THE UNIVERSITY OF TEXAS
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CANCER CENTER

"A Pilot Study of Lymphodepletion Plus Adoptive Cell Transfer with T-Cell Transduced with CXCR2 and NGFR Followed by High Dose Interleukin-2 in Patients with Metastatic Melanoma"

PI: Rodabe Amaria, MD

Abstract:

This is a pilot study to assess the feasibility and safety of producing sufficient quantities of CXCR2 and NGFR transduced tumor infiltrating lymphocytes (TILs) for treatment of metastatic melanoma. A total of 15 patients will be treated. Clinical outcomes that will be observed are tumor response by imaging , and possible grade 3 or worse toxicities not normally associated with lymphodepletion and high dose IL-2. T-cell based immunotherapy is a promising therapeutic modality for cancer. We and others have developed therapies to treat human melanomas by isolating and expanding autologous tumor infiltrating lymphocytes (TILs), followed by reinfusion into patients. Although tumor regression can be dramatic in some patients, in other patients we see no clinical response. We hypothesize that inefficient migration of T cells to tumors is one of the rate limiting steps in the generation of an effective anti-tumor response. Therefore, we are investigating methods to improve T-cell trafficking to tumors through the introduction of specific receptor genes that can enhance migration to tumor sites. Melanomas specifically express the chemokines CXCL1 and CXCL8, which are thought to promote autocrine growth and angiogenesis. However, we have found that tumor antigen-specific T-cells fail to express the chemokine receptors specific for these ligands, including CXCR2. In a strategy designed to direct T cells toward chemokines expressed by tumors, we have developed a system to genetically modify T cells using retroviral vectors encoding CXCR2. We have demonstrated that murine T-cells transduced to express CXCR2 exhibited enhanced trafficking to CXCL1 expressing tumors and this led to improved anti-tumor responses and survival. We now propose to translate our findings from our mouse models to humans by treating melanoma patients with autologous tumor-reactive TILs genetically modified to express CXCR2. To compare the ability of CXCR2 transduced TIL to migrate to tumor compared to control TIL, each patient will also receive TIL transduced with the truncated NGFR (nerve growth factor receptor) marker gene.

	Cell type						
Chemokine	RCC	FB	M71	M73	M72	Mean	SEM
CCL5 (RANTES)	0.25	0.31	0.20	0.29	0.28	0.27	0.02
CXCL1 (GRO-α)	0.43	1.12	1.59	0.96	0.50	1.04	0.22
CXCL2 (GRO-β)	0.90	0.90	1.04	0.75	0.76	0.86	0.07
CXCL8 (IL-8)	0.47	0.47	0.50	0.50	0.44	0.48	0.01
CXCL9 (Mig)	1.11	1.11	1.20	1.22	1.04	1.14	0.04

	Melanoma patient number										Mean	
Chemokine	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10		SEM
CCL5 (RANTES)	0.26	0.10	0.24	0.20	0.42	0.57	0.28	0.32	0.24	0.22	0.29	0.04
CXCL1 (GRO-α)	0.69	**65.26**	**4.37**	0.18	**12.98**	1.00	**9.89**	**23.21**	**17.77**	0.22	**13.56**	5.97
CXCL2 (GRO-β)	0.46	1.09	**2.14**	1.12	**2.72**	0.68	**3.57**	**5.12**	1.67	1.27	1.98	0.44
CXCL8 (IL-8)	0.54	0.68	0.98	0.36	**2.53**	0.53	1.03	3.62	0.54	0.38	1.12	0.33

Table 1. Chemokine expression analysis of human cancers and normal cells. *(RCC: human renal cell carcinoma; FB: fibroblasts; Mxx: normal melanocytes;P1-P10: fine needle aspirates from melanoma patient tumors.*)

CXCL9 (Mig)	1.30	**5.27**	**15.69**	1.70	**4.89**	**5.83**	**4.69**	1.45	1.32	**2.75**	**4.49**	1.30

1.0 Background and Drug Information: ***Human melanoma tumors secrete CXCL1 and CXCL8***

T-cell migration into tissues is largely dependent on expression of specific chemokines that signal through corresponding chemokine receptors expressed by lymphocytes. In order to identify chemokines that are expressed constitutively by tumor cells, we first investigated chemokine expression in human melanomas freshly isolated
from patients. Fine needle aspirates of melanomas from ten patients were obtained and subjected to comparative cDNA microarray analysis. For controls, we prepared cDNA from a renal carcinoma (RCC) cell line, cultured normal fibroblasts and cultured melanocytes. Table 1 shows ratios of the signal intensity in the various normal and tumor tissues compared to PBMC. Differences are considered significant **(in bold, Table 1)** when ratios are greater than 2 or less than 0.5 and varied from normal tissues and renal cell carcinoma (RCC) by a factor of greater than 2 or less than 0.5.

Human melanoma tumors secrete CXCL1 and CXCL8 T-cell migration into tissues is largely dependent on expression of specific chemokines that signal through corresponding chemokine receptors expressed by lymphocytes. In order to identify chemokines that are expressed constitutively by tumor cells, we first investigated chemokine expression in human melanomas freshly isolated
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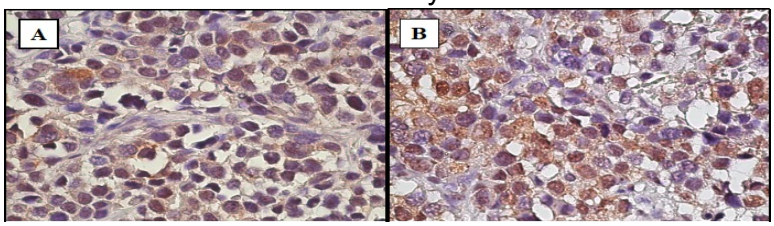
Table 2. CXCL1 production by human tumor lines	
Cell line	**Human CXCL1 (pg/ml)**
397 melanoma	606
624 melanoma	1652
A375 melanoma	2038
888 melanoma	403
1300 melanoma	648
SK23 melanoma	2801
SW480 colon CA	165
Colo 320 colon CA	0

renal carcinoma (RCC) cell line, cultured normal fibroblasts and cultured melanocytes. Table 1



shows ratios of the signal intensity in the various normal and tumor tissues compared to PBMC. Differences are considered significant **(in bold, Table 1)** when ratios are greater than 2 or less than 0.5 and varied from normal tissues and renal cell carcinoma (RCC) by a factor of greater than 2 or less than 0.5.

CXCL1 expression, in particular, stood out amongst the other chemokines as being markedly upregulated with a high relative signal intensity in a significant proportion of melanomas (6 of 10) when compared to levels in normal tissues and RCC (mean value 13.56 $\pm$ 5.97 versus 1.04 $\pm$ 0.22). CXCL9 was also upregulated in a majority of melanomas tested, although the mean value of 4.49 $\pm$ 1.30 was lower than that for CXCL1. The analysis of cDNA from fresh melanoma samples suggests that CXCL1 is expressed at the tumor site *in vivo*. To confirm that melanoma cells, as reported, could produce CXCL1 protein, we next evaluated the production of CXCL1 by several human melanoma cell lines. For these purposes, cell lines were plated at $5x10^5$ cells/ml and, 24 hours later, supernatants were harvested and CXCL1 protein was detected using a standard ELISA. With these studies, we confirmed that most human melanoma cell lines also constitutively produced significant amounts of CXCL1. **(Table 2)**(Kershaw, Wang et al. 2002).

Table 3. IL-8 Production by human melanoma tumor lines	
Tumor Line	**IL-8 pg/ml**
Mel 624	121
Mel 888	2943
Mel 888-A2	4754
Mel 938-A2	845
A375	9692
WM239	1889
WM2264	684
Mel 526	3080
Medium	0

CXCL1 binds to the chemokine receptor CXCR2. In addition, CXCL8 (IL-8) can bind to CXCR2. Because CXCL8 (IL-8) was also seen in some of the tumor samples and has been reported to be highly expressed in some melanoma cells, we evaluated a panel of melanoma cell lines for their ability to produce IL-8 **(Table 3),** and 6 of 8 melanoma lines produced significant levels of CXCL8. Moreover, we also analyzed paraffin-embedded melanoma tissue samples from patients for the expression of CXCL1 and CXCL8 by immunohistochemistry (Figure 1). Both chemokines were found to be present in the majority of lymph node and metastatic lesions analyzed, consistent with published findings (Richmond, Lawson et al. 1985; Payne and Cornelius 2002). Thus, the majority of human melanomas produce ligands for CXCR2, providing a strong rationale for improving tumor homing of T cells through ectopic expression of this chemokine receptor.

Figure 1. Human metastatic melanoma tumors express ligands for CXCR2. Paraffin-embedded, melanoma lymph node metastasis analyzed by immunohisto-chemical staining for (A) chemokine CXCL8 (B) chemokine CXCL1. Magnification, 40X.

Murine tumors produce CXCL1

As reported, the expression of CXCL1 appears to be restricted to tumor and inflammatory tissues. This was further confirmed for murine tissues by analysis of the Gene Expression Omnibus (GEO) database and the SAGEmap database (both available at http://www.ncbi.nlm.nih.gov/). Evaluation of the murine transcriptome in the GEO database, which included the evaluation of transcript levels from 122 samples representing 61 normal murine tissues using 31373 distinct probes, revealed that murine CXCL1 RNA levels were not significantly higher in any tissue compared to that seen in lung, liver, and kidney. In addition, in our own studies, CXCL1 was undetectable in lysates of minced kidney or lung from normal mice (not shown), as determined by ELISA.

We next assessed whether murine tumor cells could produce CXCL1. Fresh tumor explants derived from 24JK sarcoma, cultured overnight in medium, produced large amounts of CXCL1, as determined by ELISA (not shown)**.** In addition, cultured MC38 tumor cells expressed high levels of CXCL1 **(Figure 4B)**. While B16 tumor explants expressed CXCL1, cultured B16 tumor cells did not. Therefore, the CXCL1 gene was introduced into B16 for some of our models, as discussed below.

Activated, tumor-reactive T cells do not express CXCR2

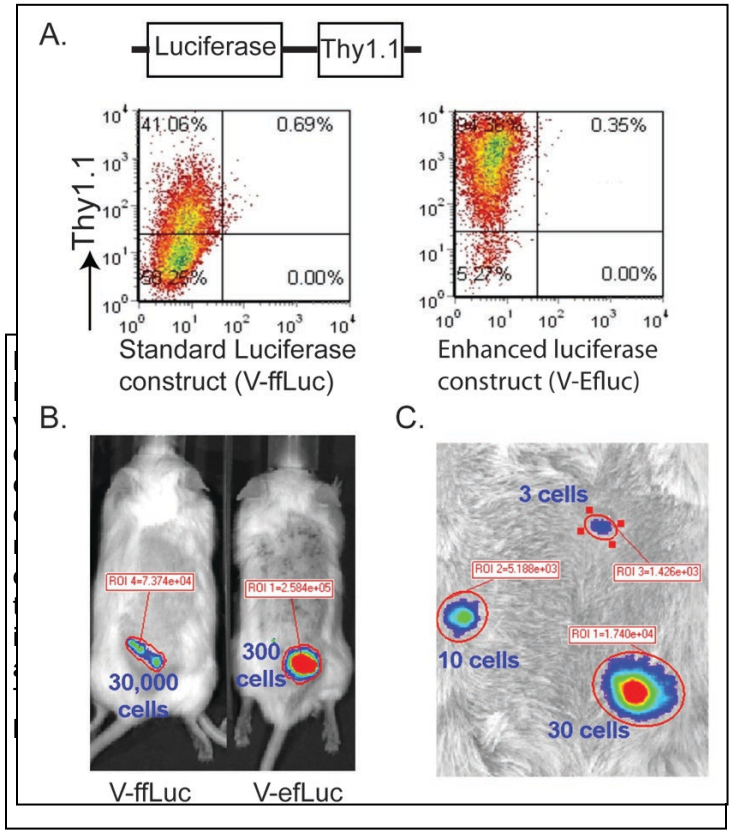
We next analyzed the chemokine receptors expressed by tumor-reactive human and murine T cells. Using an RNAse protection assay, cultured human T cells appeared to express the chemokine receptors CCR1, CCR2, CCR4, CCR5, CXCR3, and CXCR4 (data not shown; (Kershaw, Wang et al. 2002)). Notably, human T cells did not express the CXCL1-binding chemokine receptor, CXCR2. To confirm this pattern of receptor expression, we evaluated the ability of tumor reactive human T cells to respond functionally by measuring calcium flux in response to the exogenous addition of various chemokines. We found that the T cells could generate a calcium flux in response to the chemokines CCL5 (a ligand for CCR1, 3, and 5), CCL2 (ligand for CCR2), and CXCL12 (ligand for CXCR4), but not CXCL1, results that were consistent with the expression pattern observed using the RNAse protection assay.

