

Emerging Roles for RIPK1 and RIPK3 in Pathogen-Induced Cell Death and Host Immunity

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Abstract Receptor-interacting protein kinases 1 and 3 (RIPK1 and RIPK3) are homologous serine–threonine kinases that were recognized for their roles in directing programmed necrotic cell death or necroptosis under a broad range of pathologic settings. Emerging evidence suggests new physiologic roles for RIPK1 and RIPK3 in mediating cell death of innate immune responses. Our review discusses current evidence on the mechanisms and the impact of RIPK1- and/or RIPK3-dependent cell death in responses to a variety of viral and bacterial pathogens. Furthermore, the discussion also summarizes emerging roles for RIPK1 and RIPK3 in other facets of host immunity, including the maintenance of epithelial barrier function and pro-inflammatory processes that may, in some cases, manifest independent of cell death. Finally, we briefly consider the therapeutic opportunities in targeting RIPK1- and RIPK3-dependent processes in infection and immunity.

Contents

1	Introduction	38
2	Pathogen-Induced Cell Death	40
2.1	Viral-Induced Cell Death	40
2.2	Bacterial-Induced Cell Death	47
2.3	Summary	53

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Current Topics in Microbiology and Immunology (2017) 403:37–75
DOI 10.1007/82_2015_449

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Published Online: 15 September 2015

3	Epithelial Barrier Function	53
3.1	Intestinal Epithelia.....	53
3.2	Skin	57
3.3	Summary	58
4	Inflammation	58
4.1	RIPK1	59
4.2	Kinase-Dependent Inflammatory Functions of RIPK1	60
4.3	RIPK3.....	62
4.4	Summary	62
5	RIPK1 and RIPK3 as Therapeutic Targets in Light of New Innate Immune Roles	63
6	Discussion	63
	References	66

1 Introduction

Receptor-interacting protein kinase 1 and 3 (RIPK1 and RIPK3) are homologous serine–threonine kinases belonging to the RIPK family of enzymes. These kinases garnered initial interest as key regulators of death-receptor-induced programmed necrosis pathway, termed “necroptosis” (Vanden Berghe et al. 2014; Christofferson et al. 2010). Because necrotic cell death plays an important role in human disease, pathologic contributions of RIPK1 and RIPK3 have attracted major interest. Experimental approaches utilizing genetic deletion of RIPK3, expression of kinase-dead RIPK1, or use of small molecule RIPK1 inhibitors, necrostatins, in models of human disease, indeed suggested important contributions of these kinases in many pathologic states, including ischemia–reperfusion injuries, atherosclerosis, pancreatitis, multiple sclerosis, inflammatory bowel diseases, and others (Linkermann and Green 2014; You et al. 2008; Degterev et al. 2005; Ofengeim et al. 2015). While physiologic contexts involving RIPK1/3 regulation remain less clear, developing evidence, summarized in this review, suggests that these molecules may emerge as important players in regulation of the pathogen response and host immunity.

RIPK1 is comprised of three functionally distinct domains: an N-terminal kinase domain, an intermediate domain, and a C-terminal death domain. Kinase function of RIPK1 is important for tumor necrosis factor receptor (TNFR)-dependent necroptosis as well as apoptosis (Christofferson et al. 2014; Ofengeim and Yuan 2013). The serine/threonine kinase domain includes an aspartate–leucine–glycine (DLG) motif-containing binding pocket for the allosteric RIPK1 kinase inhibitor, Necrostatin-1 (Nec-1) (Degterev et al. 2008, 2013; Xie et al. 2013a, b). Nec-1 has been widely used to define kinase-dependent functions of RIPK1; however, the recent generation of RIPK1 kinase-inactive mouse models has expanded the repertoire of tools available to distinguish kinase dependent from kinase-independent functions of this protein (Polykratis et al. 2014; Berger et al. 2014). The intermediate domain of RIPK1 contains a RIP-homotypic interaction motif (RHIM), which facilitates RIPK1

interaction with other RHIM-domain-containing proteins, including DAI, TRIF, and RIPK3. RHIM-domain interactions are required for “amyloid-like” RIPK1/RIPK3 necrosome complex formation and RIPK1- and/or RIPK3-dependent death signaling (Kaiser et al. 2013; Li et al. 2012; Wu et al. 2014; Upton et al. 2012). Lastly, the C-terminal death domain mediates interaction of RIPK1 with death domain containing receptors such as Fas and TNFR1 (Christofferson et al. 2014; Ofengeim and Yuan 2013). The distinct properties associated with the various domains of RIPK1 enable the enzyme to act as a dynamic regulator of cell death signaling.

RIPK3 shares many similarities to RIPK1, including highly homologous kinase and RHIM-domains. Early work suggested that kinase activities of both proteins are required to initiate necroptosis through phosphorylation-driven assembly of a necrosome complex (Xie et al. 2013a, b; Cho et al. 2010). However, more recently, distinct roles for RIPK3 have been identified setting it apart from RIPK1 functionally. For example, RIPK3 lacks the death domain present in RIPK1. Additionally, the field now appreciates that execution of TNF- α -induced necroptosis requires RIPK3-dependent phosphorylation of mixed-lineage kinase domain-like (MLKL), a downstream effector of necroptosis, with RIPK1 playing a more upstream role in initiating RIPK3 signaling (Xie et al. 2013a, b; Wu et al. 2013). MLKL is a requisite executioner of necroptosis: Phosphorylation-dependent oligomerization and translocation of MLKL to the plasma membrane are directly linked to increase in plasma membrane permeability and reactive oxygen species generation (Hildebrand et al. 2014; Li et al. 2014; Murphy et al. 2013; Cai et al. 2014; Zhao et al. 2012). Notably, RIPK3 can also activate necroptosis independent of RIPK1 (Kaiser et al. 2013; Upton et al. 2012; Wu et al. 2014). However, kinase activity of RIPK3 is not required for RIPK1 kinase-dependent apoptosis (Dondelinger et al. 2013).

Cell death and inflammation are critical components of host immune response against invading microbial pathogens. Programmed death of infected cells releases intracellular microbes for clearance by immune cells and restricts microbial proliferation (Lamkanfi and Dixit 2010; Sridharan and Upton 2014). Pathogen-associated inflammatory changes modify host cell programming and activate cell-mediated immunity, both of which promote systemic antimicrobial states. Localized inflammatory responses also promote tissue repair. In fact, pro-inflammatory necroptotic cell death uniquely induces inflammation and promotes tissue repair concurrent with cellular demise (Kaczmarek et al. 2013; Moriwaki et al. 2014).

Epithelial barrier integrity is also a component of the innate immune system (Kumar et al. 2010; Nestle et al. 2009; Elias 2007; Macdonald and Monteleone 2005; Kaser et al. 2010). Appropriate barrier function serves to exclude microbial pathogens from entering and threatening the host. In circumstances of epithelial injury or dysfunction, the host susceptibility to infection is immediately raised.

The forthcoming review explores emerging roles of RIPK1 and RIPK3 in three facets of host immunity. The chapter begins by discussing mechanisms of RIPK1/3-dependent cell death in limiting pathogen dissemination and affecting host organism outcomes. Moreover, the discussion explores other roles for RIPK1 and

RIPK3 in host immunity, including epithelial barrier function and inflammation. Specifically, this chapter includes newly identified roles for RIPK1 and RIPK3 in shielding the host against invading pathogens by regulating epithelial barrier function. Furthermore, this text addresses emerging roles of RIPK1 and RIPK3 in directing inflammatory processes that may, in some cases, manifest independent of cell death. In conclusion, the chapter also comments on therapeutic opportunities in targeting RIPK1- and RIPK3-dependent processes during infection.

2 Pathogen-Induced Cell Death

2.1 Viral-Induced Cell Death

The pathogenesis of viral species is uniquely dependent on their ability to replicate within host cells. Accordingly, viruses have evolved mechanisms inhibiting cell death and prolonging survival of host cells to ensure reliable replication (Galluzzi et al. 2010; Roy and Mocarski 2007; Lamkanfi and Dixit 2010). Viruses equipped to inhibit apoptosis are commonplace and are described extensively in the literature (Mocarski et al. 2012; Lamkanfi and Dixit 2010). Among known examples, Kaposi's sarcoma herpesvirus (HSV-8) and human poxvirus molluscum contagiosum encode viral FLICE-inhibitory protein (v-FLIP) family members; these molecules act in a dominant-negative fashion to inhibit caspase-8 and caspase-10 recruitment to the death-inducing signaling complex (DISC) (Bertin et al. 1997; Hu et al. 1997; Thome et al. 1997; Shisler and Moss 2001; Thureau et al. 2006). Orthopoxviruses, including cowpox, vaccinia, ectromelia, and rabbitpox viruses bear homologous serine protease (serpin) inhibitors of caspases (Dobbelstein et al. 1996; Macen et al. 1996; Turner et al. 2000; Best 2008). These peptides are among a broad class of serpin protease inhibitors that bind within the active site of target enzymes rendering them inactive. Herpesvirus family members, HSV-1 and HSV-2, carry a caspase-8 inhibitor gene, UL-39, which encodes ribonucleotide reductase subunit protein, R1, also known as ICP6 or ICP10 for HSV-1 and HSV-2, respectively (Dufour et al. 2011; Mocarski et al. 2012). A third herpesviridae, cytomegalovirus (CMV), encodes a distinct inhibitor of caspase-8 function and viral inhibitor of caspase-8 activation (vICA) (Skaletskaya et al. 2001). Caspase-8 inhibitors are also present in adenoviruses and human papillomavirus-16 (HPV-16), a strain commonly associated with cervical cancer (Kabsch and Alonso 2002). Baculoviruses express inhibitors of apoptosis, including a homologue of human X-linked inhibitor of apoptosis (XIAP) and a pan-caspase inhibitor, p35 (Birnbaum et al. 1994; Deveraux and Reed 1999; Crook et al. 1993). Apoptosis inhibitors have also been identified in the influenza A virus (Yatim and Albert 2011). Although this list is not exhaustive, it serves to illustrate that apoptosis inhibitors are common in viral species.

