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Inflammatory Cell Death, PANoptosis, Mediated by Cytokines in Diverse Cancer Lineages Inhibits Tumor Growth

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ABSTRACT

Resistance to cell death is a hallmark of cancer. Immunotherapy, particularly immune checkpoint blockade therapy, drives immune-mediated cell death and has greatly improved treatment outcomes for some patients with cancer, but it often fails clinically. Its success relies on the cytokines and cytotoxic functions of effector immune cells to bypass the resistance to cell death and eliminate cancer cells. However, the specific cytokines capable of inducing cell death in tumors and the mechanisms that connect cytokines to cell death across cancer cell types remain unknown. In this study, we analyzed expression of several cytokines that are modulated in tumors and found correlations between cytokine expression and mortality. Of several cytokines tested for their ability to kill cancer cells, only TNF-α and IFN-γ together were able to induce cell death in 13 distinct human cancer cell lines derived from colon and lung cancer, melanoma, and leukemia. Further evaluation of the specific programmed cell death pathways activated by TNF-α and IFN-γ in these cancer lines identified PANoptosis, a form of inflammatory cell death that was previously shown to be activated by contemporaneous engagement of components from pyroptosis, apoptosis, and/or necroptosis. Specifically, TNF-α and IFN-γ triggered activation of gasdermin D, gasdermin E, caspase-8, caspase-3, caspase-7, and MLKL. Furthermore, the intratumoral administration of TNF-α and IFN-γ suppressed the growth of transplanted xenograft tumors in an NSG mouse model. Overall, this study shows that PANoptosis, induced by synergism of TNF-α and IFN-γ, is an important mechanism to kill cancer cells and suppress tumor growth that could be therapeutically targeted. *ImmunoHorizons*, 2021, 5: 568–580.

INTRODUCTION

Cancer is a group of diseases defined by abnormal growth of cells associated with pathological manifestations and significant morbidity and mortality globally (1). Because cancers are characterized by dysregulated cell death and inflammatory responses (2–4), many current therapeutic approaches aim to preferentially induce cell death in cancer cells (5–7). However, cancer cells acquire mutations that subvert the programmed cell death (PCD) pathways, making them refractory to anti-cancer therapies, and evasion of PCD mechanisms is one of the hallmarks of cancer (3). Therefore, gaining detailed understanding of these

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R.K.S.M., R.K., B.S., B.K., and T.-D.K. designed the study. R.K.S.M., R.K., B.S., B.K., S.L., and P.S. performed the experiments and analyzed the data. R.K.S.M., R.K., and T.-D.K. wrote the manuscript with input from the other authors. T.-D.K. oversaw all aspects the project.

Abbreviations used in this article: Cockt-1, cocktail-1; CRC, colorectal cancer; gRNA, guide RNA; GSDMD, gasdermin D; GSDME, gasdermin E; ICT, immune checkpoint blockade therapy; PCD, programmed cell death; SJCRH, St. Jude Children's Research Hospital; TME, tumor microenvironment.

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PCD mechanisms and identifying novel agonists remains a critical area of research to develop promising new strategies to treat cancers.

Elimination of transformed cancer cells by the immune system through PCD is an important checkpoint that can block cancer progression. Based on this strategy, immunotherapy such as immune checkpoint blockade therapy (ICT), which activates the immune-mediated killing of tumor cells by blocking suppressor proteins such as programmed death 1 (PD-1) and CTL associated protein 4, has improved treatment outcomes for some patients with cancer (8). Yet a significant proportion of patients remain refractory to ICT, primarily because of the failure to induce immunogenic cell death (8–10). The key effectors of ICT include cytotoxic T cells and NK cells, which can kill tumor cells directly through cytotoxic perforins and granzymes or indirectly by releasing potent proinflammatory cytokines (11). However, cancer cells can subvert the granzyme- and perforin-mediated cell death mechanisms (12), making it particularly important to understand the mechanisms of cytokine-mediated killing of tumor cells. Among the cytokines, previous studies have indicated that TNF-α has potent anti-tumor activity; however, tumor cells have evolved evasion mechanisms that allow them to suppress the expression of TNF and to block its effector cell death function of apoptosis (13). TNF-α–mediated cytotoxicity can be augmented by TLR stimulation or by specific cytokines, including IFN-γ (14–18). Several models have been proposed to explain this augmentation and suggest that the synergism may exist at the level of transcriptional regulation of MHC class I expression and/or NO production to promote cell death (19, 20). Additionally, recent studies have indicated that tumor sensitivity to cell death is associated with both TNF-α and IFN-γ expression profiles, which are particularly important in the context of tumor resistance or loss of sensitivity to granzyme- and perforin-mediated anti-tumor immune responses (11, 21). However, to date, the molecular mechanism of cell death induced by TNF-α and IFN-γ, or other cytokines, in human cancer cells remains understudied. Therefore, identifying and characterizing different cytokines capable of inducing PCD remains a critical area of research to develop promising strategies to treat cancers.

Several different PCD pathways have been described in recent years. The PCD pathway of apoptosis is a widely studied cell death mechanism executed by caspase-3 and -7 downstream of initiator caspases caspase-8/10 or -9 (22). Although loss of apoptosis is one of the founding hallmarks of cancer (3), cancer cells can also acquire resistance to apoptosis through genetic and epigenetic mechanisms (23–28). Mutations in tumor suppressor genes p53, PTEN, and caspases often promote the development of tumorigenesis (29). When apoptosis is not effective, alternative PCD pathways of pyroptosis or necroptosis can be beneficial to eliminate cancer cells (30–35). Pyroptosis is executed by gasdermin family members (35–38), whereas necroptosis is mediated by RIPK3-dependent MLKL oligomerization (39, 40). Recent studies have shown extensive cross-talk among these PCD pathways. For instance, caspase-8 has been historically linked to immunologically silent death through apoptosis, and the tumor-suppressive function of caspase-8 was initially attributed to its ability to drive apoptosis (41). However, several recent studies have found caspase-8 to be a critical component of PANoptosis (42, 43), which is defined as an inflammatory PCD pathway activated by specific triggers and regulated by the PANoptosome, a molecular scaffold for contemporaneous engagement of key molecules involved in pyroptosis, apoptosis, and/or necroptosis; PANoptosis has key features of each of these PCD pathways but cannot be accounted for by any of these PCDs alone (14, 42, 44–57). PANoptosis has been implicated in infectious and autoinflammatory diseases, but its specific role in human cancer cells remains to be defined.

