

# Interleukin-7 Permits Th1/Tc1 Maturation and Promotes *Ex vivo* Expansion of Cord Blood T Cells: A Critical Step toward Adoptive Immunotherapy after Cord Blood Transplantation

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## Abstract

Donor leukocyte infusions (DLI) in the allogeneic hematopoietic transplant setting can provide a clinically relevant boost of immunity to reduce opportunistic infections and to increase graft-versus-leukemia activity. Despite significant advances in applicability, DLI has not been available for single-unit recipients of unrelated cord blood transplant. *Ex vivo* expansion of cord blood T cells can be achieved with interleukin (IL)-2 and CD3/CD28 costimulatory beads. However, significant apoptosis occurs in proliferating T cells, diminishing the yield and skewing the CD4/CD8 ratio in the T-cell population, jeopardizing the potential efficacy of DLI. In this study, we show that interleukin (IL)-7 not only reduces apoptosis of activated T lymphocytes and enhances their proliferation but also promotes functional maturation, leading to secretion of IFN- $\gamma$  and other key cytokines. Recognizing that infused T lymphocytes will need to meet microbial antigens in secondary lymphoid organs to generate effectors, we also show that expansion with IL-7 promotes the preservation of a polyclonal broad T-cell receptor repertoire and a surface phenotype that favors lymph node homing. Expanded lymphocytes lack alloreactivity against recipient and other allogeneic cells, indicating a favorable safety profile from graft-versus-host disease. Nevertheless, expanded T cells can be primed subsequently against lymphoid and myeloid leukemia cells to generate tumor-specific cytotoxic T cells. Taken together, our findings offer a major step in fulfilling critical numerical and biological requirements to quickly generate a DLI product *ex vivo* using a negligible fraction of a cord blood graft that provides a flexible adoptive immunotherapy platform for both children and adults. *Cancer Res*; 70(13): 5249–58. ©2010 AACR.

## Introduction

Lymphopenia, particularly affecting T cells, is an almost uniform consequence of hematopoietic cell transplantation extending sometimes beyond the first year. Adoptive transfer of naturally primed and *ex vivo* restimulated T lymphocytes in the form of donor leukocyte infusions (DLI) has shown efficacy to prevent/treat EBV-associated lymphomas and posttransplant viral infections (1) and could augment graft-versus-leukemia (GVL) activity. Despite significant recent

advances in the applicability and outcome following unrelated cord blood transplantation (UCBT), currently there is no obviously available posttransplant source for DLI from the transplanted unit. Not only is there a shortage of leukocytes available for DLI but cord blood T lymphocytes are also antigen inexperienced/naïve cells. Moreover, multiple placental factors bias against Th1/Tc1 development *in utero* (2, 3), impairing Th1/Tc1 cytokine production, particularly IFN- $\gamma$  (4) and cytotoxicity (5), collectively leading to impaired antiviral immunity that leads to significant transplant-related mortality in the first 3 to 6 months after UCBT (reviewed in ref. 6).

The tempo of cellular recovery is quite variable after UCBT. Although mitogenic responses may reach reference range in children 6 to 9 months after UCBT, T-cell reconstitution is gradual and typically does not reach age-appropriate numbers before 9 to 12 months. This is in contrast with adults where T-cell reconstitution typically extends beyond the first year, presumably as a result from age-dependent decline in pretransplant thymic function (7). A detailed analysis in a cohort of adults who underwent UCBT for hematologic malignancies showed extremely severe T-cell lymphopenia that extended throughout the first year (8).

Attaining a vigorous cellular immune profile, particularly against common pathogens such as herpes viruses, affects survival after UCBT. Investigators from the Cord Blood

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doi: 10.1158/0008-5472.CAN-09-2860

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Transplantation Study found that development of antigen-specific proliferation in the first 3 years to cytomegalovirus (CMV), herpes simplex virus, or VZV led to a lower probability of leukemia relapse and a higher overall survival (9). Robust proliferative T-cell responses likely represent a powerful surrogate marker for functional T-cell immune reconstitution, leading to more effective GVL activity. These data underscore the high clinical relevance to design novel approaches aimed at alleviating posttransplant lymphopenia and the Th1/Tc1 functional deficits displayed by cord blood T cells infused in the graft.

Recently, we and others have shown the feasibility of *ex vivo* cord blood T-cell expansion (10, 11), drawing from the pioneering work by June and colleagues (12), using paramagnetic Dynal beads coated with anti-CD3 and anti-CD28 stimulatory antibodies. These artificial antigen-presenting cells (APC) simultaneously provide agonistic T-cell receptor (TCR) and costimulatory signals triggering sufficient T-cell proliferation *in vitro* to generate clinically relevant DLI products from living donors (13–15). Although in our previous work (10) robust T-cell expansion and even partial Th1/Tc1 maturation were evident starting with frozen/thawed cord blood specimens, significant apoptosis (~16%) resulted in an inverted CD4/CD8 ratio and diminished yield despite relatively low concentrations of interleukin (IL)-2 in the medium. The high degree of apoptosis was likely the result of activation-induced cell death (AICD) following strong TCR signaling on cord blood T cells as previously described (16). Infusion of overstimulated, apoptosis-prone DLI product would likely lead to a narrow T-cell repertoire and shortened T-cell survival *in vivo*. Moreover, it could falsely suggest futility of *ex vivo* expanded DLI strategies.

In the current study, we tested and confirmed our hypotheses that IL-7, acting in concert with a new clinical-grade CD3/CD28 costimulatory bead and IL-2, would not only enhance *ex vivo* cord blood T-cell proliferation while retaining a broad TCR repertoire as predicted (17) but would also reduce AICD.

We also show that IL-7 permits Th1/Tc1 maturation of the proliferating cord blood T cells to progress until a clinically desired intermediate state while preserving the naïve surface phenotype of the starting population conducive to lymph node homing. The absence of cytotoxicity against recipient and other allogeneic cells indicates a favorable safety profile from graft-versus-host disease (GVHD). Importantly, expanded CD3/CD28-expanded T cells can be subsequently primed against lymphoid and myeloid leukemia cells to generate tumor-specific CTL. Taken together, the presented experimental strategies for the first time fulfill critical numerical and biological requirements to generate a DLI product *ex vivo* in ~14 days from a negligible ( $\leq 3\%$ ) fraction of a cord blood graft, offering a flexible adoptive immunotherapy platform for pediatric as well as adult clinical trials.

