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Synergistic activation of p53 by actinomycin D and nutlin-3a is associated with the upregulation of crucial regulators and effectors of innate immunity

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ABSTRACT

Actinomycin D and nutlin-3a (A + N) activate p53, partly through induction of phosphorylation on Ser392. The death of A549 cells induced by A + N morphologically resembles inflammation-inducing pyroptosis - cell destruction triggered by activated caspase-1. The treatment with A + N (or camptothecin) strongly upregulated caspase-1 and its two activators: IFI16 and NLRP1, however, caspase-1 activation was not detected. A549 cells may have been primed for pyroptosis, with the absence of a crucial trigger. The investigation of additional innate immunity elements revealed that A + N (or camptothecin) stimulated the expression of NLRX1, STING (stimulator of interferon genes) and two antiviral proteins, IFIT1 and IFIT3. IFI16 and caspase-1 are coded by p53-regulated genes which led us to investigate regulation of *NLRP1*, *NLRX1*, *STING*, *IFIT1* and *IFIT3* in p53-dependent mode. The upregulation of *NLRP1*, *NLRX1* and *STING* was attenuated in p53 knockdown cells. The upsurge of the examined genes, and activation of p53, was inhibited by C16, an inhibitor of PKR kinase. PKR was tested due to its ability to phosphorylate p53 on Ser392. Surprisingly, C16 was active even in PKR knockdown cells. The ability of C16 to prevent activation of p53 and expression of innate immunity genes may be the source of its strong anti-inflammatory action. Moreover, cells exposed to A + N can influence neighboring cells in paracrine fashion, for instance, they shed ectodomain of COL17A1 protein and induce, in p53-dependent mode, the expression of gene for interleukin-7. Further, the activation of p53 also spurred the expression of SOCS1, an inhibitor of interferon triggered STAT1-dependent signaling. We conclude that, stimulation of p53 primes cells for the production of interferons (through upregulation of STING), and may activate negative-feedback within this signaling system by enhancing the production of SOCS1.

1. Introduction

Actinomycin D and nutlin-3a (A + N) synergistically activate the p53 pathway in various cell lines [1,2]. Actinomycin D is an anticancer drug, which at low concentration inhibits RNA polymerase I (RNA Pol I). RNA Pol I inhibition induces nucleolar stress and has been shown to trigger phosphorylation of p53, although the mechanism of action is unclear. Nutlin-3a prevents the interaction between p53 and its negative regulator MDM2 which leads to an increased steady state level of p53 [3]. This occurrence may help actinomycin D-activated kinases phosphorylate p53. The most conspicuous signs of treatment synergy is seen in the increase in p53 with phosphorylated serine 46 (Ser46) and the increase in mRNA levels of p53-regulated genes. The phosphorylation of Ser46 is difficult to observe in the presence of actinomycin D and is undetectable in the presence of nutlin-3a. However, when both substances are applied together, the status of phospho-Ser46 p53 reaches very high levels [1,2]. The phosphorylation of Ser46 is considered a marker of activated p53, which efficiently stimulates the expression of proapoptotic genes [4]. In the A549 lung cancer cell line, Ser46 is phosphorylated upon treatment with A + N, also observed with camptothecin (CPT) treatment, an anti-cancer drug which inhibits topoisomerase I and is a strong inducer of apoptosis.

CPT induces death of A549 cells via characteristic of apoptosis mechanisms including morphology, sub-G1 DNA content and activation

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of caspase 3. Alternatively, cell death induced by A + N treatment morphologically resembles necrosis, showing characteristics such as the swelling and bursting ("cell ballooning") of the cytoplasm without the extensive cell blebbing that is characteristic of apoptosis [1]. There are several forms of regulated cell death (RCD) including apoptosis, which is characterized by apoptotic morphology. Another mode of RCD, characterized by necrotic morphology, is referred to as pyroptosis [5]. Pyroptosis is triggered by the conversion of inactive pro-caspase 1 (CASP1) into active caspase-1, which cleaves gasdermin D. This protein cleavage in turn forms pores in the cell membrane leading to permeabilization, "cell ballooning," and death. The activated caspase-1 triggers the destruction of cells and also cleaves pro-interleukin-1β and pro-interleukin-18 into biologically active signaling molecules, which are strong mediators of inflammation. Interleukin-1β is a major pyrogen which promotes influx and activation of neutrophils as well as the activation of T-cells and B-cells [6]. Interleukin-18 promotes the release of IFN-γ by NK-cells and T-cells [6]. Based on our previously reported observations [1,2] and recent work by other investigators [5,7], we hypothesized that activation of p53 by A + N co-treatment induces pyroptosis of A549 cells. We started this study with testing of this hypothesis.

2. Methods

2.1. Cell culture, reagents and treatment

A549 (lung adenocarcinoma, American Type Culture Collection [ATCC]) and U-2 OS (osteosarcoma, ATCC) cells were grown as previously described [1].

The stock solutions of chemicals were prepared in DMSO: actinomycin D (10 μM; Sigma-Aldrich, St. Louis, MI, USA), camptothecin (10 mM; Calbiochem-Merck, Darmstadt, Germany), nutlin-3a (10 mM; Selleck Chemicals LLC, Houston, TX, USA), imidazolo-oxindole PKR inhibitor C16 (6 mM, Sigma-Aldrich). Stock solution of nigericin sodium salt (20 mM, Tocris Bioscience, Minneapolis, MN, USA) was prepared in ethanol. Stock solutions were diluted in culture medium to the following concentrations: 5 nM actinomycin D, 5 μM nutlin-3a, 15 μM nigericin and 5 μM camptothecin. C16 was diluted to concentrations indicated in the Results section. Control cells were mock-treated with medium containing DMSO. Human interferon-α1 (with carrier, stock solution 100 μg/ml) was purchased from Cell Signaling Technology (Danvers, MA) and the final concentration is indicated in the Results section. Control and p53 knockdown A549 cells were prepared as previously described utilizing lentivirus-delivered shRNA molecules [8]. Control and PKR knockdown A549 cells were prepared with lentivirus-delivered shRNA molecules using transduction-ready lentiviral particles (sc-36263-V for PKR and sc-108080 for control) from Santa Cruz Biotechnology according to manufacturer's protocol. Due to the fact that most cells were positively transduced (puromycin-resistant) the selection of clones was not necessary.

Culture medium from cells exposed to A + N (or mock-treated controls) was concentrated by centrifugation (2900 rcf, 20 °C) in Vivaspin Turbo 4 (3,000 MWCO) concentrator from Sartorium Stedim Lab (Stonehouse, UK). We centrifuged medium for time required to concentrate it from 4 ml to 350 μl. Subsequently, 175 μl of loading buffer [1] was added to the concentrated medium, the mixture was incubated at 95 °C for 5 min, chilled on ice and stored at -80 °C. Thirty five microliters were taken for Western blotting.

The apoptotic cells were analyzed using PE Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA) according to manufacturer's protocol using BD FACSCanto II cytometer.

2.2. Semi-quantitative real-time RT-PCR

Total RNA samples were isolated from cells using the RNeasy mini kit (Qiagen, Hilden, Germany). The cDNA was synthesized with MuLV reverse transcriptase and random hexamers (Applied Biosystems, Foster City, CA, USA). Measurements of mRNA levels were performed using Real-Time 2 × PCR Master Mix SYBR (A&A Biotechnology, Gdynia, Poland). The following oligonucleotides were used as primers: for *CASP1*: CASP1-Q1L 5'-TCG CTT TCT GCT CTT CCA CA, CASP1-Q2R 5'-TCC ACA TCA CAG GAA CAG GC; for *IFIT1*: IFIT1-Q1L 5-TGG CAG AAG CCC AGA CTT AC, IFIT1-Q1R 5'-TCA GGG TCC ACT TCA AGC AC; for *IFIT3*: IFIT3-Q1L 5'-CTG ATG CGT GCC CTA CTC TC, IFIT3-Q1R 5′-TGA CCT CAC TCA TGA CTG CC; for *NLRP1*: NLRP1-Q1L 5′-TTG GCA GAT TCT CTT CTC CGT C, NLRP1-Q1R 5′-TGA GCA CAT TGA AGC TCA GGT C; for *NLRX1*: NLRX1-Q3L 5′-TGA TCA AGG TGG TTC CAC GA, NLRX1-Q3R 5'-AAC ATG GGG AAG AGC TCG AA; for *STING* (*TMEM173*): STING-Q1L 5'-TAC ATC GGA TAT CTG CGG CT, STING-Q2R 5′-TGG GGC AGT TTA TCC AGG AA; for *SOCS1*: SOCS1-Q1F 5'-CCC TTC TGT AGG ATG GTA GCA C, SOCS1-Q1R 5′-GAA GAG GAA GGT TCT GGC; for *IL7*: IL7_2F 5′-GGT GAA GCC CAA CCA ACA AA, IL7_2R 5′- GGA GGA TGC AGC TAA AGT TCG. The primers for β-actin (internal reference gene) were: 5'-GCA AGC AGG AGT ATG ACG AG and 5'-CAA ATA AAG CCA TGC CAA TC (BioTeZ). Amplification was performed on a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). In each PCR run, cDNA samples were amplified in triplicate. Relative quantitation of mRNA was carried out using the ΔΔCT method with β-actin as a reference. Mean and standard deviation were calculated from three biological replicates.

2.3. Western blotting

Whole-cell lysates were prepared using IP buffer, supplemented with protease and phosphatase inhibitors as described previously [1]. Aliquots of lysates (35–50 μg) were separated by SDS-PAGE on 8% or 13% gels and electro-transferred onto PVDF membranes. Before incubation with primary antibody, the membranes were incubated for 1 h at room temperature in blocking solution (5% skim milk in PBS with 0.1% Tween-20). The following primary antibodies were obtained from Cell Signaling Technology: anti–phospho-Ser15 p53 (rabbit polyclonal antibody), anti–phospho-Ser46 p53, anti-phospho-Ser392, anti-IFIT1 (D2X9Z), anti-NLRX1 (D4M3Z), anti-STING (D2P2F), anti-PKR (D7F7), anti-CASP1 (D7F10), anti-cleaved caspase-1 (Asp297) (D57A2), anti-IFI16 (D8B5T), anti-PYCARD (*alias* TMS1) (E1E3I), anti-phospho-STAT1 (Tyr 701)(D4A7), anti-STAT1 (rabbit polyclonal), anti-caspase-8 (1C12), anti-caspase-9 (rabbit polyclonal). Anti-IFIT3 antibody (ab95989), anti-CASP1 antibody (ab179515) and anti-COL17A1 antibody (ab184996) were from Abcam (Cambridge, UK). Anti-SOCS1 antibody (clone 4H1) was from EMD Millipore (Temecula, CA, USA). Anti NLRP1 (*alias* NALP1) sheep polyclonal antibody was from R&D systems (Minneapolis, MN, USA). Anti-p53 (DO-1), anti-$p21^{WAF1}$ (F-5), and loading control anti-HSC70 (B-6) antibodies were obtained from Santa Cruz Biotechnology. All incubations with primary antibodies were performed overnight at 4 °C in blocking solution. HRP-conjugated secondary antibodies (anti-mouse, anti-rabbit or anti-sheep) were detected by chemiluminescence (SuperSignal West Pico or SuperSignal West Femto Chemiluminescent substrate, Thermo Fisher Scientific). When necessary, bands on Western blots from at least three independent experiments were quantitated using the GeneTools software (Syngene, Cambridge, UK). Student's *t*-test was used to calculate the statistical significance of differences.

2.4. Molecular cloning, site-directed mutagenesis and luciferase reporter assay

The regulatory elements of *NLRX1* and *NLRP1* were cloned into the pGL3-Basic reporter vector, which encodes firefly luciferase (Promega, Madison, WI, USA). The human *NLRX1* alternative promoter was amplified by PCR from a genomic DNA sample (A549 cells) using primers: 5′-TTTT GAGCTC ACC TTC TCT GTG TCC AGA CC and 5′-TTTT AAGCTT CCC CAT GGG TAC GAC AAC. The primers were designed to contain the restriction sites (underlined) for *Sac*I and HinDIII, respectively. Amplified DNA was ligated into the SacI and HinDIII sites of pGL3-Basic. The human *NLRP1* promoter was amplified by PCR from a genomic DNA sample (A549 cells) using primers: 5′-TTTT GAGCTC AGA TCT TGC CAC TGC ACT CC and 5′-TTTT CTCGAG CTC CCA GGT TTC TTC AGA C. The primers were designed to contain the restriction sites (underlined) for *Sac*I and *Xho*I, respectively. Amplified DNA was ligated into the SacI and XhoI sites of pGL3-Basic. PCRs was performed with PfuPlus! DNA polymerase mix (EURx, Gdańsk, Poland) to ensure high fidelity DNA amplification. The inserted DNA was sequenced to ensure that the clone contained no mutations.

The mutations of CWWG (W - A or T) sequence in the putative p53 response element (RE) from *NLRX1* and *NLRP1* promoters were created using GeneArt Site-Directed Mutagenesis PLUS kit (Life Technologies, Carlsbad, CA, USA) with forward (5’ TCAGACAACAGAGGAGCGTCCC ACGGCATGACTC 3′) and complementary reverse (5’ GAGTCATGCCG TGGGACGCTCCTCTGTTGTCTGA 3′) primers for *NLRX1* and the forward (5’ GAGTCCTTGTCCAAGGCGTCCGTGGGTTGAAGCC 3′) and reverse (5’ GGCTTCAACCCACGGACGCCTTGGACAAGGACTC 3′) primers for *NLRP1* (the sites of mutation are underlined).

The luciferase reporter assay was performed as described recently [2]. In short, U-2 OS cells were co-transfected using FuGene6 (Promega) with a combination of reporter vector, encoding firefly luciferase under the control of *NLRX1* or *NLRP1* regulatory elements (wild type or mutant), and expression vector pC53-SN3, encoding wild-type p53 or pC53-SCX3 encoding Val143Ala p53 mutant (a gift from Dr. Bert Vogelstein and Dr. Kenneth W. Kinzler from Johns Hopkins University, Baltimore, MD, USA) [9]. As a negative control, the p53 plasmid was replaced by empty vector. The transfection mixture also contained pRL-TK, encoding *Renilla* sp. luciferase under the control of HSV-TK promoter (internal control). The next day, the cells were washed with culture medium and incubated with fresh medium for an additional 24 h. The cells were lysed with PLB buffer from the Dual Luciferase Reporter Assay system (Promega) and the activity of the luciferases were measured. Firefly luciferase activity was normalized against *Renilla* sp. luciferase activity. Each transfection was performed in triplicate in three independent experiments.

3. Results

3.1. A + N treatment increases the expression of pro-caspase 1

Our earlier study demonstrated that treatment modalities employed by us induce cell cycle arrest at G1 or G2/M phases (A + N) or cell cycle arrest at G1 and apoptosis (CPT) [1]. Moreover, in cells exposed to A + N we observed molecular signs of autophagy, namely, the conversion of LC3B protein from cytosolic to lipidated, membrane-bound form [1]. We started this study from better characterization of fate of cells exposed to CPT or A + N. The *Western blotting* confirmed stronger induction of apoptosis (as determined by activation of executioner caspase-3, Fig. 1A) in cells treated with CPT when compared with other treatment modalities. Cleavage of caspase-9 and caspase-8 indicate that both intrinsic and extrinsic signals (apparently in autocrine fashion) play role in the induction of apoptosis by CPT. These results are confirmed by cytometric analysis. Early apoptotic cells are frequently detected only in cells exposed to CPT (Fig. 1B). In cells exposed to A + N we observed slight increase of the percentage of necrotic cells, what is consistent with our morphological observations published previously [1].

