MATERIALS AND METHODS

Leukapheresis and T cell Expansion. T cells in PBMC were activated with 20 ng/mL of soluble OKT3 and expanded in 100 IU/mL of IL-2 under cGMP conditions.[25, 26] The cells were grown in breathable flasks (FEP Bag Type 750-C1, American Fluoroseal Corporation, Gaithersburg, MD) in RPMI 1640 medium (Lonza) supplemented with 2% pooled heat inactivated human serum (Valley Biomedical, Winchester, VA). ATC were split approximately every 2-3 days based on cell counts. After 14 days of culture, ATC were harvested and armed with a pre-titrated dose of 50 ng of CD20Bi/10^6 ATC, washed, and cryopreserved in multiple aliquots. Aliquots of each bag were sent for bacterial and fungal cultures (Detroit Medical Center Microbiology laboratory), endotoxin testing (Lonza, Inc., Walkersville MD), and mycoplasma testing (Bionique Testing Laboratories, Inc., Saranac Lake, NY). Aliquots were also obtained for quality control testing for cytotoxicity directed at B9C or DAUDI lymphoma cell targets and phenotyping.

Production of heteroconjugated CD20Bi. Rituximab (Rituxan®; Genentech, SF, CA) was heteroconjugated to anti-CD3 (OKT3, Centocor, Ortho-Biotech, Raritan, NJ) to produce the CD20Bi[24]. OKT3 and rituximab that were cross-linked with Traut's reagent (2-iminothiolane HCl, Pierce Biotechnology Inc., Rockford, IL) and sulphosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulpho-SMCC), respectively, were purified on PD-10 columns (Pharmacia, Uppsala, Sweden) (**Figure 1B).** The cross-linked OKT3 and rituximab were heteroconjugated overnight and the heterconjugation was verified by SDS-PAGE gels (4-15% gradient) that was stained with Gelcode Blue (**Figure 1C**). The heterconjugated mixture was tested and revalidated regularly for clinical use. The heterconjugated CD20Bi has been stable up to 2 years after manufacture.

Mobilization of stem cells. Unless otherwise mentioned, patients received granulocyte colony stimulating factor (G-CSF) stimulation for 4 days for stem cell mobilization prior to leukapheresis

to obtain a minimum CD34+ cell dose of 2 x 10^6 cells/Kg. The median CD34+ cell dose/kg was 4.03 x 10^6 with a 95% confidence interval (CI) of 3.3,6.9 x 10^6/kg.

Infusions of aATC. On day +4 after SCT, cryopreserved aATC were thawed and infused at the bedside. aATC were given over 5-15 minutes with monitoring of vital signs and O_2 saturations before and every 15 minutes up to 1 hr after infusion. All vital signs and adverse events were recorded on the patient's chart using the NCI immunotherapy toxicity table. Patients were routinely pre-medicated with diphenhydramine (50 mg, orally or intravenously) and acetaminophen (1000 mg, orally) 30 min prior to ATC infusions to prevent fever and chills. IV hydration with 500 mls was started 1 hour prior to each infusion and IV hydration continued up to 6 hrs after infusions in the clinic. Patients received 500 ml boluses of fluid if the systolic BP dropped below 100 mm Hg. Dose limiting toxicity (DLT) was defined as a grade 4 non-hematologic toxicities or persistent grade 3 cell-based toxicity for > 24 hours based on the NCI Immunotherapy Toxicity Tables. A standard 3 + 3 dose escalation was performed. If one of the first three patients had persistent grade 3 non-hematological toxicity or received < 80% of planned aATC dose, the number of patients at that dose level would have been expanded to 6. The dose of aATC was escalated to the next level if no more patients experienced persistent grade 3 or greater non-hematological toxicity. Encountering grade 4 non-hematologic toxicities or persistent grade 3 cell-based toxicity for > 24 hours was defined as a DLT. If 2 patients in the expanded cohort at a dose level had their infusions stopped due to toxicities, this dose would be defined as DLT and the trial would be stopped. The maximally tolerated dose (MTD) was defined as the dose level below the DLT. Treatment was held until toxicity improved to grade 0 or 1. Toxicity was assessed daily for 7 days after each infusion. Standard supportive care was provided for SCT according to institutional standard of care. If patients did not receive at least 80% of the planned dose, they were considered not evaluable for dose-escalation and toxicity related to that dose.

Criteria for removal from the study. Patients would be removed from protocol for: 1) grade 4 or persistent grade 3 (> 24 hours) cell-based related toxicity; 2) initiation of other anti-tumor therapies; 3) patient decision to stop; 4) circumstances when it was deemed in the best medical interest of the patient; or 5) an unexplained delay in treatment.

