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In Vitro Evaluation of Graft-versus-Graft Alloreactivity as a Tool to Identify the Predominant Cord Blood Unit before Double Cord Blood Transplantation

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The transplantation of two cord blood (CB) units obtained from unrelated donors (double CBT) is an effective strategy for adult patients with hematologic malignancies. Sustained hematopoiesis after double CBT is usually derived from a single donor, and only a few transplantation recipients displaying a stable mixed donor–donor chimerism have been reported. We investigated the mechanisms underlying single-donor predominance in double CBT by studying in vitro the role of the graft-versus-graft cell-mediated immune effect in two-way mixed-lymphocyte culture, along with the contribution of differential hematopoietic progenitor (HP) potency in HP mixed cultures. Results for the two-way mixed-lymphocyte culture showed that despite the weak and variable alloantigen-specific cytotoxic potential displayed by CB mononuclear cells, an immune-mediated dominance for one of the two CB units was detected in the majority of experiments. Alloantigen-induced cytotoxic activity was directed toward both CB-HP and phytohemagglutinin (PHA)-activated T lymphoblastoid cells. The CB unit with the higher fold expansion of $CD34^+$ cells in single-expansion culture was prevalent in the HP mixed-expansion culture, as shown by DNA chimerism evaluation. Based on these data, we hypothesize that the dominant CB unit is able to develop prevalent cytotoxic activity toward activated lymphocytes of the other CB unit, thereby preventing them from exerting alloantigen-specific cytotoxic potential against both activated lymphocytes and HPs of the dominant unit. In accordance with this hypothesis, we propose the evaluation of alloantigen-induced cytotoxic activity generated in two-way mixed-lymphocyte culture and directed toward PHA-activated T lymphoblastoid cells as a tool to identify the potentially predominant CB unit before double CBT.

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INTRODUCTION

The infusion of two cord blood (CB) units obtained from unrelated donors has been proposed as a strategy for adult patients with life-threatening hematologic diseases who are candidates for CB transplantation (CBT), to overcome the limitation of low cell dose present in a single CB unit [1-3]. Double CBT has been shown to be effective after both myeloablative and reduced-intensity conditioning regimens [4-7]. In the majority of double CBTs, the two CB units are partially HLA matched with the recipient, as well as with each other, and the use of two partially HLA-matched CB units has been shown to possibly reduce the risk of relapse in patients with leukemia without increasing the risk of severe acute graft-versus-host disease (aGVHD) [3,8-10].

Sustained hematopoiesis after double CBT is usually derived from transplantations involving a single donor. Barker et al [4] reported that at 1 month after transplantation, >70% of double CBT recipients displayed hematopoiesis from one CB unit, whereas by day 100, 100% of patients displayed hematopoiesis from a single donor. In contrast, other studies have reported a state of stable mixed chimerism with contributions from both CB units in some patients [6,11-13]. Gertow et al [14,15] recently reported two individuals

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in which both CB units coexisted for more than 2 years after transplantation and showed that the two stable CB units were phenotypically and functionally different in terms of the number and secretome profile of T and natural killer (NK) cells. It is conceivable that a condition of stable mixed chimerism might be favored by reduced-intensity conditioning regimens.

The biological mechanisms responsible for single-donor predominance after double CBT remain incompletely understood. Barker et al [4] suggested that neither the total number of nucleated or $CD34^+$ cells nor donor–recipient HLA disparity predicts which of the two CB units will predominate; in contrast, the predominant CB unit was found to contain a significantly higher number of $CD3^+$ T lymphocytes. This latter observation was not confirmed in an analysis of a larger cohort of patients, however [1].

Preclinical studies suggested that an immune-mediated effect could play a role in promoting the predominance of one CB unit [16-18]. A recent study documented that single CB unit dominance after double CBT is concomitant with the presence of a $CD8^+$ T cell response specific for the nonengrafted CB unit in the peripheral blood of transplantation recipients [12]. Moreover, an in vivo murine model of double CBT that correlates with clinical engraftment suggested that single-unit dominance is an in vivo phenomenon likely associated with a graft-versus-graft immune interaction [19]. However, the number of postthaw granulocyte-macrophage colony-forming units has been shown to be the only significant factor predicting engraftment of the predominating unit after double CBT [20].

In this context, we investigated an in vitro approach considered potentially suitable (and realistically applicable in a clinical setting) for predicting the predominant CB unit before transplantation based on the graft-versus-graft alloreactive potency. We evaluated the possibility that an immune-mediated mechanism is responsible for single CB unit predominance using a two-way mixed-lymphocyte culture (MLC) approach. We also evaluated the potential significance of the proliferation capacity of purified CB $CD34^+$ hematopoietic progenitors (HPs) in determining the single CB unit predominance.

MATERIALS AND METHODS

Experimental Design

Two in vitro culture approaches were optimized to evaluate both the cell-mediated alloantigen-specific graft-versus-graft immune response and the differential HP proliferation capacity of the CB units (Figure 1). We evaluated the graft-versus-graft immune response through primary and/or secondary two-way MLCs in eight pairs of unrelated CB units. All pairs were HLA mismatched; this was formally proven in three pairs of CB units tested in the two-way MLC for which HLA genomic typing was performed. Alloantigen-induced cytotoxic activity generated in the two-way MLC was evaluated against the target cells in phytohemagglutinin (PHA)-activated T lymphoblastoid cells (PHA-TLCs) and expanded CB-HPs, target cells derived from the two CB units assessed in the two-way MLC. We also evaluated the preferential proliferation capacity of each CB unit in the two-way MLC by analyzing microsatellite DNA sequences (chimerism analysis).

To evaluate the differential HP potency of CB units, we assessed three of the eight pairs of unrelated HLA-mismatched CB units by expanding in mixed cultures the purified CB CD34 HPs derived from the two CB units used for the two-way MLC. We evaluated the preferential HP proliferation rate of each CB unit in the mixed-culture expansion by chimerism analysis.

