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

Kiera Rycaj¹,²,³, Joshua B. Plummer¹,³, Bingnan Yin¹,³, Ming Li¹,³,⁴, Jeremy Garza¹, Laszlo Radvanyi³,⁵,⁶, Lois M Ramondetta⁷, Kevin Lin¹, Gary L. Johanning¹,³, Dean G. Tang²,³, and Feng Wang-Johanning¹,³,⁴,⁶

¹Department of Veterinary Sciences, Michale E. Keeling Center for Comparative Medicine and Research, The University of Texas MD Anderson Cancer Center, TX, USA;
²Department of Molecular Carcinogenesis, The University of Texas MD Anderson Cancer Center, TX, USA;
³Graduate School of Biomedical Sciences, The University of Texas Health Science Center at Houston, Houston, TX, USA;
⁴Viral Oncology Program, SRI International, 333 Ravenswood Avenue, Menlo Park, CA, USA;
⁵Department of Melanoma Medical Oncology, The University of Texas MD Anderson Cancer Center, TX, USA;
⁶Department of Immunology, The University of Texas MD Anderson Cancer Center, TX, USA;
⁷Division of Gynecologic Oncology and Lyndon Baines Johnson Hospital, The University of Texas MD Anderson Cancer Center, TX, USA.

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Corresponding author: Feng Wang-Johanning, MD, PhD, SRI International: 333 Ravenswood Avenue, Menlo Park, CA USA, 650-859-3271, feng.wang-johanning@SRI.com

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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 (OC) 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 OC tissues and patient blood samples were analyzed and compared to samples from patients with benign ovarian diseases and normal female donors.

Results: OC 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 RT activities were higher in OC compared to adjacent normal and benign tissues. The OC patient plasma also had high RT activities and the OC patient sera contained HERV-K immunoreactive antibodies. HERV-K-specific T cells generated from autologous dendritic cells (DCs) pulsed with HERV-K env antigens exhibited phenotypes and functions consistent with a cellular immune response including T cell proliferation, IFN-γ 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 OC patients than patients with benign diseases and further enhanced lysis was observed if T regulatory cells were depleted.

Conclusion: Endogenous retroviral gene products in OC may represent a potentially valuable new pool of tumor-associated antigens for targeting of therapeutic vaccines to OC.
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 (OC) detection, diagnosis and immunotherapy. Our findings suggest anti-HERV-K plasma antibodies and HERV-K reverse transcriptase activity as two potential novel biomarkers for OC detection and diagnosis using readily available body fluids and a minimally invasive procedure. From a clinical standpoint, the availability of new biomarkers to detect OC at an early stage is critically important, because frequently this cancer is not detected until advanced stages when treatment options are limited. Additionally, HERV-K-specific T cells induced cellular immune responses towards autologous patient OC cells, providing evidence that the re-activated endogenous retroviral gene products represent a potentially valuable new pool of tumor-associated antigens with great specificity for targeting of therapeutic vaccines to OC.
Introduction

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

Human endogenous retroviruses (HERVs) comprise ~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 OC 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 interferon-γ (IFN-γ), macrophage-derived chemokines and secondary lymphoid-tissue chemokines are significantly increased as compared with tumors lacking T-cells (10, 11). Strong positive correlations have also been observed between levels of CD8+ T cells and granzyme B within ovarian tumors, indicating that the majority of CD8+ T cells are cytotoxic (12). OCs 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 OC vaccines is the lack of clearly defined TAAs that are capable of being recognized by T cells (i.e., T cell epitopes).

Overcoming suppressive mechanisms in the tumor microenvironment in order to enhance efficacy is another major challenge for current approaches to OC vaccination. Various studies have shown that OCs escape immune surveillance with high efficiency via creating a tolerogenic microenvironment (14). The tumor microenvironment mediates induction of an immunosuppressive programmed 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 (DCs). 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 OC patients (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+ T cell subpopulation that can be divided into two subsets: naturally occurring Tregs with CD4+CD25+FOXP3+ phenotype and induced Tregs with variable CD25 expression (17). Tregs depend on PD-1, PD-L1 (programmed cell death ligand 1), or CTLA-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^{+}\text{CD25}^{+}\text{FoxP3}^{+} Treg cells correspond to poor clinical outcome in epithelial OC, and these cells are preferentially concentrated in the tumor mass rather than in tumor draining lymph nodes (20). Additionally, 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^{+} 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 OC.

