



Inhibition of DAI-dependent necroptosis by the Z-DNA binding domain of the vaccinia virus innate immune evasion protein, E3

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Vaccinia virus (VACV) encodes an innate immune evasion protein, E3, which contains an N-terminal Z-nucleic acid binding (Z α) domain that is critical for pathogenicity in mice. Here we demonstrate that the N terminus of E3 is necessary to inhibit an IFN-primed virus-induced necroptosis. VACV deleted of the Z α domain of E3 (VACV-E3L Δ 83N) induced rapid RIPK3-dependent cell death in IFN-treated L929 cells. Cell death was inhibited by the RIPK3 inhibitor, GSK872, and infection with this mutant virus led to phosphorylation and aggregation of MLKL, the executioner of necroptosis. In 293T cells, induction of necroptosis depended on expression of RIPK3 as well as the host-encoded Z α domain-containing DNA sensor, DAI. VACV-E3L Δ 83N is attenuated *in vivo*, and pathogenicity was restored in either RIPK3- or DAI-deficient mice. These data demonstrate that the N terminus of the VACV E3 protein prevents DAI-mediated induction of necroptosis.

necroptosis | vaccinia | RIPK3 | type 1 interferon | Z-DNA binding domain

Vaccinia virus (VACV) is a double-stranded DNA virus that replicates in the cytoplasm of the host cell. Of the 200 genes encoded by VACV, one-third are dedicated to host immune evasion. One of the prime VACV immune evasion genes is E3L. The E3L-encoded protein has two conserved domains that are essential for pathogenesis, presumably by preventing the activation of the type I IFN pathway (1, 2). E3 contains a C-terminal double-stranded RNA (dsRNA)-binding domain that functions by sequestering dsRNA to avoid activation of IFN-inducible, dsRNA-dependent antiviral enzymes such as PKR (2–5). The N terminus of E3 has a Z-form nucleic acid binding (Z α) domain whose function is not well understood. While the N terminus is required for pathogenesis in mice, it is dispensable for replication and IFN resistance (IFN^R) in most cell culture settings. Among N-terminal mutants of VACV E3, pathogenesis correlates with binding to Z-DNA *in vitro*. Virulence of N-terminal mutants was restored in IFN receptor 1-deficient mice (*Ifnar*^{-/-}), indicating that the N terminus is necessary to inhibit type I IFN action *in vivo* (6). The N terminus is also necessary for PKR-dependent IFN^R in 129 mouse embryonic fibroblasts (MEFs). However, virulence of a VACV N-terminal deletion mutant was not restored in PKR-deficient mice, indicating a potential distinct requirement for this domain to block another consequence of IFN signaling *in vivo* (6).

Necroptosis plays a significant role in limiting the pathogenesis of wild-type (WT) VACV in mouse models. A previous study demonstrated that WTVACV-infected cells become sensitized to TNF-driven necroptosis (7). Cells infected with WTVACV, and then treated with TNF, undergo necroptosis at late times post-infection, suggesting that unlike herpesviruses, VACV does not encode a protein that can directly inhibit TNF-mediated necroptosis (7). Additionally, mice deficient in RIPK3 are more susceptible to VACV and succumb to lethal infections faster than do WT mice.

Necroptosis is an inflammatory form of programmed cell death that is an alternative host defense pathway initiated during the course of some viral infections (8–10). Activation of necroptosis leads to a signal cascade that is dependent on the receptor interacting protein kinase 3 (RIPK3) and the downstream pseudokinase, mixed lineage kinase-like protein (MLKL) (11). This signaling pathway leads to a breakdown in membrane integrity in a caspase-independent manner (12, 13). Necroptosis induced through the utilization of classical death receptors (DRs) results in the RIPK homotypic interaction motif (RHIM)-dependent RIPK1–RIPK3 complex, called a necrosome (7, 14–16). Both RIPK1 and RIPK3 are protein kinases, and the kinase activity of these proteins is required in the classic TNF-driven death where RIPK3 activation is dependent on RIPK1 (15, 17). RIPK3 not only plays a central role in transducing signals that drive this DR-induced process, but is also involved in signals for necroptosis initiation through nonclassical pathways such as pattern recognition receptor (PRR) activation, independent of RIPK1 (18). RIPK1-independent activation of RIPK3 has been demonstrated by other adaptor proteins, including DNA-induced activator of IFN (DAI, also known as DLM and ZBP1) (16) and TIR-domain-containing adaptor-inducing IFN β (TRIF) (18). Exposure of RIPK3 to a RHIM adaptor (RIPK1, TRIF, or DAI) induces a conformational change that activates both autophosphorylation kinase activity and transphosphorylation of MLKL, the downstream executor of necroptosis (11, 19, 20).

Significance

The host interferon system is a potent antiviral system, responsible for protecting vertebrate organisms against virus infection. In this paper we demonstrate that the N terminus of the vaccinia virus innate immune evasion protein, E3, is necessary to inhibit the action of interferon. Interferon-treated cells infected with virus encoding an E3 deleted of the N-terminal domain die rapidly. This death is dependent on the host RIPK3 protein, which is a mediator of programmed necrotic death, and on DAI, which is a sensor of virus infection. Since both E3 and DAI contain Z-DNA binding domains, we hypothesize that the cellular DAI virus sensor and the viral antiinterferon E3 protein compete for binding to a vaccinia virus-induced Z-nucleic acid, pathogen-associated molecular pattern.

