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PolyI:C-induced, TLR3/RIP3-dependent necroptosis backs up immune effector-mediated tumor elimination *in vivo*

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Running title: **TICAM-1-RIP3 necroptosis in tumor**

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Abbreviations:

DAMP, damage-associated molecular patterns, DC: dendritic cells, ds: double-stranded, DRP-1: dynamin-related protein 1, HMGB1: high-mobility group protein B1, IFN: interferon, IRF: interferon regulatory factor, MAVS: mitochondrial antiviral-signaling protein, MDA5: melanoma differentiation-associated protein 5, MHC, major histocompatibility complex, NALP: a type of NOD-like receptor (NLR), nec-1: necrostatin-1, NK, natural killer, PGAM5: mitochondrial serine/threonine phosphatase, PI: propidium iodide, polyI:C: polyinosinic-polycytidylic acid, RIP: receptor-interaction protein (kinase), ROS: reactive oxygen species, TICAM-1: toll-interleukin 1 receptor domain (TIR)-containing adaptor molecule-1, TIR: toll-interleukin 1 receptor domain, TLR: toll-like receptor, TNF: tumor necrosis factor, zVAD: z-Val-Ala-Asp fluoromethyl ketone.

Abstract

Double-stranded RNA directly acts on fibroblast and myeloid lineages to induce necroptosis as in TNF- α . Here we investigated whether this type of cell death occurred in cancer cells in response to polyI:C and the pan-caspase inhibitor z-Val-Ala-Asp fluromethyl ketone (zVAD). We found the colon cancer cell line CT26 highly susceptible to necroptosis, judged by annexin V/propidium iodide. CT26 cells possess RNA sensors, TLR3 and MDA5, which are up-regulated by interferon (IFN)-inducing pathways and linked to receptor-interacting protein kinase (RIP) 1/3 activation via TICAM-1 or MAVS adaptor, respectively. Although exogenously-added polyI:C alone marginally induced necroptosis in CT26 cells, a combinational use of polyI:C and zVAD accomplished ~50% CT26 necroptosis *in vitro* without secondary effects of TNF- α or type I IFNs. CT26 necroptosis depended on the TLR3-TICAM-1-RIP3 axis in the tumor cells to produce reactive oxygen species, but not on MDA5, MAVS or the caspases/inflammasome activation. However, the RNA-derived necroptosis was barely reproduced *in vivo* CT26 implant Balb/c mouse models with administration of polyI:C + zVAD. Significant shrinkage of CT26 tumors was revealed only when polyI:C intraperitoneally and zVAD subcutaneously (s.c.) were injected to tumor-bearing mice with depletion of cytotoxic T lymphocyte and natural killer cells. The results were confirmed with immune-compromised mice with no lymphocytes. Although necroptosis-induced tumor growth retardation appears mechanistically complicated and dependent on the injection route of polyI:C and zVAD, anti-caspase reagent directed to tumor cells will make RNA adjuvant immunotherapy more effective by modulating formation of tumoricidal microenvironment and dendritic cell-inducing antitumor immune system.

Introduction

A cell death response frequently occurs in malignantly transformed cells accompanied with infection or inflammation. Viral infection also induces programmed cell death, which is believed to be a host defense to restrict viral spread (1). However, recent advance on cell death studies may offer us an alternative interpretation of cell death in tumor cell biology: the products of dead cells profoundly modulate the immune system and microenvironment around the established tumor, which is largely relied on the mode of cell death (2,3). The programmed cell death was categorized into apoptosis, pyroptosis and necroptosis based on the difference of caspases involved (4). Typical apoptosis is induced by activation of caspase-8 or -9. Pyroptosis is a form of caspase-dependent cell death that caspase-1 or -11 activation initiates (4). Although necroptosis is usually triggered by death receptors including FAS, tumor necrosis factor (TNF) receptor and TNF-related apoptosis-inducing ligand receptor, Toll-like receptors (TLRs) also induce necroptosis when caspase-8 is inhibited by gene depletion or pharmacological inhibitors (4). Caspase inhibitors such as z-Val-Ala-Asp fluoromethyl ketone (zVAD) block TNF-induced apoptosis in many cell lines, whereas some lines respond to TNF+zVAD by activating necroptosis pathways (5). Necroptosis but not apoptosis is believed to cause modulation of tumor microenvironment that affects tumor progression and invasion (6). Exosomes, proteins and nucleic acids, released from dying cells, may be an important extracellular source of the environmental effectors (7). Double-stranded (ds) and stem-structured RNAs representing host and viral patterns of innate immunity also serve as modifiers for inflammatory environment and host immune response (8-10).

Polyinosinic-polycytidylic acid (polyI:C), a synthetic analog of dsRNA, has been known to harbor a cytolytic aspect via direct-acting to fibroblasts and macrophage lineages. Myeloid cells in tumor are highly susceptible to dsRNA compared to normal cells (7,11), while antigen-presenting dendritic cells (DCs) mature in response to dsRNA followed by immune activation (8,10), suggesting the presence of cell type-specific RNA-sensing machinery that determines the live and dead signal. PolyI:C appear to trigger both apoptosis and necroptosis (8,10). *In vitro* studies on innate immunity suggested that the several signaling pathways could be involved in polyI:C-derived cell death although there are cell type-dependent mode variations in cell death (12,13). However, in tumor cells the substantial function of the signal that

induces cell death by polyI:C remains largely undetermined in *in vivo* models.

PolyI:C is a ligand for both endosomal TLR3 and cytoplasmic Melanoma differentiation-associated protein-5 (MDA5) and to induce activation of NF- κ B and interferon regulatory factor (IRF) 3 transcription factors followed by production of inflammatory cytokines and type I/III IFNs (8, 14). TLR3 and MDA5 are upregulated by polyI:C or IFN stimulation, suggesting they are IRF3- and IFN-inducible factors (14). MDA5 and TLR3 recruit different adaptors, mitochondrial antiviral signaling protein (MAVS) or Toll-interleukin 1 receptor domain (TIR)-containing adaptor molecule (TICAM)-1, respectively (15,16), which confers distinct functional properties on the two pathways. In response to polyI:C, TLR3/TICAM-1 activate IRF3 as well as receptor interacting protein kinase (RIP) 1/3 in a cell type-dependent manner (17). Upon malignant transformation, cells usually express high levels of TLR3 and MDA5, which sense polyI:C and initiate RNA-sensing signals that are sometimes linked to cell death or live output including IFN/cytokine production (13,18). What factors discriminates between the death and live signal is yet unknown, but both IRF-3-derived and IFN receptor (IFNAR)-derived cell death have been reported so far (13,18).

Here we found that polyI:C and zVAD induced cell death in the mouse colon carcinoma cell line, CT26. Since the dead cells turned propidium iodide (PI)/annexin V-double positive and a necroptosis inhibitor (necrostatin, nec-1) blocked the cell death (12), we concluded that the polyI:C/zVAD-induced CT26 death was necroptosis. This cell death was abrogated in CT26 after depletion of *Ticam-1* or *Ripk3* or treatment with nec-1. In contrast, *Mavs* knock down barely affected tumor cell death. Notably, blocking IFNAR or TNF- α hardly affected the degree of polyI:C-induced CT26 necroptosis. Thus, necroptosis was virtually induced in CT26 *in vitro* directly by polyI:C and zVAD through the TLR3-TICAM-1-RIP3 pathway, independent of IFN or TNF- α . Neither the cytoplasmic RNA-sensor pathway (15) nor the TICAM-1-mediated inflammasome-caspase activation (19) participates in this type of tumor necroptosis.

In wild-type mice, polyI:C adjuvancy promotes cross-priming of CD8⁺ T lymphocytes, activation of natural killer (NK) cells, and IFN/cytokine production by DCs (14,20). We detected NK/cytotoxic T lymphocyte (CTL)/cytokine-independent tumor-necroptotic shrinkage in CT26-bearing immune-compromised Balb/c mice by injection of polyI:C and zVAD *in vivo*.

Materials and Methods

Cell culture and reagents

Following cell lines were provided; EG7 (lymphoma) and C1498 (acute myeloid leukemia) from American Type Culture Collection; 3LL (Lewis lung carcinoma), YAC-1 (lymphoma) and colon26 (CT26, colon carcinoma) from Summit Pharmaceuticals International Corporation (Tokyo, Japan); L929 (fibroblast) from RIKEN cell bank (Ibaragi, Japan); EL4 (lymphoma) from Dr. N. Sato (Sapporo Medical University School, Sapporo, Japan); B16F1 (melanoma) and B16F10 (melanoma) from Dr. O. Hazeki (Hiroshima University, Hiroshima, Japan); G1 (hepatocellular carcinoma) and G5 (hepatocellular carcinoma) from Dr. Y. Saeki (Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan); Renca (renal adenocarcinoma) from Dr. Y. Matsushita (Iwate Medical University School of Medicine, Iwate, Japan); MC38 (colon adenocarcinoma) from Dr. H. Tahara (University of Pittsburgh Medical Center, PA 15261, USA). A B16 subline, B16D8, was characterized as an NK-sensitive one in our laboratory (21). All cell lines were confirmed as mycoplasma-free.

