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Regulation Of Cell Death And Inflammation In Antibacterial Immunity

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Abstract
Many pathogens interfere with the activation of innate immune signaling responses. However, pro-survival and cell death-inducing signals are coupled downstream of innate immune receptors, such that survival signals prevent cell death in the context of inflammatory stimuli. Blockade of key signaling pathways by pathogen virulence factors uncouples this coordinate regulation, resulting in activation of programed cell death. Thus, cell death may act as a conserved host protective mechanism for inducing inflammation in response to pathogens that interfere with immune signaling pathways. The YopJ virulence factor of the gram-negative bacterial pathogen Yersinia pseudotuberculosis potently inhibits NF-κB and MAPK signaling, resulting in death of infected innate immune cells. This cell death occurs through a pathway involving caspase-8 and receptor-interacting serine/threonine kinase 1 (RIPK1) downstream of toll-like receptor 4 (TLR4) and TIR-domain-containing adapter-inducing interferon-β (TRIF). Our studies reveal that TNFR1 signaling additionally acts to promote Yersinia-induced cell death, via caspase-8 and RIPK1. Importantly, cell-extrinsic TNF, produced by cells that escape the effects of YopJ inhibition, is necessary for promoting this response. Thus, innate immune cytokine signaling acts to potentiate the apoptotic response to pathogen inhibition and perhaps reflects a coordinated response by heterogeneous cell populations to counter infection. We further demonstrate an in vivo function for RIPK1 kinase activity in promoting host protection and inflammatory cytokine responses to Yersinia infection that is consistent with a function of Yersinia-induced apoptosis in promoting host protective anti-bacterial immunity. Together, these data demonstrate that the apoptotic response induced during infection with pathogens that inhibit host signaling is promoted by cytokine signaling and plays an important role in potentiating host protection. Understanding the regulation of these responses to infection provides novel mechanistic insight into the pathways that may regulate cell death and inflammation in diverse pathologic states and could be targeted to treat disease.

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DEDICATION

I would like to dedicate this work to my wife, Katrina, who has given me the love and support to pursue my goals, for believing in me and for all the sacrifices made to help me along my way.

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ABSTRACT

REGULATION OF CELL DEATH AND INFLAMMATION IN ANTIBACTERIAL IMMUNITY

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Many pathogens interfere with the activation of innate immune signaling responses. However, pro-survival and cell death-inducing signals are coupled downstream of innate immune receptors, such that survival signals prevent cell death in the context of inflammatory stimuli. Blockade of key signaling pathways by pathogen virulence factors uncouples this coordinate regulation, resulting in activation of programed cell death. Thus, cell death may act as a conserved host protective mechanism for inducing inflammation in response to pathogens that interfere with immune signaling pathways. The YopJ virulence factor of the gram-negative bacterial pathogen *Yersinia pseudotuberculosis* potently inhibits NF-κB and MAPK signaling, resulting in death of infected innate immune cells. This cell death occurs through a pathway involving caspase-8 and receptor-interacting serine/threonine kinase 1 (RIPK1) downstream of toll-like receptor 4 (TLR4) and TIR-domain-containing adapter-inducing interferon-β (TRIF). Our studies reveal that TNFR1 signaling additionally acts to promote *Yersinia*-induced cell death, via caspase-8 and RIPK1. Importantly, cell-extrinsic TNF, produced by cells that escape the effects of YopJ inhibition, is necessary for promoting this response. Thus, innate immune cytokine signaling acts to potentiate the apoptotic response to pathogen inhibition and perhaps reflects a coordinated response by heterogeneous cell populations to counter infection. We further demonstrate an *in vivo* function for RIPK1 kinase activity in promoting host protection and inflammatory cytokine

responses to *Yersinia* infection that is consistent with a function of *Yersinia*-induced apoptosis in promoting host protective anti-bacterial immunity. Together, these data demonstrate that the apoptotic response induced during infection with pathogens that inhibit host signaling is promoted by cytokine signaling and plays an important role in potentiating host protection. Understanding the regulation of these responses to infection provides novel mechanistic insight into the pathways that may regulate cell death and inflammation in diverse pathologic states and could be targeted to treat disease.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

CHAPTER 1

Introduction

In order to mount an appropriate immune response, the immune system must be able to discriminate between stimuli that warrant inflammation and those that should be ignored or tolerated. There are two proposed models by which this is achieved by innate immune cells ((1, 2). The first relies on the recognition of 'non-self' signals associated with pathogens and e mouseetermed pathogen-associated molecular patterns (PAMPs) (1). This involves the recognition of pathogens through germline-encoded receptors specific for highly conserved microbial components that are often indispensable for microbial survival. These receptors were termed pattern recognition receptors (PRRs) (1). For example, the essential cell wall component of gram-negative bacteria, lipopolysaccharide (LPS), is recognized by toll-like receptor 4 (TLR4) and results in the induction of innate and adaptive immune responses (3, 4). However, there are many examples of how this model alone is insufficient to account for aspects of immune activation. For instance, this model does not explain how pathogens are recognized as warranting inflammatory responses, while commensal microbes, which share many of the same PAMPS and greatly outnumber pathogens at mucosal sites of infection, do not. Furthermore, transformed cells are recognized by the immune system and eliminated, despite the fact that they are not readily detected by PRRs directed at microbial products.

An alternative model of how immune responses are regulated postulates that pathogenic threats are distinguished through the sensing of 'danger' (2). This 'danger' can take many forms, but represents a departure from homeostasis and normal

physiology. Notably, these two models for how the immune system is activated are not mutually exclusive. The recognition of microbes through PRRs represents a specific mechanism of sensing ‘danger’, in the form of microbial products. The ‘danger model’ differs, however, by allowing that the recognition of microbes by PRRs at sites that are normally sterile may represent ‘danger’, in the form of an invasive pathogen, while PRR signaling at the intestinal barrier would be interpreted as safe. Furthermore, it suggests that transformed cells may be recognized by their ‘dangerous’ behavior, such as uncontrolled replication or altered metabolism. Cell death, in certain circumstances, may also represent an important ‘danger’ signal. Although some forms of cell death are a continuous part of homeostatic cellular turnover, other forms may differ significantly in the signals they send or release. Thus, how a cell dies may be indicative of pathologic ‘danger’.

During infection, patterns of cell death are profoundly altered. Cell death may be a direct effect of pathogen toxins or virulence factors that mediate host cell lysis. Alternatively, cell death can be induced as part of the immune response (5). In recent decades, much has been learned about the signaling pathways that regulate different cell death modalities such as intrinsic apoptosis, extrinsic apoptosis, programmed necrosis and pyroptosis. In this introduction, I will first discuss the regulation of cell death signaling pathways in the context of inflammation and infection, with a particular focus on cell death driven by cytokine receptor signaling and microbial recognition by PRRs. I will then review how the immune system recognizes and responds to cell death during homeostasis and how this response is altered in the context of necrosis or ‘immunogenic cell death’. Finally, I will provide a context for my work using a model of bacterial

infection with *Yersinia pseudotuberculosis* and highlight the important questions surrounding cell death and inflammation that exist in this model.

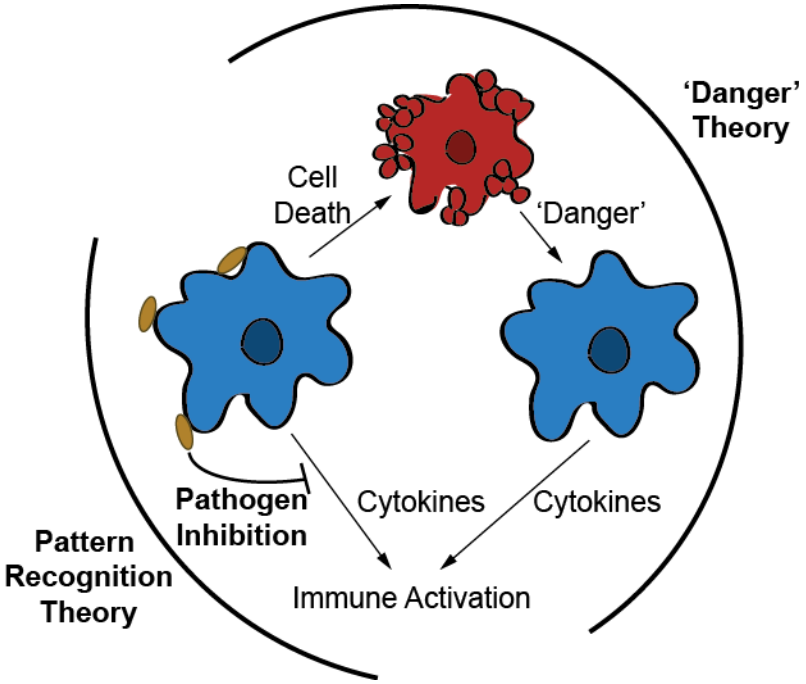

Figure 1. Cell death-associated 'danger' signals promote immune activation. Inhibition of cytokine signaling allows pathogens to escape immune activation during infection. Cell death, through the release of 'danger' signals may allow cells to bypass pathogen inhibition to elicit immune activation.

Cell Death Modalities

The mechanisms by which cells undergo cell death vary dramatically between states of homeostasis and infection or inflammation. The differences between these cell death modalities allow the immune system to recognize cell death as either a stimulus that warrants an inflammatory response or that should be tolerated. Homeostatic cell death is primarily mediated by intrinsic apoptosis (6). During infection and inflammation, cell death can be additionally driven by signaling through so-called 'death receptors' and

PRRs (5). Both of these receptor pathways can lead to pro-survival, apoptotic or necrotic signaling responses. The switch from pro-survival signaling to cell death is determined by cellular conditions that may be reflective of 'danger'. For example pathogen inhibition of inflammatory cytokine production also blocks the pro-survival response to death receptor and PRR signaling. This may result in one of several cell death pathways that may act as a proxy for the 'danger' posed by a pathogen, particularly when other important host defenses are inhibited. Here, I will briefly touch on salient features of the intrinsic apoptosis pathway, before describing the regulation of pro-survival and cell death signals downstream of death receptors and PRRs. I will end by highlighting the importance of a central regulator of these cell fates, receptor interacting protein kinase 1 (RIPK1).

Intrinsic apoptosis

The intrinsic apoptosis pathway is induced in response to numerous signals that converge upon proteins regulating the mitochondrial membrane (7). Developmental cues or stressors including DNA damage, growth factor deprivation and ER stress regulate the activity of pro-apoptotic effector proteins through anti-apoptotic Bcl2-family proteins and pro-apoptotic BH3-only proteins (8). When the activity of pro-apoptotic factors dominate over anti-apoptotic factors the effector proteins, BAX and BAK, oligomerize to promote the irreversible process of mitochondrial outer membrane permeability (MOMP) (9). Release of cytochrome c from the mitochondrial intermembrane space allows for its interaction with apoptotic protease-activating factor 1 (APAF1) (10) and the formation of the caspase-9 activation complex, termed the 'apoptosome' (11). Cleavage of effector caspases-3, -6 and -7 by active caspase-9 leads to the terminal apoptotic response.

‘Death receptor’ pro-survival signaling

Death receptors are members of the tumor necrosis factor receptor superfamily and contain a conserved cytoplasmic ‘death domain’ (DD) necessary for the recruitment of proteins to the active receptor complex (12). Alternatively, TNFR1-like receptors do not contain a DD themselves, but recruit TNFR-associated DD (TRADD) to mediate signaling. Upon receptor ligation, the DD recruits RIPK1 and TNF receptor associated factor 2 (TRAF2) (13-15). This is followed by the polyubiquitination of RIPK1 by cellular inhibitors of apoptosis proteins (cIAPs) (16-19), and subsequently by the linear ubiquitin chain assembly complex (LUBAC) (20-23). These ubiquitin chains allow for the recruitment and activation of TAK1- and IKK-containing complexes for the induction NF-κB and MAPK signaling responses (Fig 2A). These signaling pathways culminate in a transcriptional response, including the expression of pro-inflammatory cytokines and pro-survival factors that sustain cellular viability during persistent death receptor activation.

Ubiquitination is essential for the stability of the NF-κB and MAPK activating complex and cell survival (24). Supporting this, the expression of both of the ubiquitin ligases cIAP1 and cIAP2 protects against apoptosis(25), while their absence or disruption promotes cell death and embryonic lethality (26-28). Also important are negative regulators of NF-κB and MAPK activation that allow for inflammatory signaling to be turned off or toned down (Fig 2B). Negative regulators of death receptor signaling include important deubiquitinases that promote the disassembly of ubiquitin chains (29, 30). Interestingly, two deubiquitinases that both act to disassemble death receptor signaling complexes, A20 and CYLD, can differentially promote either survival or cell death, respectively (29, 30). This ability of deubiquitinases to promote diverse outcomes

demonstrates the complexity and highly regulated nature of ubiquitination in determining survival and cell death signaling.

‘Death receptor’ extrinsic apoptosis signaling

In contrast to the cell survival signaling complex induced by death receptors, formation of an alternative signaling complex, known as the ‘death-inducing signaling complex’ (DISC), results in the induction of cell death through extrinsic apoptosis (12, 31). Like the pro-survival death receptor signaling complex described above, the DISC contains RIPK1, but critically also contains the proteins FAS-associated protein with a DD (FADD), pro-caspase-8 and cFLIP (Fig 2B-C) (32-36). Importantly, the cell death activity of the DISC requires internalization of the signaling complex induced by death receptor activation (37, 38). In this intracellular DISC complex, processing and activation of caspase-8 allows for the cleavage of downstream apoptotic targets. These targets include executioner caspases (caspase-3, -6 and -7) and factors regulating alternative cell death pathways, such as RIPK1, RIPK3 and CYLD (39-42). Executioner caspases go on to irreversibly dismantle the cell through the conserved process of apoptosis, including nuclear fragmentation, changes to plasma membrane polarity and membrane blebbing. In some cell types, termed ‘type II cells’, caspase-8-dependent cleavage of the mitochondrial protein BID and the induction of MOMP is required for inducing extrinsic apoptosis (32, 43, 44). This is in contrast to ‘type I cells’ that do not require MOMP to undergo apoptosis, although it may occur. This represents an overlap in the pathways required for inducing intrinsic and extrinsic apoptosis in some cell types.

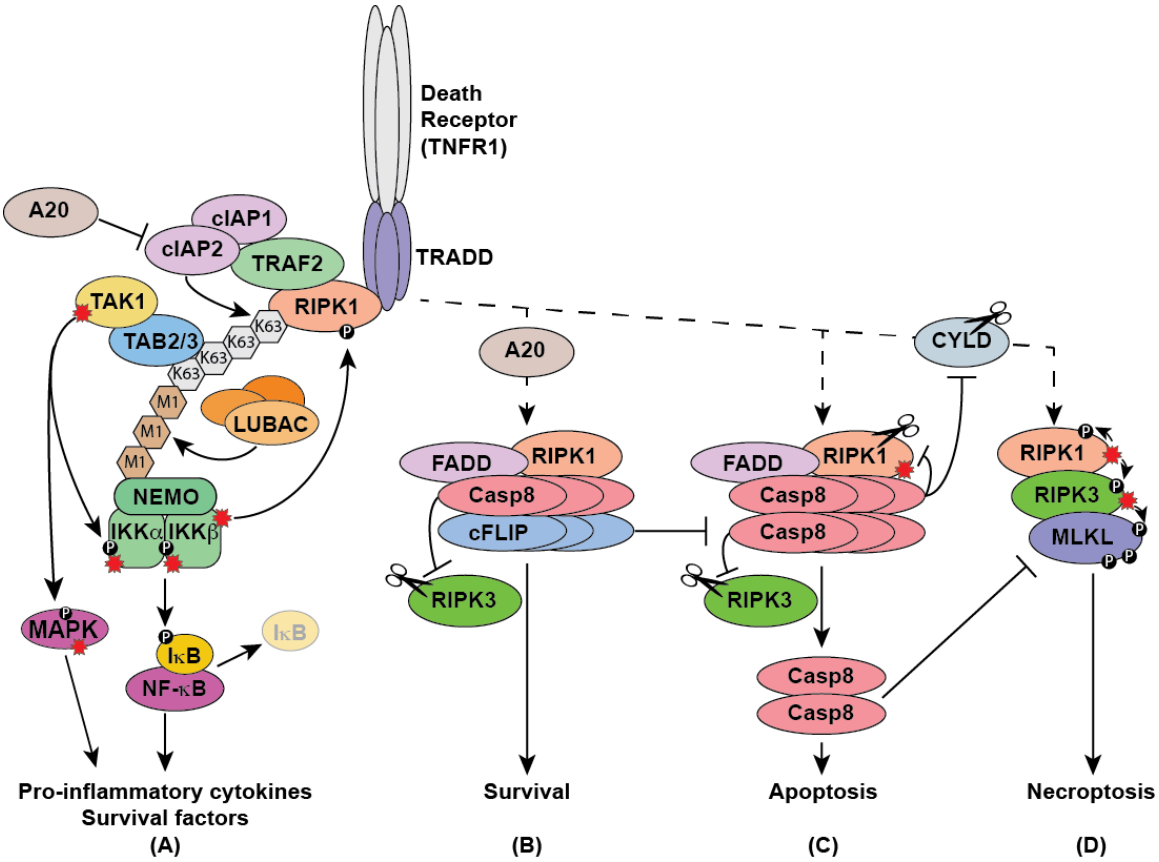

Figure 2. 'Death receptor' survival and cell death signaling.
'Death receptor' ligand binding promotes RIPK1 recruitment and ubiquitination by cIAPs and LUBAC. This allows for the activation TAK1- and IKKα/β-containing complexes. Phosphorylation of IκB promotes its degradation and the release of active NF-κB transcription factors that promote pro-inflammatory cytokine and survival factor expression **(A)**. Parallel pathways promote the activation of MAPK signaling and transcription factors that collaborate with NF-κB. A20 acts as a negative regulator of this signaling complex and deubiquitinates RIPK1 and other proteins to terminate NF-κB and MAPK signaling **(B)**. In the absence of pro-survival factors, such as cFLIP, the kinase activity of RIPK1 promotes caspase-8 processing and activation, leading to apoptosis and the cleavage of RIPK1, RIPK3 and CYLD **(C)**. In the absence of caspase-8 activity, RIPK1 promotes RIPK3 and MLKL activation through phosphorylation, resulting in programmed necrosis or 'necroptosis'. **(D)**. CYLD promotes the deubiquitination of RIPK1, necessary for promoting this response.

The activation of caspase-8 and the formation of the DISC are highly regulated processes. RIPK1 plays an important function in both promoting and inhibiting caspase-8 activity (45) and is discussed in detail later on. cFLIP (cellular FADD-like IL-1β-converting enzyme-inhibitory protein) is a catalytically inactive homologue of caspase-8 that is expressed as either a long (cFLIP(L)) or short (cFLIP(S)) isoform that binds to caspase-8 and prevents apoptosis (32, 46). Importantly, the full-length (uncleaved) caspase-8 more readily forms heterodimers with cFLIP than homodimers with itself, such that caspase-8 likely exists primarily as a heterodimer under non-apoptotic conditions (47, 48). Upon caspase-8 cleavage, homodimers are formed with a higher affinity than that between cleaved caspase-8 and cFLIP. While cFLIP(S) completely blocks the catalytic activity of caspase-8, heterodimers of caspase-8 and cFLIP(L) have the ability to process at least some caspase-8 substrates important for cell survival, particularly RIPK3 (see below)(40, 49, 50). It is not entirely clear whether the caspase-8 and cFLIP(L) heterodimer has a unique specificity for non-apoptotic substrates or whether cFLIP(L) simply restrains the activity of caspase-8 below a threshold necessary for inducing apoptosis (51).

Notably, mice deficient in any of the DISC components described above succumb to embryonic or postnatal lethality (14, 52-55). In the absence of cFLIP, apoptosis is induced by unrestrained caspase-8 activation (55). In contrast, loss of caspase-8 or FADD results in the uncontrolled activation of an alternative cell death pathway known as programmed necrosis or ‘necroptosis’ (52-54, 56). Thus, in addition to the pro-apoptotic function of caspase-8 and FADD in this complex, it also servers a pro-survival function during homeostasis (57-59).

‘Death receptor’ necroptosis signaling

Although necrosis was until recently believed to be an entirely passive process of ‘accidental cell death’, recent advances have highlighted the presence of an active form of programmed necrosis termed ‘necroptosis’ (60, 61). Importantly, the activation of necroptosis depends on the genetic deletion or pharmacologic inhibition of caspase-8 (54, 56, 62, 63), which cleaves and inactivates factors required for necroptosis, including RIPK1, RIPK3 and CYLD (39-42). Also required for the induction of necroptosis is the disassembly of the pro-survival, NF-kB-activating signal complex by the deubiquitinase CYLD (30, 64, 65). Deubiquitination of RIPK1 by CYLD allows for interaction with and phosphorylation of RIPK3 (Fig 2D) (42, 66-68). In turn, RIPK3 phosphorylates mixed linkage kinase domain-like (MLKL) (69-71), which goes on to acts as the executioner of necroptosis by interacting with and permeabilizing the plasma membrane (72-77). Thus, the kinase activities of both RIPK1 and RIPK3 are involved in a chain reaction required for death receptor-induced necroptosis. Selective inhibitors of these kinases have been explored for their potential to limit inflammation-associated cell death (60, 78-80).

Pattern recognition receptor signaling

As a first line of defense against microbial pathogens, innate immune recognition of microbes occurs through germline encoded receptors that recognize conserved microbial features (1). These receptors, termed pattern-recognition receptors (PRRs), include members of the Toll-like receptor (TLR), C-type lectin receptor (CLR), NOD-like receptor (NLR) and RIG-I-like receptor (RLR) families (81). Their signaling results in the expression of pro-inflammatory cytokines and anti-microbial effectors through the activation of transcription factors, including NF-κB, interferon-regulatory factors (IRFs),

and those downstream of MAPK signaling. Recognition of microbial products through PRRs allows for the propagation of an immune response from cells that directly encounter microbial ligands to neighboring cells, necessary for eliciting protective innate and adaptive immune responses (82). Many of the signaling pathways utilized by PRRs share features with those used by death receptors. This includes the activation of similar transcriptional responses and, importantly, the transduction of cell death signals.

Pro-survival response to pattern recognition

Toll-like receptors (TLRs) are membrane-associated receptors that recognize microbial products including bacterial cell wall components (TLR1, TLR2, TLR4, TLR6), bacterial flagellum (TLR5), and viral DNA or RNA (TLR3, TLR7, TLR8, TLR9). TLRs depend on adaptor proteins, MyD88 and TRIF, to mediate their downstream signaling responses (81). While TLR3 and TLR4 are uniquely able to signal through the adaptor TRIF, the rest of the TLRs signal through the MyD88 adaptor. TLR4 is able to pair with MyD88 in addition to TRIF, representing an exception to the other receptors in its ability to pair with more than one adaptor. TLR ligand binding and MyD88 activation result in the recruitment of proteins that promote the activity of TAK1- and IKK-complexes (83-85). These complexes, in turn, phosphorylate target proteins to activate NF-κB and MAPK-dependent transcription factors and the expression of pro-inflammatory cytokines and pro-survival factors. Endosomal TLRs can also activate a set of anti-viral response genes, including type-I interferons and cytokines such as CCL5, that expand the diversity of the response to TLR stimulation (86).

TLR3 and TLR4 signaling through the adaptor TRIF share many features with death receptor signaling pathways. TRIF is able to interact directly with RIPK1 through a

RIP homotypic interaction motif (RHIM) (87). Interactions with TRAF6 promote the ubiquitination of RIPK1 and the formation of a signaling platform analogous to that downstream of death receptors (88). This complex brings together and activates TAK1- and IKK-complexes for the induction of NF-κB and MAPK signaling. Additionally, TRIF interaction with TRAF3 promotes the activation of the transcription factors IFR3 and IRF7, allowing for an additional set of transcriptional responses to be activated downstream of TLR stimulation (86, 89, 90). Interestingly, the subcellular localization of the TLR4 signaling complex regulates its ability to engage this IRF-dependent response. Although TLR4 can signal through MyD88 at the plasma membrane, receptor endocytosis is required for TRIF to interact with TRAF3 and mediate IRF activation (91). Interestingly, this requirement for endocytosis of TLR4 limits the activation of IRF-dependent responses to activation by only intracellular pathogens.

Some cytosolic NLRs, including nucleotide-binding oligomerization domain containing 1 (NOD1) and NOD2, and RLRs, including RIG-I and MDA5, recognize microbial products to promote the activation of overlapping, yet distinct transcriptional programs through NF-κB, MAPK and IRF activation (81). Recognition of microbes by PRRs described thus far relies on ligands that are highly conserved across groups of microbes. Thus, they do not allow for the discrimination between pathogens and commensal microbes. To make this distinction the immune system must recognize unique features of pathogenic microbes, or 'patterns of pathogenesis' (92).

Inflammasome signaling

Recognition of microbial ligands and pathologic cellular signatures by an additional group of cytosolic NLRs, leads to another form of cell death known as

‘pyroptosis’, which is mediated by the so called ‘inflammasome’ complex (93). As cytosolic proteins, NLRs allow cells to recognize either microbial products or pathogenic signatures that offer a more useful distinction between pathogens and commensals than the recognition of conserved structural components alone. By acting as a direct sensor or as an adaptor protein to sensor, NLRs bring together and activate a complex known as the inflammasome, which culminates in the activation of caspase-1 through cleavage from a pro-form to a catalytically active cleaved form. Activation of caspase-1 results in a form of cell death known as pyroptosis and the processing of IL-1 family cytokines, including IL-1α, IL-1β and IL-18 (94). These NLRs specifically recognize pathogens through the recognition of microbial signals associated with virulence activities rather than structural features alone (92). For example, the NLRC4 inflammasome forms in response to the recognition of bacterial flagellin and specific type-III secretion system proteins, through which pathogens access the host cytosol for the delivery of virulence factors that can alter host-signaling response to infection (95-98). As such, the inflammasome has been described as a ‘defender of cytosolic sanctity’, because it relies not only on the presence of microbial products, but on the location of those products in the cytosol, which acts as a proxy for the virulence activity of pathogens over commensals (99).

