Donor/recipient mixed chimerism does not predict graft failure in children with β-thalassemia given an allogeneic cord blood transplant from an HLA-identical sibling

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ABSTRACT

Background
Donor/recipient mixed chimerism has been reported to be associated with an increased risk of graft failure in patients with β-thalassemia given a bone marrow transplant. We investigated the relationship between the degree of mixed chimerism over time and clinical outcome of children undergoing cord blood transplantation for β-thalassemia.

Design and Methods
Twenty-seven consecutive children given a cord blood transplant from a related donor were analyzed by short tandem repeat polymerase chain reaction and their chimerism results were compared with those of 79 consecutive patients who received a bone marrow transplant from either a relative (RD-BMT, n=42) or an unrelated donor (UD-BMT, n=37). Cord blood and bone marrow recipients received comparable preparative regimens.

Results
All cord blood recipients engrafted and displayed mixed chimerism early after transplantation; 13/27 converted to full donor chimerism over time, while 14 maintained stable mixed chimerism; all patients are alive and transfusion-independent. Twenty-four of the 79 bone marrow-recipients (12 UD- and 12 RD-BMT) exhibited full donor chimerism at all time points examined, 4/79 (2 UD- and 2 RD-BMT) did not engraft and 51/79 (23 UD- and 28 RD-BMT) displayed mixed chimerism at the time of hematologic reconstitution. Forty of 51 bone marrow recipients with mixed chimerism converted to full donor chimerism (17 UD- and 23 RD-BMT), 3/51 maintained stable mixed chimerism (1 UD- and 2 RD-BMT), while 8/51 (5 UD- and 3 RD-BMT) progressively lost the graft, and became transfusion-dependent again.

Conclusions
Mixed chimerism is a frequent event and does not predict the occurrence of graft failure in children with β-thalassemia given a cord blood transplant from a relative.

Key words: cord blood transplantation, β-thalassemia, donor/recipient mixed chimerism, MC, graft failure, tolerance.

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Introduction

A high proportion of patients affected by β-thalassemia major can be cured by allogeneic hematopoietic stem cell (HSC) transplantation, a procedure usually performed using bone marrow (BM) as the source of hematopoietic stem cells, mainly from a related (RD)[1-3] or, less frequently, from an unrelated donor (UD).[4-6] In the last two decades, cord blood (CB) has progressively become an extensively used alternative source of hematopoietic stem cells for transplanting patients with either malignant or non-malignant hematologic diseases.[7,9] As compared to bone marrow transplantation (BMT), cord blood transplantation (CBT) offers significant advantages, including easy and safe cell collection, low risk of transmitting viral infections, immediate availability of hematopoietic stem cells, elimination of risk and discomfort to donors, as well as a reduced incidence and severity of both acute and chronic graft-versus-host disease (GvHD), this last advantage translating into the possibility of using unrelated donors HLA disparate with respect to the recipient.[10-15] In particular, in patients with β-thalassemia, RD-CBT has been reported to be associated with a low risk of both acute and chronic GvHD, leading to a decreased probability of fatal/life-threatening transplantation-related complications.[16,17]

Previously reported data indicate that the detection, at a certain time point, of hematopoietic and immune cells of host origin, commonly referred to as donor/recipient mixed chimerism, is not uncommon in patients undergoing BMT for β-thalassemia major and is associated with a low incidence of GvHD.[18-20] The majority of these patients with mixed chimerism convert to full donor chimerism weeks or months after BMT, some maintain stable mixed chimerism over time even after discontinuation of immunosuppressive therapy, while a not negligible number of patients develop secondary graft failure, in particular when increasing proportions of recipient cells are observed in serial monitoring.[18-20]

Few data are available on chimerism in β-thalassemia patients undergoing CBT. The Eurocord study[17] found that stable mixed chimerism was present in seven out of 36 children who underwent RD-CBT and did not experience either primary or secondary graft failure. Two more recent reports documented full donor engraftment in a few children undergoing UD-CBT for β-thalassemia.[21,22]

The aims of the present analysis were to study the proportion of children with β-thalassemia major experiencing either transient or long-lasting persistence of hematopoietic cells of host origin after RD-CBT, and to investigate the relationship between the evolution of mixed chimerism over time and clinical outcome. For a more meaningful comprehension of the phenomenon, results of RD-CBT recipients were compared with those observed in patients who underwent RD- or UD-BMT at the same Center, in the same period. In some patients with persistence of host cells after transplantation, chimerism analysis was performed also on specific cell subsets, in order to improve our understanding of the mechanisms involved in the development and maintenance of persistent mixed chimerism.

