Interferons Transcriptionally Up-Regulate MLKL Expression in Cancer Cells

Anne-Kathrin Knuth*,2, Stefanie Rösler*,2, Barbara Schenk*, Lisa Kowald*, Sjoerd J.L. van Wijk* and Simone Fulda*†,‡

*Institute for Experimental Cancer Research in Pediatrics, Goethe-University, Frankfurt, Germany; †German Cancer Consortium (DKTK), Partner Site, Frankfurt, Germany; ‡German Cancer Research Center (DKFZ), Heidelberg, Germany

Abstract

Interferons (IFNs) are key players in the tumor immune response and act by inducing the expression of IFN-stimulated genes (ISGs). Here, we identify the mixed-lineage kinase domain-like pseudokinase (MLKL) as an ISG in various cancer cell lines. Both type I and type II IFNs increase the expression of MLKL indicating that MLKL up-regulation is a general feature of IFN signaling. IFNγ up-regulates mRNA as well as protein levels of MLKL demonstrating that IFNγ transcriptionally regulates MLKL. This notion is further supported by Actinomycin D chase experiments showing that IFNγ-stimulated up-regulation of MLKL is prevented in the presence of the transcriptional inhibitor Actinomycin D. Also, knockdown of the transcription factor IFN-regulatory factor 1 (IRF1) and signal transducer and activator of transcription (STAT) 1 as well as knockout of IRF1 significantly attenuate IFNγ-mediated induction of MLKL mRNA levels. Up-regulation of MLKL by IFNγ provides a valuable tool to sensitize cells towards necroptotic cell death and to overcome apoptosis resistance of cancer cells.

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Introduction

One hallmark of cancer is the evasion of regulated modes of cell death such as apoptosis [1]. Necroptosis is a recently identified type of regulated cell death which is activated when caspases are inhibited or absent [2]. Typical inducers of necroptosis such as tumor necrosis factor alpha (TNFα) or IFNs activate the receptor-interacting protein kinases (RIPK) 1 and 3 by phosphorylation [2]. Subsequently, RIPK3 phosphorylates the pseudokinase MLKL which oligomerizes upon its activation, translocates to the plasma membrane and executes necroptosis, for example by forming pores in the plasma membrane or by interacting with ion channels [3–8].

IFNs are cytokines with antiviral and growth-inhibitory functions and can be divided into three major classes, type I (α and β), type II (γ) and type III (λ) [9,10]. IFNγ plays a crucial role in coordinating the tumor immune response and the recognition and elimination of tumor cells by immune cells [10]. Binding of IFNs to their cell surface receptor leads to activation of the Janus kinase STAT (JAK–STAT) pathway. As a result, STAT1 is phosphorylated by JAK, translocates to the nucleus and induces the expression of ISGs [9]. IFNs are able to stimulate the expression of hundreds of genes, some of which are regulated by all IFNs, while some others only by specific IFNs [9,11]. The expression of IRF1 is preferentially induced by IFNγ [12]. IRF1 is a member of the IRF family of transcription factors, a regulator of IFNs and ISGs [13] and also exerts antiviral functions by restricting the replication of certain classes of viruses [14,15].

Abbreviations: AML, acute myeloid leukemia; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen; FCS, fetal calf serum; FDA, Food and Drug Administration; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFN, interferon; IRF1, IFN-regulatory factor 1; ISG, IFN-stimulated gene; JAK, Janus kinase; MEF, mouse embryonic fibroblast; MLKL, mixed-lineage kinase domain-like; ns, non-silencing; NSA, necrosulfonamide; PI, propidium iodide; pSTAT1, phospho-STAT1; RIPK, receptor-interacting protein kinase; STAT, signal transducer and activator of transcription; TNFα, tumor necrosis factor alpha; zVAD.fmk, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

Address all correspondence to: Prof. Dr. Simone Fulda, Institute for Experimental Cancer Research in Pediatrics, Goethe-University, Komturstrasse 3a, 60528 Frankfurt. E-mail: simone.fulda@kgu.de

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IFNs have been reported to induce necroptosis by activation of the JAK–STAT pathway [2,16–20]. We have previously shown that in various cancer cell lines IRF1 contributes to Smac mimetic/IFN-γ-induced necroptosis [21]. However, the exact mechanisms of IFN-mediated necroptosis remain so far elusive [16,19]. To better understand how IFN signaling regulates necroptosis in the present study we analyze the effect of IFN-γ on MLKL expression.

