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Young donor white blood cell immunotherapy induces extensive tumor necrosis in advanced-stage solid tumors

Dipnarine Maharaj^{a,*}, Pedro G. Vianna^{a,b}, Wendy Ward^a, Anthony J. Messina^a, Trevor Rayborn^a, Jacqueline V. Gouvea^a, Richard D. Hammer^c, Zheng Cui^d

^aSouth Florida Bone Marrow Stem Cell Transplant Institute, Boynton Beach, Florida 33437, USA

^bDepartment of Medicine, Stanford University School of Medicine, Stanford, California 94305, USA

^cDepartment of Pathology, University of Missouri School of Medicine, Columbia, Missouri 65212, USA

^dDepartment of Pathology, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157, USA

* Corresponding author.

E-mail address: dmaharaj@bmscti.org (D. Maharaj).

Abstract

Background: In the past decade, a variety of immunotherapy approaches focused predominantly on the adaptive immune system have shown unprecedented responses in patients with advanced-stage malignancies. However, studies in spontaneous regression/complete resistance (SR/CR) mice and humans have shown a novel innate cancer-killing activity mediated by granulocytes, which is completely transferable for prevention or therapy against established malignancies.

Methods: Three patients with advanced, relapsed or refractory solid tumors for which no standard therapy was available or was refused were enrolled into this ongoing combined phase I/II open label clinical trial testing the safety, dose tolerance, and possible antineoplastic efficacy of sequential infusions of HLA-mismatched non-irradiated allogeneic white cells (68–91% granulocytes) collected by leukapheresis from young, healthy donors (age 18–35) following mobilization with granulocyte colony stimulating factor (G-CSF) and dexamethasone.

Results: Besides fevers and flushing, no infusional toxicities were observed. All patients remained clinically stable following infusions with mild cytokine release syndrome and no evidence of transfusion-associated graft-versus-host disease,

acute tumor lysis syndrome, or transfusion-associated acute lung injury. Pathological examination of all cases post-mortem revealed extensive tumor necrosis up to 80% in patients 1–2, 40–50% in patient 3, and leukocyte infiltration in all cases, which could not be attributed to disease progression.

Conclusions: Allogeneic white cell immunotherapy (AWIT) from young, healthy donors is well tolerated with minimal side effects and shows antitumor activity against advanced-stage solid tumors. AWIT represents a novel, safe, and cost-effective immunotherapy that can be administered in an outpatient cancer clinic.

Keywords: Health sciences, Medicine, Internal medicine, Evidence-based medicine, Oncology, pharmaceutical science, Systems biology, Cancer research

1. Introduction

Immunotherapy represents a paradigm shift in the treatment of cancer patients by harnessing or augmenting the power of the immune response against malignant cells. Various components of the immune system have been shown to play pivotal roles in protecting humans from cancer [1]. As a result, a wide spectrum of treatments, including immune checkpoint inhibitors, monoclonal antibodies, adoptive cellular therapy, cytokines, oncolytic viruses, and cancer vaccines, have been evaluated [1, 2, 3]; each with different approaches to selectively boost or restore the antitumor activity of the immune system. While most recent clinical successes in advanced-stage cancers have focused on the adaptive immune system by stimulating T-cell function through regulatory receptors or their *ex-vivo* manipulation [3], the innate immune system and recently discovered cancer killing activity (CKA) of human granulocytes [4] may represent a novel immunotherapy approach independent of T-cell function [5].

Granulocyte transfusions (GT) have been used safely in clinical practice for over 40 years to treat cancer patients with chemotherapy-induced neutropenia and infections [6, 7, 8, 9]. However, the efficacy of GT as a therapeutic anti-cancer agent was not determined until recent studies in colonies of spontaneous regression/complete resistance (SR/CR) mice and humans suggested that the innate immune system may have a previously unrecognized role in immune surveillance and inheritable cancer resistance [4, 10]. Cui et al. showed that SR/CR mice were resistant to repeated challenges of transplantable lethal cancer cells and revealed age-dependent regression of advanced cancer mediated by a rapid and selective infiltration of host granulocytes at the cancer site with rapid cytolytic destruction of cancer cells without appreciable damage to normal cells [10, 11]. This cancer resistance mechanism is preexisting, independent of T and B lymphocytes, requires no prior cancer exposure, but can be strengthened by repeated tumor cell challenges [11]. Further studies revealed that this CKA is also present in human circulating leukocytes predominantly granulocytes, monocytes,

and natural killer (NK) cells with higher antitumor activity in healthy controls versus cancer cases [4] and aging had a profound inhibitory effect on the anticancer activity in SR/CR mice [9, 11]. Most importantly, these studies revealed that this innate cancer resistance mechanism was completely transferable to wild type (WT) recipient mice without the SR/CR phenotype either for prevention against subsequent cancer challenges or eradication of established malignancy at distant sites [5], suggesting that adoptive transfer of human peripheral blood leukocytes to patients with advanced-stage malignancies may potentially represent a new, viable anti-cancer therapy.

