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Immunohematologic Reconstitution in Pediatric Patients after T Cell-Depleted HLA-Haploidentical Stem Cell Transplantation for Thalassemia

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To analyze immunohematologic reconstitution, particularly of natural killer (NK) cells, we evaluated 13 β-thalassemia patients after 20 and 60 days and 1 year posttransplantation with T cell-depleted HLA-haploidentical stem cells. We assessed lymphocyte and bone marrow (BM) progenitor cell phenotype and differentiation capacity, spontaneous BM cytokine production, stromal cells, and stromal cell interleukin (IL)-7 production. A reduced clonogenic capability manifested at day +20. Patients had significantly lower $CD4^+$ T cells versus controls, mainly in the $CD45RA^+CD62L^+$ subset. NKs were among the first lymphocytes to repopulate the peripheral blood. At day +60, an increase in primitive BM progenitor cells paralleled small increases in $CD4^+$, naïve $CD4^+$, and thymic naïve Th cells. A significant increase in $CD4^+$ and $CD8^+$ markers paralleled an increase in $CD3^-CD16^+$ NKs, especially with full engraftment. In patients with stable mixed chimerism we observed very low levels of $CD3^+$ donor chimerism early after transplant that increased over time, but a stable population of high donor NK cells, suggesting a role of these cells on donor engraftment. Stromal cells secreted less IL-7 and displayed "macrophage-like" morphology. Patients initially manifested impaired stem/progenitor cell growth and differentiation capacity in parallel with altered T cell homeostasis and a reduced T cell naïve compartment. We hypothesize that T cell compartment damage partly arises from altered new T cell production from the hematopoietic stem/progenitor cells under stromal cytokine influence. NNK subset analysis might be useful for determining transplant outcome.

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KEY WORDS: Thalassemia, Bone marrow, Cytokines, Stem cells, Stromal cells

INTRODUCTION

Approximately 25% to 30% of patients with thalassemia could have a human leukocyte antigen (HLA)-matched related donor. Because hematopoietic stem cell transplantation (HSCT) is the only cure for thalassemia, there is need to develop alternative stem cell donation sources. We have past experience with bone marrow (BM) transplant from alternative donors for 29 patients with β-thalassemia major who received phenotypically matched grafts or haploidentical grafts mismatched for 1, 2, or 3 antigens; the results were characterized by higher graft failure (55%) and low thalassemia-free survival (21%) [1]. Haploidentical HSCT (HaploHSCT) from a mismatched family member donor offers an alternative for patients who lack an HLA-matched donor. The main complications are graft rejection, delayed immune reconstitution, graft-versus-host disease (GVHD), and a higher frequency of opportunistic infections. Several groups

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have overcome the HLA barrier in HaploHSCT by administering large doses of $CD34^+$-selected stem cells [2-4]. However, the loss of other immune components, especially natural killer (NK) cells, during the selection process makes this approach less than optimal for generating antiinfection effects and for delayed immune reconstitution, rejection, or severe GVHD.

Haploidentical T cell-depleted HSCT from mother to child, based on the assumption that the immunotolerance established during pregnancy might help bypass the HLA disparity, is under study at our center. Preliminary results suggest that HaploHSCT is a realistic therapeutic option for thalassemic patients without an HLA-identical donor. In HaploHSCT, donor-versus-recipient NK cell alloreactivity derives from a mismatch between donor NK clones bearing inhibitory killer cell Ig-like receptors for self HLA class I molecules and their HLA class I ligands on recipient cells. The mechanism by which alloreactive NK cells exert their benefits in transplantation has been elucidated, as follows: the infusion of alloreactive NK cells (1) ablates recipient T cells, which reject the graft, and (2) ablates recipient dendritic cells (DCs), which trigger GVHD, thus conferring protection against GVHD [5]. NK cell alloreactivity also boosts rapid rebuilding of donor adaptive immunity to infections.

In vivo development of lymphoid progenitors requires a strict interaction of these cells with the BM stromal microenvironment, which provides a rich milieu of cytokines, extracellular matrix proteins, and adhesion molecules [6,7]. Impaired stromal function, alteration of the hematopoietic growth factor network, and abnormal apoptosis may all be involved in impaired hematolymphopoiesis after transplant.

To investigate immunohematologic reconstitution post transplant, we analyzed BM progenitor cell growth and differentiation capacity, the functional and morphologic characteristics of BM stromal cells, and the phenotype of circulating lymphocyte subsets, particularly NK subsets, at different times posttransplant.

MATERIALS AND METHODS

Patient Population

The study group consisted of 13 consecutive β-thalassemia patients, followed as out-patients at the Mediterranean Institute of Hematology (IME), University of Rome "Tor Vergata." They were enrolled for T cell-depleted HLA HaploHSCT. The age range was 3 to 12 years (median: 5 years). All patients received hydroxyurea (60 mg/kg) and azathioprine (3 mg/kg) from day −59 until day −11; fludarabine (30 mg/m^2) from day −17 to day −11; busulfan (14 mg/kg) starting on day −10; and cyclophosphamide (200 mg/kg), thiotepa (10 mg/kg), and antithymocyte globulin (ATG-Fresenius S) (12.5 mg/kg) daily from days −5 to −2, followed by $CD34^+$ T cell-depleted (CliniMacs System, Germany), granulocyte colony stimulating factor (G-CSF)-mobilized peripheral blood (PB) stem cells from their HLA-haploidentical mother.

