Chapter 10
Programmed Necrosis in Immunity and Inflammatory Diseases

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10.1 Introduction

Necrotic cell death is characterized by extensive organelle and cell swelling and rupture of the plasma membrane. These morphological changes are entirely distinct from those of apoptotic cell death, which show organelle and cell shrinking, nuclear chromatin condensation, and nuclear and cytoplasmic blebbing to form membrane-bound fragments known as apoptotic bodies (Kerr et al. 1972; Schweichel and Merker 1973). Necrosis was once considered to be an accidental and unregulated type of cell injury. However, emerging evidence shows that necrosis can be induced in a regulated manner like apoptosis. Regulated necrosis has been called "programmed necrosis" or "necroptosis" to distinguish it from necrosis induced by physical trauma (Vandenabeele et al. 2010). Programmed necrosis can be induced by plasma membrane-associated death receptors in the TNF receptor (TNFR) superfamily (Laster et al. 1988; Vercammen et al. 1998a, b; Holler et al. 2000), T cell receptor (TCR) (Ch'en et al. 2008, 2011; Cho et al. 2011), and toll-like receptors (TLRs) (He et al. 2011; Fortes et al. 2012; McComb et al. 2012). Necrotic cell death is pro-inflammatory because it releases intracellular contents or the so-called danger-associated molecular patterns (DAMPs) (Kono and Rock 2008). The released DAMPs from necrotic cells such as HMGB1 can activate TLRs on the surface of innate immune effector cells to promote inflammatory cytokine expression (Lamkanfi et al. 2010; Yang et al. 2010). These observations imply that programmed necrosis is an important cell death module in the immune system. In fact, recent studies show that the programmed necrosis is closely associated with

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infectious and noninfectious inflammatory diseases. In this chapter, we discuss the emerging roles of programmed necrosis in biology with a specific emphasis on its role in immunity and inflammation. For simplicity sake, we will use the term necrosis to refer to regulated programmed necrosis hereafter.

10.2 Molecular Regulation of Necrosis

The most extensively characterized pathway leading to necrosis is initiated by ligation of TNF receptor 1 (TNFR-1/TNFRSF1a/CD120a). We will therefore use the pathway regulated by TNFR-1 ligation to illustrate the salient principles that govern necrosis. When TNF binds to TNFR-1, the membrane-associated TNFR-1 signaling complex termed "Complex I" is formed. Complex I comprises multiple protein adaptors including TNFR-associated death domain (TRADD), receptor-interacting protein kinase 1 (RIPK1), cellular inhibitor of apoptosis 1 (cIAP1), cIAP2, TNFR-associated factor 2 (TRAF2), and linear ubiquitin chain assembly complex (LUBAC) (Micheau and Tschopp 2003) (Fig. 10.1). This complex primarily triggers the NF-κB signaling pathway. RIPK1 ubiquitination is an essential event that mediates NF-κB activation (Walczak 2011). Although early reports show that K63 ubiquitination of RIPK1 at K377 is essential for recruitment of NEMO and activation of the IKK complex (Ea et al. 2006; Li et al. 2006), recent studies indicate that ubiquitination at sites other than K377 as well as other types of ubiquitin linkages can also occur (Dynek et al. 2010; Gerlach et al. 2011). RIPK1 ubiquitination prevents assembly of the cytoplasmic death-inducing signaling complex, also known as "Complex II," through NF-κB-dependent and -independent mechanisms (O'Donnell et al. 2007). Consistent with an inhibitory role for RIPK1 ubiquitination in cell death signaling, ubiquitin hydrolases such as cylindromatosis (CYLD) have been shown to facilitate apoptotic and necrotic responses (Hitomi et al. 2008; Vanlangenakker et al. 2011) (Fig. 10.1).