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Necroptosis is a potentially critical host defense mechanism to limit virulence by inducing death of host cells. Some viruses have adapted to regulate necroptosis, allowing the infectious cycle to continue as demonstrated in murine cytomegalovirus (MCMV) (16, 21). Normally, necroptosis is initiated through RHIM domain interactions between RIPK3 and an adaptor protein. MCMV interferes with necroptosis signaling by utilizing the M45 protein, which acts as a viral RHIM signaling inhibitor, as a viral inhibitor of RIPK activation (vIRA). The RHIM domain of vIRA binds to and sequesters RIPK3 (18, 22–25). Similar to MCMV, both herpes simplex virus (HSV)-1 and HSV-2 have the ability to regulate necroptosis by encoding homologs to M45 that also function as vIRAs in human cells (26). Studies with HSV-1 also revealed the indication that necroptosis may present a barrier to cross-species infection (27, 28).

In this study, we show that the N-terminal α domain of the E3 protein is necessary for conferring resistance to IFN in murine L929 cells, preventing the rapid induction of RIPK3-dependent necroptosis. Necroptosis was independent of RIPK1 kinase activity and dependent on the host DNA sensor, DAI/DLM/ZBP1, which like RIPK1, functions to activate RIPK3-dependent necroptosis. Attenuation of VACV-E3L Δ 83N was reversed in both *RIPK3*^{-/-} mice and *ZBP1*^{-/-} mice, suggesting that DAI-dependent necroptosis plays a role in limiting the virulence of VACV-E3L Δ 83N. Notably, VACV likely does not encode a direct necroptosis inhibitor, but the VACV E3 protein likely represents a mechanism to inhibit sensing of virus infection by DAI. Since the N terminus of both E3 and DAI encode Z-NA binding domains, E3 protein may function as a competitor of Z-form nucleic acid sensing or signaling.

Results

The N Terminus Is Required for Type I IFN Resistance in L929 Cells. The VACV E3 protein plays an essential role in counteracting the host innate immune system. While the C-terminal dsRNA binding domain has been extensively characterized, the role of the N-terminal Z-NA BD in innate immune evasion has been difficult to characterize, due to the lack of a cell culture system where the phenotype of N-terminal E3 mutants in mice can be reproduced. Virulence of VACV in mice is dependent on the presence of a full-length E3 protein. A mutant virus encoding an N-terminal Z-NA BD truncation (VACV-E3L Δ 83N) is highly attenuated in WT mice (1, 4, 6) but not in *Ifnar*^{-/-} mice, implicating the N terminus in subverting type I IFN signaling (6). While characterizing VACV mutants in several mouse cell lines, we identified L929 cells as having a phenotype consistent with the IFN-sensitive (IFN^S) phenotype seen in vivo.

L929 cells were pretreated with increasing doses of mouse IFN, then infected with equivalent plaque forming units (pfu) of WTVACV or VACV-E3L Δ 83N. As shown in Fig. 1A, plaque formation by WTVACV was IFN^R, while plaque formation of VACV-E3L Δ 83N was IFN^S. This is consistent with rescue of VACV-E3L Δ 83N pathogenesis in *Ifnar*^{-/-} mice.

We began characterizing IFN sensitivity of VACV-E3L Δ 83N in L929 cells by performing a [³⁵S]-methionine labeling experiment to determine if viral protein translation was altered in IFN-treated cells. Viral protein synthesis appeared reduced in IFN-treated, VACV-E3L Δ 83N-infected cells (Fig. S1). However, visualization of the Coomassie blue-stained gel revealed a strong reduction in total protein on the gel compared with controls (Fig. 1B and Fig. S1), suggesting that protein was lost from the mutant virus-infected cells. This pattern suggested that VACV-E3L Δ 83N virus-infected cells, but not WTVACV-infected cells, might leak their contents, leading to a reduced recovery of proteins from VACV-E3L Δ 83N-infected cells.

Type I IFN Treatment Leads to an Alteration in Morphology in VACV-E3L Δ 83N-Infected Cells. To assess morphological changes in mutant virus-infected cells, live imaging was conducted. L929 cells treated with mouse IFN and then infected with VACV-E3L Δ 83N showed distinct morphological changes not seen in cells infected with WTVACV (Fig. 1C and Movies S1 and S2). Starting at ~4 h postinfection (HPI), VACV-E3L Δ 83N-infected cells underwent progressive cytoplasmic enlargement and plasma membrane dis-