Collection of Human CB

Human CB from full-term newborns was collected after obtaining written informed consent from the mother. In detail, after delivery, the umbilical cord was clamped and disinfected, and cells were collected (with the placenta in utero) into sterile CB collection bags containing 29 mL of citrate-phosphate dextrose (Macopharma, Mouvaux, France) as an anticoagulant.

Freezing and Thawing of CB Cells

CB cells used for the ex vivo HP expansion were processed within 24 hours of collection. The CB samples used in MLC experiments were cryopreserved following our standard procedure (vol:vol) in 10% DMSO (Cryoserv; Edward Lifesciences, Irvine CA) and then thawed according to the method of Rubinstein et al [21], as described previously [22]. After cryopreservation and thawing, CB cell viability was 89.6% ± 11.08%.

CB $CD34^+$ Cell Selection

Human CB mononuclear cells (CBMCs) were isolated by centrifugation after 1:3 (vol:vol) dilution with phenol red-free RPMI 1640 (Sigma-Aldrich, Schnelldorf, Germany) and 2 mM EDTA (Sigma-Aldrich) on a density gradient (Lympholyte-H, 1.077 g/mL; Cedarlane, Ontario, Canada) at 1,600 rpm for 30 minutes at room temperature. The low-density cell fraction was collected and washed twice with PBS (Euroclone, Milan, Italy), 2 mM EDTA and 0.5% human serum albumin (Kedrion, Castelvecchio Pascoli Barga, Italy) at 1,400 rpm for 10 min and at 800 rpm for 10 min. Approximately 5×10^6 CBMCs were used for the two-way MLCs; the remaining CBMCs were incubated for 30 minutes with microbead-conjugated anti-CD34 mAb (100 μL/10^8 cells;

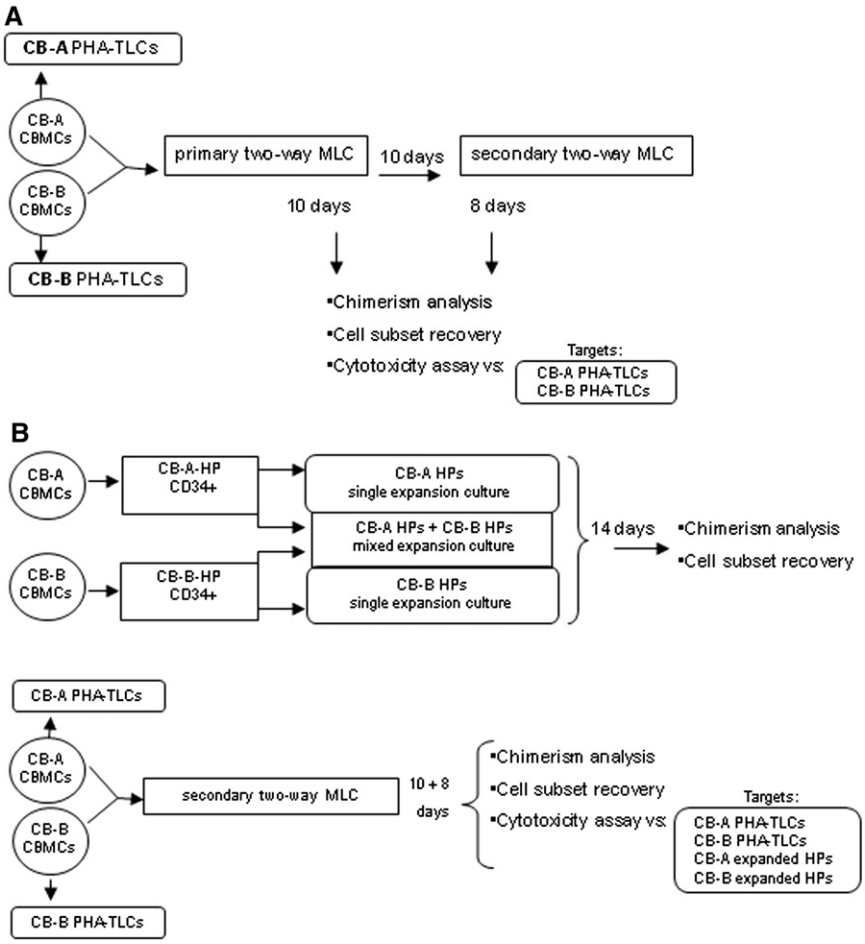

Figure 1. Experimental design scheme. CBMCs were obtained from each CB unit pair (CB-A and CB-B). (A) In experiments 1-5, a portion of CBMCs from each CB unit were used to obtain PHA-TLCs, and the remaining portion was used to carry out primary and secondary two-way MLCs. Effector cells harvested from MLCs were tested for DNA chimerism, cell subset recovery, and alloantigen-specific cytotoxic activity toward both CB-A and CB-B PHA-TLCs. (B) In experiments 6-8, a portion of CBMCs from each CB unit was used to select $CD34^+$ cells and perform single and mixed CB-HP expansion cultures and to obtain PHA-TLCs and carry out a secondary two-way MLC. Effector cells harvested from MLCs were tested for DNA chimerism, cell subset recovery, and alloantigen-specific cytotoxic activity toward both PHA-TLCs and expanded CB-HPs, and single and mixed CB-HP expansion cultures were tested for DNA chimerism and cell subset recovery.

Miltenyi Biotec, Gladbach, Germany). CB $CD34^+$ cell selection was then performed following the manufacturer's instructions. The purity of selected CB $CD34^+$ cells was evaluated by flow cytometry.

