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Supplemental Information

Differential Induction of Immunogenic Cell Death and Interferon Expression in Cancer Cells by Structured ssRNAs

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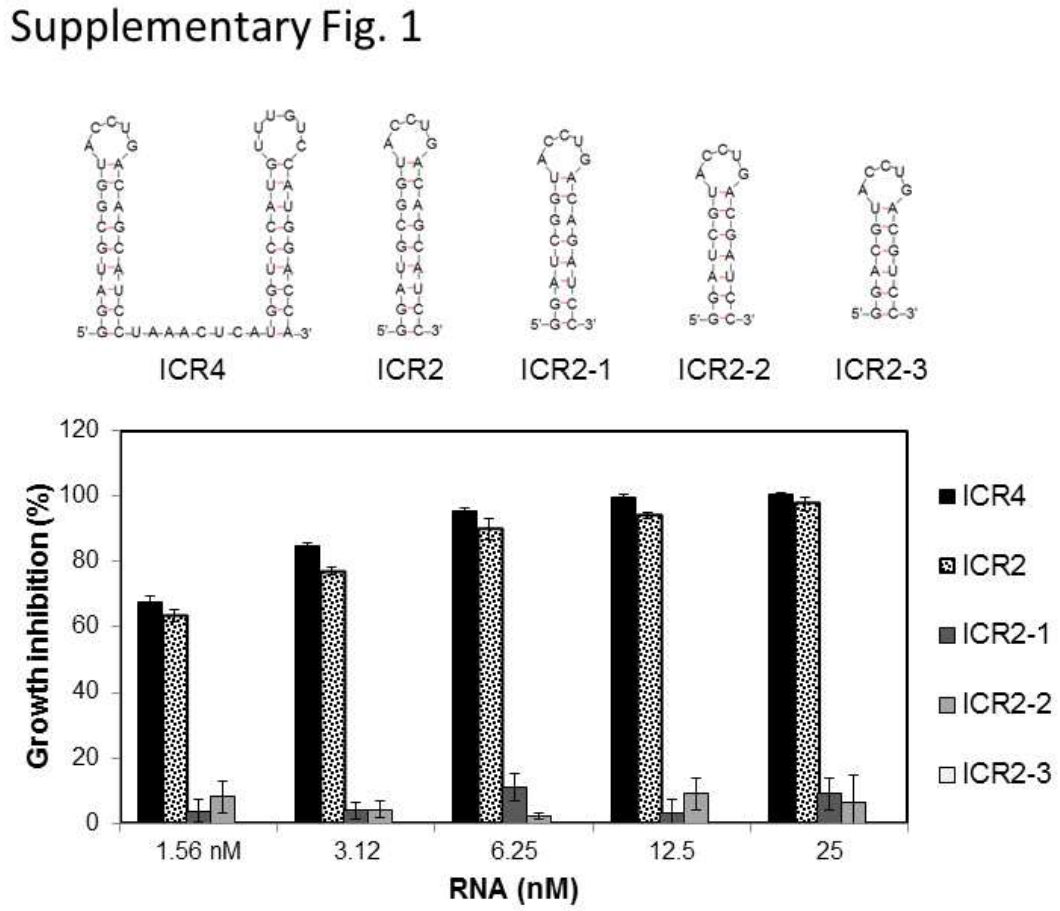
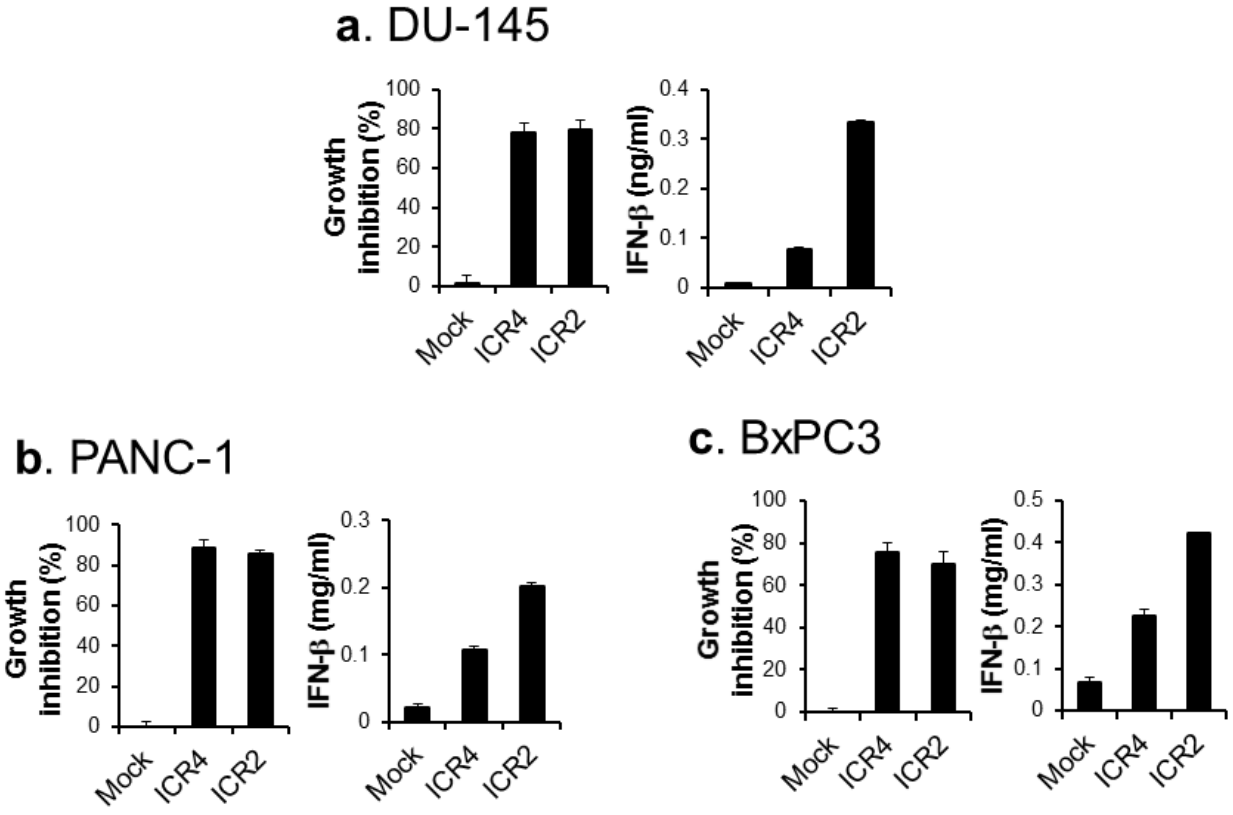
Supplemental Figures and Legends



