Cytotoxicity of Human Endogenous Retrovirus K–Specific T Cells toward Autologous Ovarian Cancer Cells

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Abstract

Purpose: To determine whether HERV-K envelope (ENV) protein could function as a tumor-associated antigen and elicit specific T-cell responses against autologous ovarian cancer cells.

Experimental Design: The expression of HERV-K transcripts and ENV protein, the presence of serum antibodies against HERV-K, reverse transcriptase (RT) activities, and cellular immune responses in primary ovarian cancer tissues and patient blood samples were analyzed and compared with samples from patients with benign ovarian diseases and normal female donors.

Results: Ovarian cancer cells in primary tumors and ascites expressed markers of cancer stem cells and markers of both mesenchymal and epithelial cells. Expression of HERV transcripts and HERV-K ENV protein and reverse transcriptase activities were higher in ovarian cancer compared with adjacent normal and benign tissues. The ovarian cancer patient plasma also had high reverse transcriptase activities and the ovarian cancer patient sera contained HERV-K immunoreactive antibodies. HERV-K–specific T cells generated from autologous dendritic cells pulsed with HERV-K ENV antigens exhibited phenotypes and functions consistent with a cellular immune response including T-cell proliferation, IFNγ production, and HERV–K–specific cytotoxic T lymphocyte (CTL) activity. Significantly higher CTL lysis of autologous tumor cells than of uninvolved normal cells was demonstrated in patients with ovarian cancer than patients with benign diseases and further enhanced lysis was observed if T regulatory cells were depleted.

Conclusion: Endogenous retroviral gene products in ovarian cancer may represent a potentially valuable new pool of tumor-associated antigens for targeting of therapeutic vaccines to ovarian cancer. Clin Cancer Res; 21(2); 471–83. © 2014 AACR.

Introduction

Ovarian cancer is the most common cause of death from gynecologic malignancies and advanced ovarian cancer remains highly lethal with >90% of patients developing tumor recurrence, resulting in 5-year survival rates of only 30% (1). Currently, there is no test with sufficient predictive value for use in screening and detection of premalignant or localized ovarian cancer, and 70% of patients with ovarian cancer present with advanced disseminated disease at the time of initial diagnosis (2). Therefore, identification of biomarkers for ovarian cancer detection, diagnosis, and immunotherapy is imperative for improving the survival of patients with ovarian cancer.

Human endogenous retroviruses (HERVs) comprise approximately 8% of the human genome and are believed to have germline-integrated their DNA into the human genome over 30 million years ago. HERVs exist as integrated retroviral DNA genomes called proviruses that inevitably accumulate mutations over time, disrupting viral genomic components and inactivating viral activity. The most biologically active HERVs are members of the HERV-K superfamily, which are transcriptionally active and encode proteins that retain functionality in several human cancers (3, 4). The expression, promoter activity, and epigenetic regulation of HERV-Ks seem to be quite different between malignant and normal cells. However, the specific role that HERV-K proteins play in cancer remains an enigma. The envelope and additional proteins of several HERVs may contribute to the development of cancer via their fusogenic properties, and the accessory proteins REC and NP9, alternative splicing products of the HERV-K (HML2) ENV gene, have been shown to play oncogenic functions via interacting with other proteins (5–7).

The central premise of successful ovarian cancer immunotherapy is that tumor cells express specific antigens that can be recognized by cytolytic T cells leading to tumor destruction (8, 9). T-cell infiltration into ovarian tumors is associated with improved survival. In tumors with high numbers of tumor-infiltrating T cells, the expression of monokines induced by IFNγ,
Translational Relevance

In this study, we provide evidence that the human endogenous retrovirus K (HERV-K) envelope (ENV) protein may function as a tumor-associated antigen useful for ovarian cancer detection, diagnosis, and immunotherapy. Our findings suggest anti-HERV-K plasma antibodies and HERV-K reverse transcriptase activity as two potential novel biomarkers for ovarian cancer detection and diagnosis using readily available body fluids and a minimally invasive procedure. From a clinical standpoint, the availability of new biomarkers to detect ovarian cancer at an early stage is critically important, because frequently this cancer is not detected until advanced stages when treatment options are limited. In addition, HERV-K-specific T cells induced cellular immune responses towards autologous ovarian cancer cells, providing evidence that the reactivated endogenous retroviral gene products represent a potentially valuable new pool of tumor-associated antigens with great specificity for targeting of therapeutic vaccines to ovarian cancer.

Macrophage-derived chemokines, and secondary lymphoid tissue chemokines are significant markers as compared with tumors lacking T cells (10, 11). Strong positive correlations have also been observed between levels of CD8<sup>+</sup> T cells and granzyme B within ovarian tumors, indicating that the majority of CD8<sup>+</sup> T cells are cytotoxic (12). Ovarian cancer s are immunogenic (13), suggesting that immunotherapy strategies utilizing these cytotoxic T cells might prove effective if an appropriate tumor-associated antigen (TAA) could be identified. However, a major obstacle in the current development of ovarian cancer vaccines is the lack of clearly defined TAs that are capable of being recognized by T cells (i.e., T-cell epitopes).

