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A Phase I Dose Escalation Safety and Feasibility Study of WT1-Specific T Cells for the Treatment of Patients with Advanced Ovarian, Primary Peritoneal, and Fallopian Tube Carcinomas

MSKCC THERAPEUTIC/DIAGNOSTIC PROTOCOL

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Please Note: A Consenting Professional must have completed the mandatory Human Subjects Education and Certification Program.

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1.0 PROTOCOL SUMMARY AND/OR SCHEMA

This is a phase I dose escalating trial designed to identify tolerable, clinically active doses of Wilms' tumor gene (WT1) peptide sensitized T cells when administered alone or with non-myelosuppressive chemotherapy in patients with recurrent or persistent, evaluable $WT1^{+}$ ovarian, primary peritoneal, or fallopian tube carcinomas. Patients eligible for this study have recurrent ovarian, primary peritoneal or fallopian tube cancer that expresses the WT1 antigen. In this phase I trial, eligible patients will be treated with autologous T cells sensitized with WT1 peptides. Initially, one dose of the WT1 cells alone will be tested specifically for any toxicities as well as to determine the levels of WT1 specific T cells achieved in the blood and the persistence of these cells post infusion. In association with these studies, patients will be evaluated for any effects these T cells might have on their disease. Thereafter, certain patients will receive the T cells following lymphodepletion with cyclophosphamide. Previously, Dudley *et al* (1,54) described lymphoablative chemotherapy with cyclophosphamide and fludarabine which was then followed by T cell infusion. Childs *et al* also developed a similar lymphoablative regimen for conditioning renal carcinoma patients for allografts.(61) Many of the patients in our study will already have been heavily pretreated with chemotherapy and therefore have a reduced bone marrow reserve. We therefore, propose using lymphodepletive chemotherapy with single agent cyclophosphamide. The proposed treatment dose has been studied extensively at our institute in patients will CLL, and found to be well tolerated and safe.(104) Furthermore, cyclophosphamide is an active agent in ovarian cancer.(105)

TREATMENT PLAN

A. **Initial Evaluation.** Patients with ovarian, primary peritoneal, or fallopian tube carcinomas will be identified. Patients with these tumor types will be offered Informed Consent 1 that allows for immunohistochemical (IHC) testing of their banked tumor for WT1 expression. To be eligible, patients must have WT1 expressing tumors. Once WT1 expression has been verified, a leukapheresis will be performed on eligible patients to collect peripheral blood mononuclear cells to establish autologous Epstein Barr Virus (EBV) transformed B cell lines (BLCL) and for the generation of the autologous WT1-specific T cell lines.

B. **Pretreatment Evaluation**. Patients will be offered Informed Consent 2 for treatment with autologous WT1-specific T cells if they meet the following criteria: (1) recurrent or persistent, evaluable ovarian, primary peritoneal or fallopian tube cancer, (2) autologous EBV BLCL established, (3) and WT1 tumor expression. Approximately eight to twelve weeks will elapse between Informed Consent 1 and 2, while patients' banked tumor specimen will be tested for WT1 expression, as well as generation of EBV BLCL and autologous WT1-specific T cell lines. Patient EBV serology will be assessed at the time of leukapheresis. Autologous T cells will be sensitized *in vitro* to recognize WT1 with the patient's autologous EBV B lymphoblastoid cell line (BLCL) or cytokine-activated monocytes (CAMs) pulsed with WT1 peptide(s). Autologous WT1-specific T cell populations will be generated in quantities sufficient for adoptive therapy. Blood samples will be obtained immediately prior to adoptive therapy to quantitate baseline levels of WT1

peptide reactive T cells, by quantitation of T cells secreting IFNγ in response to peptide, T cells binding WT1 peptide HLA tetramers, if applicable, and WT1 cytotoxic T lymphocyte precursors (CTLp) by limiting dilution analysis.

C. Generation and Isolation of WT-1 Peptide-specific T cells for Adoptive Therapy. The patient's EBV BLCL or CAMs, loaded with WT1 peptide(s), known to induce WT1-specific cytotoxic T cells in normal donors and in patients, will be used for sensitization of the patient's T cells. Prior to use as sensitizers, the EBV BLCL are irradiated to 9,000 rads in a calibrated certified blood irradiator to eradicate the capacity of these cells to grow within co-cultures. In addition, EBV BLCL will be cultured in media containing acyclovir to eliminate the risk of transferring infectious EBV in the T cells transferred. After sensitization, T cells will be expanded with IL2, IL15 and OKT3 according to a modification of the technique of Dudley and Rosenberg using autologous WT-1 peptide-loaded EBV BLCL and autologous PBMC as irradiated feeders (1). The T cells will be assessed for WT-1 specific cytotoxic reactivity in standard Cr51 release assays.

D. Treatment with WT1 Peptide-specific T cells.
Once the requisite doses of T cells have been generated, the patients will undergo treatment with the T cells. The WT1 peptide-specific T cells will be administered intravenously. Patients will be treated in 4 sequential groups of 3-6 patients. In this phase I trial, dose escalation will be based on dose limiting toxicity (DLT). In cohort 1 patients will receive WT1 specific T cells dosed at 5 x $10^6/m^2$ (level I) without cyclophosphamide lymphodepletion. The first two patients in cohort 1 received only a single administration of WT1 peptide-specific T cells, whereas the third patient is to receive infusions every two weeks for four doses. The next three cohorts of patients (levels II, III, and IV) will undergo lymphodepletion first with cyclophosphamide (Cytoxan®, CTX) at 750mg/m^2 administered intravenously, followed 48 hours later by treatment with an infusion of WT1 peptide sensitized autologous T cells. For patients receiving cyclophosphamide (levels II, III, and IV) only a single dose of cyclophosphamide will be administered 48 hours prior to the first infusion of T cells. No further doses of cyclophosphamide will be administered during the study. These patients will then receive additional infusions of T cells every 2 weeks for a total of 4 doses. These three dose levels represent administration of WT1 specific T cells at escalated doses (2 x $10^7/m^2$ [level II], 5 x $10^7/m^2$ [level III] and 1 x $10^8/m^2$ [level IV]). This is summarized in Table 1. Each cycle comprises of 28 days or 2 WT1 peptide-specific T cell infusions.

All patients (apart from the first two patients on cohort I who have already been treated) will receive WT1 peptide-specific T cell infusions once every two weeks for up to 4 doses (2 cycles) as outlined in Table 7c.

If 2 x $10^7/m^2$ T cells represents the DLT during treatment of patients on level II, the dose below the DLT, that i.e. 5 x $10^6/m^2$ T cells will be used for the next cohort of patients level III. If 5 x $10^7/m^2$ T cells represents the DLT during treatment of patients on level III, the dose below the DLT, that i.e. 2 x $10^7/m^2$ T cells will be used to complete protocol requirements

within the current cohort. It is therefore possible that the dose level: 1 x $10^8/m^2$ WT1 peptide sensitized autologous T cells may not be reached.

One dose level of WT1–specific T cells alone and three dose levels of cyclophosphamide conditioning followed by WT1-specific T cells will be tested. Groups of three patients will be accrued sequentially as summarized in Table 1:

Table 1: Dose Levels of T cells and Conditioning Chemotherapy

Level	Patients	Cyclophosphamide	WT1 Specific T cells
Level I	**3-6**	**NO**	**5 x $10^6/m^2$**
Level II	**3-6**	**Yes**	**2 x $10^7/m^2$**
Level III	**3-6**	**Yes**	**5 x $10^7/m^2$**
Level IV	**3-6**	**Yes**	**1 x $10^8/m^2$**

In this phase I trial, dose escalation will be based on the DLT, defined as a grade 3-4 toxicity developing after infusion of the T cells as graded by the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events Scale (CTCAE) Version 4.0. Only toxicities that are possibly, probably, or definitely related to T cell infusions will be considered DLTs. Patients will be observed for DLTs for 2 weeks following each T-cell infusion and for 4 weeks following the last T-cell infusion. The first two patients treated on the study in cohort one received a single T cell infusion at Level I. All subsequent patients treated on study will receive up to four T cell infusions, each infusion being separated by 2 weeks. Each patient will then be followed for four weeks after the last infusion. If no patients experience DLT during the 28-day period following the patient's initial T cell infusion, we will proceed to the next dose level. If any patient at a given dose level is not evaluable through the first 28 days post the initial T cell infusion, the patient will be replaced such that three patients are evaluated prior to dose escalation to the next level.

For patients receiving cyclophosphamide (Levels II,III, or IV), hematopoietic suppression will not be considered a DLT during the first 21 days post cyclophosphamide, since cytopenia is an expected toxicity of the pretreatment course of cyclophosphamide. However, severe grade 3 cytopenia extending beyond 21 days of the first 28-day cycle will be graded as a severe DLT.

The dose escalation scheme for this study is as follows:

Dose Escalation and DLT

1. If 0 of 3 patients experience DLT at a given dose level, then proceed to the next dose level.

2. If 1 of 3 patients experience DLT at a given dose level, 3 additional patients will be treated at that level. If 0 of those 3 develop DLT, the dose will be further escalated.

3. If 2 or more of the 6 patients experience DLT at a given dose level, the maximum tolerated dose (MTD) is defined as the previous dose level.

4. If only 3 patients are treated at the MTD, an additional 3 patients will be treated at that level. If among these 6 patients, 2 or more experience DLT, the MTD will be lowered and the same criterion will be applied to the previous dose level.

E. **Additional WT1 specific T cell infusions**

Only a single infusion of WT1 peptide-specific T cells was administered to the first two patients at dose level I. Additional patients treated on dose level I (cohort 1) and patients treated on dose level II,III, or IV who have been observed for 2 weeks post infusion and who do not develop a grade 3 or greater toxicity (with the exception of grade 3 hematopoietic suppression during the first 21 days after the cyclophosphamide) may receive at least 4 infusions of WT1 peptide sensitized T cells administered at two week intervals (i.e. on approximately days 1, 15, 29 and 43). If, after 4 infusions, the patient has a clinical response or at least stable disease, additional infusions of WT1 peptide sensitized T cells may continue to be administered once every 2 weeks until disease progression, DLT, withdrawal of consent or until the stock of the patient-specific WT1 peptide sensitized T cells has been exhausted. These additional infusions of WT1 peptide sensitized T cells will be administered at the same dose level as the patient's initial infusion.

F. **Post-Treatment Evaluation.**

Patients will be evaluated clinically by vital signs pre-infusion and hourly for 6 hours post infusion of T cells. During cycle 1 patients will undergo physical examinations and toxicity assessments the day of and the day following each WT1 peptide-specific T cell infusion. A phone toxicity assessment will be performed on Day 3 post each T cell infusion. A +72 hour window to contact patient via phone is permissible. In addition, during cycle 1 patients will also undergo physical examinations and toxicity assessments on the non-treatment weeks 2 and 4.

Patients who undergo further WT1 peptide-specific T cell infusions will undergo physical examinations and toxicity assessments on the first day following each infusion and then at least once every two weeks thereafter. A phone toxicity assessment will be performed on Day 3 post each T cell infusion. This schedule of assessment will continue until 8 weeks following the patient's final T cell infusion. Patients will also have at least weekly complete blood

counts (CBCs) with differentials and comprehensive metabolic panels (CMPs) to monitor for renal or hepatic toxicity for the first four weeks and then every two weeks thereafter to complete 4 weeks following completion of the final T cell infusion. Finally, patients will undergo physical examinations, toxicity assessments and bloods as above at the 8-week safety follow-up visit (i.e. 8 weeks following the final T cell infusion).

Subjects will have 2 safety follow-up visits approximately 4 to 8 weeks after final WT1 T cell infusion. In the case that a patient has an overall decline of health status, the follow-up safety visits will at the discretion of the investigator.

Tumor responses will be monitored radiographically using RECIST criteria by computed tomography scans (CT scans) of the chest, abdomen and pelvis. A baseline CT scan will be performed. A second CT scan will be performed at approximately 8 weeks post the first infusion. Thereafter, CT scans will be performed approximately every 8 weeks if the patient continues on study. Patients requiring surgery for management of their disease any time after T cell infusion may have biopsies. If such biopsies are performed, samples will be obtained and tumors examined by immunohistology for T cell infiltration and for residual $WT1^{+}$ tumor cells. Serum CA125 levels will be measured prior to the first infusion of WT1 specific T cells. For patients who continue to receive additional infusions of WT1 specific T cells, serum CA125 will be monitored prior to each WT1 specific T cell infusion, and if applicable at 4 and 8 weeks after the final WT1 specific T cell infusion. CA125 levels will not be used to make treatment decisions, as progression will be assessed using RECIST criteria.

In addition, patients will be tested for circulating levels and phenotype of T cells. All patients will have these levels assessed at baseline. The two patients treated with a single T cell infusion were assessed for T cells responding to WT1 peptide pool prior to the WT1 specific T cell infusion, 24-hours post infusion and at 1, 2, 4 and 8 weeks post infusion, when applicable. For patients who will receive multiple T cell infusions, frequencies of WT1 specific T cells will also be assessed prior to each WT1 specific T cell infusion and 24-hours post first and last infusion and at weeks 4 and 8 post the last infusion, when applicable.

The frequencies of the WT1 and EBV specific CTLp will be assessed by limiting dilution assay (LDA). The proportion of T cells generating intracellular IFNγ in response to WT1 and autologous EBV BLCL (if applicable) will be assessed by FACS analysis. Concurrent quantitation of T cells binding tetramers presenting WT1 and EBV epitopes in the context of the HLA alleles expressed on the T cells will be performed whenever relevant tetramers are available, as a second marker of the persistence and expansion of adoptively transferred T cells as well as a marker of recovery of autologous immune function.

Estimated time to completion: 48 months.

2.1 OBJECTIVES AND SCIENTIFIC AIMS

1) To assess the safety and tolerability of *in vitro* expanded autologous WT1 specific T cells, when administered alone or with non-myeloablative, immunosuppressive conditioning to patients with recurrent ovarian, primary peritoneal or fallopian tube cancer.

2) To determine the maximum tolerated dose (MTD) of autologous WT1 specific T cells, when administered alone or with non-myeloablative, immunosuppressive conditioning.

3) To quantitate alterations in the concentration of WT1 specific T cells in the blood at defined intervals post infusion with or without non-myeloablative, immunosuppressive conditioning in order to gain estimates regarding their survival and proliferation in these patients.

4) To assess the effects of the adoptively transferred T cells on the growth and progression of advanced ovarian, primary peritoneal or fallopian tube cancer.

3.1 BACKGROUND AND RATIONALE

Ovarian Cancer as a Target for Immunotherapy

Epithelial ovarian cancer (EOC) is the leading cause of death from gynecologic malignancies in the United States. Over 23,000 new cases are diagnosed annually and an estimated 14,000 deaths each year result from EOC (2). Ovarian cancer is the fifth leading cause of cancer death in women in the United States. Furthermore, more than 70% of patients have stage III or IV disease at the time of diagnosis (3).

The standard therapy for advanced ovarian cancer is surgical removal of as much malignant tissue as possible, followed by cytotoxic chemotherapy that includes a platinum-coordination complex and a paclitaxel derivative. Although a temporary response rate to chemotherapy of 70% is anticipated, ovarian cancer tends to recur, even in patients who achieve a complete response (4). Despite improvements in surgery and chemotherapy, the 5-year survival rate remains at about 20-30% for advanced ovarian cancer (5).

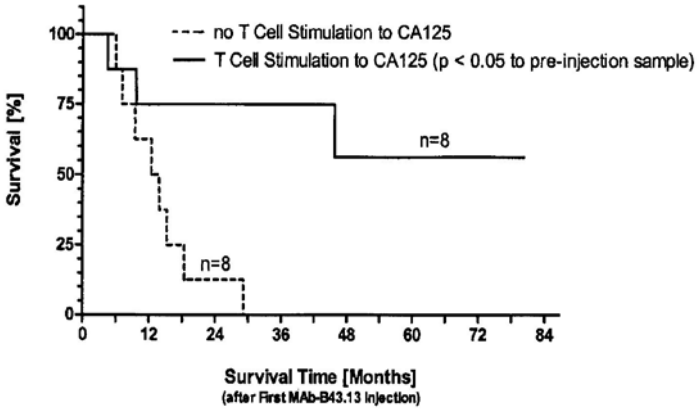
With current treatment approaches, the natural history of early stage ovarian cancer is also one of complete clinical remission, followed by relapse, with a return to remission in many patients. Although overall response rates of close to 80% are achieved in patients receiving platinum-based combination chemotherapy, only 47% of patients who are clinically free of disease will have no evidence of disease at second-look laparotomy (performed after initial chemotherapy) (6). Almost half of these patients will eventually recur with a mean interval of 24 months from second-look surgery to recurrence, with 60% of these recurrences occurring in the peritoneal cavity (7).

Given the high recurrence rate, new therapeutic approaches need to be developed for the treatment of advanced ovarian cancer, particularly tolerable approaches which could be introduced and sustained for targeted long-term eradication or control of minimal residual disease. Over the last decade, advances and clinical successes with the use of tumor-selective

monoclonal antibodies, such as rituximab in the treatment of lymphomas (8), CD33 specific antibodies in myeloid leukemias (9), the HER/neu-specific monoclonal antibody, Herceptin, in breast cancer (10), the GD-2-specific monoclonal 3F8 in neuroblastoma (11), and recently, the Ca125 specific monoclonal antibodies, Mab-B43.13 (Ovarex®) and ACA 125, in the treatment of ovarian cancer (12-14), have demonstrated the potential of immunotherapies directed against antigens differentially expressed on tumor cells.

Monoclonal antibody therapy has been successful in the treatment of ovarian cancer. Mab-B43.13, a monoclonal antibody therapy targeting the Ca125 antigen on ovarian cancer, has induced an immune response against both B43.13 and against Ca125 antigen that correlates with an improved prognosis. Recently, a randomized trial of low repetitive doses of mAb B43.13 versus placebo in patients with a clinical remission (CR) after primary therapy for ovarian cancer revealed a prolonged time to treatment failure in the treated group. Three hundred and forty-five patients with stage III and IV ovarian cancer post chemotherapy were enrolled in the study. Progression-free-survivals for treated versus untreated patients were 13.4 and 4.7 months ($p < 0.0001$), respectively (12). In addition, a paper by Noujaim *et al* also revealed that patients treated with B43.13 had an improved survival and, strikingly, that responding patients appeared to have a T cell response to Ca125 antigen (see Figure 1) (13).

Figure 1: **Survival Curves of Ovarian Cancer Patients With and Without a T cell Response to Ca125** (13)



A two-step study was conducted with ACA 125 in ovarian cancer patients and reported by Wagner *et al* (14). Initially, 18 patients were enrolled in the phase I portion, which was extended into a phase II study, with 42 patients treated in total. All patients had tumors that strongly expressed Ca125. Twenty-eight of 42 patients (67%) developed specific anti-anti idiotype antibodies (Ab3) during ACA 125 administration. The IgG subclass was predominantly IgG1 and IgG2. Cell mediated cytotoxicity from PBLs against Ca125 expressing and non-expressing human ovarian cancer cell lines in 18 patients were evaluated with an increase in cell kill in 9 of 18 patients from 19.6% ± 11.7% to 52.7% ± 13.6% at an effector:target ratio of 100:1. Cell mediated lysis was accompanied by the induction of Ab3 in 8 of 9 patients prompting only

humoral response evaluation in the remaining patients. Overall survival of all patients vaccinated with ACA 125 was 14.9 ± 12.9 months; for patients with a positive response to ACA 125 it was 19.9 ± 13.1 months; and for patients with no detectable response 5.3 ± 4.3 months ($p < 0.0001$). Once again, improved clinical outcome appeared to correlate with T cell responses to ACA 125 vaccination.

