Integrated stress response restricts macrophage necroptosis

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The integrated stress response (ISR) regulates cellular homeostasis and cell survival following exposure to stressors. Cell death processes such as apoptosis and pyroptosis are known to be modulated by stress responses, but the role of the ISR in necroptosis is poorly understood. Necroptosis is an inflammatory, lytic form of cell death driven by the RIPK3-MLKL signaling axis. Here, we show that macrophages that have induced the ISR are protected from subsequent necroptosis. Consistent with a reduction in necroptosis, phosphorylation of RIPK1, RIPK3, and MLKL is reduced in macrophages pre-treated with ISR-inducing agents that are challenged with necroptosis-inducing triggers. The stress granule component DDX3X, which is involved in ISR-mediated regulation of pyroptosis, is not required for protecting ISR-treated cells from necroptosis. Disruption of stress granule assembly or knockdown of Perk restored necroptosis in pre-stressed cells. Together, these findings identify a critical role for the ISR in limiting necroptosis in macrophages.

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Introduction

Given the broad range of potential stressors in the environment, cells must carefully balance stress-induced cell death with responding to and recovering from stressors. Programmed cell death pathways are activated by cellular stressors including host-derived inflammatory cytokines, microbial agents, and many other exogenous stresses (Fuchs & Steller, 2011; Galluzzi et al, 2018; Place & Kanneganti, 2019; Tang et al, 2019; Samir et al, 2020; Kesavardhana et al, 2020a; Place et al, 2021). Whereas programmed cell death is critical for organismal development and control of microbial infection, dysregulated and excessive cell death can contribute to autoinflammatory disease, developmental defects, and cancer (Fuchs & Steller, 2011; Place & Kanneganti, 2019; Kesavardhana et al, 2020a; Place et al, 2021). Inhibitory regulation of programmed cell death is therefore critical for limiting cell death-induced pathology. One pathway involved in such regulation, the integrated stress response (ISR), has been shown to inhibit two central programmed cell death pathways, apoptosis and pyroptosis (Arimoto et al, 2008; Thiedieck et al, 2013; Samir et al, 2019), but its role in necroptosis is unknown.

Necroptosis is a form of lytic programmed cell death which is initiated in cells with defective apoptosis signaling. In cells where caspase-8 activity is inhibited, inflammatory signaling through TNFR1 results in interaction between RIPK1 and RIPK3 via RIP homotypic-interaction motif (RHIM) domains, phosphorylation of RIPK3, and RIPK3-dependent phosphorylation of MLKL. MLKL oligomerization mediates cell death through disruption of plasma membrane integrity (Sun et al, 2012; Zhao et al, 2012; Wang et al, 2014). Although necroptosis-deficient mice (Ripk3<sup>−/−</sup> or Mlk1<sup>−/−</sup>) appear developmentally normal, necroptosis can be activated under various disease states. Physiologically, necroptosis mediated by RIPK3 and MLKL promotes embryonic lethality in Csp98<sup>−/−</sup> mice (Kaiser et al, 2011; Oberst et al, 2011; Alvarez-Diaz et al, 2016). Similarly, RIPK1 RHIM domain mutant mice exhibit perinatal lethality driven by spontaneous, ZBP1-dependent activation of RIPK3 and MLKL (Newton et al, 2016; Kesavardhana et al, 2020a). Necroptosis also drives dermatitis in an epithelial cell-specific RIPK1 knockout mouse model (Dannappel et al, 2014). Recently, a gain-of-function mutation in MLKL, Mlkl<sup>H139P</sup>, was found to result in lethal postnatal inflammation in mice, and similar mutations in the human MLKL brace region are associated with chronic recurrent multifocal osteomyelitis, demonstrating MLKL-driven inflammation is a key inflammatory cell death regulator (Hildebrand et al, 2020). MLKL is also important for mediating pathogen clearance in mice (Kitur et al, 2016; Yu et al, 2018; Zhang et al, 2020). In addition to causing necroptosis, MLKL-dependent plasma membrane disruption can also result in activation of NLRP3-dependent pyroptosis, suggesting necroptosis may be important in promoting inflammation in NLRP3 inflammasome-mediated diseases (Conos et al, 2017). Because spontaneous or excessive necroptosis results in significant autoinflammation, we hypothesized that the ISR, which is important for modulating cell fate decisions in response to multiple exogenous cell stressors (Arimoto et al, 2008; Thiedieck et al, 2013; Samir et al, 2019), may act as a cell-intrinsic negative regulator of necroptosis.