In Vitro Expansion of CB $CD34^+$ Cells

CB $CD34^+$ cells were cultured for 2 weeks in serum-free medium (CellGro SCGM; CellGenix, Freiburg, Germany) with IL-6 (10 ng/mL; PeproTech EC, London, UK), stem cell factor (50 ng/mL; Endogen Pierce, Rockford, IL), Flt-3 ligand (50 ng/mL; Endogen Pierce), thrombopoietin (10 ng/mL; PeproTech) and 10% heat-inactivated AB allogeneic plasma in cell culture chambers (Opticell; BioCrystal, Westerville, OH) at 37°C and 5% CO_2 in humidified air, as described previously [23]. The cytokines were added at the onset of the culture and replaced twice each week. $CD34^+$ cells obtained from two HLA-mismatched CB units were expanded in single culture at a concentration of 5×10^3 cells/mL and in mixed-expansion culture at a concentration of 2.5×10^3 cells/mL for each sample (final concentration, 5×10^3 cells/mL). After in vitro single culture or mixed- expansion culture procedures, CB-HPs were harvested, manually counted, and characterized by flow cytometry.

MLCs and Cytotoxicity Assays

MLCs were carried out with CBMCs from eight pairs of unrelated CB units (CB-A, CB-B) or peripheral blood mononuclear cells (PBMCs) from five pairs (PB-A, PB-B) of unrelated adult volunteers as controls. Primary two-way MLC was performed as described previously [24], by incubating a mixture of 5×10^4 CBMCs/microwell from each of the two units of the pair. Recombinant IL-2 (5 U/mL, Proleukin; Chiron, Emeryville, CA) was added to each well on days +3 and +6. Secondary two-way MLC was performed as described previously [24,25], by adding 2.5×10^4

irradiated (3,000 cGy) CBMCs from each of the two subjects of the pair (stimulators) directly to the same microwells of primary MLC effectors after 10 days of primary two-way MLC, followed by recombinant IL-2 (5 U/mL) on day +3. Primary and secondary two-way MLCs were incubated for 10 and 8 days, respectively and then tested in the cytotoxicity assay. The same MLC procedures were carried out with PBMCs from healthy unrelated adult individuals. T and NK lymphocyte subset propagation was evaluated by counting the numbers of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells/mL of culture, as well as the number of $CD3/CD56^+$ NK cells/mL of culture, recovered after both primary and secondary two-way MLC and comparing those values with the initial numbers (recorded on day 0). In addition, for each two-way MLC experiment, one-way MLC, using CBMCs or PBMCs from one of the two subjects of the pair as responder cells and irradiated (3,000 cGy) CBMCs or PBMCs of the other subject as stimulator cells, was performed to evaluate the capacity of CBMCs or PBMCs to generate alloantigen-specific cytotoxic activity in the two-way MLC.

Alloantigen-specific cytotoxic activity was tested in 5-hour and 18-hour ^{51}Cr-release assays as described previously [24]. Results are expressed as lytic units (LU)/10^6 effector cells, with 1 LU defined as the number of effector cells required to induce 10% lysis of ^{51}Cr-labeled target cells (LU10). For some experiments, results are expressed as percentage of lysis of target cells [24]. ^{51}Cr-labeled target cells included PHA-TLCs, prepared as described previously [26] and CB-HPs (see Experimental Design). PHA-TLCs used as the target in cytotoxicity assays included >95% T lymphocytes and <5% monocytes, B or NK lymphocytes.

DNA Chimerism Analysis

DNA was isolated from CBMCs or PBMCs before in vitro culture, from effector cells recovered after two-way MLC, and from CB-HPs after mixed-expansion culture. DNA chimerism was evaluated by microsatellite analysis using a previously described polymerase chain reaction (PCR)-based assay [27]. An initial screening was carried out to identify an informative marker for genotype discrimination of the two CB units or PBMC samples of each analyzed pair. After screening, at least two of the most informative short tandem repeat loci for each pair were used for chimerism analysis.

Flow Cytometry Analysis

CB $CD34^+$-enriched cells and expanded CB-HPs were washed and stained for 20 minutes at room temperature in the dark with the following monoclonal antibodies: CD34-PE, CD45-FITC, and 7AAD viability dye (Stem-Kit Reagents, Beckman Coulter, Fullerton, CA). Cells labeled with CD45 FITC/isotypic control PE and 7AAD viability dye were used as negative controls. The cells were analyzed for the expression of the following surface markers: CD34-PE (BD Biosciences, San Diego, CA), CD19-FITC (BD Biosciences), CD61-FITC (BD Biosciences), CD56-PE (BD Biosciences), CD133-APC (Miltenyi Biotec), CD3-APC (Beckman Coulter), CD45 PC7 (Beckman Coulter), and CD33-APC (BD Biosciences). After staining, the cells were washed once with PBS containing 0.1% BSA. At least 100,000 events were acquired with a Cytomics FC500 flow cytometer (Beckman Coulter) and analyzed using the CXP Analysis software.

Lymphocyte subsets were evaluated using FITC, PE, PerCP, and PerCPCy5.5 mAbs specific for antigens CD45, CD3, CD4, CD8, and CD56 (BD Biosciences), with appropriate isotype-matched controls (BD Biosciences). Two-color or three-color cytometry by direct immunofluorescence with a FACSCalibur flow cytometer (BD Biosciences) was performed as described previously [28].

HLA Typing

Low-resolution molecular typing was used to characterize HLA-A, -B, and -DRB1 loci, whereas the HLA-C locus was typed using high-resolution PCR amplification with sequence-specific primers [29]. DNA was isolated from blood specimens using magnetic beads and the robotic station that combines a Hamilton liquid handling workstation with AGOWA chemicals (Hamilton Robotics, Reno, NV). HLA-C alleles were assigned using the sequence-specific primers included in Pel Freez (Brown Deer, WI) and Genovision (Vertriebsges, Austria) commercially available kits. The amplified products were visualized on 2% agarose gels and stained with 0.5 μg/mL of ethidium bromide using the E-Gel Precast Agarose Electrophoresis System (Invitrogen Life Technologies, Paisley, UK). This system essentially contains all of the elements needed to perform electrophoresis of DNA in a "ready-to-go" format.