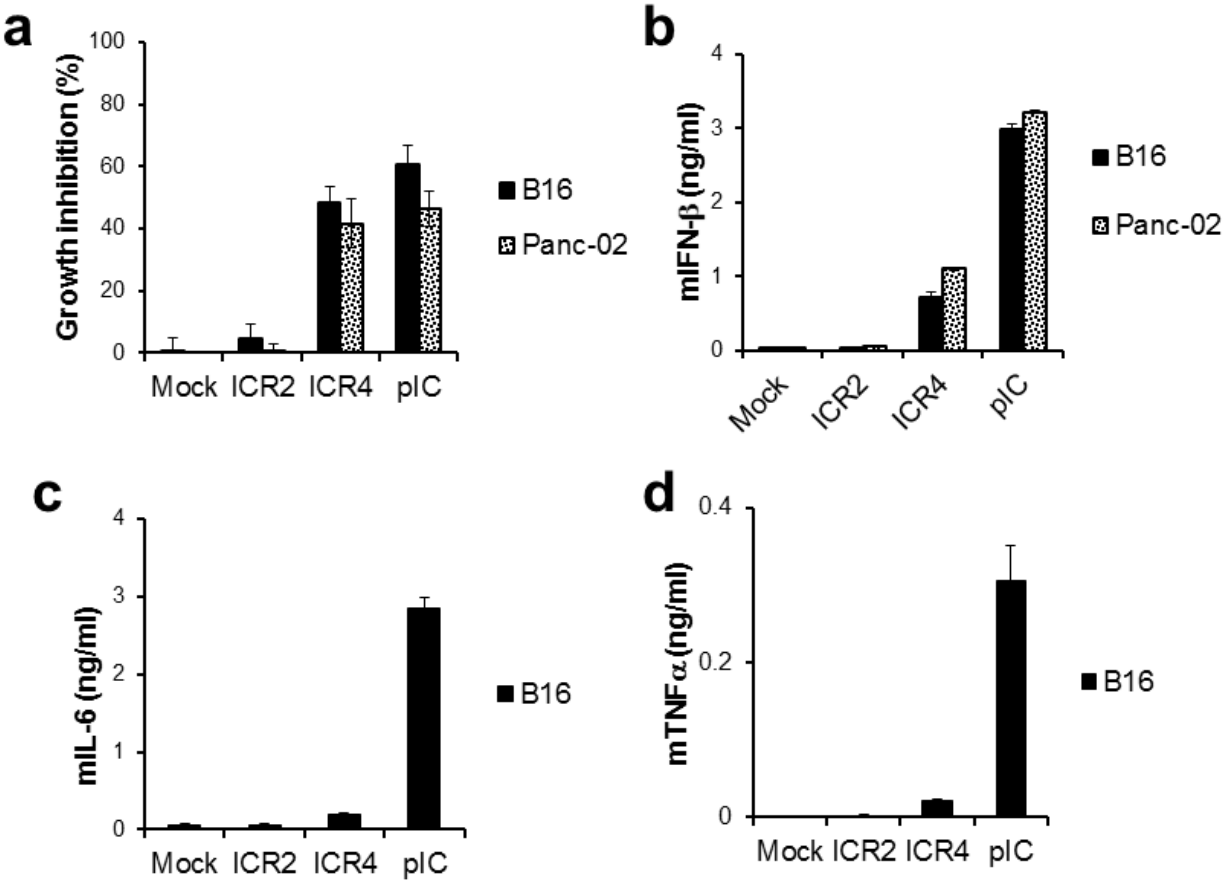
Supplementary Figure S1. Cytotoxicity of 5'ppp 2'F hairpin RNAs with 6-9 bp. WM266-4 cells were transfected for 4 h with ICR4, ICR2 and ICR2 with various truncations at indicated concentrations. Cell growth rate were determined at 3 days after transfection by MTS assay. Error bars are S.D..

