Interferons Transcriptionally Up-Regulate MLKL Expression in Cancer Cells[1]

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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].

Figure 1. IFNγ increases MLKL mRNA and protein levels over time. Cells were treated for indicated time points with 1.5 ng/ml IFNγ (EFM-192A) or 20 ng/ml IFNγ (HeLa). (A) mRNA levels of MLKL and IRF1 were quantified via RT-PCR and are shown as fold increase relative to untreated control cells with mean and SEM of at least three independent experiments performed in duplicate; *$P < .05$; ***$P < .001$. (B) Protein expression of MLKL, IRF1, phospho-STAT1 (pSTAT1), STAT1 and GAPDH was analyzed by Western blotting.

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/IFNγ-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 IFNγ 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), IFNβ from Biochrom (Ltd., Berlin, Germany), IFNγ 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:
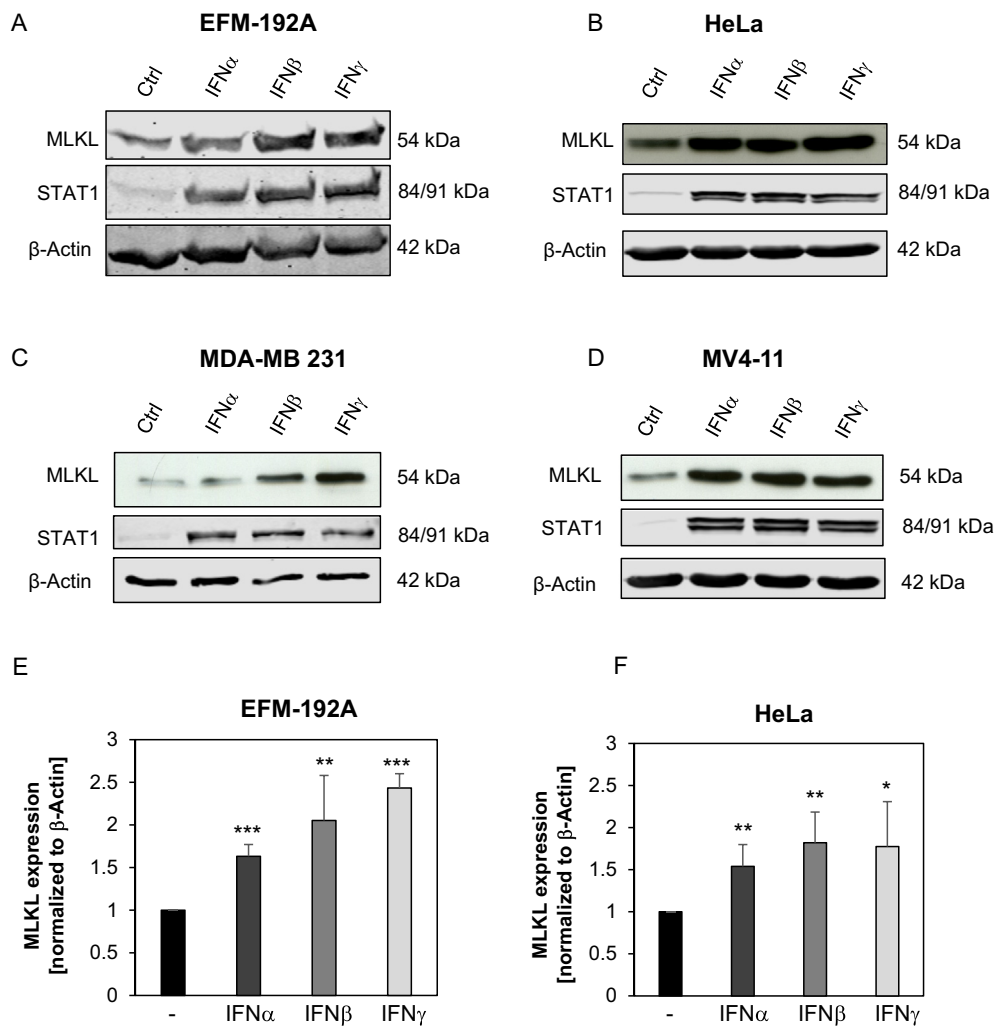
Figure 2. Type I and type II IFNs increase MLKL expression in different cancer cell lines. EFM-192A cells were treated with 1.5 ng/ml IFNα, β or γ for 24 hours (A), HeLa cells (B), MV4–11 (C) and MDA-MB-231 cells (D) were treated with 20 ng/ml IFNα, β or γ for 24 hours. Protein expression of MLKL, STAT1 and β-Actin was analyzed by Western blotting. (E and F) Western blots in A and B were quantified, and protein levels of MLKL are shown normalized to β-Actin expression of at least three independent experiments.

