SUPPLEMENTARY APPENDIX

Unexpected macrophage-independent dyserythropoiesis in Gaucher disease

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Online Supplementary Methods

Colony forming unit assay

Erythro-myeloid colony formation was assayed by culturing $CD34^+$ cells in complete methylcellulose (MethoCult H4435 enriched medium; Stem Cell Technologies): 1,000 cells were seeded per plate. The plates were incubated for 14 days at 37°C in 5% CO2, then scored based on morphology as follows: CFU-GEMM, colony-forming unit–granulocyte, erythrocyte, monocyte, megakaryocyte; BFU-E, burst-forming unit–erythrocyte; CFU-E, colony-forming unit–erythrocyte; and CFU-GM, colony-forming unit–granulocyte, monocyte. BFU-E colonies were scored based on the number of clusters and cells as early (large colonies containing more than 1 cluster and 1,000 cells) and late (small colonies with a single cluster and less than 1,000 cells) colonies.

***In vitro* differentiation of human macrophages and co-culture with erythroblasts**

$CD34^-$ PBMCs from patients and healthy individuals were suspended in RPMI complete medium (RPMI, 10% FBS, 100 µg/mL of streptomycin, 100 U/ml penicillin and 2 mM L-Glutamine - Gibco cell culture) supplemented with 10 ng/mL of hr MP colony-stimulation factor (hrM-CSF, Peprotech) at $3x10^6$ cells/mL overnight. 1 or 3 mL of $CD34^-$ PBMC suspension were plated in each well from 12 or 6 well plates respectively, and monocytes were allowed to adhere to the plastic overnight at 37°C in 5% CO_2. The following day, supernatants were removed and replaced with MP differentiation medium (RPMI complete supplemented with 25 ng/mL of hrM-CSF). The medium was changed every 3 days during 7-10 days of differentiation. EB differentiation was carried out at day 7-8 with the second phase differentiation medium (10 ng/mL hrIL-3, 100 ng/mL hrSCF and 2U/mL hrEPO) at $0.5x\ 10^6$ cells/mL and in the presence or absence of differentiated MP.

Morphological analysis

The cells were stained with May-Grünwald-Giemsa (MGG) after cytospin. The numbers of proerythroblasts, basophilic, polychromatophilic, acidophilic cells and reticulocytes (Acido+Retic) are expressed as the percentages of the total cells. The terminal maturation index was defined as the number of Acido+Retic per slide x100 divided by the number of polychromatophilic cells per slide as described by Arlet *et al*.[1]

Flow cytometry

Erythroid differentiation markers, Allophycocyanin (APC)-conjugated c-Kit (CD117) (eBiosciences) and FITC-labeled Glycophorin A (GpA) (BD Biosciences), have been used to follow the erythroid differentiation at several points during the erythroid culture, as described in Gabet *et al*.[2] PE-labeled Band3 antibody (PE-BRIC6 conjugate, Bristol Institute for Transfusion Sciences) was used to follow erythroid maturation. Cells expressing high levels of Band3 marker are considered as the highly mature acidophilic cells and reticulocytes (for gating strategy of cells see *Online Supplementary Figure S2*). APC-conjugated CD14 (Immunotech), FITC-labelled CD68, PE-conjugated CD163 and CD169 (eBiosciences), PE-conjugated CD49e (α_5 integrin) (BD Biosciences), were used to determine monocytes-MP phenotype. Corresponding Ig antibodies have been used as negative controls. Cells were incubated with antibodies in PBS supplemented by 0.2% FBS for 30 minutes on ice, except for CD68. Cytofix Cytoperm Kit (BD Biosciences) has been used for the CD68 intracellular staining. GCerase activity was measured by using the fluorogenic GCerase substrate PFB-FDGlu (5-Pentafluorobenzoylamino Fluorescein Di-β-D-Glucopyranosid) (Life Technologies). MP and erythroid progenitors were incubated with 1mM of the GCerase substrate or DMSO as control, for 20 minutes and 1 hour respectively,

at 37°C in 5% CO2. The reaction was stopped, and fluorescence intensity was measured within 15 minutes. Acquisition and analysis were performed with the FACS Canto II flow cytometer (BD Biosciences) and with the BD FACS Diva and FlowJo softwares (Version 6.1.3 and Version 7.6.5 respectively).

