Targeted Activation of RNA Helicase Retinoic Acid–Inducible Gene-I Induces Proimmunogenic Apoptosis of Human Ovarian Cancer Cells

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Abstract

Most malignant cells are poorly immunogenic and fail to elicit an effective antitumor immune response. In contrast, viral infections of cells are promptly detected and eliminated by the immune system. Viral recognition critically hinges on cytosolic nucleic acid receptors that include the proinflammatory RNA helicase retinoic acid–inducible gene-I (RIG-I). Here, we show that targeted delivery of RIG-I agonists induced ovarian cancer cells to upregulate HLA class I and to secrete the proinflammatory cytokines CXCL10, CCL5, interleukin-6, tumor necrosis factor-\(\alpha\), and IFN-\(\beta\). Ovarian cancer cells stimulated via RIG-I became apoptotic and were readily phagocytosed by monocytes and monocyte-derived dendritic cells, which in turn upregulated HLA class I/II and costimulatory molecules and released CXCL10 and IFN-\(\alpha\). Our findings offer proof of principle that mimicking viral infection in ovarian cancer cells triggers an immunogenic form of tumor cell apoptosis that may enhance immunotherapy of ovarian cancer. Cancer Res; 70(13); 5293–304. ©2010 AACR.

Introduction

Epithelial ovarian cancer (EOC) is the leading cause of death from gynecologic malignancies in developed countries. Radical cytoreductive surgery followed by platinum/taxane-based chemotherapy constitutes the current standard of care. Despite an initial response rate of 65% to 80% to frontline therapy, recurrence arises in the majority of cases, leading to a relative 5-year survival rate of only 47%. This situation warrants new therapeutic strategies aimed at consolidating the initial clinical response. Indeed, currently explored approaches to the management of EOC include neoadjuvant chemotherapy (1), molecular-based treatments, and dose-intense approaches such as intraperitoneal therapy (2). Moreover, given the consistent observation that the presence of tumor-infiltrating lymphocytes (TIL) in EOC patients is correlated with improved survival (3–5), efforts to design effective immunotherapies are under way (6, 7). In this context, the targeted activation of innate immune receptors to induce and promote antitumor immune reactivity to prevent tumor recurrence and progression seems promising (8). In recent years, a range of viruses has been manipulated toward oncolytic function and evaluated for the capacity to selectively induce cancer cell apoptosis and to elicit antitumor immune activity (9). We hypothesized that engaging the same potent antiviral immune mechanisms by delivering noninfectious agonists of the helicase retinoic acid–inducible gene-I (RIG-I) could be similarly effective while avoiding the potential risk of infection of healthy tissue.

Virus detection critically depends on pattern recognition receptors that recognize viral nucleic acids in distinct cellular compartments. In the cytosol, these receptors include the RNA-sensing receptors RIG-I (10) and melanoma differentiation antigen-5 (MDA-5; ref. 11) as well as DNA-identifying receptors, namely, absent in melanoma 2 and other yet unidentified receptors (12, 13). Engagement of these cytosolic nucleic acid receptors not only triggers the release of antiviral and proinflammatory cytokines and chemokines but also leads to the induction of mitochondrial apoptosis in susceptible cell types, including tumor cells, in a process mediated by IRF-3, Apaf-1, and caspase-9, as well as the proapoptotic Bcl-2 family member Noxa (14–16).

In this study, we used short double-stranded RNA (dsRNA) with an uncapped 5′-triphosphate (3pRNA; refs. 17, 18), a direct RIG-I agonist, as well as the synthetic dsDNA polydeoxyadenosine-deoxythymidine [poly(dAdT)], which has been shown to stimulate human cells via RIG-I following a DNA polymerase III–mediated transcription into 5′-triphosphate.
RNA (19, 20). We found that EOC cells stimulated by either RIG-I ligand undergo an immune activating form of cell death. Monocytes and monocyte-derived dendritic cells (MoDC) that engulfed such apoptotic cancer cells matured and secreted proinflammatory chemokines as well as type I IFN. Thus, RIG-I agonists were able to directly destroy EOC cells while, at the same time, enhancing immune reactivity, opening new perspectives for the treatment of EOC.