Phenotyping. The leukapheresis product, the ATC product and PBMC from the patients undergoing SCT were evaluated to assess changes in phenotype by staining for $CD3^+$, $CD4^+$, $CD8^+$, $CD25^+$, $CD19^+$, $CD20^+$, $CD45RO^+$, $CD45RA^+$, and $CD56^+$ cells. Cryopreserved PBMC from the time of leukapheresis served as baseline controls.

Specific Cytotoxicity and IFN-γ EliSpots. Specific cytotoxicity was performed using fresh PBMC mixed with ^{51}Cr labeled DAUDI and K562 cells.[22] The interferon gamma (IFN-γ) Elispots were used to measure of CD8-mediated memory CTL activity and CD4-mediated helper responses. IFN-γ EliSpots produced by PBMC were assessed after 18 hrs of stimulation with DAUDI or K562 as well as spontaneous IFN-γ EliSpots produced by PBMC at an effector to target ratio (E/T) of 10:1 as previously described.[28]

Serum cytokines. Cytokines were measured in the serum at selected time points using a 25-plex human cytokine Luminex Array (Invitrogen, Carlsbad, CA) using Bio-Plex system (Bio-Rad Lab., Hercules, CA). The multiplex panel included IL-1β, IL-1 receptor antagonist (IL-1RA), IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-13, IL-17, tumor necrosis factor (TNF-α), interferon-alpha (IFN-α), IFN-γ, GM-CSF, macrophage inhibitory protein (MIP-1α), MIP-1β, IFN-γ-induced protein-10 (IP-10), monokine induced by IFN-γ (MIG), Eotaxin, Regulated on Activation Normal T Cell Expressed and Secreted (RANTES) and monocyte chemotactic protein (MCP)-1. The limit of detection for these assays is < 10 pg/mL. The cytokine levels were calculated from a standard curve.

Quantitative IgG levels and anti-tetanus toxoid (anti-TT) IgG levels. A human anti-tetanus toxoid IgG (anti-TT) standard was produced by affinity purification of anti-TT from a

nitrocellulose membrane (Invitrogen, Carlsbad, CA) coated with pure tetanus toxoid. High titer anti-TT human serum was adsorbed onto the membrane and specific anti-TT antibodies were eluted in a low pH buffer. The amount of anti-TT was calibrated against a pure IgG standard (Southern Biotech, Birmingham, Al) by ELISA.[29] The serum anti-TT levels were reported as ng/ml of anti-TT. For the specific TT ELISA, a total of 50 ng of TT antigen (LIST Biological Lab, Inc, Campbell, CA) was plated in 100 μl of carbonate/bicarbonate buffer (pH 9.6) in ELISA plates.

RESULTS

ATC characteristics. The planned aATC dose, the actual aATC dose, the percentage of the target dose of aATC dose administered, time to progression, and survival are presented in **Table 1**. ATC were expanded up to 1.40 $x10^{11}$ cells. One patient underwent a second leukapheresis to obtain T cells due to poor *in vitro* expansion and one patient underwent a second leukapheresis to re-grow product due to contamination of the product. In the harvest product, the mean percent (± SD) viability was 91.1 ± 4.18% and the mean proportion (95% CI) of CD3, CD4, and CD8 cells were 96.5 (21.7, 76.8), 78.2 (11.3, 57.1), and 48.2% (9.6, 52.0), respectively. Greater than 90.8% of the T cells were CD45RO+ after expansion. The ATC products were enriched for CD4+ cells with the exception of one patient who had product enriched for CD8+ cells (**Table 2**). ATC derived from the NHL patients armed with CD20Bi exhibited a mean (± SD) specific cytotoxicity of 12.1% ± 6.7% (range 1.5-25%) at an E:T of 25:1 directed at CD20+ DAUDI targets by ^{51}Cr release cytotoxicity assay.

Infusions of aATC. The patients received 4 infusions of 5 (level 1), 10 (level 2), 15 (level 3), and 20 (level 4) x 10^9 aATC for total doses of 20, 40, 60, and 80 x 10^9 aATC, respectively (**Table 1**). The median total dose of aATC delivered was 6.7 x 10^{10} (95% CI of 4.24, 6.73 x 10^{10}). The maximum tolerated dose (MTD) was not reached and there were no DLTs. The most

frequent side effects included fever, chills, malaise, nausea and/or vomiting, tachycardia, hypotension, headache, transient hypoxia, hypertension, and dyspnea (**Table 3**). A typical pattern consisted of chills at ~1 hr, nausea at 1-2 hours, headaches at ~ 2-4 hrs, hypotension at ~2-3 hrs, and fever at 2-4 hrs after an infusion. The chills were controlled with meperidine and fever was controlled with alternating doses of ibuprofen 600 mg and acetaminophen 600 mg given orally every 4 to 6 hours for 24 hours.

