Granulocyte–colony stimulating factor plus plerixafor in patients with β-thalassemia major results in the effective mobilization of primitive CD34+ cells with specific gene expression profile

Elena Baiamonte,1 Rita Barone,1 Flavia Contino,2 Rosalia Di Stefano,1 Anna Martia,3 Aldo Filosa,4 Emanuela D’Angelo,5 Salvatore Feo,2 Santina Acuto,1 Aurelio Maggio1
1Campus of Hematology F. and P. Cutino, Villa Sofia-Cervello Hospital, Palermo; 2Department of Biological, Chemical and Pharmaceutical Sciences and Technologies and ATeN Center, University of Palermo; 3Division of Hematology, Villa Sofia-Cervello Hospital, Palermo; 4Center for Anemias, A. Cardarelli Hospital, Napoli; 5Pediatric Clinic De Marchi, Policlinico Hospital, Milan, Italy

Abstract
Successful gene therapy for β-thalassemia requires optimal numbers of autologous gene-transduced hematopoietic stem and progenitor cells (HSPCs) with high repopulating capacity. Previous studies suggested superior mobilization in these patients by the combination of granulocyte-colony stimulating factor (G-CSF) plus plerixafor over single agents. We mobilized four adult patients using G-CSF+plerixafor to assess the intra-individual variation of the circulating CD34+ cells number and subtypes pre- and post-plerixafor administration. The procedure was well-tolerated and the target cell dose of ≥8×10^6 CD34+ cells/kg was achieved in three of them with one apheresis procedure. The addition of plerixafor unani- mously increased the number of circulating CD34+ cells, and the frequency of the most primitive CD34+ subtypes: CD34+/38- and CD34+/133+/38- as well as the in vitro clonogenic potency. Microarray analyses of CD34+ cells purified from the leu- kapheresis of one patient mobilized twice, with G-CSF and with G-CSF+plerixafor, highlighted in G-CSF+plerixafor-mobilized CD34+ cells, higher levels of expression genes involved in HSPC motility, homing, and cell cycles. In conclusion, G-CSF+plerixafor in β-thalassemia patients mobilizes optimal numbers of HSPCs with characteristics that suggest high capacity of engraftment after transplantation.

Introduction
Autologous hematopoietic genetically modified stem and progenitor cells (HSPCs) represent an emerging therapeutic option for curing β-thalassemia, particularly in patients who lack a suitable donor for allogeneic bone marrow transplantation (BMT). In recent years, clinical trials of gene therapy for β-thalassemia are ongoing in multiple centers and proof-of-principle of efficacy and safety has already been obtained in a number of patients.1

The success of gene therapy for β-thalassemia, in which engrafted gene-corrected stem cells do not have a selective advantage over unmodified cells, greatly depends on the number and the quality of autologous HSPCs collected for genetic modification. Blood mobilization using granulocyte-colony-stimulating factor (G-CSF) and collection of HSPCs by apheresis, are relatively easier and lesser invasive procedures than BM withdrawal and yield high numbers of CD34+ cells. For these reasons HSPCs mobilization represents a preferred source for allogeneic and autologous BMT in malignancies2 and, more recently, in stem cell gene therapies.1,3-4

It has been reported that patients with inherited hematological disorders responded differently to G-CSF stimulus. Indeed, significantly impaired HSPC-mobilization has been reported in patients with primary immunodeficiency disorders.7,8 In addition, in patients with sickle cell disease, the use of G-CSF has been shown to precipitate severe sickle cell crises and, in one case, death.9,10 In patients with β-thalassemia, G-CSF-induced hyper-leukocytosis and spleen enlargement were found to be the major dose-limiting factors ultimately resulting in poor CD34+ cell yields.11-14

Plerixafor, is a recently available mobilizing agent that promote HSC trafficking by a mechanism different than G-CSF. Plerixafor is a CXCR4 antagonist that mobilizes HSCs within a few hours by disrupting the engagement of the CXCR4 stem cell receptor with its ligand (SDF-1), which is expressed on marrow osteoblasts. In contrast, G-CSF indirectly mobilizes stem cells by down-regulating the expression of SDF-1 and by releasing proteolytic enzymes, which degrades important HSC trafficking and adhesion molecules.