Overcoming suppressive mechanisms in the tumor microenvironment to enhance efficacy is another major challenge for current approaches to ovarian cancer vaccination. Various studies have shown that ovarian cancers escape immune surveillance with high efficiency via creating a tolerogenic microenvironment (14). The tumor microenvironment mediates induction of an immunosuppressive programme (15). The tumor microenvironmentmediates induction of an immunosuppressive programme cell death (PD-1) pathway, with PD-1 functioning as an inhibitory surface receptor expressed by T cells, B cells, natural killer T cells, monocytes, and dendritic cells (DC). Tumors can exploit the PD-1 inhibitory pathway to silence the immune system (15), and expression of this molecule is inversely correlated with survival of patients with ovarian cancer (16). Along with myeloid cells, regulatory T cells (Tregs) within the tumor microenvironment are a crucial component of the tumor immunosuppressive network. These cells are a heterogeneous CD4<sup>+</sup> T-cell subpopulation that can be divided into two subsets: naturally occurring Tregs with CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> phenotype and induced Tregs with variable CD25 expression (17). Tregs depend on PD-1, PD-L1 (programmed cell death ligand 1), or CTL-associated antigen 4 (CTLA-4) to carry out antitumor immune responses (18, 19). Indeed, the presence of Tregs in ovarian tumors has been associated with reduced overall survival (20–22). Specifically, CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> Treg cells correspond to poor clinical outcome in epithelial ovarian cancer, and these cells are preferentially concentrated in the tumor mass rather than in tumor draining lymph nodes (20). In addition, the therapeutic effect of blocking the PD-1/PD-L1 pathway was shown to correlate with increased numbers of polyfunctional ovarian tumor antigen–specific CD8<sup>+</sup> T cells (23). These data suggest that strategies aimed at Treg depletion used in combination with vaccine stimulation of effector T cells could lead to more effective treatments for ovarian cancer.

We have previously demonstrated that patients with breast cancer, but not normal female donors, can mount immune responses against expressed human endogenous retroviral elements using autologous DCs pulsed with the HERV-K ENV surface antigens (24). These findings provide strong evidence that retroviral gene products are capable of acting as TAs to activate both T-cell and B-cell responses. In addition to breast cancer, we were the first to report the expression of multiple HERV gene and protein products in ovarian cancer cell lines and tissues (25), suggesting that HERVs might also be able to serve as TAs to enable targeting of ovarian cancer. In our current study, we utilize a new patient cohort to identify targets or biomarkers that could potentially be used as diagnostic or prognostic tools in ovarian cancer or as new immunotherapeutic targets for the disease. Most important, we explore the development of a cancer vaccine for ovarian cancer based on HERV-K viral ENV surface protein functioning as a TAA, using patient cells cultured from matched blood, freshly resected tumors, adjacent uninvolved tissues, and benign tissues.

Materials and Methods

The basic procedures for many experiments in this study have been described previously in refs. (24–29) and in Supplementary Information.

Clinical samples and cell lines

Tissue and/or blood samples from patients with ovarian cancer and patients with benign diseases were obtained from the MD Anderson Cancer Center (Houston, TX) according to approved Institutional Review Board protocols (LAB04-0083) under informed consent. Clinicopathologic characteristics are listed in Supplementary Table S1. Normal donor blood samples (Supplementary Table S2) were obtained from Gulf Coast Regional Blood Center (Houston, Texas). Ovarian cancer cell lines SKOV3, DOV13, and OVCAR3, and the immortalized human ovarian epithelial cell line, T29, were gifts from Dr. Robert C. Bast Jr. (University of Texas M.D. Anderson Cancer Center, Houston, TX).

Harvesting of primary samples and preparation of tumor cells

A piece of tissue in 1 to 2 mL of cold complete NOE medium (250 mL of Medium 199 + 250 mL DMEM) supplemented with 10% FBS, 1% penicillin–streptomycin, and 1% Glutamax was minced into small pieces. The tissue pieces and dissociated cells were pelleted and resuspended in Accumax solution at a concentration of 0.5 g/10 mL of tissue and incubated at room temperature for 60 minutes on a shaker. The cells were transferred to a Stomacher 80 bag that was inserted into the Stomacher (Seward Co.) and run for 15 minutes at high speed. The cells were then passed through a 40 µm nylon mesh and separated from dead cells, debris, and red blood cells on a Ficoll-1077 gradient. The cells were spun at 900 rpm for 5 minutes, resuspended in low serum NOE media, and plated in a 6-well plate. To culture tumor spheres, 6-well plates were coated with 1 mL of 0.8% agarose (30), and cells were added in 2 mL per well of Mammocult media.

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supplemented with heparin and hydrocortisone. To culture ascites, the ascites were pelleted and live cells were separated from dead cells, debris, and red blood cells on a Ficoll-1077 gradient. Cells were pelleted, resuspended in low serum NOE media, and plated in a 6-well plate.

**Measuring reverse transcriptase activity in tumor tissues and plasma**

Patient plasma was isolated using Histopaque 1077 and separated (10 ml) on isopycnic gradients using OptiPrep (Iodixanol, AxisShield) as per the manufacturer's instructions. Primary ovarian tissues were also collected during surgery and frozen at −80°C until processed for reverse transcriptase assay. Tissue samples were crushed in culture media using a mortar and pestle. Five microliter aliquots of fractionated plasma or tissue samples were analyzed for reverse transcriptase activity using the EnzChek Reverse Transcriptase Assay Kit (Invitrogen, Life Technologies). The EnzChek reverse transcriptase assay kit uses a PicoGreen dsDNA quantitation reagent that preferentially detects dsDNA or RNA-DNA heteroduplexes over single-stranded nucleic acids or free nucleotides. The reverse transcriptase activity in a biologic sample generates long RNA–DNA heteroduplexes from a mixture of a long poly(A) template, an oligo-dT primer, and dTTP. The RNA–DNA heteroduplexes formed are then detected by the PicoGreen reagent. A reverse transcriptase standard curve was generated using serial dilutions of Moloney Murine Leukemia Virus Reverse Transcriptase standard curve was generated using serial dilutions of Moloney Murine Leukemia Virus Reverse Transcriptase.

