SUPPLEMENTARY APPENDIX

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

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Supplementary Methods

Inclusion criteria

Inclusion criteria were a diagnosis of SS or S beta thalassemia SCD, eligibility for HSCT, the lack of a HLA-genoidentical donor, one or more vaso-occlusive crises (VOCs) in the previous year, two or more episodes of acute thoracic syndrome, joint osteonecrosis, RBC allo-immunization, and/or cardiomyopathy. Apart from the classical causes, non-inclusion criteria included previous allogenic HSCT, major organ failure and granulocyte counts $\geq 10x10^9$/litre. Eligible patients were identified in the cohort of adult patients with SCD of Necker-Enfants Malades hospital and enrolled consecutively between May 2015 and January 2017. Because of the absence of a direct benefit and the risks incurred by the patients, enrollment was stopped when three SCD patients were analysable and exhibited reproducible results in terms of safety and efficacy. Primary and secondary endpoints were the absence of SAEs and the efficacy of mobilization of HSPCs (defined as ≥ 20 $CD34^+$ cells/µlitre), respectively.

Apheresis procedure

During the HSPC collection, we noticed that separation of mononuclear cells from granulocytes and red blood cells was not efficient and resulted in an unstable layer of mononuclear cells. This phenomenon has also been observed during the collection of BM mononuclear cells in SCD and in thalassemic patients.[1,2] In thalassemic patients, this problem was partly solved by collecting mononuclear cells at a deeper layer (toward the RBC layer).[2] Our apheresis procedure was adjusted in the same way (details of the procedure are available upon request). $CD34^+$ cells were further purified by immunomagnetic selection with the CliniMACS System (Miltenyi Biotec, Bergisch Gladbach, Germany) after immunostaining with CliniMACS CD34 Reagent (Miltenyi Biotec). To provide the patients with individual benefit, mobilized $CD34^+$ cells were cryopreserved as a back-up for their inclusion in a subsequent gene therapy trial.

RNA-Seq analysis

The reads were mapped to the human reference genome (hg38) using STAR. Raw gene counts were obtained using featureCounts and GENCODE 25 basic gene annotation. The edgeR R package was used for differential gene expression analysis. Hierarchical cluster analysis was performed using the pvclust R package. Functional enrichment analysis of gene ontology (GO) biological process (BP) categories was performed using the clusterProfiler R package. Gene set enrichment analysis (GSEA) was performed using GSEA software and the Molecular Signature Database (MSigDB). Gene lists for heat map generation were obtained from Lidonnici et al.[3] The datasets supporting the results of this article are available in the Gene Expression Omnibus repository under the accession number GSE102881.

Analysis of engraftment and humanhematopoiesis into non-obese diabetic severe combined immunodeficiency gamma (NSG) mice

Three months after transplantation, the recipients were sacrificed, cells harvested from the femurs, thymus and spleen, stained with antibodies against murine and human markers (Supplementary Table 2) and analyzed with flow cytometry using a Gallios analyser and Kaluza software (Beckman Coulter). Human chimerism was calculated according to the following equation: % human $CD45^+$ cells/(% human $CD45^+$ cells + % murine $CD45^+$ cells). For secondary transplantation, 7 to 10 x 10^6 total BM cells (containing around 250,000 human $CD34^+$ cells) were injected into busulfan-conditioned NSG mice, as described above. Analysis was performed three months after transplantation.

Colony Forming Cell (CFC) assay

Plerixafor-mobilized and BM SCD $CD34^+$ cells were plated at $1x10^3$ cells/ml in methylcellulose medium supporting the growth of erythroid (BFU-E) and myeloid (CFU-GM) progenitors (GFH4435, Stem Cell Technologies). Before plating, cells were pre-activated for 24 h, as usually performed in our gene therapy protocols, in X-VIVO 20 supplemented with the following recombinant human cytokines (Peprotech): 300 ng/ml SCF, 300 ng/ml Flt-3L, 100 ng/ml TPO and 20 ng/ml IL3. After 14 days, we scored BFU-E and CFU-GM colonies.