T and B cell depletion was carried out with $CD34^+$-coated magnetic microbeads and the CliniMACS device (Miltenyi Biotec©) from PB and BM of donors (the mothers) and resulted in grafts consisting of stem cells and effector cells (NK cells, monocytes) with the addition of BM mononuclear cells (BMMCs) (3×10^5/kg of the recipient). The purity of $CD34^+$ cells after MACS sorting was 98% to 99%.

Patients received cyclosporine after transplant for GVHD prophylaxis during the first 2 months after the BM transplantation.

Signed informed consent was received for the patients before transplantation, and all procedures were performed according to our center's established protocols. The study protocol was approved by the center's institutional review board.

Flow-Cytometric Analysis of Peripheral Blood Mononuclear Cells (PBMCs)

Whole-blood phenotype analysis consisted of lysing 500 μL blood with 10 mL of Ortho-mune Lysing Reagent (Ortho Diagnostic Systems Inc., Raritan, NJ, USA) at room temperature, and washing and labeling with a cocktail of 4 monoclonal antibodies (mAbs) for 30 minutes at 4°C. Anti-CD3-fluorescein isothiocyanate (FITC), anti-CD4-allophycocyanin (APC), anti-CD8-peridinin chlorophyll protein (PerCP), anti-CD56 peridinin chlorophyll protein (PerCP), anti-CD16 APC, anti-CD45RA-FITC, anti-CD62L-phycoerytrin (PE), and anti-CD19-PE were purchased from Becton Dickinson (San Diego, CA, USA). To identified thymic naive Th cells, we used the following antibodies as previously described [8]: anti-CD4-PerCP, anti-CD45RA-FITC, and anti-CD31-PE, all from Becton Dickinson. After staining, cells were washed once in phosphate-buffered saline (PBS) containing 2% fetal bovine serum and analyzed on a FACSCalibur cytofluorometer (Becton Dickinson, Mountain View, CA, USA) using Cell Quest software. Absolute lymphocyte counts were calculated by a standard hemocytometric technique.

Preparation of BMMCs

BM aspirates were initially collected into a tube containing ethylenediaminetetraacetic acid (EDTA) as the anticoagulant. The BM samples were diluted 1:3 with PBS 1× plus EDTA 5 mM, then separated after centrifugation by Ficoll (Lymphoprep, Nycomed

Pharma, Oslo, Norway) and resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), L-glutamine (2 mmol/L), and penicillin (250 U/mL) (all from Life Technologies s.r.l., Milan, Italy).

Colony-Forming Cell (CFC) Assay and Long-Term BM Cultures (LTBMC)

BMMCs (1×10^5) were plated in duplicate cultures in 1 mL methylcellulose assay medium containing recombinant human (rHu) erythropoietin (3 U/mL), rHu stem cell factor (50 ng/mL), rHu granulocyte/macrophage colony stimulating factor (GM-CSF; 10 ng/mL), and rHu interleukin-3 (IL-3, 10 ng/mL) (BIOPSA, Stem Cell Technologies, Vancouver, Canada). According to standardized morphologic criteria, the growth of the multipotent hemopoietic progenitor was evaluated as colony forming unit (CFU) granulocyte-, erythrocyte-, monocyte-megakaryocyte (CFU-GEMM, or CFU-MIX), a burst-forming unit erythroid (BFU-E), a CFU-erythroid (CFU-E), or a CFU granulocyte-monocyte (CFU-GM) after 2 weeks of incubation at 37°C in an atmosphere of 5% CO_2.

To analyze the most immature progenitors and the stem cell compartment, we used LTBMCs. Stromal cell cultures and determination of long-term culture-colony-forming cell numbers were performed according to a modification of previously described methods [9]. The murine BM stromal cell line M210B4 was cultured until cell confluence, then trypsinized, irradiated (8000 rad), washed, and placed in 6-well plates. Total BMMCs (1×10^6 cells, in duplicate cultures) were applied on the preestablished, irradiated stromal feeder layers and cultured at 37°C for 5 weeks.

The half-medium liquid was changed weekly. After 5 weeks, the nonadherent and adherent cells were harvested by treatment with trypsin (Life Technologies), washed, and replaced in duplicate in methylcellulose to evaluate the number of cells able to determine secondary colonies. The number of CFCs generated after 5 weeks of cultures on stromal cells gives an indirect but consistent measurement of the content of LTC-initiating cells (LTC-ICs).

Spontaneous Cytokine Production from BMMC Cultures

To evaluate the cytokine production at BM level, BMMC short-term cultures were performed with freshly collected BM samples. Briefly, isolated BMMCs were cultured in RPMI 1640 medium supplemented with 10% FCS, L-glutamine (2 mmol/L), and penicillin (250 U/mL) in 5% CO_2 atmosphere at 37°C, in the absence of stimuli, to verify the spontaneous production of cytokines IL-2 and tumor necrosis factor (TNF)-α. After 24 hours of culture, supernatants were collected and measurement of cytokines was performed by ELISA, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). For each cytokine determination a standard curve was generated.