RESULTS

Cytotoxic Activity Induced in Two-Way MLCs

In a first set of experiments, five primary and/or secondary two-way MLCs (experiments 1-5) were carried out, with CBMCs from five pairs of unrelated HLA-mismatched CB units tested for alloantigen-specific cytotoxic activity with PHA-TLCs as the target. Evaluation of the cytotoxic capacity directed against PHA-TLC targets, which were obtained from CBMCs from each of the two CB units assessed in the two-way MLC, revealed a dominant CB unit

Table 1. T and NK Lymphocyte Subsets, Chimerism Status, and Cytotoxic Activity after Two-Way MLCs with CBMCs

		T and NK Lymphocyte Subsets[a]				% DNA		Cytotoxicity versus PHA-TLC Targets[b]	
	Total Cells	T $CD3^+$	T $CD4^+$	T $CD8^+$	NK $CD3^{neg}$ $CD56^+$	CB-A	CB-B	CB-A PHA-TLC Target	CB-B PHA-TLC Target
Experiment 1: CB-1A + CB-1B									
Day 0[c]	500	275	110	35	45	50	50		
MLC I	350	280	105	171	49	40	60	1,111	0
MLC II	700	539	287	210	84	20	80	33	0
Experiment 2: CB-2A + CB-2B									
Day 0	500	200	165	50	35	50	50		
MLC I	220	99	ND	42	26	40	60	166	0
MLC II	110	96	45	36	7	20	80	333	0
Experiment 3: CB-3A + CB-3B									
Day 0	500	315	225	65	120	50	50		
MLC I	ND	ND	ND	ND	ND	ND	ND	ND	ND
MLC II	280	224	165	28	8	10	90	166	0
Experiment 4: CB-4A + CB-4B									
Day 0	500	355	335	60	20	50	50		
MLC I	800	536	400	104	80	40	60	0	0
MLC II	140	70	62	11	14	30	70	0	0
Experiment 5: CB-5A + CB-5B									
Day 0	500	350	100	85	55	50	50		
MLC I	200	176	90	100	10	50	50	4,170	1,111
MLC II	ND	ND	ND	ND	ND	ND	ND	ND	ND
Experiment 6: CB-6A + CB-6B									
Day 0	500	385	270	60	40	50	50		
MLC II	160	157	138	18	2	90	10	0	86
Experiment 7: CB-7A + CB-7B									
Day 0	500	420	395	65	25	50	50		
MLC II	200	168	ND	34	4	30	70	4,761	0
Experiment 8: CB-8A + CB-8B									
Day 0	500	370	ND	70	30	50	50		
MLC II	260	159	120	55	31	40	60	66	133

ND indicates not determined.

Primary (I) and secondary (II) two-way MLCs, performed with CBMCs from 8 pairs (CB-A, CB-B) of unrelated HLA mismatched CB units, are reported.

[a]Results expressed as cell number $\times$ 10^3/mL.

[b]Results are expressed as lytic units/10^6 effector cells.

[c]Day 0 refers to CBMC-A and CBMC-B culture mix at the beginning of the culture.

in three of the five experiments (Table 1). In particular, in experiments 1, 2, and 3, only one of the two PHA-TLC targets was lysed (see representative experiment 2 in Figure 2A), whereas in experiment 5, both targets were lysed, even though a predominant lysis of one of the targets was observed (Figure 2B). Neither of the two CB units used in experiment 4 showed detectable cytotoxic activity. Results obtained from one-way MLC, performed as a control for the respective two-way MLC, showed that CB units 1B (experiment 1), 2A and 2B (experiment 2), 3B (experiment 3), and 5A and 5B (experiment 5) had the capacity to lyse allogeneic stimulator PHA-TLCs used in the MLC, whereas the remaining CB units did not have this capability (data not shown). The two-way MLC cytotoxicity data were also compared with data from experiments using PBMCs from five pairs of healthy, unrelated adult volunteers (Table 2). In this case, evaluation of cytotoxic activity against PHA-TLC targets showed dominance for one of the two adult donors in four out of five experiments, whereas in one experiment, both targets were consistently lysed, although a predominant lysis of one of the two targets was observed (see the representative experiments shown in Figure 2C and D). Control one-way MLCs documented a lytic capacity, directed toward the allogeneic stimulators used in the MLC, of PBMCs from all adult donors (data not shown).

CBMCs displayed slow killing kinetics in the cytotoxicity assays, with significant levels of lysis ($\geq$10% specific lysis of the target) detectable only in the 18-hour ^{51}Cr-release assay. Moreover, a suppressive effect was detected in the majority of experiments with CBMCs. Figure 3A shows how CB effector cells usually exhibited greater cytotoxic activity at low effector:target (E:T) ratios. This observation suggests the presence of suppressor cells within bulk cultures, the activity of which is overrun by cytotoxic cells at low E:T ratios. Compared with CBMCs, PBMCs exhibited more rapid killing kinetics in the cytotoxicity assay, with measurable levels of lysis already detectable in the 5-hour ^{51}Cr-release assay; no suppressive effect was observed. A representative experiment is shown in Figure 3B.

Chimerism analysis, performed on cells recovered at the end of each two-way MLC, showed a greater