Emerging evidence suggests that caspase-independent death may become paramount for host defense in the presence of anti-apoptotic signals or caspase inhibitors. For example, fibroblasts infected with vaccinia virus underwent necrotic cell death that was reduced in cells infected with a mutant lacking caspase inhibitor B13R (Li and Beg 2000). In vaccinia infection, RIPK1- and RIPK3-dependent necroptosis is required to contain viral dissemination; RIPK1 kinase-inactive mice of the D138N flavor (RIPK1^{D138N}) and RIPK3 knockout (RIPK3^{-/-}) mice displayed a 10-fold to 100-fold increase in viral titers compared to wild-type (WT) counterparts (Cho et al. 2010; Polykratis et al. 2014). RIPK3^{-/-} mice were also observed to have diminished survival and increased viral dissemination compared to that observed in RIPK1^{D138N} mice suggesting that RIPK3 may play a more prominent role in virus-induced necroptosis. Similarly, cell death induced by MCMV virus, lacking RIPK inhibitor M45, has been found to involve direct activation of RIPK3 by viral DNA sensor DAI (Upton et al. 2012). These data suggest that RIPK1 and RIPK3 may play significant roles in anti-viral responses, but their activities may not be linearly connected as has been proposed in TNF- α -induced cell death (Vandenabeele et al. 2010a).

2.1.1 HSV

Herpes simplex viruses, HSV-1 and HSV-2, are viral pathogens commonly recognized for causing tissue ulceration around the oral cavity and genitals, respectively (Klein 2015a; Albrecht 2014). More severely, HSV-1 can also manifest as a life-threatening encephalitis following inoculation of the oropharyngeal cavity. Notably, HSV-1 encephalitis is regarded as the most common cause of sporadic encephalitis in the world (Klein 2015b).

Molecular studies have demonstrated that in murine cells, HSV-1 induces programmed necrosis which limits viral replication (Wang et al. 2014b; Huang et al. 2015). RIPK3 deletion completely protected mouse embryonic fibroblasts (MEFs) and L929 cells from HSV-1-induced cell death. This function of RIPK3 is attributed to its kinase activity as cells expressing a kinase-dead mutant were just as viable as RIPK3^{-/-} counterparts. L929 cells lacking RIPK1 were partially protected from cell death indicating that RIPK1 is also important for HSV-1-induced cell death. Not surprisingly, HSV-1-induced cell death also required canonical effector of necroptosis, MLKL. Knockdown of MLKL in WT MEFs or L929 cells protected against HSV-1-induced cell death. Importantly, HSV-1-induced cell death effectively limited viral propagation as RIPK3^{-/-} L929 cells generated larger viral titers than WT counterparts post-infection (Huang et al. 2015; Wang et al. 2014b).

Mechanistic analyses revealed that HSV-1-induced cell death was dependent on RHIM-domain interactions. MEFs expressing RHIM-domain mutant of RIPK3 were protected from HSV-1 infection-induced cell death. Authors identified that the viral RHIM-domain containing protein, ribonucleotide reductase subunit 1 (R1), also known as ICP6, induced necrosis in target cells (Wang et al. 2014b; Huang et al. 2015). Furthermore, HSV-1 carrying a RHIM-domain mutant of ICP6

(ICP6mutRHIM) did not cause cell death. RIPK1 and RIPK3 were co-immunoprecipitated with ICP6 in HSV-1 infected MEFs; however, no interaction was detected upon infection with virus carrying ICP6mutRHIM (Huang et al. 2015). Huang and colleagues further demonstrated that ICP6 can induce RIPK1–RIPK3 heterointeractions and RIPK3–RIPK3 homointeractions in HEK293T cells. These findings suggest that HSV-1-induced programmed necrosis requires RHIM-domain interactions of ICP6 with RIPK1 and RIPK3, which leads to induction of necroptosis in infected cells and limits viral infections (Table 1). This presents an interesting mechanism linked to the life cycle of HSVs as expression of ICP6 has been found to be essential for the reactivation of the virus in quiescent cells and has also been reported to inhibit caspase-8 activation (Goldstein and Weller 1988; Langelier et al. 2002; Dufour et al. 2011).

Necroptosis improved host health and survival while limiting viral titers and propagation *in vivo*. RIPK3^{−/−} animals lost more body weight following infection compared to WT counterparts. Moreover, HSV-1 viral DNA was detected in greater abundance in serum, liver, and nervous tissue in RIPK3^{−/−} animals suggesting increased viral propagation. Greater viral dissemination corresponded to worse outcomes as RIPK3^{−/−} animals had diminished survival compared to controls. Concordantly, viral dissemination was increased and animal survival decreased in WT mice infected with ICP6mutRHIM HSV-1 compared to WT HSV-1.

Interestingly, while two groups reported that HSV-1 is able to effectively induce cell death in murine cells, HSV-1-induced cell death was not similarly observed in human cells (Guo et al. 2015; Huang et al. 2015). Data showed that RHIM-domains of ICP6 in HSV-1 and ICP10 in HSV-2 inhibited TNF- α -induced RIPK3-dependent necroptosis in human HT29 cells by competing for RHIM-domain interactions of RIPK1 and RIPK3 (Guo et al. 2011). In suit, ICP6mutRHIM HSV-1 unleashed TNF- α -induced RIPK3 kinase-dependent cell death and limited viral titers (Table 1) (Guo et al. 2015).

In sum, ICP6 induced necroptosis in a RHIM-dependent manner in murine cells while serving the opposite function, by blocking necroptosis, in human cells. These seemingly contradictory observations stress the versatility of the RHIM-domain in necroptosis signaling. These observations also suggest that important, yet currently unknown, mechanistic differences must exist in how human and mouse RHIM-domains interact. Irrespective of further mechanistic clarification, these findings may be reconciled by the fact that HSV-1 is a natural human pathogen and thus may have evolved to evade human and not mouse RIPK1- and RIPK3-dependent necroptosis. Conversely, HSV-1 may have emerged as a human pathogen because of its ability to block necroptosis specifically in human cells. Although differences between the abilities of host–pathogen interactions in humans and mice to induce necroptosis are not yet well-established, these differences may materialize as important clues in defining innate immune roles of RIPK1 and RIPK3.

Table 1 Specific roles of RIPK1 and/or RIPK3 in microbial pathogenesis

Microbe	Host species	Caspase-8 inhibitor	Mechanism of RIPK inhibition	Mechanism of RIPK activation	RIPKs	Function of RIPK-dependent signaling in host cell	Impact of RIPK-dependent signaling on organism
Vaccinia virus	Murine	SPI-2 or B13R (Serpin)		Undefined	RIPK3	Induces host cell death	Reduced viral titers and improved host survival
Reoviruses (T3D strain)	Murine (L929 cells)	Undetermined		Undefined	RIPK1	Induces host cell death	Undefined
Influenza-A	Human	NS1		Death-receptor ligation/FasL-Fas	RIPK1 and RIPK3	Induces tissue injury/death (in the absence of cIAP2)	Diminished survival (in the absence of cIAP2)
HSV-1	Murine	UL-39/ICP6		RHIM-domain interaction of ICP6	RIPK1 and RIPK3	Induces host cell death	Reduced viral titers and improved survival
HSV-1	Human	UL-39/ICP6	ICP6 competing for RHIM-domain interactions between RIPKs		RIPK1 and RIPK3	Inhibits host cell death	Enhanced viral propagation
HSV-2	Human	UL-39/ICP10	ICP10 competing for RHIM-domain interactions between RIPKs		RIPK1 and RIPK3	Inhibits host cell death	Undefined
MCMV	Murine	vICA, Bax, Bak	M45 disrupting RHIM-domain interactions between DAI and RIPK3		RIPK3	Inhibits host cell death	Enhanced viral propagation and diminished organism survival
hCMV	Human	vICA, Bax, Bak	IE1		Undefined	Inhibits host cell death	Enhanced viral propagation and increased cell death

(continued)

Table 1 (continued)

Microbe	Host species	Caspase-8 inhibitor	Mechanism of RIPK inhibition	Mechanism of RIPK activation	RIPKs	Function of RIPK-dependent signaling in host cell	Impact of RIPK-dependent signaling on organism
<i>Yersinia</i>	Murine	Undefined		YopJ	RIPK1	Induces host cell death	Reduced bacterial propagation, tissue damage, and improved survival
<i>Salmonella</i>	Murine	Undefined		Salmonella-induced IFN- γ	RIPK1 and RIPK3	Induces host cell death	Undetermined
<i>E. coli (EPEC)</i>	Murine and Humans	Undefined	Death domain interactions of NleB1		RIPK1	Inhibits host cell death	Undetermined
<i>Clostridium Perfringens</i>	Porcine	Undefined		CPB	RIPK1	Induces host cell death	Undetermined
<i>Staphylococcus Aureus (Methicillin-Resistant, Strain USA300)</i>	Murine	Undefined		Toxins (Undefined)	RIPK1 and RIPK3	Induces host cell death	Increased tissue destruction, decreased tissue macrophages, and increased bacterial titers
<i>Mycobacterium tuberculosis</i>	Zebra fish	Undefined		Infection-induced TNF- α	RIPK1	Induces ROS generation and host cell death	Undetermined

2.1.2 CMV

CMV is highly prevalent in the general population. Although it is benign in healthy individuals, the virus can be lethal in the immunocompromised, such as AIDS patients and transplant recipients (Friel 2014). Due to its prevalence, CMV is the most common cause of congenital infection in newborns. Mothers experiencing primary CMV infection or viral reactivation are at increased risk for vertical transmission to their unborn children. Congenital infection can lead to permanent damage, marked by hearing loss, vision impairment, and or cognitive retardation (Demmler-Harrison 2015).

In 2010, Kaiser and colleagues reported that murine CMV (MCMV) M45 protein, a RHIM-domain-containing viral inhibitor of RIP activation (vIRA), was required for viral dissemination and decreased host survival (Upton et al. 2010). Further investigation resolved that the RHIM-domain of vIRA inhibited RHIM-domain interactions between DNA-dependent activator of interferon regulatory factors (DAI), an intracellular sensor of viral DNA, and RIPK3 that were required for RIPK3-dependent cell death. In accord, mice infected with MCMV carrying a mutation in the RHIM-domain of M45 protein (M45mutRHIM) had improved survival and lower viral titers in lymphoid organs compared to mice infected with WT MCMV. Similarly, deletion of DAI or RIPK3 rescued the pathogenicity of the M45mutRHIM virus, suggesting that RIPK3-dependent necrosis mitigated viral-induced health decline of the organism and minimized viral dissemination (Upton et al. 2010, 2012). These findings are consistent with a model in which RIPK3-dependent necrotic cell death limits viral replication by triggering necroptosis in the infected cells to protect the host against a pathogen fortified to suppress pro-apoptotic signals (Table 1).