In this study, we showed that the expression profiles of key inflammatory cytokines are modulated in patients with cancer and that the expressions of multiple cytokines are associated with disease severity. When we analyzed the effects of these differentially expressed cytokines in human cancer cell lines derived from colon and lung cancer, melanoma, and leukemia, we found that only TNF-α and IFN-γ induced cell death in all the lines tested. Mechanistically, combined treatment of TNF-α and IFN-γ activated PANoptosis. Inhibition of the unique upstream regulatory JAK signaling, but not NO (42), prevented cell death induced by TNF-α and IFN-γ. In vivo, the intratumoral administration of TNF-α and IFN-γ reduced tumor growth in transplanted COLO-205 cancer cells. Identifying the triggers of PANoptosis in cancer cells and the underlying mechanisms provides important insights for designing and improving therapeutic approaches.

MATERIALS AND METHODS

Mice

The NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG, JAX stock no. 005557) mice were acquired and maintained at St. Jude Children's Research Hospital (SJCRH). All mice were housed at SJCRH animal facilities under strict specific pathogen-free conditions (sterilized water, food, bedding, and cages), and aseptic handling techniques were used to avoid any unwanted infections. Animal studies were conducted under protocols approved by SJCRH's committee on the use and care of animals.

Analysis of TCGA cytokine expression data from patients with colon cancer

Cytokine expression and survival data for patients with colorectal cancer (CRC) from the Cancer Genome Atlas Program were downloaded from Human Protein Atlas (58). Normal expression data were downloaded from Genomic Data Commons Data Portal of the National Cancer Institute (59). The heat map was generated using Morpheus (https://software.broadinstitute.org/morpheus). Survival analysis and graph generation was done in GraphPad Prism.

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Cell culture and stimulation

The fully authenticated human NCI-60 cancer cell lines (National Cancer Institute, Bethesda, MD) used in this study were cultured in RPMI 1640 medium (Corning, 10–040-CV) supplemented with 10% FBS and 1% penicillin and streptomycin. Cancer cells were seeded 1 d before stimulation. On the day of stimulation, the cells were washed once with warm and sterile Dulbecco's PBS, followed by treatment with different cytokines for 48 h. The concentration of the cytokines used were 25 ng/ml of IL-6 (PeproTech, 200-06), IL-8 (PeproTech, 200-08M), IL-18 (R&D Systems, B001-5), IL-15 (PeproTech, 200-15), IL-1α (PeproTech, 200-01A), IL-1β (PeproTech, 200-01B), IL-2 (PeproTech, 200-02), TNF-α (PeproTech, 300-01A), and IFN-γ (PeproTech, 300-02). For the inhibition of NO, cancer cells were cotreated with 1 mM L-NAME hydrochloride (Tocris Bioscience, 0665) or 100 μM 1400W dihydrochloride (Enzo Life Sciences, ALX-270–073-M005) along with the cytokines. For inhibition of the JAK signaling pathway, the cancer cells were cotreated with 5 μM baricitinib (Advanced ChemBlocks, K12360) all through the experiment.

Generation of IRF1*-knockout HCT116 cells using CRISPR–Cas9 system*

HCT116 colon cancer cells (American Type Culture Collection CCL-247; fully authenticated by American Type Culture Collection) were transduced with lentiviral *Streptococcus pyogenes* Cas9-GFP and flow sorted for GFP-positive cells with optimal expression levels of Cas9-GFP. Lentiviral particles expressing two different *IRF1* guide RNAs (gRNAs, 5′-CTTGGCAGCATGCTTCCATGGG-3′ and 5′-TTGCTCTTAGCATCTCGGCTGG-3′) were generated by using the HEK293T packaging system. The HEK293T cells were transfected with *IRF1* gRNAs, along with the packaging plasmids pPAX2 and pMD2. Lentiviral particles were collected 48 h posttransfection. The HCT116 cells that express the Cas9-GFP protein were transduced with *IRF1* gRNA lentiviral particles in combination with 8 μg/ml polybrene for 24 h. The HCT116 cells carrying successful integration of the gRNAs were selected using puromycin (2.5 μg/ml). The *IRF1* knockout and the corresponding control Cas9-expressing wild-type HCT116 cells were used in further experiments.

Incucyte cell death analysis

The kinetics of cell death were measured using a two-color Incucyte S3 imaging system (Essen BioScience). Different lines of NCI-60 cancer cells were seeded in 12-well (0.25×10^6 cells/well) or 24-well (0.125×10^6 cells/well) cell culture plates and stimulated with the indicated cytokines in the presence of the cell-impermeable DNA binding fluorescent dye SYTOX Green (S7020; Life Technologies, 20 nM), following the manufacturer's protocol. A series of images were acquired at 1-h time intervals for up to 48 h posttreatment with a 20× objective and analyzed using the Incucyte S3 software, which allows precise analysis of the number of dye-positive dead cells present in each image. A minimum of three images per well was taken for the analysis of each time point. Dye-positive dead-cell events for each of the cancer cells were plotted using GraphPad Prism version 5.0 software.

Western blotting

For the Western blotting analysis of caspases, cancer cells were seeded 1 d before stimulation at a density of 0.5×10^6 cells/well in 6-well cell culture plates. The proteins from the indicated cell types were collected by combining cell lysates with culture supernatants with caspase lysis buffer (with 1× protease inhibitors, 1× phosphatase inhibitors, 10% NP-40, and 25 mM DTT) and 4× sample loading buffer (containing SDS and 2-ME). For the Western blot analysis of all other signaling proteins, the cells were lysed in radioimmunoprecipitation assay buffer, supplemented with protease inhibitor and phosphoStop as per the manufacturer's instructions, and sample loading buffer. Samples were denatured by boiling for 10 min at 100°C and separated using SDS-PAGE, followed by the transfer on to Amersham Hybond P polyvinylidene difluoride membranes (10600023; GE Healthcare Life Sciences) and immunoblotted with primary Abs against IRF1 (rabbit mAb, 8478; Cell Signaling Technology), STAT1 (rabbit mAb, 14994; Cell Signaling Technology), caspase-1 (rabbit pAb, 22915-1-AP; ProteinTech), caspase-3 (rabbit pAb, 9662; Cell Signaling Technology), cleaved caspase-3 (rabbit pAb, 9661; Cell Signaling Technology), caspase-7 (rabbit pAb, 9492; Cell Signaling Technology), gasdermin D (GSDMD) (rabbit pAb, 96458; Cell Signaling Technology), gasdermin E (GSDME)/DFNA5 (rabbit mAb, ab215191; Abcam), caspase-8 (mouse mAb, clone 12F5, ALX-804-242-C100; Enzo Life Sciences), MLKL (rabbit mAb, clone EPR17514, ab184718; Abcam), RIPK3/RIP3 (rabbit pAb, NBP2-24588; Novus Biologicals), and β-actin (rabbit mAb, 13E5, 4970; Cell Signaling Technology), followed by secondary anti-rabbit or anti-mouse HRP Abs (Jackson ImmunoResearch Laboratories), as previously described (60).

