A Role for Tumor Necrosis Factor Receptor-2 and Receptor-interacting Protein in Programmed Necrosis and Antiviral Responses

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Members of the tumor necrosis factor (TNF) receptor (TNFR) superfamily are potent regulators of apoptosis, a process that is important for the maintenance of immune homeostasis. Recent evidence suggests that TNFR-1 and Fas and TRAIL receptors can also trigger an alternative form of cell death that is morphologically distinct from apoptosis. Because distinct molecular components including the serine/threonine protein kinase receptor-interacting protein (RIP) are required, we have referred to this alternative form of cell death as “programmed necrosis.” We show that TNFR-2 signaling can potentiate programmed necrosis via TNFR-1. When cells were pre-stimulated through TNFR-2 prior to subsequent activation of TNFR-1, enhanced cell death and recruitment of RIP to the TNFR-1 complex were observed. However, TNF-induced programmed necrosis was normally inhibited by caspase-8 cleavage of RIP. To ascertain the physiological significance of RIP and programmed necrosis, we infected Jurkat cells with vaccinia virus (VV) and found that VV-infected cells underwent programmed necrosis in response to TNF, but deficiency of RIP rescued the infected cells from TNF-induced cytotoxicity. Moreover, TNFR-2−/− mice exhibited reduced inflammation in the liver and defective viral clearance during VV infection. Interestingly, death effector domain-containing proteins such as MC159, E8, K13, and cellular FLIP, but not the apoptosis inhibitors Bcl-xL, p35, and XIAP, potently suppressed programmed necrosis. Thus, TNF-induced programmed necrosis is facilitated by TNFR-2 signaling and caspase inhibition and may play a role in controlling viral infection.

Tumor necrosis factor (TNF) is a pleiotropic cytokine that mediates diverse biological responses ranging from inflammation to cell death. It is a critical pro-inflammatory cytokine in innate immunity and has been shown to be important for protection against certain bacterial and viral infections (1–3). TNF exerts its biological functions mainly through binding to its two cell-surface receptors, the p55/p60 TNF receptor-1 (TNFR-1) and the p75/p80 TNF receptor-2. Whereas most of the biological effects induced by TNF have been attributed to TNFR-1, TNFR-2 has been reported to enhance TNFR-1 signaling under certain conditions (4).

Signaling of the pre-assembled TNFR-1 results in the recruitment of the death domain-containing TRADD adapter. Subsequent binding of TNFR-associated factor-2 (TRAF2) or the serine/threonine protein kinase receptor-interacting protein (RIP) is critical for TNF-induced JNK and NF-κB activation, respectively (reviewed in Refs. 5 and 6). In addition, binding of FADD and caspase-8 or caspase-10 to TRADD can initiate the caspase cascade, which results ultimately in cell death by apoptosis. Although TNFR-2 does not contain a cytoplasmic death domain and cannot directly engage the apoptotic machinery, it can enhance the cell death signal of TNF-1, possibly through TRAF2 degradation (7). TRAF2 degradation may cause reduced steric hindrance, thereby leading to enhanced recruitment of FADD and RIP to TNFR-1 and increased cell death (8). However, direct competition among FADD, RIP, and TRAF2 for TNFR-1 binding has not been tested previously, and the mechanism by which TNFR-2 signaling enhances TNFR-1-mediated cell death remains unknown.

Although caspases are critical for the mediation of classical apoptosis, they do not appear to be obligatory for all forms of cell death (9). Interestingly, blockade of caspases can result in sensitization of necrotic death induced through Fas and TNFR-1 (reviewed in Ref. 10). Recently, Holler et al. (11) reported that the RIP kinase is essential for Fas-, TRAIL-, and TNF-induced programmed necrosis. However, the physiological significance and the signals that differentially turn on programmed necrosis as opposed to apoptosis are unknown.