In a similar fashion, we determined whether cultured murine T cells could naturally respond to CXCL1. As with human cells, murine T cells did not naturally migrate towards CXCL1, or exhibit calcium flux when exposed to CXCL1. Consistent with this, in an RNAse protection assay, murine alloreactive and peptide stimulated T cells were found to express CCR1, CCR2, and CCR5, but not CXCR2 (data not shown).

To test whether expression of CXCR2 would enhance the migration of tumor antigen-specific T cells to the tumor and provide better anti-tumor responses, we established a sensitive imaging system to track the migration of T cells in a murine tumor model using standard ecotropic packaging lines and moloney derived retroviral vectors. We combined gene transfer with imaging vectors and CXCR2 to study the migration and anti-tumor effects of these cells *in vivo.*

Visualizing T cell migration in vivo

To evaluate the effects of CXCR2 gene transfer on T-cell migration in a preclinical study, a sensitive and efficient system to trace tumor-specific T cells *in vivo* was required. Many methods have been established to monitor cells *in vivo,* including bioluminescence imaging (BLI). Compared with other technologies, BLI has several advantages that made it a powerful tool for the preclinical studies. First, BLI is a non-invasive imaging method. It allows researchers to continuously monitor the same animal for the duration of the experiment. Second, BLI does not require radioisotopes and is inexpensive. Third, multiple experimental animals can be imaged by BLI at the same time, thus saving valuable time and resources.



However, in murine adoptive T-cell therapy studies, the number of transferred T cells migrating to the tumor site is typically only a few hundred, which was below the level of detection of most bioluminescence technologies. Therefore, in order to monitor the small T cell population *in vivo*, our first priority was to develop a sensitive *in vivo* imaging system based on bioluminescence technology. To improve the expression of current virus based firefly luciferase constructs in mouse T cells, we optimized the codon usage to the highest frequency of *Mus musculus*, removed the cryptic splice sites and added the woodchuck hepatitis virus pre-element to augment export of the viral mRNA into the cytosol. These modifications resulted in >80% transduction efficiency in mouse T cells and high levels of expression **(Figure 2A)**. After sorting the firefly luciferase transduced T cells, we observed a 100 fold increase in signal intensity of T-cells transduced with our enhanced luciferase construct compared to those transduced with the standard luciferase construct **(Figure 2B)**. By using this modified firefly luciferase construct, as few as three transduced T cells could be visualized after subcutaneous injection into the mice **(Figure 2C)**.

Establishing a new murine tumor model to monitor the migration of transferred T cells in vivo

Following optimization of firefly luciferase expression in primary murine T-cells(Rabinovich, Ye et al. 2008), we next focused on studying T-cell migration in murine tumor models. However,

we found that BLI could not be used optimally with the B16 melanoma tumor model, due to the blockade of the luciferase signal by the dark skin and fur of the C57BL/6 mice and the black pigment of the B16 tumor. Therefore, we needed to develop a new murine tumor model to monitor tumor-specific T cell migration *in vivo* by BLI. To reduce the absorption of the luciferase signal by the dark skin and fur of C57BL/6 mice, the C57BL/6 mouse strain was replaced with an albino C57BL/6 mouse strain, which shares the same genetic background with C57BL/6 mice, but contains a spontaneous mutation of the tyrosinase gene. Additionally, the melanoma tumor antigen, gp100, was cloned into a lentiviral vector containing mIL-4R as a reporter gene, and transduced into a colon carcinoma derived from C57BL/6 mice, MC38. A stable gp100-expressing MC38 tumor cell line (MC38/gp100) was generated by sorting transduced tumor cells based on the expression of the mIL-4R reporter. We also transduced Pmel T cells with retroviral supernatants containing the mCXCR2 and luciferase constructs (**Figure 3A**). We routinely obtained more than 90% transduction efficiency (**Figure 3B**). Furthermore, MC38/gp100 tumor cells could activate Pmel T cells, which express a transgenic TCR recognizing an H-2D^{b} restricted gp100 peptide, to exhibit cytolytic activity (**Figure 3C**) and produce effector cytokines such as IFN-γ (**Figure 3D**). Because MC38/gp100 tumor cells, unlike B16 tumor cells, express consistent levels of MHC class I molecules, the activation of Pmel T cells by MC38/gp100 does not require IFN-γ pretreatment of tumor to induce class I expression. In this respect, the MC38/gp100 tumor cells are more similar to human melanoma than B16, since IFN gamma is not required by most human lines for recognition by CD8+ antigen-specific T-cells. The luciferase signal from the transferred T cells in the MC38/gp100 tumor site, could be appropriately detected (data not shown). Therefore, the MC38/gp100 tumor model combined with our modified BLI system is a powerful and sensitive tool to study tumor-specific T cell migration.

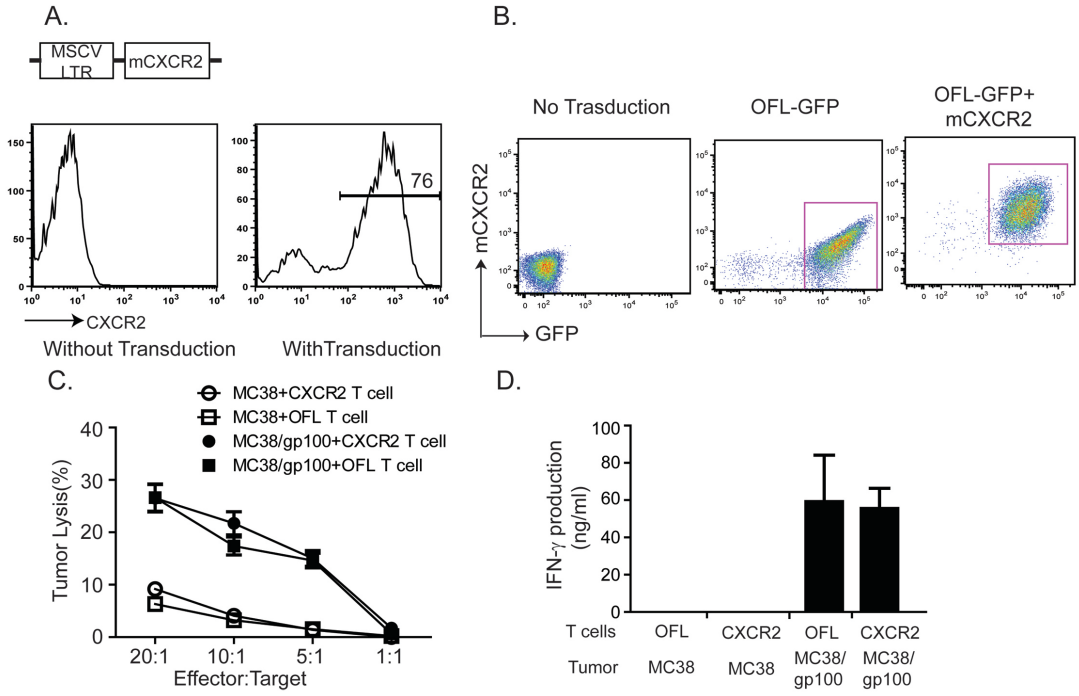


Figure 3. Generation and functional characteristics of CXCR2 expressing Pmel T cells. (A) Schematic representation of viral vector containing mCXCR2. Long terminal repeat (LTR) from the murine stem cell PCMV virus drives high-level, constitutive expression of mCXCR2 in Pmel T cells. (B) FACS analysis of transduced Pmel T cells. Pmel T cells were transduced with modified luciferase (OFL) or OFL and mCXCR2. Cells were sorted based on the expression of GFP or CXCR2. Sorted cells were cultured for 3 days and used for transfer therapy. Before transfer, the purity of transferred cells was evaluated by flow cytometry. More than 90% of transferred cells are either GFP positive or GFP and CXCR2 double positive, as expected. **(**C) Cytotoxic reactivity of CXCR2 expressing Pmel T cell against MC38/gp100 tumor. MC38 or MC38/gp100 cells were labeled with Cr^{51}. After labeling, 5000 tumor cells were cocultured with either CXCR2 expressing Pmel T cells or OFL-expressing Pmel T cells for 6 hours. The supernatants were monitored for gamma-activity. (D) IFN-γ secretion by CXCR2 expressing Pmel T cells in response MC38/gp100 tumor. Similar cell lysis rate and similar amount of IFN-gamma secretion were found between CXCR2-expressing Pmel T cells and control Pmel T cell in response to MC38/gp100 tumor.

CXCR2-transduced Pmel T cells migrate towards CXCL1 gradients in vitro

To test whether the exogenous expression of CXCR2 in Pmel T cells could enable these cells to respond to CXCL1, Pmel T cells were transduced with either a luciferase construct, OFL-GFP, or OFL-GFP and CXCR2. Sorted transduced T cells were used to perform a migration assay using a transwell system. T cell migration to the lower well was measured *in vitro* by measuring luminescent activity after addition of D-luciferin. Higher levels of luciferase intensity were observed from Pmel T cells expressing both CXCR2 and luciferase than from Pmel T cells expressing luciferase alone. Furthermore, increased migration of CXCR2-transduced Pmel T cells was dependent on CXCL1 as the migration of CXCR2-expressing Pmel T cells was similar vc. control T cells in the absence of CXCL1 **(Figure 4A)**. Moreover, we confirmed the migratory ability of CXCR2-expressing Pmel T cells towards CXCL1 produced by murine tumor cells. Our results indicated that the concentration of mCXCL1 in overnight cultured medium from $2x10^6$ MC38 or MC38/gp100 tumor cells was higher than 10ng/ml. Production of mCXCL1 was comparable between MC38 and MC38/gp100 tumor cells **(Figure 4B)**. Although mCXCL1 was present in B16 tumor explants (data not shown), mCXCL1 produced by *in vitro* cultured B16 tumor cells was undetectable by ELISA. Therefore, we also established a stable CXCL1-expressing B16 tumor cell line (Figure 4B). By using conditioned medium from these tumor cells, we investigated the migratory ability of CXCR2-expressing Pmel T cells *in vitro.*

Conditioned medium from either CXCL1-expressing B16 or MC38 tumor cells induced enhanced migration of CXCR2 expressing Pmel cells, but not the migration of control T cells **(Figure 4C)**. Additionally, the number of migrating T-cells correlated with the concentration of CXCL1 in the cell culture supernatants. By contrast, the cell culture medium from B16 tumor cells didn't alter the migration of CXCR2-expressing T cells when compared to medium alone, confirming that the migration of CXCR2-expressing T cells was dependent on CXCL1. Taken together, these results suggest that the introduction of the CXCR2 gene into Pmel T cells enhanced the migratory ability of those T cells towards a CXCL1 gradient *in vitro* in a dose-dependent manner, but did not alter the activation of T cells upon antigenic stimulation, in the presence or absence of CXCL1 (data now shown).

CXCR2-expressing Pmel T cells possess an enhanced ability to traffic to tumor in vivo

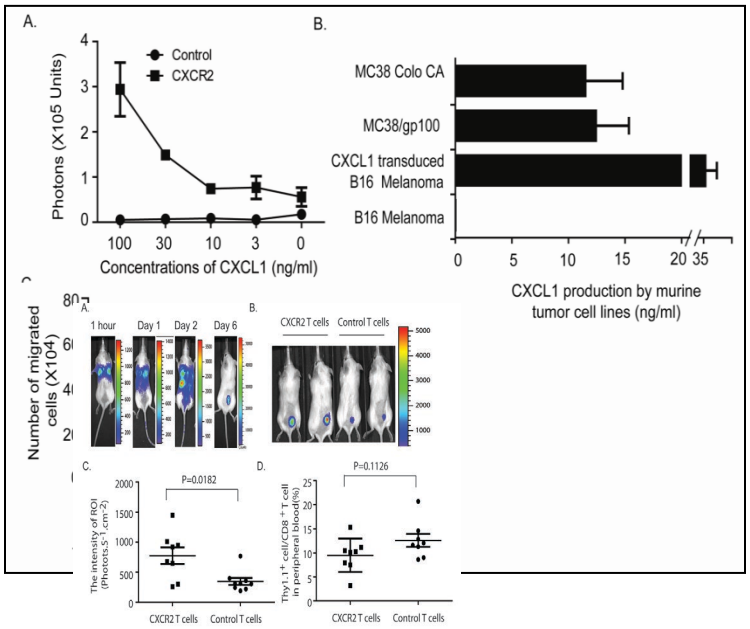


Figure 4: Enhanced migration ability of CXCR2 expressing T cells towards CXCL1 gradients (A) Migration of CXCR2 expressing T cells towards CXCL1 gradients provided by recombinant CXCL1. (B) CXCL1 production by murine tumor lines**.** (C) The migration of CXCR2 expressing T cells towards CXCL1 gradients provided by murine tumor cells.

