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## HUMAN ENDOGENOUS RETROVIRUS K AS A NOVEL TUMOR- ASSOCIATED ANTIGEN FOR DEVELOPMENT OF AN OVARIAN CANCER VACCINE

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**HUMAN ENDOGENOUS RETROVIRUS K AS A NOVEL TUMOR-ASSOCIATED  
ANTIGEN FOR DEVELOPMENT OF AN OVARIAN CANCER VACCINE**

by

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**HUMAN ENDOGENOUS RETROVIRUS K AS A NOVEL TUMOR-ASSOCIATED  
ANTIGEN FOR DEVELOPMENT OF AN OVARIAN CANCER VACCINE**

A

DISSERTATION

Presented to the Faculty of  
The University of Texas  
Health Science Center at Houston  
and  
The University of Texas  
M. D. Anderson Cancer Center  
Graduate School of Biomedical Sciences  
in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Kiera Rycaj, B.S.  
Houston, Texas

May, 2012



## DEDICATION

To my loving family and Jorge. To Babcia, Cory, Jay and Cinnamon.

“We shall not cease from exploration  
And the end of all our exploring  
Will be to arrive where we started  
And know the place for the first time”  
-T.S. Eliot



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I would like to thank everyone who made this dissertation possible. I owe the most gratitude to my advisor, Dr. Feng Wang-Johanning, who gave me this extraordinary research opportunity and who has taught me so many invaluable, incredible things about cancer biology. I'm thankful to her for providing me invaluable insights and incredible guidance throughout this journey. I am especially grateful for my supervisory committee members for sharing their wealth of knowledge with me. I also thank my colleagues who supported me and were always willing to help me.



# **HUMAN ENDOGENOUS RETROVIRUS K AS A NOVEL TUMOR-ASSOCIATED ANTIGEN FOR DEVELOPMENT OF AN OVARIAN CANCER VACCINE**

Publication No. \_\_\_\_\_

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Ovarian cancer (OC) is the fourth most common cancer in women, and the most lethal gynecologic malignancy in the United States. Adequate screening methodologies are currently lacking and most women first present with either stage III or IV disease. To date, there has been no substantial decrease in death rates and the majorities of patients relapse and die from their disease despite response to first-line therapy. Several proteins, such as CA-125, are elevated in OC, but none has proven specific and sensitive enough to serve as a screening tool or for tumor cell recognition and lysis. It has been proposed that human endogenous retrovirus sequences (HERVs) may play a role in the etiology of certain cancers. In a previous study, we showed that HERV-K envelope (env) proteins are widely expressed in human invasive breast cancer (BC) and ductal carcinoma in situ (DCIS), and elicit both serologic and cell-mediated immune responses in BC patients. We also reported the expression of multiple HERV genes and proteins in OC cell lines and tissues. In this study, we strengthened our previous data by determining that HERV-K env mRNAs are expressed in 69% of primary OC tissues (n=29), but in only 24% of benign tissues (N=17). Immunohistochemistry (IHC) staining revealed HERV-K positive cancer cells detected in endometrioid adenocarcinoma and serous adenocarcinoma but not in benign cyst or normal epithelium biopsies. Immunofluorescence staining (IFS) showed greater cell surface expression of HERV-K in OC samples compared to adjacent uninvolved samples. Enzyme-



linked immunosorbent assay (ELISA) data confirmed that a humoral immune response is elicited against HERV-K in OC patients. T-cell responses against HERV-K in lymphocytes from OC patients stimulated with autologous HERV-K pulsed dendritic cells included induction of T-cell proliferation and IFN- $\gamma$  production. HERV-K-specific cytolytic T cells induced greater specific lysis of OC target cells compared to benign and adjacent uninvolved target cells. Finally, upon T regulatory cell (T-reg) depletion, 64% of OC patients displayed an increase in the specific lysis of target cells expressing HERV-K env protein. These findings suggest that HERV-K env protein is a tumor-associated antigen capable of activating both T-cell and B-cell responses in OC patients, and has great potential in the development of immunotherapy regimens against OC.



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## ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ADCC	antibody-dependent cell-mediated cytotoxicity
BC	breast cancer
BRCA	BReast CAncer1 gene
CML	chronic myelogenous leukemia
CTL	cytolytic T lymphocyte
CTLA-4	cytotoxic T lymphocyte-associated antigen
DAPI	4',6-Diamidino-2-phenylindole
DC	dendritic cell
DCIS	ductal carcinoma in situ
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunosorbent spot
EMT	epithelial-mesenchymal transition
EpCAM	epithelial cell adhesion molecule
env	envelope
FACS	fluorescence-activated cell sorting
FcR	Fc receptor
FDA	food and administration
FBS	fetal bovine serum
gag	group-specific antigen
GM-CSF	granulocyte macrophage colony-stimulating factor



HBV	hepatitis B virus
HER2/neu	human epithelial growth factor receptor 2
HERV-K	human endogenous retrovirus K
HML2	human mouse mammary tumor-like type 2
HPV	human papilloma virus
ICAM-1	intercellular adhesion molecule
IDC	invasive ductal carcinoma
IDO	indoleamine 2,3-dioxygenase
IF	intermediate filament
IgG	immunoglobulin G
IFN- $\gamma$	interferon gamma
IFS	immunofluorescence staining
IHC	immunohistochemistry
IL	interleukin
IVS	<i>in vitro</i> stimulation
KLH	keyhole limpet hemocyanin
LINE	long interspersed nuclear elements
LFA-3	lymphocyte function-associated antigen
LTR	long terminal repeat
mAb	monoclonal antibody
MAGE	melanoma-associated antigen
MART-1	melanoma-associated antigen recognized by T cells
MET	mesenchymal-epithelial transition



MUC-1	mucin glycoprotein
NAHR	non-allelic homologous recombination
NK	natural killer cell
ORF	open reading frame
OC	ovarian cancer
OCCC	ovarian clear cell carcinoma
PAP	prostatic acid phosphatase
PLZF	promyelocytic leukemia zinc finger
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
pol	polymerase
pro	protease
PSA	prostate-specific antigen
PSMA	prostate-specific membrane antigen
RT	reverse transcriptase
RT-PCR	reverse transcription-polymerase chain reaction
SINE	short interspersed nuclear elements
SU	surface
TA	thyroid adenoma
TIL	tumor-infiltrating lymphocyte
TM	transmembrane
TNF- $\alpha$	tumor necrosis factor- $\alpha$
VEGF	vascular endothelial growth factor





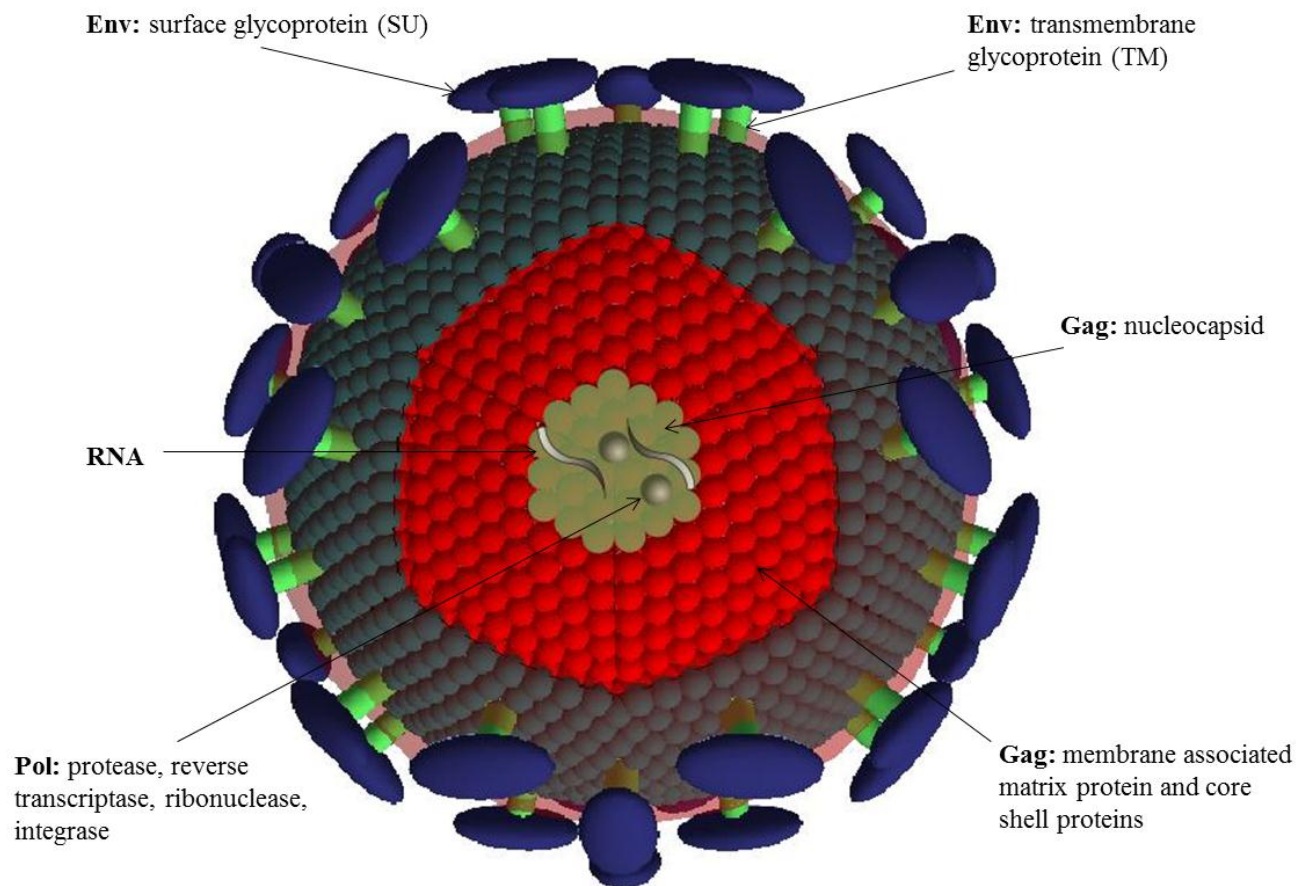


## **Chapter 1: Introduction**

### **1.1 Retroviruses**

Retroviruses are RNA viruses that are duplicated in a host cell via reverse transcriptase (RT) to produce viral genomic DNA, which is then incorporated into the host's genome. Subsequent retroviral replication occurs concomitantly with the host cell's DNA. This reverse flow of genetic information from RNA to DNA and the establishment of DNA in an integrated form in the host genome are defining aspects of retroviruses. The virion particles, approximately 100nm in diameter, are spherical and are surrounded by an envelope consisting of a lipid membrane bilayer. The surface is studded by projections of an envelope glycoprotein. Beneath the membrane is a spherical layer of protein, called the capsid protein, and an internal nucleocapsid. Based on new genera named by the International Committee on Taxonomy of Viruses, simple retroviruses encode only the Gag, Pro, and Env gene products. In addition to these gene products, complex retroviruses encode an array of small regulatory proteins with a range of functions. The cleavage of Gag, Pol and Env precursors forms the products in the mature infectious virions including the following proteins: membrane-associated matrix protein, capsid, nucleocapsid, protease, dUTPase, reverse transcriptase, integrase, surface protein (SU), and transmembrane (TM) protein<sup>1</sup>. Virus particles contain 2 copies of RNA of ~8 to 10 kb in length (Figure 1).





**Figure 1:** Schematic structure of a retroviral particle. The surface is studded by projections of an envelope glycoprotein consisting of the SU and TM proteins. The matrix protein and core shell derived from gag are depicted in the center of the virus. The 2 RNA molecules, polymerase, RNase, and integrase are shown within the core shell.



Typically, the targets of retroviruses are somatic cells, however, when the target is a gamete, the provirus becomes a permanent fixture within the offspring's genome. Literally meaning "born within", these endogenous retroviruses (ERVs) are then inherited across generations. Some of these elements first entered the primate genome more than 40 million years ago. However, new proviral copies have been generated more recently that are human specific <sup>2,3</sup>. Human endogenous retroviruses (HERVs) are believed to have entered the human genome around 30 million years ago via infection of the germline, and since then have persisted as stably integrated, vertically transmitted proviruses <sup>4,5</sup>. When we examine the collective genome of *Homo sapiens* we find that humans share most of their ERVs with chimpanzees, great apes, gibbons, and old world monkeys <sup>6,7</sup>. HERVs comprise up to 8% of the human genome and are widely dispersed <sup>8</sup>. HERVs lack mutagenic activity and in fact nearly 90% of HERV elements exist as recombined solitary long terminal repeats (LTRs) <sup>8,9</sup> and the remainder have accumulated inactivating mutations during evolution <sup>10</sup>. Specifically, the majority of HERVs are incomplete and faulty in terms of replication due to multiple termination sequences <sup>11-15</sup>. In most cases HERVs are transcriptionally silenced by epigenetic mechanisms including methylation, histone modifications, and small RNAs. However, our knowledge of how these modifications demarcate and silence HERVs in the human genome is limited.

HERVs belong to the class of retroelements, which can be subdivided into elements with regulatory LTRs (8.3% of our DNA;  $0.3 \times 10^6$  copies) and elements without LTRs (33.9%;  $2.4 \times 10^6$  copies) <sup>16</sup>. Short and long interspersed nuclear elements (SINEs and LINEs), members of the non-LTR group, are present in very high copy numbers. LINEs are abundant retrotransposons that comprise approximately 20% of mammalian genomes.



LINEs encode a RT that can be utilized for retrotranspositions of SINEs and LINEs or for the formation of pseudogenes <sup>16</sup>. The LTR containing retroelements can further be divided into 6 superfamilies <sup>17</sup>, based solely on sequence relationship: class I (gamma retroviruses) representing most families and the largest genomic fraction <sup>9, 18</sup>, class II (beta retroviruses) which contain the HERV-K elements and older families <sup>19</sup>, and class III (spuma retroviruses) <sup>9</sup> which contain the oldest recognizable HERV elements <sup>8</sup>. The other superfamilies MER4, MST and MLT represent ancient retrotransposons with no known functions in humans <sup>16</sup>.

## **1.2 HERV-K(HML2) (human MMTV-like 2) Family**

HERVs are classified into families according to the single-letter amino acid code corresponding to the tRNA primer used for reverse transcription of the HERV genome <sup>20</sup>. Thus, HERV-K's tRNA primer begins with lysine, HERV-W's begins with tryptophan and so on. The type K family (HERV-K) proviruses are present at approximately 30-50 copies per haploid human genome, but many are polymorphic in humans. HERV-K is located on chromosome 19 and possesses large open reading frames in the genes putatively encoding Gag, Pol, Env and a protease, flanked by two non-coding LTRs <sup>21</sup> (Figure 2). Therefore, HERV-Ks contain all structural features necessary for viral replication <sup>21</sup>. The *gag* gene encodes matrix, capsid (core shell proteins) and nucleocapsid proteins necessary for viral RNA encapsidation and particle formation. The *pol* gene encodes the viral enzyme machinery needed for conversion of the viral RNA to the proviral DNA form, including RT and integrase. The *env* gene encodes for two different proteins important for virus infectivity: an outer surface membrane (SU) protein and a transmembrane (TM) protein.



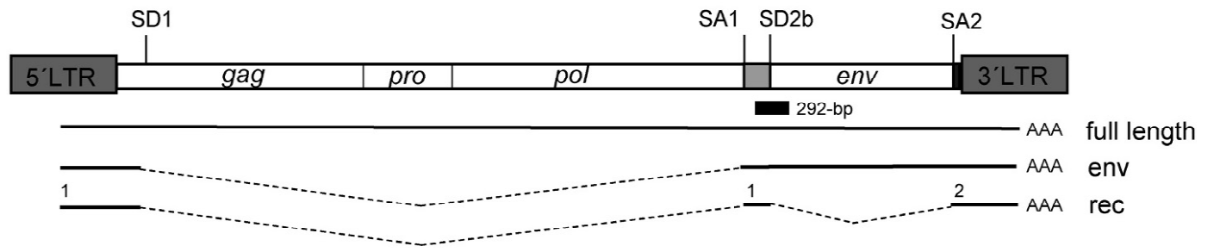
The LTRs contain elements involved in transcription initiation and termination <sup>22</sup>. HERV-K possesses 2 accessory proteins termed Rec and Np9. Rec, a protein functionally related to HTLV Rex and HIV Rev, is responsible for the nuclear export of unspliced or single-spliced viral mRNA into the cytoplasm. Np9 is a nuclear protein.

Several studies have led to the identification of 18 coding *env* genes, with the most important contributor being the HERV-K(HML2) family<sup>23-25</sup> which contains six coding *env* genes. The entry dates of all six copies with a coding *env* are less than five million years ago.<sup>26, 27</sup>

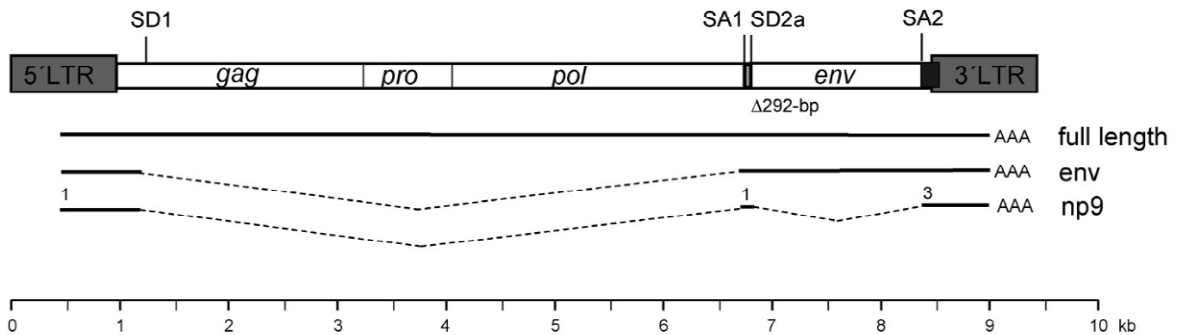
In 1986, Ono M, Yasunaga T, Miyata T, and Ushikubo H, determined the complete nucleotide sequence of the human endogenous retrovirus genome HERV-K10, a member of the human endogenous retrovirus HERV-K family. The sequence was shown to be a full-length 9.2-kb provirus with LTRs at each end <sup>21</sup>. HERV-K22, another member, contained an additional 292-bp sequence between the *pol* and *env* genes. Subsequently, the genome missing the 292-bp sequence was designated “type 1” and the genome containing the 292-bp sequence “type 2” <sup>28</sup>. The 292 bp deletion found in HERV-K type I leads to the fusion of the *pol* and the *env* gene and the absence of the *rec* gene. HERV-K type II, the HERV-K prototype, has open reading frames (ORFs) for the structural proteins Gag and Env, RT <sup>29</sup>, and the regulatory protein Rec <sup>30</sup>. These two types contain ORFs encoding genes required for retroviral replication (Figure 2).



## HERV-K(HML-2.HOM, type 2)



## HERV-K101 (type 1)



**Figure 2:** Schematic representation of full-length HERV-K(HML-2) type 1 and 2 proviruses and their respective mRNA transcripts. There are three genetic domains, flanked by the regulatory LTRs. The *gag* domain encodes for matrix and core shell (capsid) proteins, the *pol* encodes for reverse transcriptase, protease, integrase and ribonuclease, and the *env* encodes for the surface and transmembrane proteins. Splice donor (SD) and splice acceptor (SA) sites are shown. There are two types of HERV-K (HML-2) proviruses in the human genome differing by the presence (type 2; black rectangle) or absence (type 1;  $\Delta 292$ -bp) of a 292-bp sequence at the *pol*-*env* boundary<sup>28</sup>. HERV-K(HML-2.HOM) is an example of a type 2 provirus, HERV-K101 an example of a type 1 provirus. In type 2 proviruses, the 292-bp sequence harbors a splice donor site (SD2b) involved in the generation of doubly spliced transcripts coding for the two exons (light grey and dark grey boxes) of the accessory ~15kDa protein Rec by some type 2 proviruses<sup>30,31</sup>. In type 1 proviruses, an alternative splice donor (SD2a) located upstream of the 292-bp stretch is used to generate mRNAs coding for Np9<sup>32</sup>. Np9 is an ~9 kDa, 74-amino acid protein that shares its 14 N-terminal amino acids (exon 1, light grey box) with Rec and Env, whereas the 60 C-terminal amino acids (exon 2, dark grey box) are derived from the third (non-Rec, non-Env) ORF. Numbers on top of rec and np9 transcripts indicate the reading frames. Adopted from reference<sup>33</sup> with permission from Springer.



### 1.3 Normal physiological effects of HERV

A very small proportion of HERVs can potentially encode proteins, however this proportion has led to a multitudinous amount of data showing that these and other defective copies are actively transcribed. This insinuates that many LTRs possess retained transcriptional regulatory functions that confer a beneficial function. Indeed, the fact that HERVs constitute approximately 8.3% of the human genome efficaciously asserts the theory that HERVs have provided some evolutionary advantages at the population level, and in fact several advantages have been proposed. First, HERVs constitute sequences that are substrates for genomic rearrangements and therefore could contribute to modeling and the plasticity of the genome<sup>33</sup>. Also of importance, retroviral LTRs naturally possess enhancer, promoter, and polyadenylation activities<sup>34</sup>. Therefore HERV LTRs can provide tissue-specific enhancers, alternative promoters, or alternative polyadenylation signals for nearby genes<sup>18</sup>. Indeed, LTRs function as promoters of retroviral genes and sometimes function as promoters of nearby cellular genes<sup>35-38</sup>. In fact, at least 50% of the human-specific HERV-K LTRs act *in vivo* as active promoters for non-repetitive DNA transcription<sup>39</sup>. For example, HERV LTRs account for over 30% of all p53 binding sites genome-wide, therefore contributing to the anti-oncogenic function of p53<sup>40</sup>. Also, Mager DL, Hunter DG, Schertzer M, and Freeman JD, identified two human genes, HHLA2 and HHLA3, for which HERV-H LTR served as the primary polyadenylation signal. Although the functions of the particular genes were unknown, this data supports the fact that retroviral LTRs assume gene regulatory functions<sup>41</sup>.



Another advantage is the possibility that the presence of ERVs may protect organisms from infections by related exogenous retroviruses via receptor interference<sup>42, 43</sup>. For example, HERV-W envelope glycoproteins have been shown to confer cellular resistance to superinfection by exogenous retroviruses<sup>44</sup>. Finally, to support the idea of physiological *in vivo* functions of HERV-encoded proteins, Mi S, Lee X, Li X, Veldman GM, Finnerty H, Racie L, LaVallie E, Tang XY, Edouard P, Howes S, Keith JC Jr, and McCoy JM, showed in 2000, that the env protein of HERV-W, termed Syncytin-1, mediates the formation of syncytiotrophoblasts from cytotrophoblasts which is a key step in normal placental morphogenesis<sup>45-47</sup>. Another example of beneficial functions of HERV-encoded proteins involves HERV-E, which is activated in a proportion of renal cancer cells. In a recent clinical study, HERV-E provided target antigens recognizable by cytotoxic T cells from donors after allogenic hematopoietic stem cell transplantation, leading to complete or partial tumor regression in patients<sup>48</sup>.

#### **1.4 HERV and Human Disease**

One hundred years after Francis Peyton Rous demonstrated that cancer could be caused by a virus, science has elucidated seven human viruses that have been found to cause 10-15% of human cancers worldwide. The International Agency for Research on Cancer estimates that one in five cancer cases worldwide are caused by infection, with most caused by viruses<sup>49, 50</sup>. These cancers are serious public health problems but most importantly these cancers have readily identifiable targets for diagnosis, prevention and therapy<sup>51</sup>. Vaccination programs against hepatitis B virus (HBV) and human papilloma-virus (HPV), both human tumor viruses, have already made huge impacts on worldwide cancer rates<sup>52-54</sup>.



However, modern science has since shown that in human cancer, virus infection alone is not sufficient for tumorigenesis. Cancer is a complex multistep process and it is the culmination of various molecular events that generate the final transformed cellular phenotype. Viruses can be divided into two groups based on the roles they play in tumorigenesis. Direct carcinogens express viral oncogenes in each cancer cell that directly contribute to cancer cell transformation and indirect carcinogens cause cancer through chronic infections and inflammation, ultimately leading to carcinogenic mutations<sup>55</sup>. With respect to HERV-K, the exact role it plays in cancer is unknown and future studies are needed to determine its carcinogenic effects.

For many decades, HERV expression at the protein and RNA levels was found to be associated with cancer and autoimmune diseases<sup>56-59</sup>. Indeed, the expression, promoter activity, and epigenetic regulation of HERV-Ks are extremely different between malignant and normal cells. Numerous studies have documented an increased expression of HERVs in terms of both transcriptional and protein levels in tumors, with the majority of research on the HERV-K family (Table 1).



Tumor type <sup>a</sup>	HERV type	Detection <sup>b</sup>	Gene(s)	Expression <sup>c</sup>	Reference <sup>d</sup>
Breast cancer	HERV-K	P	<i>gag</i>	+	[89]
+T47D	HERV-K,E,F,W,T,FRD,I	RNA	<i>pol</i>	, N/A	[77,78,99*,121*]
+T47D, MCF7, others	HERV-K	RNA	<i>env</i>	+	[93,122-125]
T47D	HERV-K	RNA, P	<i>gag, pol, env</i>	+	[92*]
+T47D, MCF7	HERV-K	RNA	<i>gag</i>	+	[126]
Leukemia/lymphoma	HERV-K	RNA, P	<i>gag, pol, env</i>	+	[93*]
	HERV-K	P	<i>gag</i>	+	[89]
	HERV-K	RNA	<i>gag</i>	+	[87,127]
+H9	HERV-K,-H	RNA	<i>pol</i> or <i>env</i>	+,	[81,125,128,129]
	HERV-K	RNA	<i>LTR</i>	+	[80]
K562, Jurkat, others	HERV-E	RNA	<i>gag, pol, env</i>		[79]
HL60, Jurkat, others	HERV-H	RNA	<i>gag, env</i>	+	[130]
Melanoma	HERV-K	P	<i>gag, pol, env, rec</i>	+	[97*]
	HERV-K	P	<i>gag</i> &/or <i>env</i>	+	[86,87,131*]
	HERV-K	RNA, P	<i>gag, env, rec</i>	+	[132*]
	HERV-K	RNA, P	<i>env, rec, np9</i>	+	[94*]
	HERV-K	RNA, P	<i>env</i>	+	[133]
Gastro-intestinal	HERV-K	P	<i>gag</i>	+	[87]
	HERV-K	RNA	<i>env</i>	+	[125]
	HERV-H	RNA	<i>gag</i>	+	[73,134,135]
Pancreatic	HERV-K	RNA	<i>env</i>	+	[136]
	HERV-H	RNA	<i>gag</i>	+	[73]
Lung	HERV-K	P	<i>gag</i>	+	[87]
	HERV-E	RNA	<i>LTR</i>	+	[137]
	HERV-R	RNA	<i>env</i>		[82]
Prostate	HERV-K	RNA, P	<i>gag</i>	+	[87]
	HERV-E,-R	RNA	<i>env</i>	+	[138]
Ovarian/endometrial	HERV-K	RNA, P	<i>gag</i>	+	[87]
	HERV-K,-E,-R,-W	RNA, P	<i>env</i>	+	[71,88,95]
	HERV-E	RNA	N/A	+	[83]
PA-1	HERV-K	P	<i>gag</i>	N/A	[52]
Jeg, Jar	HERV-H	RNA	<i>LTR</i>	N/A	[139]
Testicular/seminoma +GH	HERV-K	P	<i>gag</i> &/or <i>env</i>	+,	[52,89-91,140,141*]
	HERV-K	RNA	<i>gag</i>	+	[142,143]
+GH, Tera-1, others	HERV-K,-H	RNA	<i>LTR</i>	+	[139,144]

<sup>a</sup> All examples describe data from primary samples, unless a cell line is listed (indent).

<sup>b</sup> Detection of viral genes at the transcriptional (RNA) or translational level (P).

<sup>c</sup> Cancer-specific up-regulation of HERV is denoted by a '+', but examples where only a specific family of HERV among a number of analyzed families are demarked by '|'.

<sup>d</sup> An asterisk indicates those studies that identified/purified virus-like particles; N/A denotes insufficient information.

**Table 1:** Expression of HERVs in human cancer: Expression of various HERV types is shown in various tumor types. The detection of viral genes at the transcriptional (RNA) or translational levels (P) is shown. Adapted from reference <sup>10</sup> with permission from Elsevier.



At the time the complete nucleotide sequence of HERV-K10 and HERV-K22 was determined <sup>21</sup>, the types, structures, and functions of human endogenous retroviruses and their relationships to human cancer were not known. However, Ono and his colleagues advocated the possibility that a particular antigen expressed in human BC tissue and T47D cells was the env gene product of a potentially active HERV-K provirus. The following year, Ono M, Kawakami M, and Ushikubo H determined that stimulation of HERV-K genome expression was observed in the human BC cell line T47D after estradiol treatment <sup>60</sup>. Correspondingly, Lower R, Lower J, Tondera-Koch C, and Kurth R demonstrated that upon steroid hormone treatment, human teratocarcinoma cells showed an increase in expression of the HERV-K genome and production of retroviral-like particles <sup>61</sup>. Similarly to the teratocarcinoma cells and supporting the data from the previous studies, T47D cells also showed an increase in the expression of the HERV-K genome and the production of retroviral-like particles upon hormone stimulation <sup>62-64</sup>. In addition, Etkind PR, Lumb K, Du J, and Racevskis J determined that T47D contains the type 1 HERV-K genome which is spliced to subgenomic transcripts <sup>65</sup>.

Willer and his colleagues were the first to characterize the HERV-K env transcription pattern in several malignant tissues and in peripheral blood mononuclear cells (PBMCs) via RT-PCR <sup>66</sup>. HERV-K env transcripts were found in all of the following tissues: BC, Hodgkin's disease, thyroid adenoma (TA), colon cancer, chronic myelogenous leukemia (CML), high and low grade non-Hodgkin's lymphomas, and myelodysplastic syndrome. HERV-K env transcripts were also found in PBMCs from patients with gastric cancer, and BC.



In 2001, Wang-Johanning F, Frost AR, Johanning GL, Khazaeli MB, LoBuglio AF, Shaw DR, and Strong TV investigated the expression of HERV sequences in BC and determined that indeed HERV-K transcripts are specifically and frequently expressed. The group further substantiated their results by determining that some of these transcripts contained ORFs that produced env protein <sup>67</sup>. To build on this knowledge, the same group investigated the expression of type 2 HERV-K *env* mRNAs and the splicing patterns of both types of HERV-K in human BC tissues. They discovered that there was an increase in HERV-K *env* and spliced *env* expression in human BC tissues. Specifically, type 2 HERV-K *env* transcripts were detected in human BCs. Of great importance, expression was significantly higher in most BC tissues than in normal breast tissues <sup>68</sup>.

In an exceptional study by Dewannieux M, Blaise S, and Heidmann T, it was demonstrated that a member of the HERV-K family, termed K108, encoded a functional env protein <sup>69</sup>. They discovered that the env protein was exported to the cell surface, cleaved into SU and TM subunits, and generated infectious pseudotyped particles. Combined with the data that HERV-K-related particles had been detected in culture supernatants from teratocarcinoma and melanoma cell lines, these findings gave credence to the idea that infectious retroviruses could be produced if they incorporated the HERV-K108 env protein <sup>3, 24, 28, 70</sup>.

Another study by Dewannieux and his colleagues helped to unify the preceding results by showing it was possible to derive the sequence of HERV-K and construct a consensus HERV-K provirus, which they termed Phoenix <sup>71</sup>. The element produced viral particles complete with structural and functional properties of a retrovirus and had the ability



to infect human cells. Upon infection, Phoenix integrated with the exact signature of the presently found endogenous HERV-K progeny. Significantly, they showed that *in vitro* recombination among present-day human HERV-K loci can generate functional elements, indicating the possibility that human cells can produce infectious retroviruses <sup>71</sup>. Similarly, in an exciting 2008 paper, Boller and his colleagues proved that the HERV-K provirus is capable of producing intact retroviral particles when cloned into a baculovirus expression vector, exposing a real-life active human endogenous retrovirus of today <sup>72</sup>.

Studies reporting the presence of retrovirus-like particles in human melanomas date back to the 1970s <sup>73, 74</sup>, however it wasn't until 2003, that Muster T, Waltenberger A, and Grassauer A made a novel discovery by showing that retrovirus-like particles, which exhibit RT activity and have homologous sequences to HERV-K, are produced by human melanoma cells. This group also showed expression of the *pol* gene and Gag, Env, and Rec proteins in human melanomas. Most importantly, these expressions were not seen in melanocytes or normal lymph nodes <sup>75</sup>. To supplement this data, Buscher K, Trefzer U, Hofmann M, Sterry W, Kurth R, and Denner J, showed expression of viral proteins in melanomas and the presence of HERV-K-specific antibodies in patients with melanomas <sup>76</sup>. Hahn S, Ugurel S, Hanschmann HM, Strobel H, Tondera C, Schadendorf D, Lower J, and Lower R expounded on the discovery that expression of HERV-K proteins induce humoral immune responses in melanoma, and showed that serological response to HERV-K correlated with survival probability in melanoma patients. The hope at that point was the possibility of using anti-HERV-K reactivity as a way to monitor patients suffering from melanoma <sup>77</sup>.



A key paper in the discovery of potential mechanisms of HERV-K came from Serafino A, Balestrieri E, Piermarchi P, Matteucci C, Moroni G, Oricchio E, Rasi G, Mastino A, Spadafora C, Garaci E, and Vellabona PS <sup>78</sup>. He and his colleagues demonstrated that human melanoma cell lines undergo a variety of morphological and molecular alterations typical of malignant cells in response to a massive HERV-K expression and subsequent production of HERV-K-related viral-like particles. This strongly indicated that activation of HERV-K expression is a key element in morphological and functional cell changes during melanoma progression <sup>78</sup>.

Several groups have found cytotoxic T-lymphocyte (CTL) responses against HERV-K antigens in melanoma patients and seminoma patients <sup>79, 80</sup>. The first evidence that HERV-encoded peptides expressed in human tumor cells could also be targets for antitumor cytotoxic CD8<sup>+</sup> T cell responses, came from a study that identified a HERV-K (HML-6)-encoded HLA-A2-restricted peptide (HERV-K-Mel) which was recognized in autologous melanoma cells by CTLs from two patients with melanomas <sup>80</sup>. This suggested that HERV-K proteins can function as tumor antigens that are targeted by CTLs and could potentially be used for vaccination.