There are several considerations in designing an allogeneic white cell immunotherapy (AWIT). First, the therapeutic cells must come only from young, healthy individuals. Animal studies have shown that young mice leukocytes resulted in the highest overall cancer survival when challenged with lethal cancerous cells compared to aged donors [9]. These studies also revealed that the loss of CKA with age was primarily due to loss of cancer recognition and migratory function but not necessarily a loss in the number of granulocytes [9, 12]. Therefore, it is highly unlikely that an expansion of granulocyte number, i.e. by the stimulation with granulocyte colony stimulating factor (G-CSF), would reconstitute the innate CKA in cancer patients. Instead, AWIT is needed in order to supplement the defective immune function. In addition, leukocytes should be non-irradiated. While irradiation helps to minimize the risk of GVHD [13], Stehle Jr. et al. and other studies showed that irradiation of donor leukocytes had a profound suppressive effect on the CKA and survival of irradiated compared to non-irradiated granulocytes [11, 14].

Second, long-term engraftment of donor leukocytes, especially donor T-lymphocytes, should be avoided through complete HLA-mismatch between donor and recipient in order to minimize the possibility of transfusion-associated graft-versus-host disease (TA-GVHD). All of a granulocyte's actions, including chemotaxis, surface recognition and degranulation are completed within hours to 2 weeks [15]. Therefore, there should be a sufficient therapeutic time window in AWIT to allow for the granulocyte's effector mechanisms to take place before their functional decline or before HLA-mismatched leukocytes, including T lymphocytes, are rejected.

Third, prior mice studies have suggested that the best therapeutic effector:target cell ratio is between 3:1 to 10:1 [11, 12], however doses of previous GT have rarely exceeded 1×10^{10} cells [16]. The goal of AWIT is to transfuse a donor granulocyte dose equivalent to the entire granulocyte load of a healthy person in order to replace the functionally deficient immune system, which in an average human adult is approximately 2×10^{11} cells [17].

The goal of this phase I/II study is to determine the safety, dose tolerance, feasibility and possible efficacy of donor white blood cell infusions predominantly of granulocytes administered in the outpatient setting to patients with advanced, relapsed or refractory solid tumors for whom no standard therapy is available or is refused. The white cells are collected by leukapheresis following G-CSF and dexamethasone mobilization from young, healthy, unrelated 18 to 35-year-old donors. The intent is to determine if a cumulative dose of 2×10^{11} non-irradiated HLA-mismatched white cells over two weeks is well tolerated without transfusion or necrosis-related toxicities.

This article describes the significant tumor necrosis and minimal infusion side effects in a subset of three out of 11 patients in whom post-mortem pathology was available for review in this ongoing clinical trial of AWIT.

2. Methods

2.1. Clinical protocol

All patients were enrolled in an FDA investigational new drug (IND) and institutional review board (IRB)-approved, interventional single group (target of 29 patients), open label, combined phase I/II clinical trial entitled “A Study Using White Blood Cells from Healthy Donors to Treat Solid Cancers” (ClinicalTrials.gov, protocol number NCT00900497). The institutional review board which approved this trial was the Western Institutional Review Board. The primary outcome measure was safety with a secondary outcome of treatment efficacy, if available. Subjects were followed for 3 months after white blood cell infusions were completed. Response at 90 days was based on comparison to tumor measurements at baseline. Outcome measures included short-term safety monitoring and dose tolerance of transfusions. All evaluations, procedures, treatments, and follow-ups on donors and patients were performed at the South Florida Bone Marrow Stem Cell Transplant Institute, an outpatient cancer program. All authors discussed and interpreted the results. No commercial sponsor was involved in the study.