Design and Methods

Patients

We retrospectively studied 106 consecutive pediatric patients affected by β-thalassemia major who were transplanted at the Pediatric Hematology and Oncology Unit, Fondazione IRCCS Policlinico San Matteo (Pavia, Italy) between June 1998 and April 2006. One other patient, who had undergone RD-CBT, had to be excluded from the analysis, as the set of short tandem repeats (STR) loci used for chimerism analysis was not informative for genotype discrimination of the recipient from the donor. Consequently, in this patient chimerism could not be checked. This child is alive and thalassemia-free.

The study was formally approved by the Ethical Committee of the Fondazione IRCCS Policlinico San Matteo, Pavia, as required for all studies involving people, medical records and human tissues. Informed consent from patients or parents was obtained according to institutional guidelines.

Children received cord blood from an HLA-identical sibling (n=27), or bone marrow from an HLA-identical sibling (n=42) or an HLA-compatible unrelated volunteer (n=37). Details on the patients' and donors' characteristics are listed in Table 1. The median age at transplantation, patients' and donors' sex and donor/recipient gender combinations, did not differ between the three groups examined. Prior to transplantation all patients were assigned to one of three classes of risk according to the factors (namely presence of hepatomegaly, or portal fibrosis on liver biopsy; regularity of pre-transplantation iron chelation) proposed by Lucarelli *et al.*[23] Forty-two patients were assigned to class 1, 54 to class 2 and ten (all undergoing BMT) to class 3. The median number of nucleated cells infused/kg of recipient's body weight was one log less in patients undergoing CBT than in those undergoing BMT, as shown in Table 1.

In all patients undergoing either RD-CBT or RD-BMT, histocompatibility was determined by serology for HLA-A and -B antigens and by DNA typing for the DRB1 locus. For patients undergoing UD-BMT, donor/recipient alleles of the HLA-A, B, Cw, DRB1, and DQB1 loci were identified by polymerase chain reaction (PCR)-single strand polymorphism analysis and sequence-based typing. Amplification and sequencing of HLA class I and class II genes were performed as previously described.[24-26] Alleles were assigned according to DNA sequences.[27]

Transplantation regimen and graft-versus-host disease prophylaxis

Data regarding conditioning regimens and GvHD prophylaxis are given in Table 1. In the majority of patients (37 RD-BMT; 30 UD-BMT; 24 RD-CBT), the preparative regimen included busulfan (16 mg/kg over 4 days), thiotepa (10 mg/kg in two divided doses), and fludarabine (40 mg/m^2/day for 4 consecutive days).[4,16,28] Twelve patients (4 RD-BMT, 5 UD-BMT and 3 RD-CBT) received busulfan, thiotepa and cyclophos-

Table 1. Characteristics of the patients, donors and transplants.

	RD-BMT n=42	*UD-BMT n=37*	*RD-CBT n=27*	*p value*
Median age at transplantation, years (range)	9 (0.6 - 23)	8 (1 - 24)	6 (0.8 - 18)	N.S.
Patients' sex, male/female	21/21	23/14	17/10	N.S.
Donors' sex, male/female	21/21	18/19	16/11	N.S.
Donor -> recipient sex combination				
Female -> Male	8 (19%)	11 (30%)	8 (30%)	N.S.
Other	34 (81%)	26 (70%)	19 (70%)	
Pesaro class at time of transplantation				
Class 1	11 (26%)	15 (41%)	16 (59%)	N.S.
Class 2	26 (62%)	17 (46%)	11 (41%)	
Class 3	5 (12%)	5 (13%)	0 (0%)	
HLA-matching				
Identical	42 (100%)	37 (100%)	27 (100%)	N.S.
Class I disparity	0	0	0	
Class II disparity	0	0	0	
Median number of nucleated cells infused x10^8/kg of recipient body weight (range)	5 (2.1-7.7)	6.4 (2.2-15)	0.33 (0.15-0.6)	<0.005
Conditioning regimen				
Bu/Cy/TT	4 (10%)	5 (14%)	3 (11%)	N.S.
TT/Treo/Flu	1 (2%)	2 (5%)	0 (0%)	
Bu/Flu/TT	37 (88%)	30 (81%)	24 (89%)	
Use of ATG				
Yes	0	37 (100%)	0	<0.001
No	42 (100%)	0	27 (100%)	
GvHD prophylaxis				
CsA	13 (31%)	0	27 (100%)	<0.001
CsA+MTX	29 (69%)	37 (100%)	0	

Bu: busulfan; Cy: cyclophosphamide; TT: thiotepa; Treo: treosulfan; Flu: fludarabine; ATG: anti-thymocyte globulin; CsA: cyclosporine A; MTX: methotrexate.

phamide (60 mg/kg/day for 2 consecutive days); the remaining patients (1 RD-BMT, 2 UD-BMT) were given a novel preparative regimen containing thiotepa, treosulfan (14 g/m^2/day for 3 consecutive days) and fludarabine. Bone marrow or cord blood was infused 36 and 72 hours following the last dose of cyclophosphophamide and fludarabine, respectively.