In a time-course experiment, A549 cells were treated with either CPT or A + N to assess the ability of these substances to induce expression of pro-caspase 1 (CASP1). We observed strong accumulation of this protein following 48-h treatment with CPT or A + N (Fig. 2A). The expression of CASP1 and the degree of p53 activation were determined in conjunction. p53 activation was determined by assessing the amount of total p53 and the amount of p53 with phospho-Ser15, phospho-Ser46 and phospho-Ser392. Serine 15 can be phosphorylated by various kinases and, together with other phosphorylated residues, promotes stabilization of p53 while inhibiting nuclear export (reviewed in [10]). Serine 392, phosphorylated by several kinases, promotes formation of an active arrangement of p53 molecules into a tetramer which then binds to DNA and regulates the expression of target genes [11]. The p21 protein coded by *CDKN1A* is a marker of activation of p53 pathway [10]. Both treatment modalities induced expression of CASP1, however, it appeared late, synchronously with strong accumulation of phosphor-Ser46 p53. Our observation is consistent with the data published by Gupta et al. [12], who demonstrated that *CASP1* gene is regulated by p53. However, the coincidence of CASP1 accumulation and p53 phosphorylation on Ser46 suggests that only strongly activated p53 efficiently stimulates *CASP1* gene transcription. The semi-quantitative RT-PCR confirmed *CASP1* gene stimulation at the transcriptional level and demonstrated a strong synergy between actinomycin D and nutlin-3a leading to the upregulation of *CASP1* (Fig. 2B). While actinomycin D or nutlin-3a acting alone upregulated *CASP1* mRNA, approximately 20- and 35-fold, respectively, when working together these treatments stimulated *CASP1* expression to increase more than 1000-fold. Camptothecin acting alone also caused strong stimulation *CASP1* mRNA, although slightly weaker (850-fold) than A + N. The Western blot showed that, consistent with RT-PCR, actinomycin D and nutlin-3a synergistically stimulated CASP1 protein expression. The synergy was also very strong in the induction of Ser392 phosphorylation (Fig. 2C). Thus, our data suggest that A + N or CPT treatment primes A549 cells for pyroptosis because they start to produce the executioner caspase required for this form of cell death.

3.2. Activation of pro-caspase-1 is undetectable in A + N or CPT treated cells, despite treatment-associated increased expression

The pyroptotic cell death starts with the activation of pro-caspase-1 by self-cleavage into small and large subunits (p10 and p20), which subsequently form a tetramer (two p10 and two p20 molecules) [13]. The p20 subunit can be detected by an antibody, used in Fig. 3A, whereas the p10 subunit can be detected by another antibody (Fig. 3B). Neither antibody detected conspicuous activation of caspase 1 despite strong expression of uncleaved pro-caspase 1 (the form with 48 kDa, Fig. 3A and B). Thus, despite strong accumulation of pro-caspase-1 upon treatment with A + N or CPT, the signal for activation of this executioner caspase is apparently missing.

3.3. Treatment with A + N or CPT upregulates proteins associated with innate immunity, including IFI16 and PYCARD

The cleavage of pro-caspase 1 is triggered by its recruitment to multiprotein structures known as inflammasomes. The crucial elements within the inflammasomes are pattern recognition receptors (PRRs), which detect pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). At least three major types of inflammasomes are activated by bacterial molecules or mechanical irritation. Other inflammasomes are stimulated by cytosolic, double-stranded DNA (dsDNA). These DNA molecules usually originate from genomes of some viruses (e.g. EBV) or from damaged chromosomes. Cytosolic dsDNA can be recognized by two inflammasome proteins AIM2 and IFI16 (interferon gamma inducible protein 16). IFI16 indirectly activates pro-caspase-1 through adaptor protein PYCARD, which has two active isoforms (19 and 22 kDa). PYCARD, binds AIM2 or IFI16 via its PYRIN domain and binds pro-caspase 1 via its CARD domain (reviewed in [14]). Interestingly, the gene for IFI16 has been shown to be a p53-regulated gene [15]. Hence, we explored possibility that the molecules forming at least one inflammasome type were expressed in A549 cells upon treatment with A + N or CPT, results are shown in Fig. 3C. Expectedly, we detected major upregulation of IFI16 following strong activation of p53. As in the case of pro-caspase-1,

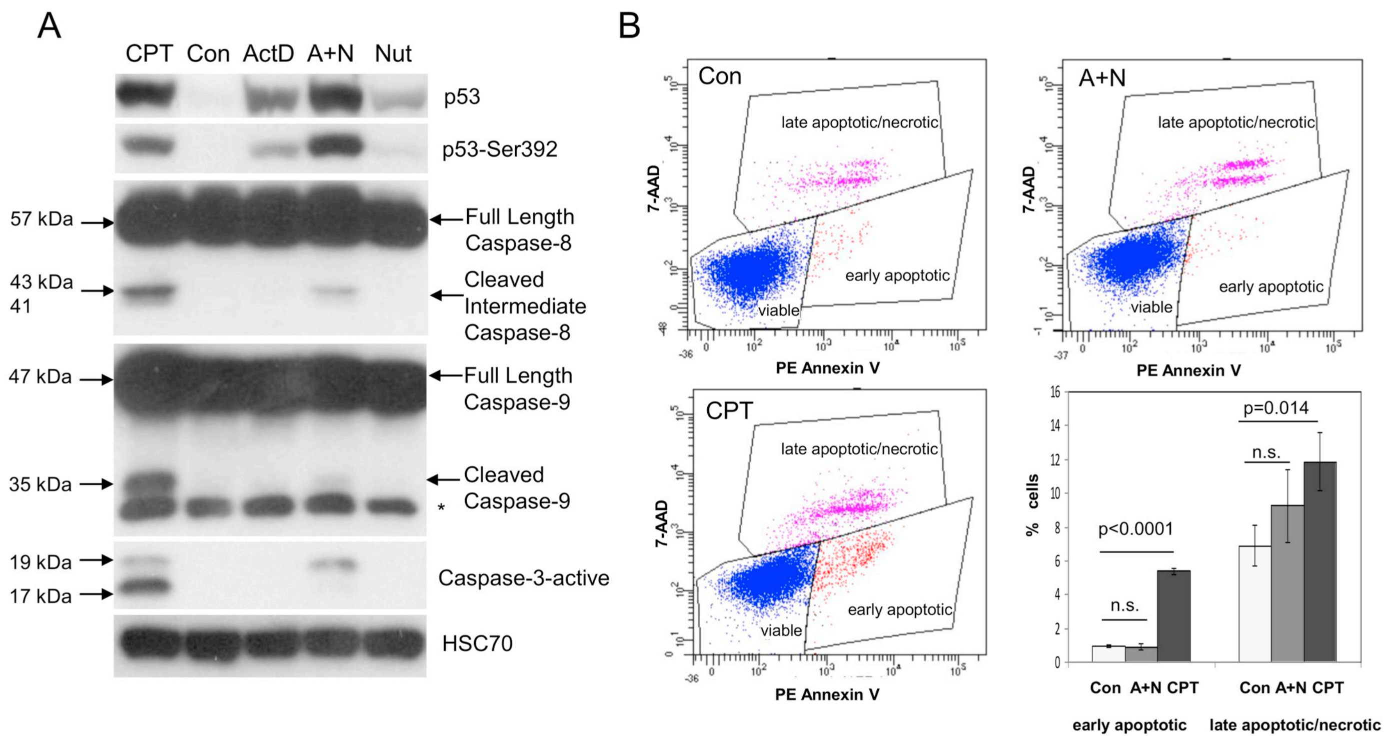

Fig. 1. Treatment with A + N does not induce extensive apoptosis of A549 cells. A. Western blot showing expression of indicated proteins in A549 cells mock-treated (Con) or incubated for 48 h with actinomycin D (ActD), nutlin-3a (Nut), both substances A + N, or CPT (* - protein unspecifically detected by ani-Caspase-9 antibody). B. Cytometric analysis of cell populations mock-treated (Con) or exposed to CPT or A + N for 48 h. Viable cells are 7-AAD (7-Amino-Actinomycin) and PE Annexin V negative, cells in early apoptosis are PE Annexin V positive and 7-AAD negative, whereas late apoptotic or dead cells are both PE Annexin V and 7-AAD positive. The graph shows the frequency of indicated cell types calculated from three biological repeats (p values were calculated by Student's *t*-test; n.s. - non significant).

actinomycin D and nutlin-3a stimulated *IFI16* expression in synergistic manner. Moreover, we noticed that the aforementioned adapter protein, PYCARD, was expressed at relatively constant level (Fig. 3C). The smallest isoform of PYCARD (15 kDa), which is an inhibitor of pro-caspase-1 activation [16] was repressed upon exposure to actinomycin D, A + N or CPT (Fig. 3C). Thus, the three proteins, able to form at least one caspase-1-related inflammasome (pro-caspase 1, IFI16 and PYCARD) were expressed in A549 cells exposed to A + N or CPT. The absence of conspicuous caspase-1 activation may be the result of the lack of at least one crucial, stimulating signal. However, A549 cells appear to be primed to undergo pyroptosis.

Most of knowledge about pyroptosis and activation of caspase-1 comes from studies on macrophages or cell lines derived from leukemias (e.g. THP-1 cells). The induction of pyroptosis in cells derived from carcinomas is poorly explored. The commonly used protocol for induction of pyroptosis in macrophages involves pretreatment of cells with lipopolysaccharides to induce expression of NLRP3 (inflammasome element) and subsequent treatment with bacterial toxin nigericin to lower cytosilic potassium level, what is required for inflammasome assembly [17]. Because A549 cells treated with A + N contain elements of at least one inflammasome type (CASP1, IFI16, PYCARD), we decided to find out if lowering cytosolic potassium level with nigericin can induce caspase 1 activation. The cells were first exposed to A + N for 49 h (control cells were mock-treated) and subsequently, after washing, the cells (control and experimental) were either mock-treated or exposed for 70 min with 15 μM nigericin (Fig. 3D). Cells were harvested by trypsinization and protein lysates were prepared as described in Material and Methods. The blot was exposed to antibody, which recognizes endogenous level of caspase 1 only upon cleavage at Asp297. Thus, this antibody recognizes the activated (cleaved at Asp297) p20 subunit of caspase 1. Subsequently, this blot was reprobed with antibody recognizing a different epitope within the p20 subunit of caspase 1 (this antibody was used in experiment presented on Fig. 3A). The Western blots are shown on Fig. 3D. Consistent with data presented on Fig. 3A, caspase-1 was induced in cells treated with A + N but its activation was not observed (no p20 band was detected). Unexpectedly, the antibody raised against caspase 1 cleaved at Asp297 detected a protein band in cells exposed to A + N and nigericin, however the band was located between 63 and 75 kDa markers, way above expected size (20 kDa). Moreover, larger than expected protein bands (one between 75 and 100 kDa and one about 180 kDa) were also detected by the second antibody in cells exposed to A + N and nigericin (Fig. 3D). Thus, two different antibodies, raised against different epitopes within p20 subunit of caspase 1 recognized larger than expected protein species in A549 cells pretreated with A + N and exposed to nigericin. This is consistent with extensive posttranslational modifications of caspase 1, which reduce mobility of the protein during electrophoresis. However, without extensive and detailed analyses any firm conclusions can not be drawn. In short, our experiments show no evidence of “classical” activation of caspase 1 in cells pretreated with A + N and exposed to nigericin.

IFI16 protein not only activates pro-caspase-1, but also may be a part of signaling system that stimulates the expression of antiviral proteins. For example, IFI16 together with another cytosolic dsDNA binding molecule, cGAS (cyclic GMP-AMP synthase), cooperates in activation of STING protein (stimulator of interferon genes), which activates TBK1 kinase, and in turn phosphorylates and activates IRF3 and IRF7 transcription factors. IRF3 and IRF7 bind to the response elements of many antiviral genes and stimulate their transcription, e.g. genes coding for interferon-α1 and interferon-β1, *IFNA1* and *IFNB1*, respectively [18]. Some genes coding for innate immunity proteins are regulated by p53. In addition to the aforementioned *CASP1* [12] and *IFI16* [15], p53 stimulates transcription of *IRF5* [19], *IRF7* [20], and *ISG15* [21] among others. Thus, we hypothesized that p53 activated by

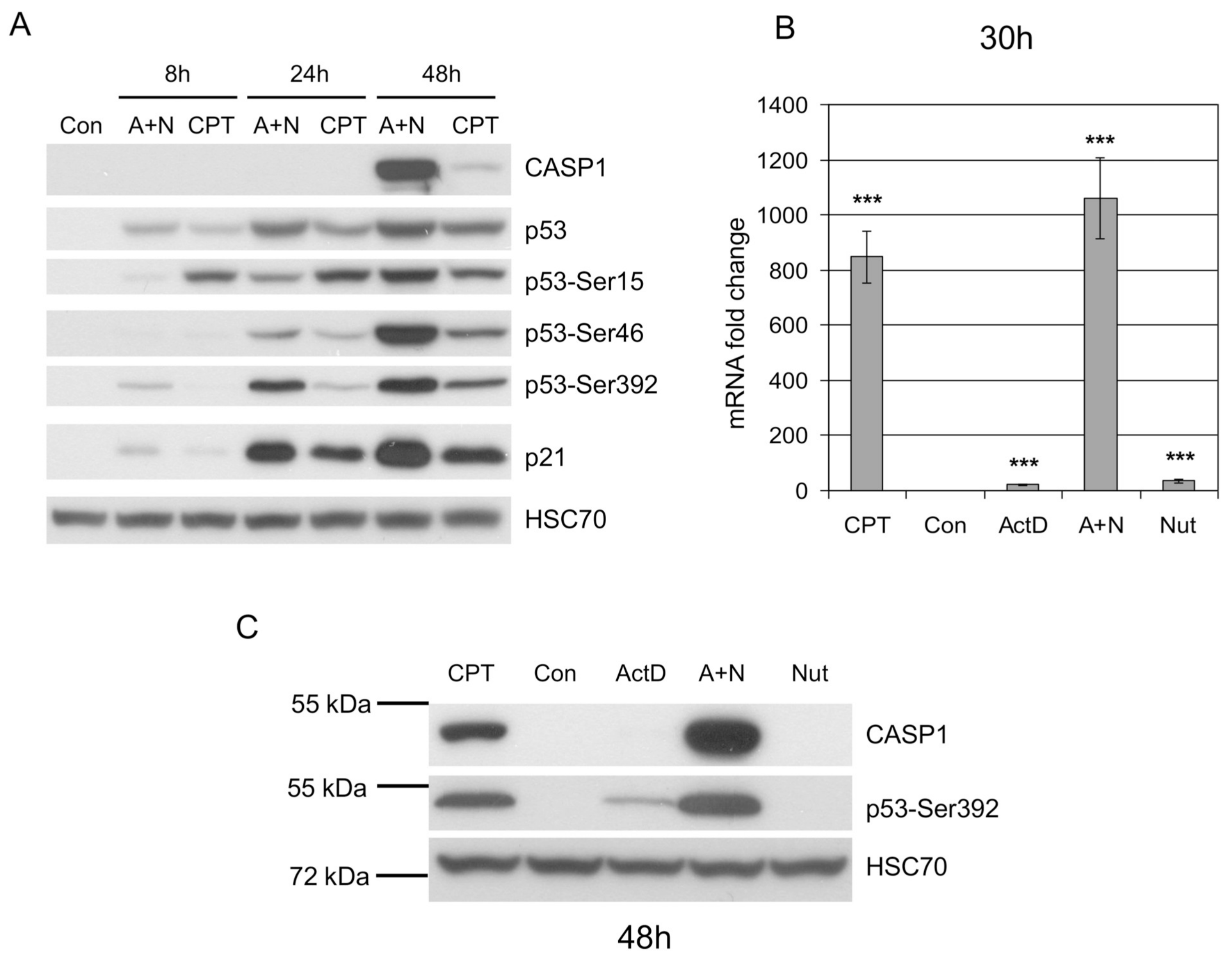

Fig. 2. Synergy between actinomycin D and nutlin-3a induces pro-caspase-1 (CASP1) expression. A. Levels of pro-caspase-1 (CASP1), p53, phospho-p53, p21, and loading control (HSC70) in A549 cells treated with A + N or CPT for indicated time. Con - mock-treated control. B. Changes in the levels of *CASP1* mRNA, measured by semi-quantitative real-time PCR of RNA samples isolated from mock-treated A549 cells (Con) or from cells incubated for 30 h with actinomycin D (ActD), nutlin-3a (Nut), both substances A + N, or CPT. The mRNA level in the mock-treated population was defined as 1. Results represent mean and standard deviations from three biological replicates, *** $p < .001$ by two-tailed Student's *t*-test, treatment versus control. C. Levels in whole-cell lysates of pro-caspase-1 protein, p53 phosphorylated on Ser392 and loading control (HSC70). A549 cells were treated as in A for 48 h. The location of molecular weight markers is indicated.

