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Infusion of CD3/CD28 costimulated umbilical cord blood T cells at the time of single umbilical cord blood transplantation may enhance engraftment

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Abstract

Limited cell numbers in umbilical cord blood (UCB) grafts present a major impediment to favorable outcomes in adult transplantation, largely related to delayed or failed engraftment. The advent of UCB transplantation (UCBT) using two grafts successfully circumvents this obstacle,

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Authors Contributions

EOH, BLL, CHJ, SGE, and DLP designed the study. EOH, SML, RR, JKM, NVF, MR, AWL, EAS were involved in the clinical care of patients and analysis of clinical data. BLL and CHJ contributed to preclinical studies and prepared the cellular product. DMF analyzed morphology and prepared photomicrographs. AC was responsible for trial design oversight and operations. EOH, RR, RVH, NAA, YZ contributed to the design of correlative studies. EOH, RR, GRJ, LPR, NAA, BLL, and executed and analyzed correlative studies. EOH, YZ, SGE, and DLP interpreted clinical and correlative studies. GRJ prepared table and figures. EOH wrote the manuscript, which was edited by RR, GRJ, NVF, DMF, BLL, CHJ, and DLP. All authors reviewed and approved the final manuscript.

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despite the engraftment of only one unit. Preclinical models suggested that the addition of UCB T cells at the time of transplant can enhance engraftment. We tested whether ex vivo activation by CD3/CD28 costimulation and expansion of T cells from a single UCB graft would be safe and feasible in adults with advanced hematologic malignancies, with an overall objective of optimizing engraftment in single unit UCBT. In this phase 1 study, recipients of single UCB units were eligible if the unit was stored in two adequate fractions. Dose limiting toxicity was defined as grade 3 or grade 4 GVHD within 90 days of UCBT. Four patients underwent UCBT; all were treated at the first dose level (10^5cells/kg). At the 10^5cells/kg dose level two subjects experienced grade 3 intestinal GVHD, thus meeting stopping criteria. For three subjects, neutrophil engraftment was early (12, 17, and 20 days), while one subject experienced primary graft failure. We observed early donor T cell trafficking and found that expanded T cells produced supraphysiologic levels of cytokines relevant to engraftment and to lymphoid differentiation and function. Taken together, these preliminary data suggest rapid engraftment in recipients of a single UCBT combined with relatively low doses of activated T cells, though potentially complicated by severe GVHD.

Introduction

Delayed engraftment and compromised immune reconstitution are the major obstacles to successful umbilical cord blood transplantation (UCBT), limitations that may be attributable to the uniquely antigen inexperienced, primarily naive T cells in UCB grafts. These same properties confer a lower risk of acute graft versus host disease (aGVHD) and greater tolerance across HLA barriers compared with other stem cell sources [1,2]. While comparative studies are lacking in adults, time to engraftment in UCBT using two partially mismatched grafts appears to be shorter than single UCBT, despite only a single engrafting unit in virtually all dual-graft recipients. The observation that T cells are the critical determinant of the engrafting unit suggests an immunologic basis for enhanced engraftment [3–6] This phenomenon of single unit dominance appears to be related to a CD81 T cell mediated interaction between units [7] although the mechanism by which the alloresponse hastens engraftment is not well understood. T cells play a critical role in normal hematopoiesis and in hematopoietic recovery following stem cell transplantation [8–12]. In transplantation, donor T cells overcome host barriers and may more directly influence stem and progenitor cell homing and differentiation/proliferation to facilitate engraftment [13] We hypothesized that activation of T cells in single UCBT would augment engraftment and tested the safety and feasibility of infusion of CD3/CD28 co-stimulated UCB T cells at the time of transplantation. Because immunotherapeutic options following relapse in UCBT are limited, we also tested whether expanded cells could be cryopreserved for future use as donor leukocyte infusions (DLI).

Methods

This was a phase 1 study testing safety and defining the maximum tolerated dose (MTD) of ex vivo CD3/CD28 costimulated UCB-derived T cells coinfused with single UCB grafts in patients with advanced hematologic malignancies using a standard 3 1 3 design. A secondary objective was to test the feasibility of ex vivo expansion through CD3/CD28

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costimulation and cryopreservation of UCB T cells for administration as DLI in the event of disease relapse. Eligible subjects had no suitable related or unrelated donor, and had a single 4/6 (or better) HLA-matched UCB graft containing at least 2.5×10^7 nucleated cells/kg. All patients gave informed consent in accordance with the Declaration of Helsinki. The trial is registered with Clinical-Trials.gov (identifier NCT00891592) where complete inclusion/ exclusion criteria are listed. See Supporting Information figures for study schema.

T cell expansion.

Single UCB units stored in two fractions were eligible for T cell expansion: the smaller fraction was thawed prior to infusion and cultured following activation by magnetic beads conjugated with antibodies directed against CD3 and CD28 [14] in the Clinical Cell and Vaccine Production Facility at the University of Pennsylvania as previously described [15]. Final cell product release criteria as specified in the FDA IND included cell viability >80%, CD31 cells >80%, bacterial and fungal cultures sampled two days prior to harvest as negative to date, gram stain negative, endotoxin <1 EU/mL, and <100 residual magnetic beads per 3 million cells.

Transplant procedure.