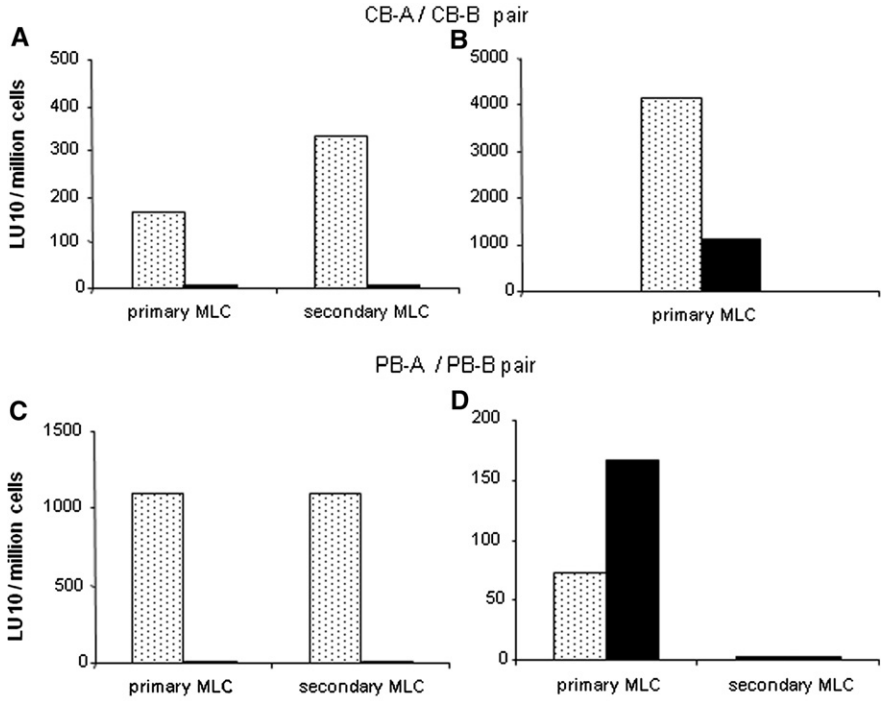

Figure 2. Alloantigen-specific cytotoxic activity generated in primary and secondary two-way MLCs. Cytotoxic activity was measured toward PHA-TLC targets obtained from CBMCs (A and B) or PBMCs (C and D) for each of the two subjects assessed in the two-way MLC. (A) Experiment 2: CB-2A/CB-2B pair. (B) Experiment 5: CB-5A/CB-5B pair, primary MLC. Results from the secondary two-way MLC are not available because of the very low cell recovery after primary culture that precluded the possibility of carrying out the assay. (C) Experiment 1: PB-1A/PB-1B pair. (D) Experiment 3: PB-3A/PB-3B pair. Results are expressed as LU10/million effector cells. Dotted columns represent CB-A and PB-A targets; black columns, CB-B and PB-B targets.

DNA percentage for one of the two CB units in four out of five experiments (Table 1). In experiments 1-3, the prevalent CB unit in terms of DNA percentage was also dominant in terms of alloantigen-directed cytotoxic activity toward PHA-TLCs. The higher DNA percentage for one of the two units in experiment 4, in which no significant cytotoxic activity was measured, conceivably can be explained by a greater alloantigen-induced proliferation capacity of the CB-4B unit compared with the CB-4A unit. Comparable data were observed when chimerism analysis was performed on cells recovered at the end of each primary and/or secondary control two-way MLC performed with PBMCs (Table 2).

Cell recovery and the lymphocyte subset distribution of two-way MLC effectors are reported in Table 1 for CB units and in Table 2 for adult controls. In the majority of both the CBMC and adult PBMC experiments, the number of effector cells recovered from secondary two-way MLC was lower than the number of input cells at day 0. These data do not agree with the findings of the respective secondary one-way MLC, in which effector cell propagation was 1.5- to two-fold greater than the number of input cells in CBMCs and two- to 2.5-fold greater than that in adult PBMCs (data not shown). This observation suggests that during the early phase of the two-way MLC, activated cytotoxic cells from each of the two units of the pair killed cells from the other unit. As the culture progressed, the unit with the prevalent alloantigen-specific cytotoxic capacity overgrew the other unit. In terms of effector cell phenotype, our data suggest that $CD8^+$ cells, a population believed to include mostly cytotoxic T lymphocytes, were preferentially activated in two-way MLC carried out with adult PBMCs (Table 2), whereas propagation of the same subset was observed only infrequently when evaluating CB effector cells in two-way MLC (Table 1). This observation could help explain the lower alloantigen-specific cytotoxic potential developed in two-way MLC by CBMCs compared with adult PBMCs.

In a second set of three CBMC experiments, effector cells recovered from secondary two-way MLC were tested for cytotoxic capacity not only against PHA-TLCs, but also against CB-HP targets. In all three experiments, CB-HPs from both CB units were lysed. Nonetheless, we observed a predominant cytotoxic capacity toward CB-HPs of one of the two CB units (Figure 4A-C). Simultaneous evaluation of the cytotoxic activity toward PHA-TLC and HP target cells showed concordance in the cytotoxic prevalence for both CB-HP and PHA-TLC targets in two of the three experiments (experiments 6 and 8; Figure 4A and C). In experiment 7, we observed a predominant lysis of CB-7B-HP targets and a dominant lysis of CB-7A PHA-TLC (Figure 4B). Chimerism analysis performed in cells recovered at the end of each two-way MLC revealed a higher percentage of

Table 2. T and NK Lymphocyte Subsets, Chimerism Status, and Cytotoxic Activity after 2-Way MLCs with PBMCs

		T and NK Lymphocyte Subsets[a]				% DNA		Cytotoxicity versus PHA-TLC Targets[b]	
	Total Cells	T $CD3^+$	T $CD4^+$	T $CD8^+$	NK $CD3^{neg}$ $CD56^+$	PB-A	PB-B	PB-A PHA-TLC Target	PB-B PHA-TLC Target
Experiment 1: PB-1A + PB-1B									
Day 0[c]	500	380	210	115	45	50	50		
MLC I	420	319	ND	281	50	40	60	1,111	0
MLC II	400	332	ND	316	16	30	70	1,111	0
Experiment 2: PB-2A + PB-2B									
Day 0	500	385	210	95	60	50	50		
MLC I	400	348	ND	276	28	30	70	111	8
MLC II	340	299	ND	282	3	10	90	333	0
Experiment 3: PB-3A + PB-3B									
Day 0	500	350	220	95	80	50	50		
MLC I	380	228	ND	87	137	50	50	74	166
MLC II	600	540	ND	222	24	50	50	0	0
Experiment 4: PB-4A + PB-4B									
Day 0	500	375	220	115	40	50	50		
MLC I	ND	ND	ND	ND	ND	ND	ND	ND	ND
MLC II	600	360	174	186	180	90	10	3	28
Experiment 5: PB-5A + PB-5B									
Day 0	500	325	135	110	70	50	50		
MLC I	ND	ND	ND	ND	ND	ND	ND	ND	ND
MLC II	550	451	198	286	77	10	90	476	0

ND indicates not determined.