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 pLenti-CRISPRv2 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

A

B
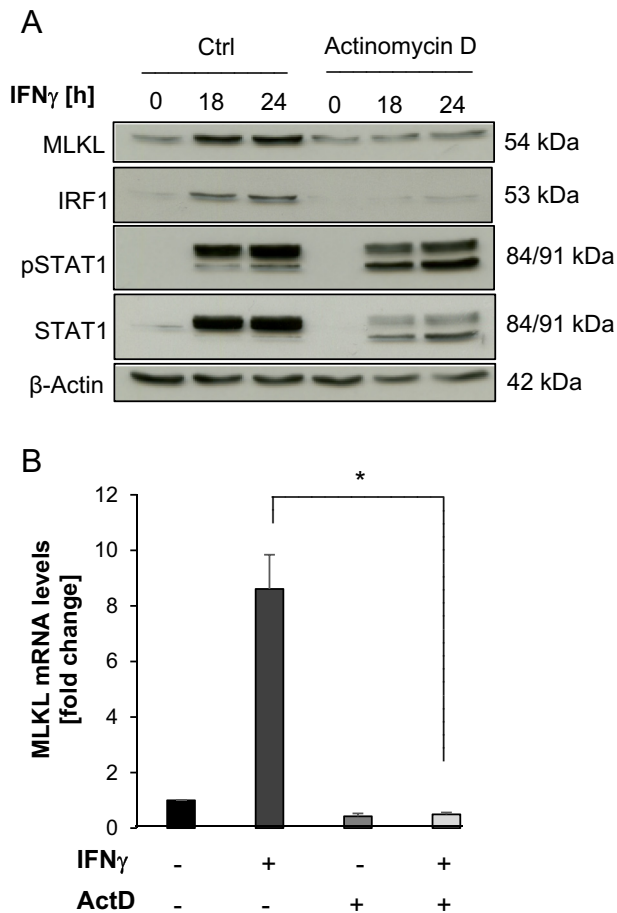
Figure 3. Inhibition of transcription prevents IFNγ-induced MLKL expression. (A) EFM-192A cells were treated with 1.5 ng/ml IFNγ for indicated time points with or without pretreatment with 100 nM Actinomycin D for 2 hours. Protein expression of MLKL, IRF1, pSTAT1, STAT1 and β-Actin was analyzed by Western 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 three independent experiments performed in duplicate; *P < .05.
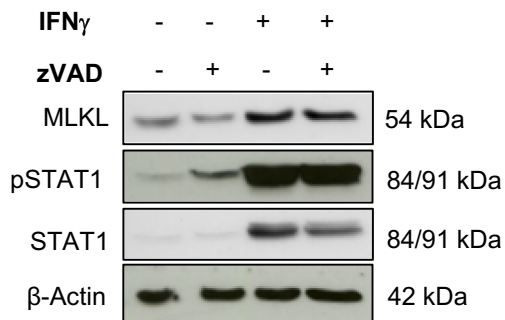
Figure 4. Caspase activity is dispensable for IFNγ-induced MLKL expression. EFM-192A cells were treated with 1.5 ng/ml IFNγ and/ or 20 μM zVAD.fmk for 24 hours. Protein expression of MLKL, phospho-STAT1 (pSTAT1), STAT1 and β-Actin was analyzed by Western blotting.

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.