2.2. Patient eligibility

Informed consent was obtained from all patients. Each participating patient was terminally ill and had a histologically or cytologically confirmed non-hematological malignancy that was metastatic or unresectable and for which standard or palliative measures did not exist or were no longer effective. Patients must have had an Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 2, and ≥ 4 weeks since prior medical or radiation therapy or surgery. Patient eligibility also included having adequate organ function, including absolute neutrophils $\geq 1,500/\mu\text{l}$, platelet transfusion independent, platelet count $\geq 100,000/$

μl , serum bilirubin ≤ 2 mg/dl, AST/ALT less than 3x upper limit of normal and serum creatinine ≤ 2 mg/dl. HLA typing was performed for recipients by polymerase chain reaction (PCR) to ensure HLA-mismatch. Exclusion criteria included patients with evidence of brain tumors or metastases, prior history of stem cell transplantation, prior history of fludarabine therapy, negative for neutrophil or HLA Class I and II antibodies, women of childbearing potential who had a positive serum pregnancy test prior to treatment, pregnant or nursing women, HIV infection, use of immunosuppressive agents in the last 30 days other than steroids or patients with uncontrolled comorbidities, including diabetes mellitus, significant cardiac disease or active serious infection.

2.3. Donor eligibility

Informed consent was obtained from all donors. Donors were selected from a young and healthy volunteer pool with age requirements between 18 and 35 years old and who had completed a full-length universal donor history questionnaire. Donors were required to be willing to undergo granulocyte leukapheresis, and have an HLA profile (A, B, C, DR, DP, DQ) via PCR in order to ensure donated granulocytes were HLA-mismatched with recipients in at least 7 out of 10 HLA subtypes. Donors must have had negative anti-neutrophil antibodies, HLA Class I and II antibodies, ABO and Rh compatible with recipient, and CMV sero-matched to the recipient. Donors were also required to meet all the routine criteria for blood transfusion, such as infectious disease panel and leukocyte counts. Exclusion criteria included a genetic relationship to the recipient, positive infectious disease workup within 30 days of leukapheresis/donation, or known cardiac illness that could cause a potential risk associated with leukapheresis. These selected donors became part of a donor registry. The test results were used to match donors with specific patients.

CKA screening was omitted from the donor selection criteria. Blanks et al. showed that CKA levels in healthy controls was higher than in cancer cases [4]. Stehle Jr et al. further demonstrated that young donor leukocytes had greater functionality and performed better [9]. Therefore, the cancer-free history of young and healthy donors and their first-degree relatives was used as a means of donor selection in addition to the other criteria described above.

2.4. Leukapheresis collection

Donor leukocytes were mobilized with 300 micrograms of G-CSF (Neupogen[®], Amgen, Thousand Oaks, CA) subcutaneously and 6 mg of dexamethasone orally 12 hours before leukapheresis collection. A COBE Spectra apheresis machine (Terumo BCT, Lakewood, CO) was specifically optimized by the manufacturer for the granulocyte collection. Peripheral veins in the arms were accessed for the

collection procedures. Each collection lasted three hours on average. Up to 15 liters of blood were processed for each donor. The average volume of the collected concentrate was 300 ml. An aliquot of each final collection product was taken for CBC with differentials. The entire collection and mobilization procedures were well tolerated by the donors.

2.5. Leukocyte dose calculation and treatment protocol

The goal of AWIT is to transfuse a donor granulocyte dose equivalent to the entire granulocyte load of a healthy person in order to replace the functionally deficient immune system, which in an average human adult is approximately 2×10^{11} cells [17]. Therefore, our target collection dose was at least 2×10^{11} . Mobilization of granulocytes in donors with G-CSF and dexamethasone can elevate the granulocyte count in the circulation by an average of 5-fold [18] and allows a leukapheresis collection of an average of 5×10^{10} cells from each donor. Thus, to achieve a target dose of 2×10^{11} , an average of 4 donors from our donor registry were needed for one treatment regimen (Table 1). Each patient received leukocyte infusions within 2–3 hours of collection without cryopreservation at a rate of no

Table 1. Donor Cells Collection and Infusion.

Infusion No.	Patient No.	1	2	3
1st	Infusion Day	1	1	1
	Cell No.	0.13×10^{11}	0.62×10^{11}	0.57×10^{11}
	% Granulocyte	68%	88%	87%
	% Mononuclear	32%	12%	13%
	Degree HLA mismatch	8 of 10	9 of 10	9 of 10
2nd	Infusion Day	3	3	3
	Cell No.	1.13×10^{11}	0.72×10^{11}	0.56×10^{11}
	% Granulocyte	91%	91%	79%
	% Mononuclear	9%	9%	21%
	Degree HLA mismatch	9 of 10	9 of 10	10 of 10
3rd	Infusion Day	7	5	5
	Cell No.	0.76×10^{11}	0.54×10^{11}	0.73×10^{11}
	% Granulocyte	84%	81%	78%
	% Mononuclear	16%	19%	22%
	Degree HLA mismatch	9 of 10	10 of 10	9 of 10
4th	Infusion Day	9	7	7
	Cell No.	0.04×10^{11}	0.3×10^{11}	0.48×10^{11}
	% Granulocyte	78%	79%	76%
	% Mononuclear	22%	21%	24%
	Degree HLA mismatch	9 of 10	8 of 10	9 of 10
Total Granulocytes		2.06×10^{11}	2.18×10^{11}	2.34×10^{11}

Abbreviations: No. represents numbers.