## Materials and Methods

### Specimens

Frozen umbilical cord blood samples not eligible for clinical use were obtained from research units at the Duke Stem

Cell Laboratory. Eligible patients were consented on an Institutional Review Board-approved protocol to obtain fibroblasts and  $\leq 3\%$  aliquots of their UCB grafts.

### T-cell enrichment and expansion system

CD3<sup>+</sup> T cells were enriched by negative immunomagnetic selection with EasySep (StemCell Technology) as described (10). Expansion was started at  $6 \times 10^5$  to  $8 \times 10^5$  T cells/mL in gas-permeable VueLifeTeflon bags (American Fluoroseal). T cells were cocultured with ClinExVivo Dynabeads (Dynal/Invitrogen Corp.) at a cell/bead ratio of 3:1 in X Vivo-15 (BioWhittaker) supplemented with  $5.5 \times 10^{-5}$  mol/L  $\beta$ -mercaptoethanol, 10 mmol/L HEPES, 5% pooled human serum (Valley Biomedical), and 100 units/mL IL-2 (Proleukin, Novartis)  $\pm$  10 ng/mL IL-7 (R&D Systems) as indicated. Medium and cytokines were replenished three times a week to maintain a cell concentration of  $\sim 1 \times 10^6$  total nucleated cell count/mL (10). After 12 to 14 days, after vigorous agitation, the beads were removed by a Dynal magnet MPC-2 or Dynal ClinExVivo MPC.

### Immunophenotypic and functional characterization of the CD3/CD28-expanded T cells

Surface and intracellular immunophenotyping, cytokine secretion, and T-cell enumeration were performed by four-color fluorescence-activated cell sorting (FACS) as described (10, 18, 19). Acquisition of  $>5,000$  CD3<sup>+</sup> events was performed on FACSCalibur or FACSCanto II (BD Biosciences). All antibodies, including isotype-specific controls, were purchased from BD Biosciences, except anti-granzyme B, which was from Serotec. Cytotoxicity was measured against IM9 cell line (20) or recipient fibroblasts by Delfia EuTDA cytotoxicity assay on a Victor 2 microplate reader (both from Perkin-Elmer). Following 7 to 9 days of prestimulation with mitomycin C-treated targets (100  $\mu$ g/mL; Sigma) in the presence of IL-7 (5 ng/mL) and IL-2 (25 units/mL), effectors were washed and cultured in serial dilution with 5,000 fresh untreated targets for 2 and 3 hours. Percent specific europium chelated ligand (EuTDA) release was calculated as follows: [experimental release (counts) – spontaneous release (counts)]/[maximum release (counts) – spontaneous release (counts)]  $\times$  100. Percent spontaneous release = [spontaneous release (counts) – background (counts)]/[maximum release (counts) – background (counts)]  $\times$  100.

TCRV $\beta$  spectratyping and Kullback-Leibler divergence analysis were performed as previously published (21).

### Leukemia-specific CTL generation from CD3/CD28-expanded cultures

CD3/CD28-expanded “day 14” T cells generated from cord blood grafts were cultured in parallel with two different mitomycin C-treated leukemia cells (IM9 and U937) at a stimulator/responder ratio of 1:10 in 24-well plates (Costar) at  $\sim 1 \times 10^6$  cells/mL. IM9 leukemia cell line represents a lymphoid malignancy (20), whereas U937 monoblastoid leukemia cells are of myeloid origin (22). CTLs were cultured in X Vivo-15 medium (Lonza) supplemented with 5% FCS (Life Technologies, Invitrogen), 5 ng/mL IL-7, 5 ng/mL IL-15, and

10 ng/mL IL-12 (all from R&D Systems) for 9 to 10 days. U937 cells were treated *in vitro* with IFN- $\gamma$  (R&D Systems) at 500 units/mL for ~48 hours before mitomycin C treatment to enhance their immunogenicity. Cultures were restimulated with mitomycin C-treated leukemia cells twice: first in the presence of IL-7 and IL-15 and second with IL-15 alone, each for 6 to 7 days. CTL cultures were refed with medium alone after half the medium was removed the night before culture termination. Washed effectors were tested in Delfia EuTDA cytotoxicity assay as described above against fresh, unmanipulated BATDA-loaded targets that included IM9 and U937 leukemia cells and phytohemagglutinin (PHA) blasts of the cord blood transplant recipient. Percent specific EuTDA release was calculated as described above.

#### Analysis of human TRC gene rearrangement

The signal-joint TCR excision circle (sjTREC) assay was performed using real-time quantitative PCR by quantifying the episomal circles generated as a by-product of TCR $\alpha$  gene rearrangement as we previously published (7). For each cell suspension prepared for TREC analysis, total nucleated cell count and absolute T cell content were enumerated by Trucount FACS as previously described (10, 18), and thereafter, dry pellets were prepared and kept frozen at  $-80^{\circ}\text{C}$  until batch-matched pairs were thawed. TREC content was expressed after adjustment for  $10^5$  T cells per sample.

#### Statistical analysis

Two-sided paired Student's *t* test was used to compare conditions  $\pm$  IL-7 and to compare T-cell cytotoxicity against the described targets. Statistical significance was set at  $P < 0.05$ .

## Results

#### Favorable effect of IL-7 on cord blood T-cell survival, proliferation, and TCRV $\beta$ repertoire during CD3/CD28-mediated expansion

