GM-CSF Secreting Cellular Immunotherapy in Combination with Autologous Stem Cell Transplant (ASCT) as Post-Remission Therapy for Acute Myeloid Leukemia (AML)


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ABSTRACT

Preclinical models have demonstrated the efficacy of GM-CSF-secreting cancer immunotherapies (GVAX® platform) accompanied by immunotherapy-primed lymphocytes following ASCT in hematologic malignancies. We conducted a phase 2 study of this combination in adult patients with AML. Immunotherapy consisted of autologous leukemia cells admixed with GM-CSF-secreting K562 cells (K562/GM). “Primed” lymphocytes were collected following a single pre-transplant dose of immunotherapy and re-infused with the stem cell graft. Fifty-four subjects were enrolled, 46 (85%) achieved a complete remission (CR) and 28 (52%) received the pre-transplant immunotherapy. For all patients who achieved CR, the 3-year relapse-free survival (RFS) was 47.4% and overall survival (OS), 57.4%. For the 28 immunotherapy treated patients, the RFS and OS was 61.8% and 73.4%, respectively. Post-treatment induction of delayed-type hypersensitivity (DTH) reactions to autologous leukemia cells was associated with longer 3-year RFS (100% vs. 48%). Minimal residual disease (MRD) was monitored by quantitative analysis of WT1, a leukemia-associated gene. A decrease in WT1 transcripts in blood was noted in 69% of patients following the first immunotherapy dose and was also associated with longer 3-year RFS (61% vs. 0%). In conclusion, immunotherapy in combination with primed lymphocytes and ASCT shows encouraging signals of potential activity in AML (ClinicalTrials.gov: NCT00116467).
INTRODUCTION

Intensive chemotherapy regimens induce complete remission in the majority of adults under age 60 with AML, but maintenance of durable remissions remains a challenge. The optimal post-remission treatment to eradicate residual leukemia is controversial. Treatment options include repeated cycles of intensive cytarabine-based consolidation chemotherapy, autologous stem cell transplant (ASCT) and allogeneic stem cell transplant (alloSCT). Mixed results with investigational immunotherapies (ie. interleukin-2, histamine dihydrochloride) added to the aforementioned aggressive treatment approaches have been noted. Several large randomized studies have failed to demonstrate a clearly superior overall strategy for post-remission treatment of AML, but alloSCT is often considered the treatment of choice for patients with high risk disease and a suitable HLA-matched donor. ASCT offers the advantage over alloSCT of a lower transplant-related mortality due to the absence of graft-vs-host disease (GVHD), but is associated with a higher relapse rate, attributed to the absence of an immune-mediated graft-vs-leukemia (GVL) effect. Strategies that combine the cytoreductive power of pre-ASCT myeloablative preparative regimens with immunotherapy, to induce an anti-leukemia immune response, could potentially result in durable remissions without the morbidity and mortality associated with allogeneic GVHD.

GVAX refers to an immunotherapy platform in which whole tumor cells are modified to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF). Preclinical studies have clearly demonstrated the induction of anti-tumor immunity and tumor regressions with the GVAX platform. Clinical trials exploring both autologous GVAX, derived from individual patient tumors, as well as allogeneic GVAX,
derived from established tumor cell lines, have been conducted in multiple tumor types. In addition, several clinical studies have explored a hybrid approach of autologous tumor cells admixed with a GM-CSF-secreting allogeneic tumor cell line 19-22. This approach offers the potential advantage of including patient-specific tumor antigens with an “off-the-shelf” GM-CSF-secreting tumor cell immunotherapy product.

Preclinical studies exploring the use of a GM-CSF-secreting cellular immunotherapy in combination with myeloablative stem cell transplant and adoptive transfer of immunotherapy “primed” lymphocytes have demonstrated enhanced anti-tumor immune and clinical activity with this approach as compared to immunotherapy administered in the non-transplant setting 23. This phase 2 trial was undertaken to explore the safety, immune, and clinical activity of immunotherapy based on the “mixed” GVAX platform (autologous leukemia cells mixed with GM-CSF-secreting K562 cells (CG9962 or K562/GM) combined with ASCT and the adoptive transfer of immunotherapy-primed lymphocytes as post-remission therapy for AML. In addition to immune response and clinical endpoints, analysis of minimal residual disease in blood and bone marrow was conducted throughout the study using quantitative analysis of Wilms Tumor-1 (WT1) gene expression. WT1 is a pan-leukemia marker over-expressed in the vast majority of acute and chronic leukemias. At the time of complete hematologic remission, despite morphologic clearance of leukemia cells from the blood and bone marrow, WT1 transcript levels remain detectable in many patients, and its persistence in the blood portends a poor prognosis 24-26. This analysis was included to monitor the immunotherapy-associated reductions in residual leukemia as a potential signal of anti-
tumor activity in study patients without hematologic evidence of leukemia at the time of immunotherapy administration.

**PATIENTS AND METHODS**

**Study Design**

This was a phase 2, open-label, single-arm, multicenter study evaluating the addition of immunotherapy to an AML treatment program including induction and consolidation chemotherapy followed by ASCT. The pre-specified objectives were to assess safety, feasibility, GM-CSF pharmacokinetics, immune response, WT1 response, relapse-free and overall survival. The trial was approved by all participating institutional review boards and all enrolled subjects gave written informed consent in accordance with the Declaration of Helsinki. The trial is registered at ClinicalTrials.gov (NCT00116467).