Materials and Methods

Cell Culture and Chemicals
EFM-192A, HeLa, MV4–11 and HT-29 cells were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and MDA-MB-231 and HEK 293 T cells from ATCC (American Type Culture Collection, CEM, Manassas, VA, USA). EFM-192A and MV4–11 cells were cultured in RPMI Medium 1640 GlutaMAX-I (Life Technologies, Inc., Eggenstein, Germany), HeLa, HEK 293 T and MDA-MB-231 cells in DMEM Medium (Life Technologies) and HT-29 cells were cultured in McCoy medium (Life Technologies), each supplemented with 10–20% fetal calf serum (FCS, Life Technologies), 1% penicillin/streptomycin (Life Technologies) and 1% sodium pyruvate (Life Technologies). Cell lines were authenticated by STR profiles and negatively tested for mycoplasma contamination. N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) was obtained from Bachem (Heidelberg, Germany), IFN-β from Biochrom (Ltd., Berlin, Germany), IFN-γ from Merck & Co., Inc. (Darmstadt, Germany) and necrosulfonamide (NSA) from Calbiochem Merck & Co. Inc. The bivalent Smac mimetic BV6 was kindly provided by Genentech, Inc. (South San Francisco, CA, USA) [22]. All other chemicals were obtained from Sigma-Aldrich (Taufkirchen, Germany) or Carl Roth (Karlsruhe, Germany), unless otherwise indicated.

siRNA Transfection and Quantitative Real-Time PCR
Cells were transfected with 20 nM Silencer Select siRNA (Invitrogen, Karlsruhe, Germany), i.e. non-silencing siRNA (no. 4390844) or targeting siRNA (#1: s7501 and #2: s7502 for IRF1, #1: s7501 and #2: s7502 for IRF1), respectively.
s277 and #2: s278 for STAT1) using Lipofectamine RNAi MAX (Invitrogen) and OptiMEM (Life Technologies, Inc.). Total RNA was isolated using peqGOLD Total RNA kit (Peqlab, Erlangen, Germany) according to the manufacturer’s instructions; 1 μg of total RNA was used for cDNA synthesis using the RevertAid H Minus First Stand cDNA Synthesis Kit (MBI Fermentas GmbH, St. Leon-Rot, Germany) according to the manufacturer’s protocol with the use of random primers. For quantification of gene expression levels, SYBR green-based quantitative real-time PCR (Applied Biosystems, Darmstadt, Germany) was performed using the 7900GR fast real-time PCR system (Applied Biosystems). Data were normalized to 28S-rRNA expression. Relative expression levels of the target transcript were calculated compared to the reference transcript by using the \( \Delta \Delta C_T \) method [23]. At least three independent experiments in duplicates are shown. All primers were purchased by Eurofins (Hamburg, Germany; suppl. Table 1).

**Generation of IRF1 CRISPR/Cas9 Knockout MDA-MB-231 Cells**

MDA-MB-231 IRF1 knockout cells were generated as described previously [24]. Briefly, three IRF1 guide RNAs were designed with 5’ and 3’ BsmB1 restriction site overhangs (suppl. Table 2), annealed and ligated into pLentiCRISPRv2 (Addgene plasmid # 52961). Lentiviral particles were generated by co-transfecting pLentiCRISPRv2 IRF1 gRNAs with pPAX2 (Addgene plasmid # 12260) and pMD2.G (Addgene plasmid # 12259) in HEK293T cells and used to transduce MDA-MB-231 cells with puromycin selection. IRF1 knockout efficiency was confirmed using Western blot analysis with IRF1 antibodies.

