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Herpes simplex virus 1 ICP6 impedes TNF receptor 1–induced necroosome assembly during compartmentalization to detergent-resistant membrane vesicles

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Receptor-interacting protein (RIP) kinase 3 (RIPK3)–dependent necroptosis directs inflammation and tissue injury, as well as anti-viral host defense. In human cells, herpes simplex virus 1 (HSV1) UL39-encoded ICP6 blocks RIP homotypic interacting motif (RHIM) signal transduction, preventing this leakage form of cell death and sustaining viral infection. TNF receptor 1 (TNFR1)–induced necroptosis is known to require the formation of a RIPK1–RIPK3–mixed lineage kinase domain–like pseudokinase (MLKL) signaling complex (necrosome) that we find compartmentalizes exclusively to caveolin–1–associated detergent-resistant membrane (DRM) vesicles in HT-29 cells. Translocation proceeds in the presence of RIPK3 kinase inhibitor GSK’840 or MLKL inhibitor necrosulfonamide but requires the kinase activity, as well as RHIM signaling of RIPK1. ICP6 impedes the translocation of RIPK1, RIPK3, and MLKL to caveolin–1–containing DRM vesicles without fully blocking the activation of RIPK3 or phosphorylation of MLKL. Consistent with the important contribution of RIPK1 RHIM-dependent recruitment of RIPK3, overexpression of RHIM-deficient RIPK3 results in phosphorylation of MLKL, but this does not lead to either translocation or necroptosis. Combined, these data reveal a critical role of RHIM signaling in the recruitment of the MLKL-containing necrosome to membrane vesicle–associated sites of aggregation. A similar mechanism is predicted for other RHIM-containing signaling adaptors, Z-nucleic acid–binding protein 1 (ZBP1) (also called DAI and DLM1), and TIR domain–containing adapter–inducing interferon-β (TRIF).

Signal transduction triggered by the inflammatory cytokine TNF contributes to antiviral host defense (1, 2), as well as to cell death and proliferation, induction of inflammation, and inflammatory disease (3). TNF receptor 1 (TNFR1), the principal death receptor recognizing secreted TNF, regulates alternate responses, triggered by spatially distinct compartments, controlled by caspase 8 (Casp8) and sphingomyelinase activities (4–6). During NF-κB–dependent survival signaling, TNFR1 translocates to detergent-resistant membrane (DRM) fractions (7). It appears that NF-κB activation depends on plasma membrane–associated TNFR1 signaling machinery (complex I), whereas receptor internalization forms a clathrin–associated signaling platform (receptosome) leading to complex II and cell death independent of membrane compartments (8, 9). In some experimental models, disruption of receptor internalization inhibits apoptosis (6, 10). Following activation, the TNFR1 death domain recruits receptor interacting protein kinase 1 (RIPK1), an adaptor whose activity is regulated by polyubiquitation to support NF-κB–responsive dominant survival and alternate death/inflammatory programs (11, 12). These outcomes are controlled by soluble intracellular complex II, also known as a ripoptosome, which includes RIPK1, FADD, and Casp8, and triggers extrinsic apoptosis (4). Casp8 protease activity prevents the RIPK1–containing complex from recruiting RIPK3 to execute necroptosis. When Casp8 activity is compromised, RIPK1 recruits RIPK3 via RIP homotypic interaction motif (RHIM) to form an insoluble necrosome (13–15). Based largely on cell-free studies, the necrosome proceeds via amyloid–like aggregates to control MLKL phosphorylation and oligomerization that precede plasma membrane disruption (16–18). MLKL requires a highly phosphorylated type of inositol phosphate to execute necroptosis (19). The necrosome is regulated both positively and negatively by RIPK1. Although this protein kinase is able to trigger necroptosis, homeostatic RIPK1 scaffold function inhibits necroptosis, as well as apoptosis (20–22). Thus, RHIM interactions suppress cell death during development but promote necroptosis when triggered by TNFR1 signaling (23). RIPK1 similarly dictates outcomes downstream of receptors involved in pathogen recognition (24), T-cell activation (25), and interferon signaling (26, 27), as well as during inflammatory tissue injury (17). A comparable RIPK3 necro
some may also be nucleated by the pathogen sensor NLRx1-binding protein 1 (ZBP1) (also called DAI and DLM1) during infection with herpes simplex virus 1 (HSV1), murine cytomegalovirus, vaccinia virus, and influenza virus (26, 28–33), or by Toll-like receptors 3 and 4 via TIR domain-containing adapter--inducing interferon-β (24, 34). RIPK3 itself has the potential to nucleate a ripoptosome to drive apoptosis as well as necroptosis (35). Thus, RHIM signaling has the potential to dictate complex alternate death outcomes that likely evolved in response to pathogen cell death suppressors (29). Because of cell lysis as well as the nondeath consequences of RHIM-dependent signaling pathways, necroptosis is likely to enhance immunogenicity (28, 36), making it an efficient strategy for pathogen elimination.

TNFR1-dependent apoptosis and necroptosis contribute to host defense against viruses in an evolutionary arms race against virus-encoded cell death suppressors (2, 37). Herpesviruses such as rodent betaherpesviruses and primate alphaherpesviruses encode inhibitors of RHIM signaling that block necroptosis (26, 31, 37–42). HSV-1 ICP6, the large subunit of the ribonucleotide reductase (R1), carries out two additional functions as a suppressor of both Casp8-mediated apoptosis and RIPK3-mediated necroptosis (29, 37, 39, 43). In contrast to the RHIM-signaling suppression observed in human cells, ICP6 drives activation in mouse cells (40, 41), suggesting that species differences in way this viral RHIM adaptor is recognized (37).

Here, we show that TNFR1-induced necroosome associates with intracellular vesicles defined by the caveola scaffold protein Cav1. ICP6 inhibits the translocation of this necroosome to DRM fractions in human cells and, as a result, prevents the final assembly of aggregated death complexes. This process depends on RHIM interactions between RIPK1 and RIPK3. The translocation of RIPK3 to DRM fractions is independent of its activity or the consequent activity of the executor of necroptosis, MLKL, but requires RIPK1 kinase activity. Moreover, death machinery components interact exclusively in DRM fractions. By dissecting RIPK3-RHIM regulation of phospho-(p)MLKL oligomer formation and translocation to detergent-resistant fractions, our work sheds light on the steps involved in the execution of necroptosis that are the target of RHIM signaling suppression. These data are relevant to other herpesviruses, as well as to broader RHIM signaling pathways.