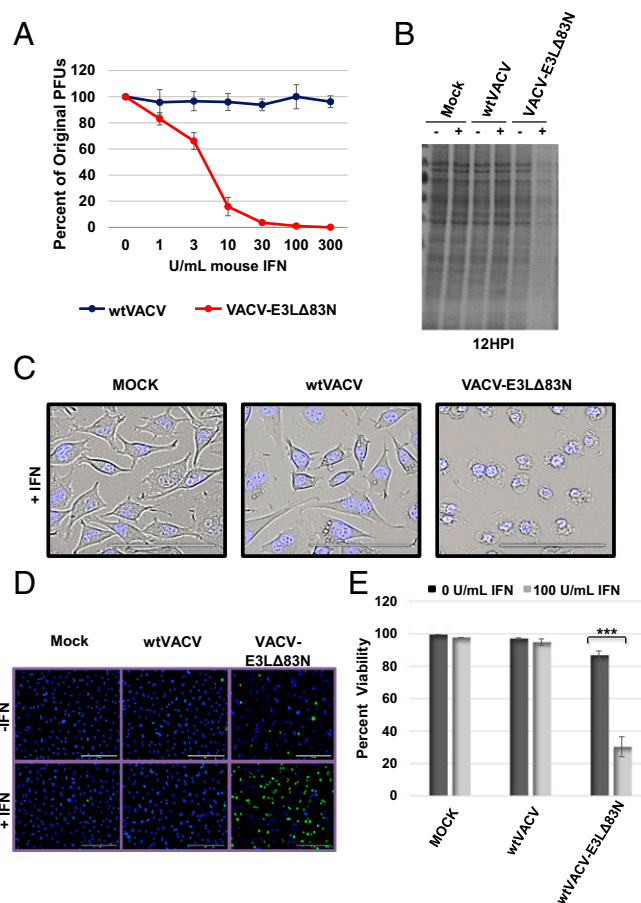


Fig. 1. E3 N-terminal truncations result in IFN sensitivity and rapid cell death in L929 cells. (A) Plaque reduction assay was performed by pretreating a monolayer of L929 cells with increasing concentrations of mouse IFN for 18 h and infecting with 100 pfu of the indicated viruses. (B) Total protein present in cell lysates was evaluated with Coomassie stain. Lysates were harvested at 12 HPI from L929 cells pretreated with IFN for 18 h and subsequently infected at an MOI of 5. (C) Transmitted live imaging at 6 HPI of L929 cells pretreated with 100 units/mL of mouse IFN and then infected with an MOI of 5. (D and E) IFN^S N-terminal mutants undergo a rapid loss of membrane integrity and cellular death. Cell viability was determined using SYTOX exclusion by taking the average percentage of viable cells in 10 fields. Loss of membrane integrity and cell death is indicated by the uptake of both dyes. ****P* < 0.001.

ruption, patterns that were not observed in cells infected with WTVACV, irrespective of IFN treatment (Fig. 1C and Movies S1 and S2). Such a pattern of cellular swelling and membrane disruption suggests that a rapid death occurs in cells infected with VACV-E3L Δ 83N, where leakage may underlie the global loss of protein recovery seen in Fig. 1B and Fig. S1.

IFN Sensitivity Results in a Rapid Death Characterized by Membrane Permeability. To establish that leakage was occurring in VACV-E3L Δ 83N-infected cells, we evaluated cellular permeability using a membrane-impermeable nuclear stain. This assay revealed that L929 cells pretreated with IFN and infected with VACV-E3L Δ 83N became permeable, while the uninfected control cells or cells infected with WTVACV did not (Fig. 1D and E). The induction of death was dependent on both the cells being primed through IFN treatment and being infected with VACV-E3L Δ 83N. Combined with the live imaging results, this pattern suggests that mutant virus in IFN-primed cells is triggering a programmed cell death pathway.

IFN-Primed Death Is Dependent on RIPK3 and Is Caspase Independent. Programmed cell death pathways can be broadly separated into

those that are caspase dependent (apoptosis and pyroptosis) and those that are caspase independent (necroptosis, or programmed necrosis). To determine whether VACV-E3L Δ 83N-induced death was caspase dependent, we employed a pan-caspase inhibitor, Z-VAD-FMK (zVAD). zVAD globally inhibits all known caspases (21). However, zVAD failed to reverse IFN sensitivity of VACV-E3L Δ 83N (Fig. 2A). Similarly, zVAD treatment failed to reverse the inability to recover proteins from infected cells (Fig. 2B) and did not reverse IFN-primed cell death in VACV-E3L Δ 83N-infected L929 cells (Fig. 2C and D). This suggests that the cell death induced by VACV-E3L Δ 83N in IFN-treated L929 cells was independent of caspases and therefore was neither apoptosis nor pyroptosis.

Necroptosis occurs independently of caspase activity and depends on the protein kinase, RIPK3. Thus, we asked if a RIPK3-specific inhibitor, GSK872, could reverse the cell death induced in IFN-treated VACV-E3L Δ 83N-infected L929 cells. Treatment with GSK872 inhibited E3L Δ 83N-induced cell death in IFN-treated cells (Fig. 2C and D), reversed the failure to recover proteins from infected cells (Fig. 2B), and restored the IFN^R of plaque formation (Fig. 2A).

VACV-E3L Δ 83N Infection in IFN-Treated L929 Cells Results in MLKL Activation. MLKL phosphorylation and aggregation have been shown to be the downstream consequences of RIPK3 activation (10, 11). To confirm that the cell death in IFN-treated, VACV-E3L Δ 83N-infected cells was from programmed necrosis, MLKL activation was examined in mock-, WTVACV-, or VACV-E3L Δ 83N-infected L929 cells, and in TNF/zVAD-treated cells. Western blots were performed to assay for the presence and phosphorylation of MLKL (Fig. 3). Phosphorylation of MLKL was specific to IFN-treated, VACV-E3L Δ 83N-infected cells and cells treated with the positive control, TNF+zVAD (Fig. 3A). Phosphorylation of MLKL was detectable by 3 HPI (Fig. 3B). In non-reducing conditions, MLKL also migrated as an aggregate in extracts from IFN-treated VACV-E3L Δ 83N-infected cells (Fig. 3A), consistent with MLKL activation. These results indicate that necroptosis, involving the aggregation and phosphorylation of MLKL, was occurring in IFN-treated VACV-E3L Δ 83N-infected L929 cells. We have seen similar death and MLKL phosphorylation in human HT29 cells infected with VACV-E3L Δ 83N (Fig. S2).