Each patient received leukocyte infusions within 2–3 hours of collection without cryopreservation at a rate of no more than one donor per day and no more than 5 infusions per week.

more than one donor per day and no more than 5 infusions per week. Thus, typical treatments spanned 1–2 weeks.

2.6. Post treatment follow-ups

After each infusion, patients were monitored carefully for adverse effects according to the NCI Common Terminology Criteria for AE (Version 3.0) [19], including fevers, flushing, transfusion-associated graft-versus-host disease (TA-GVHD), transfusion related acute lung injury (TRALI), tumor lysis syndrome (TLS), cytokine release syndrome (CRS), and macrophage activation syndrome (MAS). Blood chemistry, liver function tests and renal function were monitored every other day for 3 weeks. If adverse effects occurred at any time point during or after each individual infusion, the treatment could be stopped until the adverse events were managed. The most frequent reaction to AWIT was a febrile response. Fever is not only an indicator but also a stimulator of granulocyte functions [4, 10]. Thus, the patients were given directions to use physical means, such as ice towels and cooling fans and self-administer Tylenol 500 mg PO only if their temperature increased above 39.4 °C.

Day + 1 was designated as the first day of white blood cell infusion. Patient response status would be evaluated between Days +90 to +100 after the last infusion using the Response Evaluation Criteria In Solid Tumors (RECIST) criteria (version 1.1) [20]. However, radiographic evidence of tumor response at 90–100 days post-treatment was unable to be obtained since all three patients expired prior to the three-month assessment. Autopsies were performed and the cause of death in all three patients were determined to be without direct correlation to study treatment. Patient 1 expired on day +36 due to septic shock from *Clostridium cadaveris*. Patient 2 expired on day +57 due to complications of metastatic basaloid carcinoma of unknown primary. Patient 3 expired on day +74 due to acute bilateral bronchopneumonia.

2.7. Pathological response

Although the availability of post-mortem pathology is low in cancer patients, consent was provided for routine hospital-based pathological examination in all three cases. In addition, outside histological examination was blindly performed by two expert board certified independent surgical pathologists. Specimens from metastases and primary tumor were stained with routine H&E and the slides were examined for semi-quantitative estimates of the percent tumor necrosis and GVHD post-treatment. Example images of pathological response were taken, unaltered, and labeled (Fig. 1).