YAC-1, EL4, B16F1, B16F10, B16D8, Renca, G1, G5, 3LL, L929, EG-7, C1498 and CT26 cells were maintained in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. MC38 cells were maintained in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum, antibiotics, 2 mM glutamine, 50 μ M 2-mercaptoethanol, 1 mM sodium pyruvate and non-essential amino acid. Necroptosis resistant CT26 cells were survival cells after stimulation with 25 μ g/ml polyI:C and 25 μ M zVAD, and maintained in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. For induction of bone marrow-derived dendritic cells (BMDCs), bone marrow cells from C57B6/J WT mice were cultured in RPMI1640 with 10% heat-inactivated fetal bovine serum and antibiotics containing J558 supernatant for 7 days with replenishment of the medium every other day.

Following antibodies were purchased. Anti-RIP1 (BD Biosciences), anti-RIP3 (QED Bioscience), anti-FLAG monoclonal (SIGMA), anti-FLAG polyclonal (SIGMA), anti-tubulin (Biolegend), anti-HA monoclonal (COVANCE), anti- β -actin (SIGMA), APC anti-TLR3 (Biolegend) and FITC anti-TLR4 (MBL) antibodies. PolyI:C was from Amersham Biosciences, zVAD, butylated hydroxyanisole (BHA) and nec-1 were from SIGMA. Anti-IFNAR and anti-TNF- α antibodies were from Biolegend.

Mice

Balb/cAJcl and C57B6/J WT female mice were purchased from CLEA Japan (Tokyo, Japan). *Rag-2/Jak3* double-KO mice in Balb/c background (22) were housed and monitored in our animal research facility according to institutional guidelines. All mice were maintained under specific pathogen-free conditions in the Animal Facility in Hokkaido University Graduate School of Medicine (Sapporo, Japan) and used when they were 7 to 9 weeks of age. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments in the Animal Safety Center, Hokkaido University, Japan. All mice were used according to the guidelines of the Institutional Animal Care and Use Committee of Hokkaido University, who approved this study as no.13-0043. All efforts were made to minimize suffering.

Tumor challenge and antibody treatment

Mice were shaved at the back and injected 200 μ l of 1×10^5 or 5×10^5 CT26 cells in PBS. Tumor size was measured using caliper. Tumor volume was calculated using the following formula: tumor volume (cm^3) = (long diameter) $\times$ (short diameter)² $\times$ 0.4. PolyI:C (usually 100 μ g) was injected in mice by i.p. or s.c. while zVAD (1 mg) was s.c. injected per mouse. For depletion of CD8⁺ T cells and NK cells, anti-murine CD8 β antibody prepared from H35.17.2 hybridoma which was kindly provided by Dr. Toshitada Takahashi (Aichi Cancer Center, Japan) (23) and anti-asialo-GM1 antibody (WAKO) were i.p. injected before polyI:C treatment (21). Optimal doses of the antibodies were determined in preliminary studies (anti-asialo-GM1 30 μ l/body) and the same lot of anti-CD8 β antibody was used in the experiment.

Water soluble tetrazolium salts (WST)-1 assay

WST-1 Cell Counting kit (Dojindo) was used following manufacturer's instructions. 2×10^4 cells were plated in 96-well plate. The following day, cells were stimulated with 25 or 50 μ g/ml polyI:C, 25 μ M zVAD and 50 μ M nec-1. After 24 h, 10 μ l of WST-1 reagent was added to each well and incubated at 37 $^{\circ}$ C for 1 to 4 h. The absorbance at 450 nm was measured by a microplate reader.

Detection of reactive oxygen species (ROS)

CT26 cells (2×10^4) were plated in a 96-well plate. The following day, cells were stimulated with 50 $\mu\text{g/ml}$ polyI:C, 25 μM zVAD, 100 μM BHA and 50 μM nec-1. After 6 h, CT26 cells were incubated with 5 μM CM-H₂DCFDA (Invitrogen) at 37 °C for 15 min. Cells were washed with culture medium, and incubated at 37 °C for 15 min. Cells were washed with FACS buffer and analyzed by flow cytometry.

Measurement of high-mobility group protein B1 (HMGB1)

HMGB1 ELISA kit (Shino-test) was used manufacturer's instructions. CT26 cells (2×10^4) were plated in 96-well plate. The following day, cells were stimulated with 25 $\mu\text{g/ml}$ polyI:C, 25 $\mu\text{g/ml}$ zVAD and 50 μM nec-1. After 24 h, HMGB1 in culture supernatants was quantified with HMGB1 ELISA kit (Shino-test) following manufacturer's instructions.

Cytometric Bead Array (CBA) assay and ELISA

Production of Cytokines was measured by CBA assay (BD Biosciences). Culture supernatants or sera were incubated with capture beads for 1 h at room temperature following incubation with PE-labelled detection reagents. The intensity of beads bound to cytokines was detected by flow cytometry. Data analysis was performed by FCAP Array Software. Culture supernatants of CT26 cells or concanavalin A-stimulated splenocytes (5×10^5) were analyzed for IFN- γ levels using ELISA. IFN- γ ELISA kit was from eBiosciences. Assay was performed according to the manufacturer's instructions. IFN- β levels in sera were measured by mouse IFN beta ELSA kit (PBL) following manufacturer's instructions.

TdT-mediated dUTP-biotin nick end-labeling (TUNEL) staining

Tumors isolated from CT26-bearing *Rag2*^{-/-}/*Jak3*^{-/-} mice were fixed with 4% paraformaldehyde/PBS for 30 min at 4 °C. Fixed tissues were impregnated with 15 % sucrose/PBS for 2 h following 30 % sucrose/PBS for overnight at 4 °C. Tissues were then embedded in O.T.C. compound (Sakura Finetek Japan) and the frozen tissue blocks were sectioned by using cryotome (LEICA CM1850). TUNEL staining of frozen sections was performed using in situ cell death detection kit (Roche) following

manufacturer's instructions. Stained sections were monitored at x20 or x40 magnification using LSM510 META microscopy (Zeiss).

FACS analysis of dead cells

For detection of dead cells, 1×10^5 CT26 cells were plated in 24-well plates. The following day, cells were stimulated with 25 or 50 $\mu\text{g/ml}$ polyI:C, 25 μM zVAD and 50 μM nec-1. After 24 h, cells were stained with PI and annexin-V-FLOUS staining kit (Roche) following manufacturer's instructions. Cells were stained by intracellular staining with APC anti-TLR3 (11F8), FITC anti-TLR4 (UT49) or isotype control antibody with or without permeabilization. Stained cells were analyzed by flow cytometry.

Results

Necroptosis induced by polyI:C and zVAD

Necroptosis is induced in macrophages by TLR stimulation when caspase-8 is inhibited by caspase inhibitors, for example zVAD (4). To determine whether polyI:C or combination of polyI:C/zVAD induced cell death in tumor cell lines, we added these reagents to the culture of mouse tumor cell lines including YAC-1, EL-4, B16F1, B16F10, B16D8, Renca, G1, G5, 3LL, MC38 and CT26. The L929 fibroblast cell line was used as a positive control for cell death (24). Cell viability was measured by WST-1 assay (Supplemental Table 2). Cell death was detected after polyI:C and zVAD treatment only in the CT26 cell line of the tumor cell lines tested. Little cell death was detected by treatment with either polyI:C or zVAD alone (Fig. 1A and B). PolyI:C/zVAD-dependent cell death was observed in the CT26 as well as the control L929 cell line (Supplemental Table 2), suggesting that polyI:C-induced factors other than caspases are involved in polyI:C-derived cell death in most tumor lines. Although polyI:C activates NALP3 inflammasome via TICAM-1 and caspase-11 in bacterial infections (19), no zVAD-affecting factor participates in caspase-11 or IL-1 β levels in CT26 cells (Supplemental Fig. 1).

In CT26 cells, comparing cells treated with polyI:C and zVAD with cells treated with polyI:C alone, the population of cells with annexin V positive (10.5% vs. 0.3%) and double-positive for annexin V and PI (23.4% vs. 3.7%) were increased by the addition of zVAD to polyI:C (Fig. 1C). Although dead cells were stained with PI (Fig.

1D), rate of single positive for annexin V was increased after polyI:C and zVAD treatment (Fig. 1C), indicating polyI:C/zVAD-induced cell death was involved, in part, in apoptosis. HMGB1, a necrosis marker, was produced by polyI:C/zVAD stimulation (Fig. 1E). Hence, both necrosis and apoptosis are induced in CT26 cells by treatment with polyI:C independent of caspases.

The properties of CT26 necroptosis induced by polyI:C and zVAD

To further analyze the features of cell death after polyI:C and zVAD stimulation, we examined whether cell death induced by polyI:C and zVAD was prevented by treatment with nec-1, an inhibitor of RIP1 kinase for the necroptosis pathway. Consistent with previous observations (25), when 50% of cell death was induced by polyI:C and zVAD, nec-1 treatment fully restored cell viability (Fig. 2A and B). The population of PI-positive cells was reduced with nec-1 treatment (Fig. 2C). Moreover, treatment with nec-1 reduced the population of cells that were double-positive for annexin V and PI (Fig. 2D). By imaging analysis with PI, we confirmed that nec-1 inhibited cell death (Fig. 2E). Initiation of pyroptosis is mediated by caspase-11 expression that is dependent on TLR4-TICAM-1 (26), but neither expression of caspase-11 and IL-1 β production (Supplemental Fig. 1) nor TLR4 protein was induced by treatment with polyI:C and zVAD in CT26 cells (the latter compared with control JAWSII cells) (Supplemental Fig. 2). No evidence thus far endorses that cell death induced by polyI:C and zVAD involves NALP3-inflammasome- or caspase-mediated pyroptosis.