In terms of research on expression of HERV families in other cancers, a paper by Cherkasova E, Malinzak E, Rao S, Takahashi Y, Senchenko VN, Kudryavtseva AV, Nickerson ML, Merino M, Hong JA, Schrupp DS, Srinivasan R, Linehan WM, Tian X, Lerman MI, and Childs RW showed that HERV-E is expressed in renal cell carcinoma (RCC) and is highly immunogenic <sup>81</sup>. HERV-E expression has also been implicated in prostate cancer <sup>82</sup> and a recent study by Ishida T, Obata Y, Ohara N, Matsushita H, Sato S,



Uenaka A, Saika T, Miyamura T, Chayama K, Nakamura Y, Wada H, Yamashita T, Morishima T, Old LJ, and Nakayama E identified the HERV-K gag protein in serum from prostate cancer patients<sup>83</sup>. HERV-H expression has been shown in gastrointestinal cancer<sup>84</sup>. Transcripts containing the HERV-A LTR sequence were even found in a lung cancer cell line with no transcripts detectable in normal lung tissue<sup>85</sup>. HERV-K-MEL was found to be expressed with a high prevalence in pancreatic cancer patients<sup>86</sup>. In terms of diseases other than cancer, it has been shown that HERV-K viral load can be detected in plasma samples from rheumatoid arthritis patients. Specifically, viral load is higher in plasma samples from those with active disease<sup>87</sup>. HERVs have also been shown to be expressed in brain from patients with multiple sclerosis<sup>88, 89</sup>. In an innovative study, Contreras-Galindo R, Kaplan MH, Markovitz DM, Lorenzo E, and Yamamura Y illustrated that type 1 and type 2 HERV-K viral RNA genomes were found to coexist in the same plasma of HIV-1 patients<sup>90</sup>. The same group later showed that HIV-1 viral particles upregulated HERV-K RNA in a dose-dependent manner<sup>91</sup>. Extending these results, it was also observed that HERV (-H, -K, and -L family)-specific T cell responses in HIV-1-infected individuals were identified and inversely correlated with HIV-1 plasma viral load and positively correlated with CD4<sup>+</sup> T cells counts. These results generated the idea that HERV-specific T cells may potentially participate in controlling HIV-1 replication and that therapeutics targeting HIV-1-infected cells with HERV specific cytotoxic T cells could be beneficial<sup>92, 93</sup>.

### **1.5 HERV-K in BC in our laboratory**

For many years our laboratory has focused on BC and HERV-K env protein expression. In 2008, we demonstrated that HERV-K env proteins are strongly expressed in



human invasive BC and DCIS, and are correlated with lymph node metastasis in comparison with adjacent benign tissues. We further showed that HERV-K env protein elicits both serologic and cell-mediated immune responses. Specifically, CD8<sup>+</sup> T-cell proliferation specific for HERV-K was detected in PBMCs in a high proportion of BC patients after *in vitro* stimulation with the HERV-K protein. CD8<sup>+</sup> cytotoxic T-cells also specifically killed HERV-K expressing established target cells. More importantly, little or no responses were detected in lymphocytes from normal donors <sup>94</sup>.

To reinforce these conclusions, we obtained samples from new patient cohorts from the U.S. and China and illustrated that the HERV-K *env* gene is expressed in the majority of BC samples from both cohorts but not in adjacent noncancerous tissue or normal breast tissue. HERV-K env protein expression was significantly associated with disease stage, a positive node status, and poor disease outcome, suggesting expression may enhance metastatic spread in women diagnosed with BC. However, HERV-K env expression was not associated with age, ER or PR protein expression, or histological grade. We therefore concluded that activation of HERVs appear to be a common event in BC regardless of geographic location.<sup>95</sup>

In 2012, we combined our HERV-K knowledge with cancer immunotherapeutics and were the first to show that monoclonal and single-chain antibodies against the HERV-K env were capable of blocking growth and proliferation, and inducing apoptosis of human BC cells *in vitro*. We also demonstrated that treatment with an anti-HERV-K monoclonal antibody (mAb) significantly inhibited tumor growth in mice bearing BC xenograft tumors <sup>96</sup>.



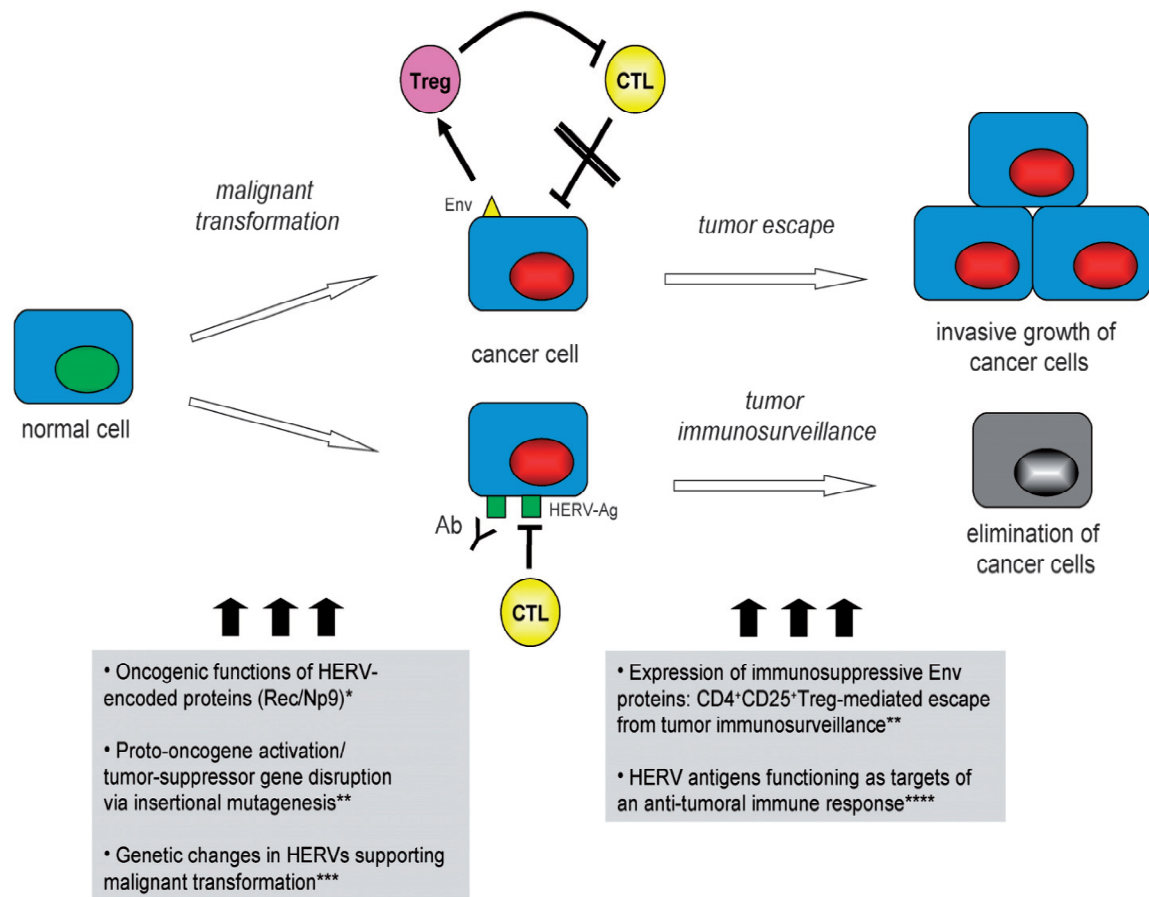
## 1.6 Proposed functions of HERVs in Human Disease

HERVs have been implicated in the pathogenesis of diseases, in particular cancer and autoimmunity<sup>97</sup>, however the specific role they play remains an enigma. HERVs have resided in their host genomes for millions of years and still show similarities with exogenous infectious retroviruses with respect to internal genome organization and the presence of LTRs. The idea that endogenous retroviral elements play key biological roles is bolstered by their significant distribution in mammalian genomes. Therefore, much consideration had been given to the specific functions of HERVs in these biological roles, producing several diverse mechanisms (Figure 3). Firstly, HERVs could be acting as intra or inter-chromosomal recombination sites causing genomic plasticity and chromosomal aberrations. Non-allelic homologous recombination (NAHR) between members of the same endogenous viral element family that are located on the same or different chromosomes may lead to genomic rearrangements such as deletion, duplication, inversion or translocation events<sup>98</sup>. A recent study of HERV-K shows that 6 (17%) of the 35 full-length copies examined have undergone some form of NAHR following insertion<sup>99</sup>. Indeed somatic rearrangements might be relevant for tumorigenesis, for example, a recurrent translocation event that creates an oncogenic HERV-K-ETS translocation variant 1 (ETV1) fusion gene has been implicated in prostate cancer<sup>100</sup>. Secondly, proviral insertions may produce somatic or germ line mutations resulting in a loss of cellular gene function. Even though no replication-competent HERV has been identified in the human genome to date, the idea of insertional mutagenesis continues to be of considerable interest. Thirdly, solitary or proviral LTRs could possibly modulate adjacent cellular gene expression. Finally, expression of HERV specific proteins may have a pathological influence<sup>22</sup> (Figure 3).



Two processes implicate the mechanism of tumor immune escape mediated by immunosuppressive endogenous retroviral Env proteins. One process is in the induction of immune tolerance at the materno-fetal barrier via a physiological expression in the placenta<sup>101</sup>. The other process is the suppression of an antitumoral immune response through aberrant expression in cancers. Also, data has shown that HERV-K accessory proteins, both Rec and Np9, have demonstrated oncogenic properties. The first strong evidence that Rec expression causes the development of germ cell tumors *in vivo*, was by Boese A, Sauter M, Galli U, Best B, Herbst H, Mayer J, Kremmer E, Roemer K, Mueller-Lantzsch N in 2000<sup>102</sup>. To understand the molecular mechanisms underlying the oncogenic effect seen in Rec expression, studies were executed to discover proteins that interacted with Rec. One of these proteins was the promyelocytic leukemia zinc finger (PLZF) protein<sup>102</sup>, which is a tumor suppressor, transcriptional repressor of the *c-myc* proto-oncogene, and spermatogonial stem cell regulator. Cells stably transfected with PLZF and Rec showed increased cell proliferation and a reduced apoptosis rate compared to cells stably transfected with PLZF alone<sup>103</sup>. After Np9 was identified<sup>103</sup>, *np9* transcripts were detected among malignant cells but not in normal cells<sup>32</sup>. Np9 was later found to directly interfere with an ubiquitin ligase that regulates the transcription factor Notch via degradation of the Notch-antagonist Numb<sup>104</sup>. The Numb/Notch pathway is an essential part of the proproliferative Ras signaling, and more importantly has been suggested to be involved in germ cell tumors by causing genetic instability<sup>105</sup>. The regulation and effector pathways of Rec and Np9 need to be further explored in order to determine the complete contribution of these proteins in human disease.





**Figure 3:** Possible mechanisms by which HERVs could be involved in tumorigenesis. A simplified scheme of tumorigenesis and potential fates of malignant cells is depicted to indicate steps in tumorigenesis in which HERVs may play a role. The process by which a normal cell (green nucleus) turns into a cancer cell (red nucleus) is referred to as malignant transformation. Possible mechanisms by which HERVs contribute to malignant transformation are listed. Malignant cells are normally controlled by the immune system (tumor immunosurveillance). Expression of HERV antigens (HERV-Ags, green squares) on tumor cells may elicit an antitumor immune response involving, among others, antibodies (Ab) and CD8<sup>+</sup> cytotoxic T cells (CTL). Ideally, antitumor immune responses result in the elimination of cancer cells. Expression of immunosuppressive endogenous retroviral envelope (Env, yellow triangle) proteins on tumor cells may lead to a CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell (T-reg)-mediated suppression of antitumor CTL, leading to escape of tumor cells from immunosurveillance (tumor escape) and consecutive invasive cancer growth. The level of evidence of the outlined mechanisms for human cancers is indicated. \* Some evidence for potential relevance in human cancer (germ cell tumors). \*\* Evidence in animal models, so far no evidence in humans. \*\*\* Hypothetical mechanism. \*\*\*\* Some evidence for anti-HERV immune responses in certain human cancers; very limited data concerning their functional relevance. Adapted from reference<sup>33</sup> with permission from Springer.



## 1.7 Ovarian Cancer

Ovarian cancer (OC) is the most common cause of death in gynecologic malignancies and its prognosis has only modestly improved over the last few decades<sup>106</sup>. Epithelial OC, the main type of this disease, accounts for more than 90% of all malignant ovarian tumors. Despite improvements in systemic therapy, advanced OC remains highly lethal with more than 90% of patients developing tumor recurrence, resulting in five-year survival rates of only 30%<sup>107</sup>. The incidence and mortality rate of OC has remained unchanged over the past decade and there is an obvious need for more sensitive markers to detect OC at an early stage and to monitor disease progress. Currently there is no test with sufficient predictive value for use in screening and detection of pre-malignant or localized OC<sup>108</sup>, and 70% of patients with OC present with advanced disseminated disease at the time of initial diagnosis<sup>109</sup>. Therefore, the identification of novel diagnostic serum biomarkers specific to and widely expressed by early-stage OCs is urgently needed for the future development of diagnostic tests for early detection and for development of new immunotherapy regimens<sup>108</sup>.

There are six tumor subclasses distinguishing patients with epithelial OC<sup>110</sup>. The most common form is termed serous carcinomas and represents 40% of all cases. The remaining epithelial ovarian cancer subclasses are endometrioid, mucinous, clear cell, transitional, and undifferentiated. Approximately 90% of epithelial ovarian cancers are sporadic, and the remaining 10% are associated with inherited mutations in the BRACA1 or BRACA2 functions<sup>111, 112</sup>. Due to their anatomical location, a high percentage of ovarian tumors disseminate throughout the peritoneal cavity and implant on various organs, thereby



forming ascites. The following stages represent degrees of tumor dissemination: stage I-II disease is confined to one or both ovaries, stage III disease shows peritoneal spread, and stage IV disease shows distant metastases <sup>113</sup>. Stage III/IV serous ovarian cancer is the most lethal and unfortunately the most common.

In early stages, small OCs are typically difficult to detect and therefore OC is often discovered in the late stages when it has spread beyond the ovaries. Current treatment consists of surgical removal of the tumor, hysterectomy, salpingo-oophorectomy, and omentectomy, followed by systemic chemotherapy. Specifically, the treatment involves aggressive tumor cytoreductive surgery followed by platinum- and taxane-based chemotherapy, with a response rate to first-line chemotherapy in approximately 65-80% of patients <sup>114</sup>. Unfortunately, most ovarian carcinomas relapse. First-line chemotherapy with cisplatin and platinum is able to achieve complete remission in 65-80% of late stage OC patients but these treatments are ineffective in OC relapse, and drug resistance contributes to the comparatively low five-year survival rate.

The central premise in OC immunotherapy is that tumor-associated antigen expression can be recognized by CTLs leading to tumor destruction <sup>115, 116</sup>. Examples of ovarian tumor antigens recognized by CTL include HER2, CA125, and MUC-1 <sup>117-122</sup>. These antigens have been incorporated into cancer vaccines currently in clinical trials <sup>123-126</sup>, however clinical efficacy of these vaccines has exhibited only modest results. CA-125 is the only marker approved for monitoring OC progression and treatment response, but it has a very low sensitivity for early stage OC and accounts for a high rate of false positive values <sup>127</sup>.



Several other OC antigens have been identified, however, but which antigens are tumor rejection antigens is unknown (EGF receptor-derived peptide p971-980, FR $\alpha$ , IGFBP-2, MUC16, p53, EpCAM, and some members of the CTA family). Ioannides and his colleagues discovered CD8<sup>+</sup> T cells, isolated from ovarian malignant ascites, capable of recognizing the human EGF receptor (EGFR)2 (HER-2/neu)-derived peptide, p971-980 <sup>128</sup>. HER-2/neu is an attractive immunologic target because of its low-level expression in peripheral tissues. However, the percentage of ovarian tumors with HER-2/neu expression has ranged from 5 to 66% <sup>129-132</sup>, and data concerning the prognostic relevance of its gene amplification or protein overexpression in OC has been contradictory. In another study, People and his colleagues found that tumor-associated lymphocytes isolated from the malignant ascites of OC patients recognized naturally processed and presented peptides derived from folate receptor (FR $\alpha$ ) <sup>133, 134</sup>. It was also determined that FR $\alpha$  expression in nonmucinous ovarian tumors increased approximately 90-fold compared with normal epithelial cells <sup>135</sup>. Expression of FR $\alpha$  is limited to a few specific tissues such as the apical surface of kidney tubule epithelium <sup>136</sup>. Thus, FR $\alpha$  is a promising therapeutic target not only due to its tumor specificity and high-level expression, but also because it is naturally immunogenic. However, there have been no follow-up reports confirming that FR $\alpha$  is an effective tumor-associated antigen. Mutation in the p53 gene is one of the most common, single genetic alterations in sporadic human epithelial ovarian carcinoma <sup>137</sup>. As a result of mutation, p53 is overexpressed in nearly 50% of OCs. However, in a recent immunotherapeutic study, small peptides derived from TP53 delivered in an emulsion-adjuvant resulted in a weak T cell response and no tumor regression in advanced OC patients <sup>138</sup>.



T cells in the tumor microenvironment are key players in patient outcomes. T cell infiltration into ovarian tumors is associated with improved survival and in tumors with high numbers of tumor-infiltrating T lymphocyte cells (TILs), the expression of monokines induced by IFN- $\gamma$ , macrophage-derived chemokines and secondary lymphoid-tissue chemokines, is significantly increased as compared with tumors lacking T cells <sup>139</sup>. Thus, the immune infiltrate has an active role in the clinical course of OC <sup>139</sup>. It has also been determined that there is improved survival in OC patients who have higher numbers of intraepithelial CD8<sup>+</sup> T cells compared with patients without intraepithelial CD8<sup>+</sup> T cells <sup>140</sup>. A recent study shows a strong positive correlation 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 <sup>141</sup>. Concerning CD4<sup>+</sup> helper T-cell infiltration, Kryczek and colleagues found high levels of IL-17 associated with improved outcome suggesting that a subset of CD4<sup>+</sup> Th cells, Th17 cells, have a role in eradicating ovarian tumors <sup>142</sup>. In another example, Tomosova and his colleagues have shown that patients exhibiting higher CD3<sup>+</sup> TIL cell numbers had an improved overall survival than those patients with lower CD3<sup>+</sup> cell numbers <sup>143</sup>. These conclusions provide possible insight needed for further immune therapies designed to influence recruitment of helpful T cell subsets.

## **1.8 Immune suppression and microenvironment in ovarian cancer**

A major reason for the current lack of effective immune-based treatments is that ovarian tumors establish a complex multilayered immune suppression network which suppresses any antitumor efforts <sup>144</sup>. There are several mechanisms by which tumors can arrest the immune response. First, tumors can increase the numbers of T regulatory cells (T-



regs) in the peripheral blood of OC patients <sup>145</sup>. Secondly, tumors can recruit or induce T-reg tumor infiltration. Indeed, a study by Curiel and his colleagues demonstrated that the accumulation of intratumoral T-reg was associated with a high mortality rate <sup>146</sup>.

As mentioned previously, it is believed that the presence or absence of specific populations of T cells within epithelial OC tumors is associated with important differences in prognosis. Importantly, the detection of antitumor immune response in the form of intraepithelial tumor infiltrating lymphocytes (TILs) predicts significantly longer survival in OC <sup>139</sup>. Alternatively, the presence of T-reg in ovarian tumors has been associated with reduced overall survival <sup>146, 147</sup>. Indeed, Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, and Zou W found evidence that CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> regulatory T cells in patients with ovarian carcinoma suppress tumor-specific T-cell immunity and contribute to growth of human tumors *in vivo* <sup>146</sup>. Also, the presence of T-reg was found in OC ascites but not in normal ascites <sup>148</sup>. This insinuates that T-reg depletion can possibly become a promising strategy for boosting TAA-specific immunity<sup>149</sup>.

Vaccines have been the main approach to OC immunotherapy <sup>108, 146, 150, 151</sup>, however much work is needed to improved their performance. Depletion of T-reg is a critical maneuver to enhance vaccine therapy. To test this, a pilot study at the University of Pennsylvania is currently administering partially mature dendritic cells (DCs) pulsed with autologous tumor cell lysate to subjects with recurrent OC in combination with immune modulation with oral metronomic cyclophosphamide (to deplete T-reg) and bevacizumab



(to disrupt the blood tumor endothelial barrier)<sup>152</sup>; partial objective responses have been observed.

In addition to infiltration by T-regs, expression of the negative regulatory co-receptor B7-H1 by tumor cells and infiltrating myeloid DCs, expression of B7-H4 by tumor-associated macrophages, and expression of indoleamine 2,3-dioxygenase (IDO) all correlate with increased morbidity and mortality in OC. Strategies for depletion of myeloid suppressor cells, blockade of B7-H1 signaling, or inhibition of IDO activity are all currently under investigation as free-standing treatments or adjuvants for active immunotherapy for OC<sup>153</sup>. Overcoming suppressive mechanisms found within the tumor microenvironment in order to enhance efficacy is a major challenge for current approaches to cancer vaccination.

Recent studies have determined that the immune system plays a critical role in controlling growth and spread of ovarian tumors and therefore it is critical for future immunotherapies to overcome these immune inhibitory mechanisms. Various studies have shown that OC escapes immune surveillance with high efficiency via creating a tolerogenic microenvironment. OC-infiltrating plasmacytoid DC and macrophages produce the chemokine CCL22 which acts via CCR4 expressed by Th2 cells<sup>154</sup>, memory T cells<sup>155</sup> and regulatory T cells<sup>156</sup>. High levels of CCL22 have been detected in OC ascites and OC but not in normal ovaries<sup>146</sup>. This group specifically found that CCL22 expression in the tumor microenvironment triggers T-reg migration into the tumor lesion. They also determined that CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T-regs suppress tumor-specific T-cell immune responses in OC patients, thus promoting tumor growth *in vivo*, and that tumor T-reg infiltration is correlated



with reduced survival <sup>146</sup>. This indicates that OC tumor progression exploits different types of mechanisms to hamper the immune response and evade immune surveillance.

Limited success of cancer vaccines are also due to other negative influences in the tumor microenvironment. One particular example pertains to the lack of T-cell costimulatory molecules in most solid tumors. This results in activated T cells becoming anergized and losing lytic capacity. It has also been shown in preclinical models of chronic viral infection that T cells chronically exposed to viral antigen can become exhausted <sup>157, 158</sup>. Interstitial pressure within a large tumor mass is another negative influence as it diminishes diffusion of macromolecules such as antibodies and T cells <sup>159, 160</sup>. Also, T-reg, tumor cells, and other cells in the tumor microenvironment have the potential to release soluble immunosuppressive factors including TGF- $\beta$ , IL-10, indoleamine-pyrrole 2,3 dioxygenase (IDO) and VEGF <sup>161-163</sup>. Finally, the phenotype of tumors also has the potential to change as seen with the tendency for IL-8 to drive carcinoma cells to the EMT phenotype <sup>164</sup>.

Many tumors display antigenic heterogeneity at both the level of antigen expression and at the level of the antigen-peptide MHC complex <sup>165</sup>. Defects in MHC-peptide complexing machinery has also been detected in tumor cells <sup>166-170</sup>. An example of this was seen when cloned carcinoma cells reverted to express a more heterogeneous antigenic population upon passage. This phenomenon has also been witnessed in clinical trials in which vaccinated patients develop higher levels of T cell responses to tumor-associated antigens compared to the antigen in the vaccine <sup>171-173</sup>.

As stated above, recent studies have identified a survival advantage associated specifically with the presence of intraepithelial TILs, located in specific tumor islets <sup>174</sup>.



The intraepithelial TILs in OC specimens are a robust biomarker for overall survival of women with this disease. If validated, TIL scores could be used not only to select women for clinical trials of cancer vaccines or adoptive T cell therapies, but could also be used to select combinations of chemotherapeutic drugs that interact positively with antitumor immune mechanisms<sup>174</sup>.

## **1.9 Involvement of HERV-K in Ovarian Cancer**

In a keystone 2006 paper from our laboratory, we turned our focus to another perilous female cancer and produced cogent results demonstrating expression of multiple HERV-K SU env proteins in OC. We also showed that in contrast to most cancers which express 1 or 2 HERVs, at least three HERV *env* genes were expressed in OC, including HERV-K Types 1 and 2, ERV3, and HERV-E<sup>175</sup>. Specifically, Type 1 (1,105 bp) and Type II (1,195 bp) HERV-K surface domains were detected at a higher frequency in established OC cell lines compared to normal epithelial cells or immortalized normal ovarian cell lines. Also in this study, expression of HERV-K *env* mRNA was greater in ovarian epithelial tumors compared to normal ovarian tissues (n=254). We also determined both cytoplasmic and cell surface expression of HERV-K env protein in established OC cell lines but not in immortalized normal ovarian cell lines. FACS analysis further confirmed cell surface expression by revealing greater surface expression of HERV-K env protein on established OC cell lines but not in immortalized normal ovarian cell lines. We also confirmed HERV-K SU env protein expression in ovarian tumor epithelial cells and correlated this significant increase with tumor histotype. Finally, we ascertained that 55% of OC patient samples (n=60) had positive titers for anti-HERV-K antibodies compared to only 5% of normal female controls (n=20).



Upon publication of this study, we were the first to report that HERV-K env protein was expressed on the surface and in the cytoplasm of HERV-K-positive OC cells. Most importantly, no expression was detected in normal or immortalized normal or immortalized normal surface ovarian epithelial cells. The detection of anti-HERV-K antibodies in OC patient sera samples showed a lack of tolerance and confirmed that OC patients possessed the ability to mount an immune response against specific HERVs.

### **1.10 Mechanisms by which HERV-K is selectively expressed in ovarian cancer**

The majority of HERV insertions are transcriptionally silenced in most embryonic and adult tissues through repressive epigenetic marks <sup>176</sup> that are deposited and maintained by the host surveillance system <sup>177</sup>. Failure to maintain these marks results in the reactivation of dormant HERV insertions <sup>178</sup>. Dysregulated expression of HERVs may be related to hypomethylation of HERV genes in tumor cells <sup>179, 180</sup>, therefore the repressive role of DNA methylation on HERV activity in human cells and tissues is being increasingly studied. Epigenetic aberrations, including genome-wide hypomethylation, are crucial in the development and progression of OC <sup>181</sup>. In contrast to the overall DNA methylation patterns observed in normal cells, a global hypomethylation of CpG dinucleotides in combination with hypermethylation of CpG islands occurs in cancer <sup>182</sup>. CpG islands tend to associate with the promoters of housekeeping genes <sup>183</sup> and therefore their methylation in malignancy causes repression of tumor suppressor genes <sup>184</sup>. In contrast, DNA hypomethylation renders HERVs transcriptionally active <sup>10</sup>. HERV-K hypomethylation can lead to several biological consequences including genomic instability <sup>185, 186</sup>, aberrant gene expression due to effects from LTRs <sup>187</sup>, and cancer development via HERV RNA or



proteins. Selective transcription of HERV-K could be explained by changes in tumor-specific transcription factors or tissue specific factors<sup>188-190</sup>. Activation could also be attributable to selective hypomethylation of these genes as seen with HERV-W in human OC<sup>180, 191, 192</sup>.

The expressions of HERV-K and HERV-E are increased in tissues from patients with ovarian clear cell carcinoma (OCCC), a particular type of epithelial OC. Long interspersed repetitive sequence-1 (LINE-1), a specific type of intersperse repetitive sequence (IRS), is hypomethylated in most cancers including epithelial OC<sup>193, 194</sup>. Therefore in a recent study, Iramaneerat K, Rattanatunyong P, Khemapech N, Triratanachat S, Mutirangura A evaluated if the loss of DNA methylation of HERV sequences can be an epigenetic signature to predict the prognosis and treatment response of OCCC<sup>195</sup>. Indeed they found that both HERV-K and HERV-E lose methylation during OCCC progression and only HERV-K hypomethylation is significantly associated with poor survival and platinum resistance<sup>195</sup>. Determining HERV-K methylation levels in OC before chemotherapy may be critical for proper treatment plans in the future. Further studies must be conducted in order to determine the mechanisms involved in HERV-K hypomethylation.

### **1.11 Mounting effective antitumor immunity**

There are three distinct steps that must be achieved in order to mount effective antitumor immunity. First, to initiate immunity, DCs must sample antigens derived from the tumor such as mutated proteins typical of cancer, the products of non-mutated genes that are preferentially expressed by cancer cells, or differentiation antigens associated with the cancer's tissue of origin<sup>196, 197</sup>. Within the realm of therapeutic vaccines, these antigens are



delivered exogenously. When a DC ingests this antigen, it will also require an activation signal to differentiate and promote immunity<sup>198, 199</sup>.

Secondly, tumor-antigen loaded DCs must generate protective T-cell responses in lymphoid organs<sup>200</sup>. The specific type of T-cell response needed for antitumor immunity is unknown. However, it will unquestionably include CD8<sup>+</sup> effector T cells with cytotoxic potential. It is imperative that the DCs received the maturation signal at this point in order to elicit the desired T cells, otherwise un-matured DCs promote tolerance by T-reg production<sup>201-204</sup>.

Finally, cancer-specific T cells must enter the tumor bed to perform their function. Due to the complex tumor microenvironment, this step in antitumor immunity is a key challenge. One major obstacle is local accumulation of T-regs that oppose the activity of effector T cells. Tumors also have the ability to down-regulate their expression of MHC class I molecules or their expression of target tumor antigens. Additionally, tumors can produce surface molecules that engage receptors on the surfaces of activated T cells, causing T-cell anergy or exhaustion<sup>205, 206</sup>. Tumors can also release immunosuppressive molecules limiting T-cell function<sup>207, 208</sup>. Hypoxia in the tumor microenvironment may also inhibit T-cell function via the generation of adenosine<sup>209</sup>. Finally, tumor stroma cells can suppress the functions of T-cells by several methods<sup>210</sup>.

## **1.12 Immunotherapy**

Immunotherapy is the treatment of disease by inducing, enhancing, or suppressing an immune response. There are two types of manipulation based on the functionality of the patient's immune system. If the patient's immune system is functional, immunotherapy can



be used to activate or enhance the patients' immune system via exposing patients to tumor-associated antigens displayed by their tumors. If the patient's immune system is not functional, passive immunization via introduction of antibodies or adoptive transfer of immune cells needs to be employed. Cancer immunotherapy combines the specificity of the adaptive immune system with the cytotoxic capabilities of both the adaptive and innate immune systems. Immunotherapy strategies include the following: antitumor monoclonal antibodies, cancer vaccines, adoptive transfer of ex vivo activated T and natural killer cells, and administration of antibodies or recombinant proteins with the potential to either costimulate immune cells or block immune inhibitory pathways<sup>211</sup>.

Tumor-specific monoclonal antibodies (mAb) have had a major impact on immunotherapy. Numerous mAbs, including bispecific mAbs and multispecific fusion proteins, mAbs conjugated with small-molecule drugs, and mAbs with optimized pharmacokinetics, are in clinical trials. Importantly, it is the interaction between mAb and Fc receptor (FcR) that contributes to the clinical antitumor activity of mAbs. For example, changing the sequence or glycosylation of Fc regions can enhance interactions with FcR in immune effector cells<sup>212, 213</sup>. There are extensive interactions among various mechanisms of action that modulate the immune effects of mAbs. Many studies have shown data supporting the role of antibody-dependent cell-mediated cytotoxicity (ADCC) as a major mechanism of mAb activity. Complement-mediated cytotoxicity (CMC) may contribute to antitumor activity in solid tumors but there is little evidence. MAbs have shown great promise and are currently the three top-selling cancer drugs. The majority of mAbs approved for clinical use contain a human immunoglobulin (Ig) G1 heavy chain. Trastuzumab is a recombinant humanized mAb that targets the HER2 receptor, inhibits cell



proliferation and DNA repair, induces apoptosis, and promotes DNA damage and immune modulation, thus causing cell-cycle arrest. This mAb has been approved by the US FDA for the treatment of metastatic BC with HER2 overexpression. Cetuximab is a chimeric mAb raised against epidermal growth factor receptor (EGFR) and inhibits endogenous ligands binding, cell motility, invasiveness, metastasis, and the promotion of apoptosis<sup>214</sup>.

Cetuximab is also FDA approved for treating advanced colorectal cancer and squamous cell carcinoma of the head and neck. Bevacizumab is a humanized mAb that inhibits the biological activities of vascular endothelial growth factor (VEGF) and blocks binding of VEGF to its receptor on vascular endothelium. This mAb has been FDA approved since 2004 as first-line therapy in metastatic colorectal cancer.

The beauty of cancer vaccines is that they demonstrate minimal toxicity in comparison to other forms of cancer therapy which kill tumor cells and normal cells alike. However, the success of immunotherapy depends on overcoming several significant barriers. First, tumor-associated antigens are typically closely related or identical to self-antigens, making unwanted autoimmune responses a risk. Secondly, the immunosuppressive factors within the tumor microenvironment need to be avoided<sup>215</sup>.

The idea of a therapeutic cancer vaccine originated with the discovery that patients produce CD8<sup>+</sup> and CD4<sup>+</sup> T cells specific for antigens expressed in their tumors<sup>196</sup>. Common tumor-associated antigens include oncoproteins, oncofetal antigens, differentiation-associated protein, viral proteins, and others. The goal of vaccination was therefore to amplify the frequency and strength of these responses or induce new reactions. Studies have shown a strong association between the presence of intratumoral CD3<sup>+</sup> or CD8<sup>+</sup> cytotoxic T cells and the IFN- $\gamma$  gene signature and prolonged patient survival<sup>139, 216</sup>.



This idea was an expedient one, however many initial attempts failed due to a poor understanding of the immune system. Presently, the importance and function of DCs has been determined to play a key role in improving therapeutic efficacy. A recent study of peptides derived from the HPV-16 E6 and E7 viral oncoproteins showed clinical responses in women with vulvar intraepithelial neoplasia due to the presence of HPV-specific IFN- $\gamma$ -producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells <sup>217</sup>. It is believed that these propitious results are due to the fact that viral proteins are recognized as foreign by the host, leading to a stronger T-cell response.