Analogous to MCMV, human CMV (hCMV) is able to block necrotic death in target cells. In 2015, Mocarski's group reported that low passage, newborn human foreskin fibroblasts transduced with hRIPK3 and infected with hCMV were protected from necrotic cell death induced by either a combination of TNF- α , smac mimetics (SM), and zVAD.fmk (zV) or M45mutRHIM MCMV infection. Importantly, in the absence of hCMV, both conditions induced death dependent on the kinase activity of hRIPK3 as death was not observed in cells transduced with a kinase-dead mutant of RIPK3 (hRIPK3 K50A). Remarkably, phosphorylated forms of hRIPK3 and MLKL were still observed in hCMV-infected cells, suggesting that hCMV differs from MCMV in that it blocks programmed necrosis downstream of RIPK3 and MLKL activation through a yet-to-be-defined mechanism. An approach utilizing UV light-induced viral mutagenesis revealed that viral regulatory protein, IE1, known to modulate host gene expression and innate immune signaling, was required by hCMV to block programmed necrosis. Expectedly, deletion of IE1 increased sensitivity to hRIPK3-induced cell death and resulted in decreased viral titers in vitro (Omoto et al. 2015).

Human and murine CMVs apply different strategies in blocking RIPK3-dependent cell death. This may reflect evolutionary variations in viral species to accommodate specificities of host cell biology. For example, although

RHIM-domain interactions may be sufficient to block RIPK3-dependent cell death in murine cells, this may not be true for human hosts. Accordingly, hCMV is armed with mechanisms independent of RHIM-domain interactions to block necroptosis. Similarly, RHIM-domain containing proteins that block HSV-induced cell death in mouse cells fail to do so in human hosts. These observations suggest that fundamental differences in RIPK3-dependent cell death pathways between human and mouse systems are not yet fully understood.

2.1.3 Reoviruses

Mammalian orthoreoviruses (Reoviruses) comprise a family of double-stranded RNA viruses known to cause respiratory and enteric infections. Although most reoviruses are not associated with clinical disease, the rotavirus, a species of reovirus, is the most common cause of gastroenteritis, resulting in diarrhea and fever in children (O’Ryan and Matson 2015).

Reoviruses are known to induce caspase-dependent apoptosis in target cells (Clarke et al. 2003; Richardson-burns et al. 2002; Danthi et al. 2013). However, recent studies have suggested that these viruses may also induce necroptosis. For example, the Type-3 Dearing (T3D) strain induced cell death in murine L929 fibrosarcoma cells that could not be blocked by inhibition of caspases, but was blocked by Nec-1. This observation suggests that the T3D strain can induce cell death in a RIPK1 kinase-dependent manner (Berger and Danthi 2013) (Table 1).

Mechanisms responsible for the activation of necroptosis by reoviruses are currently unknown. Additionally, the functional value of RIPK1 kinase-dependent mechanisms in reoviral disease, including control of viral counts in vivo, duration of infection, tissue histopathology, and health outcomes, remains to be established.

2.1.4 Influenza A

Influenza viruses, part of the broader Orthomyxoviridae family, are enveloped negative-strand RNA viruses that include strains A and B. The virus is readily aerosolized and communicable in the human population, causing a self-limited, acute, seasonal respiratory tract infection, identified as *Influenza* or *Flu*. Patients manifest with upper and/or lower respiratory tract illness in conjunction with signs of systemic illness including fever, headache, muscle pain, and weakness. Certain high-risk patient populations, such as the elderly, pregnant, and immunosuppressed, may develop a more complicated illness, marked by pneumonia or infection of the nervous system (Dolin 2015; Yatim and Albert 2011).

Deletion of RIPK1 ubiquitin ligase, cellular inhibitor of apoptosis 2 (cIAP2), promotes Influenza A-induced respiratory tissue necrosis that is dependent on RIPK1 kinase activity and RIPK3. Studies also found that cIAP2 deficient mice had diminished survival without alterations in viral load (Rodrigue-Gervais et al. 2014). Notably, cIAP2 knockout mice treated with Nec-1 or also carrying a deletion of

RIPK3 were protected from pathologic features of tissue necrosis and displayed improved survival. Significantly, loss of RIPK3 in cIAP2 knockout mice did not alter viral titers, indicating that early death in cIAP2 knockout mice was likely a consequence of respiratory failure as opposed to viral burden. These data provide evidence that in the absence of cIAP2, RIPK1- and RIPK3-dependent cell death are not required to limit viral propagation, but rather to promote lethal tissue injury (Rodrigue-Gervais et al. 2014) (Table 1).

Deletion of cIAP2 may induce RIPK1 and RIPK3 hyperactivity that licenses indirect tissue injury during Influenza-A infection. It is well-established that cIAPs are important physiologic inhibitors of RIPK1- and RIPK3-dependent necroptosis (McComb et al. 2012). Accordingly, RIPKs may be inappropriately activated in the absence of cIAP2. Acute activation of RIPK1- and RIPK3-dependent cell death following viral infection suggests that RIPK1 and RIPK3 functions are specifically modified following viral infection, either directly, as a consequence of viral regulation, or indirectly, secondary to local inflammation and death-receptor ligation. Indeed, deletion of death-receptor ligand, FasL, normalized animal survival, suggesting that RIPK1 and RIPK3 are inappropriately activated downstream of FasL in cIAP2 knockout mice (Rodrigue-Gervais et al. 2014). Furthermore, fluorescence-activated cell sorting (FACS) analysis demonstrated that both uninfected and infected respiratory epithelia of cIAP2 knockout mice were more likely to undergo necrotic cell death than their WT counterparts, suggesting that cell death is induced by an exogenously released factor(s) rather than the virus itself (Rodrigue-Gervais et al. 2014). Lastly, authors found that RIPK3^{-/-} mice did not have surplus viral burden, deficits in viral clearance, or excess tissue injury compared to WT counterparts (Rodrigue-Gervais et al. 2014). Collectively, these data suggest that Influenza-A infection, in the absence of cIAP2, promoted RIPK3-dependent cell death and tissue destruction indirectly, as a consequence of death-receptor ligation.

2.2 *Bacterial-Induced Cell Death*

Bacteria are able to replicate independently of the host cell, and thus, bacterial pathogenesis is oftentimes not strictly dependent on host cell survival. Nevertheless, certain bacterial species survive and proliferate intracellularly. For these species, host cell integrity and longevity may be paramount (Pujol and Bliska 2005). Recently, RIPK-dependent cell death has been identified as an important player in bacterial infection. However, it remains to be fully determined whether infection-associated activation of RIPK1 and RIPK3 signaling serves as a feature of the host response or an exploitable weakness in host defense. Examples of RIPK1- and RIPK3-dependent regulation of the host response against specific bacterial pathogens are examined below.

2.2.1 *Yersinia*

Yersinia species are Gram-negative enteric pathogens that include the human pathogenic forms *Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica*. *Pestis* is notorious for its ferocity and was the root of the black plague in medieval Europe (Sexton 2014). *Pseudotuberculosis* and *Enterocolitica* produce similar symptoms with fever, abdominal pain, and diarrhea (Tauxe 2013). Uniquely, *Pseudotuberculosis* can also manifest with tuberculosis-like symptoms, including tissue necrosis and granulomas in the spleen, liver, and lymph nodes (Viboud and Bliska 2005). Virulence of these bacteria is dependent on the translocation of a series of pathogenicity factors, called *Yersinia* outer proteins (Yops), which are exported from the bacteria into host cells by a bacterial-encoded protein translocation system, the type-III secretion system (TTSS) (Viboud and Bliska 2005; Zhang et al. 2011; LaRock and Cookson 2012).

Phagocytic cells, in particular macrophages and dendritic cells, play important roles in the pathogenesis of *Yersinia* species. During infection, these host immune cells engulf bacteria and facilitate their systemic spread, specifically to mesenteric lymph nodes, spleen, and liver (Viboud and Bliska 2005). Significantly, all three human pathogenic *Yersinia* species have also been shown to replicate within macrophages and dendritic cells (Pujol and Bliska 2005; Bliska 2003; Pujol et al. 2009). In accord, programmed cell death of phagocytic cells in *Yersinia* infection may be an important feature of the host response.

Recent studies have uncovered important roles for RIPK1 in *Yersinia*-induced macrophage cell death. Pharmacologic inhibition of RIPK1 prevented *Yersinia*-induced cell death in macrophages (Weng et al. 2014; Philip et al. 2014). RIPK1-deficient fetal liver-derived macrophages were also resistant to *Y. pestis*-induced cell death. Notably, *Yersinia*-induced cell death was also shown to require caspase-8 and kinase activity of RIPK3 (Weng et al. 2014). Although macrophages lacking caspase-8 or RIPK3 or treated with RIPK3 inhibitor, GSK'872, were not protected from *Pestis*-induced death, combined loss of caspase-8 and deletion or inhibition of RIPK3 resulted in complete protection (Weng et al. 2014). The sufficiency of RIPK1 kinase and either caspase-8 or RIPK3 for cell death suggests that RIPK1 may function to activate either caspase-8-dependent apoptosis or RIPK3-dependent necroptosis in response to *Yersinia* infection.

Caspase-8 and RIPK3 double-knockout animals had increased susceptibility to infection with *Yersinia* (Weng et al. 2014; Philip et al. 2014). These mice had increased bacterial colony-forming units in spleens, increased inflammatory infiltrates in hepatic tissue sections, and diminished survival. As noted during viral infection, inhibition of cell death during *Yersinia* infection was detrimental to organism survival. Accordingly, elimination of infected host cells is likely important for the control of *Yersinia* as it is for viruses because both pathogens survive and replicate intracellularly. Altogether, these observations suggest that RIPK1-dependent apoptosis requiring caspase-8 or RIPK1-dependent necroptosis requiring RIPK3 may be utilized by the host to combat *Yersinia* infection (Table 1).

Caspase-1-dependent pro-inflammatory cell death, pyroptosis, may also play a role in *Yersinia*-induced, RIPK1-dependent, cell death of phagocytic cells. Experiments showed that in the absence of RIPK1, *Yersinia*-infection resulted in reduced production of the cleaved or active form of caspase-1 (Weng et al. 2014). Although this finding suggests a role for RIPK1 in *Yersinia*-induced caspase-1 activation, it remains to be determined whether pyroptosis actually plays a role in *Yersinia* infection.