Since ROS generation is a major inducer of necroptosis, we examined the possibility that ROS is involved in polyI:C/zVAD-derived necroptosis. ROS generation was induced in CT26 cells by polyI:C and zVAD treatment (Fig. 2F). Nec-1 treatment suppressed the ROS generation induced by polyI:C and zVAD (Fig. 2F). To investigate the effect of ROS generation on cell death induced by polyI:C and zVAD, CT26 cells were pretreated with BHA, a ROS scavenger. Both cell death and ROS generation induced by polyI:C and zVAD was suppressed with BHA in parallel (Fig. 2F, G). These data indicates that ROS production is crucial for necroptosis triggered by polyI:C and zVAD in tumor cells.

Type I IFN, IFN- γ and TNF- α are inducers of necroptosis (27,28). To examine the participation of type I IFN and TNF- α in necroptosis induced by polyI:C and zVAD,

CT26 cells were pretreated with anti-IFNAR antibody or anti-TNF- α antibody (Fig. 3A). Blocking IFNAR or TNF- α signaling by specific antibody did not affect cell viability (Fig. 3A). TNF- α protein was detected less than the detection limit in the culture supernatant of stimulated CT26 cells (Fig. 3B). CT26 cells were resistant to IFN- γ -induced cell death (Fig. 3C) and barely produced IFN- γ with polyI:C treatment in CT26 cells (Fig. 3D).

The signal pathway for necroptosis in CT26 cells.

To determine the pathway involved in polyI:C and zVAD-induced necroptosis, genes encoding molecules that might participate in polyI:C recognition were silenced by siRNA in CT26 cells with knockdown efficiencies higher than 50% (Fig. 4A). *Tlr3* or *Ticam-1* knockdown resulted in a recovery of cells from necroptosis induced by polyI:C and zVAD (Fig. 4B). RIPK3 is the key molecule that interacts with mixed lineage kinase domain-like protein (MLKL) and RIP1 to transmit necroptosis signal (25,28). Cell viability was recovered in *Ripk3* knockdown cells (Fig. 4B). *Mavs* knockdown did not affect cell viability in polyI:C/zVAD-induced cell death (Fig. 4B). Collectively, necroptosis initiated by polyI:C and zVAD critically depends on the TLR3-TICAM-1-RIP3 pathway. In confirmation experiments, CT26 cells expressed TLR3 protein (Fig. 4C) and *Tlr3* knockdown cells did not induce *Ifn- β* mRNA in response to polyI:C (Fig. 4D), suggesting that TLR3 mainly signals the presence of polyI:C through the TICAM-1 pathway in CT26 cells.

To identify the molecular mechanism of necroptosis induced by polyI:C and zVAD, we examined the physical interaction among TICAM-1, RIP1 and RIP3 in cells stimulated with polyI:C. CT26 cells were transfected with HA-tagged TICAM-1 and FLAG-tagged RIP3. Immunoprecipitation assay using anti-FLAG antibody after 24 h of transfection showed that RIP3 interacted with TICAM-1 but not RIP1 in steady state conditions (Fig. 5A). Upon polyI:C stimulation, RIP3 interacted with both TICAM-1 and RIP1 in CT26 cells (Fig. 5A). However, in necroptosis-resistant B16D8 cells, RIP3 did not bind TICAM-1 after polyI:C stimulation and little necroptosis was induced after treatment with polyI:C and zVAD (Fig. 5B and Supplemental Table 1). We sometimes found a minute TICAM-1-RIP3 interaction in the absence of polyI:C stimulation in B16D8 cells (Fig. 5B), though the reason as yet unknown. Even when the RIP3 protein was overexpressed, extrinsic RIP3 did not interact with TICAM-1 in B16D8 cells

stimulated with polyI:C (Fig. 5B) and cell death was not induced by polyI:C and zVAD in RIP3-overexpressing cells (Supplemental Fig. 3).

To identify the molecules that define sensitivity to cell death induced by polyI:C and zVAD, we established necroptosis-resistant CT26 cells by culturing in medium with polyI:C and zVAD (Fig. 5C). Expression of RIP3 was decreased in necroptosis-resistant CT26 cells (Fig. 5D, 5E). The expression levels of negative regulatory molecules of necroptosis including cellular FLICE (FADD-like IL-1 β -converting enzyme)-inhibitory protein (cFLIP)_s, cFLIP_L and inhibitor of apoptosis (cIAP) (4) remain unchanged between parent and death-resistant CT26 cells (Supplemental Fig. 4). Expression of 5-azacytidine induced 2 (*Azi2*), dynamin-related protein (*Dnm1l*), *Mkl1* and phosphoglycerate mutase family member 5 (*Pgam5*), which are critical as necroptosis factors downstream of RIP1 and RIP3 (4), were unaltered in necroptosis-resistant CT26 cells (Fig. 5D). RIP3-mediated cell death was observed with mouse bone marrow-derived macrophages (BMDMs) (25) and L929 (24), but not with EL4 (Supplemental Table 1). *Tlr3* mRNA and *Ifn- β* mRNA expressions were not induced in EL4 cells in response to polyI:C (Takaki, unpublished data), suggesting unresponsiveness of our EL4 to polyI:C, which remains to be reasoned. *Ripk3* mRNA was hardly expressed in B16D8, B16F10, 3LL, MC38, C1498 and Renca cell lines that were resistant to cell death by polyI:C and zVAD (Fig. 5F and Supplemental Table 1). Thus, the interaction between TICAM-1 and RIP3 is a regulatory step in polyI:C-induced necroptosis in some types of tumor cells.

Tumor shrinkage induced by polyI:C/zVAD in CT26-implanted mice

To investigate whether polyI:C/zVAD-induced necroptosis was involved in tumor retraction *in vivo*, CT26 cells were implanted in Balb/c mice. Constant tumor growth was observed as expected (Fig. 6A). PolyI:C i.p. injection effectively and dose-dependently suppressed tumor growth (Fig. 6A). Injection of 100 μ g polyI:C resulted in >80% regression of CT26 implant tumors compared to injection of a PBS control by day 20, and tumoricidal action of 1 mg zVAD only was negligible (data not shown). Tumor regression by polyI:C treatment was partially abrogated when NK cells or CD8⁺ T cells were depleted by anti-asialo GM1 or CD8 β -specific antibodies (Fig. 6B), suggesting that tumor growth retardation by polyI:C was mediated by NK cells and CD8⁺ T cells. Of note, DCs did not directly induce cytotoxicity in CT26 cells

(Supplemental Fig. 5). Based on these results, we assessed the effect of zVAD on polyI:C-treated, NK/CD8 β -depleted, CT26-bearing mice. Mice were s.c. inoculated with 1 mg zVAD, which suppresses death receptor-mediated liver injury *in vivo* (29). Injection with s.c. zVAD and i.p. polyI:C resulted in tumor regression in immune effector-depleted mice (Fig. 6C). In the presence of CD8 T and NK cells, the zVAD effect was less than expected from the *in vitro* results. Therapeutic use of polyI:C promotes DC maturation but combination with zVAD tends to decrease BMDMs viability (25). S.c. administration of polyI:C alone effectively induced growth retardation of CT26 tumors but s.c. administration of polyI:C together with zVAD hampered the antitumor activity in NK/CD8 T cell-depleted tumor-bearing mice (Fig. 6D). In our setting (Fig. 6D) NK and CD8 T cells preferentially kill the tumor cell population that can be targeted by polyI:C/zVAD. Although the reason why the polyI:C/zVAD tumor-suppression is abrogated by s.c. administration is unknown, these reagents might target epidermal myeloid and fibroblastic cells in this route, which alters tumor microenvironment to barely promote tumor retardation (Fig. 6D).

To confirm anti-tumor effect of zVAD and polyI:C in the absence of immune cells, CT26-bearing *Rag2*^{-/-}/*Jak3*^{-/-} mice, which lacks T, B and NK cells (22), were injected with i.p. polyI:C and s.c. zVAD (Fig. 7A). Although polyI:C alone injection did not decrease tumor volume, additional s.c. zVAD treatment could significantly suppress tumor growth (Fig. 7A). Serum IFN- β and TNF- α levels in polyI:C/zVAD-treated mice were comparable to those with polyI:C alone (Fig. 7B). IFN- γ was not detected in sera by treatment with polyI:C or polyI:C/zVAD (Fig. 7B), indicating that IFN- β , TNF- α or IFN- γ is dispensable for tumor retardation *in vivo* as in *in vitro*. Upregulation of *Ripk3*, *Ripk1*, *Mkl1* mRNA, which is a maker of necroptosis *in vivo* (30), was observed in tumors prepared from polyI:C/zVAD-treated mice as compared to those of polyI:C alone (Fig. 7C). TUNEL positive cells also increased in tumors in polyI:C/zVAD-treated mice as compared with those with polyI:C alone (Fig. 7D and Supplemental Fig. 6). Taken together, polyI:C/zVAD treatment induces cell death in CT26 tumors in mice without participation of immune cells, resulting in tumor retardation.