Mechanistically, the ISR responds to a range of cellular stressors by modulating the activity of the translational machinery while also initiating gene expression required for resolving stress-induced...
changes to normal cellular homeostasis (Costa-Mattioli & Walter, 2020; Riggs et al, 2020). The ISR is initiated by activation of ISR sensor kinases (including PERK, HRI, GCN2, and PKR) that phosphorylate eIF2α, resulting in sequestration of translational machinery in liquid organelles called stress granules (SGs) made up of proteins including the core component G3BP1 (Wek et al, 1995; Tourrière et al, 2003; McEwen et al, 2005; Taniuchi et al, 2016). Under stress conditions, SG formation can also sequester apoptosis-inducing proteins and limit apoptosis (Arimoto et al, 2008; Thiedieck et al, 2013). Recently, recruitment of DDX3X into SGs was also shown to inhibit NLRP3-dependent pyroptosis by sequestration of DDX3X molecules, which are required for NLRP3 inflammasome activation in unstressed cells (Samir et al, 2019). Although sometimes cell protective, dysregulated SG assembly can also promote cell death (Reineke & Neilson, 2019). SG dysfunction is thought to contribute to disease progression in cancer and neurodegeneration (Anderson et al, 2015; Wolozin & Ivanov, 2019). Although SGs have been shown to reduce cell death via apoptosis and pyroptosis (Arimoto et al, 2008; Thiedieck et al, 2013; Samir et al, 2019), their influence on necroptosis remains unknown. Many previous studies on the ISR and SGs have used immortalized cell lines which are inherently dysfunctional in normal cell death pathways, notably necroptosis (Su et al, 2016). We therefore examined the potential role for the ISR in regulating necroptosis in primary macrophages in this study.

Here, we identify a critical role for the ISR in protecting macrophages from necrotic cell death. Macrophages pretreated with stress-inducing agents were resistant to subsequent death when challenged with necroptosis triggers. Mechanistically, pre-stressed macrophages treated with necroptosis triggers failed to activate the key necroptosis signaling proteins RIPK1, RIPK3, and MLKL. Stress-mediated protection from necroptosis did not require the SG component DDX3X, unlike stress-mediated protection from NLRP3-dependent pyroptosis. Disruption of SG assembly or knockdown of PERK-dependent ISR signaling restored necroptosis in stressed cells. Together, these findings suggest a critical role for the ISR in regulating necroptosis.

Results

SG triggers inhibit macrophage necroptosis

To determine whether the ISR can inhibit necroptosis, we pre-treated BMDMs with the SG-inducing agent thapsigargin, which induces ER stress (Fig 1A) (Harding et al, 2000). To trigger TNFR1-dependent necroptosis, caspase-8 activity must be inhibited during TNF treatment; therefore, we also added the pan-caspase inhibitor zVAD to BMDMs before the addition of TNF (Fig 1A). In BMDMs pretreated with thapsigargin, necrototic cell death was significantly reduced compared to unstressed cells (Figs 1B and C and S1A). To determine whether acute thapsigargin-induced stress also limited necroptosis, we pretreated cells for 1 h and washed off thapsigargin before stimulating BMDMs with the necroptosis trigger zVAD plus TNF (Fig 1D). Consistent with prolonged thapsigargin treatment (Fig 1A–C), acute thapsigargin pretreatment also inhibited cell death after stimulation with necroptosis triggers (Fig 1E and F). To further determine whether the ISR inhibits necroptosis, we pre-stressed BMDMs with additional SG inducers including brefeldin A, tunicamycin, MG132, arsenite, and roccaglimate A. Consistent with the thapsigargin-induced stress-mediated reduction of necroptosis, brefeldin A, tunicamycin, MG132, arsenite, and roccaglimate A pre-stressed BMDMs were protected from TNFR1-dependent necroptosis (Figs S1A and B and S2A–G). MEFs also undergo necroptosis after zVAD and TNF treatment; however, pretreatment with thapsigargin or arsenite enhanced cell death rather than reducing it as in BMDMs, suggesting cell type–specific responses guide the cell fate choice after stress and necroptosis signaling (Fig S3A and B).