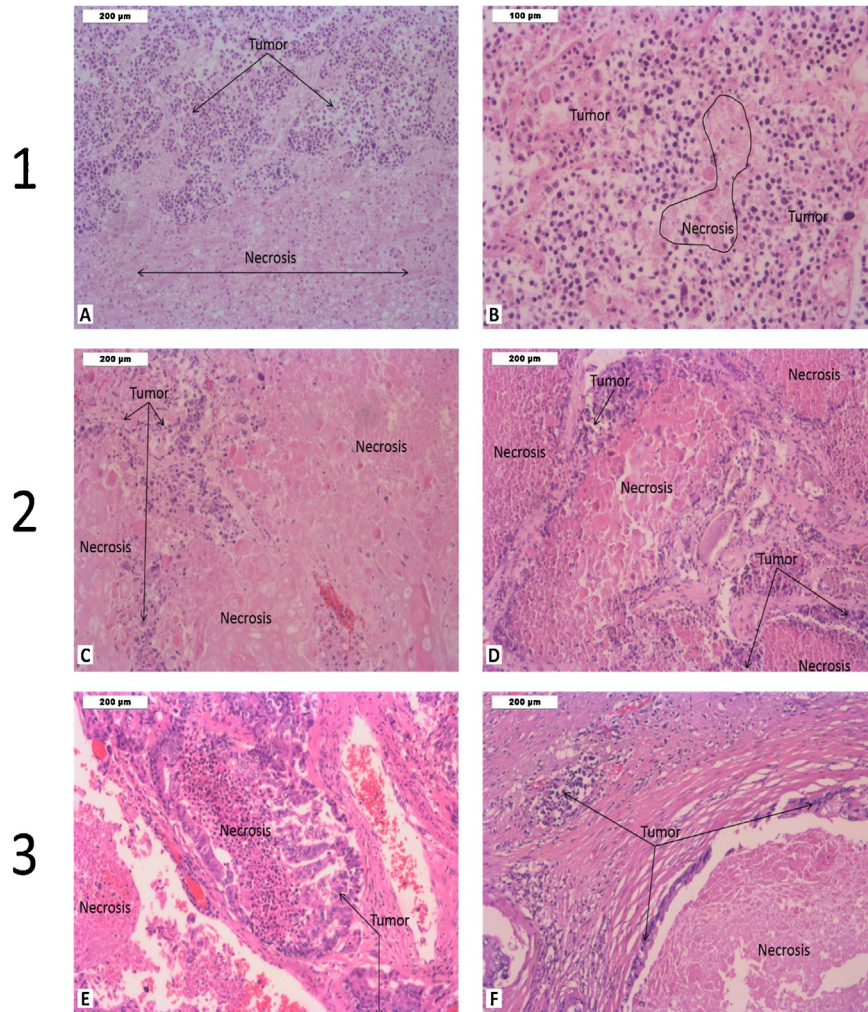


Fig. 1. Pathological antitumor response after administration of four infusions of HLA-mismatched, non-irradiated allogeneic granulocytes in patients 1–3. Hematoxylin and eosin stain shows areas of necrosis and tumor in liver (A) and lung (B) for patient 1, lung (C) and thyroid (D) for patient 2, and lung (E,F) for patient 3 at 100–200 μm magnification.

3. Results

3.1. Patient characteristics

A subset of three patients (designated patients 1, 2, 3 in whom post-mortem pathology was available) of 11 treated so far, are described as part of an ongoing clinical trial of allogeneic non-irradiated HLA-mismatched white cell infusions for patients refractory to conventional therapy (Patients 2, 3) or who refused treatment (Patient 1).

Patient 1 was diagnosed with a diffusely metastatic poorly differentiated ovarian carcinoma (Table 2) with bilateral pulmonary metastases, multiple intrahepatic

Table 2. Patient Characteristics and Results of Treatment.

Patient No.	Age (yrs)	Sex	Disease	Prior Treatment	Donor's HLA Status	No. of Infusions	Blood Chemistry*	Pathological Response
1	71	F	Metastatic Ovarian Carcinoma	Refused palliative care	Complete HLA-Mismatch	4	↑AKP 50% at 10d ↑LDH 45–50% at 40d	~80% tumor necrosis
2	66	F	Metastatic tumor of unknown primary	Bilateral mastectomy, axillary lymphadenectomy, radiation	Complete HLA-Mismatch	4	No Δ AKP ↑LDH 45–50% at 40d	~80% tumor necrosis
3	44	M	Metastatic Colon Adenocarcinoma	sigmoid colectomy, partial hepatectomy, adjuvant chemotherapy. experimental AUX-701 vaccine	Complete HLA-Mismatch	4	↑AKP 60% at 9d and 175% at 40d ↑LDH 45–50% at 40d	40-50% tumor necrosis

Abbreviations: No, number; yrs, years; HLA, human leukocyte antigen; F, female; M, male; AKP, alkaline phosphatase; LDH, Lactate dehydrogenase(LDH). Alkaline phosphatase(AKP)* In comparison to pre-treatment levels.

masses, splenic hilar nodules, bulky retroperitoneal adenopathy, a markedly enlarged uterus, and right pelvic abscess two months prior to this study. Palliative chemotherapy with carboplatin/taxol was offered but refused.

Patient 2 had a tumor of unknown primary. She was diagnosed 26 years prior to this study with an ER/PR negative infiltrating ductal carcinoma of the right breast that was treated with segmental resection, axillary lymphadenectomy, and radiation. She relapsed ten years later with an ER/PR negative intraductal carcinoma comedo-type in the left breast, treated with wide excision and radiation. One year later, extensive residual intraductal carcinoma of the right breast was diagnosed and treated with bilateral mastectomy. Fifteen years later, biopsies of a lung mass and rapidly growing neck mass revealed evidence of poorly differentiated metastatic disease for which she was treated with chemotherapy to which she was resistant.

Patient 3 had a metastatic colonic adenocarcinoma to the liver treated with sigmoid colectomy, partial hepatectomy, adjuvant chemotherapy and experimental AUX-701 vaccine. Recurrent rectal bleeding at presentation revealed a near obstructing mass 18-to-24 cm from the anal verge with pathology consistent with relapsed colon cancer along with 5 of 21 positive lymph nodes and a 1.5 cm liver lesion.