Supplementary Fig. S2



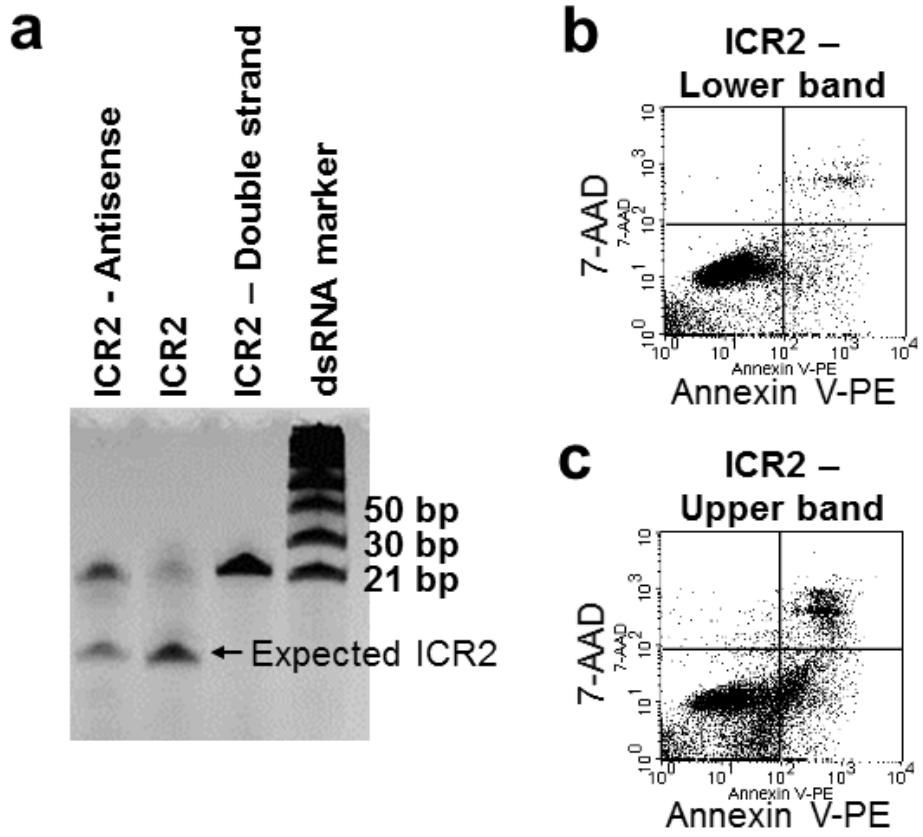
Supplementary Figure S2. Differential induction of IFN-β expression and growth inhibition of human prostate and human pancreatic cancer cells by ICR2 and ICR4. DU-145 human prostate cancer cell line (**a**), PANC-1 human pancreatic cancer cell line (**b**) and BxPC3 human pancreatic cancer cell line (**c**) were transfected with ICR2, ICR4 (1 μg/ml each) or transfection reagent alone (Mock). Cell growth and IFN-β production were determined by MTS assay and ELISA, respectively. Error bars are S.D..