Caspase activity is a critical parameter that controls necrosis. Early studies show that the broad caspase inhibitor zVAD-fmk can facilitate RIPK1-dependent necrosis in certain cell types (Vercammen et al. 1998a; Holler et al. 2000). However, RIPK1 does not act alone to drive necrosis. Another serine/threonine kinase, RIPK3, was identified in several RNA interference screens to be a critical partner of RIPK1 in necrosis (Cho et al. 2009; He et al. 2009). Caspase 8 inhibits necrosis by cleaving and inactivating RIPK1, RIPK3, and CYLD (Lin et al. 1999; Chan et al. 2003; Feng et al. 2007; O'Donnell et al. 2011). When the activity of caspase 8 is inhibited or in caspase $8^{-/-}$ or $Fadd^{-/-}$ cells, the integrity of RIPK1 and RIPK3 is preserved. This allows the two kinases to form a tight and stable complex termed the "necrosome" (Cho et al. 2009; He et al. 2009). Necrosome formation requires the RIP homotypic interaction motif (RHIM) that is present in both RIPK1 and RIPK3 (Sun et al. 2002). *Trans*-phosphorylation of RIPK1 and RIPK3 appears to be crucial for necrosome assembly, as kinase-inactive RIPK1 or RIPK3, and the RIPK1-specific inhibitor necrostatin-1 potently inhibits TNF-induced necrosis (Degterev et al. 2008).

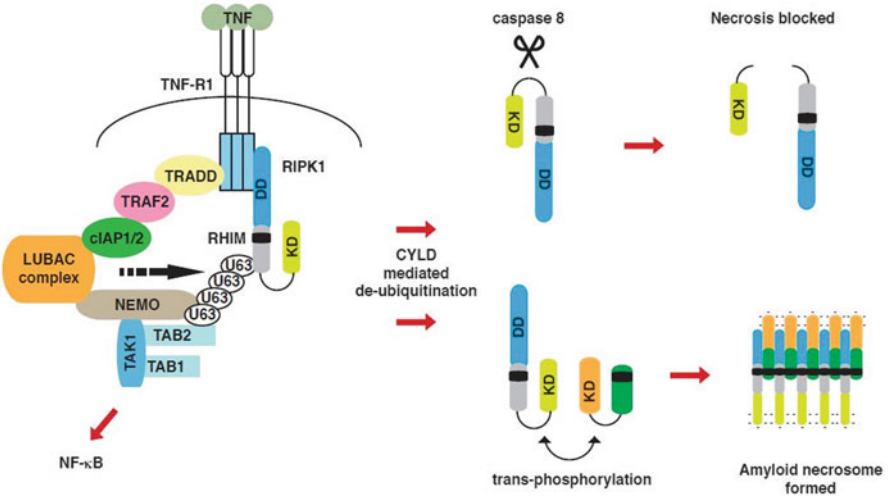

Fig. 10.1 The necrosis signaling pathway is regulated by protein ubiquitination, phosphorylation, and proteolytic cleavage. The TNFR-1-associated membrane complex (Complex I) is composed of many adaptors. Many of the molecular interactions within this complex require protein ubiquitination. RIPK1 ubiquitination through K63 linkage (U63), which is crucial for downstream NF-κB activation, is *highlighted*. Removal of ubiquitin chains from RIPK1 by the de-ubiquitinase cylindromatosis (CYLD) is important for transition of Complex I to the cytosol and assembly of Complex II. In the presence of active caspase 8, RIPK1, RIPK3, and CYLD are cleaved and inactivated (only RIPK1 cleavage is shown for simplicity sake). Cleavage of RIPK1 removes the kinase domain, thereby preventing phosphorylation of downstream substrates (e.g., RIPK3) that are important for necrosis induction. When the integrity of RIPK1 and RIPK3 is preserved, they *trans-phosphorylate* each other. The resulting negative charge may be critical in "opening up" the RHIM domain to facilitate amyloid fibril assembly and recruitment of downstream RIPK3 substrates

Hence, necrosis is regulated by at least three distinct mechanisms: protein ubiquitination, caspase cleavage, and phosphorylation.

The RHIM is an emerging protein–protein interaction domain found in several other adaptors including TIR domain-containing adaptor molecule 1 (TICAM1/TRIF) and DNA-dependent activator of interferon regulatory transcription factors (DAI/ZBP1) (Moquin and Chan 2010). Thus, the RHIM-containing adaptors all have important functions in innate immune and cell death signaling. The RHIM is defined by a highly conserved tetra-peptide core sequence of mostly hydrophobic residues that are predicted to be β-sheet (IQIG for RIPK1 and VQVG for RIPK3). Recent biophysical studies show that the RHIMs of RIPK1 and RIPK3 assemble in an amyloid-like filamentous fibrillar complex (Li et al. 2012). Mutagenesis of the RHIM core sequences shows that this amyloidal assembly is crucial for activation of RIPK1 and RIPK3 kinase activity, necrosome cluster formation, and necrosis induction (Fig. 10.1).