Membrane proteins and MLKL oligomerization

The membrane proteins were isolated using the Thermo Fisher Scientific Mem-PER Plus Membrane Protein Extraction Kit (catalog no. 89842). For Western blot analysis of MLKL oligomerization (activation), the cells were lysed in NP-40 lysis buffer (1.0% NP-40, 150 mM NaCl, and 50 mM HEPES) containing complete protease inhibitors and phospho-STOP (Roche) and divided in two fractions. One half of the lysate was mixed with a sample loading dye containing the disulfide bond–reducing reagent 2-ME (25 mM) and the other half with a sample buffer without any reducing agents. Both of these lysate preparations were boiled for 10 min at 100°C and subjected to immunoblotting analysis of total MLKL protein.

NSG mouse xenograft tumor model

The NSG mice (NOD/SCID-γ null mouse strain that lacks IL-2–γ-chain) were used as the model recipients for the s.c. transplantation of human COLO-205 cancer cells. Briefly, 8- to 10-

wk-old NSG mice were shaved on their lower backs and transplanted with 2×10^6 COLO-205 cells in 200 μl of medium prepared by mixing (1:1) RPMI and Matrigel (BD Biosciences, 354230). The mice were monitored regularly for engraftment and growth of COLO-205 cancer cell tumors. The recipient mice were divided into four groups and treated intratumorally with injection of the indicated cytokines or PBS [groups: 1) PBS, 2) TNF-α, 3) IFN-γ, or 4) TNF-α and IFN-γ] on days 12, 14, and 16 posttransplantation of the tumor cells. The tumor growth was monitored and measured using digital calipers, and the volumes of the tumors were calculated using the following formula: volume = (length × width^2) × 1/2.

Statistical analysis

GraphPad Prism version 5.0 and 8.0 softwares were used for data analyses. Data are shown as mean ± SEM. Statistical significance was determined by *t* tests (two-tailed) for two groups and one- or two-way ANOVA (with Dunnett or Tukey multiple comparisons tests) for three or more groups. The numbers of experimental repeats and technical replicates are indicated in the corresponding figure legends; *n* in the figure legends represents the number of biological replicates used in the experiments. A p value <0.05 was considered statistically significant and is represented by an asterisk.

RESULTS

TNF-α and IFN-γ cooperatively induce cancer cell death

Expression and modulation of cytokines has been associated with tumor growth and therapeutic outcomes (61–63). To determine which proinflammatory cytokines are highly modulated in tumors, we reanalyzed a publicly available TCGA dataset from healthy volunteers and patients with different stages of CRC (Stage I to IV, low to high grade tumors). The analyses showed altered expression of TNF-α, IFN-γ, IL-1α, IL-1β, IL-18, IL-6, IL-8, and IL-15 in the tumor microenvironment (TME) (Fig. 1A). We also observed that the decreased expression of proinflammatory cytokines was often associated with a decreased probability of survival (Supplemental Fig. 1). These findings suggested a strong inverse correlation between proinflammatory cytokine profiles and cancer progression. However, the role of specific cytokines in modulating tumorigenesis is not clear. It is possible that impaired cell death in the absence of proinflammatory cytokines contributes to the reduced probability of survival. To investigate this possibility, we treated the human colon cancer cell line HCT116 with the proinflammatory cytokines we identified as being differentially modulated in the TME of patients with CRC. We did not detect cell death in response to any of these cytokines individually (Fig. 1B). However, combining all the selected cytokines, referred to as cocktail-1 (Cockt-1), induced robust cancer cell death (Fig. 1B), suggesting that synergistic cytokine signaling is required for this process. Previously, we have identified that the combination of TNF-α and IFN-γ induces robust cell death in murine immune cells (42). Similarly, we found that TNF-α and IFN-γ alone or the cytokine combination that contained TNF-α and IFN-γ (Cockt-1), but not a cytokine combination which lacks both TNF-α and IFN-γ (Cockt-2), induced human cancer cell death (Fig. 1C). These results indicate that TNF-α and IFN-γ are required to trigger cancer cell death in HCT116.

We next sought to determine the efficacy of the TNF-α and IFN-γ combination in triggering cell death in a broad range of human colon cancer cell lines. The NCI-60 colon cancer cell lines (HT-29, SW-620, HCC2998, and COLO-205) were susceptible to TNF-α and IFN-γ cotreatment–mediated cell death (Fig. 1D). To study the relative contribution of TNF-α or IFN-γ in inducing cell death, we treated the NCI-60 colon cancer lines with TNF-α or IFN-γ alone or in combination. The cell death induced by cotreatment of TNF-α and IFN-γ was more robust than that induced by individual treatment with TNF-α or IFN-γ (Fig. 1E). We further extended our findings to other cancer lines from the NCI-60 panel. Comprehensive screening demonstrated that TNF-α and IFN-γ cotreatment induced cell death in a range of cancer cells, including the NCI-60 melanoma lines SK-MEL-2, M14, SK-MEL-5, and UACC-62 (Supplemental Fig. 2A, 2B); lung cancer lines HOP-92 and H226 (Supplemental Fig. 2C, 2D); and leukemia lines HL-60 and RPMI-8226 (Supplemental Fig. 2E, 2F). These findings suggest that various cancer cells may have varying sensitivities to TNF-α and IFN-γ individually, but the combined treatment triggers robust cell death across a wide range of cancer cells.

TNF-α and IFN-γ induce PANoptosis in cancer cells

We have previously demonstrated that synergism of TNF-α and IFN-γ induces inflammatory cell death, PANoptosis, by activating GSDME, caspase-8, -3, and -7, and MLKL in murine bone marrow–derived macrophages (42). To determine whether PANoptosis was activated in human cancer cells, we first analyzed the biochemical markers of PANoptosis in colon cancer lines. Consistent with the cell death data (Fig. 1), we found that the combination of TNF-α plus IFN-γ activated PANoptosis in the HCT116 colon cancer cells, whereas either cytokine treatment alone induced only weak activation of these biochemical markers (Fig. 2A–C).