Recently, considerable additional evidence has been generated supporting an important potential role for tumor reactive T cells in the targeted therapy of ovarian cancer. Early analyses of T-lymphocytes derived from ascites or tumor nodules (tumor infiltrating lymphocytes, TILs) of patients with ovarian cancer have documented the presence of cytotoxic T cell clones reactive against autologous tumor cells in a proportion of patients (15). Furthermore, the presence of T cells in the ovarian tumors has been associated with a significantly improved disease-free survival (15). Early clinical trials exploring the clinical potential of autologous TILs expanded *in vitro* and adoptively transferred to patients with advanced disease also demonstrated clinical responses. However, such responses were observed in only a small subset of these patients (16). Consistent with this finding, characterization of the expanded TIL cells from these patients suggested that the cells generated were predominantly $CD4^+$ T cells and that $CD8^+$ T cells capable of lysing autologous tumor cells could be generated only from a minority of patients.

Antigens Differentially Expressed by Ovarian Carcinomas Targetable by T cells

In pursuit of better strategies to stimulate and sustain effective cytotoxic T cell responses against ovarian cancer, subsequent investigations of cell-mediated responses to ovarian cancer have focused on three areas:

1) Identification of proteins differentially expressed by ovarian cancers in comparison with normal tissues
2) Definition of immunogenic peptide epitopes derived from these proteins that could be used to elicit effective T cell responses
3) Exploration of alternative sensitization strategies designed to preferentially stimulate the generation of tumoricidal T cells *in vitro* or *in vivo*

Several genes are now recognized that encode proteins differentially expressed by ovarian carcinoma cells, particularly WT1, HER2/neu (27,28), and MUC1 (34), as well as CA125, Folate binding protein (FBP), MUC1 and MAGE3 (22-25). Recent studies also indicate that degradation of these proteins yields immunogenic epitopes which bind HLA class I alleles and can stimulate the generation of peptide specific, tumor cytotoxic $CD8^+$ T cells (29-33).

The most extensively studied of the immunogenic peptides derived from proteins differentially expressed by ovarian carcinoma are those of the HER2/neu protein. HER2/neu is over-expressed in 20-30% of patients with ovarian cancer but its expression is correlated with a poor prognosis (27,28). Five HLA-A2 binding peptides and an HLA-A24 binding peptide have been identified (29-33). Analysis of tumor associated T cells in $HLA\text{-}A2^+$ patients with $HER2/neu^+$ ovarian cancer also suggest that T cell responses to the E75 HER2/neu peptides are immunodominant (30). Based on these studies, Brossart *et al* performed a pilot vaccination study with HER2/neu peptide-pulsed autologous dendritic cells (DC) in six patients with

advanced HER2/neu^{+} breast or ovarian cancer (34). Of these patients, two developed peptide-specific immune responses, detectable by quantitation of peptide stimulated T cells generating intracellular IFN-γ and T cells lysing peptide-loaded targets. One of these patients, with rapidly progressive ovarian carcinoma, experienced stabilization of disease for more than eight months post vaccination.

The epithelial mucin, MUC1, is highly expressed in more than 90% of ovarian cancers, and, to a lesser extent, by normal epithelial cells of the gastrointestinal and genitourinary tracts (35). The MUC1 protein, conjugated to KLH, has been evaluated by P. Livingston *et al* at Memorial Sloan-Kettering Cancer Center (MSKCC) as part of a polyvalent vaccine in patients with ovarian carcinoma. This vaccine has elicited antigen-specific antibody responses in each of seven patients immunized. The MUC1 protein also contains at least six nonamer peptide epitopes that bind HLA-A2 (n=6) or HLA-A11 (n=1). Brossart *et al* has used two of the high affinity HLA-A2 binding peptides, loaded on autologous DC to vaccinate 4 patients with advanced ovarian (n=2) or breast (n=2) (34). The vaccinations have induced peptide-specific cytotoxic responses in ¾ patients. Of the responders, one patient experienced transient stabilization of ovarian cancer. A second patient with breast cancer experienced regression of subcutaneous and visceral metastases and a striking reduction in circulating Ca125 levels, which persisted for over eight months. Biopsies of tumor sites revealed infiltration of the tumor site with CD8^{+} and CD4^{+} T cells.

A third protein highly expressed in a large proportion of ovarian cancer cells but not expressed in most adult normal tissues is the Wilms' tumor gene product (WT1). WT1 was initially isolated as a mutated gene responsible for the childhood neoplasm, Wilms' tumor (36-38). WT1 encodes a zinc finger transcription factor, which binds early growth factor gene promoters, such as platelet-derived growth factor A chain, colony stimulating factor-1, transforming growth factor-β1, and insulin-like growth factor II (37). Unlike tumor suppressor genes Rb and p53, which are expressed ubiquitously, the expression of the WT1 gene is restricted to a limited number of normal tissues including fetal kidney, ovary, testis, spleen, hematopoietic precursors, and the mesothelial cell lining of visceral organs (38). After birth, expression of WT1 in these tissues is still detected but at markedly reduced levels (38). In contrast, WT1 is aberrantly expressed at higher levels in most adult leukemias including AML, ALL, CML and MDS and in several solid tumors including: mesothelioma, desmoplastic small round cell tumor, Wilms' tumor, colon cancer, renal cell carcinoma, and non-small cell lung cancer (39). Importantly, human ovarian cancers also over-express WT1. Quenneville *et al* showed that WT1 is a sensitive and specific marker of serous, but not mucinous, ovarian carcinomas (24). We have performed a study of human ovarian tumor microarrays and we have determined that 64% of serous ovarian cancers express WT1 while other histological subtypes of ovarian cancer express the antigen to a lesser degree (40).

The basis for the increased expression of WT1 in these tumor types is unknown. Initially, WT1 was categorized as a tumor suppressor gene. Several studies by Haber *et al* demonstrated that activation of WT1 induces normal CD34^{+} CD38^{-} early hematopoietic cells to enter a prolonged G0 phase while concomitantly inducing terminal differentiation of precursor cells later in their differentiation (90). However, it is now recognized that WT1 is expressed in several non-mutated isoforms, and that the isoform dominantly expressed in leukemias and

certain solid tumors differs from that expressed in normal precursor cells (91). Recent studies have also demonstrated that high expression of WT1 in AML, MDS and certain solid tumors is associated with poor prognosis (92-95). Furthermore, when $WT1^+$ leukemic cells are treated with WT1 antisense oligonucleotides, they are consistently induced into apoptosis. These findings support the hypothesis that the isoform of WT1 dominant in these tumors is acting as an oncogene (96).

Studies from investigators in Japan, England, and MSKCC (Confer Preclinical Studies Section), indicate that peptides derived from the WT1 protein are immunogenic in man (42-44,47). Ohminami *et al* and Oka *et al* were the first to identify peptides of WT1 which, when presented by HLA A2402 and HLA A0201, could elicit WT1 peptide specific T cell clones with *in vitro* leukemocidal activity (42,43). The immunogenic HLA A0201 restricted peptide nanomers from WT1 are: 1) $_{126\text{-}134}$RMFPNAPYL and 2) $_{187\text{-}195}$SLGEQQYSV. The HLA A2402 presented nanomers are: CMTWNQMNL$_{235\text{-}243}$ and RWPSCQKKF$_{417\text{-}425}$. Studies at MSKCC demonstrated that the two WT1 peptides presented by HLA A0201 elicited $IFN\gamma^+$ cytotoxic WT1 specific HLA A0201 restricted $CD8^+$ T cells in each of 16 normal HLA $A0201^+$ normal donors tested. Furthermore, the T cells generated consistently exhibited cytotoxic activity specific for $WT1^+$ HLA $A0201^+$ leukemias, including AML, ALL and CML blasts *in vitro*, and suppressed the growth of HLA $A0201^+$ $WT1^+$ leukemic xenografts in NOD/SCID mice *in vivo* (47). Studies of Gao *et al* have also shown that a T cell clone specific for one of these peptides was capable of selectively inhibiting the clonogenic activity of Ph^+ $CD34^+$ CML blasts *in vitro* but did not affect normal Ph^- CD34 cells (44). Furthermore, when the T cell clone was co-administered with these Ph^+ $CD34^+$ blasts into SCID mice, leukemia development was prevented. These studies support the hypothesis that WT1 is expressed by clonogenic leukemic stem cells and that such cells are susceptible to WT1 specific cytotoxic T cell-mediated lysis.

Scheibenbogen *et al* have demonstrated that patients with $WT1^+$ malignancies may be able to generate T cell responses against these peptides (46). They have detected T cell responses in up to 30% of patients with AML in remission. Our own studies suggesting that WT1 specific T cells can be generated *in vitro* from a majority of women with ovarian carcinoma further support these findings (46).

Ohno *et al* conducted a small phase II trial of 12 patients with WT1 HLA **A2402** positive gynecological cancer. Patients received intradermal injections of HLA A2402-restricted modified 9-mer WT1 peptide every week for 12 weeks. The treatment was well tolerated with only local erythema at the injection site. Three of the patients had stable disease for at least a 3-month period. (106)

Adoptive T cell Therapy

Until recently, investigations of immunogenic peptides derived from tumor-associated proteins have focused on their use as vaccines, either as isolated peptides administered with

adjuvant or as peptides loaded on autologous dendritic cells isolated from the blood, for immunization of tumor bearing hosts. Unfortunately, responses to these vaccines have been observed in only a small fraction of patients. Furthermore, while cytotoxic T cell responses against peptide-loaded targets have been detected, these cells have exhibited limited activity against autologous tumor cells (51). These limitations have been ascribed to the types of T cells generated and the potential of tumor cells in the host to foster T cell tolerance to inhibit the tumoricidal activity of the T cell effectors through modulation of the expression of HLA alleles or co-stimulatory molecules, or the secretion of inhibitory cytokines such as IL-10, IL-6 or TGF-β.

An alternative approach is to adoptively transfer antigen-specific T cells sensitized and expanded *in vitro*, under conditions promoting the generation of a preponderance of cytotoxic $CD8^+$ T cells and T_{h1} $CD4^+$ T cells. This cellular immunotherapy, explored by our own laboratory and by others, has demonstrated striking efficacy in the treatment of CMV and adenoviral infections as well as certain hematologic malignancies (particularly CML and virus-associated tumors such as marrow or organ allograft-associated EBV lymphomas) (52, 107). Unlike the TIL cells expanded after nonspecific activation with IL-2, these T cells have been sensitized with antigenic extracts or immunogenic peptides loaded on antigen-presenting cells (APCs) expressing required co-stimulatory molecules, such as isolated dendritic cells or EBV transformed cells, or APCs transduced to express the relevant antigenic peptides. When T cells are sensitized with such professional APCs loaded with peptides or transduced to express a viral or tumor protein, the T cells generated are enriched for antigen-reactive $CD8^+$ T cells which exhibit strikingly greater cytotoxic activity against autologous cancer cells (34,52-56).

Clinical experience with the adoptive transfer of such T cell lines or clones is still very limited. However, in allogeneic marrow transplant recipients, adoptive transfer of donor-derived CMV-specific T cell clones or EBV-specific T cell lines has reduced or abrogated the incidence of CMV infection or EBV lymphoma post transplant (57-58). Studies at MSKCC and others have also demonstrated that adoptive transfer of as few as 10^5-10^6/kg EBV specific T cells can also induce durable regressions of multifocal monoclonal EBV^+ lymphomas in both marrow and organ allograft recipients (59). Similarly, adoptive transfer of 10^6 T cells expanded from clones specific for an HLA-2 binding peptide of the minor alloantigen HA-1 induced remission of AML in a HA-1 disparate host who relapsed post transplant (97). Strikingly, in these allogeneic hosts, the transferred, donor-derived T cells have increased in frequency in the circulation following transfer, with peak frequencies detected at the initiation of tumor regression. Furthermore, such cells have persisted for years post transplant.

In contrast to this experience with allogeneic donor derived adoptively transferred T cells, until recently, studies of adoptive transfer of large numbers (e.g. 10^{10} T cells) of autologous tumor associated antigen-specific T cell clones, expanded *in vitro* from tumor-bearing patients, and transferred back into these patients, have rarely recorded clinical effects. Furthermore, the highest frequencies achieved in the circulation have been immediately after infusion. Indeed, the persistence or survival of the transferred T cells has been measured at no more than 1-2 weeks (54). These findings may be due to one or more of the following: 1) T cells generated from tumor-bearing hosts against self antigens, such as gp100, differentially expressed on tumors are qualitatively different from those generated against even poorly immunogenic alloantigens like HA-1 or viral antigens such as CMV pp65 or EBNA3C; 2) T cells generated *in vitro* against

tumor-associated antigens from tumor-bearing hosts differ qualitatively from those generated from normal individuals; 3) autologous T cell lines and clones are often generated over extended periods *in vitro*, and may have a very limited proliferation potential after adoptive transfer; 4) autologous T cells reactive against such antigens in the tumor-bearing host may be subject to regulatory influences inducing apoptosis and clearance that are less effective or inoperative against allogeneic HLA matched cells; or 5) the space available for seeding the autologous lymphocytes in an otherwise untreated host may be limited when compared to that available to lymphocytes expanding in a lymphoablated and myeloablated marrow transplant recipient.

Generation and Characterization of WT1 Peptide Reactive T cells From Donors Inheriting HLA Alleles Other Than HLA A0201 or HLA A2402

While T cells specific for WT1 peptides presented by HLA A0201 or HLA A2402 can be regularly generated and demonstrate activity against $WT1^+$ leukemias both *in vitro* and *in vivo*, only 40% of our transplant patients inherit these alleles. To broaden the applicability of adoptive therapies specific for this oncofetal protein that is differentially expressed in leukemia cells, we have recently explored the use of autologous antigen-presenting cells loaded with a pool of overlapping pentadecapeptides (15-mers) spanning the sequence of WT1 for sensitization of T cells from individuals inheriting HLA alleles other than HLA A0201 or A2402. This approach is based on our recently reported success with this approach in generating T cells specific for CMV-pp65 (101). The use of a pool of 15-mers, each overlapping the next by 11 amino acids to span the WT1 sequence, has several potential advantages. First, the pool includes the full array of epitopes of WT1 including epitopes that can be bound and presented by both HLA class I and class II alleles permitting generation of both CD4 helper and CD8 cytotoxic T cells. Secondly, the 15-mers do not require intracellular processing, but bind directly to the HLA alleles and are then edited to appropriate size (9-mers for class I; 11-12-mers for class II) by ectopeptidases (102,103). Third, the use of the pool of 15-mers permits the donor T cells to respond to those epitopes that they have recognized as most immunogenic in prior exposures. In our studies of T cell responses to pentadecapeptides of the CMV-pp65 protein, this *in* vitro selection has lead to the consistent generation of T cells from CMV seropositive donors that are capable of lysing autologous targets infected with CMV virus. Accordingly, we have developed a pool of 141 overlapping 15-mers spanning the sequence of WT1. These pentadecapeptides are both 95% pure and microbiologically sterile. We have subsequently used this pool of peptides, loaded on autologous dendritic cells, to sensitize T cells from a series of 15 normal donors inheriting other HLA alleles. After 35-42 days of expansion, these T cells specifically generate IFNγ in response to the pool of peptides and usually to one or several dominant peptides in the pool, which we have identified by an epitope mapping strategy based on secondary responses of these total pool-sensitized T cells against a matrix of 15-mer subpools. In the example presented in Appendix A, the T cells of the donor sensitized with the pool generated in response to subpools 10 and 16, suggesting reactivity against pentadecapeptide 60. Subsequent testing (Appendix B-1) confirmed this reactivity. Thereafter, pentadecapeptide 60 was loaded onto a panel of EBV transformed B cells sharing single HLA alleles with the donor and used as targets of the WT-pool reactive T cells. In the example shown in Appendix B-2, the T cells recognized penta-

decapeptide 60 only when presented by cells expressing HLA B3501. The T cells also exhibited HLA B3501 restricted cytotoxic activity against $B3501^+$ acute lymphoblastic leukemia co-expressing WT1. The specific nonamer epitope within the pentadecapeptide recognized was subsequently identified as $HSFKHEDPM_{300-308}$. Using the same strategy, we have been able to generate WT1 specific CD8 and/or $CD4^+$ T cells from 13 of 15 normal donors. These T cells only respond to WT1 peptides; they have no reactivity against autologous or HLA-partially matched $WT1^-$ cells (Appendix C-1). The epitopes recognized have been mapped and their restricting class I and class II HLA alleles identified. Most importantly, the T cells generated from 12/13 cases after sensitization with the pool of WT1 peptides, have exhibited peptide-specific, appropriately HLA-restricted cytotoxic activity against leukemia cells expressing WT1 and the restricting HLA allele (Appendix C-2).

Lymphodepletion to Foster Expansion and Persistence of Adoptively Transferred T cells

In order to address possibilities 4 and 5 above, Dudley *et al* recently conducted trials in which melanoma patients were treated with a lymphoablative chemotherapeutic regimen consisting of cyclophosphamide 60 mg/kg/day x 2 days followed by fludarabine 25 mg/m^2/day x 5 days(1,54). Thereafter, patients were infused with 10^{10} of either extensively expanded clones of $CD8^+$ T cells specific for either tyrosinase or MART1, or tumor infiltrating T cells expanded for a much shorter period by stimulation with a mitogenic antibody OKT3 and IL2, each at doses of 10^{10} cells per patient (1,54). Following adoptive transfer of the cloned $CD8^+$ T cells, the T cells persisted for only two weeks, and never achieved levels higher than the peak levels detected immediately after infusion (54). Not surprisingly, clinical responses were not observed (54). However, in striking contrast to this experience with cloned T cells and all prior studies of adoptively transferred TIL cells, infusion of TIL cells into these lymphoablated patients resulted in a striking expansion of the cells within the patients (99). Persistence of these T cells for periods of at least four months and, in certain patients, up to 16 months was observed (89, 99). More importantly, adoptive transfer of TILs after lymphoablation has been associated with a higher tumor response rate. In the original report of Dudley et al, 6/13 patients achieved partial responses (PRs) extending from 2-24 months (1). In their updated series of 35 patients, 15 patients had achieved PRs with a mean duration of 11.5 $^+/^-$ 2.2 months (54).