Purified T cells obtained from frozen/thawed cord blood specimens were split and cultured in parallel with and without IL-7. Matched pair analysis showed significantly more viable T cells when IL-7 was added to IL-2 in the medium, leading to an average of 165-fold T-cell expansion (Table 1; Fig. 1A). Following 14 days of expansion, striking dilution of TRECs was noted as the sjTREC content in CD3 $^+$  T cells was depleted by  $\sim 2$  log in both culture conditions compared with the starting population of pre-expansion cord blood T cells (Fig. 1B), irrespective of IL-7 exposure. There was no significant difference in trypan blue viability between the culture conditions, typically  $>85\%$  by days 12 to 14. This was in stark contrast once cellular events at the end of 12 to 14 days of culture were examined by flow cytometry. Significantly more viable CD45 bright T lymphocytes were identified in cultures supplemented with IL-7 ( $71 \pm 10\%$ ) compared with cultures with IL-2 alone (mean,  $46 \pm 15\%$ ; Fig. 2A; Table 1). CD3/CD28-costimulated lymphocytes that have recently undergone apoptosis can be accurately enumerated with the aid of a simple flow cytometry gating strategy (10). Recently apoptotic/dead cells display altered physical properties, as defined by

forward scatter/side scatter, additionally stain less intensely with CD45 (and CD3) compared with "viable" lymphocytes (Fig. 3A). We also tracked T cells that have recently entered the apoptosis pathway, identified/gated in the viable CD45/FSC/SSC region by intracellular expression of activated caspase-3 (Fig. 2C) and 7-aminoactinomycin D (7-AAD)/Annexin stain (Fig. 3B). As determined by intracellular activated caspase-3 expression, there were significantly fewer T cells undergoing active apoptosis in the presence of IL-7 (median, 4% versus 8%; Table 1; Fig. 2C). The antiapoptotic effect if IL-7 was evident in both CD4 $^+$  and CD8 $^+$  subsets (Table 1). To test T-cell survival-promoting effects of IL-7 beyond the *in vitro* expansion period, expanded cells were frozen on day 14 and subsequently thawed and rested for 24 hours in culture medium devoid of cytokines. Although the rest period *in vitro* cannot mimic the *in vivo* postinfusion conditions exactly, these experiments show that T cells after expansion retain the potential to upregulate IL-7 receptor/CD127 (Fig. 3A), and that the majority of IL-7 + IL-2-expanded T cells are still alive after freeze, thaw, and 24-hour culture in medium (Fig. 3B). Independent of the described antiapoptotic effects, IL-7 promoted significantly greater T-cell proliferation. About two thirds of all T cells were still actively cycling at the termination of the expansion period, as detected by intracellular Ki67 expression (Fig. 2B; Table 1). Because naïve T cells with recent thymic emigrant (RTE) phenotype (CD28 $^+$ /CD27 $^+$ /CD45RA $^+$ /CD62L $^+$ ) represent the vast majority of unmanipulated cord blood T cells, these findings corroborate earlier studies showing the proliferative and antiapoptotic effects of IL-7 to be operational predominantly in the naïve/CD45RA $^+$  T-cell compartment (17, 23). In addition to superior T-cell proliferation and reduced apoptosis in IL-7 supplanted conditions, we also found higher TCRV $\beta$  diversity per family ( $P = 0.04$ ,  $n = 3$ ), displaying a broad polyclonal spectrum (Fig. 4).

#### Limited Th1/Tc1 "maturation" during expansion and low expression levels of 4-1BB/CD137, CD40L, and perforin correlate with absent alloreactivity

Once we had shown the salutary effects of IL-7 on T-cell viability, expansion, and overall TCR diversity, we sought to determine its effect on surface and intracellular phenotype and overall T-cell function as measured by cytokine secretion profile and cytotoxicity. Despite undergoing several cycles of cell division triggered by IL-2 + IL-7 in concert with TCR and CD28 costimulation, significantly more cord blood T cells retained the naïve starting phenotype, CD45RA $^+$ /CD62L $^+$ , in the IL-7-containing condition ( $90 \pm 5\%$ ) compared with cells cultured in IL-2 alone ( $73 \pm 14\%$ ;  $P = 0.03$ ; Fig. 2D). Surface expression of L-selectin (CD62L) is essential for effective T-cell homing to secondary lymphoid organs, a desired destination for antigen inexperienced, unprimed adoptive T-cell infusions. CCR-7, a chemokine receptor implicated in both the entry and the retaining of T cells in lymph nodes, was also expressed on the majority of expanded T cells (data not shown). Interestingly, although the surface of postexpansion T cells seemed identical to unmanipulated fresh cord blood T cells in terms of CD28 $^+$ /CD27 $^+$ /CD45RA $^+$ /CD62L coexpression, expanded T cells displayed several upregulated surface

