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Immunotherapy Program
Breast Oncology Program
Barbara Ann Karmanos Cancer Institute

Protocol # 2009-085 A Phase II Study of anti-CD3 x anti-HER2/*neu* Armed Activated T Cells for Patients with HER2/*neu* (0, 1+ or 2+) Metastatic Breast Cancers.

IND 9985

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SUMMARY

Patients with locally advanced, unresectable and metastatic breast cancer (MBC) , having HER2/*neu* amplification of 0-2+ [HER2 (0-2+)], will be entered into a phase II trial to evaluate whether infusions of HER2Bi-armed activated T cells (aATC) anytime after developing metastatic disease will improve progression free survival beyond previously published clinical trials. This is supported by the observations that our immune evaluation studies clearly show that even Her2 negative, 1+, and 2+ patients have been induced to produce cytotoxic responses to breast cancer cell lines and that armed ATC infusions induced a partial clinical response in a Her2/neu negative patient. Prior to chemotherapy (chemoT), peripheral blood mononuclear cells will be collected using 1 leukapheresis, activated with anti-CD3 and expanded in IL-2 to generate armed ATC. After culture, ATC will be harvested, armed with HER2Bi, washed, and cryopreserved in aliquots for subsequent infusions. After leukapheresis, patients will receive four cycles or 4 months worth of chemoT (primary oncologist choice). Patients who are rapidly progressing through chemoT may receive additional chemoT/hormonal therapy to make an attempt to control disease prior to immunotherapy. Following chemoT, **patients will be restaged and evaluated for chemo-related toxicities, and then given immunotherapy.** We have selected a dose of 20 billion HER2Bi-armed ATC per infusion (a total dose of 80 billion HER2Bi-armed ATC) based on immune and clinical responses in the phase I. Armed ATC will be thawed and infused once a week for 3 weeks, for 3 doses, and then a boost of 20 billion armed ATC 12 weeks after the 3rd infusion. Low dose granulocyte-macrophage colony stimulating factor (GM-CSF, 250 μg/m^2/twice per week) will start 3 days before the first aATC infusion and end with the third dose of aATC.
A total of 26 evaluable patients will be accrued in this study. Patients must receive both chemotherapy and at least 3 armed ATC infusions to be considered evaluable. Patients who are not evaluable will be replaced. We expect that the additional armed–ATC treatment given after traditional chemotherapy will improve the median progression free survival (PFS) by 2 months (estimated 4-month PFS is 50%). In the one-stage phase II design, we will test a 4-month ***PFS*** ≤25% versus a 4-month ***PFS*** ≥ 50%. In addition to progression-free (primary) and overall survival (secondary) endpoints, functional and phenotypic changes in immune cell populations, in blood and tumor samples from treated patients as a function of immunotherapy, will be evaluated before and after treatment.

1.0 OBJECTIVES

1.1 To determine in a phase II trial whether Her2Bi armed ATC infused after ChemoT for patients with HER2 0-2+ MBC or locally advanced, unresectable breast cancer would improve median PFS by 2 months beyond the median PFS of 2 months estimated from published trials in a one stage design.

1.2 To determine the overall survival (OS) of patients with HER2 0-2+ MBC and locally advanced, unresectable breast cancer who receive aATC infusion after ChemoT.

1.3 To confirm the toxicity profile for Her2Bi armed ATC given after ChemoT for patients with HER2 0-2+ MBC.

1.4 To measure functional and phenotypic changes in immune cell populations (blood and tumor sites, if accessible) as a consequence of armed ATC (tumor biopsies done at KCI only). Cytokine responses, phenotypic markers of differentiation, and anti-tumor cytotoxicity will be examined.

1.5 To develop correlates between the systemic or tumor site immune responses monitored in objective 1.3 and clinical outcomes in objective 1.1.

1.6 To collect correlative data using exploratory ^{18}F-FDG PET/CT imaging studies in select patients at KCI only.

2.0 BACKGROUND AND RATIONALE

2.1 Overall Rationale for Treatment Strategy. Metastatic Breast Cancer **(**MBC) represents a significant clinical challenge. About 20-85% of the patients who present with localized breast cancers will progress to MBC in 5 years and approximately 6-10% of the patients present with metastatic disease. Once depending on the stage at metastasis is detected the median survival is in the range of 18-24 months.[1] Because unresectable locally advanced breast cancer is not curable, patients with this diagnosis have a similar survival to patients with metastatic disease. Both chemotherapy and hormonal therapy have been used to treat (MBC) and unresectable locally advanced breast cancer with different levels of success. Most patients experience objective responses associated with palliation of symptoms,[2-4] , but complete responses (CR) are uncommon and short-lived. Initial responses may last between 8 and 14 months[5-7] , however, progression of disease is inevitable, and durable responses to subsequent therapies are progressively fewer. For the 20-25% of women with MBC who are HER2/*neu* (HER2) 3+, Herceptin® has become a mainstay of treatment in combination with chemotherapy. New treatment strategies, however, are needed to prolong progression-free and overall survival in the 75-80% of the MBC patients who are HER2 0-2+ and thus, not eligible for Herceptin®. In our phase I clinical trial, we have administered anti-CD3 activated T cells (ATC) armed with anti-CD3 x anti-HER2/neu bispecific antibody (HER2Bi) to women with both HER2-positive and HER2-negative MBC to determine their safety and the maximum tolerated dose. Arming ATC with HER2Bi makes every T cell into a HER2-specific cytotoxic T lymphocyte (CTL) with the potential to induce high levels of specific cytotoxicity that is independent of HER2 receptor-mediated mechanisms and thus, HER2 expression levels[8]. Indeed, evaluation of immune responses in our phase I clinical trial patients suggests that infusions of Her2Bi-armed ATC induce robust immune responses regardless of the patient's HER2 status. Consistent with this observation, log rank analyses comparing Kaplan-Meier estimates of PFS and OS between HER2(3+) patients (n =7) and HER2(0-2+) patients (n = 7) enrolled in the phase I trial found no significant difference between the stratified groups. In the HER2(3+) group, median overall survival was 21 months, but did not differ significantly ($p = 0.98$) from the OS curve of the HER2(0-2+) group for which median OS remained undefined (> 50% of patients still survive) strongly suggesting that armed ATC may prolong survival independent of HER2/neu status. From a mechanistic standpoint, this is not surprising. For example, unlike monoclonal antibody therapy with Herceptin®, which induces cellular death through interference with receptor-mediated pathways and is thus, more effective in cells heavily reliant on those signaling pathways, low levels of receptors may be sufficient to redirect enough armed ATC to induce T cell mediated mechanisms of cellular death. A subset of clinical situations thus exists where tumors of MBC patients with low level HER2 expression/amplification—a characteristic that would preclude therapy with Herceptin®—may still possess enhanced susceptibility to the directed cytotoxicity of HER2Bi armed-ATC.

2.2 Chemotherapy for treatment of metastatic breast cancer: The efficacy of the first-line chemotherapy in MBC is established by the results of numerous clinical trials and retrospective studies. Anthracyclines and taxanes containing regimens administered as first line chemotherapy have demonstrated their capabilities to prolong patient's survival.[9-11] The role of subsequent line chemotherapy in prolonging survival is variable and controversial. In 2002, Cardoso et al reviewed 8 phase three randomized trials of second and subsequent lines chemotherapy both single agent and doublets, with a time to progression (TTP) ranging from 3 months to 6.3 months, five of them had patients with anthracycline resistant disease[1]. Dufresne et al retrospectively evaluated the benefit of

second line chemotherapy in 505 patients with MBC in four French cancer centers between 1992 and 2002. He showed an average time to progression of 5.9 months[12]. O'Shaunghnessy et al reported in a phase III trial a TTP of 6.1 months with capcitabine and Docetaxel in MBC patients pretreated with anthracycline (about 40% of which was in the adjuvant setting).[13] Keller et al reported a randomized phase III trial of pegylated liposomal doxorubicin in patients who had progressed on both anthracyclines and taxanes a TTP of 5.8 months.[5] Martin et al reported a median PFS of 6 months in a randomized phase III trial comparing vinorelbine alone with gemcitabine and vinorelbine with no significant improvement in the OS between the two arms.[14] **In summary, regardless of the chemoregimen used for second line chemotherapy, the average median PFS is about 6 months from the time of starting second line chemotherapy. Therefore, strategies to prolong PFS both in the first line and later line settings is needed to improve quality of life among MBC patients. Additionally, since MBC is not yet curable new therapies to prolong OS are also needed.**

Table 1: Patient Characteristics

	No.	%
Age		
< 50	11	58
≥ 50	8	42
Cancer Stage		
Stage IV	19	100
Performance Status (ECOG)		
0	16	84
1	3	16
2	0	0
ER/PR Status		
Positive	12	63.1
Negative	6	31.5
Unk	1	5.2
HER2/neu Status		
0	8	42.1
1+	1	5.2
2+	2	10.5
3+	7	36.8
Unk	1	5.2
Prior Treatment w/ Herceptin		
Yes	7	36.8
No	12	63.2

2.3 HER2/*neu*Bi-armed ATC Therapy for Metastatic Breast Cancer.

2.3.1 Pre-Clinical Studies: Targeting Cancer Cells with Her2Bi-armed ATC

In preclinical studies, we have shown that HER2Bi, created by heteroconjugating anti-HER2 (Herceptin®; purchased from Genentech) to anti-CD3 (OKT3; purchased from Orthobiotech) can be used to arm ATC that have been cultured for 8-16 days. Specific cytolytic activity of ATC armed with HER2Bi (50 ng/10^6 armed ATC; dose-titration optimized) has been demonstrated against HER2-expressing cell lines derived from breast cancers (SK-BR-3, MCF-7); pancreatic cancers (MIA PaCa-2, COLO 356/FG); and prostate cancers (LNCaP, DU 145, PC-3).[15]

At effector:target ratios (E/T) from 3 to 50, both HER2Bi-armed normal and patient armed ATC are significantly more cytotoxic against HER2/neu expressing cells over armed ATC, anti-HER2, anti-CD3 alone, or ATC armed with irrelevant BiAb directed at CD20 (a B-cell antigen). HER2Bi-armed ATC also secrete significantly higher levels of some T_{H1}/T_{H2} cytokines compared to ATC alone. In mice, intravenous infusions of HER2Bi-armed ATC significantly delay growth of established PC-3 tumors compared to mice that receive ATC alone or vehicle ($p < 0.001$) without inducing toxicities.[15] in studies evaluating the long-term activity and fate of HER2Bi-armed ATC, expansion and division of HER2Bi-armed ATC versus unarmed ATC have been determined by comparing survival and ability of cells to divide and kill target cells when repeatedly exposed in vitro to SK-BR-3 cells over 336 hours of culture. Up to 2 weeks after a single arming, ex vivo expanded HER2Bi-armed ATC cocultured with SK-BR-3 targets increase in number, undergo multiple cell divisions, mediate specific cytotoxic activity, and secrete both cytokines and chemokines without undergoing Fas/FasL-induced apoptosis or activation-induced cell death.[16]

Interestingly, we have observed that HER2Bi armed ATC enhance cytotoxicity against MCF-7 cells, a cell line expressing very low levels of HER2 (fewer than 1.0×10^5 receptors/cell) that are under the limits of detection by standard immunohistochemistry, over the cytotoxicity of ATC alone. It

is most likely that arming ATC with HER2Bi enhances cytotoxicity by promoting targeting of MCF-7 cells even though they have very low levels of HER-2/*neu* expression. Unlike monoclonal antibody therapy with Herceptin®, which induces cellular death through interference with receptor-mediated pathways and would be more effective in cells heavily reliant on those signaling pathways, low levels of receptors may be sufficient to redirect enough armed ATC to induce target cell death. A subset of clinical situations thus exists where tumors of MBC patients with low level HER2 expression/amplification—a characteristic that would preclude therapy with Herceptin®—may still possess enhanced susceptibility to the directed cytotoxicity of HER2Bi armed-ATC.

Table 2: Dose Escalation Schedule

Dose Level	Her2Bi-armed ATC[a] (# cells/infusion)	IL-2[b] (IU/m²/day)	GM-CSF[c] (µg/m² twice/wk)	Patients	
				No.	%
1	5×10^9	300,000	250	6	31.5
2	10×10^9 *	300,000	250	4	21.0
3	20×10^9	300,000	250	8	42.1
4	40×10^9	300,000	250	1	5.2

[a] Administered IV twice/week for 4 weeks; [b] Administered SQ beginning 3 days prior to armed ATC and ending 7 days after the last infusion; [c] Administered SQ beginning 3 days prior to armed ATC and ending 7 days after the last infusion
* Dose level selected for Phase II trial

Table 3: Phenotyping and Viability

Dose Level	%CD3	%CD4	%CD8	Viability
1	97	90	8	95%
1	61	42	18	90%
1	95	50	45	96%
1	93	62	32	94%
1	98	50	48	97%
1	93	61	32	94%
2	96	37	60	91%
2	97	62	37	95%
2	63	46	21	87%
3	95	74	21	95%
3	87	34	55	93%
3	80	55	24	91%
3	57	31	35	92%
3	94	44	37	93%
3	92	40	60	72%
3	91	63	25	98%
4	85	50	31	89%
4	ND	ND	ND	98%
Average (%)	86.7 ± 13.5	52.4 ± 15.2	34.6 ± 15.0	92.2 ± 5.9

2.3.2 Clinical Progress Report: Preliminary Results from the Phase I Clinical Trial using Her2Bi-armed ATC to Target MBC

2.3.2.1 Patients and Treatment Schema. We performed a phase I dose escalation clinical trial in women with stable HER2+ or HER2- MBC to determine the maximum tolerated dose (MTD) for Her2Bi-armed ATC given in combination with IL-2 and GM-CSF. **Table 1** shows the characteristics of 16 women enrolled in this trial. Median age of enrolled patients was 50 years (range: 31-68 years). All 16 patients were enrolled onto this study at Roger Williams Medical Center from September 2002 to June 2006. Patients were leukapheresed to obtain T cells that were activated with anti-CD3 and expanded for 14 days in culture. Activated T cells (ATC) were harvested, armed with HER2Bi, cryopreserved, and infused in 8 divided doses. Patients were accrued to each dose level (**Table 2**) with escalation based on the criteria that grade 3 non-hematological toxicity had not occurred in 3 of 3 patients or 5 of 6 patients enrolled at each given dose level.

2.3.2.2 Characteristics and Activity of Patients' Expanded Products.

Patients' leukopheresis products were activated with a single dose of anti-CD3 (20 ng/ml) and their ATC were expanded in RPMI culture medium supplemented with 2% human serum in the presence of IL-2 (100-500 IU/ml) for 14-16 days. Cells were maintained at a concentration of 1×10^6 ATC/ml by adding supplemented medium as required. Cells were harvested and concentrated prior to arming with HER2Bi and cryopreserving to await quality control (QC) testing and clinical release. Phenotyping of expansion products was performed by double staining cells with fluorescence-labeled monoclonal antibodies and analyzing by flow cytometry. Activity of ATC products before and after arming with HER2Bi was evaluated by labeling HER2/neu-positive breast adenocarcinoma cells, SKBR-3, with ^{51}Chromium (^{51}Cr), incubating the effector ATC population overnight with the target

cells, and then quantitating ^{51}Cr-release as an indicator of target cell lysis. Average cytotoxicity (±SD) induced by patients' HER2Bi-armed ATC (60.3% ± 20.2) was significantly greater ($p < 0.0001$) against target cells compared to average cytotoxicity of patients' unarmed ATC (3.0 ± 2.8). **Table 3** shows the percentages of each T cell subpopulation comprising the expansion products of each patient enrolled in this study and the % cytotoxicity induced against SKBR-3 by patients' unarmed or HER2Bi-armed ATC. A significant, inverse correlation was found between cytotoxicity of patients' HER2Bi-ATC and the % CD4 cells comprising their expansion products (Spearman r = -0.55 with a two-tailed p – 0.03).

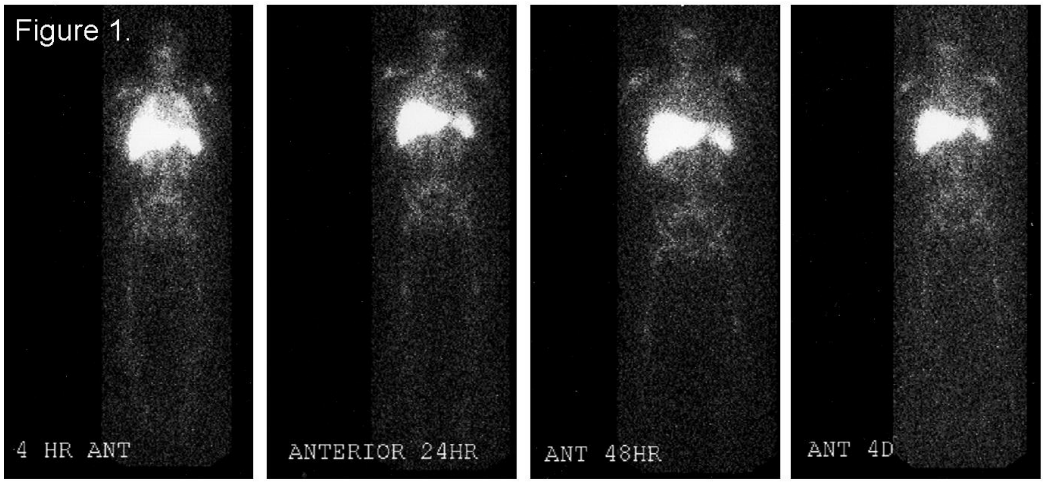
2.3.2.3 Evaluation of MTD and Toxicities. The primary endpoint of this study was to determine the MTD of HER2Bi-armed ATC and to define the toxicity profile at the MTD. The MTD is defined as the dose below the dose at which dose limiting toxicity occurred in 2 of 6 patients. Currently, the highest dose level completed is the 20 x 10^9 Her2Bi-armed ATC per infusion (160 x 10^9 total dose of armed ATC); the MTD has not yet been reached. We are now accruing at the dose level of 40 x 10^9 Her2Bi-armed ATC per infusion (320 x 10^9 total dose of armed ATC). The most frequent side effect experienced by patients receiving Her2Bi-armed ATC infusions was Grade 3 chills (68.8% of all enrolled patients). Grade 3 headache emerged as the second most common side effect (62.5% of all patients). Fewer than 50% of the total patients enrolled experienced each of the remaining symptoms. **Table 4** shows the frequency of toxic side effects in the study as a function of dose level based upon the NCI Immunotherapy Protocol Toxicity Table. By episode per infusion, incidence of chills and headache at dose level 1 (8.6% and 3.1%, respectively) increased for dose level 2 (20.8% and 8.3%, respectively) and then again at dose level 3 (43.1% and 19.6%, respectively). All patients with grade 3 chills responded to intervention with Demerol and Benadryl. Of the patients experiencing headache as a side effect, one patient experienced headache that failed to respond to interventional therapy (grade 4); this patient also experienced grade 4 hypertension. No fatalities occurred as a result of receiving Her2Bi-armed ATC infusions; however, the aforementioned patient at dose level 3 who experienced grade 4 hypertension and headache was removed from the study after receiving only 3 infusions (65.7 x 10^9 total armed ATC administered). This patient was initially suspected of developing progressive brain metastases based upon diagnostic imaging, but upon surgical follow-up was found to have a right frontal subdural hematoma. Because this condition could not be completely ruled-out as occurring in association with Her2Bi-armed ATC infusions, an adverse event was documented and this patient was removed from the protocol. Accordingly, three additional patients were added to dose level 3; none experienced persistent grade 3 toxicities and thus, we are now enrolling at dose level 4.

