

**GAMMA/DELTA T-CELLS IN PATIENTS WITH EPITHELIAL OVARIAN
CANCER**

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ABSTRACT

Title: Gamma-delta T-cells in Epithelial Ovarian Carcinoma

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Ovarian cancer is the fifth most common cancer among women and cause more deaths in the western world than any other gynecological cancers. The epithelial ovarian carcinomas (EOC) represent approximately 90% of all human ovarian malignant neoplasm. The five-year survival rate for patients with EOC is attributed to late diagnosis and poor response to therapy.

T-cells play an important role in tumor immunity of EOC, evidence includes infiltrating CD3+ T-cells in EOC lesions and a specific antigen driven immune response. Human $\gamma\delta$ TCR + T-cells are a minor subset of T-cells (1-10%) in the peripheral blood. The majority of the T cells in the peripheral blood are $\alpha\beta$ TCR + T-cells. Like the $\alpha\beta$ T-cells, $\gamma\delta$ TCR + T-cells bear a T-cell receptor that functions in antigen recognition. Most importantly most $\gamma\delta$ TCR+ T cells recognize mainly whole proteins. In contrast, $\alpha\beta$ TCR+ T cells primarily recognize peptide in association with MHC. Upon specific antigen recognition, these T cells undergo clonal expansion, generating multiple identical T cell clones. Previously in our lab clonal expansion of $\alpha\beta$ TCR+ T cells was observed in patients with EOC. Also preliminary data indicate that clonal expansion of $\gamma\delta$ TCR+ T cells in patients with EOC.

The hypothesis to be tested in this study is whether $\gamma\delta$ TCR+ T-cells are clonally expanded in tumors from patients with EOC and whether they recognize tumor cells. Data from recent studies show that tumor infiltrating $\gamma\delta$ TCR+ T cells recognize and have antitumor activity towards epithelial derived cancer cells. Following V-specific amplification of the various $\gamma\delta$ T-cell receptors (TCR) chains we observed the presence of statistically significant populations of oligoclonal γ -chain and δ -chain TCR+ transcripts in the EOC samples studied.

To further characterize the $\gamma\delta$ TCR+ T-cells in EOC lesions, full-length transcripts of clonally and non-clonally expanded γ I-, γ II-, δ 1 and δ 2-chain TCR transcripts from EOC tumors were constructed. These full-length transcripts were transduced into a mutant TCR-negative Jurkat T cell line. The transduced cells were analyzed by flow cytometry (FACS) for expression of $\gamma\delta$ TCR on the cell surface. $\gamma\delta$ TCR+ CD3+ transduced T cells were then incubated with the ovarian cancer cell lines, SKOV3, CAOV3 or OV2774. Following co-incubation experiments of these cancer cells with $\gamma\delta$ TCR+ CD3+ transduced T cells we observed killing of the target cell (SKOV3, CAOV3 and OV2774) by various $\gamma\delta$ TCR + T cell transduced cell lines. This killing was not observed by control T cell lines transduced either with vector only or single chain of TCR. Furthermore, the production of cysteine proteases such as caspase 3/7, procaspase 8 and 9 involved in target cell death were also observed following co-incubation experiments. These data suggest that our $\gamma\delta$ TCR+ transduced T cells induce SKOV3, CAOV3 and OV2774 cancer cell death measured by cytotoxicity and activation of cell death proteases.

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CHAPTER 1 BACKGROUND INFORMATION

Ovarian carcinoma is one of the most deadly gynecological cancers with the highest mortality rate in the Western countries. Most often patients are diagnosed at an advanced stage of the disease. EOC involves uncontrolled cell proliferation and metastasis resulting in organ failure. Currently, first line agents have a 70-80% response rate; most patients eventually die of recurrent chemotherapy-resistant disease, leaving the overall 5-year survival rate around 30%. Therefore, if diagnosed early ovarian cancer is a chemo-sensitive tumor, however an initial platinum-sensitive based chemotherapy can eventually produce chemo-resistant tumor cells. Researchers are continuing to develop novel therapy to combat EOC.

Definition of Epithelial Ovarian Cancer (EOC)

Tumors cells arise from normal cells by a process that involves many genomic changes. In normal epithelial cells genomic sequences are subject to corruption by various mechanisms that alter their sequences often leading to structure or regulatory modifications. The resulting mutations/modifications may result in cells acquiring novel, often highly abnormal phenotypes. Furthermore, sustained cell proliferation in an environment rich in inflammatory cells, growth factors, activated stroma, and DNA-damage-promoting agents have the potential to promote and enhance neoplastic risk (Coussens et. al., 2002). Genomic changes resulting in cellular phenotypic changes frequently cause abnormal signal processing and may result in deregulation of proliferating programs. Cells

with alterations in their growth properties and morphology are undergoing malignant transformation, a process that occurs over many years.

There are three main cell types that ovarian tumors are derived from: germ cells, stromal cells, and epithelial cells. The majority of human ovarian cancer tumors arise from epithelial tissues. These tumors are responsible for more than 80% of the cancer-related deaths in the Western world. The epithelial and stromal cells of the ovary work together in forming and maintaining the epithelial sheets. Epithelial tumors start from the cells that cover the outer surface of the ovary. The two major functions of epithelia are 1) to seal the cavity or channel that they line and to protect the underlying cell populations and 2) to provide specialized cells that secrete substances into the ducts or cavities that they line. Tumors that arise from the epithelial cells of the former are termed squamous cell carcinomas. Tumors that arise from epithelia of the latter are termed adenocarcinomas (Weinberg, 2007).

Common stages of EOC

EOC prognosis is based on type (I or II), grade (high or low), stage (I-VI), and is determined by the amount and aggressiveness of cancer cells during diagnosis or following surgery. EOC can be grouped into two categories: type I low grade neoplasm that arise in a stepwise manner from borderline tumors and type II which are high grade neoplasm in which morphology initiation has not yet been determined (Ueda et. al., 2006). The grade is on a scale of 1, 2, or 3. Grade 1 epithelial ovarian carcinomas look similar to normal tissue and tend to have a better prognosis. Grade 3 epithelial ovarian carcinomas look less like

normal tissue and usually have a poor prognosis. EOC tumor stage (I-IV) describes how far the tumor has spread from where it started in the ovary.

Classification of EOC

Many epithelial ovarian tumors are benign, do not spread, and generally do not lead to serious illness. These types of tumors include serous adenomas, mucinous adenomas, and Brenner tumors. However, some ovarian epithelial tumors may be termed as borderline epithelial ovarian carcinomas in that they do not grow into the supporting tissue of the ovary (i.e. the ovarian stroma). EOC cells have several appearances identified microscopically. These features are used to classify EOC into serous, mucinous, endometrioid, and clear cell types. Serous is the most common type of EOC.

Based on the five main histological subtypes of ovarian cancer (serous, mucinous, clear cell, endometrioid and transitional cell/Brenner tumors) as well as undifferentiated adenocarcinomas many ovarian cancer cell lines have been derived. Each subtype is thought to differ in its etiology and genetic aberrations making these differences useful to the progression of ovarian cancer research. The following ovarian cell lines have been established and provide powerful research tools to study the various subtypes of ovarian cancer: OV-MZ 1-6 are recognized as serous cystadenocarcinoma, A2780 and H134 form undifferentiated tumors, OVCAR3 form poorly differentiated serous adenocarcinomas, SKOV3 tumors were derived from clear cell carcinomas, OVCA 429 form papillary adenocarcinomas with clear cell characteristics and OV2008 form tumors of endometrioid differentiation.

Clinical signs and symptoms of EOC

Most patients with EOC are asymptomatic. When symptoms are present they include bloating, pelvic or abdominal pain, difficulty eating or feeling full quickly, urinary symptoms, indigestion, and a change in bowel habits. Women with these symptoms daily for more than two weeks should consult a gynecologist. Many women complaining of these symptoms are treated and diagnosed for the wrong disease. In addition to the above symptoms ovarian cancer patients often complain of fatigue, back pain, pain with intercourse and menstrual irregularities at the outset of the disease. The onset of EOC signs and symptoms require medical attention due to the aggressiveness generally associated with the late onset of symptoms. Furthermore EOC spreads rapidly and throughout the organs of the body.

Diagnosis of EOC

When women are diagnosed in stage I of the disease there is a 90% survival rate. However, poor diagnosis of EOC in patients with solid tumors is due to late detection and stage progression. Therefore, once diagnosed with advanced ovarian cancer 30-40% of patients have a five-year survival rate and respond poorly to therapy. Initially patients undergo non-laboratory testing which include an ultrasound (pelvic and/or transvaginal), CT scan, and an X-ray of the GI tract. Next, patients undergo laboratory testing such as the OvaCheck, which involves a method of low-molecular-weight serum protein pattern recognition. This test is based on certain patterns “signature” that are distinct for EOC. In some cases patients may receive antigen testing for epithelial tumor,

germ cell tumor, or stromal tumor associated antigens. Examples of which may include, CA-125, BRCA-1/BRCA-2, Alpha feto protein (AFP), and inhibin. Once patients are diagnosed a combination of positron emission tomography (PET) scans and CT scans are used to track and detect recurrences. Scientist are searching for the origins of the disease that may assist in the development of a reliable screening test.

Treatment of EOC

1. Surgery

Once diagnosed treatment is of most concern. Growth inhibition before the cancer travels throughout the body is ideal. Surgery is the first step in treatment and allows the type and stage of cancer to be determined. A vertical cut in the abdomen exposes the pelvic region where samples are taken and all visible tumors are removed. Most likely at least one ovary will be removed. However, if advanced stage is obvious both ovaries and fallopian tubes are removed.

2. Chemotherapy

Chemotherapy combinations that include an alkylating agent and a platinum coordination complex have high response rates in women with advanced ovarian cancer. After initial surgery patients with advanced ovarian cancer receive chemotherapy. Cisplatin-based combination therapy has been shown to be more effective compared to alkylating agents alone (Thigpen et. al., 1989). The survival was 24 to 38 months in advanced ovarian cancer patients treated when combinations containing platinum were used (McGuire et. al., 1996

and Skarlos et. al., 1997). On the other hand, advanced ovarian cancer patients treated with combinations not containing platinum the median survival was 12 to 15 months (Thigpen et. al., 1989). Therefore following adequate surgical therapy a combined drug therapy containing platinum based chemotherapy is standard in the United States. Still, many patients with EOC have developed tumors that have become resistant to these chemotherapeutic agents as a result of repeated courses of these agents. Patients with recurrent EOC are generally treated with estrogen-blocking tamoxifen, which blocks blood vessel growth. Researchers are continuously discovering and developing alternative treatments to combat recurrent and advanced EOC.

3. Adoptive Cellular Immunotherapy

Adoptive cellular immunotherapy (ACT) is a process in which a large number of autologous T cells are expanded in vitro and infused into patients for the treatment of disease. The first evidence of efficacy using adoptive T cell transfer in cancer patients was identified in the early 1990s. This type of therapy enhances the immune response initiated and prorogated by the host. Tumor infiltrating lymphocytes (TILs) expanded in vitro in rIL-2 were used for the treatment of ovarian carcinoma by adoptive immunotherapy (Freedman et. al., 1994).

Although ACT has been shown in ovarian cancer to transfer antigen-specific T cell populations with the ability to restore immunity and eradicate tumors the limitations are quickly noted (Dudley et. al., 2002). Cancer regression and autoimmunity was observed in patients following ACT with antitumor

lymphocytes. Patients respond well to ACT following lymphodepleting chemotherapy as demonstrated in patients with metastatic melanoma (Dudley et. al., 2005). Furthermore, the acquisition of CTLs specific for tumor antigens is time-consuming and laborious failing on many occasions. Due to the lack of antigen specific CTLs and other limitations scientist have identified an alternate cellular therapy involving the use of genetically engineered lymphocytes for cellular immunotherapy.

4. ACT using engineered T-cells

Most recently $\alpha\beta$ TCR + T cells have been genetically engineered with the ability to recognize and kill tumor cells. Recent evidence has shown the ability of primary human lymphocytes transduced with cDNAs encoding TCR $\alpha\beta$ specific for HLA-A2 restricted cancer testis Ag NY-ESO-1 to efficiently recognize NY-ESO-1 positive nonmelanoma tumor cell lines (Zhao et. al., 2005). Furthermore, studies showed that an anti-p53 TCR transduced into PBL recognized fresh human tumors. Cancer regression was observed in patients following transfer of genetically engineered lymphocytes. Using retroviral vectors with antigen specific TCR gene patients produced in vivo populations of antigen-specific lymphocytes. TCR gene expression was stable and modified lymphocytes developed into memory helper T lymphocytes. This modified T cell provides an attractive strategy to develop antigen-specific immunotherapy with autologous lymphocytes as a genetic treatment option (Morgan et. al., 2006).

5. Vaccines

The availability of molecularly cloned tumor antigens permitted the development of peptide based or recombinant tumor vaccines. The use of oregovomab a murine derived monoclonal antibody directed against CA125 conjugated to CA125 antigen as an immune complex was shown to elicit a humoral T cell immune response (Gordon et. al., 2004). CD8+ and CD4+ lymphocytes underwent TCR genes transfer providing an opportunity to use the same TCR specificity to produce antigen-specific killer and cell mediated immune response in patients with recurrent ovarian cancer. The results indicate tolerance to oregovomab and induced multiple antigen-specific immune responses and a significant survival benefit. Anti-idiotypic (ID) vaccines such as murine anti-Id ACA125 mAb mimic an epitope of CA125 have been shown in both animals (Schlebusch et. al., 1995) and humans (Wagner et. al., 2001 and Reinartz et. al., 1999) to effectively induce a specific anti-anti-Id antibody (Ab3) response against CA125+ tumor patients. Peptide-based vaccines have been shown to induce immunity in patients with breast and ovarian cancer (Disis et. al., 1999 and Knutson et. al., 2002).

Prevalence and incidence of EOC

Ovarian carcinoma (Oca) continues to be the leading cause of death due to gynecologic malignancies. Over 23,300 will be diagnosed and 13,900 patients will die each year from OCa. The incidence of OCa varies widely in frequency among different geographic regions and ethnic groups, with high incidences observed in Scandinavia, Western Europe and North America and low incidences found in Asian countries. Epithelial ovarian cancer (EOC) generally

occurs in women fifty years of age or older. Approximately 1 in 57 women in the U.S. and 1 in 108 in Australia are diagnosed with EOC. EOC results in a 5-year survival rate for 25-30% of patients with stage III and IV disease, contrasting with the 90% survival rates of patients with stage I disease.

EOC risk factors

The etiology of ovarian cancer is poorly understood however; certain factors have been implicated in ovarian cancer, such as, environmental agents, racial, geographical, hormonal, infertility, social, and family history (Wong and Auersperg, 2003). Most often, family history accounts for 5-10 % of all cases.

Caffeine, alcohol and smoking have all been speculated in the risk of developing EOC. Recent studies have shown a link between cigarette smoking and ovarian cancer. Evidence indicates that smoking may only increase the risk for mucinous ovarian tumors. Furthermore, the risk associated with EOC and caffeine was prevalent in women not previously taking oral contraceptives (Tworoger et. al., 2008). However, these preliminary studies warrant the need for future research to further understand the link between environmental and social factors associated with EOC.

EOC Pathogenesis

1. Putative genes involved in EOC pathogenesis

Cancer associated genes include the following: growth factor, growth factor receptors, signal transducers, and transcription factors. Normally expression of growth factors and growth factor receptors are carefully regulated. However, overexpression of growth factor receptors such as HER2/neu are

observed in 20% of ovarian cancer specimens and results in uncontrolled proliferation (Skirnisdottir et. al., 2001). Furthermore, genomic alteration of both the HER2-neu and EGFR genes is frequent (25%) in ovarian cancer. High polysomy of the EGFR gene was observed in 13% of the invasive epithelial cancers and amplification of the HER2-neu gene was found in 10% and correlated with a high expression level. EGFR/HER2-neu targeted therapies may potentially be a benefit for patients with invasive EOC (Vermeij et. al., 2008).

Cancer associated genes termed tumor suppressor genes, or anti-oncogenes encode proteins that inhibit cellular proliferation. The normal activity of genes within this group is cell cycle regulation. The suppressor of retinoblastoma, *Rb*, is a prototype of this group. Loss of heterozygosity at the *Rb* gene locus occurs in 30% of ovarian cancers compared to *Rb* pathway abnormalities which occur in 80% of ovarian cancer patients. Likewise, in advance ovarian cancers 50-70% of patients had gene alterations or expression in a nuclear phosphoprotein, p53. p53 is a transcription factor that regulates the cell cycle and thus functions as a tumor suppressor (Hashiguch et. al., 2001). Loss of heterozygosity at 17p13 was identified in a high proportion of advanced stage serous adenocarcinomas (Eccles et. al., 1992).

Genes that regulate programmed cell death and encode proteins that either block or induce apoptosis are believed to be involved in EOC. The prognostic importance of the biologic factors p53, bcl-2, and bax, which are key regulators of apoptosis, was identified in patients with early stage EOC (Skirnisdóttir et. al., 2002). Furthermore, mutations in tumor suppressor genes

such as BRCA1/BRCA2 (Pal et. al., 2005) are also found in EOC patients. In addition such genes have been used as biomarkers for treatment of patients with EOC using chemotherapy (Yurkovetsky et. al., 2006). Investigations show that inhibition of endogenous BRCA1 expression in ovarian cancer cell lines results in increased sensitivity to platinum therapy and decreased sensitivity to antimicrotubule agents. In addition, it is shown that patients with low/intermediate levels of BRCA1 mRNA have a significantly improved overall survival following treatment with platinum-based chemotherapy in comparison with patients with high levels of BRCA1 mRNA (Quinn et. al., 2007).

Conversion of proto-oncogenes to oncogenes has been associated with cellular transformation and/or disease progression. The resulting altered gene products and enhanced expression of gene products may initiate and/or propagate events leading to transformation.

Frequent ovulation may increase the risk of EOC as a result of repeated rupture and repair of ovarian surface epithelium. Recent studies indicate that inflammation may also play a role in developing cancer. Inflammation is characteristic of the peritoneum of EOC and understanding the linkage between the coagulation cascade and the cytokines/chemokines involved in inflammation may lead to a new approach to cancer therapeutics. In addition, recent evidence supports the role of the coagulation pathway and inflammation in promoting tumor cell progression and the formation of ascites. Studies have shown that a number of genes involved in the coagulation cascade and genes encoding proteins associated with inflammatory responses demonstrated altered

expression in the pelvic peritoneum and stroma, as well as the vicinity of EOC implants. Coagulation pathway proteins involved in tumorigenesis consist of thrombin, protease activated receptor, tissue factor, fibrinogen and fibrin. Several other coagulation regulatory proteins including heparin cofactor-II and epithelial protein C receptor (EPCR) were also upregulated in the peritoneum of EOC (Wang et. al., 2005).

2. Changes in pathology leading to EOC

Pathological changes in ovarian surface epithelium (OSE) have been indicated in EOC. Researchers believe that these changes in OSE may result in development and/or progression of epithelial ovarian carcinomas. OSE is a monolayered squamous-to cuboidal epithelium. OSE is characterized by keratin types 7, 8, 18, and 19, which represent the keratin complement typical for simple epithelia. OSE is distinguished from extraovarian mesothelium, apical microvilli, and a basal lamina due to the expression of mucin antigen MUC1, 17B-hydroxysteroid dehydrogenase, and cilia. Normal OSE contact and epithelial integrity is maintained by incomplete tight junctions, integrins, and cadherins.

A dense collagenous connective tissue layer, tunica albuginea, provides an inherent barrier against bioactive agents between the ovarian stroma and OSE. With age the human ovary assumes irregular contours and forms OSE-lined surface invaginations and epithelial inclusion cyst in the ovarian cortex. Within these clefts and cyst ovarian cells assume columnar shapes. Over time, the OSE within these surface invaginations are susceptible to metaplastic changes. OSE secrete IL-6 and IL-1 both of which result in proliferation of

ovarian cells and/or the release of autocrine growth regulators resulting in changes in gene expression which promotes neoplasia. IL-1 induces TNF α which is a mitogen for OSE leading to immortalization of cells and selective growth advantages. Furthermore, autocrine mechanisms via OSE derived cytokines and hormones within inclusion cyst allow accumulation of blood borne bioactive agents that would otherwise diffuse on the ovarian surface. (Auersperg et. al., 2001).

3. Tumor Antigens

The significance of identifying tumor antigens and the immune response to these antigens may help increase the effectiveness of cancer therapy. Prior to the development of cancer vaccines researchers have identified and characterized of tumor antigens. During the past decade many tumor antigens have been identified (Ghazizadeh et. al., 1997, Wang et. al., 1996, Odunsi et. al., 2003, Yakirevich et. al., 2003). Tumor antigens may be categorized into two main groups: tumor-specific antigens (TSA) and tumor-associated antigens (TAA). Tumor specific antigens are expressed only on tumor cells and not on normal cells. TSA can be presented with class I HLA molecules and induce a cell-mediated response by tumor-specific CTLs. TAAs are expressed on tumor and normal cells as well as embryonal or fetal cells (Kawakami et. al., 1994, Bakker et. al., 1994 and Wang et. al., 1996).

Cancer testis (CT) antigens are promising targets for the development of human cancer vaccines. Important characteristics of CT antigens are high expression levels in adult male germ cells, absence of expression in other normal

adult tissues, and aberrant expression in a variable proportion of a wide range of different cancer types. They are also expressed in fetal ovarian cells (Pal et. al., 2005 and Nelson et. al., 2007). The CT antigens reported to be expressed frequently in EOC include: NY-ESO-1, MAGE-A1, and MAGE-A4 (Odunsi et. al., 2003, van den Eynde et. al., 1995, and Yakirevich et. al., 2003). In addition to CT antigens sialyl-Tn and CA125 are antigens currently used as ovarian cancer markers. Sialyl-Tn is a carbohydrate associated with MUC1 mucin, which is expressed by up to 90% of ovarian tumors, and has been a popular choice of antigen for several past clinical trials (Ghazizadeh et. al., 1997). Cancer Antigen 125 (CA125), also known as MUC16, is most often used to determine tumor growth *in vivo*. Doubling or halving of the CA125 serum values correlated with tumor progression or regression, respectively (Yurkovetsky et. al., 2006). Many ovarian antigens have been identified as well as the ability of TIL to recognize peptides from these antigens in association with HLA. However, there are currently no studies that have identified definitive ovarian cancer antigens recognized by $\gamma\delta$ TCR+ T-cells.

Tumor Immunity

1. Immune cells involved in EOC

Tumor antigens have been shown to induce both humoral and cell mediated immune responses to tumor antigens and both *in vivo* and *in vitro* experiments have shown tumor cell death due to immunological effector mechanisms. Tumor-infiltrating monoclear cells derived from the inflammatory infiltrate in human solid tumors and/or ascites. These cells consist of T

lymphocytes, natural killer cells (CD56+), B-lymphocytes, and macrophages (CD68+). T lymphocytes can be grouped into two major lymphocyte populations CD3+ CD4+CD8-, CD3+CD4-CD8+. CD4+ helper T cells and CD8+ cytotoxic T cells play a role in anti-tumor responses.

a. Innate immunity cells

Natural killer (NK) cells and macrophage/monocytes have been implicated in antitumor effector response. In epithelial ovarian cancer the ovarian tumor marker MUC16 (CA125) inhibits the cytotoxic responses of human natural killer (NK) cells and down-regulates CD16 (Belisle et. al., 2007). NK cells belong to the family of cytotoxicity cells. NK cells have been shown to recognize tumor cells and induce killing of these cells. Furthermore following ligand binding activation of the NKG2D receptor on the surface of NK cells may lead to NK-cell-mediated tumor cell effector functions. Ligands for NKG2D have been found on several tumor cells. Stimulated macrophages have been shown expression of NKG2D on the cell surface as well. Activation through NKG2D leads to the production of NO and TNF- α in macrophages. In the case of EOC tumoricidal activity was reduced by inhibition of nitric oxide synthesis in vivo using the human epithelial ovarian cancer cell line, NIH:OVCAR3 for intraperitoneal transplantation (Farias-Eisner et. al., 1994). Monocytes and macrophages are involved in antibody-mediated mechanisms of tumor cell killing, such as antibody-dependent cell-mediated cytotoxicity (ADCC) and phagocytosis. These activated mononuclear leukocytes are important to the innate immune system and more importantly can be directly cytotoxic to tumor cells. Defective antitumor functions

involving monocyte-derived macrophages were shown in patients with EOC. This population of monocyte-derived macrophages were involved in suppression of T-cell proliferation through the production of interleukin (IL)-10 and transforming growth factor- α (Loercher et. al., 1999).

b. B-cells

Hybridomas have been prepared from the B cells of tumor patients that produce monoclonal antibodies. Furthermore, monoclonal antibodies have been developed against ovarian cancer cell surface antigens. Bevacizumab, anti-VEGF antibody has been shown to neutralize vascular endothelial growth factor (VEGF) (Burger et. al., 2007). These antibodies are not specific for antigens expressed exclusively on tumor cells. Bevacizumab has also been used in patients with colorectal carcinoma as well as other solid malignancies (Shih and Lindley, 2006). Antibody mediated destruction of tumor cells is attributed to complement activation, or antibody-dependent cell-mediated cytotoxicity in which Fc receptor-bearing macrophages or NK cells mediate the killing. Patients with EOC have shown production of anti-tumor antibodies following vaccination with tumor-associated antigens such as CA-125.

c. T-cells

CD8+ tumor associated lymphocytes isolated from patients with ovarian carcinoma demonstrate cytolytic activity towards autologous tumor cells in a TCR dependent manner (Ioannides et. al., 1991). Cytotoxic T lymphocytes derived from breast and ovarian cancer patients have been shown to recognize peptides from HER2/neu (Peoples et. al., 1995). Following analysis of different cytokine

profiles released by CD4+ and CD8+ T cells data suggest IFN- γ , GM-CSF, and IL-2 contribute to tumor regression (Aruga et. al., 1997). CD4+ helper T cells are not generally cytotoxic to tumors, however they may secrete cytokines as well as tumor necrosis factor (TNF α) or interferon- γ (IFN- γ). IFN- γ has been shown to induce apoptosis in ovarian cancer cells in vivo and in vitro (Wall et. al., 2003). To identify whether tumor infiltrating lymphocytes in patients with ovarian cancer are clonally expanded, V-specific PCR analysis were carried out using primers for the β -chain of $\alpha\beta$ TCR + T cell infiltrates. Experimental evidence indicates a substantial proportion of β -chain transcripts are present at the tumor site showing oligoclonal/monoclonal expansion in patients with ovarian cancer (Pappas et. al., 2005). The presence of clonally expanded $\alpha\beta$ TCR + T cells in patients with ovarian cancer is evidence that an antigen driven immune response has taken place in situ at the tumor site. A component of tumor infiltrating mononuclear cells in ovarian carcinoma is CD8+ and CD68+ cells. However, the frequency of these cells is suggested to relate with tumor type rather than the degree of tumor differentiation (Helal et. al., 2004). Patients with higher frequencies of intraepithelial CD8+ T cells demonstrated improved. In addition, favorable prognosis did not correlate to the amount of NY-ESO-1 or MAGE, CT antigen expression (Sato et. al., 2005).

Suppression of the host immune function plays an important role in the progression of cancer. In fact patients with advanced ovarian malignancies exhibit progressively impaired immune responses. More specifically epithelial cancers are frequently coated by a layer of carbohydrate-rich mucins (ie CA-125

and Sialyn Tn) that play a role in immune escape by blocking T cells interaction, shedding attacking antibodies from the cell surface and hindering access for CTL.

Suppressed T-cell activation is one characteristic of advanced ovarian cancer (Berek et. al., 2003). Regulatory T cells T(reg)s mediate homeostatic peripheral tolerance by suppressing autoreactive T cells. Disregulation or failure of host antitumor immunity may be caused by suppression of tumor-associated antigen-reactive lymphocytes mediated by T(reg) cells; however, definitive evidence that T(reg) cells have an immunopathological role in human cancer is lacking. Evidence indicates CD4(+)CD25(+)FOXP3(+) T(reg) cells in 104 individuals affected with ovarian carcinoma, show suppressed tumor-specific T cell immunity and these T(reg) cells contribute to growth of human tumors in vivo. Researchers show that tumor T(reg) cells are associated with reduced survival. Human T(reg) cells preferentially move to and accumulate in tumors and ascites, but rarely enter draining lymph nodes in later cancer stages. Tumor cells and microenvironmental macrophages produce the chemokine CCL22, which mediates trafficking of T(reg) cells to the tumor. This specific recruitment of T(reg) cells represents a mechanism by which tumors may foster immune privilege. Therefore, researchers believe, blocking T(reg) cell migration or function may help to defeat human cancer and lead to effective alternative immunotherapy (Curiel et. al., 2004).

Heat shock protein-10 (HSP-10) was detected in both sera and ascites of patients with ovarian cancer. HSP-10 appeared to be produced and released

form ovarian tumor cells. This circulating HSP-10 suppressed T-cell expression of CD3-zeta, a key activation component in T cells. Researchers conclude that release of HSP-10 may be an important factor allowing the tumor to escape immune surveillance by suppression of T cell activation (Akoyl et. al., 2006). To design effective immune-based antitumor therapies depletion of Tregs may help achieve increased antitumor effects with immunotherapy. Other important factors involved in tumor escape include TGF-B1 and IL-10. Host problems also involve development of tolerance to self-antigen(s) and impaired immune function from previous chemoradiotherapy.

Although the precise evaluation of the immune response at the primary tumor site is currently being studied progress in the field will help to develop therapies for EOC and other related cancers.

2. Soluble factors involved in EOC

TGF- β has been shown be an important cytokine involved in inflammation in ovarian cancer. to stimulate tumor cell attachment and invasion by upregulation plasminogen activator inhibitor type I. TGF- β also deregulates expression of the MHC complex and deregulates costimulatory antigen expression by dendritic cells. Suppression of Th1-Th2 cells and the conversion of pro-cytotoxic T lymphocytes to cytotoxic T lymphocytes is also contributed to the presence of TGF- β . Other cytokines such as IL-6 upregulates tumor cell attachment and suppress Th1-Th2 transformation. IL-10 has been shown to suppress Th1-Th2 transformation and cytotoxic T lymphocytes activation, as well as inhibit T cell and IFN production (Curiel et. al., 2004).

Cytokines have been used for the treatment of tumors. IFN- γ directly inhibits human tumor cell growth and induces apoptosis. IFN- γ has been used in the treatment of patients with advanced ovarian cancer. 108 patients with residual disease following first-line cisplatin-based chemotherapy upon second look laparotomy were treated with i.p. IFN- γ twice a week for 3-4 months. Out of 98 patients assessed 23 were completely responsive and 8 patients were partially responsive to IFN- γ . In ovarian cancer IFN- γ had antiproliferative activity in 8 of 8 ovarian cancer cell lines and 11 Of 14 primary cultures. In two of six patients, there was a 90% reduction in tumor cells in ascites after IFN- γ treatment, and this was related to clinical benefit as assessed by intervals between paracentesis (Wall et. al., 2003).

Alpha-interferon (IFN- α) is a type I interferon, produced largely by leukocytes and also have antiproliferative effects on cells *in vitro*. IFN- α has been shown to increase class I MHC expression on various cell types. In EOC down regulation of HLA-1 has been shown as one of many mechanisms of tumor cell escape.

High doses of IL-2 administered in conjugation with adoptive cellular immunotherapy are associated with increased blood lymphocytes, NK and LAK cell activity. IL-2 may work by stimulating anti-tumor activity of NK cells and/or CTLs. However, the treatment of patients with high levels of IL-2 alone can be extremely toxic causing fever, pulmonary edema, and often shock. This is due to the production of other cytokines by T cells as a result of IL-2 activating CD25 ligand receptor. TNF has potent anti-tumor effects but like IL-2 having highly

toxic effects at doses necessary for tumor cell killing. In addition ovarian cancer cells have developed resistance to TNF receptor mediated induction of apoptosis as shown in ovarian cancer cell lines such as SKOV3.