**Western Blot Analysis**

Western blot analysis was performed as described previously [25] using the following antibodies: Mouse anti-STAT1 (Cell Signaling, Beverly, MA, USA), rabbit anti-phospho-STAT1 (Cell Signaling), mouse anti-IRF1 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), rabbit anti-MLKL (GeneTex, Inc., Irvine, CA, USA), rabbit anti-phospho-MLKL (Cell Signaling) mouse anti-GAPDH (HyTest, Turku, Finland), mouse anti-β-Actin (Sigma-Aldrich), mouse anti-Vinculin (Sigma-Aldrich). Goat anti-mouse IgG or goat anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnologies) and enhanced chemiluminescence (Amersham Bioscience, Freiburg, Germany) or infrared dye-labeled secondary antibodies and infrared imaging (Odyssey Imaging System, LI-COR Bioscience, Bad...
Western blotting.

β-phospho-STAT1 (pSTAT1), STAT1 and μ or 20

Figure 4. Caspase activity is dispensable for IFN

Inhibition of transcription prevents IFN

Figure 3. Inhibition of transcription prevents IFNγ-induced MLKL expression. (A) EFM-192A cells were treated with 1.5 ng/ml IFNγ for indicated time points with or without pretreatment with 100 nM Actinomycin D for 2 hours. Protein expression of MLKL, IRF1, pSTAT1, STAT1 and β-Actin was analyzed by Western blotting after indicated time points. (B) mRNA levels of MLKL were quantified via RT-PCR 9 hours after IFNγ treatment and are shown as fold increase to untreated control cells with mean and SEM of at least two independent experiments performed in duplicate; *P < .05.

Figure 4. Caspase activity is dispensable for IFNγ-induced MLKL expression. EFM-192A cells were treated with 1.5 ng/ml IFNγ and/or 20 μM zVAD.fmk for 24 hours. Protein expression of MLKL, phospho-STAT1 (pSTAT1), STAT1 and β-Actin was analyzed by Western blotting.

Determination of Cell Death

Cell death was assessed by propidium iodide (PI)/HOECHST staining to determine plasma membrane permeability using ImageXpress Micro XLS system (Molecular Devices, LLC, Biberach an der Riss, Germany) according to the manufacturer’s instructions.

Statistical Analysis

Statistical significance was assessed by Student’s t-test (two-tailed distribution, two-sample, equal variance) using Microsoft Excel (Microsoft Deutschland GmbH, Unterschleißheim, Germany); *P < .05; **P < .01; ***P < .001.

Results

Type I and Type II IFNs Increase MLKL mRNA and Protein Expression

To investigate whether IFNs can stimulate expression of MLKL we assessed MLKL levels upon treatment with IFNγ using EFM-192A breast carcinoma and HeLa cervical carcinoma cells as model systems. Importantly, IFNγ significantly increased MLKL mRNA levels in a time-dependent manner in both cell lines (Figure 1A). This increase in MLKL expression was accompanied by a massive up-regulation of mRNA expression of IRF1, one of the key transcription factors of the IFN pathway [13]. Next, we determined whether the increase in MLKL mRNA levels also results in elevated protein expression. Indeed, Western blot analysis showed a time-dependent up-regulation of MLKL protein expression in IFNγ-treated EFM-192A and HeLa cells (Figure 1B). This was preceded by an increase in IRF1 and STAT1 expression as well as phosphorylation of STAT1 (Figure 1B), consistent with activation of IFN signaling.

To investigate whether both type I and type II IFNs can stimulate MLKL expression we tested also IFNα and IFNβ in addition to IFNγ. Similarly, IFNα and IFNβ caused enhanced MLKL protein expression, accompanied by an increase in STAT1 expression (Figure 2A). Furthermore, we extended our experiments to additional cancer cell lines to test the general relevance of our findings. Importantly, IFNα, IFNβ and IFNγ stimulated expression of MLKL protein also in HeLa, MV4-11 acute myeloid leukemia (AML) and MDA-MB-231 breast carcinoma cells (Figure 2, B–D). Quantification of protein expression levels confirmed significant up-regulation of MLKL protein upon treatment with IFNα, IFNβ or IFNγ (Figure 2, E and F). By comparison, IFNγ had no or little effects on expression levels of other necroptosis signaling proteins such as RIPK1 and RIPK3 (suppl. Figure 1). To determine whether up-regulation of MLKL leads to its activation, we analyzed MLKL phosphorylation. We detected little MLKL phosphorylation after IFNγ treatment as compared to a positive control treated with a prototypic necroptotic stimulus (suppl. Figure 2), indicating that IFNγ-stimulated up-regulation of MLKL alone may not be sufficient to initiate necroptotic cell death.