Plerixafor promotes a more robust mobilization when used in combination with G-CSF in both healthy volunteers and patients with hematologic diseases.15,16

Plerixafor, alone or in combination with G-CSF, has also been used to improve the yield of HSPCs in β-thalassemia patients. Plerixafor, when used as single agent, was safer than G-CSF. However, a third of patients failed to achieve the pre-determined cell dose. Conversely, a combination including G-CSF and plerixafor was shown to be synergistic and to induce successfully mobilization in four patients in whom single agent-mobilization had previously failed.13,17

Here, we report a clinical study of mobilization via the use of G-CSF+Plerixafor in four adult transfusion-
Materials and Methods

Study approval

The study was approved by the local Institutional Ethics Committee and conducted in the Division of Rare Diseases of Blood and Hematopoietic Organs-Campus of Hematology F. and P. Cutino at the Villa Sofia-Cervello, hospital in Palermo. Informed consent for privacy and research use was obtained from all eligible patients in accordance with the Declaration of Helsinki.

Patient characteristics and hematopoietic genetically modified stem and progenitor cells mobilization

Four patients with β-thalassemia major who were similar in age and weight were enrolled in the study based on the criteria listed in Table 1. Patient characteristics are listed in Table 2. The patients received subcutaneous injections of 10 μg/kg of G-CSF (Filgrastim, Amgen, Thousand Oaks, CA, USA) in the morning from day 1 to day 4 and 240 μg/kg of plerixafor (Mozobil, Sanofi-Genzyme, Cambridge, MA, USA) on day 4 at 12 hours after treatment with G-CSF. Leukapheresis was performed 10-12 h later, on day 5. If fewer than 8×10⁶ CD34+ cells/kg were collected during the first apheresis, an additional dose of plerixafor was administered on the evening of day 5, and a second apheresis procedure was performed 12 hours later (on day 6). The leukapheresis product was harvested using a cell separator (COM.TEC® multi-procedural apheresis platform, Fresenius KABI, Bad Homburg, Hessen, Germany) according to institutional standards. The volume of blood that was processed per leukapheresis was approximately three times the total blood volume. The entire process of mobilization and apheresis was performed in outpatient clinic. A minimum of 6×10⁶ cells were immune-enriched and cryopreserved for future β-globin/lentiviral vector transduction and a volume of leukapheresis containing 2×10⁶/kg CD34+ cells was stored as back-up in case of graft failure, according to JACIE guidelines, at the Bone Marrow Transplantation Unit of our hospital.

Efficacy outcomes and safety

Efficacy outcomes included the number of patients who reached the target dose, the number of apheresis that was required, and the fold increase in circulating CD34+ cells/µl after plerixafor administration.

Table 1. Eligibility criteria.

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Diagnosis of β-Thalassemia Major.</td>
<td>1. Hepatitis B and C, HIV 1 and HIV 2, HTLV 1, HTLV2 or other active infections.</td>
</tr>
<tr>
<td>3. Lack of an HLA-matched sibling to undergo an allo-traspantation</td>
<td>3. History of thrombosis or known thrombophilia (Factor V Leiden, positive anti-phospholipidic-anticardiolipin antibodies).</td>
</tr>
<tr>
<td>4. Without hydroxyurea and erythropoietin administration for at least three months before the study.</td>
<td>4. Pregnancy or breast-feeding.</td>
</tr>
<tr>
<td>5. Performance of Karnofsky status &gt; 70.</td>
<td>5. Severe pulmonary hypertension.</td>
</tr>
<tr>
<td>6. Optimal hepatic function with ALT &lt;3N, serum total bilirubin &lt;2.0 (unless secondary to haemolysis), no evidence of cirrhosis in the according to abdomen US and fibroscan.</td>
<td>6. Neoplastic syndrome familiarity.</td>
</tr>
<tr>
<td>7. FEVS &gt; 50%</td>
<td>7. Splenectomized patients with a platelet count &gt; 900.000/mmc.</td>
</tr>
<tr>
<td>8. Renal function: creatinine &lt;1.5 mg/dl, when serum creatinine is outside of the normal range, creatinine clearance (CrCl)&gt;60 ml/min/1.73 m².</td>
<td></td>
</tr>
<tr>
<td>9. Splenectomized or non-palpable spleen.</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Patient characteristics and side effects during mobilization.