Because of our previous study implicating TNF-2 in amplification of the death signal via RIP (4), we asked whether TNF-2 expression could enhance RIP-dependent programmed necrosis. Indeed, RIP expression potentiated TNF-induced programmed necrosis in a manner dependent on TNF-2, suggesting that TNF-2 may be required for programmed necrosis. This conclusion is consistent with the recent finding that RIP expression confers resistance to TNF-induced cell death (5).

To test whether TNF-2 signals can also enhance RIP activity, we used genetically engineered cells that express different levels of RIP (4). Indeed, RIP expression was sufficient to protect Jurkat cells from TNF-induced programmed necrosis in a manner dependent on TNF-2 expression. This result is consistent with the finding that RIP expression can rescue cells from RIP-dependent programmed necrosis (4).

Members of the TNF receptor superfamily are potent regulators of apoptosis, a process that is important for the maintenance of immune homeostasis. Recent evidence suggests that TNFR-1 and Fas and TRAIL receptors can also trigger an alternative form of cell death that is morphologically distinct from apoptosis. Because distinct molecular components including the serine/threonine protein kinase receptor-interacting protein (RIP) are required, we have referred to this alternative form of cell death as “programmed necrosis.” We show that TNFR-2 signaling can potentiate programmed necrosis via TNFR-1. When cells were pre-stimulated through TNFR-2 prior to subsequent activation of TNFR-1, enhanced cell death and recruitment of RIP to the TNFR-1 complex were observed. However, TNF-induced programmed necrosis was normally inhibited by caspase-8 cleavage of RIP. To ascertain the physiological significance of RIP and programmed necrosis, we infected Jurkat cells with vaccinia virus (VV) and found that VV-infected cells underwent programmed necrosis in response to TNF, but deficiency of RIP rescued the infected cells from TNF-induced cytotoxicity. Moreover, TNFR-2−/− mice exhibited reduced inflammation in the liver and defective viral clearance during VV infection. Interestingly, death effector domain-containing proteins such as MC159, E8, K13, and cellular FLIP, but not the apoptosis inhibitors Bcl-xL, p35, and XIAP, potently suppressed programmed necrosis. Thus, TNF-induced programmed necrosis is facilitated by TNFR-2 signaling and caspase inhibition and may play a role in controlling viral infection.

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The abbreviations used are: TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; TRAF2, TNF-associated factor-2; RIP, receptor-interacting protein; JNK, c-Jun N-terminal kinase; VV, vaccinia virus; DED, death effector domain; HA, hemagglutinin; Z-VAD-fmk, benzoylcarbonyl-Val-Ala-Asp-fluoromethyl ketone; rhTNFRα, recombinant human tumor necrosis factor-α; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling.
FIG. 1. TNFR-2 facilitates programmed necrosis in the absence of FADD and caspase-8. A, caspase-8−/− Jurkat cells (I9.2 cells) or I9.2 cells expressing TNFR-2 (J3.2 cells) were treated with different doses of rhTNFα for 16 h. Live and dead cells were determined using flow cytometry by propidium iodide exclusion. B, FADD−/− Jurkat cells with (I42 cells) or without (I2.1 cells) TNFR-2 were stimulated with rhTNFα and analyzed as described for A. C, caspase-8−/− J3.2 cells were stimulated with the indicated antibodies (3 μg/ml each) or 10 ng/ml rhTNFα, and cell loss was determined as described for A. Antibody (AB) 225 and monoclonal antibody (Mab) 225 are agonistic antibodies against TNFR-1 (R1). Antibody 226...
fying the apoptotic signal of TNFR-1 (7), we sought to determine whether TNFR-2 signaling may also play a role in mediating programmed necrosis. We found that, in addition to enhancing apoptosis, TNFR-2 also switches on caspase-8 activation (11), indicating amounts of time. In some experiments, cells were preincubated with geldanamycin for 12 h or Z-VAD-fmk for 30 min prior to stimulation with rhTNFa.