Materials and Methods

Cell lines
The human EOC cell lines IGROV-1 (21) and SKOV-3 (22) were cultured in cell line medium (see Supplementary Materials and Methods) at 37°C in a humidified 5% CO₂ atmosphere under strictly endotoxin-free conditions. The identity of each cell line was verified by flow cytometry at regular intervals.

Ascites-derived EOC cell cultures
Ascites was collected at the time of surgical treatment from therapy-naive patients with EOC (serous papillary). Cell cultures were established modifying a previously published technique (23). Briefly, cells isolated from ascites were cultured in cell culture medium (see Supplementary Materials and Methods), and on the formation of confluent cell monolayers, cancer cells were enriched using anti–epithelial cell adhesion molecule (EpCAM) microbeads (Miltenyi). A total of five ovarian cancer cell (OVCARE-1 to OVCARE-5) cultures was newly derived for this study. Morphology of cancer cells was visualized using Axio Observer.D1 microscope and AxioVision Rel.4.7 software (Carl Zeiss). The identity of each culture was verified by flow cytometry and immunocytochemistry. The ethics committee of the University of Bonn approved the study protocol, and all patients gave written consent.

Monocytes, MoDCs, and FastDCs
Monocytes were enriched from buffy coat–derived peripheral blood mononuclear cells (PBMC) of healthy blood donors using the monocyte isolation kit II (Miltenyi). A purity of >98% was achieved, as determined by flow cytometry. Monocytes were maintained in monocyte culture medium (see Supplementary Materials and Methods), and on the formation of confluent cell monolayers, cancer cells were enriched using anti–epithelial cell adhesion molecule (EpCAM) microbeads (Miltenyi). A total of five ovarian cancer cell (OVCARE-1 to OVCARE-5) cultures was newly derived for this study. Morphology of cancer cells was visualized using Axio Observer.D1 microscope and AxioVision Rel.4.7 software (Carl Zeiss). The identity of each culture was verified by flow cytometry and immunocytochemistry. The ethics committee of the University of Bonn approved the study protocol, and all patients gave written consent.

Stimulation
The RIG-I ligand 3pRNA was generated by in vitro transcription as described previously (17). Poly(dAdT) (Sigma-Aldrich) was used as RIG-1/cytosolic DNA receptor agonist (13, 19, 20, 25). The synthetic dsRNA polyriboinosinic-polyribocytidylic acid (InvivoGen) was used as MDA-5 agonist (26). Cytosolic stimuli were transfected using FuGENE HD (Roche), TransIT-LT1 (Mirus), or Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. Polyadenosine [poly(A); Sigma-Aldrich] was used as nonstimulatory control for transfection. Toll-like receptor (TLR) ligands were bacterial lipopolysaccharide from Escherichia coli (InvivoGen) for TLR4 (27), R848 for TLR7/8 (28, 29), and the CpG oligodeoxynucleotide M362 (Metabion) for TLR9 (30). The TLR3 agonist polyadenosine-uridine [poly(A-U); ref. 31] was prepared by dissolving equal amounts of poly(A) and polyuridine (Sigma-Aldrich) and heating the mixture to 55°C for 3 minutes. UV irradiation and chemotherapeutic drugs were used at indicated doses (see Supplementary Materials and Methods; 32).

Cell viability and apoptosis assays
Viable cells were quantified using the CellTiter-Blue reagent according to the manufacturer’s instructions (Promega). Apoptosis was assessed by staining with Annexin-V-FLUOS (Roche) in conjunction with propidium iodide or Hoechst 33258 (33). Fragmentation of genomic DNA was evaluated by permeabilizing cells in absolute ethanol and staining with propidium iodide in the presence of DNase-free RNase A (34). Changes in mitochondrial membrane potential (ΔΨm) were assessed using the MitoProbe DiOC2 (3) assay kit according to the manufacturer’s instructions (Invitrogen).

Coculture experiments
Apoptosis was induced in tumor cells by adding ligands of RIG-I for 4 hours. Cells were then washed thoroughly and cocultured with monocytes in a 1:2 ratio or with MoDCs and FastDCs in a 1:1 ratio. The fluorescence-labeled antibody against EpCAM was used to exclude tumor cells from analysis.