$\gamma\delta$ TCR+ T-cells

While $\alpha\beta$ TCR + T cells may prove to be effective in antitumor immunity one mechanism by which ovarian cancer cells evade the immune system is via down regulated HLA expression (Kooi et. al., 1996). Therefore, $\gamma\delta$ TCR + T cells may provide an alternate mechanism for antitumor immunity indicating a need for studies similar to the aforementioned to identify EOC antigens and EOC antigen-specific $\gamma\delta$ TCR + T cells. In humans $\gamma\delta$ T cells make up only a small percentage (1-5%) of spleen and lymph node T cells. The $\gamma\delta$ TCR+ T cells account for nearly 1%-10% of T lymphocytes and thymocytes in the peripheral blood of humans.

1. Subsets of $\gamma\delta$ TCR + T cells

Two groups of $\gamma\delta$ TCR + T cells exist $V\gamma 11(9)V\delta 2$ and $V\delta 1$. $V\gamma 11(9)V\delta 2$ T cells as the major population can be found within the peripheral blood and play a role in intracellular pathogens and hematological malignancies. This group of $\gamma\delta$ TCR + T cells has been shown to kill myeloma and lymphoma cells in vitro. $V\delta 1$ are primarily line the epithelial layers of various organs, such as the small intestine, liver and reproductive tract (Bukowski et. al., 1995 and Kobayashi et. al., 2001).

2. Genetic organization of the T-cell receptor chain loci

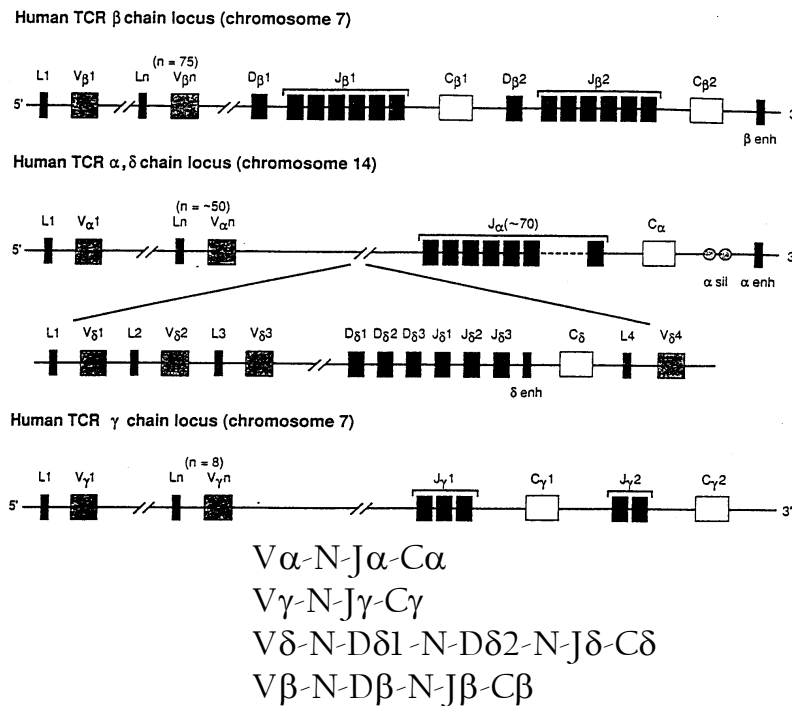
Each chain of the TCR is composed of multiple regions; the V (variable region), the J (junctional region), the D (diversity region, found only in the β - and

δ -chain), and the constant (C) region. Each region is typically coded for by multiple gene segments within the locus of the chain. The γ -chain gene locus is located on chromosome 7, and consists of approximately 12 variable region ($V\gamma$) gene segments (and only 8 out of the 12 gene segments are functional), 5 junctional region ($J\gamma$) gene segments and 2 constant region ($C\gamma$) gene segments. The $V\gamma$ consists of four groups; $V\gamma I$ includes $V\gamma 2$, $V\gamma 3$, $V\gamma 4$, $V\gamma 5$ and $V\gamma 8$, $V\gamma II$ consists of only $V\gamma 9$, $V\gamma III$ consist of only $V\gamma 10$ and $V\gamma IV$ consist of only $V\gamma 11$. The $V\gamma I$ and $V\gamma II$ subgroups consist of about 95% of the γ -chain TCR⁺ T-cell transcripts. The $C\gamma$ consists of two groups. The $C\gamma 1$ group contains a cysteine residue in the second exon of the gene that may function in disulfide linkages with the corresponding $C\delta$ chain. The second $C\gamma$ ($C\gamma 2$) contains no cysteine residues in exon 2 but has an exon 2 polymorphism; one subtype has two copies of exon 2 and the other subtype has three copies of exon 2. The TCR δ -chain gene locus is located within the TCR α -chain gene locus on chromosome 14 between the variable region and the junctional region. The δ -chain locus has three variable region ($V\delta$) gene segments, 3 joining region ($J\delta$) gene segments, 3 diversity region ($D\delta$) gene segments and one constant region ($C\delta$) segment. Because the δ -chain gene locus is within the α -chain gene locus, successful rearrangement of a δ -chain TCR transcript preferentially forms a heterodimer with a successfully rearranged γ -chain TCR transcript to form $\gamma\delta$ TCR heterodimer (Butrum et al, 1996) and is expressed on the T-cell in which it was rearranged. This expression excludes the expression of an α -chain TCR

transcript, which preferentially forms a heterodimer with a successfully rearranged β -chain TCR transcript to form $\alpha\beta$ TCR heterodimer, and vice versa. Hence, the expression of $\alpha\beta$ TCR and the $\gamma\delta$ TCR on same T-cell is mutually exclusive. The $\gamma\delta$ TCR heterodimers can either be disulfide-linked, if the γ -chain rearrangement involves $C\gamma 1$ that contain cysteine residue in its exon 2 or can be non-disulfide linked $\gamma\delta$ TCR heterodimer, if the γ -chain rearrangement involves $C\gamma 2$, which lacks the cysteine residue found in $C\gamma 1$ (Seki et. al., 1989). Frequency analysis suggests that the $J\delta 1$ segment is the most frequently used $J\delta$ segment, followed by $J\delta 3$, and then in turn by $J\delta 2$ (Davodeau et. al., 1994). Although the germ line genes of $\gamma\delta$ TCR contains fewer variable (V) and junctional (J) region gene segments than that of $\alpha\beta$ TCR+ T-cells, the potential for diversity in the $\gamma\delta$ TCR+ T-cells is potentially higher due to various factors including, a) combinational joining of V-J, V-D-J gene segment joining, b) the presence of two or more tandemly rearranged segments in the diversity (D) regions of the δ -chain transcript, c) the junctional flexibility at the V-D and D-J junctions, and d) N-nucleotide insertions catalyzed by N-terminal deoxynucleotidyl transferase enzyme (tdt). Rearrangement of the different V-D-J gene segments during maturation of lymphocytes, junctional flexibility and N-nucleotide addition creates a larger degree of diversity in the complement determining region 3 (CDR3) loops, i.e. the hypervariable region that contacts the antigen. The CDR3 loops of $\gamma\delta$ TCR+ T-cells are more similar to that of the immunoglobulin of B-cells than to that of $\alpha\beta$ TCR+ T-cells in their ligand binding domains. This is believed to be the fundamental difference in antigen recognition

between $\gamma\delta$ TCR+ T-cells and $\alpha\beta$ TCR+ T-cells, leading to speculations that $\gamma\delta$ TCR+ T-cells recognize antigens in a similar fashion to immunoglobulin receptors, i.e. whole proteins and not like the $\alpha\beta$ TCR+ T-cells that recognize peptides in association with MHC molecules.

Figure 1 – TCR genetic locus



3. Antigen recognition of $\gamma\delta$ TCR+ T-cells

Majority of $\alpha\beta$ TCR + T- recognize processed antigen(s) as peptides restricted to MHC. In contrast, the recognition of antigen(s) by majority of $\gamma\delta$ TCR+ T-cells is still being elucidated and may not be MHC-restricted. $\gamma\delta$ TCR+ T-cells may recognize whole proteins expressed at a site distal from the MHC peptide-binding groove on the surface of antigen presenting cells (APCs). The proteins recognized by $\gamma\delta$ TCR+ T-cells do not require antigen processing.

Examples of ligands recognized by $\gamma\delta$ TCR+ T-cells include MHC class Ia, Ib and II, and serpins. $\gamma\delta$ TCR+ T-cells can also recognize antigens presented by CD1 molecules as well as natural or synthetic non-peptide antigen(s) including, superantigen(s), viral proteins, lipids, phosphates, N-formylated peptides and possibly carbohydrates (Tanaka et. al., 1995). Majority of $\gamma\delta$ TCR+ T-cells in humans are typically CD3+ CD4- CD8-, although some can express the CD4 or CD8 co-receptor. Functional analysis suggest that $\gamma\delta$ TCR+ T-cells have cytolytic activity against specific stimulating target cells and is inhibited by specific monoclonal antibody to HLA class I determinants suggesting that these CD3+ CD4- CD8- TCR+ T-cell clones recognize class I MHC determinants on target cells (Rivas et. al., 1989). This finding is significant because all cells in the body express class I MHC, suggesting that $\gamma\delta$ TCR+ T-cells may play a role in surveillance of tumor and “stressed” cells leading to their destruction. Other studies suggest that $\gamma\delta$ TCR+ T-cells may also function in wound repair and maintaining the integrity of epithelial cells that are exposed to the environment, and in immuno-regulation (Girardi et. al., 2006).

4. $\gamma\delta$ TCR + T cells in tumor immunity

Several findings suggest that $\gamma\delta$ TCR + T cells recognize certain tumor related antigens, in a TCR dependent manner, and play a role in tumor surveillance. Most recent data has identified nine peptides and two proteins for $\gamma\delta$ TCR + T cells, including human mutS homolog 2 (hMSH2) and heat shock protein HSP60. All nine tested epitope peptides not only bind to $\gamma\delta$ TCR + T cells, but also functionally activate $\gamma\delta$ TCR + T cells in vitro. Identification of HSP60

confirms the use of this method for the identification of $\gamma\delta$ TCR + T cells ligands as HSP60 is an identified ligand for TCR gamma delta. Also, hMSH2 is expressed on the surface of SKOV3 tumor cells and cytotoxicity of Vdelta2 $\gamma\delta$ TCR + T cells to SKOV3 cells was blocked by anti-hMSH2 antibody, suggesting that hMSH2 may be a new ligand for $\gamma\delta$ TCR + T cells. These findings support evidence that indicate the role of $\gamma\delta$ TCR + T cells in epithelial ovarian cancer (Chen et. al., 2008). Early studies utilized TCR negative Jurkat cells transfected with cDNA for V γ II(9)V δ 2 TCR to demonstrate using more definitive methods the role of $\gamma\delta$ TCR-dependent recognition of Daudi Burkitt lymphoma and myeloma cells (Bukowski et. al., 1995). To determine the numbers of $\gamma\delta$ TCR + T cells in peripheral blood and tumor infiltrates in patients with renal carcinoma CD3+ T cells were analyzed for the presence of $\gamma\delta$ TCR + T cells compared to healthy individuals. Results showed that the $\gamma\delta$ TCR+ T cells expressing V γ II(9) and V δ 2 TCR were elevated in the peripheral blood of 10/41 patients (greater than 10% of T lymphocytes) and in 3 patients greater than 30% of T lymphocytes. The proportion of patients with elevated numbers increased with cancer stage and decreased after surgery. Following evaluation of the junctional diversity region the peripheral blood $\gamma\delta$ T cells showed oligoclonal rather than polyclonal expansion (Kobayashi et. al., 2001). During analysis of CD8+ T cells from ascites of colon cancer patients a V γ II(9)V δ 2 T cell clone was isolated showing tumor cell recognition which was HLA-class I-independent but IPP and ICAM-1 dependent (Corvaisier et. al., 2005).

V δ 1 $\gamma\delta$ TCR + T cells are believed to be important in infections and malignancies. A study using human intestinal V δ 1+ T lymphocytes showed that V δ 1 $\gamma\delta$ TCR+ T cells are able to recognize tumor cells of epithelial origins. This recognition was evident by the production of IFN γ and cytolytic activity of V δ 1 $\gamma\delta$ TCR+ T cells towards autologous and allogeneic colorectal cancer cells, renal cancer cells, and pancreatic cancer cells (Maeurer et. al., 1996).

The above evidence indicates the significance of studying $\gamma\delta$ TCR + T cells as they relate to tumor immunity. Identifying the mechanisms by which $\gamma\delta$ TCR + T cells recognized tumor antigens and their role in reducing and/or eliminating tumor growth, development, and progression will increase our understanding of T cell mediated tumor immunity. $\gamma\delta$ TCR + T cells may provide an additional approach for adoptive immunotherapy which is not subject to the expression of HLA-class I by tumor cells.

Thus far experimental evidence obtained has shown oligoclonal expansion of $\gamma\delta$ TCR+ T cells in patients with EOC. We have constructed full-length TCR transcripts of the most clonally expanded transcripts. Most recently we have generated retroviral vector containing γ -chain and δ -chain transcripts from one patient. Transduction experiments are currently underway and subsequent characterization studies will follow. Examination of T cell receptor γ -chain and δ -chain sequences showing oligoclonal expansion indicates an antigen-specific response. Identification of functional and antitumor activities will improve our understanding of the immune response during EOC disease progression and the role of $\gamma\delta$ TCR+ T cells in patients with EOC.

5. Homing signals for $\gamma\delta$ TCR+ T cells

Epithelial tumors consist of tumor infiltrating lymphocytes such as $\alpha\beta$ and $\gamma\delta$ T lymphocytes. $\gamma\delta$ TCR + T cells make up a small portion of this population. Some of these $\gamma\delta$ TCR + T cells are resident and recognize over expressed tumor antigens at the site. Furthermore, circulating $\gamma\delta$ T cells respond to IL-12 and also recognize tumor antigens. Natural killer receptor proteins 1A play a role in homing of circulating $\gamma\delta$ T cells to the site of tumor formation and also MIP1 α , MIP1 β , and RANTES. Chemotactic gradients target $\gamma\delta$ T cells to damaged tissue. The antigens are nonpeptide antigens such as phosphorylate thymidine related products involved in salvage pathway in nucleic acid synthesis and repair. This phosphorylate thymidine is over expressed by damaged or stressed cells. Other antigens may include protein antigens such as MHC I/MHC II and HSV glycoprotein or proteins with post translational modifications such as N-linked glycosylation. Malignant transformation and infections alter glycosylation patterns due to stress signals. Stress induced signals may induce expression and overproduction of antigens such as MICA/MICB both of which are molecules on neoplastic and normal epithelial and are MHC I related molecules, CD1.

6. Cytotoxic T lymphocytes

Cytotoxic T lymphocytes express either $\alpha\beta$ TCR or $\gamma\delta$ TCR+ CD3+ T-cells exhibit cytotoxicity following antigen driven TCR receptor stimulation/activation. These effector functions may be the result of intracellular infected cells, transformed cells, and rejection of transplants. The mechanisms involved in cell-mediated cytotoxicity include specific-antigen recognition and cell-to-cell contact.

During cell-to-cell contact adhesion molecules help to form stable conjugates. Once the conjugate is formed two pathways are triggered which results in death of the specific target cell. One pathway is a secretory pathway involving receptor-triggered exocytosis of preformed secretory granules, while the other uses receptor-induced surface membrane expression of Fas ligand (FasL), which cross-links Fas on target cells. CTL use both of these pathways to kill target cells. However, during mutagenesis cancer cells have undergone genetic alterations allowing the avoidance of tumor cell apoptosis.

Apoptotic signaling and execution induced by cytotoxic T lymphocytes including $\gamma\delta$ TCR+ T cells may involve the activation of caspase proteinase which cleave proteins on the basis of primary structure. The procaspases can be divided into two classes: caspase-2, -8, -9 and -10 which contain long amino-terminal prodomains and act as initiators. The second class includes caspase-3, -6 and -7. Caspase 3 is the most common activated caspase and is involved in DNA fragmentation. Procaspase-8 or -9 may activate Caspase 3.

CHAPTER 2 HYPOTHESIS AND SPECIFIC AIMS

The hypothesis to be tested in this study is whether $\gamma\delta$ TCR+ T-cells are clonally expanded in tumors from patients with EOC and whether they recognize/lyse EOC tumor cells.

The specific aims for this project are:

1. To determine whether T cells infiltrating solid EOC tumors contain oligoclonal populations of $\gamma\delta$ TCR+ T-cells. Specifically,
 - a. Fresh (not expanded in culture) $\gamma\delta$ TCR+ T-cells infiltrating the EOC specimen, contain substantial proportions of identical γ -chain TCR+ T cells.
 - b. Fresh (not expanded in culture) $\gamma\delta$ TCR+ T-cells infiltrating the EOC specimen, contain substantial proportions of identical δ -chain TCR+ T cells.
2. To construct full-length transcripts of the most clonally expanded and non-clonally expanded TCR + T-cell γ I-chain and γ II(9)-chain transcripts that are present in the EOC specimen examined, for use in expression using MSCV retroviral expression system.
3. To construct full-length transcripts of the most clonally expanded TCR + T-cell δ 1-chain and δ 2-chain transcripts that are present in the EOC specimen examined, for use in expression using MSCV retroviral expression system.

4. To determine whether T cells transduced with γ -chain and δ -chain TCR transcripts recognize ovarian tumor cells. Specifically,
 - a. Caspase 9, 8, and 3 activity on ovarian carcinoma cell line (SKOV3, CAOV3 and/or OV2774) by $\gamma\delta$ TCR+ transduced cell lines.
 - b. Cytotoxicity of $\gamma\delta$ TCR+ transduced cell lines to ovarian carcinoma cell line (SKOV3, CAOV3 and/or OV2774)

CHAPTER 3 MATERIALS AND METHODS

A) Tissue specimen collection and processing

Solid tumor and malignant ascites specimens from previously untreated patients with EOC were used in this study. Solid tumor specimens from patients with EOC were collected during therapeutic surgery and were immediately placed on ice upon removal. Fatty tissue, normal, and necrotic tissue were removed from the tumor specimens. In most specimens a small part of the tumor specimen was embedded onto optimum cutting technology (OCT) formulation, snap frozen in liquid nitrogen, and kept at -70°C to be used for Immunohistochemistry. The rest of the tumor specimen was kept in liquid nitrogen and used for RNA preparation. In other tumor specimens the entire specimen was kept in liquid nitrogen and used for RNA preparation. Ascites specimens from patients with EOC were collected under sterile conditions with heparin added to 1U/ml of ascites. The IRB of Temple University Hospital approved these studies.

B) RNA preparation

Total RNA was isolated from solid tumor specimens, single cell suspensions derived from malignant ascites of patients with EOC, and PBMC from normal by the guanidinium thiocyanate phenol-chloroform extraction method using the TRIzol® RNA isolation solution (Invitrogen) according to the manufacturer's instructions. The quality of the RNA was determined by examining the 28S and 18S bands of the rRNA after agarose gel electrophoresis.

C) cDNA synthesis

CDNA was synthesized from oligo (dT) Not I (TGCGGCCGCTTTTTTTTTTTTTT) – primed total RNA by using the Superscript II (Invitrogen) cDNA synthesis kit following manufacturer's instructions. The first strand of the cDNA of the isolated RNA was synthesized in a reaction using first-strand buffer, dNTPs (25mM), Superscript II reverse transcriptase (Invitrogen) and the primer (Genosys custom oligo, 500ug/mL) at 42°C for one hour in a 20ul reaction. The resulting cDNA/RNA hybrid underwent the second strand cDNA synthesis in a 150 ul reaction of the second-strand buffer, 25mM dNTPs, E. coli DNA polymerase, E. coli DNA ligase and RNase H (Invitrogen) to remove the RNA from the hybrid, at 16°C for 2hrs. T4 DNA polymerase was added for 45mins at 16°C followed by a phenol:chloroform extraction and ethanol precipitation of the synthesized cDNA, washing with 600ul of 100% ethanol and resuspended in nuclease free water.

D) V-specific PCR

PCR was performed using V-specific primers (γ I-, γ II(9)-, δ 1-, and δ 2-chain) to amplify the gene of interest, which was the CDR3 region of the $\gamma\delta$ TCR gene. The $V\gamma$ I subgroup ($V\gamma$ 2-5) specific primer 5'-TGCGGTGGGCCCTACTGGTGCTT-3' and 5'-GCCTTCTGGAGCTTTGTTTCAG-3' $C\gamma$ primer. The $V\gamma$ II subgroup transcripts $V\gamma$ II(9) specific primer 5'-TGCTGTCAGTCTCCACACATC-3' primer and 5'-GCCTTCTGGAGCTTTGTTTCAG-3' $C\gamma$ primer. The $V\delta$ 2 specific primer used

was 5'-GTGTGGCCCAGAAGGTTACT – 3', V δ 2 – 5' –

CATTGAGTTGGTGCCTGAA – 3', C δ 5'– CTTGGATGACACGAGATTTATT – 3'.

Each amplification reaction was conducted in a reaction containing optimized volumes of the template, 10mM dNTPs, 25mM MgCl₂, 10xPCR buffer, and 100U Platinum Taq polymerase (Invitrogen). The reaction conditions for the V-specific PCR amplification were denaturation (94°C, 1min) annealing (57°C 2min) for V γ and (55°C 2min) for V δ , and elongation (72°C, 3min), for 30 cycles; and a final extension at 72°C for 20min.

E) Cloning and sequencing

V γ I-, V δ 1-, V γ II(9)-, V δ 2-chain V-specific primers were each used to amplify the CDR3 region from the cDNA of each sample. The PCR product was gel purified, cloned into the TA vector and sequenced. Specifically, the PCR products of each transcript were separated in a 1% agarose gel in 1x TAE and purified using sodium iodide (GeneClean kit), following the supplier's instructions. This was followed by ligation into the pCR2.1 vector with overhanging single 3' deoxythymidine residues (Invitrogen). The ligation products were added to "one-shot" chemically-competent *E. coli* cells (Invitrogen) on ice followed by a 42°C heat shock treatment for 30 seconds before being placed back on ice. The cells were then incubated in 250ul SOC medium (GibcoBRL) in a horizontal shaker at 200rpm, 37°C for an hour and plated onto agar plates containing X-gal (100ug/ml) and ampicillin (100ug/ml) to facilitate blue-white screening. After 18 hours of incubation, white colonies were cultured in LB/Amp media and the plasmids were isolated, using the Perfect Prep plasmid purification mini kit

(Eppendorf) following supplier's instructions. The transcripts underwent the sequencing PCR amplification using the Big dye terminator sequencing kit v3.1 (Applied Biosystems) per manufacturer's instructions, and the PCR products were purified using Amersham Biosciences Auto SeqTM columns, denatured and analyzed using an automated 3130 DNA sequencer (Applied Biosystems). Computer analyses of the sequences and identification of the $\gamma\delta$ CDR3 region of the gene was carried out by alignment comparisons to those in the GenBank/EMBL databases using the BLAST sequence alignment program.

The maximum theoretical number of unique $\gamma\delta$ TCR+ T-cells in humans is approximately 10^{19} . Therefore, the probability of finding by chance, two identical copies of (γ I-, δ 1-, γ II- or δ 2-chain TCR+ T-cell) transcripts in a given independent sample of T-cells is negligible. In rare cases during the transformation and growth of the chemically competent *E. coli* cells, there is a possibility that the transformed cell can undergo a cell division cycle resulting in the presence of 2 identical clones after sequencing. This occurs because during the log phase growth of *E. coli* (ideal growth conditions), a cell undergoes a division in about 20-30 minutes, which could result in two doublings within 60 minutes (Hanahan et. al., 1983), the amount of time the transformed cells are grown in SOC media prior to plating. However, because of the heat shock, *E. coli* cells are found to have a longer lag phase than usual and therefore, decrease the chance of doubling within the hour incubation but in some rare cases, this may still occur. Therefore, a doublet after sequence analysis, i.e. two identical TCR+ T-cell transcripts might be due to a single transformed *E. coli*, which doubled during

transformation and growth as described above or might be evidence of a true clonal expansion.

F) Statistical analysis of sequencing data

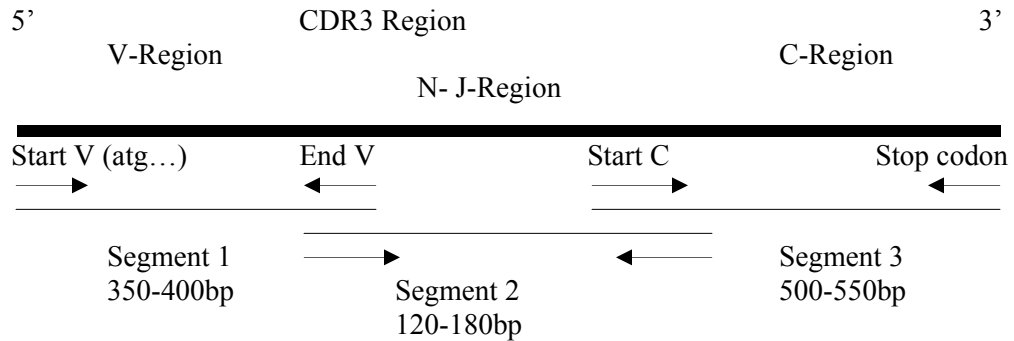
Binomial distribution was employed to calculate the probability, p , that the number of observed multiple identical transcripts were statistically significant as previously described (Lin et. al., 2005, Xu et. al., 2003, and Snedecor and Cochran, 1989). Briefly, the results obtained was tested against the probability of observing; one unique copy of each transcript analyzed, two identical copies and all other transcripts expressed once and three identical copies and all other transcripts expressed only once.

G) Full-length TCR transcript construction

$V\gamma I/V\delta 1$ -chain and $V\gamma II(9)/V\delta 2$ -chain TCR+ T-cell transcripts from patients with EOC were extracted and the full-length transcripts were constructed for each chain. The full-length constructs were designed using primers to amplify each third of the full-length transcript followed by a PCR-mediated ligation of the transcripts. Each segment was verified qualitatively by size on the gel and quantitatively by cloning and sequencing, as was the final full-length product. The first third i.e. segment 1, began from the initiating methionine (ATG) of the V-region to about 5 amino acids before the end of the V-region, to accommodate for junctional flexibility. The second third, (segment 2), extended from the last 10 amino acids of the V-region, included the N-(D in the case of δ -chain)-J region, to the first 6 amino acids of the C-(constant) region. The third segment, (segment 3) includes the beginning of the C-region to the stop codon (TAA). The three

segments of the full-length transcript overlapped sufficiently with each other to facilitate a subsequent PCR-mediated ligation. After obtaining each segment, the PCR product was verified by gel purification, cloning and sequencing. Then, the subsequent ligation PCR steps were performed to construct a final full-length transcript containing the most clonally expanded CDR3 region. The sketch of the $V\gamma I/V\gamma II(9)$ and $V\delta 1/V\delta 2$ full-length constructs is shown below in figure 2 and figure 3, respectively.

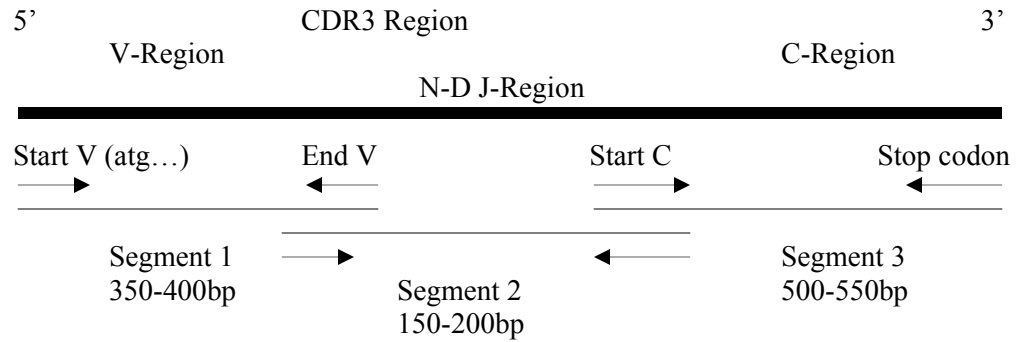
Figure 2 - V γ I/V γ II (9) Full length construction diagram



The primers for the construction V γ I were as follows; Segment 1 forward, 5'ATGCGGTGGGCCCTACTGG3', Segment 1 reverse, 5'CCGTGTCATTATCTGGGGTCTTAG3'. Segment 2 forward, 5'CTCGGAGGGTGTTCGGTGCATCAT3', Segment 2 reverse 5;ATAATGATCACGGTGGACC3', Segment 3 forward 5'GATAAACAACCTTGACGCAGATGTTTCCC3', Segment 3 reverse 5'TTATGATTTCTCTCCATTGCAGCAG3'.

The primers for the construction of V γ II(9) were as follows; Segment 1 forward, 5'ATGCTGTCACTGCTCCACACATC3', Segment 1 reverse, 5'CCGTGTCATCATCCATCGATACAG3', Segment 2 forward 5'TACTACTGTGCCTTGTGGGAGGTG3', Segment 2 reverse 5;ATAATGATCACGGTGGACC3', Segment 3 forward 5'GATAAACAACCTTGACGCAGATGTTTCCC3', Segment 3 reverse 5'TTATGATTTCTCTCCATTGCAGCAG3'.

Figure 3 - V δ 1/V δ 2-chain full-length construction diagram



The primers for the construction of V δ 1 were as follows; Segment 1 forward, 5' ATGCTGTTCTCCAG 3', Segment 1 reverse, 5'GTTTCATACAGGCAGTTCAGGGTGC 3' Segment 2 forward, 5'CCATGAAGGAGAAGCGATCGGTA ACTAC3' , Segment 2 reverse, 5'GGTATGAGGCTGACTTCTTGGTTCCAC3', Segment 3 forward, GTGGAACCAAGAAGTCAGCCTCAT, Segment 3 reverse, TTACAAGAAAATAACTTGGCAGTCAAGAG .

The primers for the construction of V δ 2 were as follows; Segment 1 forward, ATGCAGAGGATCTCCTCCCTCATC , Segment 1 reverse, GTAGTTACCGATCGCTTCTCCTTTCATGG , Segment 2 forward, GCACCCTGAACTGCCTGTATGAAAC , Segment 2 reverse, 5'GGTATGAGGCTGACTTCTTGGTTCCAC3', Segment 3 forward, GTGGAACCAAGAAGTCAGCCTCAT, Segment 3 reverse, TTACAAGAAAATAACTTGGCAGTCAAGAG .

Figure 4 – pMSCV expression vector (Clontech)

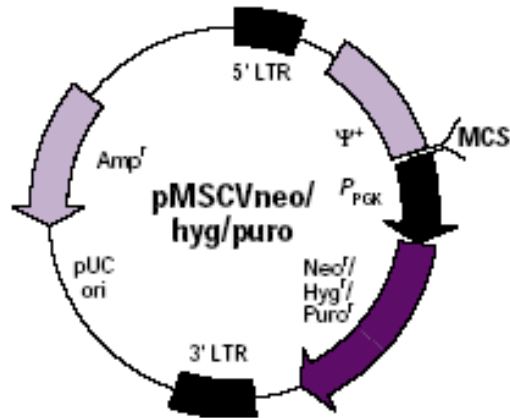
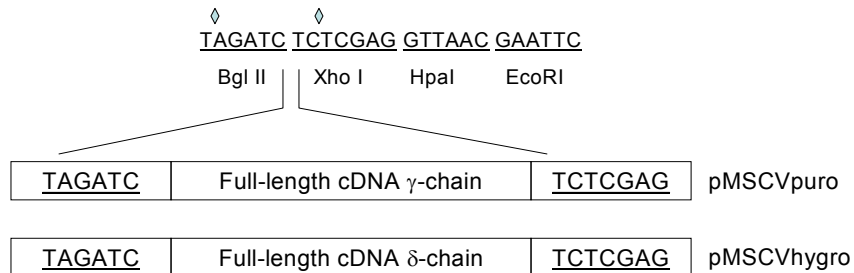


Figure 5 – γ -chain/ δ -chain TCR retroviral vector multiple cloning site (MCS)



H) MSCV expression

The retroviral vector backbone used in this study, pMSCV, is a murine stem cell virus vector, which uses an MSCV long terminal repeat (LTR) with

mutations allowing expression in a large host range (Figure 4). The NIH 3T3 fibroblast packaging cell line PT67 contains the gag-env-pol. Retroviral vectors with the packaging gene as well as containing specific γ - and δ -chain TCR transcripts were constructed (Figure 5). First, we obtained a pMSCV puromycin resistant plasmid (pMSCVpuro) and inserted our γ -chain cDNA of interest. We then constructed a similar neomycin resistant plasmid (pMSCVneo) containing our δ -chain cDNA of interest. Once our expression plasmid was constructed we transfected the NIH 3T3 fibroblast packaging cell line using the ProFection Mammalian Transfection System (Promega). Briefly, using a calcium-phosphate mediated co-precipitation with 10-20ug of plasmid vector, 2M CaCl₂, and water for a total volume of 500ul. This DNA solution was slowly added to 500ul of 2x HEPES solution until a opaque precipitate formed. Following a 30min incubation this precipitate was added to freshly cultured packaging cell line and placed in 37°C 5% CO₂ humidified incubator. Retroviral supernants were collected following 10-14 days of appropriated antibiotic selection according to the vector construct used. PT67 cells transfected with the pMSCVhygro were selected for 4-6 days with 300ug/ml of hygromycin, PT67 cells transfected with the pMSCVpuro were selected for 8-10days with 3ug/ml of puromycin and PT67 cells transfected with pMSCVneo were selected for 5-7days with 1mg/mL of G418. The retroviral supernants from each transfectant cell line was collected and used for subsequent fibronectin transduction experiments.