RIPK3 Activity Is Required for IFN-Sensitive Plaque Reduction but Is Independent of RIPK1. To investigate the adaptor that leads to RIPK3 activation in IFN-treated, VACV-E3L Δ 83N-infected cells, we evaluated the requirements for RIPK1 and RIPK3 kinase activities using specific inhibitors. The RIPK1 kinase inhibitor, GSK963, was unable to rescue plaque efficiency (Fig. 4A), cell viability (Fig. 4B), or MLKL activation (Fig. 4C) in L929 cells pretreated with IFN and infected with VACV-E3L Δ 83N, whereas, treatment with the RIPK3 inhibitor, GSK872, restored IFN^R in all assays. These results suggest that the necroptotic cell death induced by VACV-E3L Δ 83N in IFN-treated L929 cells was RIPK1 independent. WTVACV-infected cells were in fact susceptible to death induced by TNF+zVAD treatment, which is RIPK1 dependent, at early times postinfection (Fig. S3), consistent with previously published results (7, 13). Thus, VACV-E3L Δ 83N-induced death occurs independently of either RIPK1 or TNF in contrast to the necroptosis seen previously with WTVACV (7).

DAI Is Up-Regulated in L929 Cells Following IFN Treatment. As the IFN-dependent cell death induced by VACV-E3L Δ 83N is RIPK1 independent, we sought to identify the adaptor protein being utilized in its place. Several other RHIM-containing proteins have been shown to interact with RIPK3 to induce necroptosis. DAI was a potential candidate because it contains a Z α domain homologous to the one absent in VACV-E3L Δ 83N. Induction of necroptosis in L929 cells by VACV-E3L Δ 83N requires the treatment of IFN. Given that DAI is one of the most highly induced proteins following IFN treatment (29), we evaluated this transcript as well as transcripts for other important proteins in the

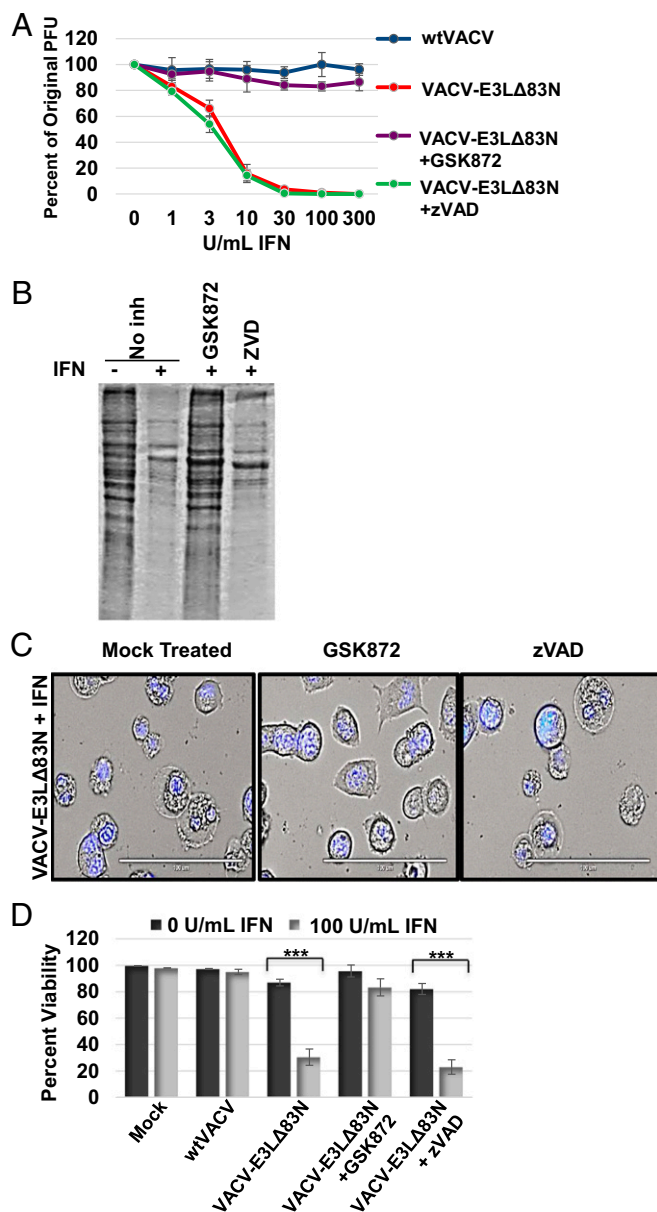


Fig. 2. IFN-primed cell death is reduced by a RIPK3 kinase inhibitor. (A) Plaque reduction assay was performed by pretreating L929 cells with increasing doses of mouse IFN for 18 h and then treating with a RIPK3 inhibitor (GSK872) at 3 μ M, or a pan-caspase inhibitor (zVAD) at 50 μ M, or mock treatment (DMSO) for 1 h before infection. Cells were infected with equivalent pfu of WTVACV or VACV-E3L Δ 83N. Once plaques formed, the percent of pfu reduction compared with mock IFN treated was calculated. (B) Total protein present in cell lysates was evaluated with Coomassie stain. Lysates were harvested at 12 HPI from L929 cells pretreated with IFN for 18 h and subsequently infected at an MOI of 5 in the presence of GSK872, zVAD, or mock treatment. (C) Transmitted live imaging at 6 HPI of L929 cells pretreated with 100 units/mL of mouse IFN for 18 h and for 1 h with either mock, zVAD, or GSK872 and then infected with VACV-E3L Δ 83N at an MOI of 5. (D) Cell viability was determined using SYTOX exclusion by taking the average percentage of viable cells in 10 fields at 5 HPI in the presence of GSK872 or zVAD or mock treated. *** P < 0.001.