Investigators attributed *Yersinia*-induced, RIPK1-dependent cell death to YopJ, one of the Yop pathogenicity factors. Macrophages infected with *Yersinia* mutants lacking functional YopJ (Δ YopJ) were protected from RIPK1-dependent cell death. YopJ is well known for inhibiting pro-inflammatory and pro-survival NF κ B and MAPK signaling in target cells. Not surprisingly, this effector has previously also been implicated in inflammasome activation and pyroptotic cell death of host cells (Weng et al. 2014; Philip et al. 2014). Accordingly, host immune cells may have evolved new modalities of YopJ-induced cell death to mitigate bacterial pathogenicity in the face of a bacterial agent, YopJ, which limits inflammatory responses (Table 1).

Regarded together, these observations cement a role for RIPK1-dependent cell death in *Yersinia* infection. Although it is unclear whether RIPK1 induces primarily an apoptosis, necroptosis, or pyroptosis, or perhaps some combination of the three, the *Yersinia* infection model uncovers novel infection-associated cell death regulation by RIPK1. Moreover, appreciating that *Yersinia* species have the capacity to replicate within phagocytic cells, it is not surprising that programmed elimination of infected immune cells protects against dissemination of *Yersinia* in the host.

2.2.2 *Salmonella*

Salmonella enterica species (*Salmonella*) are Gram-negative facultative intracellular bacilli which cause gastroenteritis in humans with symptoms including fever, diarrhea, and abdominal pains (Kotton and Hohmann 2013). Similar to other bacteria which can survive and replicate in host immune cells, *Salmonella* is able to commandeer anti-inflammatory M2 macrophages, spread systemically, and seed lymphoid tissue (Behnsen et al. 2015; Alpuche-Aranda et al. 1994; Nix et al. 2007; Kotton and Hohmann 2013; McCoy et al. 2012). Dissemination of *Salmonella* can promote tissue-specific damage including abscess formation and osteomyelitis as well as systemic infection or sepsis (Hohmann 2014).

A recent study demonstrated that *Salmonella*-induced type I interferon (IFN-I) signaling resulted in macrophage necroptosis (Robinson et al. 2012). Macrophages deficient in the interferon alpha receptor, which is required for sensing IFN-Is, or cultured with anti-IFN-I antibodies, were resistant to *Salmonella*-induced cell death. Inhibition of RIPK1 blocked over 60 % of cell death induced by *Salmonella* in WT macrophages which was similar to the extent of death observed in IFN α R knockout (IFN α R^{-/-}) macrophages. Moreover, deletion or knockdown of RIPK3 in macrophages abrogated *Salmonella*-induced cell death (Robinson et al. 2012).

Further investigation uncovered that both IFN α R^{-/-} and RIPK3^{-/-} mice had decreased bacterial load and increased numbers of circulating macrophages

compared to WT counterparts. This protective phenotype was likely conferred by the hematopoietic compartment as INFaR^{-/-} bone marrow transplanted into WT mice limited bacterial titers. Curiously, RIPK3^{-/-} mice did not have improved survival indicating that bacterial titers do not necessarily correlate with organism survival in the case of *Salmonella* infection (Robinson et al. 2012).

These paradoxical observations merit investigation into the relationship between *Salmonella* titers and manifestation of disease. One may inquire whether increased macrophage survival in the absence RIPK3 is underestimating total bacterial loads by excluding intracellular bacteria. In this case, necroptosis of anti-inflammatory M2 macrophages may serve to limit intracellular proliferation of *Salmonella* and contain infection. Nevertheless, these data strongly suggest that *Salmonella* infection presents another model of RIPK-dependent innate immune regulation. Interestingly, this regulation manifests by a mechanism that is distinct, and perhaps functionally opposite, from cell death induced by *Yersinia* (Table 1).

2.2.3 *Escherichia Coli*

Escherichia coli (*E. coli*) is a Gram-negative bacterial species that includes a diversity of strains or serotypes. Although the majority of these serotypes are benign, some have procured the capacity to cause serious infection and tissue damage. One such pathogenic strain, *Enteropathogenic E. coli* (EPEC), is associated with sporadic diarrheal illness, particularly in young children. The pathogenesis of virulent strains of *E. coli* is attributed to the acquisition of toxic effectors that are injected into target or host cells (Wanke 2013).

Nlel1 is a pathogenicity effector protein found among species of *E. coli* and *Salmonella*. Mechanistic analyses revealed Nlel1 possesses N-acetylglucosamine transferase activity that modifies a conserved arginine residue in death domain containing proteins, including TNF- α receptor (TNFR), TNF receptor-associated death domain (TRADD), FAS receptor (FASR), and RIPK1 (Li et al. 2013; Pearson et al. 2013). Nlel1 enzymatic activity blocked TRADD oligomerization and death-receptor-induced signaling complex (DISC) formation (Li et al. 2013; Pearson et al. 2013). Pearson and colleagues found that Nlel1 co-immunoprecipitated with death domain proteins, TRADD, FADD, and RIPK1, in 293T cells. In addition, Nlel1 abolished death-receptor-induced apoptosis in HeLa cells and TNF- α /zVAD/SMAC mimetic-induced necroptosis in RIPK3-expressing HeLa cells (Li et al. 2013). In murine infection models, EPEC either lacking Nlel1 or carrying enzymatically inactive Nlel1 mutants were unable to effectively colonize host gastrointestinal tracts (Li et al. 2013). These findings suggest that multiple cell death pathways, including RIPK3-dependent cell death, may be manipulated by Nlel1 expressing *E. coli* to establish infection, potentiate survival of infected cells, and minimize associated tissue destruction. Specific roles for RIPK1 and RIPK3 in Nlel1-associated infection remain to be elucidated (Table 1).

2.2.4 Pore-Forming Toxin-Producing Bacteria

Pore-forming proteins (PFPs) are bacterial toxins that increase host cell membrane permeability and thereby contribute to bacterial virulence. Some commonly recognizable virulent bacterial species and their significant clinical presentations include the following: *E. coli* (urinary tract infections and gastroenteritis), *Corynebacterium diphtheria* (diphtheria; upper respiratory infections), *Clostridium perferinges* (tissue necrosis and gas gangrene), *Clostridium septicum* (tissue necrosis and gas gangrene), *Bacillus anthracis* (anthrax; pneumonia, gastroenteritis, and/or cutaneous ulcers), *Listeria monocytogenes* (gastroenteritis and meningitis), *Staphylococcus aureus* (cellulitis, impetigo, abscess, and respiratory tract infections), *Vibrio cholera* (diarrhea and dehydration), and *Yersinia pseudotuberculosis* (gastroenteritis) (Bischofberger et al. 2012).

PFP-associated changes in membrane permeability may serve in any of a variety of functions in bacterial pathogenesis. The literature includes precedents of PFPs that direct translocation of effector proteins or other toxins into host cells, facilitate microbial invasion of the intracellular space, disrupt host cell ion homeostasis and energy balance, alter membrane compartment dynamics, and/or promote direct host cell destruction. For example, type-III secretion systems (TTSS) of Gram-negative bacteria, such as *Yersinia* and *Salmonella* species, use PFPs to translocate effector proteins into target cells (Zhang et al. 2011). Alternatively, *L. monocytogenes*' PFP, listeriolysin O (LLO) blocks acquisition of anti-bacterial proteins by *Listeria* containing host cell vacuoles (Bischofberger et al. 2012).

At high PFP concentrations, host cell death occurs by necrosis; however, at low PFP concentrations, cell death has been linked to apoptosis, necroptosis, as well as pyroptosis (Knapp et al. 2010; Kennedy et al. 2009; Lin et al. 2010; Boyden and Dietrich 2006; Craven et al. 2009). In the latter instance, the lack of uniformity in cell death suggests that host cell demise may be part of a regulated host response as opposed to a generalized manifestation of death secondary to excessive cell stress or membrane perforation. Notably, Nec-1 inhibited necrosis induced by *Clostridium perferingeas*' PFP, also known as *C. perferingeas* β -toxin (CPB), thereby specifying RIPK1 kinase-dependent regulation of CPB-induced necrosis (Autheman et al. 2013).

A study published by Kitur and colleagues suggested that RIPK1- and RIPK3-dependent necrosis may not be beneficial to the host. Kitur observed that toxin-induced programmed necrosis is a major mechanism of lung damage by methicillin-resistant *S. Aureus* (MRSA or SA), strain USA300. Authors reported that SA infection induced programmed necrosis in THP-1 human monocytes that was inhibited by Nec-1 or the MLKL inhibitor necrosulfonamide (NSF). Cytotoxicity was attributed to pore-forming toxin, α -hemolysin (Hla), and other leukotoxins as macrophage cell death could be induced by bacterial-conditioned media alone, but was abrogated by conditioned media from selective toxin-null mutant strains. Remarkably, murine lung infection models uncovered that RIK3^{-/-} or blockade of necrosis by Nec-1 enhanced bacterial clearance and limited tissue injury. Furthermore, WT mice had diminished macrophage populations in lung tissue

compared to RIPK3^{-/-} counterparts following infection (Kitur et al. 2015). These findings suggest that necrotic cell death induced by toxin-producing bacterial species, such as *SA*, may aggravate tissue damage and cause localized immunodeficiency to enhance bacterial infection and thereby compromise host survival (Table 1).

Although the collected data indicate that RIPK1 and RIPK3 may be important in models of PFP-bacterial infection models, it is unclear as to whether RIPK1- and/or RIPK3-dependent cell death generally serves as an advantageous or injurious feature of the host response. The majority of reports available do not explore the contributions of RIPK1- and/or RIPK3-dependent cell death in PFP-associated bacterial dissemination and organism survival. Accordingly, the roles of RIPK1- and/or RIPK3- dependent cell death in PFP-associated bacterial pathogenesis remain unclear.