I) Transduction of Mutant Jurkat Cells

The JRT5.3 mutant jurkat cell line (ATCC) were washed in DPBS, then resuspended at a concentration of $1 \times 10^6/\text{ml}$ in RPMI supplemented with sodium bicarbonate, 2x HEPES, glucose, 10%FBS, L-glutamine, penicillin and streptomycin. These TCR- mutant jurkat T cells were plated at $1 \times 10^6/\text{ml}$ and cultured in vitro for 48-72 h before transduction. Next, the cells were transduced with retroviral vectors by transfer to culture dishes precoated with retroviral vectors. To coat culture plates with retroviral vectors the supernatant was added to non-tissue culture-treated, six-well fibronectin coated plates (RetroNectin; Takara) and allowed to incubate at 4°C overnight. To these plates was added cells at a concentration of one half confluency in culture medium (3-4ml). Cells were then incubated overnight at 37°C , and the procedure was repeated the following day, after which the cells were expanded at 37°C in a 5% CO_2 incubator and split as necessary to maintain cell density between 0.5 and 1×10^6 cells/ml. These single chain cells were selected in 6 well non-treated tissue culture plates. Those transduced with retroviral particles with the hygromycin resistant gene were selected for 6 days in media containing 500ug/ml hygromycin, puromycin resistant gene were selected for 6 days in media containing .25ug to .35ug/ml of puromycin and neomycin resistant gene were selected for 8-10 days in media containing 3mg-5mg/ml G418.

J) FACS analysis

Cell surface expression of $\gamma\delta$ TCR and CD3 molecules on TCR+ transduced T cells was measured by immunofluorescence using PE and FITC

conjugated Abs, as directed by the supplier of anti-TCR $\gamma\delta$ (Gibco). For analysis, the relative log fluorescence of live cells was measured using a FACScan flow cytometer (The Wistar Institute).

K) Cytotoxicity Assay

The in vitro cytotoxic activity of the expanded $\gamma\delta$ TCR+ transduced T cell lines were evaluated by the Cytotox 96® a non-radioactive cytotoxicity assay. The target cells used were tumor cell lines SKOV3, CAOV3 and OV2774. The effector cells used were the, $\gamma\delta$ TCR+ transduced T cell lines. Using round bottom 96-well non-treated tissue culture plates, the following experiments were set up: constant number of target cells (1×10^4) and varying number of effector cells (100:1, 50:1, 40:1, 10:1, and 1:1) to experimental wells, effector cells at each effector cell concentration for effector cell spontaneous LDH release control, target cells with no effector cells for target cell spontaneous LDH release control, target cells and lysis solution (10x) for target maximum release control, culture medium and lysis solution for volume correction control, and culture medium for culture medium background control. The cells were then centrifuged at $250 \times g$ for 4 minutes followed by incubation at 37°C for 4.5 hours. Forty-five minutes prior to supernatant harvest, lysis solution (10x) was added to target cell maximum and culture medium volume correction control wells. 50ul of the supernatant from each well of the assay plate was harvest and added to a flat-bottom enzymatic assay plate. 50ul of the substrate/buffer mix was added to each well of the plate containing supernatant. The plate was covered and incubated at room temperature and protected from light for 30 minutes. 50ul of

stop solution was added to the plate and sample absorbance was recorded (490nm). And the percentage of cytotoxicity was calculated as:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental-Effector Spontaneous-Target Spontaneous}}{\text{Target Maximum-Target Spontaneous}} \times 100$$

L) Measurement of Caspase Activity

The Caspase-Glo® Assay (Promega) luciferase based assay was used to observe caspase 3/7, 8, and 9 activity in SKOV3, CAOv and OV2774 following co-incubation of these targets with $\gamma\delta$ TCR+ transduced T cell lines. Briefly, following caspase cleavage, the substrate luciferase (amino-luciferin) is released, resulting in the luciferase reaction and production of light measured by luminometer. Using round bottom 96-well non-treated tissue culture plates target cells were seeded at 1×10^4 cells/well and incubated for 4 hours prior to addition of the effector cells. The effector cells were added to these wells at a 10:1 and 1:1 effector to target ratio, effector cells were added to wells containing no target cells and target cells were added to wells containing no effector cells to account for background caspase production. For caspase 9 and 8 the samples (effector plus target) were added to an equal volume of reagent following a 45min co-incubation period. However, to measure caspase 3 activity the samples (effector plus target) were added to an equal volume of reagent following 5 hours of incubation.

CHAPTER 4 RESULTS

A) Sequencing results of the V γ I-chain TCR Transcripts

Previous studies done in our laboratory indicate the following results: (a) The presence of $\alpha\beta$ TCR + T-cell infiltrating solid tumor of patients with EOC (data not shown) by immunohistochemical staining using specific monoclonal antibodies to CD3+, CD4+, and CD8+ molecules, (b) The presence of statistically significant oligoclonal β -chain TCR transcripts in the solid tumor of patients with EOC (Pappas et. al., 2005) by the amplification, cloning and sequencing, (c) The presence of $\gamma\delta$ TCR+ and CD3+ T-cells infiltrating the solid tumor of patients with EOC.

To establish whether fresh (not expanded in culture) mononuclear cells infiltrating the solid tumor of patients with EOC contain oligoclonal T-cell populations, γ I-chain TCR transcripts from seven patients were amplified, cloned and sequenced. Of the results obtained, less than 2.4% of the deduced V γ I-N-J γ transcripts were nonproductively rearranged and these were not included in the analysis. The productively rearranged transcripts were analyzed using the GENBANK database. Results revealed the presence of statistically significant substantial proportions of identical V γ I-chain TCR+ T-cell transcripts in five out of seven solid tumor specimens obtained from patients with EOC, suggesting the presence of oligoclonal populations of T-cells infiltrating the solid tumor from patients with EOC.

Table 1. V γ -chain TCR+ T-cell Transcripts (CDR3 Region) Expressed in the Solid Tumor from patient OV38T

Transcript No.	V γ	N	J γ	Transcript Frequency	p-value		
					1/39	2/39	3/39
1	Y Y C A T W D G P TATTACTGTGCCACCTGGGATGGGCCA		Y Y K K TATTATAAGAAA	V γ 2J γ 1.3/2.3 15/39 38%	<0.0001	<0.0001	<0.0001
11	Y Y C A T W D TATTACTGTGCCACCTGGGAC		Y Y K K TATTATAAGAAA	V γ 2J γ 1.3/2.3 11/39 33%	<0.0001	<0.0001	<0.0001
22	Y Y C A T W G G I R N S L A V E Y Y K K TATTACTGTGCCACCTGGGGCGGTATAAGAAACTCTTTGGCAGTGGAAATATTATAAGAAA		Y Y K K TATTATAAGAAA	V γ 2J γ 1.3/2.3 8/39 20%	NS	NS	NS
15	Y Y C A T W D G Y E S D TATTACTGTGCCACCTGGGACGGCTACGAGTCGGAT		Y Y K K TATTATAAGAAA	V γ 2J γ 1.3/2.3 1/39 3%	NS	NS	NS
4	Y Y C A T W D G P R E L G TATTACTGTGCCACCTGGGACGGGCCCGTGAGTTGGGCAAAAAA		Y Y K K TATTATAAGAAA	V γ 2J γ 1.3/2.3 1/39 3%	NS	NS	NS
9	Y Y C A T W D E D TATTACTGTGCCACCTGGGAC GAAGAT		Y Y K K TATTATAAGAAA	V γ 2J γ 1.3/2.3 1/39 3%	NS	NS	NS
7	Y Y C A T W D TATTACTGTGCCACCTGGGAC		Y Y K K TATTATAAGAAA	V γ 2J γ 1.3/2.3 1/39 3%	NS	NS	NS
2	Y Y C A T W D G W G TATTACTGTGCCACCTGGGACGGGCTGGGG		Y Y K K TATTATAAGAAA	V γ 2J γ 1.3/2.3 1/39 3%	NS	NS	NS

Table 2. V γ -chain TCR+ T-cell Transcripts (CDR3 Region) Expressed in the Solid Tumor from patient OV26T

Transcript No.	V γ	N	J γ	Transcript Frequency	p-value		
					1/35	2/35	3/35
1	Y Y C A T W D TATTACTGTGCCACCTGGGAC	R L P R P AGGCTGCCGCGGCCT	Y Y K K L TATTATAAGAAACTC	V γ 2J γ 1.3/2.3 35/35 100%	<0.0001	<0.0001	<0.0001

Table 3. V γ 1-chain TCR+ T-cell Transcripts (CDR3 Region) Expressed in the Solid Tumor from patient OV42T

Transcript No.	V γ	N	J γ	Transcript Frequency	p-value	
					1/17	2/17
22	Y Y C A T W D G TATTACTGTGCCACCTGGGACGGG	P L F CCCTTGTTT	K K L F G S AAGAAACTCTTTGGCAGT	V γ 2J γ 1.3/2.3 10/17 59%	<0.0001	<0.0001
3	Y Y C A T W D G TATTACTGTGCCACCTGGGACGGG	P E K P CCGGAGAAACCC	F G S G T T TTTGGCAGTGGAACAACA	V γ 2J γ 1.3/2.3 2/17 12%		
21	Y Y C A T W D G TATTACTGTGCCACCTGGGACGGT	P G CCAGGG	N Y K K L F AATTATAAGAAACTCTTT	V γ 2J γ 1.3/2.3 2/17 12%	NS	NS
14	Y Y C A T W D G TATTACTGTGCCACCTGGGATGGG	H CAC	Y K K L F G S TATAAGAAACTCTTTGGCAGT	V γ 2J γ 1.3/2.3 2/17 12%	NS	NS
18	Y Y C A T W D G TATTACTGTGCCACCTGGGACGGG	P E CCGGAG	K L F G S AAACTCTTTGGCAGT	V γ 2J γ 1.3/2.3 1/17 6%	NS	NS

Table 4. V γ 1-chain TCR+ T-cell Transcripts (CDR3 Region) Expressed in the Solid Tumor from patient OV31T

Transcript No.	V γ	N	J γ	Transcript Frequency	p-value	
					1/44	2/44
3	Y Y C A T W D G TATTACTGTGCCACCTGGGACGGG	H F CATTTT	Y K K L F G TATAAGAAACTCTTTGGC	V γ 2J γ 1.3/2.3 44/44 100%	<0.0001	<0.0001

Table 5. V γ -chain TCR+ T-cell Transcripts (CDR3 Region) Expressed in the Solid Tumor from patient OV805T

Transcript No.	V γ	N	J γ	Transcript Frequency	p-value	
					1/19	2/19
9	Y Y C A T W D G TATTACTGTGCCACCTGGGACGGG	R E CGAGAG	D W I K T F A GATTGGATCAAGACGTTTGCA	V γ 3J γ 1.3/2.3 3/19 16%	NS	NS
23	Y Y C A T W D G TATTACTGTGCCACCTGGGACGGG	P CCC	K K L F G AAGAAACTCTTTGGC	V γ 3J γ 1.3/2.3 3/19 16%	NS	NS
22	Y Y C A T W D G TATTACTGTGCCACCTGGGACGGC	H R P CATCGACCC	K K L F G S AAGAAACTCTTTGGCAGT	V γ 2J γ 1.3/2.3 2/19 11%	NS	NS
20	Y Y C A T W D G TATTACTGTGCCACCTGGGACGGG	L L N CTCCTTAAT	K K L F G S AAGAAACTCTTTGGCAGT	V γ 5J γ 1.3/2.3 2/19 11%	NS	NS
14	Y Y C A T W D TATTACTGTGCCACCTGGGAC	A S N GCTTCCAAT	Y K K L F G S TATAAGAAACTCTTTGGCAGT	V γ 5J γ 1.3/2.3 2/19 11%	NS	NS
17	Y Y C A T W D G TATTACTGTGCCACCTGGGACGGG	P L F CCCTTGTTT	K K L F G S AAGAAACTCTTTGGCAGT	V γ 4J γ 1.3/2.3 1/19 5%	NS	NS
18	Y Y C A T W D G G TATTACTGTGCCACCTGGGACGGGGGGAAGCAAATT	G K Q N TATTATAAGAAACTCTTTGGCAGT	Y Y K K L F G S	V γ 3J γ 1.3/2.3 1/19 5%	NS	NS
11	Y Y C A T W D G TATTACTGTGCCACCTGGGACGGG	P R CCTCGT	Y Y K K L F G S TATTATAAGAAACTCTTTGGCAGT	V γ 2J γ 1.3/2.3 1/19 5%	NS	NS
4	Y Y C A T W D G TATTACTGTGCCACCTGGGACGGG	P N CCAAAT	Y Y K K L F G S TATTATAAGAAACTCTTTGGCAGT	V γ 2J γ 1.3/2.3 1/19 5%	NS	NS
5	Y Y C A T W D G TATTACTGTGCCACCTGGGACGGG	P G CCGGGG	K L F G S G AAACTCTTTGGCAGTGGA	V γ 2J γ 1.3/2.3 1/19 5%	NS	NS
21	Y Y C A T W M TATTACTGTGCCACCTGG	S V I D ATGTCGGTTATTGAT	Y K K L F G S TATAAGAAACTCTTTGGCAGT	V γ 2J γ 1.3/2.3 1/19 5%	NS	NS
7	Y Y C A T W D G TATTACTGTGCCACCTGGGACGGG		Y Y K K L F G TATTATAAGAAACTCTTTGGC	V γ 2J γ 1.3/2.3 1/19 5%	NS	NS

Table 6. V γ -chain TCR+ T-cell Transcripts (CDR3 Region) Expressed in the Solid Tumor from patient OV1T

Transcript No.	V γ	N	J γ	Transcript Frequency	p-value 1/17 2/17
11	Y Y C A T W D G TATTACTGTGCCACCTGGGATGGG		Y Y K K L F TATTATAAGAAACTCTTT	V γ 2J γ 1.3/2.3 3/17 18%	NS NS
13	Y Y C A T W D G TATTACTGTGCCACCTGGGACGGG	F TTT	Y Y K K L F TATTATAAGAAACTCTTT	V γ 2J γ 1.3/2.3 2/17 12%	NS NS
19	Y Y C A T W TATTACTGTGCCACCTGG	V N GTGAAT	Y Y K K L F TATTATAAGAAACTCTTT	V γ 2J γ 1.3/2.3 1/17 6%	NS NS
20	Y Y C A T W D TATTACTGTGCCACCTGGGAT	L T CTCACT	G W F K I F GGTTGGTTCAAGATATTT	V γ 2J γ 1.1 1/17 6%	NS NS
17	Y Y C A T W TATTACTGTGCCACCTGG	V R L G S GTCCGGCTGGGGTCTT	Y Y K K L F TATTATAAGAAACTCTTT	V γ 2J γ 1.3/2.3 1/17 6%	NS NS
22	Y Y C A T W D TATTACTGTGCCACCTGGGAT	V GTA	G W F K I F GGTTGGTTCAAGATATTT	V γ 2J γ 1.1 1/17 6%	NS NS
14	Y Y C A T W D TATTACTGTGCCACCTGGGAT	P K CCCAAG	N Y Y K K L F AATTATTATAAGAAACTCTTT	V γ 2J γ 1.3/2.3 1/17 6%	NS NS
12	Y Y C A T W D G TATTACTGTGCCACCTGGGATGGG	T TAC	N Y Y K K L F AATTATTATAAGAAACTCTTT	V γ 2J γ 1.3/2.3 1/17 6%	NS NS
10	Y Y C A T W TATTACTGTGCCACCTGG	P L V T CCCCTGGTTACG	N Y Y K K L F AATTATTATAAGAAACTCTTT	V γ 2J γ 1.3/2.3 1/17 6%	NS NS
5	Y Y C A T W D TATTACTGTGCCACCTGGGAC	K P G K AAGCCTGGGAAG	Y Y K K L F TATTATAAGAAACTCTTT	V γ 2J γ 1.3/2.3 1/17 6%	NS NS
8	Y Y C A T W D TATTACTGTGCCACCTGGGAT	N P S AATCCGTCT	Y Y K K L F TATTATAAGAAACTCTTT	V γ 2J γ 1.3/2.3 1/17 6%	NS NS
11	Y Y C A T W D TATTACTGTGCCACCTGGGAT	A R D GCCAGGGAT	K K L F AAGAAACTCTTT	V γ 2J γ 1.3/2.3 1/17 6%	NS NS
6	Y Y C A T W D TATTACTGTGCCACCTGGGAT	P S Q G CCTTCCCAGGGG	Y K K L F TATAAGAAACTCTTT	V γ 2J γ 1.3/2.3 1/17 6%	NS NS
1	Y Y C A T W D G TATTACTGTGCCACCTGGGATGGG	R AGG	Y Y K K L F TATTATAAGAAACTCTTT	V γ 2J γ 1.3/2.3 1/17 6%	NS NS

Table 7. V γ -chain TCR+ T-cell Transcripts (CDR3 Region) Expressed in the Solid Tumor from patient OV22T

Transcript No.	V γ	N	J γ	Transcript Frequency	p-value	
					1/26	2/26
26	Y Y C A T W D P V TATTACTGTGCCACCTGGGAC CCGGTG		Y Y K K TATTATAAGAAA	V γ 2J γ 1.3/2.3 11/26 42%	<0.0001	<0.00001
2	Y Y C A T W D V TATTACTGTGCCACCTGGGAC GTT		Y Y K K TATTATAAGAAA	V γ 2J γ 1.3/2.3 8/26 31%	<0.018	<0.028
24	Y Y C A T W D L P TATTACTGTGCCACCTGGGAC ACCCCG		Y Y K K TATTATAAGAAA	V γ 2J γ 1.3/2.3 3/26 8%	NS	NS
25	Y Y C A T W D P R TATTACTGTGCCACCTGGGAC CCGAGA		Y Y K K TATTATAAGAAA	V γ 2J γ 1.3/2.3 2/26 8%	NS	NS
22	Y Y C A T W D P TATTACTGTGCCACCTGGGAC CCG		Y Y K K TATTATAAGAAA	V γ 2J γ 1.3/2.3 2/26 8%	NS	NS

Table 8. Summary of clonal expansion of V γ l-chain TCR+ T -cell transcripts in the solid tumor of patients with EOC

OV38T: 15/39:#1: 38%:CDR3:YYCATWDGPYYKK
 OV38T: 11/39:#11:33%:CDR3:YYCATDYKK
 OV26T: 35/35:#1:100%:CDR3:YYCATWDRLPRPYYKK
 OV42T: 10/17:#22:59%:CDR3:YYCATWDGPLFKKLFGS
 OV31T: 44/44:#3:100%:CDR3:YYCATWDGHFYKKLFGS
 OV22T: 11/26:#26:42%:CDR3:YYCATWDPVYYKK

Table 9. Percent clonal expansion of V γ l-chain TCR+ T-cell transcripts in the solid tumor of patients with EOC

EOC Patient Sample	% Clonal Expansion
OV38T #1	38%
#11	33%
OV26T	100%
OV42T #22	59%
OV31T	100%
OV22T #26	42%

In patient OV38T, analysis of the V γ l-chain TCR+ T-cell transcripts revealed that 15 out of 39 (38.4%) transcripts sequenced each had a CDR3 region identical to that of OV38T transcript number 1 with its CDR3 region as: V γ 2J γ 1.3/2.3: YYCATWDGPYYKKLFGSGTTLVVT: p<0.05 (a statistically significant oligoclonal expansion) (see table 1). 12 out of 39 (30.7%) transcripts each had a CDR3 region identical to that of OV38T transcript number 11 with its CDR3 region as: V γ 2J γ 1.3/2.3 YYCATWDYYKKLFGSGTTLVVT: p<0.001 (a statistically significant oligoclonal expansion). 8 out of 39 (20.5%) transcripts each had a CDR3 region identical to that of OV38T transcript number 22 with its CDR3 region as: V γ 2J γ 1.3/2.3 YYCATWGYYKKLFGSGTTLVVT: p>0.05 (a statistically insignificant oligoclonal expansion). There were four unique non-

clonally expanded TCR+ T-cell transcripts, OV38T numbers 15, 4, 9, and 2, each with a unique CDR3 region as (see Table 1): V γ 2J γ 1.3/2.3

YYCATWDGYESDYYKKLFSGTLVVT: 2.6% p>0.05, V γ 2J γ 1.3/2.3

YYCATWDGPRELGYYKKLFSGTLVVT: 2.6% p>0.05, V γ 2J γ 1.3/2.3

YYCATWDGGEDYYKKLFSGTLVVT: 2.6% p>0.05, V γ 2J γ 1.3/2.3

YYCATWDGWGYYKKLFSGTLVVT: 2.6% p>0.05, respectively.

In patient OV26T, sequence analysis of V γ 1-chain TCR+ T-cell transcripts revealed that 35 out of 35 (100%) TCR+ transcripts sequenced each had a CDR3 region identical to that of OV26T number 1, each with a CDR3 region (see Table 2) V γ 2J γ 1.3/2.3 YYCATWDRLPRPYYKKLFSGTLVVT: p<0.05.

In patient OV42T, sequence analysis of V γ 1-chain TCR+ T-cell transcripts revealed that 10 out of 17 (58.8%) TCR+ transcripts sequenced each had a CDR3 region identical to that of OV42T number 22 with its CDR3 region as: V γ 2J γ 1.3/2.3 YYCATWDGPLFKLFSGTLVVT: p<0.05 (a statistically significant oligoclonal expansion) (see Table 3). 2 out of 17 (11.7%) unique transcripts with CDR3 region identical to that of OV42T number 3 with its CDR3 region as: V γ 2J γ 1.3/2.3 YYCATWDPEKPFSGTLVVT: p>0.05 (a statistically insignificant oligoclonal expansion). 2 out of 17 (11.7%) unique transcripts with CDR3 region identical to that of OV42T number 21 with its CDR3 region as: V γ 2J γ 1.3/2.3 YYCATWDPGNYKKLFSGTLVVT: p>0.05 (a statistically insignificant oligoclonal expansion). 2 out of 17 (11.7%) unique transcripts with CDR3 region identical to that of OV42T number 14 with its CDR3 region as: V γ 2J γ 1.3/2.3 YYCATWDHYKKLFSGTLVVT: p>0.05 (a statistically insignificant oligoclonal

expansion). 2 out of 17 (11.7%) unique transcripts with CDR3 region identical to that of OV42T number 18 with its CDR3 region as: V γ 2J γ 1.3/2.3

YYCATWDPEKLFSGTLVVT: $p > 0.05$ (a statistically insignificant oligoclonal expansion).

In patient OV31T, sequence analysis of V γ l-chain TCR+ T-cell transcripts revealed that 44 out of 44 (100%) TCR+ T-cell transcripts sequenced each had a CDR3 region identical to that of OV31T number 3 with its CDR3 region as:

V γ 2J γ 1.3/2.3 YYCATWDGHFYKKLFSGTLVVT: $p < 0.05$ (see Table 4).

In patient OV805T, sequence analysis of V γ l-chain TCR+ T-cell transcripts revealed that 3 out of 19 (15.9%) TCR+ T-cell transcripts sequenced each had a CDR3 region identical to that of OV805T number 9 with its CDR3 region as:

V γ 3J γ 2.1 YYCATWDREDWIKTFA: $p > 0.05$ (see Table 5). 3 out of 19 (15.9%)

TCR+ T-cell transcripts each had a CDR3 region identical to that of OV805T number 23 with its CDR3 region as: V γ 2J γ 1.3/2.3 YYCATWDPKKLFSGTLVVT: $p > 0.05$. 2 out of 19 (10.5%) TCR+ T-cell transcripts sequenced each had a

CDR3 region identical to that of OV805T number 22 with its CDR3 region as:

V γ 2J γ 1.3/2.3 YYCATWDHRPKKLFSGTLVVT: $p > 0.05$. 2 out of 19 (10.5%)

TCR+ T-cell transcripts sequenced each had a CDR3 region identical to that of OV805T number 20 with its CDR3 region as: V γ 5J γ 1.3/2.3

YYCATWGLLNKKLFSGTLVVT: $p > 0.05$. 2 out of 19 (10.5%) TCR+ T-cell transcripts sequenced each had a CDR3 region identical to that of OV805T

number 14 with its CDR3 region as: V γ 5J γ 1.3/2.3

YYCATWASNYYKKLFSGTLVVT: (10.5%) $p > 0.05$. There were seven

transcripts, each with a unique CDR3 region: OV805T number 17 V γ 4J γ 1.3/2.3
YYCATWGPLFKKLFSGTLVVT: (5.3%) p>0.05, OV805T number 18
V γ 3J γ 1.3/2.3 YYCATWGGKQNYYKKLFSGTLVVT: (5.3%) p>0.05, OV805T
number 11 V γ 2J γ 1.3/2.3 YYCATWGPRYYKKLFSGTLVVT: (5.3%) p>0.05,
OV805T number 4 V γ 2J γ 1.3/2.3 YYCATWGPNYYKKLFSGTLVVT: (5.3%)
p>0.05, OV805T number 5 V γ 2J γ 1.3/2.3 YYCATWGPGKLFSGTLVVT: (5.3%)
p>0.05, OV805T number 21 V γ 2J γ 1.3/2.3 YYCATWGMSVIDKKLFSGTLVVT:
(5.3%) p>0.05, OV805T number 7 V γ 2J γ 1.3/2.3 YYCATWG(no N
region)KKLFSGTLVVT: (5.3%) p>0.05.

In patient OV22T, sequence analysis of V γ I-chain TCR+ T-cell transcripts revealed that 11 out of 26 (42.3%) TCR+ T-cell transcripts sequenced each had a CDR3 region identical to that of OV22T number 26 with its CDR3 region as: V γ 2J γ 1.3/2.3 YYCATWDPVYYKKLFSGTLVVT: p<0.05. 8 out of 26 (30.8%) TCR+ T-cell transcripts sequenced each had a CDR3 region identical to that of OV22T number 2 with its CDR3 region as: V γ 2J γ 1.3/2.3 YYCATWDVYYKKLFSGTLVVT: p<0.05. 3 out of 26 TCR+ T-cell transcripts sequenced each had a CDR3 region identical to that of OV22T number 24 with its CDR3 region as: V γ 2J γ 1.3/2.3 YYCATWDLPYYKKLFSGTLVVT: (11.5%) p>0.05. 2 out of 26 (7.8%) non-clonally expanded TCR+ T-cell transcripts sequenced had a CDR3 region as: V γ 2J γ 1.3/2.3 YYCATWDPRYYKKLFSGTLVVT: p>0.05. 2 out of 26 (7.8%) non-clonally expanded TCR+ T-cell transcripts sequenced had a CDR3 region as: V γ 2J γ 1.3/2.3 YYCATWDPYYKKLFSGTLVVT: p>0.05.

In patient OV1T, sequence analysis of V γ 1-chain TCR+ T-cell transcripts revealed that 3 out of 17 (17.6%) transcripts sequenced each had a CDR3 region identical to that of OV1T number 11 with its CDR3 region as: V γ 2J γ 1.3/2.3
 YYCATWDG(no N region)YYKKLFSGTLVVT: p>0.05 (an statistically insignificant clonal expansion). 2 out of 17 (11.8%) transcripts sequenced each had a identical CDR3 regions as: V γ 2J γ 1.3/2.3
 YYCATWDGEYYKKLFSGTLVVT: p>0.05. There were twelve unique non-clonally expanded TCR+ T-cell transcripts with CDR3 regions as: V γ 2J γ 1.3/2.3
 YYCATWVNYYKKLFSGTLVVT: (5.9%) p>0.05, V γ 2J γ 1.1
 YYCATWDLTGWFKIF: (5.9%) p>0.05, V γ 2J γ 1.3/2.3
 YYCATWVRLGSYYKKLFSGTLVVT: (5.9%) p>0.05, V γ 2J γ 1.1
 YYCATWVGWFKIF: (5.9%) p>0.05, V γ 4J γ 1.3/2.3
 YYCATWDPKNYYKKLFSGTLVVT: (5.9%) p>0.05, V γ 2J γ 1.3/2.3
 YYCATWDGINYYKKLFSGTLVVT: (5.9%) p>0.05, V γ 2J γ 1.3/2.3
 YYCATWPLVTNYYKKLFSGTLVVT: (5.9%) p>0.05, V γ 2J γ 1.3/2.3
 YYCATWDKPGKYYKKLFSGTLVVT: (5.9%) p>0.05, V γ 2J γ 1.3/2.3
 YYCATWDNPSYYKKLFSGTLVVT: (5.9%) p>0.05, V γ 2J γ 1.3/2.3
 YYCATWDARDKLFSGTLVVT: (5.9%) p>0.05, V γ 2J γ 1.3/2.3
 YYCATWDPSQGYKKLFSGTLVVT: (5.9%) p>0.05, V γ 2J γ 1.3/2.3
 YYCATWDGRYYKKLFSGTLVVT: (5.9%) p>0.05, which were the CDR3 regions of TCR+ T-cell transcripts OV1T number 19, 20, 17, 22, 14, 12, 10, 5, 8, 11, 6 and 1, respectively (Table 6).

B) Sequencing results of the V γ II (9)-chain TCR Transcripts

Similar procedures to the V γ I sequence analysis described above were employed for the V γ II, also known as V γ 9, sequence analysis. The productively rearranged transcripts were analyzed using the GENBANK database. Results revealed the presence of statistically significant substantial proportions of identical V γ II-chain TCR+ T-cell transcripts in five out of five solid tumor specimens obtained from patients with EOC, suggesting the presence of oligoclonal populations of T-cells infiltrating the solid tumor from patients with EOC.