Myeloablative conditioning regimen combined total body irradiation (1320 cGy in 8 fractions) with fludarabine and cyclophosphamide. GVHD prophylaxis consisted of mycophenolate mofetil and cyclosporine A, starting on day —3 prior to UCBT and tapered at 30 days and 6 months, respectively in the absence of GVHD [3]. Granulocyte colony stimulating factor (G-CSF, 5 mg/ kg/day) was administered daily starting on the first day following infusion and continued through neutrophil engraftment. Antiviral prophylaxis consisted of high doses of acyclovir or valacylovir (2000 mg every 8 hours through day 1365). On the day of UCBT (day 0), the larger unmanipulated UCB fraction was infused, followed immediately by a fixed dose of the expanded CD3/CD28 costimulated T cells. The remaining cells were cryopreserved for potential future use as DLI. Four dose levels of initial co-stimulated T cells (10^5—10^8 T cells/kg) were planned. Dose limiting toxicity (DLT) was defined as any one or more of the following: (1) Grade 3/4 acute GVHD within the first 90 days following infusion, as determined by the Center for International Blood and Marrow Transplantation Research (CIBMTR) severity index; (2) Allergic reaction, grade 2 or higher, or urticaria, grade 3 or higher and deemed related to infused CD3/CD28 costimulated UCB T cells (as determined using the Common Terminology Criteria for Adverse Events v3.0); (3) Any grade 2 or greater autoimmune phenomenon; (4) Any grade 3 or greater non-hematologic organ toxicity attributable to infused CD3/CD28 costimulated UCB T cells (excluding expected toxicities of standard myeloablative UCBT); (5) Any attributable grade 4 hematologic toxicity, (with the exception of thrombocytopenia, unless it follows recovery of platelets); within the first 90 days of infusion. Peripheral blood was collected from patients at prespecified time points and plasma and mononuclear cells (MNC) were cryopreserved. For several patients, previously banked peripheral blood or bone marrow MNC were available in the Hematologic Malignancies Tissue Bank and used in correlative assays. These protocols and all amendments were reviewed and approved by the Institutional Review Board and the Abramson Cancer Center Clinical Trials Scientific Review and Monitoring Committee. All patients provided written informed consent.

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Cytokine/chemokine measurements.

MNC from unmanipulated UCB, CD3/CD28 costimulated T-cell supernatants were all assayed on a single Luminex plate according to the following procedure: cells were thawed and rested overnight at 37°C in a 5% CO_2 incubator. Cells were then counted and resuspended in media (RPMI 1640 supplemented with 10% fetal calf serum and antibiotics) to a concentration of 2×10^6/ mL; 2×10^5 cells were incubated overnight in media with or without CD3/CD28 beads. The supernatants were collected the following morning and stored frozen until they were assayed. Batched supernatants from the expanded cells and cryopreserved patient plasma were thawed and clarified by centrifugation and added to a Luminex plate with a final dilution of 1:2 in media. Results of cytokine concentrations for supernatants were normalized to cell input numbers. Protein binding and detection steps were performed as per the manufacturers instructions for the Milliplex Human Cytokine/ Chemokine Immunoassay 10-plex kit. The standard curve was prepared as a 5-fold serial dilution. The undiluted standard, or standard 1, had an initial concentration of 10,000 pg/mL for all cytokines. The limit of detection for the standards ranged between 0.53 pg/mL for GM-CSF and 38.04 pg/mL for IL-3.

Flow cytometry, CD107a degranulation assay, and MHC class I tetramer analysis.

Commercially available research grade fluorochrome-conjugated monoclonal antibodies were used for cell surface and intracellular cytokine and Foxp3 signaling (BD Biosciences, San Jose, CA; Beckman Coulter, Indianapolis, IN; BioLegend, San Diego, CA; eBioscience, San Diego, CA). The ability of T cells to degranulate when incubated with or without phorbol myristate acetate (PMA) and ionomycin was determined by staining for CD107a according to standard protocols (BD Biosciences). Antitumor potential was evaluated by degranulation in the presence of autologous tumor lysate from previously banked cells.

Soluble HLA-A2 tetramers conjugated to PE or APC were prepared with peptides and b2-microglobulin as described [16] in the Abramson Cancer Center Human Immunology Core or purchased from Beckman Coulter. Cryopreserved CD81 T cells were assayed for peptide specificity at thaw and following in vitro stimulation as previously described [17]. HTLV-1-MHC tetramer served as a negative control. All flow cytometric data were collected on a FACSCanto flow cytometer using BD FACSDiva Software (BD Biosciences). Data were analyzed with FACSDiva and FlowJo software (TreeStar, Ashland, OR).

Results

Study population and graft characteristics

Five subjects, all women, were enrolled. They ranged in ages from 25 to 48, and 4 enrolled subjects underwent UCBT, while one (ID #4) had progressive acute lymphoblastic leukemia and died prior to UCBT. One subject had high risk myelodysplastic syndrome, which was previously untreated and the remaining had high risk acute myeloid leukemia (AML). Patients with AML had 1–3 lines of prior therapy, and were in remission or in a minimal disease state (defined as primary refractory AML with fewer than 5% blasts after third induction) at the time of UCBT. One patient was CMV seropositive prior to UCBT (Table I).