Phagocytosis assay
Tumor cells were labeled with the membrane dye PKH26 (Sigma-Aldrich) following the manufacturer’s instructions; 18 hours after induction of apoptosis, tumor cells were cocultured for 4 hours with monocytes, which were then stained for CD14 and analyzed by flow cytometry.

Measurement of cytokines and chemokines
Secretion of IFN-α (Bender MedSystems), IFN-β (Invitrogen), tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-10, and CXCL10 (IP-10; BD Biosciences) was quantified in cell-free supernatants by ELISA according to the manufacturer’s instructions. The detection of CCL5 (RANTES) was performed using the cytometric bead array (CBA; BD Biosciences) following the manufacturer’s protocol.

Flow cytometry
A list of used antibodies is provided in Supplementary Table S1. Data were obtained on a LSR II flow cytometer (BD Biosciences) evaluating at least 20,000 events per sample by gating on Hoechst 33258–negative cells after excluding doublets. Analysis was performed by FlowJo software (TreeStar).

Immunoblot analysis
Cell lysates were immunoblotted following standard protocols. A list of used antibodies is provided in Supplementary Table S2.
Table S3. Analysis was performed using the Odyssey IR imaging system (LI-COR).

RNA extraction and quantification
Total RNA was extracted from cells using Trizol as described by the manufacturer (Invitrogen). RNA (1 μg) was digested with 2 units of DNase I, and cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase and oligo(dT)18 primer according to the manufacturer’s recommendations (Fermentas). Quantitative PCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) together with SYBR Green qPCR Master Mix (Fermentas). GAPDH was used as housekeeping gene. PCR products were verified by melting curve analysis, and relative quantification was done with SDS Software Rev. 2.3 (Applied Biosystems). A list of used primers is provided in Supplementary Table S4.

Immunocytochemistry
A Shandon cytopsin 4 cytocrsentrifuge (Thermo Fisher Scientific) transferred cells to microscope slides. Staining was performed on an automated staining system (TechMate 500; Dako) using the streptavidin-biotin complex method. Antibodies are summed up in Supplementary Table S2. Immunostained cells were analyzed with an IX71 microscope and CellF software (Olympus).

Statistical analysis
Values are given as mean ± SE. The F test was used to check the assumption of equal variances; the unpaired one-tailed Student’s t test or the Mann-Whitney U test was used to determine statistical significance of differences. Results with a P value of ≤0.05 were considered to be significant.

Results

EOC cell lines are sensitive to RIG-I–triggered apoptosis
Induction of apoptosis is not only a common feature of anticancer agents but also a key element in the cell-autonomous responses to virus infection. Engagement of innate immune receptors has been shown to play a pivotal role in transmitting proapoptotic signals after viral stimulation (14–16). Our objective was therefore to determine whether synthetic agonists of RIG-I were able to induce cytopathic cell death in EOC cells. We monitored two EOC cell lines (IGROV-1 and SKOV-3) for cell viability following the transfection with a direct and an indirect agonist of RIG-I, namely, 3pRNA (17, 18) and poly(dAdT) that is transcribed intracellularly into RIG-I agonistic RNA (19, 20). We found that both direct and indirect RIG-I ligands induced tumor cell death in a dose-dependent manner (Fig. 1A). To test whether the decrease in cell survival was due to apoptosis, EOC cell lines were assayed by flow cytometry using Annexin V and propidium iodide, which revealed a pronounced externalization of phosphatidylserine in response to RIG-I agonists (Fig. 1B). In addition, we measured the hypodiploid DNA peak indicative for late-stage apoptosis. In accordance with above results all RIG-I ligands induced significant apoptotic DNA fragmentation [3pRNA, P < 0.04; poly(dAdT), P < 0.009; Fig. 1C and D]. We obtained similar data for both EOC cell lines (Fig. 1C). In contrast, none of the TLR agonists tested were able to induce apoptosis of EOC cells (Fig. 1D).