TNF signaling via TNFR1 drives necroptosis through the RIPK1-RIPK3-MLKL signaling axis (Sun et al, 2012; Zhao et al, 2012). Toll-like receptor signaling via the adaptor TRIF (which contains a RHIM domain) engages necroptosis in a RIPK3-dependent manner that does not require RIPK1 (Kaiser et al, 2013; Malireddi et al, 2020a). Similar to the observed impact on TNFR1-mediated necroptosis, LPS signaling via TLR4 (Fig S4A and B) and poly I:C signaling via TLR3 (Fig S4C and D), which both signal through TRIF, induced necroptosis in cells treated with zVAD, but necroptosis was reduced in thapsigargin pre-stressed cells. Inhibition of the MAP kinase TAK1 (also known as MAP3K7) can also induce activation of pyroptotic, apoptotic, and/or necrotic molecules and cause PANoptosis (Malireddi et al, 2020a). Pretreatment of cells with zVAD followed by TAK1 inhibitor (SZ-7-oxozeanol; TAKI) results in RIPK1 kinase function-independent, but RIPK3 scaffolding function-dependent, MLKL-dependent necroptosis (Malireddi et al, 2020a). Similar to necroptosis induced by TNFR1 and TLRs, TAK1 inhibition with concurrent pan-caspase inhibition (zVAD) resulted in cell death which was limited in thapsigargin pretreated cells (Fig S5A–C). Conditional deletion of TAK1 in Lyz2creTak1fl/ff BMDMs also results in spontaneous PANoptosis which is potentiated by TNF (Malireddi et al, 2018, 2020a). Addition of thapsigargin before TNF stimulation in TAK1-deficient BMDMs also reduced cell death (Fig SSD and E). Similar to wildtype BMDMs, TAK1-deficient BMDMs stressed with thapsigargin were also protected from zVAD plus TNF-induced necroptosis (Fig SSF and G). Together, these findings show that induction of the ISR in BMDMs protects from subsequent necroptosis via TNFR1 and TLR-mediated signaling pathways.

SG inducers inhibit RIPK1, RIPK3, and MLKL phosphorylation

Necroptosis is driven by phosphorylation of RIPK3 and MLKL downstream of TNFR1 (Sun et al, 2012; Zhao et al, 2012). To determine whether the ISR inhibits necroptosis via restricting the RIPK3-MLKL signaling axis, we pretreated cells with stress-inducing agents (thapsigargin, brefeldin A, tunicamycin, or MG132) and zVAD (1 h) followed by TNF. Consistent with stress-induced protection from necrototic cell death (Figs 1 and S2), phosphorylation of MLKL and RIPK3 was reduced in cells pre-stressed with thapsigargin (Fig 2A), brefeldin A (Fig 2B), tunicamycin (Fig 2C), and MG132 (Fig 2D). We also confirmed that each stressor increased phosphorylation of eIF2α, an indicator of ISR activation (Fig 2A–D). Upstream of RIPK3, RIPK1 phosphorylation at serine residue 166 is associated with increased necroptosis signaling (Laurien et al, 2020). Consistent with the observed reduction of RIPK3 and MLKL phosphorylation, RIPK1