Anti-thymocyte globulin (10 mg/kg on days -4, -3 and -2; Fresenius, Graefelfing, Germany) was used in all patients undergoing an UD-BMT. GvHD prophylaxis consisted of cyclosporine A alone in cord blood recipients and either cyclosporine A alone (13 RD-BMT) or in combination with methotrexate in patients receiving bone marrow (see also Table 1 for details). In all patients who did not reject the graft, cyclosporine A was discontinued in the absence of chronic GvHD within the first 12 months after the transplant.

In order to prevent any risk related to persistent cytopenia in patients with poor graft function, autologous bone marrow cells were harvested and cryopreserved, for eventual rescue, before transplantation for all patients undergoing RD-CBT or UD-BMT.

Cell samples and evaluation of chimerism

Peripheral blood and/or bone marrow/cord blood samples were collected from patients and donors before transplantation; peripheral blood samples from recipients were collected at the time of hematologic engraftment (i.e. the first of 3 consecutive days at which the absolute neutrophil count was above 0.5×10^9/L), then every week between 1 and 3 months and every month between 3 and 12 months after transplantation. Then, chimerism was checked at time of each subsequent clinical control.

In 13 patients included in this analysis (6 RD-CBT, 4 RD-BMT and 3 UD-BMT), cell subset separation was performed early (2-3 months) and late (7-8 months) after transplantation, dividing the same sample employed for chimerism analysis into two aliquots. CD4$^+$, CD8$^+$ and CD19$^+$ lymphocyte subsets were selected following the manufacturer's instructions by magnetic beads (Miltenyi Biotec, Auburn, CA, USA) coupled with the appropriate specific antibody. The cell subsets obtained by the separation procedure were analyzed by means of direct immunofluorescence on a FACScalibur flow cytometer (BD Biosciences, San José, CA, USA) and data calculated using CellQuest software (BD Biosciences). The purity of all samples was more than 96%.

Chimerism was evaluated by a PCR-based assay, analyzing selected polymorphic short tandem repeat (STR) loci, as previously described.[29]

Patients who experienced primary or secondary graft failure were not investigated further.

Definitions

Graft failure was defined as either the absence of hematopoietic reconstitution of donor origin on day +45 after the allograft (primary graft rejection) or as confirmed loss of donor cells after transient engraftment of donor-origin hematopoiesis, with a return to erythrocyte transfusion dependence (secondary graft rejection).

Individuals who exhibited more than a 99% donor profile by STR-PCR analysis at all times post-transplantation were referred to as having full donor chimerism. Mixed chimerism was defined as greater than 1% recipient DNA. Individuals who exhibited mixed chimerism were further classified according to the evolution of chimerism. In detail, improving mixed chimerism was defined as a continuous increase in the proportion of donor cells over at least a 6-month period. Stable mixed chimerism was defined as fluctuations in the percentage of recipient cells over time, without complete loss of donor cells, while worsening mixed chimerism was considered as the condition in which chimerism kinetics indicated a progressive increase in the proportion of recipient cells over at least a 6-month period.

Acute and chronic GvHD were graded according to the Seattle criteria.[30,31] Patients were considered to be evaluable for acute and chronic GvHD if they survived for at least 7 and 100 days after transplantation, respectively.

Data analysis and presentation

The reference date of the analysis is September 1, 2007.

Values are expressed as medians and ranges or as absolute numbers and percentages, as appropriate.

The χ^2 test was used to compare different distributions of categorical variables among groups, while Student's t test or the Mann-Whitney rank sum test was used to compare differences in mean or median values between groups, as appropriate.

Overall survival was defined as the probability of survival, irrespective of disease status, at any time point; patients alive at their last follow-up were censored, while only death was considered as an event. Thalassemia-free survival was defined as the probability of being alive and transfusion-independent at any time point; death and either primary or secondary graft failure were considered as events, while patients alive and thalassemia-free were censored at last follow-up. Both these probabilities were calculated using the Kaplan-Meier method. Comparisons between probabilities in different groups of patients were performed using the log-rank test. Graft failure was defined as the probability of either primary or secondary graft rejection at time *t*. Hematopoietic recovery and development of acute or chronic GvHD being events which compete with death, estimations of incidence of these events relied on the non-parametric estimator of cumulative incidence curves.[32,33] In contrast, transplant-related mortality was defined as the probability of dying at time *t*, due to causes related to the transplant. Transplant-related mortality was estimated from the cumulative incidence curve. All results are expressed as a probability or cumulative incidence (%) with a 95% confidence interval (95% CI).

p values >0.1 were considered not significant and are presented in the text as NS; *p* values <0.1 but ≥0.05 were considered not significant but are shown in detail in the text; *p* values <0.05 were considered statistically significant and are presented in detail. All analyses were performed with NCSS and PASS statistical systems (Number Cruncher Statistical Systems. Kaysville, Utah, USA).