Malignant ascites contains primary EOC cells as well as tumor-associated immune cells
A common pathway of tumor spread in advanced EOC is direct dissemination into the peritoneal cavity. Triggered by this intra-peritoneal carcinomatosis, substantial populations of immune cells migrate to the peritoneal cavity. The cellular content of ascitic fluid is therefore considered a model for the tumor microenvironment. We analyzed the cellular composition of ascites in previously untreated EOC by flow cytometry. In all patient samples tested, CD45+ immune cells constituted the largest cellular population, which indicates tumor-dependent immune activation; the remaining CD45-negative cells consisted of CD90+ mesothelial cells/fibroblasts and EpCAM+ tumor cells that stained positive for the tumor markers folate receptor α (FRA) and/or CA125 (Supplementary Fig. S1A). Within the immune infiltrate, the major population was of the T-cell lineage with a mean CD4+ to CD8 ratio of 1.9 (range, 0.59 – 6.11); another significant immune cell population comprised CD14+HLA-DR+ monocytes, important effectors of innate immunity (Supplementary Fig. S1B). Thus, the presence of tumor-associated immune cells is indicative of an immune response provoked by the presence of the tumor but also provides the rationale for a therapeutic approach that aims to boost the immune system to target and destroy cancer cells within this milieu.

Because indefinitely passaged cell lines as the ones used in Fig. 1 can produce results critically different from effects obtained with primary cancer specimens, we next fractionated ascites of therapy-naive patients to derive EOC cell cultures. Tumor cells generated from five individual patients grew in culture with a uniform cobblestone pattern indicative of their epithelial origin (Supplementary Fig. S1C). Accordingly, cells stained positive for epithelial antigen and EpCAM (Supplementary Fig. S1D and E). To confirm their identity as cancer cells, we repeatedly tested up to passage 56 and consistently found that >99% of the cells in culture expressed tumor antigens FRA and CA125 at high levels (Supplementary Fig. S1D and E). Moreover, homogeneous expression of these cell surface markers indicated the absence of contaminating cell types (Supplementary Fig. S1E).

Ascites-derived EOC cells are sensitive to RIG-I–triggered apoptosis
Using ascites-derived EOC cell cultures, we next evaluated the proapoptotic effects of RIG-I ligand delivery. Consistent with results in cell lines, tumor cells derived directly from patient samples underwent apoptosis on engagement of RIG-I (Fig. 2A). Moreover, the doses required for apoptosis induction in ascites-derived EOC cell cultures were similar to those used in cell lines. A significant increase of early apoptotic populations (Annexin V positive/Hoechst 33258 negative) was observed with both direct and indirect RIG-I
Figure 1. Engagement of RIG-I induces apoptosis in EOC cell lines. IGROV-1 and SKOV-3 cell lines showed reduced viability and hallmarks of early- and late-stage apoptosis 24 h after treatment with RIG-I agonists 3pRNA and poly(dAdT) but not after treatment with TLR ligands. A, cell viability of SKOV-3 cells was assessed by fluorescence using the CellTiter-Blue reagent. Average fluorescence values of the culture medium background are subtracted. B, Annexin V–positive and propidium iodide–negative IGROV-1 cells were quantified by flow cytometry. C, to assess the hypodiploid DNA peak, fixed EOC cells were permeabilized, stained with propidium iodide, and analyzed by flow cytometry. D, the percentage of apoptotic IGROV-1 cells was quantified. Columns, mean; bars, SE. *, \( P \leq 0.05 \). Data shown in A to D are representative of at least three independent experiments.
Figure 2. Engagement of RIG-I induces apoptosis in ascites-derived EOC cells. Five independent primary EOC cultures (OVCACE-1 to OVCACE-5) were derived from malignant ascites and showed characteristics of early- and late-stage apoptosis 24 h after treatment with RIG-I agonists 3pRNA and poly(dAdT) but not after treatment with TLR ligands. A, Annexin V–positive and Hoechst 33258–negative OVCACE-1 cells were quantified by flow cytometry. B, dose-dependent apoptosis of EOC cells was quantified as in A (cumulative data obtained independently with OVCACE-2 and OVCACE-3). Columns, mean; bars, SE. *, \( P \leq 0.05 \). C, late-stage apoptosis was assessed by flow cytometry via the hypodiploid DNA content of fixed and permeabilized EOC cells stained with propidium iodide. D, the percentage of apoptotic EOC cells was quantified as in C (cumulative data obtained independently with OVCACE-1, OVCACE-2, and OVCACE-4). Columns, mean; bars, SE. *, \( P \leq 0.05 \). Data shown in A to D are representative of at least eight independent experiments.
agonists in a dose-dependent manner [3pRNA, \( P < 0.002 \); poly (dAdT), \( P < 0.01 \); Fig. 2B]. We further analyzed the degree of late-stage apoptosis and showed that treatment with each RIG-I agonist significantly increased the frequency of cells with DNA fragmentation [3pRNA, \( P < 0.03 \); poly(dAdT), \( P < 0.002 \); Fig. 2C and D]. This effect was consistent in all patient-derived EOC cell cultures (Fig. 2C). In accordance with published results of apoptosis after activation of nucleic acid receptors (14–16), we observed upregulation of Noxa (Supplementary Fig. S2A) and monitored disruption of \( \Delta \Psi \text{m} \) at an early stage in RIG-I–triggered EOC cells, which is associated with the intrinsic apoptotic pathway [8 h: 3pRNA, \( P < 0.02 \); poly(dAdT), \( P < 0.05 \); 11 h: 3pRNA, \( P < 0.008 \); poly(dAdT), \( P < 0.05 \); Supplementary Fig. S2B]. Moreover, cleaved caspase-9 and poly(ADP-ribose) polymerase were evident in lysates of RIG-I agonist–treated EOC cells (Supplementary Fig. S2C). In comparison, EOC cells were also subjected to TLR4, TLR7, and TLR9 ligands, which did not result in an increase of apoptosis (Fig. 2D). Finally, we evaluated the proapoptotic function of poly(A), which was shown to trigger cell death in human breast cancer cells via TLR3 (35, 36) and was able to promote antitumor immune reactivity in mouse tumor models (36). However, up to concentrations of 100 mg/mL, poly(A) did not exert proapoptotic function in IGROV-1 and patient-derived EOC cells (Supplementary Fig. S3A and B).