BM Stromal Cell Characterization by Immunohistochemistry

BMMCs were cultured in tissue culture chamber slides (Falcon, Lincoln Park, NJ, USA) in Iscove's modified Dulbecco's medium (IMDM) (Gibco BRL Life Technologies, Gaithersburg, MD, USA) supplemented with 20% FCS, 100 IU/mL penicillin-streptomycin, and 100 IU/mL glutamine, at 37°C in humidified air at 5% CO_2. At weekly intervals, cultures were fed by demipopulation of the nonadherent cells and replacement of 500 μL of fresh supplemented IMDM. Cultures were maintained until stromal confluence (3-4 weeks) and then analyzed by immunohistochemistry. Stromal cells were fixed with a mixture of 50:50 acetone:ethanol for 30 minutes and then incubated for 30 minutes with the following primary antibodies: anti-CD68 (1:200) and anti-CD14 (5:100) as macrophage markers; anti-CD34 (1:25) as a progenitor cell marker; anti-S100 (1:1000) as an adipocyte marker; and antivimentin (1:50) as a mesenchymal marker (all from DAKO, Dakopatts, Copenhagen, Denmark). Samples were then stained to amplify and detect the signal, according to the manufacturer's instructions, using an immunoperoxidase technique (DAKO LSAB Kit-peroxidase, Dakopatts). Slides were subsequently analyzed using light microscopy.

Spontaneous IL-7 Production from BM Stromal Cultures

BMMCs were cultured in 24-well plates in IMDM supplemented with 10% FCS, 10% horse serum, 100 IU/mL penicillin-streptomycin, 100 IU/mL glutamine, and 10^{-6} M hydrocortisone sodium succinate (Sigma, St. Louis, MO, USA), at a concentration of 1×10^6 cells/mL in a total volume of 2 mL per well. The plates were incubated at 37°C in humidified air at 5% CO_2.

At weekly intervals, cultures were fed by demipopulation of the nonadherent cells and replacement of 500 μL of fresh, supplemented IMDM. Cultures were maintained until stromal confluence (3-4 weeks), then cells were collected by trypsinization and cultured at a concentration of 1×10^6 cells/mL in a total volume of 1 mL per well. Supernatants were then collected after 24 h of culture and measurements of cytokine IL-7 were performed by ELISA, according to the manufacturer's instructions for its ultrasensitive kit (R&D Systems, Minneapolis, MN, USA).

Statistical Analysis

Nonparametric statistics were used (Mann-Whitney, Wilcoxon test) for unpaired and paired comparisons

between the parameters analyzed. A P value $< .05$ was considered significant. Statistical analyses were performed using Statview 5.0 software (SAS Institute, Cary, NC, USA).

RESULTS

Donors

Family members were assessed for HLA compatibility by serologic methods or by high-resolution molecular analysis. All donors were identical for 1 haplotype and incompatible at 3 loci (HLA-A, -B, -DR) of the other. The stem cell dose was achieved with a median of 3 leukaphereses (range: 1-5). The donors showed in PB in mean 46.1% ± 6.4% (1151 ± 650/mm^3) CD4$^+$ T cells, 25.5% ± 5.3% (671 ± 425/mm^3) CD8 T cells and 13.2% ± 3.5% (275 ± 107/mm^3) CD19$^+$. The percentage of donor's NK cells (CD3$^-$CD16$^+$CD56$^+$) was in mean 11.1% ± 8.6% (167 ± 31.7/mm^3). No correlation was observed between PB T cells of donors and engraftment in patients.

Grafts

Median infused cell doses per kilogram of recipient body weight were as follow: NC 0.208 × 10^9/kg (range: 0.009-0.650 × 10^9/kg); CD34$^+$ 18.3 × 10^6/kg (range: 7.35-28.2 × 10^6/kg); CD3$^+$ 0.736 × 10^6/kg (range: 0.008-2.38 × 10^6/kg); CD4$^+$ 0.21 × 10^6/kg (range: 0.081-0.431 × 10^6/kg); CD8$^+$ 0.531 × 10^6/kg (range: 0.08-1.46 × 10^6/kg); CD19$^+$ 0.622 × 10^6/kg (range: 0.032-2.460 × 10^6/kg); NK cells 0.403 × 10^6/kg (range: 0.201-0.821 × 10^6/kg). No correlation was observed between infused cell doses and engraftment in patients.

Engraftment

After Haplo HSCT, 6 of 13 patients manifested full engraftment, 3 of 13 were mixed chimerism (MC), and 4 of 13 rejected the transplant. Eleven patients are alive. Two patients died after full engraftment, 1, 6 months after BM transplant for Epstein-Barr virus-related central nervous system diffuse large B-cell lymphoma, and 1 from a cytomegalovirus-related opportunistic infection. Seven patients are alive and disease free with a median follow up of 15 months.

In patients who showed allogeneic reconstitution, median time for granulocyte recovery was 13 days (range: 11-17 days), whereas median time for a self-sustained platelet recovery was 12 days (range: 9-17 days). In 4 cases, donor BM was rejected with complete autologous reconstitution and return to pretransplant clinical status. In these cases, rejection occurred after transient engraftment of donor cells, as reported in Figure 1. MC was classified, according to the proportion of residual host cells present in the recipient, into MC level 1 (residual host cells <10%), MC level 2 (residual host cells between 10% and 25%), and MC level 3 (residual host cells >25%). Three patients experienced a status of mixed chimerism level 3 early after BM transplantation (BMT), which became persistent when observed respectively at 14, 38, and 42 months after the transplant. To define the condition of MC better, we analyzed the proportion of donor engraftment in different lymphoid subsets at different times after BMT. Figure 2 reported the mixed chimerism condition in each of 3 patients. The recipients with full donor chimerism had 100% of CD3$^+$ donor cells and 100% of donor NK cells, stable over time. The individuals who had stable mixed chimerism, showed to have very low levels of CD3$^+$ donor chimerism early