To analyze the *in vivo* migratory pattern of CXCR2-expressing Pmel T cells in tumor-bearing mice, we took advantage of our newly established MC38/gp100 tumor model and modified BLI system. $1x10^6$ Pmel T cells expressing OFL-GFP alone or OFL-GFP and CXCR2 were intravenously injected into C57BL/6 albino mice harboring MC38/gp100 tumors (average tumor size=20 mm^2; All the mice receiving Pmel T cells
also received DC vaccination and hIL-2 i.p injection as previously described (Lou, Wang et al. 2004).

Luciferase signals from the adoptively transferred Pmel T cells were monitored on a daily basis. Although the transferred Pmel T cells were detected by BLI in tumor sites as early as the second day after adoptive T cell transfer, most of the transferred Pmel T cells were initially found in the lung and liver which is consistent with adoptive T-cell transfer in humans (**Figure 5A**). Six days after T cell transfer, the majority of the transferred Pmel T cells were localized at the tumor site. The distribution pattern of transferred tumor-specific T cells obtained by our BLI technique is similar to the previously reported single-photon emission computed tomography (CT)-CT fusion imaging method (Pittet, Grimm et al. 2007). Therefore, we used the luciferase results from mice on day six after T cell transfer as representative data to characterize the tumor migration of transferred Pmel T cells. As shown in **Figure 5B**, mice receiving CXCR2-expressing T cells displayed a stronger luciferase signal at the tumor site than mice receiving T cells expressing luciferase alone on day 6 after T cell

transfer. The average luciferase intensity of all mice (N=9) from each group was quantified by live imaging software (**Figure 5C**). By contrast, in peripheral blood, the percentage of Thy1.1$^+$ cells, a cell surface marker for transferred Pmel T cells, among total CD8$^+$ T cells in the peripheral blood remained similar between mice receiving CXCR2-expressing Pmel T cells or control Pmel T cells (**Figure 5D**). These results suggest that transduction of T-cells with the CXCR2 gene resulted in their preferential accumulation to tumor sites expressing CXCL1.

Introduction of CXCR2 into human T cells induces calcium flux in response to CXCR2 ligands

Having demonstrated that CXCR2 transduced murine T-cells were more effective in vivo in murine tumor models, we next tested this system with human T-cells in vitro in preparation for bringing this concept to the clinic. We have attempted to utilize knowledge of the expression patterns of chemokines and chemokine receptors in tumors and T cells to design strategies to enhance T-cell migration to tumors. Our initial approaches have taken advantage of our experience in modifying T cells by retroviral gene transduction (Hwu, Shafer et al. 1993; Hwu, Yannelli et al. 1993; Hwu and Rosenberg 1994; Treisman, Hwu et al. 1994; Treisman, Hwu et al. 1995; Lam, Reeves et al. 1996; Kershaw, Wang et al. 2002; Royal, Kershaw et al. 2002). Our strategy thus far has involved transduction of human lymphocytes with specific chemokine receptor genes in order to allow them to recognize and respond to the chemokines specifically produced by tumors. In order to confer responsiveness to CXCL1, we cloned the human CXCR2 receptor gene into a retroviral vector and used it to transduce human T cells. Flow cytometric analysis demonstrated that T cells expressed surface CXCR2 following retroviral transduction (data not shown). We next evaluated the function of the CXCR2 receptor in transduced T lymphocytes by measuring calcium flux in response to the exogenous addition of CXCR2 ligands. T cells were transduced with either the CXCR2-expressing or control retroviral vector were incubated with human CXCL8.

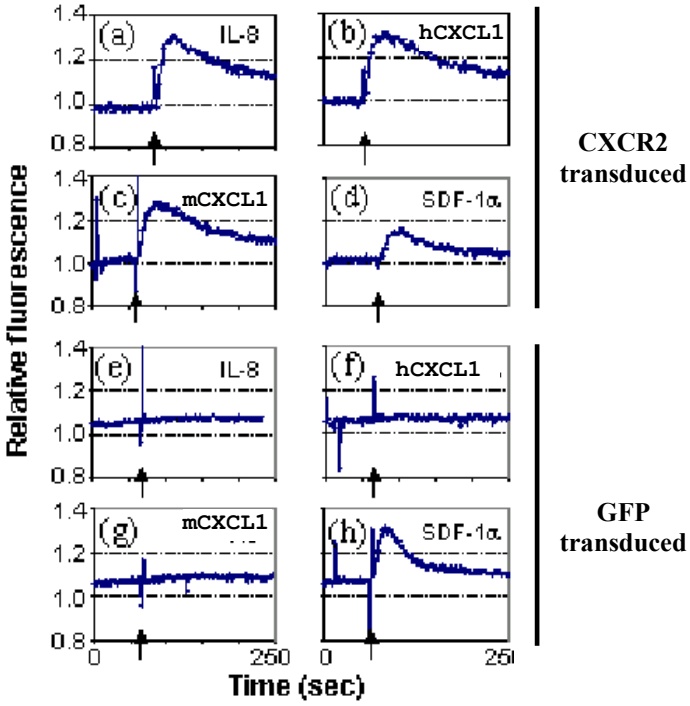


Figure 5: Enhanced migration ability of CXCR2 expressing Pmel T cells to the tumor sites (A) In vivo trafficking of OFL expressing Pmel T cells. Imaging was performed at indicated time points after T cell transfer. (B) Increased accumulation of CXCR2 expressing Pmel T cells at the tumor site. Imaging was performed at day six after T cell transfer. Data shown were from representative mice. (C) Quantitative imagng analysis of transferred T cells in tumor bearing mice. The intensities of the luciferase signal at the tumor sites in all tumor-bearing mice were quantified using live imaging software. (D) FACS analysis of the percentage of Thy1.1$^+$ in CD8$^+$ cells in peripheral blood on day six after T cell transfer. While no differences were seen in peripheral blood (D), more CXCR2

Addition of the chemokine CXCL12, which binds to the chemokine receptor CXCR4 constitutively expressed by mature T cells, was included as a positive control. CXCR2-transduced T cells responded to CXCL8 and human or mouse CXCL1 with a rapid increase in intracellular calcium **(Figures 6a, 6b, and 6c)**. The

requirement for CXCR2 was demonstrated by the lack of responsiveness to CXCR2 ligands of control vector only-transduced T cells (Figures 6e, 6f, and 6g). As expected, both chemokine receptor and vector-only transduced T cells were able to respond to CXCL12 **(Figures 6d and 6h)**. These results clearly demonstrated that introduced human CXCR2 could function in transduced primary human T cells and cause mobilization of intracellular calcium in response to binding of its chemokine ligands.

CXCR2-transduced human T cells migrate towards CXCL1 produced by tumor cells

At the time of your death, no matter the cause, we may request permission for an autopsy in order to obtain vital information concerning the safety of this experimental therapy approach. Please discuss this request with your family to inform them of your wishes. At the time of your death, no matter the cause, we may request permission for an autopsy in order to obtain vital information concerning the safety of this experimental therapy approach. Please discuss this request with your family to inform them of your wishes. At the time of your death, no matter the cause, we may request permission for an autopsy in order to obtain vital information concerning the safety of this experimental therapy approach. Please discuss this request with your family to inform them of your wishes.

We next tested CXCR2-transduced human T cells for their ability to migrate specifically toward CXCL1, as well as toward CXCL1-producing tumor cell supernatants **(Figure 7)**. Experiments were performed using 3 μm pore size transwell plates. As shown in **Figure 7a**, CXCR2-transduced T cells migrated through transwell pores in greater numbers when CXCL1 was present in the lower chamber compared to the migration in the absence of CXCL1. This specific migration was evident at concentrations of CXCL1 as low as 1 nM. The increased migration was dependent upon CXCR2 expression, as control vector-transduced T cells migrated in lower numbers irrespective of the

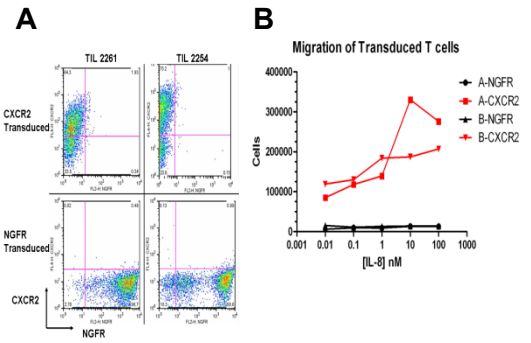


Figure 6. Calcium flux in CXCR2-transduced T cells. Human T cells were loaded with the fluorescent calcium indicator Fura-2 and assayed for calcium flux following the addition of the indicated chemokines (arrows). CXCR2-transduced T cells responded to the CXCR2 ligands CXCL8, in addition to human and murine CXCL1 **(a-c)**, to a similar degree as to the positive control CXCL12 **(d)**, the ligand for the constitutively expressed CXCR4. Control GFP vector-transduced T cells did not respond to the CXCR2 ligands **(e-g),** but did respond to the positive control CXCL12 **(h)**.

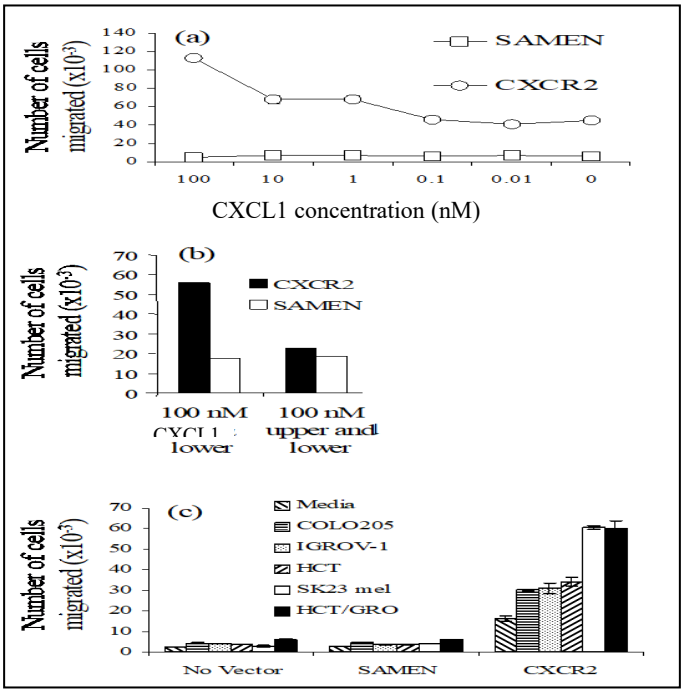


Figure 7. Chemotaxis of CXCR2-expressing T cells. T cells were assayed for chemotaxis by determining their ability to migrate from an upper chamber, through a 3 μm pore membrane towards a source of chemokine in a lower chamber in 24-well plates. **(a)** CXCR2-transduced T cells migrated towards CXCL1 in greater numbers than control vector-transduced T cells. **(b)** When CXCL1 was included in the upper and lower chambers no increase in migration was found, thereby indicating that the response to CXCL1 was chemotactic in nature and not solely chemokinetic. **(c)** CXCR2-transduced T cells migrated towards cell culture supernatant derived from the CXCL1- secreting tumor cell lines SK23 and HCT/GRO in greater numbers than towards supernatants from CXCL1 negative cells or media alone. The requirement for CXCR2 for the observed effect was evident from the comparatively lower migration levels of non-transduced or control vector-transduced T cells.

presence or absence of CXCL1. Migration was demonstrated to be a result of increased specific chemotaxis rather than increased chemokinesis since inclusion of CXCL1 in both the upper and lower chambers did not result in increased migration of CXCR2-transduced T cells through transwells **(Figure 7b)**. Chemotaxis was G-protein-dependent, as it was inhibited by pertussis toxin in a dose-dependent manner (data not shown).
In order to determine whether CXCR2-transduced T cells could respond to CXCL1 derived from tumor cells, transwell migration assays were also performed using overnight culture supernatants from tumor cell lines as the source of chemokine in the lower chamber. Significant numbers of CXCR2-transduced T cells migrated towards supernatant derived from SK23 melanoma and from a CXCL1-transduced HCT116 colon carcinoma cell line (HCT/GRO), approximately 3-fold more than towards media alone **(Figure 7c)**. An approximately 2-fold increase in migration was observed in response to CXCL1-expressing tumor cell supernatant compared to the CXCL1-negative cell supernatants derived from the colon carcinoma COLO205, the ovarian carcinoma line IGROV-1 and non-transduced HCT116.

Interestingly, migration of CXCR2-transduced T cells was greater towards CXCL1-negative supernatant derived from ovarian and colon carcinoma cell lines than towards media alone. This latter observation may be due to the presence of CXCL8 found in supernatants from these cell lines (data not shown), since CXCR2 binds to both CXCL1 and CXCL8. CXCR2 was responsible for the increased T-cell migration observed, as demonstrated by the much lower level of migration of control vector or non-transduced T cells, irrespective of the source of supernatant **(Figure 7c)**. These studies clearly demonstrate the feasibility of altering the migratory phenotype of T cells with the introduction of the chemokine receptor CXCR2 (Kershaw, Wang et al. 2002).