Post SCT infectious disease complications. There were no viral (Herpes simplex virus 1 or 2, varicella zoster virus, respiratory syncitial virus) or fungal infections during the first 100 days after SCT. One patient developed a central line infection and another patient developed *Staphylococcus aureus* bacteremia. Both were treated successfully with antibiotics.

Lymphocyte recovery. Phenotyping was performed at 0.5, 1, 2, 3, 6, and 12 months after SCT. The median percent CD3, CD4, and CD8+ cells are shown in **Figure 2B** at the indicated time points. The mean (n = 12) proportion of CD3+ and CD4+ cells was 22% and 19% at 2 weeks after SCT, respectively. By 1 month after SCT, the mean proportions of CD3+, CD4+, and CD8+ cells reached 35%, 22%, and 20%, respectively with a CD4:CD8 ratio of ~1.0. During aATC infusions the proportion of CD4+ cells was higher than the proportion of CD8+ cells in the blood but there was an increase in CD8 + population between 4 and 8 weeks after SCT **(Figure 2B)**. These data show that aATC (enriched for CD4+ cells) infusions increased the proportion of CD4+ cells in the blood during the infusions during the first 2 weeks after SCT. The median percent (interquartile range) of CD19+ B cells in PBMC at 2, 3, 6, and 12 months were 0.75 (0.26 - 2.11, n = 7), 0.27(0.16-4.84, n = 8), 3.43 (1.05 - 12.64, n = 5), and 3.85 (2.06-7.8, n = 7), respectively. Staining for CD20+ cells gave comparable results.

Post SCT levels of cytokines. Steady state serum cytokines were tested at multiple time points after SCT to assess cytokine and chemokine levels in 4 patients. **Figure 4A, upper panels** shows the levels of IL-2R and IL-12 and the **lower panels** shows levels of the IFN-γ induced chemokines CXCL10 (IP-10) and CXCL9 (MIG) at preSCT, 2 weeks, 1 month, and 2 - 3

months after SCT. These data show that levels of IL-2R, IL-12, CXCL10, and CxCL9 steadily rise during the first 3 months after SCT.

Induction of cytokines and chemokines after aATC infusion. Th1 cytokines and chemokines peaked between 1 and 4 hrs after an aATC infusion given 10 days after SCT (**Figure 4B**). There was a > 10 fold increase over serum baseline values for IL-2 (~10 pg/ml), IL-7 (~10 pg/ml), and IL-15 levels (5 -57 pg/ml), > 100 fold increase in serum baseline levels for MIP-1β (12 -129 pg/ml) and IP-10 (9 -132 pg/ml), and > 10 fold increases in serum baseline for MIP-1α (6 - 179 pg/ml) and MIG (111-281 pg/ml) levels (**Figure 4B, upper panels**). Similarly, levels of IFN-γ and IL-12 were increased at 4 hrs after the infusion above baseline (**Figure 4B, lower panels**). These changes represent production of cytokines and chemokines following aATC infusions, however, it is not known whether production of these cytokines and chemokines is resulted from the interactions between the aATC and host tissues and/or small numbers of endogenous cells developing from the graft at 10 days after SCT.

Serum IgG and anti-TT levels. Serum IgG levels were obtained at 3 months post SCT in 7 patients to determine whether targeting CD20+ B cells in the early post-SCT recovery period impaired B cell development and antibody production. Anti-TT antibody levels were measured to determine the amount of specific antibody production capacity that had been transferred in the stem cell graft. **Figures 4C and 4D** show serum IgG and anti-TT levels at pre and 3 months after SCT. The mean levels of IgG before and after SCT were 9.5 $\pm$ 1.5 mg/ml and 8.0 $\pm$ 1.5 mg/ml, respectively. The mean serum levels of anti-TT before and after SCT were 11.4 $\pm$ 7.8 μg/ml and 12.8 $\pm$ 10.1 μg/ml, respectively. Interestingly, the mean amount of anti-TT was not different from preSCT ($p = 0.059$, $n = 7$). These data show that targeting $CD20^+$ B cell after SCT did not impair the development of IgG and anti-TT antibody secreting cells during the first 100 days after SCT.