**Patient Population**

Patients were 18 – 60 years old with de novo AML (> 20% marrow blasts and no pre-existing hematologic disorder longer than 3 months), no prior leukemia therapy (except leukapheresis or < 72 hours of hydroxyurea), and creatinine < 2.0 mg/dL. Patients were excluded for acute promyelocytic leukemia, extreme obesity (weight > 200% ideal body weight), severe heart disease precluding anthracyclines, other malignancies within 5 years, active autoimmune disease, and pregnancy. Patients who achieved CR were eligible to continue on study and receive consolidation chemotherapy, immunotherapy, and ASCT. Eligibility to proceed to pre-transplant immunotherapy and ASCT included completion of protocol-directed consolidation chemotherapy, maintenance of CR, adequate autologous blood stem cell collection (> 2 x 10^6 CD34+...
cells/kg), acceptable organ function, resolution of grade 3/4 adverse events, and no systemic corticosteroids within 14 days. Post-transplant immunotherapy treatments were initiated at least 6 weeks post-transplant and required an absolute neutrophil count (ANC) > 1000/mm³, platelet > 50,000/mm³, and hemoglobin > 8 g/dL.

**Study Treatment**

The study design is shown in Figure 1. All enrolled patients underwent an initial harvest of autologous leukemia cells from blood or bone marrow. These cells were then irradiated and admixed with the K562/GM cells to create the final immunotherapy product. Thereafter, patients received induction chemotherapy with high dose cytarabine (2 gm/m² bid x 12 doses) and daunorubicin (60 mg/m² qd x 3 doses). A second cycle of alternative investigator-selected induction chemotherapy was allowed if CR was not achieved. For patients ultimately achieving CR, one cycle of consolidation chemotherapy with cytarabine (2gm/m² bid x 8 doses) and etoposide (40 mg/kg by continuous infusion over 96 hours concurrent with cytarabine) was administered followed by collection of GCSF-mobilized (5-10 mcg/kg daily beginning on day 14) peripheral blood stem cells (target > 2 x 10⁶ CD34+ cells/kg) by leukapheresis upon hematologic recovery. Approximately 2 weeks following stem cell collection patients received a single pre-transplant immunotherapy treatment followed 2 weeks later by a second leukapheresis to collect “primed” lymphocytes (target 1 x 10⁸ CD3+ cells/kg). The ASCT was performed using a myeloablative preparative regimen of busulfan (0.8mg/kg IV q 6 hours x 16 doses) and cyclophosphamide (60 mg/kg qd x 2 doses). Peripheral blood stem cells and “primed” lymphocytes were re-infused on transplant day 0 followed by treatment with daily GM-CSF (125 – 250 mcg) until neutrophil recovery (ANC >
Beginning at 6 weeks post-transplant (or upon adequate hematologic recovery as specified above) additional immunotherapy treatments were initiated. Eight post-transplant immunotherapy treatments were administered at 3 week intervals over a 6 month period (9 total treatments including the pre-transplant dose).

**Immunotherapy Production and Administration**

The targeted immunotherapy dose was $1 \times 10^8$ autologous leukemia cells admixed with $4 \times 10^7$ K562/GM cells (secreting GM-CSF at a rate of > 500 ng/10⁶ cells/24 hours). Autologous leukemia cells were harvested, processed, irradiated, tested for quality control, cryopreserved, and stored at each clinical site. Leukemia cells were harvested via blood draw, bone marrow aspiration, or leukapheresis to achieve a target of 1 - 2 x $10^9$ cells. Red blood cells (RBC) were removed by density gradient centrifugation using an established procedure (COBE 2991, DACS device, or equivalent). Assessment of tumor content was done morphologically by Wright-Geimsa staining and confirmed by flow cytometry in most cases. The light density cells were irradiated (10,000 cGy), cryopreserved in autologous plasma and dimethylsulfoxide (DMSO), and stored frozen in liquid nitrogen.

K562/GM cells, based on the GVAX® immunotherapy platform, were derived from an erythroleukemia cell line (K562) modified to secrete GM-CSF, as previously described ²². Clinical lots were prepared under Good Manufacturing Practice (cGMP) conditions (Cell Genesys, Inc, South San Francisco, CA), irradiated, cryopreserved, and stored frozen in liquid nitrogen. Six lots of K562/GM were used in the trial with a mean GM-CSF secretion rate of 3008 ng/10⁶ cells/24 hours (range 2030 to 4396). Immediately prior to immunotherapy administration autologous leukemia cells and K562/GM cells...
were thawed in a 37° waterbath, mixed together, and administered as multiple 0.5 mL intradermal injections.

**Safety and Pharmacokinetic Assessment**

Adverse events were graded according to the NCI Common Toxicity Criteria and assessed for relationship to immunotherapy treatment. Time to hematologic engraftment was assessed as a potential marker of autoimmunity directed against hematopoietic stem and progenitor cells. Time to engraftment was measured from ASCT day 0 to ANC > 500/mm³, platelet > 20,000/mm³, platelet > 50,000/mm³ and RBC transfusion independence. Serum GM-CSF levels were monitored in a subset of patients. GM-CSF levels were measured by ELISA daily for 4 days at the time of the pre-transplant immunotherapy treatment as well as the first, fourth, and eighth post-transplant immunotherapy treatments.

**Clinical Assessment**

Patients were monitored for remission status, relapse, and survival. Complete remission (CR) was defined as < 5% bone marrow blasts, resolution of any previously abnormal karyotype, ANC > 1000/mm³, platelet > 100,000/mm³, and maintenance of these criteria for at least 30 days. Relapse was defined as > 5% bone marrow blasts or recurrence of abnormal karyotype. Time to relapse was measured from the date of first documentation of CR to first documentation of relapse.

**Immune Response Analysis**

Anergy Panel: Overall immune responsiveness was assessed using an anergy panel skin test to recall antigens (Mumps, Candida, Tetanus).
Tumor DTH Testing: Induction of in vivo immune response was assessed by tumor delayed-type hypersensitivity (DTH) skin testing using $2 \times 10^6$ autologous leukemia cells per test. Skin test reagents were injected intradermally. Induration of at least 5 mm at 48-72 hours was considered a positive reaction.

In Vitro Antibody Response: Induced antibody responses reactive against autologous leukemia cells and K562/GM cells were analyzed by immunoblot as previously described. Detection of new or enhanced intensity bands on immunoblot compared to pre-treatment baseline was considered a positive antibody response.