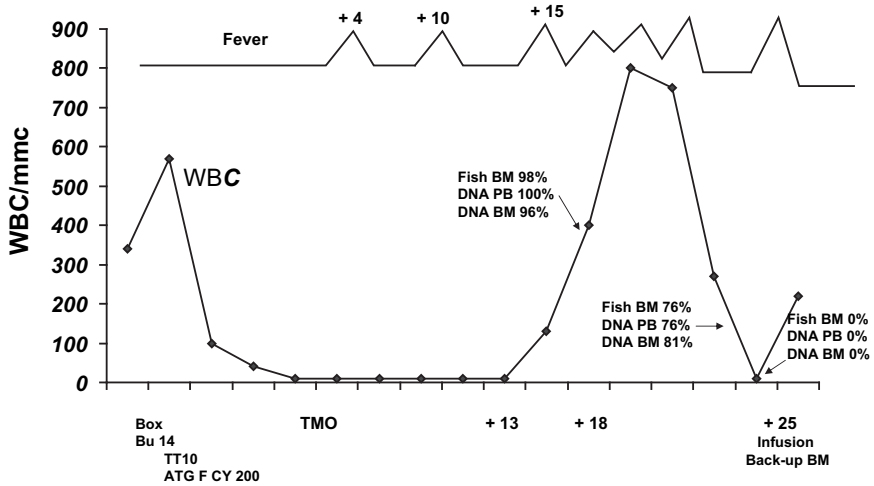

Figure 1. Transient engraftment of donor cells in a thalassemia patient after HaploHSCT. In this patient, we observed a transient engraftment of donor cells starting from 13 days post-haploHSCT. At 18 days post-haploHSCT, we did tests of marrow engraftments by FISH and DNA molecular analysis of peripheral blood (DNA PB) and bone marrow (DNA BM) with identification of a full engraftment with 100% donor cells in peripheral blood. White blood cells (WBCs) increased until 22 days, then we observed a progressively decreased number of WBC and full rejection at 25 days (FISH, DNA PB, DNA BM 0% donor cells).

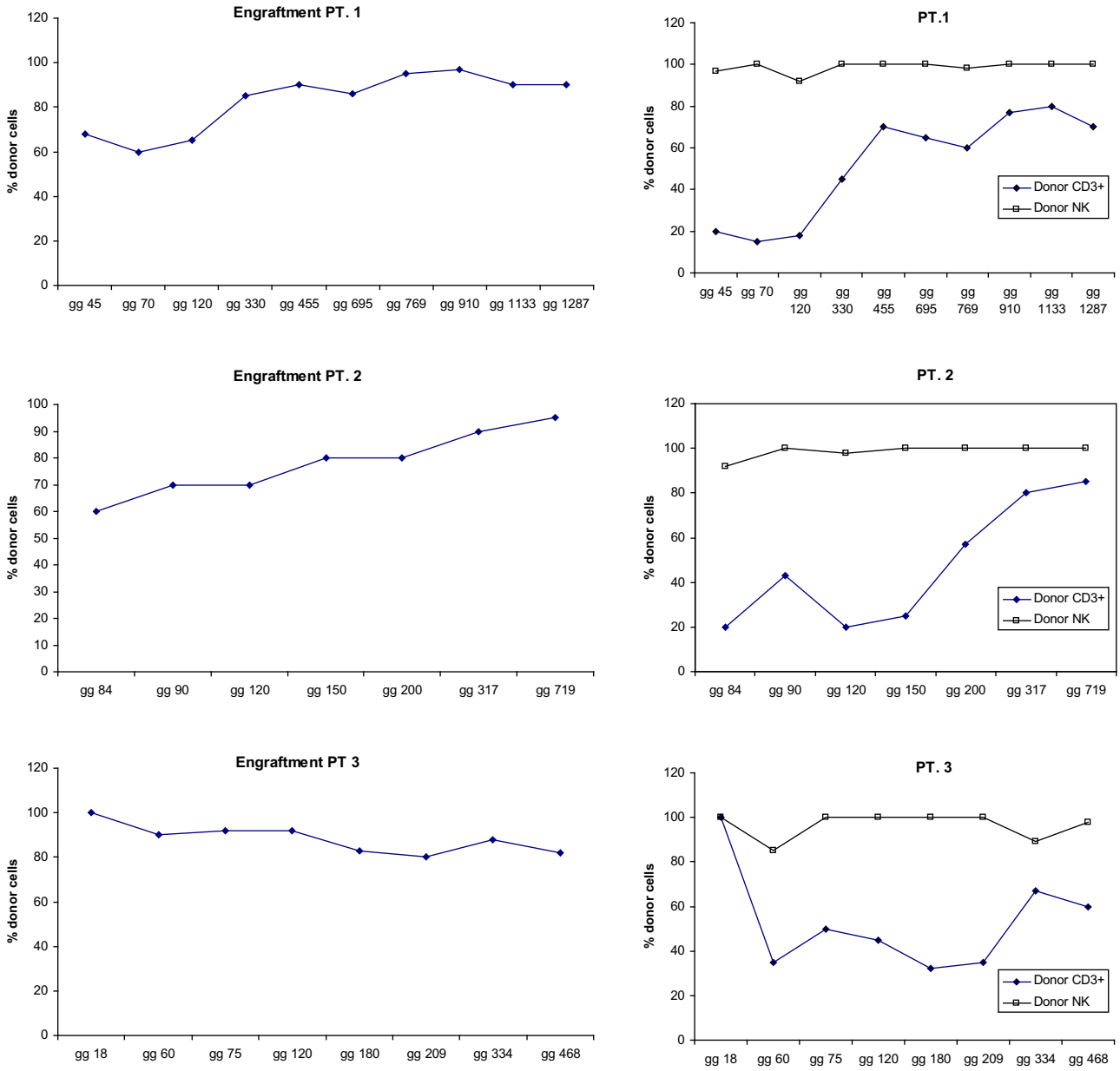

Figure 2. Mixed chimerism in 3 patients after haploHSCT. Proportion of donor engraftment in different lymphoid subsets ($CD3^+$ and NK cells) at different times after BMT in 3 patients (PT.1, PT. 2, PT. 3).