In this study, eligible patients include those with recurrent ovarian, primary peritoneal or fallopian tube cancer that expresses the WT1 antigen. In this phase I trial, these patients will be treated with autologous T cells sensitized with WT1 peptides. Initially, WT1 cells will be tested alone, specifically to test for any toxicities that such infusions might induce as well as to determine the levels of WT1 specific T cells achieved in the blood and the persistence of these cells post infusion. In association with these studies, these patients will be evaluated for any effects these T cells might have on their disease. Thereafter, two escalated dose levels will be tested in patients who have previously received lymphodepletion with cyclophosphamide. Prior experience with the proposed cyclophosphamide dose is extensive and indicates that it is well-tolerated and effective in lymphodepletion of the host.(104) As described above, this approach has permitted expression and long-term survival of the transferred T cells in tumor bearing hosts. In part, this may be due to elimination of regulatory T cells and NK cells that the body maintains

to suppress T cell clones reactive against a "self antigen" such as WT1. However, lymphodepletion may also provide space for expansion of these T cells in a largely evacuated niche within the lymphoreticular system. Furthermore, as these T cells undergo homeostatic proliferation to fill this niche, they become markedly less susceptible to the tolerogenic activity of regulatory cells and cytokines (54). Possibly, as a consequence of these alterations, the antigen-specific cytotoxic activity of the transferred cells may be sustained for prolonged periods, as has been observed in the patients treated with TIL cells by Dudley *et al* (1). Since WT1 expression, may be essential to the survival and growth of clonogenic tumor cells, the anti-tumor activity of the transferred cells may also be prolonged. Furthermore, cyclophosphamide is an active agent in ovarian cancer. (105)

Potential Toxicities of WT1 Peptide Specific T cells Following Adoptive Transfer

The short-term and long-term risks of adoptive therapy with autologous WT1 peptide-specific T cells are unknown but would be expected to involve those normal tissues which express WT1 at low levels in the post-natal period, namely, the kidneys, ovaries and testes and, possibly, the hematopoietic system, since normal $CD34^+$ hematopoietic cells express low levels of WT1. Preclinical and initial trials with WT1 peptides used as vaccines, however, suggest toxicities to these organs will be acceptably low. In studies at MSKCC, the 16 T cell lines generated from normal donors against the HLA A0201 presented RMF and SLG peptides of WT1 have not induced significant lysis of $CD34^+$ cells isolated from G-CSF mobilized peripheral blood stem cells or cord blood mononuclear cells to produce $CFU_{c,}$ BFU_E or CFU_{GEMM} colonies. Gao *et al* have also shown that while WT1 peptide-specific T cells lyse Ph^+ leukemic $CD34^+$ cells and inhibit their clonogenicity, they do not lyse Ph^- $CD34^+$ cells from normal donors and do not inhibit the growth of long-term colony initiating cells (LTCIC) which are considered the earliest clonogenic progenitors of the hematopoietic system that can be quantitated *in vitro* (44). In C57 mice, vaccination with the RMF WT1 peptide which is presented by $H\text{-}2D^b$ has induced high levels of WT1 specific T cells capable of protecting mice from leukemia following intraperitoneal inoculation of 3×10^7 leukemia following FBL-3 leukemia cells (44). In these animals, no pathologic changes were observed in the kidney or other organs. Furthermore, no suppression of blood counts was observed. Similarly, in initial clinical trials, Oka *et al* vaccinated 26 patients who had $WT1^+$ malignancies and also inherited the HLA A2402 allele, with the HLA A2402 presented WT1 peptide, $CMTWNQMNL_{235\text{-}243}$ and a higher affinity analog, CYTWNQMNL emulsified in montanide ISA51 adjuvant (45). In this group of patients, which included patients with breast (n=2) and lung (n=10) cancer as well as 14 patients with MDS or leukemia, 13 of 23 evaluable patients generated peptide specific T cell responses quantitated by measuring peptide HLA A2402 tetramer binding T cells in the blood. Reduction in the size of metastatic tumors was reported in 2/2 patients with breast cancer and 2/10 patients with lung cancer. Two patients with MDS developed leukopenia after repeated vaccinations. However, no alterations in blood counts were observed in the other patients treated including 12 patients with AML treated while in complete response. The leukopenia observed in these two patients was interpreted to reflect a reaction against the patient's $WT1^+$ myelodysplastic clone. No alterations in kidney or liver function were observed. These early clinical results thus suggest that circulating WT1 peptide-specific T cells generated in response

to vaccinations do not initiate autoaggressive response against these normal tissues that express low levels of WT1. This phase I trial will test whether WT1 peptide-specific T cells, generated *in vitro*, are associated with a low incidence of comorbidity when administered to either untreated or lymphoablated tumor-bearing hosts.

Of interest are recently published Phase I studies of adoptive T-cell therapy in patients with metastatic melanoma and ovarian cancer which have demonstrated the safety of this experimental treatment (99,100). Mackensen *et al.* conducted a phase I study assessing adoptively transferred Melan-A-specific cytotoxic T lymphocytes in patients with metastatic melanoma (99). Clinical adverse effects were mild and consisted of low-grade fever and chills (99). Three of eleven patients had anti-tumor responses (99). Kershaw *et al*. studied gene-modified autologous T cell transfer in patients with metastatic ovarian cancer (100). These authors generated α-folate receptor specific T-cells for the adoptive transfer into patients with metastatic ovarian cancer (100). Patients treated without IL-2 had mild side effects and the treatment was well tolerated (100). No patient responses were seen; however, this study, as the first description of adoptive transfer of gene-modified tumor-reactive T cells in ovarian cancer patients, provides insight into the safety and feasibility of adoptive therapy in these patients (100).

Pre-Clinical Studies at MSKCC

WT1 Expression by Ovarian Tumors

In order to define the frequency of WT1 expression in ovarian carcinoma, IHC analyses of human ovarian tumor microarrays was performed. The ovarian tumor arrays were tested for the presence of WT1 by IHC using a WT1 polyclonal antibody (Santa Cruz Biotech, Santa Cruz CA). 163 ovarian cancers were treated. Overall, 65% of ovarian carcinomas were positive for WT1. Furthermore, the WT1 expression increased with higher stage disease as 78% of stage II and IV tumors expressed WT1. Tumors of low malignant potential (LMP) had less WT1 expression (22%) (see Table 2).

Table 2: WT1 Expression in Human Ovarian Cancer as Determined by Immunohistochemical Study of Human Ovarian Tumor Microarrays.

Factor	Levels	WT1 Negative (N = 63)	WT1 Positive (N = 88)	P-value
Diagnosis	Invasive Borderline	41 22	81 6	<0.001
Stage	1A-1C IIA-IIC	40 10	13 17	<0.001

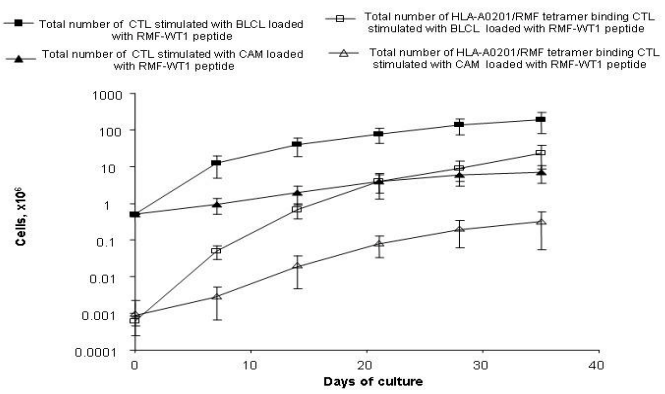
	IIIA-IIIC	11	45	
	IV	2	12	
	Missing	0	2	
Grade	1	17	6	<0.001
	2	23	17	
	3	23	64	
	Missing	0	1	
Cohort	Early	50	27	<0.001
	Advanced	13	61	

N = 151 ***Fischer Exact test**

In vitro Generation of WT1 Specific T cells from Normal Donors.

In order to induce antigen-specific T cells against WT1 or survivin, two *ex vivo* APC systems, monocyte derived dendritic cells induced by culture with IL4, GMCSF and TNFα, and EBV transformed B cell lines were explored. These APCs each express both class I and II HLA alleles as well as the necessary co-stimulatory molecules. The initial experiments have focused on the generation of WT1 reactive T cells. Therefore, the two types of APC have been loaded with WT1 peptides irradiated and used to sensitize autologous T lymphocytes. The HLA A0201 restricted peptide antigens: 1) $_{252\text{-}260}$RMFPNAPYL and 2) $_{313\text{-}321}$SLGEQQYSV or 3) an HLA A2401 binding peptide, and the recently identified, $_{428\text{-}436}$RVPGVAPTL, were used to generate WT1 specific T cells.

Figure 2: Growth Kinetics of Stimulated T cells



T cell enriched fractions were isolated by depleting adherent monocytes and $CD56^+$ NK cells from PBMC of normal or tumor bearing HLA $A0201^+$ or HLA $A2402^+$ hosts. They were then sensitized with the peptide loaded APCs at a 20:1 effector:target ratio. Cultures were presensitized at seven-day intervals over 42 days. Medium was supplemented with IL2 at three-day intervals beginning on day 11. In these initial studies, seven normal donors and two patients were evaluated. Beginning at day seven, T cell cultures were monitored for WT1 peptide-reactive T cells, by both quantitation of $CD3^+$, $CD8^+$ T cells binding the relevant peptide HLA A0201 tetramer and by quantitation of $CD4^+$ and $CD8^+$ $CD3^+$ T cells generating IFNγ after restimulation with peptide loaded autologous PBMC.

As shown in Figure 2, the total number of T cells generated was higher at each stage of the culture in the T cells stimulated with peptide loaded on EBV BLCL. The absolute number of $CD8^+$ RMF-HLA $tetramer^+$ T cells was also higher in the T cell cultures sensitized with autologous peptide loaded EBV BLCL. As shown in Figure 3a and 3b, the proportions of RMF/HLA A0201 $tetramer^+$ cells and $CD8^+$T cells generating IFNγ in response to RMF were equivalent. These studies provide evidence that peptide-loaded EBV BLCL may have several advantages as APCs including: 1) easy generation from patients while providing a continuously renewable source of APCs; 2) induce expansion of large numbers of peptide specific T cells; and 3) preferential stimulation of $CD8^+$ peptide specific cells.

Figure 3a: HLA A2 (anti-WT1 RMF) CTL

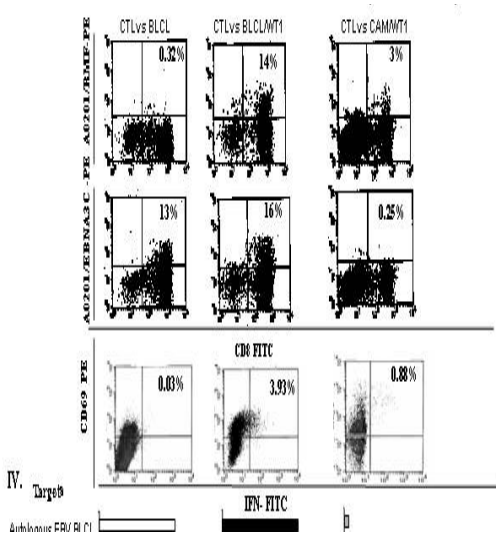
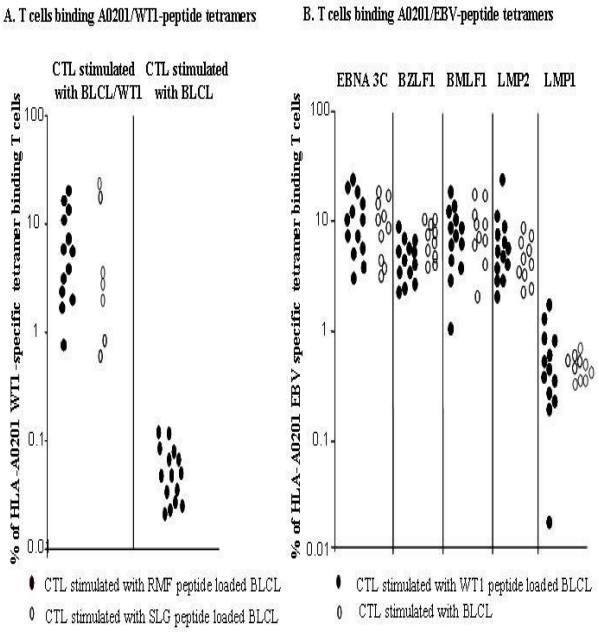


Figure 3b: Proportion of HLA A2 WT1 and EBV CTL

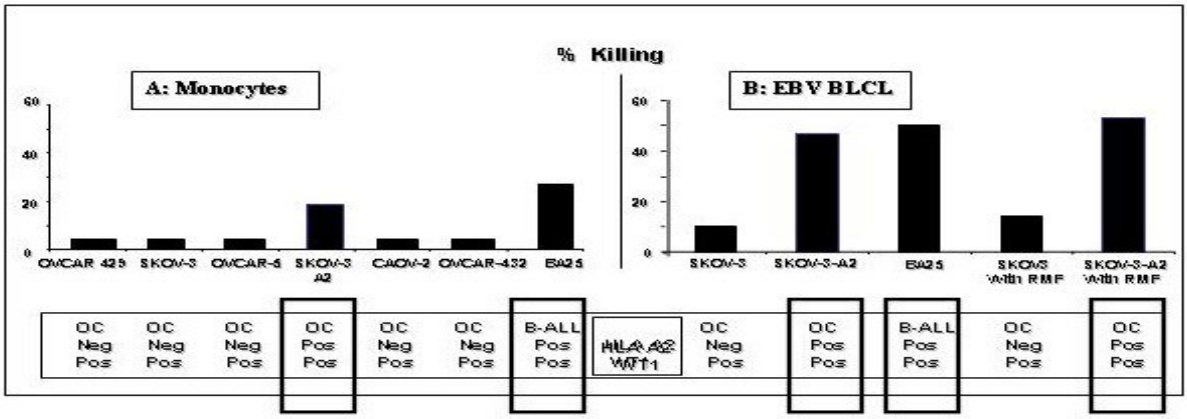


In analyses of 16 normal donors and six patients with $WT1^+$ leukemias and solid tumors expressing A0201, the T cells specific for the HLA A0201-binding RMF and SLG peptides quantitated by both tetramer binding and/or IFNγ production can be consistently generated from the blood of normal donors after *in vitro* sensitization with peptide-loaded autologous EBV BLCL. Strikingly, as shown in Figure 3, responses to RMF and SLG WT1 peptides, measured by quantitating T cells binding peptide-HLA A0201 tetramers, have been equivalent to the responses of these T cells against immunogenic peptides of EBV including EBNA3c, BZLF-1, BMLF-1 and LMP-2.

The capacity of the T cells sensitized with HLA-A0201 or HLA-A2402-binding WT1 peptide to lyse $WT1^+$ leukemic cell lines and fresh leukemia was assessed. A representative analysis of one donor is illustrated in Figure 3a. The WT1-specific T cells cultured from each normal donor lysed HLA $A2^+$ leukemic cell lines that expressed WT1. The cells killed fresh $WT1^+$ leukemia cells as well as WT1 peptide loaded targets. The tumor specificity of these T cells was confirmed by the fact that these T cells did not kill $WT1^-$ HLA $A2^+$ leukemia cells or normal hematopoietic cells or PHA blasts derived from the autologous donor. They also failed to kill HLA mismatched targets loaded with the relevant WT1 peptide or HLA disparate leukemic cells expressing WT1 indicating that these T cells are both peptide-specific and HLA restricted.

The cytolytic capacity of the WT1-specific CTLs against human ovarian tumor cells has been tested in the laboratory. The levels of HLA A2 expression and WT1 positivity have been analyzed by FACS analysis for a number of human ovarian tumor cell lines. The specific sensitivity of WT1+ HLA-A2+ ovarian cancer cells is demonstrated in Figure 4.

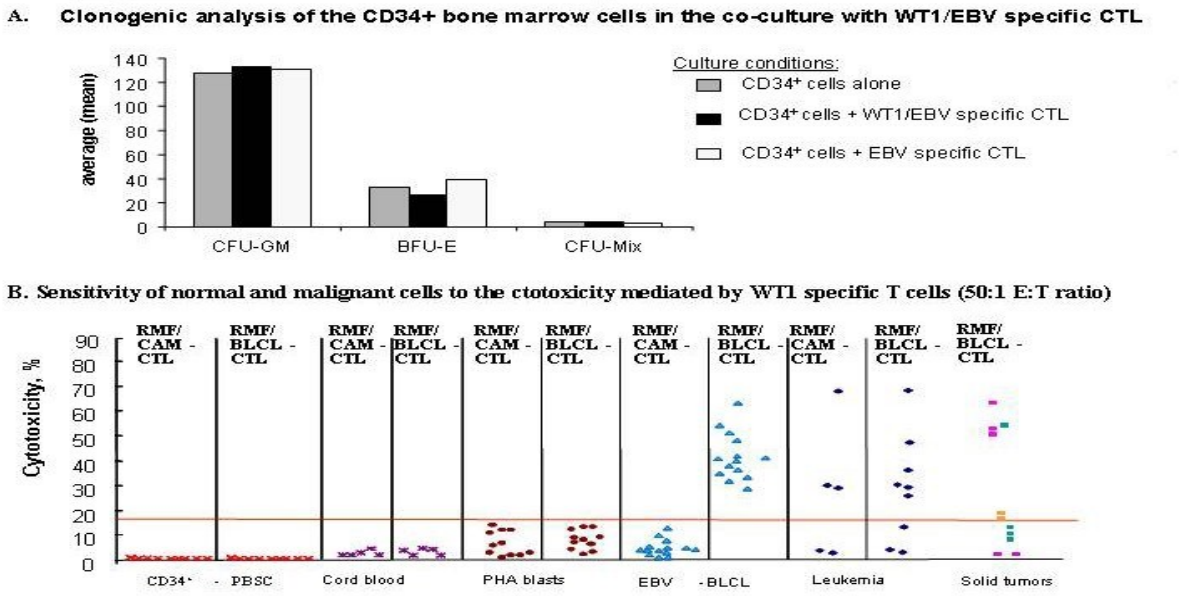
Figure 4: Anti-Tumor Activity Exhibited by HLA-A2+ CTL Stimulated *in vitro* by (A) Autologous Monocytes or (B) EBV BLCL Loaded With HLA-A2 Binding WT1 Peptide (RMF) Against Ovarian Cancer (OC) and B-Cell Acute Lymphoblastic Leukemia (B-ALL) (E:T Ratio 50:1)



WT1 specific CTLs stimulated *in vitro* with autologous monocytes loaded with a WT1 HLA-A2 binding peptide can lyse ovarian tumor cell lines that express WT1 antigen in a HLA restricted manner. The ovarian tumor cell line SKOV-3 is HLA-A2 negative and expresses low level (9%) of WT1 antigen (by FACS analysis). There is only 5% cytotoxicity seen in the ^{51}Cr Release Assay after a ten-hour incubation. However, SKOV-3 that has been transduced with HLA-A2 is lysed to a greater extent (18%). BA25 is an HLA-A2$^+$ human leukemia cell line with high WT1 expression (62%) and this was killed more (30%) by the WT1-CTL. When T cells restimulated with WT1 loaded EBV BLCL were assayed, their specific cytotoxic activity against HLA-A2+ WT1+ ovarian cancer cells was higher (50%). Thus, these experiments indicate that HLA-restricted WT1-specific CTL can lyse human ovarian tumor cells *in vitro.* The additional cytotoxicity experiments with primary ovarian tumor cells that express WT1 are currently being performed to assess their sensitivity.