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Figure 1. Stress granule triggers inhibit macrophage necroptosis.
Primary BMDMs were stimulated as indicated. (A) Schematic for inducing necroptosis (zVAD + TNF) in unstressed or pre-stressed (thapsigargin [Thaps] treated) BMDMs. (B) Representative IncuCyte images collected at indicated time-points after addition of TNF, where necroptotic cells were quantified by uptake of membrane-impermeant Sytox Green (green, with red analysis mask outline). (C) Quantification of necroptosis from automated image analysis of Sytox Green–positive nuclei at indicated time-points. (D) Schematic for acute pre-stressing of BMDMs with 1 h thapsigargin treatment before necroptosis induction with TNF. (E) Representative IncuCyte images of necroptosis quantification in acute thapsigargin pre-stressed BMDMs (green, with red analysis mask outline). (F) Quantification of necroptosis from automated analysis.
phosphorylation at S166 but not S321 was reduced in thapsigargin pre-stressed cells (Fig 2E). Together, these data suggest that the ISR limits necroptosis signaling by interfering with RIPK1-RIPK3-MLKL signaling cascade downstream of the TNF receptor.

**DDX3X-dependent SGs are not required for inhibition of necroptosis**

The ISR promotes the condensation of translational machinery and associated proteins into structures termed SGs that are composed of core and accessory proteins. The SG protein DDX3X was recently found to regulate the cell survival and pyroptotic cell fate of BMDMs (Samir et al, 2019). In sodium arsenite pre-stressed cells, DDX3X localizes to SGs; consequently, this sequestration of DDX3X protects BMDMs from NLRP3-dependent pyroptosis. In unstressed cells, DDX3X promotes the activation of NLRP3-dependent pyroptosis (Samir et al, 2019). We therefore examined whether DDX3X was required for ISR-mediated inhibition of necroptosis. First, we confirmed that BMDMs treated with thapsigargin induced the formation of SGs by staining for G3BP1 and DDX3X. Consistent with previous studies (Samir et al, 2019; Kedersha et al, 2000; Wheeler et al, 2016; Samir et al, 2019), we found in cell death between wildtype and DDX3X-deficient BMDMs, phosphorylation of MLKL was similar in wildtype and DDX3X-deficient BMDMs (Fig 3D). We further confirmed that deletion of Ddx3x limits NLRP3-dependent pyroptosis, as previously observed (Samir et al, 2019), by treating control and Lyz2CreDdx3xfl/fl BMDMs with LPS plus nigericin (Fig 3E). Together, these data suggest that the ISR restricts necroptosis in a DDX3X-independent manner, which is distinct from the role for the ISR and DDX3X in regulating pyroptosis.

**Disruption of SGs restores necroptosis in stressed BMDMs**

SG assembly is a dynamic process driven by inhibition of translation initiation, which leads to the accumulation of stalled pre-initiation complexes and liquid–liquid phase separation of RNA and proteins (Kedersha et al, 1999). To experimentally determine whether disruption of SGs restores necroptosis in BMDMs, we treated cells with the translational elongation inhibitor anisomycin. Anisomycin and other inhibitors of translation elongation stabilize polysomes, inhibit SG assembly, and disrupt pre-formed SGs (Kedersha et al, 2000; Wheeler et al, 2016; Samir et al, 2019). First, we confirmed that anisomycin treatment disrupted pre-formed SGs in thapsigargin-stressed BMDMs. As expected, anisomycin treatment (15 min) led to a rapid reduction of SGs (Fig 4A). We then compared necroptotic cell death in BMDMs that were pre-stressed, stressed and treated with anisomycin to disrupt SGs, or treated with necroptosis-inducing agents alone (Fig 4B). Disruption of SGs with anisomycin restored cell death and phosphorylation of MLKL and RIPK3 in BMDMs treated with zVAD/TNF (Fig 4C–E). In addition, we confirmed that necroptosis was restored by similarly treating wildtype, Ripk1fl/fl (K45A/K45A mutation; kinase-dead), Ripk3−/−, and Mlkl−/− BMDMs; we observed cell death in wildtype BMDMs treated with TNF and zVAD that was blocked by thapsigargin treatment and restored in response to anisomycin, whereas there was no induction of cell death in necroptosis-deficient BMDMs (Fig S7A–D). We also confirmed that cycloheximide, another translation elongation inhibitor that disrupts SGs, restored necroptosis (Fig S7E). Together, these data suggest that SG assembly limits necroptotic signaling and cell death.