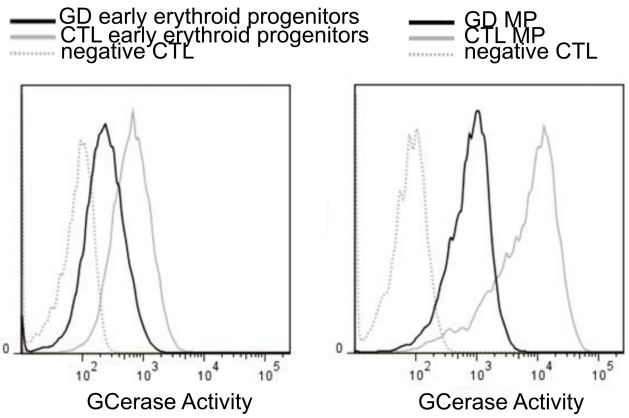
Online Supplementary Figures with legends

Figure S1. Characterization of human CD34$^+$ progenitors and macrophages from GD patients (Representative data)

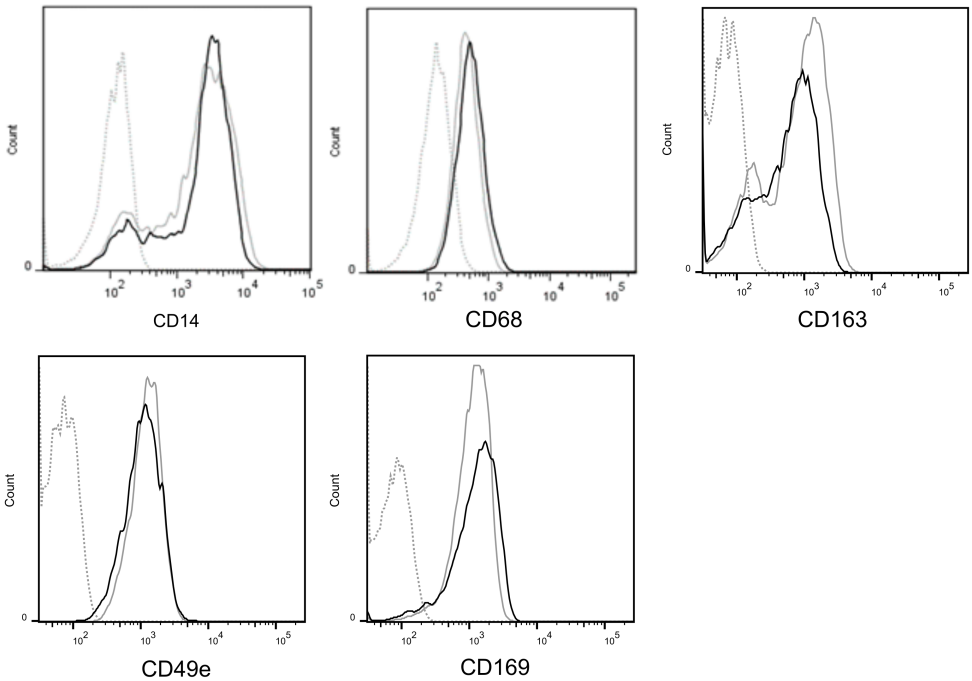
A. GCerase activity was measured in early erythroid progenitors during the Epo independent phase in the presence of the substrate (PFBFDGlu 1mmol/L) by flow cytometry (left panel, CTL control early erythroid progenitors - grey line *vs* GD erythroid progenitors - black line, or DMSO as negative CTL - dash line), and in differentiated macrophages (MP) (right panel, CTL MP - grey line *vs* GD MP - black line or DMSO as negative CTL -dash line).
B. Expression of monocytes-MP markers CD14, CD68, CD163, CD49e and CD169 was measured by flow cytometry in differentiated CTL MP (grey line) and GD MP (black line). Corresponding Ig antibodies have been used as negative controls (dash line).
C. Morphological analysis of CTL MP (left) and GD MP (right) by May–Grünwald–Giemsa (MGG) staining (magnification 60x).

Figure S1.

A.



B.



C.