A + N or CPT could stimulate the genes coding for proteins of innate immunity, which have not been so far identified as p53-regulated genes.

Because the genes for IFI16 and IRF7 proteins are both regulated by p53 [15,20], we decided to explore if the STING protein, located in the signaling pathway between IFI16 and IRF7, can also be synergistically induced by actinomycin D and nutlin-3a or by CPT. Western blot analysis showed that this was indeed the case (Fig. 4A). Moreover, the accumulation of STING was associated with phosphorylation of p53 on Ser46 and Ser392, what indicates that strong activation of p53 is needed to efficiently stimulate STING expression.

Encouraged by these results, we extended our search into other innate immunity genes. We selected genes coding various elements of defense system to bacterial or viral pathogens. One of our guides for selection was the availability of trustful, commercial antibodies against the selected proteins. Moreover, we focused on genes, which have putative binding sites for p53 identified by Tebaldi et al. [22].

IFIT1 (interferon-induced protein with tetratricopeptide repeats 1) is an antiviral effector protein [23]. By binding to the viral mRNA molecules IFIT1 out-competes the cellular translation initiation factors inhibiting the production of viral proteins. IFIT1 forms a functional complex with related proteins – IFIT2 and IFIT3. IFIT3 (interferon-induced protein with tetratricopeptide repeats 3) stabilizes IFIT1 protein expression, promotes its binding to viral mRNA and enhances IFIT1 activity as translation inhibitor [24]. Moreover, IFIT3 serves as an adapter molecule that helps in activation of the aforementioned TBK1 kinase by its upstream regulator, MAVS protein [25].

NLRX1 is a pattern recognition receptor, but unlike other PRRs, it does not participate in the formation of inflammasomes, but rather it is localized in mitochondria. The studies on its function give contradictory results, while some investigators suggest that it induces production of the reactive oxygen species (ROS) [26], others demonstrate that NLRX1 dampens the oxidative stress [27]. NLRX1 is an important but poorly understood element of innate immunity.

NLRP1 (NALP1) is the component of the first identified inflammasome [28]. It is a pattern recognition receptor activated by various bacterial toxins including *Bacillus anthracis* lethal toxin [29]. For our analysis of gene expression, we also selected PKR kinase (coded by the *EIF2AK2* gene), because it has antiviral effector functions and can participate in the activation of p53 by phosphorylating Ser392 [30].

The result of our extended analysis is presented in Fig. 4. The expression of IFIT1, IFIT3 and STING correlated with the level of phospho-Ser46 p53 and phospho-Ser392 p53. NLRX1 was upregulated by any p53 activator used in this study whereas NLRP1 was primarily upregulated by A + N (Fig. 4A). These data together with the results of semi-quantitative RT-PCR (Fig. 4B) indicate strong synergy between actinomycin D and nutlin-3a in activation of *IFIT1, IFIT3, STING* and *NLRP1* genes. *NLRX1* gene was activated by both substances in additive

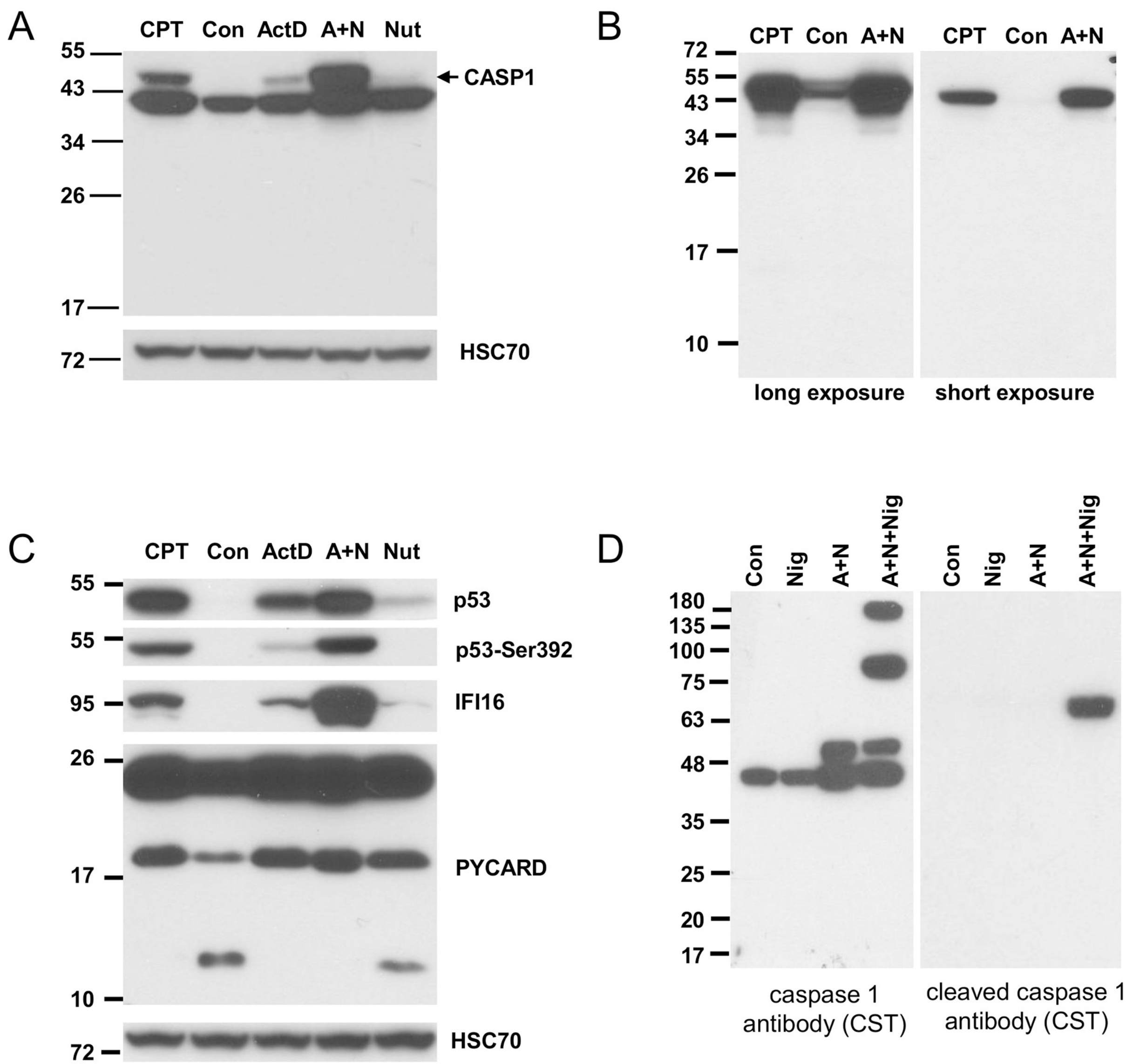

Fig. 3. The activation of pro-caspase-1 by proteolytic digestion is not detected in cells treated with actinomycin D & nutlin-3a (A + N) combination or those treated with camptothecin (CPT). A. Expression of pro-caspase-1 detected in A549 cells treated, as indicated, for 48 h. Protein was detected using the D7F10 antibody from Cell Signaling Technology. The arrow shows pro-caspase-1. The lower band is apparently off-target protein. B. The expression of pro-caspase-1 in A549 cells treated, as indicated, for 48 h. This blot was incubated with ab179515 antibody from Abcam. Both panels are results of different exposures of the same blot. This antibody is able to recognize the small subunit (p10) of active form of the enzyme. C. Expression level in whole-cell lysates of total p53, its form phosphorylated on Ser392 and the expression of IFI16 and PYCARD proteins involved in formation of inflammasomes. The PYCARD protein, known also as ASC, has four splicing isoforms, three of them can be detected by antibody used in this study, which detects epitope near carboxyl terminus of protein. Two upper bands of PYCARD (approximately 19 and 22 kDa) are activating inflammasome adaptors, while the bottom band is an inhibitory adaptor (it lacks PYRIN domain). D. Western blots on protein lysates from A549 cells: Con - mock-treated, Nig - exposed to 15 µM nigericin for 70 min, A + N - exposed to actinomycin D and nutlin-3a for 49 h and for 70 min with fresh medium, A + N + Nig - exposed to actinomycin D and nutlin-3a for 49 h and subsequently to nigericin (15 µM) for 70 min. The blot was probed with anti-cleaved Caspase-1 (Asp297) rabbit monoclonal antibody (D57A2) from Cell Signaling Technology (CST). The blot on the left was probed with anti-caspase-1 antibody (D7F10) from CST.

fashion, whereas the expression of PKR did not change detectably. Thus, we concluded that *STING* (*TMEM173*), *IFIT1*, *IFIT3, NLRP1* and *NLRX1* genes could be activated by 53, principally in response to severe stress factors.

3.4. Knock-down of p53 attenuates activation of innate immunity genes

The knockdown of p53 by shRNA molecules delivered to A549 cells by lentiviruses [8] prevented the strong accumulation of p53 with phosphorylated Ser46 or Ser392. This in turn prevented the upregulation of proteins coded by the known p53-regulated genes *IFI16* and *CASP1* (Fig. 5A). This shows that our experimental model can indicate if a gene is regulated in p53-dependent fashion. Using this model, we found that knockdown of p53 attenuated upregulation of STING, NLRX1 and NLRP1 proteins in cells exposed to A + N (Fig. 5A). In line with this, upregulation of their mRNAs was also attenuated in p53-knockdown cells (Fig. 5C). Surprisingly, at the 48-h time point, the knock-down of p53 influenced the expression of neither IFIT1 nor IFIT3 (Fig. 5A, B). However, when we performed the time-course experiment with wild-type and p53 knockdown cells, we found that at earlier time-points of treatment with A + N (18–24 h) the expression of IFIT1 or IFIT3 was lower in p53 knockdown cells (Fig. 5B). Moreover, the time-course experiment demonstrated that expression of NLRP1, NLRX1 or STING was attenuated in cells with p53 knockdown throughout the

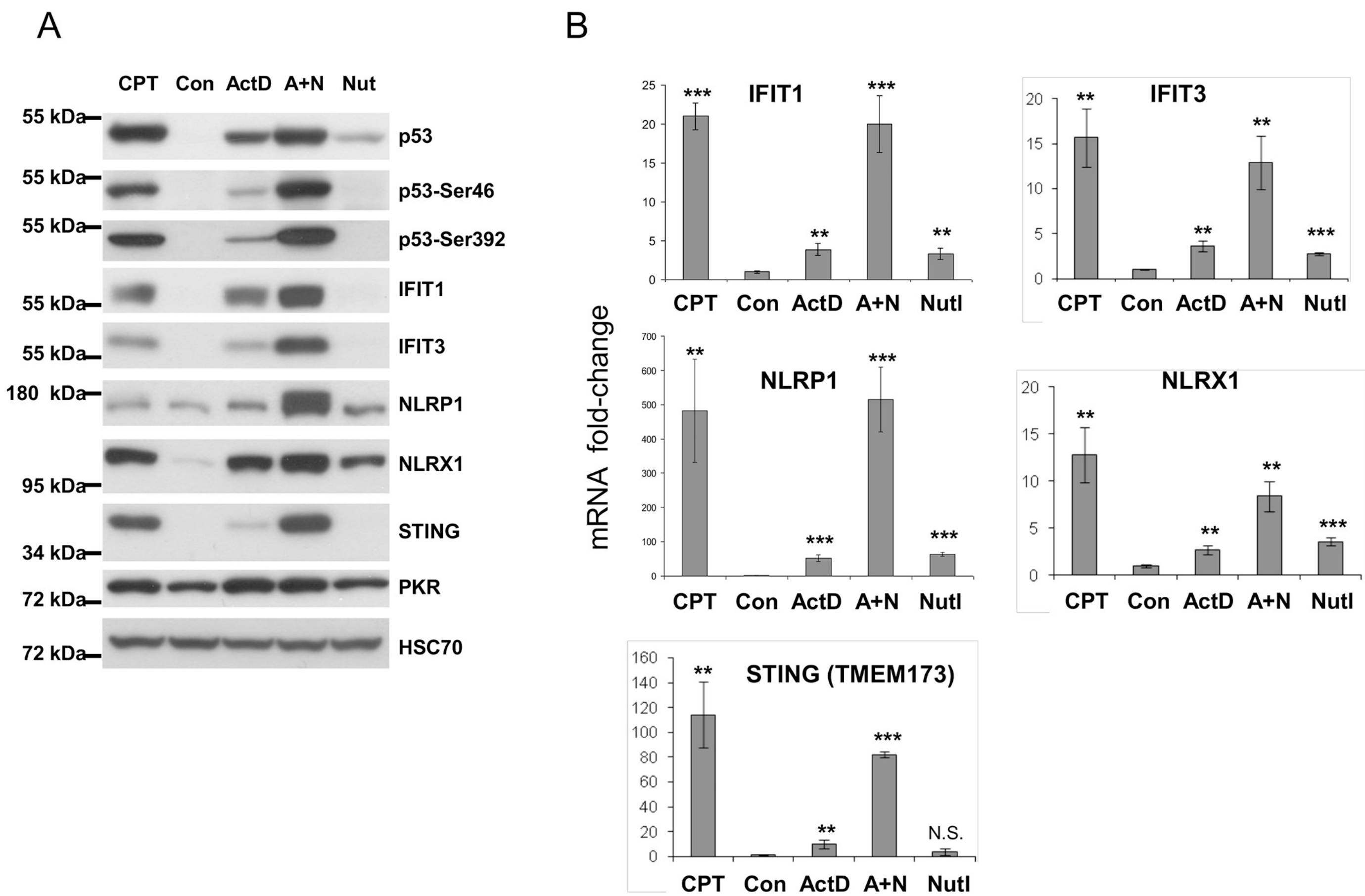

Fig. 4. Actinomycin D and nutlin-3a synergistically activate a subset of genes involved in innate immunity. A. Expression level in whole-cell lysates of p53 (total and phosphorylated) and the expression of IFIT1, IFIT3, NLRP1, NLRX1, STING (TMEM173) and PKR proteins. A549 cells were exposed as indicated for 48 h. B. Changes in the levels of *IFIT1*, *IFIT3, NLRP1, NLRX1* and *STING* mRNA, measured by semi-quantitative real-time PCR of samples isolated from mock-treated A549 cells (Con) or from cells incubated for 30 h as indicated. The mRNA level in the mock-treated population was defined as 1. Results represent means and standard deviations from three biological replicates, *** $p < .001$, ** $p < .01$ by two-tailed Student's *t*-test, treatment versus control (N.S. non significant).

treatment period, strengthening the conclusion that these three genes are regulated in p53-dependent fashion. The experiment performed with camptothecin as a stress factor yielded similar results presented in supplementary Fig. S1. The knockdown of p53 attenuated upregulation of IFIT3, NLRX1 and NLRP1 proteins (Fig. S1A). In line with this, upregulation of their mRNAs was also attenuated in p53-knockdown cells (Fig. S1B). Additionally, the knockdown of p53 was associated with attenuated upregulation of STING mRNA.