**Preparation of dendritic cells and generation of in vitro stimulated cells**

The basic procedure was depicted in Supplementary Fig. S1. Briefly, after peripheral blood mononuclear cells (PBMC) were plated and incubated for 16 hours at 37°C, floating PBMCs were rinsed off and saved for future experiments, whereas adherent cells were incubated for 6 days with granulocyte macrophage colony-stimulating factor (GM-CSF) and IL4 (1,000 U/ml; R&D Systems). The immature DCs were harvested and transfected with HERV-K ENV or HPV16 E6 cRNAs using FuGENE Transfection reagent per the manufacturer's instructions (Promega). Alternatively, cells were transfected with HERV-K ENV (KSU) or E6 fusion proteins, or keyhole limpet hemocyanin (KLH) protein using the BioPORTER lipid-based transfection reagent (Genlantis). Four hours after transfection, TNFα (1,000 U/ml) was added for an additional 16 hours at 37°C to induce DC maturation. To generate in vitro stimulated (IVS) cells, autologous PBMCs were added to DC at a ratio of 30 to 1 on day 0. Cultures were incubated in the presence of IL2 (10 U/ml) for 7 days to generate 1-week IVS cells, as described previously (24).

**T-cell proliferation, cytokine production, and CTL assays**

T-cell proliferation was evaluated by a 3H-thymidine incorporation assay. Briefly, T-cell proliferation was evaluated in patient PBMC or IVS cells by restimulation for 72 hours with DCs pulsed with no added protein, K-SU protein, or KLH control protein, at a DC to PBMC or IVS ratio of 1:30. The remaining cells were pulsed with 1 μCi/well of [3H]-thymidine and incubated for another 18 hours at 37°C. Cells were then harvested onto filter papers, transferred to scintillation vials with scintillation fluid, and analyzed on a beta counter.

Cytokine production was measured using an IFN-γ enzyme-linked immunosorbent assay (ELISPOT) assay (BD Biosciences). Briefly, ELISPOT plates were coated with 10 μg/ml of purified anti-human IFN-γ capture antibody and incubated for 24 hours at 4°C. Plates were then blocked for 2 hours with complete media and PBMC or IVS cells were plated at 1 × 10^{5} per well with DCs pulsed with various HERV-K antigens plated at 5 × 10^{4} per well. Plates were incubated for 24 hours at 37°C and then washed and incubated with the detection antibodies for 2 hours at 25°C. Plates were washed and incubated with streptavidin horseradish peroxidase for 2 hours at 25°C. Plates were washed again and developed by adding 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) substrate solution. Plates were washed, allowed to dry, and spots were counted using an ELISPOT reader (C.T.L. Technologies).

CTL assays were performed in round-bottom 96-well plates using a 4-hour 51Cr-release assay. Briefly, various target (primary tumor, adjacent normal tissue, or benign) cells were "pulsed" with K-SU or KLH control protein for 16 hours at 37°C using the BioPORTER transfection reagent. Target cells were removed from flask via EDTA buffer, pelleted, and resuspended in 500 μl media and 75 μCi of chromium. A total of 5 × 10^{4}/mL target cells and 5 × 10^{6}/mL autologous PBMCs or IVS cells (effector cells) were resuspended in media. The effector and target cells, plated at 100:1, 50:1, 25:1, and 12.5, were then combined in the plates and incubated for 4 hours at 37°C. The plates were pelleted and supernatants (100 μl) from each well were removed and counted in a gamma counter. The % specific lysis was calculated using the formula (experimental value – minimum lysis)/(maximum lysis – minimum lysis) × 100. Labeled target cells in media served as the minimum lysis value and labeled target cells in % Triton X-100 served as the maximum value.

**Results and Discussion**

**Patient ovarian cancer cells express HERV-K transcripts and ENV protein**

For many years, our laboratory has been investigating the expression and potential functions of HERV-K in various human cancers. For example, a series of studies from our laboratory have demonstrated that HERV-K ENV proteins are strongly expressed in invasive breast cancer and ductal carcinoma in situ in comparison with adjacent benign tissues (27, 28), that the HERV-K ENV protein can trigger antigen-specific immune responses in patients with breast cancer (24), and that the anti–HERV-K ENV monoclonal antibody (mAb) possesses immunotherapeutic potential (29). We have also provided preliminary evidence for the expression of HERV-K ENV transcripts in ovarian cancer (25). The main goals in the current study were to further characterize the HERV-K transcript (Supplementary Fig. S2) and protein expression in patient ovarian cancer cells and, more importantly, to determine whether the HERV-K ENV (K-SU) protein may be able to function as a potential TAA to elicit immune responses against ovarian cancer cells. To this end, we collected a new cohort of patients (n = 89) with ovarian cancer (both epithelial and germ cell tumors) or benign ovarian diseases such as cysts (Supplementary Fig. S3; Supplementary Table S1). From these patients we obtained tumor (T), the matching uninvolved normal (N), or benign (B) tissues, from which we also frequently derived single epithelial cells. Before we utilized these tissues and/or cells for HERV-K studies, we characterized the phenotypic
Ovarian cancer cells express HERV transcripts and HERV-K ENV protein. A, RT-PCR analysis of 7 HERV transcripts in the matching tumor (T) and uninvolved normal (N) tissues. Shown on the left is a representative RT experiment in Acc164 T/N. RT-PCR of no temple control (NTC) and β-actin was used as negative and positive controls, respectively. Shown on the right is densitometric quantification of Kty1 and Kty2 mRNA levels in tumor versus B/N samples. Values represent (Kty1 or Kty2 OD – corresponding β-actin OD) × 100. *P < 0.05 compared with the B/N. B, RT-PCR analysis of HERV transcripts in two benign (Acc116 and Acc143) and two tumor (Acc104 and Acc153) samples. Arrowheads point to the 1000-bp marker. C, RT-PCR analysis of HERV transcripts in ascites and spheres of Acc222 (left) and Acc65 (right) samples. Molecular ladder was indicated on the right and two arrowheads (left) point to the 1000-bp marker. D, quantitation of HERV-K ENV molecules on the surface of two each ovarian cancer patient primary tumors (T) and ascites (A). F and G, detection of surface expression of HERV-K ENV protein in primary Acc105 (F) and Acc222 (G) tumor cells derived from ascites by immunofluorescence and fluorescence microscopy using the mAb 6H5. Blue (DAPI) represents a nuclear stain and green (Alexa fluor 488) represents surface HERV-K expression. Note that the uninvolved (N) ovarian epithelial cells from Acc105 were negative for HERV-K ENV protein (F). mlG, mouse IgG.