In comparison to peptides, full-length proteins contain a broader profile of epitopes that might be presented by DCs and therefore may be more advantageous. It is known that for a cancer vaccine to be efficacious, it must induce both antigen-specific CD8<sup>+</sup> CTLs and antigen-specific CD4<sup>+</sup> cells, and protein-based vaccines usually contain both <sup>165</sup>. Indeed, a current randomize phase III trial using a recombinant fusion protein, MAGE-A3, an adjuvant, and TLR4 and TLR9 agonists, has shown a survival response of 27% in HLA-A2-positive non-small cell lung cancer patients <sup>218</sup>.

Another vaccination strategy under evaluation involves viral vectors encoding tumor antigens. These strategies exploit the fact that viral components elicit strong immune responses. In a current phase II trial, recombinant vaccinia virus encoding prostate-specific antigen and the adhesion molecules B7-1, ICAM-1 and LFA-3, were combined with GM-CSF to produce a vaccine against prostate cancer. Positive results indicate an overall survival benefit of 25.1 months versus 16.6 months in the control group <sup>219</sup>.

Numerous preclinical studies have presented data on the enhanced antitumor responses seen when vaccines are combined with inhibitors of immune suppression.



Monoclonal anti-CD25-diphtherial toxin (Ontak) has been shown to successfully reduce T-regs and to enhance vaccine efficiency<sup>220, 221</sup>. Cyclophosphamide also reduces the number of T-regs and their functionality, and has been shown to enhance vaccine efficacy in both preclinical and clinical studies<sup>222, 223</sup>. Both monoclonal and small molecule inhibitors to TGF- $\beta$  may have potential in vaccine combination therapy<sup>224-227</sup>.

### **1.13 Stem cells in ovarian cancer**

Cancer stem cells are rare chemotherapy resistant cells within a tumor which can serve to populate the bulk of the tumor with more differentiated daughter cells and potentially contribute to recurrent disease<sup>228</sup>. Within an OC, there are many unique cell populations that each have different capacities for growth, survival, metastasis and resistance to chemotherapy and radiation therapy<sup>229, 230</sup>, however the exact phenotype and characteristics of OC stem cells are still disputable<sup>231</sup>. Cancer stem cells typically make up 0.01-1.0% of malignant cells within a tumor and have unlimited division potential. Based on their resistance to traditional cancer therapies and presumed ability to recapitulate the original tumor, cancer stem cells are believed to be the source of recurrent OC<sup>228</sup>. Therefore much effort has been given the characterization of these stem cells in order to ultimately target them. One of the most widely known OC stem cell markers is CD133, a membrane glycoprotein encoded by the *CD133/Prom-1* gene. In a keystone article, Ferrandina G, Bonanno G, Pierelli L, Perillo A, Procoli A, Mariotti A, Corallo M, Martinelli E, Rutella S, Paglia A, Zannoni G, Mancuso S, and Scambia G determined that primary OC CD133<sup>+</sup> cells had greater colony forming potential and higher proliferative potential than CD133<sup>-</sup> cells<sup>232</sup>. More importantly, they determined that normal ovaries and benign tumors



had a significantly lower expression of CD133 than ovarian carcinomas. CD44, a receptor for hyaluronic acid, has been identified as a marker of cancer stem cells in breast<sup>233</sup>, prostate<sup>234</sup>, colorectal<sup>235</sup>, pancreatic<sup>236</sup>, head and neck squamous cell carcinomas<sup>237</sup>. One study analyzed tumor spheroids generated from the ascites of patients with serous ovarian cancer<sup>238</sup>. After ~10 serial passages in stem cell media, it was observed that the remaining spheroid cells were highly enriched for CD44 expression, were resistant to chemotherapy, and were able to initiate and serially propagate tumors in mice. These stem cells markers represent potential therapeutic targets and may even be functionally important for stem cell survival, making them even more attractive. One downside is that only 34-40% of ovarian tumors express CD133<sup>230, 232, 239</sup>. Also, CD44 may have a broad expression including normal tissue. Therefore, targeting these stem cells with other types of tumor specific markers has the potential to lead to localized therapeutic approaches but minimization of systemic exposure needs to be optimized.

#### **1.14 Project Overview**

In our previous work, we were the first report expression of HERV-K env protein in actual specimens of breast ductal epithelial tumors but not in normal breast in humans. We also determined that HERV-K has great potential as a BC vaccine because it induced an antigen-specific immune response in BC patients. Specifically, we showed that cytotoxic T lymphocytes from cancer patients could lyse established BC cell lines expressing HERV-K. At that time, only one other group had documented the presence of HERV-K-specific CTL responses in human cancer<sup>80</sup>. We then delved into another malevolent female cancer, OC, and were the first to report that HERV-K env protein is expressed on the surface and in the



cytoplasm of OC cells and that expression of HERV-K *env* mRNA was greater in ovarian epithelial tumors than in normal ovarian tissues. Additionally, we confirmed that the frequency of expression of HERV-K *env* protein in OC samples had a significant correlation with tumor histotype. Finally we detected anti-HERV antibodies in patients with OC, but not in normal female controls.

Based on our previous findings in BC and OC, our next goal was to reconfirm HERV-K expression in OC and evaluate the effectiveness of HERV-K as a potential tumor-associated antigen for development of an OC vaccine by examining a new patient cohort which included samples from OC patients, patients with benign disease and adjacent uninvolved samples. We obtained several patients providing both tumor and adjacent uninvolved samples, allowing an ideal situation to determine differences in HERV-K *env* expression patterns and immune responses. Most importantly, we wanted to determine if cytotoxic T cells from cancer patients could lyse autologous target cancer cells expressing HERV-K while ignoring normal cells. Therefore, we hypothesize that HERV-K is a tumor-associated antigen and that there is selective expression and distribution of these elements in OC, but not in benign samples or normal controls. We hypothesize that HERV-K *env* protein, a retroviral antigen product, will be a safe and efficient candidate for development of an OC vaccine.

Our first aim is to assess the expression profiles of HERV-K *env* proteins and anti-HERV-K antibodies in primary OC, benign patient samples, and normal controls. To achieve this goal we utilized a new patient cohort and determine expression of HERV-K *env* mRNA and protein. We specifically determined cell surface expression of HERV-K *env*



protein in primary samples from OC, benign and adjacent uninvolved tissues. We then characterized the primary cells based on IF expression and stem cell markers. Next we assessed whether activation of HERV-K correlates with clinical or histological characteristics of OC patients. We also determined and compared levels of anti-HERV-K Abs in OC and benign patient serum samples and correlated them with clinical characteristics.

Our second aim is to evaluate the immune responses triggered by HERV-K antigen in OC, benign patient, and normal donors. To achieve this we first characterized patient samples to determine specific lymphocyte populations. Next, we determined changes in T cell proliferation and IFN- $\gamma$  secretion in OC patient lymphocytes compared to benign samples, and normal donors. We also determined CTL activity using autologous OC, benign, and adjacent uninvolved patient target cells. Finally we determined the effects T-reg depletion on CTL activity.

The majority of current tumor-associated antigens represent self-antigens that have become aberrantly expressed by the cancer. Therefore, the majority of solid tumors are not immunogenic. Also, these antigens are sometimes expressed in normal tissues. Thus immunotherapeutic techniques targeted against these antigens are impeded by the need to overcome self-tolerance mechanisms that ultimately limit the immune response. Viral antigens, on the other hand, are considered “foreign” to the body and might elicit stronger antitumor T-cell responses. Therefore we believe that HERV-K, a novel tumor-associated antigen, is an ideal target for immunotherapy in OC.



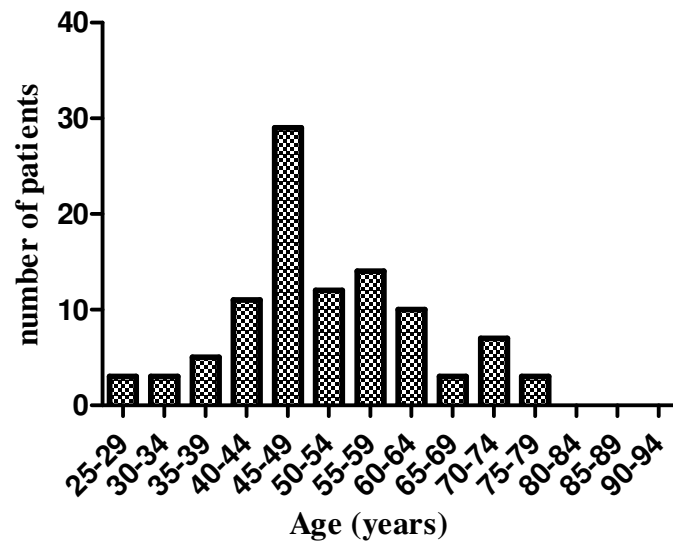
## **Chapter 2. Materials and Methods**

### **2.1 Human primary samples**

Human ovarian tissues and peripheral blood mononuclear cells (PBMC) from OC patients, patients with benign disease, patients with other cancers, or healthy female controls were obtained from The MD Anderson Cancer Center according to approved Institutional Review Board protocols. Age distribution and patient histological diagnoses are shown in Figure 4.

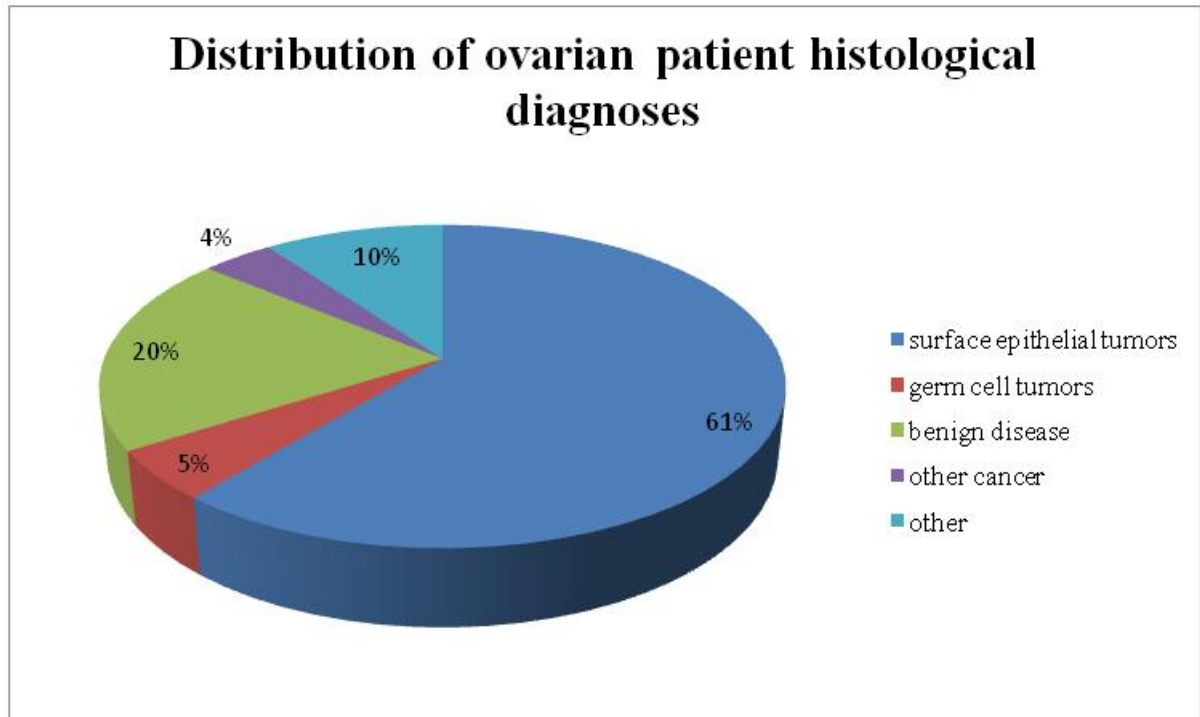


**Age distribution of patients at diagnosis**  
**N=104**



**Figure 4:** Ovarian patient age distribution. The age distribution of patients used in this study.





**Figure 5:** Ovarian histological diagnoses distributions. Examples from each histological category are listed. Surface epithelial tumors: papillary serous carcinoma, serous borderline, Mullerian borderline tumor, metastatic adenocarcinoma, mucinous adenocarcinoma. Germ cell tumors: teratoma. Benign disease: endometriotic cyst, benign cyst, follicular cysts. Other cancer: gastrointestinal stromal tumor, neuroendocrine tumor, gallbladder adenocarcinoma. Other: endometriosis, fibrotic tissue with inflammation.



## **2.2 Established cell lines**

OVCAR3, SKOV3, DOV13 human epithelial OC established cell lines were obtained from ATCC (Rockville, MD). Cells were grown in Dulbecco's modified eagle media (DMEM) supplemented with 10% FBS, 1% penicillin-streptomycin and 1% Glutamax.

## **2.3 Harvesting of primary tissue samples**

A piece of tissue in 1-2 ml of cold complete NOE culture medium (250 ml of Medium 199+ 250 ml DMEM) supplemented with 10% FBS, 1% penicillin-streptomycin and 1% Glutamax, was added to a Petri dish and chopped into small pieces with a razor blade to obtain a thick soup of tumor cells/pieces. 20X the volume of cold medium was added, inverted to mix, and spun at 900 rpm for 5 minutes. The tissue pieces and dissociated cells pelleted, while the debris remained in the supernatant. The supernatant was removed and the cells were resuspended in Accumax solution at a concentration of 10 ml/0.5 g of tissue and incubated at room temperature for 60 minutes on a shaker. The cells were washed with 1xPBS, resuspended in 20 ml of media, and transferred to a Stomacher 80 bag. The bag was inserted into the Stomacher (Seward Co.) and run for 15 minutes at high speed. The mixture was transferred to a 50 ml tube and the larger chunks were allowed to settle to the bottom. The supernatant containing the dissociated cells was transferred to a fresh tube, spun at 900 rpm for 5 minutes, and resuspended in 1-3 ml medium. The cells were then passed through a 40 $\mu$ m nylon mesh into a 50 ml tube. The live cells were then separated from dead cells, debris, and red blood cells via the use of a Ficoll-1077 gradient. The cells were spun on Ficoll-1077 at 400g for 30 minutes at room temperature and the



opaque layer of cells at the interphase of the two layers was transferred to a new tube. The cells were spun at 900 rpm for 5 minutes, resuspended in low serum NOE media to and plated in a 6-well low adherent plate. To culture tumorspheres, 6-well plates were coated with 1 ml of 0.8% agarose<sup>240</sup>, and cells were added in 2 ml per well of Mammocult media supplemented with heparin and hydrocortisone.

## 2.4 RT-PCR

For tissue samples obtained at the MD Anderson Cancer Center, RNA isolation and RT-PCR were employed as described previously<sup>67, 68</sup>. Briefly, total RNA was isolated from primary cell lines in Tri-reagent according to the manufacturer's instructions and treated with RNase-free DNase I at 37°C for 30 minutes to prevent contamination of genomic DNA. RNA was then incubated at 75°C for 5 minutes to inactivate residual DNase. A first strand cDNA was synthesized from the RNA template using Ready-To-Go<sup>TM</sup> You-Prime First-Strand Beads. 10µg RNA was added to 32µl of diethylpyrocarbonate (DEPC)-treated H<sub>2</sub>O and incubated at 65°C for 10 minutes. Following this incubation, 1 µl of random primers were added to the RNA solution and then incubated again with the beads at 37°C for 1 hour. 3.3µl of cDNA, corresponding to 1 µg of RNA, was incubated with 5 µl of 10x PCR buffer, 0.25 µl of Taq DNA polymerase, and the various primer pairs. Primers used for RT-PCR included HERV-K SU *env* type 1 and type 2 primers, HERV-E *env* primers, ERV-3 primers, and β-actin primers, which were described previously<sup>175</sup>. No template and β-actin were used as negative and positive controls, respectively. The HERV-K *env* PCR primers were designed by Sangon Biotech Co. Ltd. (Shanghai), and produced by Takara Biotechnology Co. Ltd. (Dalian), according to the HERV-K102 *env* sequence (GenBank accession no.



AF164610.1). After the initial denaturation step at 94°C for 3 minutes, 30 cycles of amplification were performed as follows: denaturation at 94°C for 1 minute, annealing at 55°C for 1 minutes, and extension at 72°C for 1 minute. The RT-PCR product was then subjected to electrophoresis on a 1% agarose gel by ethidium bromide staining to confirm size.

## **2.5 Synthesis of HERV-K env fusion proteins and antibodies and cRNA**

HERV-K env surface fusion proteins were expressed from the pGEX-6p1 plasmid (K10G17) in *Escherichia coli* (*E.coli*) BL-21(DE3) or from the pQE30 plasmid (K10Q18) in *E. coli* M15 by incubating at 18°C overnight in the presence of ampicillin (100 µg/ml), kanamycin (25 µg/ml), and 1 mmol/L isopropyl-L-thio-B-D-galactopyranoside until an OD600 of 0.8 was reached. To express the TM fusion protein, a recombinant pQE30-TM bacterial clone was grown in an identical manner. Bacterial pellets were harvested, disrupted by lysozyme treatment followed by sonication, clarified by 0.2 µm filtration, and affinity purified with glutathione-Sepharose FF using an ÄKTA fast protein liquid chromatography (FPLC; GE Healthcare). The purified K10Q18 fusion proteins were then used to immunize 6- to 8-week-old BALB/c mice. Hybridoma cells were derived from the splenocytes of immunized mice by fusion with a myeloma cell line using standard techniques, as described previously<sup>175, 241</sup>. Several monoclonal antibodies against HERV-K env SU protein were generated in our laboratory and ELISA and immunoblot screening revealed that mAb 6H5 had the highest specificity and sensitivity toward HERV-K. 6H5 hybridoma ascites was then collected from pristane-primed mice, precipitated twice with saturated ammonium sulfate, clarified by 0.2 µm filtration, and affinity purified with protein



G-Sepharose HP by FPLC. The eluate fractions containing 6H5 mAb were dialyzed against PBS, concentrated, and filter sterilized before testing. 6H5 antibody was labeled with Alexa Fluor® dyes 647 and 488 to be used in FACS analysis and IFS respectively. Keyhole limpet hemocyanin (KLH) protein (Pierce) was used as a negative control throughout our experiments. Several experiments utilized the human papillomavirus E6 protein as a negative control. HERV-K and E6 cRNA were produced by in vitro transcription<sup>67</sup>.

## 2.6 ELISA

100 µl of diluted KSU protein (10 µg/ml) was added to each well of a flat-bottom 96 well plate, and incubated overnight at 4°C. Plates were washed 6 times with 1xPBS, 100 µl of blocking buffer (1xPBS+1% BSA) was added to each well, and plates were incubated at room temperature for 60 minutes. Blocking buffer was removed by flipping the plate over and tapping it on a paper towel. *E. coli* lysate was added to serum samples at a 1:20 dilution and the mixture was incubated for 30 minutes at room temperature. Human patient serum was then added in the correct dilution to triplicate wells and plates were incubated overnight at 4°C. Plates were washed 6 times with 1xPBS, 100 µl of mouse-IgG-HRP (1:4,000 dilution in 1xPBS) secondary antibody was added to each well and incubated for 60 minutes at room temperature. Plates were again washed 6 times with 1xPBS and 100 µl of ABTS substrate solution (200 µl ABTS and 10 µl 30% H<sub>2</sub>O<sub>2</sub> in 10 ml citrate buffer) was added to each well. The absorbance was measured with a Wallac VICTOR<sup>2</sup> plate reader (Perkin Elmer) at 405 nanometers at different time points. The ELISA was performed in triplicate for each dilution.



## **2.7 FACS**

1 x 10<sup>6</sup> cells/well were added to a round-bottomed 96-well plate and labeled with 1 µg/well of primary antibody diluted in BD Stain Buffer (BD Biosciences) for 60 minutes at room temperature. After washing cells twice with 1xPBS, samples were incubated with secondary Alexa Fluor 647 conjugated mouse-IgG for 30 minutes at room temperature. After washing cells twice with 1xPBS, samples were resuspended in 250 µl 1xPBS and analyzed by a BD FACsArray (BD Biosciences).

## **2.8 QIFIKIT**

QIFIKIT (Dako) was used for the quantitative determination of cell surface HERV-K antigen by flow cytometry using an indirect immunofluorescence assay. 1 x 10<sup>6</sup> human patient ovarian cells/well were added to a round-bottomed 96-well plate and labeled with 6H5 mAb at saturating concentration. The cells were incubated, in parallel with the QIFIKIT® beads, with secondary Alexa Fluor 647 conjugated mouse IgG at saturating concentration. A calibration curve was constructed by plotting the fluorescence intensity of the individual bead populations against the number of mAb molecules on the beads. The number of antigenic sites on the specimen cells was then determined by interpolation.

## **2.9 Immunofluorescence (IFS)/Fluorescence Microscopy/Confocal Microscopy**

5x10<sup>5</sup> cells/well were seeded onto glass coverslips in a 6-well plate and incubated at 37°C overnight. The media was removed and cells were fixed with fresh 4% paraformaldehyde for 20 minutes at room temperature. Cells were blocked in 3% BSA blocking buffer for 60 minutes at room temperature, and then incubated with primary



antibody for 60 minutes at 37°C. After washing cells twice with 1xPBS, samples were incubated with secondary Alexa Fluor 488 conjugated mouse IgG for 30 minutes at 37°C. After washing cells twice with 1xPBS, cellular nuclei were stained with DAPI for 3 minutes at room temperature, and then washed again with deionized H<sub>2</sub>O. Coverslips were added to the slides with 90% glycerol in water and examined under either an Olympus IX51 fluorescent microscope with a DP70 camera, or a laser-scanning confocal microscope (Zeiss 510 Meta) with appropriate filters.

## **2.10 Immunohistochemical analysis (IHC)**

Immunohistochemical analysis was performed on tissue slides using an LV-1 Autostainer universal staining system (Dako) in combination with the VECTASTAIN ABC system and DAB kit (Vector). Slides were baked in an oven at 60°C for 1 hour and then removed to cool. Slides were placed on an Autostainer to deparaffinize and hydrate tissue sections through xylenes and a graded alcohol series. A circle was drawn with a wax pencil around the section of tissue. Peroxidase blocking solution (3% H<sub>2</sub>O<sub>2</sub>) was added to each slide and incubated at room temperature for 10 minutes. The slides were washed for 5 minutes in H<sub>2</sub>O. Blocking solution was added (AB serum (2% solution in 1XPBS), goat serum (2% solution in 1XPBS plus 3% BSA) to slides and incubated at room temperature for 30 minutes. The solution was tipped off without rinsing and 150 µl of diluted 6H5 mAb in 1 ml of blocking solution was added to each section. The slides were placed into a humidified chamber and place in a 4°C cold room for 16 hour. Slides were washed three times with 1XPBS for 30 minutes each in a glass container with a stir bar. Sections were incubated for 30 minutes with mouse-IgG-HRP (1:600 dilution in blocking buffer)



secondary antibody and then washed three times with 1XPBS for 30 minutes each in a glass container with a stir bar. Sections were incubated in DAB for 5 minutes and then washed in H<sub>2</sub>O to stop the DAB reaction. Slides were counterstained with hematoxylin for 8 minutes, washed thoroughly in H<sub>2</sub>O, and placed in the Autostainer through a graded alcohol series and xylenes. Cover slips were added with Permount, and samples were examined under a microscope Axiovert 200 (Carl Zeiss MicroImaging, Thornwood, NY).

### **2.11 Peripheral blood mononuclear cell (PBMC) isolation from human blood samples**

PBMCs were isolated from whole blood samples by density gradient centrifugation using Ficoll-1077 (Sigma, St. Louis, MO). Briefly, 10 ml of blood from a red Vacutainer tube was spun down at 2,000 rpm for 5 minutes at room temperature, and the serum layer was removed and stored at -80°C for future analysis. 10 ml of blood from a sodium heparin green Vacutainer tube was transferred to a 50 ml Falcon tube and spun down at 2,000 rpm for 5 minutes at room temperature. The upper plasma layer was removed and stored at -80°C for future analysis. 1xPBS was added to the remaining blood to reach a final volume of 40 ml. The diluted blood was then very slowly added to a 50 ml Falcon tube previously loaded with 10 ml Ficoll-1077. The tubes were spun down at 1,500 rpm for 30 minutes at room temperature using slow acceleration and deceleration rates. PBMCs were recovered by collecting the Ficol-1077 and 1xPBS layers without disturbing the pellet of erythrocytes and granulocytes and placing them in a new 50 ml tube. The cells were washed by adding 30 ml PBS and spun down at 2,000 rpm for 10 minutes at room temperature. The supernatant was discarded, 5 ml of RBC Lysis Solution (8.3g NH<sub>4</sub>Cl, 1.0g KHCO<sub>3</sub>, and 1.8 ml of 5% EDTA in 1L distilled H<sub>2</sub>O) was added to the cells and the mixture was incubated



for 5 minutes at room temperature. After washing three times with 1xPBS, the PBMCs were resuspended in complete RPMI 1640 culture medium at  $2 \times 10^6$ /ml, seeded in a 6-well plate, and placed in a 37°C incubator overnight for DC preparation.

## **2.12 Preparation of dendritic cells**

PBMCs were plated at a density of  $2 \times 10^6$  cells/well in a 6-well plate and incubated for 16 hours at 37°C. Warm media was carefully added and used to wash the cells three times. The non-adherent cells were pelleted and frozen back for future assays. The remaining adherent cells were incubated for 6 days with granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 (1,000 units/ml) (R&D Systems). The immature DCs were harvested and transfected with K-SU or KLH protein using the BioPORTER lipid-based transfection reagent per the manufacturer's instructions. After four hours, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (1,000 units/ml) was added for 16 hours at 37°C to induce DC maturation.

## **2.13 Preparation of IVS cells**

PBMCs ( $1 \times 10^6$ /ml) were added to autologous HERV-K protein transfected matured DC at a DC to PBMC ratio of 1:30 on day 0. Cultures were cultured in AIM V medium supplemented with 5% human Ab serum and IL-2 (100 units/ml) for 7 days to generate 1-week IVS cells.



## **2.14 T-cell proliferation assay**

T-cell proliferation was evaluated in patient PBMC or IVS cells by restimulation for 72 hours with DC pulsed with no added protein, K-SU protein, or KLH control protein, at a DC to PBMC or IVS ratio of 1:30. After 72 hours of incubation, the supernatants were collected and frozen for future cytokine secretion assays. The remaining cells were pulsed with 1  $\mu\text{Ci}$ /well of [ $^3\text{H}$ ]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.

## **2.15 Enzyme-linked immunospot assay**

An IFN- $\gamma$  enzyme-linked immunospot (ELISPOT) assay to measure cytokine-secreting cells in response to antigen was performed using a commercial kit (Biosource International). ELISPOT plates were coated with 10  $\mu\text{g}/\text{ml}$  of purified anti-human IFN- $\gamma$  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  $1 \times 10^5$  per well with DCs pulsed with various HERV-K antigens plated at  $5 \times 10^3$  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 and incubating for 10 to 60 minutes at 25°C until the desired color appeared. Plates were washed thoroughly with water, allowed to dry, and spots were counted using an ELISPOT reader (C.T.L. Technologies).



## 2.16 CTL assay

CTL assays were performed in round-bottomed 96-well plates using a standard 4-hour  $^{51}\text{Cr}$ -release assay<sup>242</sup>. Human primary tumor or benign cells lines (target cells) were pulsed with HERV-K or KLH control protein for 16 hours at 37°C using the BioPORTER transfection reagent described previously. Target cells were removed from flasks via EDTA buffer, pelleted and resuspended in 500  $\mu\text{l}$  media and 75 $\mu\text{Ci}$  of chromium. After incubating for 2 hours at 37°C, cells were washed with media 3 times and radioactive waste was discarded properly.  $5 \times 10^4/\text{ml}$  target cells were then resuspended in media.  $5 \times 10^6/\text{ml}$  autologous PBMCs or IVS cells (effector cells) were also resuspended in media. The effector cells and target cells were then combined in the plates and incubate for 4 hours at 37°C. Effector to target cell ratios were plated at 100:1, 50:1, 25:1, and 12.5. The plates were pelleted and supernatants (100  $\mu\text{l}$ ) from each well were removed and counted in a gamma counter. After effector and target cells are incubated for 4 hours, the amount of chromium release due to cell lysis is measured with a gamma counter. % specific lysis was calculated by the following equation= ((Experimental value-minimum lysis)/(maximum lysis-minimum lysis)) x100. Labeled target cells in media served as the minimum lysis value and labeled target cells in % Triton X100 served as the maximum value.

## 2.17 Isolation of $\text{CD4}^+\text{CD25}^+\text{CD127}^{\text{dim/-}}$ regulatory T cells

The  $\text{CD4}^+\text{CD25}^+\text{CD127}^{\text{dim/-}}$  Regulatory T Cell Isolation Kit II (Miltenyi Biotec) was used to isolate T-reg. PBMCs were pelleted and resuspended in 40  $\mu\text{l}$  of buffer consisting of phosphate-buffered saline (PBS), pH 7.2, containing 0.5% bovine serum albumin (BSA),



and 2 mM EDTA per  $1 \times 10^6$  cells. 10  $\mu$ l of CD4<sup>+</sup>CD25<sup>+</sup>CD127dim<sup>-</sup> T Cell Biotin-Antibody Cocktail II was added to the cell suspension and incubated for 10 minutes at 4°C. 30  $\mu$ l of buffer and 20  $\mu$ l of Anti-Biotin Microbeads were added to the cell suspension and incubated for 15 minutes at 4°C. Cells were washed by adding 2 ml of buffer, pelleted, and resuspended in 500  $\mu$ l of buffer. An LD Column was placed in the magnetic field of a MACS Separator and 2 ml of buffer was added to rinse the column. The cell suspension was applied to the column and the pre-enriched CD4<sup>+</sup> unlabeled cells that passed through the column were collected. Cells were pelleted, resuspended in 90  $\mu$ l of buffer plus 10  $\mu$ l of CD25 MicroBeads II, and incubated for 15 minutes at 4°C. Cells were then washed by adding 2 ml of buffer, pelleted, and resuspended in 500  $\mu$ l of buffer. A MS Column was placed in the magnetic field of a MACS Separator and 500  $\mu$ l of buffer was added to rinse the column. The cell suspension was applied to the column and unlabeled cells that passed through were collected. The column was removed from the separator and placed into a sterile collection tube. 1 ml of buffer was placed on the column and magnetically labeled cells were flushed out by pushing a plunger into the column.

## **2.18 Apoptosis Assay**

Annexin V staining precedes the loss of membrane integrity which characterizes the later stages of cell death, and therefore the vital dye 7-amino-actinomycin D (7-AAD), in conjunction with annexin V, was used to allow discrimination of early apoptotic cells. To measure the effects of 6H5 mAb on cellular apoptosis in vitro, ovarian cells DOV13, OVCAR3, and SKOV3 ( $5 \times 10^5$  cells/ml) were grown in their respective media and treated with 6H5 mAb or control mouse-IgG antibody at a concentration of 10  $\mu$ g/ml at 37°C for 16



hours. Treated cells were harvested and resuspended in 200  $\mu$ l 1 $\times$  binding buffer (BD Pharmingen) at  $1 \times 10^6$  cells per well. Cells were stained with annexin V-allophycocyanin (APC) (BD Pharmingen) at room temperature for 15 minutes followed by one wash with 1  $\times$  annexin V binding buffer. Cells were then incubated with 7-AAD-phycoerythrin-cyanide 7 at room temperature for 10 minutes, and samples were analyzed on a BD FACSArray Bioanalyzer. The percentage of annexin V-positive cells for each cell line treated with 6H5 mAb was determined using FlowJo software (Version 7.2.5).

## **2.19 Statistical analysis**

Analyses were done using GraphPad Prism version 5. Statistical significance of differences between groups was determined by a Student's *t* test or a Chi-square test. A *P* value <0.05 is considered to be statistically significant. Adjusted residual analysis, Z-test, and Chi-squared test were used in IHC data analysis.