**Table 1.** Surface and intracellular characterization of the expanded progeny (mean ± SD)

Variable	IL-2 only	IL-2 + IL-7	P*
Day 12–14 mean fold expansion	48 ± 21	165 ± 123	0.04
%Viable T lymphocytes among CD45 <sup>+</sup> events	46 ± 15	71 ± 10	0.002
%CD4 <sup>+</sup>	60 ± 20	65 ± 16	0.26
%CD8 <sup>+</sup>	52 ± 26	45 ± 20	0.15
%CD4 <sup>+</sup> /CD8 <sup>+</sup>	6 ± 5	3 ± 3	0.43
Intracellular bcl-2/CD3 <sup>+</sup> (MFI)	82 ± 19	82 ± 16	0.97
%Intracellular Ki67 <sup>+</sup> /CD3 <sup>+</sup> “proliferating”	50 ± 14	65 ± 11	0.003
%Intracellular Ki67 <sup>+</sup> /CD4 <sup>+</sup> “proliferating”	50 ± 15	62 ± 10	0.03
%Intracellular Ki67 <sup>+</sup> /CD8 <sup>+</sup> “proliferating”	52 ± 12	68 ± 11	0.001
%Intracellular activated caspase-3 <sup>+</sup> /CD3 <sup>+</sup> “apoptotic”	8 ± 3	4 ± 2	0.011
%Intracellular activated caspase-3 <sup>+</sup> /CD4 <sup>+</sup> “apoptotic”	7 ± 3	4 ± 2	0.02
%Intracellular activated caspase-3 <sup>+</sup> /CD8 <sup>+</sup> “apoptotic”	8 ± 5	4 ± 2	0.03
%CD25 <sup>+</sup> /CD3 <sup>+</sup>	62 ± 14	63 ± 17	0.95
%CD25 <sup>+</sup> /CD45RO <sup>+</sup>	43 ± 19	25 ± 28	0.08
%CD28 <sup>+</sup> /CD3 <sup>+</sup>	89 ± 5	95 ± 3	0.02
%CDRA <sup>+</sup> /RO <sup>-</sup> “naïve”	45 ± 30	78 ± 28	0.06
%CDRA <sup>-</sup> /RO <sup>+</sup> “memory”	10 ± 4	18 ± 27	0.65
%CDRA <sup>+</sup> /CD62L <sup>+</sup> “phenotypically naïve”	73 ± 14	90 ± 5	0.03
%CDRA <sup>+</sup> /CD27 <sup>+</sup> /CD8 <sup>+</sup> “naïve CD8 <sup>+</sup> ”	79 ± 7	90 ± 4	0.003
%CDRA <sup>-</sup> /CD27 <sup>+</sup> /CD8 <sup>+</sup> “central memory”	13 ± 6	4 ± 2	0.002
%CDRA <sup>+</sup> /CD27 <sup>-</sup> /CD8 <sup>+</sup> “effector CTL”	7 ± 6	6 ± 4	0.26
%CD57 <sup>+</sup> /CD28 <sup>-</sup> /CD8 <sup>+</sup> “effector CTL”	0 ± 0	0 ± 0	0.35
%HLA-DR <sup>+</sup> /CD3 <sup>+</sup> “activated”	46 ± 12	44 ± 11	0.53
%HLA-DR <sup>+</sup> /CD4 <sup>+</sup> “activated”	45 ± 12	37 ± 11	0.1
%HLA-DR <sup>+</sup> /CD8 <sup>+</sup> “activated”	45 ± 16	53 ± 15	0.003
%NKG2D <sup>+</sup> /CD3 <sup>+</sup>	38 ± 23	27 ± 20	0.03
%NKG2D <sup>+</sup> /CD137 <sup>-</sup> /CD8 <sup>+</sup> “resting”	73 ± 17	62 ± 26	0.05
%NKG2D <sup>-</sup> /CD137 <sup>+</sup> /CD8 <sup>+</sup> “activated”	1 ± 2	0 ± 0	0.32
%NKG2D <sup>-</sup> /CD137 <sup>-</sup> /CD8 <sup>+</sup> “anergic”	24 ± 16	37 ± 26	0.07
%NKG2D <sup>+</sup> /CD137 <sup>+</sup> /CD8 <sup>+</sup> “cytotoxic”	2 ± 3	1 ± 1	0.24
%Intracellular granzyme A <sup>+</sup> /CD8 <sup>+</sup>	44 ± 26	44 ± 23	0.95
%Intracellular granzyme B <sup>+</sup> /CD8 <sup>+</sup>	41 ± 35	35 ± 25	0.26
%Intracellular perforin <sup>+</sup> /CD8 <sup>+</sup>	6 ± 4	4 ± 2	0.10
%Intracellular IFN-γ <sup>+</sup> /CD3 <sup>+</sup>	20 ± 8	19 ± 10	0.64
%Intracellular TNFα <sup>+</sup> /CD3 <sup>+</sup>	50 ± 20	55 ± 27	0.65
%Intracellular IL-2 <sup>+</sup> /CD4 <sup>+</sup>	79 ± 9	54 ± 36	0.41
%Intracellular IL-4 <sup>+</sup> /CD3 <sup>+</sup>	2 ± 2	2 ± 2	0.55
%OX40 <sup>+</sup> /CD4 <sup>+</sup>	83 ± 24	78 ± 21	0.79

NOTE: Surface and intracellular phenotyping and phorbol 12-myristate 13-acetate/ionomycin-induced cytokine secretion were performed as described (1–3).

Abbreviation: MFI, mean fluorescence index.

\*Two-tailed, paired Student's *t* test.

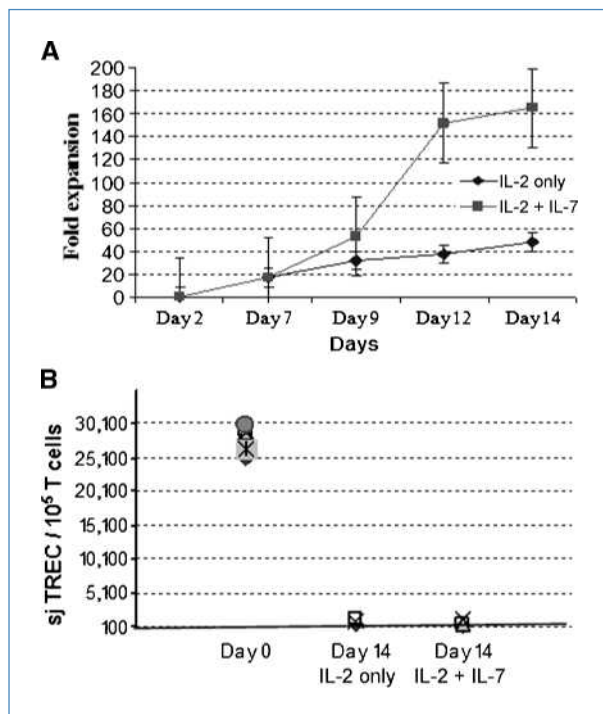
molecules commonly seen after activation, including CD25, HLA-DR, and OX40 (see Table 1). However, <10% of cells expressed CD40L (data not shown). Despite the preservation of resting, naïve, “RTE-mimicking” surface phenotype, as indicated by CD28<sup>+</sup>/CD27<sup>+</sup>/CD45RA<sup>+</sup>/CD62L coexpression, CD3/CD28 costimulation led to rapid downregulation of membrane CD127 (IL-7Rα) in parallel with surface CD25 (IL-2Rα) upregulation on the very same T cells (Fig. 3A). This

“receptor switching process” is not dependent on the presence of IL-7 in our cultures, as CD25 and CD127 expression levels were superimposable in the presence and absence of IL-7 (Fig. 3A). Moreover, because a near-complete reversal between CD25 and CD127 expression has occurred by ~24 to 48 hours of culture (Fig. 3A), this phenomenon seems independent of cell division. Numerical T-cell expansion does not begin in earnest in the cultures until days -3 to 4 (Fig. 1;



data not shown). Interestingly, when expanded T cells were frozen on day 14 and subsequently thawed and rested for 24 hours in culture medium devoid of cytokines, CD127 was reexpressed on nearly half of the viable T cells (Fig. 3A). IL-7 receptor reexpression could permit delivery of pro-survival signals to expanded T cells administered by clinical DLI infusions in the lymphopenic posttransplant state, where endogenous IL-7 level has been shown to be elevated up to 10 to 30 pg/mL after transplant (24). Although it is possible that high levels of IL-7 *in vivo* could induce downregulation of CD127 in the responding T cells, nevertheless, our results suggest that the expanded T cells retain the capacity to re-express CD127 even when rested after thaw with IL-7 at 15 to 30 pg/mL (data not shown).

