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Quantitative different red cells/nucleated cells chimerism in patients with long-term hematopoietic persistent mixed chimerism after bone marrow transplantation for thalassemia major or sickle cell disease

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Non standard abbreviations used: β-Thal, Homozygous β-thalassemia; SCD, sickle cell disease; RBCs, red blood cells; BFU-E, burst-forming unit erythroid; HSCT, hematopoietic stem cell transplantation;

Running Title: Red cells chimerism after HSCT.

Key words: thalassemia major, sickle cell disease, persistent mixed chimerism, hematopoietic stem cell transplantation.

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Abstract

**Background.** Persistent mixed chimerism represents a state wherein recipient and donor cells stably co-exist after haematopoietic stem cell transplantation. However, since in mostly of the studies reported in literature the engraftment state was observed in the nucleated cells, in this paper we determined the donor origin in the mature erythrocytes of patients with persistent mixed chimerism after transplantation for thalassemia major or sickle cell disease. Results were compared with the engraftment state observed in singularly picked-up burst-forming unit-erythroid colonies and in the nucleated cells collected from the peripheral blood and from the marrow.

**Design and Methods.** The donor origin of the erythrocytes was determined analyzing differences on the surface antigens of the erythrocytes suspension after incubation with anti-ABO and/or anti-C, -c, -D, -E and -e monoclonal antibodies by a flow cytometer. Short tandem repeats analysis was used to determine the donor origin of nucleated cells and burst-forming unit-erythroid colonies singularly picked up after 14 days incubation.

**Results.** A proportion of donor-derived nucleated cells of 71%, 46%, 15% and 25% was observed at day 1364, 1385, 1314 and 932 respectively, in four transplanted patients affected by thalassemia major or sickle cell disease. Similar results were also obtained in the erythroid precursors, analyzing the donor/recipient origin of the burst-forming unit-erythroid colonies, while on the contrary, at the same days of observation, a proportion of 100%, 100%, 73% and 90% donor-derived erythrocytes was observed in the four patients with persistent mixed chimerism.

**Conclusions.** Our results showed that mostly of the erythrocytes present in four long-term transplanted patients affected by thalassemia major or sickle cell disease, characterized by the presence of few donor engrafted nucleated cells, were of donor origin. The indication that small proportions of donor engrafted cells might be sufficient to clinical control the disease in these patients is relevant, although the biological mechanisms underlying these observations need to be further investigated.
Introduction

Notwithstanding the progress in supportive care\textsuperscript{1-2-3}, to date, hematopoietic stem cell transplantation (HSCT) represents the only today available curative treatment for patients affected by thalassemia major or sickle cell disease.\textsuperscript{4-7} Mixed chimerism, the simultaneous presence of both host- and donor-derived cells in the recipient, is often observed in a large proportion of these patients after HSCT\textsuperscript{8-11}.

A state of mixed chimerism detected early after transplantation often moves towards complete chimerism, though it may evolve into graft rejection, especially if the proportion of donor cells is lower than 25%. On the other hand, some patients have stable mixed chimerism, defined as persistent when donor- and host-derived cells coexist for periods longer than 2 years after HSCT\textsuperscript{12}. Patients with persistent mixed chimerism do not require additional RBC support and, regardless of the presence in some cases of an extremely low percentage of donor-derived nucleated cells, they are clinical controlled by an incomplete, but functional graft\textsuperscript{13-16}. These findings have tremendous implications not only in the context of allogeneic HSCT, but also in the design of gene therapy trials, based on the autologous transplantation of genetically modified CD34+ cells\textsuperscript{17}. The clinical control of the disease in the presence of persistent mixed chimerism indicates that the proportion of corrected nucleated stem/progenitor cells necessary to achieve a therapeutic level of circulating RBCs is within the frequency of gene transfer by ß-globin lentiviral vectors\textsuperscript{18}.

Most of the studies in the literature have investigated the impact of donor engraftment on nucleated cells rather than mature erythrocytes, which are functionally crucial for patients affected by thalassemia major or sickle cell disease. The evidence that the RBCs in patients with persistent mixed chimerism might originate from the donor is indirectly supported by observations relative to hemoglobin beta-globin chain synthesis\textsuperscript{12,13,19} or, more recently, by the analysis of single nucleotide polymorphisms (SNPs) expressed by genes encoding RBC antigens and structural proteins\textsuperscript{20}. We have very recently demonstrated the presence of a large majority of donor-derived RBCs in a transplanted thalassemic patient with persistent mixed chimerism in the nucleated cells by analyzing the differences between the donor and recipient surface erythrocyte markers using cytofluorimetry\textsuperscript{21}.