3.5. Ectopic expression of p53 upregulates NLRX1 and NLRP1 promoters

Our RT-PCR and Western blotting data demonstrated that *STING* (*TMEM173*), *NLRP1* and *NLRX1* genes were regulated in p53-dependent manner. The *STING* gene contains putative p53 response element (RE) more than 3700 base-pairs downstream the transcription start site, thus it is located outside of the promoter region [22]. Hence, at this stage we excluded it from the characterization of p53 RE. The *NLRX1* contains at least two plausible p53 RE identified by Tebaldi et al. [22]. One is located far downstream from the transcription start site (about 3700 bp), whereas the other is located upstream from the start of the major transcript (approximately 2200 bp). Interestingly, this site is located within the promoter of an alternative *NLRX1* transcript (NLRX-212; ENST00000482180.5). We cloned this alternative promoter into the luciferase reporter vector. This DNA fragment contains a putative p53 RE defined as the three-quarter-site (3Q) because one quarter element that binds one p53 molecule of the active tetramer is missing. The canonical p53 RE consists of two decameric half-sites (RRRCWWGYYY; R - purine, Y - pyrimidine, W - A or T) arranged tandemly. This decamer in turn consists of two pentameric quarter sites (RRRCW) arranged head-to-head. The 3Q sites are still able to bind p53 tetramers, provided that p53 molecules are properly modified post translationally [11,31]. In Fig. 6A, each quarter-site and its direction are presented as an arrow and Roman numeral. In the putative p53 RE of *NLRX1* alternative promoter, the quarter site number II is missing. In spite of this, wild-type p53 expressed from the vector significantly elevated luciferase activity controlled by *NLRX1* promoter (Fig. 6 B and D). When the critical elements of the quarter-sites III and IV were mutated as indicated on Fig. 6A, the mutant promoter was not activated by p53 (Fig. 6B). Moreover, mutant p53 (V143A), which lost its sequence-specific binding to DNA, is no longer able to activate wild-type *NLRX1* promoter (Fig. 6D). Interestingly, fragment of the cloned promoter (marked on Fig. 6A as open rectangle) contains a p53 ChIP-Seq peak (p53 binding site identified by sequencing of chromatin immunoprecipitated with anti-p53 antibody). The presence of the peak, located on chromosome 11, positions: 119,037,091-119,037,351 (genome version hg19) was reported in meta-analysis performed by Nguyen et al. [32]. The p53 ChIP-Seq peaks identified by others in this region and publically available through ChIP-Atlas tool [33] are visualized in supplementary Fig. S2. Thus, our tests combined with the ChIP-Seq data indicate that the DNA fragment, which we cloned, contains a genuine p53 RE controlling the expression of *NLRX1*. Hence, *NLRX1* appears to be regulated through direct p53 binding to the *NLRX1* promoter.

Tebaldi et al. [22] identified putative p53 RE approximately 700 bp

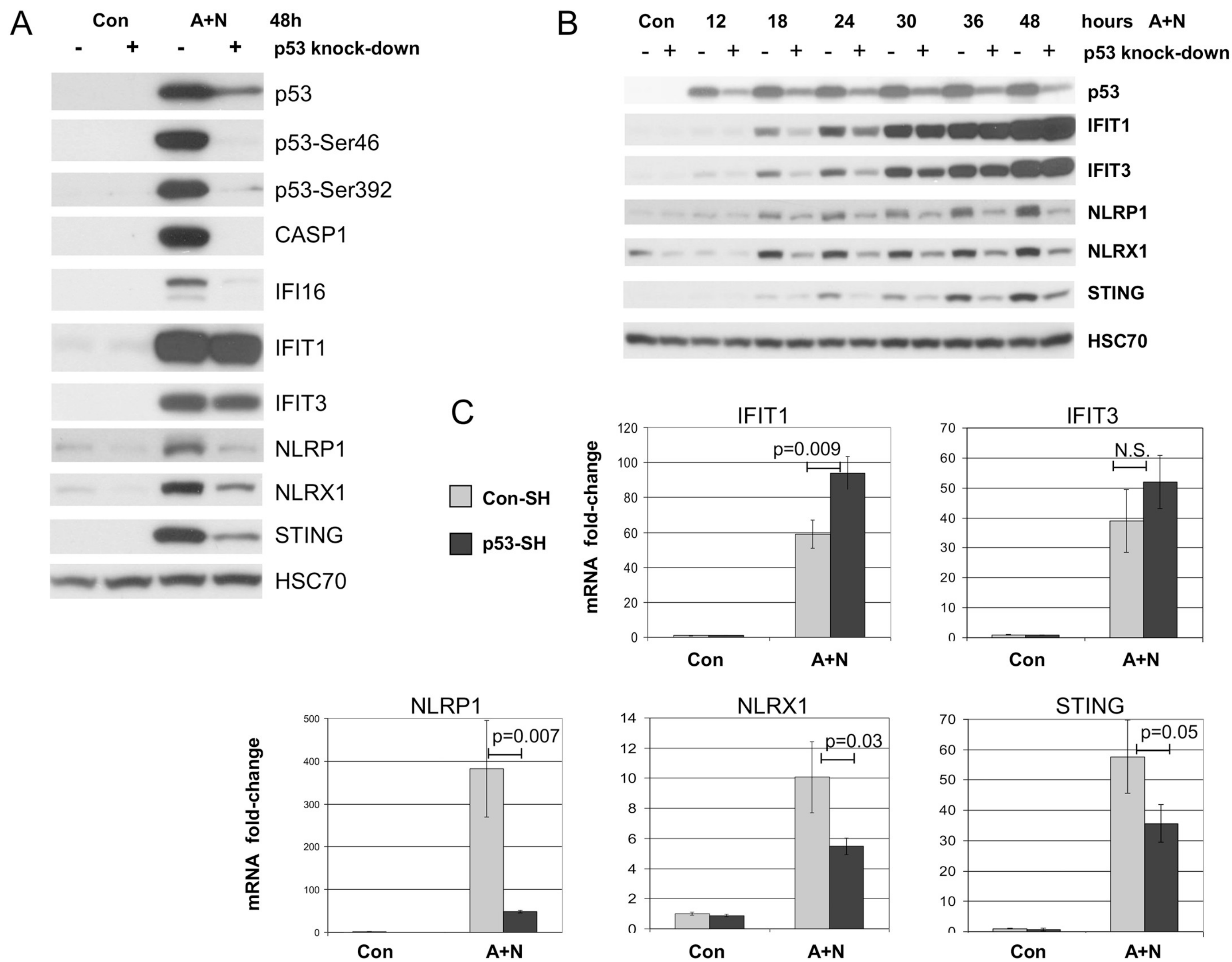

Fig. 5. Innate immunity associated genes are upregulated in a p53-dependent manner following combination actinomycin D and nutlin-3a treatment. A. Western blot showing expression of indicated proteins in A549 cells with knocked-down expression of p53 (+). These cells (+) or the controls (−) for knock-down were mock-treated (Con) or exposed to actinomycin D and nutlin-3a (A + N) for 48 h. B. The control (−) or p53 knocked-down (+) A549 cells were treated with A + N and examined by Western blotting. "Con" cells were mock-treated. C. The expression of mRNA for indicated proteins determined by semi-quantitative RT-PCR in mock-treated control cells (Con) or the cells exposed to A + N for 30 h. The A549 cells with knocked-down p53 are marked as "p53-SH", whereas the controls for knock-down are "Con-SH". The results represent mean and standard deviation from three biological replicates, *p* values were calculated by two-tailed Student's *t*-test (N.S. non significant).

upstream transcription start site of *NLRP1*. It deviates from the consensus in three positions denoted on Fig. 6A by small-case letters. Importantly, this putative p53 RE contains two CWWG elements of the consensus. We mutated one consensus element as shown in Fig. 6A. Unexpectedly, this mutation only slightly attenuated the ability of p53 to activate the promoter (Fig. 6C). Thus, while this DNA sequence may contribute to the ability of promoter to respond to p53, there is likely another p53 binding site, which plays the major role as the p53 RE within this promoter. Critically, the mutant p53 (V143A) is not able to activate this promoter. Thus, the cloned DNA sequence of *NLRP1* gene responds only to wild-type p53, but the location of the major p53 RE is not known. The aforementioned meta-analysis of the location of p53 ChIP-Seq peaks [32] may shed light on the mechanism of p53 binding to *NLRP1* promoter, which contains p53 ChIP-Seq peak located in positions 5,487,941 to 5,488,641 of on chromosome 17 (genome version hg19, marked by open rectangle on Fig. 6A and visualized in supplementary Fig. S2). This sequence overlaps a DNA fragment, found to form G4 structure (see Nguyen et al. [32] and refs therein). This alternative DNA conformation forms when single-stranded guanine-rich regions fold into stable four-stranded helical structures. The vicinity of G4 s to p53 ChIP-Seq peaks was examined [32] because there is evidence that p53 can bind to some of these structural elements and by doing so it can control the expression of nearby gene [34]. Our data generate hypothesis that p53 may control the expression of *NLRP1* from G4 element identified in its promoter region. This definitely warrants further investigation.

3.6. PKR kinase inhibitor, C16, prevents strong phosphorylation of p53

The activation of p53 by A + N or CPT was associated with strong upregulation of innate immunity genes (*CASP1*, *STING, NLRX1, NLRP1, IFI16, IFIT1* and *IFIT3*). In order to find out which kinase might be responsible for the activation of p53 under these conditions, we took a candidate-protein approach. There are many kinases that are directly or indirectly activated by PAMPs. One kinase, PKR, detects the presence of viral, double-stranded RNAs and some cellular RNA molecules appearing during stress and phosphorylates the translation initiation factor what results in inhibition of synthesis of viral proteins and

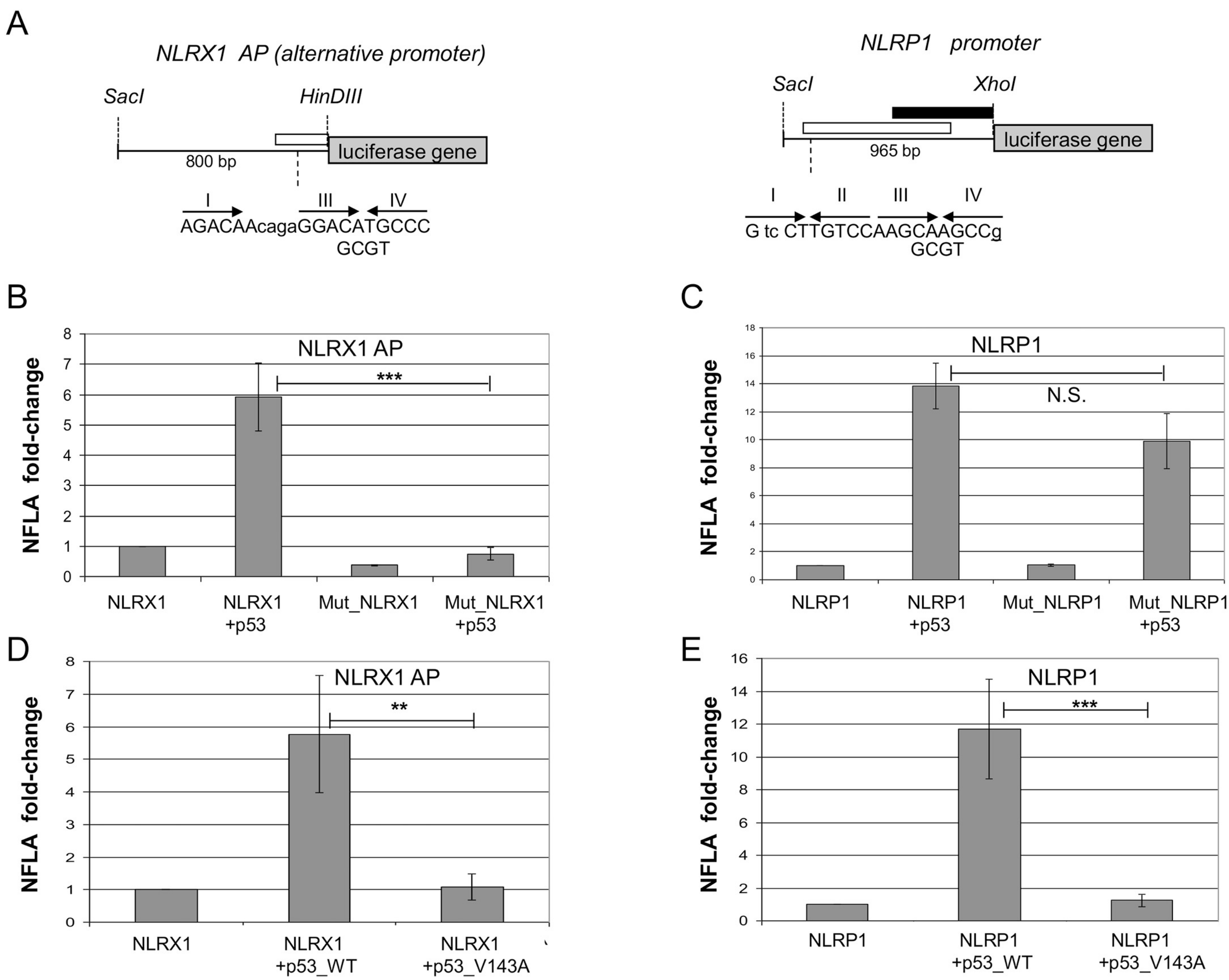

Fig. 6. *NLRX1* and *NLRP1* promoters contain a p53 response element (RE). A. The arrows and Roman numerals mark the quarter-sites of p53 RE. The nucleotides, which do not match the consensus quarter-site are marked by small-case letters. The location of p53 ChIP-Seq peaks reported by others [32] is marked by open rectangles. The *NLRP1* promoter fragment forming G4 quadruplex [32 and refs therein] is marked by black rectangle. B. & C. The fold change of the normalized firefly luciferase activity (NFLA) in U-2 OS cells transfected with a p53 expression vector and the reporter vector coding for firefly luciferase under the transcriptional control of promoters cloned from the NLRX1 or NLRP1 genes (presented on panel A). "Mut" prefix indicates the promoter version with the mutated p53 site as indicated on panels A. D. & E. The fold change of NFLA after co-transfection of the wild-type promoter reporter plasmids with expression vectors coding for either wild-type p53 or V143A mutant. The mean and standard deviation from three biological replicates performed in triplicate are presented, *** $p < .001$, ** $p < .01$ by two-tailed Student's *t*-test.

production of virus progeny [35]. Moreover, PKR can phosphorylate p53 on Ser392 [30]. Hence, we decided to test the hypothesis that PKR is involved in activation of p53 in our experimental conditions.