We also demonstrate that tumor infiltrating lymphocytes (TILs) can be readily transduced with human CXCR2 expressing vector (**Figure 8A**). Furthermore,
transduction of CXCR2 confers enhanced migration capabilities in response to CXCL1 graduent compared to NGFR transduced cells (**Figure 8B**).

Transfer of CXCR2-expressing Pmel T cells further improves T-cell mediated antitumor immune responses Since CXCR2 expression results in increased accumulation of tumor antigen specific T cells at the tumor site, we next assessed whether this increased accumulation would result in better antitumor immune responses. Seven days after tumor challenge, mice were treated with adoptive T-cell transfer (combined with TBI, gp100 peptide pulsed DC vaccination and hIL-2 injection as per our routine protocol). The size of MC38/gp100 tumor was measured every other day. Compared with untreated mice, transfer of $1x10^6$ Pmel T cells resulted in a significant decrease of MC38/gp100 tumor growth, which is consistent with our previous studies using the B16 tumor model **(Figure 9A)**. More importantly, tumor growth in mice receiving CXCR2-expressing Pmel T cells was significantly slower than in mice receiving control (luciferase only) Pmel T cells (Figure 9A). In another related experiment, tumor growth in mice transferred with $1x10^6$ CXCR2-expressing Pmel T cells was comparable to that of mice received $5x10^6$ control Pmel T cells (data not shown), suggesting that fewer T-cells need to be transferred if migration to the tumor is more efficient. Moreover, the introduction of the CXCR2 gene into adoptively transferred Pmel T cells also led to a significant increase in the survival of MC38/gp100-bearing mice **(Figure 9B)**. Although CXCR2-expressing Pmel T cells did not confer better protection in B16 tumor bearing mice than control Pmel T cells (data not shown), likely due to the lack of CXCL1 expression by native B16 tumor, the growth of CXCL1-expressing B16 tumors *in vivo* was significantly delayed in mice treated with CXCR2-expressing Pmel T cells compared with control Pmel T cells **(Figure 9C)**. Transferring CXCR2-expressing Pmel T cells was also found to significantly extend the survival time of B16-CXCL1 tumor bearing mice (Figure 6D). These data strongly suggest that expression of CXCR2 by transferred Pmel T cells enhances the in vivo antitumor immune response.

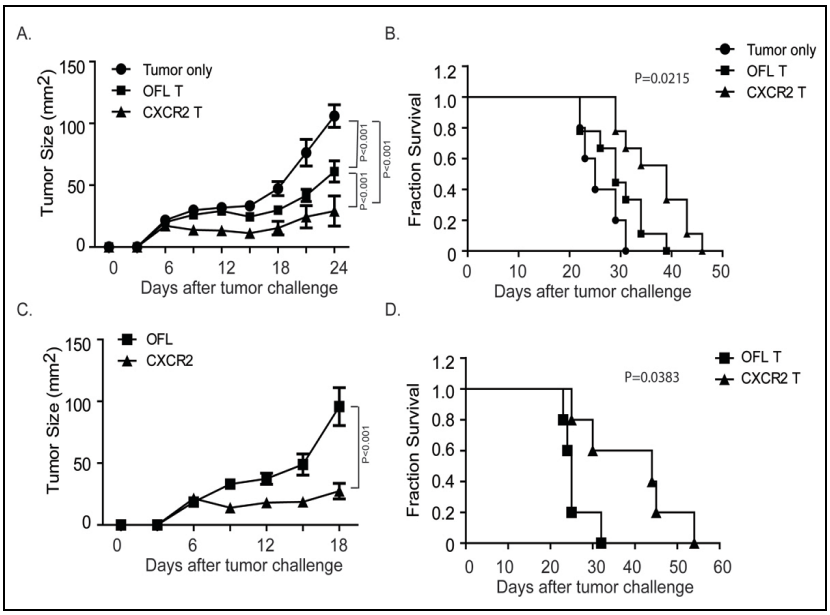


Figure 9: Regression of established tumors upon adoptive transfer of CXCR2 expressing Pmel T cells. Groups of mice were initially implanted with $5X10^5$ tumor cells in the flank at day 0, subjected to 350cGy TBI at day 6, followed by DC vaccination, systemic IL-2 and the adoptive transfer of Pmel T cells either expressing OFL alone or OFL and CXCR2. (A) Tumor growth curve of MC38/gp100 tumor-bearing mice with adoptive T cell therapy. (B) Kaplan-Meier survival curves of MC38/gp100 tumor-bearing mice with adoptive T cell therapy. (C) Tumor growth curve of B16-CXCL1 tumor-bearing mice with adoptive T cell therapy. (D) Kaplan-Meier survival curves of B16-CXCL1 tumor-bearing mice with adoptive T cell therapy. In both tumor models, CXCR2 transduction enhanced the ability of the T-cells to mediate tumor regression and increased survival.

Hence, Our results from preclinical murine models and human TILs have demonstrated the feasibility of altering lymphocyte migration *in vitro* and *in vivo* with the use of retroviral gene transfer, and also showed that improved migration of CXCR2-transduced tumor-specific lymphocytes has conferred enhanced tumor protection in murine models.

2.0 Objectives

Primary Objective

- To assess the feasibility and safety of CXCR2 and NGFR Transduced TILs for treating metastatic malignant melanoma.

Secondary Objectives

- Determine whether CXCR2 transduction enhances the ability of TIL to migrate to melanoma tumors

- Determine the levels of CXCL1 and CXCL8 chemokines produced by melanoma tumors and assess whether this correlates with the tumor localization of CXCR2 transduced TIL

- Characterize the clinical response and correlate with migration of CXCR2 transduced TIL to the tumor and levels of CXCL1 and CXCL8 at the tumor site.

3.0 Clinical Pharmacology

3.1 CXCR2 transduced TIL

CXCR2 is a 67-70 kD seven-transmembrane G-protein coupled chemokine receptor. The ligands that bind to CXCR2 include CXCL8 (IL-8) and CXCL1 (GRO-α). TIL retrovirally transduced to express CXCR2 can migrate in vitro and in vivo to CXCL1 and CXCL8 expressing tumors.

3.2 NGFR transduced TIL

Nerve growth factor receptor (NGFR) belongs to the TNF (tumor necrosis factor) superfamily of receptors and is one of the receptors for neurotrophins. A non-signaling truncated version of human NGFR, lacking the cytoplasmic domain, is routinely used as a marker for gene transduction. In this study, a human truncated NGFR will be used as a control for CXCR2 TIL transduction. Since it is of human origin and lacks a cytoplasmic domain, it will not elicit any immune responses and is incapable of eliciting intracellular signaling cascades. It acts as a marker to identify control TIL cells since human T cells normally do not express NGFR.

3.3 Interleukin-2 (Aldesleukin, Proleukin)

Interleukin-2 (IL-2) will be manufactured and supplied by Norvatis and will be purchased by the M.D. Anderson pharmacy from commercial sources. IL-2 is a 133 amino acid-long peptide primarily secreted by T-cells in response to various antigenic stimuli. The cytokine acts through a specific IL-2 receptor consisting of α, β, γ subunits. In addition to T-cell proliferation, IL-2 leads to activation and proliferation of natural killer (NK) cells, increasing their tumoricidal activity. Other actions of IL-2 include augmentation of B-cell growth and immunoglobulin production, enhancement of interferon (IFN)-γ and tumor necrosis factor (TNF)-β production from T-cells, IL-6 production by monocytes, modulation of histamine release by basophils, and upregulation of IL-2 receptors. This triggers the release of various other cytokines leading to the total immune/ inflammatory reaction and resultant toxicity.

IL-2 will be administered as an inpatient treatment. Grade III toxicities common to IL-2 include diarrhea, nausea, vomiting, hypotension, skin changes, anorexia, mucositis, dysphagia, constitutional symptoms, and laboratory changes. Additional Grade IV and V toxicities have been seen with IL-2 (See Appendix-G).

3.4 Fludarabine

Fludarabine phosphate is a fluorinated nucleotide analog of the antiviral agent vidarabine, 9-β-D-arabinofuranosyladenine (ara-A) that is relatively resistant to deamination. Fludarabine is a purine antagonist antimetabolite. Fludarabine phosphate is rapidly dephosphorylated to 2-fluoro-ara-A and then phosphorylated intracellularly by deoxycytidine kinase to the active triphosphate, 2-fluoro-ara-ATP. This metabolite appears to act by inhibiting DNA polymerase alpha, ribonucleotide reductase and DNA primase, thus inhibiting DNA synthesis. The mechanism of action of this antimetabolite is not completely characterized and may be multi-faceted.

It will be purchased by the M.D. Anderson pharmacy from commercial sources. Fludarabine is supplied as a fludarabine phosphate powder in the form of a white, lyophilized solid cake. The fludarabine powder is stable for at least 18 months at 2 – 8 degrees C; when reconstituted, fludarabine is stable for at least 16 days at room temperature. Specialized references should be consulted for specific compatibility information. Fludarabine is dephosphorylated in serum, transported intracellularly and converted to the nucleotide fludarabine triphosphate; this 2-fluoro-ara-ATP molecule is thought to be required for the drug's cytotoxic effects. Fludarabine inhibits DNA polymerase, ribnucleotide reductase, DNA primase, and may interfere with chain elongation, and RNA and protein synthesis.

Fludarabine is administered as an I.V. infusion in 100 ml 0.9% sodium chloride, USP over approximately 15-30 minutes. At doses of 25 mg/m^2/day for 5 days, the primary side effect is myelosuppression. However, thrombocytopenia is responsible for most cases of severe and life-threatening hematologic toxicity. Hemolytic anemia has been reported after one or more courses of fludarabine with or without a prior history of a positive Coomb's test; fatal hemolytic anemia has been reported. In addition, bone marrow fibrosis has been observed after fludarabine therapy. Other common adverse effects include malaise, fatigue, anorexia, and weakness. Irreversible and potentially fatal central nervous system toxicity in the form of progressive encephalopathy, blindness, and coma is rare at the currently administered doses. More common neurologic side effects at the current doses of fludarabine include weakness, pain, malaise, fatigue, paresthesia, visual or hearing disturbances, and sleep disorders. Adverse respiratory effects of fludarabine include cough, dyspnea, and allergic or idiopathic interstitial pneumonitis. Tumor lysis syndrome has been rarely observed in fludarabine treatment of Chronic Lymphocytic Leukemia (CLL).

3.5 Cyclophosphamide (Cytoxan)

Cyclophosphamide is a synthetic anti-neoplastic drug chemically related to the nitrogen mustards. It is biotransformed principally in the liver to active alkylating metabolites by a mixed function microsomal oxidase system. These metabolites interfere with the growth of susceptible rapidly proliferating malignant cells. The mechanism of action is thought to involve cross-linking of tumor cell DNA.

Cyclophosphamide is well absorbed after oral administration with a bioavailability greater than 75%. The unchanged drug has an elimination half-life of 3 to 12 hours. It is eliminated primarily in the form of metabolites, but from 5% to 25% of the dose is excreted in urine as unchanged drug. Several cytotoxic and noncytotoxic metabolites have been identified in urine and in plasma. Concentrations of metabolites reach a maximum in plasma 2 to 3 hours after an intravenous dose. Plasma protein binding of unchanged drug is low but some metabolites are bound to an extent greater than 60%. It has not been demonstrated than any single metabolite is responsible for either the therapeutic or toxic effects of cyclophosphamide. Although elevated levels of metabolites of cyclophosphamide have been observed in patients with renal failure, increased clinical toxicity in such patients has not been demonstrated.

Following conversion to active metabolites in the liver, cyclophosphamide functions as an alkylating agent and possesses potent immunosuppressive activity. The serum half-life after intravenous administration ranges from 3 to 12 hours; the drug and/or its metabolites can be detected in the serum for up to 72 hours after administration.