Discussion

Here we demonstrated that administration of a TLR3 agonist and pan-caspase inhibitor zVAD results in tumor regression in mice secondary to tumor cytotoxicity.

Notably, direct acting of these reagents on tumor cells induces a tumoricidal event. In literatures, activation of caspases usually accelerates programmed cell death and preceding polyI:C-mediated priming of TICAM-1 is crucial in promoting caspase-mediated inflammasome activation in lipopolysaccharide (LPS) signaling (19,26). In contrast to the TICAM-1 inflammasome axis, which involves caspase 11 and effector caspases 3 and 7 (19,31), the necrotic cell death observed in fibroblasts and macrophages is mainly induced through the TICAM-1-RIP3 pathway that involves no caspases (13): this pathway is activated in the absence of caspase 8 activity. This cell death process fits the definition of necroptosis, where the cell death is RIP1/3-dependent and inhibited by nec-1, and based on the production of ROS (4). We found this type of tumor death evidenced in the CT26 colon cancer cell line. Since similar RIP1/3-mediated necroptosis have been reported with neuroblastoma cell lines, most of which are caspase 8-deficient (32), RIP1/3-mediated tumor shrinkage we observed is not an isolated phenomenon in tumors.

In CT26 necroptosis induction, the TLR3/TICAM-1 pathway plays a pivotal role without involvement of TNF- α and IFNs in the process of RIP3 activation followed by cytolysis. Thus, exogenously-added polyI:C acts on TLR3 and together with zVAD induces necroptosis in the tumor cells. Exogenous polyI:C activates not only TLR3 but also MDA5 as shown by IFN- β reporter assay (33): type I IFN represents an output of the live signal induced by RNA sensors. Virtually, no up-regulation of IL-1 β is detected in CT26 cells by stimulation with polyI:C/zVAD (Supplemental Fig. 1B), suggesting that inflammasome activation is again dispensable for the CT26 cell death. Upon polyI:C stimulation, on the other hand, TICAM-1 immediately and transiently interacts with RIP1 and RIP3 to initiate necroptosis signaling, resulting in the production of ROS in CT26 cells. Although we cannot explain what mechanism is responsible for the switch for turning live to death signal in tumor cells by RNA stimulation, ROS production might reflect mitochondrial oxidative stress induced by the TICAM-1 signal, leading to the PGAM5-DRP-1 axis (4).

The function of the TICAM-1/RIP3 pathway has been shown to be involved in activation of the GTPase DRP1 that is translocated from cytosol to mitochondria to drive mitochondrial damage in macrophages, which markedly modifies inflammation (34). Since phosphorylation controls the RIP3-DRP1 activation, the phosphatase-kinase balance needs to be further investigated in tumor cells.

In CT26 tumor-bearing mice, tumor growth is abrogated with i.p. injection of polyI:C, which causes activation of antitumor NK cells and CD8⁺ CTLs (21,23). These immune effectors must be depleted in mice in order to detect an alternative mode of tumor growth retardation by direct-acting of polyI:C and zVAD on tumor cells. There appears no additive effect on immune activation and direct tumor killing induced by polyI:C and zVAD *in vivo*, suggesting that the cell death system well backs up the immune system for tumor clearance. Notably, the direct tumoricidal activity by polyI:C/zVAD can be observed only when polyI:C is administered by the i.p. route. We surmised that s.c. injection of polyI:C and zVAD directly affected viability of skin fibroblasts and Langerhans cells: decreased viability of these lineages after polyI:C and zVAD treatment is consistent with the literature (25). Taken together, tumor necroptosis induced by polyI:C makes a small contribution to CT26 growth suppression in the wild-type Balb/c mice with sufficient immune effectors *in vivo*. Yet, the tumor necroptosis activity and tumor regression by polyI:C/zVAD becomes evident in *Rag2*^{-/-}/*Jak3*^{-/-} double-deficient mice (Fig. 7). This study clarifies that the RNA-induced tumor clearance system works in tumors which is engaged in necroptosis but independent of the immune effectors or IFN/cytokines.

Although necrotic cells prepared by freeze/thaw cycles, formaldehyde fixation, or osmotic shock provoke no protective immune response in a tumor vaccination model (35-37), heat-killed necrotic cells stimulate antigen-presenting cells to increase production of IL-12 and TNF- α (38). The inconsistent results among studies might be because of differences in the composition and properties of damage-associated molecular patterns (DAMPs) containing RNA released from necrotic cells that depends on type of stimulation, resulting in induction of diverse immune responses. In this context, self RNA with incomplete stems that activates TLR3 (39) would be involved in inflammation-mediated tumor cell death.

Although RIP1 and RIP3 are required to initiate necroptotic signaling, necroptosis is induced in the absence of RIP1 (40,41). RIP1 rather protects cells from necroptosis in some tissues/organs, which reflects complex arrays of necroptosis. CT26 can be sensitized by polyI:C to induce necroptosis in the presence of zVAD similar to L929 (24). However, this result is not always generalized for the other tumor cell lines (Table 1 and Fig. 5F). The expression levels of RIP3 is lower in B16D8 cells (resistant to the polyI:C-induced cell death) than in wild-type CT26 and L929 cells (Fig. 5F), in

which necroptosis can be induced (24). The RIP3 protein expression levels are clearly different between necroptosis-resistant CT26 cells and the parent CT26 cells (Fig. 5B). In L929 cells, which are used for *in vitro* necroptosis studies (24), the *Ripk3* mRNA level is high as well as CT26 and EL4 cells. Except for EL4, these RIP3 profiles infer that RIP3 expression correlates with sensitivity to polyI:C-induced necroptosis in most tumor lines.

However, simple over-expression of RIP3 is insufficient to trigger necroptosis: an additional phosphorylation or positive regulator plays a key role to induce cell death. Recently, sirtuin-2 (SIRT2), MLKL and PGAM5 were reported to positively regulate the TNF necroptotic pathway (4,28,42). MLKL also interacts with RIP3 to trigger necrosis in fibroblasts stimulated with TLR ligands (25). The *Pgam5* and *Mlkl* mRNA levels are unchanged between parental and resistant CT26 cells (Fig. 5D). Therefore, tumor necroptotic death represents an output produced by complex signaling where RNA sensors involve.

PolyI:C, a synthetic TLR3 agonist, is used as an effective adjuvant for antitumor treatment and vaccines because of its prominent effects on DCs to induce CD8⁺ T and NK cells (21,23). In addition, stem-structured RNA from viral replicative intermediates can act as a TLR3-specific ligand (8,9). A GpC-capped dsRNA, which exclusively targets TLR3 and is named ARNAX, potently induces cross-presentation in CD8 α ⁺ DCs without a significant increase of serum cytokines (43). CD8 α ⁺ DCs, which have high expression of TLR3, are activated by i.p. or s.c. injection of these TLR3 ligands to promote cross-priming of T cells in a TLR3/TICAM-1-dependent manner (23). This treatment also induces antitumor NK activation through induction of a polyI:C-inducible gene, INAM (IRF3-dependent NK activating molecule), in DCs (44,45). Moreover, polyI:C injection induces production of type I IFNs via the MAVS pathway in stromal cells, which suppresses tumor growth (46). However, the polyI:C/zVAD tumor regression was DC-unrelated. Recently, polyI:C was found to act on tumor-associated macrophages to facilitate robust production of TNF- α resulting in hemorrhagic necrosis of the tumor (47). Tumors usually contain various types of macrophages concomitant with invasive properties (48). The recruited macrophages are obliged to support tumor progression, but often turn tumor-suppressive in response to polyI:C via TNF- α production (47). Like other myeloid species (48), ROS would be a macrophage-derived antitumor modulator induced via extracellular RNA stimulation.

Our present study further emphasize an alternative mode of RNA-mediated tumor suppression that is attributable to the direct effect of RNA on tumor cells independent of DC or macrophage responses. Tumor regresses by direct action of RNA without participation of the products of macrophages or DAMPs. In CT26 cells, tumor retardation by polyI:C depends on NK cells because of lower expression of class I MHC in CT26 cells (49). Hence, in addition to the immune or macrophage activation by polyI:C, tumor cells can be a direct target of polyI:C to induce tumor cell death. TLR3 tend to be expressed in murine and human tumor cells (50,51). TLR3 levels in tumor cells would be a biomarker for the therapeutic efficacy of dsRNA therapy in renal cell carcinoma and breast cancer (52,53). We found that tumor necroptosis by TLR3 signaling occurred only under specific conditions, in line with the findings that caspase-8 deficiency, caspase inhibition by zVAD or the presence of anticaspase viral proteins, is required for necroptosis induced by TNF- α or death receptors (54,55). Further elucidation of the molecular composition of RNA-mediated cell death, and development of a strategy to deliver an anti-caspase reagent to tumor cells will make RNA immunotherapy more potent in conjunction with dsRNA-mediated DC maturation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: T. Seya, H. Takaki, M. Matsumoto

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Acquisition of data (provided animals, provided facilities, etc.): R. Takemura, H. Takaki, S. Okada, T. Akazawa, T. Teshima,

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Takemura, H. Takaki, H. Shime,

Writing, review, and/or revision of the manuscript: H. Takaki, M. Matsumoto, H.