PKR can be inhibited by an imidazolo-oxindole compound named C16, which is considered a specific inhibitor [36]. A549 cells were treated with CPT or with A + N and additionally with C16 at concentrations from 0.2 to 1.0 μM (Fig. 7A). At 0.5 μM, C16 slightly reduced p53 phosphorylation on Ser46 and Ser392, whereas at 1.0 μM, C16 almost completely blocked phosphorylation of these residues. It was accompanied by strong inhibition (A + N) or blockage (CPT) of accumulation of p21 protein, which is encoded by the well-studied p53-regulated gene (*CDKN1A*) (Fig. 7A). Surprisingly, C16 did not impact p53 phosphorylation on Ser46 and Ser392 triggered by actinomycin D acting alone (Fig. 7B). C16 treatment did not reduce the phosphorylation of p53 on Ser15 indicating that C16 specifically influenced p53 phosphorylation on selected amino acids. Thus, C16 inhibits enzymes, which induce p53 phosphorylation on Ser46 and Ser392.

3.7. C16 can inhibit the p53 pathway in PKR knockdown cells

In order to find out if PKR is the enzyme responsible for activation of p53 upon treatment with A + N or CPT, we prepared PKR knockdown A549 cells. As shown on Fig. 8, the knockdown was successful. In cells with barely detectable expression of PKR, the activation of p53 was not changed (Fig. 8A). Moreover, C16 was able to strongly inhibit p53 phosphorylation on Ser46 and Ser392 even in cells with PKR expression considerably reduced (Fig. 8B). Thus, we conclude that in our model, p53 is phosphorylated on Ser46 and Ser392 in PKR-independent fashion. However, C16 clearly blocked not only phosphorylation of p53 but also upregulation of IFI16, NLRP1, NLRX1, STING, IFIT1 and IFIT3 induced by A + N (Fig. 8B) or camptothecin (Fig. S3), which is consistent with the hypothesis that p53 is responsible for upregulation of these genes. This activity of C16 is apparently PKR-independent as it occurs in PKR-positive as well as PKR knockdown cells (Fig. 8B and Fig. S3). Thus, our data show that p53 is activated upon CPT or A + N

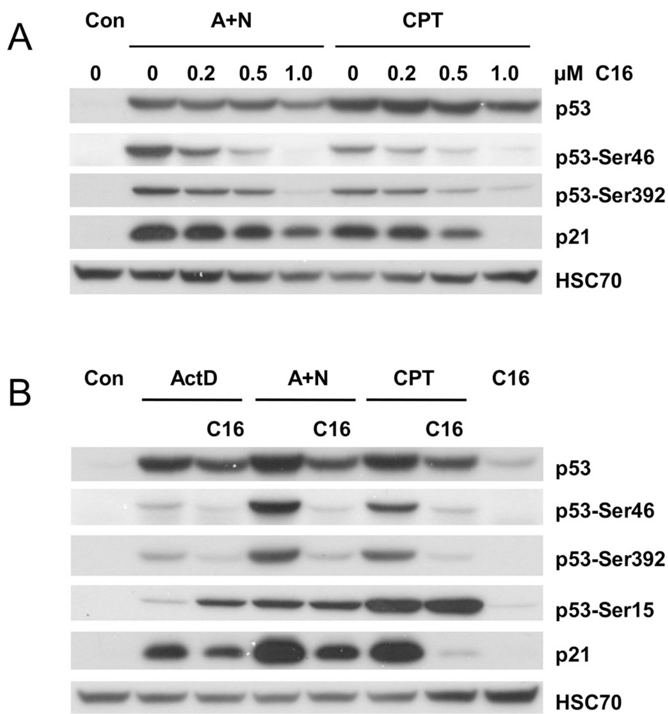

Fig. 7. C16 treatment attenuates phosphorylation of p53 on Ser46 and Ser392. A. The level of p53, its two phosphorylated forms and the expression of p21 (coded by p53 target) in A549 cells exposed for 30 h to either A + N or CPT alone or in combination with C16 at indicated concentrations. B. The Western blot showing expression of p53 and its phosphorylated forms in A549 cells exposed for 30 h to actinomycin D (ActD) actinomycin D and nutlin-3a (A + N) or to camptothecin (CPT). Some cells were also exposed to C16 used at 1 μM concentration.

treatment by an unidentified kinase(s), which is (are) sensitive to the inhibition by C16. Moreover, C16-inhibited kinase is responsible for the activation of the examined innate immunity genes. To strengthen this conclusion, we performed a similar experiment using the C16 inhibitor in the parental, unmodified A549 cell line and in the U-2 OS cell line derived from osteosarcoma (Fig. 9 A, B and C). The experiment yielded similar conclusions. The C16 compound attenuated p53 phosphorylation on Ser46 and Ser392 and prevented or attenuated upregulation of innate immunity genes (*STING, IFIT1, IFIT3, NLRX1, NLRP1*) or well-defined p53-target genes coding for p21, IFI16 and CASP1. Thus, the C16-inhibited kinase phosphorylates p53 and stimulates innate immunity genes in at least two different cell lines, suggesting a more widespread phenomenon.

3.8. The upregulation of innate immunity genes by CPT or A + N is not associated with the activation of STAT1

In principle, the accumulation of IFIT1, IFIT3 and IFI16 genes could result from autocrine stimulation of cells by interferons secreted in response to stress conditions associated with the exposure to A + N or CPT. In order to test this hypothesis, we monitored the activation status of STAT1, a transcription factor, which is phosphorylated on Tyr701 when the cells are exposed to type I (e.g. IFN-α1) or type II (IFN-γ) interferons [37]. As a positive control, we treated A549 cells with human interferon-α1 (IFN-α1) for 24 or 48 h. As expected, IFN-α1 induced accumulation of STAT1 with phospho-Tyr701, as well as accumulation of proteins coded by interferon-stimulated genes (IFIT1, IFIT3, IFI16) (Fig. 10A). Moreover, IFN-α1 treatment also led to accumulation of PKR protein coded by the *EIF2AK2* gene known to be activated by interferon [38]. However, phosphorylated STAT1 (Tyr701) did not accumulate in cells exposed to CPT or A + N suggesting that interferons signaling through STAT1 transcription factor are not induced in these experimental conditions and that these interferons are not responsible for upregulation of the examined innate immunity genes.

Because two genes (*IFIT1* and *IFIT3*) were strongly stimulated by A + N or by IFN-α1, we decided to examine whether these treatment modalities act additively or synergistically. We treated A549 cells as demonstrated on Fig. 10B. This figure shows that A + N treatment, synergistically with IFN-α1, stimulates the expression of IFIT3. The synergy is presented quantitatively in Fig. 10C. The upregulation of IFIT1 by A + N and IFN-α1 appears additive. Expectedly, IFN-α1 stimulated phosphorylation of STAT1 and A + N did not. However unexpectedly, A + N attenuated STAT1 phosphorylation induced by IFN-α1. Camptothecin had similar attenuating effect on interferon-induced STAT1 phosphorylation (Fig. 10B). We concluded that A + N (or camptothecin) may activate a mechanism that prevents strong phosphorylation of STAT1.

In interferon-treated cells STAT1 is phosphorylated by JAK1 kinase. This enzyme is inhibited by SOCS1 protein, which prevents excessive stimulation of the immune system. We hypothesized that the observed attenuated phosphorylation of STAT1 was associated with increased

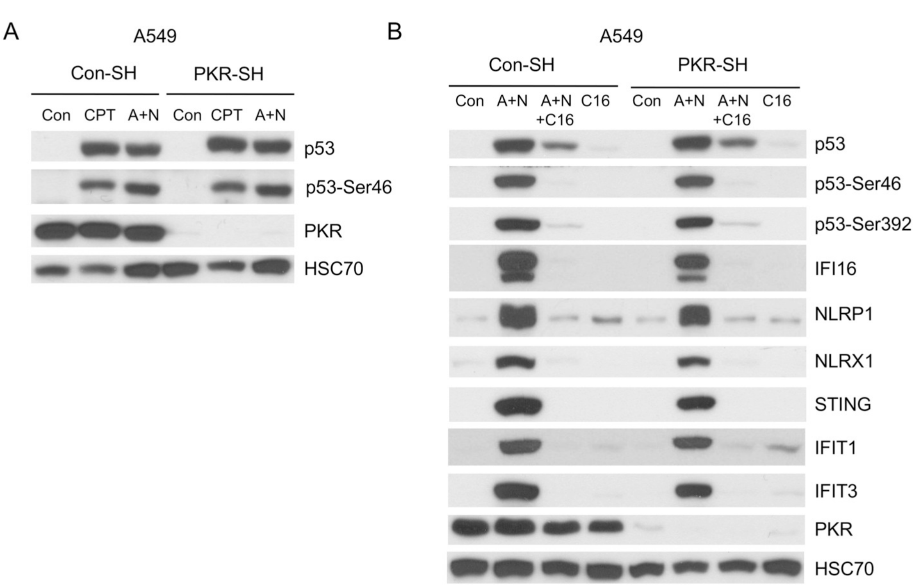

Fig. 8. C16 treatment inhibits activation of the p53 pathway in a PKR kinase-independent fashion. A. The expression of p53 and its phosphorylated form in A549 cells with knocked-down expression of PKR and in controls for knockdown exposed to A + N or CPT for 48 h. B. The expression of indicated proteins in cells with knocked-down expression of PKR and in controls for knockdown exposed to A + N and/or C16 (1 μM) for 48 h.

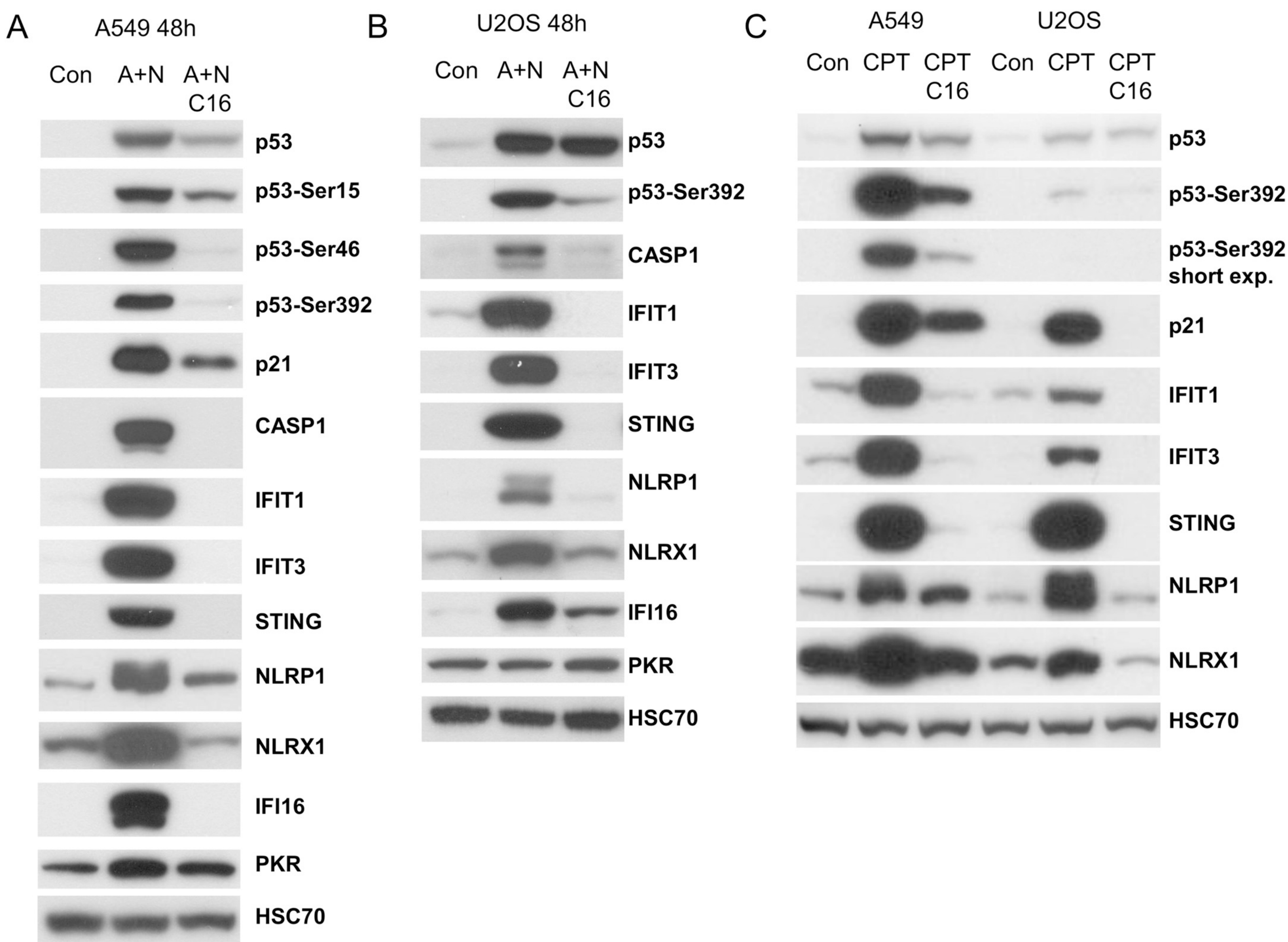

Fig. 9. Upregulation of innate immunity associated genes by A + N (or by camptothecin) is blocked by C16 treatment in A549 and U-2 OS cell lines. A549 (A) or U-2 OS (B) cells were exposed to A + N or A + N and C16 (1 μM) for 48 h. Control cells were mock-treated (Con). (C) Similar experiment was performed using camptothecin (CPT) as a stress factor. The cells were exposed for 41 h. The indicated proteins or their modified forms were subsequently detected by Western blotting.

expression of SOCS1 in cells treated with A + N (or camptothecin). Our Western blot showed that this was the case (Fig. 10B). This observation suggested that *SOCS1* may be a p53-regulated gene. Using an experimental approach employed for other genes in this study, we demonstrated that strong activators of p53 (camptothecin or A + N) stimulate *SOCS1* expression at both the mRNA (Fig. 11A) and protein (Fig. 11B) level, and that actinomycin D and nutlin-3a stimulate SOCS1 expression in a synergistic fashion (Fig. 11A and B). Further, we showed that the knockdown of p53 attenuated upregulation of SOCS1 (Fig. 11C, D, E), which indicates that *SOCS1* is regulated in p53-dependent fashion. At this moment we do not have evidence that this gene is regulated by p53 directly. The meta- analysis of Ngueyn et al. [32] shows that there are three p53 ChIP-Seq peaks within *SOCS1* promoter but they contain no p53 RE sequence motifs. Thus, strongly activated p53 may have the potential to prevent excessive stimulation of immune system. It is noteworthy that the antibody used in this experiment recognized a 37 kDa protein, whose expression did not change in any treatment conditions, in stark contrast to the RT-PCR data. The expected molecular mass of SOCS1 is 23.5 kDa. After using more sensitive detection system, the antibody recognized also a protein of approximately 24 kDa with expression pattern similar to the expression of SOCS1 mRNA (Fig. 11 A and B). Hence, we conclude that this ~24 kDa band on the blot represents SOCS1, while the 37 kDa molecule is an off-target protein.