2.3.2.4 Dose Modifications. Our dose modification plan for dealing with treatment-related toxicities is as follows: all patients with treatment related, grade 4 non-hematologic toxicity are removed from protocol. Reversible cardiac toxicity associated with Herceptin® treatment has been reported to occur in as high as 28% of treated patients,[17] therefore, if the left ventricular ejection fraction (LVEF) falls by more than 20% from the previous value (by MUGA) following HER2Bi armed-ATC, further therapy is withheld and the patient is removed from protocol treatment. Additionally, if there is persistent grade-3 toxicity at any time, treatment is held until toxicity improves to grade 0 or 1. The same dose of armed-ATC is then washed to eliminate DMSO and protein. If washing does not reduce toxicity, then the treatment is resumed with a 50% reduction in the armed-ATC dose for all subsequent doses in that course. If the toxicities continue at the reduced dose of armed-ATC, the continuous infusion of IL-2 is stopped and the armed ATC infusions are continued at the reduced dose. If grade 3 toxicity again occurs, the armed ATC infusions are stopped. Toxicities are assessed daily for 3 days after each reinfusion and then weekly between treatment courses for unresolved toxicities. Based upon this strategy, one patient at dose level 3 had her infusions stopped due to grade 4 toxicities (hypertension and headache). Of the remaining 15 patients, none had their dose reduced due to persistent grade 3 toxicities. Additionally, no cardiac toxicities occurred in any patients treated on this study. For some patients who experienced mild or moderate side effects with their first infusion, their armed ATC were washed to remove the cryopreservative, DMSO. This strategy reduced incidence of side effects by 50%.

Table 4: Side Effects by Dose Level

Dose Level	Reaction	# Patients Affected (% at Dose Level)	Total # of Episodes by Grade			
			1	2	3	4
1	Dyspnea	0 (0%)	0	0	0	0
	Chills	4 (66.7%)	0	2	9	0
	N/V	3 (50%)	4	0	0	0
	Headache	2 (33.3%)	0	0	4	0
	Fever	1(16.7%)	1	0	0	0
	Hypotension	1 (16.7%)	0	1	0	0
	Hypertension	0 (0%)	0	0	0	0
	Back Pain	0 (0%)	0	0	0	0
2	Dyspnea	0 (0%)	0	0	0	0
	Chills	2 (66.7%)	0	0	5	0
	N/V	1 (33.3%)	0	0	1	0
	Headache	2 (66.7%)	0	0	2	0
	Fever	1 (33.3%)	1	0	0	0
	Hypotension	0 (0%)	0	0	0	0
	Hypertension	0 (0%)	0	0	0	0
	Back Pain	0 (0%)	0	0	0	0
3	Dyspnea	1 (14.3%)	0	1	0	0
	Chills	5 (71.4%)	0	0	22	0
	N/V	3 (42.9%)	4	1	0	0
	Headache	6 (85.7%)	0	2	8	1
	Fever	1 (14.3%)	0	1	0	0
	Hypotension	1 (14.3%)	0	2	0	0
	Hypertension	1 (14.3%)	0	0	0	1
4	Dyspnea	0(0%)	0	0	0	0
	Chills	1 (100%)	0	2	0	0
	N/V	1 (100%)	0	0	1	0
	Headache	1 (100%)	0	1	0	0
	Fever	1 (100%)	1	0	0	0
	Hypotension	1 (100%)	1	0	0	0
	Hypertension	0(0%)	0	0	0	0
	Back Pain	0(0%)	0	0	0	0

2.3.2.5 *In Vivo* Trafficking of Her2Bi-armed ATC. For Her2Bi-armed ATC trafficking studies, a cryopreserved aliquot of Her2Bi-armed ATC were thawed, washed, and resuspended in Plasmalyte-A supplemented with 5% human serum albumen at a concentration of 5 x 10^7 cells/ml. Cells (2.5 x 10^8) were incubated with ^{111}Indium Oxine (750 μCi; Amersham, Arlington Heights, IN) for 20 minutes at 37ºC. After labeling, cells were mixed with an equal volume of human serum and washed twice to remove unincorporated radiolabel. Cells were resuspended in Plasmalyte-A supplemented with 20% human serum in a final volume of 5 ml and 1.2 x 10^8 cells (200 μCi net radioactivity) were injected intravenously into the patient 1 hour prior to the patient receiving her fourth regular infusion of Her2Bi-armed ATC (20 billion armed ATC). Whole body imaging (**Figure 1**) was performed at 4h, 24h, 48h and 96h (4 days) post administration of armed ATC. Her2Bi-armed ATC localized to the bone marrow, lung, liver, and spleen within 4h of injection. By 24h, Her2Bi-armed ATC had cleared from the lungs but persisted and remained stable in the bone marrow, liver and spleen for up to 96h post-infusion.



2.3.2.6 In Vivo Clearance of Her2Bi-armed ATC from Peripheral Blood. In *vivo* clearance of Her2Bi-armed ATC from the circulation of the patient presented in **Figure 1** was determined by obtaining 1 ml aliquots of sodium heparinized blood from the patient at 0 h (immediately after injection), 0.7 hrs, 1 hrs, 6 hrs, 8 hrs, 27 hrs, and 96 hrs post-infusion of ^{111}In-labeled Her2Bi-armed ATC. Whole blood samples were stored at room temperature after collection. After collecting all time point samples, the radioactivity was quantitated in a gamma scintillation counter. Results are presented as the % radioactivity (in cpm) at various times post-infusion compared to the radioactivity determined at time point 0h. Results suggest that 50% Her2Bi-armed ATC are cleared from circulation within ~30 min of the infusion (**Figure 2**). The patient's blood samples, however, were still positive for radioactivity (at 1.1% of the initial concentration) up to 96-h post infusion. This latter is consistent with the ability to detect cells in the lymphocyte gate of some patients' peripheral blood obtained at pre-infusion time-points (3-4 days after the last Her2Bi-armed ATC infusion) and up to 1 week after completion of infusions that stain positive for mouse IgG2a (the backbone of the anti-CD3 moiety of Her2Bi) by flow cytometry (**Figure 3**).

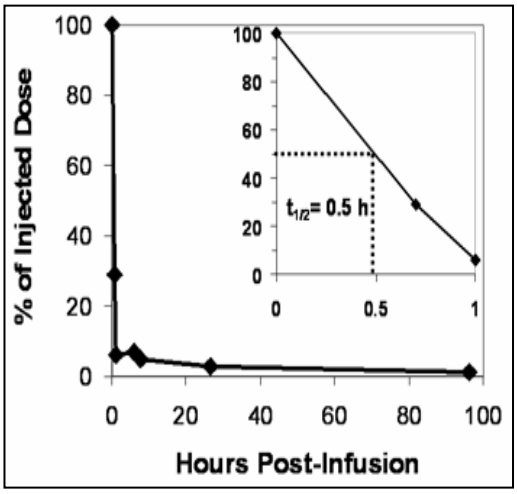


Fig 2: Clearance of ^{111}In labeled armed ATC. 1 ml aliquots of heparinized blood were drawn at 0,0.7,1,6,8,27,and 96 hrs post-infusing and counted for gamma irradiation. Results are presented as the % radioactivity in the serum (cpm) of the injected dose.

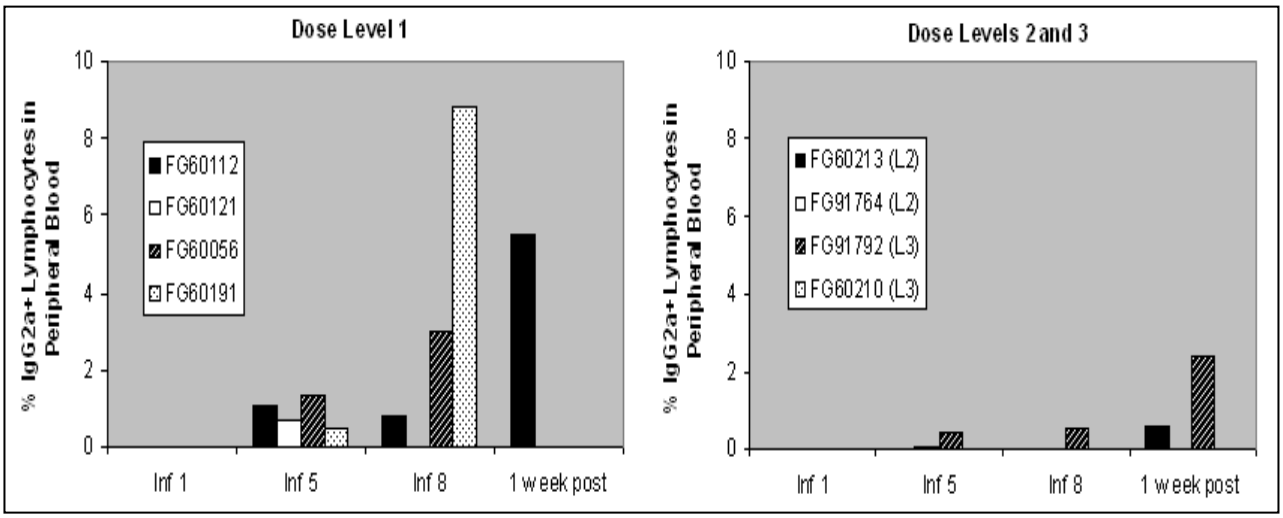


Fig 3: Ficoll-Hypaque PBL from patients were stained with anti-mouse IgG2a and quantitated by flow cytometry preinfusion #1 (Inf 1), preInf #5 (Inf 5), preinf #8 (Inf 8), and 1 week after the 8th infusion (1 week post).

2.3.2.7 Localization of Her2Bi-armed ATC to Breast Cancer Tumors. Formalin-fixed, paraffin-embedded samples were prepared from tumors after excision or needle biopsy. Tissues sample were sectioned, deparaffinized, stained with H&E and characterized for tumor content by a pathologist. Adjacent sections were then immunohistochemically stained with anti-CD3 for detection of T cells according to the manufacturer using the CSA Peroxidase System (DAKO, Carpinteria, CA) after target retrieval and endogenous biotin/avidin and peroxidase quenching with the CSA Ancillary System (DAKO). Anti-CD3 antibody (1 μg/ml) was diluted in Background Reducing Components (CSA Ancillary System) and incubated with tissue samples for 30 min at room temperature. Primary antibody was detected by incubating for 15 min with biotinylated goat anti-mouse immunoglobulins, and the signal was amplified and visualized by diaminobenzidine precipitation at the antigen site. In parallel, adjacent sections were stained with biotinylated goat anti-MIg (directed at the mouse IgG2a backbone of the anti-CD3 moiety of Her2Bi) followed by streptavidin-FITC to detect Her2Bi. Images acquired using fluorescent filters were overlayed upon images acquired by light microscopy creating composite images (**Figure 4**) to evaluate co-localization of staining.

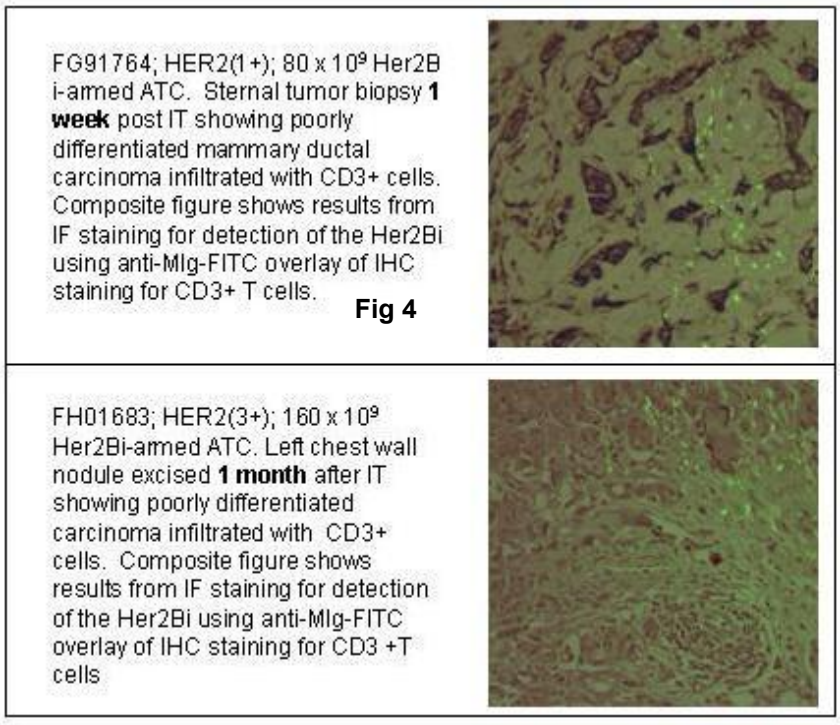


Table 5: Clinical Responses to Her2Bi-armed ATC by Dose Level[a]						
Response (%)	All Pts #	All Pts %	Dose Level 1	Dose Level 2	Dose Level 3	Dose Level 4
PR	1	5.3	0	1(100)[c]	0	0
SD	10	52.6	2(20)	2(20)	6(60)	0
PD	6	31.5	3(50)	2(33.3)	1(16.6)	0
NE	2	10.5	1(50)	0	1(50)	0

[a] At one month follow-up after the last infusion. [b]Did not complete infusion schedule or died before 1 month followup. [c]Pt received only 80 billion cells due to slow expansion.

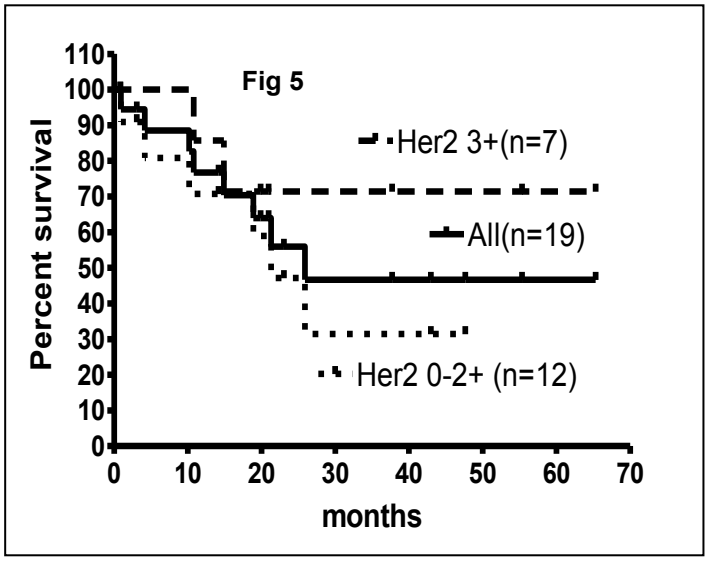
Table 6: Clinical Responses to Her2Bi-armed ATC by HER2/*neu* Expression Level [a]			
Response (%)	***All***	HER2(3+)	HER2(0-2+)
PR	**1 (6)**	0 (0)	**1 (8.3)**
SD	**10(59)**	**5 (71.4)**	**5 (50)**
PD	**6(35)**	**2 (28.5)**	**4 (41.6)**

[a] At one month follow-up after the last infusion; excluding 1 unevaluable patient and one patient with unknown HER2/*neu* status who was stable.

Table 7: Clinical Tumor Marker Responses			
Tumor Marker	No. Testing Positive Pre-TX	> 50% Reduction (No. HER2[0-2+])	15%-50% Reduction (No. HER2[0-2+])
CA 27.29	5	0	3
CEA	4	2	2
HER2/*neu*	5	2	2

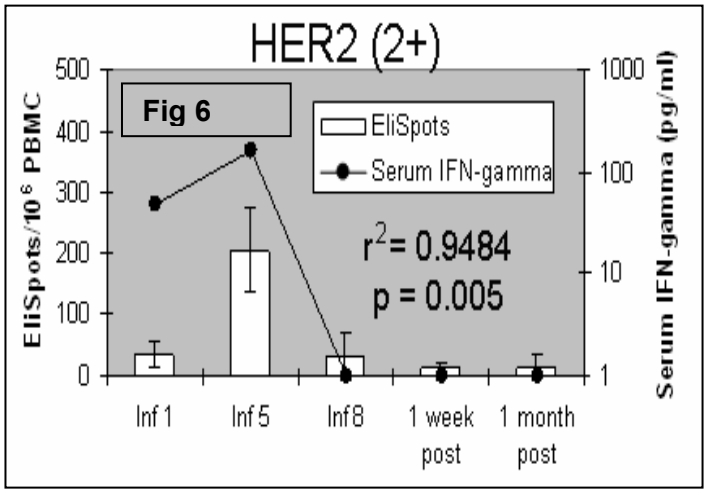
2.3.2.8 Clinical Responses to Her2Bi-armed ATC. Although clinical responses are not typically a primary endpoint for phase I clinical studies, we evaluated responses to therapy based upon tumor measurements, in those patients with measurable disease, as well as impact of treatments on levels of tumor markers in those patients who were clinically positive for tumor markers prior to initiation of treatments. No clinical tumor responses were observed, though more than half of the patients remained stable with no evidence of increase in tumor size or development of new lesions at their follow-up, one month after completion of the last Her2Bi-armed ATC infusion. Percentages of patients who remained stable during and after treatment with Her2Bi-armed ATCand their HER2/neu status were observed to increase as a function of treatment dose level (**Tables 5 and 6**). Additionally, analyses of serum tumor markers indicated impressive decreases in Carcinoembryonic Antigen (CEA), CA 27.29, and Her2/neu in the serum within one month following immunotherapy with Her2Bi-armed ATC (**Table 7**). It is important to note that the patient who experienced a partial response had Her2/neu negative expression.

2.3.2.9 Progression Free and Overall Survival. PFS was defined as the interval from starting immunotherapy to the time of progression. For these analyses, we compared patients with tumors that had been identified as having HER2(3+) expression levels to patients having HER2(0-2+) expression levels (**Fig 5**). One patient with an unknown HER2 status was analyzed with the Her2 0-2+ group (8 of these patients were documented to be Her2 0+ by IHC). *The median OS is undefined for the Her2 3+ group with 71.4% surviving, is 21.3 months for the Her2 0-2+ group with 31.4% surviving, and is 25.9 for the entire group with 46.6% surviving with median follow-ups of 20.97 (10.8-65.3), 16.54 (0.8-47.6), and 19.87 (0.8-65.3) months, respectively.*

