EUROPEAN HEMATOLOGY ASSOCIATION

Ferrata Storti Foundation

Haematologica 2018
Volume 103(5):778-786

Plerixafor enables safe, rapid, efficient mobilization of hematopoietic stem cells in sickle cell disease patients after exchange transfusion

Chantal Lagresle-Peyrou,[1,2,3*] François Lefrère,[4*] Elisa Magrin,[1,4*] Jean-Antoine Ribeil,[1,4*] Oriana Romano,[3,5,6] Leslie Weber,[2,3,7] Alessandra Magnani,[1,4] Hanem Sadek,[1,2,3] Clémence Plantier,[1,4] Aurélie Gabrion,[1,4] Brigitte Ternaux,[1,4] Tristan Félix,[3,5] Chloé Couzin,[1,4] Aurélie Stanislas,[1,4] Jean-Marc Tréluyer,[8] Lionel Lamhaut,[9,10] Laure Joseph,[4] Marianne Delville,[2,3,4] Annarita Miccio,[3,5#] Isabelle André-Schmutz[1,2,3#] and Marina Cavazzana[1,2,3,4#]

[1]Biotherapy Clinical Investigation Center, Groupe Hospitalier Universitaire Ouest, Assistance Publique-Hôpitaux de Paris, INSERM CIC 1416, France; [2]Laboratory of Human Lymphohematopoiesis, INSERM UMR 1163, Imagine Institute, Paris, France: [3]Paris Descartes University – Sorbonne Paris Cité, Imagine Institute, France [4]Department of Biotherapy, Necker Children's Hospital, Assistance Publique-Hôpitaux de Paris, France; [5]Laboratory of Chromatin and Gene Regulation during Development, INSERM UMR1163, Imagine Institute, Paris, France; [6]Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy; [7]Paris Diderot University – Sorbonne Paris Cité, France; [8]Mère-Enfant Clinical Investigation Center, Groupe Hospitalier Necker Cochin, Assistance Publique-Hôpitaux de Paris, France; [9]Intensive Care Unit, Anaesthesia and SAMU de Paris, Necker Hospital, Assistance Publique-Hôpitaux de Paris, France and [10]Paris Descartes University – Sorbonne Paris Cité, France.

**CLP, FL and EM and JAR contributed equally to this work, in alphabetical order*

[#]AM, IAS and MC contributed equally to this work

Correspondence:

isabelle.andre-schmutz@inserm.fr

Received: November 15, 2017.

Accepted: February 13, 2018.

Pre-published: February 22, 2018.

doi:10.3324/haematol.2017.184788

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/103/5/778

ABSTRACT

Sickle cell disease is characterized by chronic anemia and vaso-occlusive crises, which eventually lead to multi-organ damage and premature death. Hematopoietic stem cell transplantation is the only curative treatment but it is limited by toxicity and poor availability of HLA-compatible donors. A gene therapy approach based on the autologous transplantation of lentiviral-corrected hematopoietic stem and progenitor cells was shown to be efficacious in one patient. However, alterations of the bone marrow environment and properties of the red blood cells hamper the harvesting and immunoselection of patients' stem cells from bone marrow. The use of Filgrastim to mobilize large numbers of hematopoietic stem and progenitor cells into the circulation has been associated with severe adverse events in sickle cell patients. Thus, broader application of the gene therapy approach requires the development of alternative mobilization methods. We set up a phase I/II clinical trial whose primary objective was to assess the safety of a single injection of Plerixafor in sickle cell patients undergoing red blood cell exchange to decrease the hemoglobin S level to below 30%. The secondary objective was to measure the efficiency of mobilization and isolation of hematopoietic stem and progenitor cells. No adverse events were observed. Large numbers of $CD34^+$ cells were mobilized extremely quickly. Importantly, the mobilized cells contained high numbers of hematopoietic stem cells, expressed high levels of stemness genes, and engrafted very efficiently in immunodeficient mice. Thus, Plerixafor can be safely used to mobilize hematopoietic stem cells in sickle cell patients; this finding opens up new avenues for treatment approaches based on gene addition and genome editing. *Clinicaltrials.gov identifier: NCT02212535.*

Introduction

Sickle cell disease (SCD) is caused by a point mutation in the coding region of the *HBB* (β-globin) gene. As a result, an abnormal β-globin protein is incorporated into hemoglobin tetramers. These mutant tetramers polymerize when the local oxygen tension is low. The sickle hemoglobin (HbS) polymers rigidify red blood cells, change these cells' shape, and are responsible for structural damage to the red blood cell membrane. In turn, this modifies the cells' rheological properties, alters their flow in the microcirculation, and thus causes ischemia, stroke, multi-organ damage, severe acute and chronic pain, and chronic hemolytic anemia. Progressive chronic organ complications become the main cause of morbidity and mortality in the third decade of life.[1] SCD is endemic in Africa, and the World's Health Organization considers that 7% of the world population carries the trait.

The only curative treatment for SCD is allogeneic hematopoietic stem cell transplantation (HSCT) from matched sibling donors; the disease-free survival rate 6 years after transplantation is reportedly >90%.[2,3] Given the limited availability of suitable donors and the increase in toxicity with age, HSCT is only applied with great caution in adult SCD patients (the main target population for curative treatment).

We recently demonstrated that gene therapy is applicable to SCD patients, and that the associated toxicity and morbidity rates seem to be lower than those for allogeneic HSCT, at least in the first treated patient.[4]

As is the case with all genetic diseases, the success of gene therapy in SCD relies on several key factors; these include the source, quality and number of transduced cells, the choice of the conditioning regimen, the level of therapeutic transgene expression, and the quality of the bone marrow (BM) microenvironment at the time of harvest and transplantation. It is generally acknowledged that 2 to 3x10^6 $CD34^+$ hematopoietic stem and progenitor cells (HSPC)/kg are required for a successful outcome in autologous HSCT.[5] Considering the typical proportion of HSPC that can be corrected in gene therapy clinical trials (~50% of $CD34^+$ HSPC) and an average recovery of 70% of $CD34^+$ cells post-selection, a minimum harvest of ~6x10^6 $CD34^+$ cells/kg would be required. For reasons that have not been completely elucidated, as for thalassemic patients,[6-7] the recovery of HSPC from SCD patients' BM is peculiarly low (M. Cavazzana, *unpublished data*). In our ongoing gene therapy trial (HGB-205, ClinicalTrials.gov number, NCT02151526), two BM harvests (each requiring an exchange transfusion program before general anesthesia) were needed to obtain enough cells to transplant the three SCD patients enrolled.[4]