Pattern recognition receptor apoptosis and necroptosis signaling

The interaction of the TLR3 and TLR4 adaptor, TRIF, with RIPK1 through its RHIM domain allows it to directly couple PRRs to the extrinsic apoptosis and necroptosis signaling pathways (Fig. 3). These responses are activated through the same process as that described for death receptors. When the pro-survival, NF-kB and

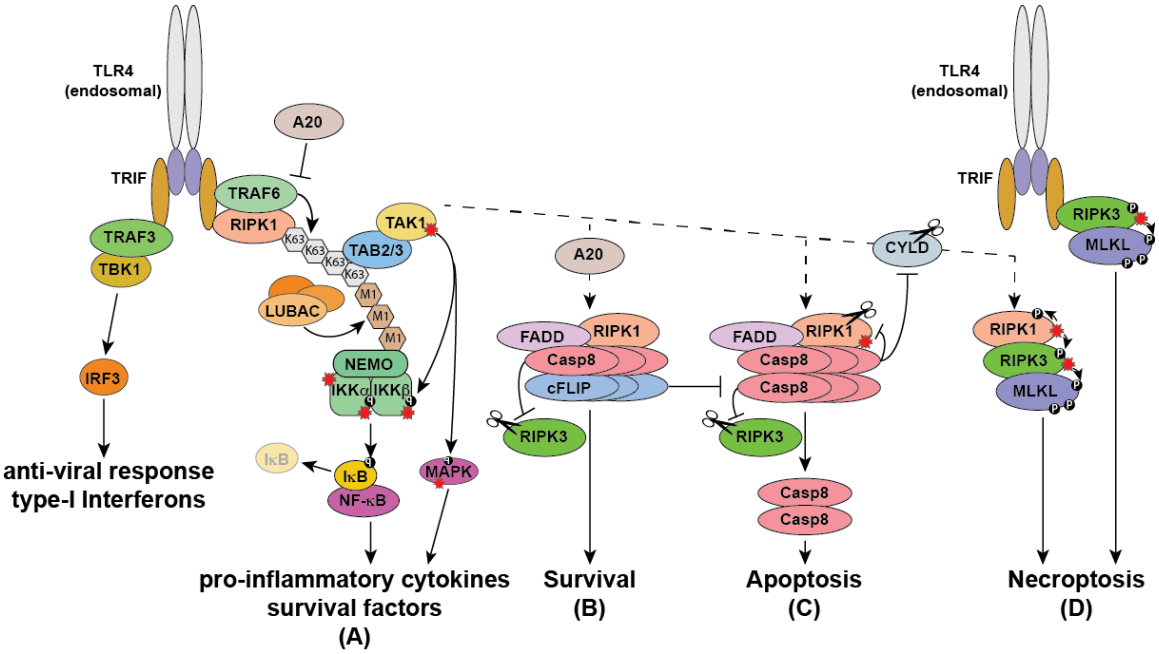

Figure 3. TLR-dependent TRIF signaling promotes survival and cell death responses.
TLR4 binding of LPS and receptor endocytosis promotes RIPK1 recruitment and ubiquitination by TRAF6 and LUBAC. This allows for the activation of TAK1- and IKKα/β-containing complexes. Phosphorylation of IκB promotes its degradation and the release of active NF-κB transcription factors that promote pro-inflammatory cytokine and survival factor expression **(A)**. Parallel pathways promote the activation of MAPK signaling and transcription factors that collaborate with NF-κB. A20 acts as a negative regulator of this signaling complex and deubiquitinates RIPK1 and other proteins to terminate NF-κB and MAPK signaling **(B)**. In the absence of pro-survival factors, such as cFLIP, the kinase activity of RIPK1 promotes caspase-8 processing and activation, leading to apoptosis and the cleavage of RIPK1, RIPK3 and CYLD **(C)**. In the absence of caspase-8 activity, RIPK1 promotes RIPK3 and MLKL activation through phosphorylation, resulting in programmed necrosis or 'necroptosis'. **(D)**. CYLD promotes the deubiquitination of RIPK1, necessary for promoting this response. RIPK3 can also interact directly with TRIF to induce MLKL activation and necroptosis.

MAPK activating complex (described above), fails to form or is disrupted, RIPK1 can form a pro-apoptotic complex with FADD and caspase-8 (13, 14), or a necroptotic-signaling complex with RIPK3 through RHIM domain interactions (87, 100, 101). Thus microbial recognition of TLR3 or TLR4 ligands can directly lead to RIPK1-dependent cell death by either apoptosis or necroptosis. TRIF can also directly bind to RIPK3, through its own RHIM domain (Fig. 3D), to bypass the requirement for RIPK1 in activating necroptosis (101). Interestingly, RIPK3-dependent necroptosis is enhanced in the absence of RIPK1 (102-104). This demonstrates an important theme for RIPK1 in promoting both survival and cell death through diverse functions.

RIPK1 is a central regulator of cell fate signaling

RIPK1 was initially identified as an apoptosis-inducing protein downstream of FAS and TNFR1 that is highly conserved between mice and humans (13, 105). Its multiple domains allow for interactions with a number of important pro-survival and cell death signaling complexes. The C-terminal death domain of RIPK1 allows its interaction directly with death receptors or their adaptor TRADD (13, 105), while its RHIM domain mediates interactions with RIPK3 and the TLR adaptor TRIF (87, 100). Although initially described as a pro-apoptotic factor it was also shown to promote NF-κB signaling (13), which required its own ubiquitination and the recruitment of TAK1- and IKK-containing signaling complexes (discussed above) (16-19). This dual function of RIPK1 suggests that it plays a unique role in regulating multiple responses to death receptor and TRIF signaling (Fig. 4A).

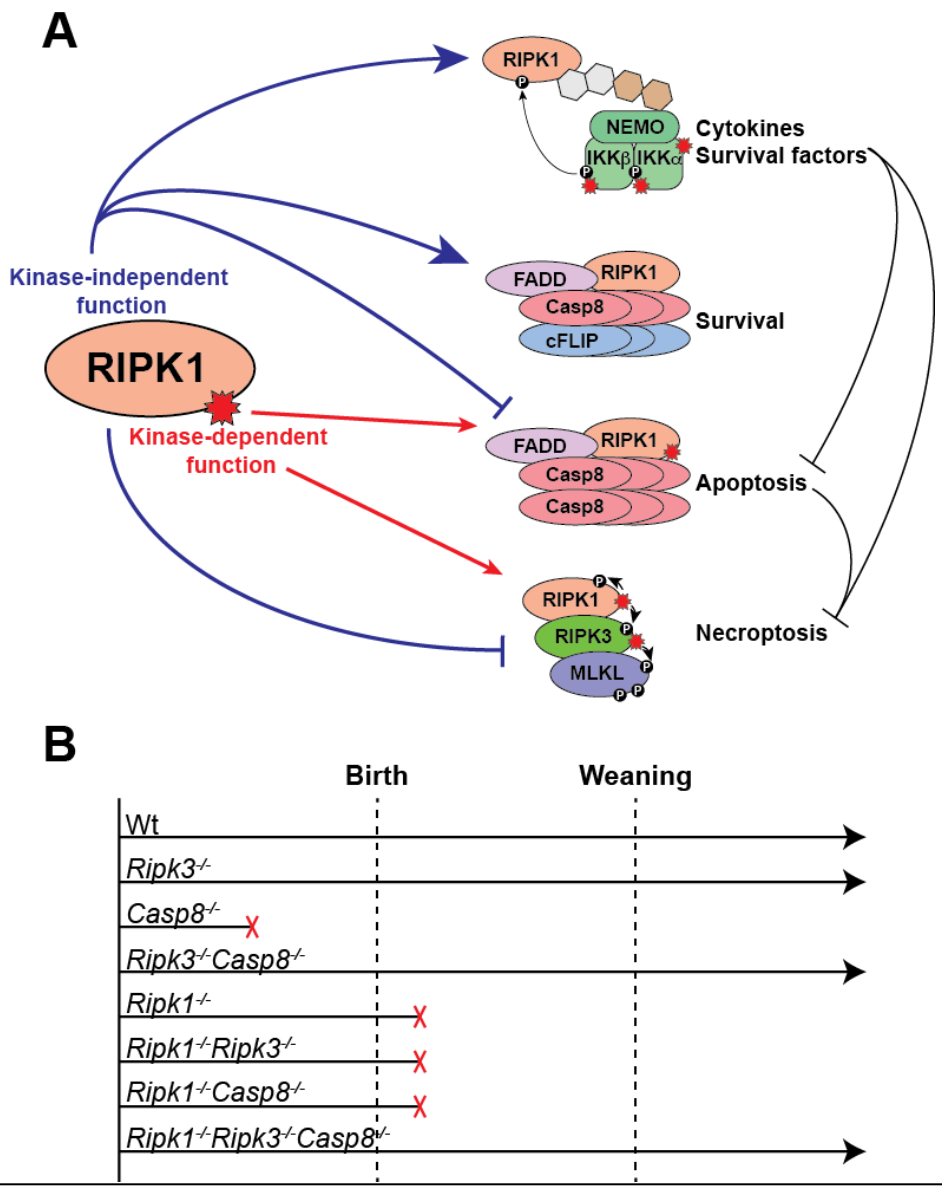

Figure 4. RIPK1 is a central regulator of both pro-survival and cell death signaling.
(A) RIPK1 functions are described as 'kinase-independent' or 'kinase-dependent'. The kinase-independent functions of RIPK1 inhibit both apoptosis and necroptosis, while promoting cell survival and cytokine production through NF-κB and MAPK pathways. In contrast, the kinase function of RIPK1 promotes both apoptosis and necroptosis. **(B)** RIPK-deficient mice ($RIPK1^{-/-}$) die shortly after birth. This lethality is mediated by increased apoptosis and necroptosis, in the absence of the kinase-independent, pro-survival function of RIPK1. Deletion of both RIPK3 and caspase-8 prevents necroptosis and apoptosis, respectively, to protect mice. Adapted from Silke et al. (45).

Genetic deletion of RIPK1 in mice leads to early perinatal lethality, associated with extensive apoptosis in lymphoid and adipose tissues (14). RIPK1-deficient cells were also defective in their ability to activate NF-κB during death receptor signaling (13, 14). This lead to the conclusion that in the absence of RIPK1, important NF-κB-dependent pro-survival gene products failed to be produced, resulting in apoptosis. Crossing RIPK1-deficient mice onto a caspase-8-deficient background provided only minimal protection against lethality, despite preventing apoptosis (Fig. 4B) (102, 103). Strikingly, RIPK1-, Caspase-8- and RIPK3-triple deficient mice were protected from the lethality seen in RIPK1 mice (102-104). Thus, while RIPK1 can promote caspase-8-dependent apoptosis and RIPK3-dependent necroptosis, the absence of RIPK1 also induces both of these responses.

In contrast to the lethality seen in mice completely lacking RIPK1, targeted mutations in the kinase domain of the RIPK1, termed ‘kinase dead’, have no effect on mouse survival or the cell death-independent activation of NF-κB and MAPK responses to death receptor and TLR signaling (78, 106, 107). Furthermore, cells from these kinase dead RIPK1 mice or cells treated with the RIPK1 kinase inhibitor, necrostatin-1, are resistant to apoptosis and necroptosis (60, 106-108). Thus, the kinase activity of RIPK1 seems to mediate its pro-apoptotic and pro-necroptotic functions, while pro-survival NF-κB and MAPK signaling are mediated by kinase-independent functions of RIPK1 (Fig 4A).

Notably, only some forms of caspase-8-dependent apoptosis seem to be promoted by RIPK1 (109). The independence of some forms of apoptosis from RIPK1 is consistent with the fact that RIPK1-deficient cells readily undergo apoptosis *in vivo* that is partially TNFR1-dependent (110). The differential requirement for RIPK1 in promoting

apoptosis suggests the presence of two distinct pro-survival checkpoints following death receptor stimulation (111). Treatment of cells with TNF and SMAC mimetics, which inhibit the ubiquitinase activity of cIAPs and destabilize pro-survival signaling, results in RIPK1-dependent apoptosis (109). Recent work suggests that this early pro-survival checkpoint in death receptor signaling is independent of NF-κB signaling (46, 112-114). In contrast, treatment of cells with TNF and cycloheximide, which inhibits protein translation, results in apoptosis that is independent of RIPK1 but inhibited by NF-κB-dependent pro-survival responses, such as the expression of cFLIP (46, 109). Thus, extrinsic apoptosis is regulated at multiple checkpoints, the disruption of which may result in distinct mechanisms of caspase-8 activation. These distinct pathways may have functional differences that influence how immune cells respond to the recognition of apoptotic cells.

Immune recognition of cell death

Recognition of cell death by the immune system can have a profound effect on the immune system through either the promotion or dampening of inflammation depending on the context (5, 115). Understanding these cell death-associated signals and the responses they promote may provide novel means of targeting infection, inflammatory disease and wound healing.

Dead cell clearance by phagocytes

Phagocytic cells of the innate immune system bear the bulk of the burden in the clearance of dead cells from the body. These macrophages and dendritic cells rely on so called ‘find-me’ and ‘eat-me’ signals released by dead or dying cells to perform their

function (115). ‘Find-me’ signals act as chemoattractants to recruit phagocytes, while ‘eat-me’ signals instruct phagocytes to engulf and clear target cells. Efficient removal of cells once they have died is particularly important for maintaining immune tolerance. The failure to clear apoptotic cells can result in secondary necrosis and the release of many inflammatory signals, capable of breaking tolerance to self antigens and inducing autoimmunity (115, 116).

During the process of apoptosis, signals are released to instruct the recruitment of neighboring phagocytic cells. These ‘find-me’ signals include nucleotides, such as ATP (117, 118), and bioactive lipids, such as lysophosphatidylcholine and sphingosine-1-phosphate (119, 120). The release of these signals from cells is regulated as an active part of the apoptotic response. For example, release of ATP from cells undergoing apoptosis is mediated by caspase-3 activated membrane channels (117). In the setting of necrosis, or other cell death pathways that involve plasma membrane permeabilization, release of many additional cellular contents occurs passively due to membrane lysis (121).

Once a phagocyte is in physical contact with a target, it must integrate a number of signals to decide whether to eat or not to eat. Encounters with normal healthy cells are accompanied by signals that inhibit clearance by phagocytes (122, 123). The simultaneous downregulation of these ‘don’t-eat-me’ signals, along with the recognition of ‘eat-me’ signals results in the initiation of cell clearance. Important focus has been placed on the composition and polarity of the plasma membrane leaflets in regulating phagocyte uptake of eukaryotic cells. In healthy resting cells, phosphatidylserine (PtdSer) is restricted to the inner layer of the plasma membrane by the activity of enzymes termed ‘flipases’ (124, 125). During apoptosis, caspase-dependent inactivation

of these flipases and activation of 'scramblases' leads to the exposure of PtdSer on the surface of apoptotic cells (126, 127). Recognition of PtdSer then acts as an instructive 'eat-me' signal to phagocytes (128). Another important 'eat-me' signal recognized on apoptotic cells is calreticulin, which is translocated from the endoplasmic reticulum to the cell surface as part of the endoplasmic reticulum stress response (123, 129, 130). In addition to promoting phagocytosis, PtdSer and calreticulin also drive tolerogenic or inflammatory immune responses to cell death, respectively.

Anti-inflammatory response to apoptosis

The majority of cellular turnover in the body is mediated by apoptotic cell death. As an integral part of immune homeostasis, this turnover is associated with anti-inflammatory and tolerogenic signaling (Fig. 5) (115). In the absence of pro-inflammatory mediators, apoptotic cell death is generally characterized as an immunosuppressive process. Recognition of apoptotic cells by engulfing phagocytes elicits the production of IL-10 and TGF-β (131, 132), as well as suppressing the expression of pro-inflammatory cytokines, such as IL-12 (133). These effects of apoptotic cells are mediated in part by PtdSer recognition, which can occur directly through numerous scavenger receptors or indirectly through molecules that engage Tyro3-Axl-Mer (TAM) receptors (132, 134). By dampening the production of pro-inflammatory cytokines and promoting production of IL-10 and TGF-β, apoptotic signals limit the priming of productive self-reactive T cell responses. This occurs through both the generation and expansion of $CD4^+$ regulatory T cells (135, 136) and the deletion of self-reactive $CD8^+$ T cells (137). Thus, homeostatic turnover of cells provides a continuous source of self-antigens for promoting peripheral tolerance.

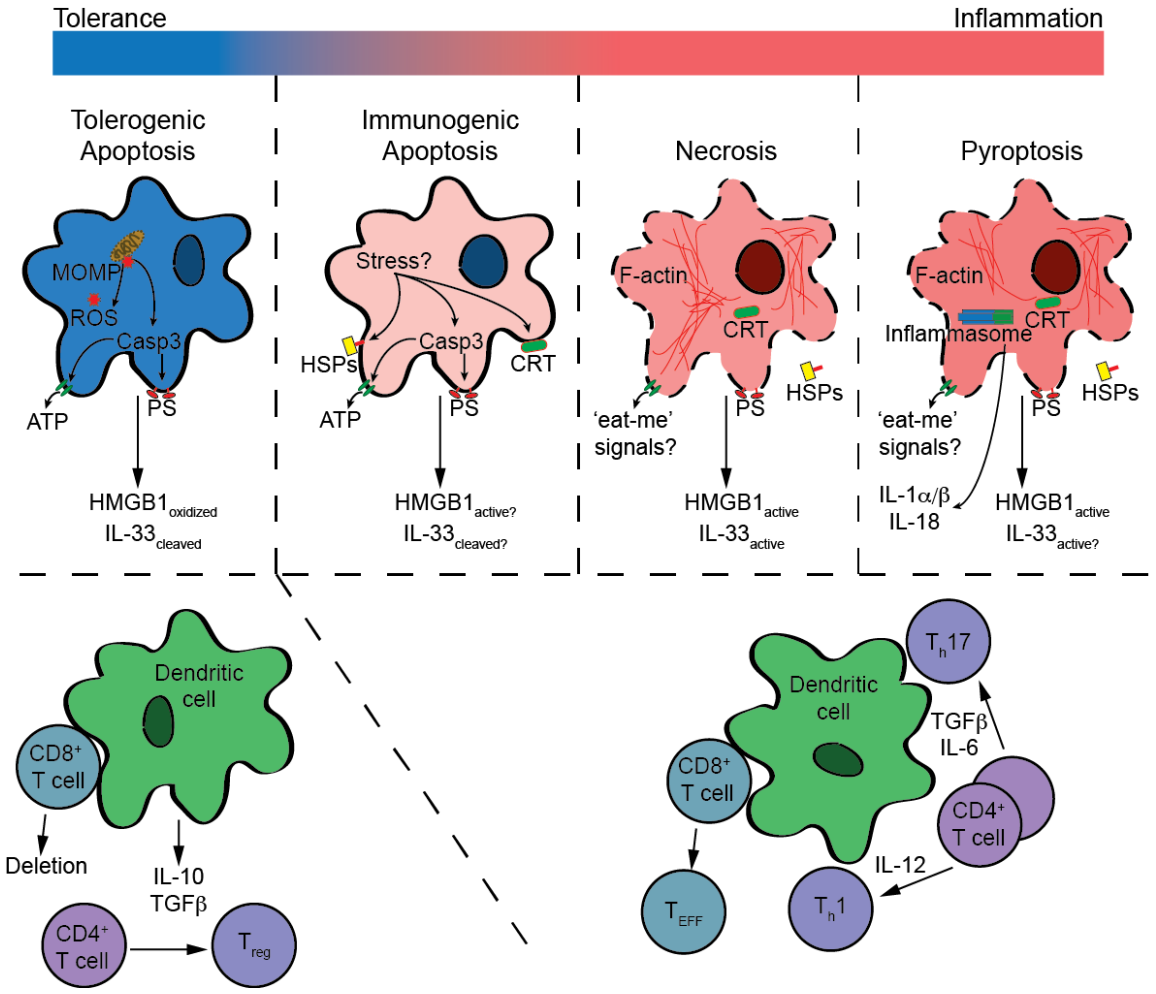

Figure 5. Diverse cell death-associated signals promote tolerogenic and inflammatory immune responses.
Cell death occurs along a continuum of tolerogenic to inflammatory. Apoptosis that occurs during homeostasis is tolerogenic and is not associated with inflammatory DAMPs. Released HMGB1 is inactivated by oxidation and IL-33 is cleaved by apoptotic caspases. Apoptosis can also be immunogenic under settings of cellular stress that results in the exposure of heat shock proteins (HSPs) and calreticulin (CRT). HMGB1 may be active, despite sensitivity to oxidation during apoptosis, and IL-33 is likely cleaved by apoptotic caspases. Necrotic and pyroptotic cell death result in the exposure of many cellular contents including F-actin. HMGB1 is released in an active form and IL-33 is not efficiently processed by caspase-1 during pyroptosis. Recognition of tolerogenic apoptosis promotes IL-10 and TGF-β production that promotes T_{reg} differentiation and self-reactive $CD8^+$ T cell deletion. Immunogenic cell death signals promote the generation of inflammatory T_h1 and T_h17 responses.

Breaking tolerance through necrotic cell death signals

In contrast to the anti-inflammatory signals associated with homeostatic apoptosis, alternative forms of cell death can elicit overt inflammatory responses. Inflammatory signaling in the context of cell death is most clearly implicated in the setting of necrosis, where membrane permeabilization results in a spillage of cellular contents into the surroundings (Fig. 5). The recognition of intracellular contents can serve as a proxy for cellular damage or 'danger', promoting the description of these signals as damage-associated molecular patterns (DAMPs) (138). These DAMPs provide a signal analogous to the recognition of foreign microbial products, in some cases sharing some of the same receptors (81, 139), and promote the production of pro-inflammatory cytokines from phagocytes and neighboring cells.

The prototypical DAMP released from necrotic cells is high mobility group box 1 (HMGB1). This nuclear protein is normally bound to DNA and chromatin, but is released following necrosis to bind the receptor for advanced glycation end products (RAGE), TLR4 and TLR2 (140-143). During apoptosis it has been proposed that HMGB1 is either not released due to tight interactions with nuclear chromatin (143) or inactivated through oxidation to prevent its ability to stimulate inflammatory responses (144). Under these circumstances it may actually have tolerogenic effects. In addition to HMGB1, other DAMPs have been described to have inflammatory effects on neighboring cells when released during necrosis. These include, but are not limited to, S100 proteins, heat shock proteins, uric acid and F-actin (139, 145, 146).

Cells that die by caspase-1-dependent inflammasome activation and pyroptosis resemble necrotic cells in the loss of plasma membrane integrity and the release of intracellular DAMPs. However, they additionally release IL-1 family cytokines that are

cleaved by catalytically active caspase-1 in the inflammasome (94). These cytokines, including IL-1α, IL-1β, IL-18 and IL-33 are released from cells that have undergone necrosis and pyroptosis (Fig. 5). IL-33 and IL-1α are bioactive in their uncleaved form, although IL-1α cleavage increases its activity (147-151). In contrast, IL-1β and IL-18 are dependent upon cleavage to promote inflammatory signaling through receptor binding (149, 152, 153). Interestingly, IL-33 is inactivated through cleavage by apoptotic caspases and high concentrations of active caspase-1 (150, 151). Thus it is possible that the relative concentration or balance of these cytokine signals allows neighboring cells to differentiate between cells that have died by apoptosis, necrosis, or pyroptosis and respond appropriately.

Immunogenic cell death – an inflammatory form of apoptosis

Although apoptosis has generally been characterized as immunosuppressive and tolerogenic under settings of homeostasis, some apoptotic stimuli can elicit an inflammatory immune response (Fig. 5) (6). This 'immunogenic cell death' is likely a means of immune activation in diverse settings of inflammation, but has been most extensively studied in the context of the cancer and is often defined based on the ability of cell death stimuli to elicit an anti-tumor response (154). Central to the induction of immunogenic cell death is the ability of certain apoptotic stimuli to promote the release or exposure of DAMPs by dying cells. One such DAMP that can confer properties of immunogenic cell death is the cell surface exposure of the endoplasmic reticulum protein calreticulin (155). The most prominent inducers of surface calreticulin include chemotherapeutics or therapies that promote endoplasmic reticulum stress (129, 130). This ability of perimortem stresses to promote the recognition of apoptotic cells as

'danger' by phagocytic cells could be important in settings other than cancer, such as infection or inflammatory disease.

During infection, cell death-associated signals may be accompanied by microbial signals that act in concert to generate novel immune responses. The recognition of apoptotic neutrophils along with LPS during infection with enteropathogenic *E. coli* results in the generation of bacteria- and self-reactive $CD4^+$ T_h17 response (156, 157). This response is mediated by the combination of apoptotic cell-dependent TGF-β and LPS-dependent IL-6 production that act together to induce T_h17 differentiation (158-160). In contrast, recognition of apoptotic neutrophils alone generates a TGFβ-dependent regulatory T cell response (156). Thus, signals that accompany the recognition of apoptotic cells can modify an otherwise regulatory immune response. Whether there are similar situations in which accessory signals could modify the response to apoptotic cells in a way that promotes T_h1, T_h2 or innate immune responses has yet to be extensively explored.

Cell death and *Yersinia* infection

The inherent stresses of mounting an immune response to a pathogen promote a significant increase in the turnover of cells. Furthermore, cell death is often a host response to pathogen recognition or result of pathogen virulence factors that target host cells. These cell death responses are diverse and highly context dependent, but the use of model systems to dissect the possible host protective versus pathologic effects of various cell death pathways during infection can promote the understanding of the evolutionary pressures that shaped the relationship between cell death, inflammation and immunity (5).

Regulation of *Yersinia*-induced death

Yersinia species are gram-negative zoonotic bacterial pathogens that include *Y. pestis*, *Y. pseudotuberculosis*, and *Y enterocolitica*. *Y. pestis* is the causative agent of both pneumonic and bubonic plague, while *Y. pseudotuberculosis*, and *Y. enterocolitica* are enteric pathogens. All three pathogenic *Yersiniae* species share a highly conserved virulence plasmid, encoding a bacterial type-III secretion system and a group of virulence factors known as *Yersinia* outer proteins (Yops) (161). These factors are injected into host target cells and play an important function in inhibiting facets of phagocyte antimicrobial activity and innate immune signaling. Importantly, these virulence factors also regulate cell death of infected host cells.