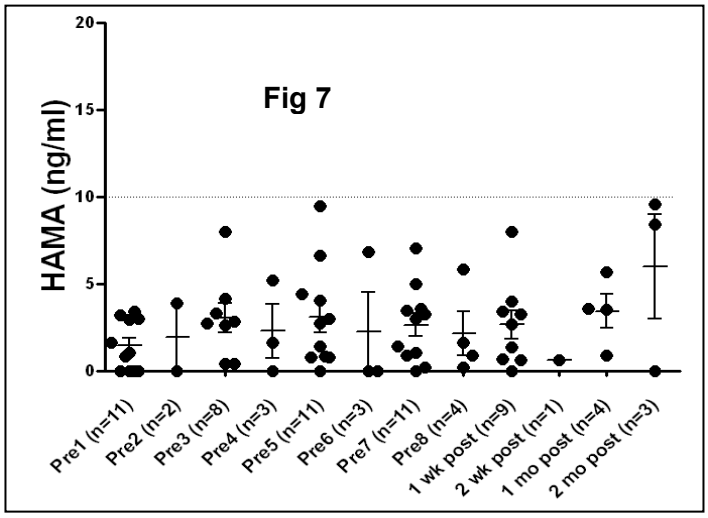


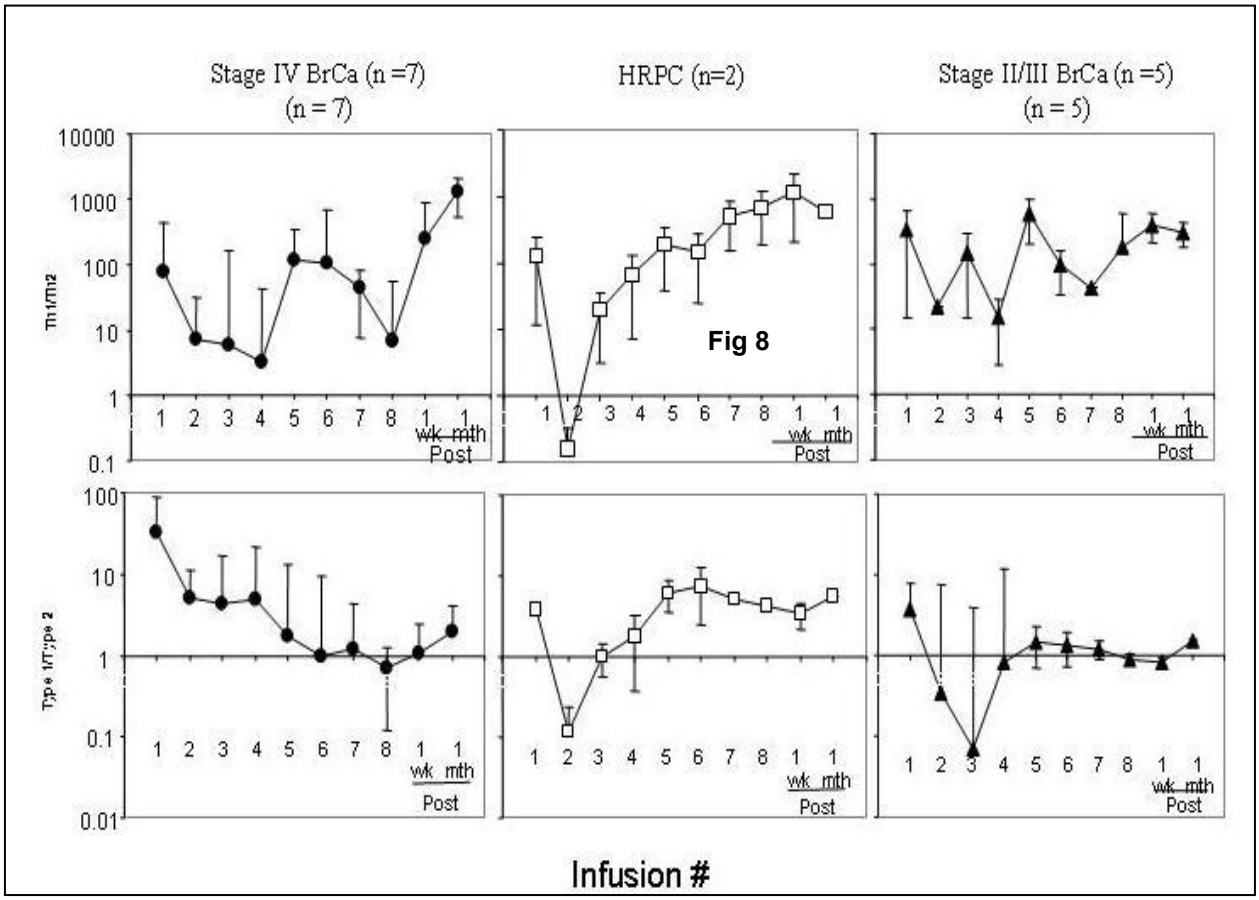
2.3.2.10 Immune Responses as a Function of Her2Bi-armed ATC infusions. Interferon-gamma (IFN-γ) EliSpot assays were used to monitor cytotoxic T lymphocyte (CTL) activity at specific time

points during the treatment regimen and 1 week after the last infusion. Blood samples were obtained just prior to the indicated infusion number (Inf #) to evaluate steady-state CTL activity (3-4 days after the last armed ATC infusion). Four patients tested by EliSpot assays had CTL activity in their peripheral blood (1-HER2[3+]; 1-HER2[2+]; and 2-HER2[0+]), and 3 of 4 of these patients had significant increases in steady-state CTL activity following Inf # 4 that persisted during the complete treatment regimen (**Fig 6**). Evaluation for antigen-specific CTL responses in 2 of these patients using co-culture with BrCa or lymphoma targets to stimulate IFN-γ production showed significant increases in BrCa-antigen-specific CTL in the HER-2(0+) patient, whereas the HER-2(3+) patient showed more generalized CTL responses. Comparison of steady-state serum IFN-γ levels in patients to their EliSpot results identified a positive correlation in 1 of the 4 patients (data not shown). No trends were observed between dose level and EliSpot results. It appears that even the lowest dose levels induce cytokine and EliSpot responses.



2.3.2.11 No Evidence of HAMA Responses to IgG2a (OKT3). Because one mAb moiety comprising Her2Bi is an unmodified mouse mAb, we evaluated patients' sera for the development of human anti-mouse antibodies (HAMA) during their treatments by ELISA (Antigenix, Inc., Huntington Station, NY). Individual patient samples are shown as solid circles (**Fig 7**), and horizontal bars represent the mean +/- standard error for the number of patients sampled at each time point (n). Of the 11 patients evaluated, none developed clinically significant HAMA levels (> 10 ng/ml) during or immediately following the treatment regimen. Average HAMA concentrations in patients did not differ significantly as a function of dose level (p = 0.55). Additionally, no correlation was found between levels of IgG2a+ cells in patients' peripheral blood and their respective HAMA levels (r^2 = 0.001; p = 0.86).





2.3.2.12 The Th1/Th2 cytokine responses in patients Her2Bi armed ATC. Cytokine analyses were performed on serum samples prior to each infusion (infusion #) from patients with Stage IV BrCA (leftmost panels), hormone refractory prostate cancer (HRPC)(middle panels), and stage II/III breast cancer (rightmost panels) who received 8 armed ATC infusions. The samples were tested for cytokines Th1, Th2, and other cytokines of interest (IL-2, IL-4, IL-5, IL-10, IL-13, and IFNγ) using a Bio-Plex Protein Array System (**Fig 8**). IL-12 p70 is summarized in **Fig 9** below. The upper three panels of **Fig 8** show the Th1/Th2 ratio for stage IV BrCA(left), HRPC(middle), and Stage II/III BrCa (right) patients as a function of Inf # . The mean Th1[IL-2+IFNγ]/Th2 [IL-4+IL-5] ratio, show a Th1-type response induced as function of armed ATC infusions, increasing from 89.1 at Inf #1 to 538.6 at Inf #8. These findings were consistent with increased specific IFNγ production observed by ELISpot analysis of patient post-Inf peripheral blood mononuclear cells (PBMC) exposed to HER2-positive BR-3 tumor cells. The lower three panels show the ratio of Type 1/Type2. Type 1 [IL-2+ IFNγ]/Type 2[IL-4+IL-5+IL-10+IL-13] remained polarized towards a Th1–type response throughout treatment. In the Stage IV BrCa patients, infusions of armed ATC skewed the ratio to ≥ 1.0 for the first 7 infusions. Together these data show that there is a clear effect on serum cytokine levels induced by infusions of armed T cells.

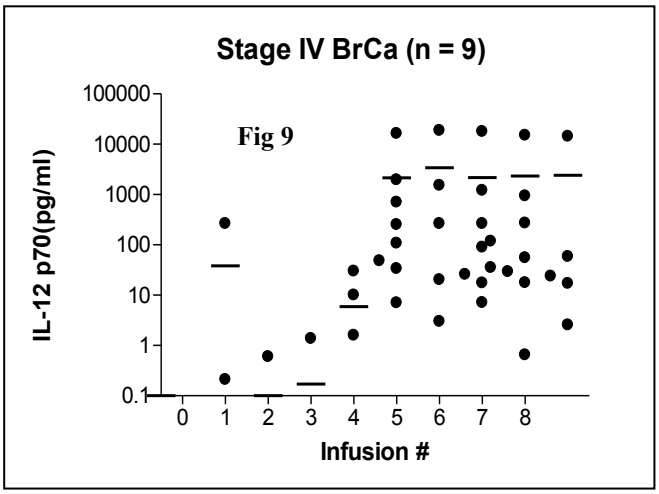


Fig 9: Sera prior to each infusion from immunotherapy patients were tested for IL-12 p70 determine level of IL-12 p70 as a function of infusion number (#).

2.3.2.13 Increased levels of IL-12 p70 in patients receiving Her2Bi-armed ATC infusions. IL-12 p70, produced mainly by activated monocyte, is the principal cytokine for polarizing T cell responses towards a Th1 phenotype. Furthermore, IL-12 has been shown

to enhance the cytotoxic functions of NK and CD8+ T cells. We have observed an increase in serum levels of IL-12 occurring around 2 weeks after 3-4 armed ATC infusions in patients with MBC (**Fig 9**). These data are critical in that they show 1) armed ATC infusions establish a systemic Th1-type anti-tumor milieu and 2) endogenous monocyte/macrophages are activated to produce IL-12 by armed ATC infusions. The induction of IL-12 provides strong evidence that endogenous immunity is induced by armed ATC infusions.

2.3.2.14 Enhanced Specific Cytotoxicity in Patient PBMC after Infusions of Her2Bi-armed ATC as a Function of Time after Infusions. Five patients undergoing Her2Bi-armed ATC infusions were studied for cytotoxicity directed at SK-BR-3 before, during, and after treatment. **Fig 10 shows 3 representative patients**. A significant increase in specific cytotoxicity directed at SK-BR-3 cells by PBMC obtained from all patients was observed during the course of treatment, with peak levels ranging from 14.15 ± 3.08% to 30.71 ± 0.54%. The tumors from FG91464, GF60210, and GF91792 were Her2/neu 1+, 3+, and 0+ by IHC expression. Although there were variations between time points, specific cytotoxicity tended to increase during treatment with 2 of 5 patients exhibiting peak cytotoxicity just before or following infusion 8. These data clearly show immune responses directed at SK-BR-3.

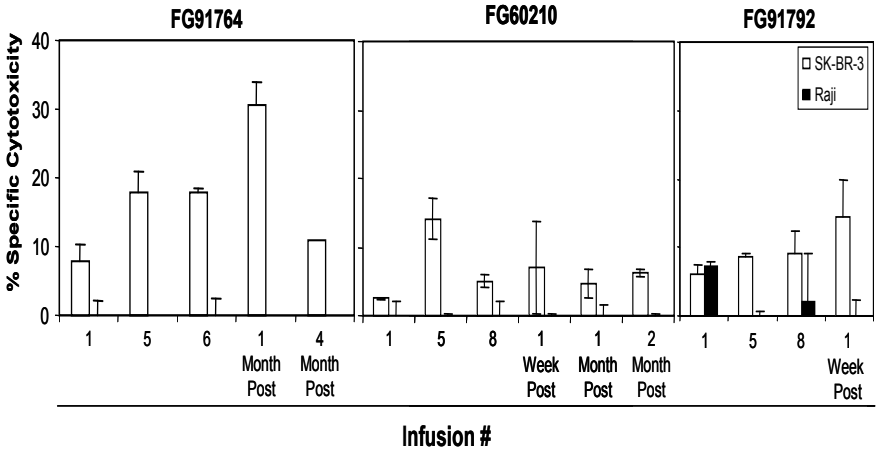


Fig 10: PBMC were acquired from whole blood collected at various time points over the course of treatment. All patient PBMC samples were tested for cytotoxic activity against HER2-expressing SK-BR-3 cells (25:1 E/T) and against HER2-negative Raji cells as a negative control.

2.3.2.15 Enhanced Specific Cytotoxicity Mediated by Endogenous Lymphocytes from Patients. PBMC were obtained from BrCa patients at the time of pheresis to obtain leukocytes for ATC expansion, prior to the 5th, 6th,and 8th infusions, 1 wk, 1 mo, 2 mo, and 4 mo after armed ATC infusions. In some patients, infusions of Her2Bi-armed ATC correlate with induction of specific cytotoxicity. Moreover, it was observed that enriched IgG2a- (endogenous) cell populations exhibited significant cytotoxic activity (**Fig 11**). Total PBMC obtained at the time of pheresis, the armed ATC product, ATC (unarmed control), PBMC at the infusion # (5 or 8), PBMC at 1 week post, 3 weeks post, or 1 month post as indicated. The endogenous ("IgG2a-") populations were prepared by depleting Her2Bi-armed ATC ("IgG2a+") by specific murine IgG2a selection. To further confirm that cytotoxicity in the endogenous IgG2a- population, we tested the IgG2a- and IgG2a+ population for IFNγ EliSpots as a surrogate marker for CTL activity. The number of, IFNγ-secreting cells significantly increased in IgG2a- (endogenous) cell populations as a function of Her2Bi-armed ATC infusions, suggesting activation and recruitment of endogenous immune effector cells into an anti-tumor-specific response (data not shown). The data in Figs 10 and 11 clearly show that the IgG2a- population exhibits high level of cytotoxicity directed at SK-BR-3 targets.

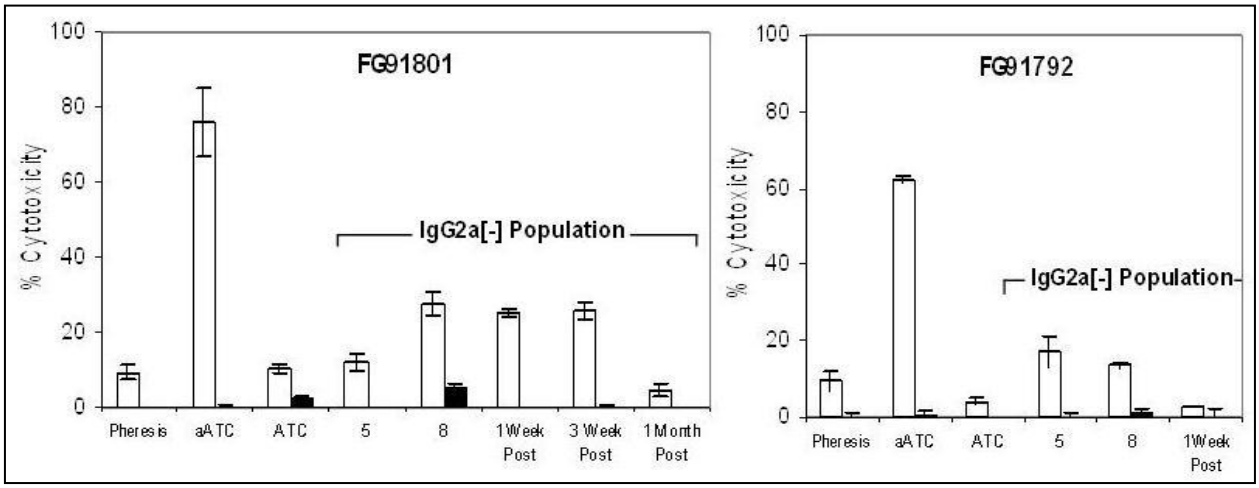


Fig 11: Circulating PBMC were depleted of IgG2a+ cells (IgG2a [-]) using Miltenyl beads. IgG2a[-] population was tested for specific cytotoxicity directed at HER2-positive SKBR-3 (25:1 E/T) or HER2-negative Raji as a function of treatment schedule. The IgG2a [-] populations were compared to the pheresis product, unarmed ATC, armed ATC product.

2.3.2.16 Specific Cytotoxicity Can Not Be ALL Due to Infused Her2Bi Armed ATC. To determine the lower limit of sensitivity for detection of armed ATC-mediated cytotoxicity, we performed a Her2Bi-armed ATC spiking experiment. PBMC were spiked to contain 5% Her2Bi-armed ATC and Miltenyi selection was performed to separate the IgG2a+ and IgG2a- populations. Cells from each population were analyzed by flow cytometry using goat anti-mouse IgG2a-PE to detect the OKT3 moiety of the BiAb (**Fig 12A**). Following Miltenyi depletion, staining for IgG2a+ cells in the IgG2a- sorted population showed only 0.87% of cells to be IgG2a+ contaminants. In a parallel study, known proportions of Her2Bi-armed ATC (0-100%) were mixed with PBMC to create a standard curve for detection of IgG2a positive cells (**Fig 12B**). Using this standard curve, flow cytometry detected as little as 0.87% IgG2a+ cells (**Fig 12A**) in samples containing known Her2Bi-armed ATC concentrations of ~0.1%, thus establishing the lower detection limit of flow cytometry for mouse IgG2a+ cells. We next performed a cytotoxicity assay (25:1 E/T) using a range of proportions for armed ATC to PBMC (**Fig 12C**). No significant increase in cytotoxicity against SK-BR-3 cells over PBMC alone was observed for PBMC populations spiked with Her2Bi-armed ATC at 0.1% or 1%, both of which are above the lower detection limit for flow cytometry and the latter of which is consistent with circulating levels of armed ATC in patients. Flow cytometry is more sensitive than cytotoxicity assay for identification of armed ATC populations. Taken together, these data show that the cytolytic activity observed in IgG2a- fractions of patient PBMC, can not be attributed to armed ATC that are too few to be enumerated by flow cytometry. Even if the level of IgG2a is below the level of detection for the circulating cells in vivo and there was 5% of the circulating cells were “armed” with Her2Bi that were below the level of detection, those cells could not provide the level of cytotoxicity observed in the PBMC of patients presented in Figs 10 and 11. *Together, the IgG2a+ phenotyping, cytotoxicity assays, IgG2a depletion assays to obtain the IgG2a- consisting of endogenous lymphocytes, and the spiking experiments provide strong evidence that Her2Bi-armed ATC infusions induced endogenous cytotoxic activity in the Ig2a- population.*

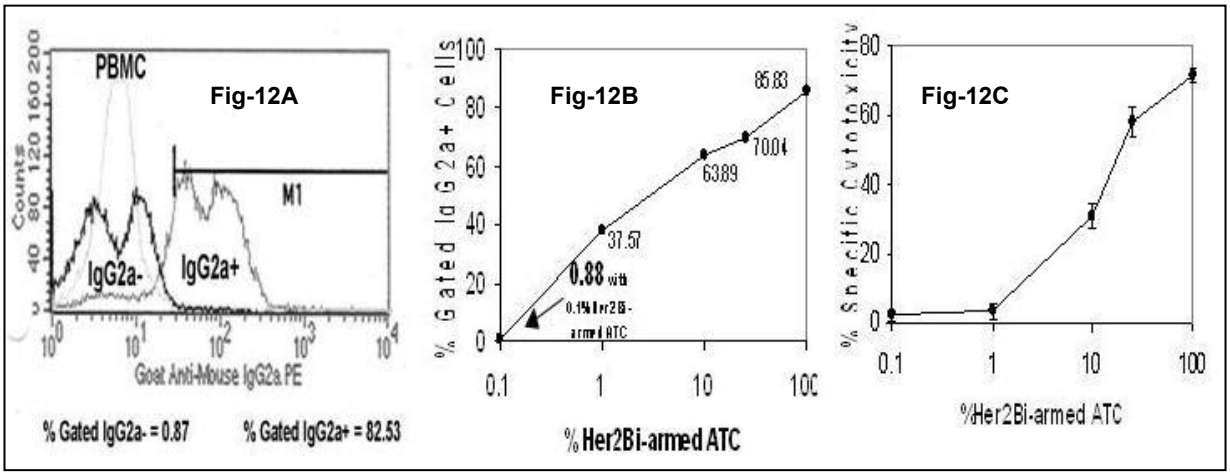
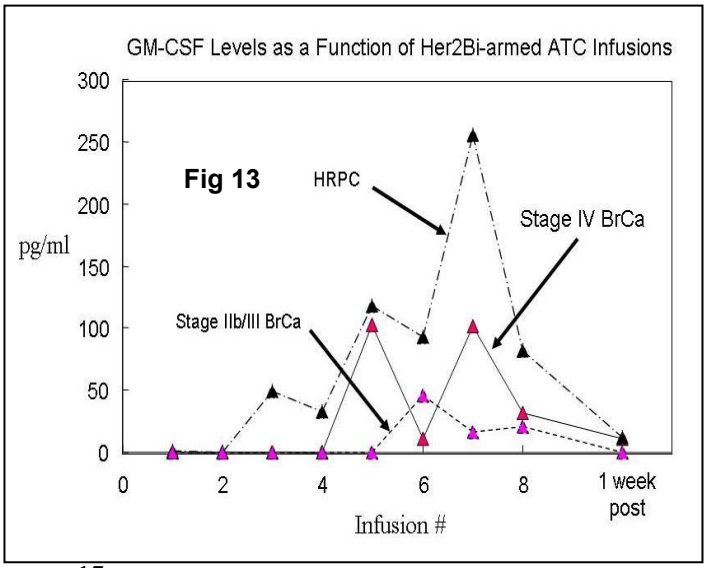


Fig-12: Shows Phenotyping of Miltenyl purified IgG2a+ and IgG2a- populations (Panel A), phenotyping for IgG2a+ cells in spiking experiment wherein various proportion of Her2Bi armed ATC were added to PBMC to determine the lower limit of sensitivity of detection for IgG2a+ cells (Panel B), and cytotoxicity of PBMC spiked with various proportions of Her2Bi-armed ATC. IgG2a+ cells were detected by flow cytometry using goat anti-mouse IgG2a-PE to detect the OKT3 moiety of OKT3 x Herceptin.