Mobilization with granulocyte colony-stimulating factor (G-CSF, Filgrastim) is widely used to increase the harvest of HSPC (relative to that obtained in a BM aspirate). However, attempts to mobilize HSPC with Filgrastim in SCD have led to severe adverse events, which hamper the cytokine's use in this setting. The first report of a severe adverse event following mobilization with low-dose Filgrastim (2.5 μg/kg/day) concerned a SCD patient who developed acute chest syndrome and an elevated white blood cell count (63,400/mm^3) as early as 3 days after the first injection.[8] The temporal relationship between Filgrastim administration, the rapid rise in the white blood cell count and the severe adverse event were strongly suggestive of a causal link. Two other severe adverse events (multi-organ failure and a death) were reported in 2001.[9-10] Between 2003 and 2008, eight other patients requiring autologous HSCT for a malignant hematopoietic disease were mobilized with Filgrastim after additional precautions had been taken: reduction of the HbS level to below 30% via red blood cell exchange, and carefully monitoring of peripheral leukocytosis and the blood ion profile.[11-13] Although all eight patients experienced bone pain, hypertension or migraine, no severe adverse events were reported - providing evidence that SCD patients can be mobilized without any major complications. The median [interquartile range] circulating $CD34^+$ cell count was 24.4/μL [21.2-48.6].[12-14]

As an alternative to Filgrastim, Plerixafor (formerly known as AMD3100) can effectively mobilize HSPC; the $CD34^+$ cell count in the circulation can reach 15 to 40/μL, depending on the dose and the study.[15,16] In contrast to Filgrastim (which acts by activating monocytes and neutrophils in both peripheral blood and the BM), Plerixafor directly inhibits the binding of stroma-cell-derived factor-1α to its CXC chemokine receptor on HSPC – thus releasing stem cells from the BM niches. This mechanistic difference explains the small increase in the white blood cell count and the short time interval between Plerixafor

Table 1. Clinical parameters of patients treated with Plerixafor.

		SCD Pler 1 (19 years)				SCD Pler 2 (20 years)				SCD Pler 3 (21 years)			
	Normal values	D-1	D+1	D+7	D+30	D-1	D+1	D+7	D+30	D-1	D+1	D+7	D+30
HbS (HPLC G8)	0%	12·1%	12·6%	15·5%	36·0%	20·2%	15·8%	ND	37·1%	6·2%*,**	13·2%**	25·2%**	36·8%**
Total bilirubin (mol/L)	0-17	112	105	109	132	32	35	ND	32	30	30	31	53
Conjugated bilirubin (mol/L)	0-5	8	7	8	9	11	13	ND	12	10	10	10	6
Lactate dehydrogenase (U/L)	125-243	399	409	361	429	566	584	ND	605	378	383	426	501
White blood cells (10^9/L)	4·0-10·0	9·9	20·1	11·8	13·2	11·9	11·2	ND	6·2	14·0	11·3	9·6	10·4
Neutrophils (10^9/L)	1·5-7	4·9	14·9	7·8	8·2	7·6	9·2	ND	2·6	9·6	9·3	6·4	6·5
Monocytes (10^9/L)	0·2-1	2·0	2·4	1·7	2·3	0·9*	0·9	ND	0·7	1·8*	1·3	1·5	1·8
Ferritin (μg/L)	22-275	2441	ND	2674	3111	ND	1009	1473	1962	ND	381	365	446
Liver quantification (at inclusion period) (μmol/g)	< 36	340 (±50)				120 (±30)				55 (±30)			

HPLC: High-performance-liquid-chromatography; D: day. ND: not determined. * D+3. **Capillary 3 (and not G8).

administration and HSPC mobilization. Furthermore, Plerixafor synergistically augments Filgrastim-induced mobilization of HSPC,[17] and so has been widely combined with Filgrastim (i) in Filgrastim-non-responsive patients, (ii) as an adjunct to increase the overall recovery of CD34$^+$ cells for autologous or allogenic HSCT, and (iii) in gene therapy trials for β-thalassemia and Wiskott-Aldrich syndrome.[18-20] In view of all the above data, we initiated a phase I/II clinical trial of Plerixafor as a single mobilizing agent (NCT02212535). Our objective was to demonstrate that SCD patients can be safely mobilized under well-defined, controlled clinical and biological conditions. Moreover, this clinical trial provided an estimate of the overall recovery of CD34$^+$ HSPC after apheresis in adult Plerixafor-mobilized SCD patients – thus providing a basis for the wider use of this protocol for gene-addition or genome-editing approaches.

Methods

Study design and human samples

This open-label phase I/II trial (ClinicalTrials.gov number, NCT02212535) was sponsored by the Assistance Publique Hôpitaux de Paris. The protocol was reviewed and approved by the French Drug Agency (*Agence Nationale de Sécurité du Médicament*) and the local independent ethics committee (*Comité de Protection des Personnes Ile-de-France II,* Paris, France). The trial was performed in accordance with the Declaration of Helsinki. Adult SCD patients and healthy donors (for control samples) provided their written, informed consent (*Online Supplementary Table S1*). The study was designed to demonstrate the safety and efficacy of the mobilization and harvesting of peripheral HSPC following a single injection of 0.24 mg/kg Plerixafor in adult SCD patients, with the inclusion criteria described in the *Online Supplementary Methods.*