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CD3/CD28 costimulation and expansion of T cells and cryopreservation is feasible and represents a potential strategy for DLI following UCBT

UCB grafts were obtained through the National Marrow Donor Program for final preclinical validation. Thawed cells were cultured with a single addition of anti –CD3/CD28 magnetic beads and expanded more than 2 logs over the culture period, sufficient expansion to meet even the highest dose level proposed in the original protocol (10^8 CD31 cells/kg). Mean cell size increased in an expected pattern following costimulation (peaking 7001 fL) and returned close to baseline (300 fL) by day 7. Phenotypic analyses demonstrated that these cells were typically CD4 biased (CD4:CD8 ratio 2:1) with virtually all CD31 cells coexpressing CD25 with moderate or bright fluorescence intensity. The majority (80%) of CD41 and a subset (25%) of CD81 cells coexpressed CD28. Virtually all cells were CD45RA1, with ~70% having the CD45RA 1 CD62L1 naive T cell phenotype (data not shown).

Recipients of UCBT all received grafts matched at 4/6 HLA loci, with precryopreservation cell doses ranging from 2.8 to 4.8×10^7 TNC/kg. Precryopreservation CD341 doses were $0.38–7 \times 10^5$ cells/ kg, while post-thaw viable absolute CD341 count (measured for 3 grafts) was within a more narrow range (67.7–166.5 cells/pL). Additional graft characteristics, including gender, ABO type, and proportional storage fractions, are presented in Table 1

All treated patients received CD3/CD28 costimulated T cells at the first dose level of 10^5 CD31 cells/kg (range $1.02–1.56 \times 10^5$, median 1.35×10^5) following UCB graft infusion on the day of transplantation. All but one expansion yielded adequate numbers of cells for cry-opreservation as future use for DLI.

Safety and outcome

There were no infusion-related adverse events. Three of four patients developed culture-negative fever and rash (<25% body surface area) temporally consistent with a pre-engraftment syndrome [18,19] and grade 1 cutaneous acute GVHD, two subjects (ID#2 and 3) were treated with a brief tapering course of systemic steroids. Patient #1 experienced grade 3 acute GVHD with stage 3 intestinal GVHD on day 140 following UCBT, and also developed a skin rash and hyperbilirubinemia, presumed to be GVHD and also possibly related to infused T cells. Pt #4 also developed grade 3 intestinal GVHD, on day 1 27. Grade 3/4 aGVHD in 2/6 patients was a predetermined criterion for MTD and the study was stopped after this event.

One subject experienced HHV-6-associated acute limbic encephalitis; other serious adverse events are listed in Table 1 and were not considered related to the infused T cells. Three patients died of transplant-related complications prior to day 1100: one from diffuse alveolar hemorrhage with possible component of idiopathic pneumonia syndrome, one from progressive pneumonia, and one from cerebral edema possibly related to opportunistic infection or posterior reversible leukoencephalopathy (Table 1).

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Neutrophil engraftment following UCBT + T cells was early

Neutrophil engraftment occurred on days 112, 117, and 120 following UCBT for the first three subjects enrolled on study. One subject also experienced platelet engraftment by day 123, while the other patients failed to achieve sustained platelet recovery. Patient #5 (who also had grade 3 intestinal GVHD) failed to engraft and received a second mismatched unrelated donor graft on day 150 following UCBT.

One subject remains in a complete remission more than five years following UCBT, without evidence for GVHD.

Early donor T cell trafficking, T cell reconstitution, and physiologic B cell expansion

We were interested in potential biologic correlates of our intervention with respect to immune reconstitution. We identified early donor T cell trafficking to the skin in subject #2 with administration of a low infused activated T cell dose. This patient developed an ill-defined rash (anterior chest/hands) on day 111 following UCBT, which was biopsied; neutrophils had appeared in the peripheral blood the day before and absolute neutrophil count (ANC) was 310/pL on the day of the biopsy. A sparse lymphocytic infiltrate was seen in the dermis, and FISH studies detected Y chromosome-containing male (donor) cells consistent with GVHD (IBMTR index A; Fig. 1 panels A and B). The ANC was >500 on day 12, and a bone marrow evaluation on day 21 documented complete remission and complete donor chimerism; immunostains identified scattered CD31 cells (Fig. 1 panels C and D). In the same subject one year following UCBT, bone marrow morphology was notable for a marked expansion (20% of cellularity) of physiologic precursor B lymphoblasts (hematogones) with a maturing B cell phenotype (Fig. 1 panels E and F and data not shown).

CD3/CD28 costimulated UCB T cells are enriched for regulatory T cells

We assayed for regulatory T cell (Treg) frequency at baseline, in the infused product and at several time points following UCBT (Fig. 2A). Tregs were enriched for in the T cell product, appeared to be slightly above baseline at day 1 10 in the two patients for whom there was a measurement, and declined by day 1 50. Of note, the activated T cell product with the lowest proportion of Tregs was infused into the subject who subsequently experienced both severe GVHD and graft failure.

CD3/CD28 costimulated UCB T cells elaborate FLT3L and B cell activating factor (BAFF)

To begin to answer the question of how T cell infusions could exert graft-promoting properties, we measured concentrations of cytokines relevant to engraftment and immune reconstitution from supernatants of CD3/CD28 costimulated T cells pre-infusion, and in serum from UCBT recipients at various time points (Fig. 2B). We found that supernatants contained supraphysiologic levels of cytokines important for engraftment/progenitor/dendritic cell development (GM-CSF, IL-3, FLT3L) as well as T and B cell differentiation/function (IL-2, IL-4, IL-10, IFN-g, BAFF). Therefore, it appears that CD3/ CD28 costimulation of UCB T cells primes them for enhanced cytokine secretion that may contribute to successful engraftment.