necroptosis signaling pathway. No significant changes were seen in transcript levels of RIPK1, RIPK3, or MLKL following IFN treatment, but a 1,000-fold induction of DAI transcripts was observed (Fig. 4D). When RIPK1, RIPK3, MLKL, and DAI levels were directly evaluated by Western blotting, an increase only in DAI levels was observed in IFN-treated L929 cells (Fig. 4E).

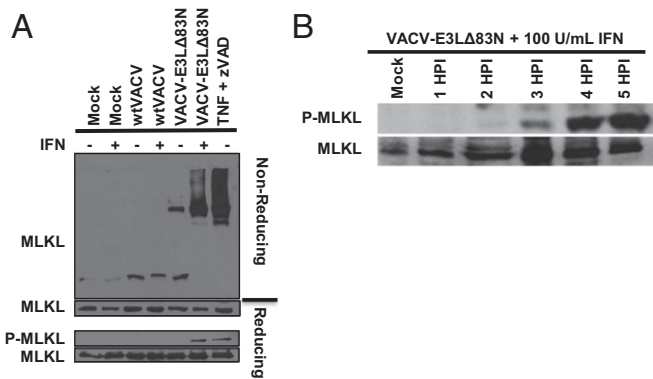


Fig. 3. VACV-E3L Δ 83N infection leads to MLKL activation. (A) Aggregation and phosphorylation of MLKL evaluated by Western blotting. Aggregation was detected by SDS/PAGE under nonreducing conditions. (B) Western blot detection of MLKL phosphorylation. P-MLKL was detected as early as 3 HPI.

DAI Contributes to Induction of Necroptosis by VACV-E3L Δ 83N. Since DAI was up-regulated by IFN treatment of L929 cells, we sought to determine if DAI expression was sufficient to induce necroptosis in cells infected with VACV-E3L Δ 83N. Utilizing a plasmid expression system, we ectopically expressed human RIPK3 alone or in combination with human DAI in HEK293T cells, which do not express DAI or RIPK3 (Fig. 5A). Following transfections, cells were either treated with TNF+zVAD or infected with WTVACV or VACV-E3L Δ 83N (Fig. 5B). Expression of RIPK3 was sufficient to sensitize the HEK293T cells to necroptosis following treatment with TNF+zVAD. This is consistent with the endogenous expression of RIPK1 in 293T cells (Fig. 5A). Expression of RIPK3 alone was not sufficient to sensitize the cells to VACV-E3L Δ 83N-induced necroptosis; the expression of both RIPK3 and DAI was required (Fig. 5B). WTVACV failed to induce death in any condition tested. We have also evaluated the effects of knockdown of DAI expression in L929 cells, where VACV-E3L Δ 83N induces necroptotic cell death (Fig. 3). Transfection of L929 cells with siRNA to DAI led to reduced induction of DAI after IFN treatment (Fig. 5C). Knockdown of DAI restored cell viability in IFN-treated, VACV-E3L Δ 83N-infected cells to the levels of viability seen in IFN-treated cells that were transfected with either siRNA to DAI or a scrambled siRNA, and subsequently infected with WTVACV (Fig. 5C). Thus, DAI expression was required for VACV-E3L Δ 83N-induced cell death, and DAI is likely the adaptor protein for RIPK3 in necroptosis induced by VACV-E3L Δ 83N.

Deficiency of RIPK3 or ZBP1 Rescues VACV-E3L Δ 83N Virulence in Mice. Given the importance of mouse studies that have defined the N terminus in subverting type I IFN signaling and virulence (6), we sought to pursue in vivo studies in WT C57BL/6, *RIPK3*^{-/-}, and *ZBP1* (*DAI*)^{-/-} mice. Mice were infected intranasally with 10⁶ pfu of either WTVACV or VACV-E3L Δ 83N [in the mouse-adapted, neurovirulent Western Reserve (WR) strain and monitored for clinical symptoms]. WTVACV infections resulted in significant pathology in WT, *RIPK3*^{-/-}, and *ZBP1*^{-/-} mice. As previously described, at this dose the VACV-E3L Δ 83N mutant was apathogenic in WT C57BL/6 mice (6). Pathogenesis of this mutant was restored in *RIPK3*^{-/-} (Fig. 5D) and *ZBP1*^{-/-} (Fig. 5E) mice to levels comparable to that of WTVACV. We have also isolated virus from tissues of infected animals (Fig. S4). WTVACV and VACV-E3L Δ 83N replicated to equivalent levels in the nose, while lower levels of VACV-E3L Δ 83N were detected in the lungs of infected WTC57BL6 mice. VACV-E3L Δ 83N replication was increased in the lungs of *RIPK3*^{-/-} or *ZBP1*^{-/-} mice, consistent with the increase in pathogenesis seen in VACV-E3L Δ 83N-infected knockout mice, although the increased titers were not statistically significant because few transgenic animals were available (Fig. S4). These results together indicate that the N

terminus of E3 is important to block RIPK3-dependent host pathways that can limit the virulence of VACV.