2.2.5 *Mycobacterium Tuberculosis*

Mycobacterium tuberculosis (*M.tb*) is the causative agent of *Tuberculosis* (*TB*), an infectious disease that is communicable by respiratory secretions in humans. Although *TB* commonly manifests in the lungs because aerosolized particles descend into pulmonary recesses, the infection can disseminate systemically if poorly contained. *TB* is established in one of three forms: primary, latent, and reactivation. Primary *TB* is active or fulminant *TB* that occurs following a new or initial exposure. Latent *TB* is established when a new infection enters a state of dormancy and is thought to occur when invading *M.tb* is confronted by a robust immune response. In this case, the host is neither contagious nor exhibits the symptoms of *TB* and is labeled a carrier. The third form, reactivation *TB*, is defined as the emergence of active or symptomatic *TB* in a carrier (Pozniak 2015; Schluger and Rom).

The genesis of *TB* is a complex process that is poorly understood; however, it is accepted that macrophages are required to contain infection (Ulrichs and Kaufmann 2006). Additionally, TNF- α is recognized as a critical cytokine in the development of *TB*; for example, patients with rheumatoid arthritis on an anti-TNF- α regimen are more likely to develop reactivation of *TB* (Miller and Ernst 2009). Patients with compromised immunity, who are immunodeficient or immunosuppressed, are also at increased risk of developing primary or reactivation *TB* (Pozniak 2015). Moreover, zebra fish models of *Mycobacterium* infection have demonstrated that exogenous TNF- α can limit mycobacterial titers in animals with deficiencies in TNF- α synthesis (Tobin et al. 2010, 2012; Roca and Ramakrishnan 2013). Roca and Ramakrishnan also report that TNF- α induces production of reactive oxygen species (ROS) and thereby augments macrophage bacteriocidal activity and promotes RIPK1- and RIPK3-dependent necroptosis. As TNF- α is the best known inducer of necroptosis in vitro and in vivo, these data may suggest involvement of RIPK1 and/or RIPK3, but this has not yet been established directly. Moreover, associations noted above prompt questions examining the impact of these RIPKs on organism health and survival in *M.tb* infection (Table 1).

2.3 Summary

Multiple lines of evidence suggest that activation of RIPK1- and/or RIPK3-dependent cell death is an important consequence of host bacterial interactions. However, available data reveal that the competitive advantage conferred by initiating RIPK1- and/or RIPK3-dependent cell death may lie with the host or the bacteria, depending on the identity of the bacteria. For example, cell death may function as part of the productive innate immune responses to mitigate infection; alternatively, cell death may be induced by bacteria to eliminate immune cells and trigger massive tissue damage and thus may be targeted for therapeutic intervention. A broader understanding of the bacterial contexts regulating RIPK1- and/or RIPK3-dependent cell death and their underlying mechanisms remains to be clarified.

3 Epithelial Barrier Function

The intestinal epithelia and skin are first-line defenses against infection by commensal and pathogenic microbial flora (Kumar et al. 2010; Bonnet et al. 2011; Kaser et al. 2010; Macdonald and Monteleone 2005). Destruction of critical epithelial barriers predispose to microbial infection and the generation of tissue inflammation. The literature has illuminated crucial roles for RIPK1 and RIPK3 in maintaining barrier tissues. For example, kinase-independent function of RIPK1 has been found to be essential in preserving intestinal epithelial integrity and skin homeostasis. Conversely, kinase activity of RIPK1 and activation of RIPK3-dependent necroptosis have been associated with epithelial tissue destruction. The ensuing discussion explores these opposing roles of RIPK1 and RIPK3 in barrier tissue biology.

3.1 Intestinal Epithelia

3.1.1 Kinase-Independent Functions of RIPK1

Two groups have reported roles for RIPK1 in maintaining epithelial integrity using independently generated mouse models of RIPK1 deletion in intestinal epithelial cells (RIPK1^{IEC}). Grossly, weight loss and diminished survival was observed in mice lacking epithelial-specific RIPK1. Histological evaluation of gastrointestinal tissue revealed atypical cell death of intestinal epithelial cells and features of inflammatory injury, including leukocyte infiltration and abnormal tissue architecture (Dannappel et al. 2014; Takahashi et al. 2014).

Loss of RIPK1 in intestinal epithelia sensitized mice to microbial injury. Takahashi and colleagues reported that broad-spectrum antibiotic treatment

prevented weight loss and improved survival in animals. Antibiotics treatment also protected against cellular apoptosis in intestinal tissue as well as signs of local tissue and systemic inflammation, shortened colon length and splenic enlargement, respectively. Conversely, Dannappel and colleagues found that antibiotic treatment was ineffective at reversing histological findings of cellular apoptosis; however, these differences may be attributed to variations in antibiotic regimen and treatment schedule.

RIPK1 deletion in intestinal epithelia was associated with increased sensitivity to inflammation-associated cell death. TNF- α treatment resulted in increased apoptosis of RIPK1^{IEC} organoids (Takahashi et al. 2014). In addition, deletion of RIPK1 was associated with increased death induced by innate immune ligand, polyinosinic: polycytidylic acid [Poly(I:C)], as well as the cytokines, interferon- β (IFN- β), and interferon- γ (IFN- γ) (Kaiser et al. 2014; Dillon et al. 2014). Congruently, deletion of TNF Receptor (TNFR^{-/-}) prolonged survival and ameliorated intestinal apoptosis in RIPK1^{IEC} mice. Although Takahashi and colleagues appreciated improved survival in RIPK1^{IEC} mice upon deletion of MYD88, a key adaptor in innate immune inflammatory signaling and downstream of multiple Toll-like receptor family members, these findings could not be affirmed by Dannappel et al. Nevertheless, together, these observations suggest that deletion of RIPK1 in intestinal epithelia increased tissue sensitivity to death by innate immune signals and cytokines.

Similarly, whole-body RIPK1 deletion results in unbridled postnatal inflammation and lethality. Notably, embryonic development is unaffected by RIPK1 deletion, highlighting the importance of RIPK1 in post-embryonic life, when the body is exposed to a variety of external factors, in particular, skin and gut colonization by microbial flora (Dillon et al. 2014; Kaiser et al. 2014; Rickard et al. 2014; Kelliher et al. 1998).

The role for RIPK1 is not exclusively limited to early development as acute deletion of RIPK1 in intestinal epithelia of adult mice also resulted in rapid death associated with apoptosis of intestinal epithelia (Takahashi et al. 2014). Moreover, the protective role of RIPK1 in intestinal epithelia was credited to its kinase-independent function or scaffold function because mouse models of RIPK1 kinase-inactivation have normal survival and fail to exhibit pathologic features associated with RIPK1 deletion (Takahashi et al. 2014; Dannappel et al. 2014).

RIPK1 was previously thought to regulate pro-inflammatory and pro-survival signaling by the regulation of downstream transcription factor NF κ B (Cusson-Hermance et al. 2005; Meylan et al. 2004). However, counter to the dogma, Takahashi and Dannappel both reported that NF κ B signaling was not impaired in RIPK1^{IEC} epithelia. Specifically, inhibitor of kappa-B (I κ B) degradation was unchanged in RIPK1^{IEC} organoids stimulated with TNF- α .

Epithelial cell death and inflammatory sequelae were attributed to caspase-8- and FADD-dependent apoptosis and/or RIPK3-dependent necroptosis. These insights were gleaned using intestinal epithelia-specific knockouts of caspase-8 (Casp8^{IEC}) or FADD (FADD^{IEC}) and RIPK3 deletion mutant mice in conjunction with RIPK1^{IEC} mice. Caspase-8^{IEC}/RIPK1^{IEC} mice were completely protected against

early lethality and histological features of intestinal pathology (Takahashi et al. 2014). Although FADD depletion in intestinal epithelia (FADD^{IEC}) of RIPK1^{IEC} mice protected against histological features of intestinal pathology in a dose-dependent manner, these mice still exhibited diminished survival. Conversely, RIPK1^{IEC}/RIPK3^{-/-} were not protected from inflammatory changes in the intestinal tissue and had diminished survival. Nevertheless, inflammatory histopathology and survival were completely rescued in RIPK1^{IEC}/FADD^{IEC}/RIPK3^{-/-} indicating that both FADD and RIPK3 were important for pathologic changes in the tissue in the absence of RIPK1 (Dannappel et al. 2014).

Dannappel and colleagues also suggested that RIPK1 maintained intestinal epithelial integrity by preserving the pro-survival or anti-apoptotic proteins, cIAP1, TRAF-2, and c-FLIP. TNF- α stimulation resulted in rapid degradation of cIAP1, TRAF-2, and c-FLIP in RIPK1 knockout (RIPK1^{-/-}) but not RIPK1 kinase-inactive MEFs. Moreover, tamoxifen-induced deletion of RIPK1 in organoid cultures was associated with reduced expression of these anti-apoptotic proteins and rapid organoid death (Dannappel et al. 2014).

3.1.2 Kinase-Dependent Regulation by RIPK1 and the Role of RIPK3

Loss of caspase-8 or death-receptor adaptor protein, Fas-associated death domain (FADD), has been shown to sensitize cells to necroptotic cell death (Zhang et al. 2009; He et al. 2009; Osborn et al. 2010; Vandenabeele et al. 2010b; Kim and Li 2013). Several groups have analyzed the consequences of genetic deletion of these factors on the integrity of the intestinal epithelium.

In 2011, Welz et al. reported that FADD prevents RIPK3-dependent intestinal epithelial cell loss in mice. Mice generated with a deletion of FADD in intestinal epithelial cells (FADD^{IEC}) displayed diminished survival, reduced weight, and diarrhea, suggesting intestinal disease (Welz et al. 2011). Evaluation of gastrointestinal tissue revealed necrotic loss of intestinal epithelia or enterocytes, enteric and colonic inflammation, and destruction of antimicrobial Paneth cells of crypts found in the small intestine. Conspicuously, FADD^{IEC}/RIPK3^{-/-} mice developed normally and were protected from intestinal epithelial cell loss, Paneth cell loss, and signs of inflammation, indicating a role for RIPK3-dependent intestinal epithelial cell loss in the generation of gastrointestinal inflammatory disease (Welz et al. 2011). Moreover, intestinal epithelial inactivation of CYLD, a cellular deubiquitinase reported to promote RIPK1- and RIPK3-dependent necroptosis, protected animals from developing gastrointestinal inflammation (Moquin et al. 2013). Significantly, antibiotic treatment, germ-free rearing, or concomitant deletion of MYD88 or TNF ameliorated or abolished signs of intestinal inflammatory disease, emphasizing the importance of gut flora and innate immune responses in the development of gastrointestinal disease upon loss of intestinal epithelia (Welz et al. 2011). Collectively, these observations suggest that activation of RIPK3-dependent necroptosis may promote intestinal epithelial cell loss and bacterial-associated gastrointestinal inflammation.