Cyclophosphamide will be obtained from commercially available sources by the M. D. Anderson pharmacy. It will be diluted in 250 ml NS and infused over approximately two hours. The dose will be based on the patient's body weight, but to prevent undue toxicity, it will not exceed a dose greater than 40% of the maximum ideal body weight for drug dosing using MD Anderson (MDACC) clinical calculator for Body Surface Area (BSA) and Body Mass Index (BMI). Hematologic toxicity occurring with cyclophosphamide usually includes leukopenia and thrombocytopenia. Anorexia, nausea, and vomiting may occur, especially after high-doses. Diarrhea, hemorrhagic colitis, and mucosal and oral ulceration have been reported in patients. Sterile hemorrhagic cystitis occurs in about 20% of patients; severity can range from microscopic hematuria to extensive cystitis with bladder fibrosis. Although the incidence of hemorrhagic cystitis associated with cyclophosphamide appears to be lower than that associated with ifosfamide, mesna (sodium 2-mercaptoethanesulfonate) has been used prophylactically as an uroprotective agent Mesna may not be effective in all patients. Patients who receive high dose cyclophosphamide may develop interstitial pulmonary fibrosis, which can be fatal. Hyperuricemia due to rapid cellular destruction may occur, particularly in patients with hematologic malignancy. Hyperuricemia may be minimized by adequate hydration, alkalinization of the urine, and/or administration of allopurinol. If allopurinol is administered, patients should be watched closely for cyclophosphamide toxicity due to allopurinal induction of hepatic microsomal enzymes. At high doses, cyclophosphamide can also result in a syndrome of inappropriate antidiuretic hormone secretion; hyponatremia with progressive weight gain without edema occurs. Cardiotoxicity has been observed at high doses of cyclophophamide. Deaths have occurred from diffuse hemorrhagic myocardial necrosis and from acute myopericarditis; in such cases, congestive heart failure may occur within a few days of the first dose. Other consequences of cyclophosphamide cardiotoxicity include arrhythmias, potentially irreversible cardiomyopathy, and pericarditis. Other reported adverse effects of cyclophosphamide include headache, dizziness, and myxedema; faintness, facial flushing, and diaphoresis.

3.6 Mesna (Sodium 2-mercaptoethanesulfonate, Mesnum, Mesnex, NSC-113891)

Mesna (sodium 2-mercaptoethanesulphonate; given by IV injection) is a synthetic sulfhydryl compound that can chemically interact with urotoxic metabolites of cyclophosphamide (acrolein and 4-hydroxycyclophosphamide) to decrease the incidence and severity of hemorrhagic cystitis.

Mesna was developed as a prophylactic agent to reduce the risk of hemorrhagic cystitis. Analogous to the physiological cysteine-cystine, mesna is rapidly oxidized to its major metabolite, mesna disulfide (dimesna). Mesna disulfide remains in the intravascular compartment and is rapidly eliminated by the kidneys. In the kidney, the mesna disulfide is reduced to the free thiol compound, mesna, which reacts chemically with the urotoxic metabolites, resulting in their detoxification.

Mesna will be obtained commercially and is supplied as a 100 mg/ml solution. Intact ampules are stored at room temperature. Diluted solutions (1 to 20 mg/dl) are physically and chemically stable for at least 24 hours under refrigeration. Mesna is chemically stable at room temperature for 48-72 hours in D5W, 48-72 hour in D5W/0.45% normal saline, or 24 hours in normal saline. It will be diluted up to 20 mg Mesna/ml fluid in D5W or normal saline and will be administered intravenously as a continuous infusion. Toxicities include nausea, vomiting and diarrhea.

3.7 G-CSF (Granulocyte Colony-Stimulating Factor)

G-CSF may be given in the form of Filgrastim or PEG-Filgrastim at the appropriate doses. The side effects of G-CSF are skin rash, myalgia and bone pain, an increase of preexisting inflammatory conditions, enlarged spleen with occasional associated low platelet counts, alopecia (with prolonged use) elevated blood chemistry levels.

3.8 Levofloxacin (Levaquin) and Trimethoprim and Sulfamethoxazole double strength (TMP / SMX DS, Bactrim)

Levaquin is used to prevent infections caused by bacteria. It is a synthetic broad spectrum antibacterial agent. The mechanism of action of levofloxacin involves inhibition of bacterial topoisomerase IV and DNA gyrase, enzymes required for DNA replication, transcription, repair, and recombination.

TMP/SMX DS will be obtained by the M.D. Anderson pharmacy from commercial sources. It will be used for the prevention of Pneumocystis carinii Pneumonia (PCP). The oral dose is 1 tablet PO bid twice a week. Like other sulfa drugs, Bactrim (sulfamethoxazole-trimethoprim) can cause allergies, fever, nausea, and vomiting. Allergies typically develop as a widespread itchy red rash with fever eight to fourteen days after beginning the standard dose. Neutropenia, a reduction in the number of neutrophils, can also occur.

3.9 Acyclovir (Zovirax) and Valacyclovir Hydrochloride (Valtrex)

Acyclovir and Valtrex will be obtained by the M. D. Anderson pharmacy from commercial sources.

Valtrex is the hydrochloride salt of *L*-valyl ester of acyclovir. It is rapidly converted to acyclovir, which has demonstrated antiviral activity against herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) and varicella-zoster virus (VZV). The inhibitory activity of acyclovir is highly selective due to its affinity for the enzyme thymidine kinase (TK) encoded by HSV and VZV. This viral enzyme converts acyclovir into acyclovir monophosphate, a nucleotide analogue. The monophosphate is further converted into diphosphate by cellular guanylate kinase and into triphosphate by a number of cellular enzymes. Acyclovir triphosphate stops replication of herpes viral DNA.

Acyclovir will be used to prevent the occurrence of herpes virus infections. It is supplied as powder for injection in 500 mg vials. Reconstitute in 10 ml of sterile water for injection for bacteriostatic water for injection to a concentration of 50 mg/ml. Reconstituted solutions should be used within 12 hours. IV solutions should be diluted to a concentration of 7 mg/ml or less and infused over 1 hour to avoid renal damage. Oral tablets of 200 and 800 mg are available, if the patient is able to tolerate medication by mouth. Reversible renal insufficiency has been reported with intravenous but not oral acyclovir. Neurologic toxicity including delirium, tremors, coma, acute psychiatric disturbances, and abnormal EEGs has been reported with higher doses of acyclovir. Should this occur, a dosage adjustment will be made or the drug should be discontinued. Stomach upset, headache, nausea, rash, hives, diaphoresis, hematuria; hypotension, and thrombocytosis have been reported. Hair loss from prolonged use has also been documented. Acyclovir will not be used concomitantly with other nucleoside analogs that interfere with DNA synthesis, e.g. ganciclovir. In renal disease, the dose is adjusted as per product labeling.

3.10 Fluconazole (Diflucan)

Fluconazole will be obtained by the M. D. Anderson pharmacy from commercial sources. It will be used for prophylaxis against fungal infections. It is available in 200 mg tablets. It can cause headache, nausea, vomiting, diarrhea or abdominal pain, and liver damage that may be irreversible. It can cause rashes and itching, which in rare cases has caused Stevens Johnson Syndrome. It has several significant drug interactions. The package insert should be consulted prior to prescribing. For IV administration in patients who cannot tolerate the oral preparation, Fluconazole comes in 2 mg/ml solution for injection, and prepared according to Clinical Center Pharmacy standard procedures. It should be administered at a maximum IV rate of 200 mg/hr.

3.11 Ondansetron hydrochloride (Zofran)

Ondansetron hydrochloride will be obtained by the M. D. Anderson pharmacy from commercial sources. It will be used to control nausea and vomiting during the chemotherapy preparative regimen. It can cause headache, dizziness, myalgias, drowsiness, malaise, and weakness. Less common side effects include chest pain, hypotension, pruritis, constipation and urinary retention. Consult the package insert for a complete list of side effects and specific dose instructions.

3.12 Furosemide (Lasix)

Furosemide, a loop diuretic, will be obtained by the M. D. Anderson pharmacy from commercial sources. It will be used to enhance urine output during the chemotherapy preparative regimen with cyclophosphamide. Adverse effects include dizziness, vertigo, paresthesias, weakness,

orthostatic hypotension, photosensitivity, rash and pruritis. Consult the package insert for a complete list of side effects and specific dose instructions.

3.13 T Cell Preparation

The procedures and reagents for expanding the human TIL cells and the Certificates of Analysis are contained in the CMC located in the IND office.

4.0 Eligibility Assessment and Enrollment

Patient evaluation for eligibility and registration will occur utilizing a two-turnstile design.

4.1 Turnstile I – Screening – Initiation of TIL Expansion

Patients must fulfill all of the following criteria to be eligible for Turnstile I of the study. All patients entering the MDACC Melanoma Clinic are tested for the BRAF mutation as standard practice and are not required to receive treatment with BRAF (and MEK) inhibitors prior to therapy with TIL on this study.

4.1.1 Turnstile I Inclusion Criteria

a. Patients must have metastatic melanoma or stage III in-transit, subcutaneous, or regional nodal disease.
b. Patients must have a lesion amenable to resection for the generation of TIL.
c. Patients must receive an MRI/CT/PET of the brain within 6 months of signing informed consent. If new lesions are present, patient must have definitive treatment. PI or his designee should make final determination regarding enrollment.
d. Age greater than or equal to 18 years.
e. Clinical performance status of ECOG 0 – 2 within 30 days of signing informed consent.
f. Patients previously treated with immunotherapy, targeted therapy, or no therapy will be eligible. Patients receiving cytotoxic agents will be evaluated by the PI or his designee as to suitable eligibility.
g. Patients with a negative pregnancy test (urine or serum) must be documented within 14 days of screening for women of childbearing potential (WOCBP). A WOCBP has not undergone a hysterectomy or who has not been naturally postmenopausal for at least 12 consecutive months (i.e. who has not had menses at any time in the preceding 12 consecutive months).

4.1.2 Screening Exclusion Criteria

a. Active systemic infections requiring intravenous antibiotics, coagulation disorders or other major medical illnesses of the cardiovascular, respiratory or immune system. PI or his designee shall make the final determination regarding appropriateness of enrollment.
b. Patients who are pregnant or nursing

4.1.3 Screening Procedures

All patients must sign an informed consent form and a negative pregnancy test (urine or serum) must be documented for women of childbearing potential before enrollment in Turnstile I and being registered in PDMS. Once enrolled into Turnstile I, patients will be screened for Hepatitis B Surface Antigen, Hepatitis B Core Antibody, Hepatitis C Virus Antibody, HIV 1/HIV 2 Antibody, HTLV I/II Antibody, RPR Qual, CMV Antibody, CMV by PCR, West Nile Virus, Chagas Disease, EBV Panel and HLA testing, within 30 days of signing the informed consent. Exception: patient does not need infectious disease panel and HLA testing repeated if it was done in Turnstile 1 on 2004-0069. An MRI/CT/PET scan of the brain will be performed within 6 months of signing the informed consent for Turnstile I. ECOG assessment will also be done within 30 days of signing informed consent.

The primary endpoint is to evaluate the safety and toxicity of CXCR2 and the secondary endpoint is to evaluate T-cell migration to tumor. TIL harvests will be obtained on patients to obtain 15 treated patients that will be required for the statistical analysis of the study. The samples will be placed in medium in the presence of IL-2. Tumor Infiltrating lymphocytes (TIL) will be transduced with CXCR2 and NGFR and expanded from the patients' tumor using standard, published techniques as described in the CMC.

While T-cells are being grown, patients may be treated with alternative therapies. However, patients must not have received other therapies for 28 days prior to lymphodepletion and T-cell infusion. If the subject is to receive fresh (not frozen and then thawed) TIL, the subject may not be treated with any alternative therapy.

Patients must have adequate TIL available. Pre-REP TIL generated in the similar trial 2004-0069 may also be utilized for Turnstile II.

4.2 Turnstile II - Treatment – Chemotherapy/Cell Infusion

Patients must sign informed consent document before Turnstile II screening procedures. Before the treatment starts and at each visit, the patient will be asked to complete two quality of life questionnaires. It should take about 15 minutes to complete the questionnaires. (FACT-G, FACT-Melanoma) Appendix D,E.
Patients must fulfill all of the following criteria to be eligible for Turnstile II of the study.