The YopJ protein of *Y. pestis* and *Y. pseudotuberculosis*, termed YopP in *Y. enterocolitica*, is an acyl-transferase that belongs to a family of secreted virulence factors injected into host cells by bacterial pathogens that infect plants, insects and higher eukaryotes (162-164). The activity of YopJ inhibits the phosphorylation-dependent activation of members of the MAPK kinase superfamily, including IKKα/β and TAK1 (Fig. 6). This results in a block in NF-κB and MAPK signaling downstream of bacterial recognition through TLRs (162, 163, 165). This inhibition of NF-κB and MAPK signaling results in not only a failure to induce key pro-inflammatory cytokines, but also cell death, due to a loss of pro-survival signals (166-170).

YopJ-dependent cell death is associated with features of apoptosis, including membrane blebbing, nuclear condensation, DNA fragmentation and cytoplasmic vacuole formation (166, 171). It is also dependent on components of the extrinsic apoptosis pathway, including caspase-8, FADD and RIPK1 (172-174). Furthermore, it has been shown that TLR4 and the adaptor TRIF, but not MyD88, promote this response (174-

177). Thus, YopJ-dependent inhibition of survival signals in the context of TLR4 and TRIF activation promotes extrinsic apoptosis of *Yersinia*-infected cells. Interestingly, however, TLR4 and TRIF deficient bone marrow derived macrophages (BMDMs) still undergo a significant, albeit reduced level of cell death compared to wild-type cells (173-177). This observation suggests that additional signaling pathways contribute to *Yersinia*-induced cell death, which have not been explored.

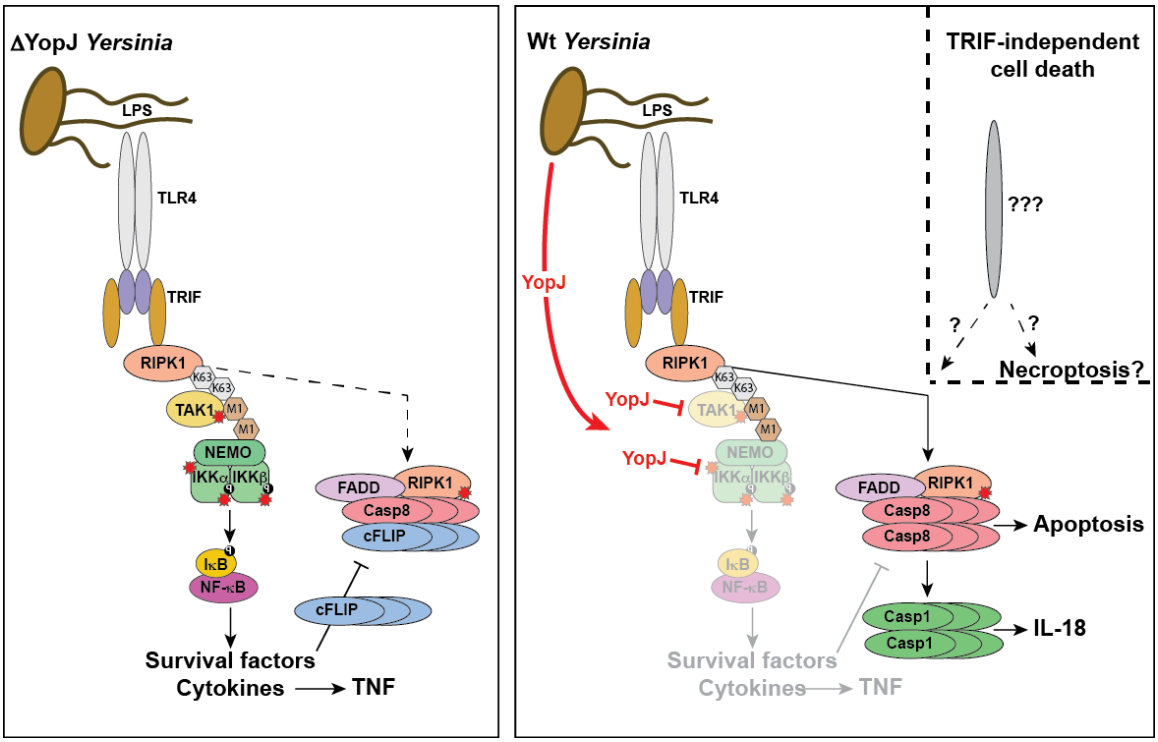

Figure 6. Regulation of *Yersinia*-induced apoptosis.
In response to LPS or YopJ-deficient *Yersinia* (ΔYopJ), TLR4-TRIF signaling promotes cytokine production, including TNF. Pro-survival responses, such as the expression of cFLIP, inhibit caspase-8 activation and apoptosis. The virulence factor YopJ acts as an acyl-transferase to inhibit TAK1 and IKKs during TLR4-TRIF signaling. This results in the activation of capsase-8-dependent apoptosis. This apoptotic response is associated with the activation of caspase-1 and release of IL-18. TLR4- and TRIF-deficient cells undergo TRIF-independent cell death that may be mediated by apoptosis or an alternative cell death response, such as necroptosis.

Inflammasome activation and pyroptosis have also been implicated in *Yersinia*-induced death (178, 179). Recognition of the *Yersinia* type-III secretion system activates the NLRP3 and NLRC4 inflammasomes, resulting in pyroptosis (180). Interestingly, the virulence factor YopK inhibits this response, potentially by limiting the translocation of virulence factors into the host cell (180, 181). Thus, cells infected with YopK-deficient bacteria, instead of undergoing apoptosis, undergo pyroptosis. Treating cells with LPS or inflammatory cytokines results in the production of inflammasome components, particularly NLRP3, that prime cells to respond to *Yersinia* through pyroptosis (178). This priming also promotes the expression of anti-apoptotic and pro-survival factors that limit the induction of YopJ-induced apoptosis. Therefore, while unactivated or naïve cells undergo apoptosis in response to *Yersinia* infection, activated cells survive or undergo pyroptosis (178, 180). This may be particularly important *in vivo*, where cells recruited to sites of infection may be activated by either bacterial LPS or cytokine signals prior to encountering live *Yersinia* and highlight the importance of a cell's prior exposures in regulating cell death responses.

YopJ-dependent apoptosis in naïve macrophages is also associated with caspase-1 cleavage and the release of IL-18 (180, 182, 183). Surprisingly, this caspase-8-dependent capsase-1 processing occurs independently of inflammasome components, including NLRP3 and ASC (174). Thus, at least two distinct pathways of caspase-1 activation exist during *Yersinia* infection. One occurs in the absence of the virulence factor YopK and requires NLRP3- and NLRC4-dependent inflammasome activation, while the other occurs in response to YopJ-dependent inhibition of NF-κB and MAPK signaling. This second pathway is consistent with the activation of caspase-1 and IL-1 family cytokine release following genetic or pharmacologic inhibition of IKKβ (174, 184).

The ability of caspase-1 activation to occur as part of apoptotic response to *Yersinia* infection may provide an inflammatory module of apoptosis to promote immunity in response to pathogen inhibition of innate immune signaling.

The induction of YopJ-induced apoptosis has, thus far, focused on signaling mediated by the recognition of *Yersinia* by PRRs, specifically TLR4 (Fig. 6). However, the presence of cell death in TLR4- and TRIF-deficient BMDMs demonstrates that additional pathways of inducing *Yersinia*-induced cell death must exist (173-177). The importance of alternative pathways of cell death may be highlighted by the fact that during *in vivo* infection, *Yersinia* alters its LPS to a form that is poorly recognized by TLR4 (185-187). It is possible that additional signals may compensate for the induction of apoptosis in this setting. In fact, TNFR1-deficient mice have decreased splenocyte apoptosis during infection, suggesting that TNF may play a role in this response (188). The block in TNF production by YopJ-dependent inhibition of cell signaling, however, raises the question of where such a TNF signal may arise. In the third chapter of this thesis, we investigate this question and find an important role for cell-extrinsic TNF in promoting apoptosis of YopJ-inhibited cells. Interestingly, the source of TNF during *in vitro* infection is a population of cells that escape YopJ injection and inhibition. Understanding these pathways that induce cell death during *Yersinia* infection will provide insight into how inflammation may contribute to cell death in a number of pathologic settings.

Consequences of *Yersinia*-induced cell death

Yersinia suppression of cytokine production by its virulence factor YopJ limits the activity of the innate immune response to infection. In fact, mice infected with lethal

doses of virulent *Yersinia* species display limited elevation in systemic cytokines, despite significant bacterial burdens in many tissues, until shortly before succumbing to death (189, 190). Consistent with the activity of YopJ in promoting bacterial virulence, *in vivo* infection with YopJ-deficient *Yersinia* (ΔYopJ) results in delayed colonization and an increased LD_{50} (Fig. 7A) (191, 192). This effect of YopJ was limited to enteric infection. Following intraperitoneal infection the loss of YopJ did not influence bacterial virulence, suggesting that the effects of YopJ are particularly important in promoting dissemination from the intestine rather than replication at sites of infection. It is also possible that the effects of YopJ on promoting bacterial virulence are mediated by its ability to induce apoptosis of cells that it encounters. The disruption of the intestinal barrier through apoptosis may potentiate pathogen dissemination (193, 194). Furthermore, elimination of innate immune cells, such as macrophages and dendritic cells, may be a mechanism by which *Yersinia* inhibits both innate and adaptive host immunity.

In contrast to studies showing a benefit of YopJ to bacterial virulence, replacing YopJ in *Yersinia pseudotuberculosis* with its ortholog, YopP, from *Yersinia enterocolitica* results in decreased bacterial virulence. Although it has similar enzymatic activity as YopJ, YopP is injected into cells at a higher rate, promoting greater apoptosis *in vitro* and *in vivo* (195, 196). Increased inhibition of host signaling and apoptosis, thus, correlates with decreased virulence (Fig. 7A). These studies suggest the ability of apoptosis to promote virulence at a normal dose and inhibit virulence at a higher dose. Thus, there appears to be an optimal range in which the costs and benefits of apoptosis and inhibition of host signaling are balanced to promote bacterial virulence over host protection. This would also suggest that apoptosis itself could be driving host immunity by stimulating inflammation or promoting the clearance of bacteria.

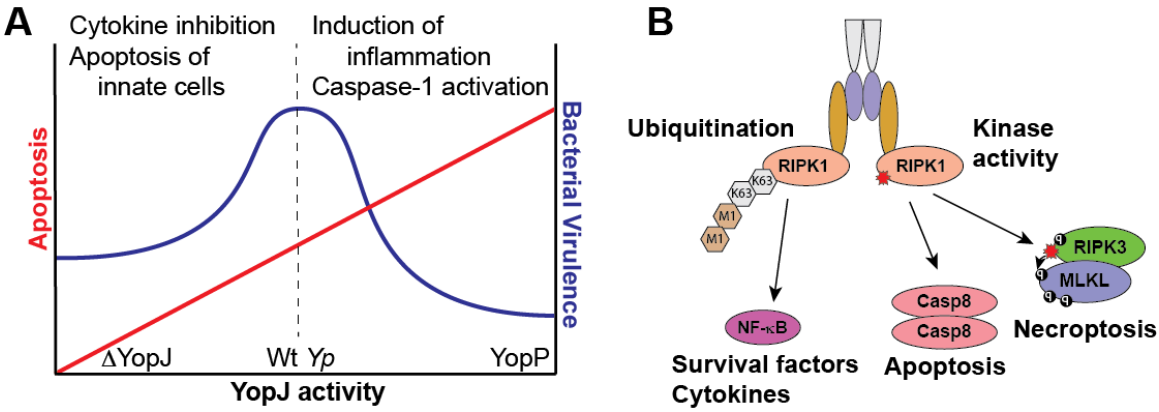

Figure 7. *In vivo* consequences of *Yersinia*-induced apoptosis.
(A) YopJ-deficient *Yersinia* have delayed dissemination and decreased virulence that correlates with decreased apoptosis during infection. The *Y. enterocolitica* homologue of YopJ, YopP, is injected in higher amounts and promotes increased apoptosis. This results in decreases bacterial virulence, despite increased inhibition of host signaling. Effects of YopJ may be evolutionarily balanced with host protective effects of apoptosis. **(B)** Pro-survival and cytokine signaling effects of RIPK1 can be separated from its kinase-dependent cell death signaling. Ubiquitination of RIPK1 promotes NF-κB and MAPK signaling but is not affected by disruption of the pro-apoptotic and –necroptotic kinase function.

Although informative, the pleotropic role of YopJ in directly inhibiting the production of pro-inflammatory cytokines and inducing apoptosis complicates the interpretation of the studies described thus far. A more direct approach at testing the role of apoptosis would target the induction of *Yersinia*-induced cell death independent of YopJ inhibition of cell signaling. This approach, however, is itself complicated by the dual role that known regulators of *Yersinia*-induced apoptosis play in promoting both transcriptional pro-inflammatory responses and regulating cell death signaling pathways. While TLR4, TRIF and TNFR1 are responsible for inducing YopJ-dependent apoptosis (173-177), they also play essential roles in stimulating innate immune responses during infection. Additionally, attempts to target *Yersinia*-induced apoptosis through regulation

of caspase-8, have demonstrated a complex relationship between the signaling pathways that regulate apoptosis and the production of pro-inflammatory cytokines downstream of PRRs (173, 174).

Yersinia-induced apoptosis is completely abrogated in cells deficient for Caspase-8 and RIPK3 (*Casp8*$^{-/-}$*Ripk3*$^{-/-}$) (173, 174), suggesting that comparing *Ripk3*$^{-/-}$ and *Casp8*$^{-/-}$*Ripk3*$^{-/-}$ mice may serve as a useful model for dissecting the role of apoptosis during infection. Strikingly, infection of *Casp8*$^{-/-}$*Ripk3*$^{-/-}$ bone marrow (BM) chimeras with *Y. pseudotuberculosis* or intact *Casp8*$^{-/-}$*Ripk3*$^{-/-}$ mice with *Y. pestis* results in increased bacterial burdens compared to either wild-type or *Ripk3*$^{-/-}$ mice (173, 174). These changes in infection were accompanied by decreased cytokine expression by *Casp8*$^{-/-}$*Ripk3*$^{-/-}$ cells *in vivo*. Infection or TLR stimulation of *Casp8*$^{-/-}$*Ripk3*$^{-/-}$ and *Fadd*$^{-/-}$ *Ripk3*$^{-/-}$ BM derived macrophages similarly demonstrated defects in cytokine expression that were independent of cell death. These findings suggest a cell-intrinsic role for caspase-8 in regulating cytokine expression (173, 174) (Philip et al., 2016 in press). In fact, this has now been definitively demonstrated through the infection of mice reconstituted with a mixture of Wt and *Casp8*$^{-/-}$*Ripk3*$^{-/-}$ BM, in which cytokine expression by *Casp8*$^{-/-}$*Ripk3*$^{-/-}$ cells is not rescued by the presence of Wt cells (Philip et al. 2016, in press). Thus, while targeting caspase-8 function provides insight into the regulation of cell-intrinsic signaling responses, this additional function of caspase-8 confounds the use of this model for isolating the effects of apoptosis during *Yersinia* infection. Given the previous challenges of isolating the role of YopJ-dependent apoptosis during *Yersinia* infection, the exact consequences of this cell death response on host immunity remain to be determined. A more targeted approach of separating apoptosis from other closely

coupled signaling responses will have to dissociate the two pathways at or below the level of caspase-8.

RIPK1 is a pleotropic regulator of both pro-survival and cell death signaling. It is required as a substrate for ubiquitination in the activation of NF-κB and MAPK signaling downstream of death receptors and TLRs (Fig. 6B) (13, 14, 87). It is also a negative regulator of both apoptosis and necroptosis (102-104). The pro-survival functions of RIPK1 have been attributed to its kinase-independent activity. In contrast, its catalytic function has been demonstrated to have an isolated effect on promoting apoptosis and necroptosis (Fig. 7B) (14, 108, 197). Thus, targeted disruption of RIPK1 kinase activity, as has been done in several *in vivo* models (106, 107, 198, 199), could serve as an invaluable tool for dissociating cell death from other cell-intrinsic signaling pathways during *Yersinia* infection. In chapter four of this thesis, we will use RIPK1 'kinase-dead' mice to test the functional consequences of *Yersinia*-induced apoptosis in the regulation of host immunity to infection. Our results demonstrate a protective role for RIPK1 kinase activity *in vivo* and suggest that *Yersinia*-induced apoptosis promotes host protective anti-bacterial inflammation.

CHAPTER 2

Materials and Methods

Mice

C57BL/6.SJL, *Tnfrsf1a*^{tm1Imx} (200) and *Tnfrsf1a*^{tm1Mak} (201) (*Tnfr1*$^{-/-}$), and *Trif*^{lps2} (202) mice were obtained from Jackson Laboratories. Ripk1 kinase-dead (*Ripk1*^{kd}) mice with K45A mutation (106) were provided by GlaxoSmithKline. *Trif*$^{-/-}$ and *Trif*$^{-/-}$*Tnfr1*$^{-/-}$ mice (103) were provided by Doug Green and T. Devi-Kanneganti (St. Jude Children's Research Hospital). Ripk3$^{-/-}$ mice were provided by Vishva M. Dixit (Genentech). Age- and sex-matched eight- to twelve-week old mice were used for all non-bone marrow (BM) chimera experiments. For generation of bone marrow chimeric mice, eight- to ten-week-old mice were lethally irradiated with 1100 rads, as either a single dose or split into two doses 24 hours apart. 3-5 x 10^6 congenic bone marrow (BM) cells were transferred i.v. by retro-orbital injection. Chimeras were allowed to reconstitute for nine to twelve weeks. All experiments were performed under Institutional Animal Care and Use Committee (IACUC) approved protocols and in accordance with the guidelines of the IACUC of the University of Pennsylvania.

Cell Culture and *in vitro* Infections.

Bone marrow-derived macrophages (BMDMs) were grown as described (174) in non-tissue culture (TC) treated petri dishes in a 37°C humidified incubator in DMEM supplemented with 10% FBS, HEPES, sodium pyruvate (complete-DMEM) and 30% L929 supernatant for 7-9 days. 16-20 hours prior to infection cells were harvested with cold PBS and re-plated into 96-well (7-10 x 10^4 cells/well), 48-well (1.5-2 x 10^5 cells/well)

or 12-well (5 x 10^5 cells/well) TC treated (LDH, western blot) or non-TC treated (flow cytometry) plates in complete-DMEM containing 10% L929 supernatant. Cells were treated with relevant inhibitors 1 hour prior to infection or stimulation: 100 μM zVAD-fmk (zVAD; SM Biochemicals), 60 μM necrostatin-1 (Nec-1; Calbiochem), 3 μM GSK2399872A (GSK'872; GlaxoSmithKline), 50μM TAPI-2 (Sigma), or 80μM dynasore (Sigma). For TLR ligand stimulation cells were treated with Pam3CK (1μg/mL), poly(I:C) (50μg/mL), CpG (1μg/mL), or LPS (50ng/mL).

Bacterial infections: Bacterial strains are described in Table S2. Bacteria were grown overnight with aeration in 2×YT broth at 26 °C. *Yersinia pseudotuberculosis* (*Yp*) were diluted into inducing media (2×YT containing 20mM sodium oxalate and 20mM $MgCl_2$) and grown with aeration for 1 h at 26 °C followed by 2 h at 37 °C. Bacteria were washed three times with warm complete-DMEM, added to BMDM cultures at a multiplicity of infection (MOI) of 20:1, unless otherwise noted, and spun onto cells at 1000 rpm for 5min. Cells were incubated at 37 °C and gentamicin (100 μg/ mL) was added 1 h after infection.

Strain name or genotype	**Description**	**Source or Reference**
IP2666 (Yp)	Wild type *Yersinia pseudotuberculosis* serogroup O:3 strain	(203)
IP26 (ΔYopJ)	IP2666 YopJ-deficient strain	(182)
32777	Wild type *Yersinia pseudotuberculosis* serogroup O:1 strain (previously known as IP2777)	(194) Provided by Jim Bliska.
327	YopJ-deficient *Yersinia pseudotuberculosis* serogroup O:1 strain	(174)

In vitro cell death assays.

Lactate dehydrogenase (LDH) release: BMDMs were plated into TC treated 96-well plates at 7 x 10^4 cells/well in complete DMEM containing 10% L929 supernatant. Lactate dehydrogenase (LDH) release was measured from cell supernatants of infected cultures and quantified using the Cytotox96 Assay Kit (Promega) according to manufacturer's instructions and as previously described (174). Cytotoxicity was normalized to Trition X-20 treated (100%) and control treated cells (0%).

Flow cytometry: Cells were plated in non-TC treated 48- or 12-well plates and infected as above. Cells harvested with cold PBS containing 2% EDTA at 2 hours post-infection and quickly stained with Zombie Yellow™ Fixable Viability Kit (Biolegend) prior to fixation and permeabilization (BD Cytofix/Cytoperm™ Kit). Cells were then stained for CD45.2 and CD45.1 (Biolegend), where indicated, and for intracellular TNF (Biolegend) and cleaved caspase-3 (Cell Signaling #9661), followed by secondary anti-rabbit Alexa Fluor 488 or Alexa Fluor 568 (Invitrogen). Samples were run on an LSRII or LSRFortessa and analyzed using FlowJo Treestar software.

CCF4-AM Injection Assay.

BMDMs were infected with YopJ-deficient bacteria complemented with beta-lactamase linked YopJ or GST control expressing plasmid (pACYC). At 1 hour post-infection cells were loaded with CCF4-AM (Invitrogen, LiveBLAzer™ FRET-B/G Loading Kit) as per manufacture's instructions, including the addition of probenecid and with the modification of diluting Solution C 4-fold in HBSS. Cells were returned to 37 °C and harvested at 2 hours post-infection for cell staining and flow cytometry analysis, as

above. Samples were run on an LSRII or LSRFortessa and analyzed using FlowJo Treestar software.

Western Blotting and ELISA.

Cell lysates were harvested from cells infected in TC treated plates with lysis buffer (20 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA) with added sample buffer and run on 4–12% NuPAGE gels (Invitrogen). Proteins were transferred to PVDF membrane (Millipore) and blotted with rabbit anti-mouse caspase-1 (sc-514, Santa Cruz Biotechnology), rat anti-mouse caspase-8 (Enzo Life Sciences, 1G12), rabbit anti-caspase-3 (Cell Signaling #9662) and β-actin (Sigma). Secondary antibodies were goat anti-rabbit, goat anti-rat (Jackson Immunoresearch) or horse anti-mouse HRP (Cell Signaling Technology). Cytokine release was measured by ELISA on cell supernatants using capture and detection antibodies against TNF (Biolegend, 430902) and CCL5 (Peprotech 500-P118 and 500-P118Bt).

Animal Infections.

For oral infection, mice were fasted for 12-16 hours and inoculated by gastric gavage with 1-2 x 10^8 CFU of wild-type *Y. pseudotuberculosis* (32777) from overnight culture in 2xYT containing irgasan (2μg/mL) and washed with PBS. For intraperitoneal infections, mice were injected with 5-20 x 10^3 CFU. Mice were euthanized and tissues were collected and weighed in 1ml of sterile PBS, bead homogenized (MP Biomedical) and plated at 10-fold dilutions on LB plates containing irgasan to determine bacterial burdens (CFU/g tissue). Remaining tissue homogenates were collected following centrifugation for measurement of tissue cytokines by ELISA. Serum was isolated from centrifuged blood collected from the thoracic cavity after cardiac puncture. Cells from

spleens and mesenteric lymph nodes were isolated for flow cytometry, as described below. All animal studies were performed in accordance with University of Pennsylvania Institutional Animal Care and Use Committee approved protocols.

Flow cytometry: Mesenteric lymph node and spleen cells were isolated by passing through a 100μM cell strainer. To measure cytokine expression by innate immune cells (neutrophils, monocytes, dendritic cells), 3-5 x 10^6 isolated cells were plated in a 96-well plate and cultured for 5 hours in the presence of brefeldin A (Sigma) and monensin (BD) in a 37°C humidified incubator in complete-DMEM containing penicillin-streptomycin and 2-mercaptoethanol. Cells were washed with PBS, stained for viability (Zombie Yellow, BioLegend) and then stained with the following antibodies from BioLegend: Ly6G (clone 1A-8 PE-Cy7), IL-6 (MP5-20F3 APC/FITC), CD3ε (clone 17A2 AF700/PE-Cy7), NK1.1 (clone PK136, FITC/AF700), BD Biosciences: CD45.1 (clone A20 APC-Cy7), CD45.2 (clone 104 FITC/PE), MHCII (clone M5/114 BV650/AF700), B220 (clone RA3-6B2 PETexasRed/APC/AF700), CD19 (clone MB19-1 Biotin, SA-BV711). ThermoFisher: CD11b (clone M1/70.15 PE-Texas Red), eBioscience: Ly6C (clone HK1.4 PerCPCy5.5), CD11c (clone N418 AF700), F480 (clone BM8 APC-eF780/Pacific Blue), TNF (clone MP6-XT22 eF450/PE-Cy7), IL-12p40 (clone C17.8 PE), and IFNγ (clone XMG1.2 AF700/APC). Singlet, live cells were gated to identify inflammatory monocytes ($CD11b^{hi}Ly6C^{hi}Ly6G^{-}$), neutrophils ($CD11b^{hi}Ly6G^{+}$), or DCs ($Ly6C^{-}Ly6G^{-}CD11c^{+}MHCII^{hi}$). Gating for cytokine positive cells was based on fluorescence-minus-one and naïve control samples. For bone marrow chimera experiments CD45.1/CD45.2 staining was used to distinguish host versus donor cell origin. Surface staining was performed in FACS buffer (PBS with 1% BSA, 2mM EDTA) and sample fixation and permeabilization performed prior to intracellular staining

according to manufacturer's instructions (BD Cytofix/Cytoperm™ Kit). Samples were run on an LSRII or LSRFortessa and analyzed using FlowJo Treestar software.