3.2. Adverse effects

All patients were treated with four white blood cell infusions with granulocyte purities ranging from 67% to 91% (Table 1) based on an automatic cell counter. All four infusions given to each of the 3 patients ranged from HLA mismatch 8 of 10 to 10 of 10 (Table 2). All three patients tolerated the transfusions well without any infusional toxicities besides flushing and fever with Tmax of 41 °C lasting from several hours to several days post transfusions. In all patients fevers were symptomatically managed with iced towels, cooling fans, and Tylenol when the fever rose above 39.4 °C. In prior studies, the CKA assay of granulocytes at an incubation temperature of 39 °C was higher than at 37 °C [4, 10], suggesting that fever is beneficial to granulocyte effector functions. No manifestations of TA-GVHD was apparent in any patient. In all three patients there was no evidence of TRALI as characterized by acute hypoxemia (SpO2 ranged from 91 to 98% on room air) or other clinical evidence of hypoxemia and no circulatory overload during or within 6 hours of transfusion. There was no transaminitis (see Table 3) or encephalopathy. While cytokines and other immune function markers were not directly measured, patients remained clinically stable post transfusions with fever being the only evidence of mild cytokine release syndrome or macrophage activation syndrome.

Biochemical parameters including electrolytes, creatinine, uric acid, alkaline phosphatase (AKP) and lactate dehydrogenase (LDH) levels were monitored

Table 3. Liver Function Tests: Before and After treatment with AWIT.

	ASPARTATE TRANSAMINASE*			ALANINE TRANSAMINASE**		
	Baseline (Day 0)	Last day of treatment (Day 4)	Post treatment	Baseline (Day 0)	Last day of treatment (visit 4)	Post treatment
Patient 1	32	49	53 (Day +13)	25	48	22 (Day +13)
Patient 2	17	34	51 (Day +54)	15	21	11 (Day +54)
Patient 3	62	51	46 (Day +66)	55	41	21 (Day +66)

Abbreviations: *AST normal range: 10–40 u/L; **ALT normal range: 10–60 u/L.

before, during, and following transfusions for signs of acute tumor lysis syndrome (TLS) and compared to pre-treatment levels (Table 1). Blood transfusion can sometimes induce a mild and transient increase of AKP or LDH that return to normal level within 24 hours [21]. In some cancer patients, there may be high levels of AKP and/or LDH [22] but these two parameters do not always coincide depending on individual differences in their removal. Electrolytes and renal function remained within normal limits throughout treatment, suggesting no signs of acute TLS. AKP and LDH were also within normal range prior to transfusions. However, AKP increased 50% at 10 days post compared to pre-treatment levels in patient 1 and returned to 20% at 37 days post. AKP level remained basically unchanged at 40 days post in patient 2. AKP level increased 60% at 9 days post, 75% at 21 days post and 175% at 40 days post in patient 3. LDH levels in all 3 patients had a 45–50% increase at 37 to 40 days post transfusions. The chronic elevation of these intracellular proteins in the serum up to 40 days after the treatment compared to pre-treatment levels is suggestive of chronic tumor lysis syndrome and consistent with findings of ongoing chronic tumor necrosis in the post-mortem examinations.

3.3. Tumor responses

The patient profiles and results of treatment are presented in Table 1. Post-mortem examination of all three patients showed similar diffuse histological evidence of tumor necrosis, which appears to be secondary to the white blood cell infusions and highly unlikely to be due to natural course of disease progression (Fig. 1). The necrosis observed appeared distinct from the coagulative type of necrosis often seen in large tumor masses secondary to ischemia. One small area of tumor showed dystrophic calcification suggestive of a coagulative necrosis component but was minimal and focal. Post-mortem examination of patient 1 at 36 days after white cell infusion revealed a partially necrotic left ovarian mass, yellow-tan partially necrotic metastatic splenic nodule, necrotic abdominal, peri-aortic, para tracheal and peri-pancreatic lymph nodes. Multiple partially necrotic lung and liver

metastases were also observed. Patient 2 showed a similar pathological response with numerous necrotic pulmonary nodules, partially necrotic tracheal mass, and multiple focally necrotic hepatic lesions at autopsy 57 days after white blood cell infusion. Post-mortem examination of patient 3 at 74 days after white blood cell infusion showed evidence of multiple areas of necrosis in metastatic lung and liver lesions. Approximately 30% of the liver was affected by necrotic masses with infiltration of macrophages and granulocytes near the necrotic sites. Pathologically, the extent of the necrosis was significantly greater than those seen in routine progression of malignancies or with treatment of conventional therapies. Overall, although there was a large tumor burden present in all patients assessed and there was extensive necrosis in about 80% of tumor in Patients 1 and 2 and 40–50% in Patient 3, which appeared to be treatment related. Evidence of leukocyte infiltration was seen in all cases, however it is unknown if these cells represent donor or host origin. There was no significant necrosis in normal tissues and necrosis was confined to areas of tumor. Pathology slides were also reviewed for evidence of TA-GVHD. Intestinal mucosa was present in one case but autolysis precluded assessing for GVHD. No definite evidence of GVHD was seen in all three cases. Radiographic evidence of tumor response at 90–100 days post-treatment was unattainable since all three patients expired prior to the planned three-month assessment.

