Purified T-depleted, CD34+ peripheral blood and bone marrow cell transplantation from haploidentical mother to child with thalassemia

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Short running head: Haploidentical transplantation for thalassemia
Abstract

Feto-maternal microchimerism suggests immunological tolerance between mother and fetus. Thus, we performed primary hematopoietic stem-cell transplantation (HSCT) from mismatched mother to thalassemic patient without an HLA-identical donor. Twenty-two patients with thalassemia major were conditioned with 60 mg/kg hydroxyurea and 3 mg/kg azathioprine from day -59 to -11, 30 mg/m² fludarabine from day -17 to -11, 14 mg/kg busulfan starting on day -10, and 200 mg/kg cyclophosphamide, 10 mg/kg Thiotepa, and 12.5 mg/kg anti-thymocyte globulin daily from day -5 to -2. Fourteen patients received CD34+ mobilized peripheral and bone marrow progenitor cells; eight patients received marrow graft selected PBSC CD34+ and BM CD3/CD19 depleted. T-cell dose was adjusted to $2 \times 10^5$/kg by fresh marrow cell addback at the time of transplant. Both groups received cyclosporine for graft versus host disease (GVHD) prophylaxis for two months post transplant. Two patients died (cerebral EBV lymphoma or CMV pneumonia), six patients reject their grafts, and 14 showed full chimerism with functioning grafts at a median follow-up of 40 months. None of the 14 patients who showed full chimerism developed acute or chronic GVHD. These results suggest that maternal haploidentical HSCT is feasible for patients with thalassemia who lack a matched related donor.
Introduction

The cure for thalassemia involves correcting the genetic defect in a hematopoietic stem cell that results in reduced or absent β-globin synthesis and an excess of α-globin dimers. Intracellular precipitation and accumulation of α-dimers results in ineffective erythropoiesis and hemolytic anemia. Replacing the abnormal thalassemic marrow with allogeneic normal or heterozygous stem cells carrying the functional gene restores appropriate β-globin chain synthesis. Eighty to ninety per cent of patients transplanted from an HLA-identical sibling or parent become ex-thalassemic after transplant \(^1,2\).

In the multiracial populations from the Mediterranean region, Middle East, and Arabian Gulf, the probability of having an HLA-identical related donor is 35-40%. Thus, the pool of potential donors must be expanded in order to cure most children with thalassemia or sickle cell anemia. The outcomes following HLA-matched unrelated donor transplant for treating thalassemia are comparable to those following HLA-identical familial transplant \(^3\). The use of mismatched, unrelated cord blood hematopoietic stem cells is still experimental.

Haploidentical hemopoietic stem cell transplantation has been explored as an option for treating patients with leukemia who lack an HLA-identical sibling or parent donor. However, severe graft versus host disease (GVHD) and high graft failure/rejection rates have limited the application of this transplant modality for patients with thalassemia. Advances using high doses of T cell-depleted peripheral blood stem cells (PBSCs) and intensive pre-transplant conditioning regimens have helped to overcome these limitations \(^4\). Grafts containing mega doses of enriched CD34\(^+\) progenitor cells can be achieved by combining bone marrow with G-CSF-mobilized PBSCs. Thereafter, T-cells can be removed by positive selection for CD34. Limiting the numbers of CD3\(^+\) cells in the graft might allow retention of rapid engraftment kinetics provided by the mobilized PBSCs while reducing the risk of extensive GVHD. In
this pilot study, we used a similar approach involving mega dose haploidentical positively selected CD34+ marrow and peripheral hematopoietic stem cell transplantation to treat patients with thalassemia who lack an HLA-identical familial or unrelated marrow donor. Positive selection of CD34+ stem cells results in an approximately 3-4 log reduction of CD3+ cells, which reduces the risk of GVHD but increases the risk of graft failure. Adding a defined dose of CD3+ marrow cells to the cellular suspension at the time of transplant can help to reduce the graft rejection rate.

In contrast to positive selection of stem cells, marrow graft depleted of CD3+ and CD19+ cells contains significant amounts of monocytes, NK cells, dendritic cells, precursor T-cells, and other cell types that may play important roles in engraftment while accelerating the post-transplant immune reconstitution. Therefore, in a second prospective phase of this pilot study, we evaluated the use of haploidentical CD3+/CD19+-depleted marrow graft combined with CD34-selected mobilized PBSCs and CD3+ marrow cells that were added back at the time of infusion.

Here, we report the outcomes of 22 children with thalassemia who were transplanted from haploidentical donors, twenty mothers and two brothers.