CHAPTER 3

CELL-EXTRINSIC TNF COLLABORATES WITH TRIF SIGNALING TO PROMOTE *YERSINIA*-INDUCED APOPTOSIS[1]

Background

Many microbial pathogens have evolved mechanisms to inhibit innate immune signaling pathways, thereby limiting the ability of infected cells to propagate inflammatory cues such as cytokine secretion (204, 205). Of the signaling pathways frequently targeted by pathogens, NF-κB and MAPK pathways elicit key host-protective antimicrobial defenses (3). However, these signaling pathways are also coupled to pro-survival signals that limit cell death pathways activated by microbial pattern recognition and cytokine receptors (5). Inhibition of innate immune signaling can, therefore, not only results in a block in cytokine and antimicrobial effector production, but also trigger cell death. This induction of cell death may be an evolutionarily ancient response to pathogen virulence factors.

The YopJ protein of pathogenic *Yersiniae* is an acyl-transferase that belongs to a family of secreted virulence factors injected into host cells by bacterial pathogens that infect plants, insects and higher eukaryotes (162-164). The activity of YopJ blocks MAPK and NF-κB signaling to interfere with the production of inflammatory cytokines (167, 170, 206). In the absence of YopJ, the virulence of *Y. pseudotuberculosis* is attenuated *in vivo* following oral infection (191). However, in addition to inhibiting

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cytokine production, YopJ-induced blockade of NF-κB and MAPK signaling also triggers cell death downstream of TLR4-dependent TRIF signaling (166-169, 175-177).

TLR4/TRIF-dependent cell death induced by YopJ requires the components of the extrinsic apoptosis pathway, specifically RIPK1, Fas-associated death domain (FADD), and caspase-8 (172-174). Interestingly, while absence of RIPK1 or caspase-8 abrogates YopJ-induced cell death, TLR4- and TRIF-deficient cells still exhibit significant, although reduced, death (173-177), implying that an additional TLR4/TRIF-independent signal contributes to *Yersinia*-induced apoptosis. However, such a pathway has not previously been described.

The TNF Receptor superfamily comprises a number of cell surface receptors that are coupled to induction of both transcriptional and apoptotic responses(12). Notably, TNFR1 can induce extrinsic apoptosis through RIPK1 and caspase-8 in a variety of immune and parenchymal tissues (207). This response can have protective roles in the context of tumorigenesis, but is pathologic in the setting of liver and intestinal damage (109, 207). In the context of infection, however, TNF is thought to act primarily as a mediator of inflammation and activator of innate immune and endothelial cells. YopJ blockade of NF-κB and MAPK activation virtually eliminates the production of TNF and other inflammatory cytokines by *Yersinia*-infected cells (167, 170, 206). Thus, the potential contribution of TNF signaling to *Yersinia*-induced cell death has never been examined.

Here, we identify a key role for TNF in promoting *Y. pseudotuberculosis*- (*Yp*) induced cell death and demonstrate that despite the greatly reduced TNF production that accompanies infection with virulent *Yp*, TNF/TNFR1 is responsible for TRIF-independent apoptosis of infected cells. Intriguingly, cells undergoing apoptosis and cells producing

TNF represented distinct populations. Unexpectedly, TNF-producing cells were injected with *Yersinia* YopJ, although to a significantly lower level than apoptotic cells. Thus, in a phenotypically heterogeneous population of infected cells, TNF production by cells that are injected but remain uninhibited by YopJ synergized with TRIF to promote maximal apoptosis in response to *Yersinia* infection. Finally, oral infection of TNFR1-deficient mice demonstrated a protective function for TNFR1 signaling *in vivo*. These findings establish an unanticipated role for cytokine responses in directing apoptosis of *Yersinia*-infected cells and a potential role for TNFR1-dependent apoptosis in promoting host protection against *Yersinia* infection.

Results

Yersinia YopJ promotes TRIF-dependent and –independent apoptosis through RIPK1 kinase activity

YopJ promotes caspase-8-dependent apoptosis of innate immune cells through the inhibition of NF-κB- and MAPK-dependent pro-survival signaling (167-169, 172-174). Although this response has been attributed to TLR4/TRIF (175-177), TRIF-deficient bone marrow-derived macrophages (BMDMs) still undergo significant *Yersinia*-induced death (173, 174, 176, 177). This implies a role for additional pathways in the induction of cell death. Both TRIF and death receptor signaling can promote survival, caspase-8-dependent apoptosis, or RIPK3-dependent programmed necrosis, depending on the nature of the genetic and environmental conditions present (208). We therefore sought to determine whether TRIF-independent death is also mediated by caspase-8-dependent apoptosis, or by an alternate death pathway, such as programmed necrosis. Similarly to wild-type BMDMs, *Trif*$^{-/-}$ BMDMs undergo YopJ-dependent cleavage of

apoptotic caspases, -8 and -3, following infection with wild-type *Y. pseudotuberculosis* (*Yp*) (Fig. 8A). As expected (174), the pancaspase inhibitor zVAD prevented the cleavage of caspase-8 and -3, but did not protect cells from death, suggesting that caspase inhibition induces a switch from *Yp*-induced apoptosis to RIPK3-dependent programmed necrosis in both wild-type and *Trif*$^{-/-}$ BMDMs (Fig. 8A-B; Fig. 9). Indeed, the RIPK3 kinase inhibitor GSK'872 inhibited death of both zVAD-treated wild-type and *Trif*$^{-/-}$ BMDMs infected with *Yp* (Fig. 8B). Importantly, single deficiency of RIPK3 (173, 174) or GSK'872 treatment alone (Fig. 8B) has no effect on *Yersina*-induced cell death of BMDMs. These data indicated that *Yp*-induced cell death in both wild-type and *Trif*$^{-/-}$ BMDMs is mediated by caspase-8-dependent apoptosis and is independent of RIPK3-programmed necrosis.

While YopJ-induced apoptosis in wild-type BMDMs requires the signaling molecule RIPK1 (173, 174), caspase-8 can mediate apoptosis via RIPK1 kinase-dependent or independent pathways (109). Whether TRIF-independent cell death depends on RIPK1 activity is unknown. Importantly, necrostatin-1 (Nec-1), a specific inhibitor of RIPK1 kinase activity, reduced YopJ-induced apoptosis of both wild-type and *Trif*$^{-/-}$ BMDMs (Fig. 8C). Altogether, these data demonstrate that both the TRIF-dependent and TRIF-independent cell death induced by *Yp* involve RIPK1 kinase-dependent apoptosis.

TNFR1 mediates TRIF-independent apoptosis and caspase-1 cleavage during *Yersinia* infection.

The TNF receptor superfamily mediates extrinsic apoptosis through recruitment and activation of FADD, RIPK1 and caspase-8 (109, 207). Given the requirement for all

three of these factors in *Yersinia*-induced apoptosis (172-174), we therefore tested the possibility that TNF might play a role in *Yp*-induced cell death. Surprisingly, despite the known blockade of TNF production by YopJ (167, 170, 206), TNFR1-deficient (*Tnfr1*$^{-/-}$) BMDMs displayed significantly decreased cell death and caspase-3 cleavage compared to wild-type BMDMs (Fig. 10A; Fig. 11A). Strikingly, macrophages deficient for both TRIF and TNFR1 (*Trif*$^{-/-}$*Tnfr1*$^{-/-}$) were also completely protected from *Yp*-induced cell death, suggesting that in the absence of TRIF, the remaining cell death is caused by the TNF Receptor pathway (Fig. 10A). BMDMs lacking both TNFR1 and TRIF signaling were completely protected from *Yp*-induced death up to 8 hours post-infection, by which time both *Tnfr1*$^{-/-}$ and *Trif*$^{-/-}$ macrophages exhibit similar levels of death to WT cells (Fig. 11B). Consistently, cleavage of caspase-8 and -3 were abrogated in the double-knockout cells, but still present in either TRIF of TNFR single-deficient cells (Fig. 10B). Critically, TNF-deficient BMDMs phenocopied *Tnfr1*$^{-/-}$ cells, in that they were partially protected from cell death in response to *Yp* infection, indicating that the role of TNFR1 is mediated by TNF itself (Fig. 10C; Fig. 9).

In addition to the activation of apoptotic caspases, *Yersinia* infection of unprimed BMDMs induces caspase-1 is activation through a RIPK1, caspase-8 and FADD pathway (173, 174, 180, 182). This pathway of inflammasome-independent caspase-1 activation occurs downstream of TLR4/TRIF signaling during *Yersinia* infection (173, 174). Interestingly, we observed that either *Trif*$^{-/-}$ or *Tnfr1*$^{-/-}$ BMDMs still showed residual, albeit reduced, processing of caspase-1 following *Yp* infection (Fig. 10D), suggesting that both pathways could independently mediate caspase-8-dependent caspase-1 processing. Indeed, only *Trif*$^{-/-}$*Tnfr1*$^{-/-}$ cells did not undergo caspase-1 cleavage following *Yp* infection, definitively demonstrating that caspase-1 processing in *Trif*$^{-/-}$ BMDMs was

mediated by TNFR1 (Fig. 10D). Interestingly, while most BMDMs infected with *Yersinia* exhibited a profound blebbing of cellular membranes prior to taking up the membrane impermeant dye PI, indicative of apoptosis (Supplemental Video 1), some cells appeared to lyse and release their cytoplasm concomitant with becoming PI positive, suggestive of pyroptosis or programmed necrosis-like morphology and kinetics (Supplemental Video 2). This is consistent with our previous findings that caspase-1 contributes to *Yersinia*-induced cell death, but is not absolutely required (174), suggesting that at least some fraction of the cells undergo pyroptosis in response to *Yp* infection. Overall, these findings provide the first direct evidence to our knowledge that TNF/TNFR1 signaling contributes to *Yersinia*-induced cell death and caspase-1 processing.

Cell-extrinsic TNF promotes *Yersinia*-induced apoptosis

Given the well established impact of YopJ on limiting NF-κB and MAPK signaling, which blocks production of cytokines, including TNF (167, 170, 206), we sought to determine the source of the TNF that was responsible for the *Yp*-induced cell death. As expected, *Yp*-infected cells secreted very low but detectable amounts of TNF compared to BMDMs infected with YopJ-deficient bacteria (Fig. 12A). *Trif*$^{-/-}$ cells secreted even less TNF compared to wild-type cells (Fig. 12A). Single-cell analysis of TNF expression by flow cytometry demonstrated that indeed, the vast majority of *Yp*-infected cells produced little to no TNF, and overlapped with uninfected cells; however, we observed a small but significant population of *Yp*-infected cells that produced low levels of TNF in response to *Yp* infection (Fig. 12B). Intriguingly, this TNF-producing population was entirely distinct from the cells undergoing apoptosis, as determined by cleaved caspase-3 staining (Fig.

12C). These data imply that a subset of cells within *Yp*-infected cultures serves as the source of TNF that promotes YopJ-induced cell death in non-producing cells. TNF could be absent in the cleaved caspase-3-positive cells because they are dying or have already released TNF due to loss of membrane integrity at the time of the analysis. This is unlikely, however, as the cells were gated on live cells that had an intact plasma membrane. Importantly, *Tnfr1*$^{-/-}$ cells exhibited no difference in their ability to produce TNF, but had greatly reduced levels of cleaved caspase-3, demonstrating a key role for TNFR signaling in promoting *Yp*-induced apoptosis (Fig. 12C).

Heterogeneous injection of YopJ leads to TNF production by a subset of infected cells.

The blockade of inflammatory innate signaling and cytokine production by YopJ limits, but does not completely abrogate, production of TNF by infected cells. These TNF-producers could be uninfected cells, cells that escaped injection of YopJ, or cells that are somehow resistant to the effects of YopJ. To distinguish between these possibilities, we utilized a YopJ-beta-lactamase (BlaM) injection reporter system (209), to determine whether cells that were injected by YopJ were capable of producing TNF. This reporter system takes advantage of a compound fluorophore, CCF4-AM, which contains a beta-lactam ring, and whose fluorescence properties are therefore altered in the presence of beta lactamase (209). YopJ-deficient Yp were complemented with YopJ protein fused to to beta-lactamase (YopJ-Bla), which reconstituted the ability to inhibit cytokine expression and to induce apoptosis (Fig. 13). Detection of CCF4-AM cleavage thus provided us with a way to quantitate cells specifically injected with functional YopJ protein because it was fused directly to beta lactamase. Intriguingly, we observed that

under these infection conditions, all of the BMDMs in culture were injected by YopJ, but that a subset of the cells exhibited low injection (Fig. 14A). Importantly, all of these low injection cells were negative for cleaved caspase-3 (Fig. 14B), but made up the entirety of the TNF-producing population of cells (Fig. 14C). Notably, all of the cells containing cleaved caspase-3 were highly injected, suggesting that apoptosis requires a high level of YopJ injection (Fig. 14B). Interestingly, lack of TNFR1 did not significantly alter the relative abundance of low injection vs. high injection cells, but significantly reduced the frequency of cleaved caspase-3-positive cells (Fig. 14B). This suggested that TNFR1 signaling increased the likelihood that a YopJ-injected cell would undergo apoptosis.

The preferential production of TNF by low injection cells could be due to levels of injected YopJ in these cells being below a threshold necessary to efficiently block cell signaling and cytokine production. To test the relationship between infections dose, cell death and TNF production, we titrated the dose of *Yp* infection down from 20 to 2.5, As expected, this resulted in a corresponding increase in frequency of low injection cells (Fig. 14D). There was a corresponding increase in the frequency of TNF-producing cells and a decrease in the frequency of apoptotic cells (Fig. 14D-F). Thus, although the number of TNF producing cells rises as the MOI decreases, cell death likely decreases due to a combination of reduced TLR stimulation at lower MOIs, together with levels of injected YopJ falling below a threshold necessary for the induction of apoptosis. Altogether, these studies demonstrate that heterogeneity in the extent of injection is present within a seemingly uniform population of cells, and that differences in injection efficiency account for the ability of a subset of cells to produce TNF, resulting in enhanced apoptosis of highly injected cells.

Membrane TNF and endocytic trafficking promote TRIF-independent apoptosis during *Yersinia* infection.

TNF secretion is regulated transcriptionally as well as at a post-translational step, in which trimeric TNF is assembled on the cell surface and subsequently released as a soluble cytokine by the metalloprotease tumor necrosis factor-α-converting enzyme (TACE) (210, 211). Interestingly, both membrane-bound and soluble TNF are able to mediate many of the inflammatory effects of TNF (212-214). We therefore utilized a specific TACE inhibitor to test whether secreted TNF was required, or whether membrane-bound TNF was sufficient to promote *Yp*-induced apoptosis. As expected, TACE inhibitor treatment dramatically reduced the amount of TNF released into the cell supernatant (Fig. 15A). Surprisingly however, TACE inhibitor treatment had no impact on *Yp*-induced cell death in Wt or *Trif*$^{-/-}$ cells, suggesting that surface TNF is sufficient to provide pro-apoptotic signals in the context of YopJ (Fig. 15B).

Although TNFR1-mediated signaling through NF-κB and MAPK pathways is initiated upon receptor ligation at the plasma membrane, internalization is thought to be required for the formation of the death-inducing signaling complex (DISC) that initiates apoptosis (38, 215, 216). Notably, activation of TRIF-dependent transcriptional responses downstream of TLR4, including the induction of type-I interferon and certain other chemokines such as CCL5, also require TLR4 endocytosis, which is mediated by dynamin (91). This is in contrast to MyD88-dependent TLR4 signaling, which occurs from the plasma membrane. As expected, blocking dynamin-mediated endocytosis potently inhibited the production of the TRIF-dependent cytokine CCL5 by wild-type cells (Fig. 15C). Interestingly, this also completely prevented *Yp*-induced apoptosis in wild-

type, TRIF- and TNFR-deficient BMDMs (Fig. 15D). These data implicate dynamin-dependent endocytosis in both TNFR1- and TRIF-dependent *Yp*-induced apoptosis.

Cell extrinsic soluble or membrane-bound TNF promote *Yp*-induced apoptosis

The TACE inhibitor studies indicated that membrane TNF was sufficient to promote *Yp*-induced cell death, but did not exclude the possibility that soluble TNF mediates this effect under normal circumstances. Indeed, addition of soluble TNF to *Tnf*$^{-/-}$ BMDMs fully restored *Yp*-induced apoptosis to wild-type levels (Fig. 16A-B). The significantly reduced levels of TNF produced by TRIF-deficient cells could potentially also account for the reduced level of apoptosis in these cells. However, while TNF addition slightly increased *Yp*-induced apoptosis of *Trif*$^{-/-}$ BMDMs, it was not fully restored to wild-type levels, demonstrating that TRIF and TNF play distinct, non-redundant roles in mediating *Yp*-induced death (6B).

In order to further test whether cell-extrinsic TNF promotes *Yp*-induced apoptosis, we infected mixed cultures of BMDMs derived from TNF-deficient and wild-type congenically marked bone marrow. When mixed at a 19:1 ratio of *Tnf*$^{-/-}$:Wt cells, just 5% of wild-type cells capable of producing TNF were sufficient to mediate maximal levels of apoptosis in *Tnf*$^{-/-}$ cells, demonstrating that minimal levels of TNF are required to induce cell death (Fig. 16C). Intriguingly, treatment of the 19:1 cultures with TACE inhibitor reduced apoptosis of *Tnf*$^{-/-}$ cells in the mixed cultures to nearly that of *Tnf*$^{-/-}$ cells alone (Fig. 16C). However, at a *Tnf*$^{-/-}$:Wt ratio of 1:1, TACE inhibition had no detectable impact on cell death in *Tnf*$^{-/-}$ cells, similar to what observed in cultures of wild-type and *Trif*$^{-/-}$ BMDMs alone following TAPI treatment (Fig. 17; Fig. 15A). This suggests that

when cell-cell contacts are limiting, TNF secreted by wild-type cells plays a key role in promoting *Yp*-induced apoptosis. However, when cell-cell contact between TNF-producing and non-producing cells is not limiting, cell-surface TNF is sufficient to mediate maximal apoptosis. Collectively these studies indicate that both cell surface and soluble TNF are capable of promoting *Yp*-induced apoptosis and likely have a common function during infection.

Finally, we sought to determine whether TNFR1-dependent apoptosis significantly impacted control of *Y. pseudotuberculosis* during *in vivo* infection. Compared to wild-type mice, *Tnfr1*$^{-/-}$ mice had increased bacterial burdens in both the spleen and liver 5 days after oral infection. This indicates an important *in vivo* role for TNF signaling in host defense that may depend on the induction of TNFR1-dependent apoptosis (Fig. 16D). Altogether, these results reveal a previously unappreciated role for cell-extrinsic TNF in macrophages killing during *Yersinia* infection and suggest that this function contributes to anti-bacterial immune defense.

Discussion

Cell death is a prominent feature of infection by many pathogens, including *Yersinia* (166, 167, 191). During *Yersinia* infection, cell death is triggered by the activity of YopJ, which promotes bacterial dissemination from mucosal tissues (191). However, *Yersinia*-induced cell death may also provide a means for infected cells to elicit inflammatory signals or induce phagocytosis of cell-associated bacteria to overcome YopJ-dependent signaling inhibition (195, 196, 217).

Although TLR4/TRIF contribute to apoptosis during *Yersinia* infection, significant cell death occurs in *Yersinia*-infected cells even in the absence of TLR4/TRIF-dependent

signals (175-177). These data suggest that an additional, as-yet-undefined, pathway contributes to *Yersinia*-induced cell death. Here, we show a key role for TNF signaling in promoting *Yersinia*-induced apoptosis, implicating innate cytokine signaling in promoting host cell death during infection. To our knowledge, this is the first direct evidence that TNF signaling contributes to *Yersinia*-induced macrophage apoptosis, and supports a model in which cell extrinsic cytokine signals play an important role in mediating death of *Yersinia*-infected cells.

The role of TNF in *Yersinia*-induced apoptosis may be particularly important because at 37 °C, *Yersinia* alters its LPS to a form that is poorly detected by TLR4 (185-187). This likely limits both anti-bacterial cytokine responses and TLR4-dependent induction of apoptosis *in vivo*. Thus, additional pathways to promote apoptosis may play critical roles in host defense. Indeed, TRIF deficiency alone does not significantly impact control of *Yersinia pseudotuberculosis* in an *in vivo* oral infection model (218) and Fas/FasL contribute to apoptosis of *Y. pestis*-infected cells in the lung and promote control of infection (219). Our studies now demonstrate an important role for TNFR1 in control of oral *Yp* infection, which contrasts both with TRIF deficiency and with previous investigations of the contribution of TNFR1 to control of *Yersinia* during systemic infection (188, 218). Prior studies with *Y. enterocolitica* found that TNFR1 played a pathological role and that *Tnfr1*$^{-/-}$ mice were more resistant to infection (188). These differences may be due to either the *Yersinia* species or route of infection.

Furthermore, while *Yp* inhibits TNF production by cells that it encounters at sites of infection, the recruitment of additional effector cells offer an abundant source of TNF for inducing apoptosis and potential clearance of cells inhibited during earlier stages of infection. Newly recruited cells would likely not have been exposed to the inhibitory

effects of *Yersinia*-injected virulence factors and may even be primed by inflammatory cues to produce TNF while entering the infected tissue. Notably, primed macrophages and monocytes respond differently when encountering *Yersinia* than naïve macrophages, particularly with respect to their mode of cell death (178). While cytotoxic lymphocytes, including NK cells and adaptive T cells, contribute to the killing of *Yersinia*-infected cells later in infection (217), our findings suggest that innate immune cells could play an early role in inducing apoptotic cell death through cytokine-dependent signaling.

Our data revealed that heterogeneity in the level of injected YopJ and bacterial inhibition of cell signaling results in a subset of cells being able to produce TNF, despite the block in NF-κB signaling imposed by YopJ. Titrating the infection of BMDMs results in both increased TNF production and decreased apoptosis, implying that a high threshold of injected YopJ is needed in order to prevent cytokine production and to potentiate TNF-induced apoptosis in infected cells. In mixed cultures, only a very small fraction of TNF-producing cells is sufficient to induce maximal cell death of TNF-deficient cells, consistent with the finding that exogenous TNF also restores cell death to *Tnf*$^{-/-}$ cells. Intriguingly, titrating the ratio of TNF-sufficient and TNF-deficient cells while blocking TNF secretion revealed that limiting cell-cell contact between TNF producers and non-producers increased the importance of secreted TNF in cell death. Thus, our data demonstrate that cell surface TNF is capable of providing the apoptotic signal if secretion is inhibited.

Our data also demonstrated a requirement for dynamin-dependent endocytosis in the induction of both TLR4/TRIF- and TNFR1-induced apoptosis. This is presumably dependent on the formation of an intracellular Death Inducing Signaling Complex, raising the question of how extrinsic TNF might mediate its apoptotic effect when membrane-

bound. TNF that cannot be cleaved from the surface of cells due to mutation of the TACE cleavage site is also capable of inducing apoptosis in TNFR-expressing cells (213). Notably, similar findings have been reported for other surface protein members of the TNF family, particularly Fas ligand (FasL) (220). FasL is expressed on activated NK cells and $CD8^+$ T cells, and contributes to their ability to induce apoptosis of infected cells (221-223). Importantly, membrane-bound FasL is sufficient to induce Fas internalization, formation of a Death Inducing Signaling Complex, and apoptosis (220). Thus, the requirement for endocytosis may apply only to a receptor signaling complex, independent of Death Receptor ligand, for the induction of apoptosis.

Whether cell surface TNF is a primary driver of apoptosis when large amounts of TNF are being secreted, and whether it is sufficient to mediate the full extent of protection from bacterial infection *in vivo* remains to be determined. Overall, our results provide new insight into mechanisms governing pathogen-induced apoptosis, and highlight a previously unappreciated synergy between TLR and TNFR that regulate the response to bacterial infection.

Figure 8

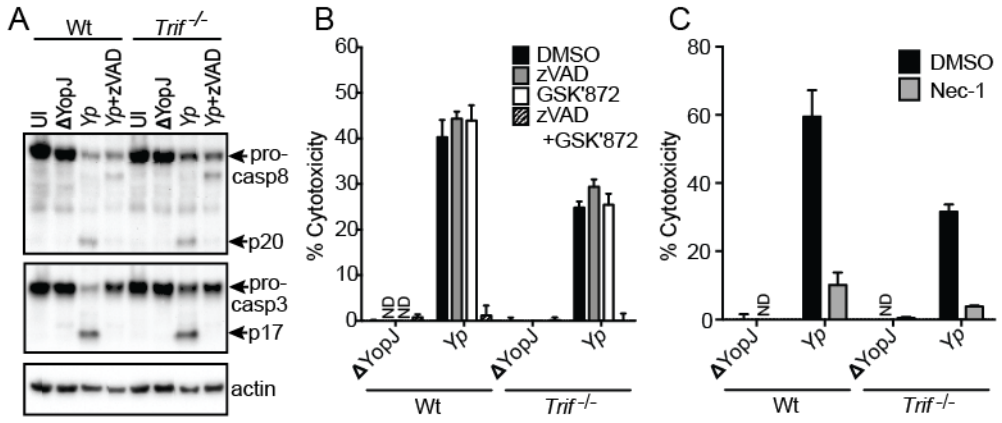

Figure 8. *Yersinia* YopJ promotes TRIF-dependent and –independent apoptosis through RIPK1 kinase activity.

(A) $_{Wild}$-type (Wt) or TRIF-deficient (*Trif*$^{-/-}$) BMDMs were left uninfected (UI) or infected with Wt *Y. pseudotuberculosis* (*Yp*) or YopJ-deficient *Yp* (ΔYopJ) for 4 h. Cell lysates were probed for caspase-8 and caspase-3 cleavage. Results are representative of two independent experiments. **(B-C)** Percent cytotoxicity was measured by LDH release from B6 and *Trif*$^{-/-}$ BMDMs infected with ΔYopJ or *Yp* for 4 h. 100μM zVAD-fmk (zVAD), 300nM GSK'872, 60μM Nec-1 were added 1h before infection where indicated. Error bars indicate mean +/- SD of triplicates and are representative of three or more independent experiments. ND, not detected.