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
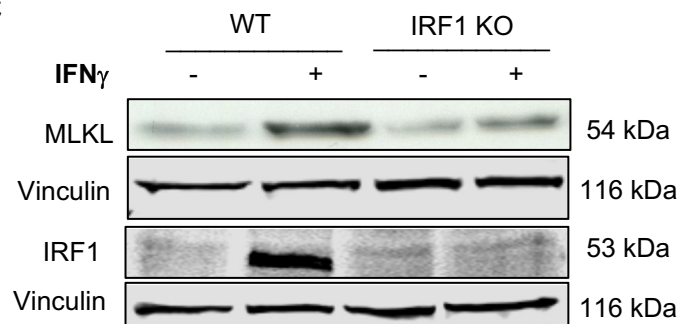
A
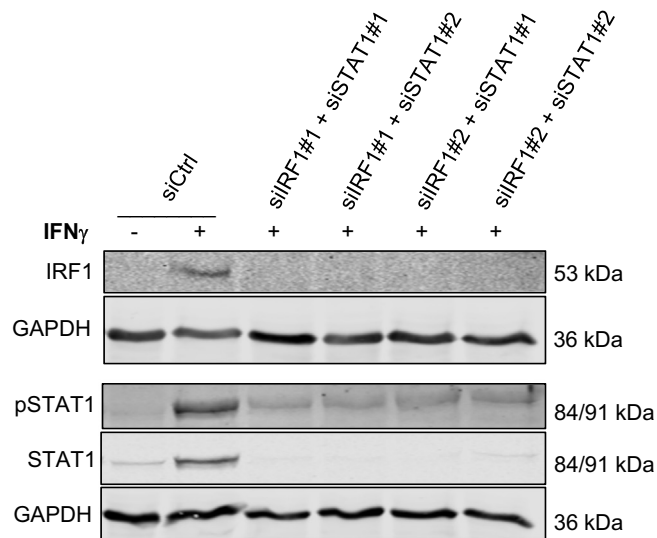
B
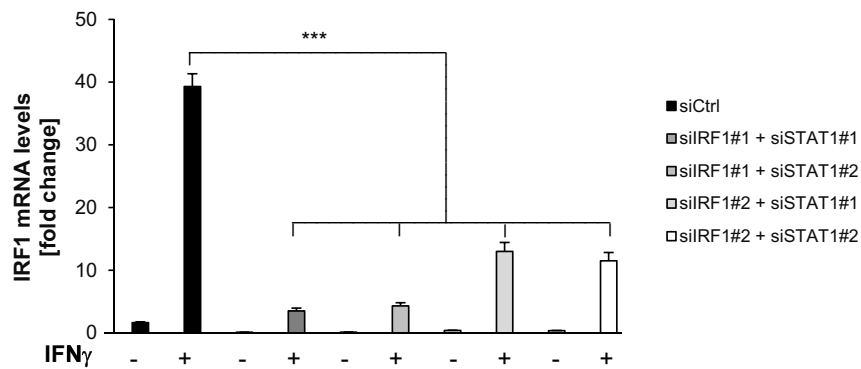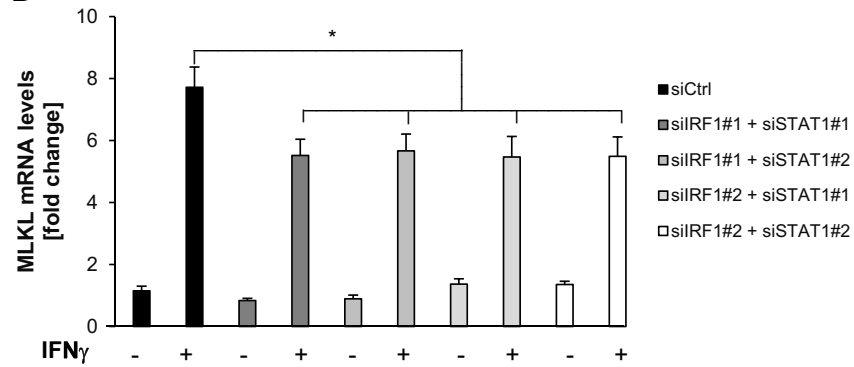
C

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).