2.4 Rationale for Monitoring Functional and Phenotypic Changes in Immune Populations. We have shown that for extended periods of time armed ATC will divide, secrete cytokines/chemokines, target and kill tumor cells *in vitro*[16]. Moreover, immune evaluation of breast cancer patients participating in our phase I clinical trial of HER2Bi-armed ATC have led to the critical observation that following armed ATC infusions, evidence of immune functions, *i.e.* cytokine production and breast cancer-specific cytotoxicity, are detected. After 4 infusions of armed ATC, MBC patients develop a Th1-type serum cytokine pattern, consisting of significant levels of IL-2, IL-7, IL-12, IFNγ, TNFα, and GM-CSF. Interestingly, although some of these responses may directly result from armed ATC functions *in vivo,* others, such as IL-12 production, cannot be attributed to T cells but rather may reflect endogenous innate immune stimulation. Based on these data, we hypothesize that in addition to the short-term direct benefits of targeting and killing tumor cells, armed ATC immunotherapy vaccinates the patients against their own "tumor antigens" and will provide additional long-term benefits of endogenous immune activity, *i.e.* local cytokine secretion and recruitment of endogenous T cells. Based on this hypothesis, we expect to see as a function of infusions: a) increased Th1-type serum cytokines, b) phenotypic changes in T cell populations consistent with activation, c) increased specific CTL function, and d) expansion of tumor-specific T cells. Moreover, we predict that evidence of endogenous immune activity will correlate with IT-associated improvements in clinical outcome.

2.5 Anti-Tumor Effects of GM-CSF. GM-CSF is known to promote the proliferation and differentiation of myeloid-committed progenitors. Many studies have shown that GM-CSF accelerates neutrophil recovery after myelosuppressive chemotherapy, PBSCT, ABMT, and allogeneic BMT[18-20] Monocytes and granulocytes clearly participate in immune responses to tumors. Preclinical studies with GM-CSF suggest that GM-CSF by itself, or in combination with other biologic response modifiers, may have anti-tumor activity as an anti-tumor agent [18-20].



2.6 Rationale for Using GM-CSF: Upon review of the serum cytokine data, there was pattern of rise in the serum levels of IL-2 and GM-CSF. **Figure 13** shows that pattern of GM-CSF levels for Stage IV BrCa, HRPC, and Stage IIb/II BrCa patients who received 8 Her2Bi armed ATC infusions. This rise occurred in the phase I metastatic breast cancer trials that began after 2 weeks (4 infusions). There was no IL-2 (data not shown) or GM-CSF increase over the baseline values until two weeks (just prior to 5th infusion GM-CSF injections of 250 μg/m^2 given twice weekly. The pattern for IL-2 increase was similar to that seen in GM-CSF. In other words, the low doses of IL-2 and GM-CSF administered to the patients did not accumulate enough in the serum to increase the levels above the pretreatment baselines but suggest that infusions of armed T cells induced the production of IL-2 and GM-CSF by endogenous immune cells. However, it was unclear whether priming the immune system with IL-2 and GM-CSF was necessary to trigger the immune responses. Based on the immune evaluation obtained thus far, our results show very low levels of cytotoxicity responses. The suggest the GM-CSF priming may be necessary to optimize the anti-tumor response of the Her2Bi armed activated T cells. Therefore, GM-CSF is being added to the protocol.

2.7 Rationale for 3 armed ATC infusions, spaced 1 week apart, and a Boost (armed ATC #4) 12 weeks after armed ATC #3: The infusion schedule was selected based on our phase I data that showed that Her2Bi armed ATC could be detected for weeks. Together with the cytokine data in the phase I, breast cancer patients developed Th1 responses after 3-4 infusions even with lower doses (5 or 10 billion twice a week). The overall timing of the responses began as early as 10 days. The total number of cells infused ranged from 15 billion to 80 billion. Nearly all of the increases in serum cytokines took place after 3-4 doses (documented in the pre5th infusion sera) regardless of the dose infused. This observation suggests that targeting induced immune responses was similar to a "vaccination" response to an injected antigen with multiple booster "immunizations".

Summary of Preliminary Data: Our *in vitro* and *in vivo* data show that armed ATC: 1) infusions are feasible and safe; 2) have well demonstrated *in vitro* activity against breast cancer cell lines with very low and high Her2 expression; 3) can traffic to sites of disease in vivo; 4) may have improved OS for the both Her2 0-2+ and Her2 3+ patients. **[The median OS is 34.7 months for the entire group, 25.9 months for the Her2 0-2+ group, and 46.4 for the Her2 3+ group];** 5) infusions induced the development of breast cancer specific CTL activity suggestive of an adaptive endogenous response; 6) infusions did not lead to development of HAMA response; 7) infusions engaged relatively intact immune systems of MBC patients leading to increased production of IL12 (an endogenous APC product); 8) infusions induced a well defined shift in the Th1/Th2 or Type 1/Type 2 cytokine patterns**; 9) retains Her2Bi on its surface while it undergoes multiple cycles of cytotoxicity, cytokine/chemokine secretion, and cell division that is dependent upon the presence of Her2Bi; and 10)** expansion of ATC from patients show that there is a shift in their Vβ specificity after expansion, which may become important for *in-vivo* tracking of these cells as well as tracking of the endogenous adaptive immune response that can develop as a result of Her2Bi-armed ATC infusions. There is clear justification for including patients even with Her2 0+ by IHC since brisk cytotoxic immune responses and the one partial remission was seen in several patients who had "no" expression of Her2 by IHC. **Together these data provide compelling evidence for the proposed clinical trial strategy and immune evaluation function tests to develop in vivo/in vitro correlates.**

3.0 TREATMENT PLAN:

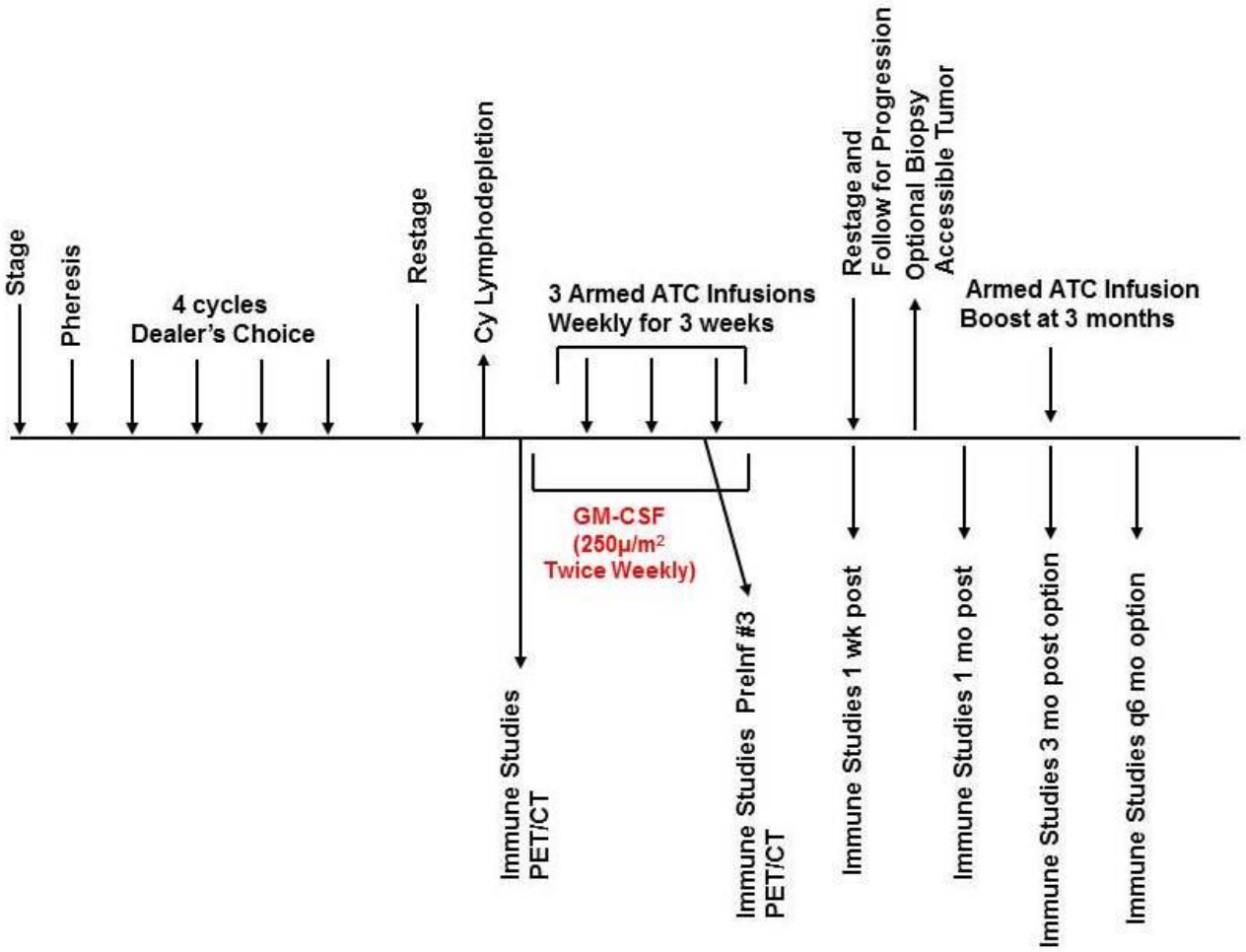
3.1 Summary of Treatment Plan: Patients who develop locally advanced, unresectable disease or metastatic breast cancer may be leukopheresed before starting ChemoT. Patients may be leukopheresed when the lymphocyte count is >500 mm^3 after holding or before starting chemoT. Peripheral blood mononuclear cells (PBMC) will be collected using 1 leukopheresis, activated with anti-CD3 (20 ng/ml of OKT3), and expanded in 100 IU/ml of IL-2 to generate ATC during 14 days of culture. After culture, ATC will be harvested, armed with HER2Bi, washed, and cryopreserved in 4

aliquots for subsequent infusions. The patients will receive 4 cycles or 4 months worth of salvage ChemoT (primary oncologist choice). Patients who are rapidly progressing through chemoT may receive additional chemoT/hormonal therapy to make an attempt to control disease prior to immunotherapy based on discussions between the primary treating oncologist and the Immunotherapy Team. Following chemoT, **patients will be restaged, preferably within 2 weeks, and evaluated for chemo-related toxicities.** These scans will serve as the baseline scans for evaluation of response of aATC treatment. Patients may receive their 1st Her2Bi-armed ATC infusion as early as 1.5 weeks after the last dose of chemotherapy and as late as 4 weeks after chemotherapy. Since the maximum tolerated dose (MTD) of Her2Bi-armed ATC has not been reached in our phase I trial and dose-limiting toxicities have not been observed in the range between 5-20 billion Her2Bi-armed ATC/infusion, we have selected a dose of 20 billion Her2Bi-armed ATC per infusion (a total dose of 80 billion Her2Bi-armed ATC). Armed ATC will be thawed and infused once a week for 3 weeks for a total of 3 doses and then a boost of 20 billion armed ATC 12 weeks (± 1 week) after the 3rd infusion. Low dose granulocyte-macrophage colony stimulating factor (250 μg/m^2/twice per week) will start 3 days before the first aATC infusion and end with the third dose of aATC. Patients who are already on the protocol will be given a choice to add GM-CSF to their treatment regimen (after reconsenting) or continue to their treatment without GM-CSF.

Patients will have met the eligibility criteria and will have been given informed consent prior to registration for the study. The ChemoT may be administered at KCI, at another institution, or in the practice of a referring MD as long as all of the eligibility criteria, consent, and follow-up criteria are fulfilled.

3.2 Summary of Imaging Plan: An optional exploratory whole body [^{18}F]-FDG-PET/CT will be performed on selected patients (only at the KCI site) after completing 4 cycles of chemotherapy. The goal is to obtain 10 evaluable patients. We plan to select patients for [^{18}F]-FDG-PET/CT who have disease still measurable on CT or MRI after completion of the Induction chemotherapy. A whole body [^{18}F]-FDG-PET/CT scan will be performed on a PET/CT machine within 7 days prior to the first armed ATC infusion. The second [^{18}F]-FDG-PET scan will be performed at least three days after the second infusion of armed ATC and before the third infusion. The patient must fast a minimum of 4 hours before the administration of the [^{18}F]-FDG for the PET scan but may freely drink water. (1) if the scan is scheduled in the morning, the patient should not eat; (2) if the PET scan is scheduled for the afternoon, the patient may have a light breakfast 4 (or more) hours before the administration of the [^{18}F]-FDG for the PET scan appointment. Glucose levels will be measured prior to the administration of [^{18}F]-FDG. The patient must have a blood glucose value of ≤150 mg/dL in order for the patient to have the [^{18}F]-FDG-PET/CT scan. All patients should be encouraged to increase fluid intake for a few hours after the scan to promote excretion of the [^{18}F]-FDG.

The FDG PET/CT scan will be done using the standardized approach as described in Appendix 2 and Appendix 3 using the methods of ACRIN 6678.



Treatment Schema

4.0 ELIGIBILITY

4.1 Metastatic Breast Cancer. Histologically confirmed breast cancer with evidence of metastatic disease (need not be biopsy proven) or locally, advanced unresectable disease.

4.2 HER2/*neu* Expression. Patients with 0-2+ HER2 expression as determined by immunohistochemistry staining and/or FISH ratio ≤2.0, with the above pathologic criteria will be eligible. Patients with HER2 overexpression by immunohistochemistry (IHC) or overamplification by FISH are not eligible and are defined as follows: IHC staining of 3+ (uniform, intense membrane staining of > 30% of invasive tumor cells), a fluorescent in situ hybridization (FISH) result of more than six HER2 gene copies per nucleus or a FISH ratio (HER2 gene signals to chromosome 17 signals) of more than 2.0.

4.3 Prior or Current Therapy

4.3.1 Hormone Therapy. Patients on prior hormonal therapy are eligible. Hormonal therapy will be stopped 2 weeks prior to leukopheresis. Hormone therapy may be restarted after leukopheresis.

4.3.2 Chemotherapy: .Patients may have received any number of prior lines of chemoT (including none). If currently receiving chemoT, patients may be responding, stable, or progressing on the current regimen at the time of registration. Prior taxanes, anthracyclines, or any other chemoT are permitted. Leukopheresis may be done when the lymphocyte count has recovered to ≥500 cells/mm^3 and there are no residual chemotoxicities that would prevent leukopheresis.

4.3.3 Radiotherapy. Leukopheresis may be done 4 weeks after radiation to the axial skeleton.

4.3.4 Biologic Agents. Patients who have received prior biological agents are eligible.

4.4 Measurable or Non-Measurable Disease. Measurable or non-measurable disease per Response Evaluation Criteria In Solid Tumors (RECIST) version 1.1. Biopsy of recurrent site(s) is not required. Biopsy of accessible sites before and after armed ATC is an optional part of this study to be performed at KCI only.

4.5 Age. ≥18 years.

4.6 Performance Status. Karnofsky ≥ 70% or ECOG 0-1

4.7 Life Expectancy. Life Expectancy ≥ 3 months

4.8 Other Malignancies. Patients with a history of another malignancy within 5 years of study entry are not eligible (except basal cell skin carcinoma and carcinoma-in-situ of the cervix).

4.9 Nonpregnant: Negative serum test for pregnancy, unless male, prior hysterectomy, tubal ligation, or postmenopausal. (Note: postmenopausal is defined as age>55 with amenorrhea for >1 year or age <55 years with amenorrhea for 2 years and FSH level within postmenopausal range of institutional parameters; patients requiring FSH level to determine menopausal status need not have this performed and may choose to proceed with serum pregnancy testing.)

4.10 No serious illness. No serious medical or psychiatric illness which prevents informed consent or intensive treatment is allowed.

4.11 Cardiac Status. Patients will be ineligible for treatment on this protocol if (prior to protocol entry):

4.11.1 There is a history of a recent myocardial infarction (within one year)

4.11.2 There is a history of a past myocardial infarction (more than one year ago) along with current coronary symptoms requiring medications and/or evidence of depressed left ventricular function (LVEF < 45% by MUGA or ECHO)

4.11.3 There is a current history of angina/coronary symptoms requiring medications and/or evidence of depressed left ventricular function (LVEF < 45% by MUGA or ECHO)

4.11.4 There is clinical evidence of congestive heart failure requiring medical management (irrespective of MUGA or ECHO results)

4.12 Pleural effusion or ascites. Patients will be ineligible if there is recurrent pleural effusion or ascites requiring drainage (through thoracentesis, paracentesis, or indwelling device) more often than once every 4 weeks.

4.13 No evidence of central nervous system (CNS) metastases. Patients with treated brain metastases (*i.e.* those who have received definitive radiation, chemotherapy, and/or underwent surgical resection) are eligible for therapy on this protocol. Patients with clinical evidence of active CNS metastases are ineligible for therapy on this protocol.

4.14 Required initial laboratory data (normal limits per treating institution):
Granulocytes $\geq$ 1,200/mm^3
Platelet count $\geq$ 50,000/µl
Hemoglobin ≥ 8 gm/dl
BUN $\leq$ 1.5 times normal
Serum creatinine < 1.8 mg/dl
Creatinine Cl ≥50 ml/mm (can be calculated utilizing the Cockcroft & Gault equation**:** Creatinine Clearance (mL/min) = {(140 - Age)} x Wt [kg] (x 0.85 if Female}/ {72 x SCr [mg/dL]}
Bilirubin < 1.5 times normal
ALT, AST and alkaline phosphatase < 5 times upper normal
Negative HIV
Negaitve Hepatitis B surface antigen
Negative Hepatitis C serology
LVEF ≥ 45% at rest (MUGA or ECHO)
PFT-FEV_1, DLCO, and FVC ≥ 50% of predicted

(Minor changes from the indicated laboratory guidelines will be allowed at the discretion of the attending team under special circumstances. The reasons for the changes will be documented.)

4.15 Medical Monitor. The medical monitor is required to review all unanticipated problems involving risk to subjects or others, serious adverse events and all subject deaths associated with the protocol and provide an unbiased written report of the event. At a minimum, the medical monitor must comment on the outcomes of the event or problem and in case of a serious adverse event or death, comment on the relationship to participation in the study. The medical monitor must also indicate whether he/she concurs with the details of the report provided by the principal investigator. Reports for events determined by either the investigator or medical monitor to be possibly or definitely related to participation and reports of events resulting in death must be promptly forwarded to the USAMRMC ORP HRPO.

4.16 Informed consent. Each patient must be aware of the nature of their disease and must willingly consent to treatment after being informed of alternatives, potential benefits, side effects, and risks. Patients who are already on the protocol may choose to have GM-CSF added to their treatment regimen. They may also choose to treated without GM-CSF. Newly consented patients will receive GM-CSF.

5.0 INFUSIONS of ARMED ACTIVATED T CELLS

5.1 Activation and Expansion of T cells with OKT3 and IL-2. ATC are prepared in the Cancer Immunotherapy Laboratory's cGMP facility (***see Section 8.1***). Immediately after pheresis, the lymphocytes are activated with soluble monoclonal anti-CD3 antibody (OKT3), which cross-links the CD3 receptors on T cells and activates them. The ATC are expanded in the presence of IL-2 for up to

14 days. After culture, ATC are harvested, armed with OKT3 x Herceptin® (Her2Bi), washed to remove unbound Her2Bi, and cryopreserved in 10% DMSO and 20% protein (human albumin or serum) using rate controlled freezing and storage in liquid nitrogen. No exogenous IL-2, OKT3, or other culture reagents (e.g. medium components) are present in the final cryopreserved product. Armed product is released for clinical use after Quality Control testing for sterility (bacterial and fungal culture, endotoxin and mycoplasma), phenotype (% of CD3 cells), and activity (*i.e*. cytolytic activity against the SKBR-3 cell line).

5.2 HER2Bi armed ATC Infusions: Armed ATC infusions will be done in an outpatient setting of the treating institution. After chemoT and before aATC infusion, the ECHO or MUGA should meet the entry criteria (LVEF > 45% by MUGA or ECHO). If the systolic blood pressure (BP) is consistently ≥140 or their diastolic BP is consistently ≥90, patients must have their BP controlled by anti-hypertensive medications for at least 7 days prior to the first armed ATC infusion.