Figure 9

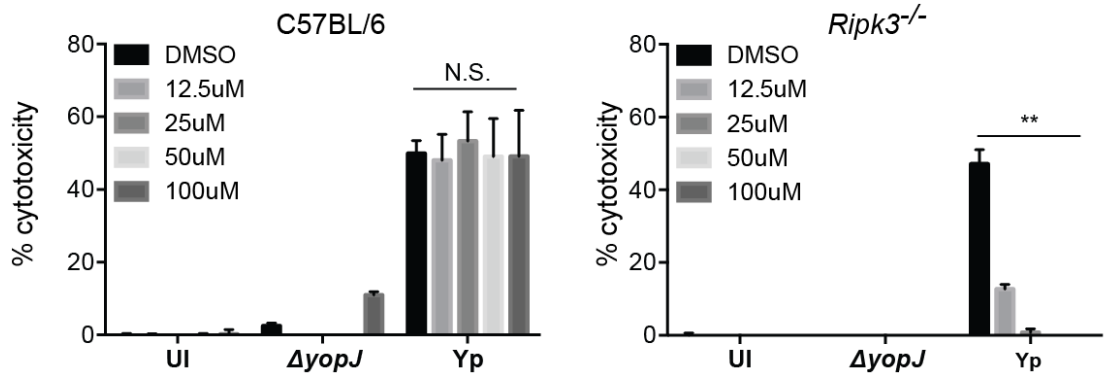

Figure 9. zVAD potentiates programmed necrosis in *Yp*-infected WT cells and prevents apoptosis in Ripk3$^{-/-}$ cells across a range of concentrations.
zVAD-fmk was added to cells at indicated concentrations one hour prior to infection with YopJ-deficient or WT *Y. pseudotuberculosis* (*Yp*). 4 hours post-infection, cytotoxicity was measured by assaying release of lactate dehydrogenase (LDH) into the supernatant. zVAD protects Ripk3$^{-/-}$ cells from *Yp*-induced cell death at all concentrations tested, in contrast to WT cells, where zVAD potentiated programmed necrosis in response to *Yp* infection, at all concentrations of zVAD tested.

Figure 10

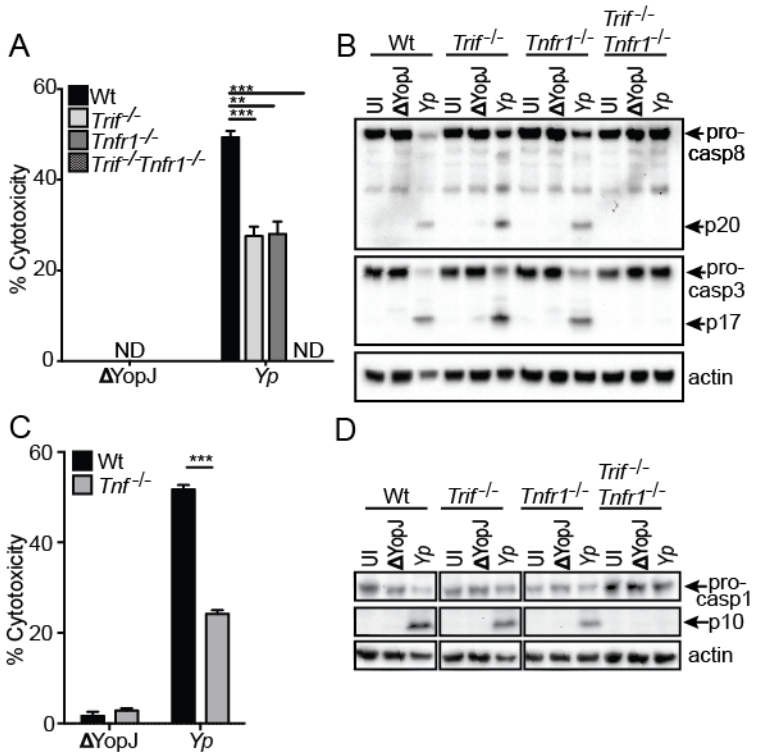

Figure 10. TNFR1 mediates TRIF-independent apoptosis and caspase-1 cleavage during *Yersinia* infection.
(A) Cytotoxicity was measured by LDH release from Wt, *Trif*$^{-/-}$, TNFR1-deficient (*Tnfr1*$^{-/-}$) and TRIF/TNFR1-deficient (*Trif*$^{-/-}$*Tnfr1*$^{-/-}$) BMDMs infected with Wt (*Yp*) or YopJ-deficient (ΔYopJ) *Y. pseudotuberculosis* for 4 h. **(B)** Cell lysates were probed for caspase-8 and caspase-3 by Western analysis. **(C)** Cytotoxicity was measured by LDH release from Wt and TNF-deficient (*Tnf*$^{-/-}$) BMDMs, as in A. **(D)** Western analysis for caspase-1, as in B. Error bars indicate mean +/- SD of triplicates. Representative of three or more independent experiments. ***p*<0.001, ****p*<0.0001. ND, not detected.

Figure 11

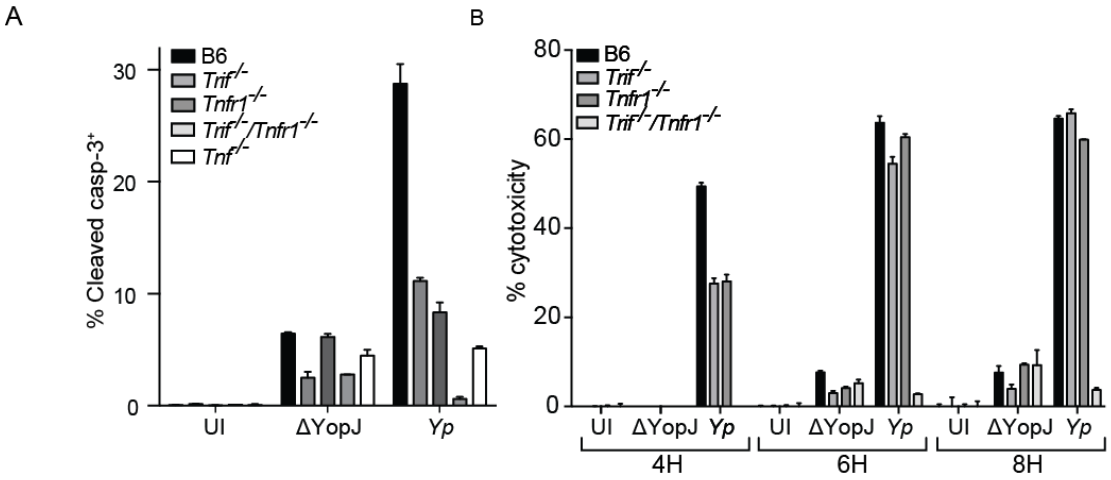

Figure 11. TRIF-independent apoptosis during Yersinia infection is mediated by TNFR1 signaling.

(A) Indicated genotypes of BMDMs were left uninfected or infected with Wt (*Yp*) or YopJ-deficient (ΔYopJ) *Y. pseudotuberculosis*. At 2 hours post-infection, cells were stained and analyzed by flow cytometry for frequency of cleaved caspase-3 (Casp3)-positive cells (gated on singlets and live cells). Results are representative of two independent experiments. **(B)** Indicated genotypes were left uninfected or infected with ΔYopJ or *Yp* and monitored for cytotoxicity as described in Materials and Methods over a timecourse of 4, 6, and 8 hours.

Figure 12

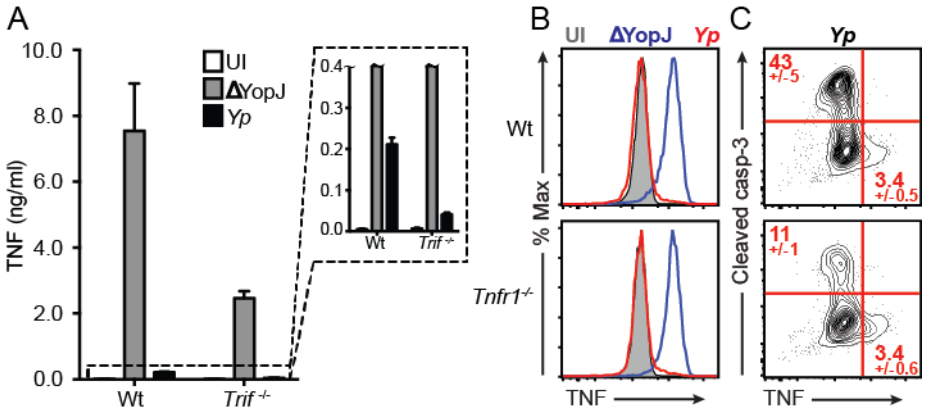

Figure 12. Cell-extrinsic TNF promotes *Yersinia*-induced apoptosis.
(A) Soluble TNF measured by ELISA from supernatants of Wt and *Trif*$^{-/-}$ BMDMs left uninfected or infected with Wt (*Yp*) or YopJ-deficient (ΔYopJ) *Y. pseudotuberculosis*. Inset indicates TNF production by *Yp* infected cells. Error bars indicate mean +/- SD of triplicates. (B) Wt and *Tnfr1*$^{-/-}$ BMDMs were left uninfected (gray filled histogram) or infected with ΔYopJ (blue histogram) or *Yp* (red histogram). Two hours post-infection, cells were stained and analyzed by flow cytometry (gated on singlet live cells). (C) Distinct populations of cells are positive for intracellular TNF or cleaved caspase-3 (casp-3). Gate frequencies represent mean +/- SD of triplicates. Data representative of three or more independent experiments.

Figure 13

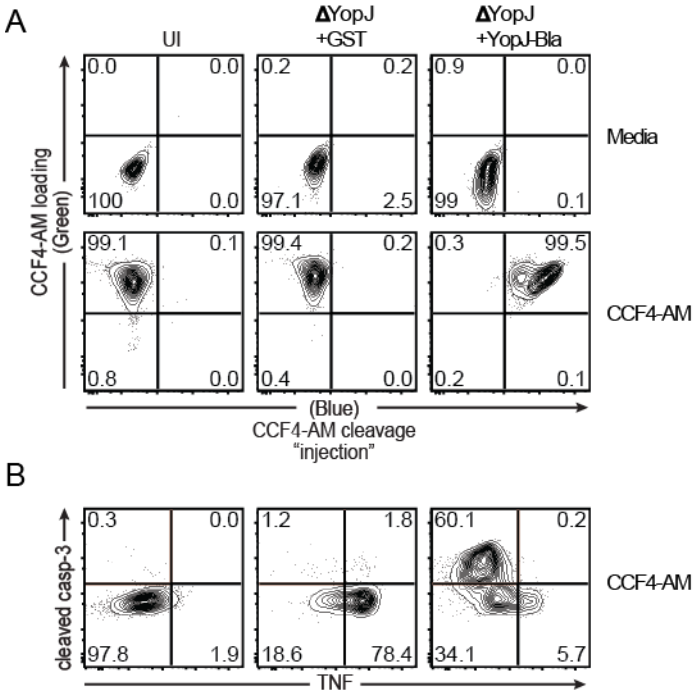

Figure 13. Yersinia injection as measured by CCF4-AM cleaveage.
Wt BMDMs were left uninfected (UI) or infected at MOI 20 with YopJ-deficient *Y. pseudotuberculosis* complemented with pACYC plasmid expressing either control GST (ΔYopJ+GST) or beta-lactamase linked YopJ (ΔYopJ+YopJ-Bla). At 1 hour post-infection, cells were loaded with CCF4-AM or control media. At 2 hours post-infection, cells were harvested and analyzed by flow cytometry for (A) CCF4-AM loading (488 excitation;515/20 emission) and CCF4-AM cleavage by injected YopJ-Bla (405 excitation; 450/50 emission) and for (B) cleaved caspase-3 and TNF staining.

Figure 14

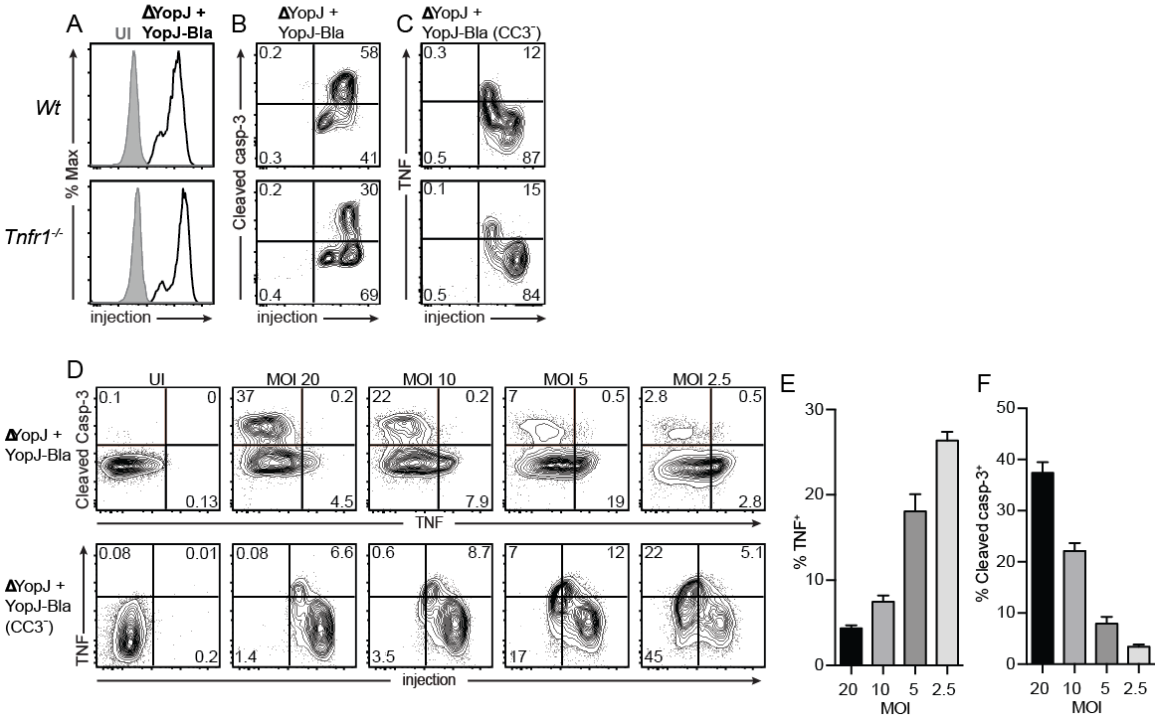

Figure 14. Heterogeneous injection of YopJ leads to TNF production by a subset of infected cells.

(A-D) Wt or Tnfr1-/- BMDMs were left uninfected (UI) or infected at MOI 20 with YopJ-deficient *Y. pseudotuberculosis* complemented with plasmid containing beta-lactamase-linked YopJ (ΔYopJ+YopJ-Bla). (A) YopJ injection was measured by CCF4-AM cleavage and compared to (B) cleaved caspase-3 in total live cells or to (C) TNF expression in cleaved caspase-3-negative ($CC3^-$) cells. (D) MOI was titrated from 20 to 2.5 and YopJ injection, cleaved caspase-3 and TNF were measured as above. (E-F) Frequency of TNF^+ and cleaved $caspase\text{-}3^+$ cells from D. Frequencies represent mean +/- SD of triplicates. Data representative of two independent experiments.

Figure 15

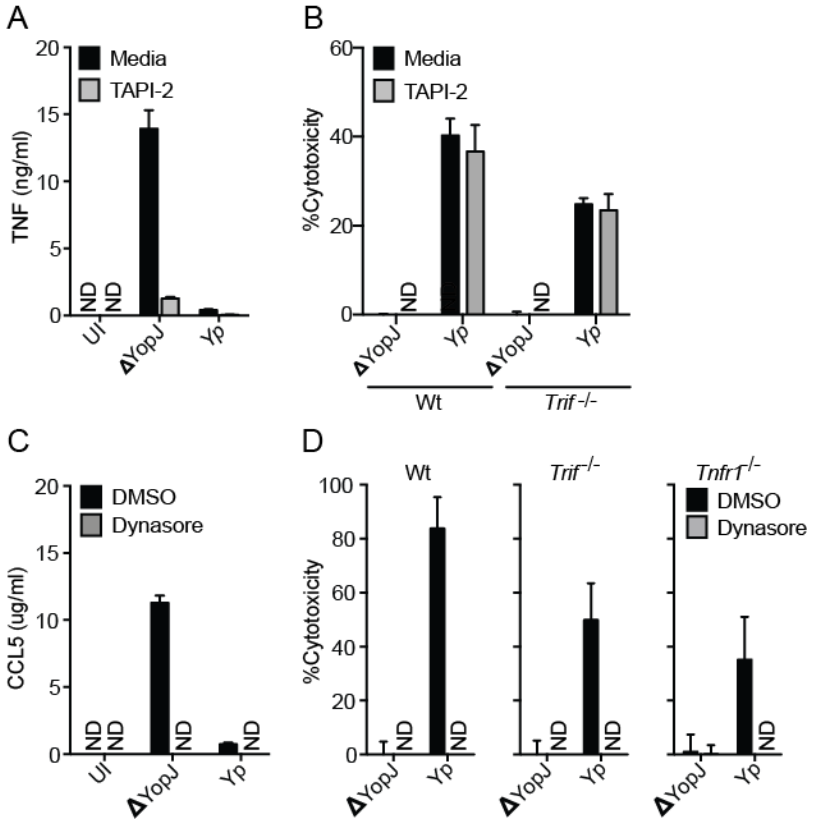

Figure 15. Membrane TNF and endocytic trafficking promote TRIF-independent apoptosis during *Yersinia* infection.
(A) Secreted TNF was measured by ELISA of supernatants of Wt BMDMs left untreated or treated with TAPI-2 and left uninfected or infected with Wt (*Yp*) or YopJ-deficient (ΔYopJ) *Y. pseudotuberculosis*. (B) LDH release from Wt and *Trif*$^{-/-}$ BMDMs treated with TAPI-2. (C) CCL5 measured by ELISA and LDH release (D) from B6 BMDMs treated with dynasore or vehicle control, as indicated. Error bars indicate mean +/- SD of triplicates and are representative of three or more independent experiments. ND, not detected.

Figure 16

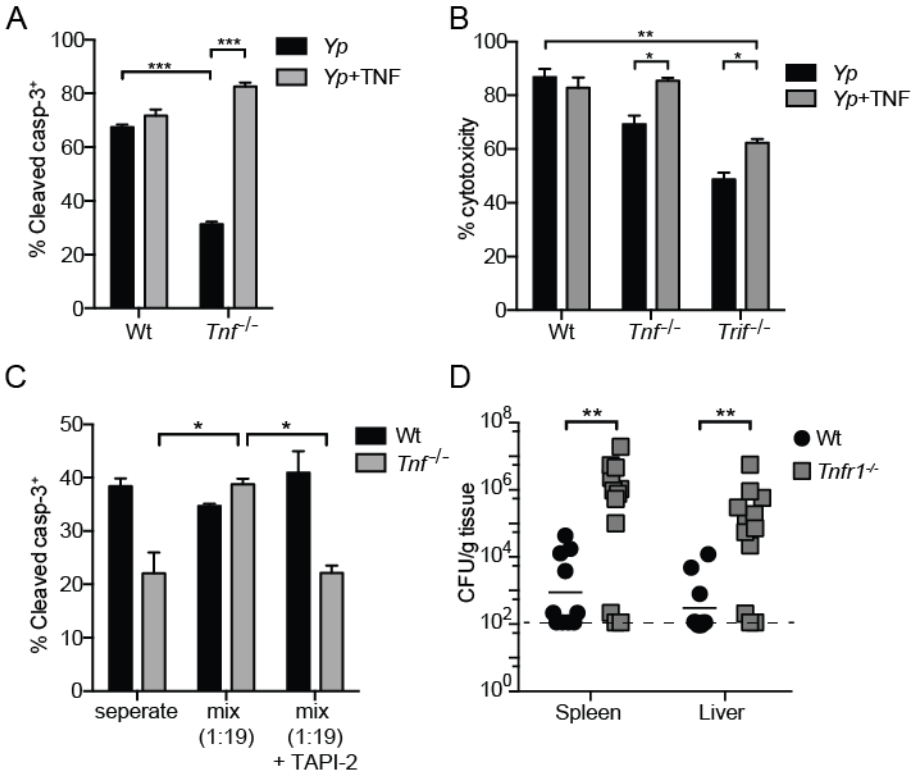

Figure 16. Cell-extrinsic TNF promotes Yersinia-induced apoptosis and *in vivo* antibacterial immunity.
(A-B) Wt, *Tnf*$^{-/-}$ and *Trif*$^{-/-}$ BMDMs were infected with Wt *Y. pseudotuberculosis* (*Yp*), treated with 1ng/mL recombinant TNF at 30 min post-infection and harvested for flow cytometric analysis of cleaved caspase-3 (A) or measured for LDH release (B). (C) *Tnf*$^{-/-}$ (CD45.2) and congenically marked Wt (B6.SJL, CD45.1) BMDMs were plated separately or mixed at a 19:1 (*Tnf*$^{-/-}$:Wt) ratio and infected with *Yp* for 2 h. Mixed cultures were analyzed by flow cytometry for cleaved caspase-3, using CD45 staining to differentiate Wt and *Tnf*$^{-/-}$ cells. Cells treated with TAPI-2 or control media 1 hour prior to infection where indicated. Error bars indicate mean +/- SD of triplicates. Data are representative of two or more independent experiments. (D) Wt and Tnfr1-/- mice were infected orally with 2x10^8 CFU *Yp* and tissue bacterial burdens were measured at day 5 post-infection. Line represents geometric mean of data pooled from two experiments, total n=10-12 mice. Dashed line indicates limit of detection. **p*<0.05, ***p*<0.01, ****p*<0.001.

Figure 17

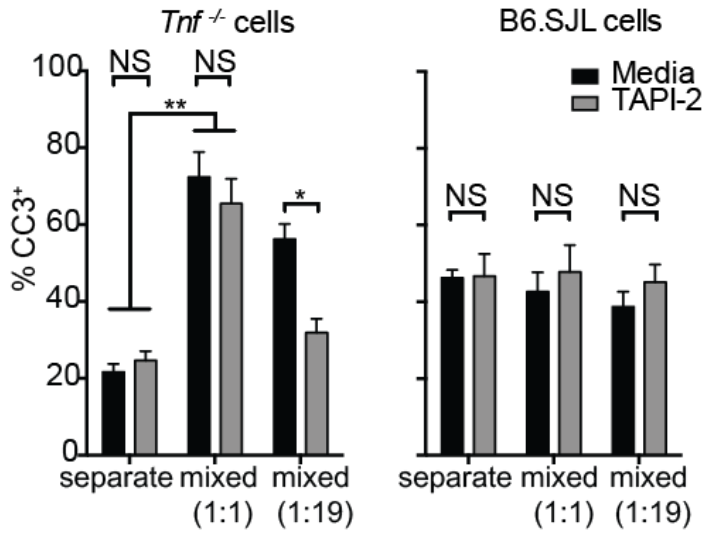

Figure 17. Mixed BMDM cultures restore maximal Yersinia-induced apoptosis in TNF-deficient cells.

$Tnf^{-/-}$ (CD45.2) and congenically marked Wt (B6.SJL, CD45.1) BMDMs were plated separately or mixed at a 1:1 or 19:1 (Tnf-/-:Wt) ratio and infected with Wt *Y. pseudotuberculosis* for 2 h. Mixed cultures were analyzed by flow cytometry for cleaved caspase-3, using CD45 staining to differentiate Wt and $Tnf^{-/-}$ cells. Cells treated with TAPI-2 or control media 1 hour prior to infection where indicated. Error bars indicate mean +/- SD of triplicates. $Tnf^{-/-}$ cells bar graph refers to % cleaved Casp3 (CC3) in CD45.2 $Tnf^{-/-}$ cells; B6.SJL cells refers to % cleaved Casp3 in CD45.1 cells in mixed cultures.

Figure 18

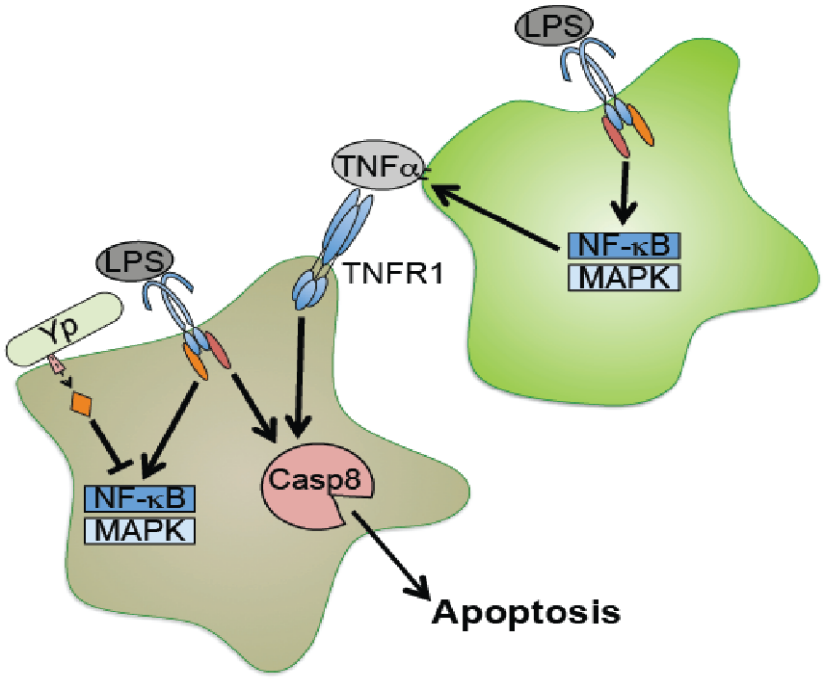

Figure 18. Cell-extrinsic TNF promotes *Yersinia*-induced apoptosis.
The *Y. pseudotuberculosis* virulence factor YopJ inhibits NF-κB and MAPK signaling, promoting caspase-8-dependent apoptosis through TLR4-TRIF and TNFR1 signaling. Inhibition by YopJ also blocks TNF expression by inhibited cells. Bystander cells able to escape the inhibitory effects of YopJ are able to act as a cell-extrinsic source of TNF for promoting apoptosis through TNFR1.