Primary (I) and secondary (II) two-way MLCs performed with PBMCs from five pairs (PB-A, PB-B) of unrelated HLA-mismatched adult healthy donors are reported.

[a]Results expressed as cell number $\times$ 10^3/mL.

[b]Results expressed as lytic units/10^6 effector cells.

[c]Day 0 refers to PBMC-A and PBMC-B culture mix at the beginning of the culture.

DNA in one of the two CB units in all three experiments (Table 1). In experiments 6 and 7, the prevalent CB unit in terms of DNA percentage was also dominant in terms of alloantigen-directed cytotoxic activity toward PHA-TLC targets, whereas in experiment 8, this type of concordance was not observed.

In these latter experiments, we also attempted to evaluate the hypothesis that unidirectional NK alloreactivity [30-32] contributes to the predominance of one of the two CB units. Potential NK alloreactivity of each CB unit toward the other unit of the pair was estimated on the basis of killer immunoglobulin-like receptor (KIR) ligand expression, according to the "missing self-recognition" model (Table 3) [31,33]. In experiments 7 and 8, the pattern of unidirectional potential NK alloreactivity was concordant with the identification of the dominant CB unit in terms of alloantigen-directed cytotoxic activity toward PHA-TLC targets (Figure 4). We could not evaluate the hypothesis that NK alloreactivity contributes to CB unit dominance in experiment 6, because each CB unit of the pair was potentially NK alloreactive toward the other unit.

Proliferation and Phenotype of CB-HPs in Mixed-Expansion Culture

In experiments 6-8, we evaluated the proliferation capacity of CB-HPs in mixed-expansion culture by culturing CB $CD34^+$ cells isolated from the same CB units assessed in two-way MLC. The results, reported in Table 4, show that the median purity of CB $CD34^+$ cells after immune selection at day 0 was 85% (range, 61%-94%), whereas the median fold expansions of total nucleated cells (TNCs) and $CD34^+$ cells in single- expansion culture were 261 (range, 119-349) and 19.4 (range, 9.5-33.4), respectively. The expansion capacity of CB-HPs in mixed-expansion culture was quite variable compared with that of the same CB-HPs of the pair in single-expansion culture. A prevalence of one CB unit over the other in terms of DNA percentage was observed in the three experiments with HP mixed-expansion culture. In experiment 6, this observation could be explained by the presence at day 0 of a higher percentage of $CD34^+$ cells in the prevalent CB unit (CB-6B) compared with the other unit (CB-6A) at day 0. However, this explanation does not hold true for experiments 7 and 8, in which the CD34 percentage at day 0 was identical. It is possible that one of the CB units in experiments 7 and 8 had stronger stem cell potency. In accordance with this hypothesis, we observed that the prevalent CB units in terms of DNA percentage also exhibited a greater capacity for $CD34^+$ cell expansion (Table 4). Of note, the prevalent CB unit, in terms of DNA percentage, identified in mixed-expansion cultures of CB-HPs did not coincide with the prevalent CB unit, in terms of DNA percentage, detected in two-way MLCs carried out with CBMCs from the same pairs of CB units (experiments 6-8; Tables 1 and 4).

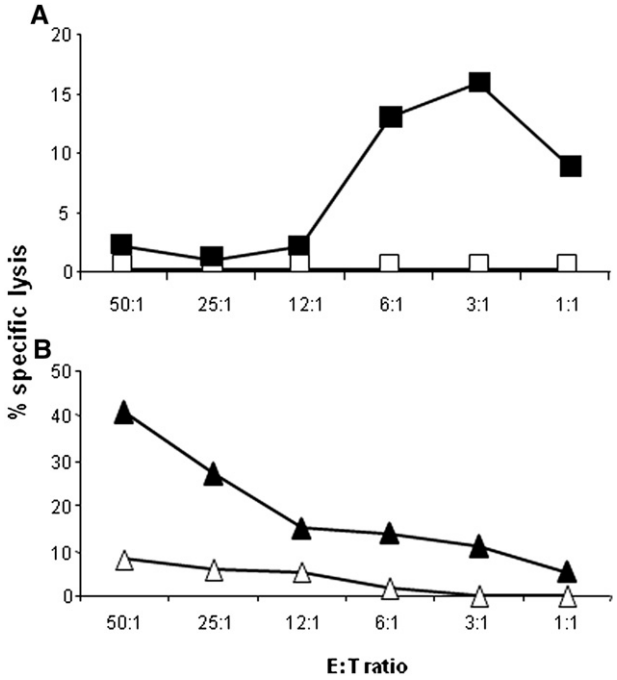

Figure 3. Alloantigen-specific cytotoxic activity of secondary two-way MLCs at different E:T ratios. Cytotoxic activity was measured toward PHA-TLC targets obtained from CBMCs (A) or PBMCs (B) for each of the two subjects assessed in the two-way MLC. (A) Experiment 3: CB-3A/CB-3B pair. Black square, CB-3A PHA-TLC target; empty square, CB-3B PHA-TLC target. (B) Experiment 2: PB-2A/PB-2B pair. Black triangle, PB-2A PHA-TLC target; empty triangle, PB-2B PHA-TLC target. Results are expressed as percent-specific lysis measured in an 18-hour (CBMCs) or a 5-hour (PBMCs) ^{51}Cr-release assay.