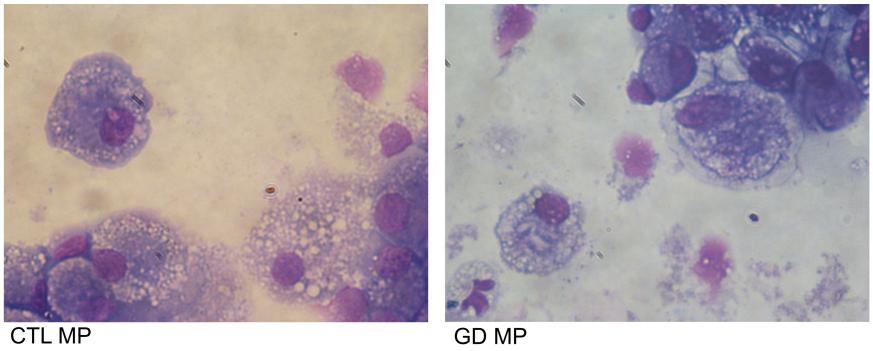


Figure S2. Band3^{hi} population gating strategy.

The population expressing high level of Band3 marker, represented by Hi (A), has been sorted and stained by MGG (B) on day 15 of culture. This population represents the mature cells (acidophilic erythroblasts and reticulocytes). IM and Low represent cells expressing intermediary and very low levels of Band3.

Figure S2.

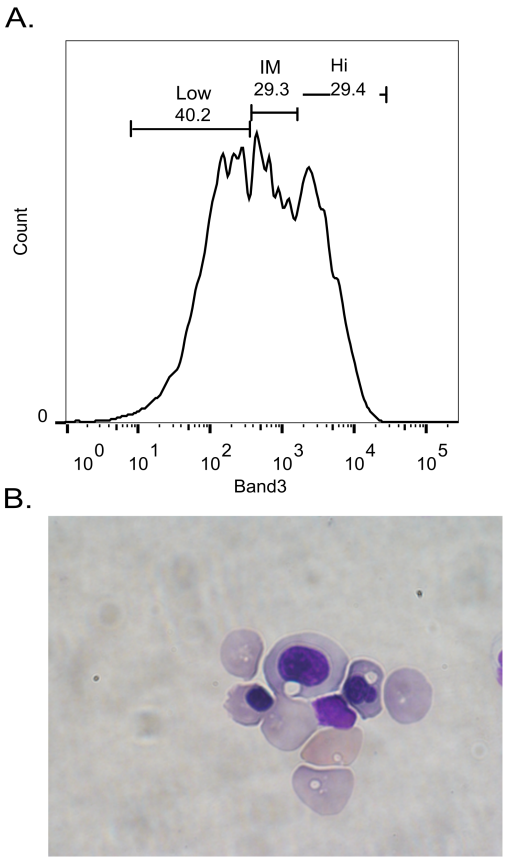
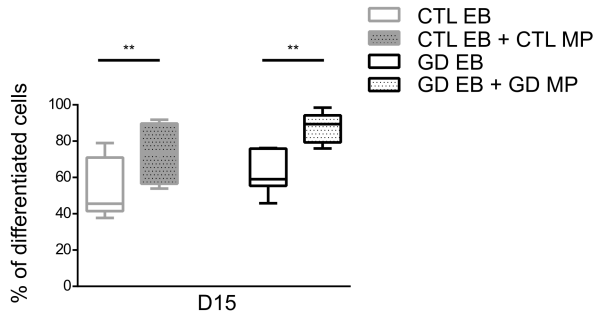


Figure S3. <u>Macrophages improve *in vitro* erythroid terminal differentiation.</u>

CTL or GD EB derived from $CD34^+$ peripheral blood cells were differentiated with or without MPs.
Differentiation was measured by the surface expression of erythroid markers during EB cell culture using flow cytometry. Results represent the percentage of differentiated EB (GPA^+ $CD117^-$ cells) at day 15.
Open grey box, CTL EB cultivated without MP; filled grey box, CTL EB cultivated with CTL MP; open black box, GD EB cultivated without MP; black dash box, GD EB cultivated with GD MP (n=8 for each condition). Medians are represented as horizontal bars (-); upper and lower quartiles are represented on the top and the bottom of the box respectively; minimum and maximum data values are on the top and the bottom of the whiskers represented as dash (-).
p values were determined by Wilcoxon signed-rank test to compare the percentage of differentiated cells between CTL EB cultivated without and with CTL MP ; or GD EB cultivated without and with GD MP (** $p<0.01$).

Figure S3.



Online Supplementary References

1. Arlet JB, Ribeil JA, Guillem F, et al. HSP70 sequestration by free alpha-globin promotes ineffective erythropoiesis in beta-thalassaemia. Nature. 2014;514(7521):242-246.
2. Gabet AS, Coulon S, Fricot A, et al. Caspase-activated ROCK-1 allows erythroblast terminal maturation independently of cytokine-induced Rho signaling. Cell Death Differ. 2011;18(4):678-689.