We next investigated whether this increased cytokine production in vitro translated into measurable differences in circulating cytokine levels in UCBT recipients. Despite a very low cell dose, we observed measurable increases in IL-10 immediately following the infusion of expanded T cells for two subjects. However, intracellular cytokine staining of preinfusion expanded cells showed that only a minority of cells secreted IL-10 (Fig. 2C), suggesting that at this cell dose level the rapid changes in serum cytokine levels observed in recipients may instead represent an innate host response to UCB and T cell infusion.

Cytokine profiles and dynamics of changes in patients undergoing UCBT have not previously been described in detail. In our small cohort, we found that a subset of the cytokines studied were significantly elevated compared to normal plasma, including FLT3-ligand (>1000 pg/ mL range), IL-5, and IL-15 (Fig. 2B). These elevated cytokines preceded the infusion of T cells and may be attributable to myeloablation and lymphodepletion [20]. However, of the two patients with evaluable samples prior to initiation of the conditioning regimen, both had high levels of BAFF and FLT3L, suggesting this milieu may be intrinsic to hematologic malignancies and/or their treatment [21].

Ex vivo alloreactivity generated by activated donor-derived UCB T cells against autologous tumor lysate

To evaluate the potential for productive donor versus host responses, cell lysates prepared from banked tumor sample (bone marrow MNC at diagnosis, ID #3) were incubated with basal autologous T cells collected prior to pre-conditioning, or with CD3/CD28 costimulated UCBT cells from the graft, together with PMA/Ionomycin (Fig. 3A). We observed a higher percentage of degranulating cells in CD3/CD28 co-stimulated UCBT cells incubated only with PMA/ ionomycin, but degranulation was markedly higher in the presence of tumor lysate, suggesting that CD3/CD28 co-stimulated UCB T cells are capable of productive anti-leukemia responses.

In vivo acquisition of tumor antigen- and viral antigen-specific cytoxic T cells

As a measure of immune reconstitution, we were interested in how and whether productive antigen specific anti-tumor and relevant antiviral responses were acquired in our UCBT recipients. At thaw, cryopreserved T cells demonstrated weak to no reactivity (data not shown). Tetramer-positive CD81 cells were more apparent following in vitro stimulation (IVS). Batched serial samples from one subject who was EBV seropositive at baseline with an HLA-A21 donor were analyzed for EBV-tetramer-positive cells (Fig. 3B). Acquisition of antigen specific CTL was not accompanied by lymphoproliferative disease, overt EBV infection, or an increase in viral load. Influenza-specific tetramer positive cells appeared after vaccination (Supporting Information Fig. S2). The leukemia-specific antigens tested were hTERT (I540) [22], WT1, PRAME, Survivin, and HoxA9 (Hox-TLD) [17]; there was convincing acquisition of HoxA9 and hTERT-specific tetramer positive cells at 1 year (Fig. 3B and data not shown).

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Discussion

Our observations demonstrate the feasibility of infusion of CD3/ CD28 costimulated UCB T cells at the time of transplantation, and suggest that even low doses of activated cells are associated with early neutrophil engraftment. This study also establishes feasibility of expanding and cryopreserving CD3/CD28 costimulated UCB T cells for future use as DLI, heretofore a major shortcoming of UCB as a graft source.

This cohort experienced significant toxicity, with three deaths before day 1100 from infection and regimen related toxicity; two deaths were in the setting of severe intestinal GVHD. Several factors may account for this observation: the overall outcome of this small group reflects, to some extent, the high-risk nature of these diseases and a high-risk intervention. However, for the two patients experiencing grade 3 aGVHD we cannot exclude the possibility that it is in part attributable to CD3/CD28 costimulated T cells. The reported risk of severe GVHD after UCBT ranges between 26–65%, and is 20% for severe disease [1–3,23–25] Therefore, the definition of DLT for this study (grade 3/4 aGHVD by day 90 in 2/6 patients at a given dose level) was conservative; discriminating between the baseline incidence and toxicity specific to the infused activated T cells is not possible in such a small cohort. Previous studies of delayed administration of CD3/CD28 costimulated cells in the allogeneic setting did not result in excessive GVHD [26], raising the possibility that, if toxicity is truly different, then timing of administration may alter the propensity for alloreactivity, potentially related to homeostatic proliferation of T cells in the lymphopenic host. In addition, the cytokine milieu and tissue damage with this intensive regimen could potentiate GVHD. Establishing the safety of these cells within this context requires further study.

Our preclinical observations suggested that graft enhancement occurred at a stage beyond the primitive stem cell, so one working hypothesis would be that activated T cells act as cytokine factories, supporting the expansion and differentiation of progenitor cells. Maintaining graft enhancement while preventing pathologic alloresponses would be the goal, isolating these interconnected phenomena the challenge. The experience with dual UCBT suggests alloreactivity both enhances engraftment and increases GVHD incidence. In theory, ex vivo expansion of Treg in UCBT is an attractive GVHD-reducing approach, as “contaminating” activated/alloreactive CD251 cells are less problematic in UCB, and feasibility of expansion has been established [27]. In practice however, in a multi-UCBT (triple product) setting, GVHD incidence did not appear to be dramatically reduced by Treg engineering [28]. Alternatively, Treg balance could be modulated in vivo. More novel GVHD prophylactic regimens, such as the mTOR inhibitor sirolimus, might confer a more strategic approach to GVHD prophylaxis. T cells are tightly regulated through the mTOR pathway, and sirolimus is known to promote Treg expansion, representing an attractive therapeutic option [29]. A promising immunosuppressive regimen in UCBT employs sirolimus in combination with the tacrolimus following UCBT [30]. Ultimately, a better mechanistic understanding of both graft enhancement and pathologic alloresponses will be the key to more strategic interventions.