**EOC cells upregulate HLA class I molecules and secrete proinflammatory cytokines and chemokines in response to RIG-I agonists**

We characterized immunogenic properties of RIG-I–stimulated cancer cells by determining expression status of HLA molecules. Constitutive expression of HLA class I antigens on EOC cells was found to be on a low level and only modestly upregulated after treatment with poly(A); stimulation via RIG-I, however, significantly enhanced the expression of HLA class I antigens in a dose-dependent manner [3pRNA, \( P < 0.05 \); poly(dAdT), \( P < 0.05 \); Fig. 3A]. We next evaluated whether treatment with RIG-I agonists initiated EOC cells to secrete cytokines and chemokines to modulate tumor-specific immune responses. We found that RIG-I ligand-treated EOC cells released significant amounts of IFN-\( \beta \) [3pRNA, \( P = 0.05 \); poly(dAdT), \( P = 0.05 \)], CXCL10 [3pRNA, \( P < 0.002 \); poly(dAdT), \( P < 0.04 \)], CCL5 [3pRNA, \( P < 0.006 \); poly(dAdT), \( P < 0.0003 \)], IL-6 [3pRNA, \( P < 0.002 \); poly(dAdT), \( P < 0.0003 \)], and TNF-\( \alpha \) [3pRNA, \( P < 0.002 \); poly(dAdT), \( P < 0.0003 \)] in a dose-dependent manner (Fig. 3B and C). In contrast, IFN-\( \alpha \) release by RIG-I–stimulated EOC cells was never detected (data not shown).

**Monocytes exposed to RIG-I agonist–induced apoptotic EOC cells become activated and secrete proinflammatory cytokines and chemokines**