Results

The results were analyzed when all patients enrolled in the study had reached a minimum follow-up of at least 15 months. The median follow-up time was 40 months (range, 15-89) in the RD-CBT group, 51 months (range, 15-110) in the RD-BMT group, and 48 months (range, 16-81) in the UD-BMT group (*p*=NS).

All patients given a cord blood transplant displayed mixed chimerism, with co-existence of donor and host hematopoietic cells, at the time of hematologic engraftment; 13/27 converted to full donor chimerism, eight within 6 months and five within 1 year after transplantation, while the remaining 14 children have stable mixed chimerism after a median follow-up of 42 months (range, 15-76, see also Figure 1 for details). All these patients are free of any immunosuppressive treatment. No patient included in the RD-CBT group experienced primary or secondary graft failure (Figure 1, Table 2).

At the time of hematologic engraftment, mixed chimerism was detected in 28 out of 42 patients undergoing RD-BMT, while 12/42 exhibited full donor chimerism and two patients had primary graft failure

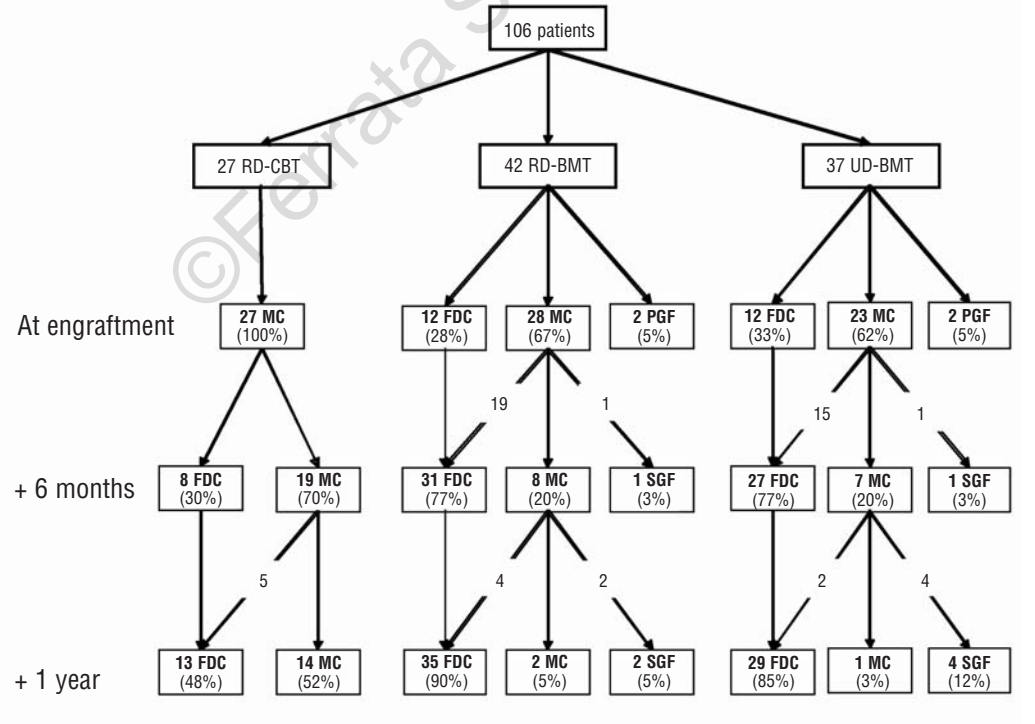

Figure 1. Evolution of chimerism status in the three groups of patients at engraftment and 6 and 12 months after transplantation. RD-CBT: related donor cord blood transplantation; RD-BMT: related donor bone marrow transplantation; UD-BMT: unrelated donor bone marrow transplantation; FDC: full donor chimerism; MC: mixed chimerism; PGF: primary graft failure; SGF: secondary graft failure.

Table 2. Clinical outcomes of the patients according to type of transplantation.

	Number of patients	Events	%	(95% CI)	p
Acute GvHD					
Overall cumulative incidence	106	10	9%	(5–17)	
RD-CBT	27	0	0%	–	<0.05
RD-BMT	42	3	7%	(2–21)	
UD-BMT	37	7	19%	(10–37)	
Chronic GvHD					
Overall cumulative incidence	99	8	8%	(4–16)	
RD-CBT	27	0	0%	–	<0.05
RD-BMT	40	2	5%	(1–19)	
UD-BMT	32	6	19%	(9–39)	
Graft failure/rejection					
Overall cumulative incidence	106	12	12%	(7–20)	
RD-CBT	27	0	0	–	<0.05
RD-BMT	42	5	12%	(5–28)	
UD-BMT	37	7	21%	(11–10)	
Transplantation-related mortality					
Overall cumulative incidence	106	5	5%	(2–12)	
RD-CBT	27	0	0%	–	N.S.
RD-BMT	42	2	5%	(1–19)	
UD-BMT	37	3	9%	(3–26)	
Survival					
Overall probability	106	5	95%	(91–99)	
RD-CBT	27	0	100%	–	N.S.
RD-BMT	42	2	95%	(89–100)	
UD-BMT	37	3	92%	(83–100)	
Thalassemia-free survival					
Overall probability	106	16	85%	(78–92)	
RD-CBT	27	0	100%	–	<0.05
RD-BMT	42	6	86%	(75–96)	
UD-BMT	37	10	72%	(58–87)	

Table 3. Univariate analysis for graft rejection.