Although amyloid fibrils are toxic to neurons, the RHIM amyloid fibril appears to be an intermediary that does not directly elicit cell damage. Rather, it has a crucial function in recruitment of downstream RIPK3 substrates. One such substrate is the

mixed lineage kinase domain-like (MLKL), which was identified by biochemical purification and by shRNA screen (Sun et al. 2012; Zhao et al. 2012). Phosphorylation of MLKL by RIPK3 is critical for necrosis induction. The significance of MLKL in necrosis is further bolstered by identification of a small-molecule inhibitor called "necrosulfonamide" (NSA). NSA inhibits TNF-induced necrosis by covalently modifying human MLKL. Surprisingly, NSA or siRNA knockdown of MLKL did not interfere with RIPK1–RIPK3 necrosome formation. Hence, MLKL is a key regulator of necrosis downstream of RIPK3 (Sun et al. 2012).

Another RIPK3 substrate is the mitochondrial protein phosphoglycerate mutase family member 5 (PGAM5). Both isoforms of PGAM5, $PGAM5_S$ and $PGAM5_L$, were reported to be downstream effectors involved in necrosis induction (Wang et al. 2012). NSA prevented the recruitment of $PGAM5_S$, but not MLKL, to the necrosome. MLKL therefore appears to function as a key adaptor that links the RIPK1–RIPK3 necrosome to downstream effectors. Interestingly, PGAM5 is a phosphatase (Takeda et al. 2009) that can dephosphorylate and activate the mitochondria fission factor Drp-1 (Wang et al. 2012). This raises the interesting possibility that the necrosome can engage the mitochondria fission machinery to execute necrosis. In addition to TNF-induced necrosis, PGAM5 also appears to have broader roles in mediating death receptor-independent necrosis, such as that induced by reactive oxygen species (ROS) or calcium ionophore (Wang et al. 2012). Whether MLKL and PGAM5 are physiologically relevant RIPK3 substrates in vivo will require examination in the relevant mutant animals.

10.3 Role of Necrosis in Innate Inflammatory Responses

10.3.1 Viral Infections

Necrotic cells are characterized by organelle and cell swelling that eventually cumulate in plasma membrane leakage. The release of endogenous adjuvants from necrotic cells is known to be immuno-stimulatory. As we have alluded to in the previous section, many protein adaptors of innate immune signaling pathways contain RHIM domains. This molecular signature suggests that the RIP kinases may have broad roles in innate immunity and inflammation. Further evidence that supports this notion comes from the fact that interferons, which are critical cytokines against viral pathogens, can greatly sensitize cellular necrosis (Kalai et al. 2002).

The first example that highlights this emerging paradigm comes from a study of host defense against vaccinia virus infection. Vaccinia virus, like other poxviruses, encodes many immune evasion genes (Moss and Shisler 2001), including those that inhibit inflammatory cytokines and TLRs (Reading et al. 2002; Harte et al. 2003; Stack et al. 2005). Despite the inhibition of inflammatory signaling, vaccinia virus elicits a strong inflammatory response in infected mice.

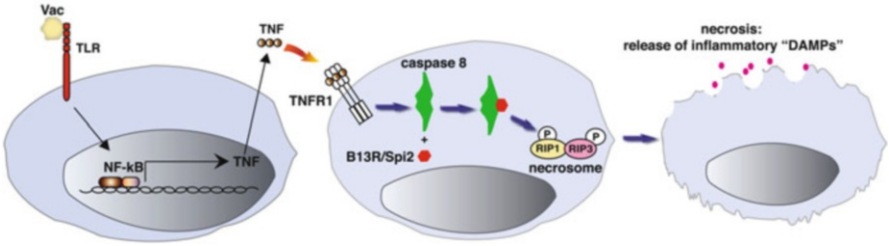

Fig. 10.2 RIP kinase-dependent necrosis is an important innate immune defense mechanism against vaccinia virus. Vaccinia virus is a large DNA virus that has been shown to activate multiple TLRs. Engagement of TLRs results in expression of inflammatory cytokines including TNF. TNF can elicit the cell death program upon binding to the receptor on the cell surface of an infected cell. Because of the virus-encoded caspase inhibitor B13/Spi2, caspase 8 is inhibited and apoptosis is suppressed. This allows assembly of the RIPK1–RIPK3 necrosome. The induction of necrosis may be advantageous in two ways. First, it serves to limit the viral factory before adaptive immunity is launched. Secondly, the release of DAMPs from the necrotic cells may further promote the antiviral inflammatory reaction