The aim of the present study was to determine the proportion of RBCs and erythroid precursors of donor-recipient origin in long-term transplanted patients with persistent mixed
chimerism, characterized by the presence of a low proportion of donor-derived nucleated cells in the peripheral blood and bone marrow.

**Design and methods**

**Patients**

The present study included 44 consecutive patients undergoing BM transplantation between June 2004 and February 2008 from a HLA identical 12/12 allele-level matched sibling, and checked at least once 2 years after the marrow infusion to determine the state of engraftment (table 1). Patients treated during the same period without a minimum follow-up of two years or that rejected the transplant were not included in the study. Forty patients were affected by β-Thalassemia major (β-Thal) and four by Sickles Cell Disease (SCD). All of the patients received a myeloablative conditioning regimen followed by the infusion of unmanipulated BM cells (median dose: 4.5x10^8/kg - range, 1.3-8.7x10^8/kg). Patients with β-Thal in class 1 or 2 (according to the Pesaro classification) were given a conditioning regimen based on 14 mg/kg busulfan (Bu) and 200 mg/kg cyclophosphamide (Cy). In addition, patients aged less than 4 years were treated with 10 mg/kg thiotepa (TT). β-Thal patients in class 3 were conditioned with 14 mg/kg Bu and a reduced dose of Cy (160 mg/kg). SCD patients were conditioned with 14 mg/kg Bu, 200 mg/kg Cy, and 10 mg/kg anti-thymocyte globulin (ATG) from day -6 to day -3 relative to infusion. BU was administered orally to 23 patients and intravenously to 21 patients. Post-HSCT GvHD prophylaxis consisted of cyclosporine (CSA), methylprednisolone (MP), and short methotrexate (MTX). Intravenous CSA was started at 5 mg/kg from day -2 to day +5, and later reduced to 3 mg/kg until post-transplant day 60 when it was tapered off 5% per week and discontinued at 365 days. The desired plasma range was 150-250 ng/mL. All patients discontinued immune-suppressive therapy with CSA at 1 year after transplantation if no signs of chronic GVHD were present. Intravenous MP was started at 0.5 mg/kg at day –1 and stopped 30 days post-transplant. Short MTX was given intravenously at 10 mg/m² on post-transplant days 1, 3, and 6 with folinic rescue. Table 1 shows the characteristics of the population studied.
Chimerism analysis of nucleated cells and BFU-E colonies
Peripheral blood and bone marrow samples were collected in EDTA at least once from donors and patients between days 20 and 60, on day 180, and thereafter during the annual routine follow-up examinations. Recipient and donor DNA samples were extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) or using the automatic DNA extractor (Promega, Madison, WI, USA), and the DNA typed by short tandem repeats (STR) and the amelogenin locus using the AmpF/STR Profiler Plus kit (Applera, Foster City, CA, USA). Amplification reactions were carried out using 1-2 ng of input DNA following the manufacturer’s recommendations. PCR products were run on an ABI Prism 3130xl Genetic Analyzer (Applera, Foster City, CA, USA). Informative loci in post-transplant samples were screened to quantify the percentage of donor cells in mixed chimeras. HSCT engraftment was quantified using fluorescent PCR primers for human identity markers based on the ratio between the peak areas of donor and recipient alleles. The mean value obtained after performing calculations for each informative STR was taken as the percentage of mixed chimerism. BFU-E colonies were grown in agar and singularly picked for STR evaluation.

Clonogenic assay
Assays for clonogenic hematopoietic progenitors were performed in methylcellulose semisolid cultures. Briefly, 1-2x10^5 low-density BM/PB cells were plated in duplicate in 35 mm tissue culture dishes, suspended in 1 mL methylcellulose medium supplemented with stem cell factor (SCF), granulocyte/macrophage colony stimulating factor (GM-CSF), interleukin (IL)-3, and erythropoietin (EPO) (Methocult GFH4434, Stem Cell Technologies, Vancouver, British Columbia, Canada). Cultures were incubated at 37°C in a fully humidified atmosphere containing 5% CO2. Plates were scored for BFU-E growth after 14 days incubation. Using an inverted microscope, individual colonies were picked up from the petri dishes and dispersed to single cell suspensions in 100 μL saline to assess the donor/recipient origin of the individual colonies using STR.