RIPK3-dependent intestinal tissue damage was also observed upon the genetic deletion of caspase-8. Weinlich and colleagues administered oral tamoxifen gavage to Rosa-CRE-expressing mice that were bred to $\text{casp8}^{\text{fl/fl}}$ mice to generate mice lacking caspase-8 in adult gastrointestinal tissue (Casp8^{GI}) (Weinlich et al. 2013). In an alternative approach, Günther and colleagues generated mice lacking caspase-8 in intestinal epithelial cells ($\text{Casp8}^{\text{IEC}}$) by breeding caspase-8 floxed ($\text{Casp8}^{\text{fl/fl}}$) mice with mice expressing CRE-recombinase under the regulation of intestinal epithelial-specific *villin* promoter.

Acute deletion of caspase-8 in adult mice promoted rapid weight loss and diminished survival (Weinlich et al. 2013). Casp8^{GI} mice also manifested with histological features of intestinal inflammation, marked by cell death of enterocytes, tissue inflammation, and infiltration of immune cells. Importantly, concomitant deletion of RIPK3 protected mice from intestinal disease noted in Casp8^{GI} mice. $\text{Casp8}^{\text{GI}}/\text{RIPK3}^{-/-}$ mice maintained body weight and had normal survival, fewer dying enterocytes, and intact gastrointestinal tissue architecture, indicating that intestinal damage was likely a consequence of RIPK3-dependent enterocyte death in Casp8^{GI} mice (Weinlich et al. 2013). Similarly, $\text{Casp8}^{\text{IEC}}$ mice were found to have spontaneous inflammatory lesions in the terminal ileum, marked by bowel wall thickening, loss of intestinal crypts, and increased cellularity, suggesting immune cell infiltration. Notably, $\text{Casp8}^{\text{IEC}}$ mice had increased epithelial cell death associated with necrotic features. Authors also observed loss of specialized epithelial cells, specifically goblet and Paneth cells, which protect enteric epithelia and aid immunity by producing mucus and secreting antimicrobial peptides, respectively (Günther et al. 2011). Not surprisingly, intestinal epithelia of these mice had increased sensitivity to TNF- α -induced necroptosis. Intravenous TNF- α resulted in high lethality associated with severe destruction of the small bowel and an increased number of dying epithelial cells that were negative for cleaved caspase-3. Significantly, inhibition of RIPK1 kinase-dependent cell death using Nec-1 blocked TNF- α -induced lethality and destruction of the small bowel (Günther et al. 2011).

Together, these studies demonstrate that activation of necroptosis in intestinal epithelia leads to loss of intestinal barrier integrity and induction of tissue inflammation. These phenotypes may be associated with commensal bacterial-induced innate immune responses. Notably, the data also suggest that inappropriate activation of RIPK3-dependent signaling in human intestinal epithelia may be linked to chronic inflammatory diseases of the bowel, namely Crohn's disease. Immunohistochemical analysis of tissue samples from human patients revealed increased RIPK3 expression in Paneth cells of the distal small bowel. Crohn's disease tissue specimens also had decreased numbers of Paneth cells and increased Paneth cell death with necrotic features as determined by electron microscopy. Moreover, Paneth cells from tissue biopsies of patients with Crohn's disease were susceptible to TNF- α -induced cell death that could be blocked with Nec-1 (Günther et al. 2011). These data suggest that aberrant activation of necroptosis and subsequent loss of barrier function may be important factors underlying chronic inflammation in the gut.

3.2 Skin

3.2.1 Kinase-Independent Functions of RIPK1

Similar to the intestinal epithelia, RIPK1 in keratinocytes and epidermal tissue is important for homeostasis of the skin. RIPK1-deficient primary keratinocytes have increased susceptibility to TNF- α -induced apoptosis; however, in contrast to intestinal epithelia, injury *in vivo* entirely dependent on necroptosis as RIPK3 deletion protects against pathological features of skin inflammation. Moreover, ablation of RIPK1 in epithelia (RIPK1^E) results in a pro-inflammatory phenotype with increased epidermal thickness and inflammation (Dannappel et al. 2014). Localized inflammation of the skin may disrupt integrity of the organ, thereby increasing susceptibility to infection and exacerbating inflammatory pathology. This theory is supported by the observation that RIPK1^E mice also lacking the Toll-like receptor adaptor protein TRIF were partially protected from inflammatory injury of the skin, likely in part due to decreased activation of innate immune pathways by microbial flora (Dannappel et al. 2014). Additionally, much of the inflammatory injury observed upon epithelial deletion of RIPK1 may be attributed to increased sensitivity to TNF- α signaling as was observed in intestinal epithelia. RIPK1^E/TNFR^{-/-} prevents inflammatory features in the skin, including epidermal thickness (Dannappel et al. 2014). Analogous to observations made in intestinal epithelia, anti-apoptotic ubiquitin ligase, cIAP1, is rapidly diminished following TNF- α exposure to RIPK1^E to primary keratinocytes (Dannappel et al. 2014). Lastly, inflammatory phenotypes associated with RIPK1 deletion in the epithelia appear to be independent of kinase activity of RIPK1 as kinase-inactive models of RIPK1 have not been found to manifest with spontaneous inflammation of the skin (Polykratis et al. 2014; Berger et al. 2014).

3.2.2 The Role of RIPK3

Deletion of FADD in epidermal keratinocytes resulted in development of RIPK3-dependent skin lesions and early death (Bonnet et al. 2011). Histologically, these mice displayed patchy pathologic skin signs, marked by epidermal hyperplasia and thickening, keratinocyte death, and immune cell accumulation. Importantly, keratinocyte death appeared to be caspase independent, as a large fraction of dying keratinocytes did not contain active caspase-3 but showed necrotic morphology by electron microscopy. Similar to FADD deficiency in intestinal epithelium, FADD^E/RIPK3^{-/-} mice neither exhibited early lethality nor developed skin lesions during development and into adulthood. In fact, FADD^E/RIPK3^{-/-} mice had normal skin, with normal epidermal thickness and an absence of dying keratinocytes and pathologic inflammatory infiltrates, indicating that inflammatory disease observed in FADD^E mice was entirely dependent on RIPK3-mediated cell death. Concordantly, FADD^E mice with concomitant deletion of TNF- α -receptor or MYD88 or an inactivating

mutation of CYLD had delayed onset of inflammatory skin lesions, stressing that compromise of barrier function and microbial-associated innate immune activation may be responsible for the disease pathogenesis (Bonnet et al. 2011).

Constitutive loss of caspase-8 in epidermal keratinocytes produced an inflammatory skin disorder with epidermal hyperplasia, dermal inflammatory cell infiltration, and premature lethality in mice (Kovalenko et al. 2009). These findings were corroborated by studies in which acute deletion of caspase-8 in adult murine skin by application 4-hydroxytamoxifen to Rosa-CRE, Casp8^{fl/fl} mice resulted in local tissue inflammation and damage (Welz et al. 2011). Importantly, 4-hydroxytamoxifen treatment of Rosa-CRE, Casp8^{fl/fl}, RIPK3^{-/-} mice resulted in minimal dermal inflammation, epidermal hyperplasia, and keratinocyte death (Welz et al. 2011). In accord, RIPK3-dependent signaling promoted skin inflammation and injury upon deletion of caspase-8 in epidermal tissue. Although the role of RIPK1 has not been specifically established in these studies, it could be anticipated that RIPK1 kinase activity may be important as an inducer of RIPK3-dependent cell death.

3.3 Summary

Loss of essential barrier cells lends the organism to infection by commensal microbiological flora, tissue injury, and diminished survival. The studies described above highlight an important new role for kinase-independent function of RIPK1 in maintaining epithelial homeostasis of the intestinal tract as well as in the skin. RIPK1 serves this function by two apparent means that may be fundamentally intertwined. First off, the presence of RIPK1 directly prevents uncontrolled activation of caspase-8 and FADD-associated apoptosis and RIPK3-dependent necroptosis at the epithelial surface. Secondly, RIPK1 promotes a survival-like state in the presence of pro-inflammatory and pro-injury signals such as TNF- α , IFN γ , and other ligands associated with infection. Indeed, dual roles of RIPK1 in both activating and inhibiting necroptotic cell death have been described in the literature (Kearney et al. 2014; Orozco et al. 2014). Furthermore, evidence reported here also demonstrates that aberrant or inappropriate activation of necroptosis, driven by RIPK1-kinase function or RIPK3, can result in loss of barrier function in the skin and intestinal epithelia. Deletion of caspase-8 or FADD in barrier tissue sensitizes cells to necrotic death and chronic inflammatory disease that may be attributed to microbial activation of the innate immune host response.

4 Inflammation

Inflammation is an integral part of the host response (Kumar et al. 2010). RIPK1 and RIPK3 are well recognized for inducing a pro-inflammatory form of cell death; however, accumulating evidence suggests that in some cases, these enzymes may also regulate inflammation independent of necroptosis. It should be noted that this

distinction may be difficult to draw in many cases due to a highly intertwined nature of cell death and inflammation. The ensuing discussion highlights emerging, but still poorly characterized roles for RIPK1 and RIPK3 in amplifying the host inflammatory response following cytokine stimulation, activation of innate immune receptors, and microbial infection.

4.1 *RIPK1*

TNF- α is well recognized for regulating RIPK1 kinase-dependent cell death; however, TNF- α is also a major pro-inflammatory cytokine that promotes nuclear factor kappa B (NF κ B)-associated inflammation (Christofferson et al. 2012; Vandenabeele et al. 2010a; Hitomi et al. 2008; Kelliher et al. 1998). Kinase-independent function of RIPK1 has been linked to NF κ B activation in response to TNF- α . Expression of kinase-inactive RIPK1 restores defective TNF- α -induced NF κ B activation in RIPK1^{-/-} Jurkat cells (Ting and Pimentel-muiffios 1996). Similarly, analysis of nuclear extracts from embryonic fetal liver-derived transformed pre-B cells demonstrated decreased NF κ B–DNA binding in electrophoretic mobility shift assays in RIPK1^{-/-} cells (Kelliher et al. 1998). Conspicuously, reports have found that TNF- α -induced NF κ B activation is associated with covalent modification of RIPK1 (Zhang et al. 2000). These findings highlight an important role for kinase-independent function of RIPK1 in promoting TNF- α -induced NF κ B-driven inflammation.