CD3/CD28 costimulation with ClinExVivo Dynabeads in this series of experiments enhanced in a larger fraction of postexpansion T cells the capacity for intracellular expression of IFN- $\gamma$ , tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), and granzyme B (Table 1) than we previously reported using different arti-



**Figure 1.** A, CD3<sup>+</sup> T-cell expansion is superior in the presence of IL-7. Frozen/thawed cord blood T cells were enriched by negative selection, enumerated by lyse/no wash multitest T cell staining in Trucount tubes (BD Biosciences) as previously described (18, 50), and then split equally into two under identical culture conditions except for the presence of IL-7 as indicated. Cells were cultured for 12 to 14 d with ClinExVivo Dynabeads, whereas medium and cytokines were replenished three times a week. A 50- $\mu$ L aliquot was removed from the bags at indicated time points and absolute T cell number was enumerated in Trucount tubes. B, irrespective of IL-7 in the culture medium, expansion leads to dilution and near-complete loss of sjTREC in day 14 progeny. sjTRECs were measured before and after expansion ( $n = 4$ ), as published previously (7). For each sample, total nucleated cell count and absolute T cell content were enumerated by Trucount FACS method (10, 18). TREC content was expressed after adjustment for  $10^5$  T cells per sample.

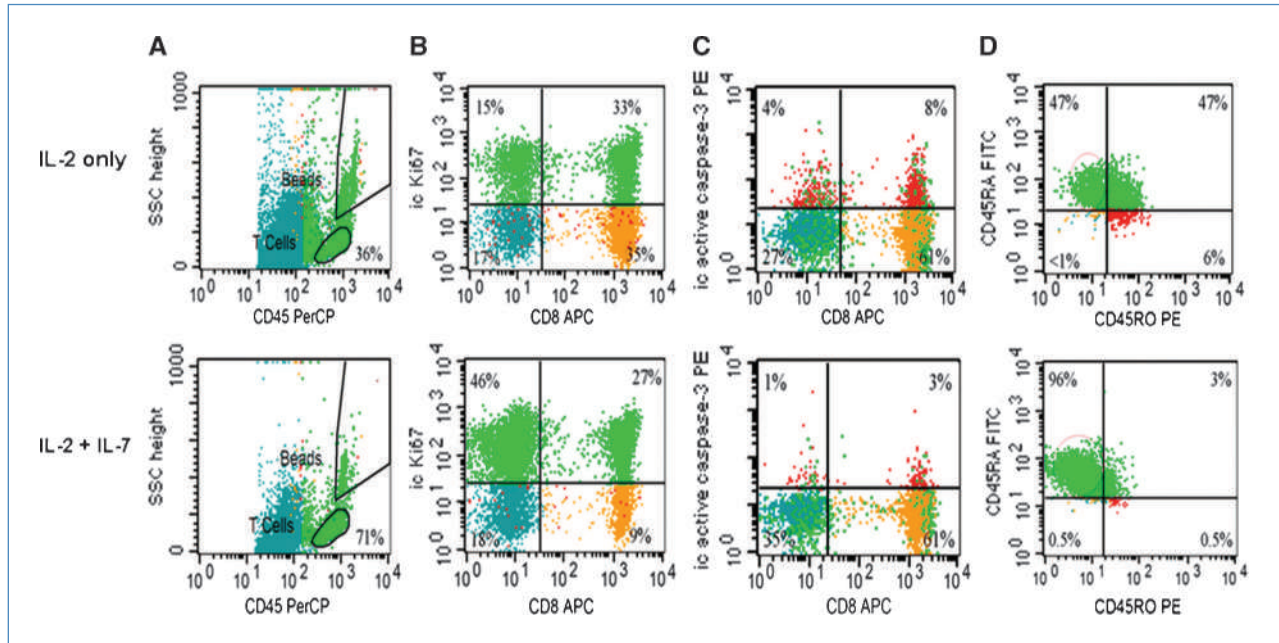
ficial APC beads (10). Nevertheless, despite the potential for an increase in alloreactivity (25) after the more robust expansion in the presence of IL-7, the expanded progeny lacked cytotoxicity against a highly immunogenic (CD40<sup>+</sup>, CD80<sup>+</sup>, CD86<sup>+</sup>) EBV<sup>+</sup> allogeneic lymphoblastoid cell line (IM9;  $n = 7$ ), or recipient fibroblasts ( $n = 2$ ), despite a week long presensitization before performing the CTL assay (Fig. 5). Interestingly, absent cytotoxicity coincided with low expression of 4-1BB/CD137, CD40L, and perforin (Table 1). Together, these features support a favorable safety profile of day 14 ClinExVivo expanded T cells with reduced likelihood for inducing GVHD *in vivo* on adoptive transfer.

### Ex vivo expanded, CD3/CD28-costimulated cord blood T cells can be primed *in vitro* against lymphoid and myeloid leukemia

DLI with day 14 ClinExVivo + IL-2 + IL-7-expanded T cells generated from the originally infused cord blood graft could alleviate posttransplant lymphopenia and qualitative T-cell defects until thymic regeneration could contribute new T cells. However, such DLI would be antigen nonspecific and will require microbial and/or tumor antigens to *in vivo* prime infused T cells in the transplant recipients. In a series of experiments, we evaluated the potential of day 14 CD3/CD28-costimulated/expanded T cells to undergo *in vitro* priming against specific leukemic targets. *In vitro* generated tumor-specific CTL responses could be adoptively infused to treat leukemia patients with minimal residual disease and/or relapse. CD3/CD28-expanded day 14 T cells were stimulated *in vitro* for 3 weeks in parallel cultures with killed, mitomycin C-treated lymphoid leukemia cells (IM9) and IFN- $\gamma$ -treated myeloid leukemia cells (U937). U937 cells by themselves cannot induce allogeneic T-cell response unless a stimulating anti-CD3 antibody is added to cultures (22). In addition, they do not provide costimulation via the CD80/CD86-CD28 pathway but likely via the ubiquitously expressed CD147 and CD98 molecules (22). CTL priming was performed in the decreasing presence of IL-12, IL-7, and IL-15, drawing from our published experimental strategy to *in vitro* prime antiviral responses from cord blood (26). Robust T-cell expansion ( $195\times$ ,  $\pm 115$ ;  $n = 4$ ) ensued over the course of  $\sim 3$  weeks when killed leukemia cells rather than ClinExVivo beads served as APC. After the course of two to three repeated stimulations, strong leukemia-specific cytotoxicity was detected in CTL assays, killing the stimulating leukemia cells but not the other leukemia or, most importantly, cord blood transplant recipient PHA blasts ( $n = 4$ ;  $P < 0.01$ ; Fig. 6A and B). Failure to recognize and kill cord blood transplant recipient PHA blasts indicates future clinical safety from the potential toxicity of GVHD.