One of the immune evasion genes encoded by vaccinia virus is B13R or Spi2, which is a serpin that inhibits caspase 1 and caspase 8 and is functionally similar to the cytokine response modifier A (CrmA) from cowpox virus (Zhou et al. 1997). Despite inhibition of caspase 8 by B13R/Spi2, vaccinia virus-infected cells are still sensitive to the cytotoxic effect of TNF (Li and Beg 2000). TNF-induced cell death of vaccinia virus-infected cells exhibits morphology that resembles necrosis and is dependent on intact RIPK1 and RIPK3 functions (Chan et al. 2003; Cho et al. 2009) (Fig. 10.2). Consistent with results from in vitro infections, RIPK3$^{-/-}$ mice exhibit reduced necrosis and inflammation and greatly increased viral replication in multiple tissues. Eventually, RIPK3$^{-/-}$ mice succumb to the infection 4–5 days post-infection (Cho et al. 2009). In wild-type mice, elevated TNF expression was detected by 24 h post-infection, which coincided with the appearance of RIPK1–RIPK3 complex in the liver (Cho et al. 2009). Because these events occur prior to induction of adaptive T cell responses, which usually peaks at days 7–8 post-infection, we conclude that RIPK3 is critically important for innate immune protection against vaccinia virus. This early control of viral replication is likely to be crucial for host control of the viral factory before virus-specific T and B cells are mobilized in high enough number to fully eradicate the virus.

For vaccinia virus, host cell necrosis is an effective innate immune antiviral defense. Could viruses have developed strategies to inhibit necrosis as a means to escape elimination from the host? Murine cytomegalovirus (MCMV) encodes three different types of viral cell death inhibitors, vICA (inhibitor of caspase 8-induced apoptosis), vMIA (mitochondria inhibitor of apoptosis), and vIRA (inhibitor of RIP activation) (reviewed in Mocarski et al. 2012). Productive infection and replication of viral progenies require the action of all three inhibitors. Although no enzymatic activity can be detected (Lembo et al. 2004), vIRA or M45 exhibits homology to ribonucleotide reductase (Brune et al. 2001). Interestingly, M45 contains a RHIM at the amino terminus that is crucial for binding to RHIM-containing cellular adaptors

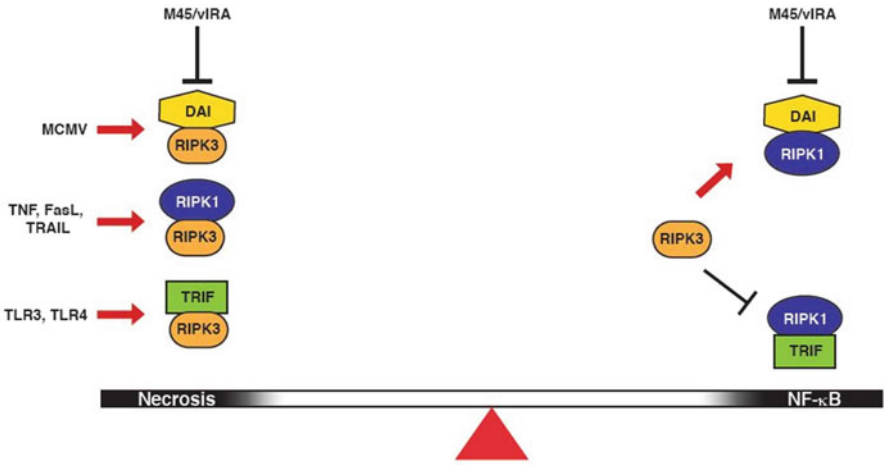

Fig. 10.3 Different RHIM domain-containing adaptors can partner with RIPK3 to induce necrosis. The three known RHIM-mediated interactions that lead to necrosis are shown on the *left*. In the case of TRIF–RIPK3, genetic and pharmacological evidence suggests that RIPK1 is also involved. However, biochemical evidence for RIPK1–RIPK3–TRIF complex is lacking at present. Hence, RIPK1 is not included in the necrotic TRIF–RIPK3 complex. On the *right*, the different types of RHIM-mediated interactions that regulate NF-κB activation are shown. Note that RIPK3 has been shown to have positive and negative effects on NF-κB activation by different complexes (Kaiser et al. 2008; Rebsamen et al. 2009)