***In vitro* erythroid differentiation**

Preactivated, plerixafor-mobilized and BM SCD $CD34^+$ cells were differentiated in mature RBCs using a 3-phase protocol adapted from Giarratana *et al.*[4] From day 0 to day 6, cells were grown in a basal erythroid medium supplemented with the following human recombinant cytokines: 100 ng/mL SCF (Peprotech), 5 ng/mL IL3 (Peprotech), and 3 IU/mL of Epo (Janssen-Cilag), and 10^{-6} M hydrocortisone (Sigma). From day 6 to day 9, cells were cultured onto a layer of murine stromal cells (MS-5) in a basal erythroid medium supplemented only with 3 IU/mL Epo. Finally, from day 9 to day 20, cells were cultured onto the MS-5 layer in basal erythroid

medium without cytokines. Erythroid differentiation was monitored by May Grunwald-Giemsa staining and FACS analysis of the erythroid surface markers CD36 (CD36-V450, BD Horizon), CD71 (CD71-FITC, BD Pharmingen) and GYPA (CD235a-PECY7, BD Pharmingen) and of the DRAQ5 (eBioscience) nuclear staining using the Gallios FACS analyzer and the Kaluza software (Beckman-Coulter, Brea, CA).

Supplementary references

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Supplementary Table 1. Characteristics of SCD and healthy donors

samples	Gender	Age (years)	Ethnicity
SCD Pler 1	male	19	West African
SCD Pler 2	male	20	West African
SCD Pler 3	male	21	Central African
HD Pler 1	male	25	Asian
HD Pler 2	male	29	Caucasian
HD Filg 1	male	36	unknown
HD Filg 2	male	61	Caucasian
HD Filg 3	unknown	unknown	unknown
SCD BM 1	female	12	West African
SCD BM 2	female	18	Central African
HD BM 1	female	27	North Africa
HD BM 2	male	44	North Africa
HD Filg 1 (*in vivo* experiments)	male	36	unknown
HD Filg 2 (*in vivo* experiments)	male	28	North Africa

Supplementary Table 2. Enrichment analysis of gene sets related to HSC-specific markers and proliferation genes.

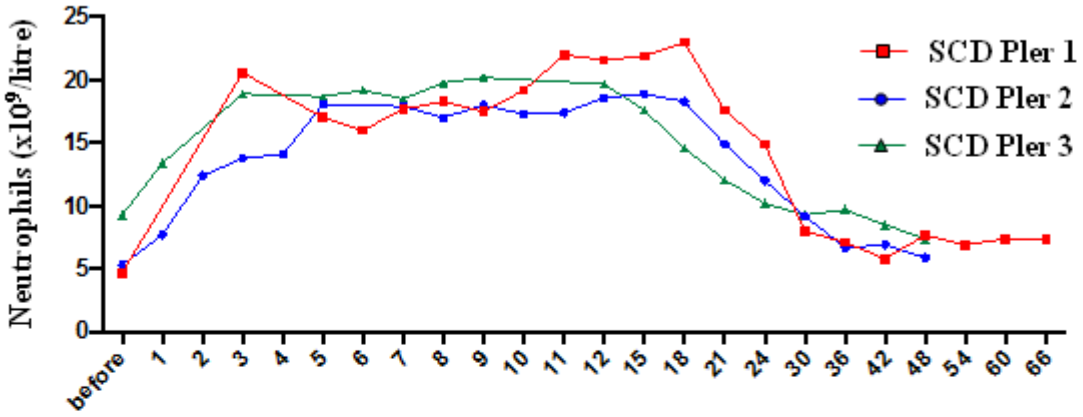
Gene Set	SCD Pler vs. HD BM	SCD Pler vs. SCD BM	HD Pler vs. HD BM	HD Pler vs. SCD BM	HD Filg vs. HD BM	HD Filg vs. SCD BM
GEORGANTAS_HSC_MARKERS	4.07	5.00	2.95	3.76	3.03	4.19
EPPERT_HSC_R	3.70	4.71	2.09	3.40	2.15	3.73
CHANG_CYCLING_GENES	-7.88	-7.57	-8.23	-8.02	-8.33	-7.85
GRAHAM_NORMAL_QUIESCENT_ VS_NORMAL_DIVIDING_DN	-7.85	-7.45	-7.78	-7.39	-8.40	-8.14

SCD, sickle cell disease. HD, healthy donor. BM, bone marrow. Pler, Plerixafor. Filg, Filgrastim
The normalized enrichment score (NES) is reported for each comparison. Positive and negative values indicate enrichment in up- and downregulated genes, respectively (FDR < 0.05 for all comparisons).

