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Poly(I: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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Key words: RNA (polyI:C), TLR3, TICAM-1, necroptosis, RIP kinases
**Abbreviations:**

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 fluoromethyl 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 fluromethyl 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-κB 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 μM 2-mercaptoethanol, 1 mM sodium pyruvate and non-essential amino acid. Necroptosis resistant CT26 cells were survival cells after stimulation with 25 μg/ml polyI:C and 25 μM 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-tublin (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 µl of $1 \times 10^5$ or $5 \times 10^5$ CT26 cells in PBS. Tumor size was measured using caliper. Tumor volume was calculated using the following formula: tumor volume (cm$^3$) = (long diameter) x (short diameter)$^2$ x 0.4. PolyI:C (usually 100 µg) 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 CD8β 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 µl/body) and the same lot of anti-CD8β 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 x $10^4$ cells were plated in 96-well plate. The following day, cells were stimulated with 25 or 50 µg/ml polyI:C, 25 µM zVAD and 50 µM nec-1. After 24 h, 10 µl of WST-1 reagent was added to each well and incubated at 37 °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 x 10⁴) were plated in a 96-well plate. The following day, cells were stimulated with 50 μg/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 x 10⁴) were plated in 96-well plate. The following day, cells were stimulated with 25 μg/ml polyI:C, 25 μg/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 x 10⁵) 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 form CT26-bearing Rag2<sup>−/−</sup>/Jak3<sup>−/−</sup> 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 x 10⁵ CT26 cells were plated in 24-well plates. The following day, cells were stimulated with 25 or 50 μg/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-1β 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.
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-1β 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-1β-converting enzyme)-inhibitory protein (cFILP)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), Mlkl 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-\(\beta\) 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 \(\mu\)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\(\beta\)-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.
Based on these results, we assessed the effect of zVAD on polyI:C-treated, NK/CD8β-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<sup>-/-</sup>/Jak3<sup>-/-</sup> 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, Mlkl 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 cytolysis.
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 RIR1/3-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-1β 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$^+$ DCs without a significant increase of serum cytokines (43). CD8$^+$ 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

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Takemura, H. Takaki, H. Shime,
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References
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 ± 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 ± SD of three independent samples. (D) IFN-γ protein in culture supernatants of CT26 cells or 5 μg/ml concanavalin A (ConA)-stimulated splenocytes was measured by ELISA. N.D., not detected. Data are means ± 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 ± SD of two independent samples. (B) Cells were stimulated with 25 μg/ml polyI:C and 25 μM zVAD for 24 h. Cell viability was measured by WST-1 assay. Data are means ± 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 x 10⁵ CT26 cells and (B) 5 x 10⁵ B16D8 cells were transfected with 2 μg of pCMVRIP3-FLAG and 2 μg of pcDNA4TICAM-1-HA, following stimulation of 25 μM zVAD. Twenty-four hours after transfection, cells were stimulated with 50 μg/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 μg/ml polyI:C and 25 μM zVAD for 24 h and cell viability was measured by WST-1 assay. Data are means ± 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 x 10⁶ CT26 cells or necroptosis resistant CT26 cells were lysed with
lysis buffer, and then analyzed by immunoblot with anti-RIP3 and anti-tublin 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 ± SD of two independent samples. N.D., not detected.

**Figure 6.** Antitumor effect of polyI:C against CT26 cells. 
(A) 1 x 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 ± 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-CD8β following injection i.p. of 100 μg polyI:C. These treatment was repeated on day 14. Results represent means ± 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 x 10^5) were injected i.p. with anti-asialo GM1 and anti-CD8β antibodies and then injected i.p. (C) or s.c. (D) of 100 μg 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 ± 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 x 10^5) were injected with or without i.p. of 100 μg polyI:C and s.c. of 1 mg zVAD at day 12 and 16 after inoculation. Results represent means ± SD of three mice. ** = p<0.01. (B, C) Rag2^−/−^/Jak3^−/−^ mice inoculated with CT26 cells (5 x 10^5) were injected with or without i.p. of 100 μg 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 ± 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 ± SD of three samples. ** = p<0.01. (D) Rag2^−/−^/Jak3^−/−^ mice inoculated with CT26 cells (5 x 10^5) were injected with or without i.p. of 100 μg 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.
Figure 1

A) Cell viability (%)

B) Untreated

C) Untreated

D) Hoechst 33342

E) HMGB1 (ng/ml)

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Figure 2

A

B

C

D

E

F

G
Figure 3
Figure 4
Figure 6

1. **PBS**
2. **Poly I:C 10 μg**
3. **Poly I:C 50 μg**
4. **Poly I:C 250 μg**

- **Days after tumor inoculation**
- **Tumor volume (cm³)**

**C**
- **PBS**
- **Poly I:C (i.p.)**
- **Poly I:C (i.p.) + NK/CD8β depletion**
- **Poly I:C (i.p.) + NK/CD8β depletion + zVAD**

- **Days after tumor inoculation**
- **Tumor volume (cm³)**

**D**
- **PBS**
- **Poly I:C (s.c.)**
- **Poly I:C (s.c.) + NK/CD8β depletion**
- **Poly I:C (s.c.) + NK/CD8β depletion + zVAD**

- **Days after tumor inoculation**
- **Tumor volume (cm³)**

**Legend**
- CT26 bearing mice
- anti-CD8β depletion antibody
- anti-asialo GM1 antibody
- poly I:C 100 μg (C) i.p. or (D) s.c. +/− zVAD 1 mg (s.c.)
- Day 10 and 14
- measure tumor volume

** Statistical analysis:**
- PBS vs. Poly I:C 10 μg, 50 μg, 250 μg
- Poly I:C 10 μg vs. Poly I:C 50 μg, 250 μg
- Poly I:C 50 μg vs. 250 μg
- Poly I:C (i.p.) vs. (s.c.)

* indicates significance at p < 0.05; ** indicates significance at p < 0.01;
N.S. indicates no significant difference.