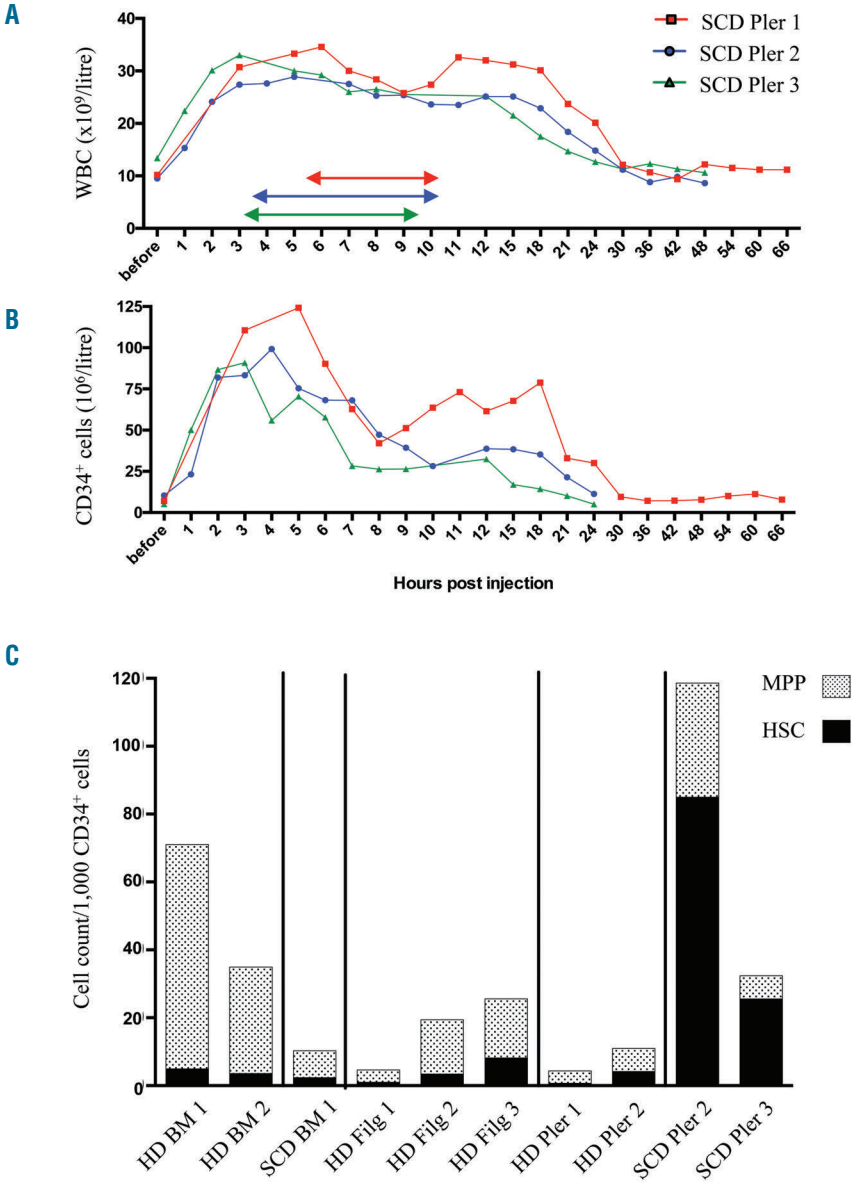

Figure 1. Plerixafor is highly efficient at mobilizing hematopoietic stem and progenitor cells from sickle cell disease patients. (A) Changes in white blood cell (WBC) and (B) CD34$^+$ hematopoietic stem/progenitor cell (HSPC) counts over the 66 h following Plerixafor administration in SCD Pler 1 (red squares), SCD Pler 2 (blue circles) and SCD Pler 3 (green triangles). Arrows indicate time and duration of apheresis. (C) Number of hematopoietic stem cells (HSC, black bars) and multipotent progenitors (MPP, dotted bars) per 1,000 CD34$^+$ cells in samples of various origins. HD: healthy donor, BM: bone marrow, SCD: sickle cell disease, Pler: Plerixafor, Filg: Filgrastim.

The patients were carefully monitored in the Intensive Care Unit and a few hours before the procedure started, they were given prophylaxis for vaso-occlusive crisis [hyperhydration 60 mL/kg/day of a 0.9% saline solution and oxygen therapy (2 L/min)], as recommended by French guidelines.[21] These guidelines recommend oxygen therapy to prevent sickling phenomena in particularly stressful conditions. As mobilization can be considered stressful, we decided to treat all patients with oxygen therapy. Baseline O_2 saturation levels were normal and remained stable during the procedure. The patients received a subcutaneous injection of 0.24 mg/kg of Plerixafor. We then monitored whole blood HbS level, plasma bilirubin, conjugated bilirubin and lactate dehydrogenase levels, and white blood cell, neutrophil and monocyte counts for 30 days after the Plerixafor injection. Plerixafor-mobilized HSPC in peripheral blood were collected by using a COBE® Spectra Optia apheresis system (Terumo BCT, Lakewood, CO, USA) with modifications (*Online Supplementary Material and Methods*). The apheresis lasted from 4 to 6 h. Product volumes and total blood volumes are indicated in Table 2. Egress of the CD34$^+$ cells was monitored hourly for at least 24 h after the Plerixafor injection: total CD34$^+$/kg collected and CD34$^+$ collection efficiency (CE1) [total CD34$^+$ collected/[L processed x (CD34$^+$ pre + CD34$^+$ post)/2] were evaluated following apheresis (Table 2).

Flow cytometry analysis

Cells were stained with specific antibodies (*Online Supplementary Table S2*) and analyzed on a BD FACSCanto™ II system, with gating on viable, 7AAD-negative cells. The data were processed using FlowJo software (version 10.2, Treestar, Ashland, OR, USA).

RNA-Seq extraction

Total RNA was extracted from 0.1-1x10^6 CD34$^+$ cells using an RNeasy Micro kit (QIAGEN). RNA-Seq libraries were prepared from ~10 ng of total RNA, using the Ovation Human FFPE RNA-Seq Multiplex System kit (Nugen) after DNase treatment (ArcticZymes) and >40 million paired-end reads/sample. Samples were generated on a HiSeq 2500 instrument (Illumina). RNA-Seq analysis was performed as described in the *Online Supplementary Methods*.

Transplantation into non-obese diabetic severe combined immunodeficiency gamma mice

The non-obese diabetic severe combined immunodeficiency gamma (NSG) mice (NOD.CgPrkdcscid Il2rgtm1Wj/SzJ, Charles River Laboratories) were housed in a pathogen-free facility. All experiments were performed in compliance with the French Ministry of Agriculture's regulations on animal experiments, and were approved by the regional Animal Care and Use Committee (APAFIS#2101-2015090411495178 v4). Six- to 8-week-old mice were conditioned with intraperitoneally injected busulfan (Sigma, 25 mg/kg body weight/day) 72 h, 48 h and 24 h before transplantation. CD34$^+$ cells (300,000 cells/mouse) from Filgrastim-mobilized healthy donors or Plerixafor-mobilized cells from the three SCD patients were transplanted into the NSG mice via retro-orbital sinus injection. Neomycin was added to the animals' acidified drinking water. Engraftment was analyzed as described in the *Online Supplementary Methods*.

Statistical analysis

Statistical tests are indicated in the Figure legends. The threshold for statistical significance was set at $P<0.05$.

Results

Patients' characteristics

Four SCD homozygous patients followed at Necker-Enfants Malades hospital and meeting our inclusion criteria were enrolled between May 2015 and January 2017. The first one was excluded from the study during the enrollment stage because of elevated granulocyte counts exceeding the limits established in the protocol (<10x10^9 granulocytes/L). The other three patients (P1 - P3) had been monitored in a reference center after the diagnosis of SCD within the first 4 years of life. All suffered from severe SCD, with a history of acute chest syndrome and more than two vaso-occlusive crises per year requiring hospitalization. P1 had undergone cholecystectomy and tonsillectomy. P2 had papillary necrosis and osteonecrosis of both femoral heads. P3 had undergone cholecystectomy, and had chronic asthma and a history of osteomyelitis events. P1 and P2 were transfused monthly because years of hydroxyurea treatment had proven to be ineffective. Hydroxyurea treatment was stopped in P3 3 months before mobilization. P3 was then transfused monthly until mobilization. In view of iron overload caused by the transfusions (Table 1), treatment with deferasirox (P1) and deferiprone (P2) was ongoing.

Table 2. Characteristics of apheresis and CD34$^+$ immunoselection.