The median percentage of $CD34^+$ cells on day 14 of in vitro expansion was 7% (range, 5%-9%). Phenotypic characterization of the $CD34^+$ cell subpopulation revealed 71% (range, 70%-87%) $CD133^+$ cells, 1.1% (range, 1%-1.2%) $CD38^{neg}CD33^{neg}$ cells, and 15% (range, 10%-23%) $CD61^+$ cells, demonstrating both the maintenance of a more immature stem cell compartment and the presence of some already committed megakaryocytic progenitors. The remaining CB-$CD34^{neg}$ cells exhibited mainly a myeloid phenotype ($CD33^+$). The proportion of contaminating lymphocytes was <1%, including $CD3^+$ T lymphocytes (median, 0.3%; range, 0.19%-0.3%), $CD19^+$ B-lymphocytes (median, 0.06%; range, 0-0.08%), and $CD56^+CD16^+$ NK-lymphocytes (median, 0.1%; range, 0.08%-0.5%). Moreover, no difference in immune phenotype between the CB-HPs expanded in single culture and mixed culture was observed.

DISCUSSION

In the context of double CBT, several mechanisms have been investigated to explain the biological interactions between the two grafted CB units that cause the phenomenon of CB unit dominance, but a consensus has yet to be reached [4,13,19,20,34]. In this article, we describe a putative role for the two-way MLC as an indicator of the dominant CB unit after double CBT. Moreover, we show that the CB unit endowed with the strongest HP potency in terms of $CD34^+$ proliferation capacity exhibits a greater capacity for extensive growth of HP cells compared with its competitor in the HP mixed-expansion culture.

In agreement with the results of seminal studies that investigated fetal-maternal immune interactions [35-37], our findings indicate that CBMCs have weaker alloantigen-specific cytotoxic potential compared with PBMCs from healthy adults and may exert immunosuppressive activity. In particular, it was previously shown that several CB T lymphocyte subsets and CB monocytes may contribute to the alloantigen-directed immunosuppressive effect [35,37]. Nevertheless, despite the weak and variable alloantigen-specific cytotoxic potential exhibited by CBMCs, we observed an immune-mediated dominance for one of the two CB units in the majority of experiments.

Cytotoxic activity generated in two-way MLC was directed toward both PHA-TLCs and in vitro expanded CB-HPs. However, the latter were apparently more susceptible to lysis mediated by effector cells generated in two-way MLC. Indeed, CB-HPs from both CB units of a pair were lysed, even though a prevalent cytotoxic capacity toward CB-HPs from one of the two CB units was seen in all experiments. In contrast, one of the two CB units was strongly dominant in the majority of experiments when PHA-TLCs, a cell population including mainly activated T lymphocytes, served as the target cells.

In consideration of the observed patterns of cytotoxic activity mediated by CB effector cells emerging in two-way MLC, we hypothesize that the predominant CB unit in the setting of double CBT is the unit able to develop a prevalent cytotoxic activity directed against activated lymphocytes of the other CB unit, thus preventing them from exerting alloantigen-specific cytotoxic potential against both activated lymphocytes and HPs of the dominant unit. The time needed for the predominant CB unit to overcome the other unit in vivo could depend on the degree of reciprocal alloantigen-specific cytotoxic potency of the two CB units. Our preliminary data also suggest that the potential NK alloreactivity of one CB unit might contribute to the CB unit dominance in the double CBT setting [30-32].

An advantage of our in vitro model is its ability to simultaneously evaluate the proliferation rate of CB-HPs derived from both single and double CB cultures. In this system, the CB unit with the greater $CD34^+$ cell expansion rate in single culture was also the prevalent unit in the mixed-expansion culture. The