Furthermore, we used the database Interferome [26] to search for IFN-dependent up- or down-regulation of MLKL in published databases. Interestingly, we found a more than 2-fold up-regulation of MLKL in 71 datasets (suppl. Table 3). Together, these data show that

Homburg, Germany) were used for detection. Representative blots of at least two independent experiments are shown. Protein expressions of Western blots were quantified using ImageJ 1.52e and normalized to β-Actin protein expression.
A

IFNγ - + - + - + - +
IRF1
GAPDH
53 kDa
36 kDa
pSTAT1
STAT1
84/91 kDa
84/91 kDa
GAPDH
36 kDa

B

MLKL mRNA levels [fold change]

IFNγ - + - + - + - +
siCtrl
siIRF1#1 + siSTAT1#1
siIRF1#1 + siSTAT1#2
siIRF1#2 + siSTAT1#1
siIRF1#2 + siSTAT1#2

C

IFNγ - + - +
WT
IRF1 KO
MLKL
Vinculin
IRF1
Vinculin
54 kDa
116 kDa
53 kDa
116 kDa
type I and type II IFNs increase MLKL expression in several cancer cell lines.

**IFNγ Transcriptionally Increases MLKL Expression**

To explore whether IFN-triggered up-regulation of MLKL occurs via increased transcription we performed an Actinomycin D chase experiment. Actinomycin D is a well-known inhibitor of transcription that blocks RNA synthesis [27]. To this end, we incubated cells for 2 hours with Actinomycin D to inhibit RNA synthesis before exposure to IFNγ. Intriguingly, IFNγ-stimulated up-regulation of MLKL was abolished in the presence of Actinomycin D compared to control cells treated with IFNγ in the absence of Actinomycin D both on the protein and mRNA level (Figure 3, A and B). Consistently, the increase in IRF1 and STAT1 expression upon exposure to IFNγ was substantially reduced in the presence of Actinomycin D (Figure 3A). This demonstrates that transcription is required for IFNγ-induced expression of MLKL and confirms that IFNγ transcriptionally increases MLKL expression.

**Caspase Activity is Dispensable for IFNγ-Induced Up-Regulation of MLKL**

As IFNγ is known to induce and activate caspases [28,29], we investigated whether caspase activity is necessary for the up-regulation of MLKL upon IFNγ treatment. To this end, we blocked caspase activity using the broad-range caspase inhibitor zVAD.fmk. Addition of zVAD.fmk did not prevent the IFNγ-induced increase in MLKL expression (Figure 4). In parallel, IFNγ similarly stimulated phosphorylation and expression of STAT1 in the presence and absence of zVAD.fmk (Figure 4). This indicates that caspase activity is dispensable for IFNγ-induced up-regulation of MLKL.

Also, we explored whether IFNγ induces necroptotic cell death when caspase activation is simultaneously blocked. Indeed, treatment with IFNγ caused a significant increase in cell death in the presence of the broad-range caspase inhibitor zVAD.fmk (suppl. Figure 3). As we observed that the IFNγ-induced up-regulation of MLKL is accompanied by an increased expression of IRF1 and STAT1, we next asked whether these transcription factors are required to up-regulate MLKL. To address this question we silenced in parallel IRF1 and STAT1 by siRNA, using two independent sequences for each target gene. As control we used a non-silencing siRNA sequence with no counterpart in the human genome. Western blot experiments confirmed efficient knockdown of IRF1 and STAT1 (Figure 5A). Importantly, silencing of IRF1 and STAT1 significantly reduced IFNγ-induced increase of MLKL expression compared to control cells transfected with non-silencing siRNA (Figure 5B). This indicates that IRF1 and STAT1 contribute to MLKL up-regulation by IFNγ.