Monocytes were the dominant phagocytic cell type attracted into malignant ascites (Supplementary Fig. S1B). In coculture experiments, we therefore asked whether monocytes reacted to an exposure to RIG-I agonist–induced apoptotic EOC cells and analyzed monocyte expression of HLA molecules and activation markers. We found that contact to RIG-I–triggered apoptotic EOC cells augmented HLA class I and II expression on monocytes and upregulated surface levels of CD69 and CD86 (Supplementary Fig. S4A). Further, we characterized the cytokine and chemokine response of monocytes exposed to apoptotic tumor cells. Incubation of monocytes with RIG-I–stimulated EOC cells resulted in significantly higher levels of CXCL10 [coculture with poly(A)-treated tumor cells [3pRNA, \( P < 0.04 \); poly(dAdT), \( P < 0.0006 \)], directly treated monocytes [3pRNA, \( P < 0.02 \); poly(dAdT), \( P < 0.002 \)], and directly treated tumor cells [poly(dAdT), \( P < 0.004 \); Fig. 4A] and IFN-\( \alpha \) [coculture with poly(A)-treated tumor cells [3pRNA, \( P < 0.004 \); poly(dAdT), \( P < 0.0009 \); Fig. 4B]. Increase of CXCL10 concentration following incubation with 3pRNA-induced apoptotic cells was clear; however, it did not reach significance when contrasted to directly treated tumor cells (\( P = 0.35 \)). Importantly, the low chemokine secretion by monocytes stimulated directly was not due to monocyte apoptosis because RIG-I agonist–treated monocytes showed very low percentages of Annexin V–positive or DNA-laddered cells (Supplementary Fig. S5A; data not shown) compared with apoptosis in ascites-derived EOC cells [3pRNA, \( P < 0.05 \); poly(dAdT), \( P < 0.002 \); Supplementary Fig. S5B]. We further observed that in the presence of apoptotic EOC cells, monocytes secreted IL-10, a major mediator of immunosuppression in the tumor microenvironment. We found, however, IL-10 secretion was significantly decreased when monocytes were cocultured with RIG-I agonist–induced apoptotic tumor cells [3pRNA, \( P < 0.005 \); poly(dAdT), \( P < 0.002 \); Supplementary Fig. S4B].

In contrast to these findings, most forms of apoptosis are thought to lack the inflammatory signals required to induce activation of antigen-presenting cells (APC). However, anthracycline chemotherapy agents such as doxorubicin have previously been shown to induce an immunogenic form of cell death that involves cell surface exposure of calretulin by tumor cells (37). We therefore compared monocyte activation following the ingestion of RIG-I–triggered and doxorubicin-induced apoptotic tumor cells. Treatment with either doxorubicin or RIG-I agonists induced apoptosis of EOC cells (data not shown). At the doses used, both types of tumor cell death inducers similarly enhanced the phagocytic uptake of apoptotic material by monocytes [3pRNA, \( P < 0.02 \); poly(dAdT), \( P < 0.04 \); doxorubicin, \( P < 0.02 \); Fig. 5A and B]. Our results suggest that fluorescence was acquired by phagocytosis rather than by mere surface binding of apoptotic cells, as doublet events were excluded, the acquired fluorescence of monocytes always remained lower than that of tumor cells alone, and the process was considerably inhibited at 4°C, a temperature that blocks engulfment but not surface adhesion (Fig. 5A and B; data not shown). Despite the similar characteristics of monocyte uptake, surface expression of calretulin on EOC cells was provoked by doxorubicin treatment (\( P < 0.002 \)) but not by RIG-I stimulation (Supplementary Fig. S6). Conversely, exclusively the engulfment of
Figure 3. Engagement of RIG-I induces EOC cells to enhance immunogenic properties. A, surface expression of HLA class I was determined by flow cytometry 48 h after stimulation with RIG-I ligands. Cumulative data obtained independently with OVCACE-1, OVCACE-2, OVCACE-3, and OVCACE-4 are given as geometric mean fluorescence intensity (MFI) values. Columns, mean; bars, SE. *, $P \leq 0.05$. B, secretion of IFN-β was quantified in the supernatant by ELISA 18 h after EOC cell stimulation (cumulative data obtained independently with OVCACE-1, IGROV-1, and SKOV-3). Columns, mean; bars, SE. *, $P \leq 0.05$. C, secretion of CXCL10, CCL5, IL-6, and TNF-α was quantified in the supernatant by ELISA or CBA 18 h after EOC cell stimulation (cumulative data obtained independently with OVCACE-1 and OVCACE-4). Columns, mean; bars, SE. *, $P \leq 0.05$. Data shown in A to C are representative of at least three independent experiments.
RIG-I–triggered apoptotic tumor cells induced monocytes to release CXCL10 [3pRNA, \( P < 0.05 \); poly(dAdT), \( P < 0.03 \)] and IFN-\( \alpha \) [3pRNA, \( P < 0.02 \); Fig. 5C]. We also observed a clear increase in the secretion of IFN-\( \alpha \) following the incubation with poly(dAdT)-mediated apoptotic tumor cells that did not reach significance (\( P = 0.068 \)).