Table 10. V γ II (9)-chain TCR+ T-cell Transcripts (CDR3 Region) Expressed in the Solid Tumor from patient OV27T

Transcript No.	Transcript Frequency	p-value V γ			N	J γ
		1/18	2/18	3/18		
13	Y Y C A L W E V G E V γ 9J γ 1.2 TACTACTGTGCCTTGTGGGAGGTGGGGGAG 15/18 84%	<0.0001	<0.0001	<0.0001		L G K K I TTGGGCAAAAAAATC
3	Y Y C A L W E V G V γ 9J γ 1.2 TACTACTGTGCCTTGTGGGAGGTGGGG 3/18 26%	NS	NS	NS		L G K K I TTGGGCAAAAAAATC

Table 11. V γ II (9)-chain TCR+ T-cell Transcripts (CDR3 Region) Expressed in the Solid Tumor from patient OV42T

Transcript No.	Transcript Frequency	p-value V γ			N	J γ
		1/16	2/16	3/16		
7	Y Y C A L W E V P C V γ 9J γ 1.3/2.3 TACTACTGTGCCTTGTGGGAGGTGCCGTGT 7/16 44% <0.0001<0.0001<0.0001					Y Y K K L F G TATTATAAGAAACTCTTTGGC
15	Y Y C A L W E V H V γ 9J γ 1.3/2.3 TACTACTGTGCCTTGTGGGAGGTG 6/16 38% <0.0001<0.0001<0.0001				CAT	Y Y K K L F G TATTATAAGAAACTCTTTGGC
5	Y Y C A L W E V V γ 9J γ 1.3/2.3 TACTACTGTGCCTTGTGGGAGGTG 1/16 6% NS NS NS					Y Y K K L F G TATTATAAGAAACTCTTTGGC
10	Y Y C A L W E V V γ 9J γ 1.3/2.3 TACTACTGTGCCTTGTGGGAGGTG 1/16 6% NS NS NS				S E AGTGAG	Y Y K K L F G TATTATAAGAAACTCTTTGGC
2	Y Y C A L W E V V γ 9J γ 1.3/2.3 TACTACTGTGCCTTGTGGGAGGTG 1/16 6% NS NS NS				R AGG	Y Y K K L F G TATTATAAGAAACTCTTTGGC

Table 12. V γ 11 (9)-chain TCR+ T-cell Transcripts (CDR3 Region) Expressed in the Solid Tumor from patient OV45T

Transcript No.	Transcript Frequency	p-value V γ			N	J γ
		1/18	2/18	3/18		
9	Y Y C A L W E V I V γ 9J γ 1.3/2.3 TACTACTGTGCCTTGTGGGAGGTGATT 8/18 44% <0.0001<0.0001<0.0001					Y Y K K L F G TATTATAAGAAACTCTTTGGC
3	Y Y C A L W Y P R V γ 9J γ 1.3/2.3 TACTACTGTGCCTTGTGG TACCCTAGG 7/18 39% <0.0001<0.0001<0.0001					K L F G AAACTCTTTGGC
11	Y Y C A L W P R V γ 9J γ 1.3/2.3 TACTACTGTGCCTTGTGG CCTAGG 2/18 11% NS NS NS					K L F G AAACTCTTTGGC
17	Y Y C A L W E V V γ 9J γ 1.3/2.3 TACTACTGTGCCTTGTGGGAGGTG 1/18 6% NS NS NS					Y Y K K L F G TATTATAAGAAACTCTTTGGC

Table 13. V γ II (9)-chain TCR+ T-cell Transcripts (CDR3 Region) Expressed in the Solid Tumor from patient OV38T

Transcript No.	Transcript Frequency	p-value V γ			N	J γ
		1/18	2/18	3/18		
12	Y Y C A L W E V R V γ 9J γ 1.3/2.3 TACTACTGTGCCTTGTGGGAGGTG 11/20 55% <0.0001<0.0001<0.0001				CGA	Y Y K K L F G TATTATAAGAAACTCTTTGGC
17	Y Y C A L W E V R V γ 9J γ 1.3/2.3 TACTACTGTGCCTTGTGGGAGGTG 3/20 15% NS NS NS				R F V AGGTTTGTG	Y Y K K L F G TATTATAAGAAACTCTTTGGC
2	Y Y C A L W E V V γ 9J γ 1.3/2.3 TACTACTGTGCCTTGTGGGAGGTG 2/20 10% NS NS NS				L CTG	Y Y K K L F G TATTATAAGAAACTCTTTGGC
16	Y Y C A L W E V V γ 9J γ 1.3/2.3 TACTACTGTGCCTTGTGGGAGGTG 1/20 6% NS NS				E W E GAGTGGGAG NS	Y Y K K L F G TATTATAAGAAACTCTTTGGC
19	Y Y C A L W E V V γ 9J γ 1.3/2.3 TACTACTGTGCCTTGTGGGAGGTG 1/20 6% NS NS NS				R F AGGTTT	Y Y K K L F G TATTATAAGAAACTCTTTGGC
14	Y Y C A L W E V V γ 9J γ 1.3/2.3 TACTACTGTGCCTTGTGGGAGGTG 1/20 6% NS NS NS					Y Y K K L F G TATTATAAGAAACTCTTTGGC
1	Y Y C A L W V γ 9J γ 1.3/2.3 TACTACTGTGCCTTGTGG 1/20 6% NS NS NS					K K L F G AAGAAACTCTTTGGC

Table 14. V γ II (9)-chain TCR+ T-cell Transcripts (CDR3 Region) Expressed in the Solid Tumor from patient OV26T

Transcript No.	Transcript Frequency	p-value V γ			N	J γ
		1/27	2/27	3/27		
2	Y Y C A L W E V L P V γ 9J γ 1.3/2.3 TACTACTGTGCCTTGTGGGAGGTGCTTCCT 24/27 89% <0.0001<0.0001<0.0001				Y Y K K L	TATTATAAGAAACTC
1	Y Y C A L W E V G E L G K K V γ 9J γ 1.3/2.3 TACTACTGTGCCTTGTGGGAGGTGGGTGAGTTGGGCAAAAAA 1/27 4% NS NS NS					
6	Y Y C A L W E V C V γ 9J γ 1.3/2.3 TACTACTGTGCCTTGTGGGAGGTGTGT 1/27 4% NS NS NS				Y Y K K L F G	TATTATAAGAAACTCTTTGGC
9	Y Y C A L W E V V γ 9J γ 1.3/2.3 TACTACTGTGCCTTGTGGGAGGTG 1/27 4% NS NS NS				K K L F G	AAGAAACTCTTTGGC

Table 15. Summary of clonal expansion of V γ II (9)-chain TCR+ T -cell transcripts in the solid tumor of patients with EOC

OV27T:15/18:#13:84%:CDR3:YYCALWEV GELGKKI
 OV42T:7/16:#7:44%:CDR3:YYCALWEVHYYKKLFG
 OV42T:6/16:#15:38%:CDR3:YYCALWEVYYKKLFG
 OV45T:8/18:#9:44%:CDR3:YYCALWEVIYYKKLFG
 OV45T:7/18:#3:39%:CDR3:YYCALWYPRKLFG
 OV38T:11/20:#12:55%:CDR3:YYCALWEVRYYKKLFG
 OV26T:24/27:#2:89%:CDR3:YYCALWEVLPYYKKL

Table 16. Percent clonal expansion of V γ II-chain TCR+ T-cell transcripts in the solid tumor of patients with EOC

EOC Patient Sample	% Clonal Expansion
OV27T #13	84%
OV42T #7	44%
OV42T #15	38%
OV45T #9	44%
OV45T #3	39%
OV38T #12	55%
OV26T #2	89%

In patient OV27T sequence analysis of V γ II(9)-chain TCR+ T-cell transcripts revealed that 15 out of 18 (83.3%) TCR+ T-cell transcripts each had a CDR3 region identical to that of OV27T number 13 with its CDR3 region as: V γ 9J γ 1.2: YYCALWEVGELGKKI: p<0.05 (a statistical significant oligoclonal expansion) (Table 10). There was one non-clonally expanded TCR+ T-cell transcript OV27T number 3 with a CDR3 region as: YYCALWEVGLGKKI: p>0.05.

In patient OV42T sequence analysis of V γ II(9)-chain TCR+ T-cell transcripts revealed that 7 out of 16 (43.8%) TCR+ T-cell transcripts each had a CDR3 region identical to that of OV42T number 7 with its CDR3 region as: V γ 9J γ 1.3/2.3: YYCALWEVPCYYKKLFG: p<0.05 (a statistical significant oligoclonal expansion) (Table 11). 6 out of 16 (37.5%) TCR+ T-cell transcripts each had a CDR3 region identical to that of OV42T number 15 with its CDR3 region as: V γ 9J γ 1.3/2.3: YYCALWEVHYYKKLFG: p<0.05 (a statistically significant oligoclonal expansion). There were three non-clonally expanded TCR+ T-cell transcripts with a CDR3 region as: V γ 9J γ 1.3/2.3: YYCALWEV (no N region) YYKKLFG: (6.3%) p>0.05, V γ 9J γ 1.3/2.3: YYCALWEVSEYYKKLFG: (6.3%) p>0.05 and V γ 9J γ 1.3/2.3: YYCALWEVRYYKKLFG: (6.3%) p>0.05 which were the CDR3 regions of the TCR+ T-cell transcripts numbers 5, 10 and 2, respectively (see Table 11).

In patient OV45T, sequence analysis of V γ II (9)-chain TCR+ T-cell transcripts revealed that 8 out of 18 (44.4%) TCR+ T-cell transcripts each had a CDR3 region identical to that of OV45T number 9 with its CDR3 region as:

V γ 9J γ 1.3/2.3: YYCALWEVIYYKKLFG: p<0.05 (a statistically significant oligoclonal expansion): (Table 12). 7 out of 18 (38.9%) TCR+ T-cell transcripts each had a CDR3 region identical to that of OV45T number 3 with its CDR3 region as: V γ 9J γ 1.3/2.3: YYCALWEVYPRKLFG: p<0.0001. 2 out of 18 (11.1%) TCR+ T-cell transcripts each had a CDR3 region as: V γ 9J γ 1.3/2.3: YYCALWEVPRKLFG: p>0.05. One additional transcript which was non-clonally expanded had a CDR3 region as: V γ 9J γ 1.3/2.3: YYCALWEV (no N region) YYKKLFG: (5.6%) p>0.05.

In patient OV38T, sequence analysis of V γ II (9)-chain TCR+ T-cell transcripts revealed that 11 out of 20 (55%) TCR+ T-cell transcripts sequenced each had a CDR3 region identical to OV38T number 12 with its CDR3 region as: V γ 9J γ 1.3/2.3: YYCALWEVRYYKKLFG: p<0.05 (a statistically significant oligoclonal expansion) (Table 13). 3 out of 20 (15%) TCR+ T-cell transcripts sequenced each had a CDR3 region identical to OV38T number 17 with its CDR3 region as: V γ 9J γ 1.3/2.3: YYCALWEVRFVYYKKLFG: p>0.05 (a statistically insignificant oligoclonal expansion). 2 out of 20 (10%) TCR+ T-cell transcripts sequenced each had a CDR3 region as: V γ 9J γ 1.3/2.3: YYCALWEVLYYKKLFG: p>0.05. There were four non-clonally expanded TCR+ T-cell transcripts each had a CDR3 region as: V γ 9J γ 1.3/2.3: YYCALWEVEWEYYKKLFG: (5%) p>0.05, V γ 9J γ 1.3/2.3: YYCALWEVRFYYKKLFG: (5%) p>0.05, V γ 9J γ 1.3/2.3: YYCALWEV(no N region)KLFG: (5%) p>0.05 and V γ 9J γ 1.3/2.3: YYCALW(no N region)KLFG: (5%) p>0.05 which were transcripts number 16, 19, 14 and 1, respectively.

In patient OV26T, sequence analysis of V γ II(9)-chain TCR+ T-cell transcripts revealed that 24 out of 27 (89%) TCR+ T-cell transcripts sequenced each had a CDR3 region identical to OV26T number 2 with its CDR3 region as: V γ 9J γ 1.3/2.3: YYCALWEVLPYYKKL: p<0.05 (statistically significant oligoclonal expansion) (see Table 14). There were three non-clonally expanded TCR+ T-cell transcripts each had a CDR3 region as: V γ 9J γ 1.3/2.3: YYCALWEVGELGKK: (4%) p>0.05, V γ 9J γ 1.3/2.3: YYCALWEVCYYKKLFG: (4%) p>0.05, V γ 9J γ 1.3/2.3: YYCALWEV(no N region)KK: (4%) p>0.05 which were transcripts number 1, 6, and 9, respectively.

C) Sequencing results of the V δ 1-chain TCR Transcripts

Similar procedures to the V γ I and V γ II sequence analysis described above were employed for the V δ 1 sequence analysis. The productively rearranged transcripts were analyzed using the GENBANK database. Results revealed the presence of statistically significant substantial proportions of identical V δ 1-chain TCR+ T-cell transcripts in four out of six solid tumor specimens obtained from patients with EOC, suggesting the presence of oligoclonal populations of T-cells infiltrating the solid tumor from patients with EOC.

Table 17. V δ 1-chain TCR+ T-cell Transcripts (CDR3 Region) Expressed in the Solid Tumor from patient OV27T

Transcript No.	V δ	N	D δ 2	N	J δ	Transcript Frequency	p-value	
							1/45	2/45
3	C A L G D TGTGCTCTTGGGGAC	Y G S T F S W V TACGGAAGTACTTTTTTCATGGGTC			I F V δ 1/D δ 2/J δ 1 ATCTTT	23/45 51%	<0.0001	<0.0001
9	C A L G D E A F L N L V P D S Y T TGTGCTCTTGGGGACGAGGCCTTCCCTAAATCTAGTACCCGATTCGTACACC				D K V δ 1/D δ 2/J δ 1 GATAAA	21/45 47%	<0.0001	<0.0001
5	L G E L P P G Y W G I V S P V G N T CTTGGGGAACTTCCCCCTGGGTACTGGGGGATTGTCTCTCCTGTAGGTAACACC				D K V δ 1/D δ 2/J δ 1 GATAAA	1/45 2%NS	NS	NS
7	C A L G E S D Y E V G G V L V TGTGCTCTTGGGGAATCCGACTACGAAGTGGGGGGGTCCTAGTC				D K V δ 1/D δ 2/J δ 1 GATAAA	1/45 2%NS	NS	NS

Table 18. Vδ1-chain TCR+ T-cell Transcripts (CDR3 Region) Expressed in the Solid Tumor from patient OV33T

Transcript No.	Vδ	N	Dδ2Dδ3	N	Jδ	Transcript Frequency	p-value		
							1/20	2/20	3/20
10	Y F C A L G	D Y G S T F S W V			D K	Vδ1/Dδ2/Jδ1			
	TACTTTTGTGCTCTTGGG	GACTACGGAAGTACTTTTTCATGGGTC			GATAAA	7/20 35%	<0.0001	<0.0001	
8	Y F C A L G D E A F L N L V P D S Y T				D K	Vδ1/Dδ2/Jδ1			
	TACTTTTGTGCTCTTGGGGACGAGGCCTTCCTAAATCTAGTACCCGATTCGTACACC				GATAAA	3/20 15%	NS	NS	
6	Y F C A L G E L T G G W Y E V D T				D K	Vδ1/Dδ2/Jδ1			
	TACTTTTGTGCTCTTGGGGAACTTACTGGGGGGTGGTATGAGGTTGACACC				GATAAA	3/20 15%	NS	NS	
1	Y F C A L G	D L P V L G D S E			D K	Vδ1/Dδ2/Jδ1			
	TACTTTTGTGCTCTTGGG	GACCTACCCGTAAGTGGGGGATAGTGAG			GATAAA	2/20 10%	NS	NS	
Transcript No.	Vδ	N	Dδ2	N	Jδ	Transcript Frequency	p-value		
							1/20	2/20	3/20
18	Y F C A L G	D L P V L G D			D K	Vδ1/Dδ2/Jδ1			
	TACTTTTGTGCTCTTGGG	GACCTACCCGTAAGTGGGGGAT			GATAAA	1/20 5%	NS	NS	NS
2	F C A L G D T R D E L Y W G I R Q C P T				D K	Vδ1/Dδ2/Jδ1			
	TTTTGTGCTCTTGGG	GACACCAGGGACGAACCTTACTGGGGGATACGCCAATGCCCAACC			GATAAA	1/20 5%	NS	NS	NS
3	Y F C A L G	T P P G D T K I P Y P			D K	Vδ1/Dδ2/Jδ1			
	TACTTTTGTGCTCTTGGG	ACCCCGCCCGGGGATACGAAGATCCCTTACCCC			GATAAA	1/20 5%	NS	NS	NS
14	Y F C A L G	D R H P Y T T S L A			D K	Vδ1/Dδ2/Jδ1			
	TACTTTTGTGCTCTTGGG	GACCGACATCCCTACACTACCTCCCTGGCC			GATAAA	1/20 5%	NS	NS	NS
22	Y F C A L G E K R L P T P L V P D H T				D K	Vδ1/Dδ2/Jδ1			
	TACTTTTGTGCTCTTGGGGAAAAACGACTTCTACGCCCTTGGTCCCAGACCACACC				GATAAA	1/20 5%	NS	NS	NS

Table 19. Vδ1-chain TCR+ T-cell Transcripts (CDR3 Region) Expressed in the Solid Tumor from patient OV39T

Transcript No.	Vδ	N	Dδ2	N	Jδ	Transcript Frequency	p-value		
							1/17	2/17	3/17
2	C F C A L G E GCTTTTGTGCTCTTGGGGAAT	S D Y E V G G V L V			D K Vδ1/Dδ2/Jδ1	GATAAA12/17 70.6%	<0.0001	<0.0001	<0.0001
18	Y F C A L G E TACTTTTGTGCTCTTGGGGAA	L P F L L W G I T			D K Vδ1/Dδ2/Jδ1	GATAAA3/17 17.6	NS	NS	NS
8	Y F C A L G E L W P P T K R I L G T TACTTTTGTGCTCTTGGGGA	CTCCCCTTCTACTCTGGGGGATCACC			D K Vδ1/Dδ2/Jδ1	GATAAA1/17 5.8%	NS	NS	NS
12	Y F C A L G E TACTTTTGTGCTCTTGGGGAA	P V G D W G Q D P F			D K Vδ1/Dδ2/Jδ1	CCCGTGGGGGACTGGGGCCAAGACCCCTTCGATAAA1/17 5.8%	NS	NS	NS

Table 20. Vδ1-chain TCR+ T-cell Transcripts (CDR3 Region) Expressed in the Solid Tumor from patient OV1T

Transcript No.	Vδ	N	Dδ2	N	Jδ	Transcript Frequency	p-value		
							1/30	2/30	3/30
9	Y F C A L G	V P G G G P P A D T			D K Vδ1/Dδ2/Jδ1				
	TACTTTTGTGCTCTTGGG	GTCCCCGGGGGGGACCGCCTGCCGACACC			GATAAA	22/30 73.3%	<0.0001	<0.0001	<0.0001
16	Y F C A L G E P K I L P P N G D Y T				D K Vδ1/Dδ2/Jδ1				
	TACTTTTGTGCTCTTGGGGAACCCAAAATCCTTCCCCCAACGGGGATTACACC				GATAAA	1/30 3.3%	NS	NS	NS
19	Y F C A L G E V Q G S R T T T G G H T				D K Vδ1/Dδ2/Jδ1				
	TACTTTTGTGCTCTTGGGGAAGTTCAGGGCTCCCGAACAACTACTGGGGGGCACACCCGATAAA				GATAAA	1/30 3.3%	NS	NS	NS
17	Y F C A L G E G G I A R G P R				D K Vδ1/Dδ2/Jδ1				
	TACTTTTGTGCTCTTGGGGAAGGGGGAATCGCACGGGGACCCCGC				GATAAA	1/30 3.3%	NS	NS	NS
23	Y F C	P Q Q R V G V P R G Y T			D K Vδ1/Dδ2/Jδ1				
	TACTTTTGT	CCTCAACAGCGAGTGGGGGTTCCCCGCGGTACACCCGATAAA			GATAAA	1/30 3.3%	NS	NS	NS
14	Y F C A L G	Q P L G L P S T G G D T			D K Vδ1/Dδ2/Jδ1				
	TACTTTTGTGCTCTTGGGA	CAACCCCTAGGCCTTCCCTCTACTGGGGGAGACACC			GATAAA	1/30 3.3%	NS	NS	NS
12	Y F C A L G D T R L G D T D T				D K Vδ1/Dδ2/Jδ1				
	TACTTTTGTGCTCTTGGGGATACGAGGCTGGGGGATACCGACACC				GATAAA	1/30 3.3%	NS	NS	NS
8	Y F C A L G E L A F P P Y W G S S Y T				D K Vδ1/Dδ2/Jδ1				
	TACTTTTGTGCTCTTGGGGAACCTCGCCTTTCCTCCCTACTGGGGGTCTTCGTACACC				GATAAA	1/30 3.3%	NS	NS	NS
2	Y F C A L G E G G I A R G P R				D K Vδ1/Dδ2/Jδ1				
	TACTTTTGTGCTCTTGGGGAAGGGGGAATCGCACGGGGACCCCGC				GATAAA	1/30 3.3%	NS	NS	NS

Table 21. V δ 1-chain TCR+ T-cell Transcripts (CDR3 Region) Expressed in the Solid Tumor from patient OV1C

Transcript NO.	V δ	N	D δ 2	N	J δ	Transcript p-value			
						Frequency	1/26	2/26	3/26
22	Y F C A L G D E A	F L N L V P D S Y T			D K V δ 1/D δ 2/J δ 1				
	TACTTTTGTGCTCTTGGGGACGAGGCCTTCCTAAATCTAGTACCCGATTCGTACACC				GATAAA2/26 7.8%NS	NS	NS		
21	Y F C A L G E L	V A P G W D I R F			D K V δ 1/D δ 2/J δ 1				
	TACTTTTGTGCTCTTGGGGAACTA	GTCGCTCCTGGGTGGGATATACGGTTT			GATAAA2/26 7.8%NS	NS	NS		
7	Y F C A L G E L	S T P D W G I L S			D K V δ 1/D δ 2/J δ 1				
	TACTTTTGTGCTCTTGGGGAACTA	TCAACGCCCCGACTGGGGGATACTCTCC			GATAAA2/26 7.8%NS	NS	NS		
2	Y F C A L G E L	G S S S P K P N			D K V δ 1/D δ 2/J δ 1				
	TACTTTTGTGCTCTTGGGGAACTA	GGATCTTCCTCCCCTAAACCAAAC			GATAAA1/26 3.8%NS	NS	NS		
4	Y F C A L G E L	P W S T G G Y A P			D K V δ 1/D δ 2/J δ 1				
	TACTTTTGTGCTCTTGGGGAACTC	CCATGGAGTACTGGGGGATACGCACCC			GATAAA1/26 3.8%NS	NS	NS		
5	Y F C A L G E L P	L I Q L G D L R E T			D K V δ 1/D δ 2/J δ 1				
	TACTTTTGTGCTCTTGGGGAACTGCCCTCATTCAACTGGGGGACCTCCGAGAACACC				GATAAA1/26 3.8%NS	NS	NS		
6	Y F C A L G E R S	H Y W G I Q W R S R S			D K V δ 1/D δ 2/J δ 1				
	TACTTTTGTGCTCTTGGGGAGCGAAGCCATTACTGGGGGATACAATGGAGGAGCCGGAGC				GATAAA1/26 3.8%NS	NS	NS		
8	Y F C A L G E L	P G S T G G Y A R			D K V δ 1/D δ 2/J δ 1				
	TACTTTTGTGCTCTTGGGGAACTC	CCAGGGAGTACTGGGGGATACGCACGC			GATAAA1/26 3.8%NS	NS	NS		
9	Y F C A L G E L	D Q T T T E G Y G D T			D K V δ 1/D δ 2/J δ 1				
	TACTTTTGTGCTCTTGGGGAACTA	GACCAGACGACTACGGAGGGATACGGGGACACC			GATAAA1/26 3.8%NS	NS	NS		
11	Y F C A L G E	P R M D W G I L H T			D K V δ 1/D δ 2/J δ 1				
	TACTTTTGTGCTCTTGGGGAA	CCTCGGATGGACTGGGGGATCCTACACACC			GATAAA1/26 3.8%NS	NS	NS		
12	Y F C A L G	F F L T G G Y R T			D K V δ 1/D δ 2/J δ 1				
	TACTTTTGTGCTCTTGGG	TTCTTCCTAACCGGGGGATATTGACACC			GATAAA1/26 3.8%NS	NS	NS		
14	Y F C A L G D Y G	S T F S W V I F G K G T			D K V δ 1/D δ 2/J δ 1				
	TACTTTTGTGCTCTTGGGGACTACGGAAGTACTTTTTCATGGGTCATCTTTGGAAAAGGAACCCGATAAA				1/26 3.8%NS	NS	NS		
18	Y F C A L G E L	S T G G Y A P			D K V δ 1/D δ 2/J δ 1				
	TACTTTTGTGCTCTTGGGGAACTC	AGTACTGGGGGATACGCACCC			GATAAA 1/26 3.8%NS	NS	NS		

Table 21. Continued

Transcript No.	V δ	N	D δ 2	N	J δ	Transcript Frequency	p-value	1/26	2/26	3/26	
15	Y F C A L G E L TACTTTTGTGCTCTTGGGGAAGT	L G D CTGGGGGAC			D K	V δ 1/D δ 2/J δ 1 GATAAA	1/26	3.8%	NS	NS	NS
16	Y F C A L G E TACTTTTGTGCTCTTGGGGAG	W G I TGGGGGATA			D K	V δ 1/D δ 2/J δ 1 GATAAA	1/26	3.8%	NS	NS	NS
17	Y F C A L G E TACTTTTGTGCTCTTGGGGAA	P D W G I L CCCGACTGGGGGATACTC			D K	V δ 1/D δ 2/J δ 1 GATAAA	1/26	3.8%	NS	NS	NS
25	Y F C A L G E L TACTTTTGTGCTCTTGGGGAAGT	P L I Q L G D E T CCCCTCATTCAACTGGGGGACGAAACC			D K	V δ 1/D δ 2/J δ 1 GATAAA	1/26	3.8%	NS	NS	NS
27	Y F C A L G E TACTTTTGTGCTCTTGGGGAG	R S H Y W G I Q W R S CGAAGCCATTACTGGGGGATACAATGGAGGAGC			D K	V δ 1/D δ 2/J δ 1 GATAAA	1/26	3.8%	NS	NS	NS
8	Y F C A L G E L TACTTTTGTGCTCTTGGGGAAGT	P G S T G G Y A R CCAGGGAGTACTGGGGGATACGCACGC			D K	V δ 1/D δ 2/J δ 1 GATAAA	1/26	3.8%	NS	NS	NS
19	Y F C A L G E L TACTTTTGTGCTCTTGGGGAAGT	D Q T T T E G Y G D T GACCAGACGACTACGGAGGGATACGGGGACACC			D K	V δ 1/D δ 2/J δ 1 GATAAA	1/26	3.8%	NS	NS	NS
3	Y F C A L G E TACTTTTGTGCTCTTGGGGAA	P R M D W G I L H T CCTCGGATGGACTGGGGGATCCTACACACC			D K	V δ 1/D δ 2/J δ 1 GATAAA	1/26	3.8%	NS	NS	NS
13	Y F C A L G TACTTTTGTGCTCTTGGG	L T G G G G CTAACCGGGGGGGGGGG			D K	V δ 1/D δ 2/J δ 1 GATAAA	1/26	3.8%	NS	NS	NS
20	Y F C A L G D TACTTTTGTGCTCTTGGGGAC	Y G S T F S W V TACGGAAGTACTTTTTCATGGGTC			D K	V δ 1/D δ 2/J δ 1 GATAAA	1/26	3.8%	NS	NS	NS

Table 22. Summary of clonal expansion of Vδ1-chain TCR+ T -cell transcripts in the solid tumor of patients with EOC

OV27T: 23/45:#3:51%:CDR3:CALGDYGSTFSWVIF
 OV27T: 21/45:#9:47%:CDR3:CALGDEAFLNLVPDSYTDK
 OV33T: 7/20:#10:35%:CDR3:YFCALGDYGSTFSWVDK
 OV39T: 12/17:#2:70.6%:CDR3:FCALGESDYEVGGVLVDK
 OV1T: 22/30:#9:73.3%:CDR3:YFCALGVPGGGPPADTDK

Table 23. Percent clonal expansion of Vδ1-chain TCR+ T-cell transcripts in the solid tumor of patients with EOC

EOC Patient Sample	% Clonal Expansion
OV27T #3	51%
OV27T #9	47%
OV33T #10	35%
OV39T #2	70.6%
OV1T #9	73.3%

In patient OV27T, sequence analysis of Vδ1 -chain TCR+ T-cell transcripts revealed that 23 out of 45 (51%) TCR+ T-cell transcripts each had a CDR3 region identical to that of OV27T number 3 with its CDR3 region as: Vδ1Dδ2Jδ1 CALGDYGSTFSWVIF p<0.0001 (a statistically significant oligoclonal expansion) (see Table 14). 21 out of 45 (47%) TCR+ T-cell transcripts sequenced each had a CDR3 region identical to OV27T number 9 with its CDR3 region as: Vδ1Dδ2Jδ1 CALGDEAFLNLVPDSYT p<0.0001 (a statistically significant oligoclonal expansion) (see Table 14). There were 2 non-clonally expanded TCR+ T-cell transcripts each had a CDR3 region as Vδ1Dδ2Jδ1 CALGELPPGYWGIVSPVGNTDK: (2%) p>0.05 and Vδ1Dδ2Jδ1

CALGESDYEVGGVLV: (2%) $p > 0.05$ which were transcripts 5 and 7, respectively.

In patient OV33T, sequence analysis of V δ 1 -chain TCR+ T-cell transcripts revealed that 7 out of 20 (35%) TCR+ T-cell transcripts each had a CDR3 region identical to that of OV33T number 10 with its CDR3 region as: V δ 1D δ 2J δ 1 YFCALGDYGSTFSWVDK $p < 0.0001$ (a statistically significant oligoclonal expansion) (see Table 18). 3 out of 20 (15%) TCR+ T-cell transcripts sequenced each had a CDR3 region identical to OV33T number 8 with its CDR3 region as: V δ 1D δ 2J δ 1 YFCALGDEAFLNLPDSYTDK $p > 0.05$ (a statistically insignificant oligoclonal expansion) (see Table 18). 3 out of 20 (15%) TCR+ T-cell transcripts sequenced each had a CDR3 region identical to OV33T number 6 with its CDR3 region as V δ 1D δ 2J δ 1 YFCALGELTGGWYEVDTDK $p > 0.05$ (a statistically insignificant oligoclonal expansion). 2 out of 20 (10%) TCR+ T-cell transcripts sequenced each had a CDR3 region identical to OV33T number 1 with its CDR3 region as V δ 1D δ 2J δ 1 YFCALGDLPVLGDSEDK $p > 0.05$ (a statistically insignificant oligoclonal expansion). There were five non-clonally expanded TCR+ T-cell transcripts each had a CDR3 region as V δ 1D δ 2J δ 1 YFCALGDLPVLGDDK: (5%) $p > 0.05$, V δ 1D δ 2J δ 1 FCALGDTRDELYWGIRQCPTDK: (5%) $p > 0.05$, V δ 1D δ 2J δ 1 YFCALGTPPGDTKIPYPDK (5%) $p > 0.05$, V δ 1D δ 2J δ 1 YFCALGDRHPYTTSLADK (5%) $p > 0.05$, V δ 1D δ 2J δ 1 YFCALGEKRLPTPLVPDHTDK (5%) $p > 0.05$ which were transcripts 18, 2, 3, 14, and 22, respectively.

D) Sequencing results of the V δ 2-chain TCR Transcripts

Similar procedures to the V γ I, V γ II and V δ 1 sequence analysis described above were employed for the V δ 2 sequence analysis. The productively rearranged transcripts were analyzed using the GENBANK database. Results revealed the presence of statistically significant substantial proportions of identical V δ 2-chain TCR+ T-cell transcripts in four out of five solid tumor specimens obtained from patients with EOC, suggesting the presence of oligoclonal populations of T-cells infiltrating the solid tumor from patients with EOC.