In Vitro VJ Infections—Jurkat cell derivatives were infected with 20 plaque-forming units of virus/cell at 37 °C in RPMI 1640 medium containing 2.5% fetal bovine serum. For all infections, cells were incubated for 1 h with virus before changing the medium. At 6 h after infection, cells were incubated in fresh medium with or without 10 ng/ml rhTNFa. Twelve hours later, cell death was detected by forward/side scatter or propidium iodide exclusion upon flow cytometry. Apoptosis was detected by TUNEL staining using an in situ cell death detection kit (Roche Applied Science). Fixed cells were stained with a monocolonal antibody to an early protein of VV (E3L) to monitor infectivity, followed by incubation with a Cy5-conjugated anti-mouse antibody (Jackson Immunoresearch Laboratories, Inc.). Using FACScan (BD Biosciences) for analysis, only individual cells positive for E3L staining were analyzed for cell death.

In Vivo VJ Infection—C57BL/6 mice and TNFR-2−/− mice in the C57BL/6 background (8–10 weeks old) were infected via intraperitoneal administration of 106 plaque-forming units of wild-type VJ. Fat pads, spleens, and livers were harvested from VV-infected mice 4 days later. Tissues were divided into two portions. Half of the tissues were fixed in 10% formalin for histological analysis. Paraffin-embedded tissues were sectioned at 4 μm and stained with hematoxylin and eosin. The remaining tissues were homogenized in 1–2 ml of medium for determination of viral titers. 10-Fold serial dilutions were made and overlaid on Vero cells. After a 90-min incubation at 37 °C, a 0.4% agarose solution in Eagle’s minimal essential medium was overlaid on the cells. On day 2, the plates were stained with neutral red. Plaques were counted on day 3.

RESULTS

TNFR-2 Facilitates Caspase-independent Programmed Necrosis—Recently, death receptors including TNFR-1 and Fas have been shown to activate an alternative death program that resembles programmed necrosis when caspase activity is suppressed (reviewed in Ref. 10). Holler et al. (11) reported that signaling through TNFR-1 and Fas and TRAIL receptors can trigger programmed necrotic death that requires a functional RIP kinase. We have shown previously that TNFR-2 can facilitate TNFR-1-mediated apoptosis in Jurkat T cells (7). To examine whether TNFR-2 signaling may also potentiate TNF-induced programmed necrosis, we introduced TNFR-2 into FADD−/− or caspase-8−/− Jurkat cells (15, 16). Although caspase-8−/− cells (I9.2 cells) are normally resistant to TNF-induced death (15), the introduction of TNFR-2 (J3.2 cells) led to a striking sensitivity to TNF-induced cytotoxicity (Fig. 1A). In a similar fashion, the introduction of TNFR-2 into FADD−/− Jurkat cells also led to enhanced sensitivity to TNF-induced cytotoxicity, although FADD−/− cells also underwent TNF-induced death at a lower level in the absence of TNFR-2 (Fig. 1B) (11, 17).

The cytotoxic effects of TNF in J3.2 cells could be mimicked by using a combination of agonistic antibodies against TNFR-1 and TNFR-2. However, either antibody alone was much less effective in triggering cell death compared with TNF (Fig. 1C). When used alone, greater death was obtained with two TNFR-1-specific antibodies compared with the anti-TNF-2 antibodies (Fig. 1C). Moreover, an antagonistic TNFR-2-specific antibody (monocolonal antibody 226) failed to synergize with either anti-TNF-1 antibody (Fig. 1C). Because TNF can induce programmed necrosis in TNFR-2-negative FADD−/− cells, we conclude that programmed necrosis is triggered through TNFR-1, and monoclonal antibody 226 are agonistic and antagonistic antibodies against TNFR-2 (R2, respectively). D, TNFR-2-expressing wild-type (WT; 4E3), FADD−/− (142), or caspase-8−/− (Casp8−/−; J3.2) Jurkat cells were treated with different doses of rhTNFα, and cell loss was analyzed as described for A. TNFR-2 cell-surface expression in all three lines was similar as determined by fluorescence-activated cell sorter staining (Supplemental Fig. 1). E, TNFR-2-positive FADD−/− cells (142 cells) were transiently transfected with pcDNA3 (Vector), full-length FADD (FADD-FL), or the FADD death domain alone (FADD-DD) and pEGFP-N1 as a transfection marker. Cells were stained with TNF or anti-Fas antibody for 1 h, and cell loss was determined in the presence of fluorescent protein-positive cells as described for A, F, and G. Shown are electron micrographs of TNF-stimulated caspase-8−/− J3.2 cells and anti-Fas antibody-treated wild-type 4E3 cells, respectively. Note the swelling of cellular organelles (white arrowhead) and the extensive loss of membrane integrity (black arrowhead) in F. Classical apoptosis as distinguished by chromatin condensation (white arrowhead) and the preservation of membrane integrity (black arrowhead) is shown for comparison in G.
but requires an enhancing signal from TNFR-2.