<table>
<thead>
<tr>
<th>Pt 1</th>
<th>Age (yrs)</th>
<th>Gender</th>
<th>Weight (kg)</th>
<th>Genotype (mutation)</th>
<th>Chelation</th>
<th>Ferritin (ng/ml)</th>
<th>Spleen</th>
<th>Side effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>22</td>
<td>F</td>
<td>47</td>
<td>+/+</td>
<td>DFP</td>
<td>1938</td>
<td>Not palpable</td>
<td>Bone pain, Warm feeling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pulse-pounding.</td>
</tr>
<tr>
<td>Pt 2</td>
<td>22</td>
<td>M</td>
<td>50</td>
<td>+/+</td>
<td>DFX</td>
<td>1143.9</td>
<td>Splenectomy</td>
<td>Bone pain, Warm feeling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pulse-pounding, Leukocytosis</td>
</tr>
<tr>
<td>Pt 3</td>
<td>21</td>
<td>F</td>
<td>56</td>
<td>+/+</td>
<td>DFX</td>
<td>603.4</td>
<td>Not palpable</td>
<td>Bone pain, Fatigue</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Anemia</td>
</tr>
<tr>
<td>Pt 4</td>
<td>23</td>
<td>F</td>
<td>51</td>
<td>+/+</td>
<td>DFX</td>
<td>485.4</td>
<td>Not palpable</td>
<td>Bone pain, Thrombocytopenia</td>
</tr>
</tbody>
</table>

[page 12] [Thalassemia Reports 2017; 7:6392]
Safety was evaluated as the incidence of adverse events in daily clinical and hematological follow-ups that were performed during and after mobilization, until the complete normalization of blood cell counts was achieved. In non-splenectomized patients, the degree of enlargement of the spleen was evaluated daily using ultrasonography. If the spleen volume increased by more than 80% from baseline, G-CSF was discontinued, and the patient was withdrawn from the study. Hyperleukocytosis (WBC ≥50×10^6/µl) led to a reduction in the G-CSF dosage or the discontinuation of treatment in cases of excessive leukocytosis (WBC ≥100×10^6/µl).

**CD34+ cell counts and immune-phenotyping**

Absolute numbers of PB-CD34+ cells were measured daily using a flow cytometer (FC-500 Beckman-Coulter, Cassina de Pecchi, Milano, Italy) according to the ISHAGE protocol using a single-platform method (Stem-Kit, Beckman-Coulter).

CD34+ cell subtyping was performed after 4 doses of G-CSF (on the morning of day 4) and at 10-12 hours after plerixafor administration (on the morning of day 5). Subtyping was done using a four-colour method with antibody combinations in a Beckman Coulter cytometer according to the manufacturer’s recommendations. Cells were stained using the following monoclonal antibodies or corresponding isotype controls: FITC-conjugated CD45, ECD-conjugated CD34, PC5-conjugated CD38 (Beckman Coulter) and PE-conjugated CD133 (Miltenyi Biotec, Bergisch Gladbach, Germany). Data were analysed using the software (Cxp Cytometer analysis software and Kaluza; Beckman Coulter).

** Colony forming unit assay**

Blood samples, containing 3×10^3 CD34+ cells, collected after G-CSF or G-CSF+plerixafor mobilization, were centrifuged, and the cellular portion lysed to eliminate mature erythrocytes. The remaining cells were plated in triplicate in complete methylcellulose medium (MethoCult H4434 Enriched - StemCell Technology, Vancouver, British Columbia, Canada) and incubated at 37°C in 5% CO2 for 14 days. Identification and counts of colony forming unit-granulocyte/macrophage (CFU-GM) and burst-forming unit-erythroid (BFU-E) were assessed using an inverted microscope. The erythroid colonies consisting of maximum 8 clusters were classified as small BFU-E, and those that consisted of 9 or more clusters were classified as large BFU-E.

**Microarray hybridization and analysis**

Whole gene expression profiling was performed on immuno-selected CD34+ cells (purity higher than 90%) using a CliniMacs CD34 Kit (CliniMacs, Miltenyi Biotec, Bergisch Gladbach, Germany). CD34+ cells were derived from the leukapheresis of patient 1 that was mobilized twice, once with G-CSF+plerixafor (CD34/G+pl) and once, one year later, with G-CSF alone (CD34/G).

In particular, CD34/G cells were enriched from the combined leukapheresis products that were collected, at MSKCC, NY, USA, for gene therapy purposes on days 5 and 6 of mobilization using G-CSF (10 µg/kg/day).