Supplementary Fig. S3



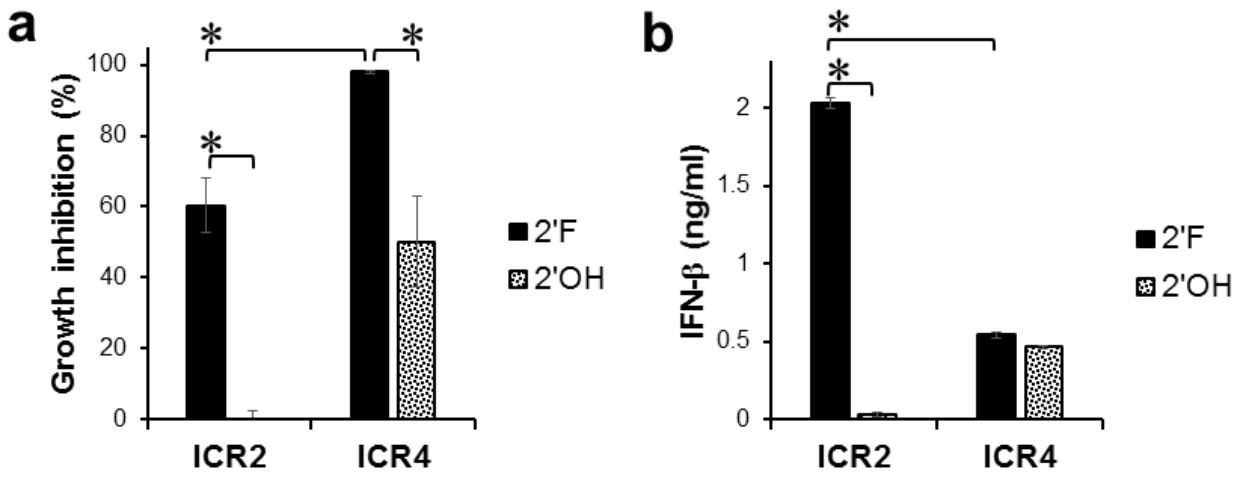
Supplementary Figure S3. Cytotoxicity and cytokine production by mouse cancer cells transfected with ICR2, ICR4 and polyI:C. B16 mouse melanoma cell line and PANC-02 mouse pancreatic cancer cell line were transfected with transfection reagent alone (Mock), ICR2, ICR4 or polyI:C (pIC) (1 μg/ml each). Cells and culture supernatants were harvested at 3 days after transfection. (**a**) Cell growth and production of (**b**) IFN-β , (**c**) IL-6 and (**d**) TNFα were determined by MTS assay and ELISA, respectively. Error bars are S.D..