In Vitro T Cell Response: Induction of T cell responses to autologous leukemia cells was performed using an Elispot assay measuring interferon-γ and Granzyme-B production (H. Levitsky). A 2-fold increase over pre-treatment baseline with at least 5 spots measured was considered a positive reaction. Analysis was limited to patients who received at least 4 post-transplant immunotherapy treatments. Detailed methods are provided in Supplemental Data (available on-line).

Minimal Residual Disease Monitoring by WT1

The presence of minimal residual leukemia was assessed by monitoring of WT1 transcript levels in blood and bone marrow using a quantitative RT-PCR assay (W. Stock). Detailed methods are provided in Supplemental Data available on-line. Briefly, total RNA was extracted from blood and bone marrow and cDNA synthesized using standard techniques. Amplifications of patient samples, K562 cell line cDNA, and no template controls were performed in triplicate. WT-1 expression levels were detected using a transcript specific primer and probe set. In order to compensate for differences in RNA integrity and cDNA synthesis efficiency, the absolute WT1 transcript copy number
was normalized to the endogenous control gene, ABL. The limit of normalized WT1 transcript quantification was $10^{-3}$ WT1/ABL. WT1 monitoring was performed at the following time points: enrollment, CR, pre-transplant immunotherapy (days 0 and 14), post transplant immunotherapy (treatments 1, 4, and 8), and 3 months and 6 months after completion of immunotherapy.

**Statistical Analysis**

It was estimated that approximately 50% of enrolled patients would both achieve CR and proceed to immunotherapy treatment and ASCT leading to a target of 50 enrolled patients to yield an evaluable population of 25 immunotherapy-treated patients. Relapse-free and overall survival was estimated using the method of Kaplan and Meier. Normalized WT1 values recorded as zero were set at $10^{-4}$ for the purpose of WT1 log change from baseline analyses. Exploratory multivariate analysis of RFS in the following subgroups was performed: WT-1 decline (present or absent), autologous tumor DTH response (more or less than 5 mm induration), antibody response to autologous tumor or K562/GM cells (present or absent), and cytogenetic risk group. Cytogenetic risk groups were defined as: good - t(8:21), inv 16; poor - complex, t(6:9), and del 7; and intermediate – normal, other. All authors had access to the primary clinical trial data for review.
RESULTS

Patients and Treatment

Fifty-four patients enrolled in the study at 4 investigational sites and underwent leukemia cell harvest; 53 (98%) received induction chemotherapy and 46 (85%) achieved CR. One patient received dose reduction of daunorubicin (from 60 to 45 mg/m²) during induction chemotherapy and 5 received an additional induction cycle (cytarabine + anthracycline +/- etoposide) following protocol-specified therapy. Thirty-four patients (63%) received consolidation chemotherapy, all per protocol; 28 (52%) received the first pre-transplant immunotherapy treatment and 27 (50%) proceeded to ASCT. Twenty-one patients (39%) initiated and 19 (35%) completed all available post-transplant immunotherapy treatments. Disposition of trial subjects and reasons for study withdrawal are outlined in Supplemental Figure 1. The primary reasons for study withdrawal included relapse, death, and triage to alternative therapies. The median time from ASCT to initiation of post-transplant immunotherapy was 66 days (range 43 – 459) with delays predominantly due to slow platelet engraftment. Of the 21 patients who received post-transplant immunotherapy treatments, 5 ultimately relapsed; 3 of these initiated post-transplant immunotherapy on schedule and two were delayed.

Baseline characteristics for all enrolled patients who underwent leukemia cell harvest (n=54) and those proceeding to pre-transplant immunotherapy (n=28) are shown in Table 1. Since most of the attrition between enrollment and ASCT occurred among patients with high risk cytogenetic abnormalities, 75% of immunotherapy-treated patients fell into an intermediate cytogenetic risk group.

Cell Processing Feasibility


The majority of enrolled patients underwent leukemia cell harvest from blood by either a simple blood draw (41%) or apheresis procedure (52%). The median number of viable leukemia cells harvested was $5.6 \times 10^9$ cells (range $0.73 - 65.91 \times 10^9$); apheresis procedures were, not surprisingly, associated with the highest leukemia cell yields. In only 3 patients (5.6%) was the tumor cell harvest less $1 \times 10^9$ cells, the minimum dose required for 9 immunotherapy treatments plus immune monitoring tests. Adequate stem cell harvest ($> 2 \times 10^6$ CD34+ cells/kg) was achieved in 25/28 (89%) patients with a median stem cell dose of $6.9 \times 10^6$ CD34+ cells/kg. A median dose of “primed” lymphocytes of $1.2 \times 10^8$ CD3+ cells/kg (range $0.7–96 \times 10^8$) was collected.

**GM-CSF Pharmacokinetics**

Serum GM-CSF concentrations were monitored following pre- and post-transplant immunotherapy treatments in 10 patients (Supplemental Table 1). Six patients had detectable GM-CSF levels at one or more time points. A similar pattern was observed after each immunotherapy dose, with peak levels (ranging from 6.1 to 71.1 pg/mL) occurring one day following administration in most patients, and measurable levels for up to 4 days. There was no clear evidence of accelerated GM-CSF clearance with repeated immunotherapy administration.

**Immune Response**

Immune response was assessed through anergy panel skin testing, immunotherapy injection site reactions, DTH skin reactions to injections of autologous irradiated leukemia cells, in vitro T cell response to autologous leukemia cells, and induction of antibody reactivity against autologous leukemia and K562/GM cells. In vivo skin testing results and in vitro T cell and antibody responses are summarized in Table 2. A response
to Candida and Mumps was evaluated at multiple time points throughout the study and was present in approximately 50% of patients at any given time point; Tetanus reactivity was evaluated at the last post-transplant immunotherapy treatment only and was present in 29% of patients. In contrast, skin reactions at immunotherapy injection sites were noted in 100% of patients with at least 94% responding at each time point. DTH reactions to injections of autologous leukemia cells were present in 2/26 (7.7%) patients prior to the first immunotherapy treatment and were induced in 7/18 (39%) additional patients at either the fourth or eighth post-transplant immunotherapy treatment. In each case induction of a positive tumor DTH response was transient. Six of the 7 patients with induced tumor DTH responses also had responses to Candida but one had no response to either Candida or Mumps.