Results

The TNFR1-induced necroosome associates with Cav1-containing intracellular vesicles

Many receptors recruit downstream signaling machinery components into specialized membrane sites, exhibiting reduced density caused by the association with lipids. These changes may be visualized by preparing DRMs using flotation–fractionation (44). To assess differences in the way TNFR1-induced death complexes associate with membranes during necroptosis and apoptosis, the human colon carcinoma cell line HT-29 was used to investigate translocation of the death machinery. HT-29 cells showed the expected (45) susceptibility to TNF + SMAC mimetic (T/S)–induced apoptosis, with slow secondary necrosis resulting in membrane permeability (Fig. 1A). Rapid necroptosis followed treatment with SMAC mimetic + z-VAD-fmk, without (S/V) or with TNF (T/S/V), where >50% of cells became permeable within 6 h post treatment (hpt) with T/S/V. We examined the recruitment of the death machinery to DRM fractions under these conditions. Both Cav1 and β-actin showed the expected association with DRM fractions (Fig. 1B), with Cav1 predominant under all conditions, whereas β-actin showed a more variable distribution dependent on treatment. Regardless of whether we used apoptotic (T/S) or necroptotic (T/S/V) conditions, treated DRM fractions took on a more uniform density, with Cav1 predominating in fractions 2 and 3. There was modest recruitment of P42-Casp8 (P42-C8) and RIPK1 following T/S treatment but robust recruitment of MLKL, pMLKL, P42-C8, RIPK1, RIPK3, and β-actin to Cav1-containing membrane fractions following T/S/V treatment, indicating that apoptotic complexes form independently of DRM, whereas necroptotic complexes associate with DRM. This is consistent with the recruitment of the necroptotic machinery (RIPK1, RIPK3, and MLKL) to detergent-insoluble fractions previously identified in high-speed centrifugation pellets (13, 14, 46). These observations also align with previous reports (7, 8, 47) showing complex II (or ripoptosome) is soluble, whereas the necroosome appears insoluble (13, 14, 46). Furthermore, these results emphasize the different biophysical properties between the ripoptosome and the necroosome, as determined by DRM flotation–fractionation assay.
To investigate the timing and events leading to necrosome translocation to membrane fractions, we employed GSK/H11032 (G963) (48), GSK/H11032 (G840) (35), or necrosulfonamide (NSA) (13) to assess the respective role of RIPK1, RIPK3, or MLKL (Fig. 2A). As expected, T/S/V drove MLKL and RIPK3 phosphorylation (pMLKL and pRIPK3, respectively) within 6 hpt in both the detergent-soluble and -insoluble (pellet) fractions. Slower-migrating (modified) forms of RIPK3 and RIPK1 were present in the insoluble fractions (Fig. 2B). RIPK1 inhibitor G963 effectively blocked the appearance of both pRIPK3 and pMLKL, whereas G840 reduced but did not eliminate pRIPK3 and pMLKL in the soluble fraction. This RIPK3 kinase inhibitor effectively blocked the translocation of both proteins to the pellet. As expected (13), NSA reduced pMLKL translocation to the pellet without altering pRIPK3 or pMLKL levels in the soluble fraction or the translocation of pRIPK3 to the pellet. None of these inhibitors impacted levels or modification of RIPK1, although all blocked the induction of necroptosis (Fig. 2C). These results align with prior observations showing the recruitment of necrosome components (RIPK1, RIPK3, and MLKL) to detergent-insoluble (pellet) fractions.

Figure 2. Time course of TNFR1-induced necroptosis. A, schematic depiction of necroptosis inhibition by RIPK1 inhibitor GSK’963 (G963), RIPK3 inhibitor GSK’840 (G840), and MLKL inhibitor NSA. B, IB of pMLKL, pRIPK3, and total RIPK1, in 1% Triton X-100–soluble (Sol.) and insoluble (Pellet) HT-29 cell fractions (Frac.) following treatment with T/S/V (6 h) in combination with 1 μM DMSO, 1 μM G963, 1 μM G840, or 10 μM NSA. β-Actin is used as loading control (n = 3). C, representative results of three typical experiments of HT-29 cells viability 22 hpt with T/S/V, either alone or in combination with G963 (T/S/V/G963), G840 (T/S/V/G840), or NSA (T/S/V/NSA). **, p < 0.005.

To investigate the timing and events leading to necrosome translocation to membrane fractions, we employed GSK’963 (G963) (48), GSK’840 (G840) (35), or necrosulfonamide (NSA) (13) to assess the respective role of RIPK1, RIPK3, or MLKL (Fig. 2A). As expected, T/S/V drove MLKL and RIPK3 phosphorylation (pMLKL and pRIPK3, respectively) within 6 hpt in both the detergent-soluble and -insoluble (pellet) fractions. Slower-migrating (modified) forms of RIPK3 and RIPK1 were present in the insoluble fractions (Fig. 2B). RIPK1 inhibitor G963 effectively blocked the appearance of both pRIPK3 and pMLKL, whereas G840 reduced but did not eliminate pRIPK3 and pMLKL in the soluble fraction. This RIPK3 kinase inhibitor effectively blocked the translocation of both proteins to the pellet. As expected (13), NSA reduced pMLKL translocation to the pellet without altering pRIPK3 or pMLKL levels in the soluble fraction or the translocation of pRIPK3 to the pellet. None of these inhibitors impacted levels or modification of RIPK1, although all blocked the induction of necroptosis (Fig. 2C). These results align with prior observations showing the recruitment of necrosome components (RIPK1, RIPK3, and MLKL) to detergent-insoluble (pellet) fractions.
Fig. 2

1% Triton-insoluble fractions in concert with signaling (15), which has been associated with the formation of amyloid under cell-free conditions (15, 16). To examine the link between these insoluble fractions and DRMs, we evaluated the impact of inhibitors on phosphorylation and translocation of MLKL and RIPK3, as well as the accumulation of modified RIPK1 and P42-C8 together with TNFR1. Fractionation studies were carried out with the P65 subunit of NF-kB as a marker for soluble fractions and Cav1 as a marker of DRM (Fig. 2D). Consistent with cell death inhibition and sphingomyelinase activation (49), RIPK1 or RIPK3 blockade during T/S/V resulted in more dense DRMs, attenuated the translocation of RIPK3, reduced levels of pRIPK3 and pMLKL, and enhanced recruitment of P42-C8 to DRM fractions without reducing patterns or modification of DRM-associated RIPK1. Like G963, G840 enhanced the recruitment of P42-C8 to DRM fractions without reducing levels of modified RIPK1. Unlike G963, G840 reduced the pattern of DRM-associated pMLKL in DRM fraction 3 despite a modest impact on RIPK3 phosphorylation. The distribution of TNRF1 with the P65 subunit of NF-kB was not influenced by any of the inhibitors, indicating that inhibitor-associated changes did not result from global alteration of DRM properties. These results suggest that the translocation of RIPK3 to DRM is a prerequisite for its appearance in the insoluble pellet.

We next determined the time course of treatment necessary to observe these events. When cells were treated with T/S/V for as short as 1 h prior to removal and incubation without T/S/V, there was still a ~20% loss of viability 19 h later, consistent with rapid activation of the necroosome (Fig. 2E). The cells were then treated with T/S/V for 1–5 h before this treatment was stopped, and a necroptosis inhibitor was added. G963 was able to inhibit necroptosis even when added after 2 h of T/S/V, whereas G840 or NSA completely blocked death even when added after 3 h of T/S/V treatment (Fig. 2F), suggesting that TNFR1-induced necroptosis depends on earlier RIPK1 phosphorylation followed by RIPK3 and MLKL phosphorylation in a pattern consistent with the known roles of pronecrotic protein kinases (13–15).

To evaluate the physical interaction between RIPK1 and RIPK3 in DRM fractions, we employed FLAG-tagged RIPK3-expressing HT-29 (HT29–FLAG–wtR3) cells (39). Following treatment with T/S/V/NSA to enhance the interaction between RIPK1 and RIPK3 (13), co-immunoprecipitation of FLAG–RIPK3 revealed interactions with RIPK1, P42-C8, and modified TNFR1 (46), predominantly associated with DRM fraction 3, where Cav1 was highly represented (see run-through DRM fractions; Fig. 3A). We next followed MLKL oligomerization and DRM association in response to T/S/V, using parental HT-29 cells, which develop membrane permeability dependent on the formation of MLKL oligomers (14). We observed pMLKL oligomers translocated exclusively in DRM fractions (Fig. 3B). Confocal immunofluorescence micrographs of treated HT29–FLAG–wtR3 cells revealed a diffuse intracellular pattern of FLAG–RIPK3 (green), whereas Cav1 (red) showed modest surface localization in controls treated with NSA alone for 4 h (Fig. 3C). T/S/V/NSA treatment led to formation of intracellular RIPK3 puncta that co-localized with Cav1 (Fig. 3C, compare arrowheads and arrows). These RIPK3 puncta co-localized with BODIPY493/503 positive intracellular vesicles (Fig. 3D). Taken together, these results indicate that the TNFR1-induced necrosome forms in collaboration with intracellular membrane vesicles. Although our observations imply DRM, further studies will be needed to evaluate the contribution of amyloid-like intracellular aggregates (21) with membrane association and compartmentalization.