All appropriate assurances for identification of product, patient, sterility, etc. will be performed prior to infusion. Frozen armed ATC will be thawed at the bedside of the patient just prior to infusion. If there is evidence of infusion-related toxicities, subsequent armed ATC will be thawed, washed, resuspended in medium and infused. Armed ATC infusions will begin on day 0, subsequent doses will be administered weekly (± 3 days) for 3 doses and then a boost of 20 billion armed ATC 12 weeks (± 7 days) after the 3rd infusion. Armed ATC will be infused intravenously (IV) with the rate of infusion based on the endotoxin content of the product. All patients will be observed for at least 2 hours after an infusion. If stable, patients will be discharged home.

5.3 Granulocyte-macrophage colony stimulating factor (GM-CSF) Injections: All patients who have not started armed ATC infusions will be eligible to receive SQ GM-CSF (250 μg/m^2/twice weekly), to start 3 days before the first ATC infusion and ending 1 week after the last armed ATC infusion. Patients already on the protocol may elect not to receive GM-CSF and still receive armed ATC infusions.

5.4 Recommended Concomitant Medications: All patients may be pre-medicated with diphenhydramine (50 mg po or IV) and acetaminophen 650 mg , 30 minutes prior to each Her2Bi-armed ATC infusion. Patients may receive prehydration of 500 ml of 0.9 normal saline prior to the armed ATC infusion. The amount of prehydration will be adjusted clinically based on the patient's clinical response to the armed ATC infusions. These medications may be repeated every 4-6 hrs as needed. Meperidine (25-50 mg IV) will also be administered if the patient develops grade 3 chills and then may be administered 30 minutes prior to subsequent armed ATC infusions dependent upon the severity of the initial reaction.

5.5 Ancillary Therapy: Patients will receive full supportive care including transfusion of blood and blood products, antibiotics, and anti-emetics, when appropriate. The reason(s) for treatment, dosage and the dates of treatment will be recorded.

5.6 Steroids/Other Therapy: With the exception of steroids for adrenal failure, septic shock, or pulmonary toxicity,steroids will not be administered. Hydrocortisone (50-100 mg IV) for severe adverse reactions, related to armed ATC infusions, is allowed. G-CSF's should not be routinely used without consulting the PI.

5.7 If the harvested total cell dose is less than 48 billion, it will be up to the PI to determine how many cells to place into as few as 2 bags and as many as 4 bags. This approach will optimize the vaccination effect of the infused targeted T cells.

6.0 POTENTIAL TOXICITIES, DOSE MODIFICATIONS, AND MANAGEMENT

6.1 Monitoring for chemotherapy toxicity: Dose reduction or adjustment, for toxicity during the oncologist's choice chemotherapy is allowed and will be based on the treating oncologist decision. The regimen for the oncologists choice will be reported. Toxicities related to oncologist's choice of chemotherapy will not be reported. For example, expected myelosuppression, GI toxicities, neutropenic fever, chills, and neuropathies need not be reported. Hospitalizations for neutropenic fever during the oncologists choice chemotherapy will be reported but will not be considered an adverse event. If there are toxicities that occur that prevent the patient from receiving their planned doses of armed T cells, the toxicities and the specific reason for removal from the protocol will be recorded and reported to the coordinating site.

6.2 Leukaheresis Procedure. Reported toxicity from leukapheresis is minimal. Patients have a small potential for infection from the placement of central catheters used for leukapheresis. Use of the anticoagulant citrate dextrose may cause symptoms of mild hypocalcemia, which are controlled with calcium replacement. Each subsite will perform leukapheresis as per their standard of care procedures defined by the institutional guidelines to obtain lymphocytes with unique patient identifiers for the subsite institution. Each subsite institution will have the KCI Standard of Care leukapheresis operating procedure for reference. Shipping and handling of the product is found in Appendix IV, which identifies the operating procedures and log forms required for the handling of the product.

6.3 Her2Bi-armed Activated T Cells (Armed ATC). Severity of toxicities associated with Her2Bi-armed ATC has been minimal (***see Table 4, Section 2.4.2.3***) and symptoms have been responsive to interventional therapy with antihistamines and analgesics. The most common side-effects are chills and headache. Other reported side-effects included mild gastrointestinal (nausea) symptoms and backache. Adverse symptoms associated with cryopreservative (i.e. DMSO) accounts for approximately 50% of all infusion-related side-effects. DMSO-related side effects include fever, nausea and fatigue. No fatalities have occurred as a result of receiving Her2Bi-armed ATC infusions; however, one patient at dose level 3 (20×10^9 Her2Bi-armed ATC per infusion) who experienced grade 4 hypertension and headache was removed from the study after receiving only 3 infusions (65.7×10^9 total armed ATC administered). This patient was initially suspected of developing progressive brain metastases based upon diagnostic imaging, but upon surgical follow-up was found to have a right frontal subdural hematoma. Because this condition could not be completely ruled-out as occurring in association with Her2Bi-armed ATC infusions, an adverse event was documented and this patient was removed from the protocol.

6.4 Dose Modification for HER2Bi Armed-ATC Toxicity. All patients with treatment related, grade 4 non-hematologic toxicity will be removed from protocol. On the cardiac evaluation after chemoT, the ECHO or MUGA should meet the entry criteria (LVEF > 45% by MUGA or ECHO).

With regard to cardiac toxicity during the immunotherapy portion of the protocol, if the left ventricular ejection fraction (LVEF) falls by >10% from the previous value (by MUGA or ECHO) following HER2Bi armed-ATC, further therapy will be withheld and the patient will be removed from protocol treatment. If there is persistent (lasts greater than 24 hrs) grade-3 toxicity (non-hematologic) at any time, treatment will be held until toxicity improves to grade 0 or 1. The same dose of armed-ATC will be washed to eliminate DMSO and protein. If washing does not reduce toxicity, then the treatment will be resumed with a 50% reduction in the armed ATC for all subsequent doses in that course. If a persistent grade 3 toxicity occurs again, the armed ATC infusions will be stopped. Toxicity will be assessed during the entire interval between each infusion and 7 days after the last infusion (Note each subsite will be provided with the SOP document for cell washing).

6.5 Retreatment Criteria. Patients removed from the study due to toxicities may be re-entered upon complete resolution of toxicity, providing the toxicity appears to be unrelated to immunotherapy. Re-

entry criteria are the same criteria required for initial treatment. Patients re-entered into the study will continue with their therapy at the same point at which it was discontinued, however, these patients will be considered off-study and data resulting from these patients will be analyzed separately.

6.6 Granulocyte-Macrophage-Colony Stimulating Factor (GM-CSF). Therapeutic Classification: Colony stimulating factor.

Known Side Effects and Toxicities: Patients receiving GM-CSF (Leukine-Sagramostim) have experienced fever 60-90 min after administration (duration 1-4 hrs); chills; nausea; vomiting; diarrhea; fatigue; weakness; headache; decreased appetite; thrombosis; rapid or irregular heartbeat or other heart problems; feeling of faintness; facial flushing; pain in the bones, muscles, chest, abdomen, or joints; local reaction at the site of injection; rashes; and kidney and liver dysfunction. Eosinophilia or other blood component abnormalities may occur. There have been infrequent reports of fluid accumulation or worsening of preexisting fluid accumulation in the extremities, in the lungs, and around the heart which may result in breathing problems or heart failure. Rarely, patients have developed acute allergic reactions. There have also been reports of low blood pressure, hypoxia, transient loss of consciousness, and difficulty in breathing after the first injection of Sargramostim. These signs may or may not recur with additional injections of Sargramostim. Patients with prior heart, lung, kidney, or liver problems may have worsening of their symptoms following administration of Sargramostim. There may be other side effects that could occur.

6.7 Potential risks from optional exploratory PET/CT scans. The imaging study involves exposure to radiation from an injection of a radioactive sugar (FDG) for the FDG-PET/CT scans. The radiation exposure is equal to a uniform whole-body exposure of approximately 8 mSv (a measure of radiation exposure) for each PET/CT scan. Patients completing the full study will receive approximately 16 mSv radiation exposure from all two (2) FDG-PET/CT scans. This is about 32% of the allowable annual dose of 50 mSv for radiation workers (for example, x-ray technicians). The risk from this level of radiation exposure is too small to be measured and is small when compared with other everyday risks.

6.8 Other less likely risks from PET/CT scans. Patients may feel discomfort from lying still on the enclosed scanning table. Bruising or bleeding may occur at the site of injection of FDG. Infection may occur at the site of injection of FDG, but sterile technique reduces this risk nearly completely. An allergic-type, or other adverse reaction, may occur related to the radioactive drug (FDG). Topical lidocaine may be used for the local anesthetic. This can cause hives, itching, and localized swelling.

7.0 DRUG FORMULATION

7.1 Proleukin (IL-2) is commercially supplied by Novartis as a sterile, white to off white, preservative-free, lyophilized powder. Each vial contains 22 million IU of proleukin. Proleukin does not contain a preservative and it can not be reconstituted with any bacteriostatic agents. Proleukin should not be filtered. It should be gently swirled during reconstitution, as shaking or filtering may cause the proteins to denature. IL-2 is used to maintain T Cell growth and is not given to the patient.

7.2 OKT3. This is a murine IgG2a monoclonal antibody directed at human CD3 commercially available from Janssen Biotech, Horsham, PA. It is purchased in vials containing 5 mg/5 ml of reconstituted bacteriostatic water. OKT3 is used to activate T cells for growth and for heteroconjugation with Herceptin® to produce the Her2Bi bispecific antibody for arming patient ATC.

7.3 Anti-HER2/*neu* monoclonal antibody (Herceptin®, Trastuzumab; Genentech, Inc., CA). Herceptin is a humanized murine monoclonal antibody directed at HER2/*neu*, and is commercially

available in multi-dose vials containing 440 mg of drug. For this study, Herceptin® is heteroconjugated to OKT3 to produce the Her2Bi bispecific antibody for arming patient ATC.

7.4 Anti-CD3 (OKT3) x anti-HER2/*neu* (Herceptin) heterconjugated bispecific monoclonal antibody (HER2Bi). Anti-CD3 x anti-HER2 is produced under GMP conditions. IND #9985 was cleared for clinical trials by the FDA that specifies the production of bispecific antibody, sterility testing, and the standard operation procedures for arming of activated T cells.

7.5 Granulocyte-macrophage Colony Stimulating Factor. GM-CSF (rhu -GM-CSF, sargramostim) is a 127 amino acid glycoprotein produced in a yeast (S. cerevisiae) expression system. The drug is supplied by sanofi-aventis U.S. LLC. GM-CSF is a hematopoietic growth factor that stimulates the proliferation and differentiation of hematopoietic progenitor cells. GM-CSF is provided as a lyophilized powder in glass vials, and is suitable for parenteral administration following reconstitution with 1 ml sterile water for injection, USP without preservative. Active Ingredient: rhu GM-CSF 250 µg or 500 µg with a specific activity of approximately 5×10^7 U/mg in a normal human bone marrow colony assay. Inactive Ingredients: Mannitol, USP 40 mg, Sucrose, USP 10 mg, and tromethamine, USP 1.2 mg. Syringe: 14 day stability refrigerated. Minibag: (50cc NS/D5W) 48 hour stability refrigerated.

8.0 PREPARATION OF HER2Bi ARMED ACTIVATED T CELLS (SOPs will be provided to the sub-sites for collection and shipping of leukapheresis product. All steps i.e 8.2, 8.3. 9.4 will be performed under GMP conditions at KCI and product will be shipped to sub-sites per SOP)

8.1 Facility. Qualified personnel who are familiar with procedures which minimize undue exposure to themselves and to the environment will undertake the preparation, handling, and safe disposal of immunotherapeutic agents in a self-contained protective environment. Cells will be generated in the Cancer Immunotherapy Laboratory at KCI under FDA #BB-IND 9985 with standard operating procedures (SOPs) for growing, splitting, harvesting, arming of ATC, and cryopreservation, and infusion of armed ATC.

8.2 Activation, Culture, and Freezing of Armed-ATC. Lymphocytes are obtained by pheresis and cultured per the standard operation procedures for producing the cell product.

8.3 Preparation of anti-CD3 x anti-HER2 (Her2Bi) Bispecific Antibody. The specific details for the production, purification, and quality control testing are part of IND #9985.

8.4 Arming of ATC with HER2Bi. The harvested ATC will be counted and a dose of 50 ng of HER2Bi per million ATC will be added to the solution and incubated for 1 hr at 4^{o}C. The armed ATC will be washed, counted, and re-suspended in the final solution that will be cryopreserved in aliquots specific for each infusion.

8.5 Cytotoxicity Assay. Cytotoxicity is measured in a 20 hr ^{51}Cr -release assay to ensure activity (minimum ≥ 10% cytotoxicity) of armed ATC over their unarmed counterparts. Tumor target cells are plated in a flat-bottomed microtiter plate and incubated at 37^{o}C. The targets are washed and labeled the next day with ^{51}Cr at 37^{o}C. These wells containing tumor cells will be washed and armed or unarmed ATC will be added at different E:T ratios for 20 hr incubation at 37^{o}C. The next day, the supernatants harvested from the microtiter wells will be counted and the percent specific lysis will be calculated.

8.6 Quality Assurance of Armed ATC Cell Product. Lists of suppliers of monoclonal antibodies, heteroconjugation reagents, and culture reagents will be maintained as well as lot numbers used and

supplier-provided documentation of sterility and documentation that all reagents are free of endotoxin and mycoplasma. The final bag of cells to be administered will be tested for anaerobic and aerobic bacteria, fungus, and mycoplasma, and the level of endotoxin. The infusion rate will be based on the level of endotoxin in the cellular product. Records of all quality control measures will be maintained by the KCI laboratory.

9.0 REGISTRATION AND REQUIRED DATA

9.1 Pre-Study/Registration/Data Submission. All patients enrolled will be evaluated, consented, and meet all eligibility criteria, prior to leukapheresis. . Eligibility criteria will be assessed as stated in section 4.0. Baseline studies will be completed within 6 weeks prior to registration.

9.1.1 Pre-Study/Registration

9.1.1.1 Patients must be registered prior to initiation of leukapheresis(no more than seven working days prior to planned start of treatment).

9.1.1.2 For phone or fax registration, the individual registering the patient must have completed the appropriate study form. The completed form must be referred to during the registration, but should not be submitted as part of the patient data but a copy will be sent to the Clinical Trials Project Manager at KCI. The individual registering the patient must also be prepared to provide the treating institution's name and ID number in order to ensure that the current (within 365 days) date of institutional review board approval for this study has been entered into the data base. Patients will not be registered if the IRB approval date has not been provided or is > 365 days prior to the date of registration.

9.1.1.3 Registration procedures: Registration by phone of patients from member, affiliate and subsite institutions must be done through the Karmanos Cancer Institute by telephoning 313-576-8506 or via fax: 313-576-8767 8:00 AM to 4:00 PM-Eastern Standard Time, Monday through Friday, excluding holidays.

9.1.1.4 For either method of registration, exceptions will not be permitted.

- Patient must meet all eligibility requirements.
- Institutions must be identified as approved for registration.
- Registrations **may not** be cancelled.
- Late registrations (after initiation of treatment) will not be accepted.

9.1.2 Data Submission

9.1.2.1 Data must be submitted according to the protocol requirements for **ALL** patients registered, whether or not assigned treatment is administered, including patients deemed to be ineligible. Patients for whom documentation is inadequate to determine eligibility will generally be deemed ineligible.

9.1.2.2 Master data collections forms will be provided and must be photocopied for data submission to the Karmanos Cancer Institute Clinical Trials Office. The subsites will receive password protected access to KCI's OnCore website to enter data online. The timeline for data submission will be 14 days from the time the test or procedure was performed (with the exception of baseline testing that may be performed ≤6 weeks prior to registration).

9.1.2.3 Data Submission Procedures: Please select one option for submitting data: fax

or express mail. Data submitted via facsimile should not be followed up with a mailed version. For facsimile submission: Fax to 313-576-8368. For express mail: Please submit one copy of all data forms directly to:

Danielle Smith or Project Manager
Clinical Trial Project Manager
Karmanos Cancer Institute
Clinical Trials Office
4100 John R, Mail Code: HWO4HO
Detroit MI 48201
Phone: (313) 576-8506
Fax: (313) 576-8767

9.1.2.4 WITHIN 3 DAYS OF REGISTRATION, PLEASE SUBMIT THE FOLLOWING:

- Evidence of a diagnosis of breast cancer by pathology; clinical evidence of metastasic or locally advanced, unresectable disease;
- Completed inclusion/exclusion eligibility criteria worksheet including medical history, physical exam, and pre-study tests/exam results;
- Copies of bone scan, CT scan reports and Baseline Tumor Assessment Form;
- A copy of the consent form (including answers to the sample and tissue consent questions) and documentation of the consenting process,.

9.1.2.5 IMMEDIATELY AFTER (i.e. ON THE SAME DAY) OF EACH BLOOD DRAW: Submit to Dr. Lum's laboratory as described in section 9.3.

9.1.2.6 AFTER EVERY CYCLE OF TREATMENT UNTIL DISCONTINUATION AND AFTER RESOLUTION OF ALL TOXICITIES: Submit copies of the Treatment Form and Adverse Event Form documenting required parameters as specified in section 12.0.

9.1.2.7 AFTER EVERY TUMOR ASSESSMENT UNTIL PROGRESSION: Submit a copy of the Tumor Assessment Form.

9.1.2.8 AFTER OFF TREATMENT: EVERY SIX MONTHS UNTIL DEATH OR FOR THREE YEARS AFTER REGISTRATION (WHICHEVER OCCURS FIRST): Submit a copy of the Follow-Up Form.

9.1.2.9 WITHIN 14 DAYS OF PROGRESSION/RELAPSE: Submit a copy of the Follow-Up Form documenting date and site of progression/relapse and the Follow-Up Tumor Assessment Form.

9.1.2.10 WITHIN 14 DAYS OF DISCONTINUATION OF TREATMENT: Submit a copy of the Off Treatment Notice and Final Treatment Form.

9.1.2.11 WITHIN 4 WEEKS OF KNOWLEDGE OF non study related DEATH: Submit a copy of the Notice of Death Certificate documenting death information. Submit a Final Treatment Form and Final Follow-Up Tumor Assessment Form (if patient was still on treatment) or a Follow-Up Form (if patient was off treatment).

9.2 **Screening Assessments.** Pre-study medical history and physical examination will be performed

by one of the protocol investigators. EKG, CXR, bone scan, mammography, tumor measurements, PFT, MUGA or ECHO, laboratory tests (hematology, chemistry, urinalysis, Creatinine Cl, tumor markers) and staging (CT or PET/CT) will be performed, as listed in Section 9.3.

9.3 Tests and Observations.

For the purposes of this study 1 month = 4 weeks (± 1 week) and 1 year = 365 days (± 1 week)

9.3.1 The schedule for testing is summarized in **Appendix 1**. (For shipment instructions to and from Subsites, please see **Appendix 4.0**). The leukopheresis product will be shipped overnight in a continuously monitored insulated shipper from the subsite to Dr. Lum's laboratory in RM 723 Hudson-Webber Cancer Research Center, 4100 John R, Detroit, MI 48201(phone numbers 313-576-8320 or 313-576-8321). The cryopreserved armed ATC product will be shipped frozen in a liquid nitrogen dry shipper to the subsite with continuous monitoring. The specific Log forms and SOPs for the shipping of the leukopharesis product and frozen armed ATC have been incorporated into SOPs for BB IND 9985.

History & Progress note, PE, and performance status will be performed by a protocol investigator at baseline (registration), post-chemoT, 4 weeks post armed ATC#3, prior to and 4 weeks post armed ATC#4. Subsequent visits should be every 8 weeks (+/- 1 week), or as clinically indicated, until 1 year post-armed ATC#4. Then, clinical data collection is recommended every 6 months. These may be completed by the treating physician.

Laboratory studies including CBC with differential, comprehensive chemistry panel w/ magnesium and phosphorus, urinalysis, CrCl, tumor markers (CEA or CA 27.29 or CA 15.3) and liver function tests will be performed at baseline (registration), post-chemoT, 4 weeks post armed ATC#3, prior to and 4 weeks post armed ATC#4. Subsequent labs (except UA and CrCl) should be every 8 weeks (± 1 week), or as clinically indicated, until 1 year post-armed ATC#4. Then, clinical data collection is recommended every 6 months. These may be completed by the treating physician.

CT or PET/CT will be done at the same interval as above.