Table 24. V δ 2-chain TCR+ T-cell Transcripts (CDR3 Region) Expressed in the Solid Tumor from patient OV1T

Transcript No.	V δ	N	D δ 2	N	J δ	Transcript Frequency	p-value	
							1/25	2/25
4	Y Y C A C D T TACTACTGTGCCTGTGACACC		A G D T R GCAGGGGATACACGC		D K L I V δ 1/D δ 2/D δ 3/J δ 1 GATAAACTCATC	10/25 40%	<0.0001	<0.0001
8	Y Y C A C D T TACTACTGTGCCTGTGACACC		V L G D P R S T GTCCTGGGGGACCCGAGGAGCACC		D K L I V δ 1/D δ 2/D δ 3/J δ 1 GATAAACTCAT	7/25 28%	<0.0001	<0.004
12	Y Y C A C D TACTACTGTGCCTGTGAC	F TTC	V L G D T G Y T GTACTGGGGGATACGGGGTACACC		D K L I V δ 1/D δ 2/D δ 3/J δ 1 GATAAACTCATC	1/25 4%	NS	NS
20	Y Y C A C D TACTACTGTGCCTGTGAC	S TCT	L G D T S CTGGGGGATACG	TCC	D K L I V δ 1/D δ 2/D δ 3/J δ 1 GATAAACTCATC	1/25 4%	NS	NS
22	Y Y C A C D TACTACTGTGCCTGTGAC	K AAG	L G V T CTGGGG GTTACC		D K L I V δ 1/D δ 2/D δ 3/J δ 1 GATAAACTCATC	1/25 4%	NS	NS
17	Y Y C A C D TACTACTGTGCCTGTGAC		V L G D T P G T GTACTGGGGGATACCCCGGGCACC		D K L I V δ 1/D δ 2/D δ 3/J δ 1 GATAAACTCATC	1/25 4%	NS	NS
11	Y Y C A C D TACTACTGTGCCTGTGAC	M ATG	V G D T N A GTCGGGGATACT	AATGCC	D K L I V δ 1/D δ 2/D δ 3/J δ 1 GATAAACTCATC	1/25 4%	NS	NS
2	Y Y C A C D T TACTACTGTGCCTGTGACACCGTGAATGGGGTCCCTACACC	V N	G G P Y T		D K L I V δ 1/D δ 2/D δ 3/J δ 1 GATAAACTCATC	1/25 4%	NS	NS

Table 25. V δ 2-chain TCR+ T-cell Transcripts (CDR3 Region) Expressed in the Solid Tumor from patient OV42T

Transcript No.	V δ	N	D δ 2	N	J δ	Transcript Frequency	p-value	
							1/24	2/24
11	Y Y C A C D T TACTACTGTGCCTGTGACACCGTG	V	G T G G G T GGAAGTGGGGGGGGCACC		D K L I V δ 2D δ 2J δ 1 GATAAACTCATC	18/24 75%	<0.0001	<0.0001
17	Y Y C A C D TACTACTGTGCCTGTGAC	I	L G D T ATACTGGGGGATACG		D K L I V δ 2D δ 2J δ 1 GATAAACTCATC	3/24 13%	NS	NS
2	Y Y C A C D TACTACTGTGCCTGTGAC	V	L G D N T GTACTGGGGGATAACACC		D K L I V δ 2D δ 2J δ 1 GATAAACTCATC	2/24 13%	NS	NS
24	Y Y C A C D TACTACTGTGCCTGTGAC	G	V A S G D T S GGTGTGGCTTCGGGGGATACGTCC		D K L I V δ 2D δ 2J δ 1 GATAAACTCATC	1/24 13%	NS	NS

Table 26. Vδ2-chain TCR+ T-cell Transcripts (CDR3 Region) Expressed in the Solid Tumor from patient OV27T

Transcript No.	Vδ	N	Dδ2	N	Jδ	Transcript Frequency	p-value	
							1/30	2/30
13	Y Y C A C D T V TACTACTGTGCCTGTGACACCGTA	T L G I N T ACTCTGGGGATTAACACC			D K L I Vδ2Dδ2Jδ1 GATAAACTCATC11/30 36%	<0.0001	<0.0001	
22	Y Y C A C D T V TACTACTGTGCCTGTGACACCGTA	L G D R CTGGGGGATCGG			D K L I Vδ2Dδ2Jδ1 GATAAACTCATC8/30 27%	<0.0001	<0.0005	
3	Y Y C A C D T V TACTACTGTGCCTGTGACACCGTA	G E D T GGGGAAGACACC			D K L I Vδ2Dδ2Jδ1 GATAAACTCATC1/30 3%	NS	NS	
4	Y Y C A C D T V TACTACTGTGCCTGTGACACCGTT	G D H R H T GGGGATCACAGGCACACC			D K L I Vδ2Dδ2Jδ1 GATAAACTCATC1/30 3%	NS	NS	
1	Y Y C A C V W G I L P S P P I A TACTACTGTGCCTGT GTCTGGGGGATACTCCCCGTCACCCCCCATCGCC				D K L I Vδ2Dδ3Jδ1 GATAAACTCATC1/30 3%	NS	NS	
6	Y Y C A C D P L L G D P P N T TACTACTGTGCCTGTGAC CCC TTACTGGGGGATCCCCCAACACC				D K L I Vδ2Dδ2Jδ1 GATAAACTCATC1/30 3%	NS	NS	
7	Y Y C A C D T V G G G G S TACTACTGTGCCTGTGACACCGTTGGGGGGGGAGGGTCC				D K L I Vδ2Dδ3Jδ1 GATAAACTCATC1/30 3%	NS	NS	
8	Y Y C A C D T I G T G D N L G P TACTACTGTGCCTGTGACACGATTGGTACTGGGGACAACCTGGGGCCT				D K L I Vδ2Dδ2Jδ1 GATAAACTCATC1/30 3%	NS	NS	
5	Y Y C A C D V A G V T TACTACTGTGCCTGTGAC GTGGCCGGGGTCACC				D K L I Vδ2Dδ2Jδ1 GATAAACTCATC1/30 3%	NS	NS	
10	Y Y C A C D T L E V L G D N T TACTACTGTGCCTGTGACACCCTCGAAGTACTGGGGGATAACACC				D K L I Vδ2Dδ2Jδ1 GATAAACTCATC1/30 3%	NS	NS	
11	Y Y C A C D V L A V T TACTACTGTGCCTGTGACGTGCTCGCTGTGACC				D K L I Vδ2Dδ2Jδ1 GATAAACTCATC1/30 3%	NS	NS	

Table 27. Vδ2-chain TCR+ T-cell Transcripts (CDR3 Region) Expressed in the Solid Tumor from patient OV26T

Transcript No.	Vδ	N	Dδ2	N	Jδ	Transcript Frequency	p-value	
							1/17	2/17
6	Y Y C A C D T V A T S G G V T				D K L I	Vδ2Dδ3Jδ1 GATAAACTCATC3/17 18%	NS	NS
1	Y Y C A C D T V G T G G Y P N T				D K L I	Vδ2Dδ3Jδ1 GATAAACTCATC3/17 18%	NS	NS
2	Y Y C A C D S V L G E R N P P T				D K L I	Vδ2Dδ2Jδ1 GATAAAGCTCATC2/17 12%	NS	NS
3	Y Y C A C D T V S A G D T G S F T				D K L I	Vδ2Dδ2Jδ1 GATAAACTCATC1/17 6%	NS	NS
5	Y Y C A C D T L R T G G Y V N				D K L I	Vδ2Dδ3Jδ1 GATAAACTCATC1/17 6%	NS	NS
10	Y Y C A C D R L G D K D				W D T R	Vδ2Dδ3Jδ1 TGGGACACCCGA1/17 6%	NS	NS
11	Y Y C A C S L V G S Q R V A				D K L I	Vδ2Dδ3Jδ1 GATAAACTCATC 1/17 6%	NS	NS
12	Y Y C A C D T V V L G D T R W T				D K L I	Vδ2Dδ3Jδ1 GATAAACTCATC 1/17 6%	NS	NS
9	Y Y C A C D T V V L G D T R R N T				D K L I	Vδ2Dδ3Jδ1 GATAAACTCATC 1/17 6%	NS	NS
14	Y Y C A C D D V R G L G G				K L I	Vδ2Dδ3Jδ1 AAACTCATC 1/17 6%	NS	NS
13	Y Y C A C D T I G G T				D K L I	Vδ2Dδ3Jδ1 GATAAACTCATC 1/17 6%	NS	NS

Table 28. Vδ2-chain TCR+ T-cell Transcripts (CDR3 Region) Expressed in the Solid Tumor from patient OV31T

Transcript No.	Vδ	N	Dδ2	N	Jδ	Transcript Frequency	p-value	
							1/31	2/31
16	Y Y C A C D T V	G G G G S				D K Vδ2Dδ3Jδ1		
	TACTACTGTGCCTGTGACACCGTG	GGGGGGGAGGGTCC				GATAAA14/31 45%	<0.0001	<0.0001
24	Y Y C A C D S L L G D T					D K Vδ2Dδ2Jδ1		
	TACTACTGTGCCTGTGAC TCCTTACTGGGGGATAACC					GACAAA5/31 16%	NS	NS
20	Y Y C A W S	G G Y F P F P L W				D K Vδ2Dδ3Jδ1		
	TACTACTGTGCC TGGTCG	GGGGGATACTTCCCCTTCCCCTTGTGG				GATAAA 2/31 6%	NS	NS
5	Y Y C A C D T V A G G D H G Q D Y A					D K Vδ2Dδ2Jδ1		
	TACTACTGTGCCTGTGACACCGTGGCCGGAGGGGACCACGGACAGGACTACGCC					GATAAA 2/31 6%	NS	NS
18	Y Y C A C D T V A S G D T S					D K Vδ2Dδ2Jδ1		
	TACTACTGTGCCTGTGACACCGTG	GCTTCGGGGGATACGTCC				GATAAA2/31 6%	NS	NS
9	Y Y C A C D T V G G Y V					T D Vδ2Dδ2Jδ1		
	TACTACTGTGCCTGTGACACCGTG	GGGGGATACGTC				ACCGA 1/31 3%	NS	NS
19	Y Y C A C D T V L G D T R A R T					D K Vδ2Dδ2Jδ1		
	TACTACTGTGCCTGTGACACCGTA	CTGGGGGATACGCGGGCGAGGACC				GATAAA1/31 3%	NS	NS
10	Y Y C A C D T V S R G P Y T					D K Vδ2Dδ2Jδ1		
	TACTACTGTGCCTGTGACACCGTG	AGTCGGGGACCTTACACC				GATAAA1/31 3%	NS	NS
3	Y Y C A C D T V G L G T L S					D K Vδ2Dδ2Jδ1		
	TACTACTGTGCCTGTGACACCGTG	GACTGGGGACACTCTCA				GATAAA1/31 3%	NS	NS
14	Y Y C A C D T V T D R G					D K Vδ2Dδ2Jδ1		
	TACTACTGTGCCTGTGACACCGTG	ACGGATAGGGGC				GATAAA1/31 3%	NS	NS
18	Y Y C A C D T V G R I T					D K Vδ2Dδ2Jδ1		
	TACTACTGTGCCTGTGACACCGTG	GGGAGAATAACC				GATAAA 1/31 3%	NS	NS

Table 29. Summary of clonal expansion of Vδ2-chain TCR+ T -cell transcripts in the solid tumor of patients with EOC

OV1T: 10/25:#4:40%:CDR3:YYCACDTAGDTRDKLI
 OV42T: 18/24:#11:75%:CDR3:YYCACDTVGTGGGTDKLI
 OV27T: 11/30:#13:36%:CDR3:YYCACDTVTLGINTDKLI
 OV27T: 8/30:#22:27%:CDR3:YYCACDTVLGDRDKLI
 OV31T: 14/31:#16:45%:CDR3:YYCACDTVGGGGSDKLI

Table 30. Percent clonal expansion of Vδ2-chain TCR+ T-cell transcripts in the solid tumor of patients with EOC

EOC Patient Sample	% Clonal Expansion
OV1T	40%
OV42T	75%
OV27T	36%
OV27T	27%
OV31T	45%

In patient OV1T, sequence analysis of Vδ2-chain TCR+ T cell transcripts revealed that 10 out of 25 (40%) TCR+ T-cell transcripts each had a CDR3 region identical to that of OV1T number 4 with its CDR3 region as: VδDδ2Dδ3Jδ1 YYCACDTAGDTRDKLI p<0.05 (a statistically significant oligoclonal expansion) (see Table 24). 7 out of 25 (28%) TCR+ T-cell transcripts each had a CDR3 region identical to that of OV1T number 8 with its CDR3 region as: VδDδ2Dδ3Jδ1 YYCACDTVLGDRSTDKLI p<0.05 (a statistically significant oligoclonal expansion). There were six non-clonally expanded TCR+ T-cell transcripts each had a CDR3 region as: VδDδ2Dδ3Jδ1 YYCACDFVLGDTGYTDKLI (4%) p>0.05, VδDδ2Dδ3Jδ1 YYCACDSLGDTSKLI (4%) p>0.05, VδDδ2Dδ3Jδ1 YYCACDKLGVTDKLI (4%) p>0.05, VδDδ2Dδ3Jδ1 YYCACDVLGDTPGT DKLI (4%) p>0.05, VδDδ2Dδ3Jδ1 YYCACDMVGDTNAGYTDKLI (4%) p>0.05,

VδDδ2Dδ3Jδ1 YYCACDTVNGGPYTDKLI (4%) $p>0.05$ which were transcripts 12, 20, 22, 17, 11, and 2, respectively.

In patient OV42T, sequence analysis of Vδ2-chain TCR+ T cell transcripts revealed that 18 out of 24 (75%) TCR+ T-cell transcripts each had a CDR3 region identical to that of OV42T number 11 with its CDR3 region as:

VδDδ2Dδ3Jδ1 YYCACDTVGTGGGTDKLI $p<0.05$ (a statistically significant oligoclonal expansion) (see Table 25). There were three additional transcripts that did not reveal oligoclonal expansion with a CDR3 region as: VδDδ2Dδ3Jδ1 YYCACDILGDTDKLI (12.5%) $p>0.05$, VδDδ2Dδ3Jδ1 YYCACDVLGDNTDKLI (8%) $p>0.05$, VδDδ2Dδ3Jδ1 YYCACDGVASGDTSDKLI (4%) $p>0.05$ which were transcripts number 17, 2 and 24, respectively.

In patient OV27T, sequence analysis revealed that 11 out of 30 (36%) TCR+ T-cell transcripts each had a CDR3 region identical to that of OV27T number 13 with its CDR3 region as: VδDδ2Jδ1 YYCACDTVTLGINTDKLI $p>0.05$ (a statistically significant oligoclonal expansion) (Table 26). 8 out of 30 TCR+ T-cell transcripts each had a CDR3 region identical to that of OV27T number 22 with its CDR3 region as: VδDδ2Jδ1 YYCACDVLGDRDKLI $p>0.05$ (a statistically significant oligoclonal expansion) (Table 26). There were nine non-clonally expanded transcripts number 3, 4, 1, 6, 7, 8, 5, 10 and 11 (see Table 26).

In patient OV26T, sequence analysis revealed that 3 out of 17 (18%) TCR+ T-cell transcripts each had a CDR3 region identical to that of OV26T number 6 with its CDR3 region as: VδDδ3Jδ1 YYCACDTVATSGGVTDKLI $p>0.05$ (a statistically insignificant oligoclonal expansion) (Table 27). There were

8 non-clonally expanded transcripts number 3, 5, 10, 11, 12, 9, 14 and 13 (see Table 27).

In patient OV31T, sequence analysis revealed that 14 out of 31 (45%) TCR+ T-cell transcripts each had a CDR3 region identical to that of OV45T number 16 with its CDR3 region as: V δ D δ 3J δ 1 YYCACDTVGGGGSDKLI $p < 0.05$ (a statistically significant oligoclonal expansion) (see Table 28). 5 out of 31 TCR+ T-cell transcripts each had a CDR3 region identical to that of OV45T number 24 with its CDR3 region as: V δ D δ 3J δ 1 YYCACDSELLGDTDKLI $p > 0.05$ (a statistically insignificant oligoclonal expansion). 2 out of 31 TCR+ T-cell transcripts each had a CDR3 region identical to that of OV45T number 20 with its CDR3 region as: V δ D δ 3J δ 1 YYCACWSGGYFPFPLWDKLI $p > 0.05$ (a statistically insignificant oligoclonal expansion). 2 out of 31 TCR+ T-cell transcripts each had a CDR3 region identical to that of OV45T number 5 with its CDR3 region as: V δ D δ 3J δ 1 YYCACDTVAGGDHGGQDYADKLI $p > 0.05$ (a statistically insignificant oligoclonal expansion). 2 out of 31 TCR+ T-cell transcripts each had a CDR3 region identical to that of OV45T number 18 with its CDR3 region as: V δ D δ 3J δ 1 YYCACDTVASGDTSDKLI $p > 0.05$ (a statistically insignificant oligoclonal expansion). There were six non-clonally expanded TCR+ T-cell transcripts number 9, 19, 10, 3, 14 and 18 (Table 27).

E) Full-length construction results

Once we determined the presence of clonally expanded $\gamma\delta$ TCR+ T-cells infiltrating the solid tumor of patients with EOC the γ I-, γ II(9)-, δ 1-, δ 2-chain TCR+ T-cell transcripts containing the clonally expanded CDR3 were used for full-length construct. These full lengths were constructed using the primers mentioned previously to amplify each third of the full-length transcript followed by a PCR-mediated ligation of the transcripts.

Figure 6 - V_{γ II(9)-chain TCR+ T-cell transcript (Segment 1) used for full-length construction

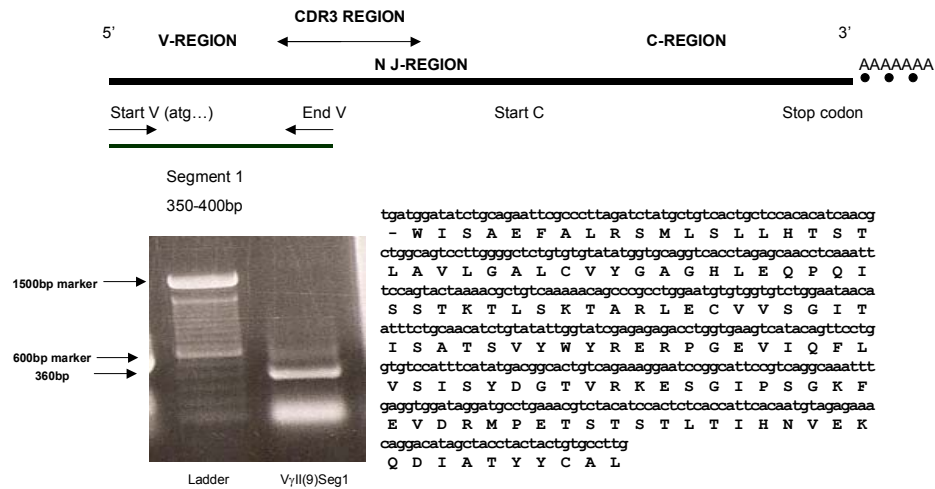


Figure 7 - C γ -chain TCR+ T-cell transcript (Segment 3) used for full-length construction

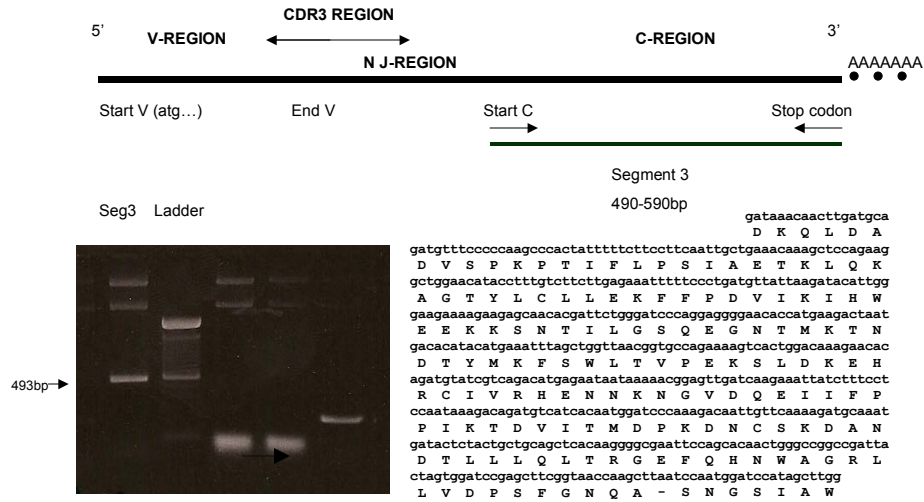


Figure 8 - V γ II(9)-chain TCR+ T-cell transcript (Segment 2) used for full-length construction of OV27T transcript 6 and 13

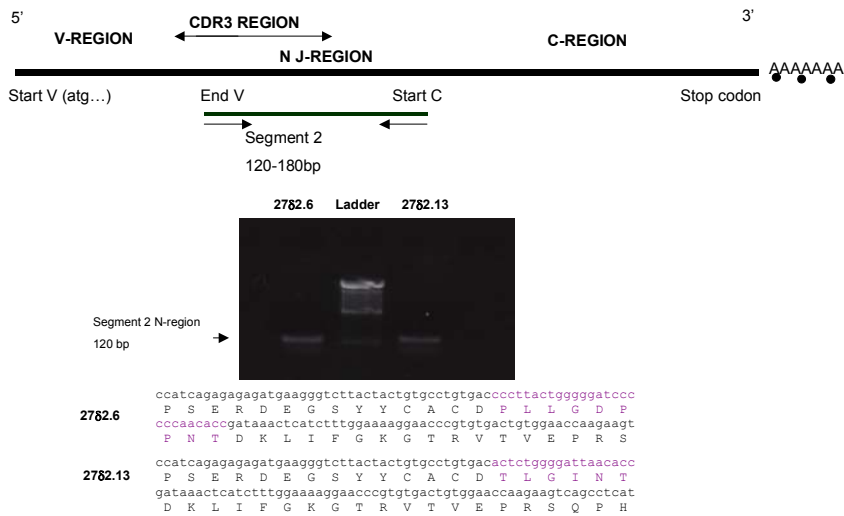


Figure 9 - V γ 9-chain TCR+ T-cell transcript (Segment 1+2+3) full-length construction of OV27T

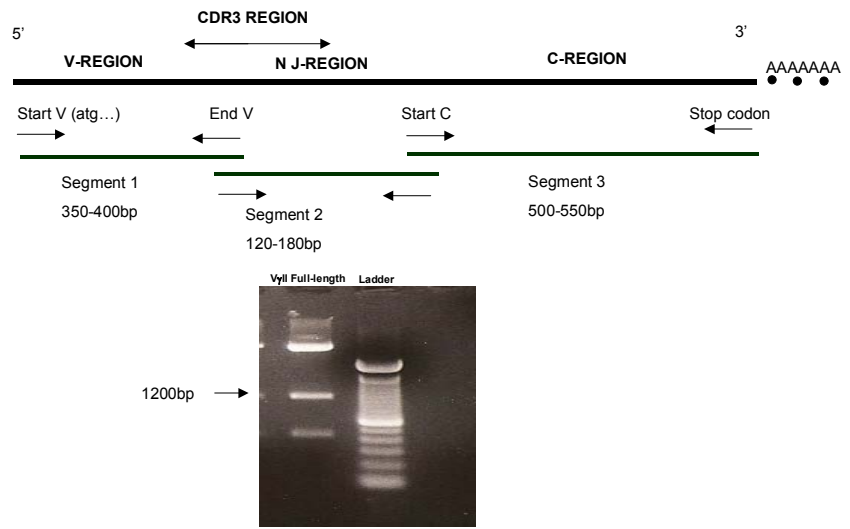


Figure 10 - Vδ2-chain TCR+ T-cell transcript (Segment 1) used for full-length construction of OV27T

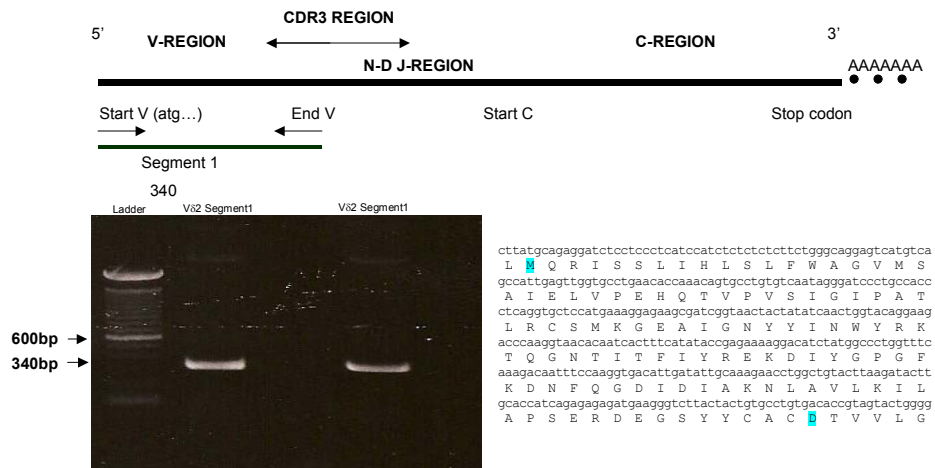


Figure 11 - Vδ2-chain TCR+ T-cell transcript (Segment 3) used for full-length construction of OV27T

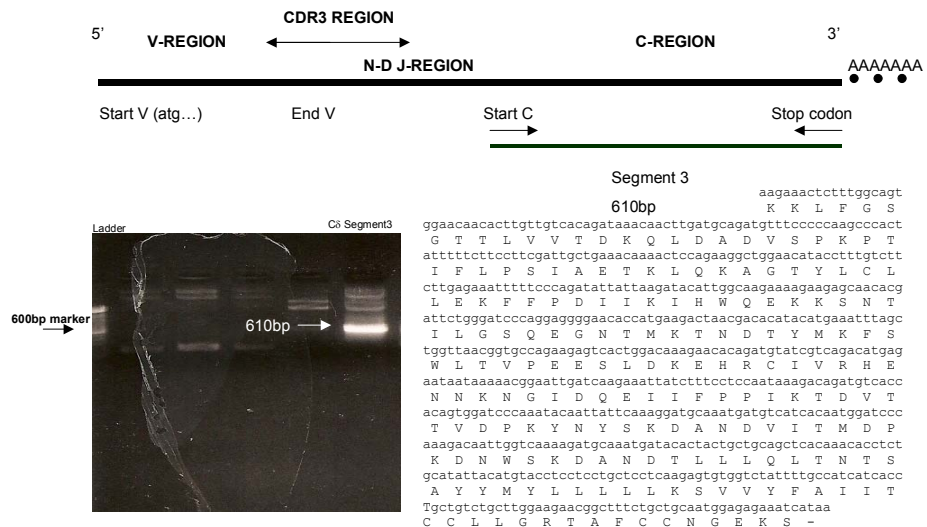


Figure 12 - V δ 2-chain TCR+ T-cell transcript (Segment 2) used for full-length construction of OV27T

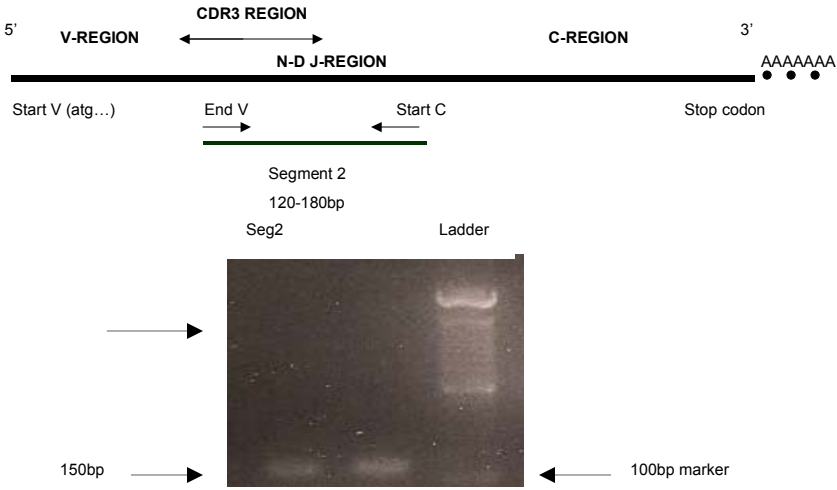


Figure 13 - V δ 2-chain TCR+ T-cell transcript (Segment 1+2+3) full-length construction of OV27T

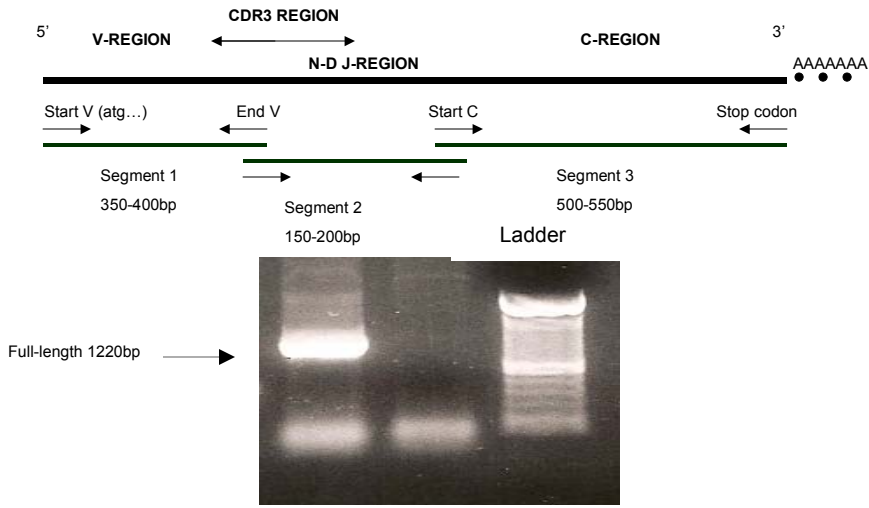


Table 32: CDR3 region of Transduced T cell lines

OV27T82.22y(9)II.13		
Solid Tumor Patient OVT27 Delta 2		
Clone V _δ	N D _δ 2 N	J _δ 1
22	Y Y C A C D T V L G D R	D K L I
	TACTACTGTGCCTGTGACACCGTA CTGGGGGATCGG	GATAAACTCATC
Solid Tumor OV27T Gamma (9)II		
Clone V _γ	N	J _γ 1.3/2.3
13	Y Y C A L W E V G E	L G K K I
	TACTACTGTGCCTTGTGGGAGGTGGGGAG	TTGGGCAAAAAATC
OV27T82.22y(9)II.3		
Solid Tumor Patient OVT27 Delta 2		
Clone V _δ	N D _δ 2 N	J _δ 1
22	Y Y C A C D T V L G D R	D K L I
	TACTACTGTGCCTGTGACACCGTA CTGGGGGATCGG	GATAAACTCATC
Solid Tumor OV27T Gamma (9)II		
Clone V _γ	N	J _γ 1.3/2.3
3	Y Y C A L W E V G	L G K K I
	TACTACTGTGCCTTGTGGGAGGTGGG	TTGGGCAAAAAATC
OV27T82.13y(9)II.3		
Solid Tumor Patient OVT27 Delta 2		
Clone V _δ	N D _δ 2 N	J _δ 1
13	Y Y C A C D T V T L G I N T	D K L I
	TACTACTGTGCCTGTGACACCGTA ACTCTGGGGATTAACACC	GATAAACTCATC
Solid Tumor OV27T Gamma (9)II		
Clone V _γ	N	J _γ 1.3/2.3
3	Y Y C A L W E V G G	L G K K I
	TACTACTGTGCCTTGTGGGAGGTGGG	TTGGGCAAAAAATC

Table 33: CDR3 region of Transduced T cell lines (Contd.)