To further explore the role of IRF1 we created IRF1 knockout MDA-MB-231 cells using CRISPR/Cas9 technology. Efficient IRF1 knockout was confirmed by Western blotting (Figure 5C). Importantly, IRF1 knockout prevented IFNγ-stimulated up-regulation of MLKL (Figure 5C), confirming that IRF1 contributes to MLKL up-regulation by IFNγ.

**Discussion**

In the present study, we show that MLKL is an ISG up-regulated by IFNs in an IRF1- and STAT1-dependent manner in cancer cells. This up-regulation of MLKL is a common feature of IFN signaling, since both type I and type II IFNs increase MLKL expression. In addition, IFN-dependent increase in MLKL expression has consistently been observed in several cell lines of different cancer entities, thus emphasizing the general relevance of this finding. The conclusion that IFNs enhance MLKL levels transcriptionally is underscored by Actinomycin D chase experiments, showing that active transcription is required for IFN-induced increase of MLKL expression. Also, prior to IFN-stimulated up-regulation of MLKL mRNA levels, the transcription factor STAT1 is rapidly phosphorylated, which marks its activation. Additional ISGs like IRF1 are transcriptionally up-regulated upon IFN treatment as well. IRF1 belongs to the IRF family of transcription factors that play an important role during IFN signaling [13]. Prediction analysis using the Eukaryotic Promoter Database confirmed that the promoter of MLKL contains both STAT1 and IRF1 binding sites, indicating that these transcription factors stimulate MLKL transcription by directly activating the MLKL promoter. Furthermore, MLKL was found to be up-regulated in a number of published datasets of IFN-regulated genes. Together, there are several lines of evidence underscoring that IFNs transactivate MLKL expression.

Our study defines MLKL as an ISG, in line with recent reports. Using DNA microarray analysis, MLKL has previously been described to be up-regulated by IFNβ or IFNγ in mouse embryonic fibroblasts (MEFs) [16]. Also, increased protein levels of MLKL have been reported upon IFN treatment in MEFs, HT-29 colon carcinoma cells and mouse dental follicle cells [30–32]. In addition, hepatotoxicity of IFNγ in a mouse model of hepatitis has recently been linked to transcriptional up-regulation of MLKL via STAT1, as shown by promoter luciferase assay [33]. In the present study, we show that, in addition to STAT1, also the transcription factor IRF1 contributes to up-regulation of MLKL by IFN. Of note, IRF1 has recently been shown to be required for 5mac mimic/IFNγ-induced necroptosis [21], suggesting that IRF1-mediated up-regulation of MLKL promotes necroptosis. By comparison, caspase activity turned out to be dispensable for IFN-induced up-regulation of MLKL.

A schematic diagram summarizes our findings (Figure 6). IFNγ treatment leads to activation and phosphorylation of STAT1, which activates the expression of ISGs like IRF1. Transcription factors such as STAT1 and IRF1 contribute to MLKL up-regulation, sensitizing cells towards necroptosis when caspases are inhibited. These findings highlight the relevance of MLKL up-regulation to overcome apoptosis resistance in cancer cells and to sensitize cancer cells that are refractory to undergo caspase-dependent apoptosis towards necrotic cell death. In line with this notion, constitutive IFN...
signaling has recently been reported to maintain a critical threshold of MLKL expression to license necroptosis [34]. Besides its function in necroptosis, MLKL has been shown to regulate endosomal trafficking and the generation of extracellular vesicles and to contribute to inflammasome activation [35–40].