Total RNA was isolated using TRIZOL according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA), and dissolved to a final concentration of 0.2-0.5 µg/µl. The quality of the RNA was tested using capillary electrophoresis in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and the samples exhibited common high quality RNA integrity numbers (RINs) ranging from 8 to 9.

Fluorescently labelled cRNA targets were generated from 150 ng of total RNA in each reaction using a Low RNA Input Linear Amplification Kit and Cyanine 3- or Cyanine 5-labelled CTP according to the manufacturer’s instructions (Agilent Technologies). Labelled cRNAs were purified using an RNeasy Mini Kit (Qiagen) and applied to a 4×44 K Whole Human Genome Oligo Microarray Kit (G4112F, Agilent Technologies).

For each hybridization, 825 ng of cyanine-labelled cRNA from each sample was mixed with an equal amount of reverse-colour cyanine-labelled cRNA from Universal Human Reference RNA (Stratagene, La Jolla, CA), which was used as a common reference. Hybridization and washing were performed using an in situ hybridization kit according to the manufacturer’s instructions (Agilent Technologies). The arrays were scanned using a dual-laser DNA microarray scanner (Sure Scan, Agilent Technologies). The data from the CD34/G+pl and CD34/G RNA samples were then normalized to the Universal Human Reference RNA (Stratagene, La Jolla, CA), and an initial gene list was created using confidence filtering at P<0.01 (t-test P-values were used as the measure of confidence, and the Benjamini-Hochberg false discovery rate was used for multiple testing correction) to eliminate genes with unreliable measurements. Consecutive lists of differentially expressed genes were generated assuming a 2-fold expression rate and using data from all independent experiments.

Gene ontology analysis was first performed using DAVID Bioinformatics Resource online software (ver. 6.7, NIAID, NIH: http://david.abcc.ncifcrf.gov/), and pathway analysis was subsequently performed using the same software.

**Statistical analysis**

The data are provided as the median, range or maximum values. Mean, standard deviation and p-value were performed with the STATA 12.0 software (StataCorp, USA) applying the Student t-test.

**Results**

**Patients and safety**

According to the Common Terminology Criteria for Adverse Events, version 4.0, the treatment was well tolerated with minimal side effects (Table 2). The splenectomized patient (Pt 2) developed hyperleukocytosis (WBC 60.2×10^6/µl) and required the G-CSF dosage dose to be halved from day 2 of mobilization (Table 3).

An average Hb reduction of 0.9 g/dl and an average reduction in platelet numbers were observed at the end of the mobilization period compared with baseline (354.5±187.8 vs 175±97.5). After the second apheresis, patient 3 developed intermediate grade thrombocytopenia (platelet count of 68.0×10^3/µl) that recovered completely within one week and a reduction in hemoglobin levels to 8.7 g/dl, which required a blood transfusion.

Other mild side effects lasted for 1-2 days after apheresis and resolved spontaneously. The patient’s hematological
parameters during mobilization are shown in Table 3.

G-CSF+plerixafor results in efficient CD34+ cell mobilization

8×10^6 CD34+ cells/kg was defined as target cell dose for gene therapy. Three out of four patients achieved the target cell dose in a single apheresis collection (8.15×10^6 cells/kg, median value), including the patient 2, despite the fact that G-CSF dose was reduced in this patient starting from day 2 to avoid hyperleukocytosis (Table 3).

In patient 3, the number of cells collected in the first apheresis was slightly below the established target and a second apheresis collection was performed on day 6, at 12 hours after an additional dose of plerixafor was administered. The total yield from the combined apheresis in this patient was 13.5 CD34+ cells/kg (Pt 3, Table 3). The kinetics of HSPC mobilization were analysed by counting the PB-CD34+ cell daily during treatment (Table 3). The addition of plerixafor mobilized a substantial portion of CD34+ cells in all of the patients, resulting in a 12-fold mean peak increase (range, 4.8- to 23-fold).

Addition of plerixafor increases the mobilization of CD34+ cell subsets with a more primitive phenotype and higher clonogenic capacity

To investigate whether plerixafor exposure causes the release of a subset of HSPCs distinct from GCSF-primed progenitor cells, we studied the co-expression of the surface antigens CD133 and CD38 in PB-CD34+ cells before and after plerixafor was administered.