**Patients and methods**

During six years, 2002 through 2008, 22 patients with thalassemia major received an HLA-haploidentical transplant. Signed informed consent was received before transplantation in accordance with the Declaration of Helsinki and all procedures were performed according to our center’s established protocols. The study protocol was approved by the institutional review board of the Mediterranean Institute of Hematology. The results for seven patients included in this study have been reported previously and are updated here with longer follow-up.6,7
Risk assignment was performed according to published criteria. The system categorizes risk based on hepatomegaly, the presence of portal fibrosis on pre-transplant liver biopsies, and the quality of prior chelation (regular: deferoxamine treatment was initiated within 18 months of the first transfusion and administered for 8-12 hours as a continuous daily subcutaneous infusion for at least 5 days per week; anything less was considered irregular chelation). The age in months when the patient first received regular chelation was recorded. A chelation index was used to describe the number of months that each patient did not receive regular chelation as a percentage of the number of months the patient should have received it. With this index, a completely satisfactory chelation history is represented by 0% and a completely unsatisfactory one by 100%.

Patient characteristics are presented in Table 1.

Donors

Family members were assessed for HLA compatibility by serological methods or by high-resolution molecular analysis. All donors (20 mothers and two brothers) were identical for one haplotype and incompatible at three loci (HLA-A, -B, -DR) of the other, except for two who were mismatched at two loci (HLA-A, -B) on the unshared haplotype. One brother was mismatched for non-inherited maternal antigens. The stem cell dose was achieved with a median of three leukaphereses (range, 1-5). Twenty-one donors had β-thalassemia minor, one had sickle cell trait.

Ten age- and sex-matched healthy donors were included as control subjects. The control subjects provided bone marrow aspirates, all of which were deemed normal. None of the control subjects had acute infections or were receiving medication at the time of the study.
Graft processing and transplant procedures

All donors received recombinant human G-CSF 15 mcg/kg/day in two daily subcutaneous boluses to mobilize PBSCs. CD34+ cells from leukaphereses and bone marrow harvests were select using the CliniMACS one-step procedure (Milteny Biotech, Germany) for 14 donors. Two-step selection (CD34 positive selection leukapheresis followed by negative selection using anti-CD3 and anti-CD19 monoclonal antibodies [mAbs]) of bone marrow cells was employed for eight donors. We attempted to suppress erythropoiesis by intensive hypertransfusion and chelation. Between day -59 and day -11 before the transplantation, 40 mg/kg deferoxamine was continuously infused through a central venous catheter each 24 hours. Red cells were transfused every 3 days to maintain the hemoglobin level between 140 and 150 g/L (14 and 15 g/dL). During this time interval hydroxyurea 60 mg/kg daily and azathioprine 3 mg/kg daily were administered to eradicate marrow, and growth factors, granulocyte colony-stimulating factor and erythropoietin, were given twice weekly to maintain stem cell proliferation in the face of hypertransfusion, thereby facilitating the effect of the hydroxyurea. Fludarabine was administered at a dosage of 30 mg/m2/d from day -17 through day -13. Starting on day -10, 14 doses of busulfan (BU) 1 mg/kg were administered orally 3 times daily over 4 days (total dose 14 mg/kg over 4 days) in the first 17 patients, and corresponding dose of busulfan give intravenous in the following 5 patients, followed by intravenous cyclophosphamide (CY) 50 mg/kg daily on each of the next 4 days (total dose 200 mg/kg), and 10 mg/kg Thiotepa, and 12.5 mg/kg anti-thymocyte globulin.

All patients received cyclosporine for GVHD prophylaxis for the first two months post transplantation. Antifungal prophylaxis included liposomal amphotericin B (1 mg/kg daily) from day +8. Cytomegalavirus (CMV) prophylaxis consisted of 5 mg/kg acyclovir three times daily through day +60.
Tests for chimerism

Fluorescent in situ hybridization. When the host and donor were sex mismatched, fluorescent in situ hybridization (FISH) was performed on peripheral blood and bone marrow to detect marrow engraftment.

DNA extraction. High molecular weight DNA was extracted from peripheral blood or bone marrow using a commercial DNA blood mini kit, in accordance with manufacturer's instructions (Qiagen, Hilden, Germany).