	Number of patients	Events	Cumulative incidence	(95% CI)	p
Overall rejection incidence	106	12	12%	(7–20)	–
Patients' gender					
Male	61	8	13%	(7–25)	N.S.
Female	45	4	10%	(4–25)	
Donors' gender					
Male	55	8	15%	(8–28)	N.S.
Female	51	4	8%	(3–20)	
Donor -> recipient gender					
Female -> Male	27	4	15%	(6–37)	N.S.
Other combinations	79	8	11%	(6–21)	
Patients' age					
<4 years	23	5	23%	(11–50)	N.S.
4–8 years	31	3	10%	(3–30)	
8–13 years	26	2	8%	(2–30)	
≥13 years	26	2	8%	(2–30)	
Pesaro class					
1	43	3	8%	(3–23)	N.S.
2	53	7	14%	(7–27)	
3	10	2	21%	(6-73)	
Type of donor					
Related donor	69	5	7%	(3–17)	N.S.
Unrelated donor	37	7	21%	(11–40)	
Stem cell source					
Bone marrow	79	12	16%	(10–27)	< 0.05
Cord blood	27	0	0%		
Type of transplant					
RD-CBT	27	0	0	–	<0.05
RD-BMT	42	5	12%	(5–28)	
UD-BMT	37	7	21%	(11–40)	
Conditioning regimen					
Bu +TT + Cy	9	2	22%	(7–75)	N.S.
Bu + TT + Flu	94	9	10%	(5-19)	
TT + Treo + Flu	3	1	33%	(7–100)	
BM cell dose infused[a]					
$<5\times10^8$/kg	35	4	12%	(5–29)	N.S.
$\geq5\times10^8$/kg	44	8	19%	(10–36)	
CB cell dose infused[b]					
$<3.5\times10^7$/kg	14	0	0%	–	N.S.
$\geq3.5\times10^7$/kg	13	0	0%	–	
Use of ATG					
Yes	41	7	18%	(11 - 39)	0.09
No	65	5	9%	(2 - 16)	
GvHD prophylaxis:					
CsA	40	0	0%	–	<0.05
CsA + MTX	66	12	19%	(11 - 32)	
Chimerism status[c]					
Full donor chimerism	24	0	0%	–	< 0.001
Improving MC	51	0	0%	–	
Stable MC	19	0	0%	–	
Worsening MC	8	8	100%		

RD: related donor; UD: unrelated donor; CsA: cyclosporine A; MTX: methotrexate; Bu, busulfan; Flu, fludarabine; TT, thiotepa; ATG: anti-thymocyte globulin; MC: mixed chimerism; [a]Analysis performed only on the 79 patients who received a bone marrow transplant. [b]Analysis performed only on the 27 patients who received a cord blood transplant. [c]For the definition, see the text. The four patients who experienced primary rejection were excluded from the analysis.

(Figure 1). One of these two patients was successfully re-transplanted from the same donor and reached sustained full donor chimerism, while the other died of a brain hemorrhage. Twenty-three out of the 28 children who showed early mixed chimerism converted to full donor chimerism, 19 within 6 months and four within 1 year after transplantation; only two patients (with follow-ups of 62 and 31 months) maintained stable mixed chimerism. The remaining three RD-BMT recipients experienced a progressive increase of circulating host hematopoietic cells (i.e. worsening chimerism), and finally developed secondary graft failure 3, 9 and 10 months after transplantation. Two of them received a second allograft from the same bone marrow donor and have full donor chimerism after follow-ups of 65 and 36 months; the other one was not re-transplanted and remains transfusion-dependent.

In the UD-BMT group, 23 out of 37 patients displayed mixed chimerism at time of hematologic reconstitution; two patients had primary graft failure (both were rescued from trilineage aplasia after infusion of autologous marrow cells) and the remaining 12 exhibited early FDC and maintained this status at all time points they were examined. Seventeen out of the 23 children in whom recipient cells were detected in the first few months after the allograft showed improving mixed chimerism and converted to full donor chimerism, 15 within 6 months and two within 1 year after transplantation; one patient maintained stable mixed chimerism after a follow-up of 41 months. The remaining five patients showed increasing host chimerism (worsening mixed

Table 4. Characteristics of patients who maintained mixed chimerism 1 year after transplantation.

