacteria inhibit apoptosis, preventing efferocytosis, and evading innate host defenses. The molecular mechanism of bacterial effectors includes secreted proteins that bind to and inhibit apoptosis cell signaling pathways. We provide an overview of the known bacterial effectors, their host cell targets and their importance for the virulence of human pathogens.

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Introduction

Certain bacterial species evade humoral immunity by entering a protected niche in an intracellular compartment. Some cell types can destroy non-pathogenic bacilli by intrinsic antimicrobial defenses. However, several bacterial species have developed virulence strategies that prevent, subvert, or overcome these defense mechanisms. Immunity to these pathogenic microbes often requires T cells to accelerate or enhance intrinsic anti-bacterial activities of infected cells. For cells that are not able to destroy or contain intracellular infection, an alternative strategy is cell death as an 'altruistic death' that can sometimes deprive an intracellular pathogen of a protected niche, interrupt its replicative cycle, and expose it to other components of the immune system. Importantly, while cell death can be detrimental for the pathogen, some pathogens exploit cell death pathways. This host–pathogen interaction is complex not only because of the large and growing number of cell death pathways [1], but also because experimental approaches vary in the use of bacterial strains, multiplicity of infection (MOI), host cell type, duration of infection, and other variables. Here, we will review the mechanisms by which human bacterial pathogens manipulate host cell apoptosis and the consequences of those interactions on host defense.

Efferocytosis of apoptotic infected cells contributes to host defense

Apoptosis is a form of cell death that is triggered by either an intrinsic (mitochondrial) or extrinsic (cell surface receptors) signaling pathway that leads to the activation of caspase-8 or caspase-9, respectively [2]. Both pathways converge by activating the executioner caspases 3, 6, and 7, which activate substrates that mediate the morphological changes associated with apoptosis (e.g. fragmentation of genomic DNA) [1]. Apoptosis is the dominant form of cell death during organismal development and homeostasis. Early during apoptosis, the plasma membrane remains intact, the cell contents are contained, and damage associated molecular patterns (DAMPs) are not released. Thus, apoptosis is a non-inflammatory death. Although apoptotic cells frequently undergo secondary necrosis *in vitro*, this is not thought to occur under normal homeostatic conditions because of a second process known as efferocytosis. Efferocytosis is the engulfment of apoptotic cells by phagocytes [1,3–5]. This evolutionarily conserved and constitutive process is one of the chief functions of macrophages. There are a variety of functionally redundant cell surface receptors and adaptors expressed by macrophages that recognize the molecule phosphatidylserine (PS), which is expressed on the cell surface early during apoptosis [1]. It is the specific recognition of PS by phagocytes that distinguishes efferocytosis from phagocytosis, macropinocytosis, or the engulfment of necrotic cells.

During microbial infection, apoptotic cell death is generally beneficial for the host and detrimental for the pathogen. While non-apoptotic cell death potentially allows pathogens to exit the host cell and disperse, apoptosis can have an antimicrobial function. How is the antimicrobial effect of apoptosis mediated? One possibility is that the same activated nucleases and enzymes that lead to the cells' demise could also help in killing pathogens.

However, many pathogens have tremendously stable cell walls and are unlikely to be damaged by the enzymes activated during apoptosis. Instead, it is increasingly recognized that the antimicrobial consequences of apoptosis are secondary to efferocytosis of infected apoptotic cells [6]. As an example of how efferocytosis enhances host defense, we will examine *Mycobacterium tuberculosis*, the etiological agent of the human chronic lung disease tuberculosis.

We and others have shown that virulent *M. tuberculosis* predominantly induces both human and murine macrophage necrosis *in vitro*. It is not precisely known how much of this macrophage cell death is primary necrosis induced by *M. tuberculosis*, and how much is secondary necrosis due to failure in efferocytosis. What is clear is that in murine macrophages, both apoptotic and necrotic cells are detected following infection with virulent *M. tuberculosis*. Furthermore, avirulent mutants exist that induce more apoptosis than necrosis, indicating that virulent *M. tuberculosis* has evolved mechanisms to inhibit macrophage apoptosis. Indeed, apoptotic death of *M. tuberculosis* infected macrophages, whether secondary to changes in bacterial virulence or in macrophage physiology, improves host resistance. Conversely, shifting the balance of the macrophage death toward necrosis is associated with increased virulence (reviewed in Refs. [7,8]). Efferocytosis of infected apoptotic macrophages was shown to synergize with apoptosis to increase host resistance. Thus, bystander macrophages engulf infected apoptotic macrophages, and the 'efferosomes' rapidly acquired characteristics of lysosomes and were associated with bacterial killing [9] (Figure 1). Interestingly, *Pseudomonas aeruginosa*, which adheres to apoptotic cells among epithelial monolayers, are engulfed and destroyed by other epithelial cells [10].