Polymerase chain reaction. To evaluate chimerism, four different minisatellite loci (33.6, SE33, APOB, and D1S80) were amplified by polymerase chain reaction (PCR). The PCR-amplified products were resolved on precast 10% non-denaturing polyacrylamide gel (NOVEX, San Diego, CA) and the gels were silver stained. Mixed chimerism was estimated semi-quantitatively by comparing recipient and donor band intensities with those of known standards.

Graft Content

Eight patients received T cell-depleted peripheral blood progenitor cells (CD34+ immunoselection) and CD3+- and CD19+-depleted bone marrow stem cells. Median infused cell doses per kilogram of recipient body weight were CD34+: \(15.2 \times 10^6\) (range, 8.2-26 \(\times 10^6\)); CD3+ T cells: \(1.8 \times 10^5\); and \(0.27 \times 10^6/kg\) CD19.

Fourteen patients received CD34+ mobilized peripheral and bone marrow progenitor cells. Positive selection was performed using the CliniMACS procedure. The CD34+ grafts contained a median of \(14.2 \times 10^6/kg\) CD34+ cells (range, 5.4-39 \(\times 10^6/kg\)), \(2 \times 10^5/kg\) CD3+ cells, and \(0.19 \times 10^6/kg\) CD19+. No side effects were associated with graft infusion.

Flow-cytometric analysis of peripheral blood mononuclear cells. For whole blood phenotype analysis, 500 µL of blood was lysed with 10 mL of Ortho-mune Lysing Reagent.
(Ortho Diagnostic Systems Inc., Raritan, NJ, USA) at room temperature, washed, and labeled with a cocktail of four mAbs for 30 min at 4°C. Anti-CD3-fluorescein isothiocyanate (FITC), anti-CD4-allophycocyanin, anti-CD8-peridinin chlorophyll protein, and anti-CD19-PE were purchased from Becton Dickinson (San Diego, CA, USA).

The NK phenotype of PBMCs was assessed by immunofluorescence and flow cytometry using FITC-conjugated anti-CD3 and PE-conjugated anti-CD56 mAbs (Becton Dickinson). After staining, cells were washed once in phosphate-buffered saline 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 standard hemocytometry. To determine marker expression on CD4+ and CD8+ cells, total lymphocytes were first identified and gated by forward and side scatter, then these cells were gated for CD4 or CD8 expression.

**Statistical analysis**

Estimates of overall survival and event-free survival were calculated by the Kaplan-Meier method. Rejection and non-rejection mortality were calculated as cumulative incidences. When estimating event-free survival, rejection, recurrence of thalassemia, and death were recorded as events. Rejection was defined as the development of complete marrow aplasia or recurrence of thalassemia (a return to the pre-transplantation pattern of globin-chain synthesis).
Results

All patients showed donor chimerism by day 14 after HSCT. Granulocytes count greater than 500 /ml occurred after a median time of 13 days (range,11-17). Six patients rejected their grafts, surviving with thalassemia. Three patients showed early mixed chimerism, which became persistent when observed respectively at 14, 38 and 42 months after the transplant. In 14 cases the transplantation was successful with complete allogeneic reconstitution. 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). There were 2 patients who died from transplantation-related causes: one of these patients died on day +114 of Epstein-Barr virus cerebral lymphoma and one died on day +92 for CMV pneumonia. In 6 cases, donor marrow was rejected with complete autologous reconstitution and return to pre-transplant clinical status. In 2 of these patients, rejection occurred after transient engraftment of donor cells. Mixed Chimerism (MC) as already described20. 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 early after bone marrow transplantation, which became persistent when observed respectively at 14, 38 and 42 months after the transplant. To define the condition of MC better, we analysed the proportion of donor engraftment in different lymphoid subsets at different times after BMT. Figures 2, 3 and 4 reported the mixed chimerism condition in each of 3 patients.

Fourteen patients developed functioning grafts at a median follow-up of 40 months. All the 14 cured children are not anymore transfusion-dependent with hemoglobin levels ranging from 10.3 gdl to 13.8 gdl and have an optimal quality of life.

Since the first transplant done by our Group in Pesaro on February 15, 1989, there are
more than 1000 ex-thalassemic after transplant. Their life is normal, this means that they are socially active, the majority has recovered fertility, more and more are having children.

A group working in Pescara has recently published their data. None of the children with full as well persistent mixed chimerism developed GVHD (Figure 1).