including RIPK1, RIPK3, and DAI (Kaiser et al. 2008; Upton et al. 2008, 2012; Rebsamen et al. 2009). Recombinant virus that encodes a defective vIRA with tetra-alanine substitutions within the core RHIM sequence fails to establish productive infection in cells and in mice due to premature cell death by necrosis. Significantly, productive infection is restored when the RHIM mutant MCMV infects RIPK3$^{-/-}$ mice (Upton et al. 2010). Surprisingly, the necrotic cell death induced upon mutant MCMV infection is not driven by TNF or RIPK1. Instead, RIPK3 pairs with another RHIM-containing adaptor DAI to induce necrosis (Upton et al. 2012). Hence, similar to poxviruses, MCMV infection sensitizes cells to necrosis. However, unlike vaccinia virus, MCMV has developed an effective strategy to inhibit host cell necrosis to ensure productive viral replication within the infected host. It will be of great interest to determine if similar viral inhibition of necrosis also occurs in human CMV infection. The MCMV studies also reveal that other than RIPK1, other RHIM-containing adaptors can partner with RIPK3 to induce necrosis (Fig. 10.3). It is noteworthy that similar RHIM-mediated interactions between RIPK1–TRIF and RIPK1–DAI/ZBP1 have been shown to mediate NF-κB activation (Meylan et al. 2004; Kaiser and Offermann 2005; Rebsamen et al. 2009) (Fig. 10.3). It will be important to determine how the different types of RHIM complexes can mediate cell survival and cell death signaling under different conditions.

10.3.2 Viral Necrosis Inhibitors

The vIRA/M45 story reveals that active suppression of host cell necrosis can be an important immune evasion mechanism for viruses. In fact, vIRA/M45 is not the first viral necrosis inhibitor identified. Viral FLICE-like inhibitor proteins (FLIPs) are orthologs of cellular caspase 8 and caspase 10. They contain tandem death effector domains but lack the enzymatic domains. Hence, they were first recognized as caspase and apoptosis inhibitors (Bertin et al. 1997; Hu et al. 1997; Thome et al. 1997). In 2003, a subset of vFLIPs, namely, MC159 from molluscum contagiosum virus and E8 from equine herpesvirus, were found to also inhibit TNF-induced necrosis (Chan et al. 2003). In contrast to M45, which inhibits necrosis through RHIM-mediated interaction with RIPK3 (Upton et al. 2010), the molecular basis by which vFLIPs inhibit necrosis is not fully understood. Because vFLIPs and vIRA/M45 are structurally unrelated, these results indicate that viral inhibitors of necrosis can come in different flavors. It will be interesting to see if additional classes of viral necrosis inhibitors will be identified in the future.

10.3.3 Bacterial Infections

Macrophages are sentinels against bacterial infections. Recent studies indicate that in the presence of caspase inhibition, the TLR4 agonist bacterial lipopolysaccharides (LPS) can induce RIPK3-dependent necrosis in macrophages (He et al. 2011). In addition, Smac mimetics, which target cIAP1, cIAP2, and XIAP for proteasomal degradation (Varfolomeev et al. 2007; Vince et al. 2007; Bertrand et al. 2008), can induce RIPK1- and RIPK3-dependent macrophage necrosis (McComb et al. 2012). Besides RIPK1 and RIPK3, another RHIM-containing adaptor TRIF also plays a crucial role in macrophage necrosis (He et al. 2011). TRIF is a TIR domain-containing adaptor that mediates type I interferon expression in response to TLR3 and TLR4 signaling (Yamamoto et al. 2002; Oshiumi et al. 2003). Like RIPK1 and RIPK3, TRIF can induce apoptosis under certain conditions (Kaiser and Offermann 2005; Weber et al. 2010). Treatment with LPS and zVAD-fmk, which mimics bacterial septic shock, causes an inflammatory cytokine storm and extensive macrophage necrosis. These effects were greatly ameliorated in $RIP3^{-/-}$ and $TRIF^{lps2/lps2}$ mutant mice (He et al. 2011). Moderately reduced inflammatory cytokine production in response to LPS was also observed in $RIP3^{-/-}$ mice treated with LPS alone (Newton et al. 2004), suggesting that necrosis-induced inflammation can occur in vivo without pharmacologic inhibition of caspases.