We have previously demonstrated that breast cancer patients, 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 TAAs 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 OC cell lines and tissues (25), suggesting that HERVs might also be able to serve as TAAs to enable targeting of OC. 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 OC or as new immunotherapeutic targets for the disease. Most important, we explore the development of a cancer vaccine for OC 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 (24-29) and in Supplementary Information.

Clinical samples and cell lines

Tissue and/or blood samples from OC patients and patients with benign diseases were obtained from the M.D Anderson Cancer Center according to approved Institutional Review Board protocols (LAB04-0083) under informed consent. Clinicopathologic characteristics are listed in Table S1. Normal donor blood samples (Table S2) were obtained from Gulf Coast Regional Blood Center, Houston, Texas. OC 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).

Harvesting of primary samples and preparation of tumor cells

A piece of tissue in 1-2 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 min on a shaker. The cells were transferred to a Stomacher 80 bag that was inserted into the Stomacher (Seward Co., Worthing, West Sussex, UK) and run for 15 min 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 min, 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 supplemented with heparin and hydrocortisone. To culture ascites, the ascites was 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 (RT) activity in tumor tissues and plasma

Patient plasma was isolated using Histopaque 1077 and separated (10 ml) on isopycnic gradients using OptiPrep® (iodixanol, AxisShield, Oslo, Norway) as per the manufacturer’s instructions. Primary ovarian tissues were also collected during surgery and frozen at -80°C until processed for RT assay. Tissue samples were crushed in culture media using a mortar and pestle. Five µl aliquots of fractionated plasma or tissue samples were analyzed for RT activity using the EnzChek RT assay kit (Invitrogen, Life Technologies, Grand Island, NY). The EnzChek RT 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 RT activity in a biological 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. An RT standard curve was generated using serial dilutions of Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) (Invitrogen). Fluorescence was measured with a Wallac VICTOR² plate reader (Perkin Elmer, Waltham, MA).

Preparation of dendritic cells and generation of in vitro stimulated (IVS) cells

The basic procedure was depicted in Fig. S1. Briefly, after peripheral blood mononuclear cells (PBMCs) were plated and incubated for 16 h at 37°C, floating PBMCs were rinsed off and saved
for future experiments, while adherent cells were incubated for 6 days with granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4, 1,000 units/ml) (R&D Systems, Minneapolis, MN). 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, Madison, WI). 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, San Diego, CA). Four h after transfection, tumor necrosis factor-α (TNF-α) (1,000 units/ml) was added for an additional 16 h at 37°C to induce DC maturation. To generate 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 IL-2 (10 units/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 $^3$H-thymidine incorporation assay. Briefly, T-cell proliferation was evaluated in patient PBMC or IVS cells by re-stimulation for 72 h 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 $[^3]$H-thymidine and incubated for another 18 h 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 immunospot (ELISPOT) assay (BD Biosciences, San Jose, CA). Briefly, ELISPOT plates were coated with 10 µg/ml of purified anti-human IFN-γ capture antibody and incubated for 24 h at 4°C. Plates were then blocked for 2 h with complete media and PBMC or IVS cells were plated at 1 x 10⁵ per well with DCs pulsed with various HERV-K antigens plated at 5 x 10³ per well. Plates were incubated
for 24 h at 37°C and then washed and incubated with the detection antibodies for 2 h at 25°C.
Plates were washed and incubated with streptavidin horseradish peroxidase for 2 h 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, Shaker Heights, OH).

CTL assays were performed in round-bottomed 96-well plates using a standard 4-h $^{51}$Cr-
release assay. Briefly, various target (primary tumor, adjacent normal tissue, or benign) cells
were “pulsed” with K-SU or KLH control protein for 16 h at 37°C using the BioPORTER
transfection reagent. Target cells were removed from flasks via EDTA buffer, pelleted and
resuspended in 500 µl media and 75 µCi of chromium. 5 X 10^4/ml target cells and 5 X 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 h 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
($\text{Experimental value-minimum lysis}/(\text{maximum lysis-minimum lysis})$ x100. 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 OC 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 lab have demonstrated that HERV-K env proteins are strongly expressed in invasive breast cancer (BC) 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 BC patients (24), and that the anti-HERV-K env mAb possesses immunotherapeutic potential (29). We have also provided preliminary evidence for the expression of HERV-K env transcripts in OC (25). The main goals in the present study were to further characterize the HERV-K transcript (Fig. S2) and protein expression in patient OC cells and, more important, to determine whether the HERV-K env (K-SU) protein may be able to function as a potential TAA to elicit immune responses against OC cells. To this end, we collected a new cohort of patients (n = 89) with OC (both epithelial and germ cell tumors) or benign ovarian diseases such as cysts (Fig. S3; 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 and certain functional properties of epithelial OC cells. The results revealed that both primary and metastatic (ascites) OCs manifested certain phenotypes (i.e., marker expression profiles) as well as properties (e.g., forming spheroids) of cancer stem cells (CSCs) and circulating tumor cells (see Supplementary Results; Table S3, Fig. S4, and Fig. 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 OC cells are generally very aggressive, invasive, drug-resistant, and metastatic.