**PERK-dependent signaling is required for stress-mediated inhibition of necroptosis**

Activation of the ISR by thapsigargin involves signaling via PERK (Harding et al, 2000). To genetically determine whether PERK was required for thapsigargin stress-mediated inhibition of necroptosis in BMDMs, we performed siRNA knockdown of Perk. Knockdown of Perk did not render BMDMs more sensitive to necroptosis following zVAD and TNF treatment (Fig 5A and B). Knockdown of Perk did, however, partially restore necroptosis in BMDMs that were pre-stressed with thapsigargin before necroptosis induction (Fig 5A and B). PERK was also required for SG assembly in BMDMs after thapsigargin treatment (Fig 5C). Perk knockdown also restored necroptosis signaling via the RIPK1-RIPK3-MLKL axis in thapsigargin pre-stressed BMDMs (Fig 5D). Together, these data suggest that PERK-dependent signaling after thapsigargin-induced stress limits necroptosis in BMDMs by inhibiting the necroptosis signaling cascade downstream of TNFR1.

**Discussion**

In this study, we identified a regulatory pathway whereby stress responses in macrophages protect cells from necroptosis. Multiple stress inducers reduced necroptotic cell death induced by necroptosis triggers that signal through TNFR1, TLRs, or by inhibiting TAK1. Consistent with the stress-induced protection from cell death, necroptosis signaling mediated by phosphorylation of RIPK1, RIPK3, and MLKL was inhibited in stressed cells. Disruption of SG formation with the translation inhibitors anisomycin or cycloheximide, or knockdown of Perk, restored necroptosis in pre-stressed BMDMs, suggesting that the ISR and downstream SG assembly mediate the
Figure 2. Stress granule inducers inhibit RIPK1, RIPK3, and MLKL phosphorylation.
Necroptosis signaling was analyzed by immunoblotting at the indicated time after the induction of necroptosis by TNF treatment. (A, B, C, D, E) Lysates from primary BMDMs pre-stressed with (A) thapsigargin (Thaps), (B) brefeldin A, (C) tunicamycin (Tunica), (D) MG132, or (E) Thaps and treated with zVAD + TNF were collected at the indicated time-points. Necroptosis signaling was examined by immunostaining for phosphorylation of MLKL and RIPK3 (p-MLKL and p-RIPK3) and phosphorylation of RIPK1 (p-RIPK1) at serine-166 (S166) or serine-321 (S321) and activation of the integrated stress response by phosphorylation of eIF2α (p-eIF2α). Immunoblots are representative of at least two independent experiments.
Figure 3. DDX3X is not required for stress-dependent inhibition of necroptosis.
Primary BMDMs were stimulated as indicated. (A) Primary BMDMs derived from control (Ddx3x<sup>fl/fl</sup>) or myeloid-specific Lyz2<sup>cre</sup>Ddx3x<sup>fl/fl</sup> mice were treated as indicated, and representative images (6 h) were obtained from automated IncuCyte analysis where necroptotic cells were quantified by uptake of membrane-impermeant Sytox Green (green, with red analysis mask outline). (B) Necroptosis was quantified by automated analysis of Sytox Green−positive nuclei. (C) Immunoblots were performed from BMDM lysates at the indicated time-points to confirm DDX3X expression was reduced and assess necroptosis signaling (via phosphorylation of MLKL [p-MLKL]) in unstressed or thapsigargin-stressed BMDMs treated with zVAD + TNF. (D) Primary BMDMs derived from control (Ddx3x<sup>fl/fl</sup>) or myeloid-specific Lyz2<sup>cre</sup>Ddx3x<sup>fl/fl</sup> mice were
保护从细胞坏死中恢复的前应激巨噬细胞。先前的研究发现，细胞坏死和NLRP3依赖的细胞坏死是由Ifosfamide介导的。