4.2.1 Chemotherapy/Cell Infusion Inclusion Criteria Turnstile II

a. Patients must have adequate TIL available
b. Patients must have at least one biopsiable measurable metastatic melanoma, lesion $\geq$ 1cm
c. Patients may have brain lesions which measure $\leq$ 1cm each. The PI or designee will approve the treatment.
d. Patients of both genders must practice birth control for four months after receiving the preparative regimen (lymphodepletion) and continue to practice birth control throughout the study. Patients must have a documented negative pregnancy test (urine or serum) for women who have menstruation in the past 12 months and without sterilization surgery.

e. Unless surgically sterile by bilateral tubal ligation or vasectomy of partner(s), the patient agrees to continue to use a barrier method of contraception throughout the study such as: condom, diaphragm, hormonal, IUD, or sponge plus spermicide. Abstinence is an acceptable form of birth control
f. Patients with a negative pregnancy test (urine or serum) must be documented within 14 days of screening for women of childbearing potential (WOCBP). A WOCBP has not undergone a hysterectomy or who has not been naturally postmenopausal for at least 12 consecutive months (i.e. who has not had menses at any time in the preceding 12 consecutive months).

g. Clinical performance status of ECOG 0-2 within 30 days of signing informed consent..
h. Absolute neutrophil count greater than or equal to 1000/m^{m3}.
i. Platelet count greater than or equal to 100,000/mm^3.
j. Hemoglobin greater than or equal to 8.0 g/dl.
k. Serum ALT less than three times the upper limit of normal.
l. Serum creatinine less than or equal to 1.6 mg/dl.
m. Total bilirubin less than or equal to 2.0 mg/dl, except in patients with Gilbert's Syndrome who must have a total bilirubin less than 3.0 mg/dl.
n. A stress cardiac test (stress thallium, stress MUGA, dobutamine echocardiogram or other stress test that will rule out cardiac ischemia) within 6 months of lymphodepletion.
o. Pulmonary function tests (FEV1>65% or FVC>65%of predicted) within 6 months of lymphodepletion.
p. MRI/CT/PET of the brain within 30 days of lymphodepletion

4.2.2 Chemotherapy/Cell Infusion Exclusion Criteria Turnstile II

a. Has had prior systemic cancer therapy within the past four weeks at the time of the start of the lymphodepletion regimen.
b. Women who are pregnant will be excluded because of the potentially dangerous effects of the preparative chemotherapy on the fetus.
c. Any active systemic infections requiring intravenous antibiotics, coagulation disorders or other major medical illnesses of the cardiovascular, respiratory or immune system, such as abnormal stress thallium or comparable test, myocardial infarction, cardiac arrhythmias, obstructive or restrictive pulmonary disease. PI or his designee shall make the final determination regarding appropriateness of enrollment.
d. Any form of primary or secondary immunodeficiency. Must have recovered immune competence after chemotherapy or radiation therapy as evidenced by lymphocyte counts (> 500/mm^3), WBC (> 3,000/mm^3) or absence of opportunistic infections.
e. Require steroid therapy or steroid-containing compounds, or have used systemic steroids in the past 4 weeks, or have used topical or inhalational steroids in the past 2 weeks prior to lymphodepletion; the exception being patients on chronic physiologic dose of steroid.
f. Presence of a significant psychiatric disease, which in the opinion of the principal investigator or his designee, would prevent adequate informed consent or render immunotherapy unsafe or contraindicated.

5.0 Pretreatment Evaluation Turnstile II

5.1 Lymphodepletion

a. Complete history and physical examination including vital signs, height, weight, noting in detail the exact size and location of any lesions that exist will be performed within 14 days prior to initiation of chemotherapy.
b. Clinical performance status of ECOG 0-2 within 14 days of lymphodepletion.
c. Chemistries to include BUN, creatinine, total bilirubin, alkaline phosphatase, LDH, ALT, sodium, potassium, chloride, and CO_2 will be performed within 14 days prior to lymphodepletion.
d. CBC, differential, PT/PTT, and platelet count will be performed within 14 days prior to initiation of chemotherapy.
e. Urinalysis (micro) within 14 days prior to initiation of chemotherapy.
f. β-HCG pregnancy test (urine or serum) on all women of childbearing potential will be performed within 14 days prior to lymphodepletion.
g. Baseline radiological studies to evaluate the status of disease must be obtained within 30 days prior to lymphodepletion (CXR, CT scan of chest, abdomen, and pelvis; MRI/CT of brain or a PET/CT may also be used).
h. EKG within 30 days prior to lymphodepletion.
i. A stress cardiac test (stress thallium, stress MUGA, dobutamine echocardiogram or other stress test that will rule out cardiac ischemia) within 6 months of lymphodepletion.
j. Pulmonary function tests (FEV1>65% or FVC>65%of predicted) within 6 months of lymphodepletion

6.0 Treatment Plan Turnstile II

6.1 Outline

- 15 patients will be treated (refer to Section 11.0 Statistical Considerations and Data Analysis). Patients will receive a T cells transduced with CXCR2 as well as T cells transduced with NGFR (as a control gene).
- T cells will be expanded in a state-of-the-art GMP facility that will allow compliance with all FDA regulations regarding investigational cell transfer products. We have demonstrated that we can successfully expand TIL on the majority of patients and have treated patients with up to 150 billion TIL.
- All patients will receive lymphodepleting chemotherapy with cytoxan and fludarabine to enhance T cell persistence and effectiveness *in vivo*. Cytoxan will be administered at 60 mg/kg/day I.V. in 250 ml NS over approximately 2 hours on Days –7 and –6. Mesna 60 mg/kg with D5W or NS at 125 ml/hr infused intravenously over 24 hours on Days -7 and -6. The dose will be based on the patient's body weight, but to prevent undue toxicity, it will not exceed a dose greater than 40% of the maximum ideal body weight for drug dosing using MD Anderson (MDACC) clinical calculator for Body Surface Area (BSA) and Body Mass Index (BMI). Fludarabine will then be infused at 25 mg/m^2 IVPB daily over approximately 15-30 minutes on Days –5 to –1.
- On day 0, all patients will receive up to 1.5x10^{11} T cells (including both CXCR2 and NGFR transduced TIL in one combined infusion) in NS, depending on the number that can be generated in the laboratory. It has been previously shown that infusion of cell numbers in this range is well tolerated. Depending on the amount of T-cells and the volume, TIL will be infused as an inpatient by I.V. for maximum infusion time of up to 4 hours, unless ordered otherwise by physician. Tylenol (acetaminophen) will be given by

mouth before the T-cell infusion to decrease the risk of these side effects. Twelve (12) to sixteen (16) hours after completing the T cell infusion, all patients will receive high dose interleukin-2 (IL-2) on an inpatient basis at the standard dose of 720,000 IU/kg as an intravenous bolus over an approximate 15 minute period every 8-16 hours for up to 15 doses on Days 1 to 5, as tolerated. Doses will be skipped if patients reach Grade III or IV toxicity due to high dose IL-2, except for the reversible Grade III toxicities common to high dose IL-2 such as diarrhea, nausea, vomiting, hypotension, skin changes, anorexia, mucositis, dysphagia, or constitutional symptoms and laboratory changes (i.e. platelets, creatinine, and total bilirubin) as detailed in Appendix **G**. If the toxicity is easily reversed by supportive measures, then additional doses may be continued.

- If the patient's platelet count is less than 80K on Day 21 (-2 days/+7 days), the second course of high dose IL-2 will be delayed until the platelet count is at least 80K. The second course of IL2 can be omitted at the discretion of PI and/or treating physician. For the evaluation of peripheral blood cells following T cell transfer, 70 ml blood samples in CPT or heparin tubes will be obtained prior to tumor harvest and/or excision, within 30 days of lymphodepletion, and again on Days6 (+/- three days), and day 21(+/- seven days)when feasible. These days were selected based on our previous studies at the National Cancer Institute. During high dose IL-2 administration (Days 1-5 and 22-26), peripheral blood mononuclear cell (PBMC) yields are low, possibly due to adhesion of immune cells to vasculature. Therefore, days 6(+/- three days)and day 21(+/- seven days)have been selected as the earliest feasible time points to obtain cells and evaluate the immediate effects of immunization. Additional time points to obtain PBMC are week 6(+/- 7 days) and week 12(+/- 7 days). To evaluate T cells at the tumor site, required tumor samples will be obtained by biopsy within 30 days of lymphodepletion and then again at approximately day 21 (+/- seven days) when feasible. Additional optional biopsies may be done at week 6(+/- 7 days) and week 12(+/- 7 days).
- The retrovirus vector is replication-defective, i.e. will not replicate after infection into the host cells. No new viral particles will be formed following infection into the TIL. The infection will be performed at the early stages of T-cell expansion. Non infecting vector will be washed away during the expansion and harvest process. The TIL for infusion will not contain any retrovirus vector; therefore there will be no danger to the nurses from the retrovirus vector. A negative pressure room will not be required; these transduced TIL will be administered in the same manner as non-transduced TIL.
- Feeder cells will be CMV negative if pre-treatment testing of the patient reveals absence of CMV seropositivity. If a patient is CMV positive at baseline, CMV positive feeder cells may be utilized. Pools of feeder cells from CMV seropositive patients will be tested by PCR to assess for the presence of CMV in the feeder cell population. Those feeder cells that have undetectable levels of CMV by PCR will be deemed eligible to be used as feeders in the TIL expansion process. We will monitor all patients receiving TIL with serum CMV pcr testing at day 3 (+/-24 hours) post TIL infusion and 3 weeks (+/-7 days) after TIL infusion. CMV pcr blood testing is drawn in a single 10mL purple top tube. The test will be performed at MDA through the molecular diagnostics lab. If there is evidence of CMV reactivation as evidenced by the CMV pcr, infectious disease specialist will be consulted and further management or therapy will be per expert recommendations.
- Administration of the lymphodepletion treatment and concomitant medications including supporting prophylactic medication will be documented in the medical record but will not be captured in the case report form for this protocol. TIL administration and IL-2 starting at Day 0 will be captured in the case report form.

- Variations from the lymphodepletion (e.g. infusion times; schedule of treatments, etc) prior to day 0 will be documented in the medical record but will not be considered protocol violations/deviations.

Treatment schema:

Turnstile 1

	Screening	Within 30 days of signing informed consent
Informed Consent	X	
CT Chest/abd/pelvis or PET[1]	X	
MRI brain[1]	X	
Donor Infectious Disease Panel[2]		X
HLA Testing		X
Pregnancy Test (serum or urine)	X	
Physical exam, Vitals, ECOG	X	
CBC with Differential	X	
Serum Chemistries[3]	X	
Research blood		X

1: CT chest/abdomen/pelvis or PET and MRI brain within 6 months of screening
2: Donor infectious disease panel includes: for Hepatitis B Surface Antigen, Hepatitis B Core Antibody, Hepatitis C Virus Antibody, HIV 1/HIV 2 Antibody, HTLV I/II Antibody, RPR Qual, CMV Antibody, CMV by PCR, West Nile Virus, Chagas Disease, EBV Panel
3: Serum chemistries: Albumin, alkaline phosphatase, total bilirubin, bicarbonate, BUN, calcium, chloride, creatinine, glucose, LDH, magnesium, phosphorus, potassium, total protein, AST, ALT, sodium
4. Pregnancy test (urine or serum) within 14 days of screening and within 14 days of lymphodepleting chemotherapy

Turnstile 2 Pretreatment Procedures

	Within 6 months of lymphodepleting chemotherapy	Within 30 days of lymphodepleting chemotherapy	Within 14 days of lymphodepleting chemotherapy
Pulmonary Function	X		

Tests			
Cardiac Stress Test	X		
Pregnancy Test (serum or urine)[4]			X
Physical exam, Vitals, ECOG			X
CBC with Differential			X
Serum Chemistries[3]			X
PT/PTT			X
Urinalysis			X
EKG		X	
CT Chest/abdomen/pelvis or PET		X	
MRI Brain/PET/CT		X	
Research Blood		X	
Tumor Biopsy		X	
Quality of Life Questionnaire		X	
RCR by PCR		X	

Turnstile 2 On-Treatment Procedures

	During Lymphodepletion and High Dose IL-2	**Day 6 (+/- 3 days)**	**Day 21 (+/-7 days)**	**Week 6 (+/- 7 days)**	**Week 12 (+/- 7 days)**	**Month 6, 9, 12**	**Yearly**
Physical exam	Daily			X	X	X	X
CBC with Differential	Every 1-2 days			X	X	X	X
Serum Chemistries	Every 1-2 days			X	X	X	X
CT Chest/abd/pelvis or PET				X	X	X	X
MRI brain/PET/CT				X	X	X	X
Research Blood		X	X	X	X		
Tumor Biopsy			X	Optional	Optional		
RCR PCR					X	Month 6, Month 12	X

Treatment:

Day -7 and –6:
Cyclophosphamide 60 mg/kg/day I.V. in 250 ml NS over approximately 2 hrs.
Mesna 60 mg/kg with D5W or NS at 125 ml/hr infused intravenously over 24 hours.

Day -5 to Day -1:
Fludarabine 25 mg/m^2 IVPB daily over approximately 15-30 minutes for 5 days.

Day 0:
Depending on the amount of T-cells and the volume, T-cells will be infused in NS with maximum infusion time of up to 4 hours, unless ordered otherwise by physician. Tylenol (acetaminophen) will be given by mouth before the T-cell infusion to decrease the risk of the side effects.

Days 1-5:
High dose IL-2, 720,000 IU/kg IV bolus (about 15 minutes) every 8-16 hours for up to 15 doses, beginning approximately 12-16 hours after T cell infusion.

Day 22-26 (-2 days/ + 7 days):
High dose IL-2, 720,000 IU/kg IV bolus (about 15 minutes) every 8-16 hours for up to 15 doses. If platelet count is greater or equal to 80K.