It is important to recognize that the role of efferocytosis is not limited to bacterial killing. A role of efferocytosis in priming T cells is well-documented. Dendritic cells (DC) take up apoptotic vesicles by efferocytosis, process and present the mycobacterial antigens to T cells [11–13]. This process increases the initial activation of T cells, particularly during infection with *M. tuberculosis* mutants that are unable to inhibit apoptosis [14], or when the host macrophage is predisposed to an apoptotic death [15]. These observations have important implications both for the developing better vaccines [16,17] and for host directed therapy of infectious disease [18].

If apoptosis and subsequent efferocytosis can eliminate the intracellular niche of microbes, it is not surprising that some have evolved to inhibit apoptosis, the penultimate event leading to efferocytosis. Identifying bacterial strategies to inhibit apoptosis can be tricky as most cells are programmed to undergo apoptosis, and most bacterial infections increase apoptotic cell death compared to uninfected cells. Therefore, it can be difficult to ascertain whether the induction of cell death is truly a bacterial virulence mechanism. To identify if and how a bacterium inhibits apoptosis induction, it is important to compare virulent and non-virulent strains, challenge infected and uninfected cells with pro-apoptotic stimuli and finally, perform genetic screens to identify anti-apoptotic bacterial genes.

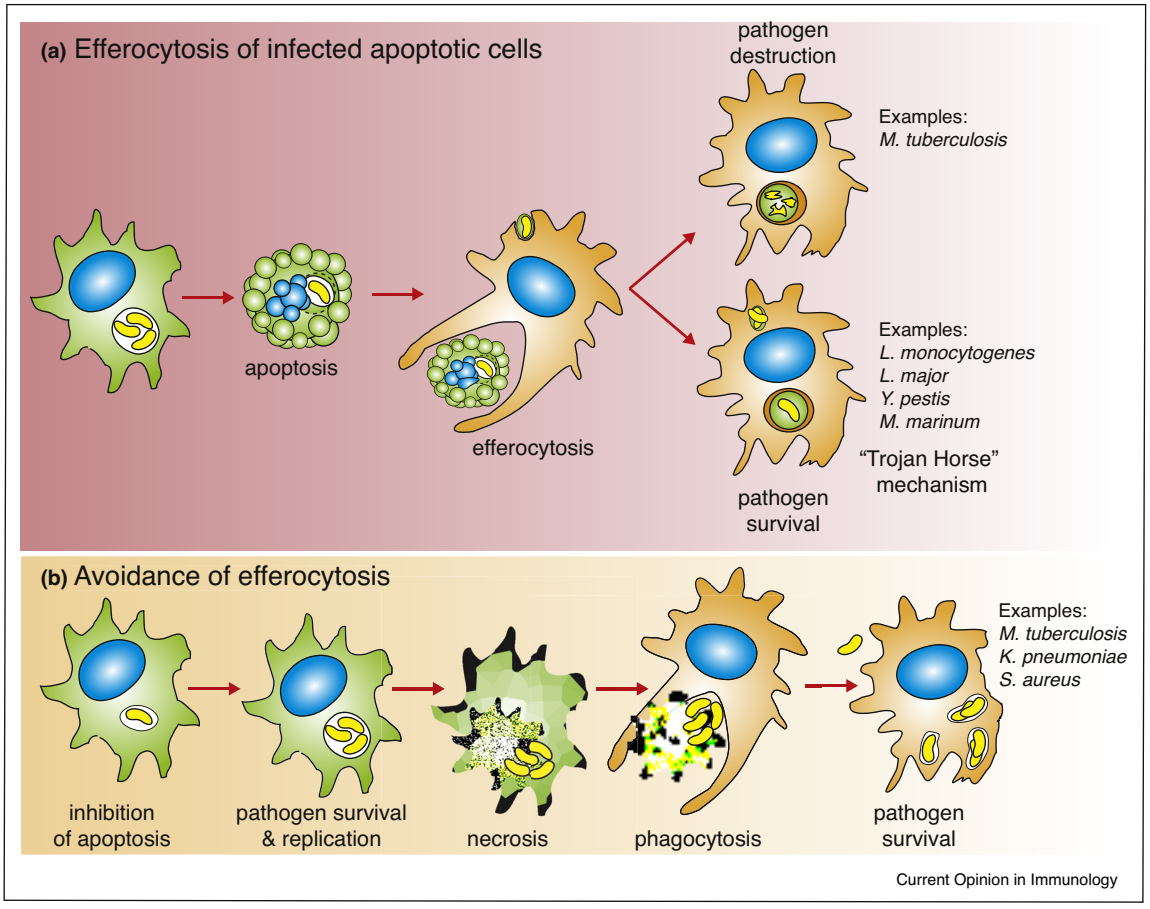
Obligate and facultative intracellular bacteria inhibiting host cell apoptosis