Apheresis	SCD Pler 1	SCD Pler 2	SCD Pler 3
Total blood volumes in liters (liters processed by the apheresis device)	3.27 (15.8)	4 (21)	4 (17.9)
Product volumes (mL)	278	382	322
Hematocrit value (%)*	4.8	5.8	8.2
CE1**	0.24	0.30	0.29
Total CD34$^+$ cells collected by apheresis x10^6	354	412	292
Total CD34$^+$ cells collected by apheresis x10^6/kg of body weight	4.6 (77)	5.8 (71)	4.5 (65)
CD34$^+$ cell product after immunoselection			
Recovery	82%	92%	31%
Purity post-selection	95%	94.7%	79.5%

*Normal value: 2-3%; **CE1 = total CD34$^+$ collected/ [L processed X (CD34$^+$pre+CD34$^+$post)/2]

Absence of Plerixafor-related toxicity

Prior to Plerixafor injection, patients underwent several erythrocytoapheresis sessions in order to decrease their HbS levels to below 30% (Table 1). Each patient received a single, subcutaneous injection of Plerixafor (0.24 mg/kg). The HbS values remained low until day +1 and increased to pre-treatment levels thereafter (Table 1). No adverse effects were observed, other than moderate hypokalemia related to the anticoagulant citrate infusion during the apheresis; this was corrected by a 2-day course of potassium chloride (Table 1). The white blood cell and neutrophil counts rose to 30.4 ± 2.8x10^9/μL and 17.8 ± 3.5x10^9/L, respectively, during the first 3 h, remained stable and then (within 24 to 36 h of Plerixafor injection) returned to pre-treatment values (Figure 1A and *Online Supplementary Figure S1*). Bilirubin, conjugated bilirubin and lactate dehydrogenase values and monocyte counts remained stable up to day +30. Serum levels of inflammatory cytokines, including interleukin-8, which is increased in SCD patients during acute crises and associated with higher numbers of circulating hematopoietic progenitors,[22] were comparable to those of healthy controls (*data not shown*). Vital parameters and blood O_2 saturation remained stable and normal during all the procedure. Monitoring of the patients between discharge and the end of the follow-up period (6 months after treatment) was uneventful.

Efficacy of Plerixafor in mobilizing hematopoietic stem and progenitor cells

Baseline CD34$^+$ cell counts were 7, 10 and 10/μL in P1, P2 and P3, respectively. The patients exhibited a very fast, intense increase in peripheral blood CD34$^+$ cell count, exceeding 80 CD34$^+$/μL 3 h after the Plerixafor injection (Figure 1B). Levels greater than 50 CD34$^+$/μL were maintained for 6 h, then decreased and returned to normal pre-treatment values (Figure 1B, and *data not shown* for day 30 and day 60). Apheresis was performed with the technical adjustments described in the *Online Supplementary Methods*. The quantities of CD34$^+$ cells harvested by apheresis (4.6 x 10^6, 5.8x10^6 and 4.5x10^6/kg body weight

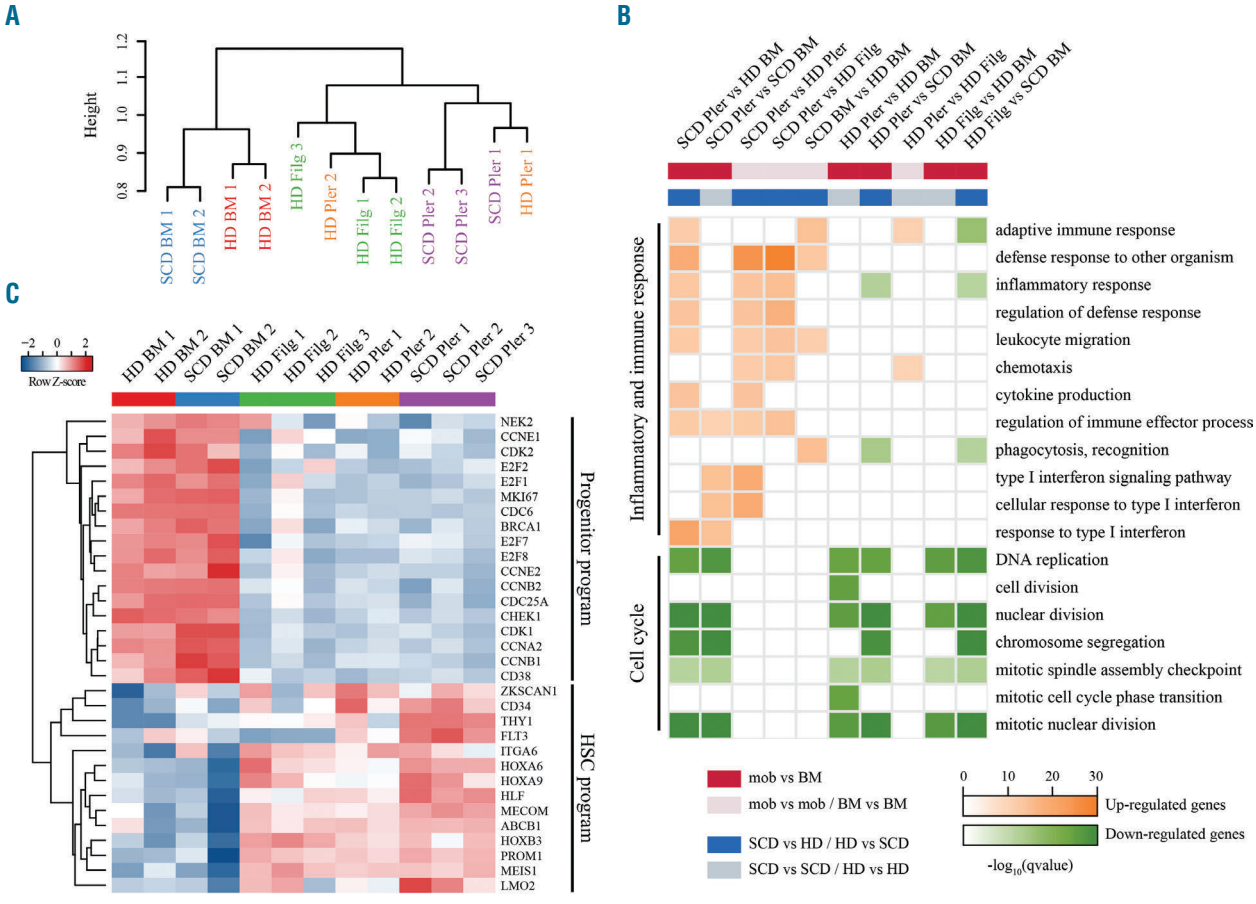

Figure 2. Analysis of the transcriptomic profiles of hematopoietic stem and progenitor cells from different sources (A) Hierarchical clustering analysis of HD BM, SCD BM, SCD Plerixafor-mobilized (Pler), HD Plerixafor-mobilized and HD Filgrastim-mobilized (Filg) HSPC (cluster method: average; distance: correlation). The color of the sample name indicates the classification. (B) Gene ontology analysis of differentially expressed genes. The most enriched biological process categories are shown on the y-axis. The x-axis shows sample comparisons, as defined in Table 3. The orange and green color gradients correspond to the statistical significance of the enrichment [expressed as $-\log_{10}$ (qvalue)] in up- and downregulated genes, respectively. The first color bar at the top indicates comparisons between HSPC from different types of source (dark red) or the same type of source (light red). The second color bar at the top indicates comparisons between HSPC from different types of donor (dark blue) or the same type of donor (light blue). (C) Heat map of genes involved in HSC and progenitor biology. A proportion of the HSC markers were highly expressed in SCD Plerixafor-mobilized HSPC compared with the other samples. The row Z-score is plotted on a red-blue color scale, where red indicates high expression and blue indicates low expression. The color bar at the top indicates the sample classification. HD: healthy donor; BM: bone marrow; HSPC: hematopoietic stem and progenitor cells; HSC: hematopoietic stem cells; SCD: sickle cell disease; Pler: Plerixafor; Filg: Filgrastim.