Our study has several important implications. First, IFN-γ-induced up-regulation of MLKL may well contribute to necroptotic cell death when caspases are inhibited, as IFN-γ significantly increased cell death in the presence of zVAD.fmk. Furthermore, the MLKL inhibitor NSA significantly rescued cells from IFN-γ-induced cell death in the presence of zVAD.fmk, as we have shown previously [21]. Second, IRF1 may facilitate IFN-induced necrotic cell death via transcriptional up-regulation of MLKL, thus providing a molecular explanation of how IRF1 contributes to IFN-γ-mediated potentiation of Smac mimic-induced necroptosis when caspases are inhibited [21]. Third, IFNs play a crucial role in coordinating the tumor immune response by recognition and elimination of tumor cells by immune cells [10]. Indeed, IFNα belongs to the agents approved by the Food and Drug Administration (FDA) for the treatment of cancer and IFN-γ has cytostatic and cytotoxic effects on cancer cells, depending on the context [41–44]. IFN-dependent up-regulation of MLKL and the resulting increased susceptibility to undergo necroptosis offer an additional explanation for their antitumor activity as immunotherapeutic agents. Taken together, up-regulation of MLKL by IFN-γ provides a valuable tool to sensitize cells towards necroptotic cell death and to overcome apoptosis resistance of cancer cells.

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Conflict of Interest
None to declare.

Appendix A. Supplementary Data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2018.11.002.

References
[1] Hanahan D and Weinberg RA (2011). Hallmarks of cancer: the next generation. Cell 144, 646–674.
[2] Vanden Berghe T, Linkermann A, Jouan-Lanhouet S, Walczak H, and Vandenabeele P (2014). Regulated necrosis: the expanding network of non-apoptotic cell death pathways. Nat Rev Mol Cell Biol 15, 135–147.
[3] Dondelinger Y, Declercq W, Montessuit S, Roelandt R, Goncalves A, Bruggeman J, Hulpiau P, Weber K, Schon CA, and Marquis RW, et al (2014). MLKL Compromises Plasma Membrane Integrity by Binding to Phosphatidylinositol Phosphates. Cell Rep 7, 971–981.
[4] Wang H, Sun L, Su L, Rizo J, Liu L, Wang LF, Wang FS, and Wang X (2014). Mixed lineage kinase domain-like protein MLKL causes necrotic membrane disruption upon phosphorylation by RIP3. Mol Cell 54, 133–146.
[5] Cai Z, Jitkaew S, Zhao J, Chiang HC, Choksi S, Liu J, Ward Y, Wu LG, and Liu ZG (2014). Plasma membrane translocation of trimerized MLKL protein is required for TNF-induced necroptosis. Nat Cell Biol 16, 55–65.
[6] Chen X, Li W, Ren J, Huang D, He WT, Song Y, Yang C, Li W, Zheng X, and Chen P, et al (2014). Translocation of mixed lineage kinase domain-like protein to plasma membrane leads to necrotic cell death. Cell Res 24, 105–121.
[7] Quarato G, Guy CS, Grace CR, Llambi F, Nourse A, Rodriguez DA, Wakefield R, Frase S, Moldoveanu T, and Green DR (2016). Sequential Engagement of Distinct MLKL Phosphatidylinositol-Binding Sites Executes Necroptosis. Mol Cell 61, 589–601.
[8] Xia B, Fang S, Chen X, Hu H, Chen P, Wang H, and Gao Z (2016). MLKL forms cation channels. Cell Res 26, 517–528.
[9] Platanias LC (2005). Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat Rev Immunol 5, 375–386.
[10] Seliger B, Ruiz-Cabello F, and Garrido F (2008). IFN inducibility of major histocompatibility antigens in tumors. Adv Cancer Res 101, 249–276.
[11] Schneider WM, Chevillotte MD, and Rice CM (2014). Interferon-stimulated genes: a complex web of host defenses. Ann Rev Immunol 32, 513–545.
[12] Der SD, Zhou A, Williams BR, and Silverman RH (1998). Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. *Proc Natl Acad Sci U S A* **95**, 15623–15628.

[13] Ikushima H, Negishi H, and Taniguchi T (2013). The IRF family transcription factors at the interface of innate and adaptive immune responses. *Cold Spring Harb Symp Quant Biol* **78**, 105–116.

[14] Kimura T, Nakayama K, Penninger J, Kiragawa M, Harada H, Matsuyama T, Tanaka N, Kaino R, Vilkiz J, and Mak TW, et al (1994). Involvement of the IRF-1 transcription factor in antiviral responses to interferons. *Science* **264**, 1921–1924.