TNF is a major inflammatory cytokine that mediates the systemic effects of LPS-induced septic shock. Consistent with a role for TNF in bacterial sepsis, $RIPK3^{-/-}$ mice are protected from TNF-induced systemic inflammatory response syndrome (SIRS) (Duprez et al. 2011; Linkermann et al. 2012a) and cecal ligation puncture-induced sepsis (Duprez et al. 2011). However, results obtained using the RIPK1

inhibitor necrostatin-1 (Nec-1) were less definitive than those obtained with RIPK3$^{-/-}$ mice. While one report shows protection by Nec-1, another report indicates that Nec-1 exacerbates TNF-induced SIRS (Duprez et al. 2011; Linkermann et al. 2012a). These opposing observations may be due to off-target effects of Nec-1 (Cho et al. 2011). Unfortunately, genetic model to assess RIPK1 function in these inflammatory diseases is currently not available because RIPK1$^{-/-}$ mice exhibit neonatal lethality (Kelliher et al. 1998). Conditional RIPK1$^{-/-}$ mice will be invaluable tools to dissect the in vivo role of RIPK1 in inflammatory diseases.

10.3.4 Necrosis in Sterile Inflammation

Besides its role in pathogen-induced inflammation, necrosis can also promote sterile inflammation. For example, retinal detachment-induced photoreceptor necrosis is blocked in RIPK3$^{-/-}$ cells (Trichonas et al. 2010). Because caspase inhibition greatly sensitizes cells to necrosis, it is no surprise that a large number of studies on necrosis-induced sterile inflammation have been performed using caspase 8$^{-/-}$ or mice deficient in FADD, an upstream adaptor that is essential for caspase 8 recruitment and activation. Similar to caspase inhibition in tissue culture, caspase 8$^{-/-}$ or FADD$^{-/-}$ mice are highly sensitive to necrosis induction. Most remarkably, germline inactivation of these genes results in extensive necrosis during embryogenesis, which results in lethality on E9.5. Embryonic lethality of caspase 8$^{-/-}$ or FADD$^{-/-}$ mice is rescued by deletion of RIPK1 or RIPK3 (Kaiser et al. 2011; Oberst et al. 2011; Zhang et al. 2011; Dillon et al. 2012). Keratinocyte- or intestinal epithelium-specific deletion of FADD or caspase 8 causes severe spontaneous inflammation in the respective tissues that can be corrected by deletion of RIPK3 (Kovalenko et al. 2009; Bonnet et al. 2011; Gunther et al. 2011; Welz et al. 2011). While the more popular view is that the inflammatory disease is caused by increased necrosis, the possibility that FADD, caspase 8, RIPK1, and RIPK3 can directly regulate innate inflammatory signaling cannot be overlooked (see below) (Rajput et al. 2011a; Wallach et al. 2011).

In addition to caspase 8$^{-/-}$ or FADD$^{-/-}$ mice, necrosis-induced sterile injury and inflammation have also been detected in wild-type animals with normal FADD and caspase 8 functions. For instance, repeated doses of cerulein can cause RIPK3-dependent acinar cell necrosis and acute pancreatitis in wild-type mice (He et al. 2009; Zhang et al. 2009). Administration of the RIPK1 inhibitor Nec-1 significantly ameliorates tissue damage in animal models of myocardial infarction, ischemia-induced brain injury, and renal ischemia/reperfusion injury (Degterev et al. 2005; Lim et al. 2007; Smith et al. 2007; Northington et al. 2011; Linkermann et al. 2012b), indicating that RIPK1-dependent necrosis is activated under these conditions in wild-type animals. Although TNF and other inflammatory cytokines are often elevated in ischemia/reperfusion-induced injury (Watters and O'Connor 2011; Lambertsen et al. 2012), it is not clear if they are the direct triggers for necrosis in these diseases. If necrosis is induced without death receptor engagement in these situations, it will be analogous to "intrinsic" apoptosis induced in response to genotoxic stress.

10.4 Direct Roles for RIPK1 and RIPK3 in Inflammation Signaling

As we have discussed in previous sections, promoting inflammation via NF-κB was the first function ascribed to RIPK1. In addition to TNFR, RIPK1 also mediates NF-κB activation by certain innate immune receptors such as TLR3 (Meylan et al. 2004), TLR4 (Cusson-Hermance et al. 2005; Ermolaeva et al. 2008) and RIG-I (Michallet et al. 2008; Rajput et al. 2011b). In contrast to RIPK1, RIPK3$^{-/-}$ cells exhibit normal NF-κB induction in response to TNFR-1 and several TLR agonists (Newton et al. 2004). However, early reports show that over-expression of RIPK3 can often inhibit or promote NF-κB activation (Sun et al. 1999; Kasof et al. 2000; Meylan et al. 2004; Kaiser and Offermann 2005). Hence, it remains possible that RIPK3 can modulate NF-κB responses in specific scenarios.