When the cytotoxic responses of different TNFR-2-expressing Jurkat cells were compared, it was clear that the loss of caspase-8 and FADD dramatically enhanced cell death responses to TNF (Fig. 1D). The enhanced response to TNF was not due to cell line idiosyncrasies, as multiple TNFR-2-positive clones of each variety exhibited consistent phenotypes (Supplemental Fig. 1). Moreover, the reconstitution of full-length FADD in FADD−/− cells resulted in the suppression of TNF-induced programmed necrosis (Fig. 1E). Unlike classical Fas-induced apoptosis, TNFα-induced programmed necrosis in caspase-8−/− J3.2 cells was distinguished by extensive loss of membrane integrity, swelling of intracellular organelles, and limited chromatin condensation (Fig. 1, F and G). This is reminiscent of the necrotic morphology reported by others (11, 18).

TNFR-2-facilitated Programmed Necrosis Requires the Protein Kinase RIP—Holler et al. (11) have shown that the protein kinase RIP is essential for Fas-induced programmed necrosis. Although the major function of RIP appears to be the induction of NF-κB upon TNF stimulation (19), RIP overexpression also causes spontaneous cell death under certain circumstances (20). To investigate whether RIP is also involved in TNFR-2-facilitated programmed necrosis, we introduced TNFR-2 into RIP−/− Jurkat cells (21). As reported previously (22), TNFR-2 expression in RIP−/− cells (R1.1 cells) failed to enhance TNF-induced death over the level observed in the parental cells (Supplemental Fig. 1). Nevertheless, TNF-induced death was completely inhibited in these cells by the pan-caspase inhibitor Z-VAD-fmk (Fig. 2A). This is in stark contrast to the caspase-8−/− and FADD−/− cells, in which Z-VAD-fmk failed to provide any protection against TNF-induced programmed necrosis (Supplemental Fig. 1). In wild-type 4E3 cells, Z-VAD-fmk had a partial protective effect, indicating that the 4E3 cells died by apoptosis, but that a significant fraction of the cells underwent programmed necrosis when caspases were suppressed. Treatment with geldanamycin, which targets Hsp90 and indirectly reduces RIP protein expression (23), also modestly decreased TNF-induced death in 4E3 cells (Fig. 2A, inset). However, Z-VAD-fmk and geldanamycin synergized to completely suppress TNF-induced death in wild-type 4E3 cells (Fig. 2A). Significantly, geldanamycin treatment completely suppressed TNF-induced programmed necrosis in caspase-8−/− J3.2 and FADD−/− I42 cells, but had no effect on TNF-induced apoptosis in RIP−/− cells (Fig. 2, A and B) (data not shown). Kinase-inactive RIP (RIP-K45A), but not the kinase-inactive apoptosis signaling kinase ASK1, strongly suppressed TNF-induced programmed necrosis in J3.2 cells (Fig. 2C). FADD−/− J3.2 cells pretreated with 0.5 μM geldanamycin or left untreated were stimulated with the indicated doses of rhTNFα, and cell loss was determined as described in the legend to Fig. 1. Analysis of RIP protein expression by Western blotting showed a >90% reduction in RIP protein levels in cells treated with geldanamycin (data not shown). B, caspase-8−/− J3.2 cells pretreated with 0.5 μM geldanamycin or left untreated were stimulated with the indicated doses of rhTNFα, and cell loss was determined as described in the legend to Fig. 1. Analysis of RIP protein expression by Western blotting showed a >90% reduction in RIP protein levels in cells treated with geldanamycin (data not shown). C, a kinase-inactive version of RIP (RIP-K45A) or ASK1 (ASK1-K709R) or the control vector pcDNA3 was cotransfected with pEGFP-N1 into caspase-8−/− J3.2 cells. Cells were treated with rhTNFα for 16 h, and live green fluorescent protein-positive cells were counted by fluorescence-activated cell sorter analysis to determine specific cell loss of transfected cells. Results are representative of three independent experiments.