4. Discussion

Recent studies on the interplay between the immune system and cancer have illustrated that in addition to adaptive immunity, the innate immune system has an inheritable naturally-occurring surveillance and antitumor mechanism capable of recognizing and removing cancerous cells as they are generated over time [4, 5, 9, 10, 11, 12, 23, 24]. Prior studies in a colony of cancer-resistant SR/CR mice support the concept that the innate immune system is capable of single handedly protecting a host against cancer in both preventative and therapeutic settings [10]. This CKA has been shown to be highly dynamic in the human population, affected by an individual's genetics, different seasons, age, and emotional stress [4, 9, 11]. In retaliation, many cancers generate an immunosuppressive tumor microenvironment through various mechanisms to avoid recognition, elimination, and to drive tumor progression [2]. However, a defective immune system lacking this immune surveillance and cancer-killing activity or ones whose function decreases over time may help to explain the rise of clinically significant neoplasms with age. Most current conventional cancer immunotherapies attempt to stimulate a damaged immune system through monoclonal antibodies directed at regulatory T-cell receptors (PD1 or CTLA4 inhibitors) or generate a targeted immune response through *ex-vivo* expression of chimeric antigen receptors (CARs). Although these therapies have shown some significant overall survival in phase III clinical trials of

patients with advanced-stage cancers and relapsed and refractory malignancies, they are costly and can have significant immune-related adverse events, such as endocrinopathies, encephalopathy, cytokine release syndrome, and death in some cases [25, 26].

The adoptive transfer of innate granulocytes from donors with validated high levels of naturally occurring cancer-killing activity to cancer patients for therapeutic purposes is a new concept. This novel immunotherapy strategy attempts to supplement the damaged components of the immune system with ones with enhanced activity instead of attempting to revive a patient's own adaptive immunity against neoplasia. This study was designed to test the adverse effect profile and possible antineoplastic effect of non-irradiated HLA-mismatched white blood cells consisting predominantly of granulocytes obtained from young and healthy donors on advanced-stage metastatic solid tumors. This trial also represents the first time that donor granulocytes were transfused at levels up to 20 times those previously reported.

Since a significantly higher dose of leukocytes was transfused for each patient, the primary concern in this trial was whether the recipients could tolerate the proposed granulocyte dose without developing TA-GVHD. Donor granulocytes per se are not known to produce TA-GVHD. However, the granulocytes collected via apheresis were only 68–91% pure (Table 2) and therefore contained donor T lymphocytes that could produce TA-GVHD in immunocompromised cancer patients. In the 3 cases reported here and all other cases yet to report in this trial, patients remained clinically stable post transfusions with minimal side effects related to this novel immunotherapy. TA-GVHD as determined by GI symptoms, liver function tests, and skin findings did not occur. We believe this is in part because patients were not severely immunocompromised as a result of not having recently received radiation or high dose myeloablative preconditioning chemotherapy regimens as are routinely done prior to hematopoietic stem cell transplants where the rates of GVHD are high. As a result, recipients were most likely able to reject the HLA-mismatched leukocytes prior to GVHD onset.

Other transfusion reactions like TRALI as determined by blood oxygen level and routine pulmonary functions were also not observed. Post-transfusion fevers were seen in all cases of this trial but the temperatures and durations of fever varied significantly in different individuals ranging from 38 °C to 41 °C and from a few hours to two weeks respectively. Coincidentally, all the long-term survivors yet to be reported in this trial have had the most severe febrile responses among all participants. This is not a complete surprise since the observed CKA of granulocytes has been shown to be higher at 39 °C versus 37 °C [4, 10].

However, widespread tumor necrosis in late stage cancer patients with large tumor burdens also pose additional risks of edema and perforation, which at critical

locations like the lung, liver or GI tract could be detrimental. One would also expect that with extensive necrosis, dramatic changes in blood chemistry could pose additional risks of electrolyte imbalances and accumulation of toxic metabolites, resulting in acute tumor lysis syndrome, acute kidney injury, cytokine release or macrophage activation syndrome. These are serious adverse events often seen with CAR T cell therapy and requiring ICU level of care [3, 26, 27]. However, these adverse events were remarkably absent following AWIT and subsequently. Nevertheless, these risk factors illustrate the importance of time and dose-dependent infusions in order to slow the rate of cell death and allow more recovery of vital organ function between infusions in order to avoid structural compromise and electrolyte imbalances.