The following Gram-negative, obligate intracellular bacterial pathogens are known to inhibit apoptosis (Table 1). *Chlamydia trachomatis* causes sexually transmitted infections in the female and male genital tract [19]. It was first reported to inhibit apoptosis in epithelial cell lines 20 years ago, which was subsequently confirmed by other investigators [19,20]. *Coxiella burnetii* causes Q fever after aerosol transmission from contaminated soil or animal products [21]. It infects and inhibits apoptosis in macrophages and epithelial cells [22,23]. *Ehrlichia chaffeensis* is the causative agent of human monocytic ehrlichiosis [24]. *E. chaffeensis* is transmitted from its zoonotic reservoir to humans via tick bites, infects mainly mononuclear and polymorphonuclear leukocytes [24], and inhibits apoptosis of neutrophil and monocyte cell lines [25,26]. *Anaplasma phagocytophilum* is another tick-transmitted pathogen that causes human granulocytic anaplasmosis [27], which inhibits apoptosis in neutrophils [28] and in tick cells [29]. *Rickettsia rickettsii* is transmitted via infected ticks and causes Rocky Mountain spotted fever [30]. It primarily infects endothelial cells and protects them from apoptosis [30,31].

Not all of the facultative intracellular pathogens inhibit host apoptosis, most likely since they are less dependent on the host cell survival (Table 1). As discussed, *M. tuberculosis* is a strict human pathogen that is transmitted via aerosol [32], and there is a direct correlation between virulence of various *M. tuberculosis* strains and their capacity to inhibit apoptosis of alveolar macrophages [33]. *Legionella pneumophila* is transmitted via aerosol from a contaminated water source and is the etiologic agent of Legionnaires' disease [34]. Although early investigations described a pro-apoptotic infection of macrophages and epithelial cells with Legionella [35], more recent studies identify the anti-apoptotic capacity of the bacterium [34,36].

Salmonella enterica serovar Typhimurium is the mouse model strain for *Salmonella typhi*, the cause of typhoid fever in humans. There are numerous studies on the induction of cell death of macrophages, dendritic cells and epithelial cells infected with Salmonella [37]. Nevertheless, there seems to be a brief anti-apoptotic phase during the infection of intestinal epithelial cells with the bacteria [37,38].

Figure 1



Apoptosis and efferocytosis constitute a linked anti-bacterial host defense mechanism. **(a)** Infected cells that undergo apoptosis are engulfed by macrophages. Following efferocytosis, most non-pathogenic bacteria are destroyed by the macrophage. However, some human pathogens have adapted and circumvented this defense mechanism, and instead, hijack it to survive and disperse or disseminate. **(b)** The inhibition of cell death (i.e. apoptosis) by bacterial pathogens can impair efferocytosis and lead to more favorable conditions for bacterial replication.