CHAPTER 4

YERSINIA-INDUCED APOPTOSIS PROMOTES ANTIBACTERIAL IMMUNITY

Background

Innate immune cells are responsible for the detection of microbial signals and mounting of protective anti-pathogen responses. However, pathogens have evolved to evade host immune responses. Thus, multiple layers of immune detection must exist for immunity to pathogens to be efficient. The recognition of conserved microbial patterns, pathogen virulence activities, and endogenous "danger" signals associated with pathology allows microbes to be recognized through multiple mechanisms and provides a means of differentiating between harmless commensals and dangerous pathogens (1, 2, 92). While the mechanisms by which pattern recognition receptors (PRRs) detect microbial products have been extensively studied (81, 224), how danger signals, such as damage-associated molecular patterns (DAMPs), are integrated into the immune response is less well understood (5). One important source of DAMPs during settings of inflammation and infection is from the process of cell death. During infection, pathogen inhibition of innate immune signaling pathways may make recognition of cell death-associated signals particularly important for host protection (204).

Yersinia species represent highly adapted pathogens capable of efficiently inhibiting host signaling pathways to establish disease (161). In contrast to *Y. pestis*, the causative agent of plague, *Y. pseudotuberculosis* and *enterocolitica* cause natural infection through the enteric route. Injection of virulence factors and inhibition of host

signaling pathways by *Yersinia* through a type-III secretion system allows dissemination and disease in susceptible host species. The efficiency of *Yersinia* species at preventing many immune signaling pathways and responses makes it a useful model system in which to study the immune response to pathogenic bacteria that evade and inhibit innate immune recognition (161).

The virulence factor YopJ of *Y. pestis* and *Y. pseudotuberculosis* is an acyl-transferase that potently inhibits NF-κB and MAPK dependent signaling pathways downstream of TLR recognition of bacterial ligands, leading to a block in the production of inflammatory cytokines (162-164, 167, 170, 206). Furthermore, the virulence factor YopK inhibits inflammasome dependent recognition of *Yersinia* (180). Thus, *Yersinia* effectively eliminates two important strategies of pathogen recognition. In addition to preventing cytokine production, YopJ inhibition leads to host cell death, due to the loss of essential NF-κB- and MAPK-dependent pro-survival signals (166, 167, 169). This cell death is mediated by receptor interacting protein kinase 1 (RIPK1) kinase-dependent apoptosis downstream of TLR4- and TNFR1-signaling (168, 172-177, 225). While YopJ activity is important for promoting *in vivo* bacterial virulence (191), it is unclear whether this effect is dependent upon its suppression of innate immune cytokine production or whether apoptosis plays a pathologic role during infection. In contrast, gain of function experiments, altering the amount of apoptosis induced by infection by increasing the injection of YopJ, demonstrate a decreased bacterial virulence associated with the enhanced induction of apoptosis (195, 196). Thus, it seems that the effects of YopJ on bacterial virulence and the host immune response are dose dependent, with normal injection of YopJ supporting bacterial virulence and increased injection of YopJ

potentiating immunity to infection. However, whether these effects are mediated by YopJ-dependent apoptosis versus the inhibition of host cytokine signaling is unknown.

To address the direct role of apoptosis during *Yersinia* infection, we investigated the possibility that specifically disrupting apoptosis without impacting cell intrinsic cytokine production would result in defective bacterial control. To test this hypothesis, we took advantage of our recent observations that multiple cell surface receptors converge on RIPK1 to mediate apoptosis in response to *Yersinia* infection (225). RIPK1 is a critical signaling adaptor downstream of the TLR and TNFR family receptors, and mediates both pro-survival and cell death responses (13, 87, 105, 226). Critically, the kinase activity of RIPK1 promotes apoptosis, but is dispensable for cell-intrinsic induction of NF-κB dependent gene expression (14, 56, 106, 107, 197). Thus, targeted disruption of RIPK1 kinase activity provides a novel model in which to block *Yersinia*-induced apoptosis, independent of innate immune signaling inhibition by the virulence factor YopJ. Intriguingly, we found that cells from mice containing a 'kinase-dead' version of RIPK1 (*Ripk1*^{kd}) do not undergo *Yersinia*-induced apoptosis. *Ripk1*^{kd} mice have a defect in their ability to control tissue bacterial burdens that is specifically dependent upon the loss of RIPK1 kinase activity in hematopoietic cells and correlated with decreased innate immune cell cytokine production. Collectively, these results demonstrate a novel role for RIPK1 in promoting apoptosis-dependent host protection during *in vivo* infection with *Yersinia*.

Results

Yersinia-induced apoptosis requires RIPK1 kinase activity

Infection with *Y. pseudotuberculosis* (*Yp*) induces apoptosis of innate immune cells both *in vitro* and *in vivo* through the activity of the virulence factor YopJ (166, 167). This cell death response occurs through TLR4/TRIF and TNFR1 signaling, which converge on the activation of caspase-8 and extrinsic apoptosis (168, 172-177, 225). Inhibition of RIPK1 kinase activity by necrostatin-1 blocks YopJ-dependent apoptosis through both TRIF- and TNFR1-dependent pathways (173, 174). Similarly, infection of BMDMs from mice with a targeted mutation in the kinase domain of RIPK1 (106), generating a 'kinase dead' protein (*Ripk1*^{kd}), induces significantly decreased YopJ-dependent cell death compared to infection of Wt cells (Fig. 19A). Importantly, RIPK1 kinase activity has been shown to be dispensable for its function in promoting cytokine production through the activation of NF-κB and MAPK signaling (78, 102, 106, 199). Consistent with these data, Wt and *Ripk1*^{kd} BMDMs produced similar amounts of TNF when infected with YopJ-deficient *Yp* (ΔYopJ) and other proinflammatory cytokines when treated with an array of TLR ligands (Fig. 19B-C). Although BMDMs infected with wild-type *Yp* produced dramatically less TNF due to YopJ-dependent inhibition of cell signaling, there was no difference in TNF production between the two genotypes of cells. This further supports the idea that RIPK1 kinase activity does not contribute to the cell-intrinsic regulation of TLR-dependent gene expression.

RIPK1 kinase activity is required for host resistance to *Yersinia* infection

The finding that disruption of RIPK1 kinase activity has an isolated effect on *Yp*-induced cell death, but not cell-intrinsic cytokine production, allows the effect of *Yp*-

induced apoptosis on *in vivo* infection to be directly tested. We, therefore, infected WT and *Ripk1*^{kd} mice orally with *Yp,* and monitored bacterial burden and inflammatory responses at day 5 post-infection. Compared to wild-type mice, *Ripk1*^{kd} mice had increased bacterial burdens in the spleen and liver (Fig. 20A). Interestingly, burdens in the mesenteric lymph node (MLN) were no different between Wt and *Ripk1*^{kd} mice, suggesting that the role of RIPK1 in regulating the colonization, replication or clearance of *Yp* during infection may be tissue specific. In fact, an alternative pathway of colonizing the spleen and liver, compared to the MLN, has been suggested for *Yp* (227). Notably, the effect of RIPK1 kinase activity on controlling *Yp* burdens was specific to the enteric route of infection, as wild-type and *Ripk1*^{kd} mice infected intraperitoneally had similar bacterial burdens in the spleen (Fig. 20B).

The kinase activity of RIPK1 is required for both caspase-8-depenent apoptosis and RIPK3-dependent necroptosis (13, 14, 45, 56, 108). However, RIPK3-deficient (*Ripk3*$^{-/-}$) BMDMs or those treated with RIPK3 inhibitor did not have any detectable defects in cell death compared to wild-type and untreated BMDMs (173, 174, 225). Furthermore, chimeric mice containing *Ripk3*$^{-/-}$ bone marrow do not have significantly different bacterial burdens following *Yp* infection when compared to those containing wild-type bone marrow (174). As expected, intact *Ripk3*$^{-/-}$ mice did not differ from wild-type mice in their bacterial burdens on day 5 post-infection (Fig 20C). Thus, early control of *Yp* infection is mediated by a RIPK3-independent function of RIPK1 kinase activity.

Hematopoietic RIPK1 kinase activity is required for optimal *in vivo* cytokine production

Yp has a tropism for lymphoid tissues *in vivo*, and has a preference for injection of virulence factors into innate immune cells (228, 229). To test the contribution of RIPK1 kinase activity in the hematopoietic compartment on the control of *Yp* infection, we generated bone marrow chimeric mice from congenically marked Wt or *Ripk1*^{kd} bone marrow. Similar to infection of intact *Ripk1*^{kd} mice, *Ripk1*^{kd} bone marrow chimeras had increased bacterial burdens in both the spleen and liver, but not the MLN, when compared to Wt bone marrow chimeras (Fig. 21A). To test whether RIPK1 kinase-activity promotes *in vivo* cytokine production by innate immune cells, we measured neutrophils, monocytes and dendritic cell production of TNF and IL-12 by flow cytometry. Intriguingly, both the frequency and number of TNF-producing neutrophils and IL-12-producing monocytes were significantly reduced in *Ripk1*^{kd} chimeras (Fig. 21B-C). Therefore, RIPK1 plays an important role in regulating *in vivo* infection and the induction of pro-inflammatory cytokines by innate immune cell populations through its function in hematopoietic cells.

RIPK1 is a cell-extrinsic regulator of *in vivo* cytokine production

Consistent with our observations with BMDMs (Fig. 19B), previous studies found no evidence to support a role for RIPK1 kinase activity in promoting cell-intrinsic signaling responses under non-necroptosis inducing conditions (78, 102, 106, 199). However, our finding that the frequency of cytokine producing neutrophils and monocytes was reduced in the absence of RIPK1 kinase activity raised the possibility that there was a cell type-specific role for RIPK1 kinase activity as a cell-intrinsic

regulator of cytokine production *in vivo*. To directly test this possibility we generated mixed bone marrow chimeras using a 1:1 ratio of congenically marked *Ripk1*^{kd} and Wt bone marrow (Fig 22A). If RIPK1 were acting in a cell-intrinsic manner to promote neutrophil and monocyte cytokine production, we would expect that the difference between cytokine production in Wt and *Ripk1*^{kd} cells should persist in the mixed chimera setting. Strikingly, both the production of neutrophil TNF and monocyte IL-12 by *Ripk1*^{kd} cells were restored to the level of Wt cells in the mixed chimeras, with no difference between *Ripk1*^{kd} and Wt cells within individual mice (Fig 22B). Thus, RIPK1 kinase activity does not play a cell-intrinsic role in promoting innate immune cell cytokine production; rather the presence of cells competent for RIPK1 kinase activity complemented *Ripk1*^{kd} cell cytokine production by providing cell-extrinsic signals. Consistent with the rescue of *Ripk1*^{kd} cytokine production in mixed bone marrow chimeras, bacterial burdens in the spleen and liver of mixed chimeras were also rescued to WT levels. There was no significant difference between mice containing a mixture of Wt and *Ripk1*^{kd} bone marrow compared to mice containing Wt bone marrow alone (Fig. 22C). Collectively, these results demonstrate a role for RIPK1 kinase activity in promoting host protective innate immune responses during bacterial infection. Moreover, our findings definitively demonstrate a cell-extrinsic role for RIPK1 that is consistent with apoptotic cell death being a driver of host protective immune responses in the context of *Yp* infection.

Discussion

In order to successfully colonize, infect, and spread within a host population, pathogens utilize diverse mechanisms of inhibiting innate immune signaling pathways

that allow for pathogen recognition and the induction of host protective immunity. While *Yersinia* efficiently escapes recognition by several mechanisms, its induction of cell death may represent an important immunogenic “danger” signal, offering an alternative mechanism of inducing inflammation in settings of cell-intrinsic signaling blockade (92). Here we have demonstrated that *Yp*-induced apoptosis promotes cytokine production and innate immunity, representing an important host defense against infection.

Although historically believed to be a primarily immunosuppressive or quiescent form of cell death, recent studies have found that under certain circumstances, apoptosis can potentiate inflammation and immunity (6, 154). Previous studies investigating the role of apoptosis during *Yersinia* infection have relied on indirect evidence from the loss or increased function of the virulence factor YopJ (191, 195, 196). These studies, while informative, are confounded by the pleiotropic effects of YopJ activity, which results in both decreased cytokine production and the induction of cell death. The demonstration that YopJ activity promotes bacterial virulence at normal doses suggests that inhibition of immune signaling and cytokine production are required for optimal infection (191, 192). In this setting, the benefits of YopJ inhibition of cytokine signaling must outweigh the immunogenic effects of YopJ-induced cell death. In contrast, enhanced activity of YopJ may drive excessive cell death, leading to inflammation that is detrimental to bacterial virulence (195, 196). These results suggest an evolutionary balancing effect of immune pressures on YopJ activity. Thus, it seems plausible that the ancestral function of YopJ and YopJ-related proteins is to suppress cell-intrinsic host signaling response to bacterial recognition, while the apoptotic response is a host defense mechanism that bypasses pathogen blockade of immune signaling. By directly disrupting host cell apoptosis, independent from the cytokine disruption mediated by YopJ, the studies

described here have for the first time isolated the effects of apoptosis on the host response to *in vivo* bacterial infection.

Importantly, the host protective effect of RIPK1 kinase-dependent apoptosis on the immune response to *Yp* infection was mediated by an extrinsic signaling response, rather than an *in vivo* cell-type specific role for RIPK1 in regulating cell-intrinsic signaling. These results are consistent with a model in which apoptosis allows for the propagation of an inflammatory response to neighboring cells that sense this cell death as a danger signal. This is supported by the ability of wild-type cells to complement the defect in cytokine production by *Ripk1*^{kd} cells in the mixed bone marrow chimera setting.

Apoptotic neutrophils, when coupled with bacterial LPS, have been demonstrated to promote an adaptive T_h17 responses in the context of enteropathogenic *E. coli* infection (156, 157). Recognition of apoptotic cells alone results in the production of TGFβ by dendritic cells (DCs), a stimulus that has potent effects on the differentiation and expansion of regulatory T cell populations (132, 135, 136) . The effect of pro-inflammatory IL-6 production, driven by the recognition of bacterial LPS, acts to switch the regulatory response induced by apoptotic cell recognition to the generation of a pro-inflammatory T_h17 response (156). In contrast to this synergy in the combined effects of apoptosis and bacterial infection on driving a distinct response, our data suggest that in the context of *Yersinia* infection apoptotic cell death simply enhances innate immune inflammation. Whether this is reflective of difference between innate and adaptive responses studied in the two models is unclear. Nevertheless, understanding the receptors that recognize cell death in the context of both of these infection models could provide insight into how multiple signals are integrated into activation of an immune response.

In the absence of RIPK1 kinase-dependent cell death, decreased TNF and IL-12 production could be responsible for the defect in bacterial control. Interestingly, TNFR1-deficient mice have a defect in bacterial control similar to that seen in *Ripk1*^{kd} and BM chimeras (225). Based on our results here, TNF may, therefore, play a role in both upstream and downstream of YopJ-dependent apoptosis. This would imply that TNF could act in a feed forward loop to potentiate further apoptosis and inflammation. IL-12 is an important driver of NK cell and T_h1 responses, promoting the production of IFNγ (230). In the case of *Yersinia* infection, IL-12 has been shown to be required for protection (231-233). Thus, it is possible that exogenous IL-12 treatment may be sufficient to protect them from *Yp* infection if the inability to control bacterial infection is dependent on the loss of IL-12.

Cytokine-independent effects of RIPK1 kinase-dependent cell death may also be responsible for mediating protection from *Yp* infection. Inhibition of phagocytosis by *Yp* limits the ability of innate immune cells to clear bacteria directly (234, 235). In contrast, apoptotic cells are typically cleared rapidly by neighboring phagocytes through efferocytosis (236). Thus, bacteria tightly adhered to or trapped by apoptotic corpses may be more efficiently cleared than in the absence of apoptosis (217, 237, 238). Thus, apoptosis may play a role in promoting bacterial phagocytosis and killing, independent of the induction of increased pro-inflammatory cytokines measured here.

In conclusion, cell death may have an important role in driving not just adaptive immunity (154, 157, 239), but in promoting essential innate immune responses during infection. Using a novel *in vivo* approach to directly disrupt cell death during infection, we have for the first time been able to separate the effects of pathogen virulence factors on the inhibition of host cell signaling from those on the induction of apoptosis. These

studies have demonstrated a host protective function for pathogen-induced apoptosis. Understanding the mechanisms by which cell death is recognized and by which immune responses to cell death promote bacterial control will be an important question that holds potential for improving the understanding of the relationship between cell death and inflammation.

Figure 19

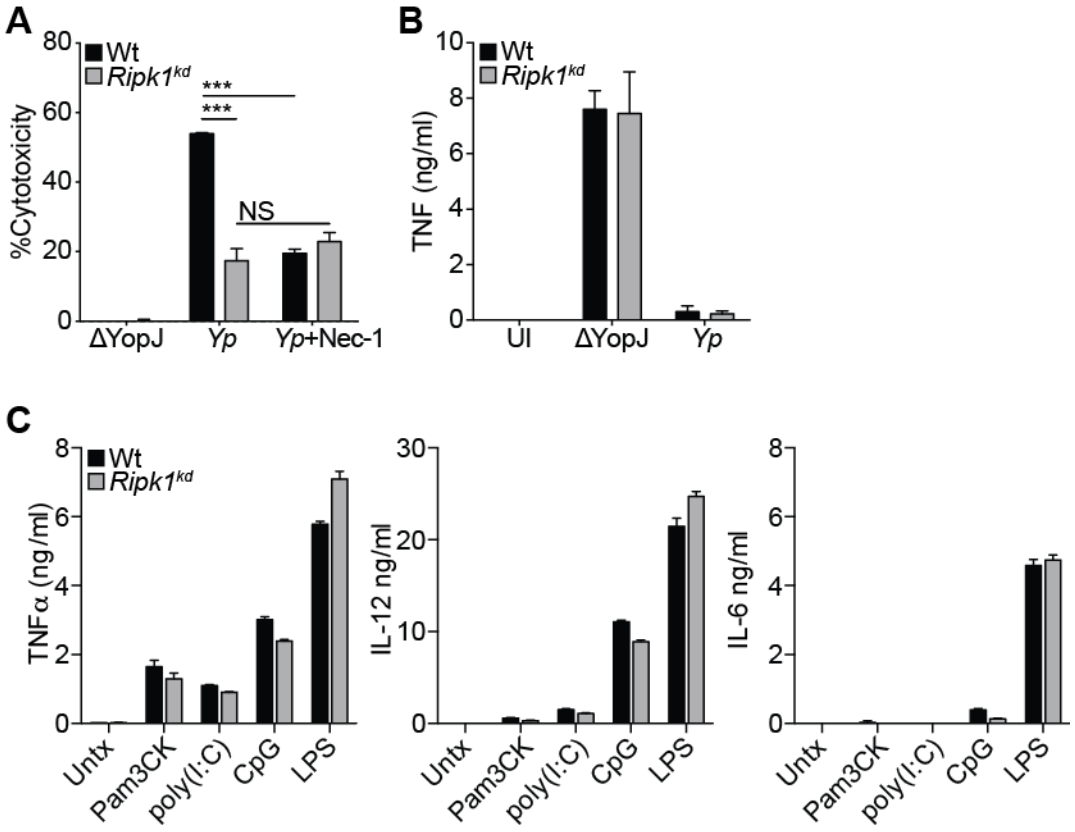

Figure 19. *Yersinia*-induced apoptosis requires RIPK1 kinase activity.
(A) Wild-type (Wt) and *Ripk1*^{kd} bone marrow derived macrophages (BMDMs) treated with the RIPK1 inhibitor, necrostatin-1 (Nec-1, 60 μM) or media control, were infected with wild-type (*Yp*) or YopJ-deficient (ΔYopJ) *Yersinia* at an MOI of 20 and cytotoxicity was measured by LDH release. Results representative of greater than 3 independent experiments. (B-C) Cytokine release was measured by ELISA from supernatants of cells infected with ΔYopJ and *Yp* (B) or treated with an array of toll-like receptor ligands (C). Pam3CK (1μg/mL), poly(I:C) (50μg/mL), CpG (1μg/mL), LPS (50ng/mL). Results representative of two independent experiments. ***$P \leq 0.001$; NS, not significant.

Figure 20

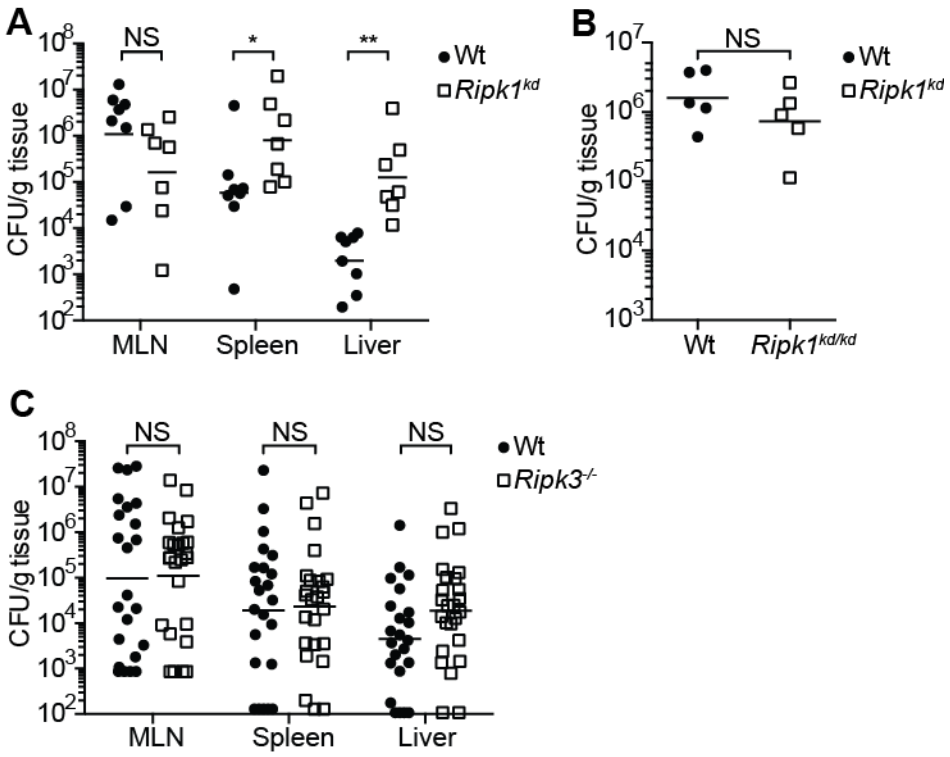

Figure 20. RIPK1 kinase activity is required for host resistance to *Yersinia* infection.
(A) Wt and *Ripk1*^{kd} mice were infected orally with 2x10^8 CFU and bacterial burdens were measured from tissue on day 5 post-infection. (B) Mice were infected by intraperitoneal injection with 5x10^4 CFU and splenic bacterial burdens were measured on day 3 post-infection. Results representative of at least 3 independent experiments. (C) Wt and *Ripk3*$^{-/-}$ mice were infected orally with 2x10^8 CFU and bacterial burdens were measured from tissue on day 5 post-infection. *P≤0.05, **P≤0.01; NS, not significant.

Figure 21

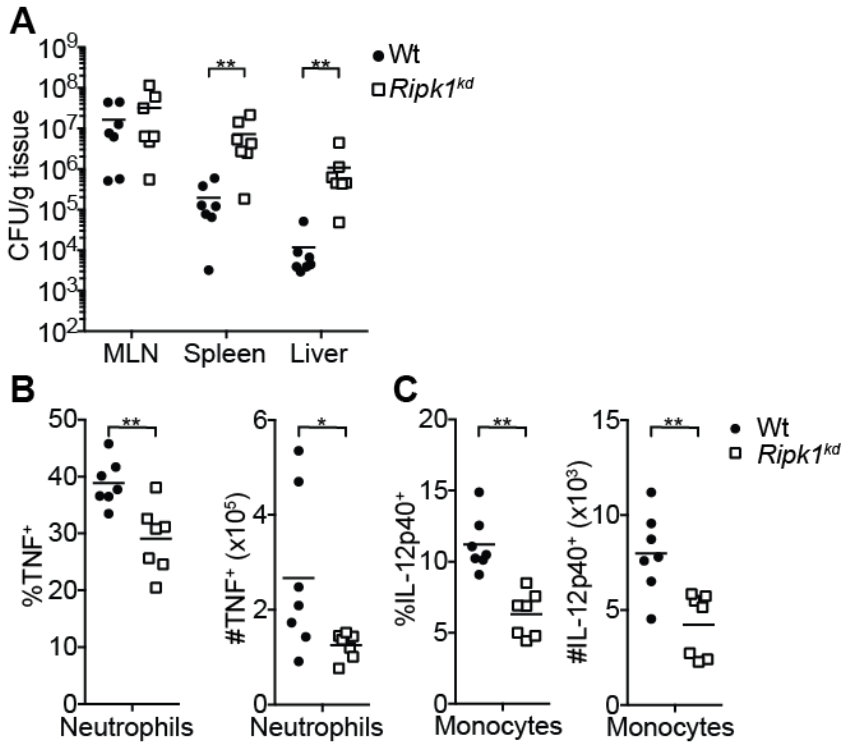

Figure 21. Hematopoietic RIPK1 kinase activity is required for optimal *in vivo* cytokine production.