[15] Hazra B, Kumawat KL, and Basu A (2017). The host microRNA miR-301a blocks the IRF1-mediated neuronal innate immune response to Japanese encephalitis virus infection. *Sci Signal* **10**, eaaf5185.

[16] Thapa RJ, Nooga S, Chen P, Maki JL, Lerro A, Andrade M, Rall GF, Degterev A, and Balachandran S (2013). Interferon-induced RIP1/RIP3-mediated necrosis requires PKR and is licensed by FADD and caspases. *Proc Natl Acad Sci U S A* **110**, E3109–3118.

[17] Lee SH, Kwon JY, Kim SY, Jung K, and Cho ML (2017). Interferon-gamma regulates inflammatory cell death by targeting necroptosis in experimental autoimmune arthritis. *Sci Rep* **7**, 10133.

[18] Legarda D, Justus SJ, Ang RL, Nikli N, Li W, Morán TM, Zhang J, Mizoguchi E, Zelic M, and Belllher MA, et al (2016). CYLD Proteolysis Protects Macrophages from TNF-Mediated Auto-necroptosis Induced by LPS and Cytokines. *Type I IFN*. *Cell* **15**, 2449–2461.

[19] McComb S, Cessford E, Alturki NA, Joseph J, Shrutinski B, Starreck JB, Gamero AM, Mossman KL, and Sad S (2012). Type I interferon induces necroptosis in macrophages in response to Salmonella enterica serovar Typhimurium. *Nat Immunol* **13**, 954–962.

[20] Cekay MJ, Roesler S, Frank T, Knuth AK, Eckhardt I, and Fulda S (2017). Smac mimetics and type II interferon synergistically induce necroptosis in various cancer cell lines. *Cancer Lett* **410**, 228–237.

[21] Varfolomeev E, Blankenship JW, Wayson SM, Fedorova AV, Kayagaki N, Garg P, Zobel K, Dynek JN, Elliot LO, and Wallweber HJ, et al (2007). IAP antagonists induce auto-ubiquitination of c-IAPs, NF-kappaB activation, and TNF alpha-dependent apoptosis. *Cell* **131**, 669–681.

[22] Livak KJ and Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2^(-Delta Delta C(T)) Method. *Methods* **25**, 402–408.

[23] van Wijk SJL, Fricke F, Herhaus L, Gupta J, Horte K, Pampaloni F, Grumati P, Kaulich M, Sou YS, and Komatsu M, et al (2017). Linear ubiquitination of cytosolic Salmonella Typhimurium activates NF-kappaB and restricts bacterial proliferation. *Nat Microbiol* **2**, 7066.

[24] Fulda S, Sieverts H, Friesen C, Herr I, and Debatin KM (1997). The CD95 (APO-1/Fas) system mediates drug-induced apoptosis in neuroblastoma cells. *Cancer Res* **57**, 3823–3829.

[25] Rusinova I, Forster S, Yu S, Kannan A, Masse M, Cumming H, Chapman R, and Hertzig PJ (2013). Interferome v2.0: an updated database of annotated interferon-regulated genes. *Nucleic Acids Res* **41**, D1040–D1046.

[26] Hurwitz J, Furth JH, Malamy M, and Alexander M (1962). The role of deoxyribonuclease acid in ribonucleic acid synthesis. III. The inhibition of the enzymatic synthesis of ribonucleic acid and deoxyribonuclease acid by actinomycin D and proflavin. *Proc Natl Acad Sci U S A* **48**, 1222–1230.

[27] Apelbaum A, Yarden G, Warszawski S, Harari D, and Schreiber G (2013). Type I interferons induce apoptosis by balancing cFLIP and caspase-8 independent of death ligands. *Mol Cell Biol* **33**, 800–814.

[28] Dai C and Krantz SB (1999). Interferon gamma induces upregulation and activation of caspases 1, 3, and 8 to produce apoptosis in human erythroid progenitor cells. *Blood* **93**, 3309–3316.