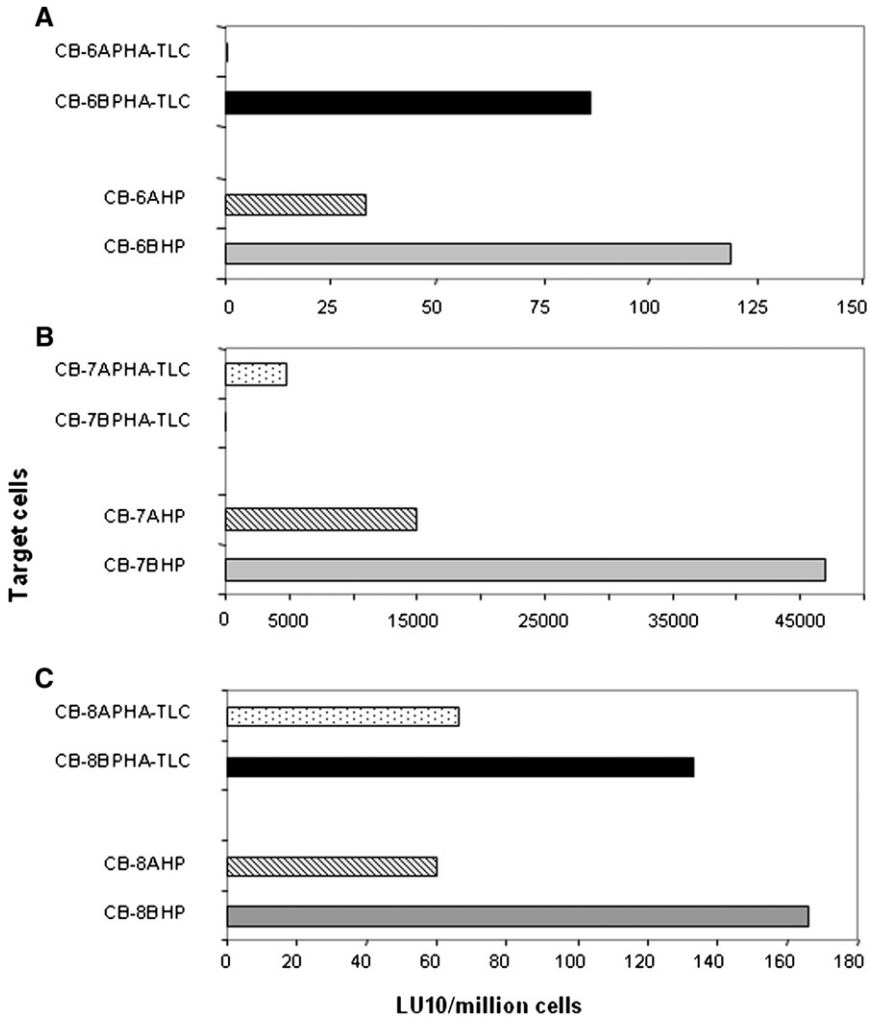

Figure 4. Alloantigen-specific cytotoxic activity generated in secondary two-way MLCs. Cytotoxic activity was measured toward both PHA-TLC and expanded CB-HP targets obtained from CBMCs for each of the two subjects used in the two-way MLC. (A) Experiment 6: CB-6A/CB-6B pair. (B) Experiment 7: CB-7A/CB-7B pair. (C) Experiment 8: CB-8A/CB-8B pair. Results are expressed as LU10/million effector cells. Dotted columns represent CB-A PHA-TLC targets; black columns, CB-B PHA-TLC targets; striped columns, CB-A expanded HP targets; gray columns, CB-B expanded HP targets.

expansion rates obtained in the single-expansion cultures were not predictive of those obtained in the coculture system. Several mechanisms potentially could explain these results, including competition for the administered growth factors due to a difference in receptor number or activation state, inhibiting or stimulating paracrine mechanisms and cell–cell contact-mediated effects. Our findings confirm recent reports indicating that in the clinical setting of double CBT, differential stem cell dose and potency may contribute to the CB unit dominance [34,38]. It is conceivable that the CB unit with the strongest HP potency

Table 3. HLA Typing of CB Unit Pairs Used for Experiments 6-8

	HLA-A	HLA-B	HLA-C	HLA-DRB1	Potential NK Alloreactivity (Missing Self)
Experiment 6: CB-6A + CB-6B					
CB-6A	23, 31	35, 40	0304[a], 1203[a]	1001, 14	CB-6A versus CB-6B (cw3)
CB-6B	03, 68	35, 3502	0401[b], 0401[b]	07, 15	CB-6B versus CB-6A (cw4)
Experiment 7: CB-7A + CB-7B					
CB-7A	03, 24	13[c], 51[c]	0202[b], 0602[b]	07, 07	
CB-7B	02, 03	50, 1517[c]	0602[b], 0701[a]	07, 13	CB-7B versus CB-7A (cw3)
Experiment 8: CB-8A + CB-8B					
CB-8A	02, 03	13[c], 40	0304[a], 0602[b]	01, 15	CB-8A versus CB-8B (cw4)
CB-8B	02, 68	44[c], 55	0303[a], 160101[a]	07, 14	

[a]Cw3 group.
[b]CW4 group.
[c]Bw4 group.

Table 4. Fold Expansion of TNC and CD34$^+$ Cells and Chimerism Status after Mixed-Expansion Culture of Purified CB CD34$^+$ HPs

	% CD34$^+$ Cells on Day 0	Fold Expansion TNC	Fold Expansion CD34$^+$ Cells	% DNA CB-A/CB-B
Experiment 6				
CB-6A	61	119	29	ND
CB-6B	93	273	33, 4	ND
CB-6A + CB-6B	ND	220	23, 4	30/70
Experiment 7				
CB-7A	76	349	16, 5	ND
CB-7B	76	269	9, 5	ND
CB-7A + CB-7B	ND	258	6, 2	80/20
Experiment 8				
CB-8A	94	239	22, 3	ND
CB-8B	94	253	15, 5	ND
CB-8A + CB-8B	ND	225	28, 7	70/30

TNC indicates total nucleated cells; ND, not determined.
Fold expansion refers to the number of cells recovered at the end of the expansion culture with respect to the number of input cells at day 0. The percentage of CD34$^+$ cells at the beginning of mixed-expansion culture of purified CB CD34$^+$ HPs derived from the two subjects used for the two-way MLC are reported; data achieved from single CB unit expansion culture are also reported.

could prevail in cases of absent immune dominance in terms of graft-versus-graft alloreactivity.

Although alloantigen-reactive lymphocyte activation and CB-HP expansion conceivably are independent phenomena, it is possible that a discordant dominance between the two CB units, in terms of stem cell potency and graft-versus-graft alloreactivity, could be detrimental in the setting of double CBT, for instance, when the dominant CB unit in terms of alloreactive capacity eradicates the CB unit able to support a more efficient hematopoietic engraftment. Moreover, as emphasized by Gutman et al [12], the selection of a potentially losing CB unit that shares host antigens not present in the dominant CB unit (ie, hematopoiesis-restricted minor histocompatibility antigens [mHAgs] [39] or noninherited maternal antigens [40]) is considered of paramount importance to further enhance the graft-versus-leukemia effect of double CBT [9,10] without increasing the risk of severe GHVD.

Another advantage of our in vitro model is that it allows evaluation of the interaction between the two CB units in a nonperturbed system devoid of the immunologic influences of the recipient. However, it is noteworthy that in the clinical setting of double CBT, major histocompatibility complex (MHC) antigens and/or mHAgs expressed by the recipient are expected to contribute to the activation of immune cells of both CB units. Therefore, in the clinical setting, this in vitro model might be adapted by adding irradiated recipient PBMCs to also evaluate the contribution of the MHC antigens and/or mHAgs on activation of the immune cells present in CB units.

In conclusion, the results of our in vitro study confirm previously reported data [12-19] supporting the key importance of graft-versus-graft cell-mediated alloreactivity as the principal mechanism promoting the engraftment of a single CB unit in patients undergoing double CBT. The novel information provided by our study is that two-way MLC might be efficiently applied in the evaluation of the potential graft-versus-graft alloreactivity for predicting the dominant CB unit before double CBT. We have set out an in vitro experimental strategy that is now ready for validation in a clinical setting to evaluate the predictive ability of this approach.

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