OV27T82.13y(9)II.13		
SOLID TUMOR PATIENT OVT27 DELTA 2		
CLONE V _δ	N D _δ 2 N	J _δ 1
13	Y Y C A C D T V T L G I N T	D K L I
	TACTACTGTGCCTGTGACACCGTA ACTCTGGGGATTAACACC	GATAAACTCATC
SOLID TUMOR OV27T GAMMA (9)IICLONE		
CLONE V _γ	N	J _γ 1.3/2.3
13	Y Y C A L W E V G E	L G K K I
	TACTACTGTGCCTTGTGGGAGGTGGGGAG	TTGGGCAAAAAATC
OV27T82.6y(9)II.3		
SOLID TUMOR PATIENT OVT27 DELTA 2		
CLONE V _δ	N D _δ 2 N	J _δ 1
6	Y Y C A C D P L L G D P P N T	D K L I
	TACTACTGTGCCTGTGAC CCC TTACTGGGGATCCCCCAACACC	GATAAACTCATC
SOLID TUMOR OV27T GAMMA (9)II		
CLONE V _γ	N	J _γ 1.3/2.3
3	Y Y C A L W E V G	L G K K I
	TACTACTGTGCCTTGTGGGAGGTGGG	TTGGGCAAAAAATC
OV42T82.11y(9)II.7		
SOLID TUMOR PATIENT OVT42 DELTA2		
CLONE V _δ	N D _δ 2 N	J _δ 1
11	Y Y C A C D T V G T G G G T	D K L I
	TACTACTGTGCCTGTGACACCGTG GAACTGGGGGGGGCACC	GATAAACTCATC
SOLID TUMOR OV42T GAMMA (9)II		
CLONE V _γ	N	J _γ
7	Y Y C A L W E V P C	Y Y K K L F G
	TACTACTGTGCCTTGTGGGAGGTGCCGTG	TATTATAAGAACTCTTGGC
OV1T81.4y.11		
CELLS ISOLATED FROM SOLID TUMOR PATIENT OVT1 DELTA 1		
CLONE V _δ	N D _δ 2 N	J _δ 1
4	Y F C A L G E L P W S T G G Y A P	D K L I
	TACTTTTGTGCTCTTGGGAACTC CCATGGAGTACTGGGGATACGCACC	GATAAACTCATC
CELLS ISOLATED FROM SOLID TUMOR PATIENT OV1T GAMMA I		
CLONE V _γ	N	J _γ
11	Y Y C A T W D G	Y Y K K L F
	TATTACTGTGCCACCTGGATGGG	TATTATAAGAACTCTTGGC

Retroviral vector construction and transduction of target cell line J.RT3-T3.5

Three retroviral vectors, pMSCVneo, pMSCVpuro and pMSCVhygro (Clontech) were used, harboring the γ -chain or δ -chain cDNAs. Both constructs used the retroviral MSCV LTR to drive the expression of either γ - chain gene or δ - chain gene governed by an IRES or an internal promoter (Figure 4). These plasmid constructs were transfected into the NIH 3T3 packaging cell lines using the calcium precipitation method. 24h following transfection media with transfection reagents was removed and replaced with fresh media and the cells were incubated 48-72h prior to selection. The cell lines were selected for stable transfectants using G418, puromycin, or hygromycin antibiotics. Retroviral supernants were collected 10-14 days following selection and used to transduce the human T cell line J.RT3-T3.5. Following transduction and antibiotic selection CD3 and $\gamma\delta$ TCR expression was analyzed by FACS analysis. Clones exhibited both high physical titer and high transduction efficiency, and those with detectable expression levels were used for additional study.

F) Expression

To determine whether the J.RT3-T3.5 T cell lines expressed on the cell surface the $\gamma\delta$ TCR transduced FACs analysis using Flow Cytometry was performed.

OV27T δ 2.22 γ (9)II.13 $\gamma\delta$ TCR+ transduced T-cell line

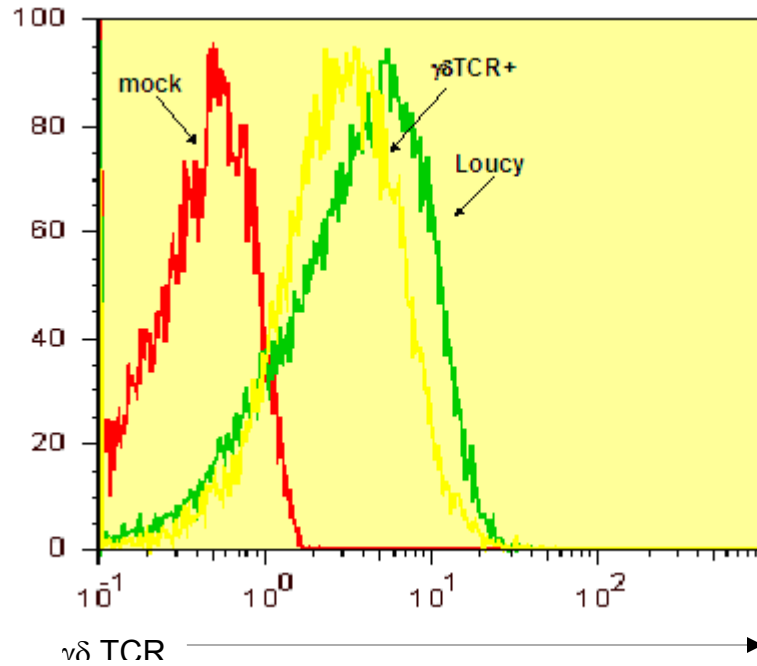


Figure 14: MSCV based retroviral expression in J.RT3-T3.5 Jurkat Mutant T cell line. Mock (red) cells infected with vector only, Loucy (green) $\gamma\delta$ TCR+ T cell line and OV27T δ 2.22 γ (9)II.13 (yellow) $\gamma\delta$ TCR+ transduced T-cell line. The results are from one representative experiment of three performed.

OV27T δ 2.22 γ (9)II.3 $\gamma\delta$ TCR+ transduced T-cell line

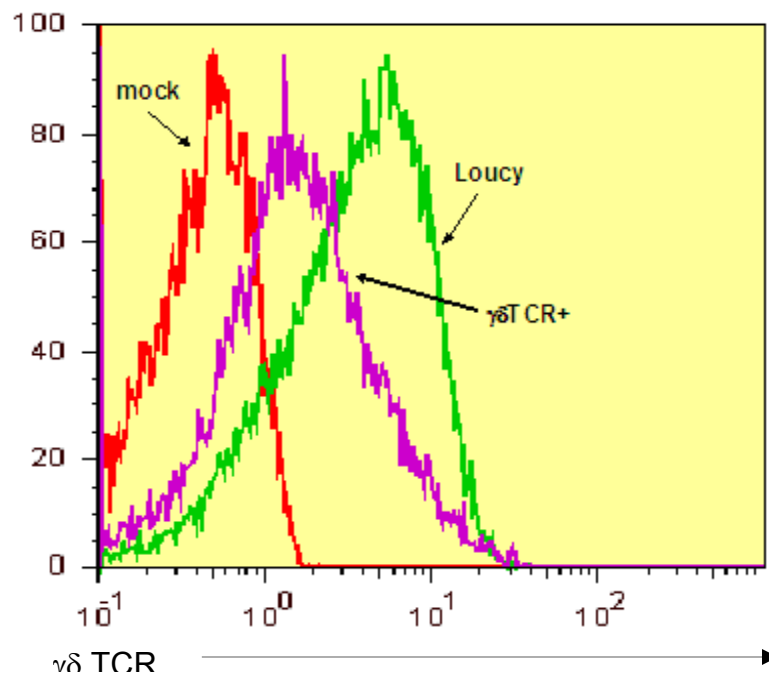


Figure 15: MSCV based retroviral expression in J.RT3-T3.5 Jurkat Mutant T cell line. Mock (red) cells infected with vector only, Loucy (green) $\gamma\delta$ TCR+ T cell line and OV27T δ 2.22 γ (9)II.3 (purple) $\gamma\delta$ TCR+ transduced T-cell line. The results are from one representative experiment of three performed.

OV27T δ 2.13 γ (9)II.13 $\gamma\delta$ TCR+ transduced T-cell line

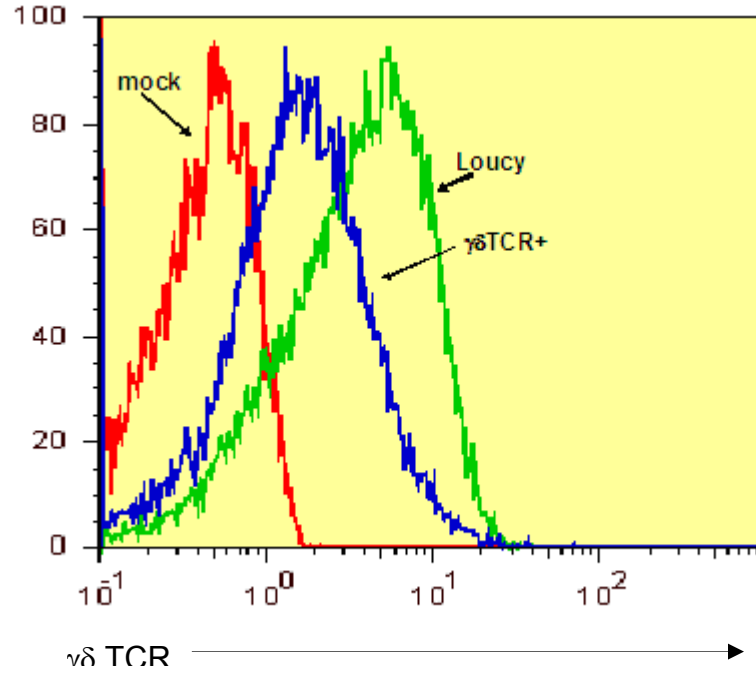


Figure 16: MSCV based retroviral expression in J.RT3-T3.5 Jurkat Mutant T cell line. Mock (red) cells infected with vector only, Loucy (green) $\gamma\delta$ TCR+ T cell line and OV27T δ 2.13 γ (9)II.13 (blue) $\gamma\delta$ TCR+ transduced T-cell line. The results are from one representative experiment of three performed.

OV27T δ 2.13 γ (9)II.3 $\gamma\delta$ TCR+ transduced T-cell line

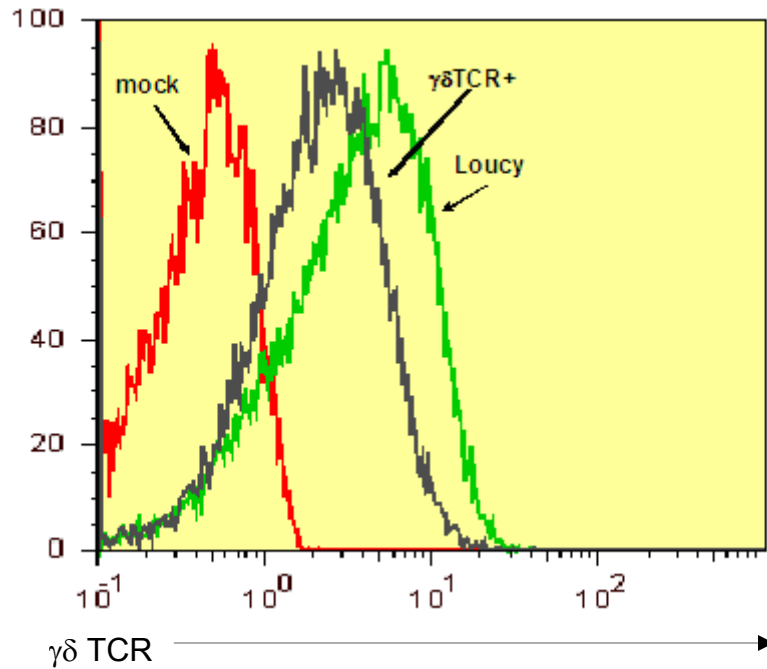


Figure 17: MSCV based retroviral expression in J.RT3-T3.5 Jurkat Mutant T cell line. Mock (red) cells infected with vector only, Loucy (green) $\gamma\delta$ TCR+ T cell line and OV27T δ 2.13 γ (9)II.3 (black) $\gamma\delta$ TCR+ transduced T-cell line. The results are from one representative experiment of three performed.

OV27T δ 2.6 γ (9)II.3 $\gamma\delta$ TCR+ transduced T-cell line

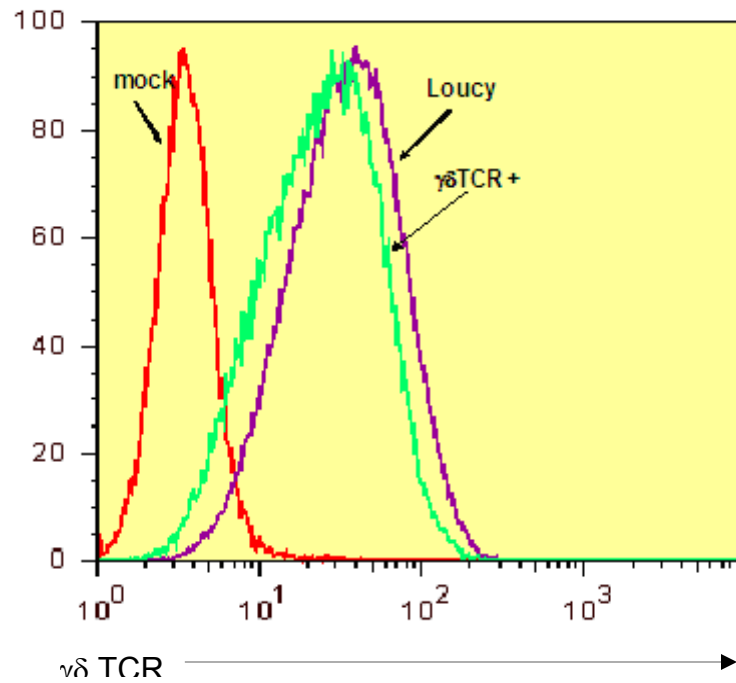


Figure 18: MSCV based retroviral expression in J.RT3-T3.5 Jurkat Mutant T cell line. Mock (red) cells infected with vector only, Loucy (purple) $\gamma\delta$ TCR+ T cell line and OV27T δ 2.13 γ (9)II.3 (green) $\gamma\delta$ TCR+ transduced T-cell line. The results are from one representative experiment of three performed.

The yield of $\gamma\delta$ TCR+ T-cells following transduction procedures varied. Under the present transduction method the $\gamma\delta$ TCR+ T-cells positive control, Loucy cell line, expressed detectable levels of $\gamma\delta$ TCR (81.4%; Figure 14, green). The cells transduced with vector only expressed undetectable levels of $\gamma\delta$ TCR (0.05%; Figure 14, red). The cell lines transduced with full-length $\gamma\delta$ TCR and expressed detectable levels of $\gamma\delta$ TCR following multiple transductions were used for subsequent cytotoxicity and caspase assays. Those include OV27T δ 2.22 γ (9)II.13 (58.32%; Figure 14, yellow), OV27T δ 2.22 γ (9)II.3 (64.3%; Figure 15, purple), OV27T δ 2.13 γ (9)II.13 (49.5%; Figure 16, blue), OV27T δ 2.13 γ (9)II.3 (53.7%; Figure 17, black), and OV27T δ 2.6 γ (9)II.3 (72.3%; Figure 18, green).

G) Caspase activation in ovarian cells following incubation with $\gamma\delta$ TCR+ transduced T cell lines

To establish whether transduced cell lines expressing $\gamma\delta$ TCR+ T cells would induce apoptosis via caspase activation ovarian cell lines were incubated with transduced $\gamma\delta$ TCR+ and caspase-8, -9, and 3 activities were measured. Caspase 9 and Caspase 8 activities were observed following 30-45min co-incubations. The luminescence was measured in relative light units (RLU), which indicates the presence or absence of caspase activity.

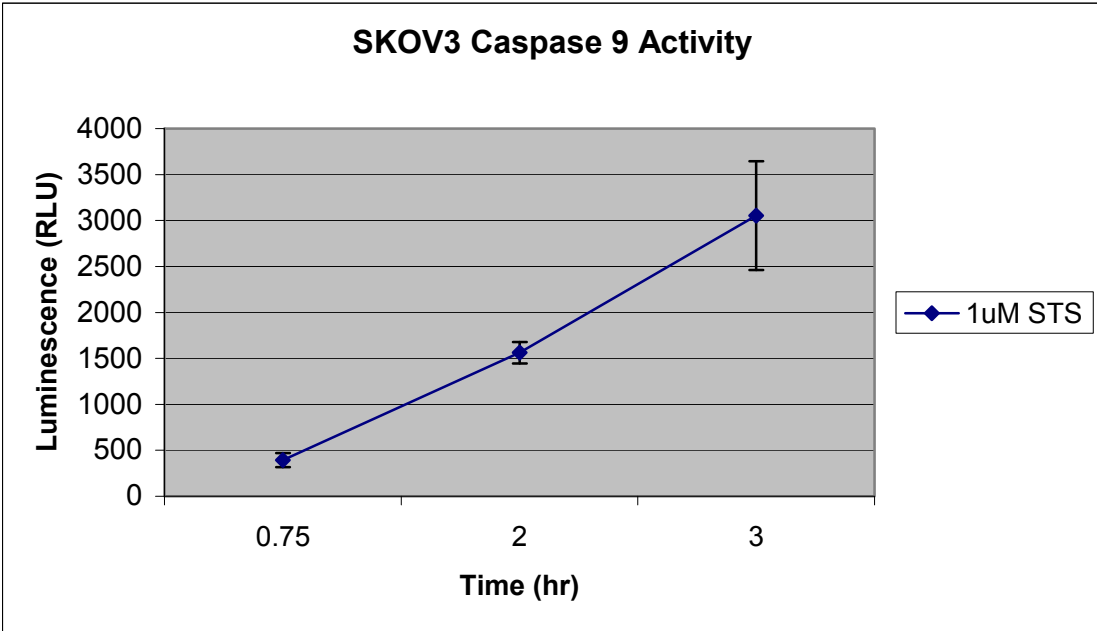


Figure 19: 1×10^4 SKOV3 cells were measured for caspase 9 activity following 1uM STS treatment at .75, 2 and 3hrs. Caspase 9 activity was measured by luminescence (RLU).

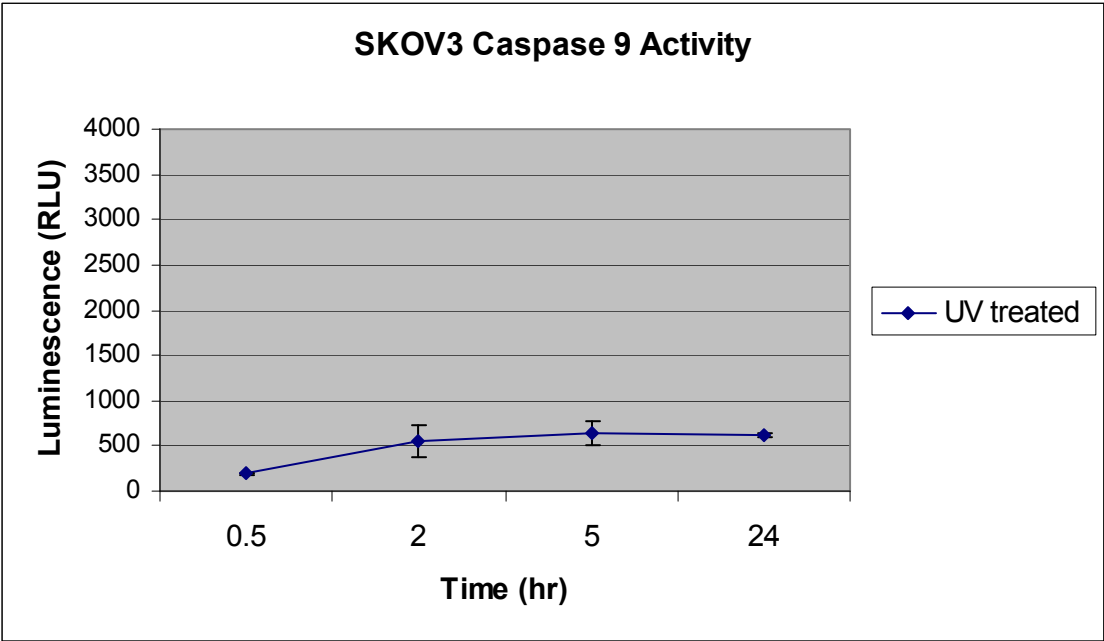


Figure 20: 1×10^4 SKOV3 cells were measured for caspase 9 activity following UV treatment at .5, 2, 5 and 24hrs. Caspase 9 activity was measured by luminescence (RLU).

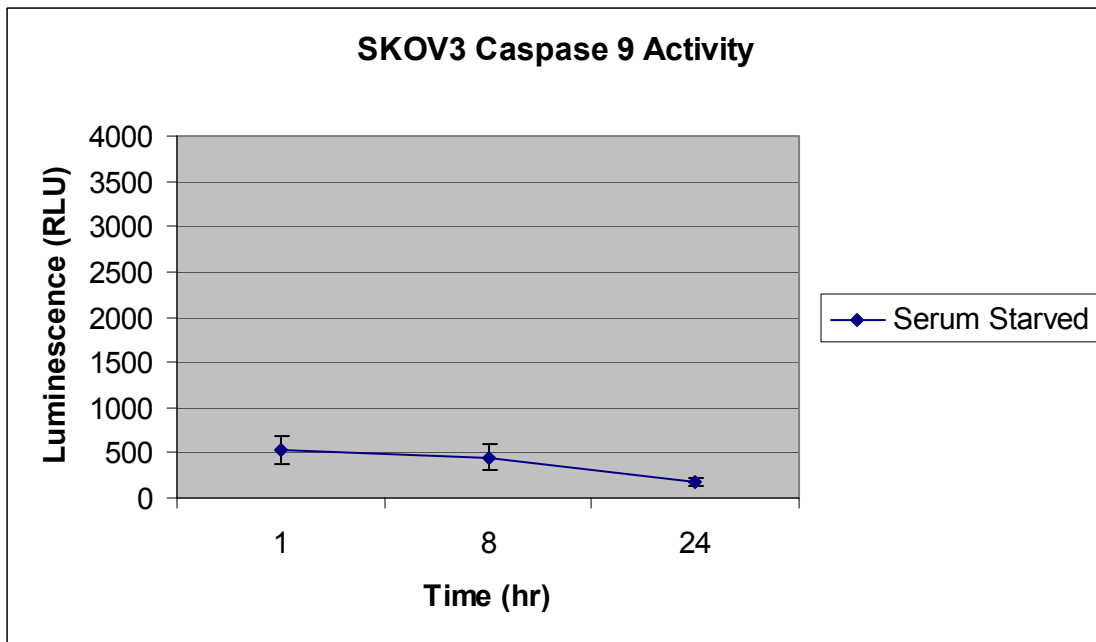


Figure 21. 1×10^4 SKOV3 cells were measured for caspase 9 activity following following serum starvation at 1, 8 and 24hrs. Caspase 9 activity was measured by luminescence (RLU).

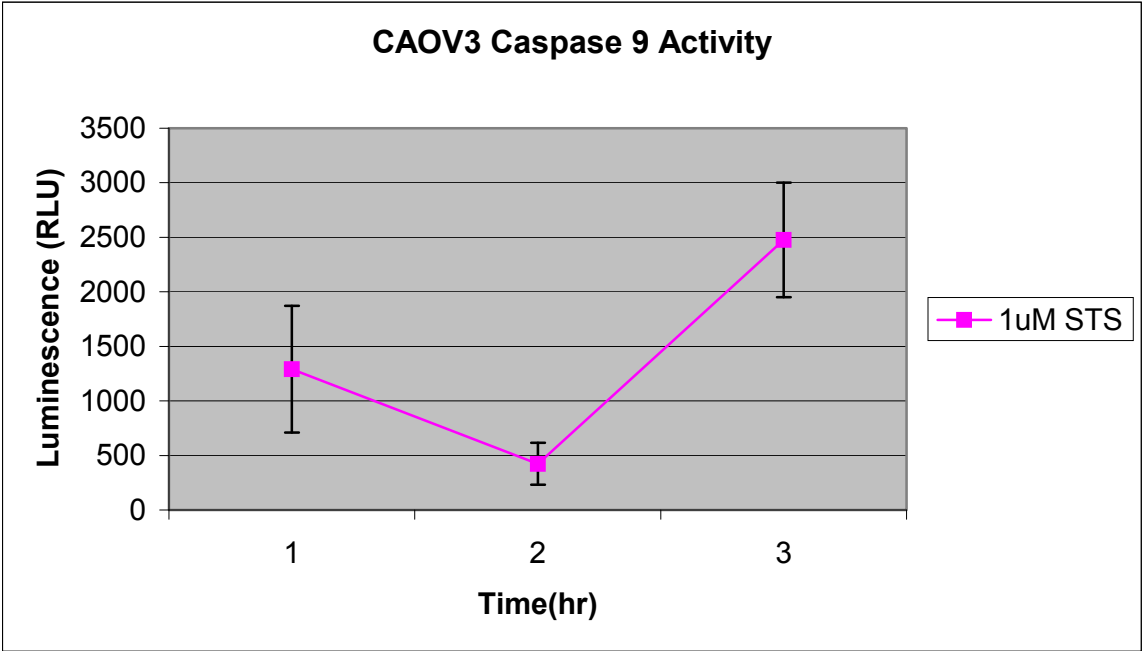


Figure 22. 1×10^5 CAOV3 cells were measured for caspase 9 activity following incubation following 1uM STS with $\gamma\delta$ TCR+ transduced T-cell line.

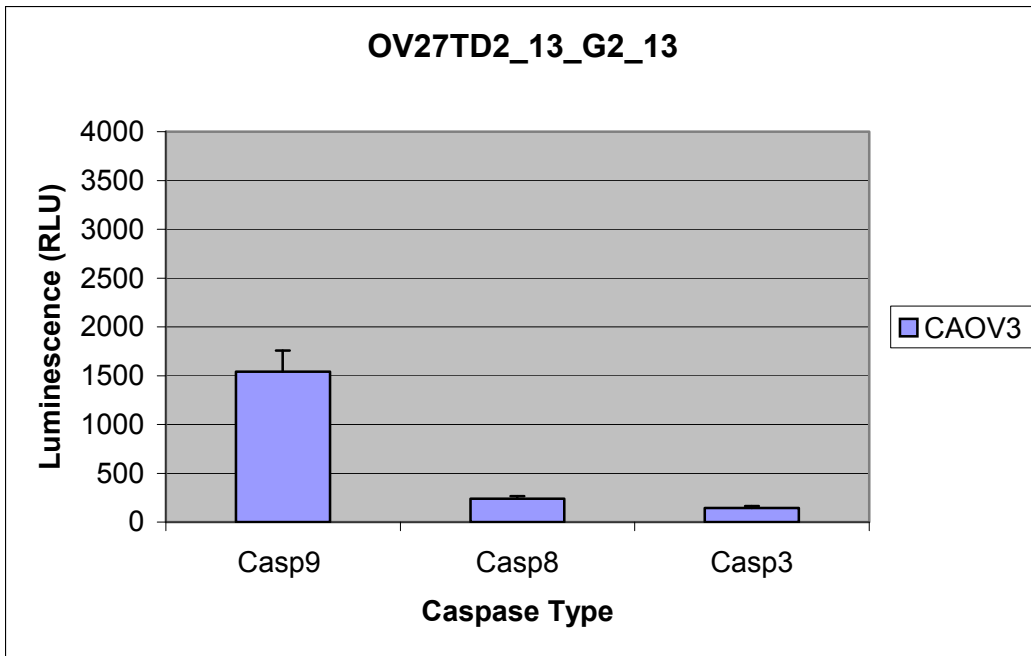


Figure 23. 1×10^5 CAOV3 cells were measured for caspase 9, 8 and 3 activity following 5 hours of incubation with $\gamma\delta$ TCR+ transduced T-cell line.

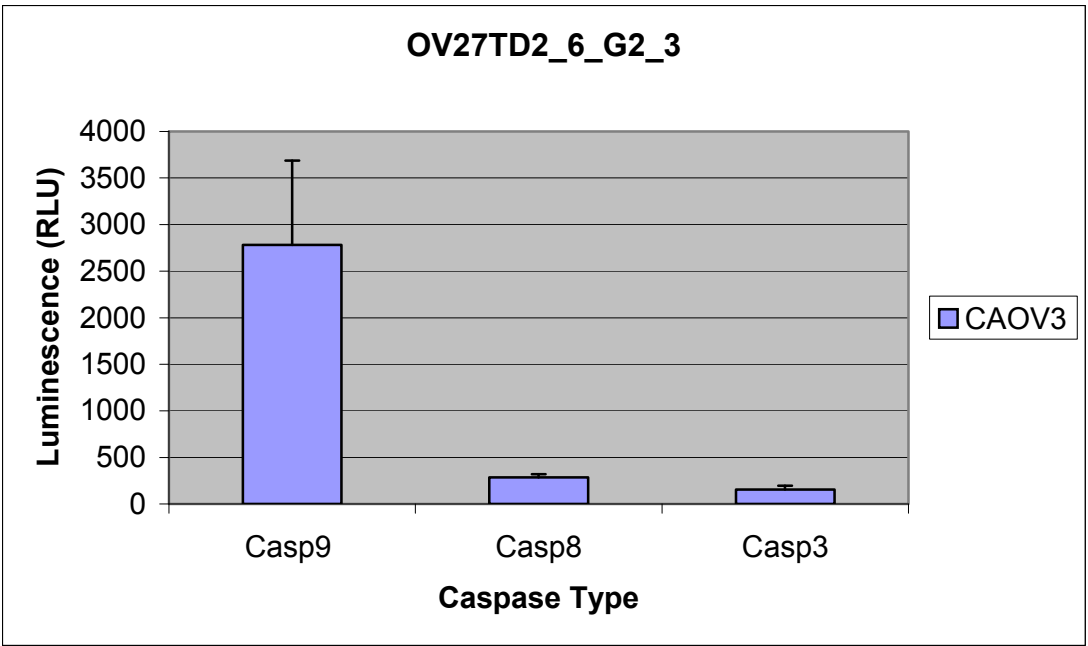


Figure 24. 1×10^5 CAOV3 cells were measured for caspase 9, 8 and 3 activity following 5 hours of incubation with $\gamma\delta$ TCR+ transduced T-cell line.

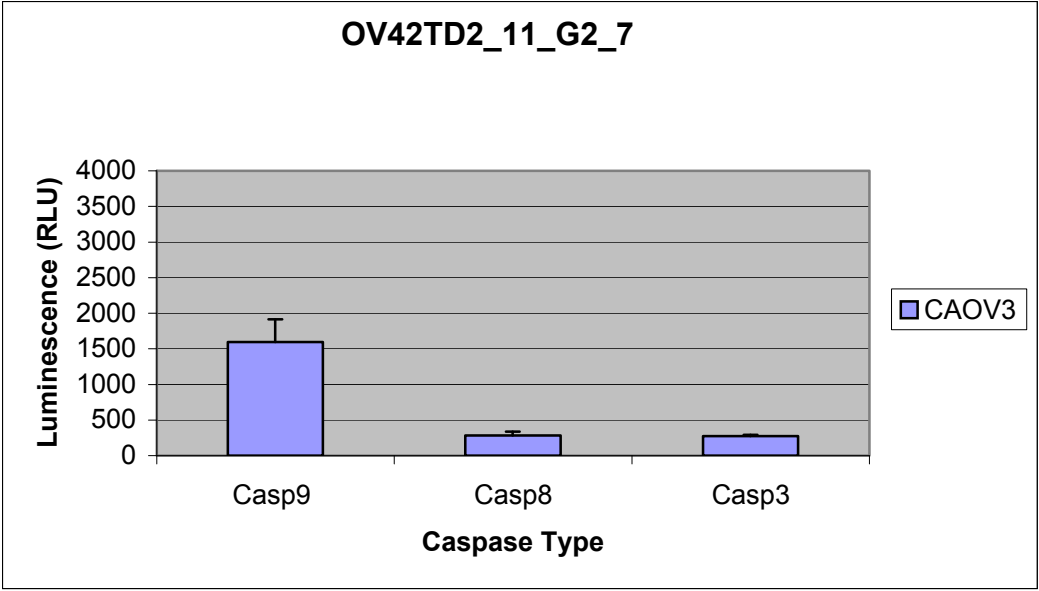


Figure 25. 1×10^5 CAOV3 cells were measured for caspase 9, 8 and 3 activity following 5 hours of incubation with $\gamma\delta$ TCR+ transduced T-cell line.