Supplementary Fig. S4



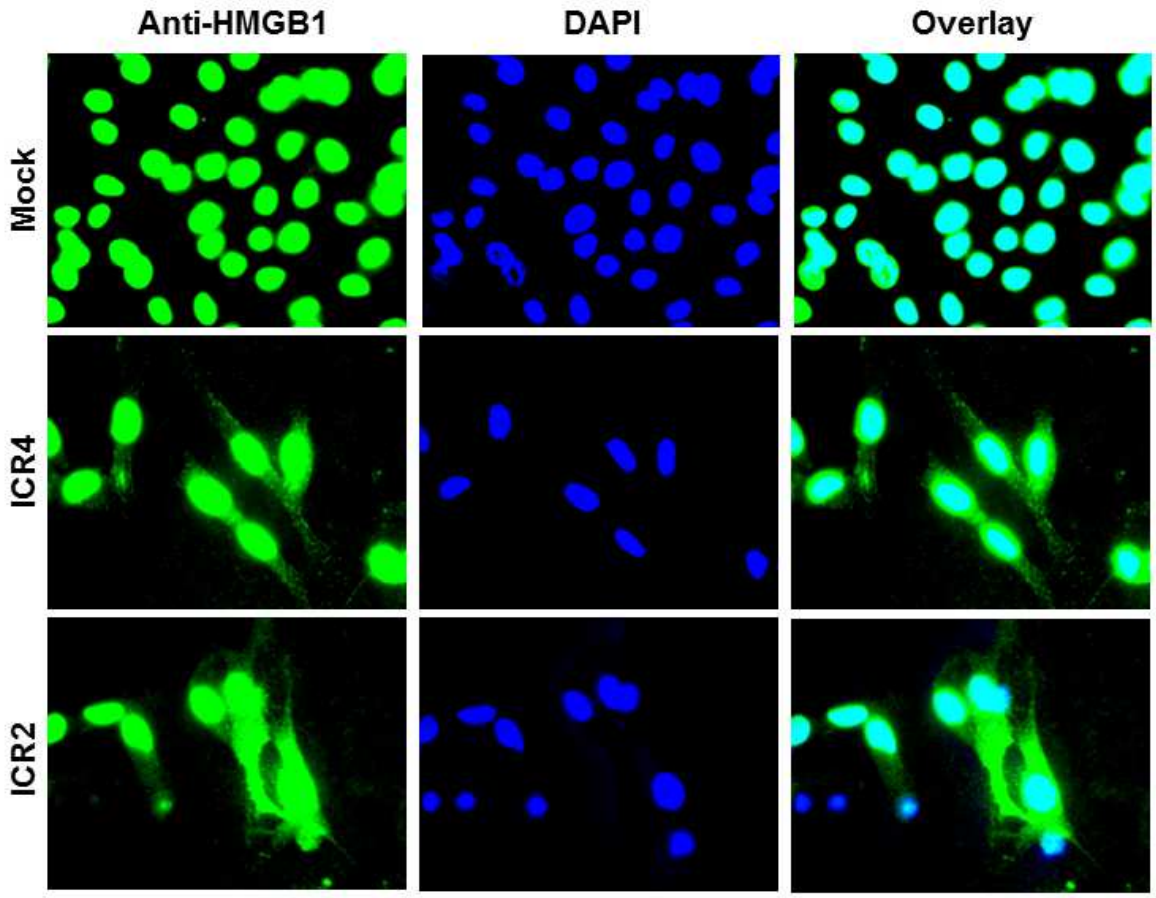
Supplementary Figure S4. Generation of T7 polymerase-induced IVT byproduct. (**a**) ICR2 and ICR2 antisense complementary to ICR2 were generated by T7 polymerase-induced IVT. ICR2-double strand was generated by hybridization of ICR2 and ICR2 antisense. RNAs were analyzed on 20% polyacrylamide gels. (**b**) Lower and (**c**) upper bands of ICR2 IVT were purified and transfected into human melanoma cell line WM266-4. Cell death levels were determined one day after transfection by Flow Cytometry-Based Annexin V-PE/7-AAD Staining Analysis.

Supplementary Fig. S5



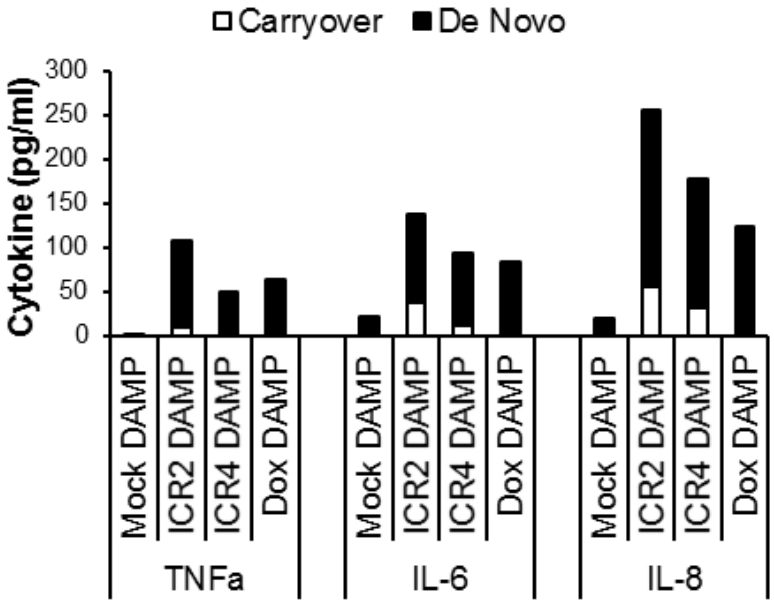
Supplementary Figure S5. Comparison of cell death- and IFN-β-inducing activities of ICR2 and ICR4 containing 2'F pyrimidine but 2'OH pyrimidine. WM266-4 cells were transfected with 2'F ICR2, 2'OH ICR2, 2'F ICR4 or 2'OH ICR4 (35 nM each). (**a**) Cytotoxicity and (**b**) IFN-β production was assessed at 72 h post transfection. Error bars represent the S.D. ∗ $P < 0.05$.