ID.N.	Age at transplant	Source of stem cells	Donor type	Conditioning regimen	GvHD prophylaxis	Nucleated cells infused/kg of recipient	Follow-up time (months)	Chimerism at last follow-up	Outcome and survival
01-4471	11	BM	RD	Bu/Flu/TT	CsA+MTX	$2.1x10^8$	62	95% donor cells	alive & well
99-2046	7	BM	RD	Bu/Flu/TT	CsA+MTX	$4x10^8$	31	40% donor cells	alive & well
03-3873	1	BM	UD	Bu/Flu/TT+ATG	CsA+MTX	$5.5x10^8$	41	10% donor cells	alive & well
99-2155	19	CB	RD	Bu/Flu/TT	CsA	$5x10^7$	76	50% donor cells	alive & well
04-5574	6	CB	RD	Bu/Flu/TT	CsA	$3.2x10^7$	27	90% donor cells	alive & well
03-2586	7	CB	RD	Bu/Flu/TT	CsA	$1.9x10^7$	33	85% donor cells	alive & well
00-3073	5	CB	RD	Bu/Flu/TT	CsA	$2.5x10^7$	72	90% donor cells	alive & well
00-2927	3	CB	RD	Bu/Flu/TT	CsA	$6x10^7$	69	90% donor cells	alive & well
04-6556	4	CB	RD	Bu/Flu/TT	CsA	$3.6x10^7$	20	80% donor cells	alive & well
99-2139	12	CB	RD	Bu/Flu/TT	CsA	$4x10^7$	61	90% donor cells	alive & well
97-1045	7	CB	RD	Bu/Cy/TT	CsA	$1.8x10^7$	24	60% donor cells	alive & well
01-4447	3	CB	RD	Bu/Cy/TT	CsA	$3.8x10^7$	62	90% donor cells	alive & well
00-3415	9	CB	RD	Bu/Flu/TT	CsA	$5.7x10^7$	66	90% donor cells	alive & well
02-1917	4	CB	RD	Bu/Flu/TT	CsA	$3.5x10^7$	39	90% donor cells	alive & well
06-419	2	CB	RD	Bu/Flu/TT	CsA	$2.2x10^7$	15	85% donor cells	alive & well
06-1065	5	CB	RD	Bu/Flu/TT	CsA	$0.8x10^7$	16	90% donor cells	alive & well
06-851	7	CB	RD	Bu/Flu/TT	CsA	$3.2x10^7$	15	90% donor cells	alive & well

BM: bone marrow; CB: cord blood; RD: related donor; UD: unrelated donor; CsA: cyclosporine A; MTX: methotrexate; Bu: busulfan; Flu: fludarabine; TT: thiotepa; ATG: anti-thymocyte globulin.

chimerism) and finally developed secondary graft failure, 5, 7, 8, 11 and 12 months after transplantation, with spontaneous recovery of normal leukocyte and platelet counts. Two of them subsequently received a second transplant from the same bone marrow donor, the first one maintaining full donor chimerism after a follow-up of 12 months, the second one showing mixed chimerism up to 4 months after transplantation which then converted to full donor chimerism (follow-up 22 months). Three patients, who for their parents' decision were not re-transplanted, are presently transfusion-dependent.

In patients who did not obtain hematopoietic reconstitution (i.e. those experiencing primary graft failure), hematopoietic cells resulted to be of host origin already at the first analysis of chimerism. Secondary graft failure occurred only in patients who underwent RD-BMT and UD-BMT with mixed chimerism at any time-point after transplantation (Table 2 and Figure 1), while none of the patients who had full donor chimerism, at any time after the allograft, subsequently experienced secondary graft failure. Secondary graft failure was always preceded by a progressive increase of the percentage of host cells. Table 3 shows all the evaluated variables potentially influencing the risk of either primary or secondary graft failure: stem cell source, type of transplant, GvHD prophylaxis and chimerism status were found to be significant in univariate analysis.

Data regarding the characteristics of the patients, donors and transplants, as well as chimerism analysis of the 17 patients maintaining mixed chimerism and transfusion independence 1 or more years after transplantation, are detailed in Table 4; at the last follow-up analysis, the percentage of donor hematopoietic cells ranged between 10% and 95%.

Donor/recipient chimerism within $CD4^+$, $CD8^+$ and $CD19^+$ cell subsets was assessed in six, four and three patients, undergoing RD-CBT, RD-BMT and UD-BMT,

Table 5. Donor/recipient mixed chimerism status in patients for whom cell subset analysis was performed.