Figure 1.

Ovarian cancer cells express HERV transcripts and HERV-K ENV protein. A, RT-PCR analysis of 7 HERV transcripts in the matching tumor (T) and uninvolved normal (N) tissues. Shown on the left is a representative RT experiment in Acc164 T/N. RT-PCR of no temple control (NTC) and β-actin was used as negative and positive controls, respectively. Shown on the right is densitometric quantification of Kty1 and Kty2 mRNA levels in tumor versus B/N samples. Values represent (Kty1 or Kty2 OD – corresponding β-actin OD) × 100. *P < 0.05 compared with the B/N. B, RT-PCR analysis of HERV transcripts in two benign (Acc116 and Acc143) and two tumor (Acc104 and Acc153) samples. Arrowheads point to the 1000-bp marker. C, RT-PCR analysis of HERV transcripts in ascites and spheres of Acc222 (left) and Acc65 (right) samples. Molecular ladder was indicated on the right and two arrowheads (left) point to the 1000-bp marker. D, quantitation of HERV-K ENV molecules on the surface of two each ovarian cancer patient primary tumors (T) and ascites (A). F and G, detection of surface expression of HERV-K ENV protein in primary Acc105 (F) and Acc222 (G) tumor cells derived from ascites by immunofluorescence and fluorescence microscopy using the mAb 6H5. Blue (DAPI) represents a nuclear stain and green (Alexa fluor 488) represents surface HERV-K expression. Note that the uninvolved (N) ovarian epithelial cells from Acc105 were negative for HERV-K ENV protein (F). mlG, mouse IgG.

and certain functional properties of epithelial ovarian cancer cells. The results revealed that both primary and metastatic (ascites) ovarian cancers manifested certain phenotypes (i.e., marker expression profiles) as well as properties (e.g., forming spheroids) of cancer stem cells (CSC) and circulating tumor cells (see Supplementary Results, Supplementary Table S3, and Supplementary Figs. S4 and S5). Considering the accumulating evidence for the importance of CSCs in therapy resistance and tumor relapse and of circulating tumor cells in dissemination and metastasis (31–40), our characterizations provide potential clues to why ovarian cancer cells are generally very aggressive, invasive, drug-resistant, and metastatic.

Subsequently, we thoroughly analyzed mRNA levels in ovarian cancer cells of seven HERV transcripts, i.e., ERV3, HERV-E4-1, HERV-K type 1 (KTY1), HERV-K type 2 (KTY2), NP9 (or REC), GAG, and P1/P3 (Supplementary Fig. S2) using specific reverse transcriptase (RT)-PCR primers (Supplementary Table S4). The first two transcripts analyzed (i.e., ERV3 and HERV-E4-1) were specific for the ENV genes of γ-retroviruses, whereas the remainder 5 transcripts were specific to HERV-K (Supplementary Fig. S2). We detected the expression of most of these transcripts in both primary tumors (e.g., 164T, 104T, and 153T; Fig. 1B) and ascites samples (e.g., Acc65 an Acc222; Fig. 1C). Of interest, tumor samples seemed to generally express higher levels of many of these transcripts than the corresponding benign (B) or uninvolved normal (N) tissues (Fig. 1A). Indeed, densitometric quantification revealed higher Kty1 and Kty2 mRNA levels in tumors than in B/N tissues (Fig. 1A, right). The ascites samples expressed high mRNA levels of these transcripts, which further increased slightly in spheres (Fig. 1C).

Subsequently, using the 6H5 mAb (29), we analyzed the HERV-K ENV protein expression in multiple cultured ovarian cancer cells

![Image](https://example.com/image.png)
as well as patient ovarian cancer samples (Fig. 1D–G; Supplementary Figs. S4B and S4D, and S5C–S5H; Supplementary Table S3). We first utilized flow cytometry (FCM) and cultured ovarian cancer cells (DOV13, OVCAR3, and SKOV3) to perform quantitative indirect immunofluorescence (QIF) assay (see Supplementary Methods) to determine the number of HERV-K ENV molecules on the ovarian cancer cell surface (Fig. 1D and Supplementary Fig. S5C). Using the titrated optimal antibody concentration, FCM analysis revealed that cultured ovarian cancer cells expressed much higher levels of ENV protein than immortalized but non-tumorigenic human ovarian epithelial cells T29 (Supplementary Fig. S5D) or the immortalized, nontransformed human mammary epithelial cells MCF-10A (Fig. 1D). Significantly, FCM analysis detected HERV-K ENV surface expression in primary ovarian cancer cells as well as ascites (Fig. 1E and Supplementary Figs. S4B and S4D; Supplementary Table S3). As observed with the HERV-K transcript mRNA expression patterns, ascites samples generally expressed high levels of ENV protein in comparison with primary tumor or benign samples (Fig. 1E and Supplementary Figs. S4B and S4D, and S5E; Supplementary Table S3). Also, sphere cultures seemed to further enrich for HERV-K ENV-positive cells (Supplementary Fig. S4B and S4D).