Fig. 3

HSV1 ICP6 RHIM-dependent block of necrosome translocation

HSV1 blocks apoptosis and necroptosis via cell death-suppression functions of ICP6 (39, 43). Therefore HSV1 lacking ICP6 (∆ICP6) permits the induction of apoptosis under apoptotic conditions and necroptosis under necroptotic conditions. Comparably, RHIM-mutated ICP6 (ICP6mutRHIM) retained Casp8 inhibitory activity but lost the capacity to block RIPK1 and RIPK3 RHIM signaling. Therefore, HT-29 cells infected with HSV1-ICP6mutRHIM under apoptotic conditions induce necroptosis (33, 39). When HSV1-infected HT-29 cells were treated with T/S/V and fractionated, Cav1 and β-actin showed reduced concentration and distribution compared with uninfected cells (compare Fig. 4A with Fig. 2D), indicating DRM disruption during infection (50). Despite RHIM inhibition, HSV1 infection failed to completely block phosphorylation of RIPK3. Infection did prevent the translocation of RIPK3, RIPK1, FADD, most of P42-C8, and MLKL to DRM fractions. As expected (39), HSV1 ICP6mutRHIM failed to block RHIM signaling, and as a result, RIPK1 recruited RIPK3 to initiate necroptosis. We observed marked translocation of modified RIPK1, pRIPK3, and pMLKL, in addition to P42-C8 and FADD, to Cav1-associated fractions in ICP6mutRHIM-infected cells (Fig. 4B). Given that HSV1 ∆ICP6 permitted apoptosis as well as necroptosis, T/S/V treatment of ∆ICP6-infected HT-29 cells resulted in the translocation of the death machinery proteins to Cav1-associated fractions, albeit with higher intensity than observed with ICP6mutRHIM virus-infected cells (Fig. 4C). A similar difference was observed when ICP6 and ICP6mutRHIM-transduced HT-29 cells were evaluated following treatment with T/S/V (data not shown). Notably, both WT and mutRHIM ICP6 remained in the soluble fractions (fractions 7–9). Taking all these results together, it appears that ICP6 RHIM-dependent interactions with RIPK1 and RIPK3 limit the translocation of the death machinery to DRM fractions. This inhibitory function was independent of the viral infection effect on the plasma membrane (50).

We next investigated the impact of viral infection on riposome formation under apoptotic conditions where the death machinery remains largely soluble (Fig. 1B). T/S treatment of HT-29 cells led to the translocation of P42-C8, modified RIPK1, FADD, P42-cFLIPs, cleaved (Cl) RIPK3 (Cl-RIPK3), and Cl-RIPK1 to the pellet, along with detectable levels of fully cleaved Casp8 subunit P18 (P18-C8) in the soluble fraction (Fig. 5A). Surprisingly, HSV1 infection did not inhibit the translocation P42-C8, FADD, P42-cFLIPs, Cl-RIPK1, or Cl-RIPK3 to the pellet 8 hpi, but blocked production of P18-C8. Notably, ICP6 distributed into both soluble and pellet fractions in a way that did not change with treatment conditions. Infection with WT HSV1 (F strain) resulted in pMLKL induction and modified
RIPK3 by 8 hpi that mostly remained in the soluble fraction. In contrast, T/S treatment slightly enhanced pMLKL induction and RIPK3 modification within the pellet fraction together with a dramatic increase in P42-C8 (Fig. 5C). Because ICP6 mutRHIM virus induces necroptosis under apoptotic conditions (39), infection with this mutant virus also resulted in pMLKL and pRIPK3 induction, where pMLKL was detected mainly in the soluble fraction. Consistent with induction of membrane permeability (Fig. 5B), T/S significantly enhanced pMLKL and pRIPK3 induction and translocation to the pellet fractions but reduced the levels of pellet-associated P42-C8. Both WT and ICP6 mutRHIM viruses showed the expected block of the fully active P18-C8 accumulation. Even though necroptosis was executed by mutant virus, as expected, these results indicate that pMLKL was induced with WT virus as well, suggesting that the viral protein ICP6 blocks the death machinery by driving an enzymatically active but incompetent complex. Inhibition takes place as the necrosome translocates to membranes.

To determine whether ICP6 bridges RIPK3 and Casp8 or associates with these proteins independently, we investigated necrosome fate under different infection conditions. We used HT-29 cells transduced with FLAG-tagged mutRHIM–RIPK3 (HT29–FLAG–mutR3, indicated as mutR3 in figures) (42), comparing to HT29–FLAG–wtR3 cells. These cells support necroptosis independent of ectopically expressed variants but enable evaluation of direct interactions between FLAG–RIPK3 and death machinery independently of cell death induction. A
similar ectopic expression strategy has been used to study RIPK1 and RIPK3 in NIH3T3 cells (51) which are resistant to RIPK1-triggered necroptosis but carry sufficient RIPK3 to support MCMV-induced necroptosis when transduced with ZBP1 (26). Treatment of HSV1 ΔICP6-infected HT29–FLAG–wtR3 cells with T/S/V/NSA resulted in P42-C8 in both soluble and pellet fractions as well as in the IP fractions (Fig. 6A). Treatment of HSV1 ΔICP6-infected HT29–FLAG–mutR3 cells with T/S/V/NSA resulted in P42-C8 in both soluble and pellet fractions as well as in the IP fractions (Fig. 6A). Treatment of HSV1 ΔICP6-infected HT29–FLAG–mutR3 cells with T/S/V/NSA resulted in P42-C8 in both soluble and pellet fractions as well as in the IP fractions (Fig. 6A). Notably, ICP6 interaction with RIPK3 in the co-IP fraction was independent of TNFR1 induction. Confocal imaging indicated that HSV1 infection disrupted RIPK3 co-localization with Cav1 in T/S/V/NSA HT29–FLAG–wtR3, whereas these RIPK3–Cav1 puncta were visible in these cells when infected with HSV1 ΔICP6 (Fig. 6B). Moreover, RIPK3 co-localization with Cav1 was lost in HT29–FLAG–mutR3 infected with HSV1 ΔICP6, indicating the role of RHIM signaling in necroptosome aggregation under viral infection conditions (Fig. 6B). Altogether, these results align with previous reports showing RIPK3 RHIM-dependent interaction with the ripoptosome. Furthermore, these results indicate that ICP6 disrupts the RHIM-dependent death machinery translocation to DRM fractions, reinforcing this as a key step in the execution of necroptosis.