We then evaluated the effect of the TNF-α and IFN-γ combination in different cancer cell lines to characterize the mechanism of cell death. Without cytokine treatment, we detected robust expression of caspase-1 in the colon cancer lines HT-29 and COLO-205 but not in SW-620 or HCC2998 (Fig. 2D). TNF-α and IFN-γ treatment further upregulated the expression of caspase-1 in all the colon cancer lines tested (Fig. 2D). We also detected active caspase-1 in TNF-α– and IFN-γ–treated COLO-205 cells (Fig. 2D), indicating that pyroptosis may be occurring in these cells. However, the expression of the pyroptotic executioner GSDMD was not observed in COLO-205 cells, suggesting that COLO-205 cells cannot undergo GSDMD-mediated pyroptosis (Fig. 2D). Similar to the varied caspase-1 expression, the expression of GSDMD and GSDME

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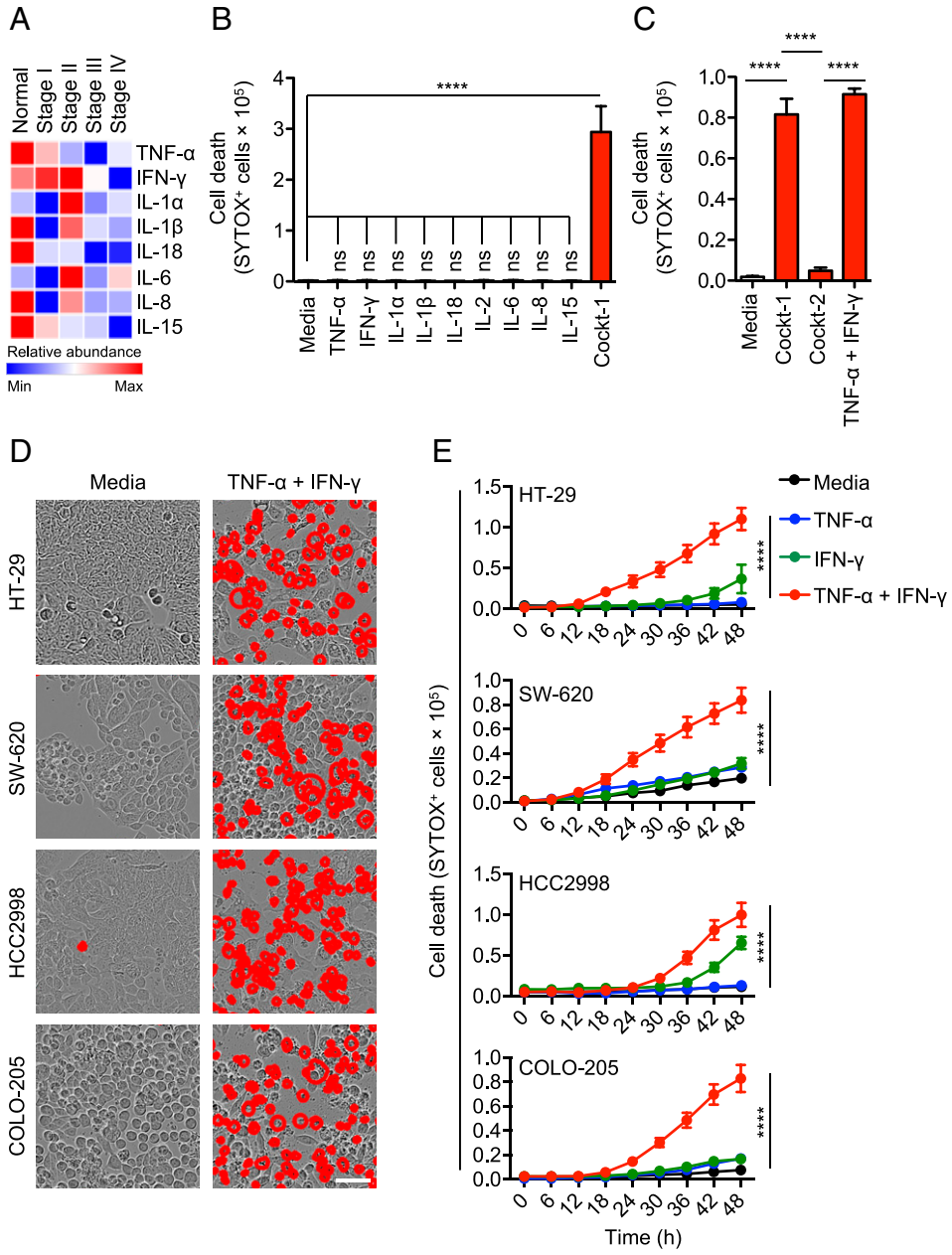

FIGURE 1. Concomitant treatment of TNF-α and IFN-γ triggers robust cell death in NCI-60 colon cancer cells.
(**A**) Heat map representing the levels of inflammatory cytokines in the TME relative to levels in healthy tissue. (**B** and **C**) Quantification of the cell death in HCT116 colon cancer cells treated with media or the indicated cytokines and assessed in culture 48 h poststimulation. Cockt-1 is a combination prepared by mixing all the individual cytokines used in this panel (TNF-α, IFN-γ, IL-1α, IL-1β, IL-18, IL-2, IL-6, IL-8, and IL-15); Cockt-2 is same as Cockt-1, except that it lacks TNF-α and IFN-γ (contains IL-1α, IL-1β, IL-18, IL-2, IL-6, IL-8, and IL-15). (**D**) Representative images of cell death detected by Incucyte imaging analysis of cancer cells treated with TNF-α and IFN-γ at 48 h posttreatment. Scale bar, 50 μm. (**E**) Time course analysis of cell death of cancer cells treated with TNF-α alone, IFN-γ alone, or TNF-α plus IFN-γ, assessed over the course of 48 h poststimulation. Data are representative of three independent experiments (B–E). Data are presented as the mean ± SEM (B, C, and E). Analyses were performed using the *t* test (B and C) or two-way ANOVA (E). ****$p < 0.0001$.

was also varied across the different cell lines. Furthermore, robust activation of GSDME, another executioner of pyroptosis known to be cleaved by caspase-3 (35, 64), was observed in response to TNF-α and IFN-γ treatment in HCT116 and SW-620 cells, whereas HCC2998 and COLO-205 cancer cells showed only minor cleavage (Fig. 2A, 2D), confirming that