Indirect immunofluorescence microscopy confirmed the expression of HERV-K ENV protein on the surface of patient ovarian cancer ascites (Fig. 1F and G and Supplementary Fig. S5F) and cultured DOV13 (Supplementary Fig. S5G) cells but not uninvolved normal ovarian epithelial cells (i.e., Acc105-N; Fig. 1F). Interestingly, permeabilized DOV13 cells showed an intracellular pool of the ENV protein (Supplementary Fig. S5G), suggesting that not all expressed ENV protein is targeted to the cell surface. Immunohistochemistry using the 6H5 mAb also detected positive HERV-K ENV protein expression in both endometrioid and serous adenocarcinomas but not in benign cyst or normal ovarian tissues (Supplementary Fig. S5H). Finally, we detected, on Western blotting analysis, the HERV-K ENV protein in fractionated plasma samples from patients with ovarian cancer but not from normal donors (Fig. 2A, see below; data not shown). Taken together, data shown here provides evidence for the expression of various HERV transcripts and HERV-K ENV protein in ovarian cancer patient tumors and ascites.

Ovarian cancer patient tumors and plasma possess reverse transcriptase activity and ovarian cancer patient sera contain HERV-K ENV immunoreactive antibodies

Current diagnostic methods for cancer, especially malignancies of female reproductive tract, mainly depend on typical symptoms and screening tests. However, as is the case with ovarian cancer, many tumors are asymptomatic at early stage, and screening tests utilizing specific biomarkers are not sensitive enough or sufficiently specific for tumor detection. Therefore, simple and accurate tests to detect early-stage breast cancer and ovarian cancer are lacking. Recent data from our group suggest that detection of anti-HERV antibodies in patient sera might be a useful method for rapidly screening women for breast cancer. We have shown the correlation between anti-HERV-K antibody titers and the type of proliferative lesion in human breast cancer serum samples, suggesting that the presence and amount of anti-HERV-K antibodies might have a diagnostic value for breast cancer (41). In fact, HERV-K serum antibody titers showed greater sensitivity and specificity than other recently proposed serum biomarkers of breast cancer (41).

In our current study, we explored the possibility that the anti-HERV-K antibodies might also be present in the ovarian cancer patient sera and their titers might have some diagnostic values for ovarian cancer. The PI/P3 primers used in our RT-PCR analyses detect a approximately 1.6 kb transcript spanning both POL and ENV genes (Supplementary Fig. S2). Detection of the PI/P3 product in many of the ovarian cancer samples (Fig. 1A–C) suggests that ovarian cancer cells may also express the pol gene product reverse transcriptase. To test this possibility, we collected blood samples from cohorts of normal female donors (ND), patients with benign ovarian diseases such as cysts and cystadenomas, and patients with ovarian cancer and used the fractionated plasma samples to perform Western blotting experiments (Fig. 2A) and to measure reverse transcriptase activity (Fig. 2B). The plasma fractionation approach was used because we were interested in identifying where the retroviruses were actually located in the plasma, and we found that retroviruses banded at a density of approximately 1.16 g/mL in isopycnic density gradients. This density corresponded to a position between gradient fraction pools A and B described below. We prepared a total of 20 isopycnic fractions (i.e., from 1 to 20), the first 8 fractions of which were used in Western blotting. As shown in Fig. 2A, the HERV-K ENV protein was detected in the density gradient-generated fractions of plasma samples from patients with ovarian cancer but much less from benign patients and not from the ND. To measure the reverse transcriptase activity, we combined fractions 1–5 into “pooled” fraction A, 6–10 into fraction B, 11–15 into fraction C, and 16–20 into Fraction D. As shown in Fig. 2B, plasma samples from patients with ovarian cancer in all fractions showed higher levels of reverse transcriptase activity than those from the ND (also see Supplementary Results) although the plasma HERV-K reverse transcriptase activities were not significantly different between the patients with ovarian cancer and the patients with benign diseases. Interestingly, plasma samples from patients with benign diseases also showed higher levels of reverse transcriptase activity than those from the ND although the ovarian cancer and benign plasma samples did not manifest any difference in reverse transcriptase activity (Fig. 2B).

Receiver operating characteristics (ROC) curves to distinguish between tumor, benign, and normal samples were used to assess the diagnostic accuracy of our reverse transcriptase activity assays. Ideally, a reverse transcriptase activity assay should effectively distinguish between a control and an ovarian cancer patient using reverse transcriptase activity as a cutoff that is not generally reached in healthy women or women with benign diseases. The area under the (ROC) curve (AUC) was calculated to determine the accuracy of a diagnostic test and the AUC scores had the following specifications: 0.90–1.0 = excellent; 0.80–0.90 = good; 0.70–0.80 = fair; 0.60–0.70 = poor; and 0.50–0.60 = fail. As presented in Fig. 2B (table on the right), all diagnostic reverse transcriptase activity screens comparing benign and normal samples were considered “good” regarding accuracy for all plasma fractions and almost all diagnostic reverse transcriptase activity screens comparing normal and tumor samples were “good.” We also measured the reverse transcriptase activity in lysates prepared from ovarian cancer, adjacent uninvolved (normal) tissues, and benign lesions. The results revealed significantly higher reverse transcriptase activity in tumors than either normal or benign tissues (Fig. 2C, also see Supplementary Results). Diagnostic reverse transcriptase activity screens comparing benign and tumor
as well as comparing normal and tumor samples were considered “excellent” regarding accuracy. Finally, we employed the recombinant HERV-K ENV protein in ELISA assays (see Supplementary Methods) to determine whether the ovarian cancer patient serum contained anti–HERV-K antibodies (Fig. 3). The results revealed higher anti–HERV-K ENV antibody titers in patients with ovarian cancer than in benign or normal female controls (Fig. 3A and B). Of potential interest, the titers in the sera of patient Acc14 decreased 12 months after surgery (Fig. 3A).