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While preliminary, the early neutrophil recovery we observed is striking, and suggests possible graft-promoting properties of costimulated T cells at low doses. The early engraftment we observed is supported by preclinical studies [13,31], and compares favorably to published studies of single or double UCBT using myeloablative conditioning, where neutrophil engraftment ranges from 23 to 60 days [2,3,24,32]. Additional studies designed to measure efficacy are needed to more accurately estimate graft-enhancing properties of CD3/CD28 costimulated UCB T cells.

We observed early (pre-engraftment) donor T cell trafficking to skin, raising the possibility that donor T cells were actively trafficking to extranodal sites of activity, including bone marrow sites. Whether the detected donor cells represented CD3/CD28 costimulated cells, or adoptively transferred mature CD31 cells in the graft is uncertain, since the infused costimulated T cells are indistinguishable from adoptively transferred CD31 cells from the unmanipulated portion of the graft. Elegant preclinical studies have shown that CD81 T cells facilitate stem/progenitor cell access to the bone marrow microenvironment following transplantation, by chaperoning along chemotactic gradients, in a manner that requires direct contact between CD81 and CD341 cells [33]. Others have observed that once there, CD41 cells are required for the maintenance of normal hematopoiesis, need to be activated, and also require cognate interactions [8]. One can speculate that defective myeloid recovery in UCBT is related to a dearth of activated cells, and that CD3/CD28 costimulation might overcome such defects.

It has likewise been speculated that earlier engraftment in recipients of two UCB grafts is related to mutual immune activation [6]. In most patients who receive dual UCBT, a single graft establishes hematopoiesis early and in a durable fashion. Clinical studies of recipients of dual UCBT have identified cytotoxic CD81 effector/effector memory cells derived from "winning" units that are specific for the non-engrafting unit, and suggest that the specific deletion of one unit by the other is one possible mechanism of single unit dominance [7]. One could speculate that paracrine effects of these interactions, if they were to occur near sites of hematopoiesis, might also positively influence collateral myeloid recovery, through the elaboration of cytokines such as those we observed, including FLT3L, IL-3, and GM-CSF. IL-3 and GM-CSF have previously been described as cytokines elaborated by activated T cells [34] and CD3/CD28 costimulated T cells specifically [14]. Expression of FLT3L, however, has not previously been specifically recognized as a critical mediator of T cell-enhanced hematopoiesis. FLT3L, among other functions, acts as a cofactor synergizing with IL-3 and GM-CSF in supporting hematopoietic colonies [35]. We observed that CD3/ CD28 costimulated T cells elaborated FLT3L at levels higher than those induced through other nonspecific T cell activation. Together, these observations could support a model whereby activated T cells function as critical cytokine "factories." Our preclinical studies suggested that the mechanism for enhancing engraftment was at stages beyond the most primitive stem cell [13], and paracrine effects of cytokines elaborated by T cells in close proximity to progenitor cells would fit such a model. FLT3L also plays an important role in dendritic cell development [36], and might thereby alter antigen presentation and indirectly lead to ongoing T cell activation/proliferation. Such alterations may be particularly accentuated in the context of the marked lymphopenia present during UCBT. We also observed a high basal concentration of FLT3L in plasma of UCBT recipients, which itself may alter hematopoietic

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and immune recovery and immune surveillance/alloreactivity, although additional comparative data from other diseases, conditioning types and intensities are lacking.

Determinants of the “winning” or dominant unit are probably numerous, but a refinement in our knowledge of modifiable factors may make UCBT safer and have important ramifications both in terms of biological activity and resource utilization-cost and otherwise —for the broader application of UCBT in adults. The relative merits of single versus dual UCBT continue to be debated in adults, with recent rigorous retrospective studies suggesting superior antileukemic activity, and not surprisingly, a higher incidence of GVHD in dual UCBT [25,37,38]. By contrast, single rather than dual UCBT is now arguably established as the standard of care in children with suitably sized grafts, with inferior outcomes related to excess GVHD and other regimen related toxicity seen in children receiving dual UCBT [39].

It is not known from our small experience whether ex vivo costimulation of UCB T cells enhanced immune reconstitution. We measured productive antigen specific anti-tumor and anti-viral responses in some patients. The early occurrence of HHV-6 associated limbic encephalitis in one subject is consistent with reports of a relatively high incidence of this complication following UCBT [40] and suggests that non-specific activation of polyclonal UCB T cells, at least at this cell dose, is not fully protective against this complication. However, in addition to ex vivo evidence for patient specific tumor cytotoxicity of donor CD3/CD28 costimulated T cells, we also saw in vivo acquisition of EBV- and leukemia-antigen specific immunity that improved following discontinuation of immunosuppressive therapy. Whether such assays can meaningfully predict outcomes, and whether ex vivo T cell costimulation will enhance immune function, will need to be defined by future studies.