整合应激反应（ISR）的细胞坏死

以前的研究表明，ISR（细胞坏死）和细胞坏死（Cell Death）之间存在联系。细胞坏死是由Ifosfamide介导的。

细胞坏死的方向

在我们的实验中，我们发现ISR和细胞坏死的分子机制不同。这些发现可能有助于我们了解细胞坏死的分子机制。


treated as indicated, and necroptosis was quantified by automated analysis of Sytox Green-positive nuclei. Ddx3x*fl/fl* and Lyz2*cre*fl/fl* quantifications overlap upon zVAD + Ars + TNF treatment. (f) Representative images (6 h) and Incubation quantification of NLRP3-dependent pyroptosis (via LPS + nigericin treatment) in BMDMs derived from the indicated genotypes. Significance was determined (B, D, E) by two-way ANOVA followed by (B, D) Dunnett’s or (E) Sidak’s multiple comparisons test, ****p < 0.0001. Data are generated from three images per replicate well (n = 3) and are representative of at least three independent biological replicate experiments. Scale bar (black) indicates 50 μm. Data are presented as mean ± SEM.

Mice

Wildtype (C57BL/6), Ripk1*fl/+* (indicated as Ripk1*KD/KD* [kinase-dead]) (Berger et al, 2014), Ripk3*−/−* (Newton et al, 2004), Mlh1*−/−* (Murphy et al, 2013), Lyz2*Mmp3*KO/R* (indicated as Tbk1*KO/F*) conditional knockout (generated by crossing B6.129P2-Lyz2*cre*fl/fl* [The Jackson Laboratory] and Tbk1*KO/F* [Xie et al, 2006]), and Lyz2*cre*Ddx3x*fl/fl* (generated by crossing B6.129P2-Lyz2*cre*fl/fl* [The Jackson Laboratory] and Ddx3x*fl/fl* [Samir et al, 2019] mice) were maintained on the B6 background. Male and female mice were used in this study at 6–10 wk of age. Mice were bred at St. Jude Children’s Research Hospital, and studies were conducted under protocols approved by St. Jude Children’s Research Hospital Committee on the Use and Care of Animals.

BMDM culture

Primary BMDMs were grown for 6 d in IMDM (12440-053; Thermo Fisher Scientific) supplemented with 10% FBS (S1620; BioWest), 30% L929-conditioned media, 1× nonessential amino acids (11140050; Gibco), and 1× penicillin-streptomycin (15070063; Thermo Fisher Scientific) (Tweedell et al, 2020). BMDMs were seeded in DMEM (11995-073; Thermo Fisher Scientific) on 12-well plates, for immunoblot analysis) or 5 × 10⁵ cells (24-well plates, for

Materials and Methods

References

Aulas et al, 2017; Markmiller et al, 2018; Yoon et al, 2018). These differences in cell types may also explain phenotypic differences in necroptosis observed across different cell types and cell lines, as we also observed.

The contribution of necroptosis, the ISR, and SGs to various diseases is still poorly understood. Necroptosis contributes to lethal developmental defects in mice lacking FADD, CASP8, and RIPK1, but mice lacking only RIPK3 or MLKL are viable, suggesting necroptosis plays an important role largely in cases where apoptosis is dysregulated (Dannappel et al, 2014; Dillon et al, 2014; Newton et al, 2016). In humans, MLKL mutations have recently been linked to autoinflammatory diseases and neurodegeneration (Faergeman et al, 2020; Hildebrand et al, 2020). Microbes which interfere with inflammatory signaling and apoptotic pathways have also been shown to induce necroptosis, suggesting necroptosis plays an important role in host protection (Kitur et al, 2016; Yu et al, 2018; Zhang et al, 2020). Similarly, pathogens often inhibit or hijack the ISR to facilitate viral replication or alter inflammatory signaling pathways (Rodrigues et al, 2018; Zhang et al, 2019). The crosstalk between the ISR and necroptosis is likely to have important implications for pathogen control. SGs are also implicated in neurological disorders such as amyotrophic lateral sclerosis, frontal temporal lobar dementia, and fragile X syndrome (Mahboubi & Stochaj, 2017; Wolozin & Ivanov, 2019; Wang et al, 2020), and they can regulate cancer cell growth and responses to chemotherapeutic drugs, suggesting a context- and cell-dependent role for SGs in regulating cancer cell death (Anderson et al, 2015; Zhan et al, 2020). In our study, we identified a significant role for the ISR in negatively regulating necroptosis signaling pathways, which improves our understanding of the mechanisms by which stress responses, SGs, and programmed cell death may broadly be targeted therapeutically.
Figure 4. Disruption of SGs restores necroptosis in stressed BMDMs. Disruption of SGs by anisomycin (Aniso) treatment in thapsigargin (Thaps) pre-stressed cells was examined. (A) Confocal microscopy images were obtained from primary BMDMs treated as indicated and stained for G3BP1 (red), DDX3X (green), and DAPI (blue), and SG signal intensities were compared. (B) Schematic for disrupting SGs in thapsigargin pre-stressed cells treated with Aniso before necroptosis induction. (C, D) Representative images of BMDMs (treated as indicated in panel B) and quantification of necroptosis (D) by uptake of membrane-impermeant Sytox Green (green, with red analysis mask outline). (E) Immunoblots were performed to determine the effect of SG disruption by Aniso on necroptosis signaling. Significance was determined (A) by Mann–Whitney test or (D) by two-way ANOVA followed by
Incucyte cell death analysis) and incubated overnight. Cells were then washed, cultured, and stimulated in DMEM containing 10% FBS.