Subsequently, we thoroughly analyzed mRNA levels in OC 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 (Fig. S2) using specific RT-PCR primers (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 (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 OC cells as well as patient OC samples (Fig. 1D-G; Fig. S4, B and D; Fig. S5C-H; Table S3). We first utilized flow cytometry (FCM) and cultured OC cells (DOV13, OVCAR3 and SKOV3) to perform quantitative indirect immunofluorescence (QIFI) assay (see Supplementary Methods) to determine the number of HERV-K env molecules on the OC cell surface (Fig. 1D; Fig. S5C). Using the titrated optimal Ab concentration, FCM analysis revealed that cultured OC cells expressed much higher levels of env protein than immortalized but non-tumorigenic human ovarian epithelial cells T29 (Fig. S5D) or the immortalized, non-transformed human mammary epithelial cells MCF-10A (Fig. 1D). Significantly, FCM analysis detected HERV-K surface expression in primary OC cells as well as ascites (Fig. 1E; Fig. S4, B and D;
Table S3). As observed with the HERV-K transcript mRNA expression patterns, ascites samples generally expressed high levels of env protein in comparison to primary tumor or benign samples (Fig. 1E; Fig. S4, B and D; Fig. S5E; Table S3). Also, sphere cultures seemed to further enrich for HERV-K env-positive cells (Fig. S4, B and D).

Indirect immunofluorescence (IF) microscopy confirmed the expression of HERV-K env protein on the surface of patient OC ascites (Fig. 1, F and G; Fig. S5F) and cultured DOV13 (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 (Fig. S5G), suggesting that not all expressed env protein is targeted to the cell surface. Immunohistochemistry (IHC) 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 (Fig. S5H). Finally, we detected, on western blotting analysis, the HERV-K env protein in fractionated plasma samples from OC patients 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 OC patient tumors and ascites.

**OC patient tumors and plasma possess reverse transcriptase (RT) activity and OC 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 OC, 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 BC and OC 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 BC. We have shown the correlation between anti-HERV-K antibody titers and the type of proliferative lesion in human BC serum samples, suggesting that the presence and amount of anti-HERV-K antibodies might have a diagnostic value for BC (41). In fact, HERV-K serum antibody titers showed greater sensitivity and specificity than other recently proposed serum biomarkers of BC (41).

In our current study, we explored the possibility that the anti-HERV-K antibodies might also be present in the OC patient sera and their titers might have some diagnostic values for OC. The P1/P3 primers used in our RT-PCR analyses detect a ~1.6 kb transcript spanning both pol and env genes (Fig. S2). Detection of the P1/P3 product in many of the OC samples (Fig. 1, A-C) suggests that OC cells may also express the pol gene product RT. 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 OC patients and used the fractionated plasma samples to perform Western blotting experiments (Fig. 2A) and to measure RT 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 ~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 OC patients but much less from benign patients and not from the ND. To measure the RT 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 OC patients in all fractions showed higher levels of RT activity than those
from the ND (also see Supplementary Results) although the plasma HERV-K RT activities were not significantly different between the OC patients and the patients with benign diseases. Interestingly, plasma samples from patients with benign diseases also showed higher levels of RT activity than those from the ND although the OC and benign plasma samples did not manifest any difference in RT activity (Fig. 2B).