We had also observed a marked expansion of physiologic B cell precursors (hematogones) in the bone marrow of one recipient; this observation has been associated with improved overall survival and lower treatment-related mortality [41]. Perhaps this is a reflection of productive T cell help, and future studies can establish its utility as a biomarker for immune reconstitution in cellular therapy studies as well. Future studies where ex vivo engineered T cells can be directly or indirectly tracked will help to define survival, proliferation, tissue trafficking, and the kinetics of immune reconstitution—and thereby better elucidate the influence of T cells on hematopoietic recovery in single unit UCBT. The contribution of these cells to GVHD, and the association of cell dose with GVL, immune reconstitution, and engraftment need to be defined in larger numbers of patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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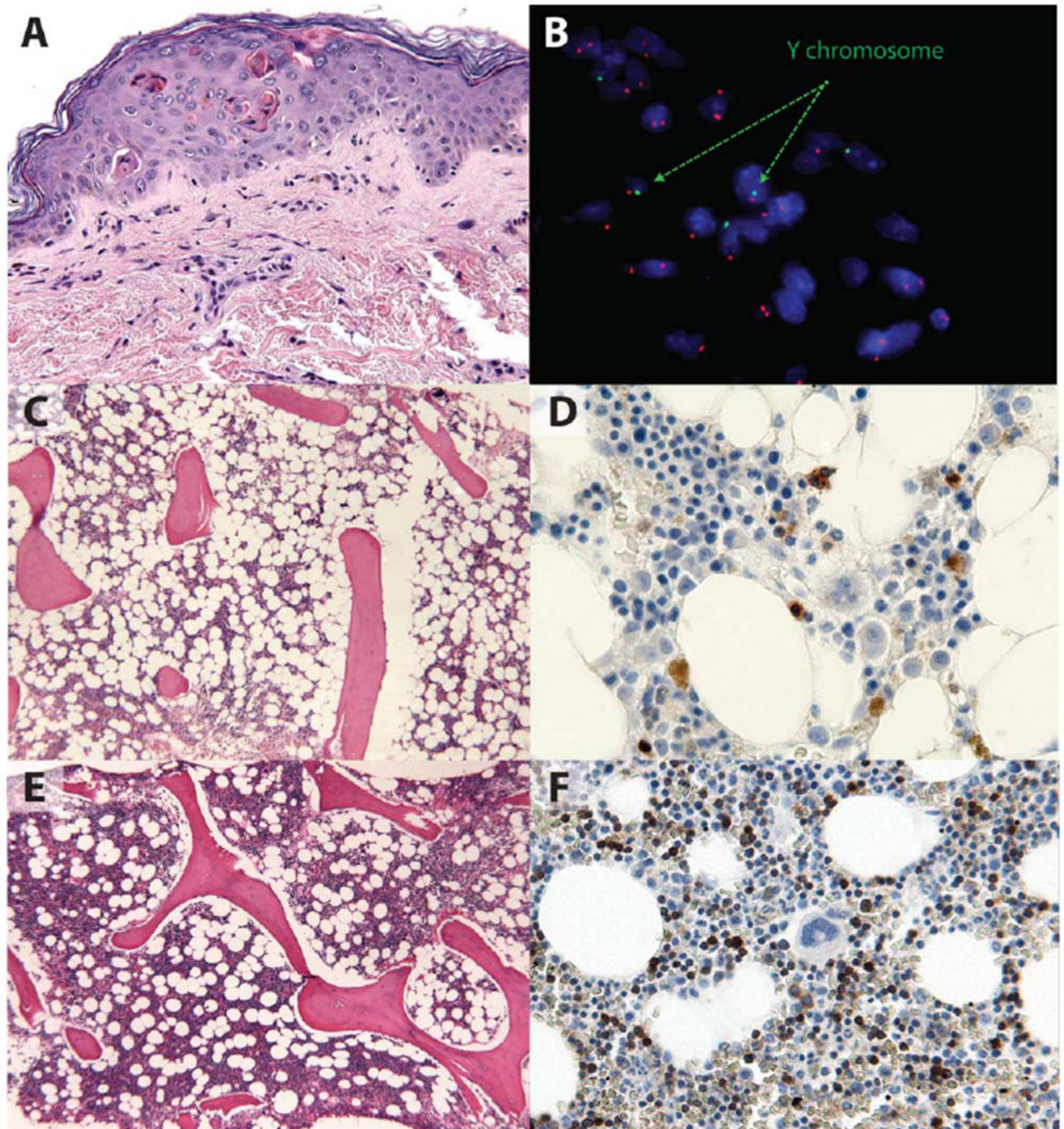

Figure 1. Early donor T cell trafficking (day +11) and hematopoietic recovery in a female recipient of UCBT and CD3/CD28 costimulated T cells

A: H&E stained sections of skin rash (20×) performed on day +11, demonstrating a sparse lymphocytic infiltrate in the superficial dermis and at the dermal-epidermal junction, associated with dyskeratotic keratinocytes. B: Fluorescence in situ hybridization (FISH) of skin (50×) detect XX (red-red) signals in stromal and epithelial cells and in some lymphocytes, of recipient (female) origin. A subset of lymphocytes contained an XY (red-green; dashed arrows) signal, compatible with male (donor) cells of origin in this sex-mismatched UCBT patient. C: H&E stained, 5× magnification and D: immunostain for