A raised concern regarding the potential use of WT1 peptide-specific T-cells for adoptive immunotherapy has been that such T cells could lyse normal CD34$^+$ hematopoietic progenitor cells expressing WT1. Accordingly, the cytolytic activity of WT1 peptide-specific T cells against HLA A0201$^+$ normal cord blood progenitors and marrow PBMC fractions enriched for CD34$^+$ hematopoietic cells by positive selection for CD34$^+$ cells on Isolex columns further depleted of T cells by E-rosette depletion have been comparatively evaluated. Similar experiments were performed with a series of primary leukemias, neuroblastomas and sarcomas that do or do not express WT1. In these analyses, each target cell population was evaluated for its level of WT1 expression. Results are summarized in Figure 5. As illustrated, the level of cytotoxicity against the WT1$^+$ leukemias and solid tumors is

Figure 5 - Tumor-restricted activity of the WT1-specific T cells

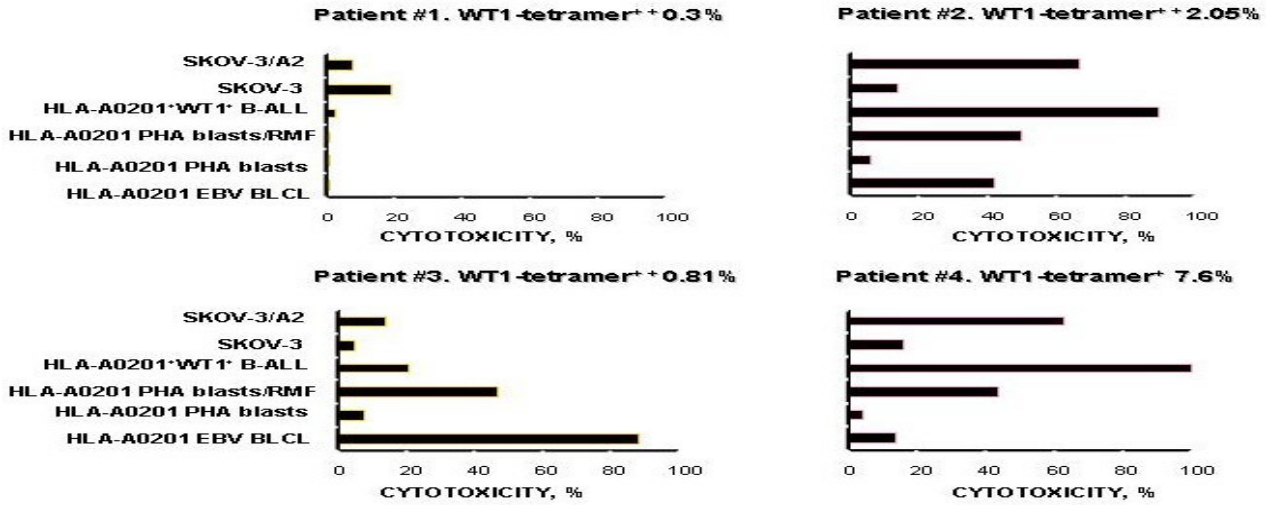


high and consistent with the high proportion of $WT1^+$ bright cells in the tumor target cells. In contrast, the proportion of cells expressing WT1 and the susceptibility of $CD34^+$ precursor enriched marrow fractions and cord blood samples to peptide-specific T cells lysis is low. Indeed, the proportion of cells lysed by WT1 peptide-specific T cells in these normal hematopoietic precursor fractions does not exceed background levels, and is comparable to the cytotoxicity generated against PHA blasts, EBV BLCL and selected leukemias which do not express WT1 (Figure 5B). To further ascertain that the small proportion of $WT1^+$ cells in the $CD34^+$ marrow and cord blood were not affected by the cytolytic T cells, collaboration with M.A.S. Moore, PhD was performed to assess the effects of WT1 peptide-specific T cells on their clonigeneity. As shown in Figure 5A, co-incubation with these T cells did not affect the yields of CFU-GM, BFU-E, or CFU mix from the $CD34^+$ marrow cell function.

Induction of WT1 Specific CTL from Ovarian Cancer Patients

Autologous EBV BLCL from six ovarian cancer patients (four of these patients are HLA A0201) have been established. The precursor frequencies of WT1 RMF-peptide tetramer specific CTLs in these patients ranges from 0.03-0.13%. WT1 specific (RMF peptide) CTLs could be generated from all patients with pulsed autologous EBV BLCL and the results of the tetramer staining and cytotoxicity experiments are illustrated in Figure 6. Interestingly, higher WT1 CTL precursor frequencies were seen in patients with WT1 positive tumors (IHC testing performed by the Pathology Core). High precursor frequencies resulted in higher percentages of WT1-CTLs after stimulation with peptide pulsed EBV BLCL. Of note, ovarian cancer patient WT1-CTLs kill WT1+, HLA-A2+ ovarian tumor cells *in vitro*.

Figure 6: Tetramer and Cytolytic activity of WT1 Specific (RMF peptide) CTLs generated from Four Ovarian Cancer Patients

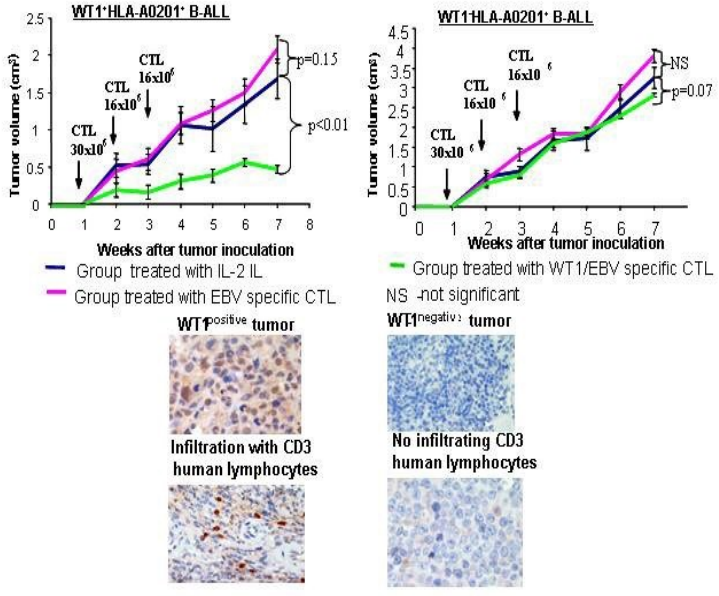


Preclinical Evaluation of WT1 Peptide-Specific T cells *In Vivo*

In order to assess the therapeutic potential of the antigen-specific CTLs, a SCID mouse model was established which was permissive to the growth of primary human myeloid and lymphoid leukemias (90,91). Initial experiments evaluated the effects of T cells sensitized with WT1 peptide loaded EBV BLCL isolated from a series of HLA A0201^{+} marrow allograft donors against subcutaneous xenografts of either their sibling's HLA matched leukemia or against allogeneic acute myeloid or lymphoid leukemias sharing the HLA A0201 allele but varying in their expression of WT1.

In the example shown in Figure 7, a single dose of 20x10^{6} T cells sensitized with EBV BLCL loaded with the RMF peptide of WT1 significantly inhibited and delayed growth of WT1^{+} HLA A0201^{+} AML xenografts when injected three weeks after initial tumor inoculation. A second dose of 35x10^{6} T cells administered at 9 weeks, when the AML xenografts again began to expand, led to complete and durable regressions of disease. As shown in Figure 7, T cells sensitized with EBV BLCL loaded with either the RMF or the SLG HLA A0201 binding peptide of WT1 were also able

Figure 7: WT1 SPECIFIC INHIBITION OF THE GROWTH OF THE HUMAN WT1+A0201+ LEUKEMIC XENOGRAFT INDUCED BY WT1/EBV CTL IN SCID MICE MODEL

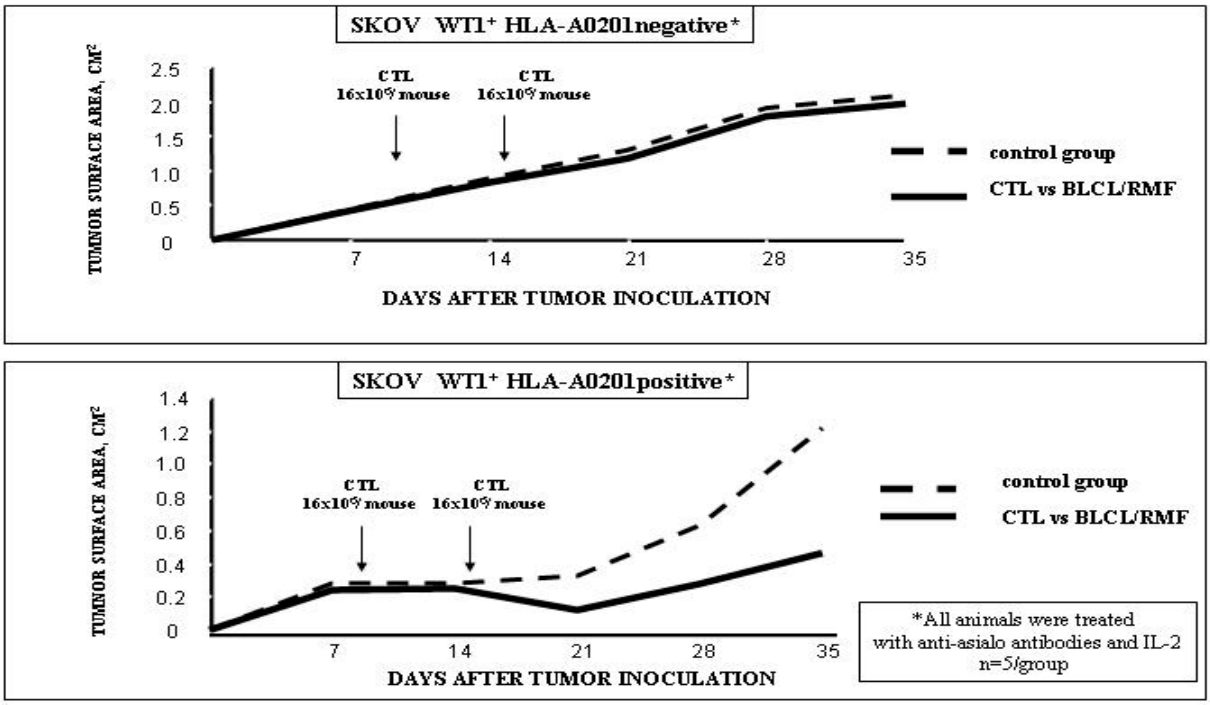


to inhibit the growth of a $WT1^+$ HLA A0201 T cell ALL.

The WT1 specificity of the *in vivo* response observed is demonstrated in the experiment presented in Figure 8. In this study, groups of animals were inoculated subcutaneously (sc) with a HLA $A0201^+$ $WT1^+$ B ALL in one thigh and a separate HLA $A0201^+$ $WT1^-$ B-ALL in the contralateral thigh. Thereafter, at day 6, 12 and 18, they received intravenous infusions of T cells sensitized with either autologous EBV BLCL loaded with the RMF peptide of WT1 or autologous EBV BLCL alone. As can be seen, the WT1 peptide sensitized T cells specifically inhibited the growth of the $WT1^+$ B-ALL, and did not affect the growth of the $WT1^-$ B-ALL in the same animals. Furthermore, the T cells sensitized with autologous EBV BLCL had no effect on either xenograft. A second important feature of the specificity in the adoptively transferred T cells is illustrated in Figure 8, namely, the selective infiltration of the T cells into the $WT1^+$ BALL xenografts, but not the $WT1^-$ BALL. This infiltration results in a striking level of apoptosis and eradication of the $WT1^+$ population.

This *in vivo* model for human ovarian cancer has also been established. SCID mice were injected subcutaneously with two human ovarian tumor cell lines that express the WT1 protein: SKOV-3 and SKOV-3-A2 (these cells are transduced with human HLA A0201 to express this molecule). After two weeks, the mice were treated with either WT1 CTL and IL-2 or IL2 alone. In the animals treated with WT1 CTL, the SKOV-3-A2 tumors regressed and their growth was inhibited (Figure 8). Pathologic evaluation of the human tumors from the mice revealed that human CD8+ T cells were present in the WT1+ A2+ tumor but not in the WT1+ A2- tumor. This suggests that WT1 CTL home to tumors if the WT1 antigen in expressed in the context of the correct HLA.

Figure 8: The Influence of WT1 peptide specific CTL (RMF) on the Size of Subcutaneous WT1 Positive, HLA A2.1 Ovarian Xenografted tumors in SCID Mice.



Tumor-Directed Migration of HLA-Restricted WT1 Specific T Cells *In Vivo*

In order to assess the homing of antigen specific CTLs to tumor targets in an in vivo system, a new technique was developed permitting sequential in vivo PET imaging of antigen-specific CTLs in SCID mice bearing human tumor xenografts (63).

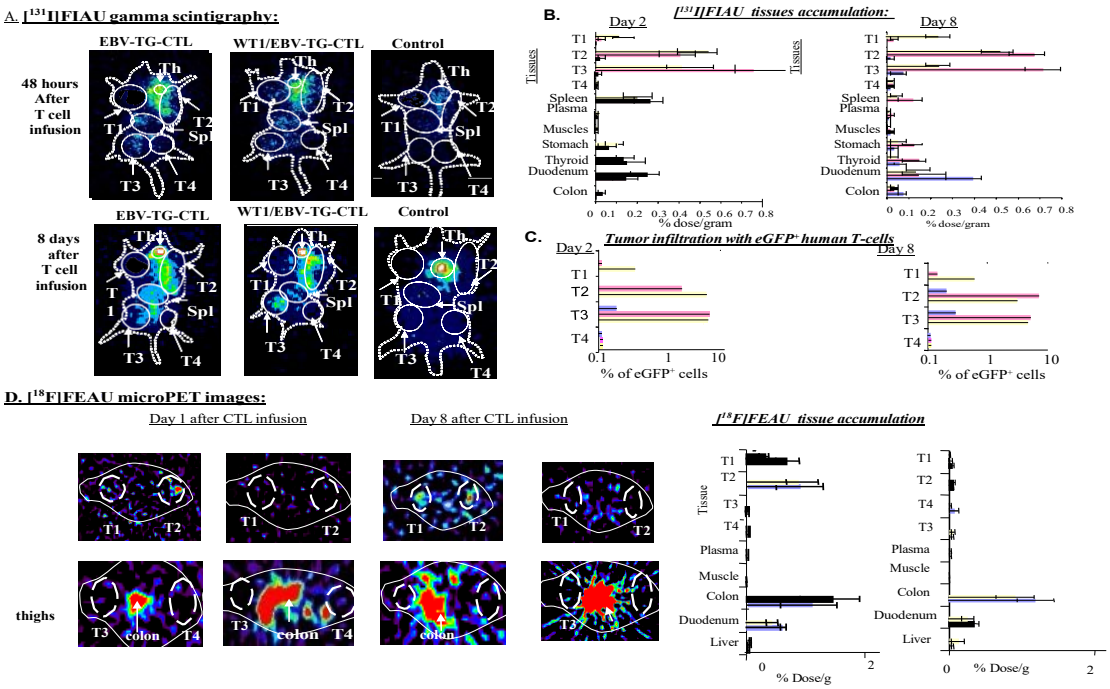
In order to image the antigen-specific T cells, a retroviral vector was developed that can efficiently transduce human T lymphocytes and direct the expression of a Herpes-Simplex Thymidine Kinase green fluorescence protein fusion gene (HSV-TK/GFP). The transduced cells can be isolated based on their expression of GFP. Transduced cells also express HSV-TK. As a result, these transduced cells will selectively incorporate ^{131}I or ^{124}I-labeled 2'-fluoro-2'deoxy-1-b-D arabinofuransyl-5-iodo-uracil (^{124}I-FIAU) into their DNA. Consequently, transduced T cells can be selectively labeled either *in vitro*, or *in vivo* at sequential times after their adoptive transfer into animals bearing tumor xenografts.

In subsequent experiments, this technique was evaluated as a method for tracking WT1 specific T cells after adoptive transfer. In the study illustrated in Figure 9, SCID mice were inoculated with a HLA A0201$^+$ WT1$^+$ BALL in the left shoulder (T1), an autologous EBV BLCL in the right shoulder (T2), and a HLA A0201$^+$ homozygous EBV BLCL (T3) and a WT1$^-$ HLA A0201$^+$ BALL (T4) in left and right thighs, respectively. After development of the tumors, groups of animals were treated with T cells sensitized either with WT1 peptide loaded autologous EBV BLCL or autologous EBV BLCL alone. The T cells were transduced with the HSV-TK/GFP vector 42 days thereafter. Post sorting, 3.1% of the transduced T cells sensitized

with WT1 peptide loaded EBV BLCL were WT1 specific and the rest reactive against EBV. *In vitro*, both the EBV-specific and the WT1 and EBV-specific transduced and sorted GFP^+ T cells were restricted by HLA A0201. One and seven days after intravenous infusion of either the WT1/EBV dual specific HSV-TK/GFP^+ T cells or the EBV-specific, HSV-TK/GFP^+ T cells, the animals received an infusion of [^{131}I]-FIAU and twenty-four hours after each dose, the animals were imaged by scintigraphy (PET could not be used due to the temporary unavailability of ^{124}I). As shown in the control animal (Figure 9) infused with non-transduced T cells followed twenty-four hours later by [^{131}I]-FIAU, label was detected by scintigraphy only in the thyroid, consistent with the absence of HSV-TK^+ T cells in the animals. Mice treated with the EBV-specific TK-GFP^+ T cells accumulated [^{131}I]-FIAU selectively in the EBV^+ HLA $A0201^+$ lymphomas (T2 and T3) and spleen both at day two and day eight post T cell infusion (Figure 9B,E). In contrast, the TK-GFP^+ T cells containing both WT1 peptide specific and EBV reactive T cells accumulated in the $WT1^+$ BALL xenografts (T1) and the EBV^+ lymphoma xenografts in proportion to the representation of WT1-specific and EBV-specific T cells in the sorted TK-GFP^+ T cells infused, but did not infiltrate into the $WT1^-$ BALL xenografts (Figure 9A,D). The scintigraphic findings were further corroborated by quantitation of the [^{131}I]-label in autopsied tissues and by FACS analysis of these tissues for transduced, GFP^+ cells obtained from mice sacrificed 24 hours after either the day 1one or day seven dose of [^{131}I]-FIAU (Figure 9).

In a separate experiment, purified TK/GFP vector transduced T cells sensitized with RMF peptide loaded EBV BLCL were infused into mice bearing four established tumor xenografts. At one, eight and 15 days post T cell infusion, the animals were injected with ^{18}F-labeled FEAU and then imaged by PET four hours thereafter. As shown in the cross sectional images at the shoulders and thighs and the tissue accumulation data presented in Figure 9D, the HLA-A2 restricted RMF-specific T cells selectively accumulated in a $WT1^+$ rhabdomyosarcoma (T1) and the $WT1^+$ BA-25 ALL (T2) xenograft, each of which also strongly expresses HLA-A0201. In contrast, the transduced T cells did not accumulate in the HLA $A0201^+$ $WT1^-$ BALL tumor (T4). Moreover, they failed to accumulate in a $WT1^+$ rhabdomyosarcoma (T3) which was genotypically HLA-A0201 but failed to express HLA A0201 detectable by an allele-specific antibody as measured by FACS. These studies suggest that down regulation of HLA expression which has been shown to protect tumor cells from T cell mediated lysis *in vitro*, can also prevent the targeted migration to and accumulation of tumor-peptide specific T cells in tumors *in vivo*.
Taken together, these *in vitro* analyses and preclinical evaluations of WT1 peptide-specific T cells *in vivo* in SCID mice bearing human $WT1^{-+}$ tumor xenografts support the potential of such T cells to lyse $WT1^+$ tumors *in vitro* and inhibit their growth *in vivo.* Based on these studies and other reports summarized in the background, these WT1 peptide-specific T cells will be evaluated in a phase I trial in patients with relapsed $WT1^+$ ovarian, primary peritoneal or fallopian tube carcinoma.