## Chapter 3: Results

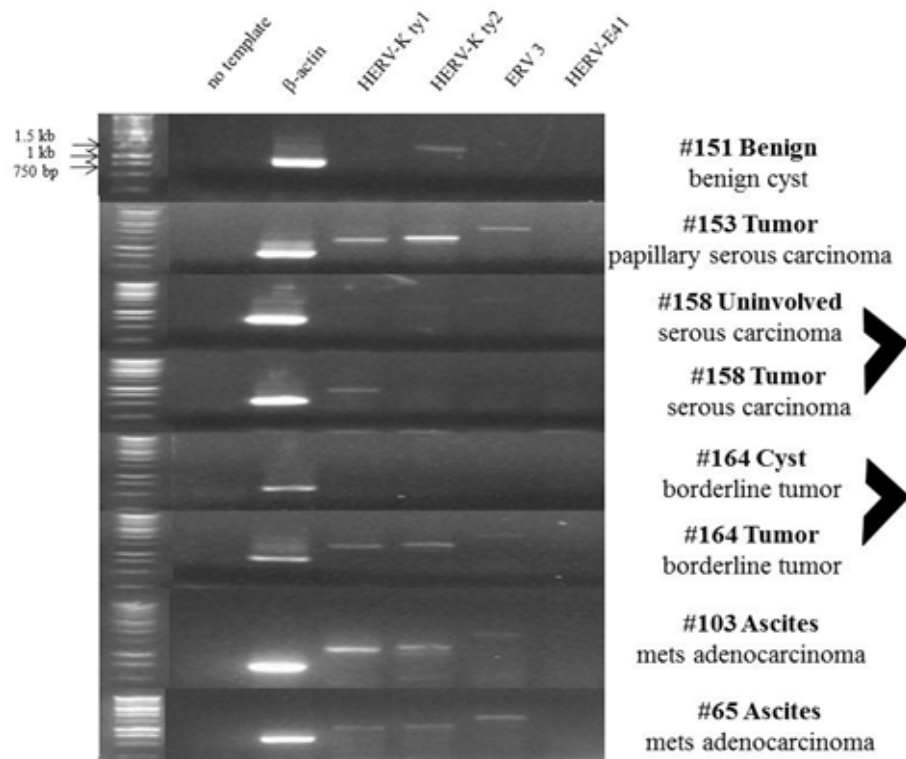
### 3.1 Expression of HERV-K env mRNA in OC, benign and adjacent uninvolved patient tissue samples

To investigate the expression of HERV-K env transcripts in patient tissue samples, RT-PCR analysis was performed using an HERV-K env SU primer pair, amplifying a 1,105 bp DNA product. HERV-K type 2, was also amplified resulting in a 1,195 bp DNA product. HERV3 (1,745 bp) and HERV-E41 (1,349 bp) primer pairs amplifying other HERV families were used as negative controls and amplification of  $\beta$ -actin was used as a positive control. Once the RT-PCR products were run on an agarose gel, band intensities were based on the following scale: +++ represent very intense bands, ++ represent strong bands, + represent average to weak bands, and – represents no bands seen. Overall, the expression of HERV-K env mRNA was assessed in cancer samples (n=29) and benign samples (n=17). Some of these samples originate from patients with both cancer and uninvolved or benign specimens. Representative gel documentation of RT-PCR products from particular patients is seen in Figure 6 depicting these band intensities. In the OC samples tested, 7 (24.14%) samples displayed an HERV-K band with +++/++ intensity, 13 (44.83%) with + intensity, and 9 (31.03%) with – intensity. In comparison, in the benign or adjacent uninvolved samples tested, only 1 (5.88%) sample displayed an HERV-K band with +++/++ intensity, 3 (17.65%) with + intensity, and the majority 13 (76.47%) with – intensity. RT-PCR products for HERV-K type 2 and HERV3 had varying results with no trend seen. The majority of patients tested had HERV-E41 bands with – intensity. As seen in the gel documentation, a patient diagnosed with a benign cyst produced an HERV-K band of – intensity. Another



patient diagnosed with papillary serous carcinoma produced an HERV-K band of + intensity. Patients #103 and #65, both diagnosed with metastatic carcinoma, produced HERV-K bands of ++ and + intensities respectively. Of great importance, mRNA from autologous tumor and benign samples showed higher expression in the tumor sample compared to the benign sample. Similarly, mRNA from autologous tumor and adjacent uninvolved sample showed a higher expression in the tumor sample compared to the adjacent uninvolved. We conclude that gene expression of HERV-K was higher in OC tissue samples in comparison to benign and uninvolved samples.





	<b>Tumor</b>	<b>Benign &amp; Uninvolved</b>
+++ / ++	7 (24.14%)	1 (5.88%)
+	13 (44.83%)	3 (17.65%)
-	9 (31.03%)	13 (76.47%)
Total	29	17
Chi-Square		P=0.0029

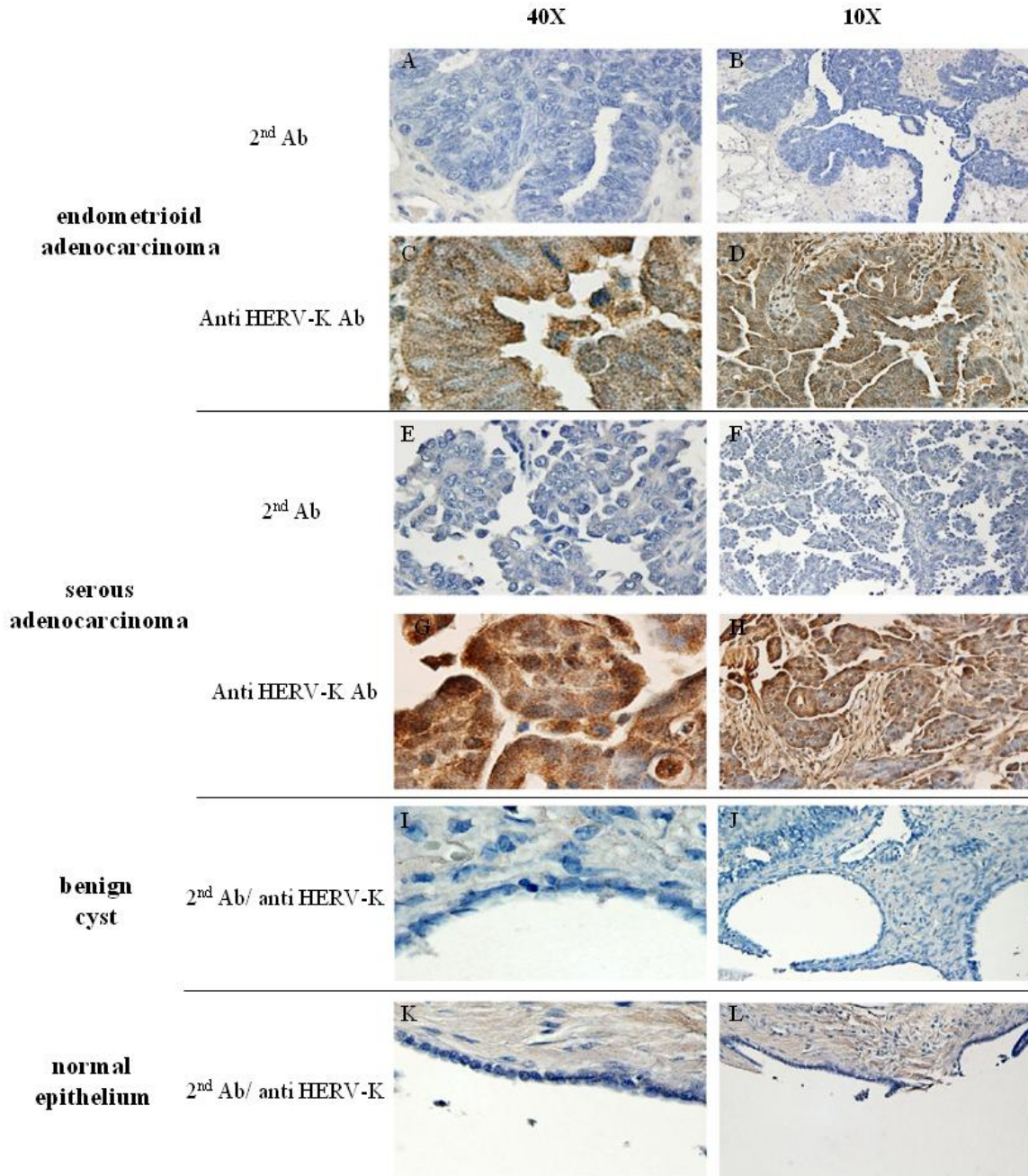
**Figure 6:** HERV env mRNA expression in OC, benign and adjacent uninvolved biopsies via RT-PCR. Each set of lanes for a given amplified gene represents the RT-PCR expression pattern using HERV-K Type 1 (HERV-K(1)), HERV-K Type 2(HERV-K(2)), ERV3, HERV-E and  $\beta$ -actin primers. Gel documentations of various samples are seen on the left. Of great importance, among tissue pairs from the same patient, HERV-K expression is seen in tumor but not in benign tissue. A table at the bottom summarizes the data from RT-PCR assays indicating that a significantly greater number of cancer samples had +++/++ and + designations when compared to benign samples. Benign samples had a statistically greater number of samples with – designations when compared to cancer samples. Black arrows represent tissues derived from the same patient. A Pearson's chi-squared test was used to calculate significance.



### **3.2 Expression of HERV-K env protein in primary samples from OC, benign and adjacent uninvolved tissues**

After detecting HERV-K genes at a higher transcriptional level in OC samples compared to benign and adjacent uninvolved samples, we next inquired whether HERV-K SU envelope protein was expressed in primary samples. To accomplish this, we used a monoclonal anti-HERV-K antibody (6H5 mAb) made using a standard hybridoma technology, as described in the Materials and Methods section, to label the cellular distribution of viral components via IHC. Representative images from various patient tissues are shown in Figure 7. Positivity is shown by the brown chromagen deposits in the images. IHC data from the tissues (n=406) show that HERV-K env expression is confined to the outer layer and to the cytoplasm in OC samples such as endometrioid adenocarcinoma and serous adenocarcinoma. No staining is seen in benign cysts or adjacent normal tissues. This data is in agreement with the RT-PCR data. We did not find a statistically significant association between envelope expression and patient age at diagnosis, race or ethnicity, disease stage or grade, or node-positive status. Statistical data is presented in Table 2.





**Figure 7:** HERV-K env protein expression in OC, benign and adjacent uninvolved biopsies via IHC. Examples of immunostaining of endometrioid adenocarcinoma (A, B, C, and D), serous adenocarcinoma (E, F, G, and H), benign cyst (I and J), and normal epithelium biopsies (K and L) with 6H5: HERV-K<sup>+</sup> cancer cells were detected in endometrioid adenocarcinoma (C and D) and serous adenocarcinoma (G and H). HERV-K expression was not detected in benign cysts or normal epithelium biopsies. The left column represents 40X and right column represents 10X power. The bottom table depicts statistical data for the samples (n=406) screened.



Table 2:

			The expression of HERV-K		
			0	1	Total
Diagnosis	Endometroid Adenocarcinoma	Count	30	6	36
		% within A-Diagnosis	83.3%	16.7%	100.0%
		% within 6H5	10.0%	5.7%	8.9%
	Mixed Type Carcinoma	Count	63	23	86
		% within A-Diagnosis	73.3%	26.7%	100.0%
		% within 6H5	20.9%	21.9%	21.2%
	OTHER	Count	50	8	58
		% within A-Diagnosis	86.2%	13.8%	100.0%
		% within 6H5	16.6%	7.6%	14.3%
	Serous Adenocarcinoma	Count	158	68	226
		% within A-Diagnosis	69.9%	30.1%	100.0%
		% within 6H5	52.5%	64.8%	55.7%
	Total	Count	301	105	406
		% within A-Diagnosis	74.1%	25.9%	100.0%
		% within 6H5	100.0%	100.0%	100.0%

	Value	df	Asymp. Sig. (2-sided)
<b>Pearson Chi-Square</b>	<b>8.134<sup>a</sup></b>	<b>3</b>	<b>0.043</b>
<b>Likelihood Ratio</b>	<b>8.840</b>	<b>3</b>	<b>0.032</b>
<b>N of Valid Cases</b>	<b>406</b>		

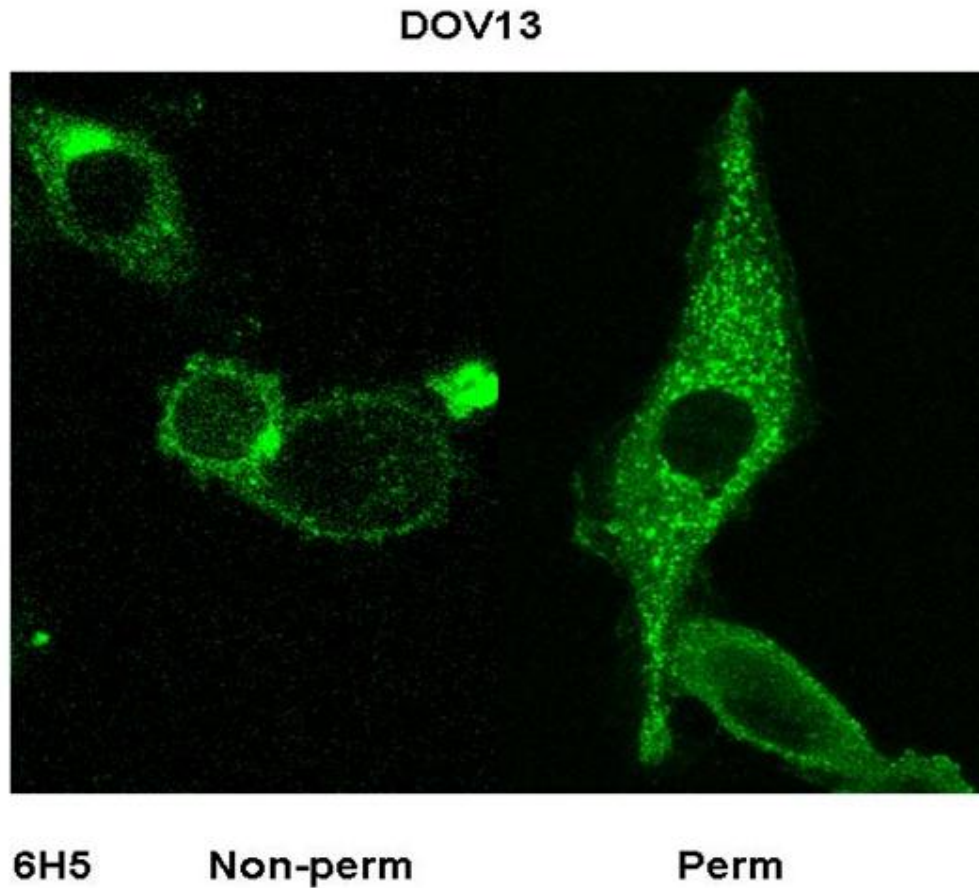
**Table 2:** The expression profile of HERV-K in ovarian tissues using anti-HERV-K monoclonal Ab (6H5). Statistical data corresponding to IHC data. Adjusted residual analysis, Z-test, and Chi-squared test were used for statistical analysis.



### **3.3 Cell surface expression of HERV-K env protein in primary samples from OC, benign and adjacent uninvolved tissues**

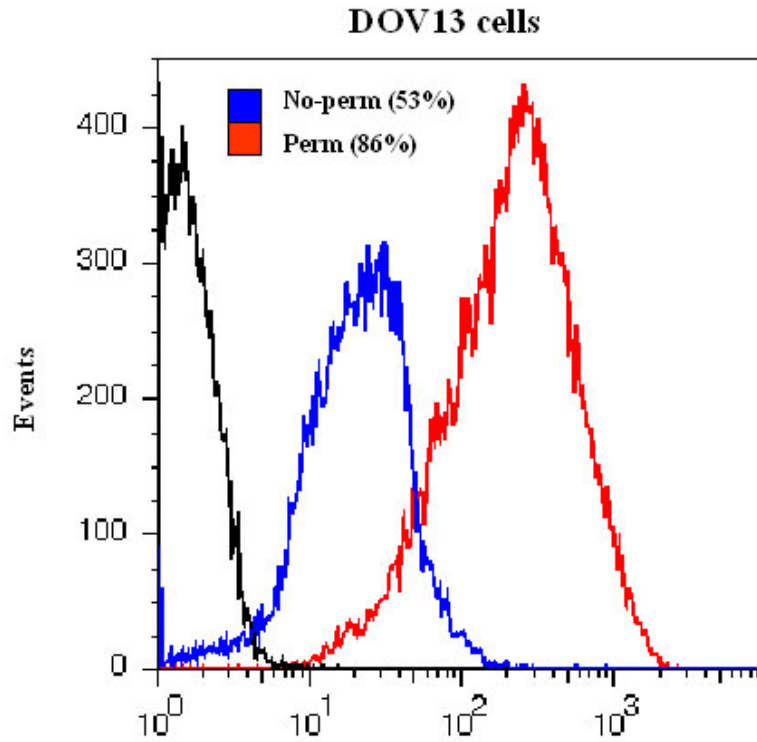
Next, we wanted to expound on the translational data and confirm HERV-K expression on the cell surface. To accomplish this, we used 6H5 mAb to label the cellular distribution of viral components via IFS and subsequent confocal microscopy. We first detected surface and cytoplasmic expression of HERV-K env protein in the established human OC cell line, DOV13. The confocal image in Figure 8 confirms that indeed HERV-K expression is seen on the surface and in the cytoplasm of these cells. FACS data presented in Figure 9 confirms this data, indicating 53% of the cell population is positive for surface and 86% of the cell population is positive for cytoplasmic expression of HERV-K. We next focused on HERV-K protein expression in our primary cell lines. Figure 10 presents confocal images of OC cells obtained from a patient diagnosed with serous carcinoma. The images show that HERV-K env expression is confined to the cell surface. In contrast, Figure 11 presents images of adjacent uninvolved cells derived from a patient diagnosed with mullerian borderline tumor with isolated foci of invasion. HERV-K env expression on the cell surface is not observed in these cells. This data also confirmed that activation of HERV-K indeed correlates with clinical or histological characteristics of OC patients. This data is in accordance with the previous RT-PCR, IHC, and FACS data.





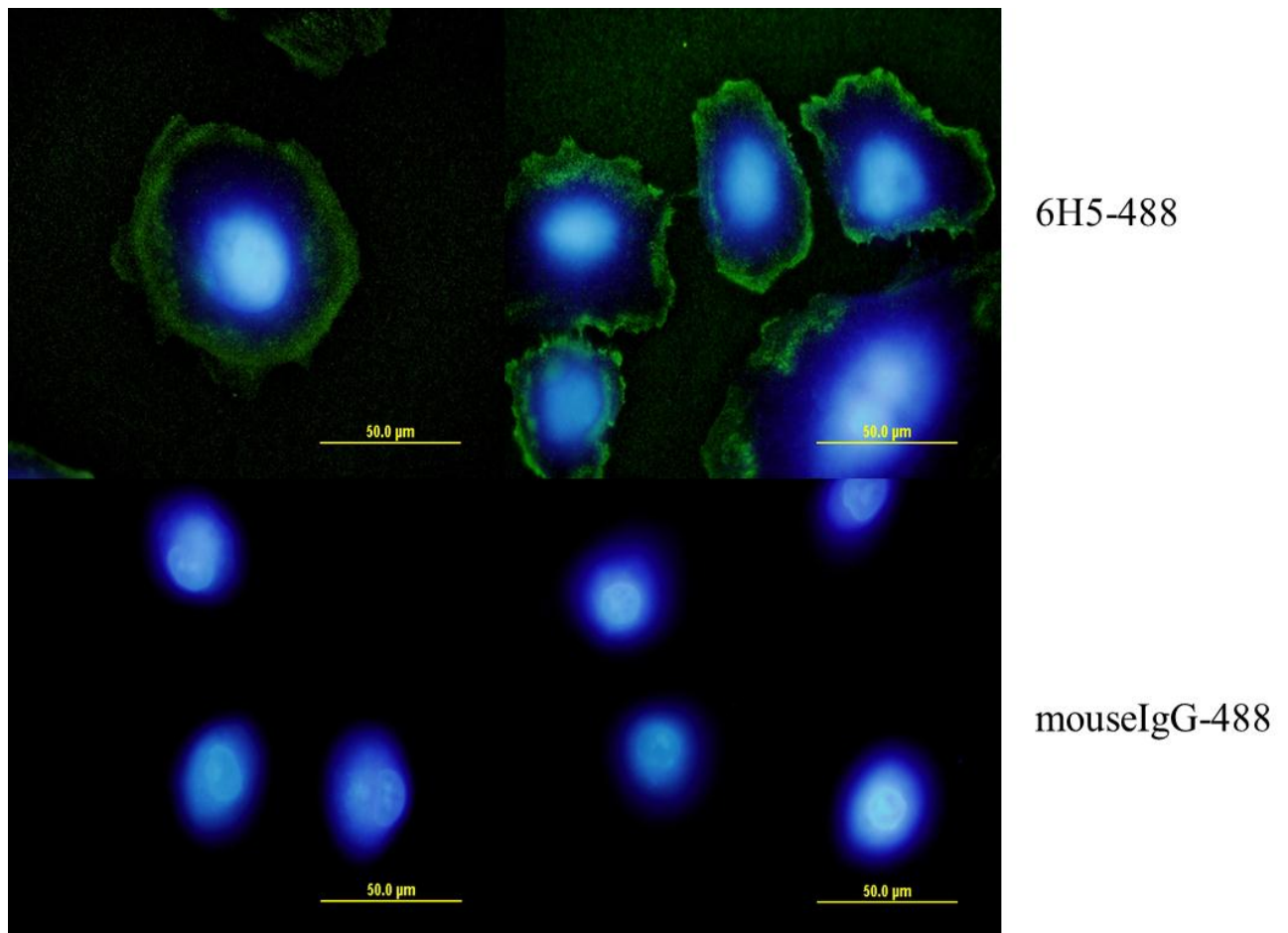
**Figure 8:** Detection of surface (Non-perm) and cytoplasmic (Perm) expression of HERV-K env protein in the established OC cell line, DOV13, via IFS and confocal microscopy. DOV13 cells were EDTA-digested, pelleted on grown on glass coverslips overnight at 37°C. Cells were either permeabilized with FACS permeabilization buffer or fixed with FACS fixation buffer and then labeled with 6H5 antibody followed by an anti-mouse-IgG Alexa Fluor 488 conjugated antibody. Confocal microscopy images show both surface and cytoplasmic expression of HERV-K.





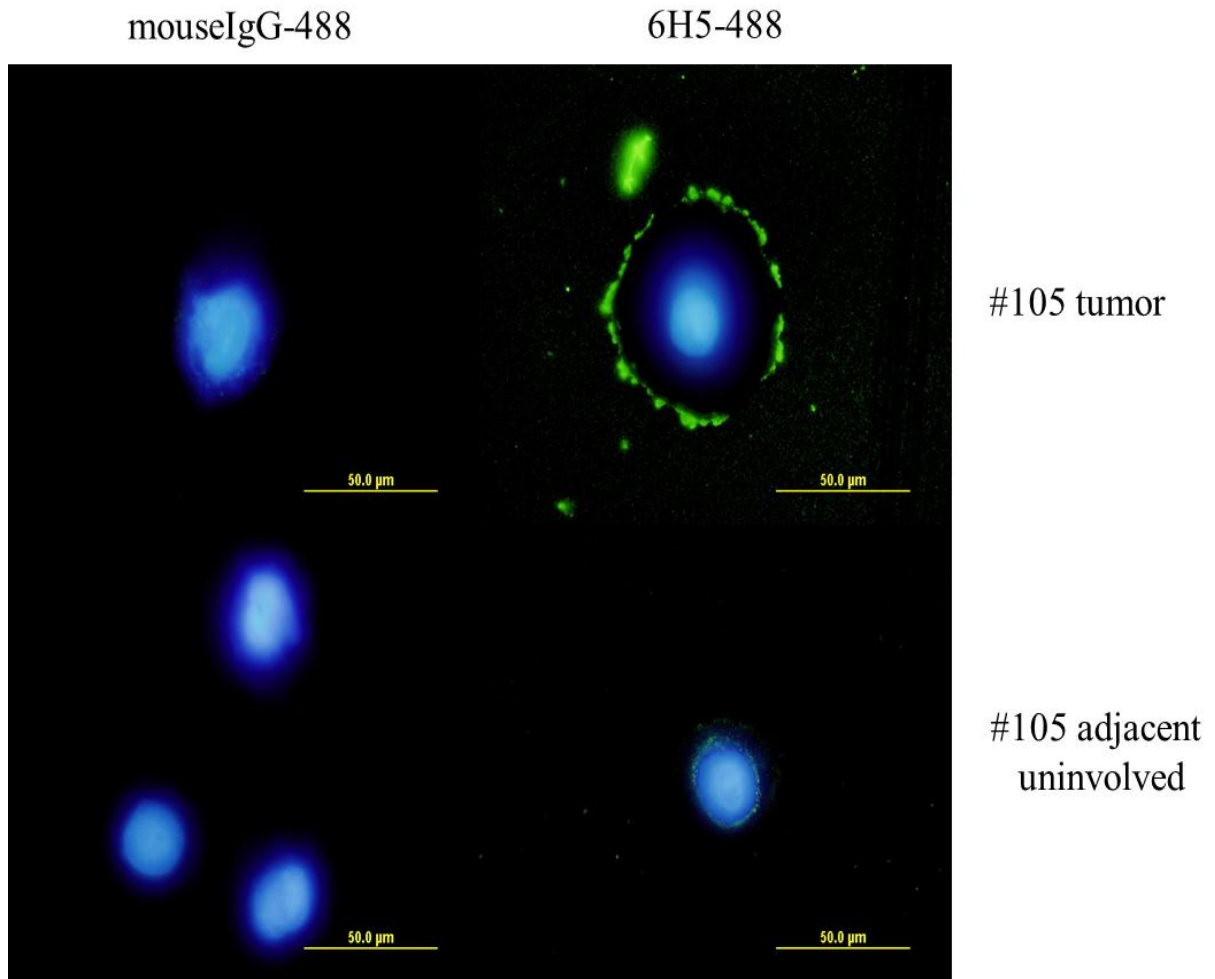
**Figure 9:** Detection of surface (Non-perm) and cytoplasmic (Perm) expression of HERV-K env protein in the established OC cell line, DOV13. DOV13 cells were EDTA- digested, pelleted and either permeabilized with FACS permeabilization buffer or fixed with FACS fixation buffer and then labeled with 6H5 antibody followed by an anti-mouse-IgG Alexa Fluor 647 conjugated antibody. FACS analysis on 6H5 conjugated to Alexa Fluor 647 labeled DOV13 cells reveals both surface (blue line) and cytoplasmic (red line) HERV-K expression.





**Figure 10:** Detection of surface expression of HERV-K env protein in primary OC cells by IFS and subsequent fluorescent microscopy. Pt#222 was diagnosed with serous carcinoma. After tissue harvest, cells were plated and allowed to expand. Cells were EDTA-digested, pelleted, and grown on glass coverslips. Cells were then fixed and labeled with 6H5 antibody, followed by anti-mouse-IgG Alexa Fluor 488 conjugated antibody. Fluorescent images depict surface expression of HERV-K in these OC patient primary cells. Cells labeled with only the secondary anti-mouse-IgG Alexa Fluor 488 conjugated antibody were used as a negative control.





**Figure 11:** Detection of surface expression of HERV-K env protein in primary OC cells by IFS and subsequent fluorescent microscopy. Tumor and adjacent uninvolved tissue were harvested from #105, diagnosed with mullerian borderline tumor with isolated foci of invasion. Cells were EDTA-digested, pelleted, and grown on glass coverslips. Cells were then fixed and labeled with 6H5 antibody, followed by anti-mouse-IgG Alexa Fluor 488 conjugated antibody. Images of tumor cells depicts cell surface staining for HERV-K. Images of adjacent uninvolved cells depict no surface expression of HERV-K in these cells. Cells labeled with only the secondary anti-mouse-IgG Alexa Fluor 488 conjugated antibody were used as a negative control.



### 3.3 Primary patient cell characterization

Recent studies have shown that non-neoplastic cells, notably the stromal cells of human carcinomas, are active and essential collaborators with neoplastic epithelial cells within tumor masses. The diverse stromal cell types within tumors are all members of several mesenchymal cell lineages that generate both connective tissue and immune cells and are biologically very different from epithelial cells whose transformation drives the growth of carcinomas<sup>243</sup>. For decades, intermediate filament proteins have been used as molecular markers in diagnostic histopathology<sup>244-246</sup>, and evidence suggests that aberrant expression of individual cytokeratins or vimentin results in abnormal cell behavior<sup>247</sup>. Indeed it has been determined that epithelial and mesenchymal cells undergo dramatic changes in their cytoskeletal scaffolds during tumorigenesis.

Vimentin is the main intermediate filament (IF) protein in mesenchymal cells<sup>248</sup>, and maintains cytoarchitecture and tissue integrity<sup>249, 250</sup>. However, a growing body of evidence has focused on the role of vimentin in cell migration and epithelial-mesenchymal transition of epithelial carcinomas<sup>248</sup>. Studies of human epithelial carcinoma cell lines have demonstrated that vimentin expression is induced in invasive cells lines<sup>251-253</sup> and in BC, its expression is correlated with poor prognosis<sup>254</sup>. Also, studies have proven that occurrence of metastasis in hepatocellular carcinoma is significantly associated with over-expression of vimentin<sup>255</sup>. In a recent study, Wang Y, Wu R, Cho KR, Thomas DG, Gossner G, Liu JR, Giordano TJ, Shedden KA, Misek DE, and Lubman DM compared protein expression in low stage (Stage 1) versus high stage (Stages 3/4) ovarian serous adenocarcinomas and determined that vimentin was significantly overexpressed in the high stage tumors as compared with the low stage<sup>256</sup>. It is unknown whether vimentin expression is a marker for



epithelial-mesenchymal transition (EMT)-like changes or if vimentin expression is required for the invasive behavior of cells<sup>248</sup>.

Cytokeratins are keratin-containing intermediate filaments found in the intracytoplasmic cytoskeleton of epithelial tissue. Cytokeratins are composed of a family of at least 20 different polypeptides and are distributed in both normal and neoplastic tissues<sup>244</sup>. Epithelial ovarian tumors are thought to arise from the ovarian surface epithelium and contain cytokeratins 7, 8, 18, and 19<sup>257</sup>. However, loss of cytokeratin expression has been found to occur in the primary tumors and is associated with factors predictive for an unfavorable prognosis for patients with BC. In one study, IHC analysis of BC tissues showed that reduced cytokeratin 18 expression was correlated with poor clinical outcome<sup>258</sup>. Also, down-regulation of cytokeratin 19 expression and overexpression of vimentin were found in highly aggressive BC cell lines exhibiting strong migratory and invasive abilities<sup>259</sup>. Loss of cytokeratin 8 and 18 expression and concurrent up-regulation of vimentin has also been found to suggest that disseminated tumor cells have acquired a mesenchymal-like, aggressive phenotype<sup>247</sup>. Alternatively, numerous others studies have shown that coexpression of vimentin and cytokeratin correlates with invasiveness and metastasis<sup>260-263</sup>.

Thus, our next goal was to characterize the primary cells harvested and used in our experiments to determine populations of epithelial and mesenchymal cells and changes in cytoskeletal scaffolds. We therefore employed the following antibodies: anti-vimentin to identify cells of mesenchymal origin, anti-pan cytokeratin to identify epithelial cells, and anti-NCL-L-5D3 (cytokeratin 8 and 18) to identify tumor epithelial cells. After labeling the primary cells, we analyzed them via FACS and confirmed these results visually via confocal



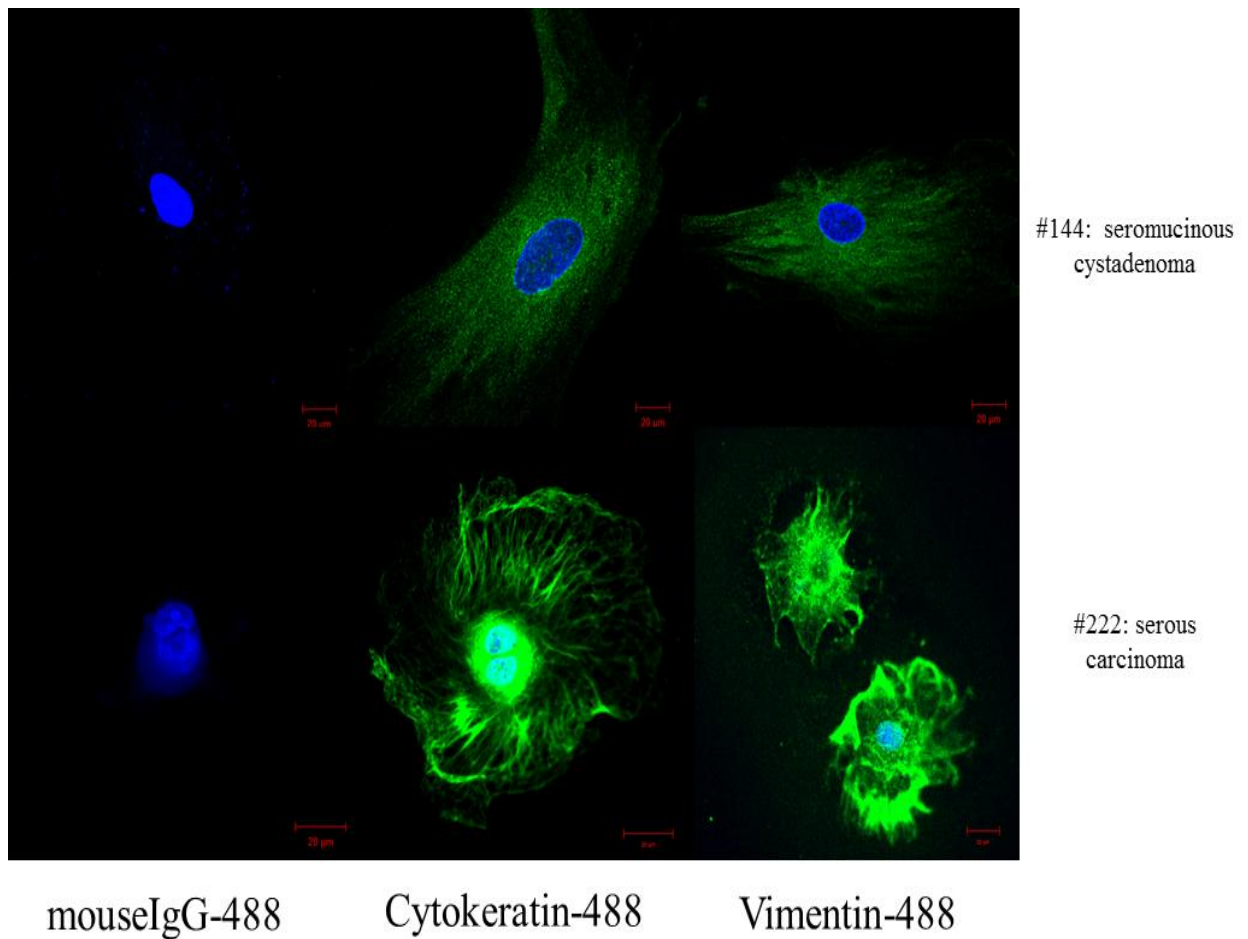
microscopy. Table 3 lists the percentage of cells positive for the respective antibody used in both tumor and benign samples. As seen in Figure 12, there is positive staining for both cytokeratin for both OC and benign samples indicating the presence of epithelial cells. Positive staining is also seen for vimentin for both OC and benign samples indicating mesenchymal cells or cells undergoing epithelial-to mesenchymal transition. Both benign and OC samples stained positively for NCL-L-5D3 indicating the presence of tumor epithelial cells. This positive co-expression of cytokeratin and vimentin corresponds to results from other studies discussed previously. Patient #65 diagnosed with metastatic adenocarcinoma displayed very high coexpression of cytokeratins and vimentins which correlated with the cell's strong migratory and invasive behavior.