ROC (Receiver Operating Characteristics) curves to distinguish between tumor, benign and normal samples were used to assess the diagnostic accuracy of our RT activity assays. Ideally, an RT activity assay should effectively distinguish between a control and an OC patient using RT 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 in order 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 RT activity screens comparing benign and normal samples were considered “good” regarding accuracy for all plasma fractions and almost all diagnostic RT activity screens comparing normal and tumor samples were “good”. We also measured the RT activity in lysates prepared from OC, adjacent uninvolved (normal) tissues, and benign lesions. The results revealed significantly higher RT activity in tumors than either normal or benign tissues (Fig. 2C; also see Supplementary Results). Diagnostic RT 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 OC patient serum contained anti-HERV-K antibodies (Fig. 3). The results revealed higher anti-HERV-K env antibody titers in OC patients than in benign or normal female controls (Fig. 3, A 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 OC cells in patient tumors also express readily detectable and high RT activity, suggesting that the HERV-K pol gene, like the env gene, is expressed into protein. The fact that we can detect the RT activity in the plasma of OC patients 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 RT activity in benign patient plasma (Fig. 2B) vs. very low RT activity from the benign tissues (Fig. 2C) could potentially be explained by the possibility that patients with benign ovarian conditions also shed HERV-K positive cells into the circulation from other diseased tissues. Our observations that the sera in OC patients contain HERV-K immunoreactive antibodies are interesting and raise the possibility that the HERV-K expressed in the OC cells can actually elicit a humoral immune response against the autologous OC cells. These findings suggest HERV-K RT activity and anti-HERV-K plasma antibodies as two potential novel biomarkers for OC detection and diagnosis. The analysis of serum for HERV-K antibodies may enable clinicians to more easily identify women with noninvasive early OC and treat these women at an early stage before the OC can spread to other tissues.

**HERV-K env protein can elicit antigen-specific T-cell proliferation and IFN-γ production.**

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

A profound biological 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 H\(^3\)-thymidine incorporation assays, of PBMCs (mainly T cells with adherent DCs depleted; Fig. S1) and the corresponding IVS T cells, prepared from 3 OC patients and 3 ND, to either un-primed DCs or DCs primed with HERV-K env cRNA or HPV16 E6 cRNA (Fig. 4A). The results showed that the OC patient PBMCs exhibited robust proliferative response to the autologous DCs primed with HERV-K env cRNA compared to unprimed DCs as well as E6-primed DCs (Fig. 4A). Important, the IVS cells showed further increased proliferative response to the HERV-K pulsed DCs and the HERV-K specific proliferative responses were stronger in OC patients compared to ND (Fig. 4A). To further corroborate these findings, we primed DCs with the recombinant KSU 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 to 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 (Fig. S1). As shown in Fig. 4C, the IVS cells from a high-grade serous adenocarcinoma patient (Acc22; Table S1) showed higher proliferative responses to KSU-pulsed
DCs compared to those from one normal donor (i.e., ND20) or from two benign samples (i.e., Acc16 and Acc21; 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, OC 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 OC patients showed significantly higher proliferation rates when combined with DCs primed with KERV-K than in patients with benign diseases (Fig. 4D).

IFN-γ 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 IFN-γ production using the IFN-γ ELISPOT (Fig. 5; see Methods for experimental details). When the IVS cells but not PBMCs from OC patient Acc103 (a moderately to poorly differentiated adenocarcinoma; Table S1) were incubated with KSU-pulsed DCs (i.e., DC+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; Table S1) blood samples collected at the time of surgery or 6 months post 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 OC patients (Fig. 5B-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-C). Finally, when we analyzed the pooled data of
IFN-γ response in KSU-pulsed DCs from OC patients (tKSU) vs. those from benign patients (bKSU), we observed significantly more IFN-γ positive spots in IVS cells co-incubated with tKSU DCs than bKSU DCs (P=0.0061; Fig. 5D).

Altogether, these results (Fig. 4; Fig. 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 OC samples than in benign patients and normal female donors, presumably because the T cells in OC patients 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 OC patients**

One of the main goals of immunotherapy is to generate cytotoxic T lymphocytes (CTLs) that can recognize antigens that are specifically expressed on the tumors, thereby leading to tumor destruction. However, there is considerable molecular diversity in OC, and a major factor limiting vaccine progress is the lack of well-characterized rejection antigens (13). IFN-γ 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 OC patient Acc115 (a malignant mixed mullerian tumor; 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 as “target” cells in a CTL (i.e., §Cr
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 OC patients revealed that percentage of CTL lysis of autologous tumor cells expressing KSU antigen was significantly higher than that of uninvolved normal cells (Fig. 6B). Similarly, significantly higher % of target cell killing was observed with tumor cells prepared from 28 OC patients than with cells from 13 patients with benign ovarian diseases (Fig. 6C). These results demonstrate the efficacy of HERV-K specific T cells in preferentially killing autologous HERV-K expressing OC cells. The results also suggest that PBMCs in circulation from OC patients contain HERV-K–specific CTLs that can be re-stimulated in vitro to induce cytolytic activity against HERV-K–expressing ovarian tumor cells.