![Fig. 2. Essential role of RIP in TNFR-2-facilitated programmed necrosis. A, TNFR-2-positive 4E3 (wild-type (WT)) and R1.1 (RIP−/−) cells were stimulated as indicated, and cells were harvested and analyzed for cell death as described in the legend Fig. 1. The inset shows the reduction in RIP protein levels when 4E3 cells were treated with geldanamycin (GA). The addition of Z-VAD-fmk (zVAD) or geldanamycin alone had no effect on cell viability (data not shown). B, caspase-8−/− J3.2 cells pretreated with 0.5 μM geldanamycin or left untreated were stimulated with the indicated doses of rhTNFα, and cell loss was determined as described in the legend to Fig. 1. Analysis of RIP protein expression by Western blotting showed a >90% reduction in RIP protein levels in cells treated with geldanamycin (data not shown). C, a kinase-inactive version of RIP (RIP-K45A) or ASK1 (ASK1-K709R) or the control vector pcDNA3 was cotransfected with pEGFP-N1 into caspase-8−/− J3.2 cells. Cells were treated with rhTNFα for 16 h, and live green fluorescent protein-positive cells were counted by fluorescence-activated cell sorter analysis to determine specific cell loss of transfected cells. Results are representative of three independent experiments.](image-url)
RIP recruitment to TNFR-1 under conditions of TNFR-2 pre-stimulation may account for the subsequent enhanced programmed necrosis, it is unlikely to explain the sensitizing effect of TNFR-2 in the absence of pre-stimulation.

Caspase-mediated Cleavage and Inactivation of RIP Dampen Programmed Necrosis

Although RIP was recruited to TNFR-1, apoptosis, but not programmed necrosis, was still the dominant response in wild-type 4E3 cells (Fig. 2A). Because genetic ablation of the apoptotic pathway in FADD/−/− and caspase-8/−/− cells or inhibition of caspases by tetrapeptide inhibitors causes increased programmed necrosis (24), we reasoned that caspase-8 activation during apoptosis may inactivate the necrotic pathway by proteolytic cleavage of RIP. Indeed, RIP was cleaved in TNF- or anti-Fas antibody-stimulated wild-type 4E3 cells, but not in either caspase-8/−/− or FADD/−/− cells (Fig. 4A) (data not shown). RIP cleavage was potently inhibited by Z-VAD-fmk. To investigate whether RIP cleavage by caspases abrogates its ability to induce programmed necrosis, we reconstituted the TNFR-2-positive RIP/−/− cells with RIP mutants corresponding to the N- and C-terminal cleavage products of RIP (RIPn and RIPc). We found that wild-type RIP