Mechanisms of host cell apoptosis inhibition by bacteria

C. trachomatis infection protects cells from extrinsic and intrinsic apoptosis pathway induced via specific activators [20], but this initial protection from apoptosis may still allow the cells to undergo necrotic death at a later time [39]. Host mitochondrial proteins are a prominent target for *C. trachomatis*-mediated apoptosis manipulation by inhibiting pro-apoptotic Bax and Bak proteins [40] and upregulating the anti-apoptotic protein Mcl-1 via increased transcription [41] and decreased protein degradation [42]. An siRNA screen identified the transcription factor HIF-1α responsible for the upregulation of Mcl-1 after *C. trachomatis* infection [43]. Interestingly, Mcl-1 is also exploited by *M. tuberculosis* to inhibit apoptosis [44] but it is induced by the PPARγ transcription factor [45]. *C. trachomatis* infection inhibits the extrinsic apoptosis pathway induced via TNF by blocking internalization of TNF–TNF-receptor complexes [46]. *M. tuberculosis* inhibits TNF signaling by promoting secretion of soluble TNF receptor 2, which neutralizes unbound TNF [47]. The *C. trachomatis* secreted protein, Pgp3, activates the PI3K/AKT/MDM2/P53 signaling pathway to suppress apoptosis and supports virulence in a mouse model [48–50] (Table 1). Another effector, CpoS was recently identified by a genetic screen in *C. trachomatis* for death-inducing mutants [51]. CpoS manipulates vesicular trafficking via interaction with host cell small GTPase, which ultimately leads to the activation of a non-apoptotic, STING-dependent cell death pathway [51] (Table 1). *A. phagocytophilum* manipulates various components of the intrinsic apoptosis pathway to inhibit cell death in mammalian or tick host cells [27]. The type IV secretion system (T4SS) translocates Ats-1 into the host cell cytosol where a mitochondrial targeting signal mediates transport to mitochondria where Ats-1 gets cleaved and then

Table 1

Inhibition of host cell apoptosis pathways by bacterial pathogens

Bacteria	Host cell	Effector	Target	Relevance (*in vivo*)	Ref.
Salmonella	Epithelial	SopB	Increased Akt activation	*sopB* mutant is attenuated	[37,38]
Mycobacterium	Macrophage	NuoG/NDH-1	Decreases phagosomal ROS	*nuoG* mutant is attenuated	[64,65]
Mycobacterium	Macrophage	Ndk	Binds to Rac1	*ndk* knock-down strain is attenuated	[66]
Legionella	Macrophage	LegK1	Phosphorylates IκBα, p100	?	[61]
Legionella	Macrophage	SidF	Binds to and inhibits BNIP3, Bcl-rambo	? *sidF* mutant is attenuated *in vitro*	[62]
Chlamydia	Epithelial	Pgp3	Activates the PI3K/AKT/MDM2/P53 pathway	*pgp3* mutant is attenuated	[48,50]
Chlamydia	Epithelial, macrophage	CpoS[a]	Binds to multiple Rab GTPases, inhibits STING-dependent cell death	*cpoS* mutant is attenuated	[51]
Anaplasma	Neutrophil	Ats-1	Translocates to mitochondria to inhibit CytC release and PARP cleavage	?	[52]
Ehrlichia	Monocyte	ECH0825	Increases MnSOD protein levels	?	[53]
Coxiella	Dendritic	AnkG	Binds to p32	?	[55]
Coxiella	Epithelial	CaeA	Inhibits caspase 7	?	[57]
Coxiella	Epithelial	CaeB	Inhibits MOMP	?	[58]

Facultative intracellular bacteria (Salmonella to Legionella) and obligate intracellular (Chlamydia to Coxiella). Shown are the best-known host cell targets of the bacterial effectors which does not necessary mean that they are directly interacting. Details on the mechanism of inhibition are given in the text.

[a] CpoS does not inhibit a known apoptosis pathway but the mutant does result in attenuation due to abrogated intracellular replication.

mediates inhibition of cytochrome C (CytC) release by the mitochondria, which is an important pro-apoptosis signaling event [52] (Table 1). *E. chaffeensis* also uses a T4SS to secrete ECH0825 which translocates into mitochondria to upregulate MnSOD protein levels in the mitochondrial matrix, neutralizing ROS and thereby inhibiting apoptosis [53] (Table 1). A subset of tandem repeat proteins (TRP) secreted by the type I secretion system (T1SS) interact with host cell proteins involved in apoptosis signaling [53]. Finally, *C. burnetii* activates the PI3K/AKT pathway to upregulate Mcl-1 expression and protect neutrophils from apoptosis [54]. The T4SS substrate AnkG binds to host cell p32 [55] and Importin-α1 [87] both of which help to translocate AnkG into the host cell nucleus, where it acts to inhibit apoptosis [56] (Table 1). Two additional T4SS substrates, CaeA, and CaeB, inhibit the intrinsic apoptosis pathway at the level of the executioner caspases (caspases 3, 6, and 7) [57] or mitochondria [58], respectively (Table 1). *R. rickettsii* infection suppresses apoptosis via the induction of NF-κB activation, which induces pro-survival genes [30,59], but the bacterial effectors mediating this effect have yet to be identified.