On day +119, one patient developed varicella zoster meningo-encephalitis (documented by PCR of cerebrospinal fluid) that responded to a combination of acyclovir and foscarnet. The two drugs were combined because the patient had been receiving acyclovir prophylaxis. On day +135, this patient also received a donor lymphocyte infusion ($5 \times 10^4$ CD3+ donor cells/kg) in order to boost anti-varicella zoster T cells. The donor, who had a history of varicella zoster, had been administered a dose of varicella zoster vaccine one week prior to peripheral lymphocyte collection. The patient recovered with no neurological sequelae or abnormalities on magnetic resonance imaging.

Fifteen patients showed CMV infection without disease that resolved with pre-emptive gancyclovir treatment. Three patients had EBV reactivation with a high viral load which resolved after treatment with retuximab.

**Immunological reconstitution**

Delayed immune reconstitution post transplant may be associated with a variety of functional and immunophenotypic abnormalities at BM level, due to augmented local production of inflammatory cytokines, increased T-cell activation, or intrinsic hematopoietic and stromal cell abnormalities.

At day +20, six of fourteen patients had significantly lower CD4+ T cell counts than did the control subjects ($1.9 \pm 1.4\%$ vs. $47.5 \pm 6\%$, respectively). This reduction was mainly in the CD45RA+CD62L+ (naive phenotype) subset ($1.3 \pm 2\%$ in patients vs. $52 \pm 12\%$ in controls).
A significant decrease in peripheral CD45RA⁺CD31⁺ Th cells (thymic naive Th cells) was observed (0.5 ± 0.3% in patients vs. 37 ± 10% in controls), whereas CD8⁺ T cells numbers were similar in patients and controls (24.2 ± 33.7% vs. 20 ± 7%). NK cells were among the first lymphocytes to repopulate peripheral blood, and up to 70% of these cells were CD56bright whereas CD56dimCD16⁺ NK cells were reduced.

On day +60, increases in the percentages of CD4⁺ T cells, naïve CD4⁺ cells, and thymic naïve Th cells were observed (3 ± 1.2%, 2.9 ± 2.1%, and 2.7 ± 1%, respectively). CD8⁺ T cells were also increased (35 ± 27.5%). As well, patients showed a significant increase in CD4⁺ cell activation markers (CD95, HLA-DR, and CCR5) that paralleled an increase in CD56dimCD16⁺ NK cells (potent cytotoxic effector cells), especially in the patients with full engraftment (47 ± 20% vs. 28 ± 31% in mixed chimerism).

The stromal layers cultured on chamber slides were positive for CD68, vimentin, and CD14, but negative for S100 and CD34, indicating cells of macrophage/monocyte lineage. In the patients with delayed immuno-hematological reconstitution, the majority (80%) of these cells appeared moderately large, rounded, and with abundant cytoplasm on light microscopy. In contrast, about 90% of stromal cells from control subjects were irregular or spindle shaped with branching cytoplasmic processes (fibroblast-like).

Spontaneous stromal cell production of IL-7 was lower in patients than in controls (0.3 ± 0.1 pg/mL vs. 0.8 ± 0.1 pg/mL, respectively; \(P = .02\)).
Discussion

Hematopoietic stem cell transplantation offers the only chance of cure for patients with thalassemia. Haploidentical transplantation may extend this possibility to the 50-60% 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 immunological tolerance may exist between mother and offspring. Van Rood et al. demonstrated a lower rate of acute GVHD in sibling transplants mismatched for non-inherited maternal antigens than in transplants mismatched for non-inherited paternal antigens.

We have reported the results of bone marrow transplantation in children with acute leukemia in relapse resistant to chemotherapy, where their haploidentical mother was used as the donor of non-manipulated bone marrow.

The combination of a megadose of purified CD34+ cells and a highly immuno-myeloablative conditioning regimen is crucial for overcoming the barrier of residual anti-donor cytotoxic T-lymphocyte precursors in T-cell depleted mismatched transplants. The immune regulatory role of CD34+ cells is supported by observation that cells within the CD34+ population are endowed with veto activity; early myeloid CD33+ cells may also have this potential.