Patient	Dignosis	Vimentin	Pan Cytokeratin	NCL-L-5D3 (cyto 8 and 18)
#65 A	metastatic adenocarcinoma	91.46%	92.14%	91.68%
#105 T	mullerian borderline tumor	98.23%	97.60%	97.43%
#118 T	serous adenocarcinoma	98.89%	95.96%	91.53%
#151 A	adenocarcinoma	96.26%	93.44%	88.81%
#153 T	papillary serous carcinoma	98.89%	88.93%	88.35%
#156 T	serous borderline tumor (LMP)	99.42%	99.22%	98.64%
#158 T	serous carcinoma	98.92%	99.28%	99.52%
#188 T	high grade serous	51.86%	55.98%	39.80%
#191 T	sclerosing stromal tumor	73.15%	74.28%	61.54%
#212 A	serous adenocarcinoma	99.14%	99.76%	99.96%
#204 T	serous adenocarcinoma	35.48%	39.57%	25.61%
#207 T	high grade serous	62.47%	51.18%	35.22%
#210 T	papillary serous	27.22%	25.88%	19.12%
#213 T	high grade serous	81.59%	89.12%	84.97%
#222 A	serous carcinoma	71.98%	75.89%	65.16%
#209 B	teratoma	98.20%	97.58%	95.66%
#144 B	seromucinous cystadenoma	28.37%	30.81%	N/A
#206 B	benign cyst	14.30%	26.50%	19.14%
Pt#215 B	benign seromucinous cystadenoma	99.10%	98.28%	93.00%
Pt#218 B	serous adenofibroma	94.57%	97.15%	95.92%

**Table 3:** Primary cell line characterization: Epithelial, mesenchymal, and tumor epithelial cell populations via FACS. After harvesting patient tissues and culturing cells, samples were characterized via FACS. Cells were stained with anti-vimentin, anti-pan cytokeratin, and anti- NCL-L-5D3 antibodies and subjected to flow cytometry analysis. Percentages indicate populations of cells positive for the respective antibody used.





**Figure 12:** Primary cell line characterization: Epithelial and mesenchymal cell populations via IFS and confocal microscopy. Benign cells from #144 diagnosed with benign disease and tumor cells from #222 diagnosed with serous carcinoma are shown. Human primary cells were characterized approximately 4 days post-harvest to determine specific cell populations. Cells were grown on glass coverslips, fixed, and labeled with anti-cytokeratin and anti-vimentin followed by a secondary anti-mouse IgG Alexa Fluor 488 conjugated antibody. Secondary antibody only was used as a negative control and indeed no staining is seen. The figure shows positive staining for cytokeratin for both OC and benign samples indicating the presence of epithelial cells. Positive staining is also seen for vimentin for both OC and benign samples indicating mesenchymal cells or cells undergoing epithelial-to-mesenchymal transition.



### 3.4 Individual cases of patient characterization

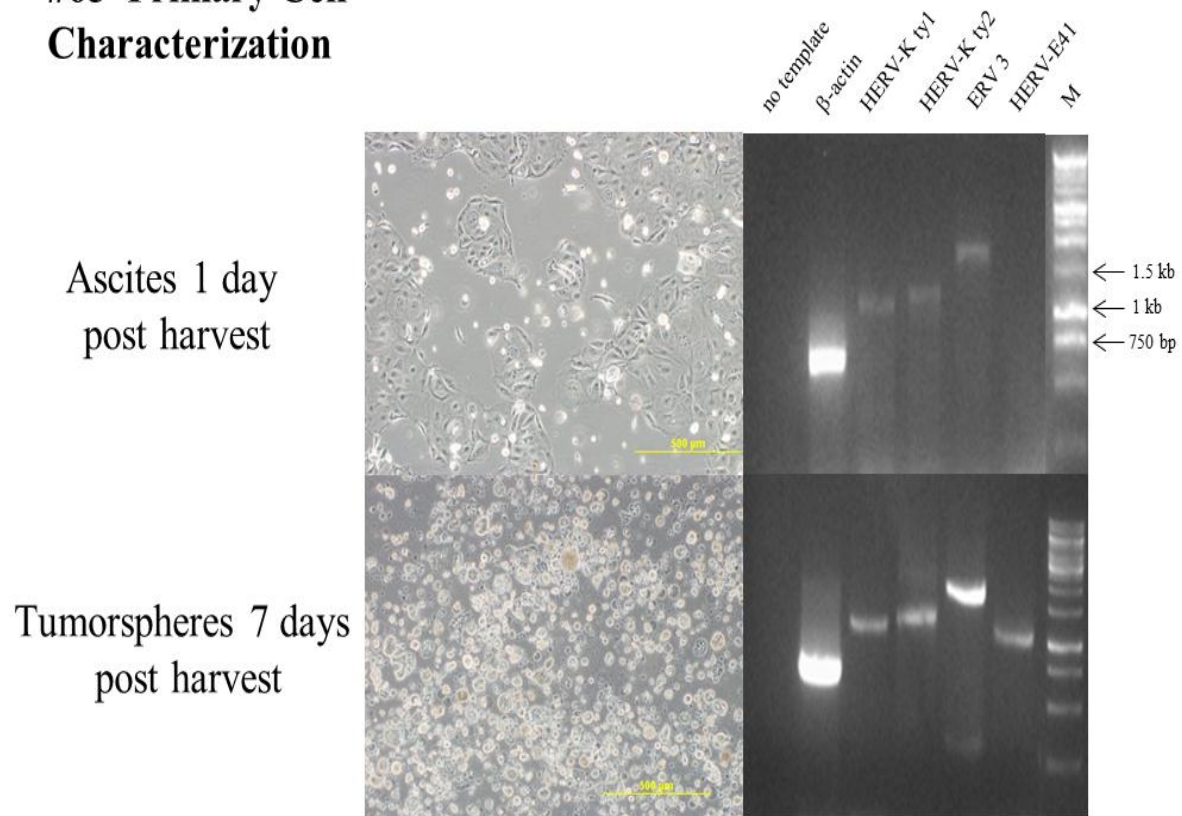
We next focused our attention on an OC patient diagnosed with metastatic adenocarcinoma. Figure 13 depicts cultured ascites cells from this patient one day post-harvest in 2-D culture to display cell morphology. These cells are morphologically similar to epithelial cells and not stromal cells which are classified by a cell body and cell processes that are long and thin. After harvesting the ascites on day 0, we cultured cells on 0.8% agarose plates in serum-free Mammocult media in order to support stem cell populations. These cells were then termed “tumorspheres” as their appearance was of suspended spheres. RT-PCR data for this patient at both time points, asserts expression of mRNA HERV-K type 1 and type 2 transcripts, and ERV-3 transcripts. However, expression is clearly increased after tumorsphere culture. Expression of HERV-E41 transcripts are seen after tumorsphere culture. Figure 14 displays a QIFIKIT assay employed in order to quantify the number of HERV-K surface molecules on ascites cells one day post-harvest. 164,528 HERV-K molecules were determined to be expressed on the cell surface. Finally, Figure 15 reveals FACS analysis of tumorspheres confirming HERV-K, epithelial, mesenchymal and epithelial cancer cell positivity after staining with 6H5 mAb, anti-vimentin, anti-cytokeratin, and anti-NCL-L-5D3 antibodies at 1 day and 7 days post-harvest. After tumorsphere culture, populations of cytokeratin, vimentin, tumor epithelial, HERV-K positive populations increased. We are also very interested in determining OC stem cell populations. Therefore, we stained cells with anti-CD133 and anti-CD44 cancer stem cell markers. FACS analysis shows that populations of cells positive for CD44 increased, but CD133 positive populations decreased slightly.



Figure 16 illustrates another patient of interest, diagnosed with metastatic serous carcinoma. After harvesting the ascites cells, we cultured them in tumorsphere forming conditions as seen in the figure. RT-PCR data for this patient at both time points, asserts expression of mRNA HERV-K type 1 and type 2 transcripts, ERV-3, and HERV-E41 transcripts . However, expression is clearly increased in all transcripts after tumorsphere culture, except for ERV3 which decreased slightly. Finally, we wanted to determine if HERV-K expression and other characteristics changed in response to this cancer stem cell supporting environment. After staining with 6H5 mAb, anti-vimentin, anti-cytokeratin, anti-NCL-L-5D3, anti-CD133, and anti-CD44, the results in Figure 17 show that the percentage of positive populations for each marker increased after tumorsphere culture, except for CD133 which decreased slightly.

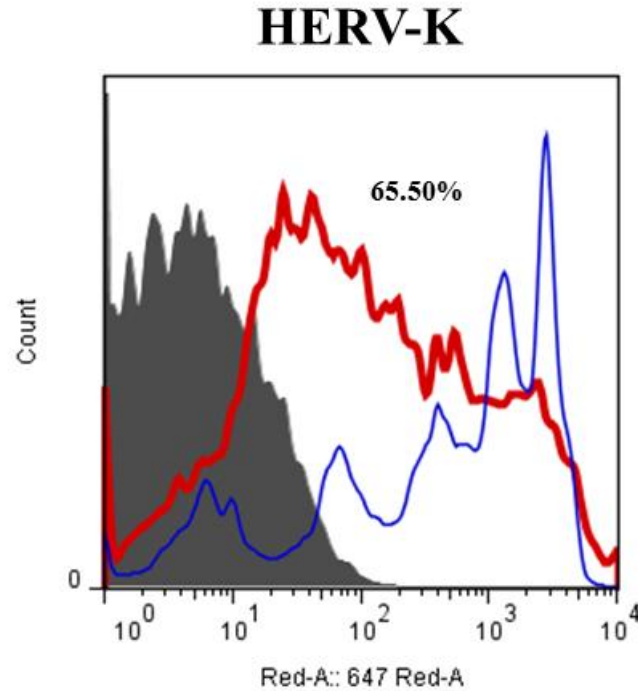


## #65 Primary Cell Characterization



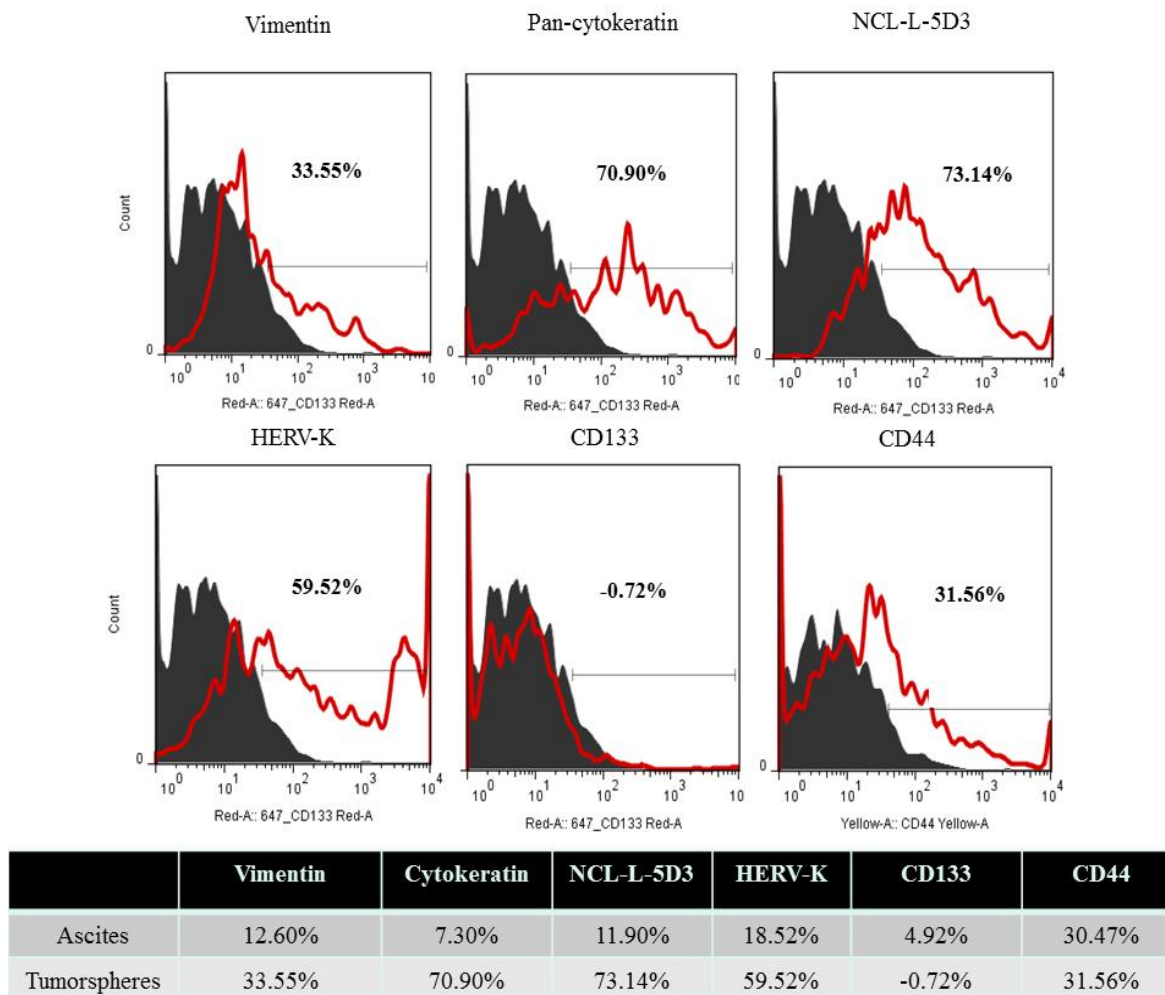
**Figure 13:** Human primary cell line characterization: HERV-K mRNA expression and cell morphology in Patient #65 ascites and tumorspheres. Patient #65 was diagnosed with metastatic adenocarcinoma. RT-PCR data for cells in culture 1 day post-harvest and 7 days post-harvest in tumorsphere conditions. 2D images of cells in culture 1 day post-harvest and 7 days post-harvest in tumorsphere conditions.





**Figure 14:** Human primary cell line characterization: Quantitation of HERV-K cell surface molecules in Patient #65 ascites. Patient #65 was diagnosed with metastatic adenocarcinoma. Quantitation of HERV-K env molecules on the surface of primary cells via QIFIKIT and FACS analysis. The gray, filled line represents the isotype control, the red line represents the HERV-K positive population and the blue line represents a series of 6 bead populations coated with well-defined quantities of a mouse monoclonal antibody. 164,258 surface molecules of HERV-K were present on the surface of these cells.

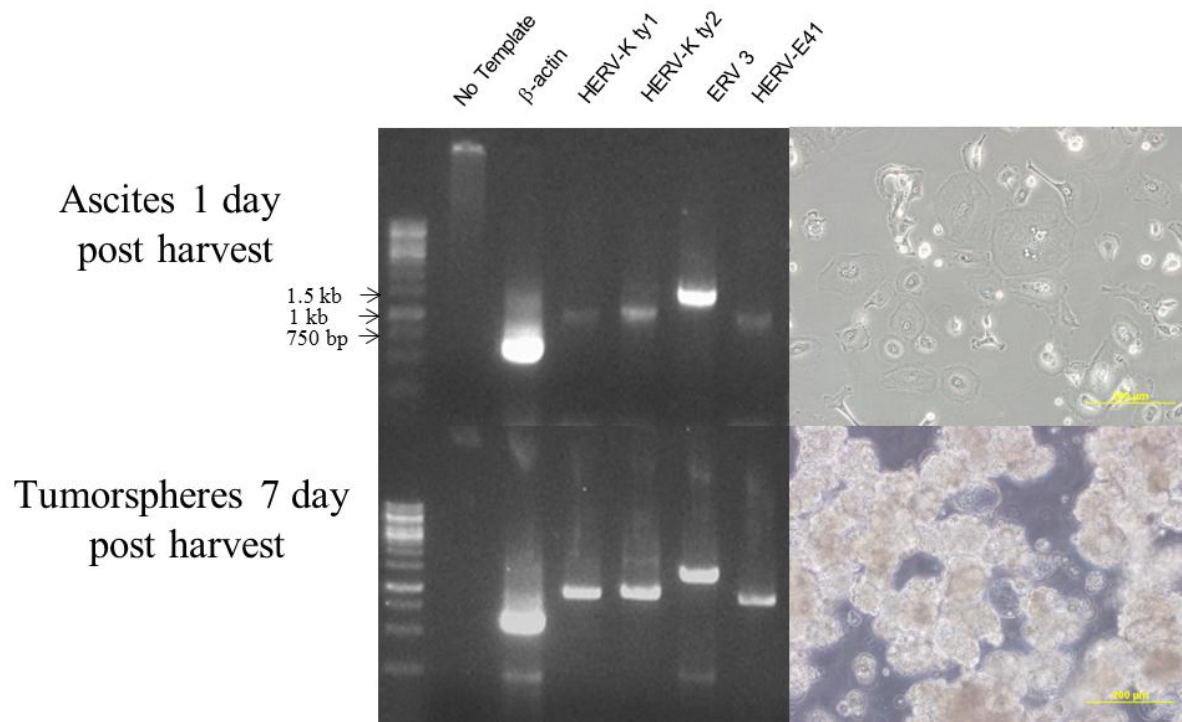




**Figure 15:** Human primary cell line characterization: Epithelial, mesenchymal, HERV-K+, and stem cell populations in patient #65 ascites and tumorspheres via FACS. Patient #65 was diagnosed with metastatic adenocarcinoma. After patient tissue was harvested, cells were grown in serum-free Epicult medium on 0.8% agarose coated plates to support tumorsphere formation. Primary cells were characterized via staining 1 day post-harvest and 7 days post-harvest with 6H5 mAb (HERV-K expression), anti-vimentin Ab (mesenchyme-derived cells), anti-cytokeratin mAb (epithelial cells), anti-NCL-L-5D3 mAb (tumor epithelial cells), anti-CD44 mAb (OC stem cells), and anti-CD133 mAb (OC stem cells), followed by FACS analysis. A table at the bottom summarizes the FACS data.

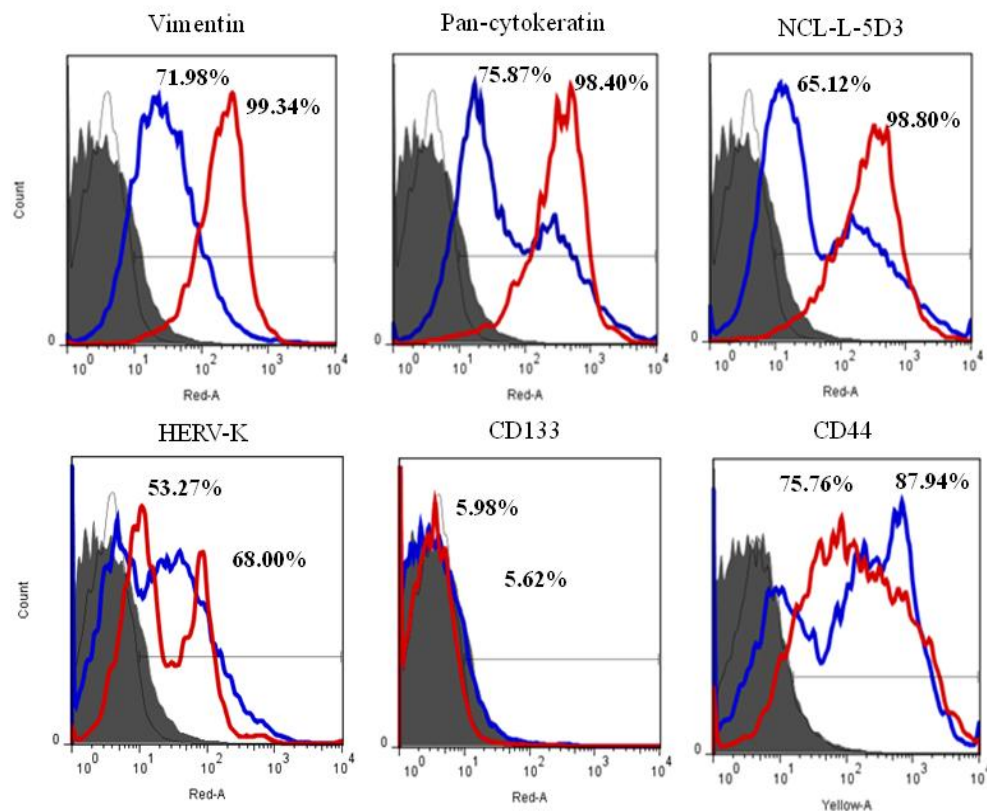


## #222 Primary Cell Characterization



**Figure 16:** Human primary cell line characterization: HERV-K mRNA expression and cell morphology in Patient #222 ascites and tumorspheres. Patient #222 was diagnosed with serous carcinoma. RT-PCR data for cells in culture 1 day post-harvest and 7 days post-harvest in tumorsphere conditions is shown. 2D images of the cells in culture 1 day post-harvest and 7 days post-harvest in tumorsphere conditions is shown.





	Vimentin	Cytokeratin	NCL-L-5D3	HERV-K	CD133	CD44
Ascites	71.98%	75.87%	65.12%	53.27%	5.98%	75.76%
Tumorspheres	99.34%	98.40%	98.80%	68.00%	5.62%	87.94%

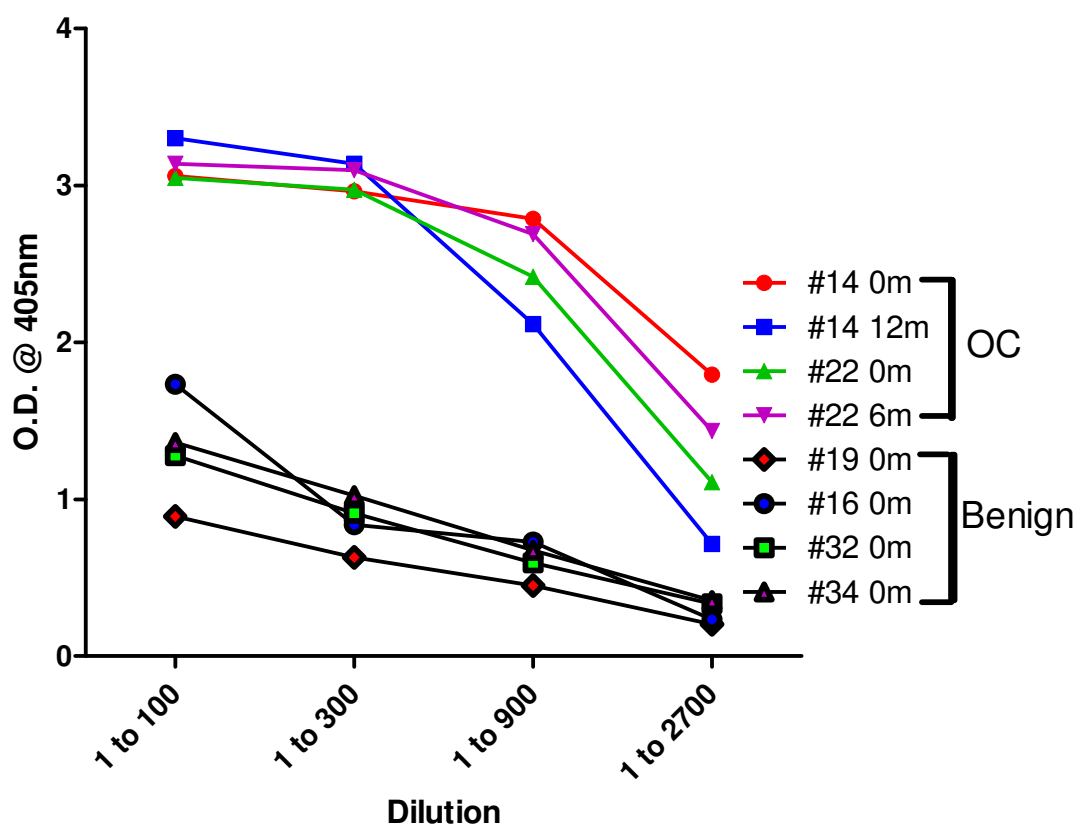
**Figure 17:** Human primary cell line characterization: Epithelial, mesenchymal, HERV-K+, and stem cell populations in Patient #222 ascites and tumorspheres via FACS. Patient #222 was diagnosed with serous carcinoma. Primary cells were characterized 1 day post-harvest and 7 days post-harvest to determine differences. Primary cells were characterized via staining with anti-vimentin Ab (mesenchyme-derived cells), anti-cytokeratin Ab (epithelial cells), anti-NCL-L-5D3 Ab (tumor epithelial cells), anti-CD44 Ab (OC stem cells), anti-HERV-K env protein (6H5) and anti-CD133 Ab (OC stem cells), and subsequent FACS analysis depicted in the bottom panel. A table at the bottom summarizes the FACS data.



### **3.5 Levels of anti-HERV-K Abs in OC, benign patient, and normal donor serum samples**

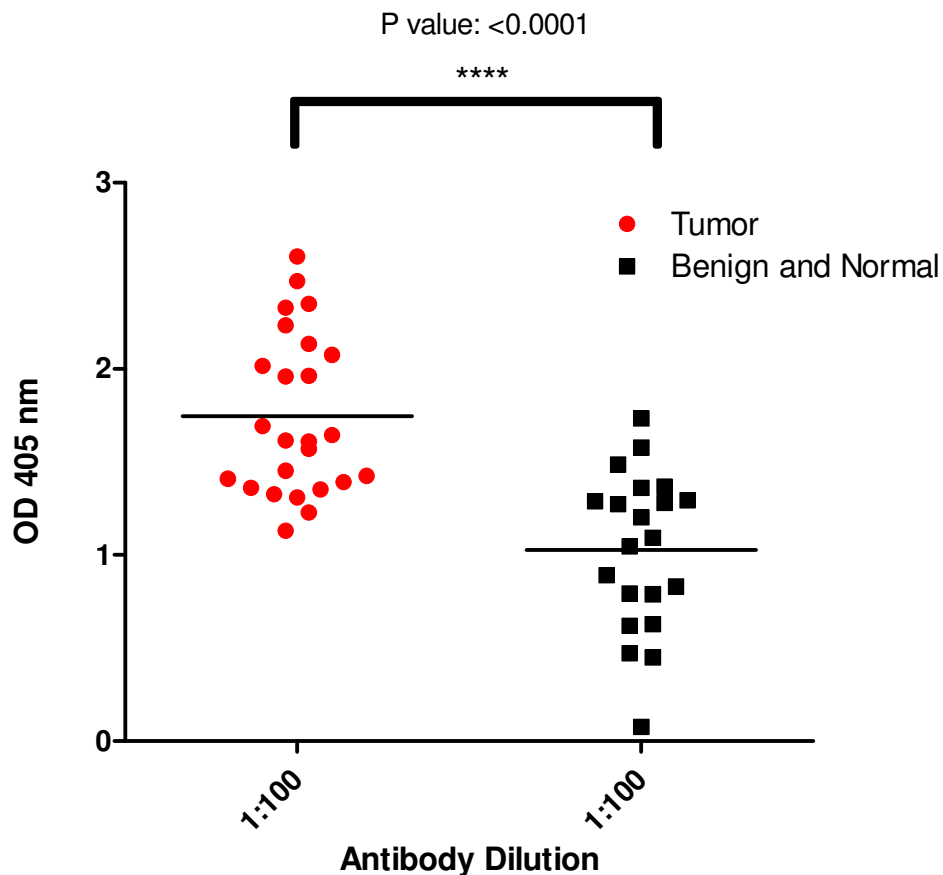
Our next objective was to determine if amounts of anti-HERV-K antibodies are different between OC, benign patient, and normal donor serum samples. We obtained several patients with various serum draws post operation. Therefore, “0m” indicated a serum draw one day before the surgery and “6m” indicated a serum draw 6 months post-surgery. Human patient serum samples were diluted and added to ELISA plates coated with purified HERV-K env protein. First we assayed 2 OC patients, #14 and #22, with two blood draw dates and 4 patients with benign disease, #19, #16, #32, #34, and several dilutions. As seen in Figure 18, titers of anti-HERV-K antibodies were significantly higher in OC patients compared to patients with benign disease. Also in this graph, serum taken the day before surgery (0m) was compared to serum taken 6 and 12 months post-surgery. No trends were seen in antibody levels and further testing needs to be completed with longer draw date span samples and in patients who experience tumor recurrence. A summary graph in Figure 19 shows that anti-HERV-K antibody titers are significantly higher in patients with OC (n=25) compared to patients with benign disease and normal donors (n=21). This data confirms that anti-HERV-K antibodies present in patients at different stages of disease and correlate with clinical and histological characteristics.





**Figure 18:** ELISA assay with sera from OC and patients with benign disease. ELISA assay was used to detect various titers of anti-HERV-K antibodies in OC patient and patients with benign disease. Patient diagnoses are as follows: #14 = serous borderline, #22 = high grade serous carcinoma, #19 = teratoma, #16 = teratoma, #32 = teratoma, #34 = teratoma. Human sera was diluted and added to blocked ELISA plates coated with purified HERV-K protein. 0m indicates a serum draw days before the surgery to remove the tumor or benign disease and 6m indicates a serum draw 6 months post-surgery. Significantly more anti-HERV-K antibodies were found in OC patient sera compared to patients with benign disease.





**Figure 19:** ELISA Summary: Detection of anti-HERV-K antibodies in serum from OC patients, patients with benign disease, and normal donors. ELISA assay was used to detect anti-HERV-K antibodies in OC patients, patients with benign disease, and normal donors. Human sera was diluted and added to blocked ELISA plates coated with purified HERV-K protein. Significantly more anti-HERV-K antibodies were found in OC patient sera (n=25) compared to patients with benign disease and normal controls (n=21). A student's *t*-test was used to calculate significance.



### 3.6 Characterization of lymphocytes obtained from OC and benign samples

Our encouraging results from the first aim served as a great precedent for our second aim, to evaluate the immune responses triggered by HERV-K antigen in OC and benign patients. Our first objective was to characterize the different types of lymphocytes present in the PBMC and IVS samples. This is important because T cell infiltrates are frequently associated with a favorable prognosis for many epithelial cancers<sup>216, 264-272</sup>. Specifically, a plethora of studies have documented infiltration by both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in epithelial OC. CD4<sup>+</sup> helper T cells have no cytotoxic or phagocytic activity but instead activate other immune cells. Examples of these activities include aiding in B cell antibody class switching, activation and growth of cytotoxic CD8<sup>+</sup> T cells, and bactericidal activity of phagocytes. There are exceptions, including T-regs, natural killer cells and cytotoxic T cells that express CD4, but these are not considered T helper cells. CD8<sup>+</sup> cytotoxic T cells induce death in tumor cells or cells that are infected with viruses or other pathogens. Studies have shown that patients with ovarian tumors containing dense infiltrates of CD3<sup>+</sup>CD8<sup>+</sup> T cells experience unexpectedly favorable progression-free and overall survival<sup>113</sup>, however the association of tumor infiltrating lymphocytes subpopulations with clinical outcome remains controversial<sup>273</sup>.

NK cells are widely known for their ability to kill cells infected by certain viruses, intracellular bacteria and tumor cells. Also, their cytolytic activity and secretion of cytokines and chemokines are crucial components of the innate immune system<sup>274, 275</sup>. In a recent study, autologous NK cells had the ability to recognize and kill human ovarian carcinoma cells<sup>276</sup>. Similarly, Alves PC, Andrade LA, Petta CA, Lorand-Metze I, Derchain



SF, and Guimarães F demonstrated that effector cells enriched with CD56<sup>+</sup> lymphocytes were the main variable affecting target cell killing in patients with ovarian neoplasia<sup>277</sup>.

As described in the Introduction, T-regs are a subpopulation of T lymphocytes that down-regulate the immune response and accumulation of intratumoral T-regs was found to be associated with a high mortality rate in OC patients<sup>146</sup>. Conversely, another group determined that the presence of T-regs and activated CD4<sup>+</sup> cells within the tumor microenvironment was associated with improved overall progression-free survival in patients with OC<sup>273</sup>. In conclusion, it is the intricate balance between these effector cells and regulatory T cells that is critical in determining patient outcome.

To classify the different populations, we stained cells with the following antibodies and analyzed them by FACS: anti-CD4 to identify T helper cells, anti-CD8 to identify CTLs, anti-CD25 to identify activated T and B cells, anti-CD56 to identify NK cells and anti-FoxP3 to identify T-regs. Table 4 shows that upon transition from PBMC to IVS cell, the percentage of T helper cells, CTLs, activated T and B cells, NK cells, and T-regs increased in a patient diagnosed with benign disease, #182. A second patient diagnosed with a benign cyst, #151, displayed an increase in the percentage of T helper cells, CTLs, activated T and B cells, NK cells, but a slight decrease in T-regs. The remaining 2 OC patients all displayed an increase in the percentage of T helper cells, CTLs, and activated T and B cells, however a decrease in NK and T-reg positive cells.



#	Diagnosis		CD8+	CD4+	CD56+	CD25+	FoxP3+
#101	teratoma	PBMC	29.80%	44.50%	14.45%	22.51%	18.74%
		IVS	45.09%	68.86%	11.70%	38.08%	37.74%
			15.29%↑	24.36%↑	2.75%↓	15.57%↑	19.00%↓
#151	cyst	PBMC	15.75%	37.18%	13.47%	39.17%	43.44%
		IVS	17.41%	52.88%	21.02%	48.44%	40.41%
			1.66%↑	15.70%↑	7.55%↑	9.27%↑	3.03%↓
#182	cyst	PBMC	18.20%	16.91%	8.09%	13.80%	8.62%
		IVS	23.61%	24.03%	13.18%	21.87%	14.72%
			5.41%↑	7.12%↑	5.09%↑	8.97%↑	6.1%↑
#192	cyst	PBMC	34.69%	31.22%	10.16%	23.36%	7.66%
		IVS	37.44%	40.39%	11.41%	28.49%	5.45%
			2.75%↑	9.17%↑	1.25%↑	5.13%↑	2.21%↓
#193	cyst	PBMC	23.99%	19.50%	4.62%	28.31%	4.09%
		IVS	44.57%	51.75%	6.19%	15.87%	6.60%
			20.58%↑	32.25%↑	1.57%↑	12.44%↓	2.52%↑
#81	serous carcinoma	PBMC	26.71%	24.85%	7.28%	12.98%	7.94%
		IVS	45.50%	55.09%	6.44%	28.31%	6.50%
			18.79%↑	30.24%↑	0.84%↓	15.33%↑	1.44%↓
#153	papillary serous carcinoma	PBMC	9.97%	42.12%	26.92%	23.15%	59.07%
		IVS	17.98%	42.61%	16.20%	52.89%	14.92%
			8.01%↑	0.49%↑	10.72%↓	29.74%↑	44.15%↓
#213	serous carcinoma	TIL	65.78%	51.79%	36.12%	N/A	42.27%

**Table 4:** Characterization of patient PBMCs and IVS cells: CD8+, CD4+, CD25+, CD56+, and FoxP3+ populations via FACS. Cells were stained with anti-CD4 (T helper cells), anti-CD8 (cytotoxic T cells), anti-CD25 (activated T and B cells), anti-CD56 (natural killer cells), and anti-FoxP3(T-regs) antibodies and then analyzed on a BD FACS array. After transition from PBMCs to IVS cells, populations of all specific cell types in both OC and patients with benign disease either increased (green ↑) or decreased (red ↓).