RIPK3 RHIM signaling regulates MLKL oligomerization

Our results show that viral inhibition of RHIM signaling could potentially inhibit necroptosis despite modest induction of pMLKL (Fig. 5, B and C). We therefore tested the consequence of high level expression of the mutRHIM–RIPK3 mutant in human cells, where RHIM signaling through this necroptotic kinase is not possible. Compared with other RIPK3 mutants, mutRHIM–RIPK3 is overexpressed (42). Our further analysis of HT29–FLAG–mutR3 cells showed that nontreated cells express high levels of pMLKL that were not affected by G963 but were eliminated by G840 treatment (Fig. 7A). Intriguingly, this pMLKL was detectable only in the soluble fraction, distinct from the pattern of T/S/V-induced pMLKL detected in both fractions. This phenomenon was not unique to HT-29 cells, because we observed pMLKL induction in the RIPK3-deficient HaCaT cells that ectopically express mutRHIM–RIPK3 (Fig. 7E) but showed no membrane permeability (data not shown). Moreover, G963 abolished pellet-associated pMLKL, whereas G840 abolished both soluble and pellet pMLKL. Membrane permeability analysis showed that the integrity of HT29–FLAG–mutR3 cells was equivalent to HT29–FLAG–wtR3 cells and parental HT-29 cells, under control (Fig. 7B, top panel) or T/S/V treatment conditions (Fig. 7B, middle panel). G963 equally rescued all cells from T/S/V-induced membrane permeability (Fig. 7B, bottom panel). A previous report showed that TNFR1 activation leads to RIPK3 oligomerization and MLKL phosphorylation, which in turn localizes in the plasma membrane as oligomers. Such experiments make use of nonreducing gel conditions where the role of disulfide bonds in the regulation of these complexes is incompletely understood (21, 52). T/S/V-treated HT29–FLAG–wtR3 cells presented pMLKL detectable as monomers and oligomers that were sensitive to G963 inhibition (Fig. 7C). The high molecular weight FLAG–RIPK3 and pRIPK3 bands detectable under T/S/V conditions were reduced when G963 was added. Nontreated HT29–FLAG–mutR3 cells presented high levels of monomeric pMLKL without detectable oligomeric pMLKL. Similar to HT29–FLAG–wtR3 cells, T/S/V treatment induced pMLKL oligomerization in HT29–FLAG–mutR3 cells, and this was sensitive to G963 inhibition, ruling out the likelihood of technical errors. Notably, unlike wtR3 cells, anti-FLAG detection revealed mutRHIM–RIPK3 variants that migrated faster than WT RIPK3 (Fig. 7C, middle panel). In agreement with previous reports, confocal micrographs of HT29–FLAG–wtR3 cells showed that FLAG–RIPK3 formed bright intracellular...
lar puncta following T/S/V/NSA treatment (Fig. 7D, lower panels), structures that were not detected in HT29–FLAG–mutR3 (Fig. 7D, upper panels). These results suggest that RHIM interactions induce RIPK3 oligomers as a requisite step leading to pMLKL oligomerization and translocation to the plasma membrane.

**Discussion**

In this study, we demonstrate that TNFR1-induced necroosome formation in human cells involves an intimate and exclusive interaction with DRM fractions, resulting in a segregated intracellular complex that assembles in association with membranes or membrane vesicles. This compartmentalization relies on RHIM signaling adaptors, RIPK1 and RIPK3. ICP6-expressing HSV1 inhibits necroosome translocation to DRM fractions, whereas ICP6-mutRHIM and ΔICP6 HSV1 fail to block this step. Thus, the accumulation of pRIPK3 and pMLKL is insufficient to drive necroptosis when an ICP6 RHIM signaling block is in effect. A similar mechanism is likely to play out in ICP6mutRHIM in ZBP1-expressing HT-29 cells (33), as well as with the MCMV M45-encoded viral inhibitor of RIP activation (vIRA), an inhibitor that blocks all forms of RIPK3 activation and is competent in human cells (42), as well as mouse cells and mice (26).

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**Figure 5. HSV1 ICP6 inhibition of Casp8 full processing.** A, IB of RIPK3, RIPK1, Casp8 (P55-C8 and P42-C8), ICP6, β-actin, cFLIP, (intact and P42), and FADD in 1% Triton X-100-soluble (Sol) and pellet fractions of HT-29 cells uninfected (Uninf) or infected for 2 h with HSV1–KOS, followed by 8 h of T/S (n = 4). B, IB of pMLKL, RIPK3, RIPK1, Casp8 (P55-C8 and P42-C8), ICP6, and β-actin in 1% Triton X-100-soluble and pellet fractions of HT-29 cells infected either with HSV1 strain F or HSV1-ICP6 mutRHIM for 2 h, followed by T/S for the indicated times. Arrows indicate RIPK3 and modified RIPK3 (n = 5). C, time course depiction of the accumulation of SYTOX Green-permeable HT-29 cells infected either with HSV1 strain F or HSV1-ICP6 mutRHIM for 2 h, following treatment with DMSO (Con) or T/S (n = 4). Mw, molecular mass.
Alternate TNF-dependent NF-κB and Casp8 activation rely on the existence of at least two separate signaling platforms downstream of TNFR1 (4, 8). Our study sheds light on the importance of membrane vesicles in the assembly of the necroosome as an alternate to the soluble ripoptosome complex (4, 53). TNFR1 undergoes two steps of activation: a plasma membrane–associated DRM-dependent step that triggers the NF-κB survival pathway, as well as a DRM-independent step where Casp8 activation takes place (5). Here we observed that the death complex converts from soluble into DRM-associated when Casp8 activity is inhibited, an observation that aligns with the importance of Casp8 activity in regulating necroptosis (4).

The contribution of DRMs following TNFR1 internalization (8), as well as in the regulation of other signaling pathways, is well-documented (44), although TNFR1 apoptosis-inducing receptosomes associate with clathrin pits rather than caveola (8). The caveola-associated process we observed is reminiscent of NF-κB signaling platforms and implicates basal activity of
Figure 7. The role of RIPK3 RHIM domain in pMLKL trimer formation. A, IB of pMLKL and FLAG in soluble and pellet HT-29–3×FLAG–RIPK3 (wtR3) or HT-29–3×FLAGmutRHIMRIPK3 (mutR3) cell fractions treated with either DMSO or T/S/V (6 h) in combination with either G963 or G840 (n = 3). B, time course of HT-29, HT-29–3×FLAG-RIPK3, and HT-29–3×FLAGmutRHIMRIPK3 cell membrane permeability following DMSO (top panel), T/S/V (middle panel), or T/S/V/G963 (bottom panel) depicted as a percentage of T/S/V-treated control (n = 4). C, IB of pMLKL, FLAG, and pRIPK3 under nonreducing conditions, in HT-29–3×FLAG–RIPK3 or HT-29–3×FLAGmutRHIMRIPK3 treated as indicated. *, monomers; **, expected multimers; †, nonspecific. RIPK1 was used for loading control (n = 3). D, confocal microscopy determination of 3×FLAG–RIPK3, or 3×FLAGmutRHIMRIPK3 (green) localization in HT-29 cells following 4 h of treatment with either NSA alone or T/S/V/NSA. The nuclei are indicated by DAPI (blue). The results are from a typical experiment repeated three times. Scale bar, 10 μm. E, IB of pMLKL, FLAG, RIPK3, and RIPK1 in HaCaT, HaCaT–3×FLAG–RIPK3 (HaCaT-wtR3) HaCaT-empty vector (Mock), or HaCaT–3×FLAGmutRHIMRIPK3 (HaCaT-mutR3; n = 5). Mw, molecular mass; Sol., Triton X-100–soluble.
Casp8 in the inhibition of internalized DRM signaling platforms necessary for necroptosis (54).