Identity number	2-3 months after transplantation				7-8 months after transplantation			
	PBMC	CD4	CD8	CD19	PBMC	CD4	CD8	CD19
RD-CBT								
04-6556	70%D	50%D	30%D	95%D	75%D	60%D	40%D	95%D
04-5574	90%D	50%D	30%D	95%D	90%D	60%D	40%D	95%D
03-2586	85%D	60%D	40%D	95%D	85%D	60%D	40%D	95%D
06-419	80%D	40%D	20%D	90%D	85%D	50%D	30%D	95%D
06-851	70%D	50%D	40%D	90%D	80%D	50%D	50%D	95%D
97-1045	50%D	30%D	20%D	95%D	60%D	30%D	20%D	95%D
RD-BMT								
03-4850	95%D	80%D	80%D	95%D	95%D	80%D	80%D	95%D
01-4471	95%D	80%D	80%D	95%D	95%D	80%D	80%D	95%D
07-213	95%D	70%D	70%D	95%D	95%D	70%D	70%D	95%D
99-2046	70%D	20%D	20%D	70%D	40%D	10%D	10%D	60%D
UD-BMT								
02-5438	90%D	70%D	60%D	95%D	95%D	80%D	70%D	95%D
03-3873	80%D	60%D	50%D	90%D	50%D	30%D	20%D	80%D
02-2251	30%D	20%D	20%D	85%D	30%D	20%D	20%D	85%D

PBMC: peripheral blood mononuclear cells; D: donor.

respectively. Both 2-3 and 7-8 months after transplantation, marked enrichment in recipient cells was detected within the $CD4^+$ and $CD8^+$ T lymphocyte subsets; by contrast, the percentage of $CD19^+$ B cells of recipient origin was significantly lower, usually in the range of 5-10%, and never exceeded 40% (Table 5).

All patients with early or late full donor chimerism, as well as the 17 children who maintained stable mixed chimerism after discontinuation of any immunosuppressive treatment, were transfusion-independent, with hemoglobin levels ranging from 9.3 to 14.7 g/dL.

Results on the cumulative incidence of acute/chronic GvHD are reported in Table 2: none of the patients in

RD-CBT group experienced grade II-IV acute GvHD, which was, by contrast, diagnosed in three and seven patients transplanted with bone marrow cells from a relative or an unrelated volunteer, respectively ($p<0.05$). Chronic GvHD was diagnosed only in patients who received a bone marrow transplant (Table 2 for details). It is noteworthy that both acute and chronic GvHD occurred only in patients with full donor chimerism at the time of hematopoietic reconstitution.

Five out of 106 pediatric patients enrolled in this study (2 RD-BMT and 3 UD-BMT recipients) died of transplantation-related complications (Table 2). With the exception of the child who died of a brain hemorrhage after having rejected the allograft, the others had full donor chimerism at engraftment, had experienced grade II-IV acute GvHD and died due to acute GvHD (3 children) or chronic GvHD (1 child). Three of these five children were in class 3 of the Pesaro classification and one each in class 1 and 2. Notably, all patients in the RD-CBT group are alive and disease-free.

Discussion

The results of our study clearly demonstrate that sustained mixed donor/recipient chimerism of circulating mononuclear cells can be found in more than half of patients with β-thalassemia given a cord blood transplant from an HLA-identical relative and that this mixed chimerism is associated with a favorable transplantation outcome, as all the 27 patients we investigated are alive and transfusion independent, without having experienced either acute or chronic GvHD.

Previous studies had documented that chimeric hematopoiesis can often be observed during the early post-transplantation period in patients undergoing BMT for non-malignant disease.[18,20,34-36] However, in the previously published reports, mainly analyzing patients given a bone marrow transplant, a status of mixed chimerism was associated with an increased risk of rejection.[18,20,34-36] Our results confirm that a relevant proportion of patients given a bone marrow transplant and who display mixed chimerism at the time of hematopoietic engraftment, irrespectively of the type of donor employed, have a remarkable rate of secondary graft failure, as eight of the 75 (11%) who did not experience primary rejection had secondary loss of the graft. By contrast, all cord blood recipients experienced mixed chimerism at the time of hematopoietic engraftment, the majority of them retaining this chimerism status 15-76 months after transplantation, but none of them experienced graft failure and all remain transfusion-independent after discontinuation of cyclosporine A. Persistent mixed chimerism was also observed in a minority of bone marrow recipients, indicating that the relationship between stable mixed chimerism and a favorable transplantation outcome is not an exclusive peculiarity of CBT. Nonetheless, our data strongly suggest that CBT, more than BMT, promotes the development of a state of reciprocal tolerance between recipient and donor cells, protecting against the risk of graft failure. It is worth noting that, while early mixed chimerism is associated with an increased risk of graft failure in BMT, all patients still displaying mixed chimerism 1 year after the allograft maintained this condition at all subsequent time points, this finding providing support to the concept that mixed chimerism persisting for more 1 year after transplantation is not followed by graft failure. Even patients with percentages of donor cells as low as 10-30% remain disease-free, suggesting that enrichment of donor cells in the mature red blood cell compartment (i.e. a different donor/recipient distribution between erythroid and other hematopoietic lineages) occurs, making patients transfusion independent.[37] Indeed, studies in a mouse model, aimed at evaluating the effect of mixed chimerism on sickle-cell pathophysiology, also showed that a significant enrichment of erythrocyte over leukocyte chimerism occurred in these mice, because of the very marked survival advantage of donor over sickle red blood cells in the peripheral blood.[38]