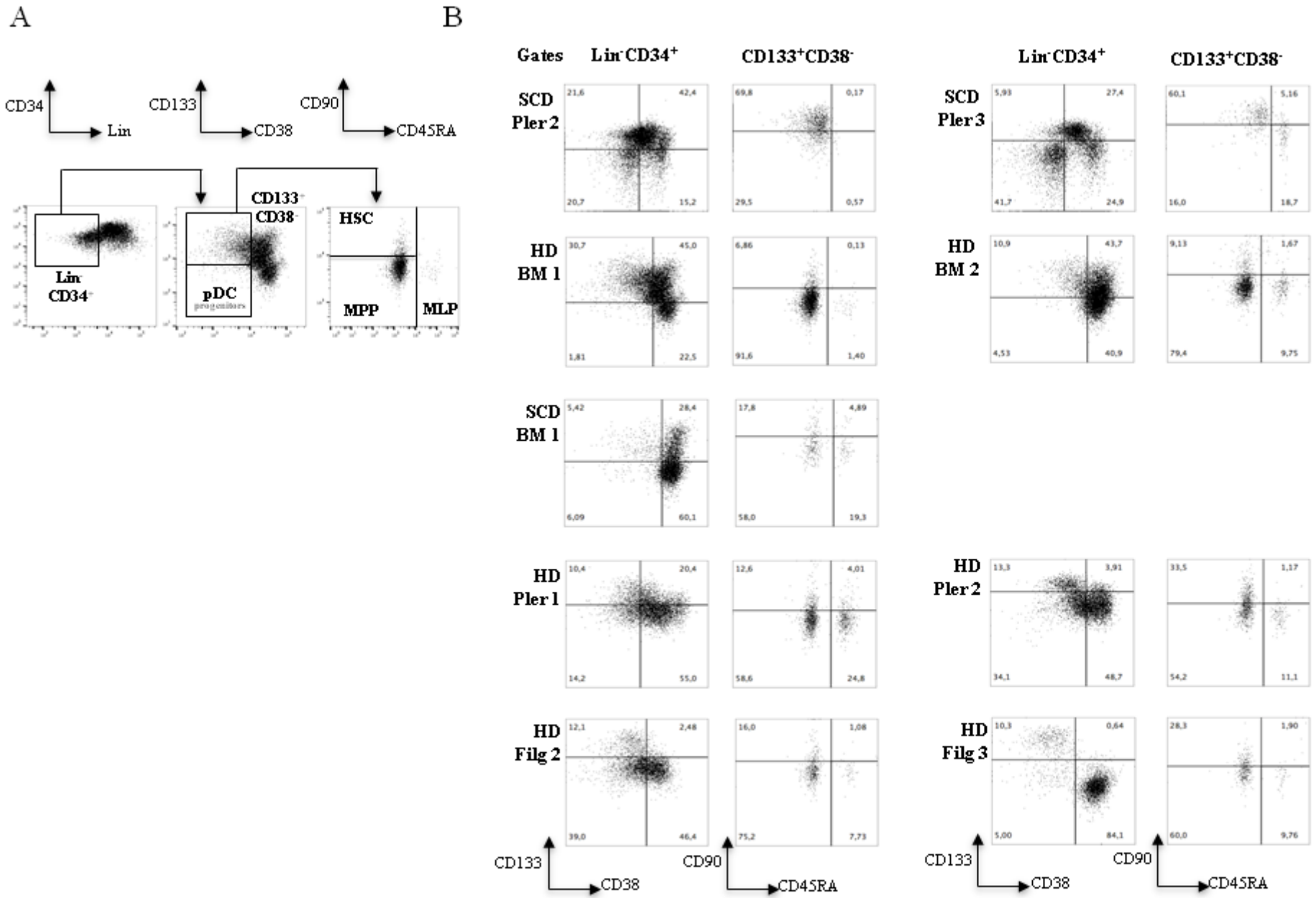
Supplementary Table 3 Antibodies used to stain human cells from different sources and to detect human engraftment in NSG mice.