Supplementary Fig. S6



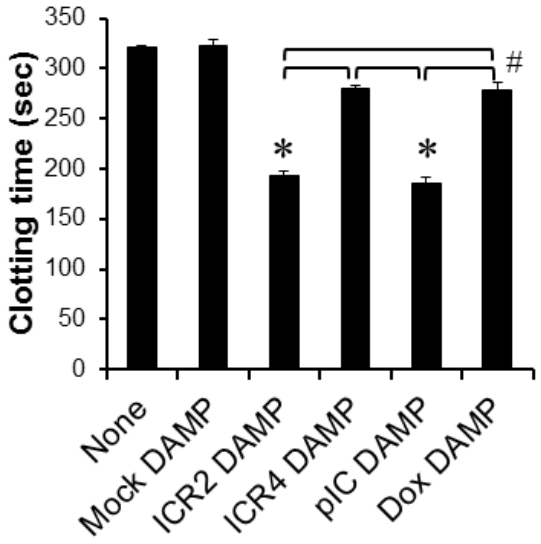
Supplementary Figure S6. Cytoplasmic translocation of nuclear protein HMGB1 in cells transfected with ICR2 and ICR4. WM266-4 cells were transfected for 4 h with ICR2, ICR4 or transfection reagent alone (Mock). Cells were harvested at 24h after transfection and co-stained with anti-HMGB1 (Green) and DAPI (blue). The expression of nuclear and cytoplasmic HMGB1 and nuclear DAPI was detected by fluoresce microscopy.

Supplementary Fig. S7



Supplementary Figure S7. Human DCs stimulated with DAMPs produced cytokines. Human PBMC-derived immature DCs were stimulated with DAMPs isolated from WM266-4 cells treated with transfection reagent alone (Mock DAMP), ICR2 (ICR2 DAMP), ICR4 (ICR4 DAMP) or doxorubicin (Dox DAMP). De novo production of TNFα, IL-6 and IL-8 by stimulated DCs (De Novo) and pre-existing cytokines in DAMPs (Carryover) were determined by ELISA.

Supplementary Fig. S8



Supplementary Figure S8. Release of pro-coagulative DAMPs from human cancer cells treated with immunogenic cell death inducing agents. Enhancement of human plasma coagulation by DAMPs released from cells treated with transfection reagent alone (Mock DAMP), ICR2 (ICR2 DAMP), ICR4 (ICR4 DAMP), polyI:C (pIC DAMP) and Doxorubicin (Dox DAMP) was determined by coagulation assay. n=3, Error bar are S.D.. # $P < 0.05$ (indicated comparison). ∗ $P < 0.05$ (*vs* normal plasma clotting time (None)).

Supplemental Table

Table S1. RNA sequences.

RNA	Sequence (5'→3')
ICR1	ggaugcgguaccugacagcauccua
ICR1A	ggaugcgguaccugacagcauccuaaagug
ICR1B	ggaugcgguaccugacagcauccuaaaguggguggaagugag
ICR1C	ggaugcgguaccugacagcauccuaaaguggguggaagugagugagugaaauaaaaa
ICR2-	ggacguaccugacgucc
ICR2-	ggaucguaccugacgaucc
ICR2-	ggaucgguaccugacagaucc
ICR2	ggaugcgguaccugacagcaucc
ICR2A	ggacgaugcgguaccugacagcaucgucc
ICR2B	ggaugcgguaccugacagcauccaccugggaugcugucagguaccgcaucc
ICR3	ggagcggaugcgguaccugacagcaucc
ICR3A	ggggaggacagcggaugcgguaccugacagcaucc
ICR3B	ggaaugaggggaggacagcggaugcgguaccugacagcaucc
ICR3C	ggguaagugaaugaggggaggacagcggaugcgguaccugacagcaucc
ICR4	ggaugcgguaccugacagcauccuaaacucaugguccauguuuguccauggacca
ICR4A	ggaugcgguaccugacagcauccuaaacucaugguccauguuuguccauggaccaacuaccgacauuguauguguugauauaaaugu
ICR5	ggaugcgguaccugacagcauccugaguuuaguuguugu + ggaugcgguaccugacagcauccacaacaacuaaaacuca
ICR-L	gguuuuuuuuuuuuuuuuuuuu