Roles for RIPK1- dependent NF κ B regulation have also been uncovered downstream of innate immune receptors. In 2004, Tschopp and colleagues noted that RIPK1 promotes NF κ B activation downstream of TLR3, the pathogen recognition receptor (PRR) for sensing viral double-stranded RNA (Meylan et al. 2004). Activation of NF κ B pathway, assessed by phosphorylation of inhibitor of kappa B (I κ B), is impaired in MEFs lacking RIPK1 following stimulation with TLR3 agonist, Poly(I:C). Exogenous protein expression in HEK293T cells demonstrated that RIPK1 associates with TLR3 adapter protein, TRIF, by common RHIM-domain interactions. Furthermore, expression of TLR3, TRIF, and RIPK1 in HEK293T cells resulted in their co-immunoprecipitation, suggesting that the three factors may complex following receptor activation (Meylan et al. 2004). Similar studies in MEFs demonstrated that RIPK1 is required for NF κ B activation following ligation of TLR4 with Gram-negative bacteria associated pathogen-associated molecular pattern, lipopolysaccharide (LPS) (Cusson-Hermance et al. 2005). Importantly, LPS-induced NF κ B activation also requires TRIF (Cusson-Hermance et al. 2005). Finally, studies found that TRIF-dependent NF κ B activation manifested in correlation with increased phosphorylation and ubiquitylation of RIPK1, suggesting importance of post-translational modification in RIPK1-dependent NF κ B activation. Together, these results find that RHIM-domain interactions likely facilitate a role of RIPK1 in NF κ B activation downstream of innate immune receptors, TLR3 and TLR4.

Apart from NF κ B signaling, kinase-independent scaffold properties of RIPK1 have also been recognized in PRR activation of the interferon-inducing

transcription factor and interferon regulatory factor 3 (IRF3) (Rajput et al. 2011). RIPK1 localizes to mitochondria in association with cytosolic RNA/DNA sensor RIG-I following viral infection with Sendai virus (SeV). These events occur in coordination with IRF3 activation and expression of downstream genes. Knockdown of RIPK1 impairs SeV-induced IRF3 activation. Moreover, MEFs reconstituted with a non-ubiquitylatable form of RIPK1 at K377 have deficits in SeV-induced IRF3 activation, indicating that ubiquitin conjugation of RIPK1 at K377 is necessary for activation of IRF3 (Rajput et al. 2011). Remarkably, in spite of an abundance of evidence linking ubiquitylation of RIPK1 to NF κ B activation, these studies found no changes in NF κ B activation following SeV infection. Accordingly, it appears as though RIPK1 may be a versatile regulator in innate immune pathways, and precise action of RIPK1 is determined in a context-dependent manner.

Few studies have ventured to uncover roles for RIPK1-dependent inflammation in bacterial infection models. However, recent investigation of RIPK1 in *Yersinia* infection discovered that RIPK1^{-/-} fetal liver macrophages carried defects in LPS as well as *Yersinia*-induced secretion of IL-6, a pyrogen that directs systemic inflammatory responses. Additionally, caspase-1 cleavage was diminished in RIPK1^{-/-} fetal liver macrophages infected with *Yersinia* species, indicating that RIPK1 may be required for caspase-1-driven inflammasome activation and consequently IL1 β and IL-18 secretion (Weng et al. 2014). It is unclear as to whether IL6 secretion and/or caspase-1 cleavage requires RIPK1 kinase activity as authors did not use inhibitors to evaluate kinase activity in these studies. Nevertheless, these observations identify roles for RIPK1 in multiple pro-inflammatory pathways in a model of Gram-negative bacterial infection.

4.2 Kinase-Dependent Inflammatory Functions of RIPK1

While kinase activity of RIPK1 is commonly associated with cell death, a number of recent reports suggest that inflammasome activation by RNA viruses, including VSV, SeV, and influenza (Flu), may occur in a RIPK1 kinase-dependent manner. The inflammasome is a molecular complex comprised of the receptor NLRP3 and caspase-1 that is responsible for the maturation and secretion of pro-inflammatory cytokines, IL1 β and IL-18. Nec-1 or knockdown of RIPK1 reduced IL1 β secretion in bone marrow-derived macrophages (BMDMs) infected with VSV (Wang et al. 2014a). This regulation was also found to require RIPK3 as RIPK3^{-/-} BMDMs also had similar defects in inflammasome activation following RNA virus infection. In a physiologic system, RIPK3^{-/-} mice produced lower levels of IL1 β and IL-18 in response to infection with RNA viruses as well (Wang et al. 2014a). Surprisingly, MLKL was not required for inflammasome activity, as determined using MLKL knockout (MLKL^{-/-}) BMDMs, suggesting that RIPK1 and RIPK3 regulate inflammasome activation independent of necroptosis pathway. Moreover, VSV-induced minimal death in target cells indicating that RIPK1 and/or RIPK3

were likely not regulating alternative death pathways such as pyroptosis. Mechanistic analysis revealed that RIPK1 and RIPK3 complexed with mitochondrial fission protein, DRP1, and were required for translocation of DRP1 to mitochondria following infection. DRP1 was required for RNA virus-induced mitochondrial fission, mitochondrial aggregates, ROS generation, and inflammasome activation as well. Poly(I:C), double-stranded RNA mimic, was found to induce NLRP3-dependent inflammasome activation in a RIPK1, RIPK3, and DRP1-dependent manner, indicating that viral nucleic acids may be the ignition for RIPK1 kinase and RIPK3-dependent inflammasome activation (Wang et al. 2014a).

Intrinsic kinase activity of RIPK1 may also underlie certain tissue-specific inflammatory processes. Ptpn6^{spin} mice harboring a Tyr208Asn mutation in the non-receptor protein tyrosine phosphatase Src-homology region 2 domain-containing phosphatase-1 (SHP-1) develop footpad inflammation that is RIPK1 kinase dependent (Lukens et al. 2013). Studies revealed that aberrant production of the cytokine IL1 α exacerbates inflammation in Ptpn6^{spin}-mediated disease. Nec-1 diminished IL1 α expression and ameliorated footpad inflammation in Ptpn6^{spin} mice. Additionally, reconstitution of WT mice with Ptpn6^{spin}/RIPK1^{-/-} fetal liver (hematopoietic precursor) cells abrogated the inflammation produced by transfer of Ptpn6^{spin}/RIPK1^{+/+} fetal liver cells. Notably, Ptpn6^{spin}/NLRP3^{-/-}, Ptpn6^{spin}/Caspase-1^{-/-}, or Ptpn6^{spin}/RIPK3^{-/-} mice were not protected from Ptpn6^{spin}-inflammatory disease. These findings suggest a critical role for kinase function of RIPK1 in hematopoietic cells to promote IL1 α -dependent inflammation that is independent of inflammasome components and RIPK3 (Lukens et al. 2013).

Similarly, mouse models of RIPK1 and RIPK3 kinase activation, such as tissue-specific caspase-8 deletion in dendritic cells, keratinocytes, and or intestinal epithelium, have spawned inflammatory disease in mice (Kovalenko et al. 2009; Günther et al. 2011; Cuda et al. 2014). Although it may be unclear as to whether inflammation in some of these models is occurring independent of necroptosis, Cuda and colleagues observed that deletion of caspase-8 in dendritic cells (DCs) generated an autoimmune condition in mice that could not be attributed to impaired cell survival or a RIPK3-mediated processes. Caspase-8 deletion in DCs crossed to RIPK3^{-/-} mice was not protected from inflammatory pathology. Rather, authors observed that systemic inflammation was partially dependent on MYD88 and also found that Nec-1 abrogated Toll-like receptor activation-induced expression of TNF- α , IL-6, and IL1 β (Cuda et al. 2014). These accounts suggest roles for kinase function of RIPK1 in regulating Toll-like receptor-induced cytokine expression in dendritic cells.

The recent availability of RIPK1 kinase-inactive mouse models has unveiled significance of RIPK1 catalytic activity in directing inflammation. For example, RIPK1 kinase-inactive mice are protected from TNF- α -induced hypothermia and shock (Polykratis et al. 2014). Another group evaluated kinase function of RIPK1 in the absence of SHARPIN, a component of the linear ubiquitin assembly complex (LUBAC). SHARPIN deletion results in gross inflammatory pathology, dermatitis, and diminished organism survival. Authors observed that crossing their independently generated RIPK1 kinase-inactive mice to SHARPIN-deficient mice protected animals from inflammatory pathology and prolonged survival (Berger et al. 2014).

4.3 *RIPK3*

Work spearheaded by Francis Chan and colleagues has marked RIPK3 as an important component of the host inflammatory response to injury and infection. In one study, the group reported impaired inflammation-associated repair in RIPK3^{-/-} mice in the dextran sodium sulfate (DSS) model of colitis (Moriwaki et al. 2014). Specifically, RIPK3 in hematopoietic cells is required for protection against DSS colitis; RIPK3^{-/-} mice with WT bone marrow lost less body weight and had reduced inflammation (Moriwaki et al. 2014). RIPK3^{-/-} mice had reduced circulating levels of IL1 β and IL23, and resupplementation of these cytokines mitigated the extent of colitis. In concordance, bone marrow-derived dendritic cells (BMDCs) had defects in LPS-induced expression of cytokines, including TNF- α , IL1 β , IL23, and monocyte chemoattractant protein-1 (MCP-1) (Moriwaki et al. 2014). It is unlikely that these observations are linked to the differences in cell death or the release of damage-associated molecular patterns as RIPK3^{+/+} and RIPK3^{-/-} BMDCs did not undergo death in response to LPS. Rather, these effects were attributed to defects in the generation of ROS and nuclear localization of subunits RelB and p50 in the NF κ B pathway (Moriwaki et al. 2014). Similar defects in inflammatory cytokine expression were not observed in BMDMs suggesting that RIPK3 may have a broad functional range that can be employed in a context or cell-specific manner.