Figure 9: In vivo imaging of WT1 CTL homing to WT1 HLA A0201 Specific tumors using FIAU



Experience with Infusion of WT-1 peptide specific T-cells and the Rationale for Repeated Dosings:

The Results to date of Phase I trials at MSKCC

At present, two Phase I trials of WT-1 peptide specific T-cells are in progress:

IRB 06-155 A2 is evaluating autologous WT-1 specific T-cells in women with advanced ovarian carcinomas. This protocol has accrued only two patients at the lowest dose, 5 x 10^6 T-cells/ m^2. Both patients ultimately had progression of disease. However, one patient had stabilization of disease for 2 months and exhibited a significant reduction in Ca-125 levels following infusion of WT-1 specific T-cells. No toxicities were observed. In particular, neither patient exhibited count depression or evidence of renal toxicity. Accrual to this trial has been limited due to the unwillingness of potential candidates to receive only one dose of the T-cells and be observed until disease progression.

Protocol 07-055 A (4) is evaluating WT-1 specific T-cells generated from normal HLA-matched HSCT donors in the treatment of patients transplanted for WT-1^+ hematologic

malignancies who have relapsed or have persistent or recurrent minimal residual disease. To date, 10 patients have been treated, 4 at dose level 1 (1 x 10^6 T-cells/ m^2), 3 at dose level 2 (5 x 10^6 T-cell/m^2) and 3 at dose level 3 (1 x 10^7 T-cells/m^2). No patient in this trial has experienced a grade III toxicity attributable to the infusion of cells. One patient experienced moderate diarrhea with streaks of blood in the stool attributable to an ongoing infection. No patient experienced renal or hematologic toxicity. No patient developed new acute or chronic GVHD, and existing GVHD was not exacerbated by this treatment.

As shown in Figure 10, of a patient with CML was treated with WT-1 specific T-cells containing both CD4+ and CD8+ T-cells recognizing epitopes in pentadecapeptides #20 presented by HLA DRB, 0102 and B4701 respectively. The infusion of WT-1 specific T-cells was followed by clearance of cells with WT-1 transcripts, as measured by quantitative RTpcr measuring WT-1 mRNA copy numbers in the blood. The infusions were also followed by the emergence of detectable WT-1 peptide specific CTL precursors (CTLp), as quantitated by limiting dilution analyses in 8 of 8 patients thus far studied. It is important to note that these patients have been treated within 1 year post transplant, at which time, they were still relatively immunodeficient by virtue of the myeloablative therapy administered for cytoreduction pre-transplant, and the T-cell depleted graft they received. Despite this, however, the increases in WT-1 specific CTLp were transient, persisting only for 7- 28 days in each of the patients tested. Increases in tetramer$^+$ T-cells were also transient except in one patient treated for CNS recurrence in whom increments in tetramer$^+$ cells could be detected as late as 100 days post infusion.

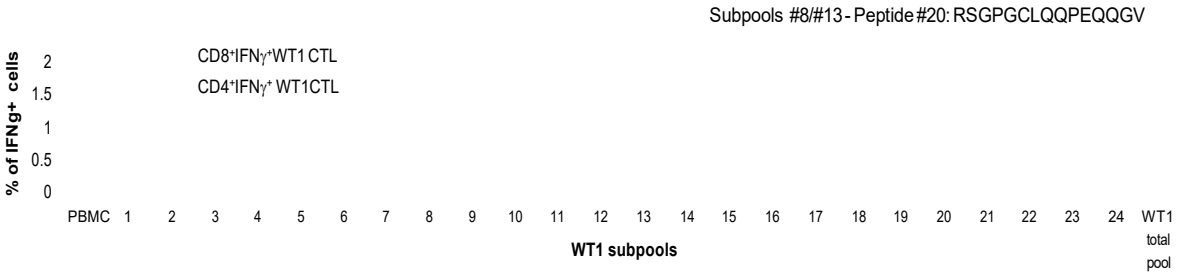
Based on these preliminary results, we suggest that infusions of autologous or allogeneic WT-1 peptide specific T-cells are associated with minimal or no toxicity. However, because these T-cells and T-cells specific for other “self” antigens that have been administered without lymphodepletion in other clinical trials survive and proliferate for periods of only 1-2 weeks after infusion, we hypothesize that repeated infusions of these cells will be required if they are to be able to exert a sustained and significant anti-tumor effect in vivo. Accordingly, the protocol has been amended to include 4 repeated doses of T-cells, at two week intervals, to better test the anti-tumor effects of the T-cells administered. The patients in dose level II will also receive cyclophosphamide prior to T-cell infusion to induce a state of lymphodepletion conducive to the growth of populations of WT-1 specific T-cells following adoptive transfer.

FIGURE 10

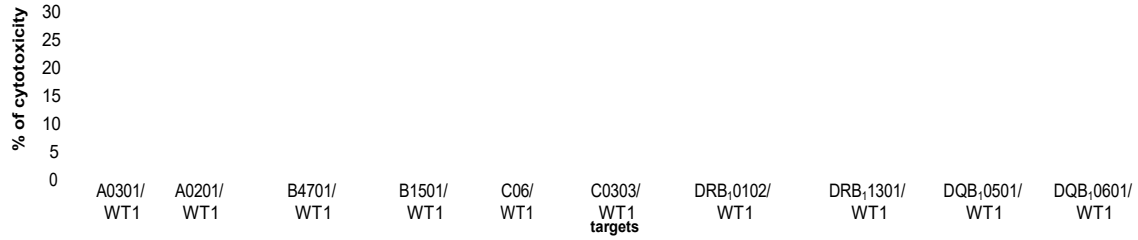
Case# 000-00-140-08:

patient with CML treated with WT1 CTL at the dose 5x10^6/m^2 (total dose 10x10^6)

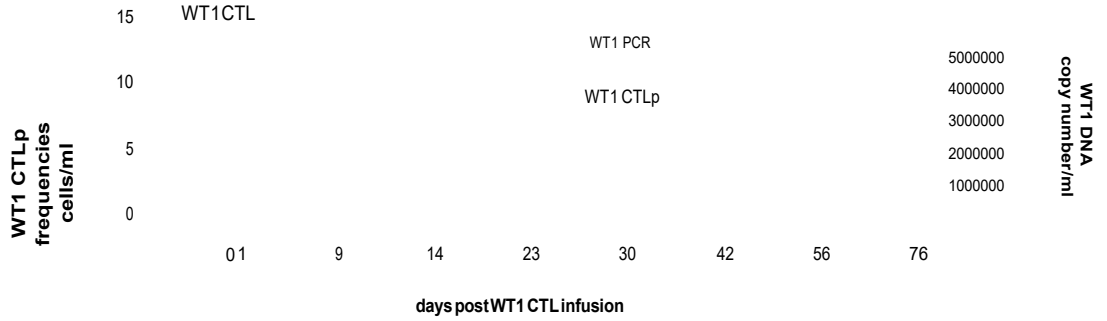
Identification of the immunogenic epitope by IFNγ FACS analysis



HLA restriction of the WT1 specific cytotoxic activity of the WT1 CTL against the EBV BLCL matching one of the HLA alleles of the T cell donor loaded with WT1 pool. The same unloaded targets were not lyzed by these T cells.



Monitoring of the WT1 PCR and WT1 CTLp in the peripheral blood of the patient after WT1 CTL infusion



4.1 OVERVIEW OF STUDY DESIGN/INTERVENTION

4.2 Design

This is a non-randomized phase I clinical study. The target population in this study are patients with recurrent or persistent, measurable $WT1^+$ ovarian, primary peritoneal, or fallopian tube carcinomas. Furthermore, the patients' ovarian, primary peritoneal or fallopian tube carcinoma must express the WT1 protein detectable by IHC analysis of the patient's tumor.

4.3 Intervention

Once the requisite dose of T cells has been generated, the patients will receive WT1 reactive T cells by intravenous infusion. Patients will be treated in 4 sequential groups of 3-6 patients. Dose escalation will be based on dose limiting toxicity (DLT). In cohort 1 patients will receive WT1 specific T cells dosed at 5 x $10^6/m^2$ (level I) without cyclophosphamide lymphodepletion. Patients in dose level II, III, and IV will receive a standard lymphodepletive regimen consisting of a single dose of cyclophosphamide 750mg/m^2 (99,104,105), administered intravenously, 48 hours prior to the first T cell infusion. No further doses of cyclophosphamide will be administered to these patients during the study. After a 48 hour rest period to permit elimination of cyclophosphamide, each patient will receive a dose of autologous T cells by an intravenous infusion from the WT1 peptide-specific T cell line by intravenous infusion.

Sequential groups of four patients will be accrued to each treatment group. The T cell and conditioning chemotherapy to be evaluated in each group are summarized in Table 3:

Table 3: Dose Levels of T Cells and Conditioning Chemotherapy

Level	Patients	Cyclophosphamide	WT1 Specific T cells
Level I	**3-6**	**NO**	**5 x $10^6/m^2$**
Level II	**3-6**	**Yes**	**2 x $10^7/m^2$**
Level III	**3-6**	**Yes**	**5 x $10^7/m^2$**
Level IV	**3-6**	**Yes**	**1 x $10^8/m^2$**

The first two patients in cohort 1 only received a single administration of WT1 specific T cells, whereas all subsequent patients treated in cohort 1 are to receive specific T cell infusions every two weeks for four doses. Patients in cohorts II, III and IV may

receive additional infusions of WT1 peptide-specific T cells once every 2 weeks for a total of 4 doses. Each cycle comprises of 28 days or two doses of WT1 specific T cells.

There will be a finite number of WT1 peptide-specific T cells and therefore a finite number of infusions possible. If, after 4 infusions, the patient (in cohort I, II,III, and IV) has a clinical response or at least stable disease, additional infusions of WT1 peptide sensitized T cells may continue to be administered once every 2 weeks until the stock has been exhausted, DLT, withdrawal of consent or disease progression. These additional infusions of WT1 peptide-specific T cells will be administered at the same dose level as the patient's initial infusion.

5.1 THERAPEUTIC/DIAGNOSTIC AGENTS

5.2 WT1 Specific T Lymphocytes

These are the patient's own (i.e. autologous) T cells (CD4 and CD8 cells) that have been sensitized with WT1 peptide loaded on autologous irradiated EBV-transformed B cell lines and expanded ex vivo as described in Section 9.1. These T cells are pre-tested to ascertain that: 1) they contain WT1 specific cytotoxic T cells; 2) they do not react against lymphocytes from the patient; 3) they are microbiologically sterile and free of mycoplasma; and 4) they contain less than five EU of endotoxin per dose of cells. One infusion of T cells was administered to the first two patients treated at dose level I. For all subsequent patients treated at dose level I and for patients treated at level II-IV infusions of T cells will be administered to each patient once every 2 weeks, according to their assigned dose level, for 4 doses. From week 2 onwards a potential +/- 72hour treatment window is permissible provided that necessary assessments and laboratory tests can still be carried out. If, after 4 T cell infusions, the patient has a clinical response or at least stable disease, additional infusions of WT1 peptide sensitized T cells may continue to be administered once every 2 weeks until the stock has been exhausted, DLT, withdrawal of consent or disease progression.

5.3 Cyclophosphamide (Cyclophosphamide (Cytoxan®, Neosar®, CTX))

- Nitrogen mustard derivative-alkylating agent
- Common Indications: Hodgkin's disease, lymphoma, multiple myeloma, chronic lymphocytic leukemia, acute lymphocytic leukemia, acute myeloblastic leukemia, mycosis fungoides, neuroblastoma, retinoblastoma, ovarian and breast carcinoma
- Supplied as: 200 mg, 500 mg, 2000 mg vials

DOSAGE, DOSE MODIFICATIONS AND ADMINISTRATION

- As previously indicated, the standard dose of cyclophosphamide will be 750mg/m^2. Baseline renal and hepatic function will be assessed prior to the single cyclophosphamide administration. No dose-reductions are permitted. Table 4 describes the parameters for standard lymphodepletive regimen consisting of a single dose of cyclophosphamide 750mg/m^2:

Table 4: Single Dose Parameters of Cyclophosphamide (750mg/m^2):

Absolute Neutrophil Count	**Platelets**	**Creatine**	**Bilirubin**	**AST**	**ALT**
≥1.5 K/mcL	**≥100 K/mcL**	**≤1.5 mg/dl**	**≤1.5xULN**	**≤ 2.5 x ULN**	**≤ 2.5 x ULN**

6.1 CRITERIA FOR SUBJECT ELIGIBILITY

6.2 Subject Inclusion Criteria

- Pathologically confirmed diagnosis of ovarian, primary peritoneal, or fallopian tube carcinoma.
- Recurrent or persistent disease following treatment with platinum-based chemotherapy.
- Evaluable disease as demonstrated by Ca125, radiologic, or pathologic studies conducted.
- Patients' carcinoma must express the WT1 protein detectable by IHC analysis of banked (paraffin embedded) or freshly biopsied tumor nodules.
    - IHC evidence of WT1 expression will be performed according to the technique described by Dupont and Soslow *et al* (40). Expression will be graded according to an adaption of the German Immunoreactive Score (IRS).
    - Only WT1 tumors with moderate to strong IRS scores (4-12) will be considered positive.
- Karnofsky Performance Status score of 70% or greater (WHO status 0-1).
- Life expectancy of at least six months.
- Adequate bone marrow, renal, and hepatic function:
    - Absolute neutrophil count (ANC) ≥ 1500/mm^3
    - Platelets ≥ 100,000/mm^3
    - Creatinine ≤ 1.5mg/dL or creatinine clearance ≥ 60ml/min

- ALT and AST all ≤ 2.5 x the institutional upper limit of normal (ULN) and total bilirubin ≤ 1.5 x the institutional ULN
- Adequate pulmonary and cardiac function.
  - No clinical evidence of cardiopulmonary disease, which, in the opinion of the investigator, precludes enrollment.
- Age greater than or equal to 18 years.
- Written Informed Consent from each patient or the patient's representative prior to study entry.
- No anti-cancer therapy (chemotherapy, biologic therapy, or immunotherapy) within three weeks prior to the initial T cell infusion (Levels I), or cyclophosphamide (Levels II, III, and IV).
- Ability to keep scheduled visits.

6.3 Subject Exclusion Criteria

- Known hepatitis B, C or HIV infection.
- History of whole abdominal radiation therapy (WART).
- Evidence of bowel obstruction.
- Active brain metastases or history of brain metastases.
- Clinically significant heart disease (NYHA class III or IV).
- Active infections requiring antibiotics within two weeks of registration.
- Serious intercurrent illness, requiring hospitalization.
- Primary or secondary immunodeficiency or active autoimmune disease.
- Other cancers (excluding non-melanomatous skin cancer) within 5 years.
- Women who are pregnant or lactating.
- Any other issue which is the opinion of the treating physician would make the patient ineligible for the study.

7.0 RECRUITMENT PLAN

This is a single institution trial. All patients will be recruited through the outpatient gynecologic medical oncology clinic of MSKCC. Patients with recurrent or persistent ovarian, primary peritoneal or fallopian tube cancer following treatment with platinum-based chemotherapy will be recruited to this study following evaluation in the outpatient or inpatient setting. MSKCC has filed HHS 441 (civil rights), HHS (handicapped individuals), 639-A (sex discrimination), and 680 (age discrimination). We also take due notice of the NIH/ADAMHA policy concerning inclusion of women and minorities in clinical research populations. Women and members of all ethnic groups are eligible for this trial.

The ethnic composition of the subject population reflects patients referred to MSKCC. Approximately 70% of the patients are Caucasians, 7% Black, 10% Hispanic and 4%

Oriental. No patient is excluded from participation in studies on the basis of ethnicity or race. Only women develop ovarian carcinoma.

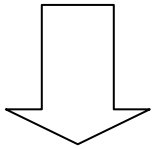
Before protocol specific procedures are carried out, the investigator will explain the full details of the protocol and study procedures and the risks involved to each patient prior to the patient's inclusion into the trial. The patient will be informed that she is free to withdraw from the study at any time. The patient will sign an IRB approved consent form indicating her consent to participate. This consent form will conform to the applicable requirement of 21 CFR 50.25 elements of informed consent. The original consent will become part of the patient's medical record. Each patient will receive a copy of the informed consent.

8.1 PRETREATMENT EVALUATION

Informed Consent 1: After signing Informed Consent 1 (Screening Informed Consent), the patients' WT1 tumor expression will be determined as previously described. In order to be eligible for this protocol, the patients' ovarian, primary peritoneal or fallopian tube carcinoma must express the WT1 protein detectable by IHC analysis of banked (paraffin embedded) tumor. After signing Informed Consent 1, several weeks will elapse while the patients' banked tumor is tested for WT1 expression. Once patients are deemed eligible, a leukapheresis will be performed for the collection of PBMC to establish autologous EBV BLCL and generation of WT1-specific T cell lines. The screening phase, as described above and outlined in Table 5, will take approximately 12-16 weeks. Patients will also have blood drawn to assess EBV serology.

Table 5: Screening Phase (Informed Consent One)

Sign Informed Consent One	**X**
WT1 Tumor Testing	**X**
HLA Testing & complete blood count	**X**



Eligible patients

Collection of 4 green top tubes for the establishment of EBV BLCL if a significant time lapse before patient can undergo leukapheresis & Collection of 1 red top tube for EBV serology	**X**
Leukapheresis for the Establishment of Autologous EBV BLCL if has not been established yet and generation of WT1-specific T cell lines	**X**

- **Screening will take approximately 12-16 weeks.**

Informed Consent 2: Eligible patients will then receive Informed Consent 2 (Treatment Informed Consent). Patients will then be treated with the following: T cell infusion alone (dose levels I) or T cell infusion with prior cyclophosphamide (dose levels II, III, and IV). Within 30 days prior to starting the T cell infusion, all patients will undergo the following:

- History and physical examination (including performance status, height, weight)
- Hematopoietic: Complete blood count and PT, aPTT
- Biochemical: Comprehensive metabolic panel, Magnesium, Ca-125 level
- Others: Urinalysis, creatinine clearance
- CT Scan of the chest, abdomen, and pelvis
- EKG
- Research Blood samples: Obtained prior to adoptive therapy to quantitate baseline levels of WT1 reactive T cells, by quantitation of WT1 specific CTLp by LDA, T cells secreting IFNγ in response to peptide and, in HLA A0201^{+} patients, T cell binding WT1 peptide HLA A2 tetramers.