**Exposure to RIG-I agonist–induced apoptotic EOC cells promotes the differentiation and maturation of DCs from monocytes**

DCs play a critical role in initiating adaptive immune responses, including antitumor immune reactivity (38). Under inflammatory conditions in vivo, monocytes are able to...
rapidly differentiate into DCs. This process is poorly mirrored in the protocols commonly used for DC derivation from monocytes in vitro. We therefore evaluated the influence of RIG-I–stimulated apoptotic tumor cells on monocytes undergoing accelerated transformation into DCs (FastDCs; ref. 24). We found that even short-time exposure of monocytes to tumor cells undergoing RIG-I–induced apoptosis showed upregulation of HLA class I and II, as well as of CD86 (Supplementary Fig. S7A). Direct engagement of RIG-I has been shown to lead to cytokine secretion [3pRNA, \( P < 0.0001 \); poly(dAdT), \( P < 0.002 \)] and maturation of immature MoDCs (Supplementary Fig. S8A; refs. 18, 19). We next evaluated whether RIG-I–stimulated apoptotic tumor cells were also able to promote the functional maturation of immature MoDCs. We found that coculture with RIG-I agonist–triggered apoptotic tumor cells significantly enhanced DC surface expression of HLA class II molecules [3pRNA, \( P < 0.02 \); poly(dAdT), \( P < 0.006 \)], the DC maturation marker CD83 [3pRNA, \( P < 0.03 \); poly(dAdT), \( P = 0.05 \)], as well as the costimulatory molecules CD86 [3pRNA, \( P < 0.002 \); poly(dAdT), \( P < 0.02 \)] and CD80 [poly(dAdT), \( P < 0.05 \); Fig. 6A]. We also noticed a clear increase in the upregulation of CD80 following the incubation with 3pRNA-mediated apoptotic tumor cells that did not reach

Figure 5. Monocytes secrete CXCL10 and IFN-\( \alpha \) upon exposure to RIG-I–induced but not in response to doxorubicin-induced apoptotic EOC cells. A, monocytes were cocultured with apoptotic PKH26-labeled OVCACE-4 cells, subsequently stained, and analyzed by flow cytometry. Control experiments with unstimulated EOC cells were performed at 4°C and 37°C. Double-positive cells indicate the uptake of apoptotic tumor cells by monocytes. B, phagocytosis of apoptotic OVCACE-4 cells was quantified as in A (cumulative data obtained with two different donors; geometric MFI values). Columns, mean; bars, SE. *, \( P \leq 0.05 \). C, monocytes were exposed to apoptotic OVCACE-3 cells. Control experiments included untreated EOC cells and monocytes, respectively. After 18 h, secretion of CXCL10 and IFN-\( \alpha \) was quantified in the supernatant by ELISA. Columns, mean; bars, SE. *, \( P \leq 0.05 \). Data shown in A and B are representative of at least two independent experiments.

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Figure 6. Exposure to RIG-I–stimulated apoptotic tumor cells initiates the maturation of MoDCs. A, immature MoDCs were incubated with RIG-I–stimulated apoptotic EOC cells. Cell surface expression of HLA class II, CD83, CD86, and CD80 was analyzed by flow cytometry. Data are given as geometric MFI values (cumulative data obtained independently with OVCACE-3 and IGROV-1). Columns, mean; bars, SE. *, P ≤ 0.05. B, immature MoDCs were cocultured with RIG-I–triggered apoptotic OVCACE-2 cells. Control experiments included untreated EOC cells and immature MoDCs. After 18 h, secretion of IFN-α was quantified in the supernatant by ELISA. Columns, mean; bars, SE. *, P ≤ 0.05. Data shown in A and B are representative of at least five independent experiments.
significance ($P = 0.33$). Similar to our observation in monocytes, incubation with RIG-I–stimulated apoptotic EOC cells, but not with doxorubicin- or UV-induced apoptotic EOC cells, induced IFN-α production in MoDCs [3pRNA, $P < 0.04$; poly (dAdT), $P < 0.02$; Fig. 6B]. MoDC upregulation of the maturation markers CD86 and CD83 was also most prominent in cocultures with RIG-I–stimulated apoptotic EOC cells compared with cultures with doxorubicin- or UV-induced apoptosis (Supplementary Fig. S9).