Oshiumi, T. Seya, Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Teshima, T. Seya

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References

1. Galluzzi L, Brenner C, Morselli E, Touat Z, Kroemer G. Viral control of mitochondrial apoptosis. *PLoS Pathog* 2008; 4: e1000018.
2. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011; 144: 646-74.
3. Criscitiello C, Esposito A, Curigliano G. Tumor-stroma crosstalk: targeting stroma in breast cancer. *Curr Opin Oncol*. 2014; 26: 551-5.
4. Moriwaki K, Chan FK. RIP3: a molecular switch for necrosis and inflammation. *Genes Dev* 2013; 27:1640-9.
5. Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G. Molecular mechanisms of necroptosis: an ordered cellular explosion. *Nat Rev Mol Cell Biol*. 2010; 11: 700-14.
6. Su Z, Yang Z, Xu Y, Chen Y, Yu Q. Apoptosis, autophagy, necroptosis, and cancer metastasis. *Mol Cancer*. 2015; 14:48.
7. Roma-Rodrigues C, Fernandes AR, Baptista PV. Exosome in tumour microenvironment: overview of the crosstalk between normal and cancer cells. *Biomed Res Int*. 2014; 2014: 179486.
8. Tatematsu M, Seya T, Matsumoto M. Beyond dsRNA: Toll-like receptor 3 signalling in RNA-induced immune responses. *Biochem J*. 2014; 458: 195-201.
9. Tatematsu M, Nishikawa F, Seya T, Matsumoto M. Toll-like receptor 3 recognizes incomplete stem structures in single-stranded viral RNA. *Nat Commun* 2013; 4:1833.
10. Green DR, Ferguson T, Zitvogel L, Kroemer G. Immunogenic and tolerogenic cell death. *Nat Rev Immunol* 2009; 9: 353-63.
11. Kono H, Rock KL. How dying cells alert the immune system to danger. *Nat Rev Immunol* 2008; 8: 279-89.
12. Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny MV, et al. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. *Cell Death Differ* 2012; 19:107-20.
13. Seya T, Shime H, Takaki H, Azuma M, Oshiumi H, Matsumoto M. TLR3/TICAM-1 signaling in tumor cell RIP3-dependent necroptosis. *Oncoimmunology* 2012; 1:917-23.
14. Matsumoto M, Seya T. TLR3: interferon induction by double-stranded RNA including poly(I:C). *Adv Drug Deliv Rev* 2008; 60:805-12.
15. Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H, et al. IPS-1, an

adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat Immunol* 2005; 6:981-8.

16. Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat Immunol*. 2003; 4: 161-7.

17. Kaiser WJ, Offermann MK. Apoptosis induced by the toll-like receptor adaptor TRIF is dependent on its receptor interacting protein homotypic interaction motif. *J Immunol* 2005; 174:4942-52.

18. Chawla-Sarkar M, Lindner DJ, Liu YF, Williams BR, Sen GC, Silverman RH, et al. Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis. *Apoptosis* 2003; 8:237-49.

19. Rathinam VA, Vanaja SK, Waggoner L, Sokolovska A, Becker C, Stuart LM, et al. TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-negative bacteria. *Cell* 2012; 150:606-19.

20. Seya T, Matsumoto M. The extrinsic RNA-sensing pathway for adjuvant immunotherapy of cancer. *Cancer Immunol Immunother* 2009; 58:1175-84.

21. Akazawa T, Ebihara T, Okuno M, Okuda Y, Shingai M, Tsujimura K, et al. Antitumor NK activation induced by the Toll-like receptor 3-TICAM-1 (TRIF) pathway in myeloid dendritic cells. *Proc Natl Acad Sci U S A* 2007; 104:252-7.

22. Ono A, Hattori S, Kariya R, Iwanaga S, Taura M, Harada H, et al. Comparative study of human hematopoietic cell engraftment into BALB/c and C57BL/6 strain of rag-2/jak3 double-deficient mice. *J Biomed Biotechnol*. 2011; 2011:539748.

23. Azuma M, Ebihara T, Oshiumi H, Matsumoto M, Seya T. Cross-priming for antitumor CTL induced by soluble Ag + polyI:C depends on the TICAM-1 pathway in mouse CD11c(+)/CD8α(+) dendritic cells. *Oncoimmunology* 2012; 1:581-92.

24. Wu YT, Tan HL, Huang Q, Sun XJ, Zhu X, Shen HM. zVAD-induced necroptosis in L929 cells depends on autocrine production of TNFα mediated by the PKC-MAPKs-AP-1 pathway. *Cell Death Differ* 2011; 18:26-37.

25. Kaiser WJ, Sridharan H, Huang C, Mandal P, Upton JW, Gough PJ, et al. Toll-like receptor 3-mediated necrosis via TRIF, RIP3, and MLKL. *J Biol Chem* 2013; 288:31268-79.

26. Kayagaki N, Wong MT, Stowe IB, Ramani SR, Gonzalez LC, Akashi-Takamura S, et al. Noncanonical inflammasome activation by intracellular LPS independent of TLR4.

Science 2013; 341:1246-9.

27. Thapa RJ, Nogusa S, Chen P, Maki JL, Lerro A, Andrade M, et al. Interferon-induced RIP1/RIP3-mediated necrosis requires PKR and is licensed by FADD and caspases. *Proc Natl Acad Sci U S A* 2013; 110:E3109-18.
28. Zhao J, Jitkaew S, Cai Z, Choksi S, Li Q, Luo J, et al. Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. *Proc Natl Acad Sci U S A* 2012; 109:5322-7.
29. Künstle G, Leist M, Uhlig S, Revesz L, Feifel R, MacKenzie A, et al. ICE-protease inhibitors block murine liver injury and apoptosis caused by CD95 or by TNF-alpha. *Immunol Lett* 1997; 55:5-10.
30. Jouan-Lanhouet S, Riquet F, Duprez L, Vanden Berghe T, Takahashi N, Vandenabeele P. Necroptosis, in vivo detection in experimental disease models. *Semin Cell Dev Biol*. 2014; 35:2-13.
31. Kang SJ, Wang S, Kuida K, Yuan J. Distinct downstream pathways of caspase-11 in regulating apoptosis and cytokine maturation during septic shock response. *Cell Death Differ* 2002; 9:1115-25.
32. Nomura M, Ueno A, Saga K, Fukuzawa M, Kaneda Y. Accumulation of cytosolic calcium induces necroptotic cell death in human neuroblastoma. *Cancer Res* 2013; 74: 1056-66.
33. Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, et al., The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol*. 2004; 5:730-7
34. Wang X, Jiang W, Yan Y, Gong T, Han J, Tian Z, Zhou R. RNA viruses promote activation of the NLRP3 inflammasome through a RIP1-RIP3-DRP1 signaling pathway. *Nat Immunol*. 2014; Oct 19. doi: 10.1038/ni.3015.
35. Scheffer SR, Nave H, Korangy F, Schlote K, Pabst R, Jaffee EM, et al. Apoptotic, but not necrotic, tumor cell vaccines induce a potent immune response in vivo. *Int J Cancer* 2003; 103:205-11.
36. Goldszmid RS, Idoyaga J, Bravo AI, Steinman R, Mordoh J, Wainstok R. Dendritic cells charged with apoptotic tumor cells induce long-lived protective CD4+ and CD8+ T cell immunity against B16 melanoma. *J Immunol* 2003; 171:5940-7.
37. Bartholomae WC, Rininsland FH, Eisenberg JC, Boehm BO, Lehmann PV, Tary-Lehmann M. T cell immunity induced by live, necrotic, and apoptotic tumor cells.

J Immunol 2004; 173:1012-22.

38. Yoon TJ, Kim JY, Kim H, Hong C, Lee H, Lee CK, et al. Anti-tumor immunostimulatory effect of heat-killed tumor cells. *Exp Mol Med* 2008; 40:130-44.

39. Bernard JJ, Cowing-Zitron C, Nakatsuji T, Muehleisen B, Muto J, Borkowski AW, et al. Ultraviolet radiation damages self noncoding RNA and is detected by TLR3. *Nat Med* 2012; 18:1286-90.

40. Roderick JE, Hermance N, Zelic M, Simmons MJ, Polykratis A, Pasparakis M, et al. Hematopoietic RIPK1 deficiency results in bone marrow failure caused by apoptosis and RIPK3-mediated necroptosis. *Proc Natl Acad Sci U S A* 2014; 111: 14436-41.

41. Dannappel M, Vlantis K, Kumari S, Polykratis A, Kim C, Wachsmuth L, et al. RIPK1 maintains epithelial homeostasis by inhibiting apoptosis and necroptosis. *Nature* 2014; 513:90-4.

42. Narayan N, Lee IH, Borenstein R, Sun J, Wong R, Tong G, et al. The NAD-dependent deacetylase SIRT2 is required for programmed necrosis. *Nature* 2012; 492:199-204.