(A) Bone marrow chimeras were generated by reconstituting lethally irradiated congenic hosts (B6.SJL, CD45.1$^+$) with bone marrow from wild-type or *Ripk1*^{kd} mice (CD45.2$^+$). Chimeras were infected orally with 2x10^8 CFU and bacterial burdens were measured from tissue on day 5 post-infection. (B) Innate immune cells from day 5 post-infection mesenteric lymph nodes (MLN) were measured for TNF and IL-12 production by flow cytometry. Numbers of cytokine positive neutrophils (CD11b^{hi}Ly6G$^+$) and monocytes (CD11b^{hi}Ly6C^{hi}Ly6G$^-$) calculated from tissue cell counts and frequencies of total singlet live cells. **P*≤0.05, ***P*≤0.01

Figure 22

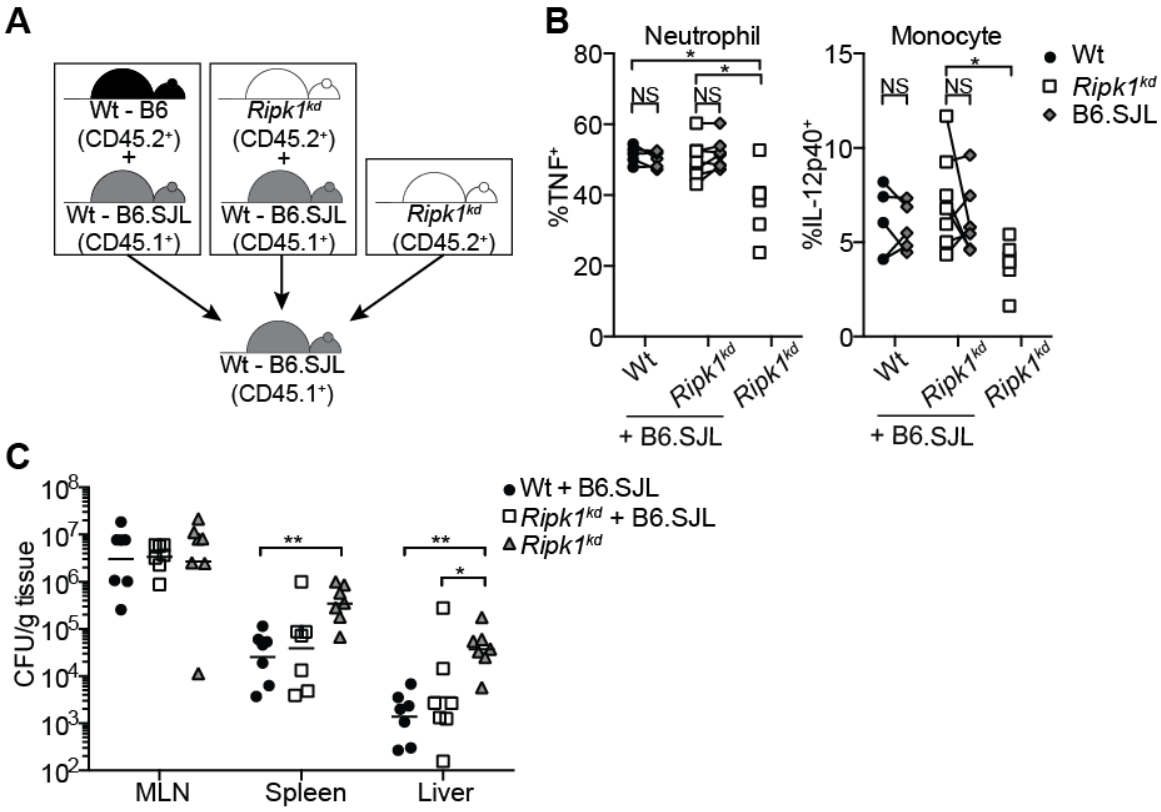

Figure 22. RIPK1 is a cell-extrinsic regulator of *in vivo* cytokine production.
(A) Mixed and *Ripk1*^{kd} bone marrow chimeras were generated by reconstituting lethally irradiated congenic hosts with *Ripk1*^{kd} bone marrow alone or a 1:1 mixture of B6.SJL (CD45.1^{+}) and wild-type or *Ripk1*^{kd} (CD45.2^{+}) bone marrow. (B) Chimeras were infected orally with 2x10^{8} CFU and innate immune cells from day 5 post-infection mesenteric lymph nodes (MLN) were measured for TNF and IL-12 production by flow cytometry. Frequency of TNF-producing neutrophils (CD11b^{hi}Ly6G^{+}) and IL-12-producing monocytes (CD11b^{hi}Ly6C^{hi}Ly6G^{-}) were compared between groups (unpaired) or within individual mice (pairwise, indicated by connecting lines). (C) Bacterial burdens were measured on day 5 post-infection. Results representative of three independent experiments. *P≤0.05, **P≤0.01; NS, not significant.

Figure 23

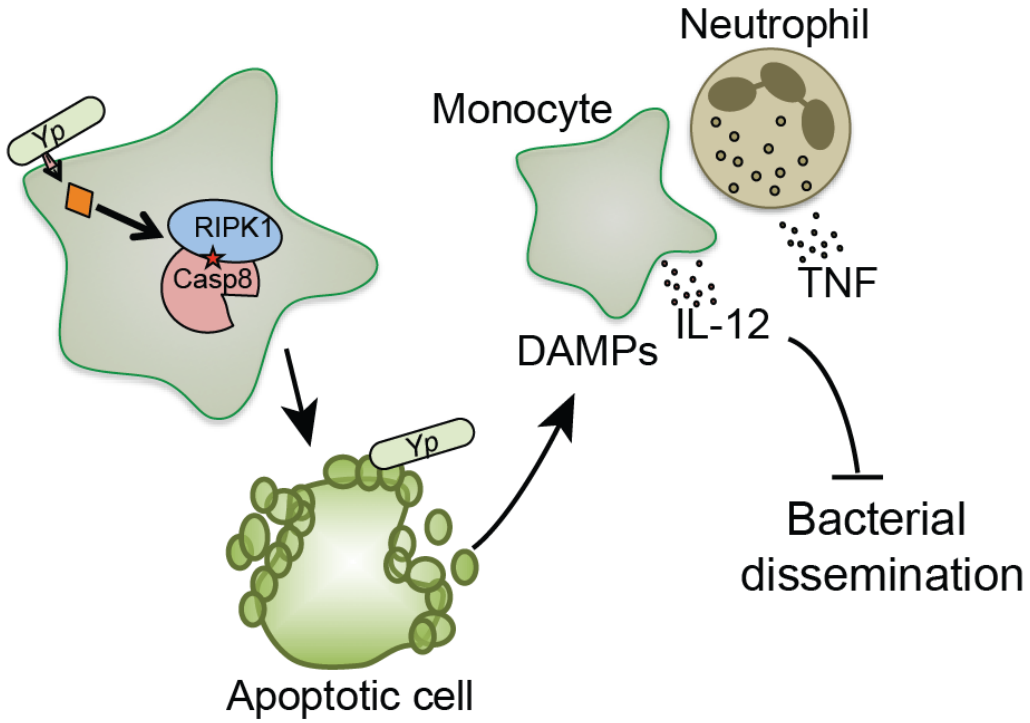

Figure 23. *Yersinia*-induced promotes host-protective antibacterial immunity. *Yersinia*-induced apoptosis mediated by RIPK1 and caspase-8 promotes the release of damage-associated molecular patterns (DAMPs). These signals promote the production of IL-12 and TNF in a cell-extrinsic manner. Increased monocyte IL-12 and neutrophil TNF production promote antibacterial immunity to limit bacterial dissemination.

CHAPTER 5

Conclusions and future directions

The innate immune system has developed to recognize pathogens through PRRs that detect conserved microbial structures or patterns present on microbes (1). The recognition of these signals represents an important stimulus for promoting immune activation. However, many pathogens evade detection by these PRRs through virulence factors that inhibit host cell signaling (204). Alternatively, innate immune recognition of endogenous 'danger' signals may allow for the indirect recognition of pathology induced during infection (2). Cell death of infected cells may allow for 'danger' to be translated into an immune response, even when pathogens inhibit host signaling. During *Yersinia* infection, the virulence factor YopJ efficiently limits pro-inflammatory cytokine production by cells it encounters. However, this inhibition also results in the induction of apoptosis that has the potential to alert other cells to the presence of a dangerous pathogen. In chapter three of this thesis, I described the previously unappreciated contribution of TNFR1 to *Yersinia*-induced apoptosis and the role of cell-extrinsic TNF production in promoting this response. In chapter four, I tested the role of apoptosis in regulating host immunity or pathogen virulence during *Yersinia* infection, demonstrating a host protective function for RIPK1 kinase-dependent apoptosis. Here I will discuss the implications of our findings in these two chapters and how these results open the door to future inquiry. I will first focus on events and signaling pathways leading to *Yersinia*-induced apoptosis. I will then discuss the implications of this cell death response on *in vivo* infection with *Yersinia* and how they provide insight into the broader interpretation of cell death signaling in the regulation of infection and inflammation.

Regulation of *Yersinia*-induced cell death

The YopJ virulence factor of *Yersinia pseudotuberculosis* potently inhibits cytokine production and induces apoptosis of host cells (166, 167, 170, 206). It was previously only appreciated that the recognition of bacterial LPS by TLR4 contributed to this cell death (168, 169, 175-177). This conclusion suggested that the extrinsic signals promoting host cell apoptosis were entirely pathogen-derived and independent of host factors. The data presented here demonstrate an important contribution of TNF signaling to *Yersinia*-induced cell death and support an active role for cytokine signaling and the immune response in inducing this cell death.

In some ways it is not surprising that TNF signaling can contribute to cell death. Death receptors, including TNFR1, are well-established inducers of apoptosis (12). Inhibition of NF-κB and MAPK signaling by *Yersinia* would predispose to these cell death outcomes. However, the potent inhibition of TNF production during *Yersinia* infection suggests that TNF would not be a contributor to this cell death response. Furthermore, inhibition of TNF production and depletion of pro-survival signaling from cells are directly linked by the activity of YopJ. In other words, those cells that are most inhibited should also not be producing TNF, making it unlikely that autocrine signaling would be able to mediate apoptosis. This is likely why the contribution of TNF to *Yersinia*-induced cell death has been overlooked until now. Our findings that cell-extrinsic TNF promotes *Yersinia*-induced cell death highlight the importance of heterogeneity and cell-cell interactions in promoting immune responses.

Despite cells being treated similarly during *in vitro* infection, it is consistently seen that only a fraction of BMDMs undergo apoptosis. Interestingly, our results using the β-

lactamase injection assay demonstrate heterogeneity in the effects of YopJ on BMDMs. At an MOI of 20 all the cells are injected (Fig. 14A). However, a fraction of the cells have a reduced amount of injected YopJ, as measured by cleaved β-lactamase substrate (Fig. 14A). This implies that some cells may be restrictive to the amount of YopJ that is injected by *Yersinia*. Furthermore, a fraction of BMDMs containing lower amounts of YopJ are able to produce TNF, while others are completely inhibited in their response. This additionally suggests that some cells may be able to resist inhibition by YopJ, even once it is injected. Thus, infection of BMDMs demonstrates heterogeneity at both the level of virulence factors injected into cells and the susceptibility of cells to the effects of these virulence factors.

Recent work with bone marrow derived dendritic cells (BMDCs) has shown significant heterogeneity in cellular phenotype of similarly treated cells (240, 241). Within a given culture, some BMDCs express markers of and behave more like macrophages and others more like classical dendritic cells. If similar heterogeneity exists in BMDM cultures at baseline, it may be the cause of heterogeneity in the response to *Yersinia* infection. Notably, BMDCs are more resistant to YopJ-dependent apoptosis than BMDMs (195, 218). If cells with more DC like properties are present in BMDM cultures, these cells might make up the population able to produce TNF following infection. Using additional cellular markers to assess BMDM heterogeneity following infection may provide insight into the features that protect from or predispose to *Yersinia*-induced apoptosis. The importance of cellular heterogeneity in BMDM cultures also provides insight into how multiple cell types may interact *in vivo* to promote diverse responses to infection. The response to PRR signaling can be propagated by the activation and recruitment of other cells through cytokine signaling. Cells unable to respond

appropriately to this inflammatory signal, due to pathogen inhibition, may be recognized as dangerously altered. In this setting, cell death may be advantageous for the host, allowing for release of DAMPs or for the removal and clearance of pathogen-inhibited cells.

Based on my *in vitro* data, TRIF- and TNFR1-dependent apoptosis signaling pathways appear to parallel each other. Both are dependent on RIPK1 kinase activity (Fig. 8C); both require receptor or signaling complex endocytosis (Fig. 15); and both result in caspase-8-dependent caspase-1 cleavage (Fig. 10D). Based on these results, it would seem reasonable to hypothesize that they have similar importance *in vivo*. However, there is evidence to suggest that this may not be the case and that TNFR1-induced cell death plays a more dominant role *in vivo* than TRIF signaling. During *in vivo* infection, and when cultured at 37°C, *Yersinia* alters the structure of its LPS to one that is poorly recognized by TLR4 (185-187, 242). Thus, *Yersinia* may partially escape direct detection, in addition to inhibiting signaling by YopJ. Signals released from apoptotic cells may, therefore, be a more important inflammatory cue for eliciting inflammation than recognition of bacterial LPS. Based on my data demonstrating a protective function for RIPK1 kinase-dependent apoptosis during *Yp* infection, the immune response would benefit from facilitating apoptosis through TNFR1. Furthermore, infection of TRIF-deficient mice with *Yp* has previously been shown not to differ from that of wild-type mice (data not shown) (218). In contrast, I show that infection of TNFR1-deficient mice results in increased bacterial burdens (Fig. 16D). Interestingly, the pattern of increased bacterial burdens in spleen and liver, but not in Peyer's patches or MLN, following oral infection matches that seen in the results from infection of *Ripk1*^{kd} mice (Fig. 20A). Although

correlative, this similarity suggests that TNFR1 signaling, but not TRIF signaling, is mediating *in vivo* protection through RIPK1 kinase-dependent apoptosis.

The increased bacterial burden seen in TNFR1-deficient mice could alternatively result from cell death-independent functions of TNF on regulating innate immunity to *Yersinia* infection. One potential method to directly test whether TRIF- or TNFR1-induced apoptosis, versus non-apoptotic functions, contribute to protection *in vivo* would be through the use of mixed bone marrow (BM) chimeras. Based on my results, the increased bacterial burden in *Ripk1*^{kd} BM chimeras can be rescued by co-transfer of wild-type BM (Fig. 22C). This implies that only a fraction of the hematopoietic compartment (~50%) must be capable of undergoing Ripk1 kinase-dependent apoptosis in order to control infection (Fig. 24). Mice with a mixture of Wt and *Tnfr1*$^{-/-}$ BM would be expected to behave similarly. If TNFR1 act in the same cell populations as RIPK1 in promoting a protective apoptotic response, then co-transfer of TNFR1-deficient (*Tnfr1*$^{-/-}$) BM along with *Ripk1*^{kd} BM should not be able to rescue the increased bacterial burden in *Ripk1*^{kd} chimeras. This would be because neither *Ripk1*^{kd} nor *Tnfr1*$^{-/-}$ would be capable of undergoing *Yersinia*-induced apoptosis and providing the protective effects of this response (Fig. 24). It is also possible that TRIF and TNFR1 perform similar functions in promoting apoptosis *in vivo*. However, the fact that TRIF-deficient mice do not have a defect in bacterial control (218), while TNFR1-deficient mice do (Fig. 16D), suggests that this is not the case. Infection of double deficient mice (*Trif*$^{-/-}$*Tnfr1*$^{-/-}$) or chimeras receiving a mixture of *Trif*$^{-/-}$*Tnfr1*$^{-/-}$ and *Ripk1*^{kd} BM could test this hypothesis. If TNFR1-induced apoptosis provides a protective function *in vivo*, this would support a model in which TNFR1 promotes RIPK1 kinase-dependent apoptosis to facilitate the innate immune response against bacterial infection.

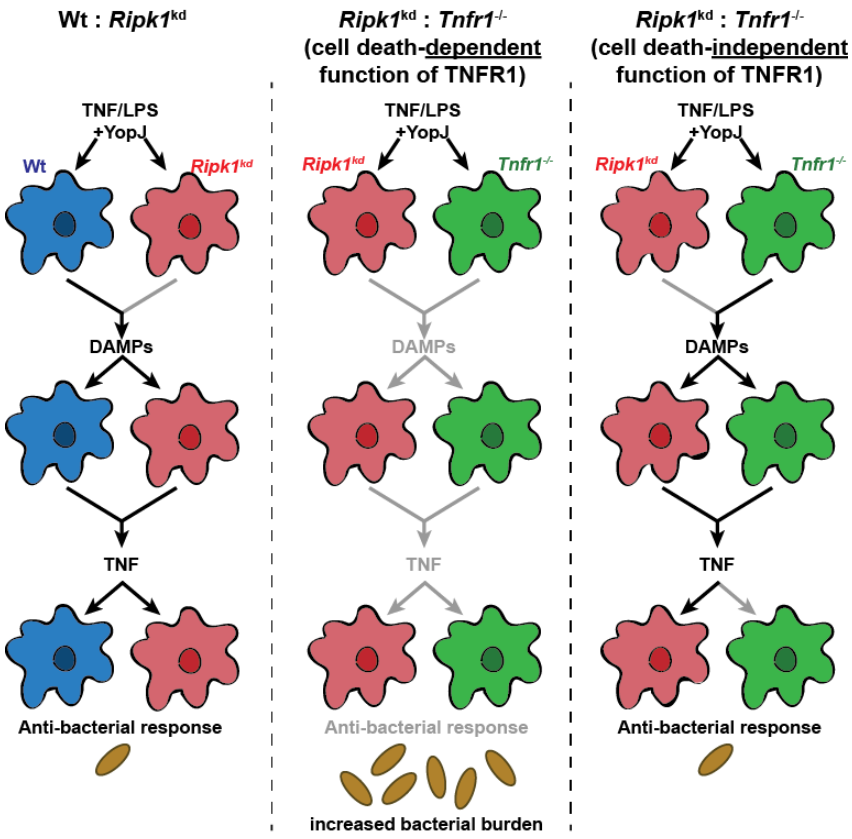

Figure 24. Does TNFR1 promote protection through regulation of *Yersinia*-induced apoptosis?
Tnfr1$^{-/-}$ and *Ripk1*^{kd} mice both increased bacterial burdens following *Y. pseudotuberculosis* infection compared to Wt mice. These defects can be rescued in mixed chimeras containing added Wt bone marrow. Wt cells provide cell death-dependent DAMPs that *Ripk1*^{kd} cells do not. If TNFR1 acts at the same stage of promoting apoptosis as RIPK1, then mixed chimeras containing *Tnfr1*$^{-/-}$ and *Ripk1*^{kd} BM in the absence of Wt cells should have a similar defect to chimeras containing *Tnfr1*$^{-/-}$ or *Ripk1*^{kd} BM alone. If the function of TNFR1 does not dependent on its regulation of *Yersinia*-induced apoptosis, then these mixed chimeras will control infection similar to Wt chimeras.

The current model for how *Yersinia* promotes apoptosis is that the inhibition of key kinases, including TAK1 and IKKα/β, by the acetyl-transferase activity of YopJ prevents induction of pro-survival signals downstream of TLR4/TRIF and TNFR1 signaling (169, 243). It has been largely assumed that the pro-survival signals blocked by YopJ are dependent on a transcriptional NF-κB- and MAPK-dependent response (167-169). However, an additional NF-κB- and transcription-independent pro-survival signal mediated by TNFR1 has recently been identified (114, 244-247). Thus, two distinct pathways of regulating extrinsic apoptosis exist. The presence of these two pathways is demonstrated by the differential requirement for RIPK1 in mediating TNF-induced apoptosis following disruption of either an 'early' or 'late' checkpoint in preventing cell death by apoptosis and necroptosis (Fig. 25) (109, 111). Genetic or pharmacologic inhibition of cIAPs, TAK1 or the IKK complex activity represents a disruption of an 'early checkpoint' that results in RIPK1-dependent apoptosis (46, 109, 112, 113). The 'late checkpoint' regulating TNF-induced apoptosis can be targeted by blocking of NF-κB-dependent responses, either through inhibition of protein translation or expression of a dominant negative form of inhibitor of κB (IκB-DN)(46, 112, 113). This pathway of apoptosis is independent of RIPK1 and results from loss of negative regulators of caspase-8 activation such as cFLIP. Importantly, the kinetics of apoptosis through these two pathways is distinct, with disruption of the 'early checkpoint' promoting more rapid apoptosis (Fig. 25).

Recent studies have attributed this 'early checkpoint' to RIPK1 phosphorylation by IKKβ, which inhibits its ability of RIPK1 to complex with caspase-8 and promote apoptosis (114). Although this regulation of apoptotic signaling has yet to be shown to

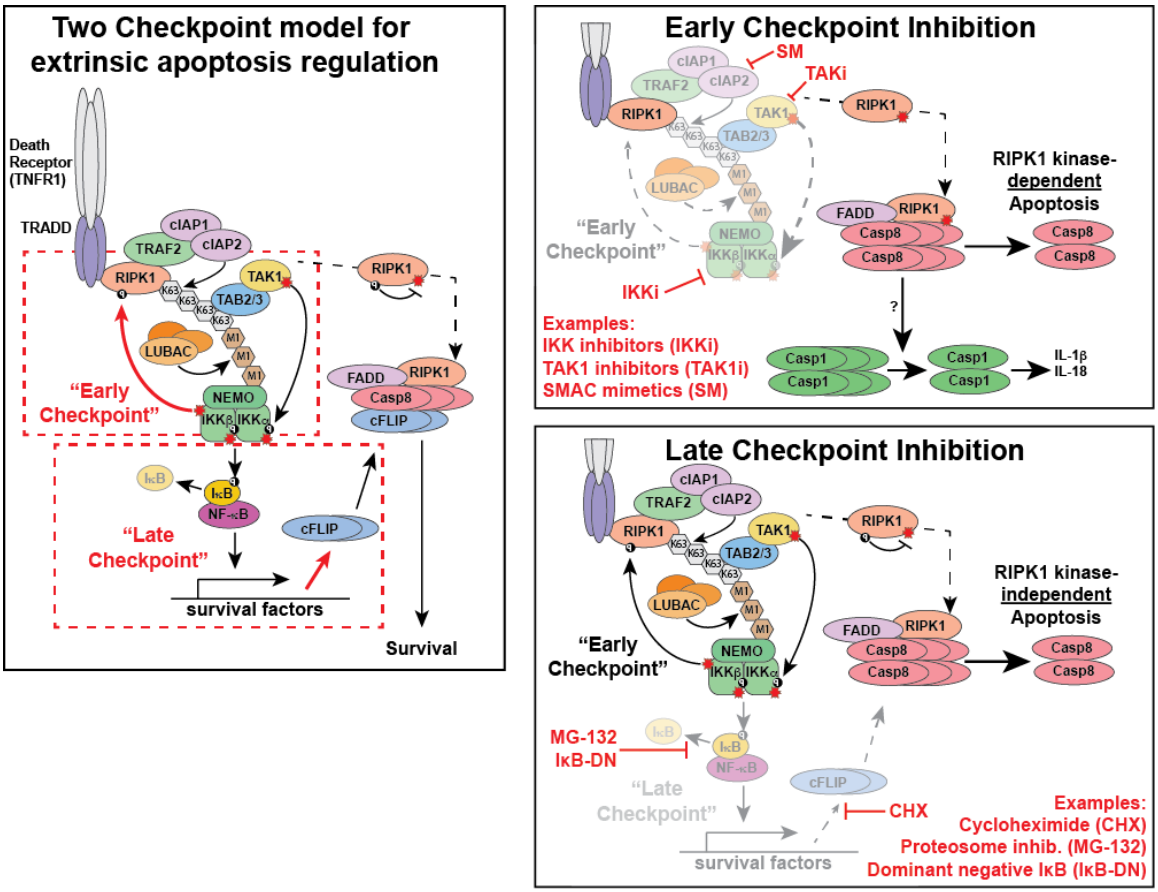

Figure 25. Two checkpoint model for regulation of extrinsic apoptosis.
(A-B) The 'early checkpoint' regulating extrinsic apoptosis requires activation of TAK1 and IKK complexes and is independent of NF-κB and transcriptional pro-survival responses. IKKβ phosphorylates RIPK1 to inhibit its kinase function and ability to promote caspase-8 activation. This response can be inhibited by IKK inhibitors, TAK1 inhibitors and SMAC mimetics that disrupt components of the signaling complex required for this early checkpoint. Caspase-1 cleavage may also occur during this apoptotic response and lead to release of IL-1 family cytokines. **(C)** The 'late checkpoint' is promoted by NF-κB-dependent expression of pro-survival factors, such as cFLIP, that inhibit caspase-8 activation. This checkpoint is blocked by proteasome inhibitors (MG-132), protein translation inhibitors (CHX) and expression of dominant negative I-κB.

exist for TRIF-induced apoptosis, the parallels between the two pathways suggest they may be analogous. Since YopJ targets both TAK1 and IKKα/β, it is likely that *Yersinia*-induced apoptosis results from failure to phosphorylate RIPK1 downstream of TRIF and TNFR1. This model is supported by results in which cells expressing a dominant negative form of IκB (IκB-DN) were infected with wild-type or YopJ-deficient *Yp* (169). In contrast to survival of wild-type cells, IκB-DN expressing cells underwent apoptosis in response to ΔYopJ infection. Interestingly, these cells still exhibited significantly less death than IκB-DN-expressing cells infected with wild-type *Yp*, suggesting that inhibition of NF-κB-mediated responses was only partially responsible for inducing YopJ-dependent apoptosis and that additional NF-κB-independent survival signals must be disrupted. RIPK1 phosphorylation by IKKβ is likely also a signal disrupted by YopJ that contributes to *Yp*-induced apoptosis. Measuring the phosphorylation of RIPK1 following infection with ΔYopJ and wild-type *Yersinia* could test this hypothesis.

Infection of IκB-DN expressing cells pre-treated with LPS could offer an alternative approach to demonstrate a functional role for the inhibition of RIPK1 phosphorylation in promoting *Yersinia*-induced apoptosis. Priming of wild-type cells with LPS prior to infection with *Yp* protects against YopJ-dependent apoptosis (178, 180). If this protection occurs through an NF-κB-independent pro-survival signal then priming of IκB-DN expressing cells should be sufficient to mediate at least partial protection from YopJ-dependent apoptosis. Priming alone would be expected to induce some cell death in IκB-DN-expressing cells. However, the kinetics and magnitude of the response would differ between apoptosis resulting from a failure in the ‘late checkpoint’ versus ‘early checkpoint’ described above. Using TRIF- or TNFR1- deficient cells for similar experiments could also test for the first time whether IKK-dependent phosphorylation of

RIPK1 acts as an NF-κB-independent survival signal downstream of TLR4/TRIF signaling in addition to TNFR1.