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CD3+; 40× magnification of bone marrow sections day +21 following UCBT, showing trilineage hematopoiesis with scattered T cells. E: H&E, 5× magnification and F: immunostain for CD79a; 20× magnification day +365 following UCBT highlights hematogones. Tandem complete white blood count 6.7 with a normal differential and absolute lymphocyte count of 2910 × 10^3/μL with normal absolute numbers of CD4+ and CD8+ cells, Hemoglobin 12.2 g/dL, Platelets 254 × 10^3/μL. Cytogenetic studies performed on bone marrow aspirate showed a normal 46,XY male (donor) karyotype in 30 metaphase cells. FISH studies were negative for anomalies of chromosomes 9 or 21, present in the ALL that preceded the diagnosis of therapy-related AML. Immunoglobulin heavy chain gene rearrangement by PCR showed a polyclonal distribution of peaks. Post-transplant chimerism studies detected 100% single donor DNA in peripheral blood (whole blood and CD3+ subset) and whole bone marrow on days +21, +28, and +365 following UCBT. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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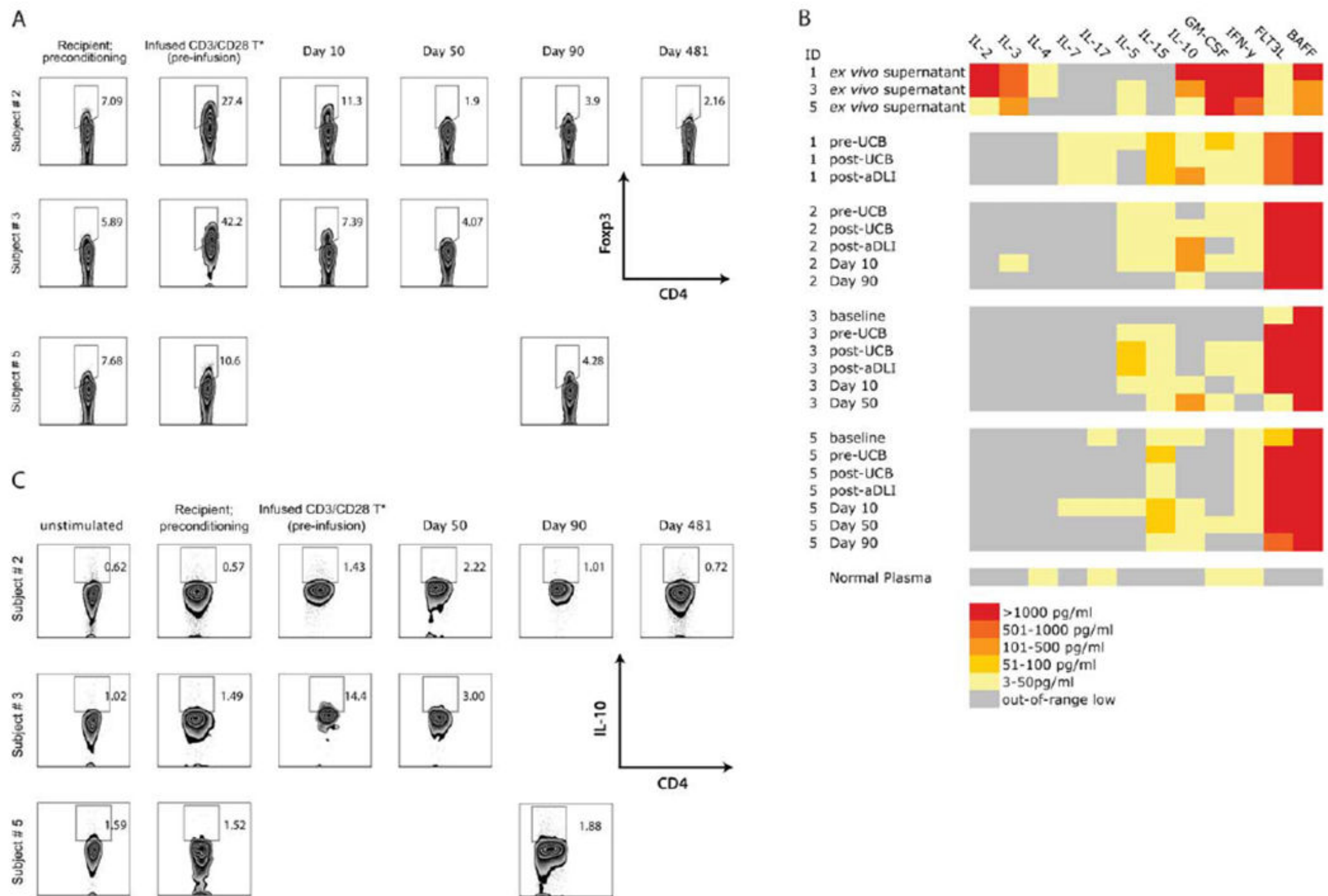

Figure 2. T cell polarization

A: Regulatory T cells are enriched in the T cell product and decline beyond day +10 following UCBT. B: Concentrations of cytokines were measured in supernatants from expanded cells (subjects 1, 3, and 5, top 3 rows; normalized per million cells) and in serum from UCBT recipients at pre-defined timepoints; baseline samples were drawn prior to day zero, pre-UCB, post-UCB, and post-aDLI were all drawn on day zero, prior to infusion of UCB MNCs, following UCB MNC infusion but prior to T cell infusion, and following T cell infusion respectively. C: Intracellular cytokine staining for IL-10 in CD4+ cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