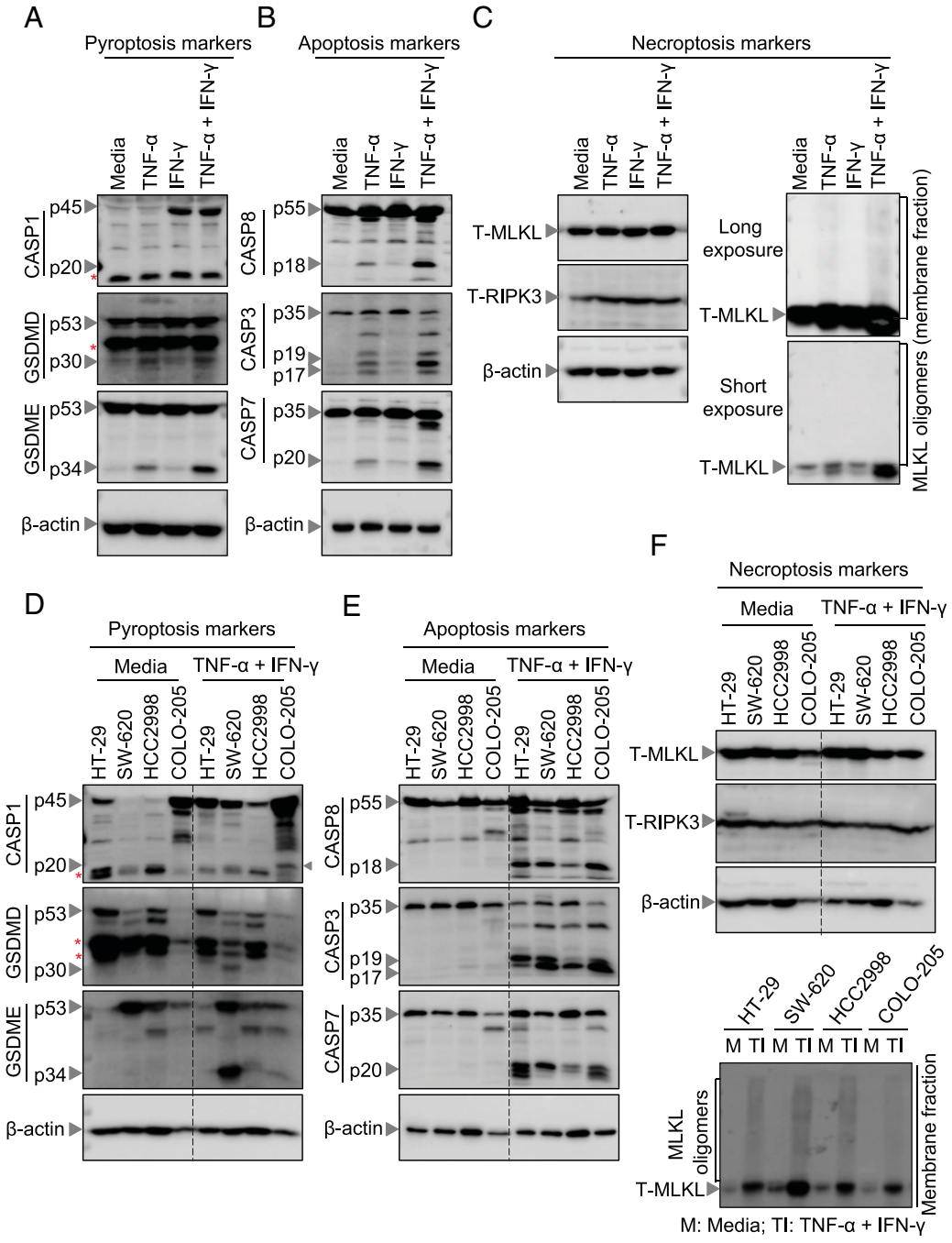

FIGURE 2. TNF-α and IFN-γ treatment triggers PANoptotic cell death in human cancer cells.

(**A**–**C**) Western blot analysis of PANoptosis components in HCT116 colon cancer cells treated with cytokines as indicated and assessed in culture at 48 h poststimulation. (A) Western blot analysis of the pyroptosis markers: pro- (p45) and activated (p20) caspase-1 (CASP1), pro- (p53) and activated (p30) GSDMD, and pro- (p53) and activated (p34) GSDME. (B) Western blot analysis of the apoptosis markers: pro- (p55) and cleaved caspase-8 (CASP8; p18), pro- (p35) and cleaved caspase-3 (CASP3; p19 and p17), and pro- (p35) and cleaved caspase-7 (CASP7; p20). (C) Western blot analysis of necroptosis components: total MLKL (T-MLKL), MLKL oligomers, and total RIPK3 (T-RIPK3). (**D**–**F**) Western blot analysis of PANoptosis components in NCI-60 colon cancer cells treated with cytokines as indicated and assessed in culture at 48 h poststimulation. (D) Western blot analysis of the pyroptosis markers: pro- (p45) and activated (p20) CASP1, pro- (p53) and activated (p30) GSDMD, and pro- (p53) and activated (p34) GSDME. (E) Western blot analysis of the apoptosis markers: pro- (p55) and cleaved CASP8 (p18), pro- (p35) and cleaved CASP3 (p19 and p17), and pro- (p35) and cleaved CASP7 (p20). (F) Western blot analysis of necroptosis components: T-MLKL, MLKL oligomers, and T-RIPK3. Western blot of β-actin was used as loading control. Asterisks indicate nonspecific bands. Data are representative of at least three independent experiments (A–F).

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pyroptotic effectors are activated in these cells. In addition, we observed proteolytic cleavage of apoptotic caspases, including the upstream caspase-8 and the downstream caspase-3 and -7 in all five colon cancer lines (Fig. 2B, 2E), indicating a universal susceptibility of these cancer cell lines to TNF-α and IFN-γ treatment–induced apoptotic effector activation. Next, we examined the role of TNF-α and IFN-γ in inducing necroptotic effectors by assessing the oligomerization of MLKL as a measure of its activation. We observed higher order oligomers of MLKL in cells treated with TNF-α and IFN-γ (Fig. 2C, 2F). Additionally, the expression of RIPK3 and MLKL was intact in all these colon cancer lines. Furthermore, we observed that TNF-α and IFN-γ cotreatment induced PANoptosis in a range of other cancer cells, including the NCI-60 melanoma lines SK-MEL-2, M14, SK-MEL-5, and UACC-62 (Supplemental Fig. 3A–C); lung cancer lines HOP-92 and H226; and leukemia lines HL-60 and RPMI-8226 (Supplemental Fig. 3D–F). Overall, the contemporaneous activation of pyroptotic, apoptotic, and necroptotic effectors observed in these cell lines indicate that TNF-α and IFN-γ induce robust PANoptosis in a wide variety of cancer cells.

TNF-α and IFN-γ drive IRF1-dependent but inducible NO synthase–independent cancer cell death

We previously observed that TNF-α plus IFN-γ treatment induces death of immune cells in murine bone marrow–derived macrophages through the JAK/STAT1–IRF1–inducible NO synthase signaling axis (42). To investigate whether TNF-α and IFN-γ treatment uses a similar mechanism to induce human cancer cell death, we generated HCT116 cells deficient in *IRF1* and treated HCT116 control and *IRF1*-deficient HCT116 colon cancer cells with the combination of TNF-α and IFN-γ (Fig. 3A). The cell death induced by TNF-α and IFN-γ was significantly reduced in *IRF1*-deficient HCT116 colon cancer cells compared with HCT116 control cancer cells (Fig. 3B, 3C). The reduced cleavage of GSDMD and GSDME and caspase-8, -3, and -7 and the reduction in high order oligomers of MLKL from the cell membrane fraction in *IRF1*-deficient HCT116 colon cancer cells confirmed that IRF1 was required for TNF-α plus IFN-γ treatment–induced PANoptosis in human cancer cells (Fig. 3D–F).