Chimerism analysis of RBCs
For cytofluorimetric analysis, RBCs were washed and diluted in saline (0.5% final dilution). Five micro liters of cell suspension were incubated with anti-ABO and anti-C, c, D, E and e monoclonal antibodies, following the manufacturer’s instructions (ABH- and RH- Erythrokit, Institute Jacque Boy SA, Reims, France). After the incubation, cells were washed with phosphate-buffered saline (PBS) and incubated with FITC-conjugated anti-human Ig. After incubation and two additional washes, analysis was performed using a FC500 flow cytometer and transferred to the CXP analysis program (Beckman-Coulter Hialeh, FL, USA).
**Ethical approval**
The study was approved by the Local Ethical Committee (*July 2008, N.109/08*) and samples were taken after obtaining written informed consent. Informed consent was obtained from all the patients before the clinical standardized treatment.

**Results**

**Analysis of engraftment in nucleated cells.**
The presence of recipient- and donor-derived nucleated cells in the PB and/or BM of the recipient was observed in 7 of the 44 (15.9%) transplant patients affected by thalassemia major or sickle cell disease on the day of their last clinical contact (follow-up range 720-1673 days post-transplant, median 1010 days post-transplant). Engraftment monitoring analysis showed that 3 patients had more than 96% donor-derived nucleated cells in PB (data not shown), whereas in patients UPN 31, UPN 35, UPN 41, and UPN 57, the proportion of donor-derived nucleated cells was 71%, 46%, 15%, and 25%, respectively (table 2). The chimerism kinetic determined analyzing the nucleated cells in the PB and in the BM of these patients is summarized in figure 1. None of these 4 patients suffered of acute or chronic graft versus host disease (GvHD). At the day of their last clinical control all the patients were in good conditions and transfusion independent, with blood count values within a range of normality (Table 3).

**Analysis of engraftment in RBCs**
We determined the proportion of donor-derived RBCs by analyzing differences in erythrocyte surface markers between the donor and recipient RBCs, using cytofluorimetric analysis in the four patients with low donor engrafted nucleated cells described above. We found a very high proportion of donor-derived RBCs, indicating the presence of quantitative different red cells/nucleated cells chimerism. In the four patients, the detection of erythrocytes to determine donor-recipient origin was performed after more than 1 year from the last RBC transfusion therapy. Studying the erythrocyte group markers “0” vs. “B” in patient UPN 31 (Figure 1A), RBCs were consistently 100% of donor origin on post-transplant days 635, 836, 1007, and 1364. Similar results were detected in patient UPN 35 on post-transplant days 575, 940, and 1371 (Figure 1B) for differences in Rh system surface markers “e” vs. “E”. The presence of quantitative different red cells/nucleated cells chimerism was also observed in patients UPN 41 and UPN 57 (Figures 1C-D), though the proportion of donor-derived erythrocytes in these cases was high, but not complete. In patient UPN 41 (ABO difference “0” vs. “B”), the proportion of donor-derived RBCs was 80% on day 935 and 73% on day 1314, whereas in UPN57, the proportion was 90% on day 935 (ABO difference “A” vs.
The proportion of donor-derived RBCs was also examined in the three patients with persistent mixed chimerism characterized by a large proportion of donor-derived nucleated cells and the four patients with full donor engraftment in the nucleated cells. As expected in all of these patients, the proportion of donor-derived RBCs was 100% (data not shown).

**Analysis of engraftment in BFU-E**

The ability of individual early progenitors to give rise to colonies of erythroid cells was assayed by plating the BM or PB cells isolated from the patients with persistent mixed chimerism in semisolid medium. Single BFU-E colonies were picked from the BM/PB cultures of patients UPN 31, 35, and 41 to determine the origin of the erythroid precursors and compare it with the characteristics of the RBCs. In patient UPN 31, the proportion of donor-derived BFU-E colonies cultured from the PB and BM was 93% on post-transplant day 1007, and 96% on days 1182 and 1364. In patient UPN 35, the proportion of donor-derived BFU-E colonies was 63% and 46% in the PB and 41% and 55% in the BM on post-transplant days 940 and 1371, respectively. A similar composition was obtained for patient UPN 41 on post-transplant day 1314 as the proportion of donor-derived BFU-E colonies obtained from the PB and BM was 11% and 35%, respectively. Due to technical problems, we could not analyze the proportion of BFU-E on the day of last contact with patient UPN 57. Table 2 summarizes the data relative to the proportion of donor-derived BFU-E on the day of last clinical contact, compared to the percentage of donor-derived RBCs and nucleated cells.