Antibodies	Clone	Supplier
Murine CD45	3-F11	BD Biosciences
Human CD45	5B1	Miltenyi Biotec
CD1a	HI149	BD Biosciences
CD2*	RPA2.10	BD Biosciences
CD3*	BW264/56	Miltenyi Biotec
CD4*	OKT4	Sony
CD7	M-T701	BD Biosciences
CD8*	RPA-T8	BD Biosciences
CD11b	M1/70.15.11.5	Miltenyi Biotec
CD13	WM15	BD Biosciences
CD14*	MφP9	BD Biosciences
CD15*	80H5	Beckman Coulter
CD16*	368	BD Biosciences
CD19*	HIB19	Sony
CD20*	2H7	BD Biosciences
CD33*	WM53	BD Biosciences
CD34	4H11	Ebioscience
CD36	CB38	BD Biosciences
CD38	HIT2	BD Biosciences
CD56*	B159	BD Biosciences
CD45RA	HI100	BD Biosciences
CD71	M-A712	BD Biosciences
CD90	5E10	BD Biosciences
CD133	ACC133	Miltenyi biotech
CD235a*	GA-R2	BD Biosciences
IgD	IA6-2	BD Biosciences
IgM	B159	BD Biosciences
TCR gd	IMMU510	Beckman Coulter
TCR ab	BW242/412	Miltenyi Biotec
Mouse IgG1 isotype	MOPC-21	BD Biosciences
Mouse IgG2a isotype	G155-178	BD Biosciences
Mouse IgG1 k isotype	MOPC-21	BD Biosciences
Viability marker	7 -AAD	BD Biosciences

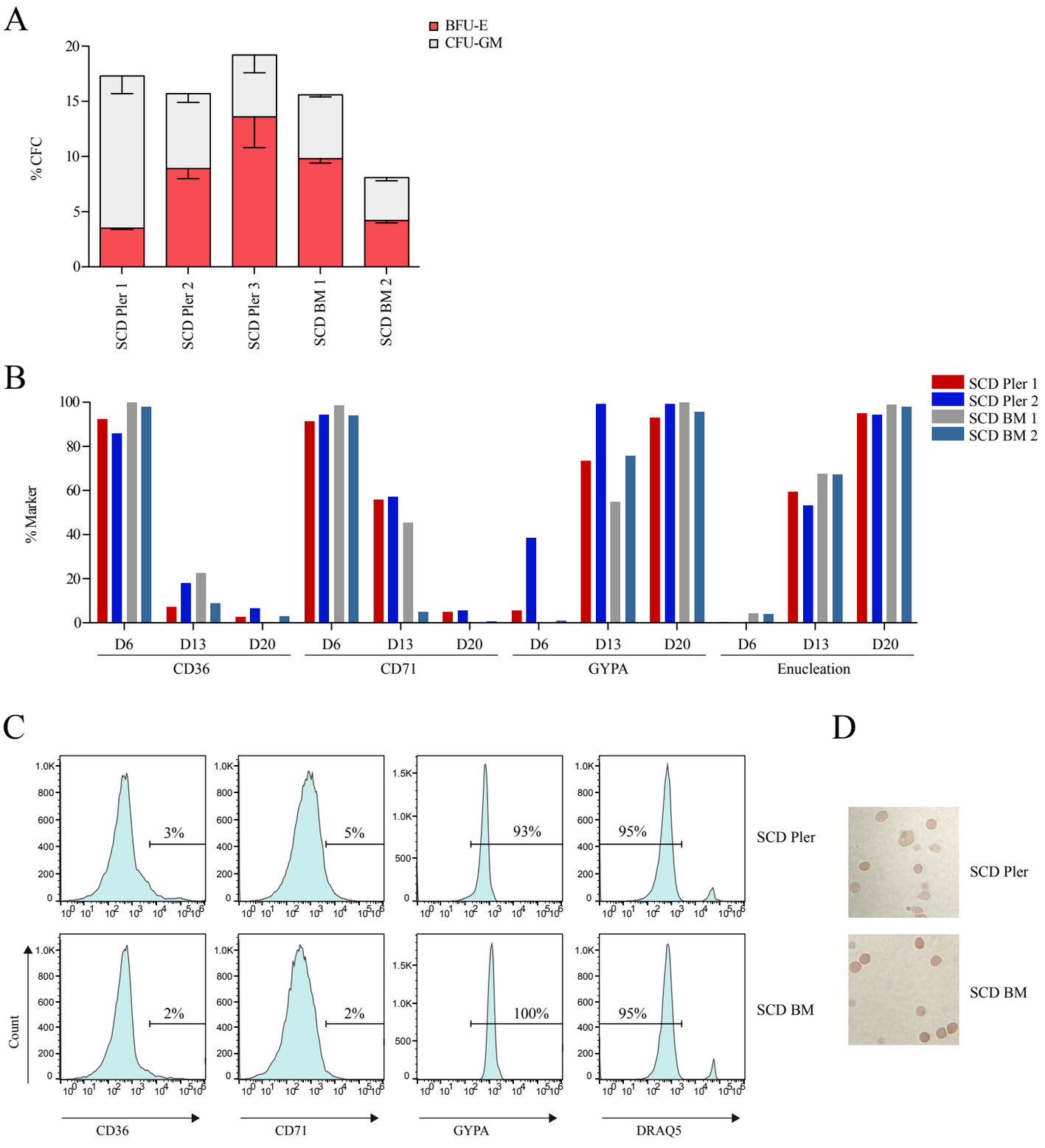
* Antibodies were mixed altogether to stain lineage positive population