Discussion

The VACV E3 protein has long been established as an essential innate immune evasion protein that confers resistance to IFN (5, 6). Although the consequences of E3 C-terminal dsRNA sequestration have been clear for decades (2–4), the dramatic IFN sensitivity of VACV E3 N-terminal deletion mutants has remained unresolved. Previous work suggested that the N terminus of E3 was essential for IFN^R by preventing the phosphorylation of PKR in MEF129 cells. However, the highly attenuated phenotype of VACV-E3L Δ 83N was not reversed in PKR-deficient mice, implicating an alternative mechanism for the IFN^S in vivo phenotype of the mutant virus (6).

In this study, we resolve the role of the E3 N terminus, providing evidence that this domain competes with DAI to prevent DAI-dependent activation of RIPK3 and consequent necroptosis. Infection of L929 cells with VACV-E3L Δ 83N led to a rapid IFN-dependent cell death characterized by cellular swelling and loss of plasma membrane integrity. This cell death was caspase independent but dependent on the kinase activity of RIPK3. Infection of IFN-treated cells with VACV-E3L Δ 83N led to MLKL phosphorylation and aggregation, indicative of necroptotic cell

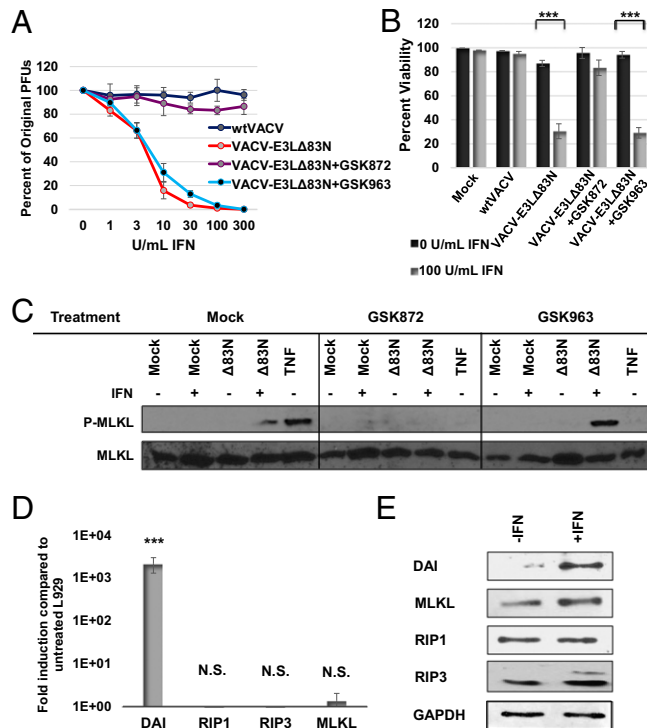


Fig. 4. IFN-primed necroptosis is independent of RIPK1. (A) Plaque reduction assay was performed by pretreating L929 cells with increasing doses of mouse IFN for 18 h and then treating with a RIPK3 inhibitor (GSK872) at 3 μ M, or a RIPK1 inhibitor (GSK963) at 3 μ M, or mock treatment (DMSO) for 1 h before infection. Cells were infected with equivalent pfu of WTVACV or VACV-E3L Δ 83N. (B) L929 cell viability was determined using SYTOX exclusion by taking the average percentage of viable cells in 10 fields at 5 HPI in the presence of GSK872, GSK963, or mock treatment. (C) L929 cells were pretreated with IFN for 18 h and either infected with VACV-E3L Δ 83N or treated with TNF and zVAD in the presence of GSK872, GSK963, or mock treatment, and evaluated for P-MLKL by Western blot. (D) qPCR evaluation of necroptosis-associated gene induction in L929 cells following 18-h treatment of 100 units/mL of IFN. (E) Western blot of protein expression level of necroptosis-associated proteins following 18-h treatment of 100 units/mL of IFN. *** P < 0.001. NS, no significance (>0.05).