after transplantation that increased over time, in parallel with high and stable levels of donor NK population, suggesting that donor NK cells might have promoted tolerance and donor engraftment. The levels of immunosuppression were uniform in all patients. No patient developed GVHD and all patients came off immunosoppression at the same time.

Immunologic and Hematologic Data on PBMCs

At day 20 posttransplant, patients had significantly lower $CD4^+$ T cell numbers in comparison to normal value (4% ± 5.4% versus 47.5% ± 6%, respectively; 16.7 ± 31.7/mm^3 versus 410-1580/ mm^3), mainly in the $CD45RA^+CD62L^+$ (naive phenotype) subset (1.6% ± 1.3% in patients versus 52% ± 12% normal value). There was a significant decrease in peripheral $CD45RA^+CD31^+$ Th cells (thymic naive Th cells) (on average 1% ± 0.3% in patients versus 37% ± 10% normal value), whereas $CD8^+$ T cell numbers did not statistically differ between patients and normal value (17% ± 20% versus 20% ± 7%). All patients displayed reduced numbers of B cells versus normal value, and 5 patients had only 0% to 1% of control levels of $CD19^+$ cells.

At day 60 posttransplant, no significant change was observed in the percentages of $CD4^+$ T cells, naïve $CD4^+$ cells, thymic naïve Th cells, or $CD8^+$ T cells

(5.8% ± 3.5%, 48% ± 32.4/mm^3 CD4$^+$; 3.2% ± 1.5% naïve CD4$^+$; 2.9% ± 0.5% CD31$^+$ and 16.8% ± 15%, 150.2 ± 161.9/mm^3 CD8$^+$).

Compared with normal values, thalassemia patients showed a significant increase in CD4$^+$ cell activation markers (CD95, HLA-DR, and CCR5), observed after 60 days posttransplant. Only a year after transplant did we identify a partial normalization of CD3$^+$, CD4$^+$, CD8$^+$, and CD19$^+$ compartments (Figure 3).

NK cells were among the first lymphocytes to repopulate the PB, and up to 45% of these cells were CD3$^-$CD56^{bright} (mean 45.6% ± 35%, 189.6 ± 192.6/mm^3), whereas CD3$^-$CD16$^+$ NK cells were reduced (mean 15.2% ± 16.6%, 56.3 ± 73.8/mm^3). Interestingly, a direct correlation was observed between the percentages of the CD3$^-$CD56$^+$CD16$^+$ NK subset and the BM engraftment with mean values of 78% ± 15.3% CD56$^+$CD16$^+$ in the 6 patients with full engraftment, 14% ± 5.1% in the 3 patients with a stable mixed chimerism after transplant (70%-80% of donor cells), and 1.4% ± 1.3% in the 4 patients with rejection (Figure 4a). This correlation was principally observed for the CD3$^-$CD56^{bright} compartment (mean 67.9% ± 27% in full engraftment versus 44.3% ± 19.3% in mixed chimerism and 2.4% ± 1.9% in patients with rejection) (Figure 4b). Interestingly, in patients with mixed chimerism at +20 days, the NK compartment was represented principally by CD3$^-$CD16$^+$ (with cytotoxic functions) (mean 30.3 ± 16.2 versus 16.5 ± 16 with full engraftment and 1.9 ± 3.3 in rejection) (Figure 4c). In all patients, the origin of the NK subsets was the mothers.

At day +60, we observed an increase in the CD3$^-$CD16$^+$ NK cells (potent cytotoxic effector cells), especially in patients with full engraftment (mean 31.5% ± 21.7% versus 14.5% ± 10% in mixed chimerism versus 0.7% ± 0.4% in patients with rejection) (Figure 4).

CFC Assay and LTBMCs

As reported in Figure 5, the reduced clonogenic capability observed at day 20, showed an initial increase of committed progenitor cells at day +60 after transplant. This finding was also observed on primitive BM progenitor cells compartment after 2 months of transplant (1.8 ± 1 at 20 days versus 3.3 ± 2.4 LTC-CFC/10^6 BMMCs; P = .0022).

Measurements of Spontaneous Cytokine Production in BMMC Cultures

In vitro production of IL-2 and TNF-α by short-term culture of BMMCs was determined in thalassemia patients 20 and 60 days posttransplant. The spontaneous production of IL-2 and TNF-α was significantly decreased in patients 60 days posttransplant (18.6 ± 9.2 pg/mL at 20 days versus 8.9 ± 5 pg/mL at 60 days IL-2; P = .00015, and 11.9 ± 4.1 pg/mL at 20 days versus 4.5 ± 3.3 pg/mL at 60 days TNF-α; P = .0003).