Analysis of in vitro T cell response to autologous tumor by Elispot was performed after a 7 day in vitro stimulation in patients who completed at least 4 post-transplant immunotherapy treatments. Seven of 17 (41%) patients showed induction of interferon-γ Elispot responses following the first pre-transplant immunotherapy; 2 of these subjects also showed induction of granzyme-B responses. A representative pre-transplant immunotherapy response is shown in Figure 2a and 2b. Fifteen of 17 patients showed induction of Elispot responses following post-transplant immunotherapy with the majority (10 of 15) demonstrating induction of both interferon-γ and granzyme-B responses. A representative post-transplant response is shown in Figure 2c and 2d. Fourteen of these 15 patients remain in continuous CR. All 7 of the patients with induction of autologous tumor skin DTH responses post-transplant also showed induction of in vitro T cell responses to autologous tumor by Elispot. The peak Elispot response,
assessed by either interferon-γ or granzyme-B production, coincided with the time point when DTH conversion was documented in 6 of 7 subjects.

Induction of antibody reactivity against the immunotherapy components was also assessed. Following immunotherapy, 5/15 (33%) of patients demonstrated induction of antibodies reactive against autologous leukemia cells whereas 15/15 (100%) demonstrated induced antibody reactivity against K562/GM cells. Preliminary analyses in 8 subjects showed no evidence of induction of antibody responses directed against WT1 (data not shown).

**Clinical Outcome**

Forty-six of 53 (85%) patients achieved CR following one or more cycles of induction chemotherapy with a high dose cytarabine-based regimen. At the time of the primary analysis, median follow-up for surviving patients was 3 years. For all patients who achieved CR, the estimated 3-year relapse-free survival (RFS) was 47.4% (95% CI: 30.7 – 62.4) and overall survival (OS) was 57.4% (95% CI: 40 – 71.5). For patients treated with immunotherapy (n = 28), of whom 27 proceeded to ASCT, the estimated 3-year RFS was 61.8% (95% CI: 40.2 - 77.6) and OS, 73.7 % (95% CI: 52.5 - 86.5).

Limited additional follow up of the immunotherapy treated group (median 5 years) showed an estimated 5-year RFS of 55.1% (95% CI: 34.6 – 71.6) and OS of 60.1% (95% CI: 37.7 -76.7).

**Minimal Residual Disease**

Minimal residual disease (MRD) was monitored in blood and bone marrow throughout the study using a quantitative molecular assay that measured expression of the gene encoding WT1. The percent of patients with detectable WT1 transcript levels
throughout the study is shown in Figure 3. All patients assessed had detectable WT1 levels in blood and bone marrow at enrollment when overt leukemia was present. Over 90% of patients who achieved CR had associated declines in WT1 in both blood and bone marrow (median of approximately 3-logs in blood). Nevertheless, 46% and 95% of patients had persistently detectable WT1 levels in blood and bone marrow, respectively. A change in WT1 transcript copy number in the blood was evaluated between the time of the pre-transplant immunotherapy treatment and collection of the leukapheresis product two weeks later. Pre-transplant immunotherapy was delivered a median of 55 days (range 36 – 165) following initiation of consolidation chemotherapy and 24 days (range 15 – 130) following stem cell collection. Over this two week interval, 11/16 (69%) patients with persistently detectable levels of WT1 in blood showed further WT1 declines, with 8/11 (70%) achieving an undetectable WT1 status (Figure 3). Of the 3 remaining patients still evaluable for further reductions in WT1 with post-transplant treatments, one showed a subsequent decline to undetectable levels, one had stable WT1 levels, and one relapsed prior to initiating post-transplant immunotherapy. In general, patients who maintained a continuous CR status throughout the study had greater WT1 declines from baseline in both blood and bone marrow than patients who ultimately relapsed (Figure 4).

Examination of Relapse-free Survival in Subgroups

Exploratory analyses were conducted evaluating RFS in subgroups based on WT1 response, autologous tumor DTH response, autologous tumor and K562/GM antibody response, and cytogenetic risk group. Results are summarized in Table 3. While subgroups were small, limiting data interpretation, several observations were made.
Patients with a decrease in WT1 levels in blood following the pre-transplant immunotherapy treatment (Figure 5a) had longer RFS than those without WT1 declines (median 37.0 vs. 9.0 months, \( p = 0.0294 \); 3-year RFS 60.6 vs 0%). In addition, those who achieved undetectable WT1 levels at any time point during the trial (Figure 5b) also had longer RFS (median 37.0 vs. 7.0 months, \( p = 0.0884 \); 3-year RFS 58.2 vs. 14.3%). In particular, failure to achieve an undetectable WT1 transcript level prior to transplant was associated with a high risk of relapse within 12 months (Figure 5c). In vivo immune response induction was also associated with prolonged RFS; the 7 patients with treatment-induced autologous tumor DTH reactions all remain in continuous CR at last follow-up (3-yr RFS 100 vs. 48.1%) (Figure 5d). Three of these 7 DTH responders converted from detectable to undetectable WT-1 transcript levels following the first immunotherapy dose and the other 4 had undetectable levels at onset of immunotherapy. Of the 17 patients analyzed for in vitro T cell response to autologous tumor, 15/17 remain in continuous CR impeding formal subgroup analysis. However, 14/15 of these CCR patients demonstrated post-transplant Elispot responses against autologous tumor. In contrast, no association was seen between induction of new antibody reactivity against autologous tumor and RFS. Finally, cytogenetic risk group was associated with leukemia outcome with a 3-year RFS of 80% in all patients who achieved CR with good risk (n=7), 51% with intermediate risk (n=33), and 0% with poor risk (n=6). Corresponding 3-year RFS data for the 28 immunotherapy treated patients was 75% with good risk (n=4), 66% with intermediate risk (n=22), and 0% with poor risk (n=2) cytogenetics.
Safety