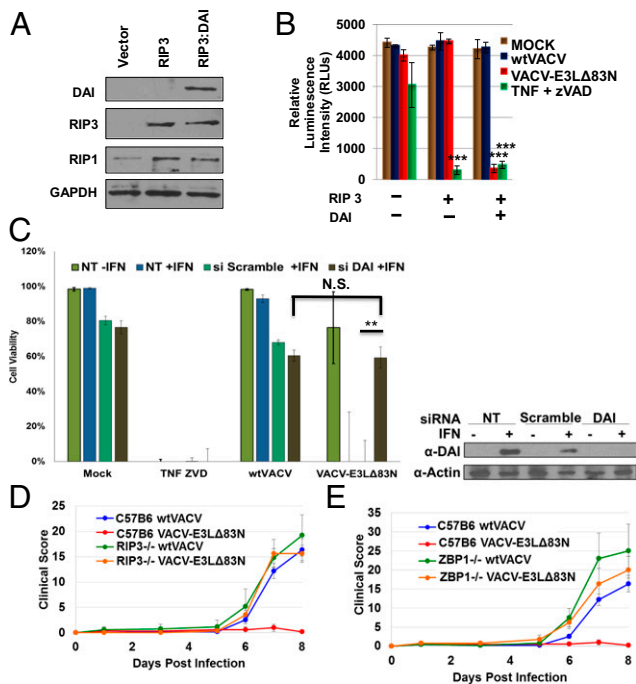


Fig. 5. Both RIPK3 and DAI are required for VACV-induced necroptosis. (A) 293T cells express RIPK3 and DAI only after transfection. (B) Viability of human 293T cells under indicated conditions was measured by Cell Titer-Glo assay. (C) L929 cells were untransfected (NT) plus or minus IFN, or transfected with either scrambled siRNA plus treated with IFN, or transfected with siRNA targeted to knockdown DAI expression, plus treated with IFN; cells were then either mock infected, treated with TNF-zVAD, infected with WTVACV, or infected with VACV-E3LΔ83N. (Right) Western blot of the cell lysates, showing the presence or absence of DAI. (D) Eight- to 10-wk-old *RIPK3*^{-/-} or WT C57BL/6 mice were inoculated by intranasal route with 10⁶ pfu of the indicated viruses (five mice per group). (E) Eight- to 10-wk-old *ZBP1(DAI)*^{-/-} or WT C57BL/6 mice were infected by intranasal route with 10⁶ pfu of the indicated viruses (five mice per group). ***P* < 0.01. N.S., no significance (>0.05).

death (20). VACV could not inhibit TNF-induced cell death, which is RIPK1 dependent (7, 30), suggesting an alternative activator for RIPK3. Since expression of DAI/DLM/ZBP1 is highly inducible in L929 cells by IFN treatment, and since DAI/DLM/ZBP1 contains a Zα domain that can substitute for the Zα domain of the E3 protein in supporting pathogenesis in mice (31), we asked if cell death was dependent on DAI/DLM/ZBP1 expression. Knockdown of DAI in L929 cells restored viability in IFN-treated, VACV-E3LΔ83N-infected cells. HEK293T cells do not express RIPK3 or DAI and are resistant to virus- or TNF-induced necroptosis. Ectopic expression of RIPK3 restored TNF-induced cell death in HEK293T cells, but expression of both RIPK3 and DAI was needed to restore death in VACV-E3LΔ83N-infected cells.

E3 and DAI are members of the same Zα binding domain-containing family of proteins. Despite the E3 and DAI Zα domains sharing less than 20% sequence homology, the DAI domain functionally replaced the E3 domain (31–33). This suggests that both DAI and the N terminus of E3 can bind to the same nucleic acid molecules in a VACV-infected cell. We therefore propose a model (Fig. 6) in which IFN priming of cells enhances the expression of DAI, which acts as a sensor of a VACV-induced Z-form nucleic acid pathogen-associated molecular pattern (PAMP). During WTVACV infection, the Zα domain of E3 is presumed to bind to this putative PAMP and mask it, preventing sensing by DAI. In VACV-E3LΔ83N-infected cells, the absence of the N-terminal Zα domain of E3 allows the homologous Zα domain of DAI to sense the VACV-induced PAMP and act as a RIPK3 adaptor protein to initiate necroptosis.

Virus-induced necroptosis has been shown to be an important innate defense mechanism against viral infections with three distinct viruses: poxviruses, herpesviruses, and influenza A virus (IAV) (8–10). WTVACV has previously been shown to sensitize cells to TNF-driven necroptosis at late times postinfection, suggesting that VACV does not produce any proteins that can inhibit TNF- and RIPK1-dependent necroptosis (7, 13). Thus, it appears that VACV can only inhibit DAI-mediated necroptosis but not RIPK1-mediated necroptosis. These earlier studies also reported that pathogenesis in mice infected with WTVACV was exacerbated in *RIPK3*^{-/-} mice. We did not detect an increase in pathogenesis in *RIPK3*^{-/-} mice infected with WTVACV. The difference in results between previous studies and the current study may be due to the different modes of infection, IP in the previous work, vs. IN in the work described in this manuscript.

The herpesviruses have also been shown to encode an inhibitor of necroptosis (26, 34, 35). MCMV is the most well characterized of the herpesviruses that can inhibit induction of necroptosis as an innate immune defense mechanism (35). The MCMV M45 protein contains a RHIM domain that can interact with the RHIM domain of RIPK3 and inhibit TNF-, TRIF- and DAI-induced necroptosis (16, 18, 21). In the absence of M45, MCMV-infected cells become susceptible to DAI-mediated necroptosis (16, 23). Unlike RHIM-dependent viral inhibition of necroptosis in the herpesvirus system, E3 inhibition is likely a different mechanism of inhibition, as E3 does not contain an identifiable RHIM domain (21).

Two groups have recently reported that IAV infection leads to DAI-mediated cell death (8, 29). IAV infection can induce RIPK3-dependent necroptosis or apoptosis, depending on the nature of proteins recruited to the complex, or it can induce pyroptosis. Unlike the poxviruses and herpesviruses, IAV does not appear to encode a necroptosis inhibitor. The consequence of DAI-mediated cell death in IAV pathogenesis is unclear, in that differing results were seen in IAV-infected *ZBP1*^{-/-} mice (8, 29).