**Discussion**

The presence of mixed chimerism early after transplant is associated with an increased risk of graft rejection in patients treated with HSCT for thalassemia major or sickle cell disease, mostly when the proportion of donor-derived cells determined in the PB or BM early after transplant is less than 75%\(^{10-13}\). On the other hand, patients that move into a state of stable mixed chimerism, which becomes persistent when the recipient overcomes a minimum post-transplant follow-up period of 2 years, are no longer exposed to the risk of graft failure. The potential role of T regulatory cells in establishing persistent mixed chimerism has been recently pointed-out\(^{21-24}\). However, undependably from the biological mechanisms involved, data from several studies showed that patients with persistent mixed chimerism have a clinical control of the disease, despite the presence of an extremely low proportion of donor nucleated cells\(^{10-13,25}\). The majority of studies concerning the assessment of persistent mixed chimerism, however, have almost exclusively focused the attention
on the percentage of donor engraftment in the nucleated cells rather than in the mature erythrocytes, cells functionally crucial for patients affected by thalassemia major or sickle cell disease.

Our results showed that in long-term transplanted patients with persistent mixed chimerism, despite the presence of few donor engrafted nucleated cells in the PB and BM, the erythrocytes were almost completely of donor origin. Three patients in particular, at the time of their last follow-up, had a proportion of donor-derived nucleated cells in the PB/BM of 46/54%, 15/25%, and 25/ND%, whereas the percentage of donor-derived RBCs was 100%, 73%, and 90%, respectively. We further investigated the proportion of erythroid precursors in the BM to assess if the distribution was similar to the nucleated cells or if it overlapped the RBCs picture. Interestingly, the donor proportion of the BFU-E colonies examined was equivalent to the nucleated cells, showing a quantitative different erythroid precursors/erythrocytes chimerism. β-Thal is characterized by ineffective erythropoiesis leading to severe anemia and extensive erythroid expansion, caused in part by accelerated apoptosis of the thalassemic erythroid precursors, roughly 15-fold above the healthy controls\(^{28-30}\). The evidence for ineffective erythropoiesis in patients affected by severe SCD, developing mixed chimerism early after allogeneic non-myeloablative stem cell transplantation was described by Wu\(^{26}\). A possible explanation for the presence of a greater proportion of donor-derived erythrocytes with respect to the BFU-E observed in the persistent mixed chimeras may be found in the improved survival of donor RBC precursors compared to their host counterparts, which might be destroyed during ineffective erythropoiesis. However, because the results of chimerism on BFU-E are comparable to those observed in BM and nucleated cells, the disappearance of the recipient erythroid cells must take place at the level of more mature BM erythroid precursors.

Recently, Serafini et al. determined the presence of quantitative different red cells/nucleated cells chimerism between nucleated cells and RBCs by analyzing erythrocyte surface markers in a long-term transplanted patient using cytometry, and showed that the majority of the patient’s erythrocytes were of donor origin, whereas the nucleated cells were mostly derived from the recipient\(^{21}\). Similar results were obtained by Felfly and Trudel in thalassemic mutant mice which were transplanted in order to determine the minimal percentage of normal BM cells necessary for correcting the thalassemic phenotype in a competitive re-population transplantation assay\(^{27}\). Their results showed a 2- to 2.5-fold amplification of normal RBCs compared to white blood cells (WBCs) in the peripheral blood of mice presenting 19–24% BM chimerism, indicating the evidence of an \textit{in vivo} selective advantage for the normal RBCs. Recently, Miccio demonstrated that in a gene therapy preclinical model there is a selective advantage for the genetically-corrected erythroid component, leading to correction of thalassemia in mice engrafted with as low as 30% of transduced
HSCs. Armistead et al. constructed a panel composed of 10 different genes uniquely expressed in RBCs and characterized by polymorphisms with high minor-allele frequencies to investigate RBCs engraftment in hemoglobinopathies. The panel increased in donor-derived reticulocyte RNA compared to the recipient’s baseline endogenous erythropoietic capacity, which manifested as recipient-derived nucleated RBC progenitors and reticulocyte RNA.