**Fig. 3.** Pre-stimulation of TNFR-2 enhances cell death and recruitment of RIP to the TNFR-1 signaling complex. A, 4E3 (wild-type (WT)), I42 (FADD/−/−), and J3.2 (caspase-8/−/− (Casp-8/−/−)) cells were pre-stimulated with an agonistic anti-TNFR-2 antibody or left untreated for 5 h, followed by stimulation with TNF for 4 h. Cell death was determined by a combination of annexin V and propidium iodide staining. B, normal Jurkat (TNFR-2-negative) and 4E3 (TNFR-2-positive) cells were pretreated with an agonistic anti-TNFR-2 antibody for 6 h to induce TRAF2 degradation prior to stimulation with rhTNFα for 5 min (7). Lysates were precleared with three rounds of washing with protein G-agarose beads prior to immunoprecipitation (IP) with anti-TNFR-1 antibody. Western blotting was performed with antibodies to TRADD and RIP as indicated. Reprobing the blots revealed a loss of TRAF2 in the cell lysates and no contaminating TNFR-2 in the immune complexes. Similar results were obtained when a TNFR-2-specific mutein was used to pre-stimulate the cells (data not shown). C, I42 (FADD/−/−) and 4E3 (wild-type) cells were stimulated with rhTNFα for the indicated times. Immunoprecipitation was performed with TNFR-1-specific antibody, and the same membranes were probed on Western blots using antibodies to RIP, TRADD, and TRAF2 as indicated. The whole cell extracts (WCE) showed that equivalent amounts of each protein were present in the cell lysates. The higher molecular weight species (*) associated with RIP in the immunoprecipitation represent ubiquitination of RIP (34).

**Fig. 4.** RIP is cleaved and inactivated in the presence of apoptotic activation of caspases. A, 4E3 (wild-type (WT)) and J3.2 (caspase-8/−/− (Casp-8/−/−)) cells were treated with rhTNFα, Apo-1 (anti-Fas antibody), and Z-VAD-fmk (zVAD) for 6 h before whole cell lysates were harvested. Western blot analysis using a C terminus-specific RIP antibody showed that full-length RIP was cleaved into a smaller fragment (RIPc) in 4E3 cells in response to TNF and anti-Fas antibody stimulation. B and C, R1.1 (RIP/−/−) cells were transfected with the indicated plasmids and stimulated with TNF as indicated. Cell loss was determined as described in the legend to Fig. 1. Results are representative of three experiments.

RIP recruitment to TNFR-1 under conditions of TNFR-2 pre-stimulation may account for the subsequent enhanced programmed necrosis, it is unlikely to explain the sensitizing effect of TNFR-2 in the absence of pre-stimulation.

Caspase-mediated Cleavage and Inactivation of RIP Dampen Programmed Necrosis—Although RIP was recruited to TNFR-1, apoptosis, but not programmed necrosis, was still the dominant response in wild-type 4E3 cells (Fig. 2A). Because genetic ablation of the apoptotic pathway in FADD/−/− and caspase-8/−/− cells or inhibition of caspases by tetrapeptide inhibitors causes increased programmed necrosis (24), we reasoned that caspase-8 activation during apoptosis may inactivate the necrotic pathway by proteolytic cleavage of RIP. Indeed, RIP was cleaved in TNF- or anti-Fas antibody-stimulated wild-type 4E3 cells, but not in either caspase-8/−/− or FADD/−/− cells (Fig. 4A) (data not shown). RIP cleavage was potently inhibited by Z-VAD-fmk. To investigate whether RIP cleavage by caspases abrogates its ability to induce programmed necrosis, we reconstituted the TNFR-2-positive RIP/−/− cells with RIP mutants corresponding to the N- and C-terminal cleavage products of RIP (RIPn and RIPc). We found that wild-type RIP
and the non-cleavable mutant RIP-D324A potently restored TNF killing (Fig. 4, B and C). Collectively, these data show that RIP is cleaved and inactivated by caspase-8 during apoptosis. Thus, in addition to TNFR-2 signaling, caspase-8 inhibition is also required to switch on the programmed necrosis machinery.