The nature of the molecules sensed by DAI in infected cells remains unclear. DAI was initially identified as a DNA sensor, and as a Zα domain-containing protein. It is capable of binding Z-form nucleic acid in vitro. DAI has been reported to sense either IAV RNA or IAV proteins in IAV-infected cells (8, 29). The nature of the molecules being sensed during herpesvirus and VACV infection are likewise unclear, although the fact that the DAI Zα domain can functionally replace the Zα domain of E3 suggests that these proteins are competing for binding to a VACV-induced Z-form nucleic acid. Further characterization of this system may lead to the identification of physiologically relevant Z-form nucleic acid in the cell.

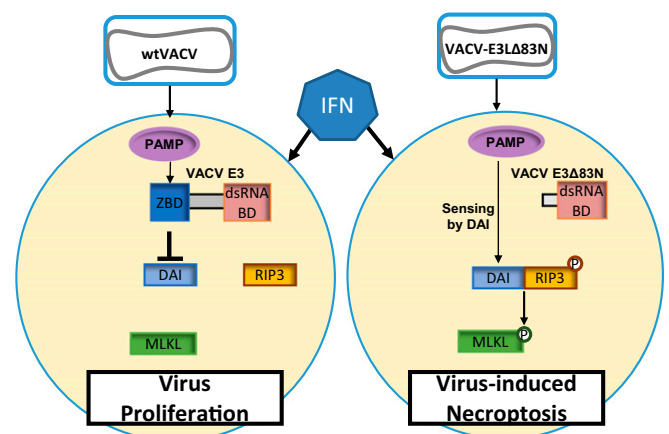


Fig. 6. Model for mechanism of VACV inhibition of necroptosis.

Materials and Methods

Cells and Viruses. L929 cells and HEK293T cells were obtained from ATCC. The WR strain of WTVACV and mutant virus deleted of the N-terminal 83 amino acids of E3 (VACV-E3LΔ83N) were described previously (4).

IFN Resistance Evaluation by Plaque Reduction. L929 cell monolayers were treated with 0–300 units/mL of mouse IFN- α and then infected with either WTVACV or VACV-E3LΔ83N virus. Plaque formation was detected by staining with crystal violet (see *SI Materials and Methods* for more details).

L929 Viability Assay. Viability was assessed with a SYTOX nuclear stain exclusion assay or by a CellTiter-Glo luminescent cell viability assay (Promega) (see *SI Materials and Methods* for more details).

Inhibitors and Treatment of Cells. All inhibitor treatments were applied for 1 h preinfections. GSK872 and GSK963 were kindly provided by GlaxoSmithKline (see *SI Materials and Methods* for more details).

RT-PCR Methods. RNA was isolated from mock- or IFN-treated cells, reverse transcribed into cDNA, and amplified. cDNA was detected on a CFX Connect Real-Time PCR detection system (see *SI Materials and Methods* for more details).

Expression of DAI and RIP3 in HEK293T. The human RIP3 and DAI proteins were expressed from plasmid expression systems (pLV-EF1a-cDNA-IRES-Puro) in HEK293T cells. At 48 h posttransfection, if indicated, the cells were treated as described and infected at a multiplicity of infection (MOI) of 5 with the indicated virus.

siRNA Transfections. L929 cells were transfected with either ON-TARGET plus Nontargeting Pool or SMARTpool: ON-TARGET plus Zbp1 siRNA (see *SI Materials and Methods* for more details). The experiments were performed 18 h later as described.

Intranasal Infections of Mice. Anesthesia and infections were as previously described (6). Mice were infected with either WTVACV or VACV-E3LΔ83N

virus intranasally (IN). C57BL/6 mice were from The Jackson Laboratory, *Ripk3*^{-/-} mice were from Genentech (36), and *Zbp1*^{-/-} mice (37) were from Shizuo Akira, Osaka University, Osaka, Japan. All procedures were approved by the Emory University Institutional Animal Care and Use Committee (see *SI Materials and Methods* for more details).

Western Immunoblot Analysis. L929 cells were pretreated with mouse IFN- α and infected with viruses at an MOI of 5. Lysates were harvested as previously described (16). For samples used for MLKL aggregation, cells were prepared as described above but lysed in 100 μ L of 1 \times SDS without 2-mercaptoethanol. Proteins were separated by SDS/PAGE (see *SI Materials and Methods* for more details).

Total Protein Staining. L929 cell monolayers were infected with WR strains of WTVACV or E3LΔ83N at an MOI of 5. Lysates were prepared and separated on denaturing gels and visualized following SDS/PAGE (see *SI Materials and Methods* for more details).

Live Imaging of L929-Infected Cells. L929 cells were pretreated with 100 units/mL of mouse IFN- α for 18 h before infection. A live nuclear stain was applied 15 min before infection. Cells were infected with either WTVACV or VACV-E3LΔ83N as described above. For images used to evaluate alterations in morphology, infected cells were incubated for an additional 5 h and imaged with an EVOS FL auto imaging system. For time-lapse imaging, cells were overlaid with minimum essential medium (MEM) containing 1 μ M SYTOX Green nucleic acid stain. Images were taken every 2 min for 5 h (see *SI Materials and Methods* for more details).

Statistics. Statistical analysis was done by using a two-tailed, unpaired *t* test. ****P* < 0.001, ***P* < 0.01, **P* < 0.05. No significance (N.S.) was used to represent *P* > 0.05. Error bars represent SE.

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