Supplemental Materials and Methods

Enzyme-linked immunosorbent assay (ELISA)

TNF-α, IL-6 and IL-8 were determined using BD OptEIA™ ELISA sets (BD Biosciences, Franklin Lakes, NJ). IFN-β production was determined using IFN-β ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ). HMGB-1 secretion was determined using HMGB1 ELISA kit (Tecan, Morrisville, NC) by following the manufacturer's instructions.

Immunoblot analysis and antibodies

Mitochondria and nuclear fractions were isolated using Mitochondria Isolation kit and NE-PER Nuclear Extraction reagent, respectively, (both from Thermo Scientific). Mitochondrial lysates, nuclear lysates and total cell lysates were prepared in 1x RIPA buffer (Sigma, St. Louis, MO) in the presence of the complete protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma). 30 µg of protein lysates were electrophoretically separated on 4-20% Mini-PROTEAN® TGX™ polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred to polyvinylidene fluoride (PVDF) membranes (PolyScreen®, PerkinElmer). After rinsing in TBST20, membranes were blocked for 1 h in 5% dry milk in TBTS20, followed by overnight incubation with primary antibodies anti-XIAP (1:1000) (3B6; Cell Signaling, Danvers, MA), anti-TRAIL (1:1000) (C92B9; Cell Signaling), anti-phospo (p)-IRF-3 (1:500) (4D4G; Cell Signaling), anti-cleaved caspase-3 (1:200) (D3E9; Cell Signaling), anti-caspase-7 (1:200) (Cell Signaling), anti-NF-κB p65 (1:1,000) (L8F6; Cell Signaling), anti-RIP (1:1,000) (Cell Signaling), anti-RIG-I (1:500) (D14G6; Cell Signaling, Danvers, MA), anti-MDA5 (1:500) (D74E4; Cell Signaling) and anti-PKR (1:350) (Catalog No 3072; Cell Signaling). When

different proteins were sequentially detected on the same membrane, membranes were treated for 8 minutes with Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL), washed, blocked and probed again, as described above. Primary antibodies were detected using horseradish peroxidase (HRP)-conjugated anti-rabbit (1:2,000) (Cell Signaling) or anti-mouse (1:2,000) (Cell Signaling) secondary antibodies. Anti-β-Tubulin (1:1,000) (9F3; Cell Signaling), anti-CoxIV (1:1,000) (3E11; Cell Signaling) and anti-Histone H3 (1:1,000) (D1H2; Cell Signaling) were used as loading controls. HRP activity was visualized using the Western Lightning Plus Kit (PerkinElmer, Waltham, MA).

Detection of surface Calreticulin and cytoplasmic HMGB-1

The expression of surface Calreticulin was determined by flow cytometry after co-staining with anti-Calreticulin-PE (1/100 dilution) (Abcam, Cambridge, MA) and 7-AAD (BD Biosciences). For the detection of HMGB1, cells were fixed with a 4% paraformaldehyde solution followed by blocking and permeabilization with 5% BSA, 0.2% Triton X-100 in PBS, and stained overnight with anti-HMGB1 (1/1000 dilution) (Abcam), Alexa Fluor 488-conjugated goat anti-rabbit IgG (1/1000 dilution) (Abcam) was used as a secondary antibody. DAPI (4',6-Diamidino-2-phenylindole) (Sigma) was used as a nuclear counterstain. The expression of HMGB1 and DAPI was observed under a Zeiss Axio Observer microscope, and the images were analyzed using MetaMorph software (Sunnyvale, CA).

Clotting assay

5 µl DAMPs or culture media were mixed with 50 µL normal pooled human plasma (George King Bio-Medical Inc., Overland Park, KS). The mixture was incubated for 3 min at 37 °C, followed by the addition of 50 µL $CaCl_2$ (25 mM). Clotting times were recorded using STart®

Hemostasis Analyzer (Diagnostica Stago, Parsippany, NJ).