Programmed Necrosis Is Required for TNF-mediated Killing of VV-infected Cells in Vitro—Inhibition of apoptosis by certain viral genes is believed to be important for the successful propagation of the virus within the host by delaying the demise of the infected cell. However, the programmed necrosis that we observed could eliminate infected cells when apoptosis is suppressed. To test this hypothesis, we infected various Jurkat cells with recombinant VV. VV encodes a gene called SPI-2, which is homologous to the cytokine response modifier A (crmA) gene and is a potent inhibitor of caspase-8- and TNF- or Fas-induced apoptosis (12, 25). However, J3.2 (caspase-8−/−) cells remained responsive to TNF-induced death upon VV infection, albeit at a lower level than uninfected controls (Fig. 5B). Deletion of SPI-2 from VV only modestly increased the cell death induced by TNF. Strikingly, substituting SPI-2 with the apoptosis inhibitor MC159 resulted in a complete abrogation of TNF-induced cell death in the infected cells (Fig. 5A) (see below). Similar results were obtained with wild-type and FADD−/− Jurkat cells (data not shown). The majority of the dying cells were not positive for the apoptosis marker TUNEL (data not shown), implying that TNF-induced death in VV-infected Jurkat cells was not apoptotic. In contrast to infection in wild-type, caspase-8−/−, or FADD−/− cells, RIP−/− cells infected with VV were resistant to TNF-induced death (Fig. 5B). Deletion of SPI-2 had no significant effect on TNF-induced death (Fig. 5B). Thus, we conclude that RIP-mediated pro-
grammed necrosis is crucial for TNF-induced cytotoxicity in VV-infected cells.

Because TNF-induced programmed necrosis in cultured cells requires TNFR-2, we tested the potential role of TNFR-2 in controlling VV infection in vivo. TNFR-2−/− mice and control C57BL/6 mice were infected with VV, and viral titers in different organs were determined 4 days post-infection. Higher viral titers were recovered from the fat pads (3-fold), livers (100-fold) and especially spleens (1000-fold) of TNFR-2−/− mice compared with wild-type mice (Fig. 5C). Examination of the infected tissues revealed a large number of inflammatory foci in the livers of the infected wild-type mice, but not in those of the uninfected control mice or the infected TNFR-2−/− mice (Fig. 6, A–C). In addition, there was a general dissolution of follicle structures in the spleens of the infected TNFR-2−/− mice (Fig. 6, D–F). Although other explanations are possible, these data are nevertheless consistent with the hypothesis that TNFR-2 facilitates programmed necrosis of the infected tissues and triggers an inflammatory reaction that is crucial for the subsequent initiation of adaptive immunity against VV infection.

Viral Inhibitors of Programmed Necrosis—Because there is a strong selective advantage to blocking host cell death, we hypothesized that certain viral gene products may inhibit programmed necrosis. Indeed, transient expression of MC159 led to strong inhibition of TNF-induced death in caspase-8−/− J3.2 cells (Fig. 7A), consistent with its inhibitory effect in VV infection (Fig. 5A). MC159 is an anti-apoptotic gene from the poxvirus Molluscum contagiosum that shares sequence homology with the DEDs of caspase-8 and caspase-10, but has no enzyme domain (12). We found that other cellular and viral DED-containing inhibitors, including the equine herpesvirus-2 E8, the Kaposi’s sarcoma-associated herpesvirus (human herpesvirus-8) K13, and cellular FLIP, also protected cells from programmed necrosis, although the protection by K13 and cellular FLIP was weaker (Fig. 7A). By contrast, several known apoptosis inhibitors, including p35 and XIAP, failed to confer protection, whereas Bcl-xL had a moderate suppressive effect on TNF-induced programmed necrosis (Fig. 7B). Collectively, these data show that the ability to suppress programmed necrosis is not common for all apoptosis inhibitors, but is re-
In this study, we have shown that TNFR-2 signaling can facilitate apoptosis and programmed necrosis through TNFR-1. Similar to a previous report describing a role for RIP in Fas-induced programmed necrosis (11), we found that RIP is essential for TNFR-2-facilitated programmed necrosis. Interestingly, we found that pre-stimulation of TNFR-2 enhances subsequent TNF-dependent RIP recruitment to TNFR-1. Because reducing cellular RIP levels using geldanamycin potently suppressed programmed necrosis, these data suggest that enhancing RIP recruitment to TNFR-1 under conditions of TNFR-2 pre-stimulation may underlie the basis of increased cell death caused by TNFR-2 pre-stimulation. However, enhanced RIP recruitment was not observed without TNFR-2 pre-stimulation. Therefore, other mechanisms are likely involved in the sensitization of cell death without TNFR-2 pre-stimulation. One possibility is that TNFR-2-induced degradation of TRAF2 may relieve the steric hindrance that normally prevents the binding of the RIP kinase substrate to the TNFR-1 complex. Also, it is noteworthy that a recent report has implicated the critical involvement of a “Complex II” that does not contain TNFR-1 in mediating TNFR-1-induced apoptosis (27). It is possible that a similar Complex II is involved in mediating TNFR-2-facilitated programmed necrosis.