Combined, data presented herein indicate that the ovarian cancer cells in patient tumors also express readily detectable and high reverse transcriptase activity, suggesting that the HERV-K pol gene, like the ENV gene, is expressed into protein. The fact that we can detect the reverse transcriptase activity in the plasma of patients with ovarian cancer and patients with benign ovarian diseases suggests that either HERV-K–expressing ovarian cells and/or HERV-K viral particles are shed into the blood circulation. The seeming discrepancy between the high reverse transcriptase

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Combined, data presented herein indicate that the ovarian cancer cells in patient tumors also express readily detectable and high reverse transcriptase activity, suggesting that the HERV-K pol gene, like the ENV gene, is expressed into protein. The fact that we can detect the reverse transcriptase activity in the plasma of patients with ovarian cancer and patients with benign ovarian diseases suggests that either HERV-K–expressing ovarian cells and/or HERV-K viral particles are shed into the blood circulation. The seeming discrepancy between the high reverse transcriptase activity in patient plasma was examined using a traditional academic point system with the following specifications: 0.90–1.0 = excellent; 0.80–0.90 = good; 0.70–0.80 = fair; 0.60–0.70 = poor; 0.50–0.60 = fail. C, reverse transcriptase activity was compared in tissues from patients with ovarian cancer (tumor) or adjacent normal tissue (normal) or from benign ovarian diseases (benign). Statistical data are displayed in the table (see the text for description).

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<th>Significance</th>
<th>AUC</th>
<th>ROC curve</th>
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<td>Yes</td>
<td>0.5000</td>
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Figure 2. Detection of HERV-K protein and reverse transcriptase (RT) activity in patient plasma. A, detection of HERV-K ENV protein in plasma fractions by immunoblot using 6H5 mAb. The recombinant HERV-K ENV protein (Env) was used as a positive control. The density gradients and fraction numbers are indicated below. Note that the HERV-K ENV proteins in Acc204 plasma fractions ran slightly slower than the recombinant Env probably due to protein glycosylations in patient tumors. B, reverse transcriptase activity was compared in pooled plasma fractions (see text for descriptions) from normal female donors or patients with ovarian cancer or benign lesions. Statistical data are presented in the table on the right. A guide for classifying the accuracy of a diagnostic test is the traditional academic point system with the following specifications: 0.90–1.0 = excellent; 0.80–0.90 = good; 0.70–0.80 = fair; 0.60–0.70 = poor; 0.50–0.60 = fail. C, reverse transcriptase activity was compared in tissues from patients with ovarian cancer (tumor) or adjacent normal tissue (normal) or from benign ovarian diseases (benign). Statistical data are displayed in the table (see the text for description).
HERV-K Expression and Immunity in Ovarian Cancer Cells

Figure 3.
Detection of anti-HERV-K antibodies in ovarian cancer patient serum. A, detection of anti-HERV-K antibodies in serially diluted serum samples obtained from 2 patients with ovarian cancer (Acc14 and Acc22) and 4 patients with benign ovarian diseases (Acc16, 19, 32 and 34). 0 mo indicates that serum was drawn before the surgery while 6 mo and 12 mo indicate serum draws 6 or 12 months after surgery, respectively. The anti-HERV-K antibody titers were presented as OD readings at 405 nm by ELISA. B, summary of the ELISA results of anti-HERV-K antibody titers were presented as OD readings at 405 nm by ELISA. 

HERV-K ENV protein can elicit antigen-specific T-cell proliferation and IFNγ production

Consistent with the abovementioned possibility that HERV-K ENV protein in ovarian cancer cells could elicit a humoral immune response, we have previously demonstrated that the HERV-K ENV protein can trigger cell-mediated immune responses in patients with breast cancer (24). In subsequent experiments, we asked whether the HERV-K ENV (KSU) protein could activate T cells prepared from the patients with ovarian cancer (Figs. 4 and 5) and could function as TAs to elicit T-cell-specific immune responses against the autologous ovarian cancer cells (Fig. 6, Fig. 7). Basic procedures in preparing peripheral blood mononuclear cells (PBMC) and various immune cells including T cells, DCs, and in vitro sensitized (IVS) cells (i.e., the T cells activated by HERV-K primed DCs) were depicted in Supplementary Fig. S1.