Interestingly, when IKKβ is genetically or pharmacologically inhibited, cells undergo caspase-1 activation and IL-1 family cytokine release during the apoptotic response to LPS or TNF stimulation (184). This is similar to the cleavage of caspase-1 and IL-18 release during YopJ-dependent apoptosis, and further suggests that YopJ acts to induce cell death by inhibition of an NF-κB-independent checkpoint for regulating apoptosis. Thus, defects in the ability of IKKβ to inhibit RIPK1 activity through phosphorylation may be key determinants of caspase-1 activation during apoptosis and the switch of some forms of apoptosis from tolerogenic to immunogenic in nature.

The immune response to *Yersinia*-induced apoptosis

Yersinia infection induces YopJ-dependent apoptosis of host cells that is mediated by RIPK1 kinase activity. RIPK1 ubiquitination and its kinase-independent functions promote cell survival and cytokine production downstream of death receptor and TLR signaling. In contrast, RIPK1 kinase activity specifically promotes both apoptosis and necroptosis when pro-survival signaling is inhibited (14, 106-108, 197). Targeted mutations in the catalytic domain allow for these two functions to be separated in a 'kinase dead' mutant version of RIPK1 (78, 102, 106, 199). In chapter four of this thesis we used mice expressing kinase dead RIPK1 (*Ripk1*^{kd}) to specifically test the contribution of *Yersinia*-induced apoptosis in the regulation of *in vivo* infection. This allowed for a system in which the function of infection-induced cell death could be specifically targeted for the first time. Strikingly, our infection data demonstrate an important function for RIPK1 kinase activity in the control of bacterial burdens and the

production of optimal inflammatory cytokines by innate immune cells (Fig. 21B). Using mixed BM chimeras we show that RIPK1 kinase activity acts in a cell-extrinsic manner to promote cytokine production, which is consistent with a cell death-mediated function (Fig. 22B-C). In addition to promoting apoptosis, RIPK1 kinase activity promotes RIPK3-dependent necroptosis. However, while necroptosis is thought to act as a more inflammatory form of cell death than apoptosis, we find no role for RIPK3 in regulating early infection with *Yersinia* (Fig. 20C). Thus, a key question that remains is how does RIPK1 kinase-dependent apoptosis promote the immune response to infection?

The combination of apoptotic and microbial signals has been shown to alter the response to one that is distinct from either signal alone (239). This has been most clearly shown by the phagocytosis of apoptotic neutrophils during infection with the enteropathogenic bacterium *Citrobacter rodentium*. Recognition of apoptotic cells alone results in the production of TGF-β and the expansion of regulatory T cell responses to self-antigen (135, 136). With the addition of LPS, antigen-presenting cells will produce the inflammatory cytokine IL-6 that, in conjunction with TGF-β, induces an antibacterial T_h17 response (156, 158-160). Thus, the addition of a bacterial induced PRR signal converts apoptosis from an immunosuppressive to an inflammatory stimulus. In the case of *Yersinia*, the adaptive response is not characterized by robust T_h17 differentiation in wild-type or *Ripk1*^{kd} mice (data not shown). However, synergistic responses integrating both apoptotic cell- and bacterial-derived signals could theoretically alter the response to *Yersinia* infection in other ways that should be considered when studying the role of apoptosis on host immunity.

The induction of immunogenic cell death is mediated by the release or exposure of DAMPs that convey cellular stress or damage to the immune system (6). While

DAMPs, including HMGB1, ATP and surface calreticulin, generate immunogenic responses in the context of cancer (154), signals associated with apoptosis that may confer immunogenicity in the setting of infection are not well understood. Whether these common DAMPs are also released during *Yersinia*-induced apoptosis, and the possibility that they contribute to the protective effects of RIPK1 kinase-dependent cell death *in vivo* are important questions to be addressed. In response to TLR stimulation alone, *Ripk1*^{kd} BMDMs do not behave differently from wild-type cells (Fig. 19B-C). However, our *in vivo* data suggest that RIPK1 kinase activity provides a cell-extrinsic signal that confers increased cytokine production by neutrophils and monocytes (Fig. 21B). If this signal is mediated by release of immunogenic stimuli from cells undergoing *Yp*-induced apoptosis, then stimulation of BMDM or BMDCs with *Yp*-infected cells or culture supernatants may provide an *in vitro* model for testing the effects of apoptotic cells on macrophage and DC activation. Intraperitoneal or IV injection of wild-type versus *Ripk1*^{kd} *Yp*-infected cells could similarly be used to test the *in vivo* contribution of RIPK1 kinase-dependent apoptosis in promoting inflammatory responses. Genetic disruption or antibody blockade could test the function of specific DAMP signals in this response.

Yersinia-induced apoptosis is associated with the cleavage and activation of caspase-1 (180, 182, 183). This allows for the release of cytokines in the IL-1 family, such as IL-1β and IL-18, which require processing by caspase-1 for their secretion and activity. The ablation of YopJ-dependent caspase-8 processing and apoptosis in *Ripk1*^{kd} cells may also prevent release of these cytokines *in vivo*. Decreased IL-1β and IL-18 release could be responsible for increased bacterial burdens seen in *Ripk1*^{kd} mice (233, 248). Measuring levels of IL-1β and IL-18 from *Yersinia*-infected mice, however, is challenging for several reasons. First, both cytokines can be released in their non-

cleaved and inactive form. Thus, measuring bioactive IL-1β and IL-18 levels requires differentiating between the two forms. Furthermore, IL-18 is quickly bound by its soluble binding proteins (249). The majority of IL-18, at least in human serum, is bound to IL-18 binding protein, requiring special considerations for the measurement of total versus free IL-18 (250). . It is not entirely clear whether current murine IL-18 ELISA reagents are capable of making these distinctions. Important experiments for linking a defect in the production of these cytokines to the phenotype of *Ripk1*^{kd} mice will be treatment of mice with exogenous IL-1β or IL-18. Whether these cytokines rescue the defects in either cytokine production or control of bacterial burdens seen in *Ripk1*^{kd} mice will be informative for understanding how *Yersinia*-induced apoptosis contributes to the immune response.

Another IL-1 family member, IL-33, is also released from cells upon membrane lysis, and its release has primarily been attributed to necrotic cell death (150, 151). IL-33 was initially described as a driver of type 2 immunity and has been extensively studied for its role in promoting T_h2 and group 2 innate lymphoid cell responses (251, 252). It has also been shown to play a role in promoting T_h1 and $CD8^+$ T cell responses (253, 254) and is implicated in a number of human inflammatory conditions (255). Unlike IL-1β and IL-18, IL-33 is active in its uncleaved form and inactivated by the apoptotic caspases-3 and -7 (150, 151). Thus, following release from apoptotic cells, it is believed to be inactive. However, studies assessing IL-33 activity have not looked extensively at RIPK1 kinase-dependent forms of apoptosis, and it remains possible that inactivation of IL-33 is not universally associated with all pathways of apoptosis (see discussion below). Thus, IL-33 release should be measured in the setting of *Yersinia*-induced apoptosis.

The contribution of different cell types *in vivo* to *Yersinia*-induced apoptosis and the subsequent induction of immune responses is an important area that remains to be explored. While macrophages undergo YopJ-dependent apoptosis (166, 167, 256), other cell types such as dendritic cells and neutrophils are more resistant to this cell death (180, 218, 257). Many more populations of cells have not been directly studied. It will first be important to determine what cell populations are, in fact, undergoing *Yersinia*-induced apoptosis *in vivo*. This could be done by flow cytometric analysis of *Yersinia*-infected tissues using apoptotic markers such as annexin V staining or antibodies against cleavage products of apoptotic caspase targets, such as cleaved caspase-3 or PARP1. However, these approaches may be limited by the loss of fragile dead cells during isolation and the background of apoptosis that exists from homeostatic turnover. An alternative would be to visualize apoptotic cells by immunohistochemistry or immunofluorescence staining, which may also provide useful information on the localization of *Yersinia*-induced apoptosis within lymphoid tissues.

Many cells may undergo cell death during infection, either as a consequence of YopJ-dependent inhibition or through independent processes more generally associated with inflammation. Not all of these cell death responses may have the same impact on the immune response. Thus, determining what cells are necessary for mediating the effects of RIPK1 kinase activity on the immune response will require specifically targeting individual cell populations *in vivo*. Unfortunately, genetic tools do not currently exist for the cell-type specific conditional ablation of RIPK1 kinase activity alone. However, the use of BM chimera models may allow the requirement for RIPK1 kinase activity in some cell populations to be tested *in vivo* (Fig. 26). For example, the role of RIPK1 kinase activity specifically in monocytes may be able to be tested by comparing

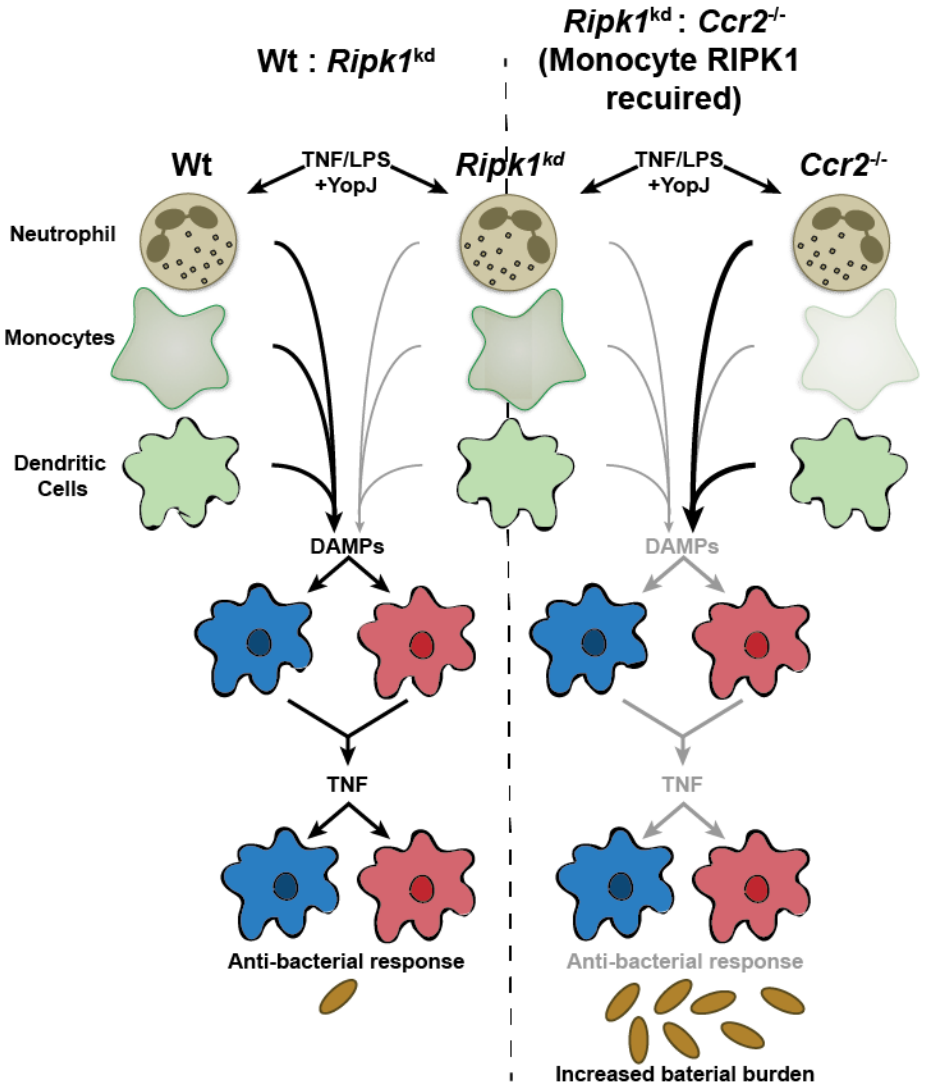

Figure 25. In what cell populations is RIPK1 kinase activity required?
Mixed BM chimeras containing Wt and *Ripk1*^{kd} BM control *Yersinia* infection normally. This implies that only a fraction of cells is required to be able to undergo RIPK1 kinase-dependent apoptosis in order to promote protection. BM from mice lacking specific cell populations of immune cells can be mixed with BM from *Ripk1*^{kd} mice in order to test the requirement for RIPK1 kinase activity in specific cell populations. In *Ripk1*^{kd} : *Ccr2*$^{-/-}$ mixed chimeras only tissue monocytes will be completely derived from *Ripk1*^{kd} cells. If monocyte RIPK1 kinase activity is required for protection during infection, then these mice will have elevated bacterial burdens.

CCR2$^{-/-}$:Wt and *CCR2*$^{-/-}$:*Ripk1*^{kd} mixed BM chimeras. Because *CCR2*$^{-/-}$ monocytes fail to egress from the BM, monocytes in *CCR2*$^{-/-}$:*Ripk1*^{kd} mixed chimeras will be derived entirely from *Ripk1*^{kd} BM. Other cell types, unaffected by the loss of CCR2, will be derived from a mixture of Wt and *Ripk1*^{kd}. Since Wt BM is able to rescue both cytokine production and control of bacterial burdens in Wt:*Ripk1*^{kd} chimeras, the contribution of RIPK1 kinase activity provided by other cell populations should remain intact, while only the effects of monocyte RIPK1 kinase activity should be disrupted (Fig. 26). Performing similar experiments with mixtures of *Ripk1*^{kd} BM and T cell-, B cell- or dendritic cell-deficient BM could test the contribution of additional immune cell lineages to cytokine production and bacterial control *in vivo*.

Understanding the cells necessary for recognizing apoptosis and promoting downstream inflammatory responses would allow for targeted therapeutic approaches of either promoting or blocking these responses in pathologic settings. In other model systems, marginal zone or CD169^{+} macrophages are important for the clearance of apoptotic cells and priming of either tolerogenic or anti-tumor adaptive immune responses against apoptotic cell-associated antigens (258-262). Whether these cells are able to recognize DAMPs and promote innate immune responses in the context of *Yp* infection would be an important hypothesis to test. Again, this could be done with mixed BM chimeras, but would also require some knowledge of the cell death-associated signals that must be recognized in order to promote the innate response. BM from mice expressing diphtheria toxin receptor from the CD169 locus and diphtheria toxin treatment could be used to deplete this specific population (261, 262). Adding BM from mice defective in receptors required for recognizing apoptosis-derived inflammatory

signals known to be important in this model could allow for a specific function to be attributed to this cell population if the immune response to infection is not restored.

Our data demonstrates that in *Ripk1*^{kd} BM chimeras there is a defect in the production of TNF and IL-12 by neutrophils and monocytes, respectively (Fig. 21B). Whether it is the disruption of these cytokines that ultimately causes increased bacterial burdens in these mice remains to be addressed. During *Yersinia* infection IL-12-dependent responses have been shown to be a determinant of host protection (231-233). IL-12 is an important cytokine in the regulation of NK cell, CD8$^+$ T cell, and T_h1 T cell responses (230) and is particularly important for promoting the production of IFNγ during infection (231). Interestingly, IL-12 acts synergistically with IL-18 to mediate the production of IFNγ (263, 264). Thus, there may be a combined defect in these two cytokines that is responsible for the immune defects we see in *Ripk1*^{kd} mice. Until day 7 post-infection, there are very few cytokine-producing CD8$^+$ and CD4$^+$ T cells following restimulation with heat-killed antigen or CD8$^+$ T cells specific for a particular immunodominant epitope of *Yersinia* (data not shown) (265). Thus, if IL-12 is playing a role in promoting IFNγ production and limiting bacterial burdens early in the response, it may be through NK cell activation. Treatment of *Ripk1*^{kd} mice or BM chimeras with exogenous IL-12 may provide protection against increased bacterial burdens by driving IFNγ-dependent responses if this is the dominant defect in these mice (231, 232).

TNFR1-deficient mice have increased bacterial burdens in the spleen and liver, supporting a role for TNF in promoting protection from *Yersinia* infection, (Fig. 16D). However, this role for TNF in promoting protection may be multifactorial if TNF acts at several steps in the immune response. Based on the contribution of TNFR1 to *Yersinia*-induced apoptosis *in vitro*, TNF may be necessary for promoting apoptosis *in vivo* (see

discussion above and chapter 4). Since RIPK1 kinase dependent apoptosis appears to support further TNF production by neutrophils, this may result in a feed-forward loop for promoting protection from *Yersinia* infection. Whether TNF is required for promoting *Yersinia*-induced apoptosis *in vivo* could be addressed using *Tnfr1*$^{-/-}$:*Ripk1*^{kd} mixed BM chimera studies (described previously, Fig. 24). Alternatively, TNF signaling could play a role in cell death-independent antibacterial responses, such as promoting cell recruitment or phagocytosis. Treatment of mice with exogenous TNF could be performed to test for this function for TNF. However, this would have to be done carefully, as systemic treatment of mice with exogenous TNF can lead to lethal inflammation and shock (266). Whether this would be a useful approach to test the role of TNF in *Yersinia* infection remains to be determined.

In addition to the effects that apoptotic cell death may have during active infection, it is possible that developmental effects of RIPK1 kinase activity could shape the immune response. For example, if RIPK1 kinase-dependent apoptosis occurs at low levels during homeostasis or development, then detection of cell death-associated signals could have an influence on the conditioning of phagocytes and other cells such that their responsiveness to infection is altered. The effect of apoptotic cells death or engulfment on developing phagocytes in mammalian hosts has not been well studied. However, recent work in *Drosophila* demonstrate that corpse engulfment by phagocytic cells was required for proper responsiveness to wounding or infection (267). This effect was mediated by increased expression of a surface receptor required for the apoptotic cell uptake and wound healing. Previous studies using the *Ripk1*^{kd} mice have not found differences at homeostasis. However, if priming by certain apoptotic cues occurs in developing mouse phagocytes, then disruption of key apoptotic signaling pathways,

such as those mediated by RIPK1, may alter the responsiveness of cells upon infection or injury in ways that have not yet been tested

Our data demonstrating a defect in cytokine expression in the absence of RIPK kinase-dependent apoptosis suggests that loss of TNF or IL-12 may be responsible for the increased bacterial burdens in *Ripk1*^{kd} mice. However, alternative mechanisms may play a role in limiting infection in this system. Apoptosis of infected cells acts to limit the replicative niche of pathogens during viral or intracellular bacterial infection (268). It is unclear whether this may apply to *Yersinia*, which is thought to exist primarily as an extracellular pathogen. Although *Yersinia* has been reported to be able to survive within select cell types (269-271), the potent inhibition of phagocytosis by *Yersinia* would seem to suggest that it actively avoids internalization within host cells. Alternatively, apoptosis of cells inhibited by *Yersinia* may allow for adherent bacteria to be trapped and cleared through the process of efferocytosis (217, 237, 238). Inhibition of macrophage phagocytosis by *Yersinia* virulence factors limits the ability of targeted cells to clear bacteria themselves. In these cases, it may be beneficial for an inhibited cell to die rather than remain a paralyzed host to adhered bacteria. Apoptosis could allow for release of 'find-me' and 'eat-me' signals in order to allow neighboring phagocytes to recognize and phagocytose apoptotic cell corpses along with associated bacteria. Thus, the anti-phagocytic effects of *Yersinia* could be bypassed.

The contribution of RIPK1 kinase activity to host protective immunity suggests that apoptosis may play an important role in promoting other immune responses to infection. It is clear from our data that, at least in the context of infection, some forms of apoptosis may be inflammatory. This is consistent with evidence that cancer cell death can occur through immunogenic apoptotic pathways that drive anti-tumor adaptive

immune responses (154). In the case of *Yersinia*, however, it is unclear whether similar mechanisms of eliciting protective immunity are involved during the early phase of infection, when an adaptive response has not yet developed, or whether unique mechanisms of cell death-induced inflammation exist for promoting innate immune responses. Furthermore, whether these early defects in innate immunity to *Yersinia* infection in *Ripk1*^{kd} mice translate into defects in the adaptive response or long lasting immunity is unknown.

Looking beyond the current cell death nomenclature

Apoptosis and necrosis were initially characterized as distinct forms of cell death with morphologic features that could be distinguished under a microscope (272). As the understanding of cell death at the molecular level has developed, the field has adapted a more complete appreciation for the diversity of regulated pathways leading to death (8, 273, 274). However, much of what we know about how cell death is recognized and integrated by the immune system is likely too often generalized based on broad classifications of cell death pathways. Despite the fact that both intrinsic and extrinsic apoptosis pathways converge on the activation of executioner caspases to complete the cell death process, each can be initiated by diverse stimuli that may have a profound effect on the recognition of cell death by the immune system. Therefore, the predominant characterization of apoptosis as immunosuppressive versus inflammatory requires much more careful consideration and attention to the molecular details defining a dead cell. Instead of looking at apoptotic cells as defined by just the terminal events in their life it may be more appropriate to consider the sum a cell's experiences preceding its demise. In other words, it may be just as important to consider factors preceding the

induction of a particular cell death program as following it. For example, apoptosis of naïve T cells generates a tolerogenic response (275, 276), versus apoptosis of activated T cells, which generates an inflammatory response dependent on the expression of activation markers (277). Thus, signals released from dying cells may be highly dependent on environmental stresses and the history of the cell.

The description of DAMPs released from dying cells as associated with either apoptosis, necrosis or pyroptosis may also require more careful consideration. Molecular changes that occur during different cell death pathways may regulate the release or immunogenicity of DAMPs. As many cell death responses occur independent of new protein translation, post-translational modifications to DAMPs may be particularly important for directing the immune response and conveying cues about the cell death processes that have occurred. Post-translational modifications that could alter the immune response to cell death include cleavage and oxidation. Specific examples of DAMPs regulated by these modifications include IL-33 and HMGB1, respectively; however, these processes may contribute to the regulation of many more unappreciated signals.

Caspase substrate specificity can influence the release of immunogenic DAMPs during different cell death modalities. An example of this is the cleavage of the IL-1 family cytokine, IL-33, by apoptotic caspases but much less efficiently by caspase-1 (150, 151). Although both intrinsic and extrinsic apoptosis converge on the activation of executioner caspases, caspase-8 and caspase-9 activity may result in the cleavage of additional targets that could impact the state of the dying cell. Furthermore, extrinsic apoptosis can be triggered by defects in multiple pro-survival signals. The differential requirement for RIPK1 in caspase-8 activation resulting from defects in either an ‘early’

or ‘late’ checkpoint in death receptor signaling (Fig. 25) suggests that these apoptotic complexes may be distinct in their composition. Whether caspase-8 has similar substrate specificity in these two complexes is not known and raises the question of whether caspase substrates, such as IL-33, are universally associated with apoptosis or whether different cellular conditions or stimuli may regulate their cleavage. Kinases activated downstream of death receptors, including TAK1, IKKα/β and even RIPK1, could also influence the cleavage products of apoptotic caspases through phosphorylation of caspase substrates (278). Inhibition of TAK1 and IKKβ may promote results in a similar pathway of RIPK1-dependent caspase-8 activation but result in distinct apoptotic cleavage events, as a result of differential phosphorylation of caspase targets.

Oxidation is another potential mechanism by which DAMPs may be modified during cell death. Release of mitochondrial reactive oxygen species (ROS) is associated with the induction of intrinsic apoptosis during mitochondrial outer membrane permeabilization (MOMP) (7). Furthermore, caspase-dependent cleavage of components of the respiratory chain complex results in the increased production of ROS (279). These changes in the redox state of the cell promote the oxidation of HMGB1 and the loss of its immunogenic potential upon release (144). During extrinsic apoptosis, cell type determines a differential requirement for MOMP in the activation of executioner caspases and apoptotic death (32, 43, 44). ‘Type I cells’ can mediate death receptor-induced apoptosis independent of MOMP. In contrast, ‘type II cells’ require caspase-8-dependent cleavage of the mitochondrial protein BID in order to induce MOMP and the amplification of the apoptotic response. While HMGB1 is oxidized during intrinsic apoptosis, it is less clear whether it may also be oxidized during extrinsic apoptosis in

type I cells that have not undergone MOMP and have lower levels of ROS. Thus, the type of cell targeted for apoptosis may have differential effects on the ensuing immune response. Furthermore, RIPK3-dependent necroptosis has also been shown to be associated with the increased production of ROS (67, 280). This raises the possibility that under some circumstances HMGB1 may, in theory, be oxidized during necroptosis as well. Whether this is actually the case is unclear, but supports the idea that the molecular events preceding various cell death outcomes should be examined more extensively, as they may have a profound effect on the immunogenicity of cell death responses.

To conclude, my studies have expanded upon the understanding of cell death during infection and inflammation. Using the gram-negative pathogen *Yersinia pseudotuberculosis* as a model system, I have described the pathways that induce apoptotic cell death in the setting of pathogen inhibition of host signaling. This apoptotic response is promoted by cytokine signaling from other immune cells that coordinate the killing of cells inhibited by pathogen virulence factors. Thus, innate immune responses play an active role in driving pathogen-induced apoptosis, consistent with a protective role for this apoptotic response in immunity to infection. Furthermore, we use a novel *in vivo* infection model that takes advantage of our understanding of the cell death pathways induced during *Yersinia* infection. Through targeted disruption of RIPK1 kinase activity we demonstrated for the first time a host protective function for *Yersinia*-induced apoptosis, independent of cell-intrinsic signaling pathways for the induction of cytokine expression. These studies demonstrate that in the setting of infection, the host apoptotic responses can bypass pathogen inhibition of host signaling for the induction of immune responses. This implies that pathogen-induced apoptosis can elicit antibacterial

inflammation rather than tolerogenic responses associated with apoptosis that occurs during homeostasis. How this functional switch in the response to apoptosis is regulated is an important question for future studies that has the potential to impact our understanding of the consequences of cell death in many pathologic settings. Ultimately, harnessing the regulation of cell death may offer novel strategies for targeted therapies in disease ranging from infection to cancer to inflammatory disease.

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