The frequency of primitive CD34+ subtypes (CD34+/CD133- and CD34+/CD133+) consistently increased in all patients after plerixafor was added. CD34+/CD133- cells were increased by 1.3-fold. A significant increase was also observed in the frequency of the most primitive CD34+ sub-type, CD34+/CD133+/CD138- cells, after plerixafor addition. The same analysis performed in PB sample obtained from patient 3 at 11 hours after the second administration of plerixafor, showed no change in the proportion of CD34+ subtypes compared to that of the day before (data not shown).

These data suggest that the difference in subset composition that was observed between days 4 and 5 can be attributed to plerixafor stimulation instead of a longer period of mobilization. In support of this hypothesis, we also did not detect substantial changes in the proportion of PB-CD34+ cell subsets in the three healthy donors on days 4 and 5 during mobilization with G-CSF (10 μg/kg/day) used as single agent (data not shown). These data are in line with those reported by others regarding the frequency of CD34+/CD38- cells in PB of donors on days 4, 5 and 6 of G-CSF mobilization.18

Clonogenic capacity of PB CD34+ cells was investigated in short-term colony forming (CFC) assay based on the numbers and types of colonies that developed, before and after plerixafor.

The absolute number of CFU-GM and BFU-E that developed from PB-CD34+ cells plated at day 5 was unanimously higher than that developed from the same number of cells plated, before plerixafor addition. In addition, the proliferative capacity of the progenitors also increased after plerixafor was added, as observed comparing the sizes of the BFU-E colonies that devel-

Table 3. Blood CD34+ cell counts and hematological parameters during G-CSF+plerixafor mobilization.

<table>
<thead>
<tr>
<th>Day</th>
<th>+1</th>
<th>+2</th>
<th>+3</th>
<th>+4</th>
<th>+5*</th>
<th>+6*</th>
<th>+7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt 1</td>
<td>WBC 10^9/L</td>
<td>6.6</td>
<td>25.0</td>
<td>32.1</td>
<td>30.0</td>
<td>60.3</td>
<td>19.4</td>
</tr>
<tr>
<td></td>
<td>ANC 10^9/L</td>
<td>3.9</td>
<td>21.4</td>
<td>27.1</td>
<td>24.3</td>
<td>40.2</td>
<td>14.1</td>
</tr>
<tr>
<td></td>
<td>HGB g/dL</td>
<td>11.5</td>
<td>10.9</td>
<td>10.7</td>
<td>10.6</td>
<td>10.6</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>PLT 10^9/L</td>
<td>294</td>
<td>260</td>
<td>262</td>
<td>279</td>
<td>318</td>
<td>211</td>
</tr>
<tr>
<td></td>
<td>CD34+/uL</td>
<td>4</td>
<td>8</td>
<td>10</td>
<td>48</td>
<td>361</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>CD34+/Kg</td>
<td>8.3×10^6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt 2*</td>
<td>WBC 10^9/L</td>
<td>12.8</td>
<td>60.2</td>
<td>56.6</td>
<td>55.8</td>
<td>92.2</td>
<td>21.5</td>
</tr>
<tr>
<td></td>
<td>ANC 10^9/L</td>
<td>5.9</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>61.6</td>
<td>15.7</td>
</tr>
<tr>
<td></td>
<td>HGB g/dL</td>
<td>10.8</td>
<td>9.7</td>
<td>9.8</td>
<td>10.8</td>
<td>10.1</td>
<td>10.2</td>
</tr>
<tr>
<td></td>
<td>PLT 10^9/L</td>
<td>633</td>
<td>587</td>
<td>580</td>
<td>588</td>
<td>530</td>
<td>292</td>
</tr>
<tr>
<td></td>
<td>CD34+/uL</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>92</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>CD34+/Kg</td>
<td>8.3×10^6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt 3</td>
<td>WBC 10^9/L</td>
<td>4.2</td>
<td>24.3</td>
<td>27.5</td>
<td>30.0</td>
<td>68.1</td>
<td>65.0</td>
</tr>
<tr>
<td></td>
<td>ANC 10^9/L</td>
<td>2.1</td>
<td>19.8</td>
<td>22.8</td>
<td>25.7</td>
<td>42.3</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>HGB g/dL</td>
<td>11.2</td>
<td>9.2</td>
<td>9.0</td>
<td>9.8</td>
<td>5.6</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>PLT 10^9/L</td>
<td>225</td>
<td>220</td>
<td>213</td>
<td>250</td>
<td>239</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>CD34+/uL</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>115</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>CD34+/Kg</td>
<td>7×10^6</td>
<td>6.5×10^6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt 4</td>
<td>WBC 10^9/L</td>
<td>6.2</td>
<td>20.6</td>
<td>21</td>
<td>25.1</td>
<td>53</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>ANC 10^9/L</td>
<td>3.3</td>
<td>18.4</td>
<td>18.3</td>
<td>21.7</td>
<td>42.9</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>HGB g/dL</td>
<td>10.7</td>
<td>10.7</td>
<td>11.5</td>
<td>10.6</td>
<td>10.7</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>PLT 10^9/L</td>
<td>266</td>
<td>263</td>
<td>238</td>
<td>211</td>
<td>217</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>CD34+/uL</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>8</td>
<td>102</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>CD34+/Kg</td>
<td>8×10^6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values obtained after administration of plerixafor to GCSF regimen are indicated in italics. WBC, white blood cell; ANC, absolute neutrophil count; HGB, hemoglobin; PLT, platelet. *Days of treatment when the phe-notype and clonogenic ability were analyzed; °in this patient, the G-CSF dosage was halved beginning on day 2 of mobilization.
oped from PB-CD34+ cells at these two time points. On day 5 of mobilization, we observed a clear increase in the portion of intermediate/large BFU-E colonies that consisted of more than 9 clusters over the total number of BFU-E colonies.