Immunohistochemistry of BM Stromal Cells and IL-7 Production

Next, we generated stromal cell layers from BMMCs of patients. The stromal layers cultured on chamber slides were positive for CD68, vimentin, and CD14 but negative for S100 and CD34, indicating that they were preferentially of the macrophage/monocyte lineage. Upon light microscopy examination, the majority (75%) of these cells appeared as

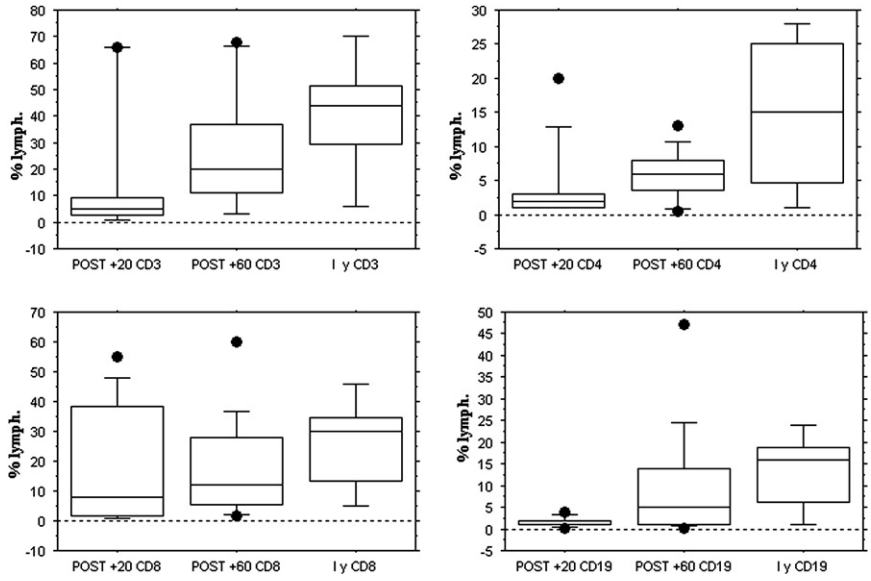

Figure 3. Mean immune indicator values before and after HaploHSCT. The bars represent the mean value (±SD) of percentages of circulating T (CD3$^+$, CD4$^+$, CD8$^+$) and B lymphocytes (CD19$^+$) after 20 days, 60 days, and 1 years of transplant in β-thalassemia patients.

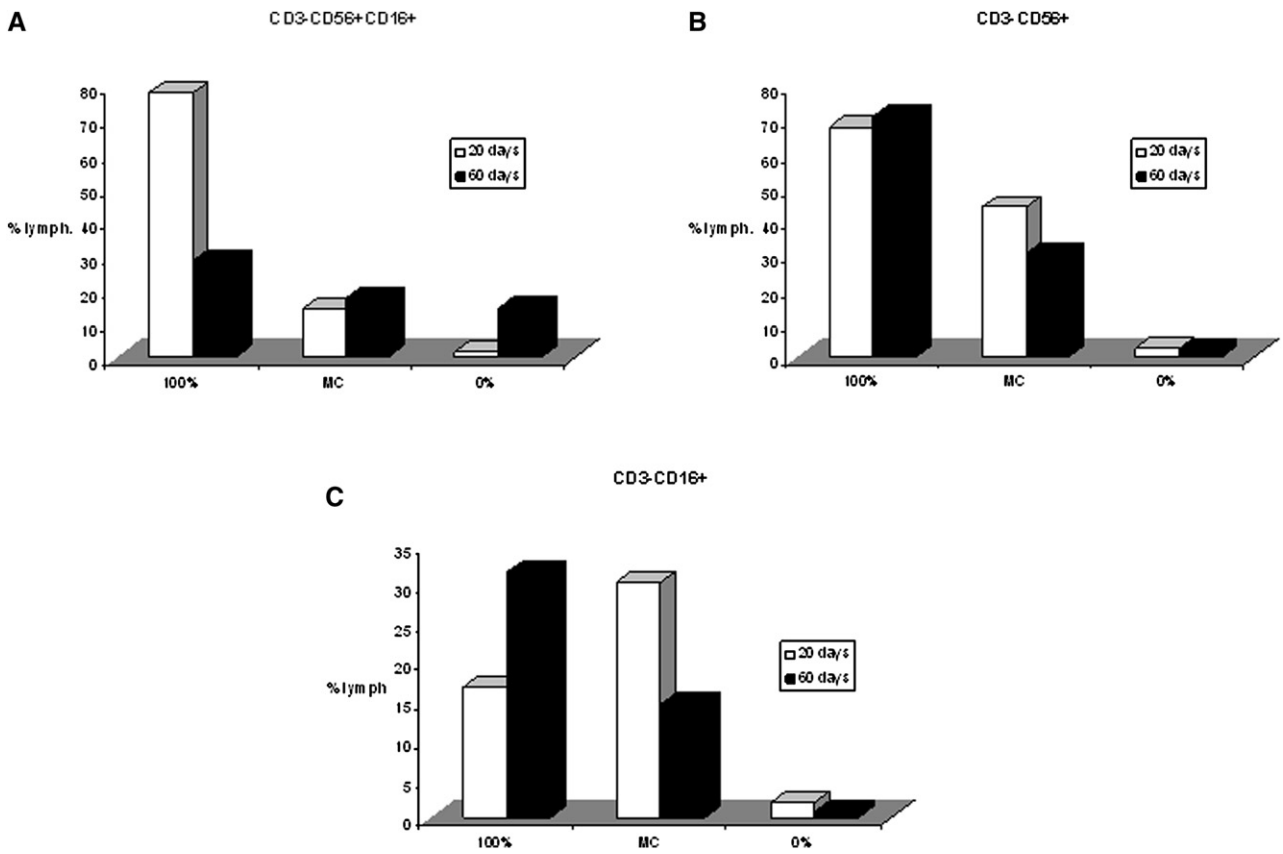

Figure 4. NK subsets after haploHSCT. The bars represent the mean value of percentages of $CD3^{-}CD56^{+}CD16^{+}$ (A), $CD3^{-}CD56^{+}$ (B), and (C) $CD3^{-}CD16^{+}$ in full engraftment, in mixed chimerism, and in rejection 20 and 60 days posttransplant.