An amyloid-like signaling platform has been implicated in RIPK1–RIPK3 RHIM signaling (16) upstream of MLKL activation. Given that RIPK1 initiates an interaction with DRM and recruits RIPK3 to these membrane sites also in advance of MLKL activation, RIPK1–RIPK3 amyloid should be evident at this stage of the process. In our studies, the association of these components with DRM appears as puncta in immunofluorescence rather than the elongated structures expected from what would occur with RHIM amyloids. Distinct from what would occur with RHIM amyloids, TNFα-mediated necroptosis promotes TNFR1 interaction directly with the ripoptosome components RIPK1, P42-Casp8, and RIPK3 in DRM vesicles as we observe from our immunoprecipitation and fluorescence imaging experiments. Our data support a vital DRM role in the regulation of TNFR1–necrosome complex but do not support the amyloid-like intermediates that have been demonstrated in cell-free conditions (16, 18). RIPK3 interacts with MLKL in a ratio of 1:1 (55), and under normal circumstances, TNFR1 binds to a trimeric soluble TNFα in a stochastic ratio of 1:1, leading to TNFR oligomerization (56). Therefore, we speculate that TNFR1-bound RIPK1 is responsible for RHIM-dependent recruitment and oligomerization of RIPK3, which in turn recruits, phosphorylates, and oligomerizes MLKL within this vesicle environment. This hypothesis is supported by recent reports showing MLKL association and regulation of endosomal receptor uptake and the interaction with flotillin-containing vesicles upon the induction of TNFα-mediated necroptosis (57, 58). Flotillins are markers of DRM fractions that have been used in combination with Cav1 (44). Therefore, the association between RIPK3 and Cav1, along with the reported association between pMLKL and Flotillins (57) together suggest that a TNFR1-bound necosome forms in DRM-internalized vesicles to phosphorylate and translocate MLKL to the plasma membrane via intracellular trafficking, leading to plasma membrane rupture. MLKL oligomerization and induction of membrane rupture relies on highly phosphorylated phosphoinositols (19). These lipids have been shown to play a pivotal role in the regulation of intracellular trafficking as secondary messengers downstream of membrane receptor (59), granting a possible role of phosphoinositols in necroptosome oligomerization and necroptosis induction. Interestingly, the ESCRT machinery was found to antagonize and delay necroptosis, a necessary step in the generation and release of signaling molecules from the dying cells (58). This indicates an important regulatory role of intracellular trafficking vesicles in the induction and regulation of necroptosis. Our data support a recent publication indicating that, unlike in mouse cells, in human cells pMLKL demands the direct binding to a necroosome complex rather than pRIPK3 to induce necroptosis (60). We observe an induction of pMLKL following viral infection or via mutRHIM–RIPK3 overexpression that did not lead to membrane rupture.

Notably, RIPK1 kinase activity controls both RIPK3 and MLKL translocation to DRM, as well as their translocation to pellet fractions. RIPK3 kinase activity controls the formation of the insoluble pellet but is dispensable for DRM association. This indicates that necroosome association with DRMs might be an intermediate stage prior to the necroosome oligomerization, a process controlled by RIPK3 kinase activity.

In addition to facilitating viral pathogenesis, viral inhibitors of necroptosis are useful tools in unraveling signaling mechanisms. HSV1 infection blocks TNF-induced necroptosis in human cells via ICP6 RHIM-signaling competition (29) to disrupt the formation of a functioning RIPK1–RIPK3 (39) or ZBP1–RIPK3 necrosome (33). As expected, HSV1 infection inhibits any association of the death machinery proteins to DRM fractions and, as a result, inhibits high molecular weight ripoptosome and necrosome formation. When we compare the effect of HSV1 infection on the translocation of the death machinery proteins to detergent-insoluble pellets under apoptotic conditions, we observe that the viral infection does not inhibit the translocation of RIPK1, P42-C8, and cleaved-RIPK3 to these fractions. Comparably, when necroptosis is triggered, virus-infected cells show reduction of Casp8, RIPK1, and RIPK3 recruitment to the insoluble fractions, suggesting that the virus employs distinct strategies to block the ripoptosome and the necrosome. This may be due to the nature of the ripoptosome and the necrosome as indicated by their different associations with DRM fractions. ICP6 has been shown to engage Casp8, as well as RIPK3, via its C and N termini, respectively, forming an inactive complex of RIPK3/ICP6/Casp8. Mutant ICP6 variants that fail to bind Casp8 do not inhibit necroptosis (39). Given the lack of such a complex during viral infection, we predict that ICP6 blocks death induction by binding each protein independently to inhibit two separate signaling platforms to secure cell viability and complete productive infection. The finding that ICP6 inhibition of Casp8 is separated from RIPK3 inhibition is not surprising. Although the ripoptosome is a membrane-independent mega complex (47), the necrosome is membrane-associated as well as compartmentalized. Further investigations will be necessary to determine why binding to Casp8 is necessary for ICP6 to block necroptosis. Notably, the viral infection appeared to compromise the integrity of the DRM fractions; however, the death machinery translocated to DRMs when we used mutant viruses that allow unleashing necroptosis, despite this disruption.

Previously, HSV1 inhibition of apoptosis was shown to result from ICP6 block of basal as well as autoactivated Casp8 activity (43, 61). Here we found an accumulation of cleaved RIPK3 in the pellet fractions after T/S treatment during viral infection (Fig. 4A). This suggests that the viral protein ICP6 forms a complex with Casp8 that benefits from some activity of this protease, sufficient to cleave RIPK3 and suppress necroptosis, but not activate apoptosis. Such an activity might serve the virus inhibiting other forms of necroptosis such as ZBP1/DAI–induced necroptosis (26, 33). Basal Casp8 activity is sufficient for receptosome internalization and association with clathrin pits (5), separate from caveola. This raises the possibility that the ICP6–Casp8 complex might inhibit necroptosis by modifying the cellular membranes with which the complexes associate.

Contrary to our expectations, HSV1 strain F infection induces pMLKL, as well as some pRIPK3, while also triggering translocation to insoluble fractions even though membranes remain intact. This aligns with our observations with the
ectopic expression of mutRHM–RIPK3. When we ectopically expressed mutRHM–RIPK3, we discovered unexpected non-necroptotic pMLKL induction, probably a result of the strong overexpression of mutRHM–RIPK3, contrasting previous reports suggesting that the minimal requirement for necroptosis induction is MLKL phosphorylation, which leads to oligomerization and translocation to the plasma membrane (15). Notably, mutRHM–RIPK3 expression in the RIPK3-insufficient cell line HaCaT also lead to pMLKL induction, a result that rules out a contribution of the endogenous RIPK3 in the induction of this nonnecroptotic pMLKL. One explanation for this difference is that the ectopic overexpression of phosphomimetic MLKL variant leads to unnatural oligomer formation. Another possibility is that the overexpression of mutRHM–RIPK3 leads to partial phosphorylation of MLKL on one of two crucial phosphorylation sites on the human MLKL (15). Either way, this result suggests a novel role for RIPK3 RHIM domain signaling in the execution of necroptosis. Recently it was shown that reconstituting MLKL-deficient U937 or HT-29 cells with the phosphomimetic T357E/S358E and T357E/S358D or with the phosphoablating T357A/S358A human MLKL fail to support necroptosis (60). These data align with our observations and suggest that unlike in mouse cells, necroptosis induction in human cells bears extra regulatory steps beyond MLKL phosphorylation. In our study we used an antibody directed to detect pSer-358 as an indicator for pMLKL induction. This phosphorylation site and Thr-357 have been described as the two critical phosphorylation events to induce necroptosis in human cells (15). However, data from mouse MLKL suggest positive and negative regulatory roles of other phosphorylation event on MLKL that could fine-tune the induction of necroptosis (62). For instance, S228A phosphomimetic mutated MLKL led to a significant cell death upon expression only in cells deficient in RIPK3, a result suggesting a negative regulatory role of RIPK3 on MLKL activation of necroptosis (62).

