Supplemental Online Methods

Manufacture of MART-1 F5 TCR transgenic lymphocytes

The retroviral vector MSGV1-F5AfT2AB was generated under good manufacturing practices (GMP) at the Indiana University Viral Production Facility (IU VPF), starting from a master cell bank provided by Dr. Steven A. Rosenberg (NCI Surgery Branch). It consisted of a 4,196 bps provirus genome including the 5' LTR from the murine stem cell virus (MSCV) promoter, the murine leukemia virus packaging signal including the splicing donor and splicing acceptor sites, human alpha chain and beta chain genes of the anti-MART-1 F5 TCR from TIL clone DMF5, and murine stem cell virus 3' LTR. The alpha and beta TCR chains were linked by a T2A self-cleaving sequence.

A non-mobilized leukapheresis processing two plasma volumes from study patients was processed in the Human Gene Medicine Program (HGMP)/JCCC GMP Suite at UCLA. PBMC were isolated by Ficoll gradient centrifugation and cultured in AIMV media (Gibco, Invitrogen, Chicago, IL) supplemented with 5% human AB serum (Omega Scientific, Tarzana, CA) in the presence of 50 ng/ml anti-CD3 (OKT3, Miltenyi Biotec, Auburn, CA) and 300 IU/ml IL-2 in order to stimulate T-cell growth to prepare for viral vector transduction. Activated PBMC were transduced with the clinical grade retrovirus vector expressing the MART-1 F5 TCR using retronectin-coated plates (Retronectin, Takara, Otsu, Shiga, Japan) in two consecutive days and kept in culture for 96 hours (up to protocol amendment 2) or up to 72 hours (after protocol amendment 3) from the
time of the first retroviral transduction. Transduced cells were harvested and either cryopreserved (up to protocol amendment 7) or infused fresh (after protocol amendment 8) as soon as the lot release criteria were cleared.

In-process and final product testing included Gram stain, fungal stain, sterility culture for bacteria and fungus, mycoplasma assay (MycoAlert assay, Lonza, Walkersville, MD), and endotoxin assay (Endosafe PTS system, Charles River, Charleston, SC). Transduction efficiency was tested with MHC tetramer analysis for MART-1 (Beckman-Coulter, Brea, CA) gated in CD3 (BD Bioscience, Franklin Lakes, NJ), CD4 (BD Bioscience) and CD8 (Beckman-Coulter) positive lymphocytes. Potency testing of the TCR transgenic cells was assessed using a co-culture system to detect MART-1-specific IFN-γ production by ELISA. Briefly, transduced and control non-transduced T cells were co-cultured with K562-A2.1 pulsed and non-pulsed with MART-1_26-35 peptide (Biosynthesis, Lewisville, TX) or HLA and antigen matched melanoma cell line (M202) and mismatched cell line (M238) overnight and supernatant collected for IFN-γ quantification by ELISA (eBioscience, San Diego, CA).

Manufacture of MART-1 peptide pulsed DCs

Autologous DC were differentiated from adherent peripheral blood monocytes in a one-week in vitro culture in media containing 5% heat-inactivated autologous plasma supplemented with GM-CSF and IL-4 as previously described. DC were pulsed with the MART-1_26-35 anchor-modified immunodominant peptide in the HLA-A2*0201
haplotype and administered i.d. Three preparations of freshly manufactured and lot release tested MART-1/DC vaccines were administered starting on day 1 separated by 14 days. In-process and final product testing included Gram stain, fungal stain, sterility culture for bacteria and fungus, mycoplasma assay and endotoxin assay. Since MART-1 TCR transgenic cells may decrease in frequency in blood over time, patients were eligible to receive a new set of 3 intradermal administrations of $10^7$ MART-126-35 peptide pulsed DC as a booster vaccination after study day 90. The level of MART-1 TCR transgenic cells had to be below 5% of total lymphocytes by MHC tetramer or dextramer assay analysis to provide the booster DC vaccinations.

**Determination of Replication Competent Retrovirus (RCR)**

A GALV quantitative PCR (qPCR) assay, developed and optimized at the IU VPF, was used to rule out the presence of replication competent retrovirus (RCR). This assay was performed retrospectively in PBMC processed from post therapy peripheral blood samples at 3, 6, 12 months. Annual samples were archived thereafter. For the first four patients, the S+/L- assay for GALV was done in a cryopreserved aliquot of the final product infused into the patients as well as 5% of the cell culture supernatant as the culture period after transduction was 4 days.

**Plasma cytokine and cellular co-culture analyses**
Baseline pre-ACT plasma samples and post-ACT plasma samples at various time points (before and after steroid therapy) were subjected to cytokine analyses using multiplex kits (Bioplex, Hercules, CA; Milliplex, Billerica, MA) as per the manufacturer's instructions. Data were analyzed using their respective software and interpreted as fold change with respect to the patient's own baseline level in order to avoid inter-kit and patient to patient variability. To test for potential neo-autoreactivity, the TCR transgenic cells used in the infusion and cells recovered from the peripheral blood of the patient post-ACT were co-cultured overnight with autologous baseline peripheral blood mononuclear cells or bone marrow cells, that were either pulsed with MART-1 peptide (as a positive control for TCR transgenic cell reactivity) or were left un-pulsed to test for autoreactivity to self antigens and supernatant collected for IFN-γ and IL-17 quantification by ELISA (eBioscience).