The infusion of $2 \times 10^5$ cell/kg bone marrow mononuclear cells freshly obtained from the bone marrow of the donor requires cyclosporine prophylaxis for GVHD during the first two months after transplant. However, the addition of bone marrow mononuclear cells (including NK cells, mesenchymal stem cells, T cell) to a T cell depleted allograft may help promote engraftment and control GVHD.
Haploidentical transplantation is associated with major post-transplant immune deficiency resulting in significant morbidity and mortality from infection. Delayed immune reconstitution post transplant may be associated with a variety of functional and immunophenotypic abnormalities at BM level, due to augmented local production of inflammatory cytokines, increased T-cell activation, or intrinsic hematopoietic and stromal cell abnormalities. After 20 days post transplant, a significant decrease in total lymphocyte counts and depletion of CD4+ T cells expressing predominantly the CD45RA+CD62L+ phenotype were observed. Also, the CD4+CD45RA+CD31+ T-cell subset *In vivo* and *in vitro*, hemato-lymphopoiesis occurs in association with the complex network of cell types found in the stroma, including non-hematopoietic (fibroblasts, adipocytes, and endothelial cells) and hematopoietic cells (macrophages and T cells). Progenitor cell growth and differentiation depend on their interaction with stromal cells. 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. Recent evidence shows that IL-7 acts as a master regulator of T-cell homeostasis, expanding both the naive and memory T-cell populations. Compared with controls, thalassemia patients exhibited altered stromal cytokine production at 20 days post transplant, 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. NK CD56+<sup>bright</sup> 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. Interestingly, we observed higher percentages of NK CD56+<sup>bright</sup> cells 20 days post transplant in patients with full engraftment, suggesting a role for donor NK cells in improved
engraftment and in prevention of rejection by an attack of the host lympho-hematopoietic cells.

After 60 days post transplant, a significant decrease in total lymphocyte counts, and depletion of CD4+ T cells expressing predominantly the CD45RA+CD62L+ phenotype were observed. Also the CD4+CD45RA+CD31+ T cell subset was significantly reduced in our cohort, suggesting a thymus involvement in these patients. Indeed, it is possible that the T-cell defect in thalassemia patients may occur at multiple levels, including egress from thymus.

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 bone marrow. The higher percentages of CD3-CD16+ in mixed chimerism patients may have a possible role on control of host cell escape and in maintainer the chimerism condition.

NK cells (CD56+) developed more rapidly than other lymphocytes, but CD56dim CD16+ NK cells were increased after 60 days post transplant, particularly in patients with full engraftment, suggesting a role for donor NK cells in bone marrow engraftment.

The prevalence of macrophage-like cells in long-term bone marrow culture as opposed to typical “fibroblast-like” cells suggests that the composition of the marrow stromal was altered, possibly due to an underlying inflammatory process within the bone marrow microenvironment. Stromal cells produce IL-7, which acts as a growth and anti-apoptotic factor for B and T cell precursors. This IL-7 production may be critical for the development of the new immune system from uncommitted progenitors infused with the graft. Stromal IL-7 production was decreased in transplant recipients, suggesting an important role for bone marrow accessory cells in immuno-hematological reconstitution post transplant. We hypothesize that the recovery of the T cell compartment resulted from deregulated production of new T cells from hematopoietic stem cells under the influence of the stromal
microenvironment. The results of this study suggest that it may be possible to boost engraftment and immune recovery via the administration of specific cytokines (i.e., IL-2 plus IL-7) and/or mesenchymal stem cells. One patient died on day +114 of Epstein-Barr virus cerebral lymphoma. The patient had low levels of CD8+ at the time of infection. No association between the number of CD19+ cells infused and occurrence of EBV reactivation was found. Despite the high incidence of CMV reactivation, only one patient died for CMV pneumonia.

In conclusion, the transplant protocol described herein, appears to be well tolerated and effective for eradicating the hematopoietic system in patients with thalassemia.

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P. Sodani designed the study and wrote the paper
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G. Adorno performed research
A. Lanti performed research
L. Faulkner performed research
M. Testi performed research
M. Andreani performed research
G. Lucarelli designed the study

The authors have no conflict of interest to declare.
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Tables

Table 1. Patients characteristics at transplantation for 22 patients younger than 17 years

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No. of Tx, number of red cell transfusions before transplantation; CI, chelation index; GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase; FS, portal fibrosis (Mi, mild; Mo, moderate; Se, severe; Ci, cirrhotic).
Figure

Figure 1. Kaplan-Meier probabilities of survival, thalassemia-free survival, and cumulative incidence of rejection and non-relapse mortality in 22 thalassemic patients younger than 17 years of age.
Figure 2. Proportion of donor engraftment in different lymphoid subsets at different times after BMT. (Patient 1)
Figure 3. Proportion of donor engraftment in different lymphoid subsets at different times after BMT. (Patient 2)
Figure 4. Proportion of donor engraftment in different lymphoid subsets at different times after BMT. (Patient 3)
Purified T-depleted, CD34+ peripheral blood and bone marrow cell transplantation from haploidentical mother to child with thalassemia

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