43. Matsumoto M, Tatematsu M, Nishikawa F, Azuma M, Ishii N, Morii-Sakai A, Shime H, Seya T. Defined TLR3-specific adjuvant that induces NK and CTL activation without significant cytokine production in vivo. *Nat Commun.* 2015; 6: 6280.

44. Ebihara T, Azuma M, Oshiumi H, Kasamatsu J, Iwabuchi K, Matsumoto K, et al. Identification of a polyI:C-inducible membrane protein that participates in dendritic cell-mediated natural killer cell activation. *J Exp Med.* 2010; 207: 2675-87.

45. Kasamatsu J, Azuma M, Oshiumi H, Morioka Y, Okabe M, Ebihara T, Matsumoto M, Seya T. INAM Plays a Critical Role in IFN- γ Production by NK Cells Interacting with Polyinosinic-Polycytidylic Acid-Stimulated Accessory Cells. *J Immunol.* 2014; 193: 5199-207.

46. Wang Y, Cella M, Gilfillan S, Colonna M. Polyinosinic:polycytidylic acid boosts the generation of memory CD8 T cells through melanoma differentiation-associated protein 5 expressed in stromal cells. *J Immunol.* 2010; 184: 2751-5.

47. Shime H, Matsumoto M, Oshiumi H, Tanaka S, Nakane A, Iwakura Y, et al. Toll-like receptor 3 signaling converts tumor-supporting myeloid cells to tumoricidal effectors. *Proc Natl Acad Sci U S A.* 2012; 109: 2066-71.

48. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. *Nat Med* 2013; 19:1423-37.

49. Masuda H, Saeki Y, Nomura M, Shida K, Matsumoto M, Ui M, et al. High levels of RAE-1 isoforms on mouse tumor cell lines assessed by anti-"pan" RAE-1 antibody confer tumor susceptibility to NK cells. *Biochem Biophys Res Commun* 2002; 290:140-5.
50. Salaun B, Lebecque S, Matikainen S, Rimoldi D, Romero P. Toll-like receptor 3 expressed by melanoma cells as a target for therapy? *Clin Cancer Res* 2007; 13:4565-74.
51. Conforti R, Ma Y, Morel Y, Paturel C, Terme M, Viaud S, et al. Opposing effects of toll-like receptor (TLR3) signaling in tumors can be therapeutically uncoupled to optimize the anticancer efficacy of TLR3 ligands. *Cancer Res.* 2010; 70: 490-500.
52. Morikawa T, Sugiyama A, Kume H, Ota S, Kashima T, Tomita K, et al. Identification of Toll-like receptor 3 as a potential therapeutic target in clear cell renal cell carcinoma. *Clin Cancer Res* 2007; 13:5703-9.
53. Salaun B, Zitvogel L, Asselin-Paturel C, Morel Y, Chemin K, Dubois C, et al. TLR3 as a biomarker for the therapeutic efficacy of double-stranded RNA in breast cancer. *Cancer Res.* 2011; 7: 1607-14.
54. Mocarski ES, Upton JW, Kaiser WJ. Viral infection and the evolution of caspase 8-regulated apoptotic and necrotic death pathways. *Nat Rev Immunol* 2012; 12:79-88.
55. Kaczmarek A, Vandenabeele P, Krysko DV. Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. *Immunity* 2013; 38:209-23.

Figure legends

Figure 1. PolyI:C and zVAD induce necroptosis in CT26 cells.

(A-E) CT26 cells were stimulated with 25 µg/ml polyI:C or 25 µM zVAD for 24 h. (A) Cell viability was measured by WST-1 assay. Data are means ± SD of three independent samples. ** = $p < 0.01$ (B) Morphology was analyzed by microscopy. Arrow heads show cells with cytoplasmic swelling. (C) Cells were stained with PI and Annexin V and analyzed by flow cytometry. The number shows percentages of the gated population. One of two experiments is shown. (D) Cells were stained with PI and Hoechst33342 and analyzed by fluorescent microscopy. One representative of three experiments is shown. (E) HMGB1 in the culture supernatants was quantified with HMGB1 ELISA kit.

Figure 2. Necrostatin-1 inhibits the necroptosis in CT26 cells.

(A-E) CT26 cells were stimulated with 25 µg/ml polyI:C and 25 µM zVAD with or without 50 µM necrostatin (nec)-1 for 24 h. (A) Cell viability was measured by WST-1 assay. Data are means ± SD of three independent samples. ** = $p < 0.01$. (B) Morphology was analyzed by microscopy. (C) Cells were stained with PI and analyzed by flow cytometry. The graph shows percentages of the PI positive cells. Data are means ± SD of three independent samples. ** = $p < 0.01$. (D) Cells were stained with PI/Annexin V and analyzed by flow cytometry. The number shows percentages of the gated population. One of two experiments is shown. (E) Cells were stained with PI and Hoechst33342 and analyzed by fluorescent microscopy. One of two experiments is shown. (F, G) CT26 cells were stimulated with 50 µg/ml polyI:C and 25 µM zVAD with or without 50 µM nec-1 or 100 µM BHA for 6 h. (F) Cells were stained with CM-H2DCFDA and analyzed by flow cytometry. One representative of three experiments is shown. (G) Cells viability was measured by WST-1 assay. Data are means ± SD of three independent samples. ** = $p < 0.01$.

Figure 3. Type I IFN, IFN-γ and TNF-α signaling barely participate in the CT26 necroptosis.

CT26 cells were stimulated with 25 µg/ml polyI:C and 25 µM zVAD in the presence of 10 µg/ml anti-IFNAR antibody or 10 µg/ml anti-TNF-α antibody for 24 h. (A) Cell viability was measured by WST-1 assay. Data are means ± SD of three independent samples. ** = $p < 0.01$. (B) TNF-α protein in culture supernatants of CT26 cells or 100

ng/ml LPS-stimulated BMDCs was measured by CBA assay. Data are means $\pm$ SD of two independent samples. (C) Cell viability of CT26 cells treated with polyI:C or 100 U/ml IFN- γ for 24 h was measured by WST-1 assay. Data are means $\pm$ SD of three independent samples. (D) IFN- γ protein in culture supernatants of CT26 cells or 5 μ g/ml concanavalin A (ConA)-stimulated splenocytes was measured by ELISA. N.D., not detected. Data are means $\pm$ SD of two independent samples. n.s., not significant, N.D., not detected.

Figure 4. The TLR3-TICAM-1-RIP3 pathway governs polyI:C-derived necroptosis. (A, B) Cells were knocked down for *Tlr3*, *Ticam-1*, *Ripk3* and *Mavs* by siRNA. (A) Total RNA was collected and the mRNA levels of *Tlr3*, *Ticam-1*, *Ripk3* and *Mavs* were determined by real-time PCR. mRNA expression levels are shown as relative expression to β -actin. Data are means $\pm$ SD of two independent samples. (B) Cells were stimulated with 25 μ g/ml polyI:C and 25 μ M zVAD for 24 h. Cell viability was measured by WST-1 assay. Data are means $\pm$ SD of three independent samples. * = $p < 0.05$ ** = $p < 0.01$. (C) Cells were stained with anti-TLR3 antibody with or without permeabilization and analyzed in flow cytometer. One of two experiments is shown. (D) *Tlr3*-silenced cells were stimulated with polyI:C for 1 h. *Ifn- β* mRNA expression was measured by real-time PCR. mRNA expression levels are shown as relative expression to β -actin.

Figure 5. PolyI:C stimulation increases the coupling of TICAM-1 with RIP1 and RIP3. (A) 6×10^5 CT26 cells and (B) 5×10^5 B16D8 cells were transfected with 2 μ g of pCMVRIP3-FLAG and 2 μ g of pcDNA4TICAM-1-HA, following stimulation of 25 μ M zVAD. Twenty-four hours after transfection, cells were stimulated with 50 μ g/ml polyI:C for 30 minutes and then lysed with lysis buffer. Total cell lysate (TCL) were subjected to immunoprecipitation with anti-FLAG antibody, and then complexes were analyzed by immunoblot with anti-FLAG, anti-HA and anti-RIP1 antibodies. (C) Parent and resistant cells were stimulated with 25 μ g/ml polyI:C and 25 μ M zVAD for 24 h and cell viability was measured by WST-1 assay. Data are means $\pm$ SD of three independent samples. ** = $p < 0.01$. (D) mRNA expression of the indicated genes was determined by real-time PCR. mRNA expression levels are shown as relative expression to β -actin. (E) 2×10^6 CT26 cells or necroptosis resistant CT26 cells were lysed with

lysis buffer, and then analyzed by immunoblot with anti-RIP3 and anti-tubulin antibodies. (F) Level of *Ripk3* mRNA in various cell lines. Total RNA was collected from CT26, EL4, EG7, C1498, Renca, L929, 3LL, B16D8, MC38, and B16F10 cells and expression of *Ripk3* were determined by real-time PCR. mRNA expression are shown as relative expression to β -actin. Data are means $\pm$ SD of two independent samples. N.D., not detected.