Our results suggest that TNFR-2 does not directly engage the cell death machinery, but rather enhances programmed necrosis indirectly. This is in contrast to the results of Cusson et al. (28), who showed that enhanced thymocyte apoptosis in RIP−/− mice can be rescued by TNFR-2 (but not TNFR-1) deficiency. It is possible that TNFR-2 could directly initiate thymocyte cell death when NF-κB activation is compromised in the absence of RIP (19, 28). Alternatively, TNFR-2 could indirectly enhance cell death in thymocytes via other death receptors in a manner similar to its effect on TNFR-1 in Jurkat cells. To this end, it is noteworthy that TNFR-2 has been shown to enhance cell death induced by Fas in immune-privileged sites such as the eye (29). CD30, another TRAF2-binding TNFR-like receptor that can induce TRAF2 degradation, has also been demonstrated to enhance TNFR-1-induced apoptosis (30). Besides TNFR-2 signaling, efficient programmed necrosis also requires inactivation of caspases because RIP is normally cleaved and inactivated by caspase-8 during apoptosis induction. Our results thus provide a molecular explanation of the observation that cells often undergo programmed necrosis when apoptosis is inhibited by tetrapeptide inhibitors of caspases or dominant-negative FADD (reviewed in Ref. 10).

Using VV infection as a model, we have shown that RIP-dependent programmed necrosis is essential for the in vitro killing of infected Jurkat cells and that TNFR-2 is required to control viral replication in vivo. TNFR-2−/− mice exhibited a dramatic reduction in the number of inflammatory foci in the liver. Because programmed necrotic cell death is highly pro-inflammatory and because an inflammatory reaction is critical for optimal priming of dendritic cells, our results suggest that programmed necrosis induced by TNF and other death cytokines may bolster the adaptive immune response against viral infections by stimulating dendritic cell maturation and by enhancing the uptake and presentation of viral antigens (31). Thus, TNFR-2-facilitated programmed necrosis may be a crucial antiviral response, especially for viruses that can block apoptosis. Whether RIP and programmed necrosis are indeed involved in controlling VV and potentially other viral infections through such a mechanism will await further in vivo experiments.

TNF has long been known as a necrosis-inducing agent. Recently, other death cytokines, including FasL and TRAIL, have been shown to be able to induce necrotic death under certain conditions. Strikingly, the protein kinase RIP is involved in the induction of programmed necrosis by all of these death cytokines. Thus, programmed necrosis induced by different cytokines appears to employ the same molecular machinery as seen in apoptosis. This conservation suggests that the programmed necrosis pathway regulates important aspects of mammalian physiology. The fact that viral inhibitors such as MC159, E8, and K13 can potently inhibit programmed necrosis argues that programmed necrosis may be an important antiviral mechanism. Interestingly, MC159 did not affect the recruitment of RIP to the TNFR-1 signaling complex. This suggests that other yet to be identified components of the programmed necrosis pathway may be the targets of inhibition. Finally, recent studies on the RIP homolog RIP2 showed that it plays a role in the immune response to *Listeria* infection. Interestingly, RIP2 deficiency dampens the inflammatory response due to defective NF-κB activation through the Toll-like receptors (32, 33). Hence, the RIP family of protein kinases may have a general role in regulating innate immune responses against bacterial and viral infections.

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