for P1, P2 and P3, respectively) were high in all three patients despite a limited collection efficiency (Table 2). This enabled the cryopreservation of $3x10^6$ unselected $CD34^+$ cells/kg as a back-up for the upcoming gene therapy trial (used in the case of absence of engraftment) and the immunoselection and further analyses of mobilized $CD34^+$ cells as detailed below. Following $CD34^+$ selection, $CD34^+$ cell purity was in the normal range (Table 2). $CD34^+$ recovery was high in P1 and P2 (82% and 92%, respectively) and lower in P3 (31%).

Characterization of mobilized hematopoietic stem and progenitor cells

To determine the hematopoietic differentiation capacity and self-renewal potential of the mobilized $CD34^+$ cells, we performed a number of phenotypic, transcriptomic and functional analyses.

Hematopoietic stem cells (HSC) and their immediate progeny (multipotent progenitors) within the $CD34^+$ subset are negative for lineage, CD38, and CD45RA markers and positive for CD133[23-25] and can, therefore, be detected using flow cytometry. We compared the numbers of HSC and multipotent progenitors among mobilized SCD $CD34^+$ cells with the values for (i) the BM of healthy donors and SCD patients, and (ii) samples from healthy donors mobilized with either Filgrastim or Plerixafor (*Online Supplementary Table S1*, Figure 1C and *Online Supplementary Figure S2*). We estimated the number of HSC per 1000 $CD34^+$ cells to be >25 in Plerixafor-mobilized SCD samples and <5 in all other samples, suggesting that Plerixafor mobilizes HSC with an unexpectedly high efficacy in SCD patients.

The frequency of erythroid and granulocyte/monocyte colony-forming cells was similar in Plerixafor-mobilized and BM SCD samples and in the range of that observed in Filgastrim-mobilized HSPC (*Online Supplementary Figure S3A* and *data not shown*). Upon erythroid differentiation, Plerixafor-mobilized as well as BM SCD HSPC gave rise to >90% of mature $GYPA^+CD36^{low}CD71^{low}$ enucleated red blood cells (*Online Supplementary Figure S3B-D*).

The transcriptome of highly purified $CD34^+$ HSPC from the different sources was analyzed using RNA-Seq. Unsupervised gene expression analysis showed that the samples clustered into two major groups, based on cell origin: the "BM" group encompassed BM samples from SCD patients and healthy donors, whereas the "mobilized" group encompassed Plerixafor-mobilized HSPC from SCD patients and healthy donors and Filgrastim-mobilized HSPC from healthy donors (Figure 2A).

Next, we identified differentially expressed genes among the different populations (false discovery rate <0.05, Table 3). In a comparison of samples from SCD patients and healthy donors, we observed that the genes upregulated in SCD samples are involved in inflammatory and immune responses (e.g. defense response to other organisms, type I interferon signaling pathway, cytokine