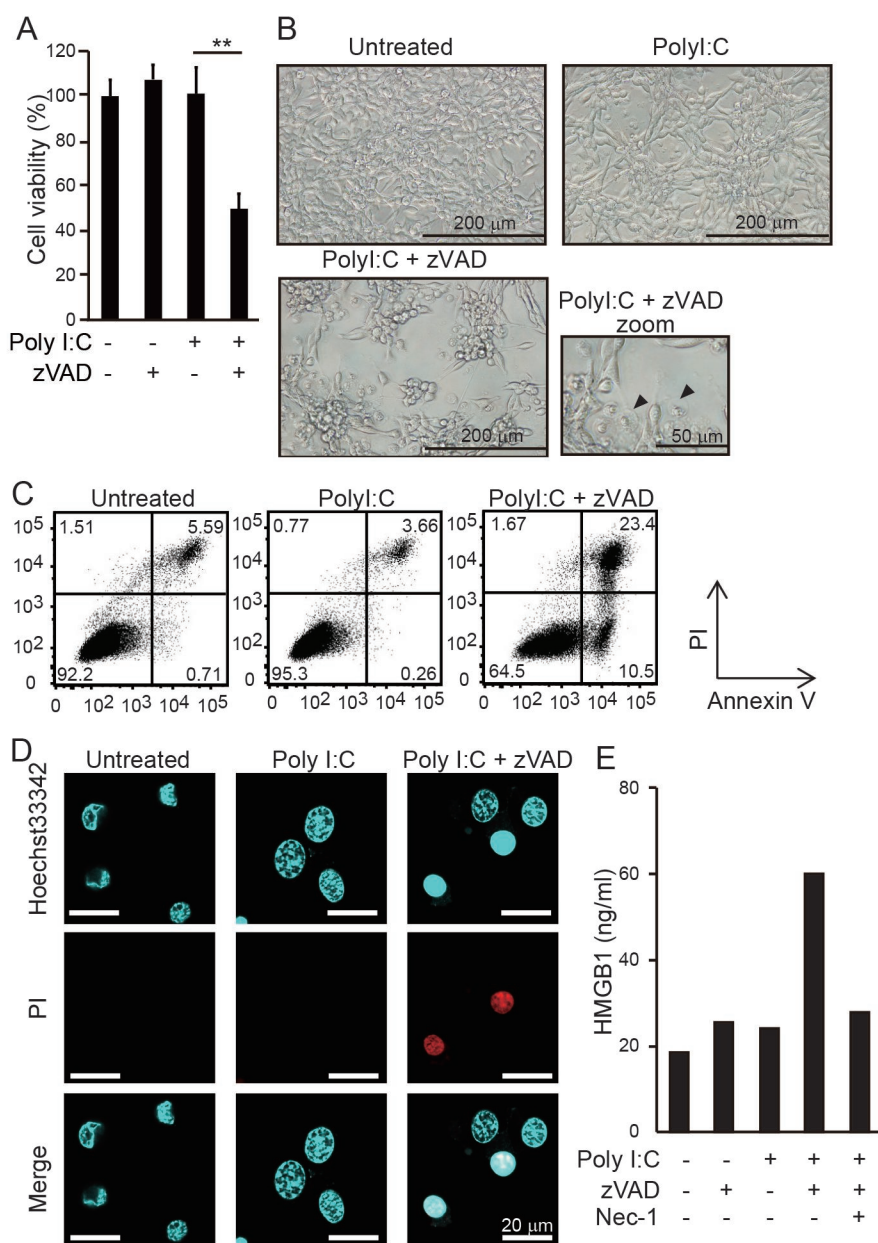
Figure 6. Antitumor effect of polyI:C against CT26 cells.

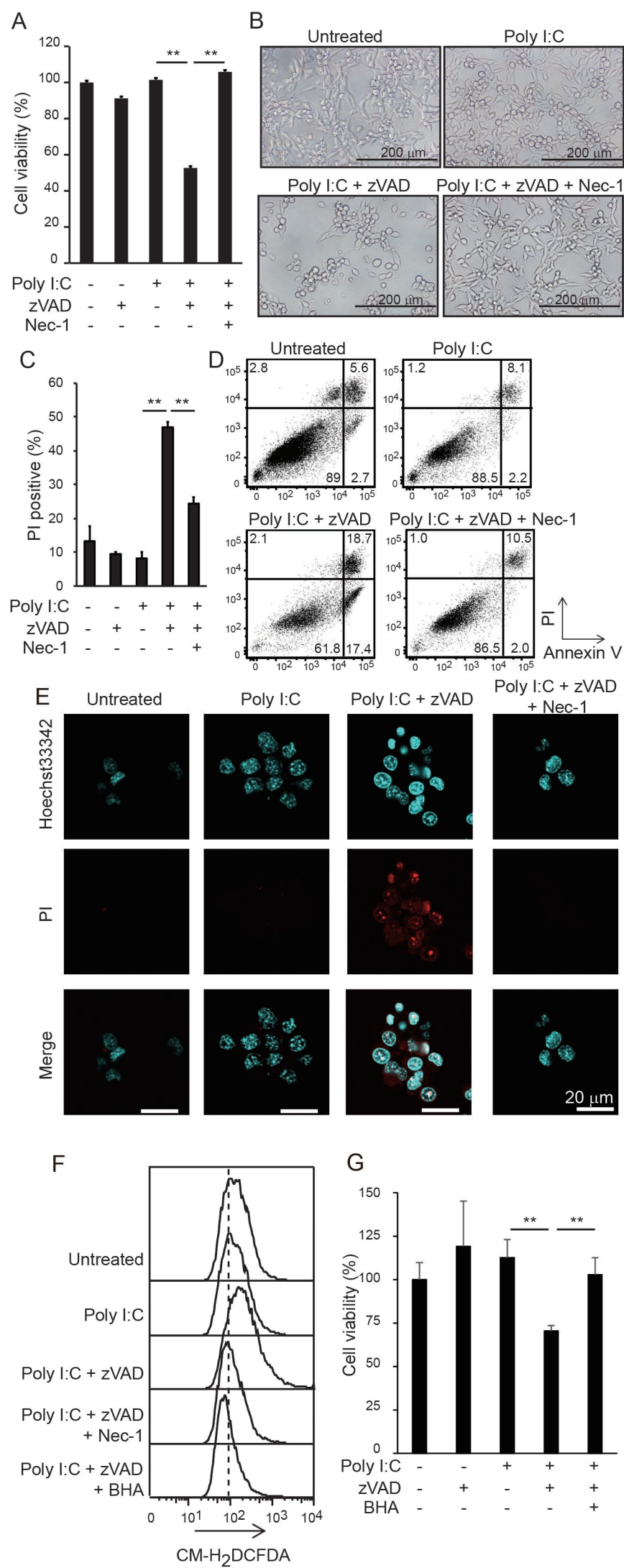
(A) 1×10^5 CT26 cells were subcutaneously inoculated into Balb/c mice. Ten days later, tumor-bearing mice were injected i.p. with the indicated doses of polyI:C. The polyI:C treatment was repeated at day 17 and 24. Results represent means $\pm$ SD of three mice. ** = $p < 0.01$. (B) Ten days after inoculation, tumor-bearing Balb/c mice were injected i.p. with anti-asialo GM1 or anti-CD8 β following injection i.p. of 100 μ g polyI:C. These treatment was repeated on day 14. Results represent means $\pm$ SD of three mice. * = $p < 0.05$ ** = $p < 0.01$ (C, D) Schedule of treatment is shown in lower panel. Balb/c mice inoculated with CT26 cells (5×10^5) were injected i.p. with anti-asialo GM1 and anti-CD8 β antibodies and then injected i.p. (C) or s.c. (D) of 100 μ g polyI:C with or without s.c. of 1 mg zVAD. These treatment was repeated 4 days after the first treatment (day 10). Results represent means $\pm$ SD of three mice. ** = $p < 0.01$. n.s., not significant.

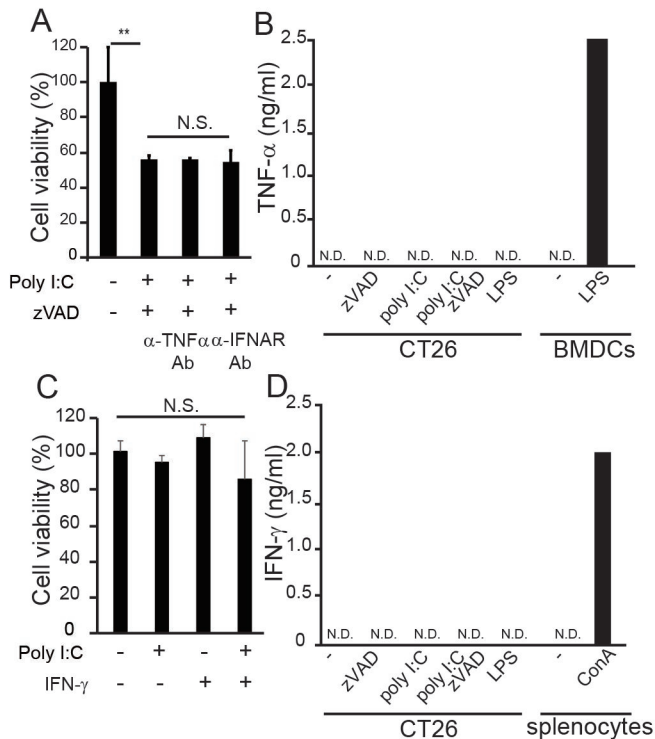
Figure 7. polyI:C and zVAD treatment induces tumor regression in CT26 bearing