Recent evidence suggests that RIPK3 has a surprising function in driving maturation of the pro-inflammatory cytokine IL-1β (Vince et al. 2012). Production of IL-1β requires two signals. The first signal, which can be provided by activation of innate immune receptors such as TNFR-1 or TLR4, activates de novo synthesis of pro-IL-1β in an NF-κB-dependent manner. Release of mature IL-1β requires a second signal that involves activation of the inflammasome and caspase-mediated processing of pro-IL-1β (reviewed in Rathinam et al. 2012a). In most cases, caspase 1 is the enzyme responsible for processing of pro-IL-1β and related cytokines such as pro-IL-18. However, noncanonical activation of the inflammasome can result in activation of caspase 8 or caspase 11 (Kayagaki et al. 2011; Gringhuis et al. 2012; Pierini et al. 2012; Rathinam et al. 2012b). Vince and colleagues show that in LPS-primed macrophages, Smac mimetics induces IL-1β processing and maturation through canonical NLRP3–caspase 1 and noncanonical NLRP3–caspase 8 inflammasome activation. Surprisingly, RIPK3 and ROS production are also required for Smac mimetic-induced IL-1β maturation. Consistent with the effects of Smac mimetics, LPS-primed cIAP1$^{-/-}$cIAP2$^{-/-}$XIAP$^{-/-}$ macrophages exhibit spontaneous IL-1β processing (Vince et al. 2012). These results suggest the tantalizing possibility that RIPK3 can promote inflammation through multiple means. On one hand, release of DAMPs from necrotic cells can activate TLRs to promote inflammatory gene expression. On the other hand, RIPK3 can directly engage the inflammasome to promote the expression of IL-1-like inflammatory cytokines.

10.5 Necrosis in Adaptive Immunity

10.5.1 T Cell Tolerance

The maintenance of immune homeostasis is critically dependent on proper cell death regulation. T cells recognize antigenic peptides bound to self major histocompatibility complex (MHC) through their TCRs. Because antigen receptors on T and

B cells are generated by random gene rearrangement, T cells that express TCRs of different affinities to MHC are generated. Lymphocytes that express TCR with little affinity for MHC are eliminated through "death by neglect" in a process termed "positive selection." T cells that survive positive selection are further subjected to "negative selection," a process that eliminates potentially autoreactive T cells with TCR that bind too strongly to self peptide–MHC complexes. The cumulative effect of positive and negative selection is a TCR repertoire that is largely devoid of autoreactive cells (reviewed in Stritesky et al. 2012). Death receptors in the TNFR superfamily do not appear to play significant roles in the thymic selection processes, since animals deficient in these receptors undergo normal thymic selection.

Once T cells leave the thymus to populate the peripheral organs, additional mechanisms, collectively termed "peripheral tolerance," are required to prevent activation of any autoreactive T cells that managed to escape thymic negative selection. In contrast to thymic selection, the death receptors Fas/CD95/APO-1 and, to a lesser extent, TNFR-1 and TNFR-2 play key roles in peripheral tolerance (Zheng et al. 1995; Lenardo et al. 1999). Naïve T cells undergo clonal expansion upon TCR engagement. However, repeated TCR stimulation can result in death of the activated T cells (Zheng et al. 1998). This phenomenon is often referred to as "activation-induced cell death" (AICD) or more appropriately as "restimulation-induced cell death" (RICD) (Snow et al. 2009). Both TCR restimulation and T cell trophic factor IL-2 can greatly enhance Fas and Fas ligand (FasL) expression in activated T cells (Zheng et al. 1998). As a result, activated T cells are eliminated through FasL–Fas interaction in a paracrine fashion. As such, deficiency in the receptor or the ligand leads to defective RICD and lymphoproliferative diseases. The well-known mouse models for autoimmunity *lpr* and *gld* are caused by mutations in Fas and FasL, respectively (Watanabe-Fukunaga et al. 1992; Lynch et al. 1994). In human, similar mutations lead to similar systemic autoimmune disease termed the autoimmune lymphoproliferation syndromes (ALPS) (Puck and Sneller 1997).