Previously, we have shown that signaling through JAK–STAT1–IRF1 is required for NO production, which in turn induces PANoptosis in macrophages (42). To investigate the role of the JAK–STAT1 pathway in PANoptosis induced by TNF-α and IFN-γ, we treated cancer cells with the JAK inhibitor baricitinib. Baricitinib-treated cancer cells showed reduced cell death (Fig. 4A, 4B), suggesting that the JAK–STAT1 pathway is required for cancer cell death. To further investigate the role of NO in this process, we treated cancer cells with the NO inhibitors L-NAME (NOS inhibitor) or 1400W (inducible NO synthase inhibitor) and monitored cell death. In contrast to their effect in macrophages (42), NO inhibitors failed to inhibit the cancer cell death induced by TNF-α plus IFN-γ treatment, both in colon cancer (Fig. 4A, 4B) and melanoma cell lines (Supplemental Fig. 4A, 4B). Together, these results suggest that the JAK–STAT1–IRF1 signaling axis plays a key role in driving TNF-α and IFN-γ treatment–induced cancer cell PANoptosis, whereas NO is dispensable for this process.

TNF-α plus IFN-γ suppresses tumor growth in vivo

We next examined the therapeutic potential of TNF-α and IFN-γ to prevent tumor development in tumor-bearing NSG mice (Fig. 5A). We observed that intratumoral administration of TNF-α alone provided a minor but significant reduction in tumor weight, whereas combined intratumoral injection of TNF-α and IFN-γ resulted in a larger reduction in tumor weight and significantly reduced the tumor volume (Fig. 5B–D). Together, these findings demonstrate that administration of IFN-γ promotes robust JAK–STAT–dependent IRF1 expression and potentiates TNF-α–mediated cell death, PANoptosis (Fig. 5E), and that the TNF-α and IFN-γ combination was superior to single agents alone in treating the established tumors. These findings provide a proof of concept for the applicability of TNF-α and IFN-γ–mediated cell death for the treatment of tumors in vivo.

DISCUSSION

Improved understanding of the mechanisms and novel agonists that can drive cell death in cancer cells is essential for developing new targeted therapeutics. Our finding in this study that the combination of TNF-α and IFN-γ acted as a potent inducer of PANoptosis in a range of different cancer cell types provides important mechanistic insights. Inhibition of JAK signaling significantly reduced the cytotoxic effects of TNF-α and IFN-γ treatment. However, in contrast to what was previously observed in murine macrophages (42), TNF-α plus IFN-γ–induced PANoptosis did not rely on the NO pathway to kill the human cancer cells. Additionally, TNF-α plus IFN-γ treatment activated caspase-1 in human COLO-205 cancer cells. Consistent with these in vitro findings, we also found that concomitant treatment of TNF-α and IFN-γ provided tumor-suppressive effects in an in vivo xenograft model of tumorigenesis.

Studies using proinflammatory cytokines or the inhibition of these cytokines for disease treatment have indicated the feasibility and potential for cytokine modulation as a therapeutic strategy (65–68). In cancer specifically, TNF-α has been shown to augment the efficacy of birinapant, a second mitochondria-derived activator of caspases mimetic and antagonist of inhibitor of apoptosis proteins–induced death, in triple-negative breast cancer cells (69). Additionally, early studies focused on IFN-γ also provided evidence for its critical role in the immunological rejection of tumors (70, 71). However, clinical trials using systemic delivery of cytokines for cancer treatment have historically identified several side effects (52). Therefore, preclinical and clinical research protocols are being developed for