IRF1 and STAT1 Contribute to MLKL Up-Regulation by IFNγ

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 5 C). 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 Smac mimetic/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 necroptotic cell death. In line with this notion, constitutive IFN

Figure 5. IRF1 and STAT1 contribute to MLKL up-regulation by IFNγ. (A, B) EFM-192A cells were transiently transfected with two distinct siRNAs targeting IRF1, STAT1 (each 20 nM) or non-silencing siRNA (40 nM). 72 hours after transfection cells were treated with 1.5 ng/ml IFNγ for 24 (A) or 9 hours (B). (A) Protein expression of IRF1, pSTAT1, STAT1 and GAPDH was analyzed by Western blotting. (B) mRNA levels of MLKL and IRF1 were quantified by RT-PCR and are shown as fold change relative to untreated control cells with mean and SEM of at least three independent experiments performed in duplicate; *P < .05; ***P < .001. (C) MDA-MB-231 IRF1 CRISPR/Cas9 knockout cells were treated with 20 ng/ml IFNγ for 24 hours. Protein expression of MLKL, IRF1 and Vinculin was analyzed by Western blotting.
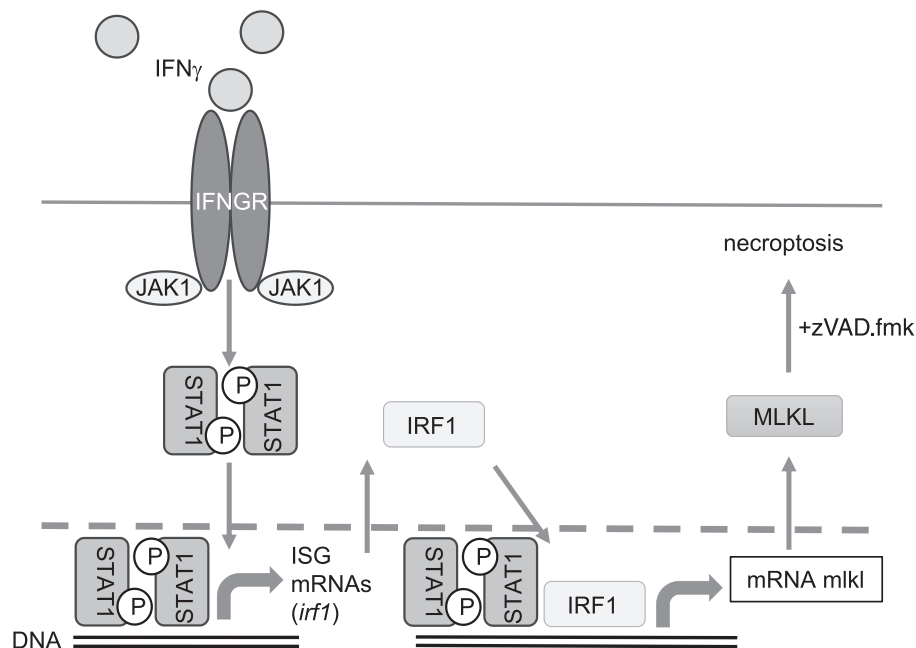
Figure 6. IRF1 and STAT1 regulate MLKL expression. IFNs like IFNγ bind to their IFN receptor and induce the activation and phosphorylation of STAT1. The transcription factor STAT1 induces the expression of ISGs such as IRF1. We hypothesize that IRF1 together with STAT1 transcriptionally up-regulates MLKL after IFN stimulation, leading to higher protein levels of MLKL that sensitize cells towards necroptosis.

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, IFNγ-induced up-regulation of MLKL may well contribute to necroptotic cell death when caspases are inhibited, as IFNγ significantly increased cell death in the presence of zVAD.fmk. Furthermore, the MLKL inhibitor NSA significantly rescued cells from IFNγ-induced cell death in the presence of zVAD.fmk, as we have shown previously [21]. Second, IRF1 may facilitate IFN-induced necroptotic cell death via transcriptional up-regulation of MLKL, thus providing a molecular explanation of how IRF1 contributes to IFNγ-mediated potentiation of Smac mimetic-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 IFNγ 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 IFNγ provides a valuable tool to sensitize cells towards necroptotic cell death and to overcome apoptosis resistance of cancer cells.

Acknowledgements

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.

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