### **3.8 Changes in T cell proliferation in OC patients compared to samples from patients with benign disease and normal donors**

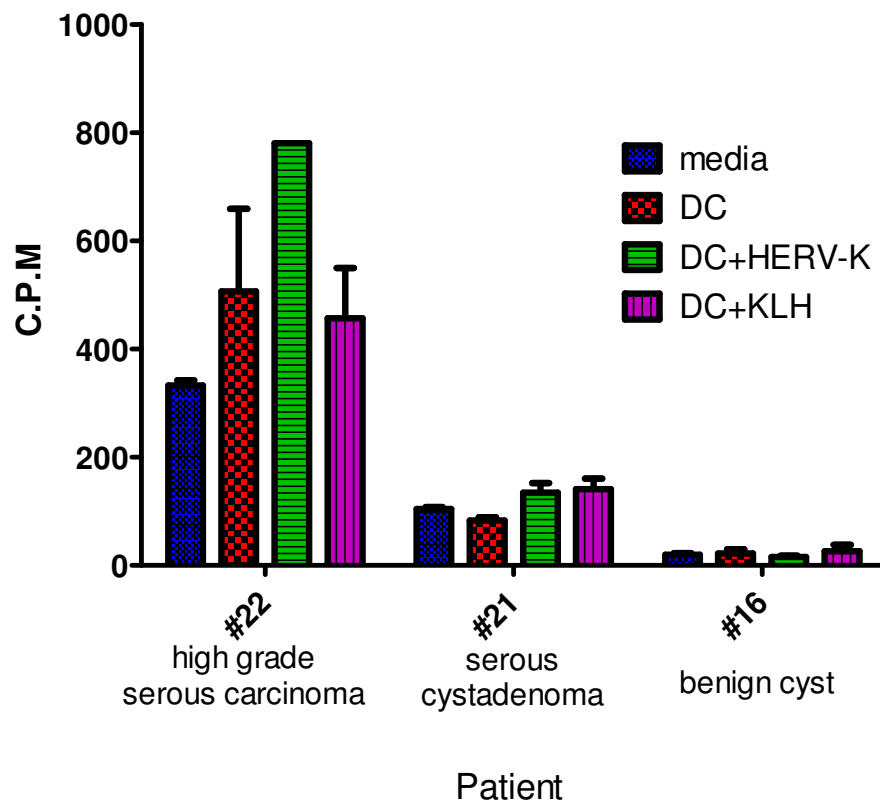
Our next goal was to focus on T-cell responses in ovarian patients. Many agents can specifically or nonspecifically induce T cell activation resulting in proliferation of activated T cells. In this work, we were interested in determining if HERV-K env protein could specifically induce T cell proliferation. To accomplish this, we isolated PBMCs from patient blood samples and generated IVS cells by combining PBMCs with autologous DCs pulsed with HERV-K for 7 days. T cell proliferation specifically induced by DCs pulsed with HERV-K env protein or KLH control protein were then compared between autologous PBMC and IVS cells and between OC, patients with benign disease or normal donors via [<sup>3</sup>H] thymidine incorporation assay. Figure 20 depicts 2 patients with benign disease and 1 with OC. T cell proliferation was clearly higher in the OC patient compared to the patients with benign disease. Also, DCs pulsed with HERV-K induced greater T-cell proliferation compared to DC pulsed with KLH, unpulsed DCs, or media alone. Another T cell proliferation assay is depicted in Figure 21 showing results for 4 patients with OC, one with benign disease and 3 normal donors. T cell proliferation was higher in OC patients compared to the patient with benign disease and the normal donors. DCs pulsed with HERV-K induced greater T cell proliferation compared to DCs pulsed with KLH, unpulsed DCs, or media alone. Finally, DCs pulsed with HERV-K induced greater T-cell proliferation in IVS cells than in PBMCs.

Figure 22 illustrates a T cell proliferation summary including OC patients (n=8) and patients with benign disease or normal donors (n=6). Overall, HERV-K antigen induced



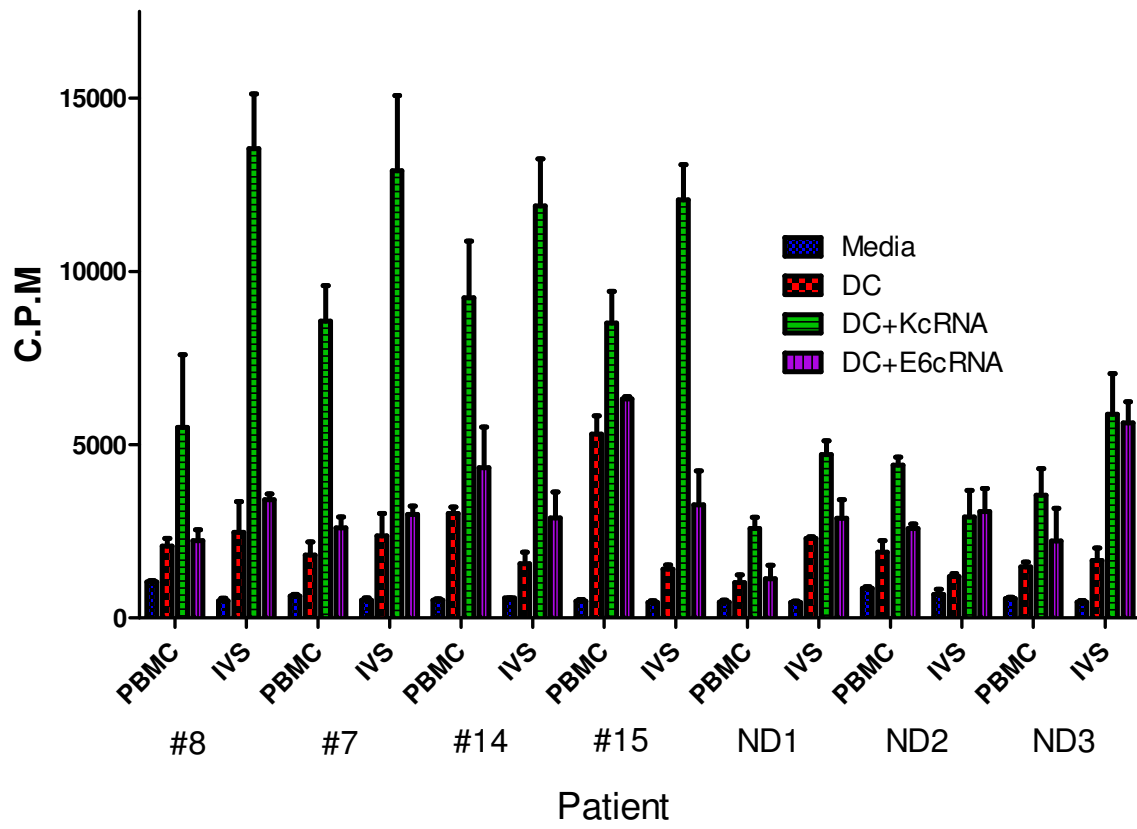
significantly greater T cell proliferation in IVS cells in OC patients compared to the control protein. HERV-K antigen also induced significantly greater T cell proliferation of IVS cells in OC patients than in benign disease patients and normal controls.





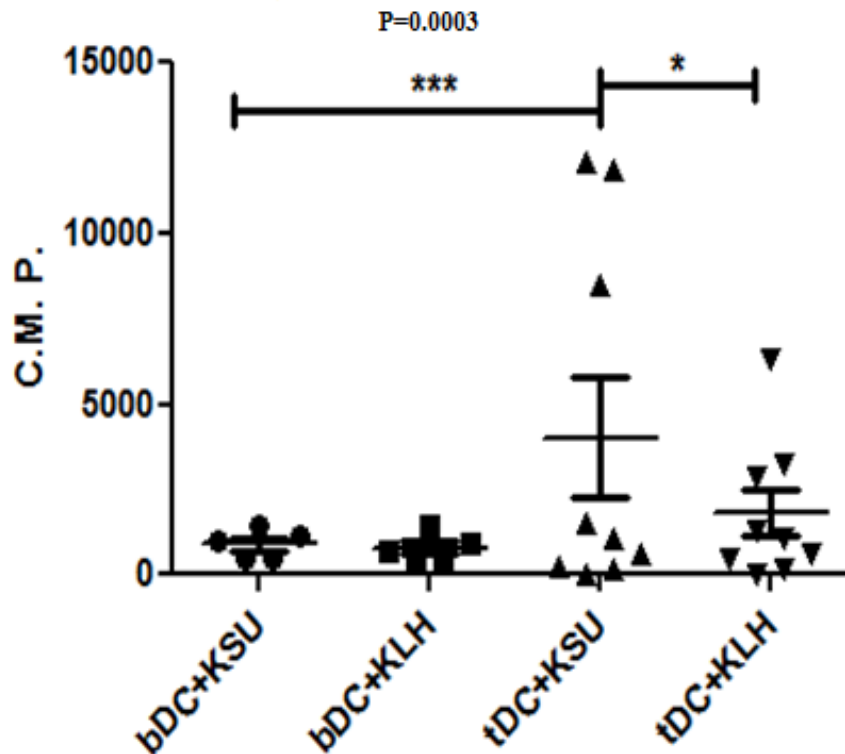
**Figure 20:** T cell proliferation assay. IVS cells were combined with DCs pulsed with HERV-K protein or with KLH control protein. Cells were also combined with media alone and unpulsed DCs as negative controls. T cell proliferation is clearly greater in patients with OC compared to benign diseases when IVS cells are combined with HERV-K pulsed DCs.





**Figure 21:** T cell proliferation assay. Patient diagnoses are as follows: #8=benign simple cyst, #7=metastatic adenocarcinoma, #14=serous borderline, #15=serous borderline, ND1-3=normal female donors. PBMCs or IVS cells were combined with DCs pulsed with cRNA for the HERV-K protein or cRNA for E6 control protein. T cell proliferation is clearly greater in patients with OC compared to benign diseases when IVS cells are combined with HERV-K cRNA pulsed DCs.





**Figure 22:** T cell proliferation summary. IVS cells were combined with DCs pulsed with HERV-K protein or with KLH control protein. T cell proliferation is significantly greater in patients with OC (n=8) compared to benign diseases or normal donors (n=6) when IVS cells are combined with HERV-K pulsed DCs. T cell proliferation is significantly greater when OC patient IVS cells are combined with HERV-K pulsed DCs as compared to KLH pulsed DCs. ). A student's *t*-test was used to calculate significance. b=benign or normal, t=tumor.



### **3.9 Changes in IFN- $\gamma$ secretion in OC patients compared to samples from patients with benign disease and normal donors**

IFN- $\gamma$  is secreted by activated CD4<sup>+</sup> and CD8<sup>+</sup> cells and is critical for innate and adaptive immunity and for tumor control. Therefore, we utilized an ELISPOT assay to analyze IFN- $\gamma$  production at the single cell level. In this particular assay, a spot forms at the site where secreted cytokine is bound; therefore, one spot is equal to one molecule secreting cell. The microplate is then subjected to an automated ELISPOT reader to determine the amount of spots per well. This allows the frequency of cells secreting IFN- $\gamma$  to be calculated. To begin this assay, we isolated PBMCs from patient blood samples and generated IVS cells by combining PBMCs with autologous DCs pulsed with HERV-K for 7 days.  $1 \times 10^5$  KSU IVS cells or PBMCs were combined with media,  $5 \times 10^3$  autologous DCs,  $5 \times 10^3$  DCs pulsed with HERV-K protein or  $5 \times 10^3$  DC pulsed with KLH protein, and incubated for 16 hours before adding the color substrate. IFN- $\gamma$  production, specific to HERV-K env protein pulsed DCs, used as antigen presenting cells, was then compared between PBMC and IVS cells obtained from the same donors. IFN- $\gamma$  production was also compared between OC patients, patients with benign disease, and normal donors.

Figure 23 illustrates an IFN- $\gamma$  ELISPOT assay with 1 OC patient, diagnosed with adenocarcinoma, and 3 normal donors. The image at the top is the actual ELISPOT plate, and the column graph below quantifies the number of spots for this plate. Clearly more IFN- $\gamma$  secretion is observed when IVS cells from the OC patient are combined with HERV-K pulsed DCs, compared to all 3 normal donors. Also, there is clearly less IFN- $\gamma$  secretion



when IVS or PBMCs are combined with media alone, unpulsed DCs, or DCs pulsed with KLH protein, as compared to HERV-K pulsed DCs.

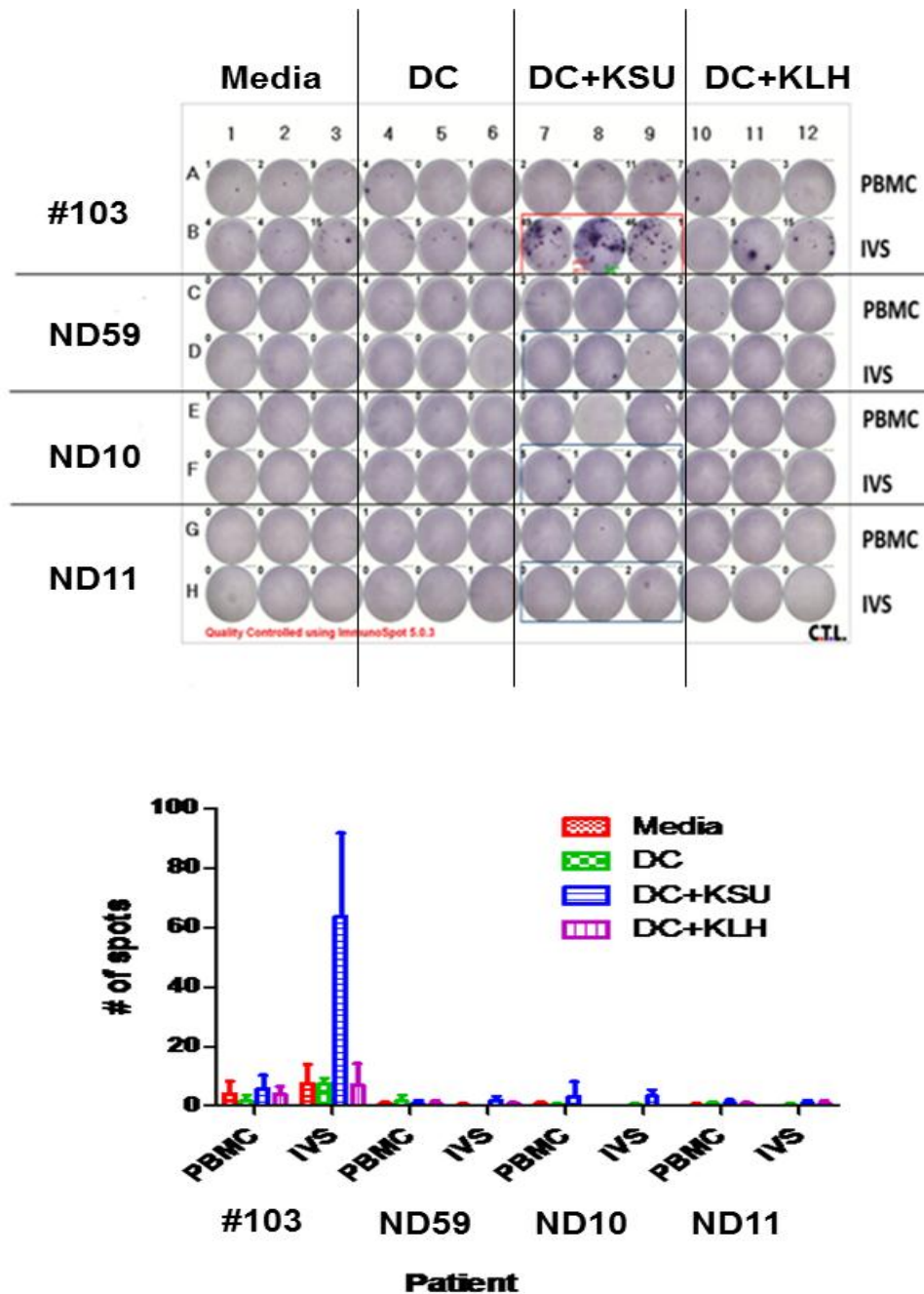
There has been great interest in the expression of retroviral transmembrane proteins in human diseases. As discussed previously in the Introduction, the *env* genes encode a surface (SU) protein and a transmembrane (TM) protein. The function of the TM protein is to hold the SU domain in the viral envelope and it aids in membrane fusion.

Transmembrane envelope proteins of different retroviruses have been correlated with immunosuppressive properties<sup>278, 279</sup>. After demonstrating the strong expression of HERV-K in melanomas<sup>76</sup>, it was established that there was cell surface expression of the TM envelope protein in melanomas and melanoma cell lines, and this protein may interact with the neighboring lymphocytes and induce immunosuppression. Alternatively, in our lab, we discerned a positive correlation between anti-HERV-K TM serum reactivity and tumor progression in human BC samples. We also discovered that the HERV-K TM envelope protein is capable of triggering strong humoral immune responses. Figure 24 illustrates another IFN- $\gamma$  ELISPOT assay including 1 normal donor and 1 OC patient whose lymphocytes were obtained 1 days before surgery (0 month) and again sampled 6 months after the tumor was removed. We additionally combined IVS or PBMCs with DCs pulsed with the TM protein. IFN- $\gamma$  production is clearly seen when the OC patient IVS sample is combined with HERV-K TM and HERV-K pulsed DC cells. There was no IFN- $\gamma$  production seen with PBMC cells or in the normal donor. This data further substantiates our previous data that TM is not immunosuppressive.



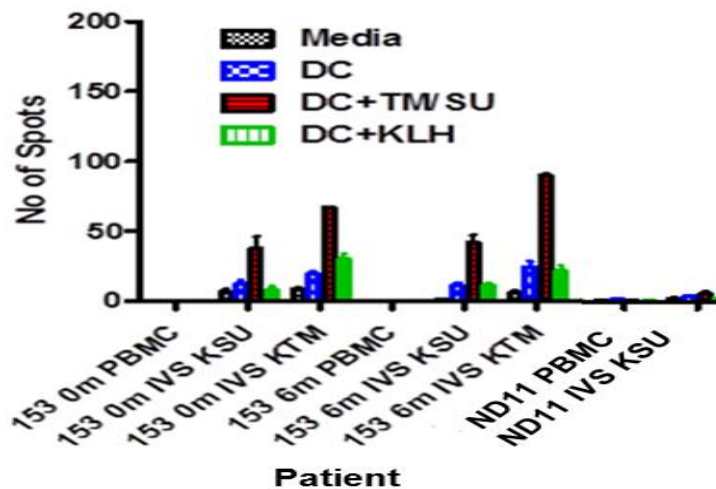
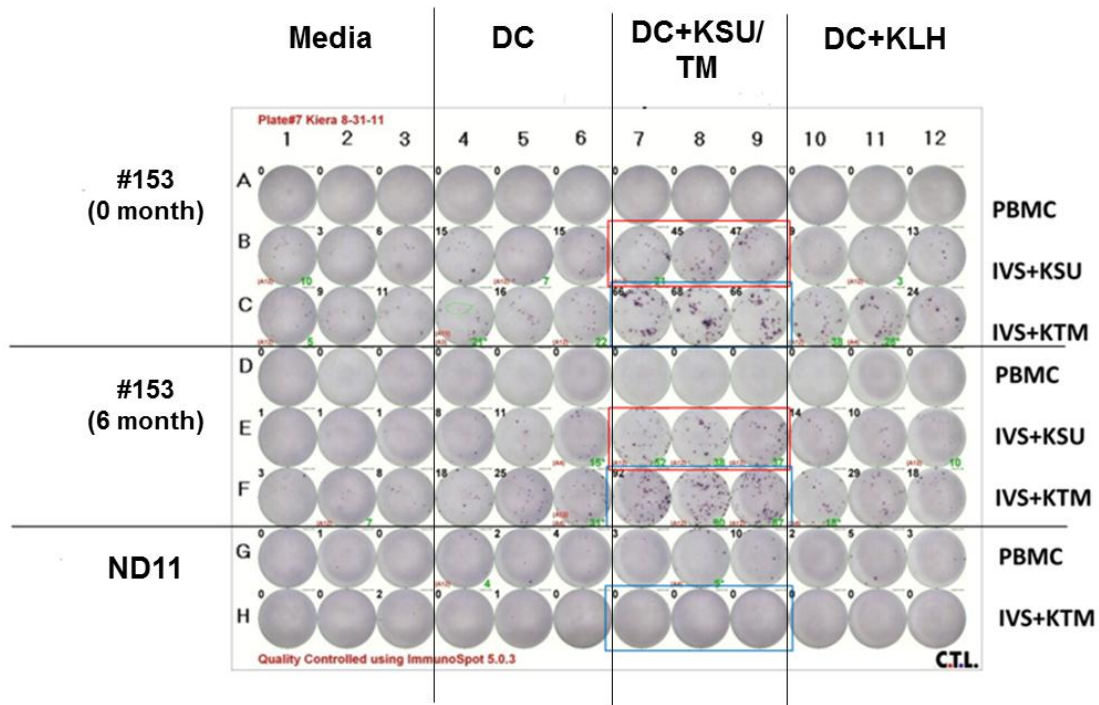
Figure 25 display a summary of IFN- $\gamma$  ELISPOT assays including OC patients (n=9) and patients with benign disease or normal donors (n=6). We concluded that significantly more IVS cells from OC patients secrete IFN- $\gamma$  than PBMCs from OC patients when stimulated with DCs pulsed with KSU protein. Also, significantly more IVS cells from OC patients secrete IFN- $\gamma$  when combined with KSU than with control protein. Finally, significantly more IVS cells from OC patients secrete IFN- $\gamma$  than IVS cells from benign disease patients and normal donors.





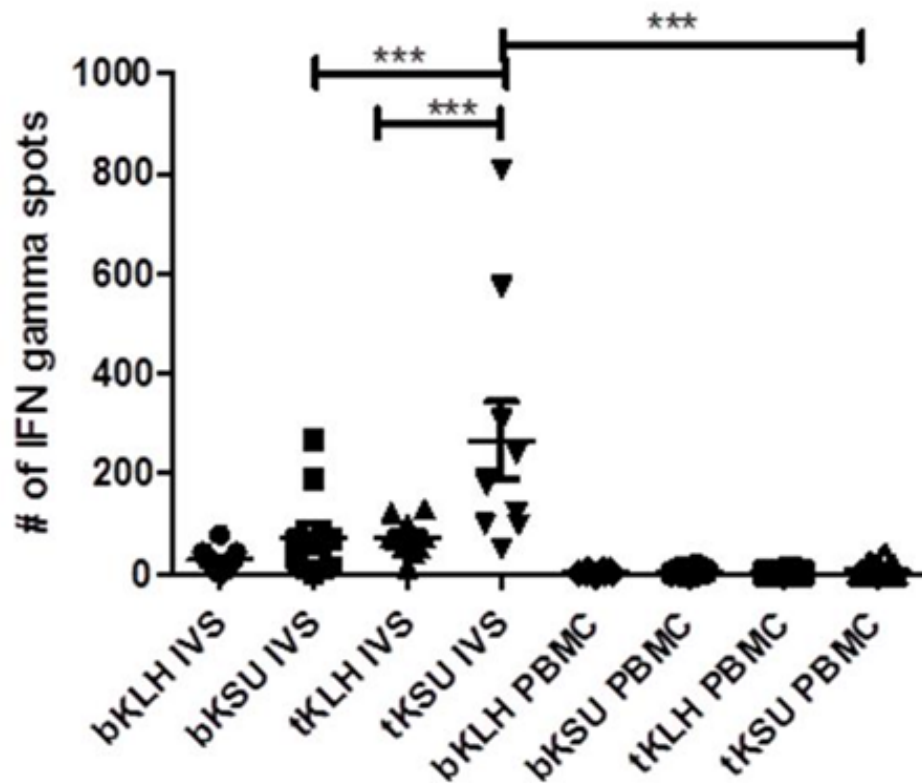
**Figure 23:** IFN- $\gamma$  ELISPOT assay. Patient #103 was diagnosed with adenocarcinoma. ND59, ND10, and ND11 represent normal female donors. An image of an ELISPOT plate is displayed at the top. KSU IVS or PBMC were combined with DCs pulsed with HERV-K protein, DCs pulsed with KLH control protein, media alone, or unpulsed DCs. A purple spot represents one molecule secreting cell. Spots are clearly seen when IVS cells are combined with HERV-K pulsed DCs but not media, unpulsed DCs, or DCs pulsed with control protein in the OC patient. Considerably less or no spots are seen in the normal donors. A column graph representing the number of spots in each patient from the same ELISPOT plate is displayed at the bottom.





**Figure 24: IFN- $\gamma$  ELISPOT:** Patient #153 was diagnosed with high grade papillary serous carcinoma and BC. ND11 represents a normal female donor. KSU IVS or PBMC were combined with DCs pulsed with HERVK protein, DCs pulsed with KLH control protein, DCs pulsed with TM protein media alone, or unpulsed DCs. A purple spot represents one molecule secreting cell. Spots are clearly seen when IVS cells are combined with HERV-K pulsed DCs but not media, unpulsed DCs, or DCs pulsed with control protein in the OC patient. Considerably less or no spots are seen in the normal donors. 0 and 6 months indicate lab blood draw dates post operation.





**Figure 25:** Summary for ELISPOT. OC patients (n=9) are compared with patients with benign disease or normal donors (n=6). KSU IVS or PBMC were combined with  $5 \times 10^3$  DCs pulsed with HERV-K protein, DCs pulsed with KLH control protein, unpulsed DCs, or media alone. Significantly more spots are seen when IVS cells are combined with HERV-K pulsed DCs compared to IVS cells combined with KLH pulsed IVS cells in OC patients. Significantly more spots are seen when IVS cells are combined with HERV-K pulsed DCs in OC patients compared to patients with benign disease. Significantly more spots are seen when IVS cells are combined with HERV-K pulsed DCs in OC patients compared to PBMCs in OC patients. A student's *t*-test was used to calculate significance.

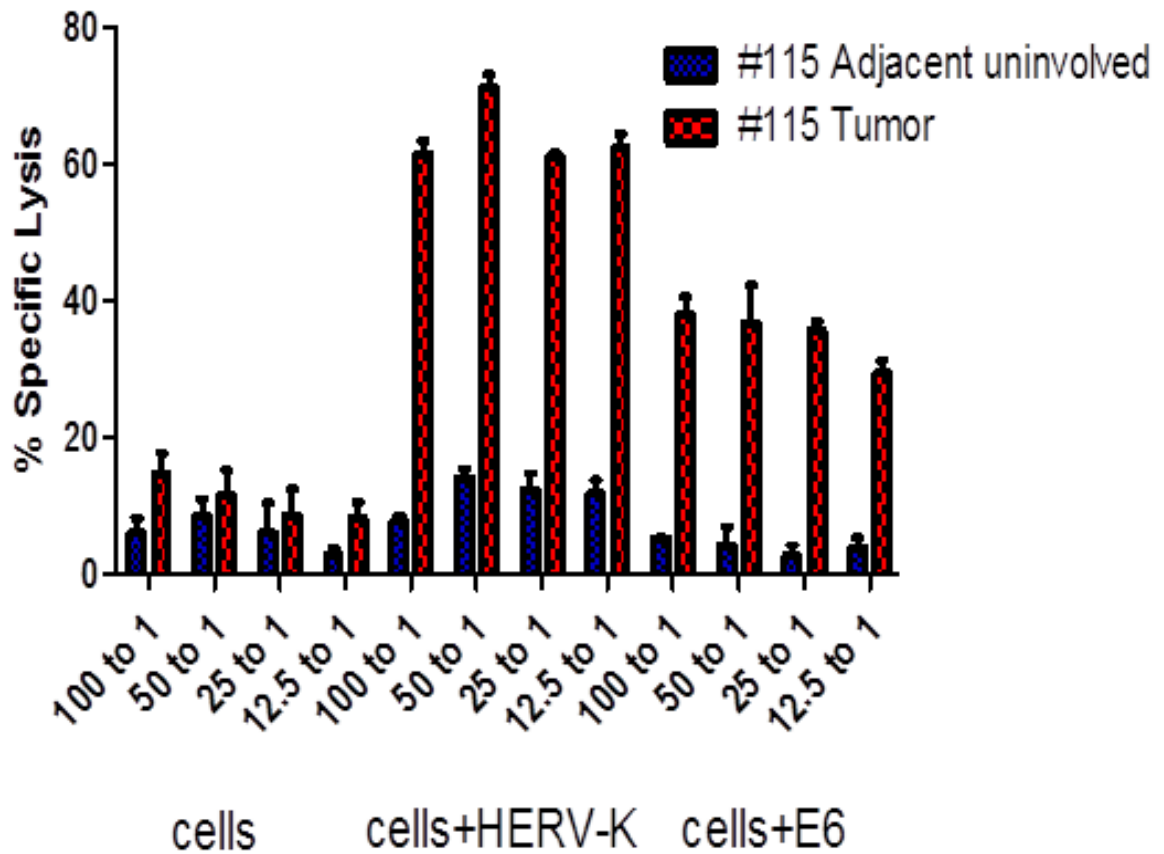


### **3.10 CTL activity using autologous OC and benign patient primary target cells**

Our paramount objective was to determine the ability of antigen specific T cells to kill autologous target cells expressing HERV-K. We wanted to ensure that these antigen-specific T cells kill only cancer cells which express HERV-K but not benign or normal cells that do not express HERV-K. We also wanted to ensure that T cells could kill target cells from the same patient instead of an established cancer line not specific to the patient. The standard CTL assay indicates the presence and cytotoxic activity of T cells to a specific antigen. Target cells, primary tumor or benign cells or adjacent uninvolved cells isolated from patient tissue, are labeled with <sup>51</sup>Chromium and combined with IVS cells isolated from autologous patient blood samples.

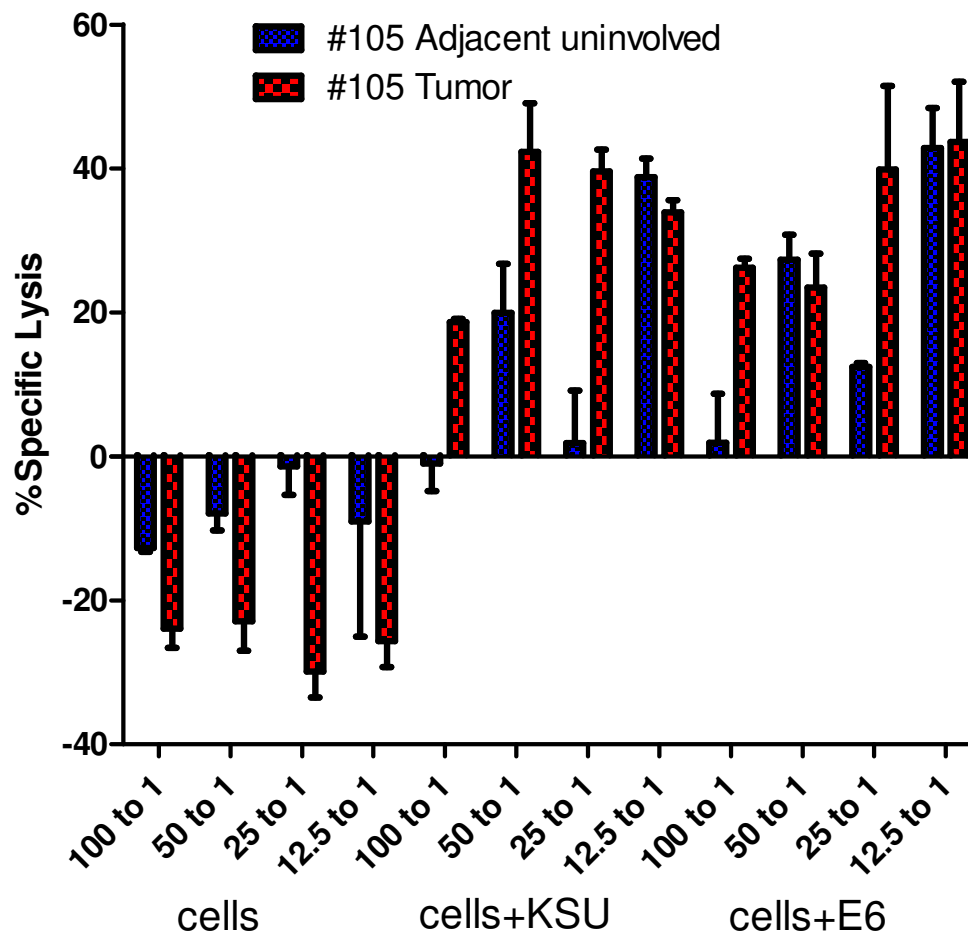
Figure 26 presents a CTL assay including both tumor and adjacent uninvolved cells obtained from an OC patient diagnosed with malignant mixed mullerian tumor. The results show that IVS cells from the OC patient exhibited higher specific lysis of tumor target cells compared to adjacent uninvolved cells. Also, specific lysis was higher when target cells were pulsed with HERV-K compared to E6 or unpulsed. Figure 27 presents a CTL assay including both tumor and adjacent uninvolved cells obtained from an OC patient diagnosed with mullerian borderline tumor with isolated foci of invasion. The results show that IVS cells from the OC patient exhibited higher specific lysis of tumor target cells compared to adjacent uninvolved cells. Also, specific lysis was higher when target cells were pulsed with HERV-K compared to unpulsed. Specific lysis was also high when target cells were pulsed with E6 protein. This possibly indicates that this female is HPV positive.