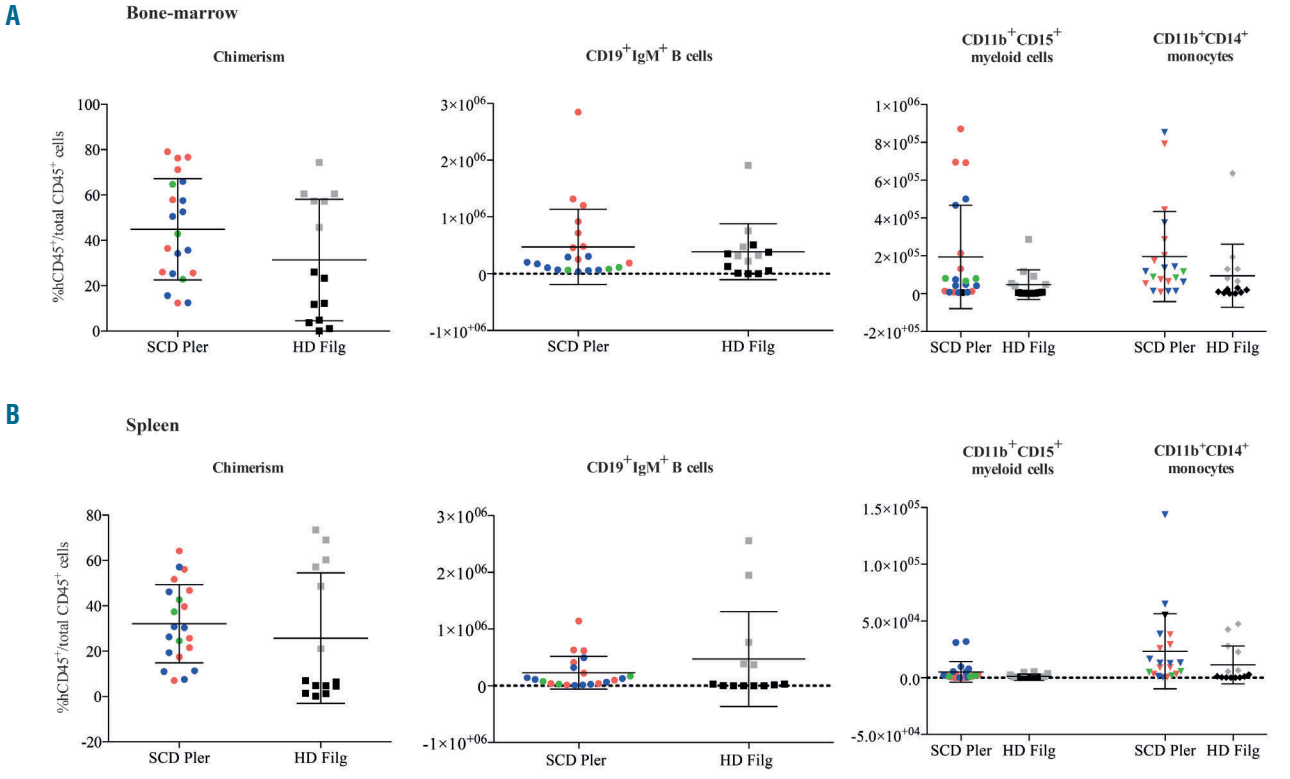

Figure 3. Plerixafor-mobilized $CD34^+$ cells from sickle cell disease patients engraft to the same degree as Filgrastim-mobilized $CD34^+$ cells from healthy donors in NSG mice. NSG mice were sacrificed 3 to 4 months after the injection of SCD (SCD Plerixafor, n=3) or HD (HD Filgrastim, n=2) $CD34^+$ cells. (A) Bone marrow cells and (B) splenocytes were isolated, stained and analyzed by flow cytometry. The chimerism (defined as % human $CD45^+$cells/total $CD45^+$cells) and the numbers of human B lymphocytes ($CD19^+IgM^+$), granulocytes ($CD11b^+CD15^+$), and monocytes ($CD11b^+CD14^+$) were evaluated in each group of mice (red circles and red triangles SCD Pler1; blue circles and blue triangles SCD Pler2; green circles and green triangles SCD Pler3; the two HD Filg control are represented by gray squares/gray diamond and black squares/black diamonds, respectively). Each dot represents an individual mouse. HD: healthy donor; SCD: sickle cell disease; Pler: Plerixafor; Filg: Filgrastim.

production, leukocyte migration, phagocytosis and adaptive immune response) (Figure 2B). Major differences in gene expression (>900 differentially expressed genes) were observed when comparing mobilized and BM samples (Table 3). The genes downregulated in mobilized *versus* BM HSPC are involved in cell cycle-related processes (e.g. DNA replication, chromosome segregation, and nuclear division) – confirming that mobilized samples contain more quiescent cells, presumably HSC, than progenitors (Figure 2B, *Online Supplementary Figure S4A* and *Online Supplementary Table S2*). Interestingly, mobilized populations poorly expressed genes that are typical of committed hematopoietic progenitors, relative to BM samples (Figure 2C, *Online Supplementary Figure S4A* and *Online Supplementary Table S2*). Conversely, a large proportion of the genes involved in HSC biology were strongly expressed in mobilized HSPC (Figure 2C, *Online Supplementary Figure S4A* and *Online Supplementary Table S2*). Importantly, some HSC markers (e.g. THY1, HLF and FLT3) were more strongly expressed in Plerixafor-mobilized SCD samples – confirming the latter's high HSC content – than in BM samples and Filgrastim- or Plerixafor-mobilized HSPC from healthy donors (Figure 2C).

Fewer than 300 genes were differentially expressed between Filgrastim- or Plerixafor-mobilized samples (Table 3). Of note, genes encoding transcriptional regulators and surface markers of plasmacytoid dendritic cell progenitors are upregulated in Plerixafor-mobilized samples compared to Filgrastim-mobilized samples (*Online Supplementary Figure S4B*). FACS analyses showed the appearance of a $CD133^{-}CD34^{dim}$ cell population preferentially in Plerixafor-mobilized samples (*Online Supplementary Figure S2* and *data not shown*). This population might contain plasmacytoid dendritic cell progenitors that are mobilized by Plerixafor, as recently described.[16]

Proof of stemness was further confirmed by transplantation into conditioned, immunodeficient mice. Human chimerism in the BM and spleen was similar in recipients of Filgrastim-mobilized $CD34^{+}$ cells from healthy donorss and Plerixafor-mobilized $CD34^{+}$ cells from SCD patients (Figure 3A,B). Similar counts of lymphoid and myeloid subsets were detected in both groups (Figure 3A,B). The numbers of human $CD34^{+}$ cells, HSC and multipotent progenitors in bone marrow were comparable between the two groups (*Online Supplementary Figure S5*). After secondary transplantation, all recipients of Plerixafor-mobilized SCD samples and Filgrastim-mobilized $CD34^{+}$ cells from healthy donors displayed engraftment - further demonstrating the presence of true HSC in HSPC mobilized with Plerixafor in SCD patients (*data not shown*).

Discussion

Successful transplantation of autologous gene-corrected cells primarily depends on the collection and effective genetic modification of a sufficient number of true stem cells. Thus, poor harvesting of BM or mobilized peripheral stem cells limits the success of this procedure. To overcome the need for two or more BM aspirates, we initiated a phase I/II clinical trial with the objective of establishing whether Plerixafor-induced stem cell mobilization in SCD patients can avoid the increased risk of vaso-occlusive crises observed with Filgrastim mobilization and of validating the efficiency of HSPC harvesting with this procedure.[26] Because of the risks incurred by the patients and the absence of a direct benefit, the trial was restricted to patients with $<10x10^9$ granulocytes/L, knowing their role in vaso-occlusive crises.

Table 3. Number of differentially expressed (up- or downregulated) genes in HSPC from different sources (false discovery rate < 0.05).

Comparison	Differentially expressed	Upregulated	Downregulated
SCD BM *vs.* HD BM	134	52	82
SCD Pler *vs.* HD Pler	165	133	32
SCD Pler *vs.* HD Filg	265	188	77
SCD Pler *vs.* HD BM	1685	761	924
SCD Pler *vs.* SCD BM	1544	753	791
HD Pler *vs.* HD BM	923	315	608
HD Filg *vs.* HD BM	1893	789	1104
HD Pler *vs.* HD Filg	47	20	27
HD Filg *vs.* SCD BM	1683	800	883
HD Pler *vs.* SCD BM	1024	398	626

SCD: sickle cell disease. HD: healthy donor. BM: bone marrow. Pler: Plerixafor. Filg: Filgrastim.

We decided to discontinue hydroxyurea treatment 3 months before the mobilization in P3 and to submit all the patients to monthly transfusions. The rationale for this decision was based on the following observations: (i) hydroxyurea has no beneficial role in $CD34^{+}$ cell mobilization in thalassemic patients;[27] (ii) hydroxyurea withdrawal is associated with an increase in the number of circulating $CD34^{+}$ cells in SCD patients;[28] and (iii) in various clinical settings hydroxyurea has been associated with myelosuppression[29,30] suggesting BM toxicity and potential impairment of HSC.