No grade 3 or 4 adverse events related to the leukemia cell harvest were reported. During immunotherapy treatment, grade 1/2 injection site reactions, reported in all patients, were the most common adverse event. Other common related adverse events included pain, pruritis, and headache (each 21%), fatigue (18%), urticaria and feeling abnormal (each 14%). Only one related grade 3 event (arthralgia) was reported. Due to a theoretical concern about induction of autoimmunity against the hematopoietic system by the pre-transplant dose of immunotherapy and subsequent reinfusion of “primed” lymphocytes with the stem cell graft, time to post-transplant hematologic engraftment was monitored. The median time to ANC > 500/mm^3 was 15 days (range 13 – 21), to platelets > 20,000/mm^3 was 12 days (range 6 – 108), and to platelets > 50,000/mm^3 was 20 days (range 9 – 435). A median of 3 platelet transfusions (range 0 – 41) and 3 RBC transfusions (range 0 – 22) were administered post-transplant.

DISCUSSION

This trial explored the combination of ASCT, adoptive transfer of immunotherapy-primed lymphocytes, and pre- and post-transplant immunotherapy based on the GVAX platform as post-remission therapy for adult AML. The results demonstrate the feasibility of immunotherapy production, a favorable toxicity profile, and encouraging signals of potential activity. These signals included induction of tumor DTH skin reactions, anti-tumor T cell and antibody responses, and reductions in minimal residual disease as measured by WT1 transcript levels. Furthermore, clinical outcomes
were at least comparable to historical single center data evaluating ASCT as post-remission therapy in AML.

Over one billion leukemia cells (median 5.6 billion) were successfully collected at diagnosis in 95% of enrolled patients with the vast majority obtained through a simple blood draw or apheresis procedure. The tumor cell yield was higher and harvested through less invasive procedures than in previous clinical trials evaluating this mixed GVAX platform in non-small cell lung cancer (NSCLC) and multiple myeloma. In the NSCLC trial, the median tumor cell dose harvested via surgical procedures was only 40 million cells. In the myeloma trial, the median tumor cell dose harvested was 3.5 billion cells, but required a high volume bone marrow harvest under general anesthesia. Clearly, acute leukemia represents an optimal clinical setting in which to harvest autologous tumor cells for immunotherapy applications.

In a subset of patients, serum GM-CSF levels were monitored as a potential biomarker of K562/GM pharmacokinetics. As observed in previous trials, the majority of subjects showed reproducible levels of serum GM-CSF following repeat dosing. K562 cells, based on their lack of HLA expression are targets of natural killer cell cytotoxicity, but this did not result in rapid in vivo clearance. Furthermore, peak levels of serum GM-CSF were below that reported to result in significant induction of myeloid suppressor cells and inhibition of immunogenicity in animal studies.

Immunotherapy was well tolerated with expected toxicities of injection site reactions and flu-like symptoms. No patients suffered from engraftment failure, a theoretical risk of this immunotherapy approach and protocol design. Time to neutrophil engraftment (median 15 days) was somewhat delayed compared with most published
studies using mobilized peripheral blood stem cells (median 9 – 10 days)\(^2\) and delayed platelet engraftment was observed in some patients. Delayed neutrophil and platelet engraftment have been reported in patients receiving autologous CD34+ stem cell doses of < 10 million cells with a median time to ANC > 500/mm\(^3\) of 12 days and platelets > 50,000/mm\(^3\) of 46 days reported in this population\(^29\). The CD34+ stem cell dose administered in this study was < 10 million cells in the majority of patients (median 6.9 million), making this a potential explanation for delayed engraftment. However, an effect of immunotherapy, particularly on neutrophil engraftment, remains possible.

Treatment-associated immune responses were demonstrated and included induction of autologous tumor DTH skin reactions, in vitro T cell responses to autologous tumor by Elispot, and autologous tumor-reactive antibodies. Of interest is that all 7 patients with positive DTH reactions post immunotherapy have remained relapse-free with a minimum follow up of 2 years. Furthermore, while the analysis was skewed toward patients still in CR following completion of 4 post-transplant immunotherapy treatments, 14 of 15 such patients demonstrated measurable Elispot responses to autologous tumor post-transplant. The transient nature of the induced tumor DTH responses may have been due to clearance of leukemia antigens in the setting of continuous CR and/or cessation of immunotherapy treatment. In most subjects, the time point of tumor DTH reactivity coincided with peak levels of Elispot reactivity. At least one prior trial using the GVAX platform in the adjuvant setting in the treatment of resected pancreatic cancer also showed a positive association between autologous tumor DTH reactions and freedom from relapse\(^15\).
Monitoring of minimal residual disease in leukemia trials is a powerful tool that has been shown to be highly predictive of response to therapy and clinical outcome in multiple hematologic cancers including acute promyelocytic leukemia (PML-RARα by RT-PCR)\textsuperscript{30,31}, chronic myeloid leukemia (bcr-abl by RT-PCR)\textsuperscript{32,33} and acute lymphoblastic leukemia (various methods)\textsuperscript{34,35}. WT1 codes for a zinc-finger transcription factor that is over-expressed in the vast majority of acute leukemias making this an attractive pan-leukemia cell marker for monitoring of MRD across the entire karyotypic spectrum of AML\textsuperscript{24,25}. Previous studies have demonstrated an association between persistent or recurrent detection of WT1 in patients with AML in CR and risk of relapse\textsuperscript{26,36}. Cilloni et al. recently reported that detectable WT1 in the blood of AML patients in first CR was a significant adverse prognostic factor, even when adjusted for other risk factors or treatments, including ASCT. In their series of 71 AML pts who entered a CR after induction therapy, 23 had persistently measurable WT1 in the blood and all 23 went on to relapse\textsuperscript{26}.