EKG, CXR, HBsAg/HIVab/HCVab, PFTs, and serum pregnancy test will be performed at baseline registration

MUGA or ECHO will be done at baseline, post-chemoT, and 4 weeks after armed ATC #4, to coincide with the clinic visit.

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Bone scan will be done at baseline, post-chemoT, and as clinically indicated.

Tumor biopsies are optional within 4 weeks post armed ATC#3

9.3.2 Immune and sHER2 studies will be done at pheresis, pre-armed ATC (within 6 days of starting), pre-infusion #3 (within 2 days), 4 weeks (± 1 week) after infusion #3, up to 7 days before infusion #4 (boost), and 4 weeks (± 1 week) post #4. If there are positive findings, additional studies will be done at 3 (± 1 week) and 6 (± 1 week) months after the last infusion of armed ATC and every 6 months as indicated based on new findings.

If clinically or scientifically indicated (determined by treating physician in consultation with Dr. Lum) additional evaluation studies (1-10ml red top tube) may be drawn at any or all of the following time points: pre-infusion, 1, 2, and 4 hours (+/- 15 min), 8 and 16 hours (+/- 1 hr), 24, 48, and/or 72 hours (+/- 2 hr)

after each infusion, to study cytokine release. "Clinically indicated" is based on the clinical judgment of the investigator.

9.3.3 Circulating Tumor Cells: If funds are available, blood for Circulating Tumor Cells (CTC) will be collected pre armed ATC and 4 weeks after infusion #3 for the KCI site only. Blood will be drawn by venipuncture and 8 ml of blood will be collected into a Veridex Cellsave tube that is necessary to preserve CTC's (this tube will replace 1-10ml green top). The Cellsave tubes must be inverted 4-5 times immediately after the blood is drawn. The blood sample should be kept at room temperature and forwarded to the KCI Translational Research Core Laboratory within 24 hours of drawing for further processing.

9.4 **Confidentiality.** All information collected about subjects during the course of this study will be kept confidential to the extent permitted by law. Patients will be identified in the research records by a code number that includes the consecutive patient number on the study and consecutive scan number. Information that identifies patients personally will not be released without written permission. Qualified representatives of the Food and Drug Administration, the National Cancer Institute, Karmanos Cancer Institute, and Wayne State University may review the records without losing the confidentiality of the records. Patient name will not be used in any publications or reports resulting from this study. The Department of Defense may review the records and the results of the [^{18}F]-FDG PET/CT scans which are supported by the U.S. Army Medical Research and Materiel Command without losing the confidentiality of the records.

9.5 **Termination or Deviation.** In the event a protocol is modified, terminated or extended the IRB is notified according to the policies and guidelines of Wayne State University and can be found at www.hic.wayne.edu/hicpol.html. In the event deviation from a protocol occurs the IRB will be notified according to the policies and guidelines of Wayne State University and can be found at www.hic.wayne.edu/hicpol.html.

10.0 Response definition

10.1 Tumor Measurements: All tumors will be measured using the metric system. prior to initiation of therapy. These measurements will consist of the longest diameter and the perpendicular diameter at the widest portion of the tumor and will be made and recorded by the physician or his designee. An estimate of overall objective and subjective response will be made and recorded at the end of treatment. Disease status will be evaluated in the patients and their responses will be confirmed a minimum of four (4) weeks after the first response has been recorded using standard **RECIST 1.1** criteria. Complete Response (CR), Partial Response (PR), Minor Response (MR), Progressive Disease (PD), Stable Disease (SD) or No Change (NC): No change in tumor size(s) and no evidence of progression. Duration of response will be measured from first observation of the response.

10.2 Tumor Biopsies (at KCI only): In order to optimize tissue sampling and minimize the number of biopsies the patients need to undergo, we will focus on obtaining a surgically accessible metastatic tumor within 4 weeks post-armed ATC#3. As described in objective 3, the immune cells will be evaluated for phenotype and function.

10.3 Optional Imaging Analysis Measurements for KCI selected patients: [^{18}F]-FDG-PET/CT Imaging data will be analyzed at Karmanos Cancer Institute. The reproducibility of tumor uptake based on standardize uptake value (SUV) analysis is estimated to be on the order of 10% and a true effect in an individual case can be defined as a decrease in tracer uptake greater than three times the standard deviation of the tracer ([^{18}F]-FDG) uptake without treatment[21],[22-24]. The main variable of interest will include SUVpeak, which includes a circle of about 1 cm diameter. Descriptive statistics will also be generated using SUVmean and SUVmax percent changes

evaluated between scan 1 and scan 2. The response is defined as a decrease in SUVpeak of >=20%. The association between the [^{18}F]-FDG PET/CT assessments (percent changes from baseline in SUVpeak) and immunologic biomarker changes as well as tumor response will be explored.

10.4 Definition of progression-free survival: Progression-free survival is defined as the time from post-ChemoT, pre-aATC staging scans to progression per RECIST, new second breast cancer primaries, or to death from any cause without documentation of progression, whichever occurs first.

11.0 CRITERIA FOR REMOVAL OF PATIENTS FROM STUDY

11.1 Disease Progression: All patients deemed healthy enough to receive aATC after ChemoT, regardless of response (or progression) will receive aATC. Patients with documented progressive disease *after* beginning aATC therapy may continue therapy, including the boost and all follow up, if deemed healthy enough to continue; however, the primary endpoint of PFS will have been met. Patients with clinical progression, as defined by increasing tumor markers or any other criteria for which the treating oncologist begins another treatment regimen, will be considered to have met the primary endpoint of PFS on the date of the first new treatment. For patients who progress post IT (after 3 infusions) and begin another antineoplastic treatment, the course of ChemoT must not be given within 2 weeks prior to and after any aATC infusion. Patients receiving aATC after such progression must be healthy enough to receive the aATC infusion(s) and the treating physicians must deem that it is reasonably safe for the patients to hold their other anti-neoplastic therapy for aATC administration. Otherwise, patients will withdraw from study treatment, but may still be followed for secondary endpoints (such as immune evaluations and overall survival).

11.2 Her2Bi-armed ATC: Patients must receive a minimum of 60% (*i.e.* at least 48 billion Her2Bi-armed ATC) of the total infusion dose (80 billion Her2Bi-armed ATC) to be considered evaluable for the primary endpoint PFS. Patients receiving less than 60% of the total dose may complete the treatment regimen. If the total dose of armed ATC produced is 60 billion or less, the number of infusions will be fixed at four and the cells will be aliquoted equally. Due to counting reproducibility, and loss of cells on freezing and thawing, we expect a potential loss of up to 20% upon thawing and infusions. Therefore, if enough cells are available, up to 10-20% more cells will be aliquoted in a freezer bag for infusion.

11.3 Extraordinary Medical Circumstances: If at any time the constraints of this protocol are detrimental to the patient's health, the patient shall be withdrawn from treatment.

11.4 ChemoT Toxicity: Patients will be removed from study if chemoT toxicities that would potentially exacerbated infusional toxicities reported for armed T cells (the immunotherapy team will evaluate and discuss these toxicities before proceeding).

11.5 Patient decision to discontinue therapy

11.6 Protocol violation: Unexplained delay in delivery of treatment.

11.7 Grade 4 non-hematologic treatment related toxicity

11.8 Unexpected or Life-Threatening Toxicity:

11.8.1 Questions regarding drug therapy will be directed to the Principal Investigator.

11.8.2 Reporting requirements and procedures depend upon: (1) whether agents are suspected of causing toxicity, (2) whether possibility of such a toxicity was reported in the consent form, or manufacturer's literature (Published Toxicity), (3) the severity or grade of the toxicity.

11.8.2.1 Expected toxicities: Grade 4 myelosuppression will be reported only as part of regular data submission. All other toxicities, Grades 3-5 will be reported to Principal and Co-Investigators within 7 calendar days. All other toxicities, Grades 1 to 2 will be reported only as part of regular data submission.

11.8.2.2 Unexpected Toxicities: Unexpected toxicities are toxicities that are not listed in the toxicity management section of the protocol, the consent form, or the manufacturer's package insert. All unexpected toxicities will be reported to Principal Investigators at the site and PI at KCI (coordinating center), within 7 calendar days. The PI at KCI will inform other site PIs about unexpected toxicities.

12.0 REPORTING ADVERSE REACTIONS

12.1 Investigators will notify the **FDA and IRB** of all serious and unexpected adverse reactions. All Adverse reactions will be graded based on CTCAE v3. All adverse reactions will be reported to the subsite IRB and the KCI Clinical Trial Project Manager. The KCI Clinical Trial Project Manager will provide centralized reporting to the WSU IRB and Dr. Lum (or his designee) will be responsible for centralized reporting to the FDA.

12.2 All reactions in a 'reportable' category will be reported unless it is documented in the medical record chart that treatment is definitely **not** responsible for the toxicity.

12.3 Serious and unexpected adverse reactions will be reported to the following sources within the stated time frame.

12.3.1 FDA: Written IND safety report (MedWatch 3500A) within 7 calendar days. Report by telephone within 3 working days. SAE reports will be included in annual reports.

12.3.2 All participating investigators: Written IND safety report within 7 calendar days.

12.3.3 WSU IRB: Written report within 5 working days.

12.4 Procedure for calling the FDA is as follows:

12.4.1 The research nurses or PI will call for ADRs.

12.4.2 The PI or his designee will call for regulatory/protocol issues.

12.4.3 The FDA contact is:

Mark Davidson
Center for Biologics Evaluation and Research
Food and Drug Administration

Division of Biological Investigational New Drugs
1401 Rockville Pike HFM 99
Rockville, MD 20852
Phone: 301-827-5358
Fax: 301-827-9796

13.0 Measurement of Functional and Phenotypic Changes in Immune Populations: To measure functional and phenotypic changes in immune cell populations as a consequence of immunotherapy. Cytokine responses, phenotypic markers of differentiation (*i.e.* CD45RA/RO) and specificity, and anti-tumor cytotoxicity will be examined. The specific procedures are well-described in the literature. The subsequent paragraphs summarize the strategy and procedures.

13.1 Serum cytokine/chemokine levels. Levels will be quantified using the Bio-Plex Protein Array system. Data analysis will focus on those factors known to be important regulators of T cell responses, such as IL-2, IL-4, IL-7, IL-10, IL-12, IL-15, IFN-γ, and TNF-α. Serum concentration of each factor, at the indicated study points, will be compared relative to pre-IT serum samples.

13.2 PBMC subsets and phenotype analysis. Peripheral blood mononuclear cell (PBMC) and tumor-infiltrating lymphocyte (TIL) samples will be stained for flow cytometric analysis. We will compare proportions of T, B, NKT and NK cells at during and post-armed ATC to proportions of those subsets pre-armed ATC. T cell proportions would be further analyzed by subset (CD4, CD8, CD25+).

13.3 Cytotoxic activity and/or IFNγ production in PBMC populations. PBMC and TIL will be tested in specific cytotoxicity and IFNγ EliSpot assays after exposure to SK-BR-3 (HER2+ BrCa) or Daudi (HER2- Burkitt's lymphoma). Specific activity of pre-armed ATC samples will be compared to those obtained during and post-armed ATC for significant increases in percent cytotoxicity and frequency of IFNγ-producing cells as indicators of armed ATC-induced *in vivo* immune functions.

13.4 Blood Draws for immune studies. Immune studies will be done as described earlier in section 9.3.2. If there are positive findings, additional studies may be done beyond a year. The cytotoxicity studies will be repeated yearly if the findings remain positive. A total of no more than 70 ml of blood will be drawn at any time point.

14.0 STATISTICAL CONSIDERATIONS

Patients will be restaged after chemoT using RECIST 1.1 criteria and divided into (1) chemo-responders and (2) chemo-non-responders. All patients given armed ATC will be analyzed for PFSas a primary endpoint after they have received the proposed combined treatment. Further analyses by disease status based on their chemo-responses will be performed at the end of the study.

14.1 Statistical Analysis for Phase II (Objectives 1.1 and 1.2). HER2(0-2+) metastatic BrCa patients will be enrolled. Eligible patients will receive combined chemotherapy with Her2Bi-armed ATC. To evaluate the efficacy of the proposed combined therapy, PFS will be investigated as the primary endpoint from the time of the first aATC infusion. All patients will be restaged after ChemoT, Results will be compared to published trials reporting on chemotherapy alone in patients matching the same eligibility criteria. Published trials on chemotherapy alone reported a median PFS of around 1.6 months when calculated from completion of 3-cycles of chemotherapy without any additional treatment[24] For Patients with HER2 0-2+, we estimated their median PFS would around 2 month under traditional treatment (estimated 4-month PFS was 25%). We expect that the additional armed–ATC treatment given after traditional chemotherapy will improve the median PFS by 2 months (estimated 4-month PFS of 50%). Since fewer toxicities were observed from armed ATC treatment, and potential benefit was shown in phase I study, we consider to use one-stage design test a 4-month ***PFS*** ≤25% versus a 4-month ***PFS*** ≥ 50%. A total of 26 evaluable patients (must have received chemotherapy and 3 out of 4 armed T cell infusions) will be accrued in this study. Not evaluable patients will be replaced. For a small sample size, this study is deigned to have a power higher than 90% by accepting a maximum type I error rate of 20%. With a total of 26 patients, a cut-off success number (the number of patients without progression by the 4-month follow-up) to reject the null hypothesis was set as 9 patients for which it would gain a power of 96% (>90%) at an alpha level less than 20% (actual value=18%). By the end of the trial, if 9 or more patients have not progressed by the 4-month follow-up, we can declare that armed-ATC treatment is effective on PFS. As a secondary endpoint, overall survival (OS) among all patients will be investigated. OS will also be measured from the time of first aATC infusion. The Kaplan-Meier method will be used to estimate PFS and OS. This phase II study will also provide toxicity profile. This number of patients will provide two-sided 95% confidence intervals for the estimates of the true toxicity rate and immune response rate within a range of ±19.2%.

In addition, PFS and OS will be examined among chemo-responsive patients (chemo-responders, including patients with CR, PR, and stable disease) separately from the patients who show evidence of progression on chemotherapy at the end of this study. Based on the estimated median PFS of 2 months for chemoT alone treatment, we expect that there will be around 12 chemo-responders among 26 patients at the time of restaging after chemoT. We expect that chemo-responders will have an even longer PFS (from the time of the first aATC infusion) after receiving armed ATC than non-chemo-responders and we assume that these chemo-responders will improve their median PFS to 6 months (estimated 4 month PFS was 63%). Twelve chemo-responders will have 83.4% power to detect the difference of 4-month PFS between 25% versus 63% with two-sided significance level of 0.05. For such a small sample size, we would consider a higher type I error rate (maximum alpha=20%) for a power higher than 90% to set a cut-off success number. Among the twelve chemo-responsive patients, if five or more patients won't progress by 4-month follow-up, we can declare that chemo-responsive patients with IT would have a longer PFS. For chemo-non-responsive patients, armed ATC response rate and OS will be explored as well as chemo-responders. The patients who receive GM-CSF will be analyzed together and separately from the other patients. Since the numbers will be too small to determine differences in the clinical endpoints for the group that will receive GM-CSF, we will evaluate whether GM-CSF injections increase immune responses in the blood as outlined in Statistical Analysis for Objective 1.4.

14.2 Statistical Analysis for Objective 1.3: This phase II study will also provide toxicity profile. This

number of patients will provide two-sided 95% confidence intervals for the estimates of the true toxicity rate and immune response rate within a range of ±19.2%.

14.3 Statistical Analysis for Objective 1.4: The functional and phenotypic changes in immune cell populations (blood and tumor sites, if accessible) as a consequence of armed ATC will be investigated. The cytokine responses, phenotypic markers of differentiation, specificity, and anti-tumor cytotoxicity will be compared between baseline and pre-armed ATC treatment and between baseline and after armed ATC in the blood and in the tumors (if accessible) among all 26 patients. **Serum cytokine responses** will be quantified focus on the levels of those factors known to be important regulators of T cell responses, such as IL-2, IL-4, IL-7, IL-10, IL-12, IL-15, IFN-γ, and TNF-α. **Phenotype analysis** will measure the percent of T, B, NKT and NK cells in peripheral blood mononuclear cell (PBMC) and tumor-infiltrating lymphocyte (TIL) samples. T cell proportions would be further analyzed by subset (CD4, CD8, CD25+). **Cytotoxicity** is measured in percentage in PBMC and TIL. Assuming these immunologic changes are independent, with 80% power and a two-sided 0.05 significant level, a total of 26 patients will detect a difference of about 0.55 standard deviation (a medium effect size) in the levels of cytokine, percent of certain phenotypic markers of differentiation, specificity, and cytotoxicity between baseline and after armed ATC[25]. For above immune measurements, means, standard deviations, medians will be calculated and distributions of data will be examined to ascertain whether normal theory methods are appropriate or whether transformation of data will be required. Paired t-test or Wilcoxon sign rank test will be used to compare the difference between baseline and after T reg depletion, and between baseline and after armed ATC in each of above immune measurements. For exploratory purpose, we will also perform these comparisons in chemo-responsive patients and chemo-non-responsive patients separately.

14.3 Statistical Analysis for Objective 1.5: The association between armed ATC-induced changes in immune functions (monitored in objective 1.4) and clinical endpoints will be analyzed. The clinical endpoints include tumor response, PFS, and OS. Tumor response is categorized into five levels including complete response, partial response, minor response, no change**,** and progressive disease. For measuring the strength of correlation between tumor responses and each quantitative variable of innate immune functions, Spearman correlation coefficients will be calculated. For power estimation, the conventional effect size is based on the correlation coefficient.[26;26] With a sample size of 26 patients, the study will have an 80% power to detect a correlation coefficient of 0.48 (a large effect size) at a two-sided 5% significance level. Thus, the study should be able to detect meaningful correlation between the changes in immune functions and tumor responses. To further examine the association between the changes in immune functions and the tumor response (complete/partial response versus others), multivariable logistic regression model will be used. To test the association between the immunologic changes and PFS or OS, multivariable Cox proportional hazard regression model will be applied. Due to small sample size, however, patients' clinical characteristics (such as age, stage, grade, Her2/neu expression, estrogen and progesterone receptor status, and size of primary lesion) can be considered to be adjusted for separately in above multivariable regression analyses. To gain more information, similar data analyses will be explored in chemo-responders and chemo-nonresponders separately.

14.4 Statistical Analysis for Objective 1.6: The correlation between the [^{18}F]-FDG PET/CT assessments (percent changes from baseline in SUVpeak) and immunologic biomarker changes as well as tumor response will be explored in select patients. Spearman correlation coefficients will be calculated. No power estimation for this exploring objective.

14.5 Accrual of Patients and Duration of Study: A total of 70 patients will be consented to obtain 26 evaluable patients to achieve the statistical design requirements. This takes into account patients deemed as screen failures whom are not enrolled as well as patients whom are not evaluable (i.e., failure to complete all chemotherapy and at least 3 aATC infusions), Depending upon the detection of

toxicities and clinical responses, the duration of the study will be 4 years with minimum of 1 year follow-up on status of the patients. The immune follow-up of study subjects may be extended based on the presence of immune changes as detailed in Appendix 1. The immune function studies may continue on patients who have positive immunologic findings beyond the 4th year of the study. Patients may continue to give blood even if they have progressed with their disease.

15.0 DATA SAFETY MONITORING. The Data and Safety Monitoring Committee (DSMC) provides the primary oversight of data and safety monitoring at Karmanos Cancer Institute (KCI) for Investigator-initiated trials.

15.1 The Investigator will schedule monthly meetings or more frequently depending on the degree of risk encountered by study participants. These meetings will include the protocol investigators and research staff involved with the conduct of the protocol.

15.2 During these meetings the investigators review all aspects of the trial conduct related to:

- Safety of protocol participants
- Evaluate compliance with requirements regarding the reporting of AEs.
- Validity and integrity of the data
- Enrollment rate relative to expectation of target accrual
- Retention of participants, protocol compliance (protocol violations and deviations)
- Data completeness and source documentation

These monthly meetings are recorded on the data and safety monitoring report forms and submitted for review on a quarterly basis to the DSMC.