3.9. Cells exposed to A + N can modulate their environment

The last question we wanted to answer concerns the ability of A + N-treated cells to modify their environment. The detailed characterization of the secretome of these cells will be the subject of another study. Here, wanted to show an example of increased secretion of an extracellular protein induced by treatment with A + N. We selected COL17A1 protein. Its gene is directly regulated by p53 [39]. We took advantage of the fact that COL17A1 is unusual collagen molecule with its full-length form anchored in cellular membrane (~180 kDa molecule) and its extracellular domain (~120 kDa ectodomain) shed to cell environment. Increased COL17A1 shedding correlates with decreased cell motility [40]. We exposed A549 cells to A + N in complete medium for 24 h. Subsequently, the cells were rinsed with PBS and exposed to A + N in serum free-medium for 24 h. The control cells were mock-treated. By removing serum, we reduced the expected background staining from serum proteins. At the end of experiment the number of control cells and exposed cells was very similar. The whole cell lysate was prepared from attached cells. The medium was centrifuged at 720 *g* for 5 min. to remove detached cells. Subsequently, 4 ml of medium was concentrated using Vivaspin Turbo concentrators. Protein lysates and equal volumes of concentrated media were analyzed by Western blotting (Fig. 12A). Expectedly, we detected strong induction of COL17A1 protein in cells exposed to A + N. In cell lysate both collagen forms

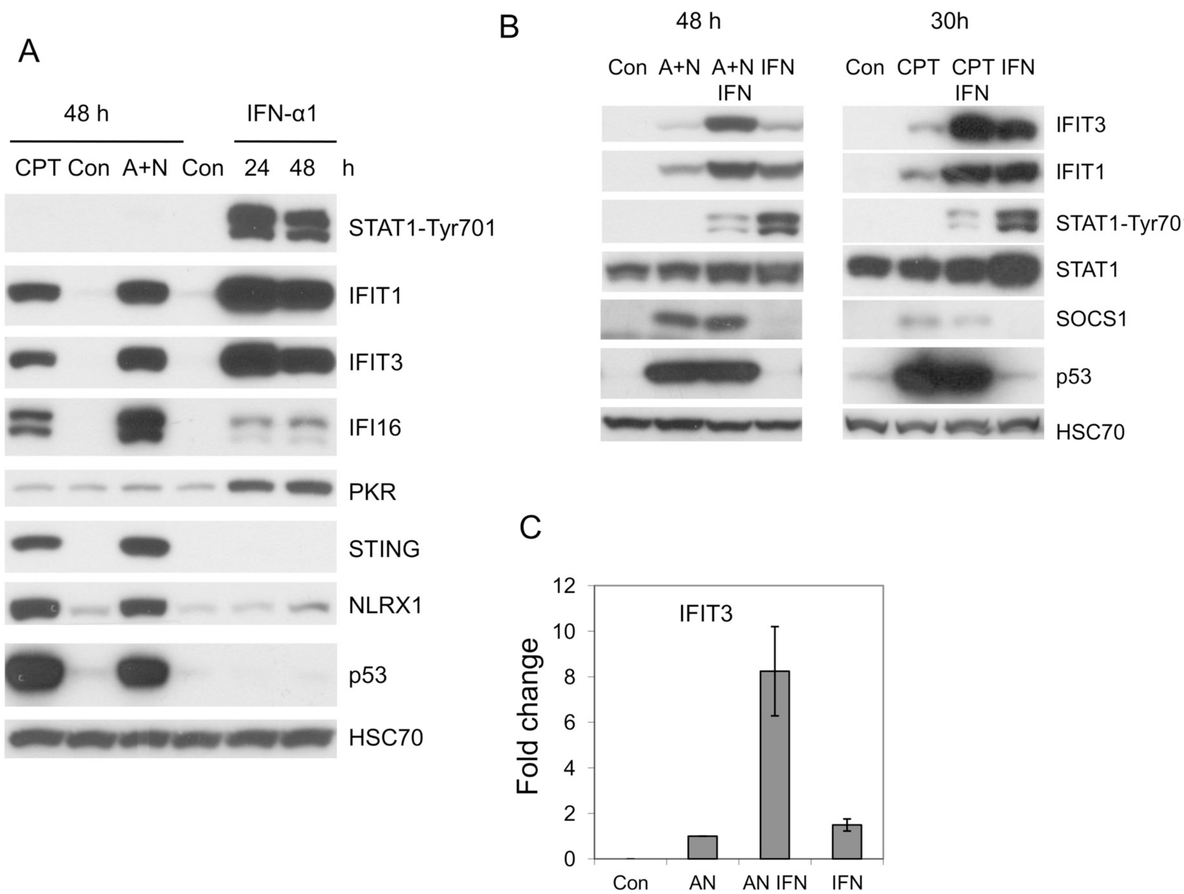

Fig. 10. Upregulation of innate immunity associated genes by A + N or camptothecin does not involve activation of STAT1. A. A549 cells were mock-treated (Con) or exposed either to A + N or to camptothecin (CPT) for 48 h. Other cells were exposed to recombinant human interferon-α1 (IFN-α1) at 2 ng/ml for 24 or 48 h. Subsequently, the expression of indicated proteins was examined by Western blotting. One of the antibodies detects activated STAT1 transcription factor with phosphorylated tyrosine 701 (STAT1-Tyr701). B. A549 cells were exposed to A + N (or CPT), IFN-α1 (IFN, 1 ng/ml) or to both treatment modalities simultaneously. The expression of IFIT3, IFIT1, SOCS1, STAT1 (total or phosphorylated) and p53 was examined by Western blotting. C. Quantitative Western blot analysis of IFIT3 in A549 cells exposed as indicated. The means and standard deviation were calculated from Western blots of protein lysates prepared from three biological replicates. Because IFIT3 was undetectable in control cells, its expression was set as 1 in cells exposed to A + N.

(180 kDa and 120 kDa) were detected. Its plausible, that cleavage of full-length molecule occurs already in vesicles transporting collagen to cell surface. However, in culture medium only the 120 kDa ectodomain was detected, the full-length form was not visible even after strong overexposure of the film. If the ectodomain in medium originated from cell lysis, we would also expect to detect the full-length molecule in the medium. Thus, we conclude that the ectodomain did not leak passively from dying cells. Hence, we infer that in cells exposed to A + N, the COL17A1 is strongly induced and its ectodomain is shed from cells modifying their environment.

To find out if A + N treatment can induce genes coding for immune-related extracellular molecules we searched for p53 ChIP-Seq peaks in genes encoding various cytokines and chemokines. Interestingly, using ChIP-Atlas [33], we found p53 ChIP-Seq peak at the exon 1/intron 1 border of interleukin-7 gene (Fig. 12B). This ChIP-Seq peak was detected in cells expressing engineered p53 molecules with strong co-operative binding of p53 monomers [31]. We found that actinomycin D and nutlin-3a strongly synergize in the induction of *IL7* (Fig. 12C). Moreover the induction of *IL7* by A + N or CPT was significantly attenuated in p53 knockdown cells (Fig. 12D). Thus, strongly activated p53 has the ability to induce expression of *IL7* what can change the activity of nearby immune cells because IL7 is non-redundant growth factor for many hematopoietic cell lineages especially for T and NK cells [41].

4. Discussion

We started our study with analysis of expression and activation status of caspase-1 in cells with strongly activated p53. Pro-caspase-1, coded by *CASP1*, was considerably upregulated by CPT or A + N, however we did not observe proteolytic activation, despite both CPT or A + N stimulating the expression of genes (*IFI16*, *NLRP1*) coding for inflammosome components required for autoproteolytic activation of pro-caspase-1. Thus, it appears that robust stimulation of p53 primes the cells to undergo caspase-1-induced death called pyroptosis, however, the bulk of cells did not die in this fashion in our experimental conditions. The fact that cells exposed to CPT or A + N express the components of inflammasomes activated by different DAMPS (NLRP1 responding to bacterial toxins and IFI16 responding to foreign, dsDNA) speaks in favor of the "priming" hypothesis. It seems that the cells vaguely experience stress conditions but they determine how to die based on the ultimate, specific trigger.

The expression of *CASP1* gene is an example of extreme synergy between actinomycin D and nutlin-3a in stimulating expression of p53-regulated gene (Fig. 2 and 3A). Using our p53 knockdown cells, we confirmed the observation of Gupta et al. [12] and Schlereth et al. [31] that *CASP1* is upregulated in p53-dependent fashion (Fig. 5A), however, p53 must be properly, post-translationally modified in order to stimulate *CASP1* expression. Phosphorylation of Ser46 and Ser392 appear to be among such key modifications (Fig. 5A, 9B), but other modifications are likely also required. Interestingly, Schlereth et al.

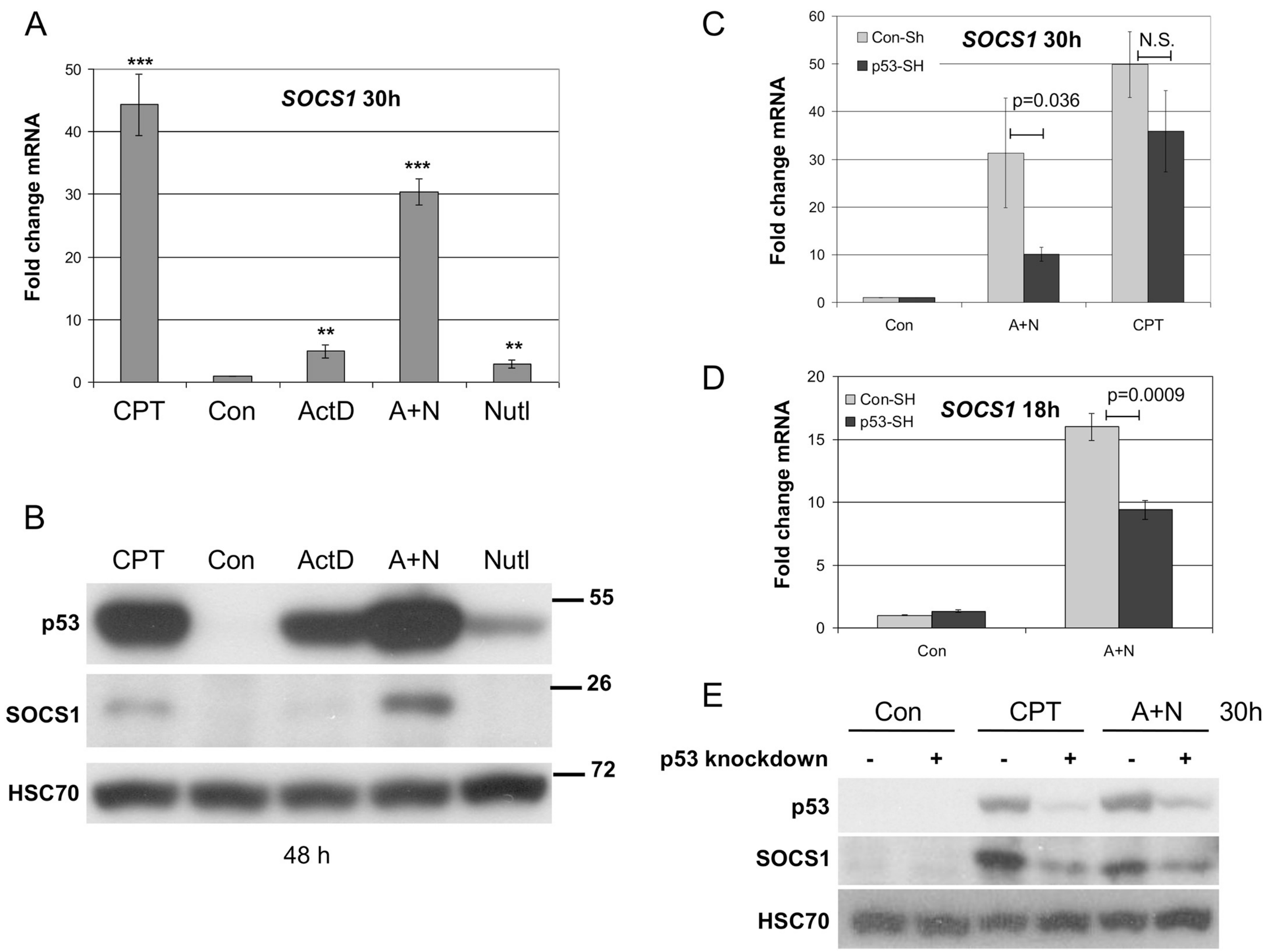

Fig. 11. *SOCS1* gene expression is upregulated in a p53-dependent manner. A. Measurement of relative *SOCS1* mRNA levels in A549 cells exposed to indicated substances or combination treatments for 30 h: CPT - camptothecin, Con - mock-treated control, ActD - actinomycin D, A + N, and Nut - nutlin-3a, *** $p < .001$, ** $p < .01$ by Student's *t*-test. B. Protein expression in A549 cells exposed to indicated substances or their combinations for 48 h. C. D. Relative SOCS1 mRNA levels in p53 knockdown A549 cells (p53-SH), and control cells (Con-SH), exposed to A + N or CPT for 30 (C) or 18 (D) hours. The results represent mean and standard deviation from three independent experiments, *p* values calculated by Student's *t*-test E. Expression of indicated proteins in p53 knockdown A549 cells (+), or control cells (−), exposed to CPT or A + N for 30 h.