moderately large and frequently rounded, with abundant cytoplasm at day +20. At day +60, we observed a tendency to normalization of stromal composition near that of normal subjects, in which about 90% of the stromal cells exhibited a different morphology characterized by an irregular or spindle shape and branching cytoplasmic processes (fibroblast-like).

Stromal cells from patients spontaneously produced lower levels of IL-7 compared with normal value (0.3 ± 0.1 pg/mL versus 0.8 ± 0.1 pg/mL, respectively; $P = .02$).

DISCUSSION

HSCT offers the only chance of cure for patients with thalassemia. Haploidentical transplantation may extend this possibility to the 50% to 60% of the patients who lack a suitably matched familial donor or an HLA-identical unrelated donor. The presence of fetal cells in maternal blood and of maternal cells in fetal blood (fetomaternal microchimerism) suggests that immunologic tolerance may exist between mother and offspring [10,11].

We have reported [12] the results of BM transplantation in children with acute leukemia in relapse resistant to chemotherapy, where their haploidentical mother was used as the donor of nonmanipulated bone marrow. The combination of a megadose of purified $CD34^{+}$ cells and a highly immunomyeloablative conditioning regimen is crucial for overcoming the barrier of residual antidonor cytotoxic T-lymphocyte precursors in T cell-depleted mismatched transplants [13] and the addition of BMMCs (including NK cells, mesenchymal stem cells, T cells) to a T cell-depleted allograft may help promote engraftment and control GVHD. The reason of adding back BMMCs of 3×10^{5}/kg in our patients was based on the assumption that a minimal threshold of lymphocytes for developing of GVHD was 10^{5}/kg [14]. The simultaneous addition of BMMCs (including stem cells, NK cells, monocytes, DCs, mesenchymal cells), may have a potential role for immunotolerance and engraftment. BM could be a site of T cell priming, because the antigen is accessible for presentation by BM DCs. It was described that antigen-specific $CD4^{+}$ and $CD8^{+}$ T cells home to BM after antigen challenge in the periphery and are maintained there over long periods of time [15]. In addition, an important role for $CD4^{+}$ T cell on BM engraftment is described, not only promoting rejection of the few host cells after the conditioning regimen, but also allowing efficient HSC differentiation and reconstitution [16]. In the presence of antigen-primed $CD4^{+}$ T cells, cytokines are secreted and terminal differentiation of hematopoietic committed

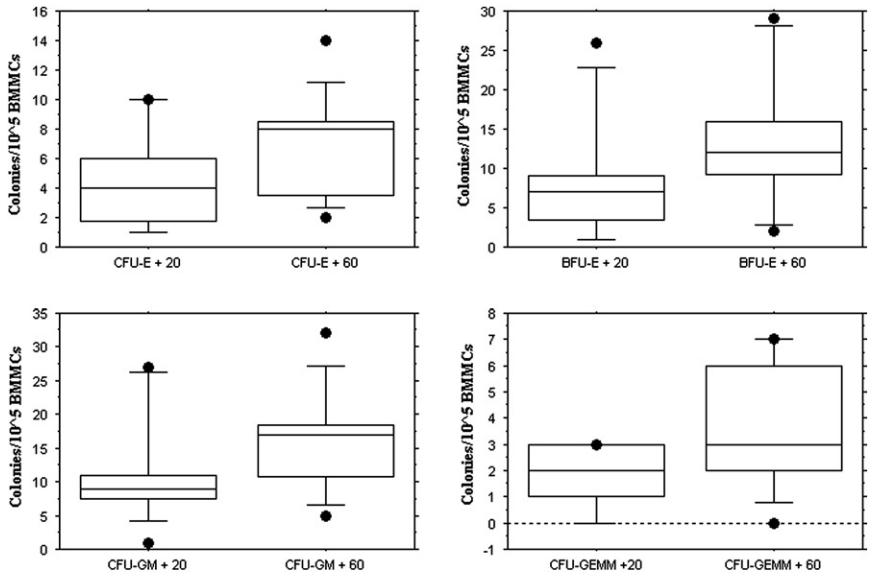

Figure 5. Clonogenic progenitor cells in thalassemia patients. The bars represent the mean number (±SD) of colonies obtained by 100,000 BMMCs plated in methylcellulose (CFC assay) from thalassemia patients +20 and +60 days posttransplant. The growth of CFU-GEMM, BFU-E, CFU-E, and CFU-GM was evaluated according to standardized morphologic criteria.

progenitors and precursors is efficient. When $CD4^+$ T cells are absent or their cognate antigens are not available, hematopoiesis is defective, resulting in immature cell accumulation in the BM or leukopenia in the PB [16].