The Data Safety Monitoring Board (DSMB) of Karmanos Cancer Institute will perform comprehensive reviews of IND clinical trials on a semiannual basis. The Study Monitor must be familiar with the protocol, the investigational product, written informed consent, Standard Operating Procedures (SOPs), Good Clinical Practices (GCPs), and Applicable Regulatory Requirements. Monitoring will be performed at six month intervals at the Sponsor's discretion, but not less than twice in a 12-month period. The Chair of the DSMC will also serve as the Medical Monitor as required by the Department of Defense.

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Appendix 1: Time Table for Testing

Study Calendar	**Pre-Chemo**	**Pheresis**	**Post-Chemo T**	**aATC #1 (day 0)**	**aATC #2**	**Pre-aATC #3**	**aATC #3**	**4 Wks Post-aATC #3**	**Pre-aATC #4**	**aATC #4b (12 wks Post-aATC #3)**	**1 Month Post-aATC #4**	**3 Month Post-aATC #4**	**5 Month Post-aATC#4**	**6 Month Post-aATC#4**	**7 Month Post-aATC #4**	**9 Month Post-aATC #4**	**1 Year Post-aATC #4**
Window	≤ 6 Weeks					≤ 3 days		± 1 week	≤ 7 days		± 1 week	± 1 week	± 1 week	± 1 week	± 2 week	± 2 week	± 2 week
H&P and PE	Xa		Xa					Xa	Xa		X	X	X		X	X	X
KPS	X		X					X	X		X	X	X		X	X	X
CBC w/diff, Chem panel, LFT	X		X					X	X		X	X	X		X	X	X
Urinalysis & CrCl / OK to calc	X		X					X	X		X						
CEA or CA27.29 or CA 15.3	X		X					X	X		X	X	X		X	X	X
CT, MRI, or PET/CT - CAP	X		X					X	X		X	X	X		X	X	X
Tumor Measurements	X		X					X	X		X	X	X		X	X	X
Immune Studies and sHER2		X		Xc			Xc	X	Xc		X	Xf		Xfg			Xfg
Pregnancy Test	X																
HBsAg / HIVab / HCVab	X																
EKG	X																
CXR	X																
PFT	X																
MUGA or ECHO	X		X								X						
Bone Scan	X		X														
CT - Head	X																
FDG-PET/CT (select pts at KCI site only; optional)			Xj			Xe											
Tumor Biopsy								(X)									
CTC (d)				Xc				X									
Pheresis		Xh															
aATC Infusion				X	X		X			X							
GM-CSF (Twice/week)n				X	X		X										

a:To be performed by one of the investigators. Exceptions will be made for non-local patients.

b:12 weeks post aATC#3

c:Drawn in AM / prior to aATC

d:If funds: draw & send to KCI Translational Core w/i 24 hours. Note: The CTCs will be collected only at KCI.

e:≥3d post aATC#2/prior ATC#3

f:If previous positive findings

g:Then every 6 months

h: After all eligibility is complete and patient is formally registered to the study and with the coordinating site (KCI).

j: Within 7 days prior to aATC#1 at the KCI site only

m:Send to Dr. Lum's lab @ KCC

n: GM-CSF injections 250 μg/m^2 twice weekly starting 3 days prior to the first aATC infusion and ending on the last aATC infusion

Appendix 2: PET Imaging Acquisition Parameters and Image Data Analysis

PET Imaging Acquisition Parameters and Image Data Analysis
(Detailed criteria/specifications for performance of PET scanning in the study)

Additional information for PET Imaging Acquisition Parameters and Analysis are available on the ACRIN web site at (www.acrin.org/6678_protocol.aspx). For more detailed information, send an email to petcorelab@acr-arrs.org.

Acquisition and Analysis of FDG-PET/CT Scans

FDG-PET/CT scans will be performed according to the guidelines for NCI-sponsored studies as recently published by Shankar et al. [1].

1. Participant Preparation

Participants must fast for a minimum of 4 hours prior to the injection of FDG for the PET scan. However, they will be encouraged to drink water to ensure adequate hydration.

- Upon arrival at the PET facility, the participant's weight and height will be measured and recorded. Serum glucose should be measured to determine that the blood glucose concentration is within the normal range.
- If the serum glucose concentration is found to be greater than 150 mg/dL, the study should be rescheduled. The referring oncologist or the primary physician of the patient will be contacted to optimize blood glucose control.
- The participant should be placed in a comfortable position, either supine or semi-recumbent. A large-bore intravenous line (21-gauge or greater) should be placed in an arm or hand vein. The room should be kept warm to avoid shivering and temperature effects that may increase muscular or fat uptake. The participant should move as little as possible and should not talk more than necessary in the first 30 min following FDG injection.
- Prior to positioning the participant on the PET scanner the participant should be asked to urinate.

2. Injection of [^{18}F] Fluorodeoxyglucose (FDG)

- The dose of FDG to be administered should be 10 to 20 millicuries (mCi), adjusted according to weight as suggested by the scanner manufacturer.
- FDG will be synthesized and prepared in accordance with USP compendial standards.
- The exact time of calibration of the dose should be recorded and the exact time of injection noted to permit correction of the administered dose for radioactive decay. In addition, the dose remaining in the tubing or syringe, or that was spilled during injection should be recorded. The injection should be performed through an intravenous catheter.

3. FDG-PET/CT Imaging

- PET scanning must begin 60 ± 10 minutes after FDG injection. The time between injection and the start of the PET scanning for the second and third scans should be matched as closely as possible to that for the first scan (less than 10 min difference in uptake times).
- Participants will generally be positioned in the PET/CT scanner with their arms raised above the head. If participants cannot tolerate this position for the duration of the PET/CT study, a different participant positioning may be chosen. However, arms should be positioned in the same way at the baseline and the follow-up studies.
- A low-dose CT scan will be acquired for attenuation correction and anatomical localization of findings in the PET scan.
- The acquisition parameter for the low-dose CT scan for attenuation correction should be: kV = 120; effective mAs = 30-80 (patient dependent); gantry rotation time ≤ 0.5 sec; maximum reconstructed width = 3-5 mm without overlap; standard reconstruction algorithm, minimum reconstruction diameter = outer arm to outer arm; and without iodinated contrast [2].
- The axial field of view of the CT scan for attenuation correction will range from the mid thighs to the base of the skull. Arm positioning will be the same as for the PET scan, typically above the head.
- The CT scan will be performed during "shallow breathing" as described previously [3]. No respiratory gating will be applied.
- After the CT scan, a PET scan covering the same axial field of view will be performed. This scan will start at the mid thighs. The number of bed positions and the acquisition time per bed position will be scanner specific. Typical parameters are 6 bed positions and an acquisition of 2-5 min per bed position.

4. Image Reconstruction

- The PET data will be corrected for dead time, scatter, randoms and attenuation using standard algorithms provided by the scanner manufacturers.
- Image reconstruction will be performed as specified in the ACRIN certification of the PET/CT scanner.

5. Blinded Central Image Analysis

- Activity concentrations in the attenuation-corrected PET images will be converted to standardized uptake values (SUVs) by dividing the activity concentrations by the decay-corrected injected dose and multiplying with the body weight of the participant.
- The intrathoracic lesions with the highest FDG uptake in the pre-chemotherapy PET scan (scan 2 in group A and scan 1 group B) will be analyzed in order to determine the metabolic response of the participant (primary endpoint).
- A circular region of interest (ROI) with a diameter of 0.75 to 1.5 cm will be centered at the site of maximum FDG uptake within this lesion. ROIs of the same size will be placed in the slices immediately above and below, at the same transverse location.
- The average SUV within the volume encompassed by these three ROIs will be determined and recorded. This approach for definition of ROIs has been successfully used for assessment of tumor response in patients with advanced NSCLC [4] and a variety of other malignant tumors [4-11]. In total these studies include more than 300 patients and more than 600 PET scans. As part of the exploratory analyses, the maximum SUV and glucose-corrected average and maximum SUVs [12] will be recorded and analyzed.
- In most patients the primary tumor will show the highest FDG uptake. However, in a small subgroup of participants the primary tumor may be small (< 2cm) and show only low FDG uptake in PET or it may have been resected in participants with recurrent NSCLC. In this case, the intrathoracic metastatic lesion with the highest FDG uptake will be used for analysis.
- As part of an exploratory analysis, ROIs will be placed in the same way in up to 6 metastatic lesions. In participants with more than 6 metastatic lesions in several organs a maximum of 3 lesions should be in the same organ. In each organ, the lesions with the highest FDG uptake will be selected for analysis.
- For quality control purposes, a large circular ROI (diameter >= 5 cm) will be placed in normal liver tissue. The mean SUV in this ROI will be recorded.
- In the case of multiple liver metastases, it may not be feasible to place one large ROI in normal liver tissues. In this case, several small ROIs, including the same number of pixels as one 5-cm ROI, may be placed in normal liver tissue. FDG uptake within these lesions will be averaged and used for further analysis.
- If the mean SUV within the liver changes by more than 1.0 between two scans, the SUV calculation will be checked for errors. Specifically, the scanner cross calibration, the decay correction of the injected activity, and the participant's body weight will be checked (see checklist below for details)
- Previous studies have shown that this approach for quantitative analysis of tumor FDG uptake is highly reproducible (9). Nevertheless, quantitative analysis will be performed independently by two observers in order to minimize random errors in SUV measurements. Discrepant findings will be discussed and resolved by consensus.

6. Local Image Interpretation

- Describe the location of the primary tumor and of metastatic lesions.
- Measure and record the maximum SUV in the primary tumor. If maximum SUV is less than 4.0, contact ACRIN Image Management Center (IMC) to determine whether the participant should remain in the study or not.
- If the diameter of the primary tumor is less than 2 cm or the primary tumor demonstrates only low FDG uptake, measure the maximum SUV of the lesion with the highest FDG uptake in the chest.
- Measure and record the mean SUV in the liver as described in Section 5 above.
- Check image quality according to the checklist below.

7. SUV Calculations

7.1 Time of Injection and Scan Start Time

7.1.1 Data Correctly Recorded and Entered?
Check whether the time of injection and the scan start time have been correctly recorded. If the PET scanner software performs decay correction for the time interval between injection and imaging, check whether the time of injection and the start time of the scanner have been correctly entered.

7.1.2 Data correctly recorded and entered: Proceed to 7.2

7.1.3 Injection Time Missing

7.1.3.1 Time between injection and start of scan known: Record time between injection and start of scan and proceed to 7.2

7.1.3.2 Time between injection and start of scan unknown: Record a protocol violation and try to repeat the scan. If the scan cannot be repeated, the participant goes off study.

7.1.4 Scan Start Time Missing

7.1.4.1 Time between injection and start of scan known: Record time between injection and start of scan and proceed to 7.2

7.1.4.2 Time between injection and start of scan unknown: Record a protocol violation and try to repeat the scan. If the scan cannot be repeated, the participant goes off study.

7.2 Is the Time between FDG injection and Start of the PET Emission Scan within the Specifications of the Protocol?

7.2.1 All Scans (Baseline and Follow-up Scans)

7.2.1.1 Time between injection and start of PET scan within 50-70 min: If it is a baseline scan, proceed to 7.3. If it is a follow-up scan, proceed to 7.2.2

7.2.1.2 Time between injection and start of PET scan >=45 and < 50 min: Record a protocol variation. Participant remains in the study.

7.2.1.3 Time between injection and start of PET scan >70 min and <= 80 min: Record a protocol variation. Participant remains in the study.

7.2.1.4 Time between injection and start of PET scan < 45 min: Record a protocol violation and try to repeat the scan. If the scan cannot be repeated, the participant goes off study.

7.2.1.5 Time between injection and start of PET scan > 80 min: Record a protocol violation and try to repeat the scan. If the scan cannot be repeated, the participant goes off study.

7.2.2 Follow-up Scans

7.2.2.1 Time between injection and PET imaging differs by 10 min or less between the baseline and the follow-up scan: Proceed to 7.3

7.2.2.2 Time between injection and PET imaging differs by more than 10 min, up to 15 min: Record a protocol variation. Participant remains in the study.

7.2.2.3 Time between injection and PET imaging differs by more than 15 min: Record a protocol violation and try to repeat the scan. If the scan cannot be repeated, the participant goes off study.

7.3 Injected Dose

Has the injected dose been correctly calculated and entered in the header of the PET data set?

7.3.1 Injected dose known and correctly entered: Proceed to 7.4

7.3.2 Injected dose unknown or incorrectly entered: Correct image header information. If injected dose is unknown, but the dose at the time of imaging is known, record this dose and use it for SUV calculations. Proceed to Section 7.4. If the dose at the time of imaging is also unknown, try to repeat the scan. If the scan cannot be repeated the participant goes off study.

7.4 Body Weight

7.4.1 Body weight of the participant correctly recorded and entered into the header of the PET data set: Proceed to Section 8.

7.4.2 Body weight incorrect or unknown: Record a protocol violation. Retrieve body weight from participant chart.

8. Check Fasting State and Blood Glucose Levels

8.1 Participant fasted for > 4 hours: Proceed to 8.3

8.2 Participant fasted for <= 4 hours

8.2.1 Blood glucose levels <= 150 mg/100mL: Participant remains in the study.

8.2.2 Blood glucose levels > 150 mg/100mL: Try to repeat the scan. If the scan cannot be repeated the participant goes off study.

8.3 Blood glucose level <= 150 mg/100mL: Proceed to 9

8.4 Blood glucose level > 150 mg/100mL: Try to repeat the scan. If the scan cannot be repeated the participant goes off study.

9. Measure Liver SUV

(Internal quality control, see Section 5 above for details of this measurement)

9.1 All Studies (Baseline and Follow-up Studies)

9.1.1 Mean liver SUV within 1.5 – 4.0 (expected variability): Proceed to 9.2.

9.1.2 Mean liver SUV < 1.5 or > 4.0: Check scanner calibration and cross calibration of dose calibrator and PET scanner. Are there signs of partially paravenous tracer administration in the images? Record protocol variation, if no explanation for the unusual liver SUV can be found or there is evidence of partially paravenous tracer administration.

9.2 Follow-up Scans

9.2.1 Difference in mean liver SUV between the baseline and follow-up scan less than 1.0: Proceed to 10.

9.2.2 Difference in mean liver SUV between the baseline and follow-up scan greater than 1.0: Check scanner calibration and cross calibration of dose calibrator and PET scanner. Are there signs of partially paravenous tracer administration in the images? Record protocol variation, if no explanation for the unusual liver SUV can be found or there is evidence of partially paravenous tracer administration.

10. Artifacts in the Reconstructed Images

10.1 Beam hardening artifacts in CT

10.1.1 Are there any metal implants or other structures with high density in the chest? No, proceed to 10.1.2

10.1.1.1 Do the implants cause beam hardening artifacts on CT that are visible on the reconstructed PET emission images? No, proceed to 10.1.2

10.1.1.2 Are the beam hardening artifacts overlying the tumor region? No, proceed to 10.1.2

10.1.1.3 Is there an alternative lesion in the chest that could be used for quantitative measurements in PET (e.g. pulmonary metastasis or lymph node metastasis): If yes, use this lesion for quantitative analysis. If no, the participant goes off study.

10.1.2Was the scan acquired with the arms raised above the head? Yes, proceed to 10.2.

10.1.2.1 Are the resulting beam hardening artifacts visible on the PET images and over the tumor region? No, proceed to 10.2

10.1.2.2 Is there an alternative lesion in the chest that could be used for quantitative measurements in PET (e.g. pulmonary metastasis or lymph node metastasis): If yes, use this lesion for quantitative analysis. If no, the participant goes off study.

10.2 Participant Movement

10.2.1 Is there any visible mis-registration between the outer contours of the tumor as seen on CT and the outer contours seen on PET? This is checked on the PET/CT fusion images.

10.2.1.1 If yes, estimate the degree of misregistration by counting the number of slices that the tumor is visible on CT, but not on PET. If there is misregistration by more than 3 slices of the PET scan (about 1 cm), report a protocol variation.

10.2.2 Can the volume of interest for quantitative analysis of the PET scans be placed in an area where PET and CT images overlap? If yes, use this area for quantitative analysis. If no, proceed to 10.2.1.

10.2.3 Is there another lesion that can be used to quantify FDG uptake (see Appendix VI for criteria)? If no, try to repeat the PET scan. If it is not possible to repeat the scan, the participant goes off study.

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CT Acquisition Parameters and Image Data Analysis

Additional Information for the CT Acquisition Parameters and Volumetric Analyses are also available on the ACRIN web site at (www.acrin.org/6678_protocol.aspx). For more detailed information, contact the Image Management Center at 215-940-8820.

Acquisition and Analysis of the CT Scans

The CT scans serve three purposes: (a) attenuation correction and registration with PET scans for anatomic localization of FDG uptake; (b) response assessment using RECIST; and (c) high spatial quality data sets for central volumetric analyses of lung lesions. Depending upon considerations of workflow and the need for intravenous contrast, individual institutions may have different processes for completing the chest CT scans used for RECIST and tumor volumetric assessments. The following are potential scenarios:

- **Single Platform**: fusion PET/CT scanner: All studies are performed on a fusion PET/CT scan, including the PET/CT scanner, and one single inspiratory CT without contrast for both RECIST criteria and tumor volumetry.
- **Single Platform**: fusion PET/CT scanner: All studies are performed on a fusion PET/CT scan, including the PET/CT scanner, an inspiratory CT with contrast for RECIST criteria, and an inspiratory CT without contrast for tumor volumetry.
- **Dual Platforms**: Fusion PET/CT scanner used to perform a PET/CT scan and an inspiratory CT without contrast for tumor volumetry as well as a dedicated, standalone CT scanner to perform a CT with contrast for RECIST criteria.

1. Low Dose CT for PET Attenuation Correction

Parameters for these CT scans are described in Appendix VI (Acquisition Parameters and Analysis of the PET/CT Scans), since the primary purpose of these scans is to correct the PET emission data for photon attenuation.

2. CT Volumetry:
Optional (Participant May Not Undergo Volumetric CT Scans/Decision Left to Clinicians and Participants)

2.1 Image Acquisition

To ensure the level of spatial quality necessary for tumor volumetric analyses, the scanner platforms must be able to perform prospective reconstructions of a single helical sequence at different slice thicknesses. The following are representative PET/CT systems:

- Siemens Biograph PET/CT (CT scanners 6,16, 64-slice); Siemens Medical Solutions | Malvern, PA.
- GE Discovery PET/CT scanners; GE Healthcare Technologies | Waukesha, WI, USA
- Philips Gemini PET/CT scanners (Brilliance CT: 6, 10, 16, 40-slice; 40 and 64 channel); Philips Medical Systems | Andover, MA, USA

Research CT scans for tumor volumetric analysis will be performed on equipment qualified for this trial, and all CT scans for an individual participant should be performed on the same platform throughout the trial. In the rare instance of equipment malfunction, follow-up scans on an individual participant can be performed on the *same type* of platform.

In Groups A and C, the optional CT component of the two baseline (pre-chemotherapy) PET-CT scans will provide a basis for determining the inter-scan precision (reproducibility) of volumetric measurements. In addition, for Group A, changes in tumor volume will be assessed post-cycle 1 in order to assess early changes in tumor volume in response to chemotherapy. In Group B, measurement of tumor volume by CT will be performed at baseline and may occur at both or either timepoint—post-cycle 1 or 2 of chemotherapy. These optional CT scans will allow us to evaluate the time course of changes in tumor volume during chemotherapy.

Each scanner platform has slightly different technical specifications and user inputs for imaging parameters. The following table provides specifications for the acquisition and reconstruction parameters for the CT series performed for tumor volumetry.

Table 1. Parameters for the CT scans used to measure tumor volume.

Parameter	Diagnostic CT Scan for Tumor Volumetry
KV	120
Gantry rotation time	≤ 0.5 sec
Scanner Effective mAs (Regular-large size patient)	100-260
Maximum scan | breath-hold time (40 cm long thorax)	≤ 15 sec
Number of active channels (N)	≥ 6
Maximum Reconstructed slice width	1-1.5 mm
Reconstruction interval	0 – 20% overlap
Reconstruction algorithm*	Standard
Reconstruction diameter (dFOV)	outer rib to outer rib
Intravenous contrast media	None
Arm positioning	Above the head

Parameters for tumor volumetry should provide an in-plane voxel size of 0.55-0.75 mm.

All CT datasets will be transmitted to ACRIN Imaging Management Center for archive and distribution to Image Volumetric Analysis CORE (V-CORE) facilities for volumetric analyses using one or more volumetric software programs.