[31] found that activation of *CASP1* gene by p53 critically depends on the cooperation of p53 molecules forming the DNA-binding tetramer. Mutations of p53, which specifically destroy the cooperativity of p53 molecules, do not significantly influence the ability of p53 tetramer to activate the cell cycle inhibitors (e.g. p21). However, these mutations can completely destroy the ability of p53 to activate *CASP1*. Hence, we suspect that CPT or A + N, which strongly stimulate *CASP1*, promote post-translational modifications that may favor cooperative binding between p53 monomers. Here, the question arises, as to what physiological conditions result in p53 activation to the degree that allows for *CASP1* gene stimulation. This level of activation can be achieved with camptothecin and possibly with anticancer drugs derived from camptothecin (topotecan, irinotecan), which are widely used in clinic. Hence, it is possible, that camptothecin-related drugs facilitate pyroptosis. However, what conditions beyond treatment with anticancer drugs are able to strongly stimulate p53 in vivo? We suspect, that these conditions occur locally within tissues and are associated with infections from various pathogens, which initially induce local tissue damage, secrete various toxins and trigger activity of immune cells, in turn releasing a plethora of locally-acting toxic factors including DNA-damaging ROS. Biologically, actinomycin D is an antibiotic, toxic also to eukaryotic cells, produced by *Streptomyces antibioticus*, a Gram-positive bacterium living in soil [42]. Is it possible that the strong activation of p53 can result from similar toxins produced by bacteria able to infect humans? Some soil bacteria from *Streptomyces* genus were found to produce infections in immunodeficient individuals or in humans living in tropical regions [43,44]. In the context of this conjecture, it is not surprising that strong activation of p53 is associated with the induction of many immunity genes detected in this study. We found that A + N or CPT treatment stimulated the expression of *IFIT1*, *IFIT3*, *NLRP1*, *NLRX1, IL7* and *STING* (*TMEM173*) genes, which code for proteins involved in innate immunity and which have not been identified so far as p53-regulated genes. In cells with knocked-down expression of p53, the upregulation of *NLRP1*, *NLRX1, IL7* and *STING* was attenuated, indicating that these four genes are regulated in p53-dependent fashion. The activation of *IL7* gene indicates that p53 can modify the microenvironment of cell by sending signals, which help in proliferation and survival of nearby immune cells. In case of *NLRP1* and *NLRX1* we were able to show that the gene promoters were activated by ectopically expressed p53. Moreover, for *NLRX1*, we identified the p53 RE within its promoter using the site-directed mutagenesis. According to published meta-analyses [45,46], some high-throughput studies

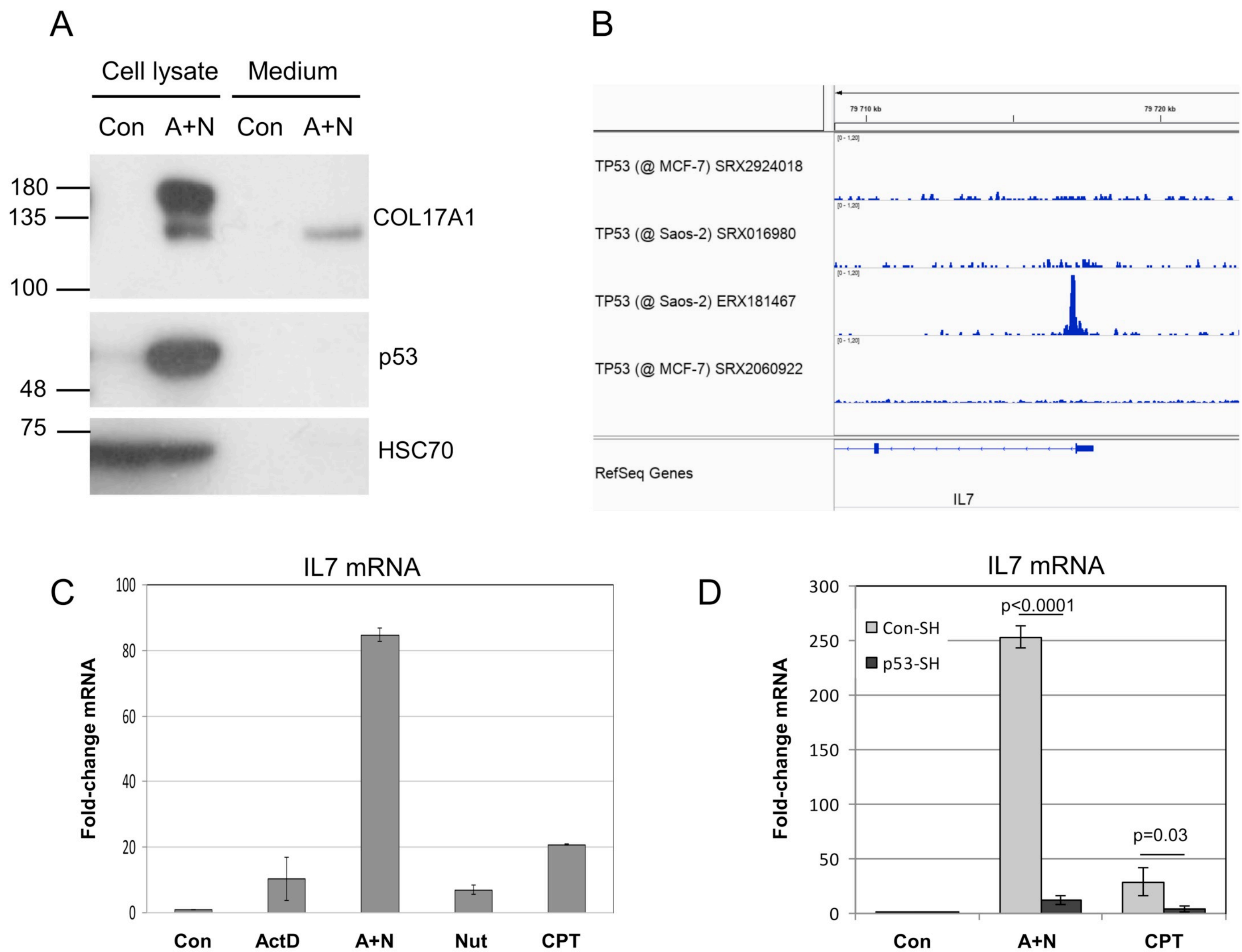

Fig. 12. Exposure to A + N upregulates expression of genes coding for secreted proteins. A. The expression of COL17A1 protein (180 kDa whole-length molecule and 120 kDa ectodomain) in cell lysates and in concentrated medium of A549 cells exposed for 48 h to A + N (24 h in complete medium + 24 h in serum-free medium). B. Genome browser (IGV) views of p53 binding peak at the exon1/intron1 border of *IL7* gene. Using ChIP-Atlas tool [33] we imported publically available coverage tracks from four ChIP-Seq experiments aimed at finding p53 binding sites in MCF-7 cell line exposed to ionizing radiation and Nutlin (sample ID SRX2924018), SAOS-2 cell line ectopically expressing wild-type p53 (sample ID SRX016980), SAOS-2 ectopically expressing pair of engineered p53 molecules with strong cooperative binding of p53 monomers [31] (sample ID ERX181467) or in MCF7 cells treated with Nutlin (sample ID SRX2060922). C. Measurement of relative *IL7* mRNA levels in A549 cells exposed to indicated substances or combination treatments for 30 h. D. Relative *IL7* mRNA levels in p53 knockdown A549 cells (p53-SH), and control cells (Con-SH), exposed to A + N or CPT for 30 h (p values from three repeats calculated by Student's *t*-test).

noticed that *NLRP1*, *NLRX1, IL7* and *STING* are regulated in p53-dependent fashion in some cell lines and in some treatment conditions. However, according to the criteria used by the authors of the meta-analyses, these genes are not p53 targets. This apparent discrepancy may result from the fact that some of the original studies did not use stress factors, which activate p53 enough to stimulate expression of these genes. Hence, probably they fell below a certain threshold in the meta-analysis to be considered a "direct p53 target".

In case of *IFIT1* and *IFIT3*, unexpectedly, we found that p53 knockdown by lentivirus-delivered shRNA particles did not strongly attenuate their expression especially at mRNA level. This is confusing based on the degree of upregulation of these genes, as this expression appears to closely correlate with the degree of p53 activation determined by the presence of phosphorylated Ser46 or Ser392. Moreover, the kinase inhibitor C16, which prevents p53 activation, also prevents induction of *IFIT1* and *IFIT3*. However, with the data at hand, it is safer to conclude that the regulation of *IFIT1* and *IFIT3* by p53 is an open issue, which needs to be clarified.

The *NLRX1* and *NLRP1* genes are new additions to the list of p53-regulated genes coding for innate immunity proteins. In addition to the aforementioned *CASP1* and *IFI16*, p53 is required for optimal upregulation of *ISG15*, which codes for a protein with direct antiviral activity [21]. *IRF5* and *IRF7*, regulated by p53, code for transcription regulators stimulating expression of interferons and inflammatory cytokines [19,20]. IRF9, which forms complex with activated STAT1 and STAT2 and stimulates expression of many antiviral effector molecules, is also regulated by p53 [47]. Thus, p53 can facilitate the production of interferons. Hence, it is not surprising that many viruses, even the ones that are not associated with cancer, e.g. SARS coronavirus [48], produce proteins, which inactivate p53. Strikingly, p53 was discovered because it formed tight complex with viral protein [49]. In this context, our finding that strongly activated p53 induces expression of *STING* is pivotal in that it points to another mechanism used by p53 to stimulate the synthesis of these antiviral cytokines. STING is located at the crossroad of two signaling pathways. One pathway emerges from the detection of DNA viruses (by cGAS or IFI16 proteins), while the second originates from the detection of RNA viruses (by RIG-I receptors) and both converge on the activation of IRF3 transcription factor, which directly stimulates the transcription of genes for type I interferons [18]. We found that in spite of strong upregulation of STING, interferons are not produced in our model, which can be inferred from the lack of STAT1 phosphorylation in cells exposed to CPT or A + N. Probably, the cells are primed for interferon production but they lack a specific trigger (e.g. infection by a virus) to actually activate transcription of interferon genes.

Our hypothesis that PKR was responsible for p53 activation leading to the induction of innate immunity genes was not supported by observations. Although the activation of p53 and induction of innate immunity genes was blocked by C16, a specific inhibitor of PKR, the near complete knockdown of PKR did not prevent activation of p53 or upregulation of innate immunity genes. Moreover, C16 was able to block p53 activation and upregulation of innate immunity genes even in cells with almost full knockdown of PKR. This is probably another example of the off-target activity of a kinase inhibitor. Whatever the target(s) of C16, the kinase(s) blocked by this compound play major role in activation of p53 in response to CPT or A + N treatment. Even in submicromolar concentrations C16 inhibited phosphorylation of p53 on Ser46 and Ser392. Moreover, C16 did not block phosphorylation of p53 on Ser15 which argues against general block of many kinases. We do not know if C16 blocks the activity of kinases that directly phosphorylate p53 on Ser46 and Ser392 or if it blocks the activity of a kinase, which phosphorylates p53 on other amino acid forming a signal for other kinases to modify Ser46 and Ser392. Some studies demonstrated that C16 protects against tissue damage and inflammation, especially in the central nervous system [36,50,51]. In light of our observation that C16 inhibits p53 activation and the fact that p53 is strong inducer of cell death, it must be considered that some cytoprotective activities of this compound are mediated through the inhibition of p53-induced apoptosis or inflammation. The off-target activity of C16 has been already noticed by other investigators [52]. In our opinion, the search for p53-activating kinase inhibited by C16 is of great importance as it may be playing major role in stimulating p53-regulated apoptosis and innate immunity.

A surprising finding of this study is the activation of *SOCS1* expression by A + N or CPT in p53-dependent manner. SOCS1 protein inhibits the signaling through the pathway stimulated by various cytokines including type I (e.g. IFN-α, IFN-β) and type II (IFN-γ) interferons [53]. Hence, *SOCS1* appears to be another gene of innate immunity system regulated by p53. *SOCS1*, in contrast with other p53-regulated genes mentioned in this Discussion, inhibits interferon-induced signaling. It may seem counter-intuitive, but in our opinion it is plausible. We suspect that the p53-SOCS1 relationship is a part of a negative feedback loop within the p53-innate immunity signaling system. A good analogy is p53-MDM2 relationship in the p53 signaling system. In this loop, p53 activates *MDM2* gene, which codes for the negative regulator of p53 [46]. We hypothesize that p53-dependent *SOCS1* has the task of quickly silencing the signaling when the stress factor disappears. It is also possible that SOCS1 prevents excessive stimulation of innate immunity when p53 is activated by a strong stress factor and the cells are additionally exposed to interferons (not unusual situation in lung epithelium for instance). It was found by others that *SOCS1* can be upregulated by nutlin-3a (a specific p53 activator) in acute myeloid leukemia cells which also supports the notion that SOCS1 is p53-dependent gene [54]. Thus, the data presented in this paper and the observations made by others support the notion that the innate immunity is the stress-response system strongly influenced by p53. Moreover, upregulation of antiviral genes by CPT or A + N is another observation in the growing body of evidence that anticancer chemotherapeutic agents have strong antiviral properties resulting from upregulation innate immunity genes [55]. This property of anticancer drugs must be carefully considered when planning for the anticancer strategies combining the use of oncolytic virotherapy with chemotherapeutic agents [56].

5. Conclusions

Strong activation of p53 by actinomycin D acting with nutlin-3a or by camptothecin is associated with upregulation of many innate immunity genes. Considering the functions of their proteins, we conclude that strongly stimulated p53 primes the cells for pyroptosis and for the induction of interferon genes. Activation of *NLRP1, NLRX1, STING, IL7* and *SOCS1* is p53-dependent, i.e., downregulation of p53 attenuates their expression. In the regulation of innate immunity, p53 plays double role, by inducing some genes (e.g. *STING*) it helps to trigger interferon production and by inducing *SOCS1* it prevents excessive stimulation of the interferon signaling. In our experimental conditions both activation of p53 and upregulation of innate immunity proteins is strongly inhibited by C16, an anti-inflammatory substance, considered a specific inhibitor of PKR kinase, but acting in our model in an apparently PKR-independent manner. Thus, an unidentified kinase inhibited by C16 plays a major role both in activation of p53 and in stimulation of a subset of innate immunity genes.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2020.109552.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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References

[1] A. Zajkowicz, A. Gdowicz-Kłosok, M. Krześniak, D. Ścieglińska, M. Rusin, Actinomycin D and nutlin-3a synergistically promote phosphorylation of p53 on serine 46 in cancer cell lines of different origin, Cell. Signal. 27 (9) (2015 Sep) 1677–1687, https://doi.org/10.1016/j.cellsig.2015.05.005.

[2] A. Zajkowicz, A. Gdowicz-Kłosok, M. Krześniak, P. Janus, B. Łasut, M. Rusin, The Alzheimer's disease-associated TREM2 gene is regulated by p53 tumor suppressor protein, Neurosci. Lett. 681 (2018 Aug 10) 62–67, https://doi.org/10.1016/j.neulet.2018.05.037.

[3] M.L. Choong, H. Yang, M.A. Lee, D.P. Lane, Specific activation of the p53 pathway by low dose actinomycin D: a new route to p53 based cyclotherapy, Cell Cycle 8 (17) (2009 Sep 1) 2810–2818.

[4] F. Mantovani, F. Tocco, J. Girardini, P. Smith, M. Gasco, X. Lu, T. Crook, G. Del Sal, The prolyl isomerase Pin1 orchestrates p53 acetylation and dissociation from the apoptosis inhibitor iASPP, Nat. Struct. Mol. Biol. 14 (10) (2007 Oct) 912–920.

[5] L. Galluzzi, I. Vitale, S.A. Aaronson, et al., Molecular mechanisms of cell death: recommendations of the nomenclature committee on cell death 2018, Cell Death Differ. 25 (3) (2018 Mar) 486–541, https://doi.org/10.1038/s41418-017-0012-4.

[6] L.I. Labzin, M.A. Lauterbach, E. Latz, Interferons and inflammasomes: cooperation and counterregulation in disease, J. Allergy Clin. Immunol. 138 (1) (2016 Jul) 37–46, https://doi.org/10.1016/j.jaci.2016.05.010.

[7] J. Shi, W. Gao, F. Shao, Pyroptosis: gasdermin-mediated programmed necrotic cell death, Trends Biochem. Sci. 42 (4) (2017 Apr) 245–254, https://doi.org/10.1016/j.tibs.2016.10.004.

[8] M. Krześniak, A. Zajkowicz, I. Matuszczyk, M. Rusin, Rapamycin prevents strong phosphorylation of p53 on serine 46 and attenuates activation of the p53 pathway in A549 lung cancer cells exposed to actinomycin D, Mech. Ageing Dev. 139 (2014 Jul) 11–21, https://doi.org/10.1016/j.mad.2014.06.002.

[9] S.J. Baker, S. Markowitz, E.R. Fearon, J.K. Willson, B. Vogelstein, Suppression of human colorectal carcinoma cell growth by wild-type p53, Science. 249 (4971) (1990 Aug 24) 912–915.

[10] B. Gu, W.G. Zhu, Surf the post-translational modification network of p53 regulation, Int. J. Biol. Sci. 8 (5) (2012) 672–684, https://doi.org/10.7150/ijbs.4283.

[11] K. Sakaguchi, H. Sakamoto, M.S. Lewis, C.W. Anderson, J.W. Erickson, E. Appella, D. Xie, Phosphorylation of serine 392 stabilizes the tetramer formation of tumor suppressor protein p53, Biochemistry. 36 (33) (1997 Aug 19) 10117–10124.