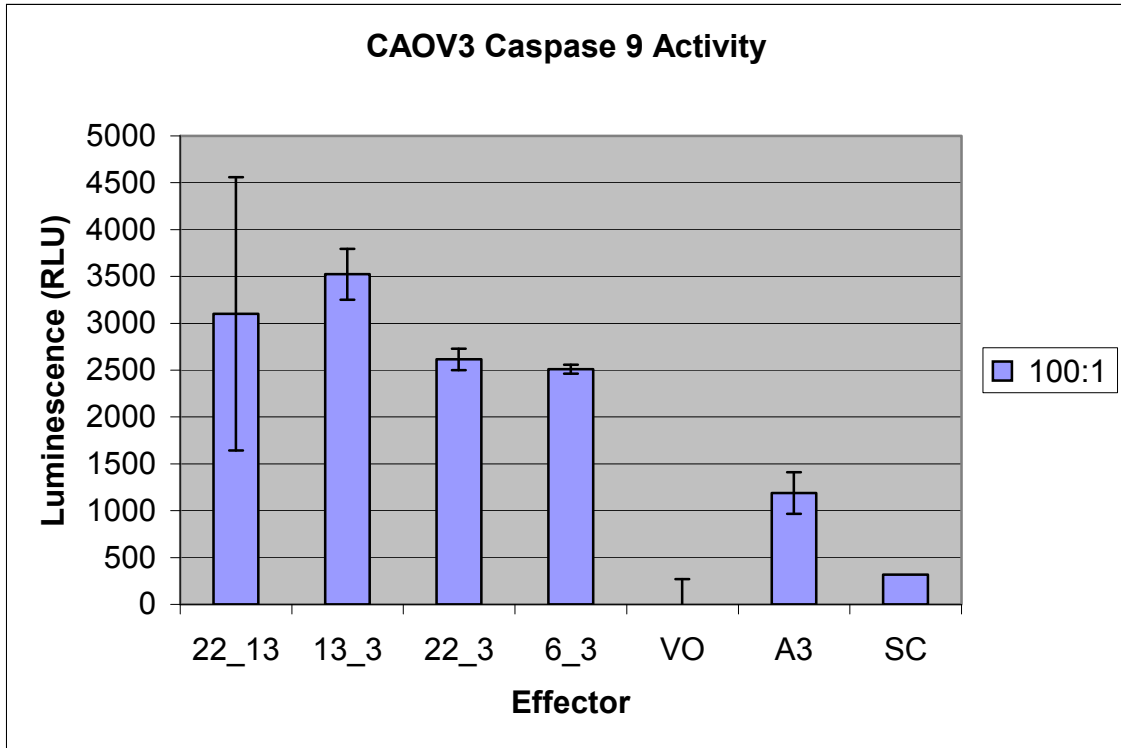


Figure 26. Summary of Caspase 9 activity in CAOV3 cells following 5hrs incubation with $\gamma\delta$ TCR+ transduced T-cell line, vector only (VO), control (A3) and single chain (SC) cells.

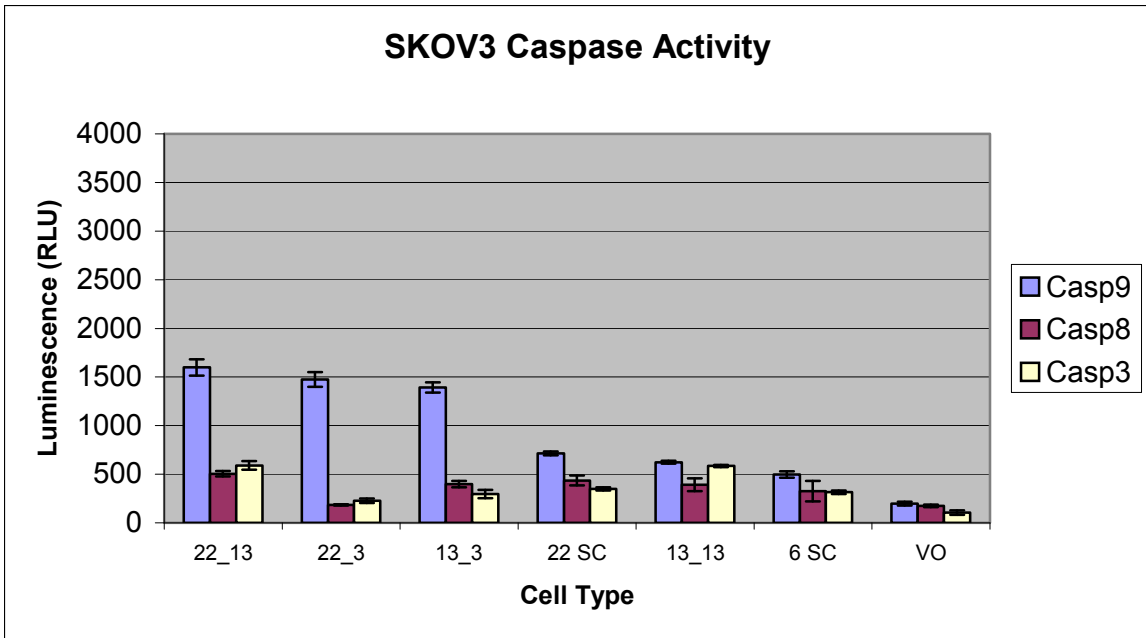


Figure 27. Summary of Caspase 9, 8 and 3 activity in SKOV3 cells following 5hrs incubation with $\gamma\delta$ TCR+ transduced T-cell line, vector only (VO) and single chain (SC) cells.

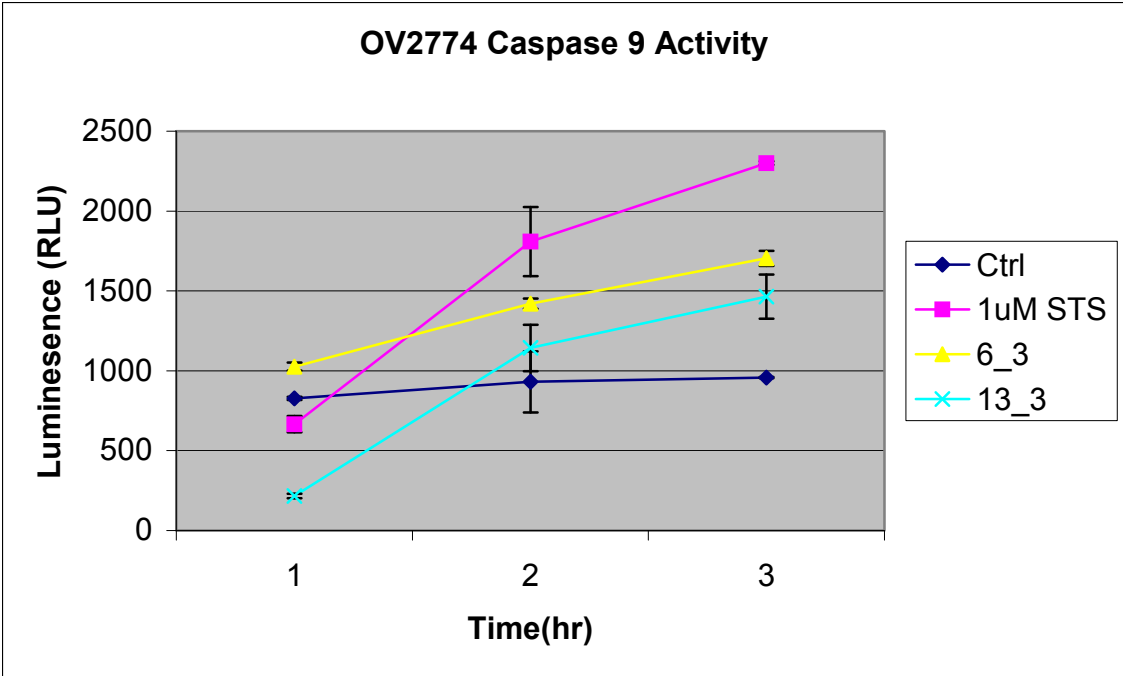


Figure 28. Caspase 9 activity in 1×10^5 OV2774 cells following incubation with 1uM STS or $\gamma\delta$ TCR+ T-cell lines.

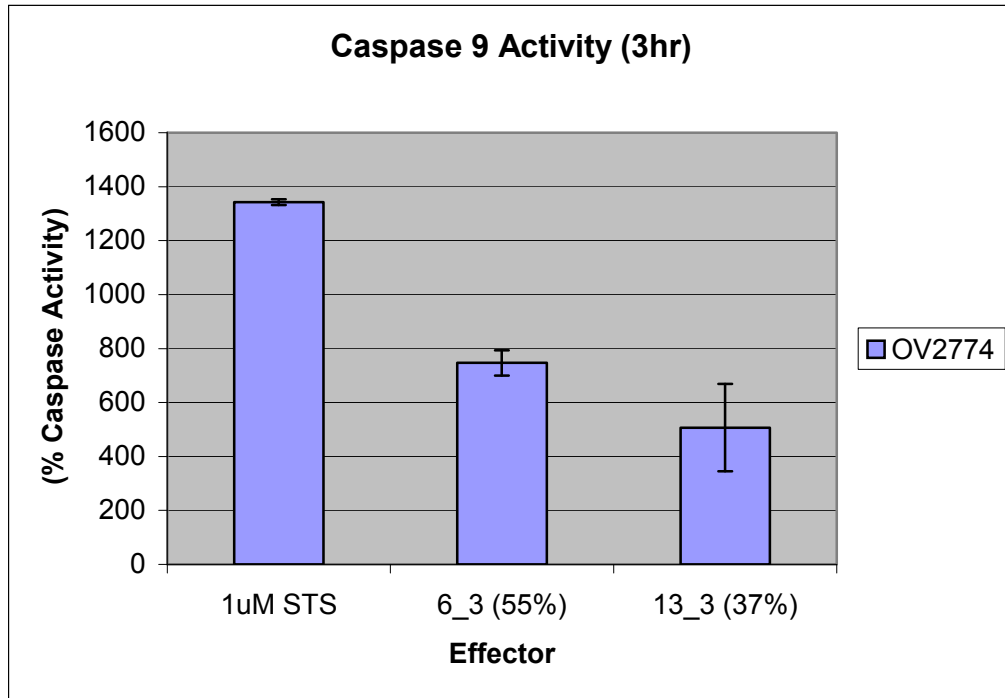


Figure 29. Caspase 9 activity following incubation with 1uM STS or $\gamma\delta$ TCR+ T-cell lines measured in percent (%) of maximum.

The ability of $\gamma\delta$ TCR+ T-cells to induce caspase activity on ovarian tumor cell lines was observed using the Jurkat transduced cells and the ovarian tumor cell lines during co-incubation experiments. However, the caspase activity induced on SKOV3 cells following staurosporine treatment, UV irradiation, and serum starvation was performed. Once we identified the amount of caspase activity generated from a known apoptotic inducing agent we compare this to the amount of caspase activation induced (Figure 19, Figure 20 and Figure 21) by our genetically engineered $\gamma\delta$ TCR+ T-cell lines). Using CAOV3 cells following 1 μ M STS the level of caspase activation measured in 2475.3 RLU (Figure 22). Using TCR transduced cell lines as effectors and CAOV3 as target tumor cells OV27TD2_13_G2_13 induced caspase activity on tumor cells in the amount of 1540.5 RLU (Figure 22), OV27TD2_6_G2_3 induced caspase activity on tumor cells in the amount of 2781.1 RLU (Figure 24), OV42TD2_11_G2_7 induced caspase activity on tumor cells in the amount of 1594.6 RLU (Figure 25), OV27TD2_22_G2_3 induced caspase activity on tumor cells in the amount of 2615.3 RLU (Figure 26), and OV27TD2_22_G2_13 induced caspase activity on tumor cells in the amount of 3100.6 RLU (Figure 26).

Using SKOV3 cells following 1 μ M STS treatment the level of caspase activation measured in luminescence (RLU) was 3053. Using TCR transduced cell lines as effectors and SKOV3 as target tumor cells OV27TD2_22_G2_13 induced caspase activity on tumor cells measured in luminescence (RLU) in the amount of 1599 (Figure 27), OV27TD2_22_G2_3 induced caspase activity on tumor cells measured in luminescence (RLU) in the amount of 1474 (Figure 27),

OV27TD2_13_G2_3 induced caspase activity on tumor cells measured in luminescence (RLU) in the amount of 1393 (Figure 27), and OV27TD2_13_G2_13 induced caspase activity on tumor cells measured in luminescence (RLU) in the amount of 623 (Figure 27).

Using OV2774 cells following 1uM STS treatment the level of caspase activation measured in luminescence (RLU) was 1343. Using TCR transduced cell lines as effectors and OV2774 as target tumor cells OV27TD2_6_G2_3 induced caspase activity on tumor cells measured in luminescence (RLU) in the amount of 747 (Figure 29), and OV27TD2_13_G2_3 induced caspase activity on tumor cells measured in luminescence (RLU) in the amount of 507 (Figure 29).

H) Recognition and cytotoxicity

Ovarian carcinoma cell lines (SKOV3, CAOV3 and OV2774) were observed for cytotoxicity as a result of incubation with $\gamma\delta$ TCR+ transduced T cell lines at ratios of effector to target cells (100:1, 50:1, 40:1, 25:1, and/or 10:1).

Previous studies have demonstrated that activated $\gamma\delta$ TCR+ T cells exhibit a lytic activity against a variety of tumor cells (Groh et. al., 1999 and Zocchi et. al., 1990). Furthermore $V\delta 1+$ T cells were shown to recognize ovarian cancer cells by production of $IFN\gamma$ and cytotoxicity (Maeurer et. al., 1996 and Chen et. al., 2001). To investigate this question, genetically engineered $\gamma\delta$ TCR+ T cell lines were incubated with SKOV3, CAOV3 or OV2774 ovarian tumor cell lines as well as control cell lines and cytotoxicity was measured.

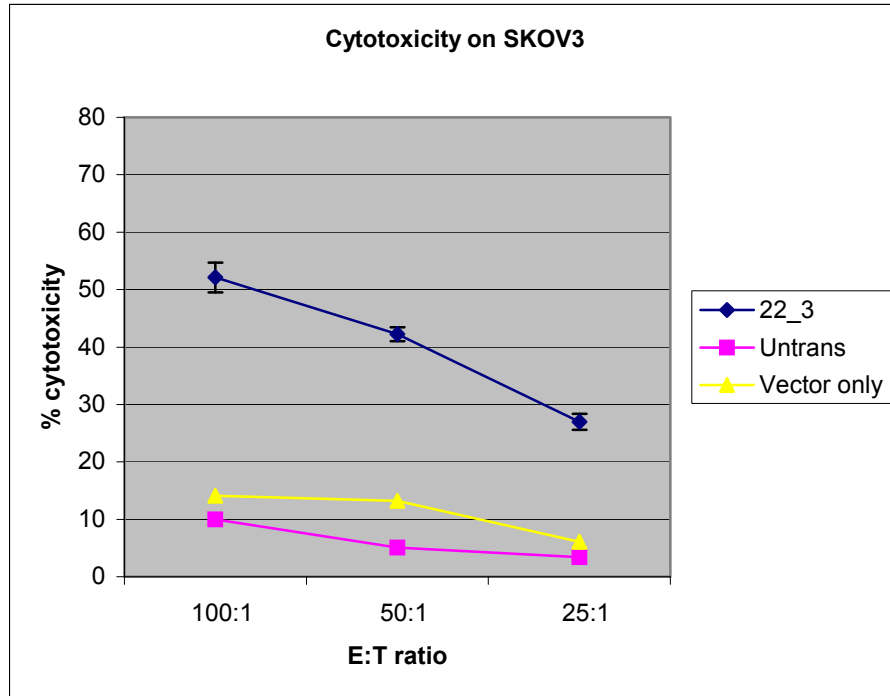


Figure 30. Cytotoxicity on 1×10^4 SKOV3 cells was measured following 5.5 hours incubation with vector only, untransduced T-cell line and genetically engineered $\gamma\delta$ TCR+ T-cell line 22_3.

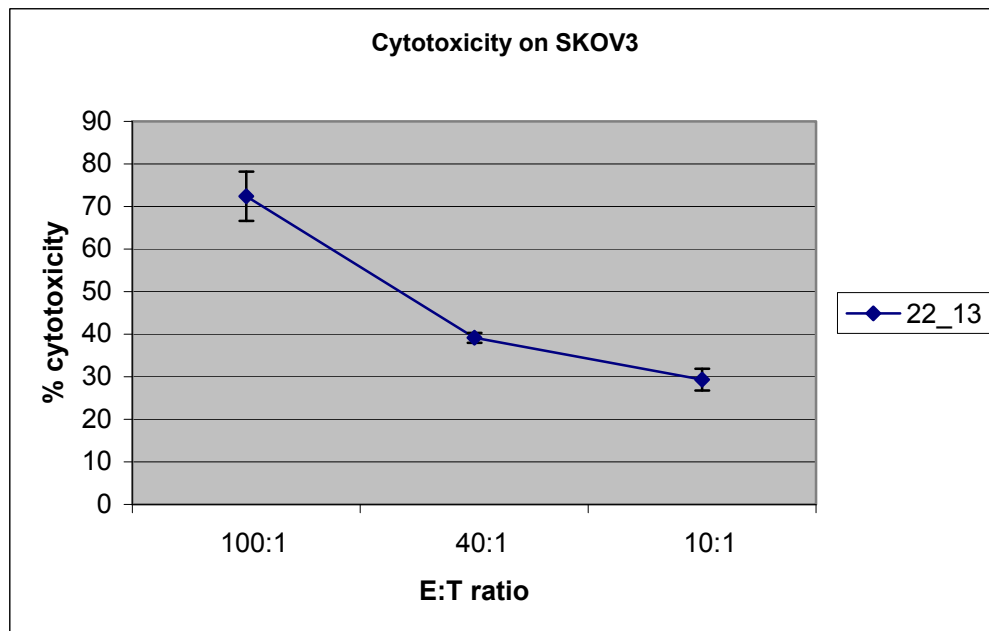


Figure 31. Cytotoxicity on 1×10^4 SKOV3 cells was measured following 5.5 hours incubation with genetically engineered $\gamma\delta$ TCR+ T-cell line 22_13.

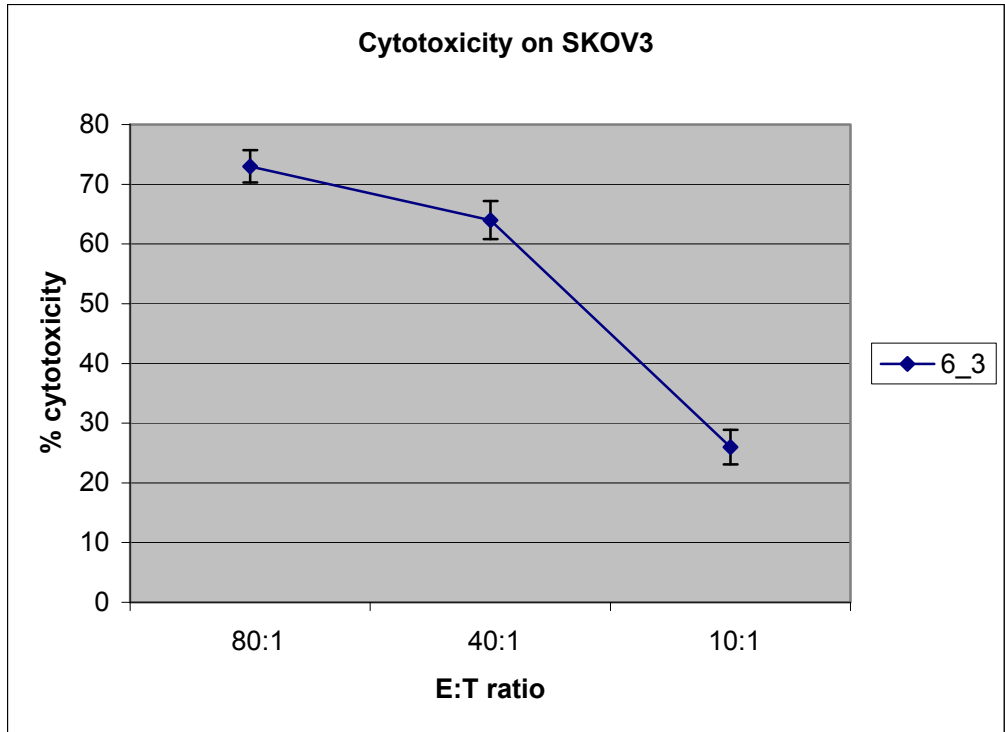


Figure 32. Cytotoxicity on 1×10^4 SKOV3 cells was measured following 5.5 hrs incubation with genetically engineered $\gamma\delta$ TCR+ T-cell line 6_3.

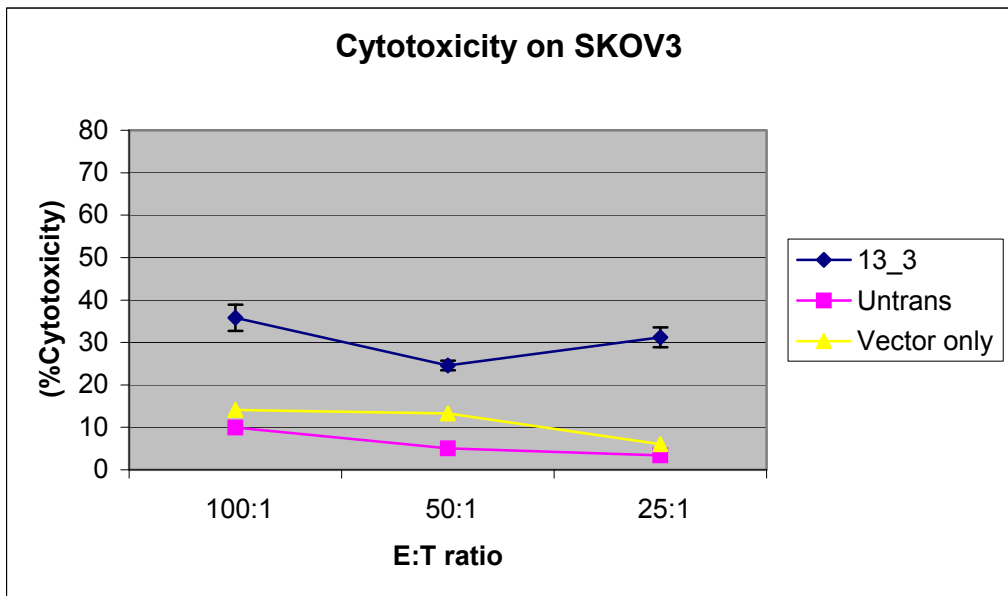


Figure 33. Cytotoxicity on 1×10^4 SKOV3 cells was measured following 5.5 hrs incubation with genetically engineered $\gamma\delta$ TCR+ T-cell lines.

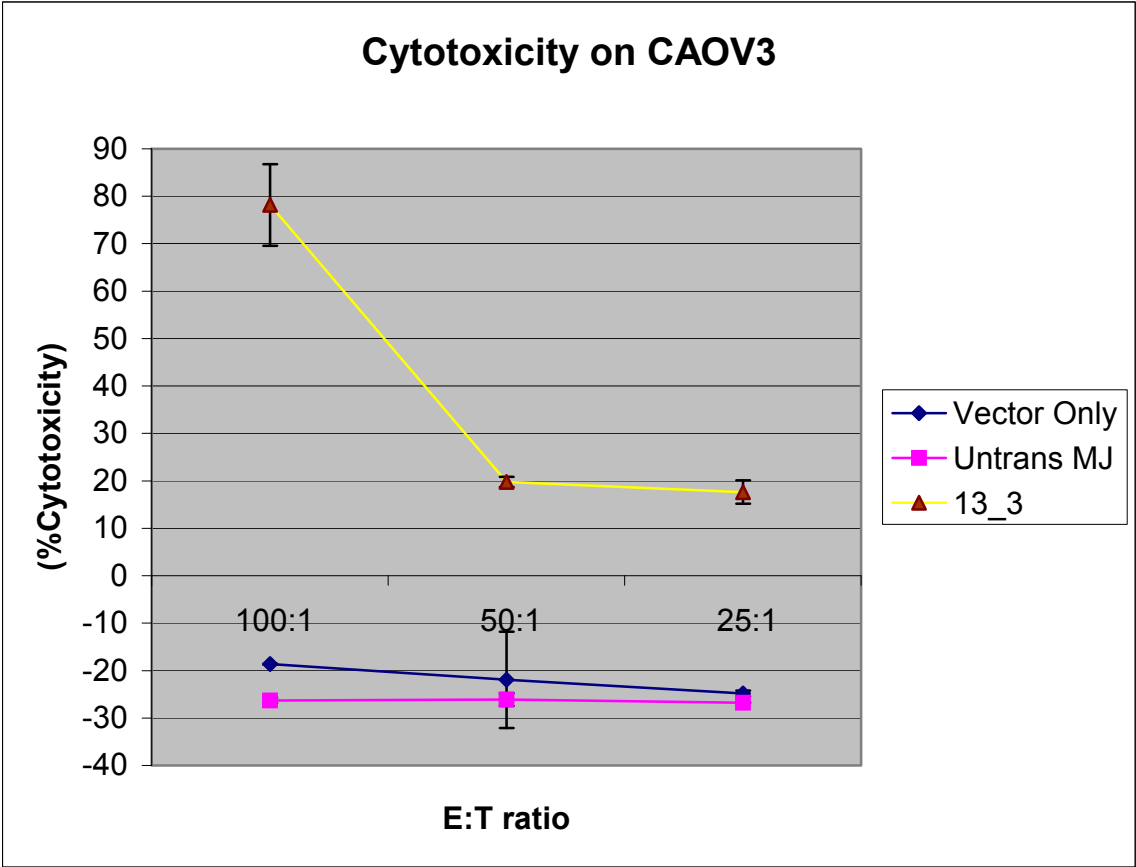


Figure 34. Cytotoxicity on 1×10^4 CAOV3 cells was measured following 5.5 hr incubation with genetically engineered $\gamma\delta$ TCR+ T-cell line 13_3.

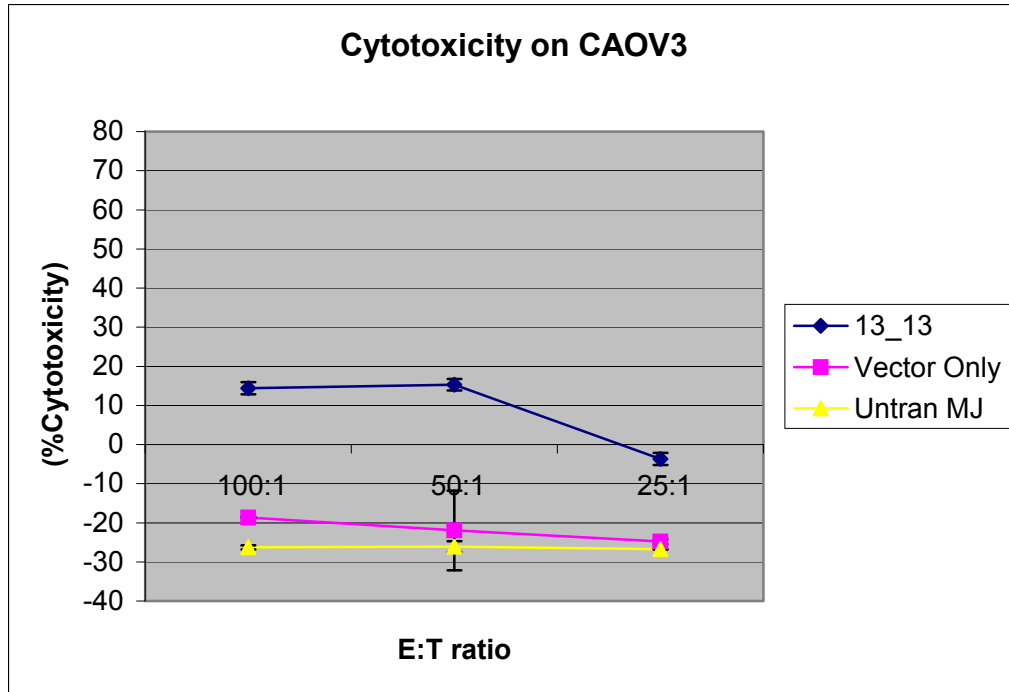


Figure 35. Cytotoxicity on 1×10^4 CAOV3 cells was measured following 5.5 hrs incubation with genetically engineered $\gamma\delta$ TCR+ T-cell line 13_13.

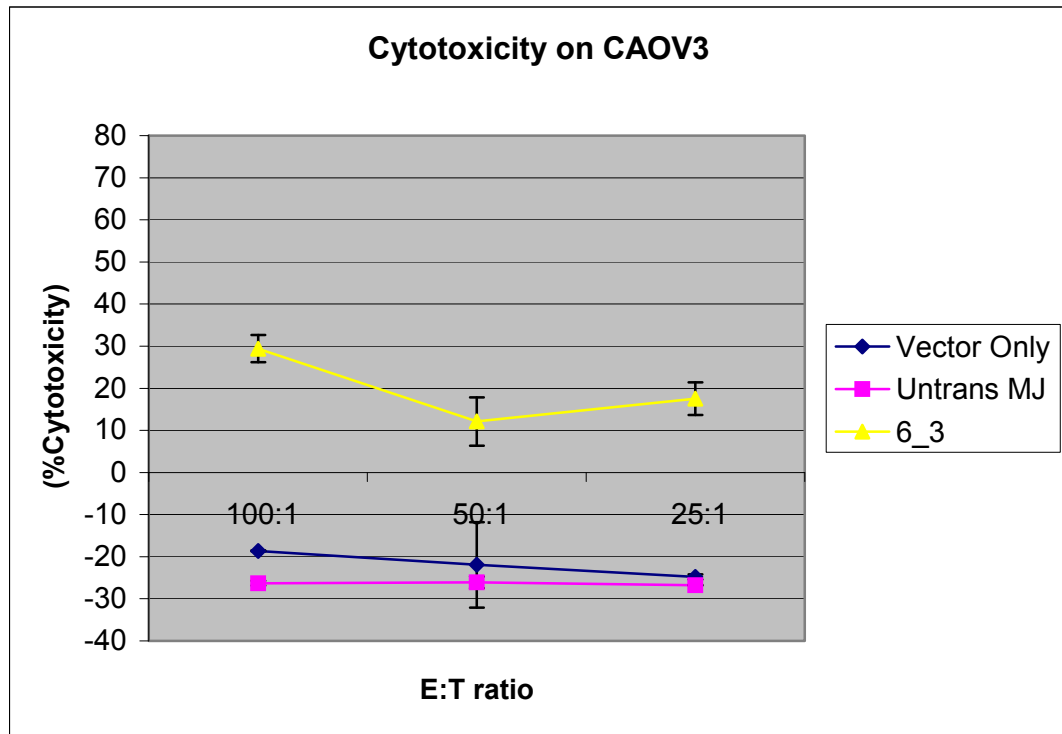


Figure 36. Cytotoxicity on 1×10^4 CAOV3 cells was measured following 5.5 hrs incubation with genetically engineered $\gamma\delta$ TCR+ T-cell line 6_3.

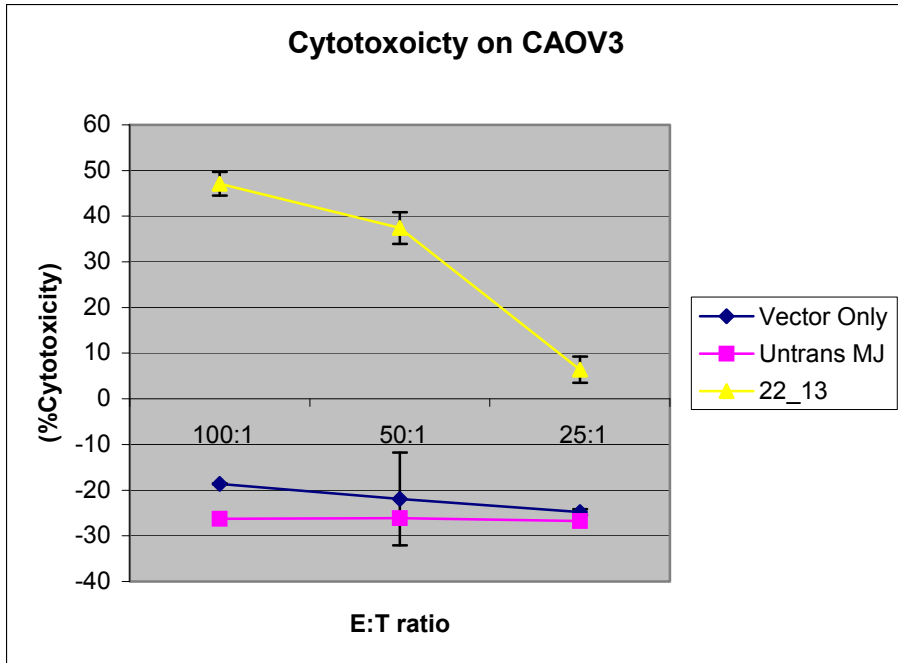


Figure 37. Cytotoxicity on 1×10^4 CAOV3 cells was measured following 5.5 hrs incubation with genetically engineered $\gamma\delta$ TCR+ T-cell line 22_13.