### Discussion

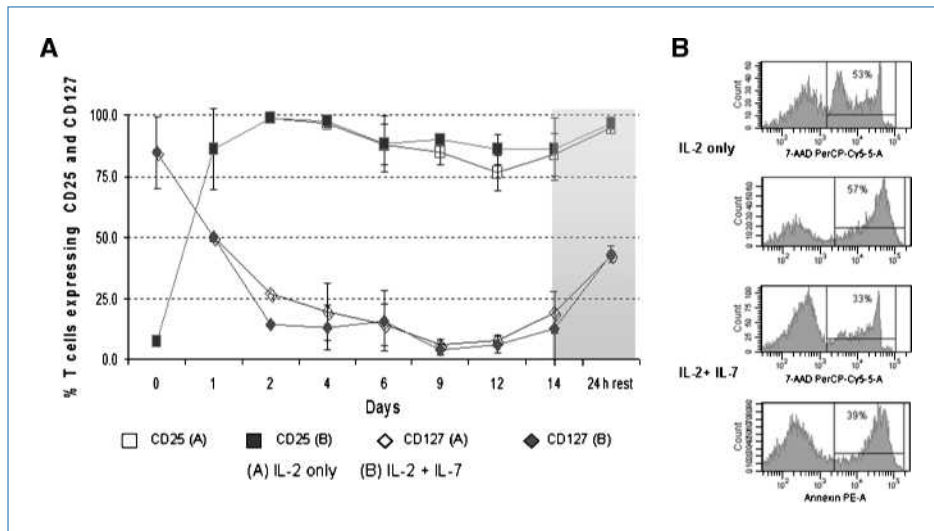
IL-7 is a very attractive immune modulator with proposed utility for *in vivo* administration to alleviate posttransplant lymphopenia (reviewed in ref. 27). It is well accepted that exogenous IL-7 can increase survival of naive T cells and promotes homeostatic expansion particularly among naive



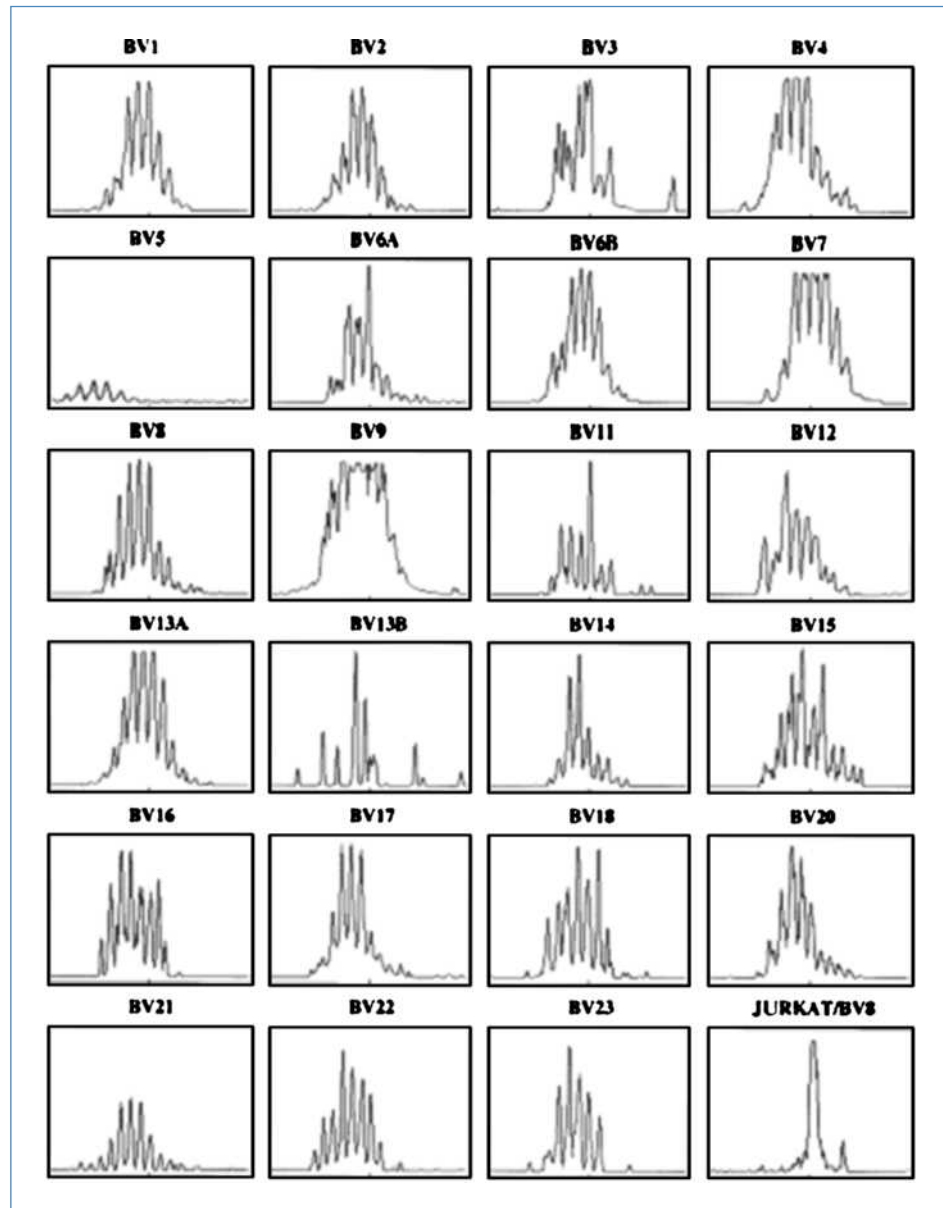
**Figure 2.** Flow cytometry profile of the expanded T-cell progeny  $\pm$  IL-7. Surface and intracellular (ic) FACS characterization was performed as shown previously (10, 18, 19). The relative size of T-cell subsets in each quadrant is expressed as the percentage of total viable T cells (see Table 1 for *P* values). A, CD45-PerCP/SSC defines an unambiguous region of viable cells. All other CD45 dim cells (recently apoptotic) also stain dim for CD3 (data not shown). B, intracellular Ki67 staining (top quadrants) identifies more proliferating T cells when expanded with IL-7 than without. C, when expanded without IL-7, more T cells undergo apoptosis and stain with intracellular active caspase-3<sup>+</sup>, although gated from the viable region of A. D, more T cells display the phenotype of naive/CD45RA<sup>+</sup>/RO<sup>-</sup> T cells when expanded with IL-7. Representative of 10 experiments.