2.2 Volume Measurements and Image Analysis Core Credentialing

Up to four software programs may be used to provide volumetric measurements of all measurable disease within the lung parenchyma. No attempt will be made to perform manual volumetric analyses of measurable disease in the mediastinum or other anatomic regions of low surrounding contrast. The software programs may include:

- Siemens Leonardo Lung Processing Program; Siemens Medical Solutions (Malvern, PA).
- Lung VCAR software; GE Healthcare (Waukesha, WI)
- R2 ImageChecker CT Lung System; R2 Technology (Sunnyvale, CA)
- Lung Imaging Database Consortium Image Analysis Software; UCLA Thoracic Imaging Research Group

Each software platform will be run as described by the vendor | software developers. Measurable disease on PET/CT scans of all participants will be measured according to the protocol schema using each software system by independent readers blinded to all clinical and imaging information. Measurable lesions will be defined on the baseline (pre-chemotherapy cycle 1) CT scan. The textual description of these lesions will be used to identify them on subsequent scans. Volumetric analyses on subsequent scans will be performed without knowledge of measurements performed at the local sites or the results of FDG PET uptake changes.

All CT image series will be transmitted to ACRIN Imaging Management Center for archive and distribution to distributed CORE facilities for volumetric analyses using one or more of the different software programs.

A distributed V-CORE includes the ACRIN CORE Laboratory as well as individual sites that maintain and have documented expertise in the use of the analysis workstations | software that will perform the tumor volumetry in this trial. One V-CORE laboratory will perform all measurements using a given image analysis platform. Volumetric measurements will be performed only by radiologists certified for protocol 6678; a single reader will analyze images for a given participant at all time points. All readers will be trained on the ACRIN V-CORE SOP and sign off to document training. The expertise of a V-CORE laboratory and its compliance with image transmission, de-identification, and mark-up procedures will require documentation of the following capabilities:

- Ability to send and receive DICOM formatted image data archived at ACRIN Image Management Center (IMC) using sFTP. Sites must be able to receive de-identified data with scrubbed headers from ACRIN IMC.
- Documentation of the specific volumetry software platform by vendor, version, and other specifications, as well as brief description of the software segmentation steps.
- Documentation of prior CORE experimental analyses using this software, anticipated number of analyses per unit time, and guaranteed access to the software for purposes of this study.

- Compliance with transmission procedures and performance of volumetric measurements using the specific image analysis software in three test cases, consisting of one ACRIN test case and two cases from the V-CORE that have been scrubbed of identifiers prior to transmission to ACRIN IMC.
- Satisfactory credentialing for completion of RECIST criteria on CT scans (see below).

Each volumetry platform achieves nodule segmentation and volumetric measurement with different proprietary image processing routines and knowledge base. A detailed understanding of the analytical basis underlying the segmentation process is beyond the scope of this trial. The objectives of the V-CORES are to perform volumetric analyses of: (a) Groups A and C two baseline CT components of PET/CT scans to assess interscan variability; and (b) Group B sequential CT components of PET/CT scans and Group A CT components of PET/CT scans immediately pre-chemotherapy and post-cycle 1, to analyze volume changes in support of the exploratory objectives and to identify differences in performance between software platforms.

2.2.1 Baseline Scans

All CT scans from PET/CT studies will be transferred from the participating sites to ACRIN IMC. IMC will distribute the scrubbed data sets to V-COREs for analyses. The cores will receive neither the results of local image interpretations nor any clinical information beyond the cycle associated with the data set. The primary lung lesion will be defined on the baseline scan; multifocal lung lesions may be defined as the primary lung lesion depending on the individual case. The target lesion will be segmented according to the requirements of the individual software program and the volume calculated. Manual editing in regions of complex anatomy, such as the juxtapleural or juxtavascular regions, will be allowed if this is a routine feature of the software. Software programs that provide 3D volumetric renditions of the nodule will be captured and these 3D volumetric renditions will be archived. In studies with more than one lesion, lesions will be numbered craniocaudally and, if multiple nodules are present in the same axial plane, from medial to lateral. The x and y coordinates of the target centroid will also be captured. This indexing will be used on all subsequent reads to ensure concordance of lesions on follow up.

2.2.2 Follow-up Interpretations

When interpreting all subsequent follow-up CT scans, the baseline (pre-chemotherapy) annotated scan will be reviewed for: the number of the target lesion, the location of the lesion (x, y coordinates) and the target volume. This will provide the reader with a basis for assessing measurement change. Readers will review all available images for the current time point prior to making a measurement. The target lesion will be segmented according to the requirements of the individual software program, the volume calculated, and target location by number and x, y coordinates recorded. All image metadata (results of image analysis) will be entered into the ACRIN analysis database.

3. Tumor Response Evaluation According To RECIST

3.1 Image Acquisition

Response assessment using RECIST criteria will be performed on: CT scans pre-chemotherapy cycle 1, the CT scans pre-chemotherapy cycle 3 and, thereafter, at every other chemotherapy treatment cycle. Scans will be performed after administration of oral and IV contrast agents according to institutional practices.

Iodinated intravenous (IV) contrast media may be administered for diagnostic CT scans per standard institutional practice, unless contraindicated. The decision not to use IV contrast for CT is at the discretion of the performing radiologist. If IV contrast is *not* administered for these scans, then these non-contrast CT series can be used for both RECIST and volumetric measurements by prospectively reconstructing the image data into both thick (2.5 to 5 mm) and thin-section (1 to 1.25 mm) series, respectively; this will alleviate the need for any additional research-related CT scans (specifically, those done for volumetrics only).

The CT scan with contrast is obtained with arms elevated above the head and at suspended maximal inspiration to provide optimal relative contrast of lung lesions within aerated lung. CT scans for RECIST interpretations may be acquired on the fusion PET/CT scanners or dedicated CT system, depending upon the workflow practices, but in all instances, the scanner platforms will be qualified for this trial by ACRIN. Typical parameters for image acquisition and reconstruction are shown in Table 2.

Table 2: Parameters for the CT scans used to assess tumor response according to RECIST

Parameter	Dedicated CT for RECIST*
KV	120
Gantry rotation time	≤ 0.5 sec
Scanner Effective mAs (Regular-large size patient)	100-260
Maximum scan | breath-hold time (40 cm long thorax)	≤ 15 sec
Number of active channels (N)	≥ 6
Maximum Reconstructed slice width	5 mm[1]
Reconstruction interval	0-20% overlap
Reconstruction algorithm	Standard
Reconstruction diameter (dFOV)	outer rib to outer rib
Intravenous contrast media	Per institution practice | indication[2]
Arm positioning	Above the head

1 **If the inspiratory scan of the PET/CT is non-contrast, it *also* should be used for tumor volumetry, and prospectively reconstructed at 1-1.25 (volumetry) and 2.5-5 mm (diagnostic interpretation using RECIST) slice thicknesses.**

2. **Intravenous contrast should be administered according to standard practices with respect to amount, flow rates, and timing with respect to image acquisition, depending upon the body parts imaged. The same method of contrast administration should be followed with all subsequent scans.**

3.2 Image Data Analysis

3.2.1 Local CT Interpretation

Local CT interpretations generally will be provided to treating physicians for purposes of managing therapy. However, treating physicians will be blinded to the results of the first follow-up CT scan (scan 3 in group A and scan 2 in group B) except when the scan shows potentially life threatening tumor progression, impending fractures or other serious complications. In order to ensure that treating physicians are blinded, sites will need to take appropriate measures to prevent these digital images from being accessible in their institution's PACS system. Also, a full written report of the results of CT should not be made available to the treating physicians, but a limited report indicating there were no life threatening or serious changes may be made available after chemotherapy cycle 1. Response assessment by CT after 2 cycles of therapy will be communicated to the physicians as part of the routine clinical care of participants with advanced NSCLC treated with chemotherapy.

3.2.2 Response Evaluation Criteria in Solid Tumors (RECIST)

The following categories of disease will be used in determining response to treatment by RECIST:

- **Measurable disease:** The presence of at least one measurable lesion. If the measurable disease is restricted to a solitary lesion, its neoplastic nature should be confirmed by cytology | histology.
- **Measurable lesions**: Lesions that can be accurately measured in at least one dimension with longest diameter ≥ 10 mm with helical CT.
- **Non-measurable lesions**: all other lesions, including small lesions (longest diameter < 20 mm with conventional techniques or < 10 mm with helical CT scan), i.e., bone lesions, leptomeningeal disease, Ascites, pleural/pericardial effusion, inflammatory breast disease, lymphangitic cutis | pulmonis, cystic lesions, and also abdominal masses that are not confirmed and followed by imaging techniques.

All measurements will be taken and recorded in metric notation on a calibrated diagnostic imaging workstation at full resolution using electronic calipers. The baseline measurements will be performed within one week of the start of treatment; measurements obtained following chemotherapy cycles will be obtained on scans performed within 1-3 days of the next cycle of treatment. The same method of assessment and the same technique will be used to characterize each identified and reported lesion at baseline and during follow-up. Lung lesions will be evaluated on lung windows; mediastinal and or soft tissue lesions will be evaluated on soft tissue windows. The acquisition parameters provided above serve as guidelines for CT technique.

"Target" and "Non-Target" lesions will be documented according to the following guidelines:

- All measurable lesions up to a maximum of five lesions per organ and 10 lesions total, representative of all involved organs, should be identified as **target lesions** and recorded and measured at baseline.
- Target lesions should be selected on the basis of their size (lesions with the longest diameter and their suitability for accurate repeated measurements.

- A sum of the longest diameter (LD) for all target lesions will be calculated and reported as the baseline sum LD. The baseline sum LD will be used as reference by which to characterize the objective tumor response.
- All other lesions (or sites of disease) should be identified as non-target lesions and should also be recorded at baseline. Measurements of these lesions are not required, but the presence or absence of each should be noted throughout follow-up.

The following summarizes the categories of response:

Response Criteria	Evaluation of Target Lesions
Complete Response (CR)	Disappearance of all target lesions.
Partial Response (PR)	At least a 30% decrease in the sum of the LD of target lesions, taking as reference the baseline sum LD.
Progressive Disease (PD)	At least a 20% increase in the sum of the LD of target lesions, taking as reference the smallest sum LD recorded since the treatment started or the appearance of one or more new lesions.
Stable Disease (SD)	Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum LD since treatment start
	Evaluation of Non-Target Lesions
Complete Response (CR)	Disappearance of all non-target lesions and normalization of tumor marker level.
Incomplete Response; Stable Disease (SD)	Persistence of one or more non-target lesions(s) or/and maintenance of tumor marker level above the normal limits
Progressive disease	Appearance of one or more new lesions and/or unequivocal progression of existing non-target lesions (1)
(1) Although a clear progression of non-target lesions only is exceptional, in such circumstances, the opinion of the treating physician should prevail and the progression status should be confirmed later on by follow-up imaging.	

Evaluation of Best Overall Response: The best overall response recorded from the start of the treatment until disease progression | recurrence (taking as reference for PD the smallest measurements recorded since the treatment started). In general, the participant's best response assignment will depend on the achievement of both measurement and confirmation criteria.

Target Lesions	Non-Target Lesions	Evaluation of Non-Target Lesions	Overall Response
CR	CR	No	CR
CR	Incomplete Response | SD	No	PR
PR	Non-PD	No	PR
SD	Non-PD	No	SD
PD	Any	Yes or No	PD
Any	PD	Yes or No	PD
Any	Any	Yes	PD

Confirmation:

- To be assigned the status of PR or CR, changes in tumor measurements must be confirmed by repeat assessments that should be performed no less than 4 weeks after the criteria for response are first met. Longer intervals as determined by the study protocol may also be appropriate.
- To be assigned the status of SD, follow-up measurements must have met the SD criteria at least once after study entry at a minimum interval (in general, not less than 6-8 weeks) that is defined in the study protocol.

Duration of Overall Response: This is measured from the time measurement criteria are met for CR or PR (whichever status is recorded first) until the first date that recurrence or PD is objectively documented, taking as reference for PD the smallest measurements recorded since the treatment started.

Duration of Stable Disease: Stable disease is measured from the start of the treatment until the criteria for disease progression are met, taking as reference the smallest measurements recorded since the treatment started. The clinical relevance of the duration of SD varies for different tumor types and grades. Therefore, it is highly recommended that the protocol specify the minimal time interval required between two measurements for determination of SD. This time interval should take into account the expected clinical benefit that such a status may bring to the study population.

3.2.3 Blinded CT Reading
Central blinded reading of all CT scans according to RECIST criteria will be used for correlation with metabolic changes seen on PET and CT tumor volumetry.

3.2.4 Baseline Interpretations
Target lesions on scans for Group A and B participants will be defined on the baseline scan. A maximum of 10 target lesions will be defined with no more than 5 lesions per single organ. The 10 target lesions will be chosen with representation from all organs with measurable disease.

A screen capture of each target lesion annotated with a pointer and a lesion reference number assigned to the target lesion will be generated and archived. This will be used on subsequent reads to ensure concordance between lesions on follow up exams. The reader will identify the axial slice on the baseline exam that represents the largest axial equator of the lesion and will choose the orientation of longest axis diameter measurement to be approximated on subsequent scans. Measurements will be made from the axial post-contrast scan that best demonstrates the lesion as distinct from background. All measurements will be made by electronic calipers; images that capture the actual measurement axis with calipers will be saved and archived with the exam.

3.2.5 Follow-up Interpretations
When interpreting all subsequent follow-up CT scans, the baseline (pre-chemotherapy) annotated scan will be reviewed for: the axial equator (slice location) from which the maximum diameter was obtained, the axis of the diameter, and the measurement. This will provide the reader with a basis for assessing measurement change. Readers will review all available images for the current time point prior to making a measurement. Measurements will be made from the axial post contrast scan that best demonstrates the lesion as distinct from background. Unless there is an obvious change in lesion shape, the reader will identify the slice on the current exam that best matches the lesion anatomy used for the prior measurement as well as an axis for diameter measurement that best approximates the axis used for prior measurements. If lesions undergo an obvious change in shape, a new axis (and axial equator) corresponding to longest diameter will be measured. All measurements will be made by electronic calipers; images that capture the actual measurement axis with calipers will be saved and archived with the exam.

3.2.6 Reader Qualification for RECIST
Central reads will be performed by certified core radiologists, and will employ a single reader for each participant for all time points. All readers will be trained on the ACRIN RECIST SOP and sign off to document training.

Training includes the following reads from one archive test case:
- One Baseline test scan to ensure compliance with | understanding of the use of RECIST criteria and the method of annotating index lesions for purposes of future comparison.
- Three test follow-up scans to ensure compliance with SOPs for follow-up reads and target definitions. All three lesion diameters in the test set must be within 10% of a standard established for the test case defined by the ACRIN Imaging Management Center.

4. Qualification of CT Scanners
Participating sites will be required to submit two (2) CT scans of the lungs using the volumetric parameters outlined on page 113 (Appendix VII, Section 2.1, Table 1), before enrolling any study participants to this trial.

4.1 Image Data Sets

Two (2) image data sets will be evaluated. Images produced at an image thickness of 3-5mm will be used for measurements employing RECIST criteria. Images produced at an image thickness of 1-1.5mm will be used for volumetric analyses. Both image data sets may be produced from a single PET/CT acquisition provided that the PET/CT scanner is capable of prospective reconstruction at varying image thicknesses.

4.2 Reconstructing Volumetric Images for RECIST Measurements

Images produced for volumetric analyses can be used for RECIST measurements if the PET/CT scanner is capable of producing prospective reconstructed slices. Images must first be prospectively reconstructed at an image thickness of 3 mm before measurements are made using RECIST criteria.

4.3 Reconstructing CT Images Produced on a PET/CT for Volumetric Analyses

CT Images produced on a PET/CT scanner also may be used for volumetric analyses if:

1. The CT scan is acquired on inspiration; and

2. The PET/CT scanner can prospectively reconstruct images at 1-1.5 mm.

4.4 Required CT Test Case Submission

The required CT test cases must be submitted to the ACRIN MR/CT Imaging Core Lab. All imaging submissions must be in DICOM format via CD/DVD-ROM or via the internet using a secure File Transfer Protocol (sFTP).

Removal of Confidential Participant Information

The header record on DICOM formatted image data, which often contains information identifying the participant by name, MUST be scrubbed before the image is transferred. This involves replacing the Participant Name tag with the ACRIN Institution ID or number, replacing Participant ID tag with the ACRIN case number and putting the study number into the Other Participant ID tag. This can be performed using a customized software program or by using TRIAD software available from ACRIN. Contact the ACRIN Image Management Center for additional details at Triad-Support@acr-arrs.org.

Appendix 4.0

General Specimen Submission Instructions

A. All submitted specimens must be labeled with the protocol number, patient number, patient's initials and date of specimen collection.

B. The Federal guidelines for shipment are as follows:

i. The specimen must be wrapped in an absorbant material.
ii. The specimen must be placed in an ***AIRTIGHT*** container (like a re-sealable bag).
iii. Pack the specimen, inside an airtight container, in a Styrofoam shipping container.
iv. Pack the Styrofoam shipping container in a cardboard box.
v. The cardboard box must be marked as "BIOHAZARD."

1.1 Submission of Blood Collection

A. Directions for obtaining, handling and shipping blood and serum:

i. After patient registration, contact Dr. Lum's laboratory at the Karmanos Cancer Center (313-576-8320 or 313-576-8321) to obtain blood collection kits.

ii. Blood is to be obtained on the day of leukophereis (prior to start of procedure), then as per section 9.0 and Appendix 1

iii. Each blood collection kit contains:

- one instruction sheet/log sheet
- one prelabeled red top (serum) Vacutainer® tube
- six prelabeled green top Vacutainer® tubes
- an absorbent pad

Each label contains a highly visible seven-character alpha-numberic unique identifier, common to all tubes in the kit. A place on the label is available for writing the draw date and (optionally) the initials of the participant.

iv. Blood Collection:
Blood samples can be collected under fasting or non-fasting conditions. Add the date of draw to the labels and the initials of the subject. Using a Vacutainer® blood collection, needle, draw 10 mL of blood into each of the seven tubes. Leave all tubes at room temperature until they are packaged for shipping.

v. Packing for Shipping: Return the tubes to the packaging from which they were taken, seal the bag securely. Be sure to check that identifying information has been placed on all tubes.

vi. Shipping: Transfer all collected blood tubes to a cardboard box. Seal the box securely with packaging tape. Put the box into a styrofoam shipping container (e.g. Thermosafe, #399; (800) 323-7442) and seal the container.

Attach labels to the outside of the box: a shipping label and a biohazard label. Write your name, address, and telephone number on the shipping label.

Add the following information to the airbill:

Dangerous goods, shippers declaration not required.

Ship by selected carrier on Monday-Wednesday only.

vii. Specimens must be mailed (via FedEx) to the following address:

- Ship all samples to:

Dr. Lum's Laboratory
Karmanos Cancer Institute
BMT/Immunotherapy GMP Facility
4100 John R.
723 HWCRC
Detroit, MI 48201
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1.2 Log Forms and Operating Procedures for Handling the Pheresis or Cell Product

A. Log Forms

i. Log 23: Leukapheresis Product Packing Log

ii. Log 24: Transport Log for Shipping Leukapheresis/ATC units to BMT/Immunotherapy GMP Facility

iii. Log 25: Shipment Delivery and Receipt log for Leukapheresis/ATC units to BMT/Immunotherapy GMP Facility

iv. Log 26: Activated T Cell Product Packing Log

v. Log 27: Activated T Cell Unit Shipping, Receipt and Inspection Information

v. Log 28: Dry Shipper Evaluation Log

v. Log 29: LogTag Temperature Evaluation Log

B. Operating Procedures

i. OP-07: ShipsLOG Data Logger for Liquid Nitrogen Vapor Dry Shipments

ii. OP-08: LogTag Analyzer

C. Quality Control Procedures

i. QCP-13: Preparation for Transport and Transportation of Non-Cryopreserved Leukapheresis Products Between the Pheresis Product Collection Facility and the Karmanos Cancer Institute GMP Facility

ii. QCP-14: Shipping Cryopreserved ATC Units to Infusion Centers

iii. QCP-15: Packaging and Shipping Peripheral Blood Samples for Immune Evaluation Testing