[29] Dillon CP, Weinlich R, Rodriguez DA, Cripps JG, Quarato G, Gurung P, Verbiatic KC, Brewer TL, Lambri F, and Gong YN, et al (2014). RIPK3 blocks early postnatal lethality mediated by caspase-8 and RIPK3. *Cell* **157**, 1189–1202.

[30] Rodriguez DA, Weinlich R, Brown S, Guy C, Fitzgerald P, Dillon CP, Oberst A, Quarato G, Low J, and Cripps JG, et al (2016). Characterization of RIPK3-mediated phosphorylation of the activation loop of MLK during necroptosis. *Cell Death Differ* **23**, 76–88.

[31] Tanzer MC, Khan N, Rickard JA, Etemadi N, Lalaooni N, Spall SK, Hildebrand JM, Segal D, Misasi M, and Chau D, et al (2017). Combination of IAP antagonist and IFNgamma activates novel caspase-10- and RIPK1-dependent cell death pathways. *Cell Death Differ* **24**, 481–491.

[32] Gunther C, He GW, Kremer AE, Murphy JM, Petrie EJ, Amann K, Vandenabeele P, Linkermann A, Poremba C, and Schleichler U, et al (2016). The pseudokinase MLKL mediates programmed hepaticellular necrosis independently of RIPK3 during hepatitis. *J Clin Invest* **126**, 4346–4360.

[33] Surhan J, Liu BC, Muendlein HJ, Weindel GG, Smitova I, Tang AY, Iylukha V, Sorokin M, Budzin A, and Fitzgerald KA, et al (2018). Constitutive interferon signaling maintains critical threshold of MLKL expression to license necroptosis. *Cell Death Differ* [E-pub ahead of print].

[34] Conos SA, Chen KW, De Nardo D, Hara H, Whitehead L, Nunez G, Masten SL, Murphy JM, Schroder K, and Vaux DL, et al (2017). Active MLKL triggers the NLRP3 inflammasome in a cell-intrinsic manner. *Proc Natl Acad Sci U S A* **114**, E961–969.

[35] Gutierrez KD, Davis MA, Daniel BP, Olsen TM, Ranji-Jain P, Tait SW, Gale Jr M, and Oberst A (2017). MLKL Activation Triggers RIPK3-Mediated Processing and Release of IL-1beta Independently of Gasdermin-D. *J Immunol* **199**, 2156–2164.

[36] Kang S, Fernandes-Alnemri T, Rogers C, Mayes L, Wang Y, Dillon C, Roback L, Kaiser W, Oberst A, and Sagara J, et al (2015). Caspase-8 scaffolding function and MLKL regulate NLRP3 inflammasome activation downstream of TLR3. *Nat Commun* **6**, 7515.

[37] 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 [e57].

[38] Yu SX, Chen W, Liu ZZ, Zhou FH, Yan SQ, Hu GQ, Qin XX, Zhang J, Ma K, and Du CT, et al (2018). Non-Hematopoietic MLKL Protects Against Salmonella Mucosal Infection by Enhancing Inflammasome Activation. *Front Immunol* **9**, 119.

[39] Zhang X, Fan C, Zhang H, Zhao Q, Liu Y, Xu C, Xie Q, Wu X, Xu Y, and Zhang J, et al (2016). MLKL and FADD Are Critical for Suppressing Progressive Lymphoproliferative Disease and Activating the NLRP3 Inflammasome. *Cell Rep* **16**, 3247–3259.

[40] Parker BS, Rautela J, and Hertzog PJ (2016). Antimutator actions of interferons: implications for cancer therapy. *Nat Rev Cancer* **16**, 131–144.

[41] Zaidi MR and Merlino G (2011). The two faces of interferon-gamma in cancer. *Clin Cancer Res* **17**, 6118–6124.

[42] Lee S and Margolin K (2011). Cytokines in cancer immunotherapy. *Cancers (Basel)* **3**, 3856–3893.

[43] Mocellin S, Pasquals S, Rossi CR, and Nitti D (2010). Interferon alpha adjuvant therapy in patients with high-risk melanoma: a systematic review and meta-analysis. *J Natl Cancer Inst* **102**, 493–501.