In our study, all patients had detectable levels of WT1 in both blood and bone marrow at study entry. Nevertheless, clearance of WT1 from the blood was only achieved in approximately half of the patients in CR, even after one additional round of consolidation chemotherapy, suggesting that the amount of additional leukemia cell kill achieved with repeated cycles of high dose cytarabine-based chemotherapy is minimal. Following receipt of a single dose of immunotherapy pre-transplant, however, two-thirds of patients showed a further decline in WT1 transcript levels in blood with 70\% of patients achieving undetectable levels. The ability to monitor further declines in WT1 following post-transplant treatments was, therefore, limited. A decline in WT1 following
the first immunotherapy dose was associated with longer relapse-free survival. In fact, all patients without a measured WT1 decline in response to pre-transplant immunotherapy went on to relapse within one year of transplant. It is unlikely that a late effect of prior consolidation chemotherapy accounted for the declines in blood WT1 levels observed after this first treatment given that: 1) the median 3-log reduction in WT1 levels following induction was observed within 6 weeks of initiation of chemotherapy, 2) no incremental reduction in median WT1 levels in blood was observed 8 weeks following consolidation chemotherapy, and 3) the pre-transplant immunotherapy was administered at a time when normal hematopoiesis had recovered from the cytotoxic effects of chemotherapy. It is also unlikely that clearance of mobilized WT1-expressing hematopoietic progenitors from the blood after stem cell collection contributed to this measured WT1 decline, given that mobilization of CD34+ progenitors into the blood typically declines to background levels within several days of cessation of GCSF mobilization and stem cells were collected a median of 24 days prior to the first dose of immunotherapy in this trial.

In general, subjects whose leukemia ultimately relapsed had higher levels of WT1 post-remission than subjects who remained in continuous CR. Furthermore, achievement of undetectable WT1 levels after induction or consolidation chemotherapy, immunotherapy, or ASCT were all associated with favorable outcome, suggesting that clearance of MRD, as measured by WT1 transcript levels, may be a useful biomarker to follow in the management of AML in general, and in the conduct of trials evaluating post-remission therapies in patients in hematologic remission, in particular.
While background WT1 expression in normal bone marrow progenitors likely accounted for persistent detection of WT1 transcripts in the marrow in the majority of patients throughout the study, expression of WT1 in normal blood is rare. Therefore, persistent expression in blood likely represents true MRD\textsuperscript{24,25}. This makes peripheral blood, not only the most convenient, but also the best source material for WT1 monitoring of MRD in AML.

In conclusion, this study demonstrated encouraging signals of activity that were positively associated with clinical outcome in younger patients with AML in the setting of ASCT. These data suggest that this immunotherapy approach may lead to significant reductions of MRD in the majority of patients with leukemia in remission. Furthermore, given the rapid WT1 response noted following a single dose of immunotherapy prior to ASCT, it would be reasonable to explore this and other platforms of leukemia-specific immunotherapy as post-remission maintenance therapy in the absence of ASCT, especially in older patients with AML for whom aggressive post-remission therapies may not be indicated.

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AUTHOR CONTRIBUTIONS

Ivan Borrello designed research, performed research, analyzed data, edited the paper, Hyam Levitsky designed research, performed research, contributed analytical tools, analyzed data, edited the paper, Wendy Stock designed research, performed research, contributed vital analytical tools, analyzed data, edited the paper, Dorie Sher contributed vital analytical tools, analyzed data, Lu Qin contributed vital analytical tools, performed research, Daniel J. DeAngelo designed research, performed research, edited the paper, Edwin Alyea designed research, performed research, edited the paper, Richard Stone designed research, performed research, edited the paper, Lloyd Damon designed research, performed research, edited the paper, Charles Linker designed research, performed research, edited the paper, Daniel Maslyar analyzed data, and Kristen Hege designed research, analyzed data, wrote the paper.

CONFLICT OF INTEREST DISCLOSURE

Kristen Hege and Dan Maslyar are past employees and stockholders of Cell Genesys, Inc. Under a licensing agreement between Cell Genesys and the Johns Hopkins University, Hyam Levitsky and Ivan Borrello are entitled to a share of milestone payments and a share of royalty received by the University on sales of GVAX. Dr. Levitsky previously served as a paid consultant to Cell Genesys. The terms of this arrangement are being managed by the Johns Hopkins University in accordance with its conflict of interest policies. Wendy Stock and Hyam Levitsky received funding to support correlative studies.
including analysis of WT-1 and immune monitoring, respectively. The other authors have no financial conflicts of interest to disclose.

REFERENCES


# TABLES

## Table 1: Baseline Patient and Tumor Characteristics

<table>
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<tr>
<th></th>
<th>Enrolled (n = 54)</th>
<th>Immunotherapy Treated (n = 28)</th>
</tr>
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<tbody>
<tr>
<td>Age (years): Median (range)</td>
<td>47 (21-60)</td>
<td>49 (24-59)</td>
</tr>
<tr>
<td>Female: No. (%)</td>
<td>31 (57)</td>
<td>18 (64)</td>
</tr>
<tr>
<td>Ethnicity: No. (%)</td>
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<tr>
<td>Caucasian</td>
<td>46 (85)</td>
<td>25 (89)</td>
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<tr>
<td>Other</td>
<td>8 (15)</td>
<td>3 (11)</td>
</tr>
<tr>
<td>Cytogenetic Risk Group (%)</td>
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<tr>
<td>Poor</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td>Intermediate</td>
<td>65</td>
<td>75</td>
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<td>54</td>
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<tr>
<td>Other</td>
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<td>Good</td>
<td>15</td>
<td>14</td>
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<td>Leukemia Cell Harvest Procedure: No. (%)</td>
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<tr>
<td>Apheresis</td>
<td>28 (52)</td>
<td>15 (54)</td>
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<tr>
<td>Blood Draw</td>
<td>22 (41)</td>
<td>11 (39)</td>
</tr>
<tr>
<td>Bone Marrow Aspiration</td>
<td>4 (7)</td>
<td>2 (7)</td>
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<td>Leukemia Cell Immunophenotyping (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD13</td>
<td>91</td>
<td>91</td>
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<td>CD33</td>
<td>98</td>
<td>96</td>
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<tr>
<td>CD34</td>
<td>75</td>
<td>68</td>
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<td>Blood Stem Cell Collection</td>
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<td>Median CD34+ cells x 10⁶/kg (range)</td>
<td>NA</td>
<td>6.94 (0.34 – 14)</td>
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<tr>
<td>Primed Lymphocyte Collection</td>
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<td></td>
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<tr>
<td>Median CD3+ cells x 10⁸/kg (range)</td>
<td>NA</td>
<td>1.17 (0.67 – 96)</td>
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Table 2: In Vivo (Skin DTH Testing) and In Vitro Immune Response Analysis