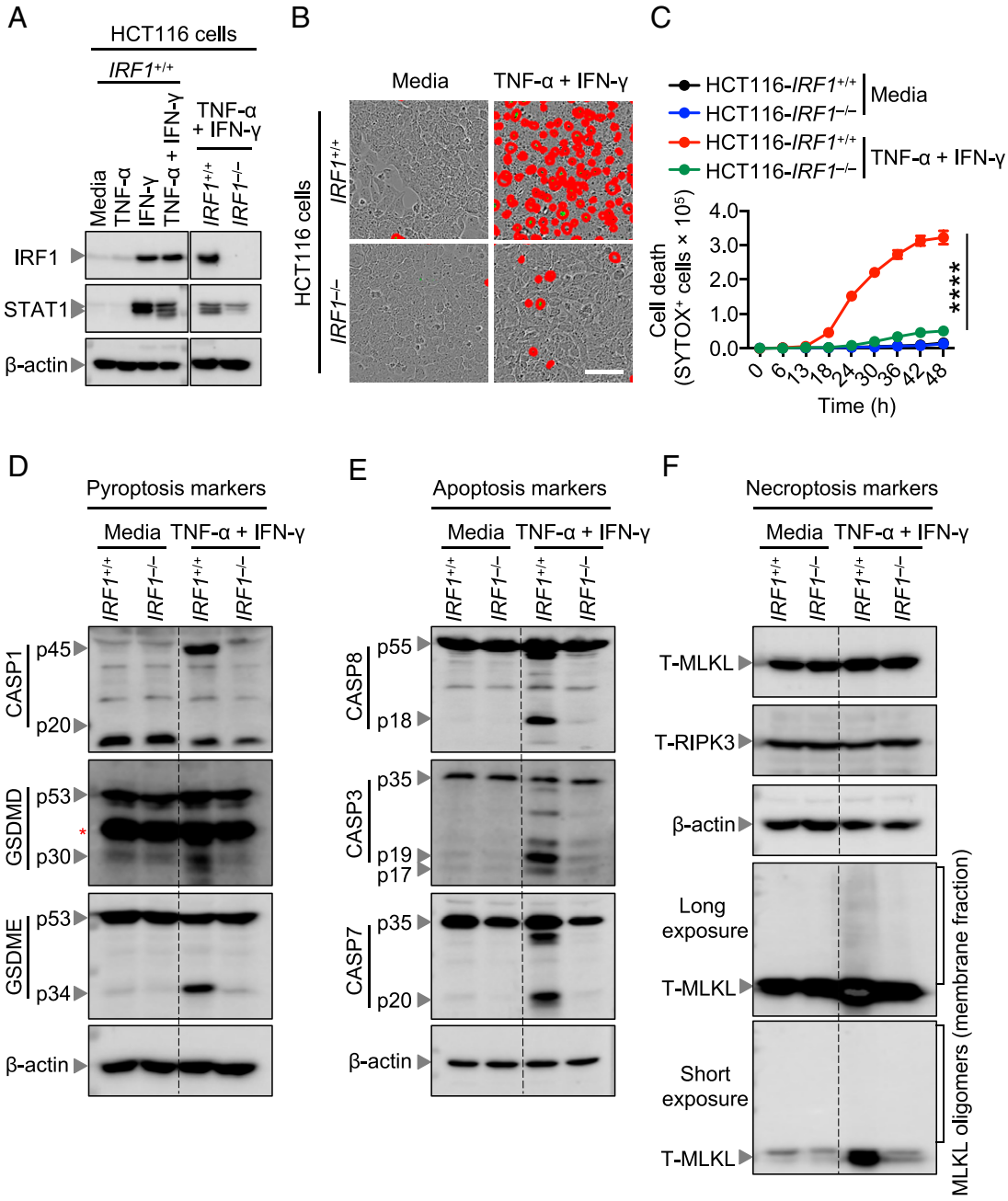

FIGURE 3. TNF-α and IFN-γ treatment triggers IRF1-dependent PANoptotic cell death.

(**A**) Western blot analysis of IRF1 and STAT1 proteins in *IRF1*$^{-/-}$ and the corresponding control *IRF1*$^{+/+}$ HCT116 colon cancer cells after treatment with the indicated cytokines for 48 h. (**B**) Representative images of cell death detected by Incucyte image analysis of indicated HCT116 cells, treated with TNF-α and IFN-γ at 48 h posttreatment. Scale bar, 50 μm. (**C**) Quantitative real-time analysis of cell death in wild-type and *IRF1*-deficient HCT116 colon cancer cells cotreated with TNF-α and IFN-γ. (**D**–**F**) Western blot analysis of PANoptosis components in wild-type and *IRF1*-deficient HCT116 cells treated with TNF-α and IFN-γ for 48 h. (D) Western blot analysis of the pyroptosis markers: pro- (p45) and activated (p20) caspase-1 (CASP1), pro- (p53) and activated (p30) GSDMD, and pro- (p53) and activated (p34) GSDME. (E) Western blot analysis of the apoptosis markers: pro- (p55) and cleaved caspase-8 (CASP8; p18), pro- (p35) and cleaved caspase-3 (CASP3; p19 and p17), and pro- (p35) and cleaved caspase-7 (CASP7; p20). (F) Western blot analysis of necroptosis components: total MLKL (T-MLKL), total RIPK3 (T-RIPK3), and MLKL oligomers. Western blot of β-actin was used as loading control. Asterisks indicate nonspecific bands. Data are representative of at least three independent experiments (A–F). Data are presented as the mean ± SEM (C). Analysis was performed using two-way ANOVA (C). ****$p < 0.0001$.

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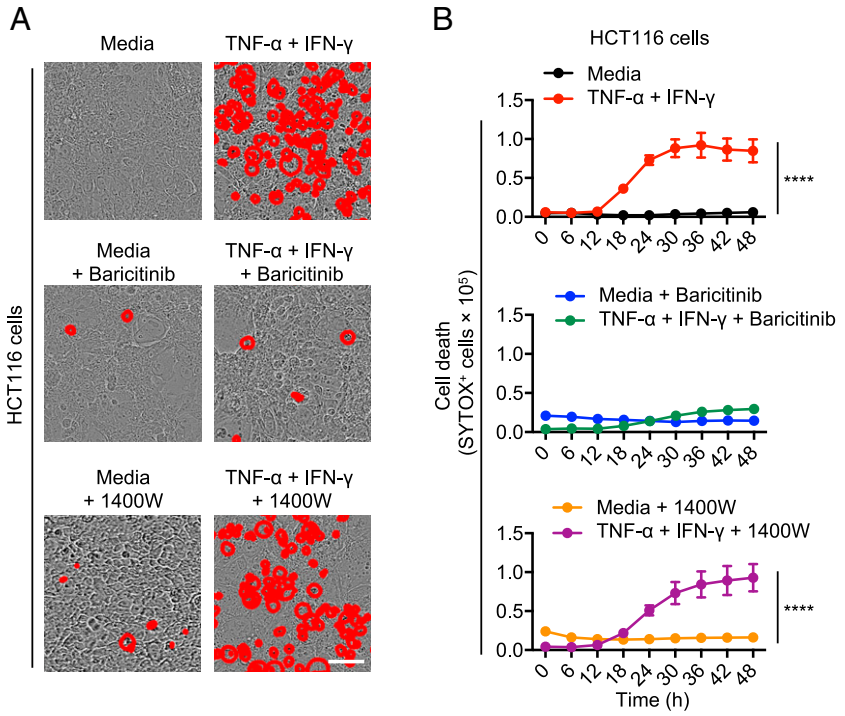

FIGURE 4. JAK inhibition, but not NO inhibition, prevents the TNF-α– and IFN-γ–dependent cancer cell death.

Representative images (**A**) or time course analyses (**B**) of cell death detected by Incucyte imaging of human HCT116 colon cancer cells treated for 48 h with TNF-α and IFN-γ, in the presence or absence of the JAK1 inhibitor, baricitinib, or inducible NO inhibitor, 1400W. Scale bar, 50 μm. Data are representative of two independent experiments (A–B). Data are presented as the mean ± SEM (B). Analyses were performed using two-way ANOVA (B). ****$p < 0.0001$.

targeted and local delivery methods to reduce toxicities while enhancing efficacy (72, 73). Our findings in this study show that TNF-α plus IFN-γ was effective against a wide range of tumor cell types, including colon and lung cancer, melanoma, and leukemias, and that this combination suppressed tumor growth via intratumoral delivery in vivo, highlighting its potential for clinical applications.