G-CSF+Plerixafor combination mobilizes of CD34+ cells with gene expression pattern distinct from that of G-CSF-primed cells

We performed a microarray analysis to analyse global gene expression in CD34+ cells that were purified from the leuka- pheresis of patient 1 by two independent mobilization regimens: one with G-CSF+plerixafor (CD34/G+pl) and one, with G-CSF used as a single agent (CD34/G).

The CD34/G and CD34/G+pl samples were compared after normalization using Universal Human Reference RNA (Stratagene) as a common reference. A subset of differentially expressed genes was selected from all of the microarray data after they were first filtered according to confidence at P<0.01 and then by expression level (≥2-fold). Using these selection criteria, we found that 873 genes were commonly up-regulated and that 1691 genes were down-regulated in the CD34/G+pl cells than in the CD34/G cells.

The genes were classified according to their function using the Gene Ontology Classification System (Figure 1A and Supplementary Table 1). As shown in Figure 1B a substantial number of genes associated with homing and engraftment processes (e.g., CXCR4, CD82, DPP4 and ROBO4) and genes known to be linked to stress resistance (e.g., CXCL4, SOD2, IL8 and PPBP) were up-regulated. Several genes, encoding chemokines that have been shown to be involved in cell mobility (e.g., CXCL2, CX3 and CXCR2) were also up-regulated, and genes involved in transcriptional regulation (e.g., GATA-2, BTG2, DUSP2, MS4A3 and TNF) were either up- or down-regulated. The array analysis also indicated in both CD34/G+pl and CD34/G cells the substantial over-expression (more than 16-fold compared to the Universal Reference RNA) of several genes (RGS18, MPO, TNFSF4, EGR1) that are involved in mobilization, homeostasis, homing and engraftment (data not shown).

Discussion

The outcome of gene therapy for β-thalassemia, in addition to requiring sufficient genetic modification of CD34+ cells, and efficient myeloablative conditioning, large-ly depends on the quantity and the quality of the autologous hematopoietic stem cell collected. Indeed, to reduce morbidity result- ing from ineffective erythropoiesis and to achieve transfusion independence, it is neces-sary to infuse an adequate number of HSPCs that are capable of generating long-term gene-corrected erythroid precursors.

In this study, G-CSF+plerixafor mobiliza-tion was shown to be safe and efficacious for collecting high numbers of HSPCs. The target cell dose was achieved in all patients, and in three of them, it was achieved in a single apheresis collection. This result was similar to that previously reported by Yannaki and co-workers, in other 4 patients mobilized using the same combination; conversely, in other studies in which G-CSF was used as a single agent, similar cell yields were achieved after two apheresis collections in the majority of the patients.

It has been shown that G-CSF and pler-i xafor promote HSPCs trafficking via dif-ferent mechanisms, that may explain differ-ent HSPC subsets.

Papers describing differences in G-CSF and G-CSF+plerixafor grafts in healthy donors and onco-hematological patients have concluded that CD34/G+pl cells show more immature phenotypes than CD34/G cells.