Haploidentical transplantation is associated with major posttransplant immune deficiency, resulting in significant morbidity and mortality from infection. Immune reconstitution posttransplant and maintenance of homeostasis in BM require a well-balanced interaction between the hematopoietic cells and the immune system. The immune system may modulate the function of BM hematopoietic progenitor cells and/or their microenvironment, either by inflammatory cytokine production or by cell-to-cell interactions [17]. Delayed immune reconstitution posttransplant may be associated with a variety of functional and immunophenotypic abnormalities at the BM level because of augmented local production of inflammatory cytokines, increased T cell activation, or intrinsic hematopoietic and stromal cell abnormalities.

Quantitative and functional defects in hematopoietic progenitor cells were observed in our patients in the initial phase posttransplant, as indicated by an altered clonogenic potential of BM-committed progenitor cells and a significant reduction in primitive progenitors. These abnormalities may represent either an intrinsic progenitor cell defect or secondary progenitor cell damage in response to an underlying inflammatory process within the BM microenvironment. The patients displayed an altered BMMC cytokine production, characterized initially by increased levels of TNF-α. This cytokine pattern changed after 60 days posttransplant, with a tendency to normalization.

These findings correlated with a significant decrease in total lymphocyte counts and depletion of $CD4^+$ T cells expressing predominantly the CD45 RA^+CD62L^+ phenotype. Also, the $CD4^+$CD45 RA^+CD31^+ T cell subset was significantly reduced in our cohort, suggesting thymus involvement in these patients. Furthermore, we cannot rule out a defective egress of naïve T lymphocytes from the thymus [18]. Indeed, it is possible that the T cell defect in thalassemia patients may occur at multiple levels, including egress from the thymus.

In vivo and in vitro, hematolymphopoiesis occurs in association with the complex network of cell types found in the stroma, including nonhematopoietic (fibroblasts, adipocytes, and endothelial cells) and hematopoietic cells (macrophages and T cells). Progenitor cell growth and differentiation depend on their interaction with stromal cells. Low levels of cytokine production can be more effective when local concentration is increased by cell-cell contacts and by the binding of cytokines to the extracellular matrix [19,20]. The prevalence of macrophage-like cells in long-term BM culture, rather than the typical "fibroblast-like" cells, suggests an altered composition of the BM stroma, possibly linked to an underlying inflammatory process within the BM microenvironment.

A central function of stromal cells is IL-7 production [21]. Recent evidence shows that IL-7 acts as a master regulator of T cell homeostasis, expanding both the naive and memory T cell populations [22,23]. IL-7 primarily acts as a growth and antiapoptotic factor for B

and T cell precursors, and its production is critical for the beginning of B and T lymphopoiesis starting from stem cells. The patients exhibited altered stromal cytokine production at 20 days posttransplant, characterized by decreased IL-7 levels. We can hypothesize that the delayed immunoreconstitution of the T cell compartment may be initially the result of altered generation of new T cells arising from hematopoietic progenitor cells with the interaction of impaired stromal cell function. Based on these results, it can be hypothesized that therapeutic administration of some cytokines (ie, IL-2 plus IL-7) and or mesenchymal stem cells (MSCs) might form a future strategy supporting T cell development posttransplant.

Because MSCs are the precursors of BMSCs, it was originally thought that they could facilitate hematopoietic stem cell (HSC) engraftment [24-26]. MSC infusion, in addition to being safe [21], improved the success of HSC transplantation and the clinical outcome [27]. When we added maternal MSCs in vitro to BMMCs of the daughter, we observed a significant increase of the more immature progenitor cells in the LTC-IC assay (data not shown), suggesting a role for MSCs in facilitating engraftment.

NK $CD56^{+bright}$ cells develop more rapidly than other lymphocytes, but $CD3^{-}CD16^{+}$ NK cells (with cytotoxic potential) require more prolonged exposure to maturation factor (IL-2) in the BM. No significant correlation has been observed between cell doses infused after T cell depletion or immunologic characteristics of the donors and engraftment in patients, but interestingly, we observed higher percentages of NK $CD56^{+bright}$ cells only 20 days posttransplant in patients with full engraftment, suggesting a role for newly generated NK cells in improved engraftment and in prevention of rejection by an attack of the host lymphohematopoietic cells. The higher percentages of $CD3^{-}CD16^{+}$ in mixed chimerism patients may have a possible role in control of host-cell escape and in maintaining the chimerism condition. The recipients with full donor chimerism had 100% of CD3 donor cells and 100% of donor NK cells, stable over time. The situation was different in patients with mixed chimerism who had high level of donor NK cells and low donor $CD3^{+}$ cells during the first month after transplantation. Subsequently, the number of donor $CD3^{+}$ cells progressively increased, probably because of donor NK cells promoted tolerance and engraftment.

Pioneering studies by Velardi and colleagues [28] revealed that patients with acute myelogenous leukemia transplanted from an NK alloreactive donor benefited from higher rates of engraftment and reduced rates of GVHD. The virtual abrogation of GVHD may be a consequence of NK cell-mediated killing of recipient antigen-presenting cells [29,30]. In our group, no patient showed signs of GVHD posttransplant [31]. The beneficial effects could also be related to depletion of patient antigen-presenting cells and facilitation of engraftment as a result of the killing of T cells, removing patient lymphohematopoietic cells, and production of growth factors required for engraftment and for accelerating recovery of myelopoiesis. Our studies may also suggest NK subset analysis as a useful measure of transplant outcome.

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