Analyses in BMDCs demonstrated that RIPK3 is required for caspase-8 and caspase-1 dependent IL1 β processing and secretion. Authors observed that LPS-induced RIPK3 complex formation with caspase-8 in BMDCs. Conspicuously, both caspase-8 cleavage as well as IL1 β secretion were decreased in LPS-stimulated RIPK3^{-/-} BMDCs suggesting that RIPK3 directs LPS-induced IL1 β secretion by facilitating maturation or activation of caspase-8 (Moriwaki et al. 2015). Kinase activity of RIPK3 appeared to be dispensable as RIPK3 kinase-inactive BMDCs did not display a deficit in IL1 β production (Moriwaki et al. 2015). Authors also observed that kinase-independent function of RIPK1 may be required as lentiviral CRE-mediated deletion of kinase-inactive RIPK1 in BMDCs reduced LPS-induced caspase-8 maturation; however, no defects were observed in absence of CRE. Paradoxically, RIPK3 kinase inhibitor, GSK'872, augmented LPS-induced IL1 β production, suggesting that this regulation may not be controlled by the catalytic activity of RIPK3 per se, but may be influenced by the conformation of the kinase domain. Consistent with this model, GSK'872 enhanced the recruitment of RIPK1 to RIPK3 (Moriwaki et al. 2015).

4.4 *Summary*

Inflammation is a crucial part of the host response during infection. Notably, RIPK1 and RIPK3 have been identified in critical roles regulating pro-inflammatory transcription factors, cytokine synthesis, and cytokine secretion in response to immunogenic ligands. Moreover, RIPK1 kinase activity and RIPK3 have been

linked to tissue-specific and systemic inflammation. As discussed, in some instances, inflammatory signaling by these proteins can be clearly separated from cell death. Accordingly, these factors may emerge as potential therapeutic targets not only in pathologic settings that are driven by significant cell death and tissue loss, but also in disease states that are primarily inflammatory in nature.

5 RIPK1 and RIPK3 as Therapeutic Targets in Light of New Innate Immune Roles

RIPK1 and RIPK3 emerged as potential targets in many human pathologies involving necrotic cell death in models of stroke, tissue infarction, atherosclerosis, ischemia–reperfusion injury, pancreatitis, inflammatory bowel disease, and others (Linkermann and Green 2014; You et al. 2008; Degterev et al. 2005). The emerging roles of these proteins in innate immunity need to be considered when evaluating therapeutic potential of targeting RIPK1 and RIPK3. However, involvement of RIPK1 and RIPK3 in responses to pathogens per se should not necessarily diminish enthusiasm for developing RIPK-targeting therapies. In particular, while RIPK1 and RIPK3 may regulate robustness of innate immune responses, it is unclear whether inhibition of these pathways may truly change the outcome of infection as opposed to just changing the kinetics of cell death and, ultimately, the death of the organism. It should also be noted that, thus far, neither RIPK1 nor RIPK3 have emerged as critical players in any of the paradigms involving reactivation of the dormant pathogens. On the other hand, in cases of acute infection or sepsis, in which cell death and inflammation are the reason for high mortality, these pathways may be targeted to transiently attenuate the intensity of the host responses to improve patient outcomes, especially when longer term therapeutic options are available to ultimately eradicate pathogens. Needless to say, better understanding of the balance of RIPK1 vs RIPK3 contributions using recently developed genetic and pharmacologic tools will also be important. Current evidence suggests overlapping functions of these regulators in a number of paradigms, including pathogen-induced necroptotic cell death, as well as possibly distinct roles in response to particular pathogens. For example, RIPK3 may be the primary mediatory of certain virus-induced responses, whereas RIPK1 is responsible for YopJ-induced apoptosis by *Yersinia* (Table 1) (Upton et al. 2012; Kaiser et al. 2013; Moriwaki et al. 2014; Lamkanfi and Dixit 2010; Weng et al. 2014; Philip et al. 2014).

6 Discussion

Emerging evidence presented here illuminates roles for RIPK1 and RIPK3 in fortifying host immunity against certain pathogens. Specifically, RIPK1 and RIPK3 appear to play a role in the host response to mitigate viral dissemination and promote host survival in viral infection. RIPK1 and RIPK3 may also play multiple,

complex roles in bacterial pathogenesis as well as contribute to other facets of innate immunity, including epithelial barrier homeostasis, and inflammation occurring independent of cell death.

RIPK1- and/or RIPK3-dependent cell death is an important escape tool for cells infected by viral species, which depend on the survival of host cells to propagate. As noted, viral pathogens are armed with caspase or apoptosis inhibitors to subvert the cell death defense and promote microbial dissemination. Only a few pathogens, such as HSV and CMV, have been analyzed with respect to regulation of necrotic cell death and, intriguingly, were found to be equipped with mechanisms to disrupt pro-necrotic signaling. Thus, the question remains as to whether inhibition of necroptosis represents a common element of viral infection, akin to targeting apoptosis, or inhibition of necroptosis reflects a recent evolutionary addition to viral–host interactions and, thus, may only be observed among a limited number of viral pathogens (Omoto et al. 2015; Guo et al. 2015; Huang et al. 2015; Wang et al. 2014b).

It is necessary to clarify that the utility of caspase or apoptosis inhibitors in viral-induced RIPK1- and/or RIPK3-dependent cell death is largely assumed. In fact, vaccinia virus is the only available viral infection model in which caspase inhibition has been shown to be required to induce necrotic cell death (Li and Beg 2000). Likewise, the occurrence of caspase inhibition in RIPK1 and RIPK3 activation associated with bacterial infection has not been examined (Faherty and Maurelli 2008). In the examples summarized here, bacterial-induced RIPK1 and/or RIPK3 activation is linked to the presence of specific bacterial effector proteins and/or toxins, namely *Yersinia* and YopJ, *E. coli* and NleB1, and *Clostridium* and CPB. Programmed necrosis in *SA* infection is associated with multiple toxins, including Hla and leukotoxins. RIPK1 and/or RIPK3 activation in *Salmonella* and *M. tb* infections is associated with host cytokine responses, IFN-I and TNF- α , respectively. In a few of these systems, RIPK1-dependent apoptosis, rather than necrosis has been reported. Thus, another key question remains as to whether activation of RIPK1 and/or RIPK3 signaling is generally a consequence of pathogen-dependent caspase inhibition or represents a cellular response independent of caspase regulation. It should also be noted that caspase inhibition or inhibition of other RIPK1 and/or RIPK3 modulators, such as cIAP ubiquitin ligases or Tak1 kinase, appears to be required for activation of RIPK1 and/or RIPK3 by many of the known triggers (i.e., TNF family, IFNs, TLR agonists) in vitro (Christofferson et al. 2014). However, it is similarly not required in many of the in vivo models studied to date, some of which clearly involve the same triggers. Thus, fully understanding the mechanism(s) silencing RIPK1 and RIPK3 signaling in vitro, but likely failing to do so efficiently in vivo, appears to be another critical step in fully revealing the roles of RIPK1 and RIPK3 proteins in innate immune regulation.

Although a significant body of evidence indicates that RIPK1- and RIPK3-dependent cell death pathways are beneficial for limiting the propagation of viral pathogens, the evidence for RIPK1 and RIPK3 in bacterial pathogenesis is equivocal. It has been proposed that bacterial-induced necrosis pathways may serve to restrain the host immune response and promote leukopenic states (Lamkanfi and Dixit 2010). This viewpoint suggests that bacterial species, unlike viruses, may not be under selective pressure to inhibit cell death because these pathogens are able to grow and replicate independent of

the host cell. This perspective is consistent with observations of *SA*-induced necrotizing pneumonia or *Salmonella* and PFP-induced death discussed here. Conversely, evidence from studies exploring the pathogenesis of *Yersinia* species suggests that RIPK1-dependent cell death pathways aid in host survival. These pathogens differ in a variety of respects; however, notably, the literature suggests that *Yersinia* may have an intracellular phase that is a feature of its pathogenesis (Pujol et al. 2009; Pujol and Bliska 2003, 2005). Under this circumstance, host cells provide nourishment for invading bugs and activation of programmed cell death limits access to this resource. Accordingly, RIPK1 and/or RIPK3-dependent cell death in bacterial infection may not be a generalizable process; however, appreciating infection-specific impact of RIPK1- and/or RIPK3-dependent cell death may be of value in managing infectious disease clinically. For example, although physiologic RIPK1 and/or RIPK3-dependent cell death may be effective in limiting certain bacterial infections, in other instances, therapeutic targeting of RIPK1- and/or RIPK3-dependent cell death may aid in limiting pathogen-induced tissue damage and improving patient outcomes.

Apart from roles in pathogen-induced cell death, kinase-independent and kinase-dependent functions of RIPK1 and RIPK3 have also ascended as important elements in epithelial barrier integrity. Kinase-independent function of RIPK1 sequesters pro-apoptotic activity of caspase 8–FADD complex and pro-necrotic function of RIPK3. Loss of surface epithelia is associated with infection-associated tissue damage, inflammation, and diminished organism survival, signifying that the role of RIPK1 in maintaining epithelial integrity is an important first-line defense against microbial species. Conversely, activation of RIPK1 and RIPK3 kinase-dependent signaling, through the loss of FADD or caspase-8, triggers loss of epithelial barrier function, and the role of this process in a variety of inflammatory pathologies remains to be elucidated from the therapeutic perspective.

Lastly, RIPK1 and RIPK3 are gaining repute as regulators of pro-inflammatory processes independent of cell death. This summary of available literature highlights roles for RIPK1 and RIPK3 in regulating inflammatory pathways in response to cytokine signals, innate immune ligands, and microbial species. However, direct evidence to implicate the role of RIPK1- and/or RIPK3-dependent inflammation in microbial pathogenesis and organism survival in vivo is currently unavailable. Importantly, its emergence would surely confirm this new role in innate immunity for these proteins. Understandably, scientists may shy away from these ventures because of the specter of underlying cell death in experimental models and the clearly established pro-inflammatory nature of cell death processes; however, these confounding factors can be addressed experimentally. For example, evaluating RIPK1- and RIPK3-dependent inflammation on a *MLKL*^{-/-} background may provide interesting insights into necroptosis-independent functions of RIPK1 and RIPK3. Alternatively, infection-associated RIPK1- and RIPK3-dependent function may be evaluated in models of cytokine depletion and inflammatory attenuation. Indeed, efforts are underway to discriminate RIPK1/3-dependent inflammation from cell death (Kang et al. 2014). Importantly, it is necessary to recognize that these answers will be important in ultimately addressing the significance of RIPK1- and/or RIPK3-dependent inflammation in the host response.

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Nagata, S.; Nakano, H. (Eds.)

2017, VII, 183 p., Hardcover

ISBN: 978-3-319-23912-5