Figure 1. Bar graph representations of the differential expression profiles of genes that were analysed in the microarray analysis. A) Bar graph of biological process overrepre-sented in CD34/G+pl cells compared to CD34/G cells. B) Log changes are shown to represent the difference in the expression level of genes differentially expressed between CD34/G+pl cells and CD34/G cells. -Log2-fold changes (FC) were calculated after nor-malization to Universal Reference RNA (P<0.01, FC cut-off ±2). Values greater than or equal to 1 represent gene up-regulation, and values less than 1 indicate gene down-regulation.
However, the hematopoietic cell repertoire in thalassemia patients may differ from that of other subjects because they endure chronic anaemia, prolonged transfusion regimens, and iron overload may alter the balance that regulates the retention/release of these cells. We were interested in characterizing intra-individual variations in CD34+ cells that were mobilized using G-CSF or G-CSF+plerixafor.

We found that the addition of plerixafor uniformly resulted in a substantial increase in the proportion of primitive CD34+ subtypes that have previously been shown to possess extensive proliferation and repopulation capacities.\textsuperscript{25,26} Moreover, CD34/G+pl cells displayed a higher capacity for clonogenicity and proliferation than CD34/G cells from the same patients. A trend towards higher frequencies of primitive cells has been previously observed in G-CSF+plerixafor grafts (compared to single-agent-mobilized grafts) obtained from thalassemia patients and from mouse model of thalassemia.\textsuperscript{14,17,27}

Our results corroborate and extend this finding at an intra-patient level that allowed us to abolish variability in cellular composition between individuals.

Expression profiling of CD34/G and CD34/G+pl cells, derived from patients with hematologic malignancies,\textsuperscript{28} and primitives\textsuperscript{29} mobilized with both modalities, revealed that CD34/G+pl cells expressed significantly higher levels of genes that promote superior engraftment after myeloablative. We found that also in thalassemia patients, gene expression profiles depended on the mobilization protocol. This result may explain published functional data that have shown that G-CSF+plerixafor grafts (compared to single-agent-mobilized grafts) obtained from thalassemia patients are more competitive than G-CSF grafts at engrafting murine recipients.\textsuperscript{30}

In particular, according to the mechanism of plerixafor mobilization, the expression level of CXCR4 was 5-fold higher in CD34/G+pl cells than in CD34/G cells. This result was analogous to that reported by Fruehauf and co-workers in patients with multiple myeloma and non-Hodgkin lymphoma.\textsuperscript{31}

CXCR4 is expressed on the surface of HSPCs, especially more primitive CD34+ cells with long-term repopulating potential, and it plays a central role in regulating the adhesion of HSPCs to their native niche in BM.\textsuperscript{32} Conversely, genes involved in the cleavage of VCAM-1 and SDF-1 (CXCL12) antigens, such as cathepsin (ELANE) and cathepsin G (CTSG), were overexpressed in both CD34/G+pl and CD34/G cells. These data are in line with a mechanism involving G-CSF mobilization.\textsuperscript{32}

The chemokines CXCL2, CX3 and CXCR2, involved in a rapid HSPCs mobilization, were also up-regulated in CD34/G+pl cells.\textsuperscript{33} Among the genes that were found to be differentially expressed in the two samples, there was a group of antigen surface genes that regulate adhesion to the microenvironment. In particular, CD82 and Robo4 were overexpressed and CD26 was down-regulated in CD34/G+pl cells. It has been reported both CD82 and Robo 4 play a key role in anchoring HSPC to BM niches and in long-term reconstitution,\textsuperscript{34-36} while inhibiting CD26 may increase the success of HSPC transplantation.\textsuperscript{37}

Several transcription regulators have also been shown to be up-regulated in CD34/G+pl cells. Among these, GATA-2, EGR1) involved in mobilization, homeostasis, self-renewal were also substantially overexpressed in CD34/G+pl cells.\textsuperscript{33} Among the genes that regulated the adhesion of HSPCs to their niche genes (RGS8, MPO, TNFSF4 and EGR1) involved in mobilization, homeostasis, homing, engraftment, cell survival and self-renewal were also substantially overexpressed in both samples.

Conclusions

Overall, the mobilization study reported here, in thalassemia patients, highlighted how the addiction of plerixafor to G-CSF-based mobilization not only enhanced cell yields, but also promoted the egress from BM of CD34+ cells with phenotypic and functional characteristics and gene expression profiles that suggest high capacity of engraftment after transplantation.

References