<table>
<thead>
<tr>
<th>Test Performed</th>
<th>Pre-transplant Baseline</th>
<th>Day 14</th>
<th>Post-transplant Treatment #1</th>
<th>#4</th>
<th>#8</th>
<th>Follow-up 9 mo</th>
<th>Off-study</th>
<th>Any time point</th>
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<td></td>
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</tr>
<tr>
<td>Immunootherapy</td>
<td></td>
<td>26/27</td>
<td>19/19 (100%)</td>
<td>18/19 (95%)</td>
<td>15/16 (94%)</td>
<td></td>
<td></td>
<td>27/27 (100%)</td>
</tr>
<tr>
<td>positive reactiona</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Candida positive</td>
<td>6/27 (22%)</td>
<td>4/23</td>
<td>7/20 (35%)</td>
<td>8/17 (47%)</td>
<td>6/17 (35%)</td>
<td>5/10 (50%)</td>
<td>6/16 (38%)</td>
<td>15/28 (54%)</td>
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<tr>
<td>reactiona</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mumps positive</td>
<td>4/8 (50%)</td>
<td>3/6</td>
<td>1/3 (33%)</td>
<td>1/2 (50%)</td>
<td>1/1 (100%)</td>
<td></td>
<td></td>
<td>4/8 (50%)</td>
</tr>
<tr>
<td>reactiona</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Tetanus positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2/7 (28.6%)</td>
<td></td>
<td>2/7 (28.6%)</td>
</tr>
<tr>
<td>reactiona</td>
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<td></td>
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<td></td>
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<tr>
<td>Tumor positive</td>
<td>2/26 (7.7%)</td>
<td>0/26</td>
<td>0/21 (0.0%)</td>
<td>3/18 (17%)</td>
<td>4/18 (22%)</td>
<td>0/11 (0.0%)</td>
<td>0/18 (0.0%)</td>
<td>9/28 (32%)</td>
</tr>
<tr>
<td>reactiona</td>
<td></td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td><strong>In Vitro</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Tumor Elispot</td>
<td></td>
<td>7/17</td>
<td></td>
<td></td>
<td></td>
<td>15/17 (88%)</td>
<td></td>
<td>16/17 (94%)</td>
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<td>positive T cell</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>responseb</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Tumor-reactive</td>
<td>4/15e (27%)</td>
<td>5/14e</td>
<td>3/13e (23%)</td>
<td></td>
<td></td>
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<tr>
<td>antibody responsed</td>
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<td></td>
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</tr>
</tbody>
</table>

aA positive reaction was defined as an induration of at least 5 mm
bA positive response was defined as at least 2-fold increase over pre-treatment baseline in either interferon-γ or granzyme-B production by Elispot against autologous leukemia cells
cPost-transplant results are summarized as patients with positive Elispot responses at any post-transplant time point
dA positive response was defined as development of new or increased intensity bands compared to pre-treatment baseline on immunoblot against lysates of autologous leukemia cells
eAnalyses conducted at post-transplant treatments 2, 3 and 7 rather than 1, 4 and 8.
Table 3: Median Relapse-Free Survival by Subgroups

<table>
<thead>
<tr>
<th>Assessment</th>
<th>Subgroups</th>
<th>Median Relapse-free Survival (months)</th>
<th>95% CI</th>
<th>Logrank p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-1 decrease after first immunotherapy treatment – peripheral blood</td>
<td>Yes (n=11) No (n=5)</td>
<td>37.0</td>
<td>(7.0, 37.0)</td>
<td>p=0.0294</td>
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<td></td>
<td></td>
<td>9.0</td>
<td>(7.0, 13.0)</td>
<td></td>
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<tr>
<td>WT-1 decrease after first immunotherapy treatment – bone marrow</td>
<td>Yes (n=12) No (n=8)</td>
<td>NR</td>
<td>(NR, NR)</td>
<td>p=0.7441</td>
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<td></td>
<td></td>
<td>37.0</td>
<td>(9.0, NR)</td>
<td></td>
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<tr>
<td>WT-1 undetectable after transplant – peripheral blood</td>
<td>Yes (n=19) No (n=6)</td>
<td>NR</td>
<td>(37.0, NR)</td>
<td>p=0.0004</td>
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<td></td>
<td></td>
<td>8.5</td>
<td>(7.0, 13.0)</td>
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<td>WT-1 undetectable at any time point post induction – peripheral blood</td>
<td>Yes (n=33) No (n=8)</td>
<td>37.0</td>
<td>(10.0, NR)</td>
<td>p=0.0181</td>
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<td>7.0</td>
<td>(2.0, 13.0)</td>
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<tr>
<td>WT-1 undetectable at any time point post induction – bone marrow</td>
<td>Yes (n=15) No (n=23)</td>
<td>NR</td>
<td>(11.0, NA)</td>
<td>p=0.0884</td>
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<td></td>
<td></td>
<td>13.0</td>
<td>(7.0, NA)</td>
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<tr>
<td>WT-1 undetectable at any time point post consolidation – peripheral blood</td>
<td>Yes (n=23) No (n=6)</td>
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<td>(37.0, NR)</td>
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<tr>
<td>WT-1 undetectable at any time point post consolidation – bone marrow</td>
<td>Yes (n=13) No (n=16)</td>
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<td>28.0</td>
<td>(9.0, 37.0)</td>
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<td>WT-1 undetectable prior to ASCT – peripheral blood</td>
<td>Yes (n=19) No (n=8)</td>
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<td>(NR, NR)</td>
<td>p=0.0028</td>
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<td>9.0</td>
<td>(7.0, 37.0)</td>
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<tr>
<td>Induced DTH response to autologous tumor</td>
<td>Yes (n=7) No (n=19)</td>
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<td>(NR, NR)</td>
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<td>28.0</td>
<td>(9.0, NR)</td>
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<tr>
<td>Induced antibody response to autologous tumor</td>
<td>Yes (n=5) No (n=10)</td>
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<td></td>
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<td>NR</td>
<td>(NR, NR)</td>
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<tr>
<td>Cytogenetic risk group – all patients who achieved CR</td>
<td>Good (n=7) Normal/other (n=33)</td>
<td>NR</td>
<td>(6.0, NR)</td>
<td>p&lt;0.0001</td>
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<td></td>
<td>Poor (n=6)</td>
<td>37.0</td>
<td>(9.0, NR)</td>
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<td></td>
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<td>4.0</td>
<td>(1.0, 7.0)</td>
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<tr>
<td>Cytogenetic risk group – all patients who received pre-ASCT immunotherapy</td>
<td>Good (n=4) Normal/other (n=22)</td>
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<td>Poor (n=2)</td>
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<tr>
<td></td>
<td></td>
<td>6.5</td>
<td>(6.0, 7.0)</td>
<td></td>
</tr>
</tbody>
</table>