9.1 TREATMENT/INTERVENTION PLAN

Treatment with WT1 specific T cells:

9.2 Generation and Isolation of WT1 Specific T cells for Adoptive Therapy

The patients qualifying and consenting to participate in this study (i.e. after signing Informed Consent One) will donate 4 green top tubes of peripheral blood to establish autologous EBV BLCL if the leukapheresis cannot be collected immediately which will be cryopreserved for potential use as peptide-loaded sensitizing cells and peptide loaded targets in assays of T cell reactivity against antigenic peptides. If the leukapheresis product is collected the 4 green top tubes are not needed. Once the EBV BLCL are established the patient will undergo leukapheresis to collect PBMC for the generation of autologous CAMs and autologous WT1-specific T cell lines. The patient's EBV BLCL or CAMs, loaded with peptide, will be used for autologous T cell sensitization. Please see Appendix A for methodology.

The use of EBV BLCL as APCs has advantages in that they can be propagated in large numbers once the EBV BLCL have been established. In addition, T cells sensitized to peptide loaded EBV BLCL also generate EBV-specific T cells, which should replenish EBV-restricted T cells in the immunosuppressed host.

Prior to use as sensitizers, the CAMs or EBV BLCL will be irradiated to 3000rads or 9,000 rads respectively in a calibrated certified blood irradiator to eradicate the capacity of these cells to grow within co-cultures. In addition, EBV BLCL will be cultured in media containing acyclovir to eliminate the risk of transferring infectious EBV in the T cells transferred. CTLs from the EBV seronegative individuals will be generated from the CD3 enriched B cell depleted lymphocytes.

To prepare T cells for sensitization and *in vitro* expansion, mononuclear cells will initially be isolated from heparinized leukapheresed white cell preparations by centrifugation on Ficoll-Hypaque density gradient. After washing, T cells will be enriched by initially depleting monocytes by adherence to sterile plastic tissue culture flasks or by the immunomagnetic depletion using the CD14 microbeads. Thereafter, B and NK cells will be depleted by incubation with clinical grade anti-CD19 and CD56 microbeads Miltenyi reagent. The T cell enriched cell fractions will be washed and suspended in medium containing 5% prescreened heat-inactivated Ab serum in preparation for sensitization.

Autologous T cells (CD4 and CD8 cells) will be sensitized in vitro to recognize WT1 peptides loaded on autologous irradiated CAMs or EBV BLCL and expanded ex vivo. After initial sensitization, the WT1/EBV peptide-sensitized T cells are cultured for 35-42 days. They are restimulated with CAMs or EBV BLCL loaded with the total pool of pentadecapeptides spanning the whole sequence of WT1 protein overlapping by 11aa at 7-day intervals. In addition, after 10 days of culture, IL2 (Chiron) is added every 3 days and IL15 (Miltenyi) is added every 7 days. After 32-42 days of culture, the T cells are washed and then acceptance tested for T cell content and for specific reactivity against targets loaded with WT1 total pool of pentadecapeptides, sterility, mycoplasma, HLA type and endotoxin. Autologous WT1 specific T cell populations will be generated in quantities sufficient for adoptive therapy.

After sensitization, the cells will be expanded in large-scale cultures with IL2 and OKT3 according to a modification of the technique of Dudley and Rosenberg, using irradiated autologous WT1 peptide loaded EBV BLCL and autologous WT1 peptide-loaded PBMC as irradiated feeders (1) The T cells will be assessed for WT-1 reactive T cells after sensitization and expansion by quantitation of T cells generating IFNγ in response to secondary restimulation with WT1-peptide loaded autologous peripheral blood mononuclear cells. In HLA-A0201^{+} patients, T cells binding WT1 peptide-HLA A0201 tetramers will also be quantitated, where applicable. The WT1 specific cytolytic activity will be assessed against autologous WT1 peptide loaded targets.

Prior to infusion, T cells will be tested and certified as microbiologically sterile and free of mycoplasma. Products that do not meet sterility testing requirements will not be released for use in patients. Each dose of cells will contain < 5 EU

endotoxin/dose of cells. Certified T cell doses will be cryopreserved until use. For administration, the cell dose is suspended in approximately 25-60 ml sterile preservative free saline for injection, and infused intravenously over 5-10 minutes. If the T cells are not used for treatment, they may be used for research purposes in the laboratory.

T cell infusions will be administered in the Adult Same Day Hospital, an outpatient center, at the main campus of MSKCC. Conditioning chemotherapy will be administered at our outpatient chemotherapy center.

9.3 Preparative Immunosuppression and Treatment with WT1 T cells

Patients will be treated with the cells in sequential groups of three patients varying by T cell dose and the addition of lymphodepletive therapy prior to T cell infusion. Level I will receive WT1 specific T cells without prior cyclophosphamide. Patients in Levels II, III and IV will undergo pre-infusion lymphodepletion with intravenous cyclophosphamide, 750mg/m^2. After a 48-hour rest period to permit elimination of cyclophosphamide, each patient in levels II, III, and IV will receive a dose of autologous T cells from the WT1 specific T cell line by intravenous infusion according to the table below.

In this phase I trial, one dose level of T cells will be tested in patients without other treatment. An escalated T cell dose will then be evaluated in the next three groups (levels II,III, and IV) that will undergo lymphodepletion first with cyclophosphamide prior to the initial T cell infusion. Only a single dose of cyclophosphamide will be administered to patients on level II, III, and IV during the study. The T cell doses evaluated in this dose escalation are listed in Table 6.

Table 6 Dose Levels of T Cells and Conditioning Chemotherapy

Level	Patients	Cyclophosphamide	WT1 Specific T cells
Level I	**3-6**	**NO**	**5 x 10^6/m^2**
Level II	**3-6**	**Yes**	**2 x 10^7/m^2**
Level III	**3-6**	**Yes**	**5 x 10^7/m^2**
Level IV	**3-6**	**Yes**	**1 x 10^8/m^2**

The first two patients treated at dose level I received a single infusion of the WT1 peptide-specific T cells. All subsequent patients may receive additional WT1 peptide-specific T cells infusions, once every two weeks for 4 doses. If, after 4 infusions, the patient has a clinical response or at least stable disease, additional infusions of WT1

peptide sensitized T cells may continue to be administered once every 2 weeks until the stock has been exhausted, DLT, withdrawal of consent or disease progression.

9.4 Dose Escalation and Dose Limiting Toxicity (DLT):

In this phase I trial, dose escalation will be based on the dose limiting toxicity (DLT). For this trial, DLT will be defined as a grade III or greater toxicity developing following infusion of the T cells. Only toxicities that are possibly, probably, or definitely related to T cell infusions will be considered DLTs. For dose Levels II, III, and IV (who receive cyclophosphamide), hematopoietic DLT will not be included in the first 21 days of the first cycle of treatment, since cytopenia is an expected toxicity of the pretreatment course of cyclophosphamide. However, severe grade 3 cytopenia extending beyond these 21 days will be graded as a severe toxicity. The dose escalation scheme for this study is as follows:

- If 0 of 3 patients experience DLT at a given dose level, then proceed to the next starting dose level.

- If 1 of 3 patients experience DLT at a given dose level, 3 additional patients will be treated at that level. If 0 of those 3 develop DLT, the dose will be further escalated.

- If 2 or more of the 6 patients experience DLT at a given dose level, the maximum tolerated dose (MTD) is defined as the previous dose level. If only 3 patients are treated at the MTD, an additional 3 patients will be treated at that level. If among these 6 patients, 2 or more experience DLT, the MTD will be lowered and the same criterion will be applied to the previous dose level.

  If any patient at a dose level is not evaluable through the first twenty-eight days post the initial T cell infusion, the patient must be replaced before dose escalation to the next level occurs so that at least three patients are evaluated at a dose level prior to dose escalation.

10.1 EVALUATION DURING TREATMENT/INTERVENTION

- Patients will be evaluated clinically with vital signs pre-and six hours post infusion of T cells.

- Patients will receive physical examinations and toxicity assessment (using CTCAE version 4.0) on the day after each T cell infusion. All patients will be

examined at least once weekly during cycle 1. Patients will also have a safety visit four and eight weeks after the final T cell infusion, when applicable.

- Patients will have at least weekly CBCs with differentials and platelets, biochemical screens and creatinine clearances to monitor for renal or hepatic toxicity for the first four weeks (i.e., cycle 1) and then every two weeks thereafter (i.e., cycle 2 or greater) until the final T cell infusion. Patients will also have these bloods assessed at 4 and 8 weeks following the final T cell infusion, when applicable.

- Serum CA125 levels will be measured at baseline, prior to each T cell infusion, and at 4 and 8 weeks after the final T cell infusion.

- In addition, during cycle 1 patients will be tested for circulating levels and phenotype of T cells as well as for proportion of T cells or generating intracellular IFNγ in response to WT1 or binding WT1/HLA A2 tetramers prior to chemotherapy (if applicable), prior to the first T cell infusion, 24 hours post the first T cell infusion and at 1 week post the initial infusion. For patients receiving multiple T cell infusions frequencies of T cells specific for WT1 will also be assessed prior to each infusion of WT1 specific T cells and 24-hours post the 4^{th}, 5^{th}, 6^{th} and 8^{th} when applicable infusions by LDA. Furthermore, frequencies of T cells specific for WT1 will also be assessed 4 and 8 weeks following the final infusion the T cells.

- Concurrent quantitation of EBV specific T cell precursors by LDA and T cells generating IFNγ in response to autologous EBV BLCL will be performed as a second marker of the persistence and expansion of adoptively transferred T cells, as well as recovery of autologous immune function.

- Tumor responses will also be monitored radiographically by CT of the chest, abdomen and pelvis performed at baseline (prior to the initial T cell infusion and if applicable cyclophosphamide) and approximately every 8 weeks (i.e. following every 2 cycles) whilst on study.

- Four weeks after the final T cell infusion will be considered the Safety Follow-Up visit and an additional follow-up visit will occur at 8 weeks post final T cell infusion.

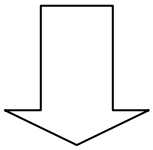
- This study will also monitor patients for alterations in circulating levels of WT1 specific T cells at sequential intervals post adoptive transfer. In addition, patients will be monitored for alterations in tumor size and levels of circulating tumor antigens in the post treatment period. These evaluations will be observational and descriptive only, since the study group size is insufficient to provide a statistically significant estimate of the proportion of patients

exhibiting significant alterations in WT1 specific T cells, biochemical indices of tumor burden or radiologic evidence of tumor response. However, this data will provide initial qualitative estimates of the T cell infusions effects, which are critical in planning subsequent phase II trials.

Timelines with all of the evaluations, tests and procedures occurring after each signed informed consent are depicted in **Tables 7A-C** below:

Table 7A: Screening Phase (Informed Consent One)

Sign Informed Consent One	**X**
WT1 Tumor Testing	**X**
HLA Testing & complete blood count	**X**



Eligible patients

Collection of 4 green top tubes for the establishment of autologous EBV BLCL, if a significant time lapse before patient can undergo leukapheresis & collection of 1 red top tube for EBV serology	**X**
Leukapheresis for the Establishment of Autologous EBV BLCL if has not been established yet and generation of WT1-specific T cell lines	**X**
Screening will take approximately 12-16 weeks	**X**

Table 7B: Treatment Phase (Informed Consent Two)

Sign Informed Consent Two	**X**
Patients will then follow Table 7C	**X**

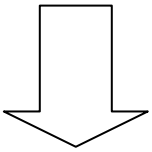


Table 7C: Treatment Phase (Informed Consent Two)*

	Prior to Cycle 1			Cycle 1[d]								Cycle 2+						Tumor Assessment	Follow-up Visits	
Time D=Day	Within 30 days of D1	D-1	D0	D1	D2	D3	D8	D15	D16	D17	D22	D1	D2	D3	D15	D16	D17	After every 2 Cycles	4 weeks after final infusion	8 weeks after final infusion
WT1 peptide-specific T cell infusion				X				X				X			X					
Cyclophosphamide		Y																		
History, Physical & Toxicity Assessment	X	Y		X	X		X	X	X		X	X	X		X	X			X	X
Phone Assessment [c]						X				X				X			X			
CBC	X	Y[b]		X	X		X	X	X		X	X	X		X	X			X	X
COMP	X	Y[b]		X	X		X	X	X		X	X	X		X	X			X	X
MAG	X			X	X		X	X	X		X	X	X		X	X			X	X
PT/PTT	X			X	X		X	X	X		X	X	X		X	X			X	X
Urinalysis	X			X	X		X	X	X		X	X	X		X	X			X	X
Creatinine Clearance (estimated)	X			X	X		X	X	X		X	X	X		X	X			X	X
Ca-125	X			X				X				X			X				X	X
Research Labs: 4 Green-Top tubes (RNB)		Y		X	X		X	X	X			X	X[a]		X	X[a]			X	X
EKG	X																			
CT Scan	X																	X		

[a] Research Labs = 4 green- top tubes with heparin (WT1 CTLp and EBV CTLp by LDA, TET, Intracellular IFN) with labels, date and time. These should be drawn prior to infusion on treatment days and 24 hours after the first, fourth, fifth, sixth and eighth infusion, if applicable.

[b] Standard MSKCC guidelines to be followed for laboratory studies pre-cyclophosphamide. These should include a cbc **and** comprehensive panel within 7 days prior to cyclophosphamide.

[c] Phone assessments have a +72hour window to contact patients, if permissible

[d] **From cycle 1 day 8 onwards a potential +/- 72 hour treatment window is permissible provided that necessary** assessments and laboratory tests can be still carried out.

***** The first 2 patients on study were treated per protocol versions 06-155A(3) and 06-155A(4). One patient was treated per protocol 06-155A(5). The next 3 patients were treated per protocol version 06-155A(6).

X = All Dose Levels (i.e. 1, 2, 3, 4) Y = Dose Levels 2, 3 and 4

11.1 TOXICITIES/SIDE EFFECTS

11.2 Risks of WT1 peptide-specific T cell infusions

- Since WT1 is expressed at low levels on normal kidney cells and marrow stem cells, there is a small but real risk of inducing nephritis or extended marrow suppression. Patients with AML in remission often have detectable levels of WT1 specific CTLs, yet do not have renal impairment or marrow

suppression (46). Furthermore, initial trials in 26 patients evaluating vaccination with two of the WT1 peptides for future use in this study have not detected kidney or marrow dysfunction in those patients who generated WT1 specific responses, except in two patients with MDS who developed leukopenia. In these patients, the leukopenia was ascribed to suppression of the $WT1^{+}$ malignant MDS clone (98). Based on these studies, the risks of adoptively transferred WT1 peptide specific T cells are expected to be acceptably low. However, in this phase I trial, patients will be closely monitored by serial creatinine clearances and serum chemistries to detect renal toxicities and by serial blood counts to assess for myelosuppression. If extended (>20 days) count depressions are observed and are associated with high levels of WT1 specific CTL in the circulation, the patients may be considered for treatment with steroids and/or ATG (anti-thymocyte globulin) to eliminate the T cells.

- There is a finite risk that the T cells generated *in vitro* could transmit an infection. Each dose of the T cells is tested extensively for microbial contaminants prior to release. In addition, the doses are tested for endotoxin. These tests are in full compliance with FDA guidelines. Therefore, the risk is expected to be small. Furthermore, T cells that do not meet sterility testing requirements will not be released for use.

- During infusion of activated T cells, a small proportion of patients may develop fever or hypotension. This will be treated with Tylenol and fluid boluses as required. There is a small risk of hypersensitivity and/or anaphylaxis during infusion of the T cells. If this occurs, the infusion would be stopped and appropriate measures taken to treat the symptoms (fluid boluses, steroids, antihistamines, etc.).

11.3 RISKS OF CYCLOPHOSPHAMIDE

- Nausea, vomiting (patients will receive antiemetics prior to and during treatment to prevent this side-effect) and diarrhea

- Acute water retention due to inappropriate antidiuretic hormone release (hydration will be closely monitored and acute water retention treated with diuretics; hydration will be maintained to insure adequate urine output)

- Cardiomyopathy (pretreatment cardiac function will be assessed to define eligibility; patients will be monitored during treatment to insure against cardiac dysfunction; at these doses, cyclophosphamide induced cardiomyopathy is rare)

- Hemorrhagic cystitis

- Fatigue, loss of fertility, nail changes, mouth sores, secondary malignancies

- Infection or bleeding induced during count nadirs. This anticipated risk is low because the count depressions that observed with this protocol rarely extend 10 days post treatment.

- Risk of opportunistic infections due to viruses such as CMV, EBV or Pneumocystis. The level of immunosuppression induced by cyclophosphamide is transiently severe. Cumulative evidence suggests that the risk of opportunistic infections is moderate. Patients will be closely monitored by PCR amplified techniques for viral antigenemia and treated if it develops.

Table 8: Common Toxicities Related to Cyclophosphamide

Hematologic	leukopenia, thrombocytopenia, anemia
Gastrointestinal	anorexia, nausea and vomiting (common with doses > 600mg/m^2), diarrhea
Hepatic	hepatitis, elevations in SGOT and SGPT
Renal	hemorrhagic cystitis
Cardiac	cardiotoxicity with high doses (> 120 mg/kg)
Respiratory	high doses may cause interstitial pulmonary fibrosis
Dermatologic	alopecia, facial flushing
Miscellaneous	nasal stuffiness, syndrome of inappropriate anti-diuretic hormone (SIADH) induced hyponatremia, fever after high-dose therapy

11.4 RISKS OF LEUKAPHERESIS

- The risks of leukapheresis can involve the occasional vasovagal responses to venipuncture and the minimal hemodynamic alterations associated with single unit phlebotomies. To protect against these risks, leukaphereses will be conducted in the Blood Bank Donor Room with full medical and nursing supervisors and support systems to prevent and/or address adverse events.

12.1 CRITERIA FOR THERAPEUTIC RESPONSE/OUTCOME ASSESSMENT

Response: We will evaluate each patient for evidence of tumor response by RECIST 1.1 criteria, as a preliminary indication of this treatment's therapeutic potential. Parameters

Please refer to the following reference for complete details: Eisenhauer EA, Therasse P, Bogarerts J, et al. New response evaluation criteria in solid tumours: Revised RECIST guideline (version 1.1). European Journal of Cancer 2009; 45:228-247.

- **Measurable disease ("Target")** is defined as at least one lesion that can be accurately measured in at least one dimension (longest diameter to be recorded). Each lesion must be ≥ 10 mm when measured by CT (CT scan slice thickness no greater than 5 mm*); > 10 mm caliper measurement by clinical exam (lesions which cannot be accurately measured with calipers should be recorded as non-measurable); and > 20 mm by chest x-ray.

*If CT scan with slice thickness > 5 mm is used, the minimum lesion size must have a longest dimension twice the actual slice thickness**.**

- An assessment of response will be made based on the radiographic imaging studies (CT scans).

Response:

Complete Response (CR): Disappearance of all target and non-target lesions. Any pathological lymph nodes (whether target or non-target) must have reduction in short axis to <10 mm. Normalization of CA125, if elevated at baseline.

Partial Response (PR): At least a 30% decrease in the sum of diameters of target lesions, taking into reference the baseline sum diameters.