In order to optimize the safety of the mobilization procedure in SCD patients, we reduced HbS levels to below 30% via erythrocyte exchanges, and we closely monitored white blood cell counts and serum levels of inflammatory cytokines. Furthermore, the use of Plerixafor avoided the adverse events associated with Filgrastim: vascular events and splenic rupture after the administration of this latter have been extensively reported in clinical populations and even in healthy stem cell donors.[31] Although these events are usually rare, their frequency may be higher in the presence of other vascular risk factors (such as SCD).[31] Thus the benefit/risk ratio of using a hematopoietic growth factor such as Filgrastim (especially at the high doses required in patients without a malignant blood disease) appears to be unacceptably low. Hence, we gave our three patients Plerixafor at the standard dose. No adverse events occurred, serum levels of inflammatory cytokines were in the normal ranges (*data not shown*) and the white blood cell counts did not exceed $40x10^9$/L (i.e. a value often reported in the literature, and far from the 50 to $75x10^9$/L often observed in healthy donors after 5 days of Filgrastim treatment).[32]

The rapid mobilization with Plerixafor alone (compared with Filgrastim) is also an important advantage. The current guidelines on mobilization in patients with a malignant disease recommend initiating apheresis 11 h after Plerixafor administration;[33] this contrasts with the 5 to 7 days required for collection after Filgrastim administration. Moreover, rapid, transient stem cell mobilization by

Filgrastim+Plerixafor (especially in very poor mobilizers) has been observed by various groups (including ours); the rapid decrease in peripheral blood stem cells may cause collection to fail when apheresis is initiated according to conventional guidelines.[34] We monitored the egress of $CD34^+$ cells into the blood every hour after Plerixafor injection. The time course of $CD34^+$ mobilization was remarkably similar in our three patients. Peak counts of over 80 $CD34^+/\mu L$ were achieved as early as 2 to 3 h after Plerixafor administration; this confirms that HSPC can be harvested immediately in SCD patients. The decrease in $CD34^+$ cell count observed after 6 h might be the consequence of a short-term mobilization of HSC by Plerixafor and therefore to the reduced egress of $CD34^+$ cells from BM combined with their return to the BM. Another possibility is that the drop in $CD34^+$ cells is due to the leukapheresis procedure. Pantin *et al.* analyzed the kinetics of $CD34^+$ counts in healthy donors treated with Plerixafor and not subjected to the apheresis procedure.[35] In their work, $CD34^+$ cell counts peaked at 6 h at lower values than the ones observed in our study and start to decrease 10 h after Plerixafor administration, with differences in kinetics and range of mobilization probably related to the clinical conditions of healthy donors *versus* SCD patients. Overall, the study by Pantin *et al.* suggests that leukapheresis *per se* does not cause a decrease in $CD34^+$ cell counts. Additionally, the limited collection efficiency (≤30% of the circulating $CD34^+$ cells) (Table 2) does not support the hypothesis that the drop is due to the leukapheresis procedure. Close monitoring of peripheral blood $CD34^+$ cell counts is therefore a crucial point for efficient apheresis in SCD patients mobilized with Plerixafor.

The leukapheresis product contained significantly more HSC than the other stem cell sources used as controls, i.e. 8- to 10-fold more than in BM from healthy donors or SCD patients and in Filgrastim- or Plerixafor-mobilized cells from healthy donors. Accordingly, HSPC from the patients' Plerixafor-mobilized samples showed elevated transcription of several HSC-associated genes. We do not have a formal explanation for this result; we can only hypothesize that sickling cycles damage the BM stroma and favor the mobilization of HSC.

Genes involved in inflammatory and immune responses were upregulated in Plerixafor-mobilized SCD samples. The inflammation-related characteristics of HSC and their environment constitute a major obstacle to both allogeneic and autologous transplantation. Significant pathological changes in hematopoiesis have been described by Weisser *et al.*[36] in a setting of murine and human chronic granulomatous disease, an inherited disease characterized by chronic, sterile, granulomatous inflammation). In mice and in humans with chronic granulomatous disease, BM and Filgrastim-mobilized grafts contain a low proportion of HSC; in transplanted mice, this feature is associated with low reconstitution potential.[36] Hence, we transplanted mobilized $CD34^+$ cells from SCD patients into immunodeficient NSG mice, in order to establish whether an inflammatory expression profile interfered with engraftment and self-renewal capacity. In fact, the cells engrafted as well as Filgrastim-mobilized samples in both primary and secondary transplantation, demonstrating that Plerixafor-mobilized SCD $CD34^+$ cells contain true stem cells that are able to reconstitute human hematopoiesis as well as their Filgrastim-mobilized counterparts. Moreover, it is possible to effectively correct Plerixafor-mobilized SCD HSC by gene addition (*manuscript in preparation*). Taken as a whole, our results show that the inflammatory characteristics of HSC do not impair self-renewal and engraftment.

In conclusion, the present results show that $CD34^+$ cells can be safely mobilized with Plerixafor in SCD patients under well-defined clinical conditions, including a 3-month interruption of hydroxyurea treatment, monthly transfusions and red blood cell exchanges. The proportion of true stem cells in the Plerixafor-mobilized $CD34^+$ population was significantly higher than the proportion from any other source, although their egress must be monitored carefully. After harvesting under specific conditions, the cells can be successfully immunoselected. In the case that the optimal dose of $CD34^+$ cells required for gene therapy ($6\text{-}9x10^6/kg$) is not reached after one apheresis and in the absence of adverse events, a second mobilization by Plerixafor, 24 h after the first one, will be considered. In our small cohort, the high numbers of cells enabled us to extend our gene-addition therapy project without having to perform several low-yield BM harvesting steps. Furthermore, the results removed the obstacle of collecting an appropriate graft for genome-editing purposes. Lastly, our study emphasizes the importance of considering the specific characteristics of a diseased BM; finding a way to put the patient's hematopoietic system into a steady-state condition may circumvent a lack of true stem cells in the harvested product or poor engraftment of genetically-modified cells.

Acknowledgments

The authors would like to thank Jean-Marc Luby for excellent, dedicated technical assistance, Valérie Jolaine, Michaela Semeraro for the logistics of the clinical trial, Michaela Semeraro for care of patients, and Christine Bole and Olivier Alibeu for the RNA sequencing. We also thank Frédéric Galacteros, Pablo Bartolucci and Susanne Matthes-Martin for revision of the clinical protocol.

Funding

This work was supported by state funding from the Agence Nationale de la Recherche as part of the Investissements d'Avenir program (ANR-10-IAHU-01 and ANR-16-CE18-0004), the French National Institute of Health and Medical Research (INSERM), and Assistance Publique-Hôpitaux de Paris (AP-HP). This work was also supported by a grant from the European Research Council (ERC 2015-AdG, GENEFORCURE).