**MEF culture**

MEFs were derived by minimal passage of cells in DMEM containing 10% FBS and 1× penicillin-streptomycin. MEFs were seeded overnight at 1 × 10^6 cells in a 24-well plate before stimulation.

**Primary BMDM and MEF cell stimulations**

Cells were pre-stressed with SG-inducing agents: thapsigargin (2 μg/ml [10522; Cayman Chemical]); brefeldin A (3 μg/ml [00-4505-5]); Thermo Fisher Scientific); tunicamycin (20 μg/ml [3516; Tocris]); MG132 (25 μM [474790; Calbiochem]); sodium (meta) arsenite (50 μM [S7400; Sigma-Aldrich]); rocaglamide A (250 nM [Hy-19356; MedChemExpress]) 1 h or 30 min (for arsenite) before addition of TNF (25 ng/ml) unless otherwise specified. To induce necroptosis, cells were pretreated where indicated with zVAD (50 μM) 1 h before TNF, LPS (100 ng/ml [tir-1-smlps; InvivoGen]), or TAK1 inhibitor (5Z-7-oxozeaenol [0.1 μM; Cayman Chemical]) treatment. In SG disruption experiments, anisomycin (25 μg/ml) or cycloheximide (25 μg/ml) were added 15 min before TNF, where indicated. Pyroptosis was induced by priming cells for 4 h with LPS (100 ng/ml) followed by addition of nigericin (20 μM [11437; Cayman Chemical]).

**siRNA knockdown of Perk**

The siGENOME siRNA SMARTpool containing four siRNAs targeting Perk (Horizon; M-044901-01-005) was used. A total of 5 nmol was dissolved in nuclease-free water to a concentration of 50 μM, and 0.5 μl siRNA was added to 1 × 10^6 BMDMs. Electroporation was performed using the Neo transfection system (Invitrogen), with parameters −1,500 V, 1 pulse and 20-μs width. Mock transfection was performed as described but without the addition of siRNA. After electroporation, BMDMs were immediately transferred into 12-well plates with a seeding density of 1 × 10^6 cells per well. BMDMs were pretreated with thapsigargin and zVAD followed by TNF to induce necroptosis as above 48 h after transfection.