Discussion
We have shown that the cytosolic innate immune receptor RIG-I can effectively be engaged in EOC cells to promote tumor apoptosis. Whereas most forms of apoptosis are considered “immunologically silent” and tolerogenic (39), cell death initiated by RIG-I agonists was highly immunostimulatory with apoptotic tumor cells not only secreting proinflammatory cytokines and chemokines but also inducing functional maturation and type I IFN secretion of APCs. By this approach, synergistically interlocking mechanisms of both cell-autonomous and immune cell–mediated antitumor reactivity are engaged.

Many immune and nonimmune cell types are able to sense RNA viral presence in the cytosol via RIG-I. We found that both established EOC cell lines and patient-derived EOC cell cultures were similarly susceptible to RIG-I–induced apoptosis, indicating that during malignant transformation this pathway remains functional and amenable to therapeutic intervention. Our data on RIG-I–triggered cell death in EOC cells are in accordance with recent studies describing proapoptotic activity of MDA-5 and RIG-I engagement in human and mouse melanoma cell lines (14, 16, 40). Yet, whereas melanomas evade immune detection and are characteristically devoid of immune cell infiltrates, EOC elicits cytotoxic immune reactivity, and tumor-infiltrating CD8 T lymphocytes are associated with improved survival (3–5). Thus, induction of immunogenic apoptosis is particularly plausible in EOC to shift the balance away from tumor-induced immune suppression toward enhancement of cytotoxic antitumor immune responses. Accordingly, although downregulation of HLA class I expression is a common feature in EOC and correlated with a poor prognostic outcome (41), we show that RIG-I ligation induced upregulation of HLA class I. RIG-I–engaged EOC cells additionally secreted proinflammatory mediators. In particular, the release of CXCL10 attracting TIL has been reported to be associated with an improved overall clinical outcome in EOC (3).

Consequently, EOC cell apoptosis triggered by pathways of virus detection is able to strongly activate human phagocytic cells that then secrete IFN-α, whose pleiotropic antiviral effects promote antitumor activity (42). Moreover, monocytes that ingested RIG-I–induced apoptotic tumor cells showed decreased secretion of IL-10, a major contributor of immunosuppression in tumor microenvironment that was shown to be induced by the mere presence of EOC cells (43). Whether monocytes and DCs matured in this fashion, also present tumor antigens, and effectively expand cytotoxic TIL will be the subject of future study.

Together, our data provide a framework for clinical studies to evaluate the potential of RIG-I agonists as immunotherapeutic agents for EOC. Because selected chemotherapeutic drugs are similarly able to cause immunogenic tumor cell apoptosis in mouse models, interest has renewed in harnessing this desirable form of tumor cell death for immunotherapy (44). The usually good initial response to surgery and combination chemotherapy in EOC points toward a window of opportunity for immunotherapeutic intervention, in which residual tumor burden and tumor-induced immune suppression are minimal. A recent study showed that cytoreductive surgery of the primary tumor relieves immune suppression and decreases the frequency of circulating regulatory T cells (45). Moreover, in EOC patients who have recovered from chemotherapy, antitumor cytotoxic T cells can be restimulated in vitro comparably well as in healthy volunteers (46). Based on our findings, clinical trials evaluating efficacy of RIG-I agonists may consider intra-peritoneal application. However, it is unclear whether apoptosis-inducing concentrations of RIG-I–stimulatory nucleic acids can safely be achieved in few residual EOC cells in vivo. An alternative approach we favor for future development is to generate an autologous DC vaccine, loaded in vitro with RIG-I–induced apoptotic EOC cells derived from the patient’s ascites.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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