We identified that individual treatments using either TNF-α or IFN-γ alone were not sufficient to induce cell death in cancer cells. However, a range of human cancer cell lines was susceptible to the PANoptotic cell death induced by the combination of TNF-α and IFN-γ. PANoptosis is a form of inflammatory cell death that uses molecular components from pyroptosis, apoptosis, and/or necroptosis but that cannot be accounted for by any of those three pathways alone. These observations are significant as engaging the versatile multicell death modality PANoptosis is highly effective in bypassing the resistance to cell death in cancer cells. The intrinsic plasticity in PANoptosis, potentiated by the PANoptosome and the engagement of effectors from multiple PCDs, allows a cell to proceed with inflammatory cell death even when a key molecule from pyroptosis, apoptosis, or necroptosis is inhibited, mutated, or repressed. Moreover, the cytokines TNF-α and IFN-γ represent two classic markers of activated cytotoxic T cells, which play central roles in eliminating tumor cells in vivo, suggesting potential implications for the development of anti-cancer therapeutic approaches. Although several cytokines were modulated in the TME in patients, the TNF-α and IFN-γ combination was unique; all other cytokines tested failed to induce any significant cancer cell death, both in isolation and in combination. Moreover, other cytokines such as IL-1β and IL-6 are detrimental and may even promote tumorigenesis (66, 68, 74–81). Lack of detectable levels of cell death in response to these other cytokines could be one of the reasons they fail to provide consistent results in preclinical and clinical applications. An additional contributing factor may be that acute treatment with proinflammatory cytokines promotes cytotoxic and anti-tumor responses, whereas chronic exposure drives a tolerogenic tumor-promoting microenvironment (82). In line with this, we found that acute treatment with TNF-α and IFN-γ substantially reduced tumor growth in vivo. Additionally, previous therapeutic approaches administering either TNF-α or IFN-γ alone have failed, most often because of inconsistent results, toxicities, and/or lack of efficacy (83–87). Together, this suggests that the anti-tumor response is largely dependent on the synergism between TNF-α and IFN-γ.

Although the TNF-α and IFN-γ combination effectively reduced tumor growth in our in vivo model, the tumors continued to grow gradually and finally emerged at extended time points, suggesting that resistance mechanisms may develop. The resistance mechanisms may help the cancer cells escape TNF-α and IFN-γ treatment–induced cell death in immune-deficient NSG mice. However, the outcome may be different in immunocompetent animals. Indeed, cell death is known to augment the development of T cells and other adaptive immune responses that promote durable tumor regression (88–92). For

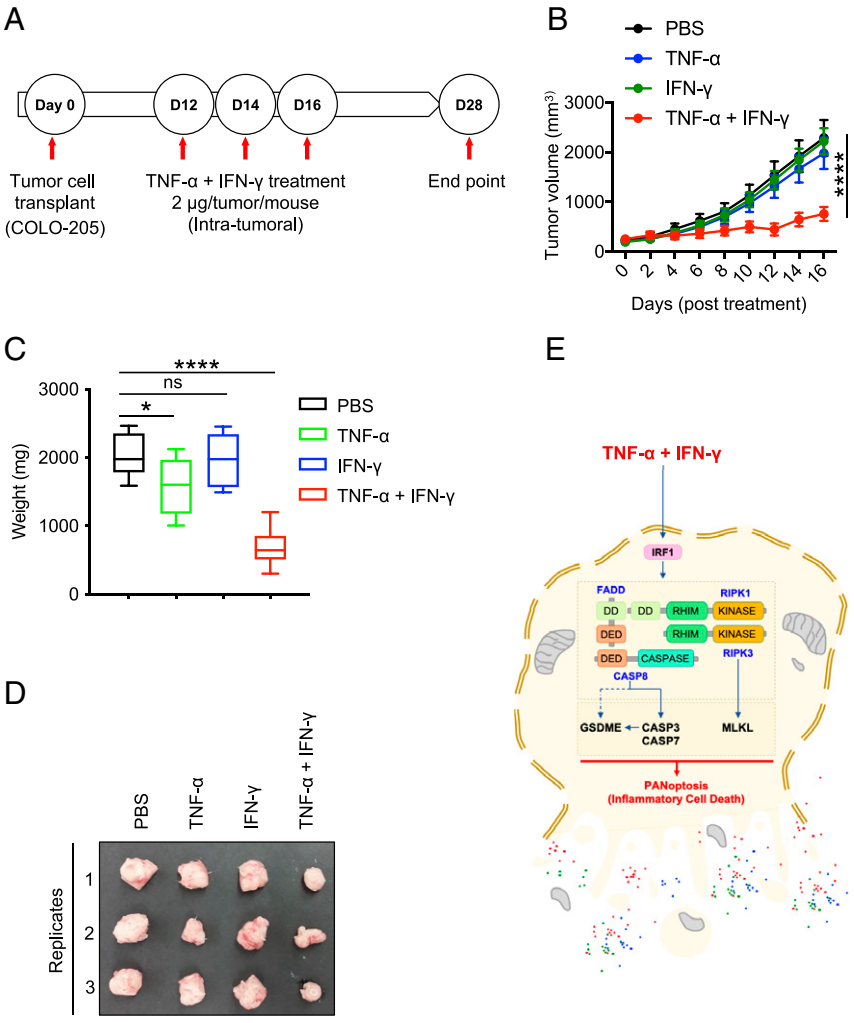

FIGURE 5. TNF-α and IFN-γ treatment suppresses transplanted tumor growth in vivo.

(**A**) Diagram representing the NSG mouse model of tumorigenesis. (**B**) Time course analysis of tumor volume of transplanted COLO-205 cell tumors in NSG mice. (**C**) Weight of the tumors measured at the end point of the experiment, 28 d postinoculation, and 16 d after the first treatment with TNF-α and IFN-γ. (**D**) Representative images of the tumors that were collected at day 28 postinoculation. (**E**) Depiction of the TNF-α plus IFN-γ–induced PANoptosis pathway based on the experimental findings from the current study. Data are presented as the mean ± SEM (B and C). Analyses were performed using one-way ANOVA (B) or the *t* test (C). *$p < 0.05$; ****$p < 0.0001$.

instance, during ICT, cytotoxic T cells and NK cells produce potent proinflammatory cytokines (11). Furthermore, the success of ICT is strongly associated with TNF-α, IFN-γ, and other proinflammatory cytokines, and low expression of TNF-α in tumors correlates with poor outcomes in nonresponding patients (93). Additionally, the development of lung cancer is associated with decreased expression of proinflammatory cytokines by the cytotoxic T cells and NK cells (94). These early findings indicate that disrupting the resistance to cell death through cytokine signaling has tremendous potential to extend the therapeutic benefits of ICT to diverse patients and cancer types (10, 95). Therefore, it is possible that the highly inflammatory nature of the PANoptotic cell death induced by TNF-α and IFN-γ would provide robust activation of anti-cancer immunity in immunocompetent animals to completely eradicate tumors and aid in the development of long-lasting anti-tumor immunity.

Overall, our study identifies the combination of TNF-α and IFN-γ as an effective approach to induce inflammatory cell death, PANoptosis, in cancer cells. These findings lay a foundation for identifying new strategies to target different cancer types and develop more effective anti-cancer regimens.

DISCLOSURES

SJCRH filed a provisional patent application on TNF-α (03B1) and IFN-γ (03B3) signaling described in this study, listing R.K. and T.-D.K. as inventors (serial no. 63/106,012). The other authors have no financial conflicts of interest.

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