T cells (28–31; reviewed in refs. 17, 23). Large animal models have established that IL-7 alone will lead not only to peripheral expansion of naive T cells but also to enrichment of antiviral CMV-specific T cells (32), showing the physiologic relevance of IL-7R $\alpha$  expression on both naive cells and central memory T cells. Recently, the first cohorts of humans received recombinant human IL-7 at the National Cancer Institute (NCI) to assess its safety and effects on T-cell

homeostasis and function (33, 34). IL-7 induced *in vivo* T-cell cycling, bcl-2 upregulation, and a sustained increase in peripheral blood and total body T-cell mass, affecting both CD4<sup>+</sup> and CD8<sup>+</sup> subsets. Moreover, T-cell expansion caused significant broadening of circulating TCR repertoire diversity, as naive T cells expanded preferentially (34). Homeostatic expansion is the primary mechanism of IL-7-mediated immune restoration in these studies accompanied by dilution



**Figure 3.** A, kinetic analysis of surface CD25 and CD127 expression. Simultaneous monitoring of IL-2R $\alpha$  (CD25) and IL-7R $\alpha$  (CD127) was performed after FACS surface staining and acquisition as described (18, 19) on serial aliquots obtained before (day 0) and during expansion on the indicated days. B, cell death after 24 h of rest in cytokine-free medium was assayed and scored by positive staining for Annexin and 7-AAD in parallel after freeze and thaw of expanded day 14 T cells. Representative of four experiments.



**Figure 4.** TCR $\beta$  repertoire of T cells expanded in the presence of IL-7 displays high TCR diversity. Day 14 progeny of an IL-7-supplemented ClinExVivo CD3/CD28-costimulated culture. TCR $\beta$  spectratyping and DKL analysis were performed as we previously published (21). Representative of three experiments.

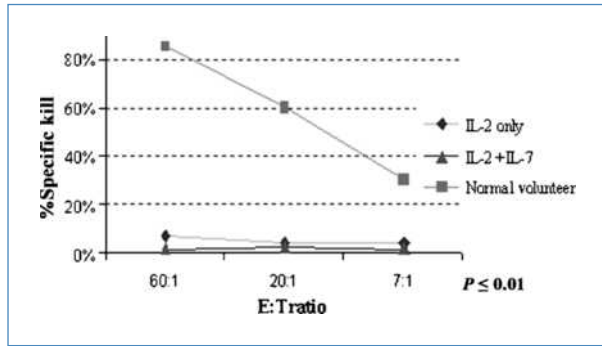
of TRECs among sorted CD4<sup>+</sup> and CD8<sup>+</sup> naïve cells (34). Importantly, IL-7 may induce T-cell maturation in nonlymphopenic circumstances as shown by another NCI study performed in rhesus macaques (35). In immunocompetent macaques, IL-7 induced the acquisition of central memory cell markers in both naïve CD8<sup>+</sup> and CD4<sup>+</sup> T-cell subsets. Moreover, both central memory and effector memory T cells entered cell cycle, showing the contribution of IL-7 to the maintenance of the entire T-cell pool. Nevertheless, taken together from the human and primate studies, there seems to be a preferential expansion of RTE and naïve T cells, thus explaining the broadened TCR $\beta$  repertoire after IL-7 therapy and dilution of TREC in the sorted naïve cells.

Despite the consensus on the proliferative and antiapoptotic effects of IL-7, there have been conflicting reports on the

functional changes induced by IL-7. Most studies agree that IL-7 signaling can sensitize naïve CD4<sup>+</sup> T cells and prime T cells for IL-2-induced IFN- $\gamma$  production (36–38); however, the variable degree of this functional maturation may have dramatic consequences in an allogeneic milieu where exaggerated T-cell activation and alloreactivity could induce and/or potentiate GVHD, a continuing barrier to the safety of allogeneic transplantation (reviewed in ref. 39). It is still vigorously debated whether donor T-cell exposure to IL-7 will worsen GVHD, as some murine studies contend (10, 25, 40, 41) or not (42, 43; reviewed in ref. 17).

The effect of IL-7 on human cord blood T cells has previously been tested *in vitro* (28, 44–46); however, these experiments were probing its effect without concomitant CD3/CD28 costimulation and without concurrent exogenous





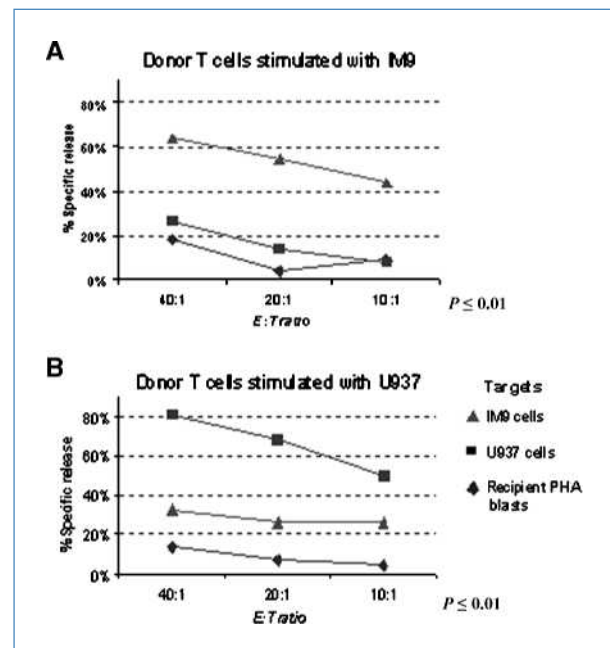
**Figure 5.** Absent cytotoxicity of the expanded cord blood T cells against allogeneic targets irrespective of  $\pm$  IL-7. Effector T cells were obtained from peripheral blood lymphocytes of healthy volunteers as positive controls and compared with CD3/CD28-costimulated cord blood T cells  $\pm$  IL-7. First, effectors were primed/sensitized against a highly immunogenic (HLA-DR<sup>+</sup>, CD40<sup>+</sup>, CD80<sup>+</sup>, CD86<sup>+</sup>) IM9 cell line for 7 to 9 d at 1:1 to 1:3 responder/stimulator ratio and then reexposed to fresh BATDA-loaded IM9 targets at the indicated E:T ratios for 2 and 3 h. Europium release was measured by the Delfia EuTDA cytotoxicity assay (10), and the calculated percent specific cytotoxicity is presented on the Y axis. Representative of seven experiments.