**Overall assessment of PET scan changes on study**

There were nine patients with a complete set of 2 scans (baseline and day 30) available for review. Patients classified as transient responders from scan 1 to scan 2 (7 out of 9) had the PET lesion count decreased by 44% (range 13-80) and the SUVmax decreased by 37% (range 13-76%). In contrast, out of the two patients who did not have evidence of antitumor activity in these scans, one (F5-4) had an increase in the lesion count while there was an overall improvement in SUVmax by 34%, and the other patient (F5-11) had no increase in the lesion count but had a worsening of SUVmax by 13%.
Supplemental Online Discussion

The NCI Surgery Branch F5 trial, with a 20% overall objective response rate and 13% durable responses, used the same TCR and gene transfer vector\textsuperscript{1,2} as the UCLA/Caltech F5 clinical trial. Both clinical trials used the same retrovirus vector construct and master cell bank, and were produced at the same GMP viral vector production facility. Both clinical trials also used the same non-myelodepleting but lymphodepleting chemotherapy conditioning regimen followed by HD IL-2. However, there were significant differences between the two protocols that likely played a role in the different clinical outcomes. The NCI approach included an \textit{ex vivo} expansion following the REP protocol that produces a higher number of cells infused, which were administered without a cryopreservation step. This may result both in higher number and functionality of the adoptively transferred TCR transgenic cells. The NCI protocol resulted in toxicities from immune responses against MART-1 positive pigmented cells in the eye and the inner ear, not noted in the UCLA/Caltech experience. Therefore, the NCI program appeared to have produced TCR transgenic preparations with greater potency either due to higher cell number or intrinsic activity. However, the UCLA/Caltech experience demonstrated brisk proliferation and peripheral blood reconstitution with TCR transgenic cells in many of the cases, which may have been facilitated by the ACT of cells with less \textit{ex vivo} manipulation and the use of DC vaccination. Evidence from preclinical models of ACT suggest that T cells which were subjected to shorter periods of \textit{ex vivo} expansion are more likely to contain long term-repopulating T cells, resulting in higher frequency of TCR transgenic reconstitution after
infusion to lymphopenic hosts. In the UCLA/Caltech experience, the toxicities increased when using freshly manufactured (non-cryopreserved) cells, in particular the skin and pulmonary toxicities, and there was evidence of boosting effects by the DC vaccines only in these cases.
Supplemental Figures

Supplemental Figure 1. TCR transgenic cell therapy manufacture protocol modifications. The initial study protocol with up to 96 hours of \textit{ex vivo} expansion after the first retroviral transduction was used for the first four patients. Cells proliferated briskly after anti-CD3 and IL-2 exposure using all the three variations of the TCR transgenic cell therapy manufacture protocols used during the study period. There was an average 28-fold expansion (patients F5-1 to F5-4). The cells from patient F5-4 had suboptimal expansion, being the only case where less than the desired $1 \times 10^9$ cell dose was administered (Table 1). A protocol modification was generated limiting the period of \textit{ex vivo} expansion to 72 hours after the first retroviral transduction, which eliminated the requirement to prospectively test for RCR by S+/L- assay for GALV in each preparation following FDA guidance. With this protocol change, the number of cells produced was slightly lower (F5-5 to F5-9). After amendment 8 was implemented allowing increased cell dose to up to $1 \times 10^{10}$ cells, the initial cell concentration and the number of plates used for cell therapy manufacture was increased. This resulted in an approximate doubling of the absolute number of final cells manufactured (F5-10 to F5-14).

Supplemental Figure 2. Transduction efficiencies of infused transgenic cells. The infused cell therapy was based on total cell counts, which included a range of transduction efficiencies from 41.7\% to 84.3\% of MART-1 MHC tetramer+CD3+ cells. Representative dot plots of MHC tetramer analysis for MART-1 are shown. The later preparations had a lower transduction efficiency due to using half the amount of retroviral supernatant and double the number of cells per well during transduction as
compared to the earlier nine patients; it may also be related to a progressive loss of retroviral titer detected in the annual vector recertification studies.

Supplemental Figure 3. Number and function of TCR transgenic cells infused. a) Absolute number of TCR transgenic cells administered to each patient calculated based on the combination of the delivered cell dose and the transduction efficiency of each preparation. b) Functionality of these cells based on antigen-specific IFN-γ release by ELISA in a co-culture with MART-1-peptide pulsed artificial antigen-presenting cells (black bar = infused cryopreserved, up to $10^9$ cells; dotted bars = infused cryopreserved up to $10^{10}$ cells; striped bars = infused fresh up to $10^{10}$ cells). In all cases, the TCR transgenic cell preparation met the lot release testing criteria for potency, producing greater than 30,000 pg/ml/million cells of MART-1-specific IFN-γ. However, there was a relatively wide range of IFN-γ production (range 1.5-87.1 x $10^5$ pg/ml/million cells).