Because Fas–FasL-induced lymphocyte cell death exhibits classical features of apoptosis (e.g., chromatin condensation, caspase activation), it is widely believed that apoptosis is the cell death module that controls peripheral tolerance. However, this notion was challenged when mice with T cell-specific deletion of FADD or caspase 8 were found to be immunodeficient rather than developing *lpr*-like autoimmune disease (Zhang et al. 1998, 2005; Ch'en et al. 2008). Similarly, human patients with caspase 8 mutations also exhibit immunodeficiency rather than ALPS-like systemic autoimmunity (Chun et al. 2002). Although these defects were originally attributed to defective TCR-induced NF-κB activation (Su et al. 2005), subsequent experiments show that TCR-induced NF-κB activation was normal in caspase $8^{-/-}$ T cells (Ch'en et al. 2008). Further examination revealed that FADD$^{-/-}$ or caspase $8^{-/-}$ T cells undergo extensive necrosis-like cell death upon stimulation through the TCR (Walsh et al. 1998; Kennedy et al. 1999; Hueber et al. 2000). Consistent with the notion that necrosis underlies the proliferative defect, treatment with Nec-1 restored normal T cell proliferation (Osborn et al. 2010). Moreover, FADD$^{-/-}$RIPK1$^{-/-}$ and caspase $8^{-/-}$RIPK3$^{-/-}$ T cells show normal TCR-induced proliferation in vitro, virus-induced clonal expansion in vivo, and cytokine expression (Kaiser et al. 2011;

Zhang et al. 2011). Results obtained from RIPK3$^{-/-}$ mice expressing a dominant negative FADD also show similar phenotypes (Lu et al. 2011). Most remarkably, mice deficient in FADD/caspase 8 and RIPK3 developed lpr-like autoimmune disease that is more aggressive than lpr itself (Ch'en et al. 2011; Kaiser et al. 2011; Oberst et al. 2011), possibly because both Fas- and TNFR-1-induced cell deaths are inhibited. These results revealed an unexpected pro-survival function for FADD and caspase 8 during T cell clonal expansion. They also highlight the fact that caspase-dependent apoptosis and RIP kinase-dependent necrosis are both required to enforce T cell tolerance and homeostasis.

10.5.2 B Cell Responses

In contrast to T cells, B cell proliferation through the antigen receptor or CD40 is unaffected in FADD$^{-/-}$ and caspase 8$^{-/-}$ B cells (Beisner et al. 2005; Imtiyaz et al. 2006). By contrast, TLR3- and TLR4-induced B cell proliferation, but not B cell proliferation induced by the TLR9 agonist CpG DNA, is impaired in FADD$^{-/-}$ and caspase 8$^{-/-}$ B cells (Beisner et al. 2005; Imtiyaz et al. 2006). Unlike TCR-induced proliferation, defective FADD$^{-/-}$ B cell proliferation was not restored in FADD$^{-/-}$RIPK1$^{-/-}$ B cells (Zhang et al. 2011). Because TLR3 and TLR4 share the unique signaling adaptor TRIF and that TRIF has been shown to interact with RIPK1 to mediate NF-κB activation (Meylan et al. 2004; Vivarelli et al. 2004), the defective TLR3/4-induced proliferation in FADD$^{-/-}$RIPK1$^{-/-}$ B cells can be attributed to defective NF-κB signaling. Taken together, these results illustrate that RIPK1 and RIPK3 have differential roles in regulating antigen receptor-induced proliferation in T and B cells.

10.6 Concluding Remarks

Genetic experiments have clearly demonstrated that the RIP kinase-driven necrosis is a biologically relevant cell death module. However, key questions remained to be answered. For example, why are RIPK1 and RIPK3 expression highly induced during T cell activation (Cho et al. 2009, 2011)? It seems counterintuitive that death-promoting molecules are upregulated at a time when lymphocyte expansion is a priority. Similarly, expression of RIPK3 was highly induced during embryogenesis (Zhang et al. 2011). The potential inflammation and damage that necrosis can lead to, such as that seen in FADD$^{-/-}$ and caspase 8$^{-/-}$ animals, is unlikely to be a desired outcome during embryogenesis. In light of these observations, one can envision that RIPK1 and RIPK3 have important biological functions other than necrosis. Discovering and deciphering the non-necrotic or normal physiological functions of the RIP kinases will be of critical relevance as the scientific community ponders the therapeutic potential of manipulating necrosis in the clinics.

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