A profound biologic issue in limiting the efficacy of cancer vaccines is the failure of T cells to expand in response to antigenic stimulation. Therefore, we first analyzed the proliferative responses, by H3-thymidine incorporation assays, of PBMCs (mainly T cells with adherent DCs depleted; Supplementary Fig. S1) and the corresponding IVS T cells, prepared from 3 patients with ovarian cancer and 3 ND, to either unprimed DCs or DCs primed with HERV-K ENV cRNA or HPV16 E6 cRNA (Fig. 4A). The results showed that the ovarian cancer patient PBMCs exhibited robust proliferative response to the autologous DCs primed with HERV-K ENV cRNA compared with unprimed DCs as well as E6-primed DCs (Fig. 4A). Importantly, the IVS cells showed further increased proliferative response to the HERV-K pulsed DCs and the HERV-K-specific proliferative responses were stronger in patients with ovarian cancer compared with ND (Fig. 4A). To further corroborate these findings, we primed DCs with the recombinant KSHV protein and carried out similar thymidine incorporation assays. As illustrated in Fig. 4B, the PBMCs and IVS cells from an adenocarcinoma patient (Acc72) showed higher proliferative responses to KSU-pulsed DCs compared with those from two benign samples (i.e., Acc70 and Acc91). It should be noted that PBMCs and/or IVS cells in some samples (such as Acc14 and 15 in Fig. 4A and Acc72 in Fig. 4B) also displayed some proliferative response to HERV-K ENV cRNA or protein pulsed DCs, which was not particularly surprising considering widespread HPV infection in the female population. Therefore, we performed yet another experiment in which we utilized DCs pulsed with the recombinant KLH protein as the control (Supplementary Fig. S1). As shown in Fig. 4C, the IVS cells from a high-grade serous adenocarcinoma patient (Acc22; Supplementary Table S1) showed higher proliferative responses to KSU-pulsed DCs compared with those from one normal donor (i.e., ND20) or from two benign samples (i.e., Acc16 and Acc21; Supplementary Table S1). In this experiment, the KLH-pulsed DCs elicited similar responses in Acc22 IVS cells to the unprimed DCs (Fig. 4C). When we analyzed the pooled data of proliferative responses, ovarian cancer patient IVS cells showed significantly higher proliferation rates when combined with DCs primed with HERV-K than with DCs primed with KLH (Fig. 4D). Importantly, IVS cells in patients with ovarian cancer showed significantly higher proliferation rates when combined with DCs primed with HERV-K than in patients with benign diseases (Fig. 4D).

IFNγ is secreted by activated CD4+ and CD8+ cells and is critical for innate and adaptive immunity and for tumor control. Therefore, we assessed HERV-K–specific T-cell responses by measuring IFNγ production using the IFNγ ELISPOT (Fig. 5; see Materials and Methods for experimental details). When the IVS cells but not PBMCs from ovarian cancer patient Acc103 (a moderately to poorly differentiated adenocarcinoma; Supplementary Table S1) were incubated with KSU-pulsed DCs (i.e.,...
DC, and KSU), a large number of IFN-γ-producing T-cell clones were observed (Fig. 5A). This effect was much less obvious in the same IVS cells stimulated with unprimed DCs or DCs pulsed with KLH (Fig. 5A). Strikingly, IVS cells (and PBMCs) prepared from 3 ND did not show any IFN-γ response to KSU-primed DCs (Fig. 5A). Similarly, IVS cells prepared from Acc153 (a high-grade papillary serous carcinoma; Supplementary Table S1) blood samples collected at the time of surgery or 6 months after surgery (Fig. 5B), or from Acc156 (LMP) and Acc158 (a serous carcinoma; Fig. 5C) also demonstrated robust IFN-γ response to KSU-primed DCs. Interestingly, the HERV-K transmembrane (KTM) protein, which has been reported to have immunosuppressive properties (42), demonstrated similar IFN-γ-stimulating effects to KSU in activating DCs in these 3 patients with ovarian cancer (Fig. 5B and C) but not in a ND-derived DCs (ND25; Fig. 5B). Again, when the IVS cells from the same patients were combined with unprimed DCs or DCs pulsed with KLH protein, minimal IFN-γ response was observed (Fig. 5B and C). Finally, when we analyzed the pooled data of IFN-γ response in KSU-pulsed DCs from patients with ovarian cancer (tKSU) versus those from benign patients (bKSU), we observed significantly more IFN-γ-positive spots in IVS cells cocultivated with tKSU DCs than bKSU DCs (P = 0.0061; Fig. 5D).

Altogether, these results (Fig. 4 and 5) suggest that the human HERV-K gene product, i.e., the ENV protein, is immunogenic and can elicit notable T-cell responses measured by T-cell proliferation and IFN-γ production. Of great interest, the KSU-triggered T-cell responses are much stronger in ovarian cancer samples than in benign patients and normal female donors, presumably because the T cells in patients with ovarian cancer are already partially stimulated by the endogenous DCs, which are partially primed by the reactivated HERV-K expression.

HERV-K–specific T-cell cytotoxicity in ovarian cancer patients

One of the main goals of immunotherapy is to generate cytotoxic T lymphocytes (CTL) that can recognize antigens that are specifically expressed on the tumors, thereby leading to tumor destruction. However, there is considerable molecular diversity in
ovarian cancer, and a major factor limiting vaccine progress is the lack of well-characterized rejection antigens (13). IFNγ production and proliferation are clear signs of specific activation; however, they do not prove cytotoxic ability. Our paramount objective was to determine the ability of antigen-specific T cells to kill autologous target cells expressing HERV-K while ignoring benign or normal cells. Our cohort of samples provided us a unique opportunity to achieve this objective in that it allowed cytolytic activity of autologous target cells (instead of an established cancer line not specific to the patient) to be assessed. To this end, we prepared KSU-primed IVS cells from ovarian cancer patient Acc115 (a malignant mixed mullerian tumor; Supplementary Table S1) and autologous tumor cells and, for comparison, normal cells from the adjacent uninvolved tissues (Fig. 6A). Using the IVS cells as the “effector” cells and autologous tumor or normal cells in a CTL (i.e.,51Cr release) assay, we observed higher specific target cell lysis in tumor cells than the normal cells (Fig. 6A). Significantly, the % specific lysis was increased dramatically in tumor cells when the target cells were also loaded with the KSU (Fig. 6A). Similar CTL assays in a total of 6 patients with ovarian cancer revealed that percentage of CTL lysis of autologous HERV-K-expressing ovarian cancer cells. The results also suggest that PBMCs in circulation from ovarian cancer patients contain HERV-K-specific CTLs that can be restimulated in vitro to induce cytolytic activity against HERV-K-expressing ovarian tumor cells.