Then why are OC 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 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 OC patients. 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 OC cells escape immune surveillance via creation of an immunosuppressive microenvironment (14) and induction of suppressive Tregs is frequent in OC tumor lesions (43). 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 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 OC samples. Of significance, FoxP3+ cells were detected in tumor-infiltrated lymphocytes (TILs) in Acc213 OC (a high-grade serous tumor; 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 OC cells. As shown in Fig. 7A, in OC patients Acc104 (a high-grade serous carcinoma; Table S1) and Acc164 (a mucinous borderline tumor; Table S1), target cell killing was greater in tumor cells than in matching normal cells and Treg depletion slightly enhanced lysis. In two other OC patients we studied, tumor cells pulsed with KSU showed more preferential killing compared to 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−) 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−, and Treg+ cells showed preferential killing of tumor cells and significantly higher percentage of lysis was observed in Treg− than Treg+ from OC patients (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 OC cells and ascites. In addition, the OC tissues and patient plasma express detectable RT activity and patient sera contain HERV-K antibodies. Furthermore, HERV-K primed DCs can activate T cell responses (i.e., T cell proliferation and IFN-γ production). Most important, HERV-K specific T cells can elicit robust cytotoxicity towards autologous OC 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 tumor-associated antigens to develop therapeutic vaccines to target OC and other tumors.

**Disclosure of Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgements**

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References


Figure legends

Figure 1. OC 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 vs. B/N samples. Values represent (Kty1 or Kty2 OD – corresponding β-actin OD) x 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 1,000 bp marker.

(C) RT-PCR analysis of HERV transcripts in ascites and spheres of Acc222 (left) and Acc65 (right) samples (Fig. 1). Molecular ladder was indicated on the right and two arrowheads (left) point to the 1,000 bp marker.

(D) Quantitation of HERV-K env molecules on the surface of ovarian epithelial and cancer cells via QIFI KIT and FCM analysis. The gray line represents the isotype control, red line HERV-K positive population and blue line a series of 6 bead populations coated with defined quantities of a mouse monoclonal antibody (high affinity anti-human CD5, clone CRIS-1). The percentages of HERV-K positive cells are indicated above and the numbers of HERV-K surface molecules in the 4 cell types (i.e., MCF-10A, DOV13, OVCAR3, and SKOV3) are 39,759, 1.86 x 10^8, 4.9 x 10^8, and 3.61 x 10^8, respectively.

(E) Quantitation of HERV-K env molecules on the surface of two each OC patient primary tumors (T) and ascites (A).
(F-G) Detection of surface expression of HERV-K env protein in primary Acc105 (F) and Acc222 (G) tumor cells derived from ascites by IF and fluorescence microscopy using the mAb 6H5. Blue (DAPI) represents nuclear 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). mIgG, mouse IgG.

**Figure 2.** Detection of HERV-K protein and 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) RT activity was compared in pooled plasma fractions (see Text for descriptions) from normal female donors or patients with OC or benign lesions. Statistical data is 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. AUC: area under the curve; ROC, Receiver Operating Characteristics.

(C) RT activity was compared in tissues from patients with OC (tumor) or adjacent normal tissue (normal) or from benign ovarian diseases (benign). Statistical data is displayed in the table (see Text for description).

**Figure 3.** Detection of anti-HERV-K antibodies in OC patient serum
(A) Detection of anti-HERV-K antibodies in serially diluted serum samples obtained from 2 OC patients (Acc14 and Acc22) and 4 patients with benign ovarian diseases (Acc16, 19, 32 and 34). 0m indicates that serum was drawn before the surgery while 6m and 12m indicate serum draws 6 or 12 months post-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 antibodies in serum samples from OC patients (n=24), and patients with benign diseases and normal donors (n=20). P value was based on Student’s t-test.