[12] S. Gupta, V. Radha, Y. Furukawa, G. Swarup, Direct transcriptional activation of human caspase-1 by tumor suppressor p53, J. Biol. Chem. 276 (14) (2001 Apr 6)

10585–10588 Epub 2001 Feb 13. PubMed PMID: 11278253.

[13] A. Malik, T.D. Kanneganti, Inflammasome activation and assembly at a glance, J. Cell Sci. 130 (23) (2017 Dec 1) 3955–3963, https://doi.org/10.1242/jcs.207365.

[14] C.M. Turner, N. Arulkumaran, M. Singer, R.J. Unwin, F.W. Tam, Is the inflammasome a potential therapeutic target in renal disease? BMC Nephrol. 15 (2014 Jan 23) 21, , https://doi.org/10.1186/1471-2369-15-21 PubMed PMID: 24450291; PubMed Central PMCID: PMC3918225.

[15] L.L. Song, F. Alimirah, R. Panchanathan, H. Xin, D. Choubey, Expression of an IFN-inducible cellular senescence gene, IFI16, is up-regulated by p53, Mol. Cancer Res. 6 (11) (2008 Nov) 1732–1741, https://doi.org/10.1158/1541-7786.MCR-08-0208 (Epub 2008 Oct 30. PubMed PMID: 18974396).

[16] N.B. Bryan, A. Dorfleutner, S.J. Kramer, C. Yun, Y. Rojanasakul, C. Stehlik, Differential splicing of the apoptosis-associated speck like protein containing a caspase recruitment domain (ASC) regulates inflammasomes, J Inflamm (Lond). 7 (2010 May 18) 23, https://doi.org/10.1186/1476-9255-7-23.

[17] D. Boucher, M. Monteleone, R.C. Coll, K.W. Chen, C.M. Ross, J.L. Teo, G.A. Gomez, C.L. Holley, D. Bierschenk, K.J. Stacey, A.S. Yap, J.S. Bezbradica, K. Schroder, Caspase-1 self-cleavage is an intrinsic mechanism to terminate inflammasome activity, J. Exp. Med. 215 (3) (2018 Mar 5) 827–840, https://doi.org/10.1084/jem.20172222.

[18] A. Zevini, D. Olagnier, J. Hiscott, Crosstalk between cytoplasmic RIG-I and STING sensing pathways, Trends Immunol. 38 (3) (2017 Mar) 194–205, https://doi.org/10.1016/j.it.2016.12.004.

[19] T. Mori, Y. Anazawa, M. Iiizumi, S. Fukuda, Y. Nakamura, H. Arakawa, Identification of the interferon regulatory factor 5 gene (IRF-5) as a direct target for p53, Oncogene. 21 (18) (2002 Apr 25) 2914–2918 PubMed PMID: 11973653.

[20] L. Yuan, Z. Chen, S. Song, S. Wang, C. Tian, G. Xing, X. Chen, Z.X. Xiao, F. He, L. Zhang, p53 degradation by a coronavirus papain-like protease suppresses type I interferon signaling, J. Biol. Chem. 290 (5) (2015 Jan 30) 3172–3182, https://doi.org/10.1074/jbc.M114.619890 (Epub 2014 Dec 10. PubMed PMID: 25505178).

[21] B.T. Hummer, X.L. Li, B.A. Hassel, Role for p53 in gene induction by double-stranded RNA, J. Virol. 75 (16) (2001 Aug) 7774–7777 PubMed PMID: 11462054.

[22] T. Tebaldi, S. Zaccara, F. Alessandrini, A. Bisio, Y. Ciribilli, A. Inga, Whole-genome cartography of p53 response elements ranked on transactivation potential, BMC Genomics 16 (2015 Jun 17) 464, , https://doi.org/10.1186/s12864-015-1643-9.

[23] A. Pichlmair, C. Lassnig, C.A. Eberle, M.W. Górna, C.L. Baumann, T.R. Burkard, T. Bürckstümmer, A. Stefanovic, S. Krieger, K.L. Bennett, T. Rülicke, F. Weber, J. Colinge, M. Müller, G. Superti-Furga, IFIT1 is an antiviral protein that recognizes 5′-triphosphate RNA, Nat. Immunol. 12 (7) (2011 Jun 5) 624–630, https://doi.org/10.1038/ni.2048 PubMed PMID: 21642987.

[24] R.C. Fleith, H.V. Mears, X.Y. Leong, T.J. Sanford, E. Emmott, S.C. Graham, D.S. Mansur, T.R. Sweeney, IFIT3 and IFIT2/3 promote IFIT1-mediated translation inhibition by enhancing binding to non-self RNA, Nucleic Acids Res. 46 (10) (2018 Jun 1) 5269–5285, https://doi.org/10.1093/nar/gky191.

[25] X.Y. Liu, W. Chen, B. Wei, Y.F. Shan, C. Wang, IFN-induced TPR protein IFIT3 potentiates antiviral signaling by bridging MAVS and TBK1, J. Immunol. 187 (5) (2011 Sep 1) 2559–2568, https://doi.org/10.4049/jimmunol.1100963.

[26] I. Tattoli, L.A. Carneiro, M. Jéhanno, J.G. Magalhaes, Y. Shu, D.J. Philpott, D. Arnoult, S.E. Girardin, NLRX1 is a mitochondrial NOD-like receptor that amplifies NF-kappaB and JNK pathways by inducing reactive oxygen species production, EMBO Rep. 9 (3) (2008 Mar) 293–300, https://doi.org/10.1038/sj.embor.7401161.

[27] G. Stokman, L. Kors, P.J. Bakker, E. Rampanelli, N. Claessen, G.J.D. Teske, L. Butter, H. van Andel, M.A. van den Bergh Weerman, P.W.B. Larsen, M.C. Dessing, C.J. Zuurbier, S.E. Girardin, S. Florquin, J.C. Leemans, NLRX1 dampens oxidative stress and apoptosis in tissue injury via control of mitochondrial activity, J. Exp. Med. 214 (8) (2017 Aug 7) 2405–2420, https://doi.org/10.1084/jem.20161031.

[28] F. Martinon, K. Burns, J. Tschopp, The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta, Mol. Cell 10 (2) (2002 Aug) 417–426.

[29] C. Lin, J. Zhang, Inflammasomes in inflammation-induced cancer, Front. Immunol. 8 (2017 Mar 15) 271, https://doi.org/10.3389/fimmu.2017.00271.

[30] A.R. Cuddihy, S. Li, N.W. Tam, A.H. Wong, Y. Taya, N. Abraham, J.C. Bell, A.E. Koromilas, Double-stranded-RNA-activated protein kinase PKR enhances transcriptional activation by tumor suppressor p53, Mol. Cell. Biol. 19 (4) (1999 Apr) 2475–2484.

[31] K. Schlereth, R. Beinoraviciute-Kellner, M.K. Zeitlinger, A.C. Bretz, M. Sauer, J.P. Charles, F. Vogiatzi, E. Leich, B. Samans, M. Eilers, C. Kisker, A. Rosenwald, T. Stiewe, DNA binding cooperativity of p53 modulates the decision between cell-cycle arrest and apoptosis, Mol. Cell 38 (3) (2010 May 14) 356–368, https://doi.org/10.1016/j.molcel.2010.02.037.

[32] T.T. Nguyen, S.A. Grimm, P.R. Bushel, J. Li, Y. Li, B.D. Bennett, C.A. Lavender, J.M. Ward, D.C. Fargo, C.W. Anderson, L. Li, M.A. Resnick, D. Menendez, Revealing a human p53 universe, Nucleic Acids Res. 46 (16) (2018 Sep 19) 8153–8167, https://doi.org/10.1093/nar/gky720.

[33] S. Oki, T. Ohta, G. Shioi, H. Hatanaka, O. Ogasawara, Y. Okuda, H. Kawaji, R. Nakaki, J. Sese, C. Meno, ChIP-Atlas: a data-mining suite powered by full integration of public ChIP-seq data, EMBO Rep. 19 (12) (2018 Dec), https://doi.org/10.15252/embr.201846255 pii: e46255.

[34] M. Petr, R. Helma, A. Polášková, A. Krejčí, Z. Dvořáková, I. Kejnovská, L. Navrátilová, M. Adámik, M. Vorlíčková, M. Brázdová, Wild-type p53 binds to MYC promoter G-quadruplex, Biosci Rep. 36 (5) (2016 Oct 14) pii: e00397.

[35] J.A. Marchal, G.J. Lopez, M. Peran, A. Comino, J.R. Delgado, J.A. García-García, V. Conde, F.M. Aranda, C. Rivas, M. Esteban, M.A. Garcia, The impact of PKR activation: from neurodegeneration to cancer, FASEB J. 28 (5) (2014 May) 1965–1974, https://doi.org/10.1096/fj.13-248294.

[36] S. Ingrand, L. Barrier, C. Lafay-Chebassier, B. Fauconneau, G. Page, J. Hugon, The oxindole/imidazole derivative C16 reduces in vivo brain PKR activation, FEBS Lett. 581 (23) (2007 Sep 18) 4473–4478.

[37] G.R. Stark, J.E. Darnell Jr., The JAK-STAT pathway at twenty, Immunity. 36 (4) (2012 Apr 20) 503–514, https://doi.org/10.1016/j.immuni.2012.03.013.

[38] I.H. Park, K.W. Baek, E.Y. Cho, B.Y. Ahn, PKR-dependent mechanisms of interferon-α for inhibiting hepatitis B virus replication, Mol Cells. 32 (2) (2011 Aug) 167–172, https://doi.org/10.1007/s10059-011-1059-6.

[39] V. Yodsurang, C. Tanikawa, T. Miyamoto, P.H.Y. Lo, M. Hirata, K. Matsuda, Identification of a novel p53 target, COL17A1, that inhibits breast cancer cell migration and invasion, Oncotarget. 8 (34) (2017 Jun 9) 55790–55803.

[40] C.W. Franzke, K. Tasanen, H. Schäcke, Z. Zhou, K. Tryggvason, C. Mauch, P. Zigrino, S. Sunnarborg, D.C. Lee, F. Fahrenholz, L. Bruckner-Tuderman, Transmembrane collagen XVII, an epithelial adhesion protein, is shed from the cell surface by ADAMs, EMBO J. 21 (19) (2002 Oct 1) 5026–5035.

[41] H.Y. Huang, S.A. Luther, Expression and function of interleukin-7 in secondary and tertiary lymphoid organs, Semin. Immunol. 24 (3) (2012 Jun) 175–189.

[42] T.M. Karpiński, A. Adamczak, Anticancer activity of bacterial proteins and peptides, Pharmaceutics 10 (2) (2018 Apr 30), https://doi.org/10.3390/pharmaceutics10020054 pii: E54.

[43] M. Kapadia, K.V. Rolston, X.Y. Han, Invasive Streptomyces infections: six cases and literature review, Am. J. Clin. Pathol. 127 (4) (2007 Apr) 619–624.

[44] P. Verma, A. Jha, Mycetoma: reviewing a neglected disease, Clin. Exp. Dermatol. (2018 May 28), https://doi.org/10.1111/ced.13642 [Epub ahead of print] Review. PubMed PMID: (29808607).

[45] M. Fischer, P. Grossmann, M. Padi, J.A. DeCaprio, Integration of TP53, DREAM, MMB-FOXM1 and RB-E2F target gene analyses identifies cell cycle gene regulatory networks, Nucleic Acids Res. 44 (13) (2016 Jul 27) 6070–6086, https://doi.org/10.1093/nar/gkw523.

[46] M. Fischer, Census and evaluation of p53 target genes, Oncogene. 36 (28) (2017 Jul 13) 3943–3956, https://doi.org/10.1038/onc.2016.502.

[47] C. Muñoz-Fontela, S. Macip, L. Martínez-Sobrido, L. Brown, J. Ashour, A. García-Sastre, S.W. Lee, S.A. Aaronson, Transcriptional role of p53 in interferon-mediated antiviral immunity, J. Exp. Med. 205 (8) (2008 Aug 4) 1929–1938, https://doi.org/10.1084/jem.20080383.

[48] Y. Ma-Lauer, J. Carbajo-Lozoya, M.Y. Hein, M.A. Müller, W. Deng, J. Lei, B. Meyer, Y. Kusov, B. von Brunn, D.R. Bairad, S. Hünten, C. Drosten, H. Hermeking, H. Leonhardt, M. Mann, R. Hilgenfeld, A. von Brunn, p53 down-regulates SARS coronavirus replication and is targeted by the SARS-unique domain and PLpro via E3 ubiquitin ligase RCHY1, Proc. Natl. Acad. Sci. U. S. A. 113 (35) (2016 Aug 30) E5192–E5201, https://doi.org/10.1073/pnas.1603435113.

[49] R. Aloni-Grinstein, M. Charni-Natan, H. Solomon, V. Rotter, p53 and the viral connection: back into the future (‡), Cancers (Basel) 10 (6) (2018 Jun 4), https://doi.org/10.3390/cancers10060178 pii: E178.

[50] C. Tronel, G. Page, S. Bodard, S. Chalon, D. Antier, The specific PKR inhibitor C16 prevents apoptosis and IL-1β production in an acute excitotoxic rat model with a neuroinflammatory component, Neurochem. Int. 64 (2014 Jan) 73–83, https://doi.org/10.1016/j.neuint.2013.10.012.

[51] S. Mangali, A. Bhat, M.P. Udumula, I. Dhar, D. Sriram, A. Dhar, Inhibition of protein kinase R protects against palmitic acid-induced inflammation, oxidative stress, and apoptosis through the JNK/NF-kB/NLRP3 pathway in cultured H9C2 cardiomyocytes, J. Cell. Biochem. (2018 Sep 27), https://doi.org/10.1002/jcb.27643.

[52] H.M. Chen, L. Wang, S.R. D'Mello, A chemical compound commonly used to inhibit PKR, {8-(imidazol-4-ylmethylene)-6H-azolidino[5,4-g] benzothiazol-7-one}, protects neurons by inhibiting cyclin-dependent kinase, Eur. J. Neurosci. 28 (10) (2008 Nov) 2003–2016, https://doi.org/10.1111/j.1460-9568.2008.06491.x.

[53] S. Chikuma, M. Kanamori, S. Mise-Omata, A. Yoshimura, Suppressors of cytokine signaling: potential immune checkpoint molecules for cancer immunotherapy, Cancer Sci. 108 (4) (2017 Apr) 574–580, https://doi.org/10.1111/cas.13194.

[54] V. Tisato, A. Norcio, C. Celeghini, D. Milani, A. Gonelli, P. Secchiero, Upregulation of SOCS-1 by Nutlin-3 in acute myeloid leukemia cells but not in primary normal cells, Clinics (Sao Paulo). 69 (1) (2014 Jan) 68–74, https://doi.org/10.6061/clinics/2014(01)10.

[55] Y. Shin, H. Lim, B.S. Choi, K.C. Kim, C. Kang, Y.S. Bae, C.H. Yoon, Highly activated p53 contributes to selectively increased apoptosis of latently HIV-1 infected cells upon treatment of anticancer drugs, Virol. J. 13 (1) (2016 Aug 16) 141, , https://doi.org/10.1186/s12985-016-0595-2.

[56] S.T. Wennier, J. Liu, G. McFadden, Bugs and drugs: oncolytic virotherapy in combination with chemotherapy, Curr. Pharm. Biotechnol. 13 (9) (2012 Jul) 1817–1833.