Progressive Disease (PD): At least a 20% increase in the sum of diameters of target lesions, taking as reference the smallest sum on study (this includes the baseline sum if that is the smallest on study). In addition to the relative increase of 20%, the sum must also demonstrate an absolute increase **of at least 5 mm**. (Note: the appearance of one or more **NEW** lesions is also considered progression. Guidance on when a lesion is to be considered new is provided in the above cited reference). **Unequivocal progression of existing non-target lesions** is also considered progression (a detailed description and examples of unequivocal progression of existing non-target lesions is provided in the above cited reference).

For equivocal findings of progression (e.g. very small and uncertain new lesions; cystic changes or necrosis in existing lesions), treatment may continue until the next scheduled assessment. If at the next scheduled assessment, progression is confirmed, the date of progression should be the earlier date when progression was suspected.

Stable Disease (SD): Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum diameters while on study.

Not evaluable (NE) is when no imaging/measurement is done at all at a particular time point. The patient is not evaluable (NE) at that time point.

Early death is defined as having NO repeat tumor assessments following initiation of study therapy resulting from the death of the patient due to disease or treatment.

Patients with global deterioration of health status requiring discontinuation of treatment without objective evidence of disease progression at that time will be recorded as '**symptomatic deterioration**'. Every effort should be made to document objective progression even after discontinuation of treatment.

Confirmation of response (for both CR and PR): Complete or partial response may only be claimed if the criteria for each are met at a subsequent time point (≥ 4 weeks later) in studies with a primary endpoint that includes response rate. When response rate is a secondary endpoint (e.g. randomized phase II or III studies with progression-free survival or overall survival as primary endpoint) confirmation is NOT required.

Special note on lymph nodes: Lymph nodes identified as target lesions should always have the actual short axis measurement recorded (measured in the same anatomical plane as the baseline examination), even if the nodes regress to below 10 mm on study. This means that when lymph nodes are included as target lesions, the 'sum' of lesions may not be zero even if complete response criteria are met, since a normal lymph node is defined as having a short axis of < 10 mm. For PR, SD and PD, the actual short axis measurement of the nodes is to be included in the sum of target lesions.

Special note on target lesions that become 'too small to measure': While on study, all lesions (nodal and non-nodal) recorded at baseline should have their actual measurements recorded at each subsequent evaluation, even when very small (e.g. 2 mm). However, sometimes lesions or lymph nodes which are recorded as target lesions at baseline become so faint on CT scan that the radiologist may not feel comfortable assigning an exact measure and may report them as being 'too small to measure'. When this occurs it is important that a value be recorded on the D2M form. If it is the opinion of the radiologist that the lesion has likely disappeared, the measurement should be recorded as 0 mm. If the lesion is believed to be present and is faintly seen but too small to measure, a **default value of 5 mm** should be assigned (Note: It is less likely that this rule will be used for lymph nodes since they usually have a definable size when normal and are frequently surrounded by fat such as in the retroperitoneum; however, if a lymph node is believed to be present and is faintly seen but too small to measure, a default value of 5 mm should be assigned in this circumstance as well).

Evaluation of best overall response is according to Tables 9.0-9.1:

Table 9.0: Time point response: patients with target (+/– non-target) disease

Target lesions	Non-target lesions	New lesions	Overall response
CR	**CR**	**No**	**CR**
CR	**Non-CR/non-PD**	**No**	**PR**
CR	**Not evaluated**	**No**	**PR**
PR	**Non-PD or not all evaluated**	**No**	**PR**
SD	**Non-PD or not all evaluated**	**No**	**SD**
Not all evaluated	**Non-PD**	**No**	**NE**
PD	**Any**	**Yes or No**	**PD**
Any	**PD**	**Yes or No**	**PD**
Any	**Any**	**Yes**	**PD**

Table 9.1: Time point response: patients with non-target disease only

Non-target lesions	New lesions	Overall response
CR	**No**	**CR**
Non-CR/non-PD	**No**	**Non-CR/non-PD**
Not all evaluated	**No**	**NE**
Unequivocal PD	**Yes or No**	**PD**
Any	**Yes**	**PD**

Duration of response is defined as the time measurement criteria are first met for CR/PR until the first date that recurrent or progressive disease is objectively documented (taking as reference for progressive disease the smallest measurement recorded on study).

Duration of stable disease is measured from the start of the treatment (in randomized trials, from the date of randomization) until the criteria for progression are met, taking as reference the smallest sum on study (if the baseline sum is the smallest, this is the reference for calculation of PD).

Progression-Free Survival is the period from study entry until recurrent or progressive disease is objectively documented (taking as reference for progressive disease the smallest measurement recorded on study), death or date of last contact.

Survival is the observed length of life from entry into the study to death or the date of last contact.

13.1 CRITERIA FOR REMOVAL FROM STUDY

In accordance with the Declaration of Helsinki, ICH Good Clinical Practice Guidelines, and the US FDA Regulations, a patient has the right to withdraw from the study at any time for any reason without prejudice to his/her future medical care by the physician or at the institution. The Investigators also have the right to withdraw patients from the study (see below). Should a patient (or a patient's legally authorized representative) decide to withdraw, all efforts will be made to complete and report the observations as thoroughly as possible.

A complete final evaluation should be made at the time of the patient's withdrawal with an explanation of why the patient is withdrawing, and an attempt should be made to perform a follow-up evaluation.

Patients may be removed from study if one or more of the following events occur:

- Significant noncompliance by the patient
- Patient refusal to continue treatments or observations
- Unacceptable toxicity
- Progressive disease that in the Investigator's opinion requires therapeutic intervention that would interfere with the interpretation of results from the study
- Decision by the Investigator that termination is in the patient's best medical interest
- The stock of the patient-specific WT1 specific T cells has been exhausted
- Unrelated medical illness or complication
- Lost to follow-up

14.0 BIOSTATISTICS

This is a phase I dose escalating trial designed to identify tolerable, clinically active doses of Wilms' tumor gene (WT1) peptide sensitized T cells when administered alone or with non-myelosuppressive chemotherapy in patients with recurrent or persistent, measurable $WT1^{+}$ ovarian, primary peritoneal, or fallopian tube carcinomas. The dose escalation scheme is detailed above. Using this dose escalation scheme, escalation to the next treatment Level (Levels I → II → III→ IV) is probable if the risk of DLT is low, and the likelihood of escalations decreases as the risk of DLT increases, as demonstrated in Table 10:

Table 10: Dose Escalation/Toxicity Risk

True risk of Toxicity		.10	.20	.30	.40	.50	.60
Probability of Escalation	.91	.71	.49	.31	.17	.08	

Six patients (dose levels I and II) have already been treated on the protocol. A maximum of 18 patients will be treated. It is anticipated that this study will accrue 4-6 patients per year and will last approximately 4 years. At the end of this study, a full phase II trial will be initiated with the dose of WT1 specific T cells selected on the results of this phase I study.

The secondary aims of the study, 3 and 4, will be addressed by descriptive exploratory statistical analyses, since the sample size is not known in advance and there are no formal hypotheses being tested. These analyses may include descriptions of time patterns for continuous variables measured repeatedly, both on the individual level and aggregated by dose level.

15.1 SUBJECT REGISTRATION AND RANDOMIZATION PROCEDURES

15.2 Subject Registration

Confirm eligibility as defined in the section entitled Criteria for Patient/Subject Eligibility.

Obtain informed consent, by following procedures defined in section entitled Informed Consent Procedures.

During the registration process registering individuals will be required to complete a protocol specific Eligibility Checklist.

All participants must be registered through the Protocol Participant Registration (PPR) Office at Memorial Sloan-Kettering Cancer Center. PPR is available Monday through Friday from 8:30am – 5:30pm at 646-735-8000. The PPR fax numbers are (646) 735-0008 and (646) 735-0003. Registrations can be phoned in or faxed. The completed signature page of the written consent/verbal script and a completed Eligibility Checklist must be faxed to PPR.

16.1 DATA MANAGEMENT ISSUES

A Research Study Assistant (RSA) will be assigned to the study. The responsibilities of the RSA include project compliance, data collection, abstraction and entry, data reporting, regulatory monitoring, problem resolution and prioritization, and coordinate

the activities of the protocol study team.

The data collected for this study will be entered into a secure database. Source documentation will be available to support the computerized patient record.

16.2 QUALITY ASSURANCE

Weekly registration reports will be generated to monitor patient accruals and completeness of registration data. Routine data quality reports will be generated to assess missing data and inconsistencies. Accrual rates and extent and accuracy of evaluations and follow-up will be monitored periodically throughout the study period and potential problems will be brought to the attention of the study team for discussion and action.

Random-sample data quality and protocol compliance audits will be conducted by the study team, at a minimum of two times per year, more frequently if indicated.

16.3 DATA AND SAFETY MONITORING

The Data and Safety Monitoring (DSM) Plans at Memorial Sloan-Kettering Cancer Center were approved by the National Cancer Institute in September 2001. The plans address the new policies set forth by the NCI in the document entitled "Policy of the National Cancer Institute for Data and Safety Monitoring of Clinical Trials" which can be found at: http://cancertrials.nci.nih.gov/researchers/dsm/index.html. The DSM Plans at MSKCC were established and are monitored by the Office of Clinical Research. The MSKCC Data and Safety Monitoring Plans can be found on the MSKCC Intra net at: http://mskweb2.mskcc.org/irb/index.htm

There are several different mechanisms by which clinical trials are monitored for data, safety and quality. There are institutional processes in place for quality assurance (e.g., protocol monitoring, compliance and data verification audits, therapeutic response, and staff education on clinical research QA) and departmental procedures for quality control, plus there are two institutional committees that are responsible for monitoring the activities of our clinical trials programs. The committees: Data and Safety Monitoring Committee (DSMC) for Phase I and II clinical trials, and the Data and Safety Monitoring Board (DSMB) for Phase III clinical trials, report to the Center's Research Council and Institutional Review Board.

During the protocol development and review process, each protocol will be assessed for its level of risk and degree of monitoring required. Every type of protocol (e.g., NIH sponsored, in-house sponsored, industrial sponsored, NCI cooperative group, etc.) Will be addressed and the monitoring procedures will be established at the time of protocol activation.

17.1 PROTECTION OF HUMAN SUBJECTS

17.1 Privacy

MSKCC's Privacy Office may allow the use and disclosure of protected health information pursuant to a completed and signed Research Authorization form. The use and disclosure of protected health information will be limited to the individuals described in the Research Authorization form. A Research Authorization form must be completed by the Principal Investigator and approved by the IRB and Privacy Board.

17.2 Serious Adverse Event (SAE) Reporting

The NCI Common Terminology Criteria for Adverse Events (CTCAE) Version 4.0 will be used to identify and grade adverse events. All serious and non-serious adverse events occurring from the time of signing the treatment phase informed consent (consent #2) until 30 days following the patient's final T cell infusion will be reported. In addition, any adverse events occurring outside of this window that are considered, by the investigator, related to study tests/procedures should also be reported.

A serious adverse event (SAE) is any sign, symptom or medical condition that emerges during treatment or during a post-treatment follow-up period that (1) was not present at the start of treatment and it is not a chronic condition that is part of the patient's medical history, OR (2) was present at the start of treatment or as part of the patient's medical history but worsened in severity and/or frequency during therapy, AND that meets any of the following serious criteria:

- Results in death
- Is life-threatening
- Requires or prolongs inpatient hospitalization
- Is disabling
- Is a congenital anomaly/birth defect
- Is medically significant or requires medical or surgical intervention to prevent one of the outcomes listed above.

Any seriousadverse events as defined above should be reported to IRB as soon as possible, but no later than five calendar days after notification of event. The IRB requires a CRDB Adverse Event (AE) report be submitted electronically to the SAE Office at sae@mskcc.org containing the following information:

Fields populated from the CRDB:

- Subject's name
- Medical record number
- Disease/histology (if applicable)
- Protocol number

Data to enter:

- The date the adverse event occurred
- The adverse event
- Relationship of the adverse event to the treatment (drug, device, or intervention)
- If the AE was expected
- The severity of the AE
- The intervention
- Detailed text that includes the following information:
  - A explanation of how the AE was handled
  - A description of the subject's condition
  - Indication if the subject remains on the study
  - If an amendment will need to be made to the protocol and/or consent form

The NCI CTCAE Version 4.0 will be used to grade all adverse events. Furthermore, all adverse events that occur while the patients are on study and are considered possibly, probably, or definitely related to study treatment and/or procedures, will be collected. The severity and relationship to the study drug will be characterized.

The PI's signature and the date that the report is signed are required on the completed form.

<u>For IND/IDE protocols:</u>

The CRDB AE report should be completed as above and the FDA assigned IND/IDE number written at the top of the report. If appropriate, the report will be forwarded to the FDA by the SAE staff through the IND Office.

18.1 INFORMED CONSENT PROCEDURES

Before protocol-specified procedures are carried out, consenting professionals will explain full details of the protocol and study procedures as well as the risks involved to participants prior to their inclusion in the study. Participants will also be informed that they are free to withdraw from the study at any time. All participants must sign an IRB/PB-approved consent form indicating their consent to participate. This consent form meets the requirements of the Code of Federal Regulations and the Institutional Review Board/Privacy Board of this Center. The consent form will include the following:

1. The nature and objectives, potential risks and benefits of the intended study.
2. The length of study and the likely follow-up required.
3. Alternatives to the proposed study. (This will include available standard and investigational therapies. In addition, patients will be offered an option of supportive care for therapeutic studies.)
4. The name of the investigator(s) responsible for the protocol.
5. The right of the participant to accept or refuse study interventions/interactions and to withdraw from participation at any time.

Before any protocol-specific procedures can be carried out, the consenting professional will fully explain the aspects of patient privacy concerning research specific information. In addition to signing the IRB Informed Consent, all patients must agree to the Research Authorization component of the informed consent form.

Each participant and consenting professional will sign the consent form. The participant must receive a copy of the signed informed consent form.

19.1 REFERENCE(S)

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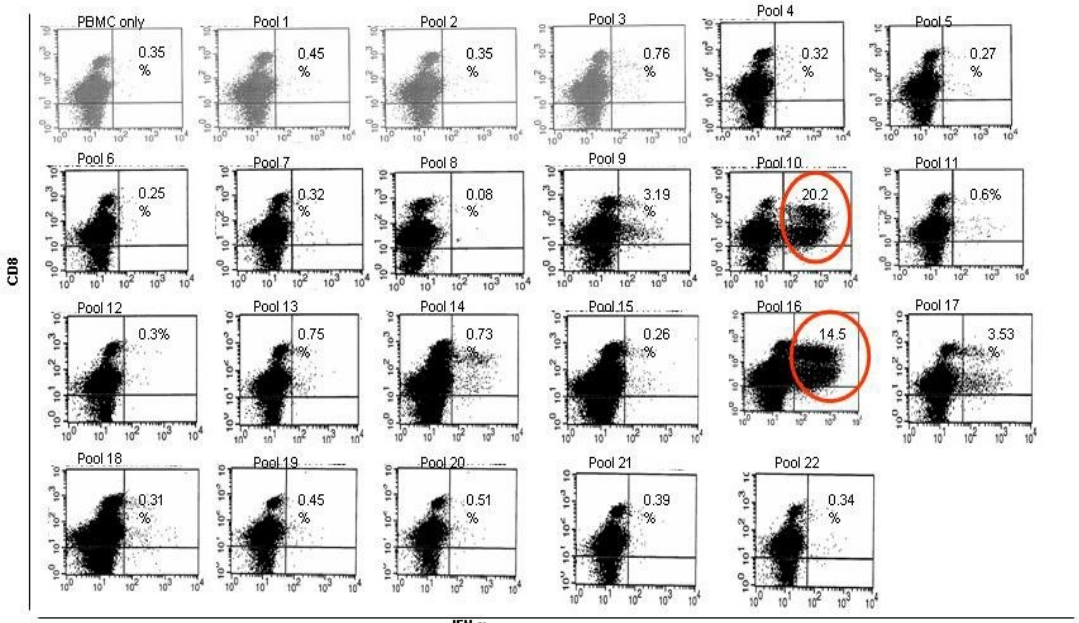
Appendix A: The T cells of the donor sensitized with the pool generated in response to subpools 10 and 16, suggesting reactivity against pentadecapeptide 60.

Appendix A



	1	2	3	4	5	6	7	8	9	10
11	1	2	3	4	5	6	7	8	9	10
12	11	12	13	14	15	16	17	18	19	20
13	21	22	23	24	25	26	27	28	29	30
14	31	32	33	34	35	36	37	38	39	40
15	41	42	43	44	45	46	47	48	49	50
16	51	52	53	54	55	56	57	58	59	60
17	61	62	63	64	65	66	67	68	69	70
18	71	72	73	74	75	76	77	78	79	80
19	81	82	83	84	85	86	87	88	89	90
20	91	92	93	94	95	96	97	98	99	100
21	101	10	103	104	105	106	107	108	109	110
22	111	11 2	113							

→ WT1 peptide pools: 10 and 16

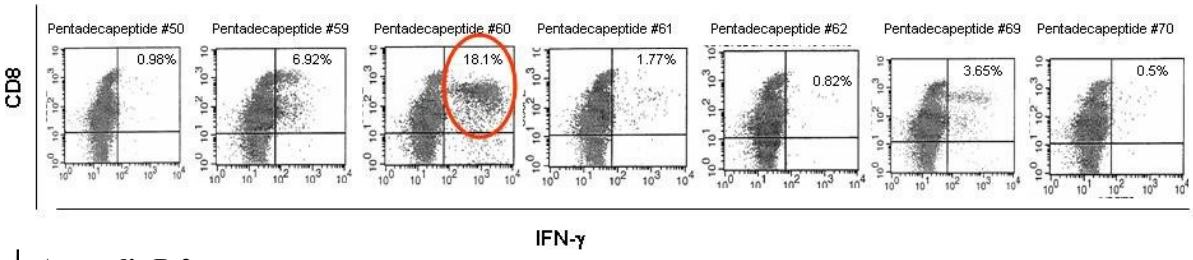
↓

Pentadecapeptide #60

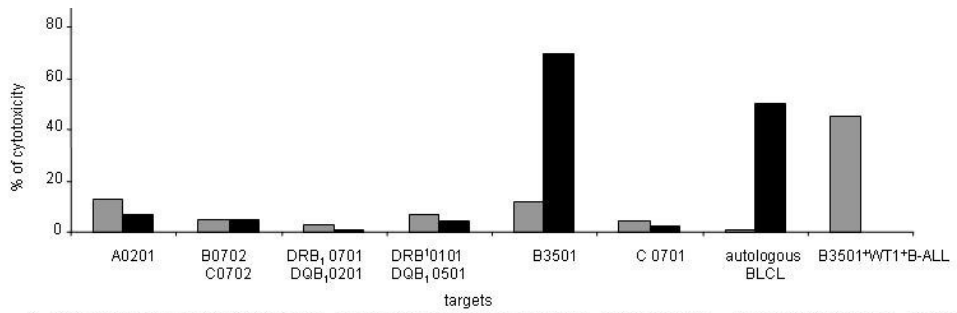
QFPNHSFKHEDPMGQ

Appendix B-1 and B-2

Appendix B-1



Appendix B-2



Appendix B-1 T cells from the same donor were analyzed for their capacity to produce IFN-γ in response to the single pentadecapeptides (№50, 59, 60, 61, 62, 69, 70) of pools 10 and 16. Only peptide №60 induced significant IFN-γ production. This peptide is contained within pools 10 and 16.