6.2 Prophylaxes

6.2.1 Infection Prevention and Pneumocystis carinii Pneumonia (PCP) Prophylaxis

Patients will receive Levaquin at 500 mg daily until ANC recovers to greater than 500/mm3 and the fixed combination of trimethoprim (TMP) and sulfamethoxazole (SMX) as double strength (DS) tablet [DS tabs = TMP 160 mg/tab and SMX 800 mg/tab] p.o. b.i.d. twice a week. TMP/SMX-DS will be taken by patients beginning on Day -7 and continuing for a minimum of 6 months post chemotherapy. For patients with sulfa allergies, Pentamidine will be given (once discharged from the hospital) 300 mg IV every 21 days for six months after lymphodepletion. If IV Pentamidine is not feasible after discharge, Pneumocystis Jirovecii Pneumonia (PCP) prophylaxis can be substituted with oral antimicrobials such as Atovaquone as per standard of care for 6 months after lymphodepletion.

6.2.2 Herpes Virus Prophylaxis

At the time of the T cell infusion, patients will be administered valtrex 500 mg p.o. daily for 6 months if the patient is able to take oral medications. If the patient needs intravenous medications give acyclovir 5 mg/kg IVPB every 8 hours, which is continued until absolute neutrophil count is greater than 1000/ml. Reversible renal insufficiency has been reported with IV administered acyclovir but not with oral acyclovir. Neurologic toxicity including delirium, tremors, coma, acute psychiatric disturbances, and abnormal EEGs has been reported with higher doses of acyclovir. If symptoms occur, a dosage adjustment will be made or the drug will be discontinued. Acyclovir will not be used concomitantly with other nucleoside analogs (e.g.

ganciclovir), which interfere with DNA synthesis. In patients with renal disease, the dose is adjusted as per product labeling. Valtrex/Acyclovir is to be continued for 6 months after lymphodepletion.

6.2.3 Fungal Prophylaxis

Patients will begin Fluconazole 200 mg p.o. daily with the T cell infusion (Day 0) and continue for 6 months after lymphodepletion.

6.2.4 Ondansetron hydrochloride (Zofran)
It will be used to control nausea and vomiting during the chemotherapy preparative regimen. It can cause headache, dizziness, myalgias, drowsiness, malaise, and weakness. Less common side effects include chest pain, hypotension, pruritis, constipation and urinary retention. Consult the package insert for a complete list of side effects and specific dose instructions.

6.2.5 Furosemide (Lasix)
It will be used to enhance urine output during the chemotherapy preparative regimen with cyclophosphamide. Adverse effects include dizziness, vertigo, paresthesias, weakness, orthostatic hypotension, photosensitivity, rash and pruritis. Consult the package insert for a complete list of side effects and specific dose instructions.

6.2.6 Empiric Antibiotics

Patients will start on broad-spectrum antibiotics, either a 3rd or 4th generation cephalsosporin or a quinolone for fevers $\geq$38.5 C with an ANC less than 500/mm^3. Aminoglycosides should be avoided unless clear evidence of sepsis. Infectious disease consultation will be obtained from all patients with unexplained fever or any infectious complications.

6.2.7 Blood Product Support

In order to reduce neutropenia following chemotherapy and T cell infusion, G-CSF will be given at 5 µg/kg/day daily subcutaneously until neutrophil counts reach >500/mm^3 Using daily CBC's as a guide, the patient will also receive platelets and packed red blood cells (PRBC's) as needed. Attempts will be made to keep Hb >8.0 gm/dl, and platelets >20,000/ml. Leukocyte filters will be utilized for all blood and platelet transfusions to decrease sensitization to transfused WBC's and decrease the risk of CMV infection. Irradiated blood and blood products should be used.

7.0 Evaluation During Study

- Before the treatment starts and at each visit, the patient will be asked to complete two quality of life questionnaires. It should take about 15 minutes to complete the questionnaires.
- During the preparative regimen and high dose IL-2 therapy, patients will have a complete blood count (CBC) and electrolytes every 1 to 2 days of treatment. Patients will be monitored with vital signs at baseline, every 15 minutes (+/- 10 minutes) during cell infusion and after cell infusion hourly (+/- 30 minutes) for 4 hours after the infusion.

- Phlebotomy will be performed prior to tumor harvest and/or excision, or within 30 days of lymphodepletion, and again on Day 6 (+/- three days) and day 21 (+/- seven days) after the cell infusion when feasible, and again at 6 week (+/- 7 days) and 12 week (+/- 7 days) after the cell infusion when feasible. These blood samples will be used to evaluate T cell persistence and function, using a variety of assays. To assess the secondary endpoint of T cell migration to the tumor, required tumor samples will be obtained within 30 days of lymphodepletion and then again at approximately day 21 (+/-7 days).Optional biopsies may be obtained at 6 weeks (+/- 7 days) and 12 weeks (+/- 7 days).
- The presence of CD4+ T-cells at the tumor site will also be evaluated. Patients" blood samples will be obtained and undergo analysis for detection of RCR by PCR prior to lymphodepletion and RCR PCR will be performed approximately at 3 and 6 months and at approximately one year post cell administration. RCR PCR assays detect the GaLV envelop gene and are performed by research staff in the Department of Medical and Molecular Genetics, Indiana University. Blood samples will be archived annually thereafter 10 years if all previous testing has been negative with a brief clinical history. If patients die or develop neoplasms during this trial, efforts will be made to assay a biopsy sample for RCR. If any post-treatment samples are positive, further analysis of the RCR and more extensive patient follow-up will be undertaken, in consultation with CBER. The results of these tests are maintained by the Department of Medical and Molecular Genetics and by the MD Anderson Cancer Center research team.
- Complete evaluation of evaluable lesions with physical examination and appropriate CT scans or PET/CT will be performed approximately at 6 weeks (+/- 7 days) and at 12 weeks (+/- 7 days) after the cell infusion, at which time patient will be restaged.
- After 12 weeks and for patients who have progression of disease at 6 weeks or at 12 weeks post cell infusion, if feasible, patients will be evaluated per Melanoma Standard of Care and follow-up visit tests and scans will be physician dependent.
- Tumor measurements will not be required for patients who have progression of disease.
- If patients are unable to return for follow-up visits, the patients will be followed for survival via phone or clinic visit approximately every 3 months for a year and then yearly for 10 years or until patient death, patient withdraws consent, patient is lost-to follow-up or the study is completed.
- At the time of the patient's death, no matter the cause, we may request permission for an autopsy in order to obtain vital information concerning the safety of this experimental therapy approach.

- We will send the patient two questionnaires (FACT-G, FACT-Melanoma)(Appendix D, E) yearly to get information regarding their quality of life for 15 years. For this reason, we will ask the patients to continue to provide us with a current address and telephone number, even after completion of this research study

Blood and tissue specimens collected in the course of this research project may be banked and provided in the future to investigators with IRB approved research protocols.

Re-Treatment

If patients have stable disease or a partial response to treatment, they may be re-treated after re-evaluation with the same schedule that they had been given safely previously [less than Grade III (except for common high dose IL-2 toxicities noted in Appendix) or Grade IV toxicity

easily reversible by standard measures]. Re-treatment benefits and risks will be carefully explained. A maximum of 1 re-treatment course may be given for each patient.

8.0 Evaluation of Toxicity

This study will utilize the National Cancer Institute Common Terminology Criteria (CTC) for Adverse Events version 4.0 for toxicity and Adverse Event reporting. A copy of the CTCAE version 4.0 can be found in Appendix A. All appropriate treatment areas should have access to a copy of the CTCAE version 4.0.

Careful evaluation to ascertain the toxicity, immunologic effects and anti-tumor efficacy of cell infusions will be performed. Toxicities will be monitored and documented on a daily basis beginning at day 0 (T-cell infusion) and continuing until discharged from the hospital following the initial IL-2 infusion. The criteria for stopping the study and halting enrollment and the administration of study drug will be as follows:

1. A Grade IV non-hematologic toxicity not explained by IL-2 infusion as outlined in Appendix G.
2. Death not due to disease progression within 3 months of the initiation of the conditioning regimen.

The principal investigator will monitor the data and toxicities to identify trends. The principal investigator will be responsible for revising the protocol as needed to maintain safety. The MD Anderson IRB will review serious adverse events as they are submitted. Serious adverse events will be submitted to the FDA by the IND Safety Project Manager in the IND Office. The principal investigator will also review serious adverse events and evaluate trends. Whenever a trend is identified, the principal investigator will determine an appropriate follow up plan. The investigator or physician designee is responsible for verifying and providing source documentation for all adverse events and assigning the attribution for each event for all subjects enrolled on the trial.
The use of the nonmyeloablative regimen in this protocol is a major procedure which entails serious discomforts and hazards for the patient. Although it is anticipated that this protocol is relatively safe because of the expected recovery of the patients' bone marrow within 2 to 4 weeks, fatal complications are possible. It is therefore only appropriate to carry out this experimental procedure in the context of life threatening metastatic cancer. The major hazards are infection and disease progression. The major discomforts are nausea, mucositis, anorexia, diarrhea, fever and malaise. Side effects of common drugs used in this nonmyeloablative regimen include:

Cyclophosphamide: Marrow suppression, nausea, mucositis, rash, hemorrhagic cystitis, myocardial damage, alopecia, infertility, nausea and vomiting, SIADH.

Fludarabine: Myelosuppression, fever and chills, nausea and vomiting, malaise, fatigue, anorexia, weakness, neurologic toxicity, and interstitial pneumonitis. Serious opportunistic infections have occurred in CLL patients treated with fludarabine.

Antimicrobials in general: Allergic reactions, renal impairment, nausea, vomiting, hepatic damage, marrow suppression.

High Dose IL-2: A variety of side effects have been associated with high-dose IL-2 administration in our experience at the NCI and a listing of these side effects in 652 patients who received 1,039 treatment courses are listed in Appendix G.

Serious Adverse Event Reporting (SAE)

- A serious adverse event is-any adverse drug experience occurring at any dose that results in any of the following outcomes:
  a. Death
  b. A life-threatening adverse drug experience – any adverse experience that places the patient, in view of the initial reporter, at immediate risk of death from the adverse experience as it occurred, it does not include an adverse experience that, had it occurred in a more severe form, might have caused death.
  c. Inpatient hospitalization or prolongation of existing hospitalization
  d. A persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions.
  e. a congenital anomaly/birth defect.

- Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered a serious adverse drug experience when,, based upon appropriate medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or development of drug dependency or drug abuse (21 CFR 312.32).

  a. Important medical events as defined above, may also be considered serious adverse events. Any important medical event can and should be reported as an SAE if deemed appropriate by the Principal Investigator or the IND Sponsor/ IND office.**,**
  b. All events occurring during the conduct of a protocol and meeting the definition of a SAE must be reported to the IRB in accordance with the timeframes and procedures outlined in "University of Texas M.D. Anderson Cancer Center Institutional Review Board Policy for Investigators on Reporting Serious Unanticipated Adverse Events for Drugs and Devices". Unless stated otherwise in the protocol, all SAEs, expected or unexpected, must be reported to the IND office regardless of attribution (within 5 working days of knowledge of the event).
  c. All life-threatening or fatal events**,** expected or unexpected, and regardless of attribution to the study drug, must have a written report submitted within 24 hours
  (next working day) of knowledge of the event to the Safety Project Manager in the IND office.
  d. The MDACC "Internal SAE Report Form for Prompt Reporting" will be used for reporting to the IND office.
  e. Unless otherwise noted, the electronic SAE application (eSAE) will be utilized for safety reporting to the IND office and MDACC IRB.

f. Serious adverse events will be captured from the time the patient signs consent until 30 days after the last dose of drug unless the participant withdraws consent. Serious adverse events must be followed until clinical recovery is complete and laboratory tests have returned to baseline, progression of the event has stabilized, or there has been acceptable resolution of the event.
g. Additionally, any serious adverse events that occur after the 30 day time period that are related to the study treatment must be report to the IND office. This may include the development of a secondary malignancy.
h. The adverse events will be recorded in CORe according to the following guidelines and all other information will be entered into PDMS.