Although these data have been obtained in a limited number of patients and that further studies on a larger cohort are needed to corroborate our preliminary findings, these results support the evidence that low levels of donor engraftment can result in significant functional improvement for patients with thalassemia major or sickle cell disease. Moreover, in the future, RBCs chimerism evaluation might become a relevant clinical information in the routinely engraftment monitoring. The observations of few engrafted cells being sufficient to clinical control patients affected by a thalassemia major or sickle cell disease are particularly interesting in light of a possible gene therapy approach, as the gene defect might be corrected by introducing the normal gene into the patient stem cells. In fact, previous experience in clinical trials, for both thalassemia major and other genetic diseases, has shown that not all cells carrying the genetic defect can be repaired. When genetically modified stem cell transplantation will be a possible option for treating thalassemia major, the co-existence of the repaired cells with those still expressing the genetic defect will be an expected scenario, not in an allogeneic, but in an autologous, environment. Also in this light a better understanding of the mechanisms underlying the establishment of quantitative different red cells/nucleated cells chimerism will be particular relevant.
Funding

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Authorship and disclosures

MA designed the research, analyzed data and wrote the paper; MT analyzed data and contributed in writing the paper. GL supervised the research and revised the paper; PT, LD, FA, DF, RC, FR and M.B. performed molecular and erythroid research experiments; JG and PS followed the clinical aspects of the transplanted patients; GF, AB and MT revised the paper. The authors declare no competing financial interests.

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Table 1. Clinical characteristics of the 44 patients in the present study

<table>
<thead>
<tr>
<th>Disease</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta Thalassemia major</td>
<td>40</td>
</tr>
<tr>
<td>Sickle Cell Disease</td>
<td>4</td>
</tr>
</tbody>
</table>

**Gender**

<table>
<thead>
<tr>
<th>Gender</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>28</td>
</tr>
<tr>
<td>Female</td>
<td>16</td>
</tr>
</tbody>
</table>

**Age:**

| Median | 8.5 years (range 2 - 24 years) |

**HLA compatibility:**

related - 12/12 alleles matched

**Follow-up:**

| Median | 1010 days (range: 720 and 1673) |

Table 2. Engraftment state in RBCs, Nucleated Cells and BFU-E at the last clinical control in patients with persistent mixed chimerism.

<table>
<thead>
<tr>
<th>UPN</th>
<th>Disease</th>
<th>Day of last clinical contact</th>
<th>% of donor nucleated cells in PB</th>
<th>% of donor nucleated cells in BM</th>
<th>% of donor RBCs in PB</th>
<th>% of donor BFU-E in PB</th>
<th>% of donor BFU-E in BM</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>β-Thal</td>
<td>1364</td>
<td>71</td>
<td>80</td>
<td>100</td>
<td>96</td>
<td>96</td>
</tr>
<tr>
<td>35</td>
<td>β-Thal</td>
<td>1371</td>
<td>46</td>
<td>51</td>
<td>100</td>
<td>55</td>
<td>46</td>
</tr>
<tr>
<td>41</td>
<td>β-Thal</td>
<td>1314</td>
<td>15</td>
<td>20</td>
<td>73</td>
<td>11</td>
<td>35</td>
</tr>
<tr>
<td>57</td>
<td>SCD</td>
<td>932</td>
<td>25</td>
<td>ND</td>
<td>90</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

UPN, unique patient number; PB, peripheral blood; BM, bone marrow; RBCs, red blood cells; BFU-E, burst-forming unit erythroid; ND, not determined; β-Thal, β-Thalassemia major; SCD Sickles Cell Disease.

Table 3. Hemocytometric blood tests at last clinical control in patients with persistent mixed chimerism.

<table>
<thead>
<tr>
<th>UPN</th>
<th>Day of last clinical control</th>
<th>RBC Mil/µl</th>
<th>WBC µl</th>
<th>Hb g/dl</th>
<th>% of Reticulocytes</th>
<th>% of Beta globin chain synthesis in PB</th>
<th>alfa/non alfa ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>1364</td>
<td>5,50</td>
<td>6500</td>
<td>10,9</td>
<td>1,5</td>
<td>100</td>
<td>1,48</td>
</tr>
<tr>
<td>35</td>
<td>1371</td>
<td>5,40</td>
<td>8800</td>
<td>10,8</td>
<td>1,4</td>
<td>100</td>
<td>1,45</td>
</tr>
<tr>
<td>41</td>
<td>1314</td>
<td>4,40</td>
<td>12000</td>
<td>10,1</td>
<td>3,7</td>
<td>100</td>
<td>1,9</td>
</tr>
<tr>
<td>57</td>
<td>932</td>
<td>3,70</td>
<td>6000</td>
<td>9,7</td>
<td>1,9</td>
<td>100</td>
<td>1,1</td>
</tr>
</tbody>
</table>

UPN, unique patient number; RBC, red blood cells; WBC, white blood cells; Hb, Hemoglobin.
**Figure 1.** Engraftment evolution over the time of patients with persistent mixed chimerism.

UPN, unique patient number; PB, peripheral blood; BM, bone marrow; RBCs, red blood cells.