Rag2^{-/-}/Jak3^{-/-} mice. (A) *Rag2^{-/-}/Jak3^{-/-}* mice inoculated with CT26 cells (5×10^5) were injected with or without i.p. of 100 μ g polyI:C and s.c. of 1 mg zVAD at day 12 and 16 after inoculation. Results represent means $\pm$ SD of three mice. ** = $p < 0.01$. (B, C) *Rag2^{-/-}/Jak3^{-/-}* mice inoculated with CT26 cells (5×10^5) were injected with or without i.p. of 100 μ g polyI:C and s.c. of 1 mg zVAD at day 14. (B) After 2h later, IFN- β , IFN- γ and TNF- α proteins in sera were measured by CBA assay or ELISA. Results represent means $\pm$ SD of three samples. N.S.; not significant, N.D.; not detected. (C) After 6h later, *Ripk1*, *Ripk3* and *Mlkl* mRNA expression in tumors was measured by real-time PCR. Results represent means $\pm$ SD of three samples. ** = $p < 0.01$. (D) *Rag2^{-/-}/Jak3^{-/-}* mice inoculated with CT26 cells (5×10^5) were injected with or without i.p. of 100 μ g polyI:C and s.c. of 1 mg zVAD. After 24 h, frozen sections of tumors were prepared. Frozen sections were subjected to TUNEL staining. Stained sections were monitored at

x40 magnification using microscopy. One representative of two experiments is shown.



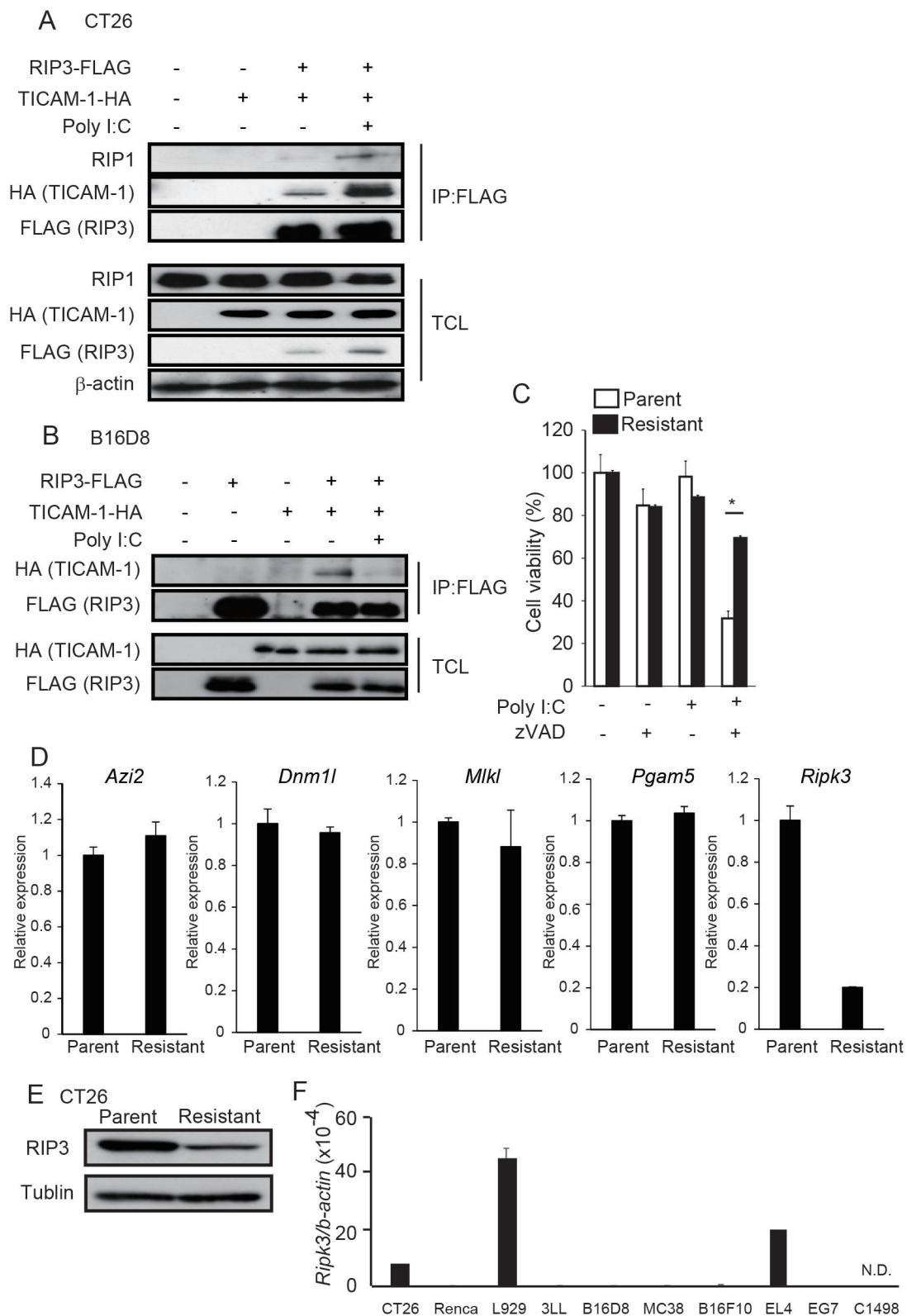


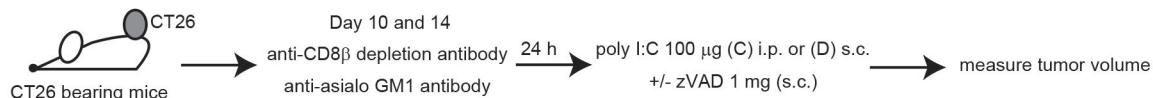
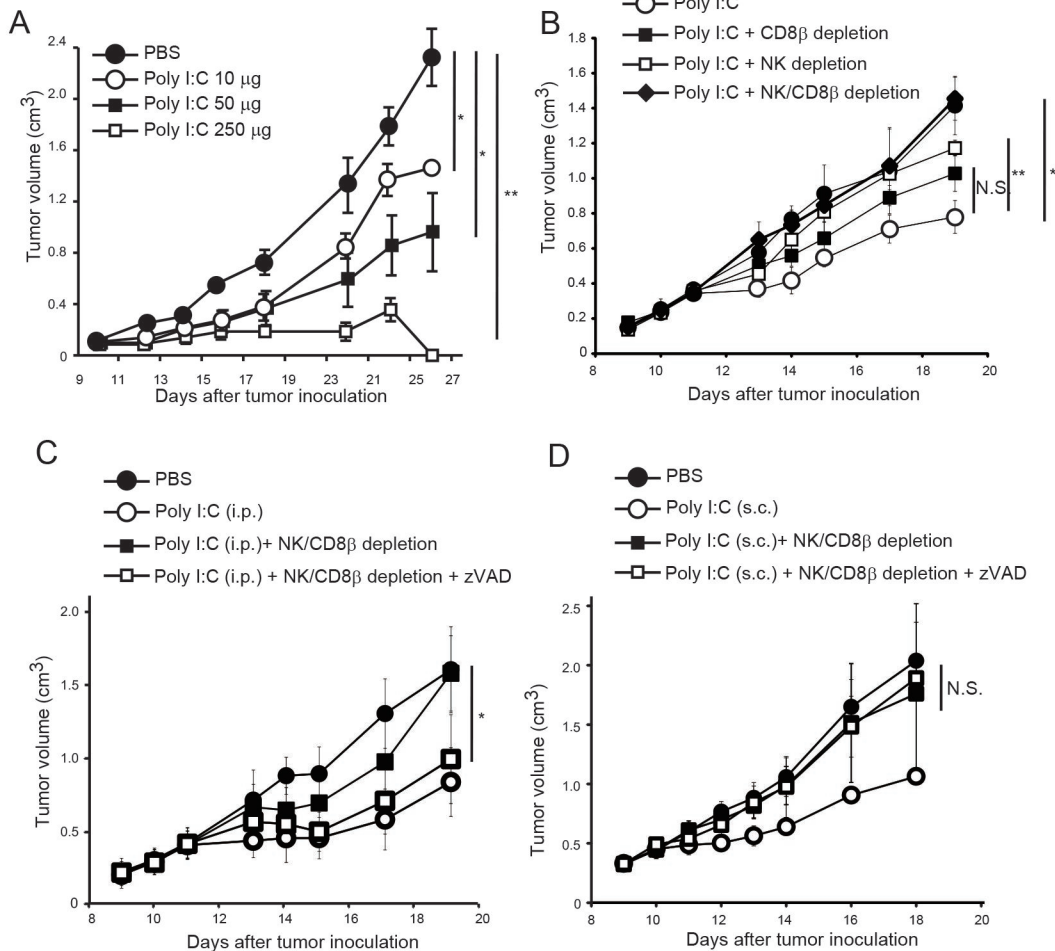


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Figure 3

Figure 4





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Figure 6