In conclusion, this work sheds new light on the mechanisms of necroptosis regulation downstream of death receptors and adds new insights on necroptosis regulation. Because of the unique nature of ripoptosome and necrosome, HSV1 utilizes separate distinct strategies to inhibit cell death pathways.

**Experimental procedures**

**Antibodies and reagents**

The following antibodies were from Cell Signaling Technology: anti-Casp8 (9746), anti-p65 NF-κB (6956), and anti-Caveolin1 (3267). The following antibodies were from Abcam: anti-pMLKL (187091), anti-RIPK3 (72106), anti-pRIPK3 (209384), and anti-FADD (108601). Protein A/G–conjugated beads were from Santa Cruz Biotechnology, anti-total MLKL (M6697) was from Sigma–Aldrich, and anti-RIPK1 (610459) was from BD Medical Technology. Anti-ICP6 was a generous gift from Sudan He (Jiangsu Institute of Hematology). z-VAD-fmk was from Enzo Life Sciences. RIPK1 inhibitor GSK’963, RIPK3 inhibitor GSK’840, and IAP antagonist SMAC007 were provided by GlaxoSmithKline (35). IAP antagonist BV6 was provided by Domagoj Vucic (Genentech), recombinant human TNF was from R&D or from PeproTech, necrosulfonamide was from CalBiochem, tosyl-1-lysyl-chloromethane hydrochloride was from Abcam, cycloheximide was from Sigma–Aldrich, and carfilzomib was from BioVision.

**Cell growth and treatments**

Cells and viruses, including human colon cancer cell line HT-29, and Vero cells were maintained at 37 °C in 5% CO₂ using Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Atlanta Biologicals), 4.5 g/ml glucose, 2 mM L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin (Invitrogen). The R1-null mutant HSV1 ΔICP6 and its parental strain KOS were kindly provided by Dr. Sandra Weller (University of Connecticut Health Center, Farmington, CT). HSV viral stocks were prepared by infecting HT-29 cells stably expressing ICP10 or ICP6 at a multiplicity of infection of 0.01 and were titered by plaque assay on monolayer cultures of Vero cells. For the induction of necroptosis, the cells were treated with T/BV6/V (30 ng/ml, 25 μM, and 0.1 μM BV6, respectively) or with T/SMA007/V (1 μM SMA007). The cells were either treated for 24 h, as indicated for viability assays, or for 6–8 h (HT-29), for IB assays. GSK’840, GSK’963, and NSA were dissolved in DMSO as supplier recommended (35) and were used as indicated in the text to inhibit necroptosis. DMSO was used as no treatment control as adequate. For viral infection experiments of HT-29 cells, all cells were infected with a multiplicity of infection of 5 and incubated for 2 h at 37 °C in 5% CO₂ for virus binding, and then the infection medium was replaced with standard growth medium supplied with treatments, as indicated.

**Plasmids and lentivirus stable transduction**

Human-3×FLAG–RIPK3 and human-3×FLAG–mutRHM–RIPK3 expression vector were previously described (42). Briefly, hRIPK3 ORF was inserted into pLV–EF1–MCS–IRE5–Puro lentiviral vector (Biosettia). Three-tandem FLAG epitope-tagged hRIP3 expression plasmid was constructed by inserting hRIPK3 ORF into p3FLAG–CMV10 vector (Sigma). Overlap extension PCR was employed to generate expression constructs of hRIP3 mutants, tetra-Ala RHIM domain (amino acids 459–462) substitution (mutRHIM). Transient transfections were performed with Lipofectamine LTX with Plus reagent (Invitrogen). Lentivirus stock was prepared from 293T cells that were transfected with plLV-hRIPIK3 or plLV-mutRHM–RIPK3 constructs along with psPAX2 and VSV-G–expressing plasmids. Low passage HT-29 cells were transduced with lentiviral vector and selected with 2 μg/ml puromycin (Invitrogen).

**Immunoprecipitation and immunoblot**

Whole-cell extracts were prepared using lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, including phosphatase and protease inhibitors (Sigma–Aldrich)), and clarified cell lysates were incubated overnight with anti-FLAG, mixed with protein A/G–agarose beads (Santa Cruz). Next day, the IP lysis mix was centrifuged, the bead fraction was collected for co-IP detection, and the supernatant was collected for run-through analysis. The co-IP beads were washed three times with cold IP wash buffer (50 mM Tris, 150 mM NaCl, 5 mM
EDTA) prior to immunoblot analysis. For immunoblots, the samples were prepared for denaturing SDS-PAGE by heating for 3 min at 98°C in Laemml buffer (Bio-Rad, 1610737) in the presence of freshly prepared 10 mM DTT. For nonreducing SDS-PAGE experiments, samples were prepared in the same fashion in the absence of DTT. Samples were resolved in 10% SDS-polyacrylamide gels; proteins were transferred to Immobilon PVDF membrane (Millipore) and developed using specified Abs. Alternatively, whole cell lysates were centrifuged at 15,000 rpm (20 min, 4°C) for the separation of 1% Triton X-100 soluble and insoluble (pellet) fractions (35).

Cell fractionation

DRM fractions were prepared as previously described (44) with the following modifications. The cells were grown in 182-cm² tissue culture flasks, up to 80% confluency, and then treated with T/S/V with or without the necroptosis inhibitors for 8 h, and then the cells were scraped and washed with cold PBS. For DRM–IP experiments, the cells were treated with T/S/V/NSA for 4 h. Then the cell pellets were lysed in a Dounce homogenizer using DRMs lysis buffer (0.1% Triton X-100, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 30 mM HEPES, pH 7.5, 1 mM Na₂VO₄, 50 μM phenylarsine oxide). Homogenates were clarified at 400 × g (3 min, 4°C). Supernatant was collected, and 400 μl of 60% Optiprep and 15% sucrose mix were added to a 200-μl sample of supernatant to a final concentration of 40% Optiprep and 10% sucrose, which was overlaid with 1-ml solutions containing 35, 30, 25, 20, and 10% sucrose, and were overlaid with 1-ml solutions containing 35, 30, 25, 20, and 10% sucrose, and were overlaid with 1-ml Optiprep and 10% sucrose solution. The sample was then centrifuged (6 h, 170,000 × g, 4°C). Nine 520-μl fractions were collected from the top of the gradient.

Cell viability

2 × 10⁴ cells were cultured in 96-well plates for cell viability and membrane permeability studies. The cells were incubated 24 h, or as indicated, and then viability was assessed using Cell Titer-Glo luminescent cell viability assay (Promega) (28). The values depict viability as a percentage of DMSO-treated cells. Alternatively, the cells were cultured with 62.5 nM SYTOX Green (Invitrogen), a live-cell impermeant nucleic acid fluorescent dye, and analyzed by an IncuCyte ZOOM live-cell imaging and analysis system (Essen Biosystems). Green cells per mm² were calculated from four images at indicated points, and the values depict mortality (membrane permeability) as a percentage of T/cycloheximide- or T/S/V-treated cells, as indicated.

Confocal microscopy

Confocal microscopy was performed using an Olympus IX 81 Fluoview 1000 microscope and a UPLSAPO 60× objective, and the images were processed and analyzed using FV-1000 software.

Statistical analyses

Statistical comparisons employed parametric evaluation using Student’s t test (GraphPad Prism software, or Microsoft Excel). All experiments were repeated at least three times with similar results, and data are represented as the means ± S.D.