The pathological examinations in all three cases provided unequivocal evidence of widespread necrosis and leukocyte infiltration after AWIT regardless of tumor type. Despite the heterogeneous tumors (ovarian, colon, and unknown primary), the extent of tumor necrosis (up to 80% in patients 1–2 and 40–50% in patient 3) was significantly greater than the cell death routinely seen during cancer progression or with conventional therapies and is therefore believed to be treatment related. Various explanations may help explain these findings. In animal studies, the tumor necrosis was attributed solely to donor granulocyte's CKA since the absence of adaptive immune system components in both donors and recipients did not ablate the cancer resistance mechanism [5]. Studies in SR/CR mice aimed at determining the effector mechanism behind this CKA revealed that SR/CR leukocytes possess a unique ability to recognize chemoattractant factors across various cancer cell lines, infiltrate the cancer site and form tight surface contact zones with cancer cells [23]. Upon direct surface contact with infiltrating SR/CR leukocytes cancer cells were killed via both cytolysis and apoptosis [24]. Studies have shown that perforin, superoxide, or nitric oxide were not functional requirements for cancer-resistance in SR/CR mice [12].

However, the AWIT transfusions in this study represented an impure population of both granulocytes and mononuclear cells including lymphocytes so donor T-lymphocytes may have contributed to the necrosis observed. In addition, participation of host T cells in the anti-tumor immune response cannot be excluded [28]. Studies by O'Donnell et al. and others have illustrated that the infusion of HLA-mismatched lymphocytes and the resulting graft rejection may stimulate the host's own T cells against the tumor [29, 30, 31, 32]. This observation may explain several reports of clinical responses in hematologic malignancies despite rejection of the donor graft. Given a granulocyte's rapid effector functions within an hour to 2 weeks of transfusion [23] but our observed lack of acute side effects related to cytolysis, this induced host-versus-tumor effect may help explain the on-going chronic (elevated LDH and ALK) rather than acute necrosis seen in these three cases at +36 to 74 days. This is also reminiscent of the

animal studies of Cui et al. which showed chronic tumor necrosis in a similar time period. Regardless of the mechanism of action, the widespread tumor necrosis following AWIT provides evidence that we are able to significantly improve antitumor immunity in cancer patients with a wide variety of tumor types, even at late stages of disease.

It has been postulated that the host immune response may be more effective during the early stages of oncogenesis, before neoplastic cells acquire the ability to suppress immune cell function [33]. It is hopeful that if these patients could have been treated at earlier stages of disease and with lower tumor burdens, not only would there be less risk of complications from large tumor burdens and or extensive chemo-radiation treatment with less damage to critical organ function but could also allow adequate time for the re-instatement of host immune surveillance prior to the development of the full tumor immunosuppressive effect. This ongoing clinical trial and similar studies have shown cases in which AWIT in early stage cancers has provided long-term survivorship and stable disease, although these are yet to be reported.

From the bench to the bedside, AWIT represents a novel step forward in cancer immunotherapy as it attempts to reinstate an innate and highly effective surveillance system to recognize and destroy cancer cells. This pilot study shows that AWIT is a safe and an easier alternative to antibody or T cell engineered immunotherapies because the product in AWIT is delivered at the point of care without any laboratory manipulation and uses at least 7 of 10 HLA mismatched donors from a database of 200 donors, which are relatively easy to find once a database is established.

One of the major challenges that immunotherapy faces is the prohibitively high costs for antibody-based treatments and engineered T cells compared with conventional cancer therapies [34]. AWIT cells represent a potentially cost-effective and logistically easier outpatient treatment option for solid tumors. However, clinical trials are still too early to determine if overall and progression-free survival will compare favorably to current immunotherapies.

In summary, AWIT from young healthy donors represents a new immunotherapy option with exceptional efficacies in killing cancer cells as seen in three cases of advanced-stage malignancies. The lack of treatment related toxicity, the logistic ease of therapy in an outpatient clinic, and the broad availability of unrelated donors makes this treatment suitable for further evaluation as an additional cancer immunotherapy.

Declarations

Author contribution statement

Dipnarine Maharaj, Pedro Vianna, Wendy Ward, Anthony Messina, Trevor Raborn, Jacqueline V. Gouvea, Richard Hammer, Zheng Cui: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

The clinical trial described in this paper was registered at <https://clinicaltrials.gov> under the registration number NCT00900497.

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