L. pneumophila inhibits apoptosis by the upregulation of anti-apoptosis gene transcription through the activation of the NF-κB signaling pathway [60]. *L. pneumophila* secretes LegK1 into the host cell cytosol via its T4SS to inhibit host cell apoptosis. LegK1 has serine/threonine kinase activity and phosphorylates IκBα and p100 leading to increased NF-κB activation [61] (Table 1). SidF is another translocated *L. pneumophila* protein which binds to the pro-apoptotic host cell proteins BNIP3 and Bcl-rambo and inhibits their activity to protect cells from intrinsic apoptosis pathway induction [62] (Table 1). Interestingly, the SdhA protein, which was identified as an anti-apoptotic effector, affects the vacuolar stability of *L. pneumophila* and does not directly manipulate host cell death pathways [63]. Salmonella uses the type III secretion system (SPI1) to translocate the phosphoinositide phosphatase SopB into the host cell cytosol to activate the pro-survival host cell kinase Akt [37,38] (Table 1). AvrA has first been described as a pro-apoptosis effector of Salmonella that mediates its activity by inhibiting NF-κB signaling [88]. Nevertheless, two subsequent studies described an anti-apoptotic role for AvrA via inhibition of JNK signaling [89,90]. NuoG is one of the 14 *M. tuberculosis* proteins forming the proton-translocating NADH-dehydrogenase and its deletion leads to a *M. tuberculosis* mutant that induces more host cell apoptosis and is attenuated *in vivo* [64]. The mechanism of apoptosis suppression involves the inhibition of reactive oxygen species (ROS) accumulation in the *M. tuberculosis* phagosome, which sensitizes cells to TNF-mediated apoptosis induction [65] (Table 1). Interestingly, *M. tuberculosis* nucleoside diphosphate kinase, Ndk, binds to and inhibits host cell Rac1, which reduces the activation of the phagocyte NADPH oxidase (NOX2) (Table 1). Consequently, less phagosomal ROS is produced and less apoptosis occurs,

similar to the results obtained with the *nuoG M. tuberculosis* mutant [66]. The deletion in *M. tuberculosis* of the probable membrane transporter Rv2456c leads to the reduced activation of NF-κB signaling and hence increased apoptosis [67], resembling the *L. pneumophila legK1* mutant phenotype. Multiple *M. tuberculosis* genes involved in modulating host cell apoptosis, pyroptosis or necrosis have been described [68,69].

Bacterial avoidance of efferocytosis can be host-detrimental

If, as argued above, *M. tuberculosis* inhibits apoptosis and induces necrosis as a virulence strategy, how does this benefit bacterial fitness? Human neutrophils undergo necrosis following *M. tuberculosis* infection by an ESX1-dependent mechanism that triggers host ROS production [70]. When these necrotic, infected neutrophils are engulfed by uninfected macrophages, the bacilli are associated with early phagosomes, where they are competent to grow intracellularly. Similar to neutrophils, *M. tuberculosis* infected hMDM undergo non-apoptotic cell death *in vitro*. These highly infected dead macrophages are cytotoxic to the engulfing macrophages leading to a positive feedback loop of *M. tuberculosis* growth, macrophage death, engulfment, and *M. tuberculosis* growth [71]. While little is known about the uptake of bacteria associated with cellular debris, there are damage-associated molecular pattern ligands such as F-actin, which can be recognized by cell surface receptors such as DNGR-1 [72]. Thus, in contrast to improved containment of bacilli in apoptotic macrophages, infected necrotic cells not only lead to the dispersal of the bacteria but may promote its subsequent growth. What is the relevance of these *in vitro* studies to an intact host? First, defective efferocytosis, caused by mutation in a lysosomal cathepsin, leads to increased susceptibility in a zebrafish model of *Mycobacterium marinum* [73]. In addition, an *M. tuberculosis* mutant in the transcriptional repressor Rv3167c induces more host cell necrosis and is hypervirulent in mice [74]. Thus, disrupting the apoptosis/efferocytosis cycle contributes to the virulence of *M. tuberculosis* (Figure 1). *Staphylococcus aureus* is a medically important Gram-positive bacterium that is generally known for its extracellular lifestyle but is killed after phagocytosis by neutrophils. However, some *S. aureus* bacilli can survive in human neutrophils. Human macrophages bind these neutrophils as they begin to undergo apoptosis but do not engulf the infected neutrophils. Instead, the infected neutrophils die in a RIP-1-dependent manner typical of necroptotic cell death, which could lead to bacterial dispersal [75]. The alpha toxin of *S. aureus* may be responsible for blocking efferocytosis of infected neutrophils by alveolar macrophages in a murine infection model [76]. Such an incomplete or 'frustrated' efferocytosis also occurs during *M. tuberculosis* infection, especially at a high MOI, which leads to an atypical form of cell death [77,78].