In agreement with the results of previously reported studies,[35,36,39,40] we observed that mixed chimerism is associated with less GvHD, irrespectively of the type of donor and stem cell source employed. These data suggest that one of the biological mechanisms contributing to the lower incidence and severity of GvHD after CBT, as compared to after BMT, could be a greater frequency of long-lasting donor/recipient hematopoietic chimerism among the cord blood recipients, conceivably favoring the establishment of reciprocal donor/recipient immune tolerance. On the other hand, our data support the concept that a condition of early and stable full donor chimerism after BMT increases the risk of developing both acute and chronic GvHD in patients transplanted for a non-malignant disease, this translating into a greater risk of transplant-related mortality. Indeed, four out of the five deaths in our study population were attributable to either acute or chronic GvHD.

Despite the absence of rejection, the greatest percentage of recipient cells in patients with sustained mixed chimerism belonged to the $CD4^+$ and $CD8^+$ T lymphocyte subsets. In order to explain this observation, it can be hypothesized that recipient T cells playing a role in the maintenance of tolerance (namely regulatory or suppressive T cells) contributed to the pool of circulating lymphocytes. This interpretation is in accordance with research by Battaglia *et al.*, who reported a skewed T-cell receptor repertoire in thalassemia patients with sustained mixed chimerism after RD-BMT, which was associated with normal immune function.[41] The preferential expansion of given T-cell clonotypes present in the peripheral blood of patients with mixed chimerism was interpreted as being correlated with the establishment of specific tolerance/anergy.[41] Further biological studies aimed at better elucidating the mechanisms at the basis of donor/recipient reciprocal tolerance are warranted.

Different factors possibly contributed to the occurrence of a high percentage of stable mixed chimerism in our RD-CBT patients, including: (i) the lower number of T lymphocytes transferred with the graft, as compared with BMT, (ii) the naïve status of the great majority of

T cells, which may be less efficient in eliminating residual recipient T cells that escaped the lytic effect of the pre-transplantation conditioning regimen, (iii) a fetal-maternal immune interaction, which influences the composition of T-lymphocyte subsets, more prone to suppressing rather than activating any immune response, including that against alloantigens.[7,42,43] We recently demonstrated that cord blood transplant recipients, in particular those who did not receive total body irradiation in the conditioning regimen and did not experience GvHD, may harbor several circulating T-lymphocyte clones of recipient origin, specific for widespread pathogens, such as human cytomegalovirus and *Candida albicans*.[29] The early post-transplantation occurrence of episodes of infection/reactivation, together with the naïve state of cord blood T lymphocytes adoptively transferred with the graft, could also have favored the establishment of mixed chimerism.

Our results confirm those of a previously published Eurocord study which documented that RD-CBT is a safe procedure, as no patient died.[16] In contrast to that study, in which a significant proportion of patients experienced either primary or secondary graft failure,[16] all our patients are disease-free. However, none of our patients was given methotrexate as part of GvHD prophylaxis after RD-CBT and all received thiotepa in the conditioning regimen, both factors found to be strictly correlated with sustained donor engraftment in the Eurocord study.[16]

In view of our and other previously published results showing that even low percentages of donor cells are enough to guarantee transfusion independence,[44,45] it could be tempting to use reduced-intensity preparative regimens also in patients with thalassemia, since such regimens are certainly capable of reducing the early toxicity and long-term complications associated with conventional allogeneic hematopoietic stem cell transplantation. However, the available evidence indicates that the barrier to stable partial donor engraftment after minimally toxic regimens employed for transplantation in patients with hemoglobinopathies seems to be much more difficult to overcome than in adults with hematologic malignancies.[46,47]

In summary, this is the first study addressing the issue of donor/recipient chimerism in patients with β-thalassemia undergoing RD-CBT from an HLA-identical relative in a longitudinal study; it shows that mixed chimerism sustained over time in circulating mononuclear cells can be found in a large proportion of these patients, without predicting the occurrence of graft failure.

Authorship and Disclosures

DL, RM, FL: designed the study, analyzed the data, and wrote the paper; EB, DM, GA: acquired and analyzed the data; MZ: performed the statistical analysis; ADC-M, GG, MZ, AM, FL: performed the transplants; DL, RC, CP, ML, PG: performed the chimerism analysis. All authors participated in the critical review and final approval of the paper. The authors reported no potential conflicts of interest.

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