**Figure 26:** CTL assay using human patient autologous cells as target cells: Patient #115 was diagnosed with malignant mixed mullerian tumor and both tumor and adjacent uninvolved tissue was harvested. CTL assays were performed using a standard 4-hour  $^{51}\text{Cr}$ -release assay. Autologous primary OC tumor and adjacent uninvolved cells lines were pulsed with HERV-K or KLH control protein and used as target cells. Cells were plated at effector to target cell ratios of 100:1, 50:1, 25:1, and 12.5:1. Specific lysis is highest when autologous target cells expressing HERV-K compared to E6 protein are combined with IVS cells from this patient. % specific lysis is considerably higher when IVS cells are combined with autologous tumors cells compared to adjacent uninvolved cells.





**Figure 27:** CTL Assay. #105 was diagnosed with mullerian borderline tumor with isolated foci of invasion. CTL assays were performed using a standard 4-hour  $^{51}\text{Cr}$ -release assay. Autologous primary OC tumor and adjacent unininvolved cells lines were pulsed with HERV-K or E6 control protein and used as target cells. Cells were plated at effector to target cell ratios of 100:1, 50:1, 25:1, and 12.5:1. Specific lysis is highest when autologous target cells expressing HERV-K compared to E6 protein are combined with IVS cells from this patient. % specific lysis is considerably higher when IVS cells are combined with autologous tumors cells compared to adjacent unininvolved cells.



### **3.11 Effects of T-reg depletion on CTL activity using autologous OC, adjacent uninvolved, and benign patient primary target cells**

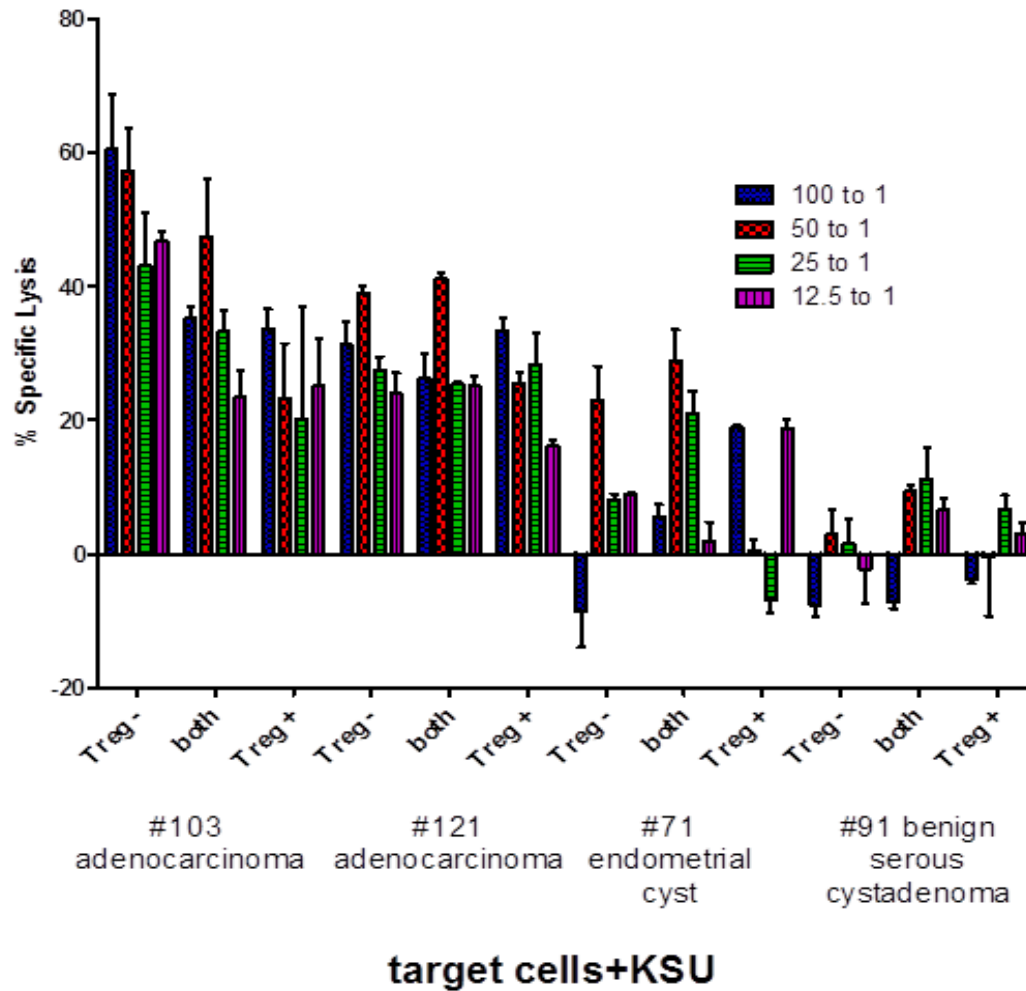
T-regs within the tumor microenvironment are a crucial component of the tumor immunosuppressive network. Therefore, our final objective was to deplete T-regs from our lymphocyte population and determine the effect on specific lysis of HERV-K expressing target cells. We utilized a CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim/-</sup> regulatory T cell isolation kit in order to magnetically isolate T-regs from our populations. Our lymphocytes fell into three groups: “T-reg-” indicated a lymphocyte population depleted of T-regs, “T-reg<sup>+</sup>” indicated a lymphocyte population consisting of isolated T-regs, and “both” indicated a lymphocyte population not subjected to the isolation kit. CTL assays were then conducted as described in the Material and Methods section.

Figure 28 includes CTL data from 2 OC patients diagnosed with adenocarcinoma and 2 patients diagnosed with benign disease. IVS cells from each patient were magnetically separated into T-reg populations as described above, and used as effector cells. The results show that overall specific lysis was higher in the OC patients compared to patients with benign disease. Specifically, T-reg depletion resulted in increased specific lysis of OC target tumor cells pulsed with HERV-K in #103. Figure 29 displays data from one OC patient. Overall, specific lysis was higher in the OC target cells compared to adjacent uninvolved cells. Also, OC target cells pulsed with HERV-K showed higher specific lysis than the unpulsed target cells. T-reg depletion did not exhibit a cytolytic advantage in this patient.



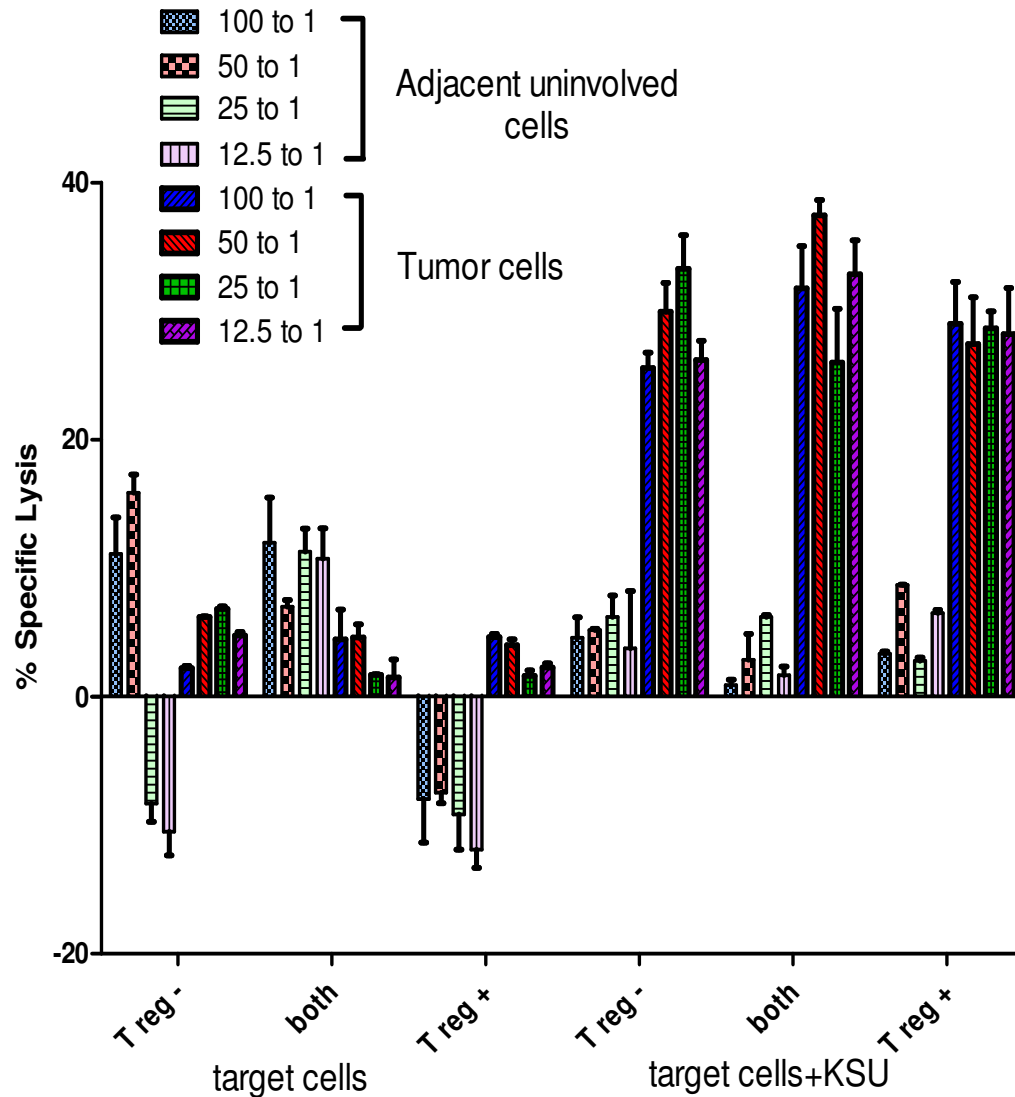
Figure 30 displays CTL data from 2 OC patients and 2 patients with benign disease. The results show that overall specific lysis was higher in the OC patients compared to patients with benign disease. Also, both OC patients benefited from T-reg depletion, manifesting in greater specific lysis of target cells expressing HERV-K. Figures 31 and 32 both include CTL data from 1 OC patient and 1 patient with benign disease. Specific lysis is clearly greater when target cells from the OC patient are pulsed with HERV-K compared to the patient with benign disease. T-reg depletion benefited the OC patients but not the patients with benign disease. Figure 33 includes CTL data from 1 OC patient and 1 patient with a teratoma. Specific lysis is clearly greater when target cells from the OC patient are pulsed with HERV-K compared to the patient with a teratoma. T-reg depletion did not benefit these patients. Finally, Figures 34 and 35 both include CTL data from 1 OC patient. Specific lysis was greater in both patients when target cells were pulsed with HERV-K compared to KLH control protein. T-reg depletion benefited 1 OC patient of the two.





**Figure 28:** CTL Assay: Human patient autologous cells as target cells. Primary human tissue was harvested from patients with various diagnoses. CTL assays were performed using a standard 4-hour  $^{51}\text{Cr}$ -release assay. Autologous primary OC tumor and benign cells were pulsed with HERV-K and used as target cells. Effector cells were separated into T-reg-, Treg+ and unseparated groups. Cells were plated at effector to target cell ratios of 100:1, 50:1, 25:1, and 12.5:1. Specific lysis is highest when autologous cells expressing HERV-K are combined with IVS cells from OC patients compared to patients with benign disease.

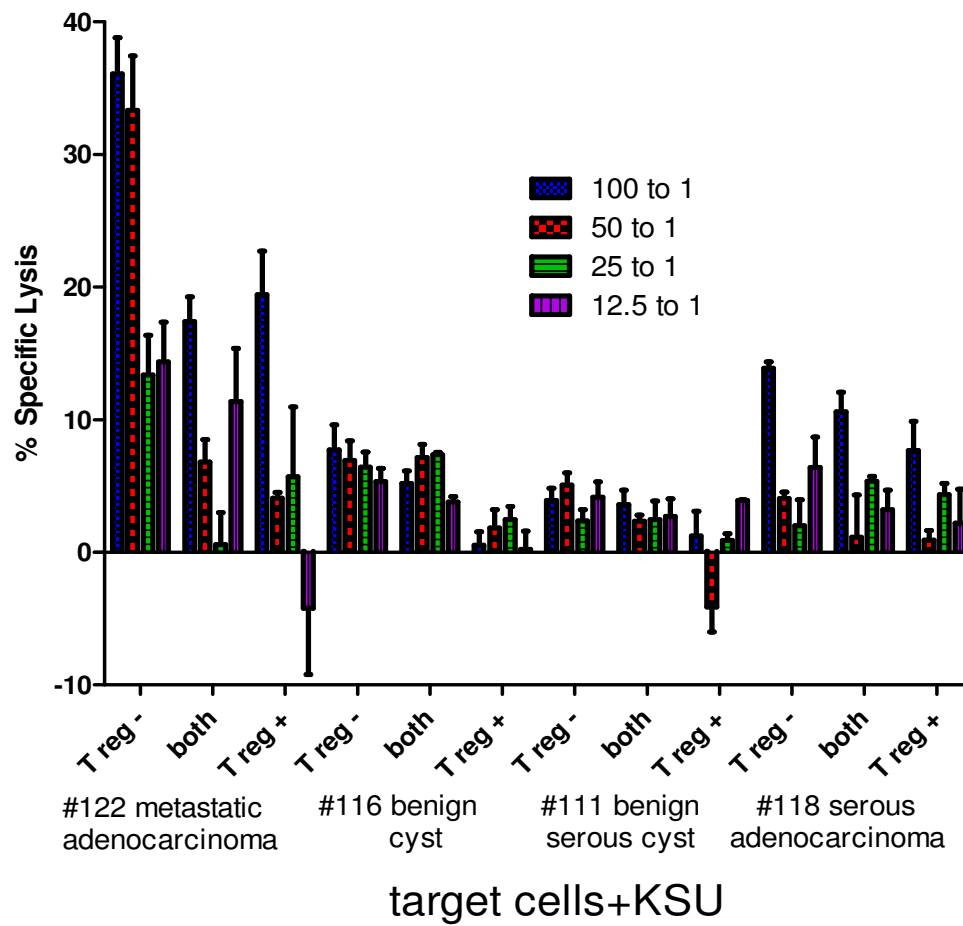




### #112 serous borderline carcinoma/mucinous borderline adenocarcinoma

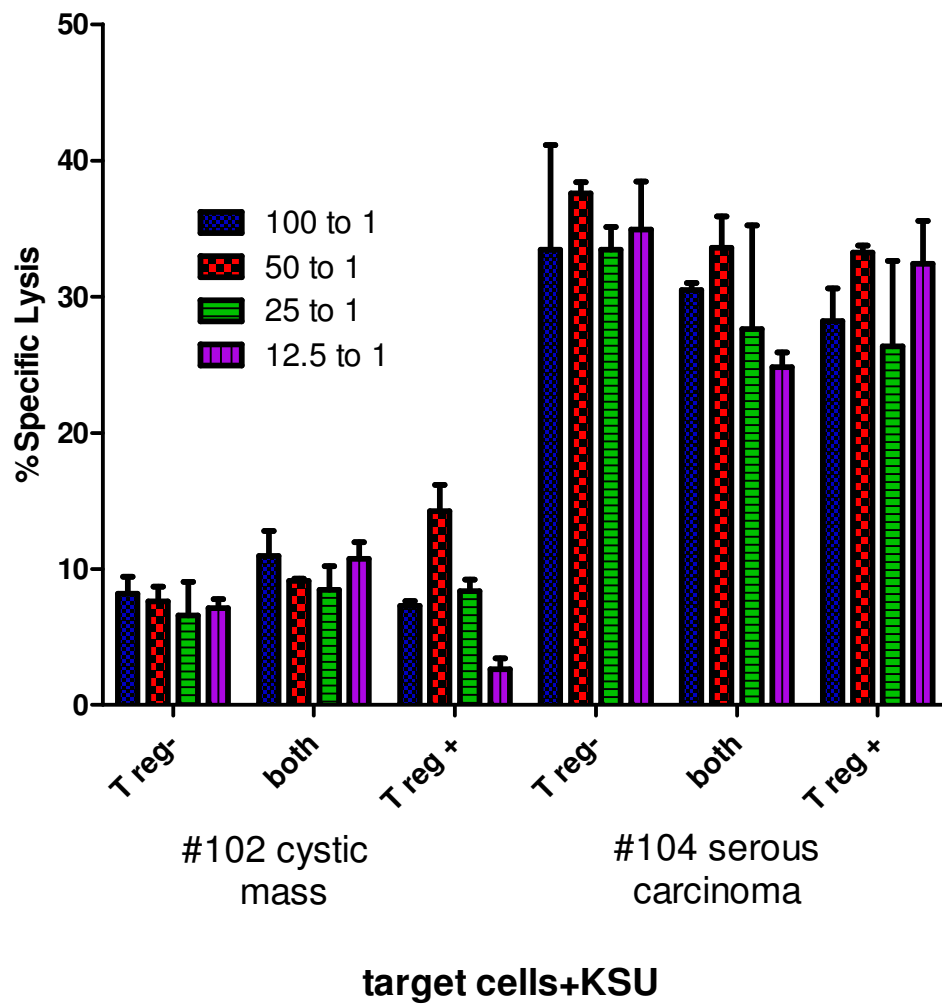
**Figure 29:** CTL Assy. #112 was diagnosed with serous borderline carcinoma/mucinous borderline adenocarcinoma. CTL assays were performed using a standard 4-hour <sup>51</sup>Cr-release assay. Autologous primary OC tumor and adjacent unininvolved cells lines were pulsed with HERV-K and used as target cells. Cells were plated at effector to target cell ratios of 100:1, 50:1, 25:1, and 12.5:1. Specific lysis is highest when autologous target cells expressing HERV-K are combined with IVS cells from this patient.





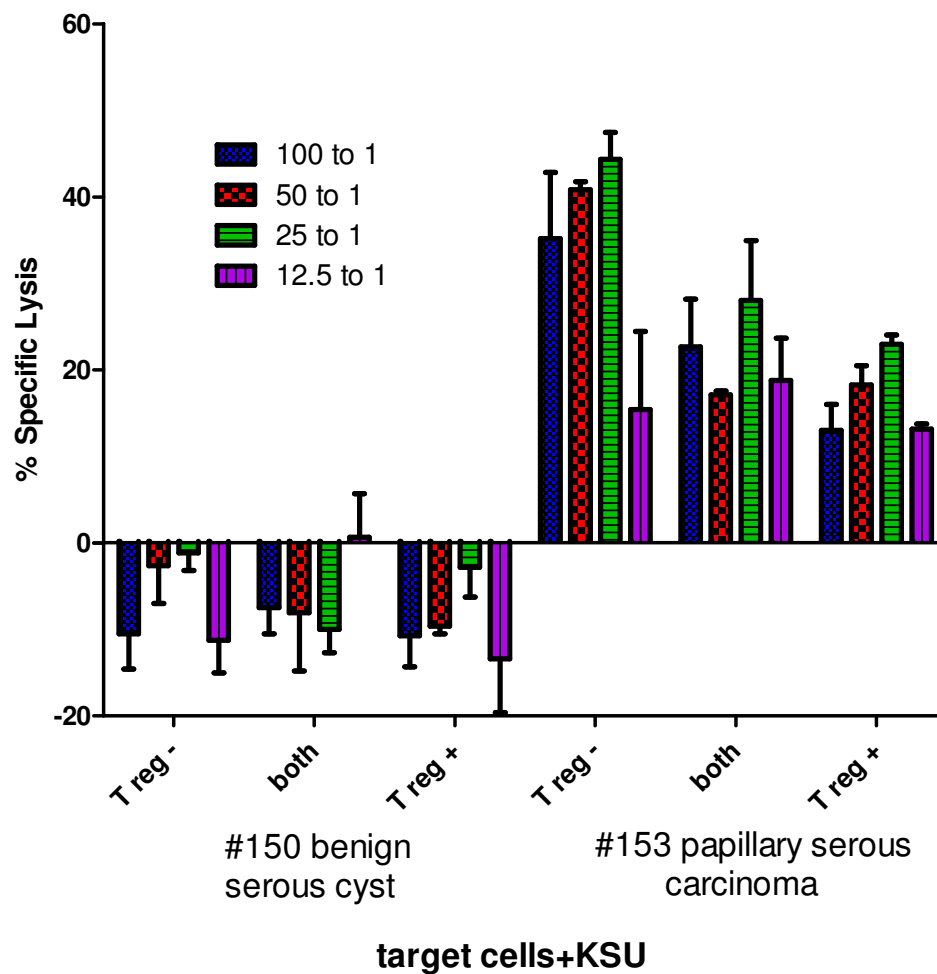
**Figure 30:** CTL assay. CTL assays were performed using a standard 4-hour  $^{51}\text{Cr}$ -release assay. Autologous primary OC tumor and adjacent uninvolved cells lines were pulsed with HERV-K and used as target cells. Cells were plated at effector to target cell ratios of 100:1, 50:1, 25:1, and 12.5:1. Specific lysis is highest when autologous cells expressing HERV-K are combined with IVS cells from OC patients compared to patients with benign disease.





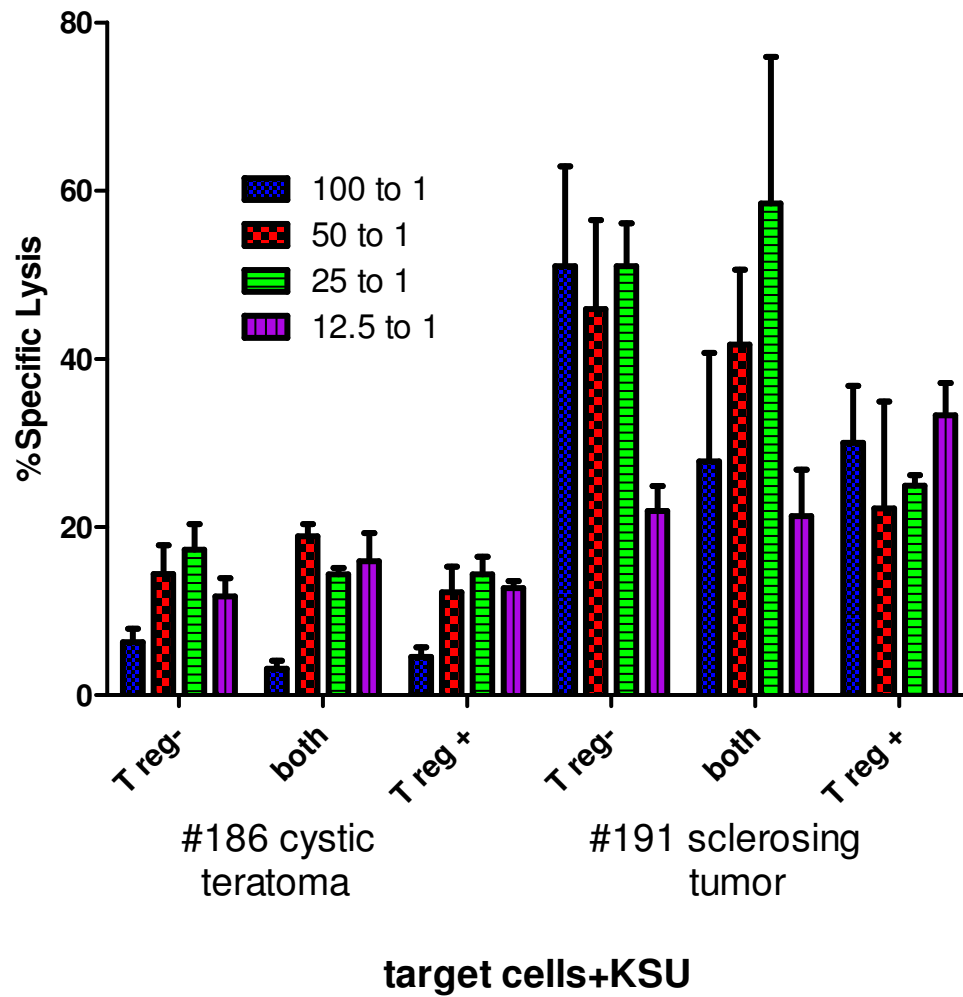
**Figure 31:** CTL Assay. CTL assays were performed using a standard 4-hour  $^{51}\text{Cr}$ -release assay. Autologous primary OC tumor and adjacent uninvolved cells lines were pulsed with HERV-K and used as target cells. Cells were plated at effector to target cell ratios of 100:1, 50:1, 25:1, and 12.5:1. Specific lysis is highest when autologous cells expressing HERV-K are combined with IVS cells from OC patients compared to patients with benign disease.





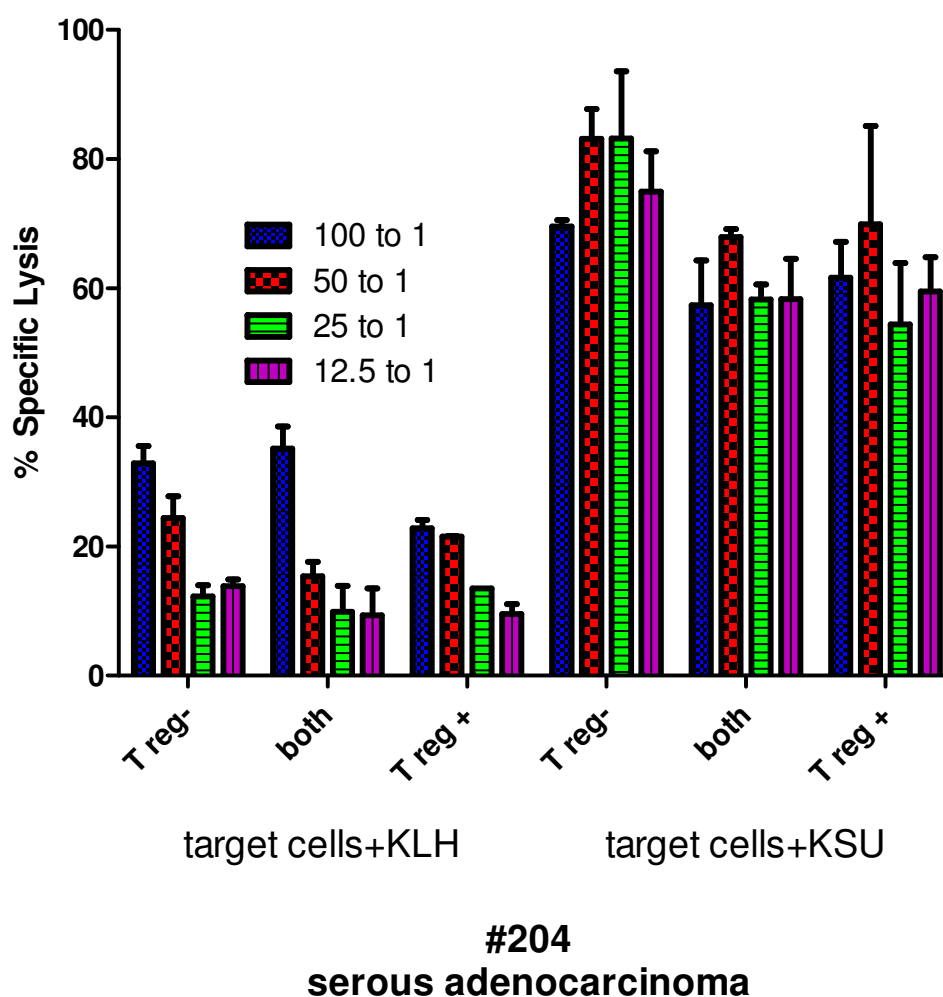
**Figure 32:** CTL assays were performed using a standard 4-hour  $^{51}\text{Cr}$ -release assay. Autologous primary OC tumor and adjacent uninvolved cells lines were pulsed with HERV-K and used as target cells. Cells were plated at effector to target cell ratios of 100:1, 50:1, 25:1, and 12.5:1. Specific lysis is highest when autologous cells expressing HERV-K are combined with IVS cells from OC patients compared to patients with benign disease.





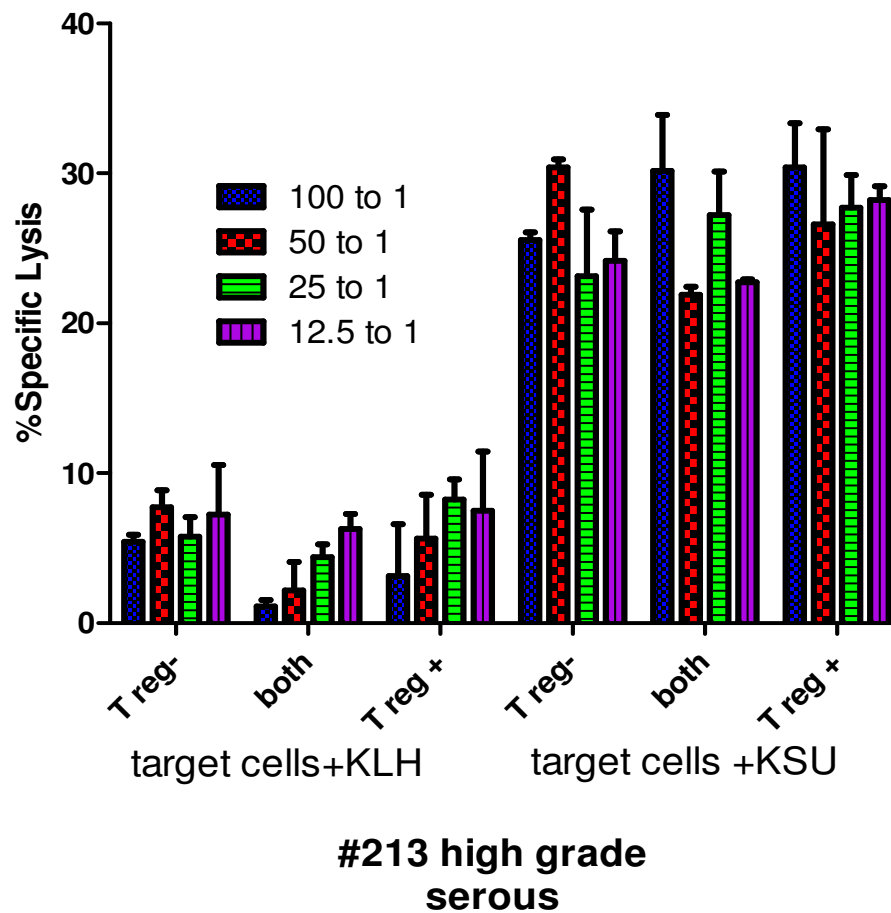
**Figure 33:** CTL assays were performed using a standard 4-hour  $^{51}\text{Cr}$ -release assay. Autologous primary OC tumor and adjacent uninvolved cells lines were pulsed with HERV-K and used as target cells. Cells were plated at effector to target cell ratios of 100:1, 50:1, 25:1, and 12.5:1. Specific lysis is highest when autologous cells expressing HERV-K are combined with IVS cells from OC patients compared to patients with benign disease.





**Figure 34:** CTL assays were performed using a standard 4-hour  $^{51}\text{Cr}$ -release assay. Autologous primary OC tumor and adjacent uninvolved cells lines were pulsed with HERV-K and used as target cells. Cells were plated at effector to target cell ratios of 100:1, 50:1, 25:1, and 12.5:1. Specific lysis is highest when autologous target cells expressing HERV-K compared to KLH protein are combined with IVS cells from this patient.



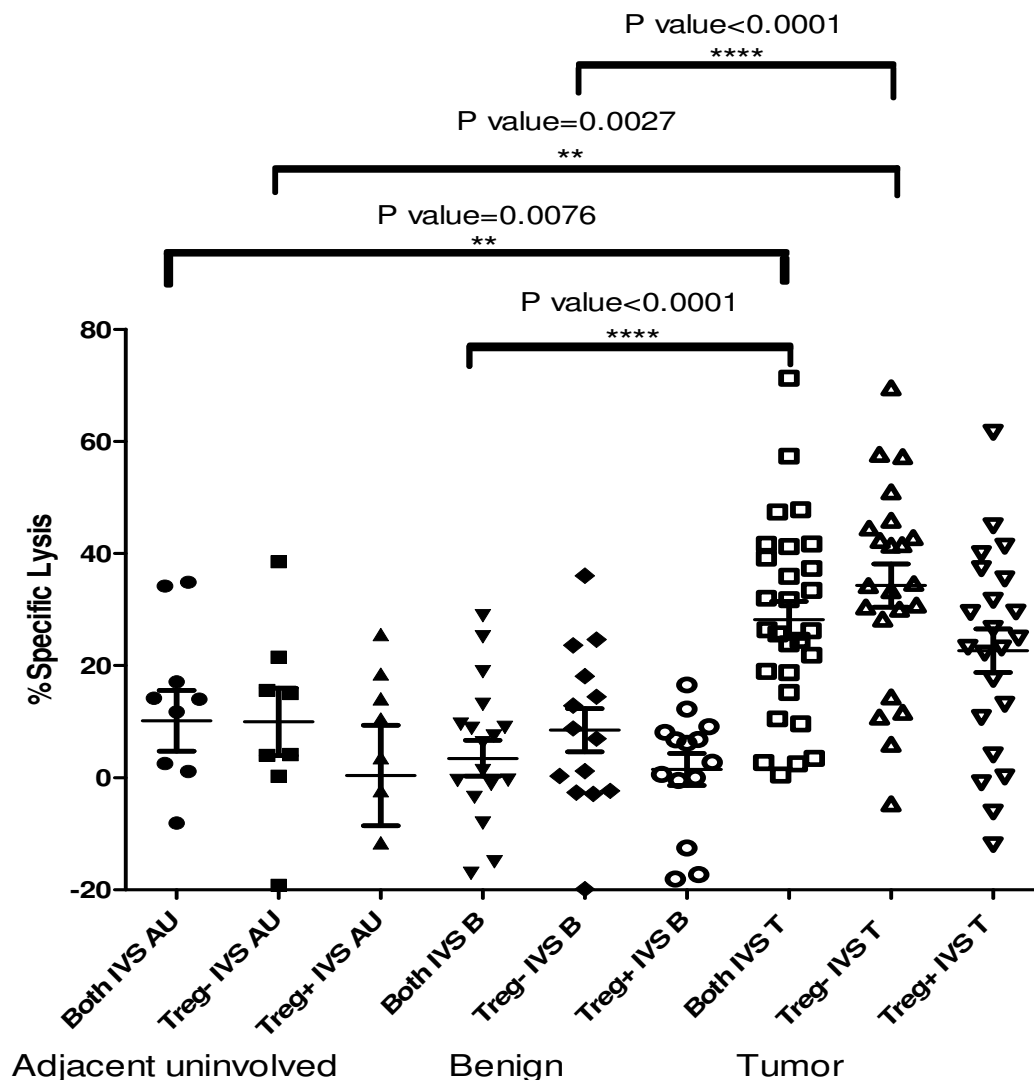


**Figure 35:** CTL assays were performed using a standard 4-hour  $^{51}\text{Cr}$ -release assay. Autologous primary OC tumor and adjacent uninvolved cells lines were pulsed with HERV-K and used as target cells. Cells were plated at effector to target cell ratios of 100:1, 50:1, 25:1, and 12.5:1. Specific lysis is highest when autologous target cells expressing HERV-K compared to KLH protein, are combined with IVS cells from this patient.