Cell death induced by *Klebsiella pneumoniae* is determined by several factors and is strain-dependent [79]. One strain of *K. pneumoniae* (A28006) led to inflammasome activation and induced pyroptosis, where as a second strain (A54970) did neither. Interestingly, A28006 was significantly more attenuated *in vivo*, and the attenuation was dependent upon caspase 1/11 and inflammasome activation. Following A28006-induced pyroptosis, bystander macrophages took up the cellular debris and bacteria, which inhibited bacterial replication. Although pyroptotic cell engulfment has been referred to as efferocytosis [79,80], it has not been shown to depend on PS recognition. Importantly, A54970 induced IL-10, which inhibited inflammasome activation and pyroptosis. A different strain of *K. pneumoniae* interferes with apoptosis induction in infected neutrophils by activating necroptosis, which also diminishes engulfment by macrophages [81]. In this case, *in vivo* treatment with necrostatin-1 (an inhibitor of RIPK-1) significantly reduced the virulence of *K. pneumoniae* in a mouse model. Thus, some virulent bacterial strains subvert cellular death pathways to avoid innate defenses.

Exploitation of efferocytosis

Just as some pathogens have evolved mechanisms to avoid efferocytosis, some pathogens have evolved counter-strategies that exploit efferocytosis. For example, *Yersinia pestis*, the etiological agent of plague, is normally thought to be killed by neutrophils. However, *Y. pestis* grown at the flea temperature of 21°C can survive in human neutrophils, which when they undergo apoptosis, are taken up by macrophages [82]. This 'Trojan Horse' mechanism of infecting macrophages reduces pro-inflammatory cytokine production and allows *Y. pestis* to evade innate immunity. Similarly, *M. marinum* disseminates after dying infected macrophages are engulfed by motile macrophages, which can lead to the spread of disease beyond the primary granuloma [83]. A 'Trojan Horse' model has also been shown for the eukaryotic pathogen *Leishmania major* in which parenteral entry in the skin and subsequent infection of neutrophils is followed by efferocytosis by myeloid cells. This facilitates *L. major* dissemination without triggering a danger alarm [84,85]. Finally, *Listeria monocytogenes* damages not only the phagosomal membrane, which allows it to enter the cytosol, but also the plasma membrane. *L. monocytogenes* apparently buds from the plasma membrane in host derived PS + vesicles, which are engulfed by macrophages in a TIM4-dependent mechanism but this does not lead to the killing of the bacteria within the efferosome [86]. Thus, various pathogens are able to exploit efferocytosis to evade innate immunity and spread throughout the host.

Conclusion

Apoptosis was the first regulated cell death pathway identified and it has a crucial role in the development

of the immune system and its return to homeostasis after T or B cell responses. Less appreciated is the role that apoptosis plays in the effector arm of immune responses. CD8 T cells kill target cells by activating extrinsic pathways of apoptosis and as reviewed here, different types of cells infected with pathogenic microbes die by apoptosis, which can enhance host resistance. The existence of additional regulated cell death pathways, such as necroptosis and pyroptosis, has provided additional insight into how pathogenic microbes evade host immunity. Here, we present evidence that many bacterial pathogens actively inhibit host cell apoptosis, which enables them to avoid the antibacterial effects of efferocytosis. Unraveling how cell death pathways are manipulated by bacteria provides a greater nuanced understanding of the host–pathogen interaction. There are still knowledge gaps, particularly for obligate intracellular pathogens, some of which are not genetically tractable. Furthermore, how efferocytosis is regulated during infection, which has important roles in anti-bacterial immunity, resolution of inflammation, and tissue remodeling, is incompletely understood. Insight into these pathways is beginning to be exploited for new therapeutic strategies for cancer. It remains to be determined whether they can be similarly manipulated to modify the virulence of bacteria or attenuate the untoward consequences of infection.

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Conflict of interest statement

Nothing declared.

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