IL-2. Compared with adult T cells, cord blood T cells have been shown to express higher surface levels of IL-7R $\alpha$  chain matched with lower amounts of the common  $\gamma$ c chain. Notably, IL-7 does not alter the surface phenotype of proliferating naïve cells (28, 47). Interestingly, whereas in adult blood only “true” RTE (i.e., the CD31<sup>+</sup> subset of CD62L<sup>+</sup>/CD45RA<sup>+</sup>/CD4<sup>+</sup> T cells) enters cell cycle in response to IL-7, in cord blood both CD31<sup>+</sup> and CD31<sup>-</sup> cells proliferate vigorously (48).

Our data presented above clearly show the salutary role of IL-7 on *ex vivo* cord blood T-cell expansion as evidenced by enhanced T-cell cycling, reduced apoptosis, and increased TCRV $\beta$  diversity per family. Just as important from a clinical immunotherapy perspective, we also show that the addition of IL-7 to *ex vivo* expansion cultures using artificial CD3/CD28 APC beads permits the limited acquisition of granzymes and secretion of Th1/Tc1 cytokines without acquisition of alloreactive cytotoxicity. Significantly, despite T-cell maturation and an activated surface phenotype, IL-7 promotes the preservation of a surface phenotype (CD28<sup>+</sup>/CD27<sup>+</sup>/CD45RA<sup>+</sup>/CD62L), typically seen on naïve T cells including RTE. These surface features together with CCR-7 expression should facilitate effective T-cell homing to secondary lymphoid tissues that will be an essential step for the infused postexpansion T cells before priming could occur by encountering their cognate antigens in the right microenvironment. In parallel, we propose that reduced/absent cytotoxicity against allogeneic cells as a manifestation of residual functional “immaturity” will be an essential parameter in order for clinical DLI products to safely cross the HLA barrier, as opposed to full “adult like” cytotoxicity that may lead to severe alloreactivity/GVHD in the HLA-mismatched, unrelated UCBT setting. The predicted safety of expanded T-cell products in moderate doses is supported by an *in vivo* NOD/SCID- $\beta$ 2m<sup>-/-</sup> mouse model, where adoptive transfer of CD3/CD28-costimulated human cord blood T cells facili-

tated engraftment in the absence of inducing xenogeneic GVHD (49).

To our knowledge, our study is the first to present a relatively simple, easily reproducible methodology to *ex vivo* expand cord blood T cells that meet both numerical and critical qualitative biological benchmarks to usher DLI to the clinical UCBT setting. DLIs with *ex vivo* expanded T cells from the originally infused graft could alleviate posttransplant lymphopenia and qualitative T-cell defects well before thymic regeneration could contribute new T cells. In fact, by starting with  $\sim 2 \times 10^6$  total cord blood T cells, obtainable easily by “sacrificing” 2% to 3% of a typical UCB graft,  $\sim 100$ -fold expansion would yield  $\sim 2 \times 10^6$  cells/kg for patients up to 90 kg. Dose escalation/phase I trials will be started at lower doses in the HLA-mismatched UCBT setting to test safety while testing for augmented cellular immune function in a prophylactic manner for high-risk patients, such as those with viral reactivation and severe lymphopenia. Significantly, as we have shown with both a lymphoid and a myeloid leukemia cell line model, the antigen nonspecific



**Figure 6.** Leukemia-specific CTL can be *in vitro* primed starting with the CD3/CD28-expanded cord blood T cells. T cells were first CD3/CD28 expanded in the presence of IL-2 + IL-7 over 14 d as described and thereafter primed/sensitized against two killed leukemia cell lines in parallel cultures for 7 to 9 d at 10:1 responder/stimulator ratio in the presence of IL-12, IL-7, and IL-15. A, CTL primed *in vitro* with mitomycin C-treated IM9 cells. B, CTL primed *in vitro* with IFN- $\gamma$ -treated and mitomycin C-treated U937 cells. Each CTL culture was restimulated two more times (first in IL-7 + IL-15 and second in IL-15 alone) for a total of 3 wk with their respective killed leukemia cells. Cytotoxicity of washed effectors after 3 wk in CTL culture was tested against fresh, unmodified, BATDA-loaded IM9 cells, U937 cells, and recipient PHA blasts at the indicated E:T ratios for 3 h, as indicated. Europium release was measured by the Delfia EuTDA cytotoxicity assay, and the calculated percent specific cytotoxicity is presented on the Y axis. Representative of four experiments.



expansion strategy will also lend itself as a starting platform to generate and expand large numbers of tumor-specific CTL available for adoptive transfer to treat residual and/or relapsed leukemia after transplantation.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Acknowledgments

We thank Mark Bonyhadi and Joanne Kurtzberg for helpful comments; Eli Lien for ClinExVivo Dynabeads; Susan Buntz, Jeff Hale, and Melissa Ventevoegel

for technical assistance; and the staff at the Duke University Stem Cell Laboratory and the Carolinas Cord Blood Bank at Duke University.

### Grant Support

R01-CA132110, 7R01HL091749 (P. Szabolcs). Immunoscope assays were performed in the Immune Reconstitution and Biomarker Analysis Shared Resource (Duke Human Vaccine Institute, G.D. Sempowski), which is housed in the Regional Biocontainment Laboratory at Duke (UC6 AI58607), and partially supported by the Duke Center for Translational Research (P30 AI51445).

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Received 07/31/2009; revised 04/26/2010; accepted 04/27/2010; published OnlineFirst 06/08/2010.

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