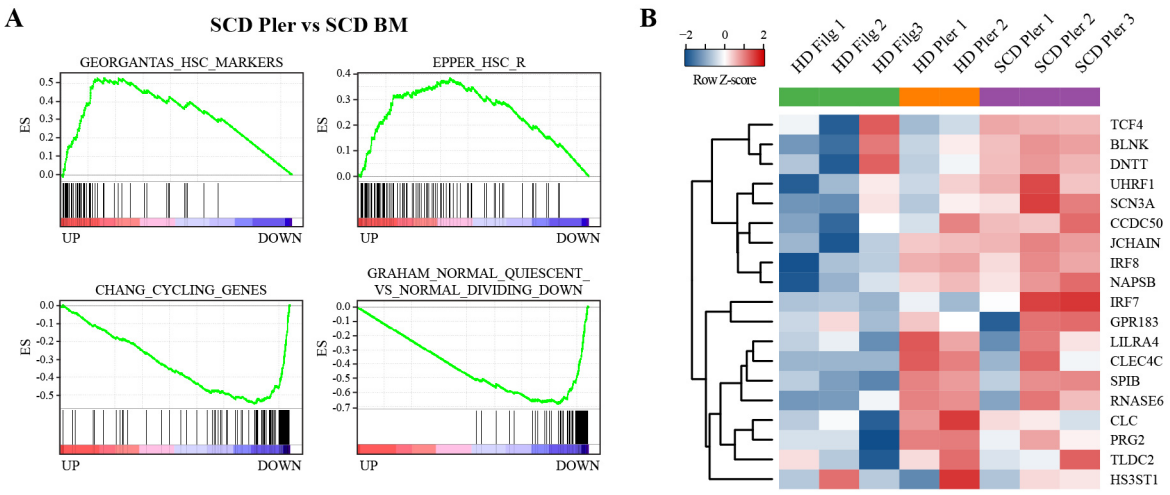
Supplementary Figure 1. Effect of Plerixafor on neutrophil counts. Changes in the neutrophil count over the 66 hours following Plerixafor administration in P1 (SCD Pler 1, red squares), P2 (SCD Pler 2, blue circles) and P3 (SCD Pler 3, green triangles).



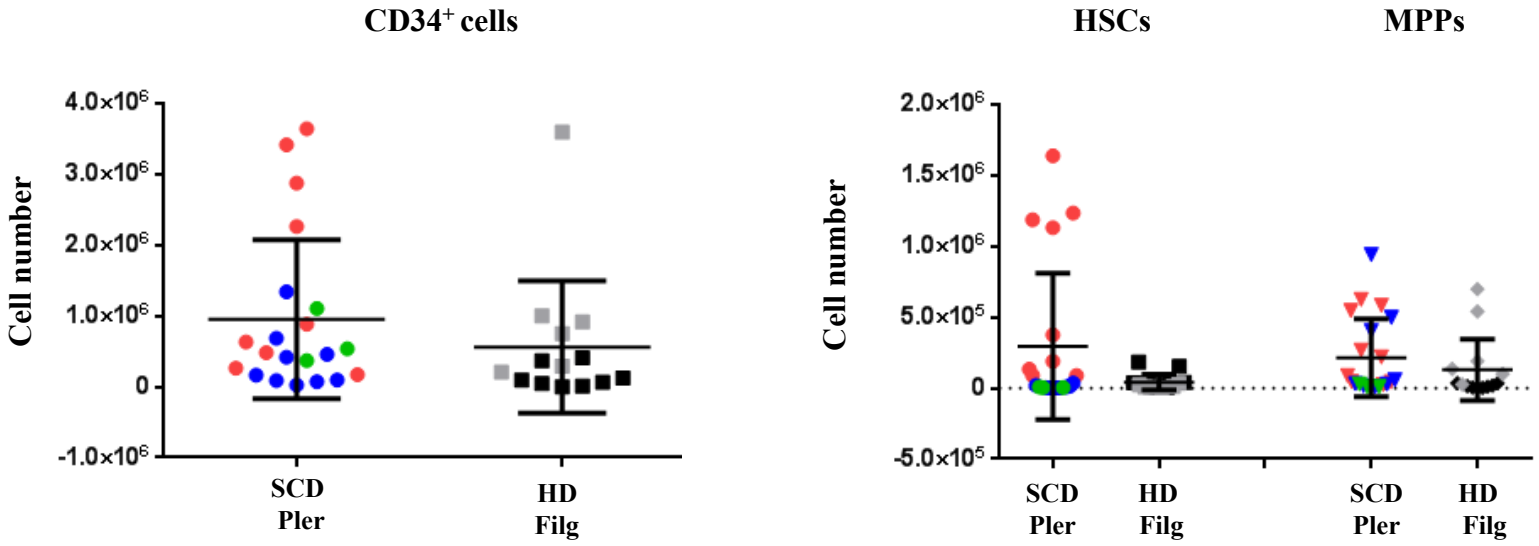
Supplementary Figure 2. Flow cytometry analysis of the proportion of HSCs and MPPs in CD34+ HSPCs from different sources. (A) Gating strategy and explanation of the various subsets; (B) Dot plot analysis of CD38 and CD133 expression in Lin$^-$CD34$^+$ gated cells and CD90 and CD45RA expression in Lin-CD34$^+$CD38$^-$CD133$^+$ cells.