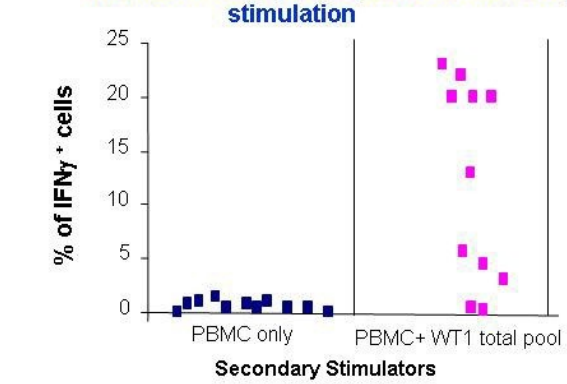
Figure 4B. Sensitized T cells from the same donor inheriting HLAA0201 B 3501/0702 C0702/0701 $DRB_1$0701$DQB_1$0201$DRB_1$0101$DQB_1$0501 were tested for their capacity to kill BLCLs sharing only one haplotype either unmodified (grey bars) or loaded with peptide #60 (black bars). As shown only BLCL with HLA B3501 loaded with peptide #60, autologous BLC loaded with the same peptide and HLA B3501+ B cell leukemia naturally expressing WT1 were killed.

Appendix C-1 and C-2

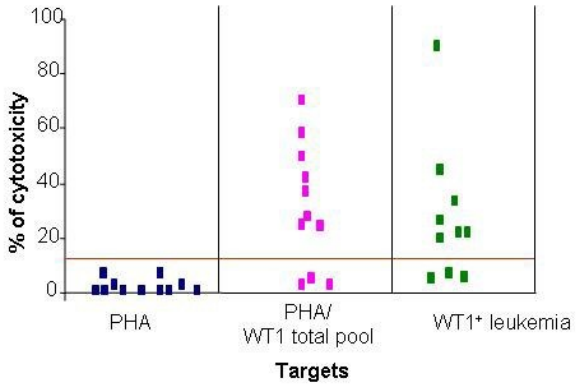
Appendix C

WT1 specific responses of CTL generated from PBMC of normal donors (n=11) by stimulation with autologous APCs loaded with total pool of WT1 derived pentadecapeptides

C-1



C-2



APPENDIX D: Generation of WT1-Specific Cytotoxic T-Lymphocytes (CTL)

STANDARD OPERATING PROCEDURE **ADOPTIVE IMMUNE CELL THERAPY FACILITY** Memorial Sloan-Kettering Cancer Center			
Document No. 03-II-1.02.	**Effective Date:** **11/06/2009**	**Previous Date:** **09/20/2007**	**Page 1 of 10**
Title: Generation of WT1-Specific Cytotoxic T-Lymphocytes (CTL)			
Approval:			
Facility Director		**QA Manager**	

1. Purpose:

1.1 WT1-specific CTL lines are generated from normal donors or recipients of T cells by co-culture with irradiated autologous cytokine activated monocytes (CAM) or EBV-transformed B cell lines (LCL). Under the culture conditions applied, outgrowing CTL should be WT1 or WT1/EBV specific. The CTL lines exhibiting WT1-specific responses are infused into patients with WT1-expressing malignancies.

1.2 Responsibility:

1.2.1. Technicians are responsible for the sample receipt, appropriate sample labeling, exact accomplishing of the procedures listed in the SOP, reporting the results to the Facility Director, keeping the records of all the procedures applied to the component during its processing/culturing.

1.2.2. Facility Supervisor is responsible for maintaining the equipment to meet the requirements of the Quality Management Plan, ordering the necessary equipment, supplies and reagents, designate and re-distributes the work load in the facility, assures that the planned processes are performed in the facility according to the schedule.

1.2.3. QA Manager is responsible for the control of all the procedures to be performed according to the Standard Operating Procedures, data entries, inventory, revision of the batch records, evaluation of the quality of the components produced, validation of the procedures, revision of the SOPs,, validation of the reagents, training of the personnel, evaluation of the personnel professional education level. QA Manager

insures quality and safety of reagents and supplies used in the manufacturing procedures by obtaining the appropriate documentation (certificates of analysis and recipes of formulation where applicable) and conducting functional assays (where applicable).

1.2.4. Facility Director is responsible for the dedication of the procedures to be applied to the samples received, individual changes in the protocols, if needed for the special cases, reports of the results to the attendings and Medical Director of the AICT Facility.

2. Definition and Abbreviations:

2.1. CTL - Cytotoxic T-lymphocytes

2.2. PBL - Peripheral blood lymphocytes

2.3. PBMC - peripheral blood mononuclear cells

2.4. LDS - Low density separation

2.3. EBV - Epstein Barr Virus

2.4. EBV BLCL - EBV transformed B lymphocyte cell line

2.5. WT1 - Wilm's tumor antigen 1

2.6. BLCL - B Lymphoblastoid Cell Lines

2.7. CAM - Cytokine-activated monocytes

2.8. LSM - Lymphocyte separation media

2.9. YH5 - YSSEL's medium containing 5% Human serum

2.10. YH10 - YSSEL's medium containing 10% Human serum

3. Materials and Equipment:

3.1. Specimen

3.1.1. NK depleted PBL from normal donor or T cell recipient

3.1.2. PBMC from the same normal donor or T cell recipient

3.1.3. CD3 enriched PBL from the same donor or T cell recipient

3.1.4. Lymphoblastoid Cell Lines (LCL) prepared from the PBMC of the same individual according to procedure "GENERATION OF B95-8 EBV TRANSFORMED LYMPHOBLASTOID CELL LINES (LCL)" and cultured for ≥2 weeks in 100μM acyclovir.

3.1.5. CAMs prepared from the PBMC of the same donor according to SOP 02-I-1.02. "Generation of cytokine activated monocytes"

3.2. Materials

3.2.1. YSSEL's	Gemini
3.2.2. Characterized human serum	Gemini
3.2.3. PBS	Gibco
3.2.4. Accuprep	Accurate Chemical & Scientific Corporation
3.2.3. 75cm flask	NUNC
3.2.4. 25cm flask	Falkon
3.2.5. Interleukin-2 (Proleukin)	Chiron Corporation
3.2.6. 15ml and 50ml centrifuge tubes	Falcon
3.2.7. Disposable pipets	Fisher
3.2.8. Conical cryovials with vented caps	Fisher
3.2.9. Synthetic overlapping pentadecapeptides spanning the sequence of CMV pp65 protein	Invitrogen
3.2.10. DMSO	Edwards Lifesciences
3.2.11. Interleukin-15	Miltenyi Biotech

3.3. Equipment

3.3.1. Biological safety cabinet, certified	Nuare
3.3.2. Centrifuge	Sorvall
3.3.3. Incubator	Nuare

3.3.4. Irradiator (maintained by the Blood Bank of MSKCC) ILB437C

4. Procedure:

4.1.*Preparation of the total pool* of the 141 synthetic WT1 derived pentadecapeptides spanning the whole sequence of WT1 protein and overlapping by 11aa:

4.1.1. Each peptide is delivered from the manufacturer in the lyophilized form in several vials. The lyophilized peptides are kept at -80°C until diluted. Dilute one of the vials of each synthetic pentadecapeptide in DMSO at a concentration 10mg/ml. Aliquot into conical cryovials at 35µl/vial, label each vial and freeze ay -80°C. Use one aliquot (25µl) of each peptide for preparation of total pool and subpools as described below. Store the leftover volume of each peptide at -80°C for future single peptide testing.

4.1.2. Take 25µl of each peptide (250µg) and transfer into 50ml tube.

4.1.3. Add 10.575ml of DMSO. Mix well.

4.1.4. Label 282 conical cryovials: total pool of WT1 pentadecapeptides Invitrogen, 50µg/20µl, 50µl/vial, date of aliquoting.

4.1.5. Aliquot prepared total pool at 50µl/vial and freeze at -80°C.

4.2.*Preparation of 24 subpools of synthetic WT1 derived pentadecapeptides:*

4.2.1. Each pool (##1-23) is made to contain 12 pentadecapeptides except pool #24 which is made to contain 9 pentadecapeptides only.

4.2.1.1. To prepare each of 23 pools (##1-23) take 25µl (250µg) of each pentadecapeptide comprising the pool from the dilutions prepared in step 4.1.1.and pool into one tube to have total 3000µg in 300µl. Then add 900µl of DMSO to make final concentration of 50µg in 20µl, containing ~4.2µg of each pentadecapeptide.

4.2.1.2. To prepare pool#24 take 30µl (300µg) of each peptide comprising the pool from the dilution prepared in step 4.1.1. and pool into one tube to have total 2700µg in 270µl. Add 810µl of DMSO to make the final concentration of 50µg in 20µl. This dilution contains ~5.6µg of each pentadecapeptide.

Pool the following peptides into the following pools using the map:

Pool #	1	2	3	4	5	6	7	8	9	10	11	12

13	1	2	3	4	5	6	7	8	9	10	11	12
14	13	14	15	16	17	18	19	20	21	22	23	24
15	25	26	27	28	29	30	31	32	33	34	35	36
16	37	38	39	40	41	42	43	44	45	46	47	48
17	49	50	51	52	53	54	55	56	57	58	59	60
18	61	62	63	64	65	66	67	68	69	70	71	72
19	73	74	75	76	77	78	79	80	81	82	83	84
20	85	86	87	88	89	90	91	92	93	94	95	96
21	97	98	99	100	101	102	103	104	105	106	107	108
22	109	110	111	112	113	114	115	116	117	118	119	120
23	121	122	123	124	125	126	127	128	129	130	131	132
24	133	134	135	136	137	138	139	140	141			

The upper horizontal row and the left vertical column contain the numbers of the pools made and the content of each pool is defined by the pentadecapeptides which numbers are presented in the corresponding horizontal or vertical rows aligned together with each pool number.

4.3.*Preparation of the antigen-presenting cells:*

Either CAM or EBV BLCL loaded with the same stimulating peptide(s) can be used for the in vitro sensitization of WT1 specific T cells

4.3.1. Preparation of CAM:

4.3.1.1.CAMs are generated according to the SOP 02-I-1.02. If CAMs are frozen thaw them in Yssel's containing 10% of HS by spinning at 1000rpm +4°C for 7 minutes. Discard supernatants. If fresh CAMs are used they should be spun down at the same conditions and supernatant should be discarded. If the BLCL are used for sensitization the cells also should be spun down at the same conditions and the supernatant should be discarded.

4.3.1.2. Add 5ml of Yssel's medium and count the cells.

4.3.1.3. Calculate the number of CAMs or BLCL required for initial sensitization at 20:1 E:T ratio. CAMs required for subsequent restimulations are used at 10:-4:1 E:T ratio. BLCL are used at 4:1 ratio..

4.3.1.4. To load the antigen wash CAMs or BLCL in serum free Yssel's at 1000rpm +4°C for 7 minutes.

4.3.1.5. Discard supernatant. Add serum-free Yssel's media to have final cell concentration $1x10^6$cells/ml.

4.3.1.6. Add 50µg/ml (20µl/ml) of complete pool of synthetic WT1 pentadecapeptides.

4.3.1.7. Incubate CAMs or BLCL with peptide for 3 or 2 hours accordingly at RT shaking every 15-20 minutes.

4.3.1.8. After 2-3 hours of incubation irradiate peptide-loaded CAM with 6000rads and BLCL with 9000rads using the irradiator specified for this protocol.

4.3.1.9. Wash the cells with 50ml of Yssel's for 7 minutes at 1000rpm +4°C before adding to the responder cells.

4.3.10. Re-suspend in 1 ml of YH5

4.3.2. Preparation of EBV BLCL:

4.3.2.1.Prepare the EBV BLCL from the same preselected donor of WT1 specific T cells as described in the SOP "GENERATION OF B95-8 EBV TRANSFORMED LYMPHOBLASTOID CELL LINES (LCL)".

4.3.2.2. Count the EBV BLCL.

4.3.2.3. BLCLs are used only for secondary restimulations at 4:1 E:T ratio. Take EBV BLCL in the amount sufficient to re-stimulate T cells at 4:1 responder:stimulator ratio and spin down in YH5 at 1200rpm RT.

4.3.2.4. Discard supernatant. Add serum-free Yssel's medium to a cell concentration $1x10^6$cell/ml.

4.3.2.5. Add 50µg/ml/$1x10^6$ cells of the stimulating WT1 peptide pool.

4.3.2.6. Incubate at RT for 2 hours shaking every 20 minutes.

4.3.2.7. Irradiate with 9000rads.

4.3.2.8. Spin down in YH5 at 1200rpm RT. Discard supernatant.

4.4. Preparation of "responder" cells

4.4.1. Wash NK- and monocyte-depleted PBL prepared in the SOP ## IV-1.02 and IV-2.02 or CD3 separated T cells prepared in the SOP ## III-1.02 and III-1.01 with YH5 for 7 minutes at +4°C 1200rpm.

4.4.2. Decant the supernatant and re-suspend the cells in YH5.

4.4. 3. Remove 20µl of cells (if needed, add 20µl of 50% red cell lysis buffer) and count using a hemocytometer according to standard operating procedure. If cell concentration is $>1x10^6$ /ml, adjust to $1x10^6$ /ml by adding YH5. If cell concentration is $<1x10^6$ /ml, pellet and re-suspend at $1x10^6$ /ml in YH5.

4.4.4. Put the cells in $25cm^2$ flask.

4.5. Mix responders and stimulators prepared in section 4.3. and 4.4. respectively in this SOP by adding WT1 peptide loaded irradiated CAMs and BLCL into the $25cm^2$ flask with prepared responders at 20:1 responder:stimulator ratio.

4.6. Culture at +37°C 5%CO2 in air for 5 days

4.7. Feed on day 5 by adding the same volume of YH5.

4.8. Day 7: count the CTL. If the viability of the T cells <60% and the total number of cells $\geq 10^7$ the dead cells should be removed by Low Density separation: cells are diluted in PBS at 1:1 ratio and layered over Accuprep in 50 ml tube at 1:2 ratio. Cells are centrifuged at 2300rpm RT for 25 minutes. The buffy coat is collected into a separate 50ml tube. Tube is filled up with PBS and centrifuged at 1200rpm at RT for 7 minutes. Supernatant is discarded. The cell pellet is resuspended in YH5. Cells are counted

4.9. Adjust the cell concentration with YH5 up to $1.0x10^6$ cells/ml. Add interleukin-2 (IL-2) at the final concentration of 10Un/ml.

4.10. 1st re-stimulation is done on day 7: if the CTL line was initiated by stimulation on CAMs restimulate on day 7 with autologous peptide-loaded CAM at 10:1-4:1 responder :stimulator ratio. Prepare the CAMs as described in step 4.3.1. of this SOP. Upon Facility Director's decision EBV BLCL can be used as APC for restimulation at 4:1 R:S ratio if the number of CAMs is insufficient or the proliferation of T cells is slow. If the WT1 CTL line has been started by stimulation on BLCL all subsequent restimulatons should be done by co-incubation with the BLCL loaded with the WT1 total pool. In this case prepare the stimulators as described in step 4.3.2. of this SOP. Repeat re-stimulations every 7-8 days as described in this section of the SOP.

4.11. Culture at +37°C 5%CO2 in air for 3 days and then feed with YH5 to adjust the concentration to $1x10^6$ cells/ml. Add 10 units/ml of recombinant human Interleukin-2 (IL-2). Repeat this step every 2-3 days. If the viability of the cells decreases and is below 75% - increase the dose of IL-2by 5 Un/ml. Count the cells every 2-3 days. If the viability increases keep feeding with the same dose of Il-2. If the viability remains below 75% - increase the dose of IL-2 by another 5 Un/ml. The increase of the IL-2 dose should be approved by the Facility Director or, in the absence of the Facilkity Director by the designated experienced senior technician.

4.12. Starting from day 14 of the CTL line add interleukin-15 (IL-15) to the feeding cocktail at 10ng/ml weekly.

4.13. Culture at +37°C 5%CO_2 and feed with YH5 and IL-2 every 2-3 days and IL-15 and restimulation every 7 days as described in steps 4.8.-4.12.

4.14. Test the cells for Interferon-gamma production and cytotoxicity approximately on day 34 (between days 32 and 34) against uutologous EBV BLCLs unmodified or loaded with the WT1 total pool pf pentadecapeptides autologous and recipient-derived PHA blasts or CAM and the same targets loaded with the stimulating WT1 peptides as well as

PHA blasts or EBV BLCL from HLA mismatched donor unmodified and loaded with the same WT1 peptides at 50:1 and 25:1 E:T ratio according to SOP “Determination of Specific Cytotoxicity of the Cytotoxic T lymphocytes”.

4.15. If the cells are cytotoxic and specific, but the total cell number is not sufficient for the therapeutic dose expand them as described in Step 4.10-4.12. for another week to achieve the therapeutic dose and repeat testing between days 40 and 42 as described in section 4.14. of this protocol.

4.16. When the sufficient number of cells for patient infusion is obtained and control tests meet the quality criteria, cells should be characterized and frozen according to SOP ”Characterization and Freezing of the Cytotoxic T lymphocytes”. Note! If the T cells are cytotoxic but the total dose obtained by day 42 is not sufficient for the therapeutic dose, the generated T cell line should be frozen for the infusion and super-expansion with OKT3 antibodies should be initiated according to the appropriate SOP. If sufficient number of PBMC/PBL is available another WT1 specific T cell line should be initiated according to this protocol to achieve the final total therapeutic dose required.

5. Notes:

5.1. Since all cells prepared are intended for infusion to patients, it is essential to adhere to proper procedures to prevent misidentification or contamination of patient samples.

5.2. ALL flasks and centrifuge tubes must be labeled with a sticky label including patient name, patient AICT number, type of the culture and culture start date. UNLABELED MATERIAL WILL BE DISCARDED.

5.3. NEVER work with more than one patient cell line at any one time.

5.4. ALWAYS use medium prepared and labeled specifically for each patient's cells.

5.5. NEVER use medium to feed more than one patient's cells

5.6. Perform all steps in a certified biological safety cabinet, using aseptic technique.

5.7. CHANGE gloves between the cultures for different patients

5.8. Procedure should be validated annually for the efficiency of the generation of WT1 specific cytotoxic T cells based on the retrospective analysis of the results of cytotoxicity assay, immunophenotyping and cell counts.

6. References:

6.1. Rooney CM, Smith CA, et al. Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation, the Lancet, 1995; 543:9-13.

6.2. Heslop HE, Ng CYC, Li C, Smith CA, Loftin SK, Krance RA, Brenner MK, Rooney CM. Long term restoration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. Nature Medicine 2:551-555, 1996.

7. Attachments:

Forms:
AICT-F-008
AICT-F-009
AICT-F-010
AICT-F-011
AICT-F-012
AICT-F-013

Memorial Sloan-Kettering Cancer Center
IRB Protocol

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PROCEDURE AUTHOR, IMPLEMENTATION DATE,
REVISION FORM

PROCEDURE: **Generation of WT1-Specific Cytotoxic T-Lymphocytes (CTL)**

AUTHOR _____**(Signature)** **Ekaterina S.Doubrovina, M.D., PhD.**

Laboratory Director _____**(Position)**

IMPLEMENTATION DATE: _____ PREVIOUS VERSION: _____

REVISIONS:
I have reviewed this protocol_____

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