NR = not reached; a median relapse-free survival has not been reached
FIGURE LEGENDS

**Figure 1. Study Design.** Design included leukemia harvest at diagnosis of AML, induction chemotherapy with high dose cytarabine (Ara-C) and daunorubicin (dauno), consolidation chemotherapy with high dose Ara-C and etoposide (VP16) followed by stem cell mobilization with GCSF, peripheral blood stem cell (PBSC) collection, single pre-transplant dose of immunotherapy followed by collection of primed lymphocytes by lymphapheresis, pre-transplant myeloablative preparative regimen with Busulfan (Bu) and Cyclophosphamide (Cy), PBSC and primed lymphocyte infusion, and post-transplant immunotherapy treatments administered every 3 weeks for 8 doses, beginning at least 6 weeks post-transplant.

**Figure 2: In Vitro T Cell Immune Response to Autologous Tumor.** Results of Elispot assay in two representative patients. Blood samples were analyzed at various study time points for T cell response to autologous leukemia cells and assessed for interferon-γ and granzyme-B production by Elispot. Patient 028-404 showed induction of T cell response by both interferon-γ (2a) and granzyme-B (2b) following pre and post-transplant immunotherapy. A typical pattern is seen with waning of the pre-transplant response early post-transplant followed by recovery with post-transplant treatments. Patient 028-405 showed induction of T cell response by both interferon-γ (2c) and granzyme-B (2d) following post-transplant immunotherapy only. This response coincided with induction of a tumor DTH skin test response at post-transplant treatment 8. Analysis time points included: pre-transplant immunotherapy baseline (Day 0) and day 14 following the first
immunotherapy dose (Day 14), prior to post-transplant immunotherapy #1 (~ 6 weeks post transplant) and at post-transplant immunotherapy #4 and #8, and follow up at month 9 and 12.

Figure 3: Detectable WT1 Transcript Levels in Blood and Bone Marrow. Percentage of patients with detectable WT1 transcript levels in peripheral blood (PB) and bone marrow (BM) at enrollment, complete remission (CR), pre-transplant immunotherapy day 0 (Day 0) and day 14 (Day 14), post-transplant immunotherapy number 1 (vax 1) and number 4 (vax 4), and follow up (post vax 4). Boxes signify chemotherapy treatments including induction, consolidation (consol), and ASCT. Arrows signify immunotherapy administration. Numbers along the bottom of the chart signify the number of patients with data at each time point.

Figure 4: WT1 Transcript Levels in Patients with Continuous Complete Remission vs. Relapse. Median log10 change from baseline in WT1 transcript levels for patients who achieved a CR and remained in continuous CR (CCR) throughout the study (dashed line) versus those who relapsed (solid line). Time points include: enrollment (enroll), complete remission (CR), pre-transplant immunotherapy day 0 (Day 0) and day 14 (Day 14), post-transplant immunotherapy number 1 (vax 1) and number 4 (vax 4), and last study measurement (off-study). Boxes signify chemotherapy treatments including induction, consolidation (consol), and ASCT. Arrows signify immunotherapy administration.
Figure 5: Relapse-free Survival in Subsets. (5a) Reduction in WT1 following pre-transplant dose of immunotherapy. (5b) Achievement of undetectable WT1 status at any time during the trial. (5c) Achievement of undetectable WT1 status prior to ASCT. (5d) Induction of Autologous Tumor DTH response. (Yes, dashed line; No, solid line).
FIGURES

Figure 1
Figure 2

IFN-γ

Granzyme-B

Pt 028-404

Pt 028-405
Figure 3

[Graph showing study time points and detectable WT-1 (%)]

Study Time Point

Detectable WT-1 (%)

Enroll
CR
Day 0
Day 14
Vax 1
Vax 4
Post Vax 4
PB
BM
PB
BM

Induction
Consol
ASCT
\(\downarrow\)
\(\downarrow\)
\(\downarrow\)
\(\downarrow\)
\(\downarrow\)
\(\downarrow\)
\(\downarrow\)
\(\downarrow\)

Values:
Enroll: 51, 40
PB: 39, 28
BM: 36, 24
Day 0: 23, 20
Day 14: 19, 18
Vax 1: 19, 17
Vax 4: 19, 17
Figure 4
Figure 5
GM-CSF secreting cellular immunotherapy in combination with autologous stem cell transplant (ASCT) as post-remission therapy for acute myeloid leukemia (AML)