References

1. Ware RE, de Montalembert M, Tshilolo L, Abboud MR. Sickle cell disease. Lancet. 2017;390(10091):311-323.
2. Walters MC, De Castro LM, Sullivan KM, et al. Indications and results of HLA-identical sibling hematopoietic cell transplantation for sickle cell disease. Biol Blood Marrow Transplant. 2016;22(2):207-211.
3. Gluckman E, Cappelli B, Bernaudin F, et al. Sickle cell disease: an international survey of results of HLA-identical sibling hematopoietic stem cell transplantation. Blood. 2017;129(11):1548-1556.
4. Ribeil JA, Hacein-Bey-Abina S, Payen E, et al. Gene therapy in a patient with sickle

cell disease. N Engl J Med. 2017;376(9):848-855.
5. Cavazzana M, Ribeil JA, Lagresle-Peyrou C, Andre-Schmutz I. Gene therapy with hematopoietic stem cells: the diseased bone marrow's point of view. Stem Cells Dev. 2017;26(2):71-76.
6. Wang TF, Chen SH, Yang SH, Su YC, Chu SC, Li DK. Poor harvest of peripheral blood stem cell in donors with microcytic red blood cells. Transfusion. 2013;53(1):91-95.
7. Constantinou VC, Bouinta A, Karponi G, et al. Poor stem cell harvest may not always be related to poor mobilization: lessons gained from a mobilization study in patients with beta-thalassemia major. Transfusion. 2017;57(4):1031-1039.
8. Abboud M, Laver J, Blau CA. Granulocytosis causing sickle-cell crisis. Lancet. 1998;351(9107):959.
9. Adler BK, Salzman DE, Carabasi MH, Vaughan WP, Reddy VV, Prchal JT. Fatal sickle cell crisis after granulocyte colony-stimulating factor administration. Blood. 2001;97(10):3313-3314.
10. Grigg AP. Granulocyte colony-stimulating factor-induced sickle cell crisis and multiorgan dysfunction in a patient with compound heterozygous sickle cell/beta+ thalassemia. Blood. 2001;97(12):3998-3999.
11. Onitilo AA, Lazarchick J, Brunson CY, Frei-Lahr D, Stuart RK. Autologous bone marrow transplant in a patient with sickle cell disease and diffuse large B-cell lymphoma. Transplant Proc. 2003;35(8):3089-3092.
12. Kamble RT, Tin UC, Carrum G. Successful mobilization and transplantation of filgrastim mobilized hematopoietic stem cells in sickle cell-hemoglobin C disease. Bone Marrow Transplant. 2006;37(11):1065-1066.
13. Rosenbaum C, Peace D, Rich E, Van Besien K. Granulocyte colony-stimulating factor-based stem cell mobilization in patients with sickle cell disease. Biol Blood Marrow Transplant. 2008;14(6):719-723.
14. Tormey CA, Snyder EL, Cooper DL. Mobilization, collection, and transplantation of peripheral blood hematopoietic progenitor cells in a patient with multiple myeloma and hemoglobin SC disease. Transfusion. 2008;48(9):1930-1933.
15. Liles WC, Broxmeyer HE, Rodger E, et al. Mobilization of hematopoietic progenitor cells in healthy volunteers by AMD3100, a CXCR4 antagonist. Blood. 2003;102(8):2728-2730.
16. Schroeder MA, Rettig MP, Lopez S, et al. Mobilization of allogeneic peripheral blood stem cell donors with intravenous plerixafor mobilizes a unique graft. Blood. 2017;129(19):2680-2692.
17. Broxmeyer HE, Orschell CM, Clapp DW, et al. Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. J Exp Med. 2005;201(8):1307-1318.
18. Devine SM, Flomenberg N, Vesole DH, et al. Rapid mobilization of CD34+ cells following administration of the CXCR4 antagonist AMD3100 to patients with multiple myeloma and non-Hodgkin's lymphoma. J Clin Oncol. 2004;22(6):1095-1102.
19. Karponi G, Psatha N, Lederer CW, et al. Plerixafor+G-CSF-mobilized CD34+ cells represent an optimal graft source for thalassemia gene therapy. Blood. 2015;126(5):616-619.
20. Ferrari G, Cavazzana M, Mavilio F. Gene therapy approaches to hemoglobinopathies. Hematol Oncol Clin North Am. 2017;31(5):835-852.
21. Lionnet F, Arlet JB, Bartolucci P, et al. [Guidelines for management of adult sickle cell disease]. Rev Med Interne. 2009;30(Suppl 3):S162-223.
22. Lamming CE, Augustin L, Blackstad M, Lund TC, Hebbel RP, Verfaillie CM. Spontaneous circulation of myeloid-lymphoid-initiating cells and SCID-repopulating cells in sickle cell crisis. J Clin Invest. 2003;111(6):811-819.
23. Doulatov S, Notta F, Eppert K, Nguyen LT, Ohashi PS, Dick JE. Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development. Nat Immunol. 2010;11(7):585-593.
24. Six EM, Bonhomme D, Monteiro M, et al. A human postnatal lymphoid progenitor capable of circulating and seeding the thymus. J Exp Med. 2007;204(13):3085-3093.
25. Handgretinger R, Kuci S. CD133-positive hematopoietic stem cells: from biology to medicine. Adv Exp Med Biol. 2013;777:99-111.
26. Fitzhugh CD, Hsieh MM, Bolan CD, Saenz C, Tisdale JF. Granulocyte colony-stimulating factor (G-CSF) administration in individuals with sickle cell disease: time for a moratorium? Cytotherapy. 2009;11(4):464-471.
27. Yannaki E, Papayannopoulou T, Jonlin E, et al. Hematopoietic stem cell mobilization for gene therapy of adult patients with severe beta-thalassemia: results of clinical trials using G-CSF or plerixafor in splenectomized and nonsplenectomized subjects. Mol Ther. 2012;20(1):230-238.
28. Richard RE, Siritanaratkul N, Jonlin E, Skarpidi E, Heimfeld S, Blau CA. Collection of blood stem cells from patients with sickle cell anemia. Blood Cells Mol Dis. 2005;35(3):384-388.
29. Charache S, Terrin ML, Moore RD, et al. Effect of hydroxyurea on the frequency of painful crises in sickle cell anemia. Investigators of the Multicenter Study of Hydroxyurea in Sickle Cell Anemia. N Engl J Med. 1995;332(20):1317-1322.
30. Alvarez-Larran A, Perez-Encinas M, Ferrer-Marin F, et al. Risk of thrombosis according to need of phlebotomies in patients with polycythemia vera treated with hydroxyurea. Haematologica. 2017;102(1):103-109.
31. Halter J, Kodera Y, Ispizua AU, et al. Severe events in donors after allogeneic hematopoietic stem cell donation. Haematologica. 2009;94(1):94-101.
32. Lack NA, Green B, Dale DC, et al. A pharmacokinetic-pharmacodynamic model for the mobilization of CD34+ hematopoietic progenitor cells by AMD3100. Clin Pharmacol Ther. 2005;77(5):427-436.
33. To LB, Levesque JP, Herbert KE. How I treat patients who mobilize hematopoietic stem cells poorly. Blood. 2011;118(17):4530-4540.
34. Lefrere F, Mauge L, Rea D, et al. A specific time course for mobilization of peripheral blood CD34+ cells after plerixafor injection in very poor mobilizer patients: impact on the timing of the apheresis procedure. Transfusion. 2013;53(3):564-569.
35. Pantin J, Purev E, Tian X, et al. Effect of high-dose plerixafor on CD34(+) cell mobilization in healthy stem cell donors: results of a randomized crossover trial. Haematologica. 2017;102(3):600-609.
36. Weisser M, Demel UM, Stein S, et al. Hyperinflammation in patients with chronic granulomatous disease leads to impairment of hematopoietic stem cell functions. J Allergy Clin Immunol. 2016;138(1):219-228.e219.