References

1. Cho, Y. S., Challa, S., Moquin, D., Genga, R., Ray, T. D., Guildford, M., and Chan, F. K. (2009) Phosphorylation-driven assembly of the RIP1–RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 137, 1112–1123. CrossRef Medline
2. Mocarski, E. S., Upton, J. W., and Kaiser, W. J. (2011) Viral infection and the evolution of caspase 8-regulated apoptotic and necrotic death pathways. Nat. Rev. Immunol. 12, 79–88. CrossRef Medline
3. Kalliolias, G. D., and Ivashkiv, L. B. (2016) TNF biology, pathogenic mechanisms and emerging therapeutic strategies. Nat. Rev. Rheumatol. 12, 49–62. CrossRef Medline
4. Brenner, D., Blaser, H., and Mak, T. W. (2015) Regulation of tumour necrosis factor signalling: live or let die. Nat. Rev. Immunol. 15, 362–374. CrossRef Medline
5. Schütze, S., Tchikov, V., and Schneider-Brachert, W. (2008) Regulation of TNFR1 and CD95 signalling by receptor compartmentalization. Nat. Rev. Mol. Cell Biol. 9, 655–662. CrossRef Medline
6. Ali, M., Fritsch, J., Zidgon, H., Pemzer-Jung, Y., Schütze, S., and Futerman, A. H. (2013) Altering the sphingolipid acyl chain composition prevents LPS/GLN-mediated hepatic failure in mice by disrupting TNFR1 internalization. Cell Death Dis. 4, e929. CrossRef Medline
7. Legler, D. F., Micheau, O., Doucey, M. A., Tschopp, J., and Bron, C. (2003) Recruitment of TNF receptor 1 to lipid rafts is essential for TNFα-mediated NF-κB activation. Immunity 18, 655–664. CrossRef Medline
8. Schneider-Brachert, W., Tchikov, V., Neumeyer, J., Jakob, M., Winoto-Morbach, S., Held-Feindt, J., Heinrich, M., Merkel, O., Ehrenschwender, M., Adam, D., Mentlein, R., Kabilitz, D., and Schütze, S. (2004) Compartmentalization of TNF receptor 1 signaling: internalized TNF receptors as death signaling vesicles. Immunity 21, 415–428. CrossRef Medline
9. Willingham, M. C., and Pastan, I. (1990) The receptosome: an intracellular organelle of receptor mediated endocytosis in cultured fibroblasts. Cell 21, 67–77. CrossRef Medline
10. Schneider-Brachert, W., Tchikov, V., Merkel, O., Jakob, M., Hallas, C., Kruse, M. L., Groth, P., Lehn, A., Hildt, E., Held-Feindt, J., Dobner, T., Kabilitz, D., Krönke, M., and Schütze, S. (2006) Inhibition of TNF receptor 1 internalization by adenovirus 14.7K as a novel immune escape mechanism. J. Clin. Invest. 116, 2901–2913. CrossRef Medline
11. Wertz, I. E. (2014) TNFR1-activated NF-κB signal transduction: regulation by the ubiquitin/proteasome system. Curr. Opin. Chem. Biol. 23, 71–77. CrossRef Medline
12. Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R., and Verma, I. M. (1996) Suppression of TNF-α-induced apoptosis by NF-κB. Science 274, 787–789. CrossRef Medline
13. Wang, H., Sun, L., Su, L., Rizo, J., Liu, L., Wang, L. F., Wang, F. S., and Wang, X. (2014) Mixed lineage kinase domain-like protein MLKL causes necrotic membrane disruption upon phosphorylation by RIP1. Mol. Cell 54, 133–146. CrossRef Medline
14. Chen, X., Li, W., Ren, J., Huang, D., He, W. T., Song, Y., Yang, C., Li, W., Zheng, X., Chen, P., and Han, J. (2014) Translocation of mixed lineage kinase domain-like protein to plasma membrane leads to necrotic cell death. Cell Res. 24, 105–121. CrossRef Medline
28. Daley-Bauer, L. P., Roback, L., Crosby, L. N., McCormick, A. L., Feng, Y., Kaiser, W. J., Sridharan, H., Huang, C., Mandal, P., Upton, J. W., Gough, P. J., Bertin, J., Hartmann, B. M., Sealfon, S. C., et al. (2016) RIPK3 activates parallel pathways of MLKL-driven necroptosis and FADD-mediated apoptosis to protect against Influenza A Virus. Cell Host Microbe 20, 13–24 CrossRef Medline

31. Upton, J. W., Kaiser, W. J., and Mocarski, E. S. (2010) Virus infection of RIP3-dependent necrosis. Cell Host Microbe 7, 302–313 CrossRef Medline

32. Nogusa, S., Thapa, R. I., Dillon, C. P., Liedmann, S., Ogui, T. H., 3rd, Ingram, J. P., Rodriguez, D. A., Kosof, R., Sharma, S., Sturm, O., Verbiest, K., Gough, P. J., Bertin, J., Hartmann, B. M., Sealfon, S. C., et al. (2016) RIPK3 activates parallel pathways of MLKL-driven necroptosis and FADD-mediated apoptosis to protect against Influenza A Virus. Cell Host Microbe 20, 13–24 CrossRef Medline

33. Guo, H., Gilley, R. P., Fisher, A., Lane, R., Landsteiner, V. J., Ragan, K. B., Dovey, C. M., Carette, J. E., Upton, J. W., Mocarski, E. S., and Kaiser, W. J. (2018) Species-independent contribution of ZBP1/DAI/DLM-1-triggered necroptosis in host defense against HSV1. Cell Death Dis. 9, 816 CrossRef Medline

34. He, S., Liang, Y., Shao, F., and Wang, X. (2011) Toll-like receptors activate programmed necrosis in macrophages through a receptor-interacting kinase-3-mediated pathway. Proc. Natl. Acad. Sci. U.S.A. 108, 20054–20059 CrossRef Medline

35. Mandal, P., Berger, S. B., Pillay, S., Moriwaki, K., Huang, C., Guo, H., Lich, J. D., Finger, J., Kasparcova, V., Votta, B., Ouellette, M., King, B. W., Wisniski, D., Lakdawala, A. S., DeMartino, M. P., et al. (2014) RIP3 induces apoptosis independent of pronecrotic kinase activity. Mol. Cell 56, 481–495 CrossRef Medline

36. Yatim, N., Justfougues-Saklani, H., Orozco, S., Schulz, O., Barreire da Silva, R., Reis e Sousa, C., Green, D. R., Oberst, A., and Albert, M. L. (2015) RIPK1 and NF-κB signaling in dying cells determines cross-priming of CD8+ T cells. Science 350, 328–334 CrossRef Medline

37. Guo, H., Kaiser, W. J., and Mocarski, E. S. (2015) Manipulation of apoptosis and necroptosis signaling by herpesviruses. Microb. Immunol. 204, 439–448 CrossRef Medline

38. Upton, J. W., Kaiser, W. J., and Mocarski, E. S. (2008) Cytomegalovirus M45 cell death suppression requires receptor-interacting protein (RIP) homotypic interaction motif (RHIM)-dependent interaction with RIP1. J. Biol. Chem. 283, 16966–16970 CrossRef Medline

39. Guo, H., Omoto, S., Harris, P. A., Finger, J. N., Bertin, J., Gough, P. J., Kaiser, W. J., and Mocarski, E. S. (2015) Herpes simplex virus suppresses necroptosis in human cells. Cell Host Microbe 17, 243–251 CrossRef Medline