Figure 4. HERV-K env induced T cell proliferation

(A) H³-Thymidine incorporation assays in PBMCs and IVS cells prepared from 3 OC patients (Acc7, 14, and 15) and from 3 normal female donors (ND). The PBMCs and IVS cells (Fig. S4) were incubated with media alone, unprimed autologous DCs, or autologous DCs primed with either HERV-K env or HPV16 E6 (control) cRNA for 72 h in the presence of H³-thymidine. Shown are the CPM (counts per minutes; mean ± S.D). Note that for unknown reasons lower CPM readings were observed in the DC wells.

(B-C) H³-Thymidine incorporation assays performed similarly to those described above in A except autologous DCs were pulsed with recombinant KSU or E6 protein (B) or KLH protein (C). See Text for more details. Note that the protein-primed DCs (B and C) generally elicited lower T cell proliferative responses than the cRNA-pulsed DCs (A), presumably due to well-known lower efficiency in protein transduction compared to RNA/DNA transfection.

(D) Analysis of pooled data of proliferative responses. See Text for detailed descriptions.

Figure 5. HERV-K env induced IFN-γ production
(A) ELISPOT assay for IFN-γ producing T cell clones. HERV-K SU (KSU) IVS or PBMCs from Acc103 OC patient or from 3 ND were combined with DCs pulsed with HERV-K protein (DC+KSU), DCs pulsed with KLH control protein (DC+KLH), media alone, or unpulsed DCs. An image of an ELISPOT plate is displayed and a purple spot represents one IFN-γ secreting cell.

(B-C) ELISPOT assays in more OC patients. Experiments were conducted as in A except with an additional condition in which DCs pulsed with the HERV-K transmembrane protein (KTM) were also used.

(D) Summary of IFN-γ ELISPOT data for OC patients (all t samples; n=10) compared with patients with benign disease (all b samples; n=10). Note the negligible IFN-γ responses in all studies with PBMCs.

Figure 6. HERV-K–specific T-cell cytotoxicity in OC patients

(A) CTL assays in Acc115 OC patient derived cells. Both tumor and adjacent uninvolved normal cells from Acc115 were harvested and employed as “target cells” and the autologous IVS cells were used as “effector cells”. Target cells were further pulsed with KSU protein. Effector to target cell ratios ranged from 100:1 to 12.5:1.

(B) The percent specific lysis from CTL assays using OC target cells vs. normal (uninvolved) target cells. Data were based on the matching samples from 6 patients.

(C) The percent specific lysis from CTL assays using OC target cells vs. benign tissue-derived target cells. Data were based on the non-matching samples from 28 OC patients and 13 benign diseases.

Figure 7. Effect of Treg depletion on HERV-K–specific T-cell cytotoxicity in OC patients
(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) OC 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 OC patients (n=8). Target cells were prepared from OC or adjacent uninvolved (normal) tissues or from benign tissues. Three types of effector cells as described in A were used. Student’s t-test was used to calculate significance of differences between groups.
Ryczaj et al, Figure 1
Comparison: B vs T: Fraction A
AUC: 0.9423
Yes: 0.0031
Good: 0.5068

Comparison: B vs N: Fraction A
AUC: 0.8890
Yes: 0.006
Good: 0.8839

Comparison: B vs T: Fraction B
AUC: 0.7967
Yes: 0.309E-01
Good: 0.006

Comparison: B vs N: Fraction B
AUC: 0.7200
Yes: 2.66E-04
Good: 0.8219

Comparison: B vs T: Fraction C
AUC: 0.8348
Yes: 0.0491E-08
Good: 0.8219

Comparison: B vs N: Fraction C
AUC: 0.8348
Yes: 0.0491E-08
Good: 0.8219

Comparison: B vs T: Fraction D
AUC: 0.7011
Yes: 0.409E-06
Good: 0.8219

Comparison: B vs N: Fraction D
AUC: 0.8219
Yes: 0.409E-06
Good: 0.8219

Rycz et al, Figure 2
A

B

C

D

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Rycaj et al, Figure 5
A

Acc115

% Specific Lysis

- Normal cells
- Tumor cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Cells + KSU</th>
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<tbody>
<tr>
<td>100:1</td>
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</tr>
<tr>
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<td>50:1</td>
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<td>12.5:1</td>
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</table>

B

% Specific Lysis

- P = 0.0006
- Normal cells
- Tumor cells

Rycal et al. Figure 6