Supplementary Figure 3. Clonogenic and erythroid differentiation potential of Plerixafor-mobilized CD34⁺ cells. (A) Frequency of eruthroid and myeloid CFC (BFU-E, Burst Forming Unit-Erythroid and CFU-GM, Colony Forming Unit- Granulocyte, Monocyte) per 1,000 Plerixafor-mobilized (3 donors) and BM SCD (2 donors) CD34⁺ HSPCs. Bars indicate mean values±SEM of technical replicates. (B-D) Analysis of erythroid differentiation of Plerixafor-mobilized (3 donors) and BM SCD (2 donors) CD34⁺ HSPCs. (B) Time-course FACS analysis of CD36, CD71 and GlycophorinA (GYPA) expression, and of enucleation in erythroid cells derived from Plerixafor-mobilized (2 donors; P1 and P2) and BM SCD (2 donors) HSPCs. We used the nuclear dye DRAQ5 to determine the proportion of enucleated cells (DRAQ5⁻). Cells progressively lost the expression of CD36 and CD71 early erythroid markers and up-regulated GYPA, a protein typically expressed at late stages of erythroid differentiation. (C) Representative FACS analysis of CD36, CD71 and GYPA expression and enucleation. RBCs were analyzed at day 20 of erythroid differentiation. (D) Representative photomicrographs of RBCs obtained at day 20 of culture and stained using May-Grünwald Giemsa.



Supplementary Figure 4. Analysis of HSC-specific markers, cell cycle- and pDC progenitors-related genes. (A) GSEA of HSC- and cell cycle-related gene sets. Plots show the gene sets positively and negatively enriched when comparing SCD Pler and SCD BM samples (FDR <0.05). ES: enrichment score. (B) Heatmap of genes encoding transcriptional regulators and surface markers of pDC progenitors. The row Z-score is plotted on a red-blue colour scale, where red indicates high expression and blue indicates low expression. The colour bar at the top indicates the sample classification.



Supplementary Figure 5. Numbers of CD34$^+$ cells, HSCs and MPPs in the bone marrow of NSG mice transplanted with Plerixafor-mobilized CD34$^+$ cells from SCD patients or Filgrastim-mobilized CD34$^+$ cells from HDs. NSG mice were sacrified 3 to 4 months after injection of SCD (SCD Plerixafor, n=3) or HD (HD Filgrastim, n=2) CD34$^+$ cells. Bone marrow cells were stained and analysed by flow cytometry. The numbers of human CD34$^+$ cells, hematopoietic stem cells (HSCs) and multipotent progenitors (MPPs) were evaluated in each group of mice (red circle and red triangle SCD Pler1; blue circle and blue triangle SCD Pler2; green circle and green circle SCD Pler3; the two HD Filg control are represented by grey square/grey diamond and black square/black diamond , respectively).
Each dot represents an individual mouse.