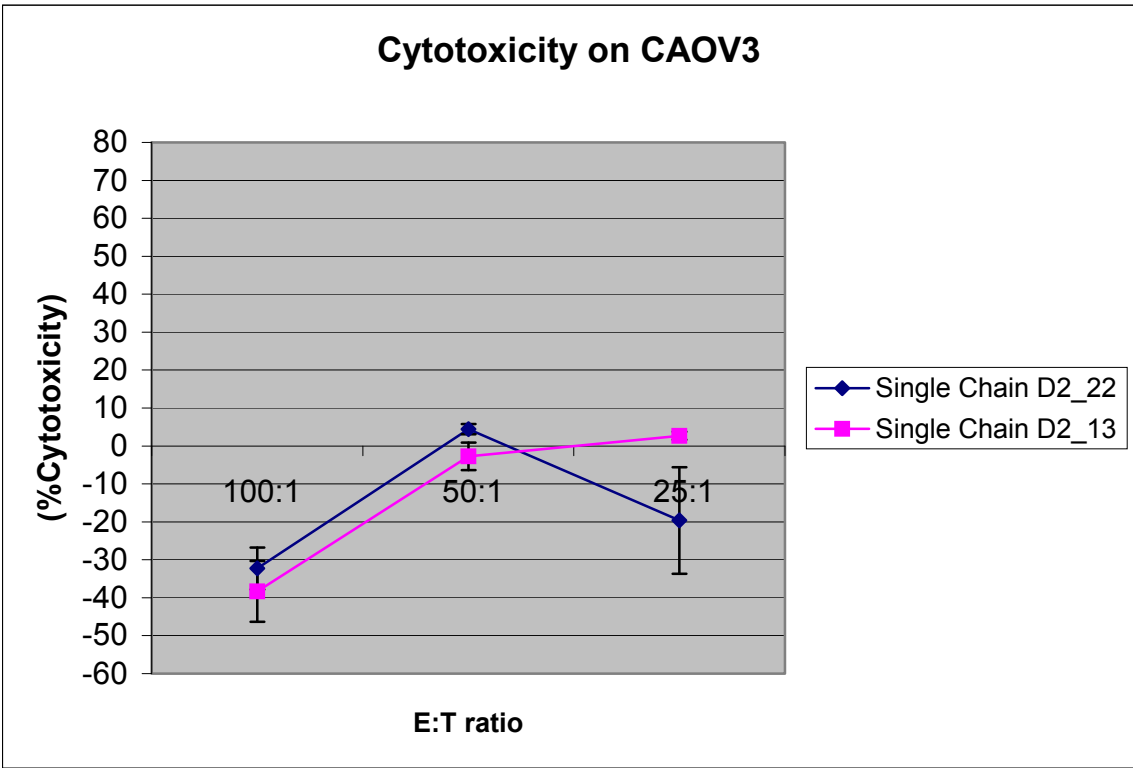


Figure 38. Cytotoxicity on 1×10^4 CAOV3 cells was measured following 5.5 hrs incubation with genetically engineered single chain δ TCR+ T-cell lines.

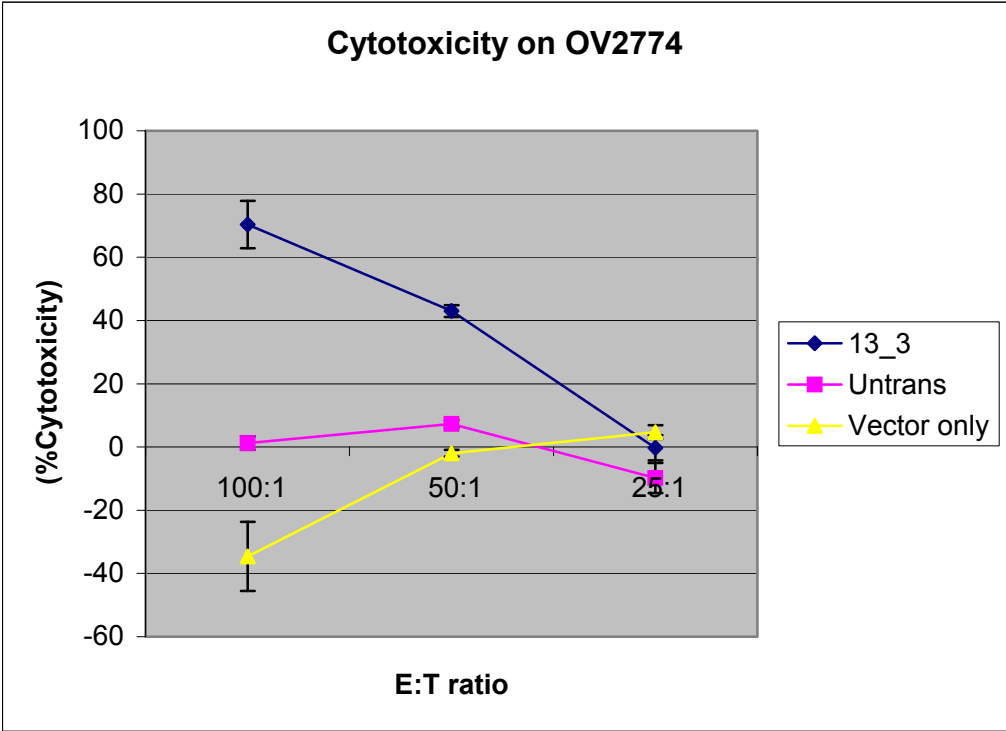


Figure 39. Cytotoxicity on 1×10^4 OV2774 cells was measured following 5.5 hrs incubation with genetically engineered $\gamma\delta$ TCR+ T-cell line 13_3.

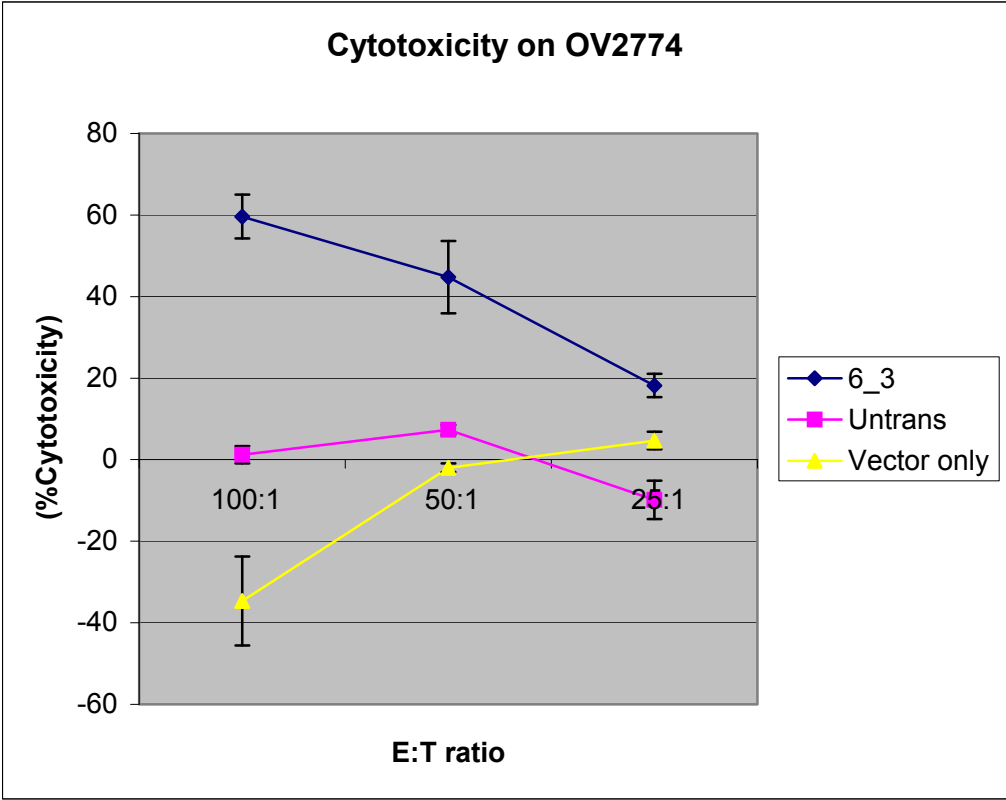


Figure 40. Cytotoxicity on 1×10^4 OV2774 cells was measured following 5.5 hr incubation with genetically engineered $\gamma\delta$ TCR+ T-cell line 6_3.

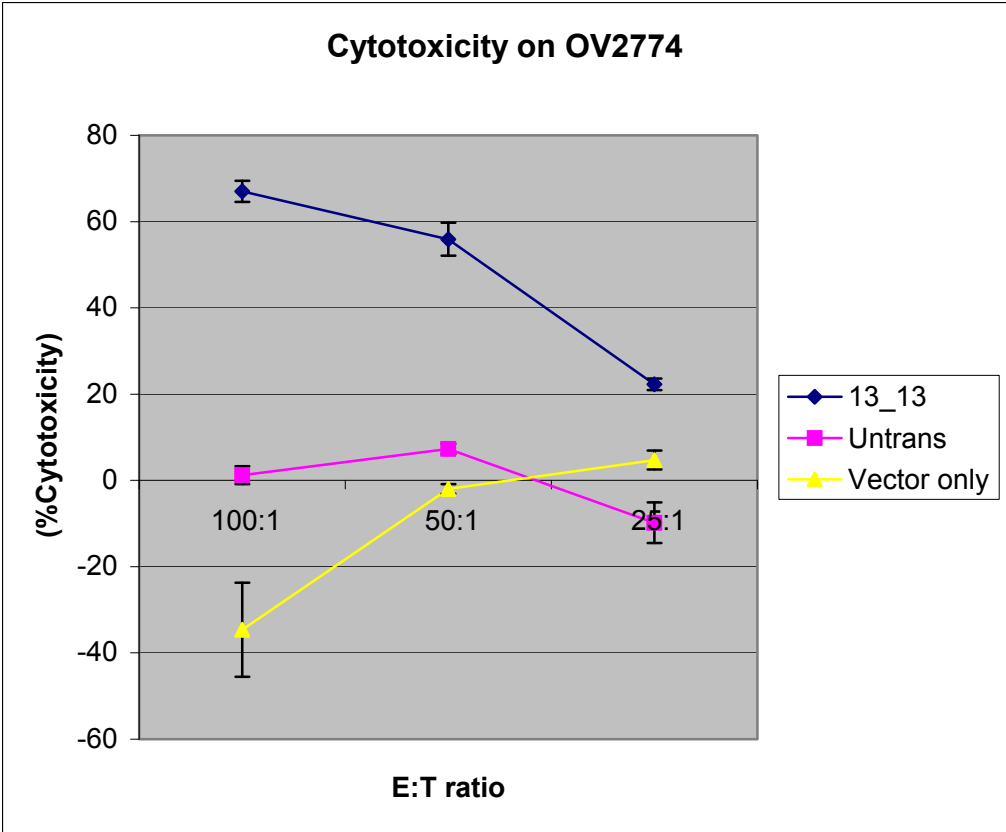


Figure 41. Cytotoxicity on 1×10^4 OV2774 cells were measured following 5.5 hr incubation with genetically engineered $\gamma\delta$ TCR+ T-cell line 13_13.

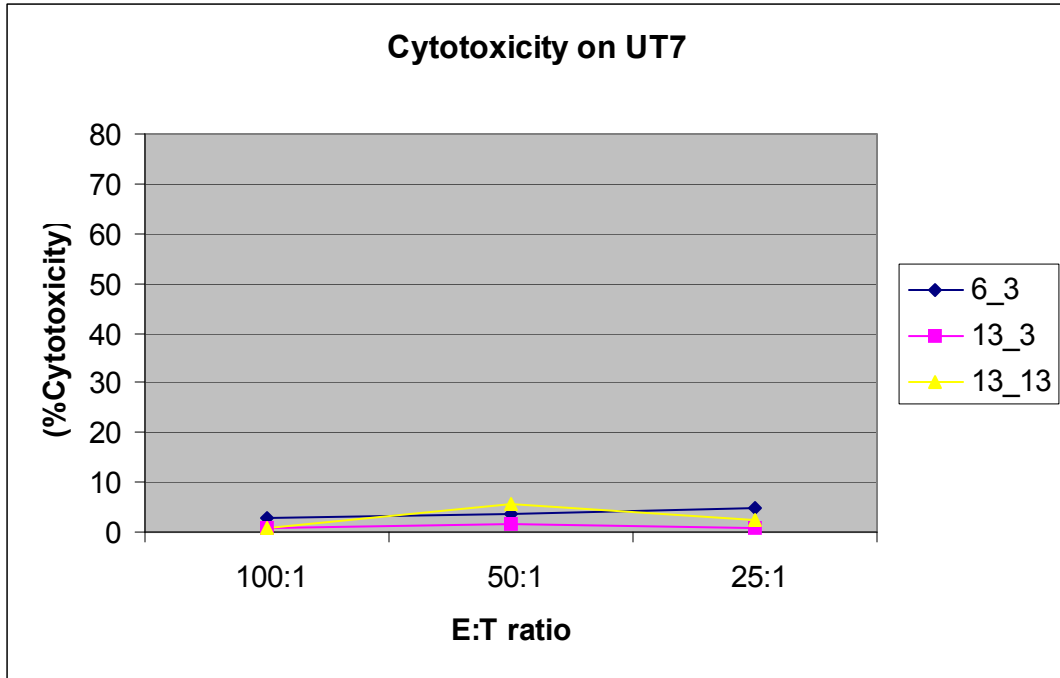


Figure 42. Cytotoxicity on 1×10^4 UT7 cells were measured following 5.5 hr incubation with genetically engineered $\gamma\delta$ TCR+ T-cell lines 6_3, 13_3 and 13_13.

Table 33. $\gamma\delta$ TCR+ T-cell lines recognition of SKOV3 cell line

%Clonal Expansion	Cell Line	%Cytotoxicity	Casp 9
24/84	22_13	39.2	3200
27/26	22_3	42.9	2949
36/26	13_3	24.6	2787
3/26	6_3	64	nd

Table 34. $\gamma\delta$ TCR+ T-cell lines recognition of CAOV3 cell line

%Clonal Expansion	Cell Line	%Cytotoxicity	Casp 9
36/26	13_3	19.8	3500
36/84	13_13	15	1499
3/26	6_3	12	2700

Table 35. $\gamma\delta$ TCR+ T-cell lines recognition of OV2774 cell line

%Clonal Expansion	Cell Line	%Cytotoxicity	Casp 9
36/26	13_3	43	1123
36/84	13_13	56	nd
3/26	6_3	42	1420

We measured lysis of SKOV3, CAOV3 and OV2774 tumor cells by genetically engineered $\gamma\delta$ TCR+ transduced T cell lines. Following analysis using an Effector:Target ratio of 100:1, 50:1 and 25:1 the genetically engineered transduced cell line OV27T δ 2_22_ γ II(9)3 induced 51, 42 and 27% cytotoxicity on SKOV3 cells. Genetically engineered TCR+ T-cell transduced cell line OV27T δ 2_22_ γ II(9)13 induced 72.4, 39.2 and 29.3% cytotoxicity on SKOV3 cells. Genetically engineered TCR+ T-cell transduced cell line OV27T δ 2_6_ γ II(9)3 induced 73, 64, and 26% cytotoxicity on SKOV3 cells. Genetically engineered TCR+ T-cell transduced cell line OV27T δ 2_13_ γ II(9)3

induced 35.8, 24.6 and 31% cytotoxicity on SKOV3 cells. The TCR- T-cell line (untransduced control) induced 10, 5 and 3% cytotoxicity (see Figure 28).

Using CAOV3 ovarian cancer cell targets the genetically engineered TCR+ T-cell transduced cell line OV27T δ 2_13_ γ II(9)3 induced 78.2, 19.8 and 17.6% cytotoxicity on CAOV3 cells. Genetically engineered TCR+ T-cell transduced cell line OV27T δ 2_13_ γ II(9)13 induced 14, 15 and <0% cytotoxicity on CAOV3 cells. Genetically engineered TCR+ transduced T cell line OV27T δ 2_6_ γ II(9)3 induced 29.4, 12 and 17.5% cytotoxicity on CAOV3 cells. Genetically engineered TCR+ transduced T cell line OV27T δ 2_22_ γ II(9)13 induced 47, 37 and 4.7% cytotoxicity on CAOV3 cells. The single chain TCR-transduced T-cell line showed minimal induction of cytotoxicity on CAOV3 cells.

Using OV2774 ovarian cancer cell line targets the genetically engineered TCR+ transduced T-cell line OV27T δ 2_13_ γ II(9)3 induced 70, 43 and .2% cytotoxicity on OV2774 cells. TCR+ T-cell line OV27T δ 2_6_ γ II(9)3 induced 59, 42 and 18% cytotoxicity on OV2774 cells. TCR+ T cell line OV27T δ 2_13_ γ II(9)13 induced 67, 56 and 23% cytotoxicity on OV2774 cells. The untransduced TCR- T-cell line induced minimal cytotoxicity on OV2774 cells.

Using UT7 leukemia cell line targets the genetically engineered TCR+ T-cell line OV27T δ 2_3_ γ II(9)3, OV27T δ 2_13_ γ II(9)3 and OV27T δ 2_13_ γ II(9)13 induced less than 5% cytotoxicity on UT7 cells.

CHAPTER 5 DISCUSSION

In this present study we examined the presence of oligoclonal populations of $\gamma\delta$ TCR+ T cells transcripts in the solid tumors specimens from patients with EOC. $\gamma\delta$ TCR+ T cells have been identified in TILs derived from patients with a variety of solid tumors and their increased presence is associated with increased survival (Kobayashi et. al., 2001 and Maeurer et. al., 1996). To determine whether $\gamma\delta$ TCR+ T cells are clonally expanded in vivo we amplified using V γ - and V δ -specific PCR γ - and δ -chain TCR transcripts from EOC tumors. The amplified transcripts were cloned, sequenced and analyzed. Analysis revealed the presence of substantial proportions of identical γ - and δ -chain TCR transcripts that were statistically significant in tumor specimens. Sequence analysis revealed the presence of statistically significant identical oligoclonal/monoclonal γ I-chain TCR transcripts in five out of seven patients. Five out of five patients revealed statistically significant identical oligoclonal γ II(9)-chain TCR transcripts. Four out of six patients revealed statistically significant identical oligoclonal δ 1-chain TCR transcripts. Four out of five patients revealed statistically significant identical oligoclonal δ 2-chain TCR transcripts. Multiple identical copies of TCR transcripts can be derived only by specific antigen-driven activation, proliferation and clonal expansion of the T-cell clones, which recognize these antigens. Two possibilities could explain the clonal expansion of $\gamma\delta$ TCR+ T cells in patients with EOC. First, the expansion may result from circulating $\gamma\delta$ TCR+ T cells, which encountered a tumor antigen leading to activation, proliferation and expansion of

a particular $\gamma\delta$ TCR+ T cell utilizing an antigen reactive CDR3 region. On the other hand, in situ $\gamma\delta$ TCR+ T cells that reside in the normal reproductive tract specifically the ovary underwent a TCR-mediated antigen driven clonal expansion within the solid tumor of patients with EOC. These results demonstrate that T cells infiltrating solid tumor specimens of patients with EOC contain oligoclonal populations of T cells, which confirm and extend previous work within our laboratory.

T cells recognize through their $\gamma\delta$ TCR mainly whole proteins, in general, unrestricted to MHC class I or II (Rock et. al., 1994). In contrast, $\alpha\beta$ TCR recognize small peptides derived from endogenous or exogenous antigens in association, in general, with MHC class I or II restriction. Furthermore, $\gamma\delta$ TCR+ T cells recognize in an MHC-independent manner, lipids, carbohydrates, glycolipids, and other ligands. T-cells each express a different TCR molecule, which serves as a unique fingerprint of the T-cell clone. Therefore, the probability of finding by chance multiple identical copies (two or more) of a particular γ - or δ -chain TCR transcript within an independent sample of T cells is negligible. Researchers have observed that in the host a specific antigen-driven proliferation and clonal expansion of the T-cell clones that recognize tumor antigen(s) takes place but in some patients this specific anti-tumor response appears to be suppressed by regulatory T cells, T(reg)s. Evidence indicates that these T(reg)s inhibit in vitro proliferative responses, cytokine production, and the cytolytic activity of autologous T cells. Therefore, the tumor has evolved to evade the host immune response.

Once we determined the presence of oligoclonal/monoclonal expansion of $\gamma\delta$ TCR+ T-cells in patients with EOC the next step was to determine their reactivity to tumor cells. Therefore, we carried out in vitro experiments to assess the ability of T-cells transduced with the oligoclonal $\gamma\delta$ TCR transcript expressed on the cell surface to recognize and kill tumor cells.

We first constructed full-length transcripts of both γ -chain and δ -chain TCR transcripts using the CDR3 regions of the clonally expanded $\gamma\delta$ TCR+ T cell transcripts amplified from the tumors of patients with EOC. Then using a MSCV retroviral expression system we expressed these transcripts in the mutant jurkat cell line. Knowing many human tumors are immunogenic and elicit an antigen-specific T-cell immune responses our goal was to generate cytotoxic T-cells that are able to recognize tumor cells. We wanted to identify the existence of an antitumor immune response in humans in which $\gamma\delta$ TCR+ T-cells play a role.

We then analyzed the induction of cell death protease activity by measuring caspase 9, 8 and 3 activity and then determined whether genetically engineered $\gamma\delta$ TCR+ T-cells that expressed the clonally expanded TCR transcripts on the cell surface induce lysis of tumor cells. For each set of $\gamma\delta$ TCR we constructed two retroviral vectors that contain either a γ -chain or δ -chain TCR full-length transcript. These retroviral vectors were used to mediate genetic transfer of the $\gamma\delta$ TCR transcripts. This procedure required multiple-vector transduction as well as antibiotic selection. The results of this investigation show that genetically engineered TCR+ T-cell lines induced caspase activity as well as cytotoxicity towards the ovarian cancer cell lines SKOV3, CAOV3 and OV2774.

Further investigations will involve the identification of the putative self or non-self antigen(s) recognized by the genetically engineered $\gamma\delta$ TCR+ T-cells. This will assist in understanding the role of antigen-driven oligoclonal expansion of $\gamma\delta$ TCR+ T-cells in EOC.

The identification of cytotoxic T cells that express natural or by genetically engineering T-cell transcripts that recognize tumor cells will serve as a novel immunotherapy. Patients with ovarian cancer are often diagnosed at an advanced stage and the cancer cells are often resistant to normal doses of chemotherapeutic agents. These patients have a survival rate of one-year. The effectiveness of adoptive immunotherapy in cancer patients has been identified in recent studies where high-dose therapy followed by autologous stem cell transplantation was shown to be effective resulting in a minimal disease state and improved survival in patients with myeloma (Rapoport et. al., 2005). Many cancer patients experience a dysfunctional tumor immune response and need an alternate to traditional adoptive cellular transfer (ACT) therapy. Primarily the lack of cytotoxic T cells that recognize tumor cells needed for in vitro expansion limits the ability to utilize traditional ACT.

The novel approach of using $\gamma\delta$ TCR+ T-cell transcripts for the retroviral transduction of patient cytotoxic T-cells may be useful as an alternate therapeutic method or in support of high-dose therapy. By using the $\gamma\delta$ TCR+ T-cell transcripts for expression in cytotoxic peripheral T cells we can generate and/or enhance the patient's immune response. Cytotoxic T lymphocytes express either $\alpha\beta$ TCR or $\gamma\delta$ TCR+ CD3+ T-cells and exhibit cytotoxicity following antigen driven

TCR receptor stimulation/activation. As stated previously the mechanisms involved in cell-mediated cytotoxicity include specific-antigen recognition and cell-to-cell contact. The secretory pathway involving receptor-triggered exocytosis of preformed secretory granules that contain granzymes or the use of receptor-induced surface membrane Fas ligand (FasL), which cross-links Fas on target cells both have the ability to result in effector cell induced cytotoxicity on tumor target cells. Since $\alpha\beta$ TCR and $\gamma\delta$ TCR+ CD3+ T-cells are capable of being cytotoxic against cancer cells an antitumor immune response involving both subsets of cytotoxic T lymphocytes may enhance and/or support one another.

Both experimental and clinical studies suggest that $\gamma\delta$ TCR+ T-cells play a role in the antitumor cell immune response (Rospollini et. al., 2005, Choudhary et. al., 1995, and Kabelitz et. al., 2004). $\gamma\delta$ TCR+ T-cells recognize tumor cell antigens and are activated and exert the aforementioned cytotoxicity and release cytokines toward the target tumor cell. The significant component of this recognition is the ability of $\gamma\delta$ TCR+ T cell to recognize whole protein in an HLA-unrestricted manner. Unfortunately there are few definitive ovarian cancer tumor cell antigens identified that are recognized by $\gamma\delta$ TCR+ T cells. Therefore, identifying clonally expanded $\gamma\delta$ TCR+ T cells in patients with EOC and the antigens they recognize will aid in the treatment of patients with EOC.

As part of identifying the antitumor immune response researchers are continuing to understand the pathogenesis of EOC (Garson et. al., 2005 and Liu et. al., 2004) but believe many factors are involved and include the following:

A) Genetic factors

As previously described, several genetic factors have been indicated to be involved in the initiation and progression of EOC. BRAC1 and BRAC2 account for 5-10% of inherited EOC. Many of these genes 1) induce cellular proliferation, 2) inhibit cellular proliferation or 3) regulate programmed cell death.

Furthermore, genetic factors that regulate the coagulation pathway and inflammation have been shown to be involved in tumor cell proliferation, angiogenesis, invasion and metastasis (Wang et. al., 2005).

B) Inflammation

There is increasing evidence that infiltrating immune cells are present in EOC and the peritoneal environment. Previous studies showed that 70% of the cells within the EOC tumors were mononuclear infiltrates (Freedman et. al., 1994). The tumor rejection process includes cytotoxic T lymphocytes (CTLs) killing the tumor cells and producing cytokines and/or chemokines. Cancer research has shown that tumor immunity and the subsequent tumor elimination process is highly complex and involves various components of the host immune system. For example, the tumor stroma is a mixture of various cell types and stromal agents. The stroma provides growth factors, blood supply, and the extracellular matrix that sustains the tissue. Furthermore, the microenvironment of the peritoneum in EOC includes cytokines/chemokines involved in inflammation (Roskelley et. al., 2002). The antiproliferative effect of cytokines such as IFN γ is important for tumor regression and the release IL-12 (Broderick

et. al., 2006) is necessary for memory T cells to proliferate, secrete IFN- γ , and kill tumor cells.

Chemokines also play an important role in the antitumor response (Schutysen et. al., 2002). Interleukin-8 (IL-8) is considered an important chemokine associated with tumor progression and appears to be the point of linkage for coagulation and inflammation in malignancy. The inflammation process may include cross talk between tumor cells and the surrounding cellular stroma. The inflammatory infiltrates are comprised of different leukocyte populations and interact with the cancer cells and their surroundings, which may influence the phenotypic changes in the tumor (Freedman et. al., 2004). More specifically $\gamma\delta$ TCR+ T-cells may function at the site of the tumor by producing soluble mediators to further recruit and activate other inflammatory cells as well as directly recognize tumor surface antigens leading to tumor cell death.

C) Autoimmunity

Research indicates that immuno-suppression may contribute to the progression of ovarian cancer. The presence of regulatory T-cells (Treg) and the secretion of soluble molecules in cancer patients could be important in inducing T-cell suppression, therefore allowing tumor growth. Suppressor cells have been thought to play a part in the progression of cancer. CD4+CD25+ regulatory T cells present in EOC tumors have been shown to be associated with poor patient survival. Evidence indicates Tregs preferentially move to and accumulate in tumors and ascites in later cancer stages (Curiel et. al., 2004). Furthermore, dysregulation of the immune response may also include a loss of signal-

transducing proteins leading to decreased T-cell activation (Choi et. al., 1998). In addition, HSP-10 appears to be expressed and released from many proliferating cell types including ovarian cancer cells. This circulating HSP-10 exhibit suppression of T-cell signaling leading to decreased T-cell activation (Akyol et. al., 2006). These factors contribute to the down-regulation of the tumor immune response, therefore allowing the tumor to grow and escape the host immune surveillance.

Over the past twenty years there has been substantial evidence demonstrating an antitumor response takes place in humans. Immunogenic tumor antigens that elicit an immune response can be used for the purpose of vaccine development to aid in the treatment of patients with various malignancies. In the microenvironment of EOC, mononuclear cells infiltrate into the pelvic peritoneum and stroma, where they may become activated, clonally expand and are targeted towards tumor cells in response to a self or non-self antigen(s). This cellular immune response is often down-regulated by cytokines such as IL-10, IL-6 and TGF- β , as well as growth factors and chemokines that may promote the progression of EOC by suppressing the antitumor response. Therefore, while discovering and developing novel immunotherapy such as $\gamma\delta$ TCR+ T-cells we must consider the significance of decreasing the number of regulatory T lymphocytes in the microenvironment of the solid tumor.

CHAPTER 6 CONCLUSION

The hypothesis tested in this investigation is that $\gamma\delta$ TCR+ T-cells play an important role in the antitumor immunity of EOC. The $\gamma\delta$ TCR+ T-cells may have undergone specific antigen-driven activation and clonal expansion to facilitate their selective lytic activity against the tumors.

EOC disease initiation and progression may be the result of many factors; environmental, genetic and immune suppression. Recent advances in the identification and characterization of the role of resident V δ 1 and circulating V δ 2 TCR+ T-cells indicate that they display unique features in terms of antigen specificities. Thus, making $\gamma\delta$ TCR+ T-cells suitable candidates as antitumor effectors in the context of EOC. In particular, $\gamma\delta$ TCR+ T-cells lack antigen-processing requirements and have the ability to recognize unconventional MHC-like molecules that might accompany the MHC-restricted tumor-specific immune response mediated by $\alpha\beta$ TCR+ T-cells. On the other hand, $\gamma\delta$ TCR+ T-cells may provide an alternative antitumor response when MHC class I molecules are down regulated, which often occurs during EOC progression (Kooi et. al., 1996).

Moreover, $\gamma\delta$ TCR+ T-cells expression of NK-like receptors, such as NKG2D and killer inhibitory receptors provide an additional mechanism for tumor antigen recognition and effector function in response to stress-induced molecules.

The current hypothesis for EOC tumors speculates that inflammatory infiltrates play a role in the antigen recognition and EOC tumor cell lysis in the defense against solid EOC tumors bearing surface tumor antigen(s). These mononuclear cells include T-cells bearing the $\gamma\delta$ T-cell receptor, and once the T-cells have infiltrated into pelvic peritoneum and stroma they proliferate and oligoclonally expand in response to specific antigenic stimulation. Both circulating and resident intraepithelial $\gamma\delta$ TCR+ T-cells secrete soluble mediators including MIP-1 α , MIP-1 β , RANTES and IL-8, chemo attractants for activated lymphocytes, professional APCs and neutrophils (Boismenu et. al., 1996 and Sallusto et. al., 1998), thereby contributing to the recruitment of circulating lymphocytes, including $\gamma\delta$ TCR+ T cells. Direct cytolysis of tumor cells in patients with EOC by the $\gamma\delta$ TCR+ T-cells and direct apoptosis of these tumor cells triggered by chemical mediators, such as IFN- γ results in tumor cell death and solid tumor destruction. When this process continues the tumor begins to minimize and is eventually eliminated. It is important to note that these mononuclear cell infiltrates may recognize and respond to both the surface antigen(s) that initiated the disease but escaped the initial immune response due to immune suppression. Therefore, the identification of the antigen(s) that elicit an immune response and the genetically engineering of $\gamma\delta$ TCR+ T-cells that recognize these antigen(s) may prove to be an alternative therapeutic option for patients with EOC.

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