Supplemental Figure 4. Pre- and post-treatment PET scans showing evidence of major tumor response and MART-1-specific TCR transgenic cell levels in patient F5-10. Cell dose escalation to $10^{10}$ resulted in an unanticipated serious adverse event (SAE) in patient F5-10, who developed a prolonged pancytopenia requiring repeated platelet and red blood cell transfusions over two months after ACT. This occurred while the patient was having a major tumor response. a) Baseline, post-treatment day 35 and 56 scans showing disappearance of many lesions. A PET CT scan on post treatment day 171 supported by a bone marrow biopsy showed massive bone marrow infiltration by S100 and MART-1 positive melanoma cells. b) MART-1 MHC tetramer analysis of
baseline PBMC, infused cells, post-infusion day 7 and 15 peripheral blood PBMC and post corticosteroid, ATG and cyclosporine administration. Given the initial possibility that the pancytopenia in patient F5-10 could be related to the infused cells, the patient received immune suppressive therapy with corticosteroids, which led to a rapid disappearance of both the MART-1-specific TCR transgenic cells and also total T cells with high CD3 expression (day +39 panel) but without improvement in the pancytopenia. The TCR transgenic T cells did not recognize bone marrow precursors nor were specifically high in IL-17 production that has been associated with aplastic anemia (Supplemental Figures 4 and 5). A PET CT scan done at 7 months after ACT (panel A, right image) and repeated bone marrow biopsy demonstrated that the prolonged pancytopenia was due to massive bone marrow infiltration by S100 and MART-1 positive melanoma cells.

**Supplemental Figure 5. Lack of autoreactivity to bone marrow cells in patient F5-10.** These studies were conducted to test for a potential autoreactivity to self-antigens due to mispairing of the TCR transgenic chains with endogenous TCRs potentially resulting in neo-specificity that may recognize self-antigens in hematopoietic lineage cells. The TCR transgenic cells used in the infusion to patient F5-10, and cells recovered from the peripheral blood of the patient on days +7 and +15 post-ACT, were used in co-culture studies. a) and b) IFN-γ quantification in the supernatant of a co-culture of the TCR transgenic lymphocytes administered to the patient with (a) autologous baseline peripheral blood mononuclear cells (BL), or (b) bone marrow cells (BM), both either pulsed with MART-1 peptide (as a positive control for TCR transgenic
cell reactivity) or were left unpulsed. c) and d) IL-17 quantification in supernatants from the same co-cultures.

**Supplemental Figure 6.** Comparison of IL-17 production by F5-10 infused TCR transgenic cells stimulated with MART-1-pulsed K562 A2.1 cells with that of patients who did not develop pancytopenia. Co-culture supernatant was subjected to ELISA assay for IL-17.

**Supplemental Figure 7.** Chest X-rays of patients F5-12 and 14 who developed SAEs of acute respiratory distress. Patchy pulmonary infiltrates within one week of cell infusion. A time-course decrease in size of lung metastases (arrows) was also observed.

**Supplemental Figure 8.** Cytokine production by multiplex assay in plasma from patients F5-12 and F5-14 to study the potential development of a cytokine storm. Plasma collected from peripheral blood at the time of respiratory distress was analyzed with a multiplexed cytokine quantification assay and results were compared to the levels reported in two patients with cytokine storm in a patient who died after receiving the Her2/neu CAR transgenic ACT (see ref. 32) and cytokine storm in a phase I trial with an activating anti-CD28 antibody (see ref. 33). Considering patient to patient and method of assay variation, we chose to compare the fold increase from the patient’s own baseline rather than the absolute values in pg/ml. The graph for anti-CD28 trial represents the average values of six patients. This comparison demonstrated that the increases in the
cytokine levels of F5-12 and F5-14 were elevated, but at much lower levels than that observed in the Her2/neu CAR ACT cytokine storm case and the six patients in the anti-CD28 trial. The panel of raised cytokine and chemokine levels observed in F5-12 and F5-14 correlates more with that seen in the acute phase of pneumonia (see ref. 34).

Supplemental Figure 9. Recall whole body rash and re-expansion of the TCR transgenic cells in peripheral blood of patient F5-13 with subsequent MART-1/DC vaccination. Boosting effect observed in the percentage of MART-1 tetramer positive T cells in the peripheral blood after the third MART-1/DC vaccination and booster MART-1/DC vaccination on Day 95 post ACT.
**Supplemental Table 1.** Toxicities and response to therapy with the subsequent protocol amendments

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Legend: SAEs: serious adverse events.
Supplemental Text References