Then why are ovarian cancer cells in patient tumors not eliminated by the HERV K-primed CTLs? One explanation may be the overall immunosuppressive tumor microenvironment that is limiting the functions of antigen-specific CTLs (8, 14, 17, 19, 42). As mentioned in the section Introduction, Tregs represent a subpopulation of immunosuppressive T lymphocytes that dampen the immune response and accumulation of intratumoral Tregs has been associated with a high mortality rate in patients with ovarian cancer. Likely, it is the intricate balance between the abundance of effector T cells and that of Tregs that determines patient outcome. In fact, some studies have shown that ovarian cancer cells escape immune surveillance via creation of an immunosuppressive microenvironment (14) and induction of suppressive Tregs is frequent in...
ovarian cancer tumor lesions (14). We first characterized the composition of cellular populations in our IVS cells by staining with antibodies against CD4 (for T helper cells), CD8 (for CTLs), CD25 (for activated T and B cells), CD56 (for NK cells), and FoxP3 (for Tregs). As presented in Supplementary Table S5, we observed an increase in both CD8+ and CD4+ cells in both tumor and benign samples, an increase in activated B and T cells in all patients (except the last benign patient), and a decrease in Tregs only in ovarian cancer samples. Of significance, FoxP3+ cells were detected in tumor-infiltrated lymphocytes in Acc213 ovarian cancer (a high-grade serous tumor; Supplementary Table S5), supporting the presence of Tregs in ovarian tumors in situ.

We subsequently determined the effect of Treg depletion (from the IVS effector cells) on the ability of HERV-K specific CTLs to lyse autologous target ovarian cancer cells. As shown in Fig. 7A, in ovarian cancer patients Acc104 (a high-grade serous carcinoma; Supplementary Table S1) and Acc164 (a mucinous borderline tumor; Supplementary Table S1), target cell killing was greater in tumor cells than in matching normal cells and Treg depletion slightly enhanced lysis. In 2 other patients with ovarian cancer we studied, tumor cells pulsed with KSU showed more preferential killing compared with the same cells pulsed with KLH control and in one patient (Acc204, a serous adenocarcinoma) Treg depletion resulted in slightly increased target cell lysis (Fig. 7B). In a cohort of 7 (unmatched) tumor and benign samples, we observed higher target cell lysis in tumor cells than in benign cells and, in general, cell killing was enhanced when Tregs were depleted (Fig. 7C). Overall, Treg depletion (i.e., Treg−/C0) led to enhanced specific lysis (Fig. 7D). A comparison of lysis of tumor cells, uninvolved normal cells, and benign cells as targets in the presence of undepleted T cells (both), Treg− cells and Treg+ cells showed preferential killing of tumor cells and significantly higher percentage of lysis was observed in Treg− than Treg+ from patients with ovarian cancer (n = 28; P = 0.0396; Fig. 7E).

In summary, in this project we have shown the expression of HERV-K transcripts and HERV-K ENV protein in primary ovarian cancer cells and ascites. In addition, the ovarian cancer tissues and patient plasma express detectable reverse transcriptase activity and patient sera contain HERV-K antibodies. Furthermore, HERV-K primed DCs can activate T-cell responses (i.e., T-cell proliferation and IFNγ production). Most importantly, HERV-K–specific T cells can elicit robust cytotoxicity towards autologous ovarian cancer cells, which is further enhanced by Treg depletion. These observations, together with the findings that HERV-K expression appears to be reactivated in a wide spectrum of human tumors, provide rationale for employing HERV-K gene products as TAAs to
develop therapeutic vaccines to target ovarian cancer and other tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Rycaj, B. Yin, M. Li, J. Garza, F. Wang-Johanning
Writing, review, and/or revision of the manuscript: K. Rycaj, J.B. Plummer, B. Yin, M. Li, J. Garza, I.G. Radvanyi, L.M. Ramondetta, G.L. Johanning, D.G. Tang, F. Wang-Johanning
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Rycaj, J. Garza, F. Wang-Johanning
Study supervision: K. Rycaj, F. Wang-Johanning

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Figure 7. Effect of Treg depletion on HERV-K–specific T-cell cytotoxicity in patients with ovarian cancer. A, tumor and adjacent normal uninvolved tissues obtained from patient Acc104 and Acc164 were harvested and employed as “target cells.” Unpurified bulk IVS cells (both) were used as “effector” cells, which were further separated into purified Tregs (Treg”) and Treg-depleted (Treg”) population. The effector to target cell ratios ranged from 100:1 to 12.5:1. B, ovarian cancer cells from patients Acc204 and Acc213 were purified and employed as target cells for the CTL assay. IVS cells were prepared and further fractionated as in A and target cells were further pulsed with KSU or KLH protein. C, CTL assays using a cohort of tumor (T) or benign (B) cells from the patients indicated as target cells. IVS cells were used as effector cells and target cells were pulsed with KSU protein. D, the percent specific lysis from CTL assays was compared among adjacent uninvolved ovarian, benign ovarian, and ovarian tumor target cells (n = 13). E, summary of percent specific lysis from CTL assays from normal female donors (n = 10), benign disease (n = 18), and ovarian cancer patients (n = 8). Target cells were prepared from ovarian cancer or adjacent uninvolved (normal) tissues or from benign tissues. Three types of effector cells as described in A were used. The Student t test was used to calculate significance of differences between groups.

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References


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