40. Huang, Z., Wu, S. Q., Liang, Y., Zhou, X., Chen, W., Li, L., Wu, J., Zhuang, Q., Chen, C., Li, Zhong, C. Q., Xia, W., Zhou, R., Zheng, C., and Han, J. (2015) RIP1/RIP3 binding to HSV-1 ICP6 initiates necroptosis to restrict virus propagation in mice. Cell Host Microbe 17, 229–242 CrossRef Medline

41. Yu, X., Li, Y., Chen, Q., Su, C., Zhang, Z., Yang, C., Hu, Z., Hou, J., Zhou, J., Gong, L., Jiang, X., Zhong, C., and He, S. (2016) Herpes simplex virus 1 (HSV-1) and HSV-2 mediate species-specific modulations of programmed necrosis through the viral ribonucleotide reductase large subunit R1. J. Virol. 90, 1088–1095 CrossRef Medline

42. Omoto, S., Guo, H., Talekar, G. R., Roback, L., Kaiser, W. J., and Mocarski, E. S. (2015) Suppression of RIP3-dependent necroptosis by human cytomegalovirus. J. Biol. Chem. 290, 11635–11648 CrossRef Medline

43. Dufour, F., Sasseville, A. M., Chabaud, S., Massie, B., Siegel, R. M., and Langelier, Y. (2011) The ribonucleotide reductase R1 subunits of herpes simplex virus types 1 and 2 protect cells against TNF-α- and FasL-induced apoptosis by interacting with caspase-8. Apoptosis 16, 256–271 CrossRef Medline

44. Park, J. W., Park, J. W., Kupperman, Y., Boura-Halfon, S., Pewzner-Jung, Y., and Futerman, A. H. (2013) Ablation of very long acyl chain sphingolipids causes hepatic insulin resistance in mice due to altered detergent-resistant membranes. Hepatology 57, 525–532 CrossRef Medline

45. He, S., Wang, L., Miao, L., Wang, T., Du, F., Zhao, L., and Wang, X. (2009) Receptor interacting protein kinase-3 determines cellular necroptotic response to TNF-α. Cell 137, 1100–1111 CrossRef Medline

46. Ali, M., and Mocarski, E. S. (2018) Proteasome inhibition blocks necroptosis by attenuating death complex aggregation. Cell Death Dis. 9, 346 CrossRef Medline

47. Fedoktistova, M., Gesericke, P., Kellert, B., Dimitrova, D. P., Langlais, C., Hupe, M., Cain, K., MacFarlane, M., Häcker, G., and Leverkus, M. (2011)
cIAPs block ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. Mol. Cell 43, 449–463 CrossRef Medline

48. Berger, S. B., Harris, P., Nagilla, R., Kasparcova, V., Hoffman, S., Swift, B., Dare, L., Schaeffer, M., Capriotti, C., Ouellette, M., King, B. W., Wisnoski, D., Cox, J., Reilly, M., Marquis, R. W., et al. (2015) Characterization of GSK’963: a structurally distinct, potent and selective inhibitor of RIP1 kinase. Cell Death Discov 1, 15009 CrossRef Medline

49. Philipp, S., Puchert, M., Adam-Klages, S., Tchikov, V., Winoto-Morbach, S., Mathieu, S., Deerberg, A., Koller, L., Marchesini, N., Kabelitz, D., Hanun, Y. A., Schütze, S., and Adam, D. (2010) The polycomb group protein EED couples TNF receptor 1 to neutral sphingomyelinase. Proc. Natl. Acad. Sci. U.S.A. 107, 1112–1117 CrossRef Medline

50. Vitiello, G., Falanga, A., Petruk, A. A., Merlino, A., Fragneto, G., Paduano, L., Galdiero, S., and D’Errico, G. (2015) Fusion of raft-like lipid bilayers operated by a membranotropic domain of the HSV-type I glycoprotein gH occurs through a cholesterol-dependent mechanism. Soft Matter 11, 3003–3016 CrossRef Medline

51. Orozco, S., Yatim, N., Werner, M. R., Tran, H., Gunja, S. Y., Albert, M. L., Green, D. R., and Oberst, A. (2014) RIPK1 both positively and negatively regulates RIPK3 oligomerization and necroptosis. Cell Death Differ. 21, 1511–1521 CrossRef Medline

52. Huang, D., Zheng, X., Wang, Z. A., Chen, X., He, W. T., Zhang, Y., Xu, J. G., Zhao, H., Shi, W., Wang, X., Zhu, Y., and Han, J. (2017) The MLKL channel in necroptosis is an octamer formed by tetramers in a dyadic process. Mol. Cell. Biol. 37

53. Tenev, T., Bianchi, K., Darding, M., Broemer, M., Langlais, C., Wallberg, F., Zachariou, A., Lopez, J., MacFarlane, M., Cain, K., and Meier, P. (2011) The ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. Mol. Cell 43, 432–448 CrossRef Medline

54. Kaiser, W. J., Upton, J. W., Long, A. B., Livingston-Rosanoff, D., Daley-Bauer, L. P., Hakem, R., Caspary, T., and Mocarski, E. S. (2011) RIP3 mediates the embryonic lethality of caspase-8–deficient mice. Nature 471, 368–372 CrossRef Medline

55. Xie, T., Peng, W., Yan, C., Wu, J., Gong, X., and Shi, Y. (2013) Structural insights into RIP3-mediated necroptotic signaling. Cell Rep. 5, 70–78 CrossRef Medline

56. Eck, M. J., and Sprang, S. R. (1989) The structure of tumor necrosis factor-α at 2.6 Å resolution: implications for receptor binding. J. Biol. Chem. 264, 17595–17605 Medline

57. Yoon, S., Kovalenko, A., Bogdanov, K., and Wallach, D. (2017) MLKL, the protein that mediates necroptosis, also regulates endosomal trafficking and extracellular vesicle generation. Immunity 47, 51–65.e7 CrossRef Medline

58. Gong, Y. N., Guy, C., Olauson, H., Becker, J. U., Yang, M., Fitzgerald, P., Linkermann, A., and Green, D. R. (2017) ESCRT-III acts downstream of MLKL to regulate necroptotic cell death and its consequences. Cell 169, 286–300.e16 CrossRef Medline

59. Saltiel, A. R., and Kahn, C. R. (2001) Insulin signalling and the regulation of glucose and lipid metabolism. Nature 414, 799–806 CrossRef Medline

60. Petrie, E. J., Sandow, J. J., Jacobsen, A. V., Smith, B. J., Griffin, M. D. W., Lucey, I. S., Dai, W., Young, S. N., Tanzer, M. C., Wardak, A., Liang, L. Y., Cowan, A. D., Hildebrand, J. M., Kersten, W. J. A., Lessene, G., et al. (2018) Conformational switching of the pseudokinase domain promotes human MLKL tetramerization and cell death by necroptosis. Nat. Commun. 9, 2422 CrossRef Medline

61. Dufour, F., Bertrand, L., Pearson, A., Grandvaux, N., and Langelier, Y. (2011) The ribonucleotide reductase R1 subunits of herpes simplex virus 1 and 2 protect cells against poly(I:C)-induced apoptosis. J. Virol. 85, 8689–8701 CrossRef Medline

62. Tanzer, M. C., Tripaydonis, A., Webb, A. I., Young, S. N., Varghese, I. N., Hall, C., Alexander, W. S., Hildebrand, J. M., Silke, J., and Murphy, J. M. (2015) Necroptosis signalling is tuned by phosphorylation of MLKL residues outside the pseudokinase domain activation loop. Biochem. J. 471, 255–265 CrossRef Medline