**Microscopy**

To image and quantify cell death over time, images were automatically collected using an IncuCyte S3 (Essen Biosciences). To quantify cell death, cells were stimulated in media containing 25 nM Sytox Green (S7020; Thermo Fisher Scientific), and Sytox+ nuclei were quantified by automated analysis using the Basic Analyzer module provided with the Incucyte software (v2018C). Three images were collected for each replicate well (n = 3) in each experiment and Sytox+ nuclei counts were exported as Object Count (Sytox+ nuclei) per well values, which extrapolates the total object count based on the count per image, area of image acquisition, and the total area of the well. For confocal microscopy, cells were seeded in chamber slides (80055 or 80826, Ibidi). BMDMs were stimulated, then fixed in 4% PFA, permeabilized with 0.1% Triton X-100, blocked in 5% BSA/PBS-T (0.1% Tween-20), and stained with indicated primary antibodies for G3BP1 (66486-1-ig; Proteintech, 1:250) or DDX3X (A300-474A; Bethyl Laboratories, 1:250) overnight at 4°C in blocking solution. Cells were then washed with PBS-T, incubated with appropriate secondary antibodies conjugated with fluorophores (A-11001, A-11004, A-11008, or A-11011; Thermo Fisher Scientific, 1:250) for 2 h at room temperature, washed, and imaged on a Nikon C2 confocal microscope. SG signal intensity analysis was determined in Imaris 9.3 (Oxford Instruments). SG objects were identified by generating a colocalization channel for G3BP1 (signal threshold 500-4,095)/DDX3X (signal threshold (2,000-4,095)) followed by SG object identification [Surfaces menu] to obtain object signal intensities [Intensity-Sum] values for all objects (threshold 100-4,095) normalized to media control.

**Immunoblotting analysis**

For signaling blots, supernatant was removed, and cells were lysed in RIPA buffer containing protease and phosphatase inhibitors plus 4× Laemmli sample buffer. Proteins were separated via SDS–PAGE with 8–12% polyacrylamide gels, transferred to PVDF membranes (IPVH00010; Millipore), and blocked with 5% nonfat dry milk. Primary antibodies against phospho-MLKL (Ser345) (37333; Cell Signaling Technologies [CST], 1:1,000), MLKL (37705; CST, 1:1,000), phospho-RIPK3 (Thr231/Ser232) (91702; CST, 1:1,000), RIPK3 (2283; ProSci, 1:1,000), phospho-eIF2α (Ser51) (3398; CST, 1:1,000), eIF2α (63212; CST, 1:1,000), β-actin (8457; CST, 1:1,000), and phospho-PI3K (Ser345) (38662; CST, 1:1,000), DDX3X (A300-474A; Bethyl Laboratories, 1:1,000), PERK (3192; CST, 1:1,000), or TAK1 (58326; CST, 1:1,000) were immunoblotted with appropriate secondary antibodies conjugated with HRP incubated for 1 h at room temperature (Jackson ImmunoResearch). Membranes were visualized using Luminata Forte Chemiluminescence substrate (WBFLU0500; Millipore) or SuperSignal West Femto substrate (34096; Thermo Fisher Scientific) on a Bio-Rad ChemiDoc.

**Quantification and statistical analysis**

GraphPad Prism 6.0 or Imaris 9.3 software were used for data analysis. Data are shown as mean ± SEM. Statistical significance was determined by t test for two groups or one-way ANOVA for three or more groups and two-way ANOVA for comparison between multiple groups. The specific statistical testing for each experiment is indicated in the figure legends.
Figure 5. Knockdown of Perk restores necroptosis in thapsigargin pre-stressed BMDMs.

Primary BMDMs were stimulated as indicated (A, B) Representative IncuCyte images of mock siRNA or Perk siRNA knockdown BMDMs treated as indicated (A) and necroptosis was quantified (B) by uptake of membrane-impermeant Sytox Green (green, with red analysis mask outline) (C) Confocal images of mock siRNA or Perk siRNA treated BMDMs treated as indicated and stained for G3BP1 (green), DDX3X (red), and DAPI (blue). (D) Immunoblots were performed to determine whether siRNA (mock or Perk) knockdown restored necroptosis signaling in pre-stressed BMDMs. Data are representative of a single independent biological replicate experiment with IncuCyte quantification performed on two replicate wells containing a total of eight image fields. Significance was determined by two-way ANOVA followed by Tukey’s multiple comparisons test (B). ****P < 0.0001. Data are presented as mean ± SEM. Scale bars indicate (black) 50 μm or (white) 10 μm.
Supplementary Information

Supplementary Information is available at https://doi.org/10.26508/lsa.202101260.

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Author Contributions

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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