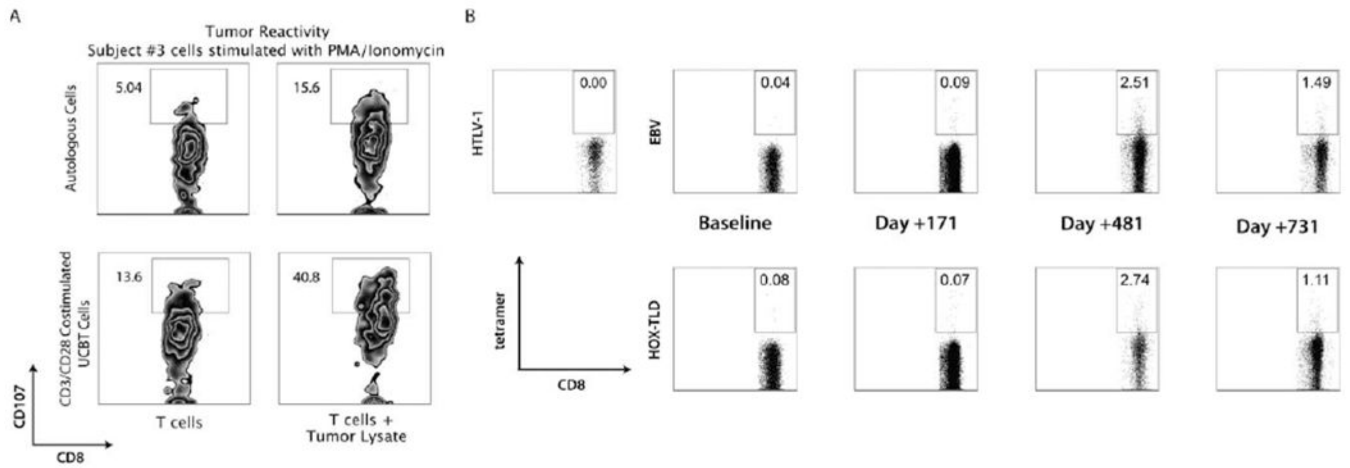

Figure 3. Antigen Responsiveness

A: Ex vivo alloreactivity generated by activated donor-derived UCB Tcells against autologous tumor lysate Autologous MNC (top row) or CD3/CD28 costimulated UCBT cells from the graft (bottom row) were incubated with PMA/Ionomycin (left) or with cell lysates prepared from banked tumor sample (bone marrow MNC at diagnosis) and PMA/ Ionomycin (right). Degranulation was assessed by flow cytometry measurement of CD107a in CD8+ gated cells. B: Acquisition of EBV- and HOX-TLD specific cytotoxic Tcells. Batched serial samples from one subject (#2) who was EBV seropositive at baseline with an HLA-A2+ donor were analyzed for EBV-tetramer-and HOX-TLD-positive cells. HTLV-1-MHC tetramer served as a negative control.

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TABLE I.

Patient and cellular product characteristics, safety and outcome

Pre-Transplant Characteristics

ID	Age	Gender	Disease	ABO/Rh	CMV
1	31	F	MDS (RAEB-1 with t(3,3)(q21;q26)	B+	neg
2	25	F	AML, therapy related	A+	neg
3	48	F	AML (M5), primary refractory	O+	neg
4	28	F	ALL, relapsed	ND	ND
5	32	F	AML (M5), primary refractory	O+	pos

Graft Characteristics

ID	TNC/kg ($\times 10^7$)	CD341 ($\times 10^8$)	341/kg ($\times 10^5$)	CD34+/ul (post-thaw)	Gender	HLA matching (of 6)	mM loci	ABO/Rh	Fractions
1	4.15	2.3	0.383	67.7	M	4	B, DRB1	AB+	80/20
2	4.8	44.4	7.01	166.5	M	4	A, DRB1	A+	60/40
3	2.8	7.4	1.12	82.3	M	4	A,B	O+	80/20
5	3.25	4.6	0·529	ND	F	4	A, DRB1	A−	80/20

Infused T cell Characteristics

ID	TNC/kg ($\times 10^5$)	CD3+/kg ($\times 10^5$)
1	1.57	1.56
2	1.56	1.46
3	1.31	1.24
5	1.07	1.02

Engraftment and Outcome

ID	Day Neutrophil >500/ul	Day Platelet >20/ul	Outcome (cause of death)
1	20	NR	Death, day +34 (TRM)
2	12	23	Alive, 5+ years in CR
3	17	NR	Death, day +69 (TRM)
5	>50[a]	NR	Death, day +96 (TRm)

Safety Data

SAE (or attributable toxicity)	Maximum Grade (# events)	Relationship to Infused T Cells	Dose Limiting Toxicity?

Rash (consistent with aGVHD and/or pre-engraftment syndrome)	1 (3)	possible	no
Diarrhea	3 (2)	possible	yes
Pericardial Effusion	4 (1)	unlikely	no
Diffuse Alveolar Hemorrhage	5 (1)	unrelated	no
Encephalitis (CSF HHV-61)	4 (1)	unrelated	no
Fever	1 (2)	unrelated	no
Varicella Zoster	3 (1)	unrelated	no
Diarrhea (colitis, CDIF1)	1 (1)	unrelated	no
Creatinine Increased	1 (1)	unrelated	no
Hypotension	3 (3)	unrelated	no
Cerebral edema (with radiologic PRES)	5 (1)	unrelated	no

[a]Primary graft failure; received 2nd mismatched unrelated transplant.

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