Recommended Adverse Event Recording Guidelines						
Attribution	**Grade 1**	**Grade 2**	**Grade 3**		**Grade 4**	**Grade 5**
Unrelated	Phase I	Phase I	Phase I Phase II		Phase I Phase II Phase III	Phase I Phase II Phase III
Unlikely	Phase I	Phase I	Phase I Phase II		Phase I Phase II Phase III	Phase I Phase II Phase III
Possible	Phase I Phase II	Phase I Phase II Phase III	Phase I Phase II Phase III		Phase I Phase II Phase III	Phase I Phase II Phase III
Probable	Phase I Phase II	Phase I Phase II Phase III	Phase I Phase II Phase III		Phase I Phase II Phase III	Phase I Phase II Phase III
Definitive	Phase I Phase II	Phase I Phase II Phase III	Phase I Phase II Phase III		Phase I Phase II Phase III	Phase I Phase II Phase III

<u>Exceptions to this rule will include events and laboratory abnormalities that represent common symptoms and abnormalities of melanoma and chemotherapy and/or have no clinical significance:</u>

- Abnormalities in hematologic parameters due to myelosuppressive therapeutic effect:

  i. Anemia, neutropenia, lymphopenia, thrombocytopenia
  ii. Epistaxis or bleeding except for catastrophic CNS or pulmonary hemorrhage

- Common symptoms of cancer (unless grade >/= 3) including:

  i. Fatigue
  ii. Weakness
  iii. Bone, joint or muscle pain

iv. Alopecia
v. Loss of appetite, Nausea, Vomiting
vi. Chemistry abnormalities (phosphorus, calcium, glucose)
vii. Coagulation abnormalities (shortened PT, PTT, increased fibrinogen)

- Laboratory abnormalities:

i. LDH (increased or decreased)
ii. Alkaline phosphatase (increased or decreased)
iii. Low levels of the following: AST, ALT, creatinine, BUN, uric acid, bilirubin, albumin, total protein
iv. Electrolyte abnormalities (sodium, potassium, bicarbonate, CO2, magnesium

- General therapy related events

i. Catheter related events
ii. Rash related to antibiotic use

Abnormal laboratory test results will be captured if intervention is required.

- Reporting to FDA

a. Serious adverse events will be forwarded to FDA by the IND Sponsor (Safety Project Manager, IND office according to 21 CFR 312.32.
b. The gene therapy reporting addendum ("Additional Reporting Form for Serious Adverse Events on Gene Therapy Trials") must be included with each SAE submitted.

- It is the responsibility of the PI and the research team to ensure serious adverse events are reported according to the Code of Federal Regulations, Good Clinical Practices, the protocol guidelines, and Institutional Review Board policy**.**

Careful evaluation to ascertain the toxicity, immunologic effects and anti- tumor efficacy of therapy will be performed.

9.0 Criteria for Response

Tumor response to therapy in this study will be done using *immune-related response criteria (irRC)* which is a modified version of WHO criteria.

9.1 Definition of Measureable and Non-Measurable Lesions

Measurable Lesions are lesions that can be accurately measured in two perpendicular diameters, with at least one diameter ≥ 10 mm. The area will be defined as the product of the largest diameter with its perpendicular. Skin lesions can be considered measurable. Cutaneous lesions that are ≥ 5 mm in diameter can be considered measurable.

Non-Measurable (evaluable) Lesions are all other lesions, including unidimensionally measurable disease and small lesions.

Definition of Index/Non-Index Lesions

All measurable lesions, up to a maximum of five lesions per organ and ten lesions in total, should be identified as *index* lesions to be measured and recorded on the medical record at baseline. The *index* lesions should be representative of all involved organs. In addition, *index* lesions should be selected based on their size (lesions with the longest diameters), their suitability for accurate repeat assessment by imaging techniques, and how representative they are of the patient's tumor burden. A sum of the products of diameters (SPD) for all *index* lesions will be calculated and considered the baseline sum of the products of diameters. Response criteria to be followed are listed below. The baseline sum will be used as the reference point to determine the objective tumor response of the *index* lesions at tumor assessment (TA). Measurable lesions, other than *index* and all sites of non-measurable disease will be identified as *non-index* lesions. *Non-index* lesions will be recorded on the medical record and should be evaluated at the same assessment time points as the *index* lesions. In subsequent assessments, *non-index* lesions will be recorded as "stable or decreased disease," "absent", or "progression."

9.2 Definition of Tumor Response Using irRC

The sum of the products of diameters at tumor assessment using the immune-related response criteria (irRC) for progressive disease incorporates the contribution of new measurable lesions. Each net Percentage Change in Tumor Burden per assessment using irRC criteria accounts for the size and growth kinetics of both old and new lesions as they appear.

Definition of Index Lesions Response Using irRC

irComplete Response (irCR): Complete disappearance of all *index* lesions. This category encompasses exactly the same subjects as "CR" by the mWHO criteria.

irPartial Response (irPR): Decrease, relative to baseline, of 50% or greater in the sum of the products of the two largest perpendicular diameters of all *index*

and all new measurable lesions (i.e., Percentage Change in Tumor Burden). Note: the appearance of new measurable lesions is factored into the overall tumor burden, but does not automatically qualify as progressive disease until the SPD increases by ≥25% when compared to SPD at nadir.

irStable Disease (irSD): Does not meet criteria for irCR or irPR, in the absence of progressive disease.

irProgressive Disease (irPD): At least 25% increase Percentage Change in Tumor Burden (i.e., taking sum of the products of all *index* lesions and any new lesions) when compared to SPD at nadir.

Definition of Non-Index Lesions Response Using irRC

irComplete Response (irCR): Complete disappearance of all *non-index* lesions. This category encompasses exactly the same subjects as "CR" by the mWHO criteria

irPartial Response (irPR) or irStable Disease (irSD): *non-index* lesions are not considered in the definition of PR, these terms do not apply.
irProgressive Disease (irPD): Increases in number or size of *non-index* lesions does not constitute progressive disease unless/until the Percentage Change in Tumor Burden increases by 25% (i.e., the SPD at nadir of the index lesions increases by the required amount).

Impact of New Lesions on irRC
New lesions in and by themselves do not qualify as progressive disease. However, their contribution to total tumor burden in included in the SPD which in turn feeds into the irRC criteria for tumor response. Therefore, new non-measurable lesions will not discontinue any subject from the study.

9.3 Definition of Overall Response Using irRC

Overall response using irRC will be based on these criteria:

Immune-Related Complete Response (irCR): Complete disappearance of all tumor lesions (index and non-index together with no new measurable/unmeasurable lesions) for at least 4 weeks from the date of documentation of complete response.

Immune-Related Partial Response (irPR): The sum of the products of the two largest perpendicular diameters of all index lesions is measured and captured as the SPD baseline. At each subsequent tumor assessment, the sum of the products of the two largest perpendicular diameters of all index lesions and of new measurable lesions are added together to provide the Immune Response Sum of Product Diameters (irSPD). A decrease, relative to baseline of the irSPD compared to the previous SPD baseline, of 50% or greater is considered an immune Partial Response (irPR).

Immune-Related Stable Disease (irSD): irSD is defined as the failure to meet criteria for immune complete response or immune partial response, in the absence of progressive disease.

Immune-Related Progressive Disease (irPD): It is recommended in difficult cases to confirm PD by serial imaging. Any of the following will constitute progressive disease:

- At least 25% increase in the sum of the products of all index lesions over baseline SPD calculated for the index lesions.
- At least a 25% increase in the sum of the products of all index lesions and new measurable lesions (irSPD) over the baseline SPD calculated for the index lesion.

Immune-Related Response Criteria Definitions

Index Lesion Definition	Non-Index Lesion Definition	New Measurable Lesions	New Unmeasurable Lesions	Percent Change in Tumor Burden	Overall irC Response
Complete Response	Complete Response	No	No	-100%	irCR
Partial Response	Any	Any	Any	≥-50% <50%to<+25% >+25%	irPR irSD irPD
Stable Disease	Any	Any	Any	<50%tp<+25% >+25%	irSD irPD
Progressive Disease	Any	Any	Any	≥+25%	irPD

9.4 Immune-Related Best Overall Response Using irRC (irBOR)

irBOR is the best confirmed irRC overall response over the study as a whole, recorded between the date of first dose until the last tumor assessment before subsequent therapy (except for local palliative radiotherapy for painful bone lesions) for the individual subjects in the study. For assessment of irBOR, all available assessments per subject are considered.

If a lesion is surgically resected or treated with definitive radiosurgery, the size of the lesion prior to the definitive local therapy will be included in the calculated irBOR.

10.0 Criteria for Removal from the Study

Patients will be taken off the study if: (a) the patient voluntarily withdraws, (b) there is significant noncompliance, or (c) there is progression of disease in those patients after being treated by

lymphodepletion, T-cell infusion, and IL-2 in Turnstile II. Any patient who develops Grade IV toxicity due to cell infusion will be taken off protocol.

11.0 Statistical Considerations and Data Analysis

This is a pilot study to assess the feasibility and safety of producing sufficient quantities of CXCR2 and NGFR transduced tumor infiltrating lymphocytes (TILs) for treatment of metastatic melanoma. A total of 15 patients will be treated. Clinical outcomes that will be observed are tumor response by imaging, and possible grade 3 or worse toxicities not normally associated with lymphodepletion and high dose IL-2.
Based on our current experience with generation of gene modified TILs, we are not able to adequately fulfill the parameters for a phase I study due to technical challenges with generating enough T cells to allow for dose escalation. In our 3 patients treated thus far, we have not been able to reach the nominal dose level 2 due to the inherent variability in quantities of T cells able to be generated for individual patients. Thus, this trial is being redesigned as a feasibility study with the primary objectives to assess the feasibility of generating TILs and safety of and treating patients with genetically modified T cells.

Secondary objectives include response, defined following immune-related response criteria. Immunological endpoints include number of CXCR2 transduced cells and number of NGFR transduced cells at infusion, number of CXCR2 transduced cells and number of NGFR transduced cells based on tumor biopsy, and the amounts of CXCR2 and CXCL8 cytokines. Regression analysis will be used to estimate the ability of the genetically transduced T-cells to differentially recognize the cancer cell cytokines. Logistic regression will be used to assess the effects of immunological variables on the probability of clinical response. A sample size of 15 patients was determined primarily by resource limitations.

12.0 Statistical Considerations. A clinical response/safety summary will be submitted to the IND medical monitor after the first five participants are treated and every five thereafter.

12.1 Outcomes. The primary clinical endpoint will be response, defined following immune-related response criteria as a 50% or greater decrease in the tumor's linear dimension post treatment, compared to baseline, evaluated by PET or CT imaging at week 6, week 12 and then every 3 month intervals for up to one year of follow up.

Immunological endpoints will include (i) at infusion, Xc = number of CXCR2 transduced cells and Xs = number of NGFR transduced (control) cells, (ii) post treatment, based on tumor biopsy, Yc = number of CXCR2 transduced cells and Ys = number of NGFR transduced cells, and (iii) post treatment, Z_{CXCR2} = amount of CXCR2 cytokine and Z_{CXCR8} = amount of CXCL8 (IL-8) cytokine.

12.2. Estimation of Immunological Effects. Denote the pre-treatment and post-treatment ratios by $R_X = Xc/Xs$ and $R_Y = Yc/Ys$. In order to test the hypothesis that the CXCR2 cells are more likely to find the tumor than the NGFR (control) cells, the linear regression model $R_Y = b_0 + b_1 R_X$ + error will be fit to the (R_X , R_Y) pairs and the hypothesis H_0: $b_1 = 1$ will be tested against the alternative H_A: $b_1 > 1$. [1] The alternative H_A says that the CXCR2 cells are more likely to enter and be present in the tumor than the control cells. This is a statistical version of the intuitive idea that, if the TIL methodology works as designed and the genetically transduced T-

cells differentially recognize the cancer cell cytokines, then the ratio of post- and pre-treatment ratios RR = R_Y/R_X should be larger than 1.

12.3. Estimation of Clinical Response Rate. Additional regression analyses will include logistic regression to assess the effects of Yc, R_Y and RR on the probability of clinical response, as well as regression fits of Z_{CXCR2} and Z_{CXCR8} as functions of Yc, R_Y and RR.

12.4. Sample Size. A total of 15 patients will be treated as determined primarily by resource limitations.

13.0 Data Entry and Protocol Management

For the purposes of this study at M. D. Anderson Cancer Center, the Protocol Data Management System (PDMS) will be employed. All patients will be registered in CORe utilizing a two-turnstile registration before any study specific tests are performed. The continual reassessment method (CRM) used in phase I will be implemented using the Biostatistics Department Clinical Trial Conduct Website.

14.0 Administrative Procedures

14.1 Changes to the Protocol:

Any change or addition to this protocol requires a written protocol amendment that must be approved by the IND Office and the IRB. A copy of the written approval of the IRB must be received by the IND Office and the principal investigator before implementation of any changes. The IRB must review and approve all amendments to the protocol.

14.2 Ethics and Good Clinical Practice:

This study must be carried out in compliance with the protocol and Good Clinical Practice, as described in:

14.2.1 ICH Harmonized Tripartite Guidelines for Good Clinical Practice 1996.

14.2.2 US 21 Code of Federal Regulations dealing with clinical studies (including parts 50 and 56 concerning informed consent and IRB regulations).

14.2.3 Declaration of Helsinki, concerning medical research in humans (Recommendations Guiding Physicians in Biomedical Research Involving Human Subjects, Helsinki 1964, amended Tokyo 1975, Venice 1983, Hong Kong 1989, Somerset West 1996).

The investigator agrees, when signing the protocol, to adhere to the instructions and procedures described in it and thereby to adhere to the principles of Good Clinical Practice.

15.0 References

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