Figure 36 displays a CTL assay summary including OC samples (n=28), adjacent uninvolved samples (n=10), and benign samples (n=18). Overall, cytolytic activity was significantly greater when IVS effector cells were combined with autologous tumor target cells than with autologous uninvolved target cells. Cytolytic activity was significantly greater when IVS effector cells were combined with autologous tumor target cells than with autologous benign target cells. Cytolytic activity was significantly greater when T-reg-effector cells were combined with autologous tumor target cells than with autologous adjacent uninvolved target cells. Cytolytic activity was significantly greater when T-reg-effector cells were combined with autologous tumor target cells than with autologous benign cells. Finally, T-reg depletion led to higher specific lysis of autologous tumor and benign target cells. Of the 22 OC patients tested, 14 (64%) displayed higher specific lysis of tumor target cells after T-reg depletion.





**Figure 36:** CTL Assay Summary. Target cells pulsed with KSU and an effector to target ratio of 50 to 1. Human patient autologous cells were used as target cells. Primary human tissue was harvested from OC patients (n=28), uninvolved normal tissue (n=10) and patients with benign disease (n=18). CTL assays were performed using a standard 4-hour  $^{51}\text{Cr}$ -release assay. Primary OC tumor and benign cells were pulsed with HERV-K and used as target cells. Cytolytic activity was significantly greater when IVS effector cells were combined with autologous tumor target cells than with autologous uninvolved target cells. Cytolytic activity was significantly greater when IVS effector cells were combined with autologous tumor target cells than with autologous benign target cells. Cytolytic activity was significantly greater when T-reg effector cells were combined with autologous tumor target cells than with autologous adjacent uninvolved target cells. Cytolytic activity was significantly greater when T-reg effector cells were combined with autologous tumor target cells than with autologous benign cells. T-reg depletion led to higher specific lysis of autologous tumor and benign target cells. A student's *t*-test was used to calculate significance between groups.



## Chapter 4: Discussion

Approximately 8.3% of the human genome is derived from HERVs, resulting from integration events that took place millions of years ago. Most HERVs are defective and unable to produce infectious particles; however, some of them have retained the capacity to encode viral proteins<sup>280</sup>. HERV-K is the only family that has maintained intact ORFs for *gag*, *pro*, *pol*, and *env*. As discussed previously, HERV genes play important roles in some normal physiological events but unfortunately have been associated with several autoimmune diseases and cancer. The expression of HERV-K mRNA and protein and production of retroviral particles has been detected in human cancer (Table 1). The primary focus in our laboratory has been on HERV-K expression in BC patients<sup>94-96</sup>. However, in 2006, our group was the first to report that HERV-K env protein is also expressed on the surface and in the cytoplasm of OC cells. We also determined that expression of HERV-K env mRNA was greater in ovarian epithelial tumors than in normal ovarian tissues. Finally, we discovered anti-HERV antibodies in patients with OC, but not in normal female controls<sup>175</sup>. In this study, we confirm and augment our previous findings by providing cogent data on HERV-K expression and the immune responses they trigger in a new cohort of patient samples.

In this study, we amassed blood and tissue samples from patients with OC and patients with benign disease. Within this new patient cohort, several patients provided both OC and adjacent uninvolved samples, which were invaluable in determining differences in HERV-K



expression within one patient. Blood samples from normal female donors were also collected and compared.

Our first objective was to determine the expression of HERV-K at the transcriptional level in our samples. Our results show that patients with OC had overall stronger expression of HERV-K transcripts compared to patients with benign disease and adjacent uninvolved samples. We had the ability to compare OC and adjacent uninvolved samples, as well as OC and benign samples from the same patient. In every patient pair, HERV-K expression was greater in OC than in adjacent uninvolved and benign samples. Our next objective was to determine if HERV-K expression at the translational level showed similar results. We implemented IHC, IFS, FACS and dry cell ELISA to achieve this. Biopsies from patients (n=406) with OC, benign disease and normal epithelium biopsies were assayed. HERV-K expression was detected in endometrioid adenocarcinoma and serous adenocarcinoma samples but not in benign cysts or normal epithelium. IFS data from DOV13, an established OC cell line, indicates both surface and cytoplasmic expression of HERV-K which substantiates data from our 2006 paper. IFS analysis shows that human primary cell lines, distinctly exhibit greater cell surface expression of HERV-K in OC samples compared to benign samples (Figure 8, 10, 11)

Of great importance was the characterization of our primary human cell lines harvested from OC and benign samples. As mentioned previously, it has been determined that epithelial and mesenchymal cells undergo dramatic changes in their cytoskeletal scaffolds during tumorigenesis that are sometimes unpredictable. These observations may be due to the fact that tumors have the potential to initiate expression of a novel IF, or alternatively,



may cease to express a normally present IF<sup>281</sup>. This infidelity of IF expression has been observed *in vitro* in specific tumor cell lines of human malignancies and includes expression of vimentin by epithelial cells, expression of cytokeratin by mesenchymal cells, or coexpression of both<sup>251, 262, 263, 281-285</sup>. Indeed Hendrix and his colleagues determined that cytokeratin and vimentin positive human BC cells displayed increased invasive potential, proliferation rates, clonogenicity, and tumorigenicity compared to cells in which vimentin and cytokeratin was transiently down-regulated<sup>263</sup>. In one study, six permanent human tumor cell lines established from patients with serous adenocarcinomas of the ovary showed co-expression of cytokeratins (7, 8, 17, 18, and 19) and vimentin<sup>286</sup>. In another study, Thomas PA, Kirschmann DA, Cerhan JR, Folberg R, Seftor EA, Sellers TA, and Hendrix MJ determined that tumor cells which expressed both cytokeratin and vimentin in approximately equal ratios, translated to poorer survival of these BC patients<sup>254</sup>. To add more complexity to this subject, in one study, epithelial cells in most benign breast disease samples tested were positive for cytokeratin 8 and 18<sup>287</sup>. Another study showed that co-expression of cytokeratin and vimentin was found in all benign prostatic epithelium and in 83% of adenocarcinomas<sup>288</sup>.

In our study, we indeed observed that the majority of OC human primary cells lines positively coexpressed vimentin and cytokeratin. Co-expression was also observed in the benign primary cells. Another group illustrated that loss of cytokeratin 8 and 18 expression and concurrent up-regulation of vimentin suggested that disseminated BC tumor cells had acquired a mesenchymal-like, aggressive phenotype<sup>247</sup>. Contrary to this, the majority our OC and benign primary cells positively expressed cytokeratin 8 and 18. IFS data displayed



the morphological appearance of these vimentin and cytokeratin expressing cells as interwoven IF fibers (Table 2 and Figure 8).

In order to understand this up-regulation of vimentin in our primary patient cells, we should observe normal epithelial behavior. In normal wound healing, epithelial cells undergo an epithelial-mesenchymal transition (EMT) whereby their phenotype changes to a fibroblastic appearance, enabling them to move into the wound site, after which they reconstruct the epithelium by reverting back to an epithelial state via mesenchymal-epithelial transition (MET). However, in epithelial carcinomas, cells do not revert back from the EMT, and take on an aggressive, invasive role <sup>248</sup>.

Based on their resistance to traditional cancer therapies and presumed ability to recapitulate the original tumor, cancer stem cells are believed to be the source of recurrent OC <sup>228</sup>. There is growing evidence that tumor initiating cells capable of forming new tumors are rare and the majority of cancer cells are nontumorigenic <sup>289</sup>. The notion that cancer is driven by tumor initiating cells has obvious therapeutic implications but curative therapy should be designed to target these initiating cells instead of the bulk of nontumorigenic cells <sup>290</sup>. Therefore, our next objective was to characterize OC stem cell populations in two previously described individual patients. Patient #65 was diagnosed with metastatic adenocarcinoma, and the tumor cells from this patient had an epithelial morphology. Cells were harvested on day 1 and then grown in tumorsphere inducing conditions for 6 days. RT-PCR confirmed that transcripts for HERV-K Type1 and Type 2, ERV-3, and HERV-E41 increased upon tumorsphere formation. QIFIKIT and FACS assay confirmed the presence of HERV-K surface expression. FACS confirmed populations for mesenchyme,



epithelial, HERV-K<sup>+</sup>, and tumor epithelial cells also increased upon tumorsphere formation. We also included two antibodies for CD133 and CD44, OC stem cell markers, and determined that populations of CD44<sup>+</sup> cells increased upon tumorsphere formation. The increased expression of HERV-K and CD44 populations indicates that targeting HERV-K in OC cells could possibly be beneficial in eradicating stem cell populations (Figure 15).

The second patient characterized was diagnosed with serous carcinoma which had metastasized and formed an ascites. One day post-harvest, cells took on an epithelial-like morphology in culture. In a recent study, ascites from a patient diagnosed with advanced ovarian adenocarcinoma grew in an anchorage-independent manner in vitro as spheroids and presented the distinctive phenotype of CD44+cKit+, expressed both epithelial and mesenchymal markers and established tumors that were histopathologically similar to those in the human disease <sup>291</sup>. Therefore to promote an anchorage-independent, self-renewing sphere population, we plated ascites cells in serum-free Epicult media on 0.8% agarose coated plates for 7 days <sup>240</sup>. To investigate the phenotypic characteristics of these tumorsphere cells, FACS was used to analyze the expression of common OC stem cell markers. Indeed, after tumorsphere culture, expression of epithelial, tumor epithelial and mesenchymal markers, as well as the cancer stem cell marker CD44 increased, as was also observed <sup>291</sup>. CD133 expression decreased slightly Figure 17. The most valuable observation was the concomitant increase HERV-K as the most alluring potential vaccine targets are molecules that are associated with cancer stem cells and/or the EMT process <sup>165</sup>. The similarities between cells undergoing EMT and the acquisition of “stem-like” characteristics have been explored by recent studies <sup>292</sup>.



Moving our focus to immune responses generated by HERV-K, we next compared the levels of anti-HERV-K antibodies in serum from OC patients to patients with benign disease. Our results show a positive correlation between anti-HERV-K serum reactivity and tumor progression. In an attempt to discern a pattern in anti-HERV-K antibody levels during post-operative spans, the data produced was inconclusive. Our patient sample cohort is limited in terms of samples available during remission, and recurrence and further studies will be necessary once additional samples become available (Figure 18-19).

After identifying a humoral response against HERV-K in OC patients, our next objective was to explore HERV-K-specific T-cell responses. To accomplish this, it was very important to first characterize our patient PBMC and IVS populations. This is critical because T cell infiltrates are frequently associated with a favorable prognosis for many epithelial cancers<sup>216, 264-272</sup>. However, it is the intricate balance between these effector cells and regulatory T cells that determine the critical outcome of the patient. Although changes in cell populations were not significant, trends were observed. In the patient diagnosed with benign disease, the positive populations of T helper cells, cytotoxic T cells, activated T and B cells, NK cells, and T-regs increased upon conversion from PBMCs to IVS cells. A second patient diagnosed with benign disease displayed the same trends except a slight decrease in T-regs. In the patients diagnosed with OC, the positive populations of T helper cells, cytotoxic T cells, activated T and B cells increased while positive populations of NK cells and T-regs decreased. These increases in particular effector cell populations are what triggered the strong cell-based immune reactions against HERV-K expressing cells in our subsequent assays discussed below. A decrease in T-regs initially appears beneficial but this



is hard to discern because the exact balance between effector cells and T-regs is unknown (Table 4).

A profound biological issue in limiting the efficacy of cancer vaccines is the limitation on the ability of T cells to expand in number in response to antigenic stimulation. Normally, strict biological limits imposed on the immune system to prevent excessive T-cell activation and expansion<sup>293</sup>. Therefore, our subsequent goal was to determine if HERV-K could induce T cell activation resulting in proliferation of activated T cells. Our results show that T cell proliferation was significantly greater when OC patient IVS cells are combined with HERV-K pulsed DC, compared to KLH pulsed DCs. T cell proliferation is also higher when HERV-K pulsed DCs are combined with IVS from OC patients, compared to patients with benign disease (Figures 20-23). IFN- $\gamma$  is secreted by activated CD4<sup>+</sup> and CD8<sup>+</sup> cells, so we next employed an ELISPOT assay, which is a common assay used to monitor T cell immune responses. Results show that there are significantly more IFN- $\gamma$ -secreting effector cells when IVS cells are combined with HERV-K pulsed DCs, compared to IVS cells combined with KLH pulsed IVS cells in OC patients. Significantly more IFN- $\gamma$ -secreting effector cells are seen when IVS cells are combined with HERV-K pulsed DCs in OC patients compared to patients with benign disease. Finally, significantly more IFN- $\gamma$ -secreting effector cells are present when IVS cell are combined with HERV-K pulsed DC in OC patients compared to PBMCs in OC patients (Figure 23-25). These results are akin to the results seen in our BC patient samples<sup>94</sup>. These results are also parallel to results published by Mullins CS and Linnebacher M who discovered that stimulation of T peripheral T cells with autologous DCs pulsed with HERV-H retroviral peptides resulted in



sustained proliferation of predominantly CD8<sup>+</sup> T cells and high numbers of IFN- $\gamma$  secreting T cells<sup>294</sup>.

IFN- $\gamma$  production and proliferation are clear signs of specific activation, however they do not prove cytotoxic ability. CD8<sup>+</sup> cytotoxic T lymphocytes are extremely important effector cells of the immune system and destroy cells that express foreign antigens on their surface. Thus, our most powerful results are seen in our CTL assays utilizing patient lymphocytes and autologous target cells. Cytolytic activity was significantly greater when IVS effector cells were combined with autologous tumor target cells than with autologous uninvolved target cells. Cytolytic activity was also significantly greater when IVS effector cells were combined with autologous tumor target cells compared to autologous benign cell (Figure 26-27). Overcoming suppressive mechanisms found within the tumor microenvironment in order to enhance efficacy is a major challenge for current approaches to cancer vaccination. Therefore our next goal was to determine the effects of T-reg depletion on cytolytic activity. We discovered that cytolytic activity was significantly greater when T-reg effector cells were combined with autologous tumor target cells than with autologous adjacent uninvolved target cells. Also, cytolytic activity was significantly greater when T-reg effector cells were combined with autologous tumor target cells than with autologous benign cells. Overall, of all the OC patients tested, 64% displayed higher specific lysis of tumor target cells after T-reg depletion (Figure 28-35). Therefore, future vaccine regimens with HERV-K have the potential of an increase in efficacy upon combination with T-reg depletion methods. This is the first report to explore the immune responses triggered by HERV-K in OC patients and these results are a beneficial contribution supporting optimism for the future of OC immunotherapeutics.



## Chapter 5: Conclusion and Future Studies

### 5.1 Conclusion

The history of cancer immunotherapy began in 1891 when William Coley, a surgeon, injected live or inactivated *Streptococcus pyogenes* and *Serratia marcescens* into the tumors of patients in order to reproduce remissions of sarcomas observed in patients with erysipelas<sup>295</sup>. The injected toxins were used to stimulate antibacterial phagocytes that might kill bystander tumor cells. However, this approach was never embraced by oncologists because of the many risks associated with the administration of infectious agents<sup>215</sup>. Significant responses in cancer immunology occurred over the following 40 years; however, successes were sporadic. Today, cancer immunotherapy is coming of age with the advent of inhibitors that target oncogenic pathways, agents that prime and guide a patient's immune response, and pharmacodynamics biomarkers to identify efficacy of a particular treatment<sup>215</sup>.

There are many impediments to success in the cancer vaccine realm. First, expression of the tumor antigen in the target tumor population may be inadequate for predicting the ability to generate effective T-cell responses. In addition, the antigen expression within the tumor bed can be heterogeneous and the relative specificity of a



tumor-associated antigen may be the same for tumor versus normal adult tissue. However, in our study, we found increased expression of HERV-K in OC samples compared to normal control or benign disease samples and evidence that HERV-K indeed generates strong T-cell responses. Secondly, the optimal adjuvant for producing antitumor CD8<sup>+</sup> T-cell responses that can be used safely and effectively in humans is not yet clear<sup>215</sup>. Finally, the effectiveness of a tumor-specific T-cell population may be limited by the multiple mechanisms of immunosuppression used by tumors to protect them from T-cells. However, in our study, we found that T-reg depletion resulted in higher specific lysis levels for target cells expressing HERV-K. The development of tumor immunotherapies is an extremely active field and characterization of novel tumor-associated antigens is essential because they minimize the risk of autoimmunity. A novel tumor associated antigen has several requirements. First, it is expressed in a significant proportion of patients with a particular cancer type. Secondly, it is not expressed or expressed at low levels in normal tissues. Thirdly, it is immunogenic<sup>211</sup>. Our data demonstrates that HERV-K is indeed a novel tumor-associated antigen that elicits a strong immune response due to its inherent viral quality, making it a strong potential as an OC vaccine. We have efficaciously asserted that impediments to a successful cancer vaccine can be overcome and we look forward with unequivocal optimism in this new era of cancer immunotherapy.

## **5.2 Future Studies**

The field of therapeutic cancer vaccines has reached several new and significant milestones in scientific empiricism. Great strides have been made in vaccine-delivery systems for the induction and enhancement of host antitumor immune responses.



Immunostimulants have been shown enhance immunogenic properties of vaccine preparations. Finally, the development and characterization of tumor-associated antigens overexpressed only in tumor cells and not normal cells has increased the efficacy and decreased the toxicity of vaccines. There are numerous types of vaccines that have been explored in preclinical and clinical trials, and some are currently being evaluated in the clinic. We feel it advantageous to detect successes in these current vaccines and apply or combine them with HERV-K in future studies.

Whole tumor vaccines are advantageous because numerous tumor associated antigens and tumor-specific antigens are present. These tumor cells can either be obtained for the same patient or from other patients. Disadvantages include high costs associated with custom vaccine production and considerable variability of tumors among patients<sup>296-301</sup>. Additionally, allogenic vaccines produce alloimmunity to nontumor components, altering the immune response. Another vaccine approach involves direct injection of cytokine genes or costimulatory molecule genes into the tumor. B7-1, a T cell costimulatory molecule on the surface of antigen-presenting cells, interacts with CD28 for up-regulation of T cell function and cytotoxic T lymphocyte-associated antigen (CTLA-4) for down-regulation of T cell function. Addition of B7-1 to an immunogenic tumor has been shown to make the tumor more immunogenic in preclinical studies<sup>302</sup>. Similar results are seen when intercellular adhesion molecule (ICAM)-1 and lymphocyte function-associated antigen (LFA)-3 have been added to tumors with marked success. The advantage of this approach is that the vaccine contains unique tumor-associated antigens and tumor-specific antigens specific to the patient. Indeed, clinical studies have shown that recombinant vectors expressing the B7-1 costimulatory molecule have been directly injected into melanoma and



carcinoma lesions<sup>303</sup>. Another clinical study utilizing recombinant vectors containing B7-1, ICAM-1 and LFA-3 injected directly into melanoma tumors has exhibited positive results<sup>304</sup>. Recombinant vectors containing cytokines can also be used and have been administered in preclinical models<sup>305, 306</sup> and in prostate tumors clinically<sup>307</sup>. Vectors expressing GM-CSF have also been administered in melanoma patients<sup>308</sup>.

Successes have also been shown with administration of peptide-based vaccines because peptides do not contain parts of the molecule shared with normal cellular proteins as compared to whole proteins. Peptides are inexpensive, easy to prepare, and minimize the potential for autoimmunity. On the other hand, the inherent specificity of peptides could lead to activation of only CD8<sup>+</sup> cells inducing a response that is short-lived due to the absence of CD4<sup>+</sup> cells. Also, peptides are beneficial only in patients who have a specific HLA allele. Numerous clinical trials are currently testing peptide vaccines including HPV<sup>309</sup>, ras<sup>310, 311</sup>, HER-2/neu<sup>312</sup>, MAGE<sup>313</sup>, MART-1, tyrosinase<sup>314</sup>, gp100<sup>315, 316</sup>, CEA<sup>317</sup>, MUC-1<sup>318</sup>, and PSMA<sup>319, 320</sup>.

Vectors as a means of vaccine delivery have also been explored<sup>321-327</sup>. Vector based vaccines are advantageous because of the flexibility of inserting the entire tumor antigen, just parts of the gene, or multiple genes. Indeed several clinical trials with recombinant vaccinia, a vaccine vector from the poxvirus group, containing tumor-associated antigens such as CEA<sup>328</sup>, MUC-1, PSA<sup>329</sup>, and HPV<sup>315, 330</sup>, have been completed and others are ongoing<sup>331-333</sup>. Unfortunately, there is the possibility of development of host-induced immunity to the vector itself, limiting continued use. Plasmid DNA vectors are another vaccine-delivery system used in studies however, how these vectors induce an immune



response is not fully understood<sup>334</sup>. Current clinical trials employing this technique include DNA vaccines encoding melanoma antigens such as MART-1<sup>335</sup>, gp100<sup>336</sup>, and PSA<sup>337</sup>.

DC vaccines have created great excitement in the cancer vaccine field because they are the most potent antigen presenting cell<sup>338, 339</sup>. This method involves loading the DC with a peptide, protein, anti-idiotypic Ab, apoptotic bodies from tumor cells or actually fusing it with a tumor cell<sup>340-343</sup>. Unfortunately, DC vaccines are extremely expensive and require great effort in isolating and culturing large amounts of PBMCs from each patient.

In April 2010, the Food and Drug Administration (FDA) approved Provenge (sipuleucel-T), the first active immunotherapy approach to cancer treatment. Provenge is comprised of an incompletely characterized, complex mixture of PBMCs supplemented with a cytokine and a tumor-derived differentiation antigen, for the treatment of advanced prostate cancer. PBMCs are obtained from the patient via leukapheresis, cultured for several days, and then reinfused into the patient. Even though clinical results showed little evidence of tumor shrinkage or delay in disease progression, a 4.1 month improvement in median survival was achieved and deemed significant by the FDA. It is believed that the professional antigen-presenting cells activated with the PA2024 fusion protein combined with GM-CSF, which produced significant results. This is a huge accomplishment in the field of cancer immunotherapy, and further studies are required to understand the therapeutic mechanism of Provenge<sup>344</sup>. Another therapy currently in late-stage phase 3 clinical trials involves immunization with autologous DCs loaded ex vivo with a recombinant fusion protein consisting of the tumor antigen prostatic acid phosphatase (PAP) linked to GM-CSF



<sup>345</sup>. Based on the success of these DC-based vaccines, it might be advantageous to try this method with autologous DCs loaded ex vivo with HERV-K in OC patients.

An imperative issue in the development of cancer vaccines involves the appropriate disease stage at which to administer vaccines. As dictated by medical ethics, all new phase 1 immunotherapies and many phase 2 trials must be administered to patients with advanced disease who have failed conventional therapy. Therefore, the majority of current vaccines focus on late-stage cancer, with little emphasis on preventative or prophylactic vaccines. Unfortunately, patients with extensive disease are not ideal candidates in which to determine new vaccine efficacy partly because previous therapy could have caused immunosuppression. Therefore, the field of cancer immunotherapy must mature to a point where vaccine can be evaluated in patients with early-stage cancers. This will allow us to determine if HERV-K could also be effective in cancers at different stages. Another concern in cancer vaccine development is the possibility of expression of tumor-associated antigens in normal cells, leading to undesirable immune reactions. This deleterious side-effect was seen experimental and clinical studies when vitiligo was induced in melanoma patients<sup>346, 347</sup>. Although HERV-K was not expressed in the normal tissues we studied, future HERV-K vaccination clinical studies need to monitor this chance occurrence.

The data presented here provides the rationale for HERV-K in clinical vaccination protocols targeting ovarian tumors expressing HERV-K, however additional assays need to be conducted with larger sample sizes in order to ensure that HERV-K is not expressed in normal tissues. If this data reconfirms our data, HERV-K specific vaccination strategies have the potential to improve OC patient outcome. Performance in the clinic is the final



arbiter of success in drug development and we believe that HERV-K vaccination should be moved into clinical trials without hesitation. In comparison to other vaccine types we believe that the use of a full length HERV-K protein is a superior method because it contains a broader profile of epitopes that might be presented by DC. As innately inquisitive scientists, our present study generated other perplexing ideas that we would like to further investigate, as discussed below.

Stromal cells in the tumor environment stimulate epithelial cell proliferation which leads to neoplastic transformation. This dependence of carcinoma cells on stromal support explains the difficulty of generating a true microenvironment *in vitro*. The interactions between these two cell types is extremely complex and involves exchanges of dozens of distinct molecular species that mediate cell-to-cell signaling<sup>243</sup>. Therefore further research is necessary in order to correctly assimilate a comparable tumor microenvironment *in vitro*, making it more representative of tumors in patients. Indeed, instead of focusing on the intracellular signaling defects within cancer cells, new cancer therapy is directed toward interrupting specific signaling, thereby depriving cancer cells of essential stromal support<sup>243</sup>.

As illustrated in Figure 13, after receiving an ascites sample from an OC patient diagnosed with serous carcinoma, our goal was to promote stem cell growth with a serum-free and low adherent environment. After 7 days in this environment, round spherical tumorspheres formed and the population of CD44<sup>+</sup> stem cells increased. Most importantly, HERV-K expression increased. This provides the exciting potential of targeting OC stem cells via immunotherapeutic methods by exploiting their increased HERV-K expression.



Further studies need to be conducted on the relationship between OC stem cells and HERV-K expression.

Numerous studies have indicated that HERVs may be involved in the process of tumorigenesis. Therefore, the next step is to determine the causal relationship between tumor development and HERV-K expression. Specifically, the regulation and effector pathways of HERV-encoded oncoproteins need to be elucidated. Currently, a causative role of HERVs in human tumors has not been demonstrated and future studies need to clarify this issue. Determining the role of HERVs in the complex multi-step process leading to cancer will shed light on the biology of cancer and also the interplay between HERVs and their human hosts <sup>33</sup>.

Another thought to contemplate is that the tumor or ascites specimens in our patient cohort were obtained at primary surgery. Therefore, the immunological changes that occur during treatment and recurrent disease are unknown and need to be explored. Also, the positive and negative regulatory components in the tumor environment, during these periods of treatment and recurrence need to be determined <sup>113</sup>.

As mentioned previously, numerous preclinical studies have presented data on the successes of vaccines in combination with other therapeutic modalities; and therefore, future studies should determine the effect of combining HERV-K with radiation, chemotherapy, hormone therapy, and other immunotherapies. Recent innovative techniques in immunotherapy have been developed in order to overcome the immunogenicity of a weak tumor-associated antigen. These include immunostimulants, adjuvant-like molecules <sup>348, 349</sup>, microbial products <sup>350, 351</sup>, cytokines <sup>352-355</sup>, and chemokines. For example, GM-CSF have



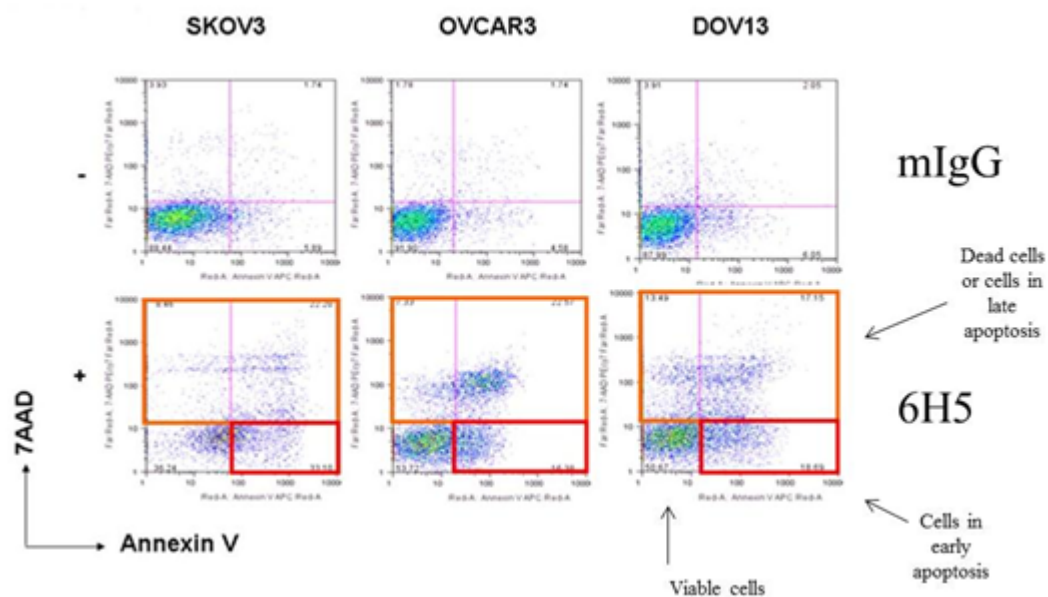
been shown to enhance vaccine efficacy in clinical trials<sup>331, 356-359</sup>. Preclinical studies with the monoclonal antibody, anti-CTLA-4 combined with vaccines results in higher-avidity antigen-specific T cells<sup>360-362</sup>. Preclinical and early clinical studies have shown that vaccine in combination with chemotherapeutic agents produces no detrimental effects in the immune response<sup>363</sup>. Future studies are needed in order to determine if these elements in combination with our vaccine will result in enhancement of T-cell responses and antitumor activity. Hopefully, the use of our vaccine regimen in combination with other therapies mentioned above, will present beneficial results.

MAbs function through several different mechanisms including inhibition of tumor-related signal transduction, induction of apoptosis, inhibition of angiogenesis, enhancing host immune response against cancer and targeted delivery of cytotoxic agents to the tumor site. The most important development for cancer immunotherapy are the results of the ipilimumab phase III trials. The FDA granted broad approval in March 2011 for the use in patients with metastatic melanoma, either as an initial therapy or after relapse. Ipilimumab is a monoclonal antibody to CTLA4, a negative regulator of T-cell activation, and therefore enhances host immune responses against cancer. Ipilimumab provides clear clinical validation for cancer immunotherapy in general<sup>344</sup>. Highlighting a different function of mAbs, we previously reported that anti-HERV-K-specific monoclonal antibodies inhibited growth and induced apoptosis of breast cancer cells *in vitro*. Also, we strengthened these results by then showing that mice treated with 6H5 antibody showed statistically reduced growth of xenograft tumors compared to mice treated with control mouse IgG, for tumors originating from the established BC cells line, MDA-MB-231<sup>96</sup>. Our results also showed that binding of the anti-HERV-K antibody to the cell surface env signals through TP53



pathway activation. TP53 is a tumor suppressor gene that plays a key role in apoptosis and senescence, activation of DNA repair proteins, and cell cycle arrest at the G<sub>1</sub>/S regulation point during DNA damage recognition. We believe that either the internalization of the complex formed between env and the antibody or signaling through surface env after binding of the antibody leads to TP53 pathway activation. Based on this recent success, we would also want to establish whether anti-HERV-K protein antibodies can successfully target ovarian tumors with the similar excellence that we accomplished in breast tumors. In Figure 37, the effect of 6H5 on the induction of ovarian cell apoptosis was assessed by annexin V flow cytometric assay. Cells treated with 6H5 mAb induced more early and late apoptosis than cells treated with mouse-IgG, thus giving us confidence that anti-HERV-K antibodies could show immunotherapeutic potential in targeting ovarian tumors. We believe that the same mechanisms of mAb action are involved as in our BC results however more extensive research is needed.





**Figure 37:** Induction of apoptosis in established OC cells treated with anti-HERV-K 6H5 antibody. The induction of ovarian cell apoptosis was assessed by annexin V flow cytometric assay. Cells were incubated with either 6H5 mAb (10.0  $\mu\text{g/ml}$ ) or mouse-IgG (10.0  $\mu\text{g/ml}$ ) for 16 hours at 37°C. The orange quadrant represents dead cell or cells in late apoptosis, the red quadrant represents cells in early apoptosis and the cells in the lower left quadrant represent viable cells. Cells treated with 6H5 mAb induced more early and late apoptosis than cells treated with mouse-IgG.



Moving forward, knowledge about the immune effects of anticancer mAbs can influence our ability to use them therapeutically. This information can identify biomarkers that can enhance our ability to select those patients who are most likely to respond to mAb therapy. Therefore we can use immunoassays in order to personalize medicine for patients. We can also modify the mAb structure to modulate effector mechanisms such as the ability to bind to FcR or fix complement. The mechanism of mAb action has not been fully elucidated, however there are several potential mechanisms that have been proposed. Firstly, the interaction between antibody and tumor antigen may induce apoptosis by activating mechanisms of complement-mediated cell death in the tumor cell <sup>364</sup>. Secondly, inhibition of ligand-receptor interaction with mAbs can lead to cell death because cells are deprived of tumorigenic stimuli such as growth factors for proliferation, differentiation, interaction with other cells, growth, and survival <sup>365</sup>. Thirdly, the formation of an entire antiidiotypic network induces immunological responses against cancer cells and therefore, the development of antiidiotypic antibodies (Ab2) against Ab1-binding sites is another important way of mAb action <sup>366</sup>. The amount of antigen on tumor cells, the subclass of the antibody, and the type of effector cell are some additional factors that regulate the ability of mAbs to induce ADCC.

Multimodality approaches, based on synergistic effects observed with the combination of antibodies with chemotherapeutic drugs and/or radiotherapy also merit further investigation. Immune-mediated effects may be further exploited with the use of



bispecific molecules. Stratification of patient sub-populations with tumors overexpressing disease-related clinical biomarkers could result in improving both efficacy and specificity of antibody-based treatment for solid tumors.

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