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ARTICLE

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Nelly Reihani,[1] Jean-Benoit Arlet,[2] Michael Dussiot,[3] Thierry Billette de Villemeur,[4] Nadia Belmatoug,[5] Christian Rose,[6] Yves Colin-Aronovicz,[1] Olivier Hermine,[7] Caroline Le Van Kim[1*] and Melanie Franco[1*]

[1]Université Sorbonne Paris Cité, Université Paris Diderot, Inserm, INTS, Unité Biologie Intégrée du Globule Rouge, Laboratoire d'Excellence GR-Ex, Paris; [2]Sorbonne Paris-Cité, Université Paris Descartes, Service de Médecine Interne, Assistance publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Inserm UMR 1163, CNRS ERL 8254, Hôpital Necker, Institut Imagine, Laboratoire d'excellence GR-Ex, Paris; [3]Sorbonne Paris-Cité, Université Paris Descartes, Inserm UMR 1163, CNRS ERL 8254, Institut Imagine, Hôpital Necker, Laboratoire d'excellence GR-Ex, Paris; [4]Sorbonne Université, Université Pierre et Marie Curie, Service de Neuropédiatrie Hôpital Trousseau, Assistance publique-Hôpitaux de Paris, Hôpital et GRC ConCer-LD, Paris; [5]Hôpitaux universitaires Paris Nord Val de Seine, Assistance publique-Hôpitaux de Paris, Hôpital Beaujon, Service de Médecine Interne, Centre de Référence des Maladies Lysosomales, Clichy; [6]Université Catholique de Lille, Hôpital Saint Vincent de Paul, Service d'Hématologie, Lille and [8]Sorbonne Paris-Cité, Université Paris Descartes, Assistance publique-Hôpitaux de Paris, Hôpital Necker, Service d'Hématologie, Inserm UMR 1163, CNRS ERL 8254, Institut Imagine, Laboratoire d'excellence GR-Ex, Paris, Inserm UMR 1163, CNRS, France

MF and CLVK contributed equally to this work

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ABSTRACT

Gaucher disease is a rare inherited disease caused by a deficiency in glucocerebrosidase leading to lipid accumulation in cells of mononuclear-macrophage lineage known as Gaucher cells. Visceral enlargement, bone involvement, mild anemia and thrombocytopenia are the major manifestations of Gaucher disease. We have previously demonstrated that the red blood cells from patients exhibit abnormal properties, which indicates a new role in Gaucher disease pathophysiology. To investigate whether erythroid progenitors are affected, we examined the *in vitro* erythropoiesis from the peripheral $CD34^+$ cells of patients and controls. CD34- cells were differentiated into macrophages and co-cultivated with erythroblasts. We showed an accelerated differentiation of erythroid progenitors without maturation arrest from patients compared to controls. This abnormal differentiation persisted in the patients when the same experiments were performed without macrophages, which strongly suggested that dyserythropoiesis in Gaucher disease is secondary to an inherent defect in the erythroid progenitors. The accelerated differentiation was associated with reduced cell proliferation. As a result, less mature erythroid cells were generated *in vitro* in the Gaucher disease cultures compared to the control. We then compared the biological characteristics of untreated patients according to their anemic status. Compared to the non-anemic group, the anemic patients exhibit higher plasma levels of growth differentiation factor-15, a marker of ineffective erythropoiesis, but they had no indicators of hemolysis and similar reticulocyte counts. Taken together, these results demonstrated an unsuspected dyserythropoiesis that was independent of the macrophages and could participate, at least in part, to the basis of anemia in Gaucher disease.

Correspondence:

melanie.franco@inserm.fr

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Introduction

Gaucher disease (GD), the most common lysosomal storage disorder, is caused by autosomal recessive mutations in the gene encoding glucocerebrosidase (GCerase). This enzyme is required for the degradation of glycosphingolipids, and its reduced activity results in the accumulation of the immediate substrates glucosylceramide (GlcCer) and its deacylated products, the glucosylsphingosines, within the macrophages.[1] GD has been classified into three types depending on the absence (type 1, the most frequent form comprising 90% of patients) or the presence (type 2 and type 3) of neurological features. Type 2 GD is the most severe form, and the afflicted subjects have a very short life expectancy (less than 2 years). In GD types 1 and 3, visceral enlargement (splenomegaly and hepatomegaly), bone involvement and hematological manifestations (anemia, thrombocytopenia) represent the major symptoms of the disease. GD type 1 is treated efficiently by enzyme replacement therapy (ERT) that leads to a specific uptake of recombinant GCerase by the deficient macrophages.[2] Substrate reduction therapy represents an alternative oral approach based on the reduced synthesis of glucosylceramide. These treatments have been demonstrated to have beneficial effects on anemia, thrombocytopenia, hepatosplenomegaly and bone crisis.[1]

Complications associated with GD have been attributed to the accumulation of GCerase substrates in cells of the monocytic lineage.[3,4] Indeed, lipid-laden macrophages transformed into Gaucher cells that infiltrate the spleen, the liver and the bone marrow are considered the basis for the major GD symptoms. However, a growing number of studies performed on murine and *in vitro* models as well as those using cells from GD patients, indicate that the pathophysiology of GD may involve a wider array of cell types, including hematopoietic and mesenchymal cells, thymic T cells and progenitors, dendritic cells and osteoblasts.[5-10] We have previously shown that the red blood cells (RBCs) of GD patients exhibit abnormal morphological, rheological and functional properties, and could be considered additional factors in the GD pathophysiology on the basis that they can trigger ischemic events.[11] Importantly, a study of a large cohort revealed that anemia was the only risk factor for avascular osteonecrosis, the most debilitating skeletal complication for GD type 1 patients.[12] Anemia affects 36% of GD patients.[13] The underlying mechanism has not been fully described, although it has been generally attributed to hypersplenism and splenic sequestration.[14] However, the severity of the anemia is not directly associated with the degree of splenomegaly, and the anemia may sometimes persist after splenectomy.[15,16] Recent data have shown both bone marrow and hematopoietic abnormalities in GD, and bone marrow infiltration by Gaucher cells was observed in GD patients.[17-20] It has also been reported that GD may affect the growth of erythroid progenitors and myelopoiesis of induced pluripotent stem cells (iPSCs).[21] Moreover, an important extramedullary hematopoiesis with the presence of erythroid cells was observed in the spleen of a murine model of GD.[9,10] We previously detected GCerase activity in normal erythroblasts but not in circulating RBCs.[11] High lipid levels have been frequently reported in the plasma and RBCs of GD patients,[22-25] which raises the possibility that the RBCs are overloaded with lipids due to the passive incorporation of GlcCer and/or that the erythroid progenitors are primarily affected. To evaluate the alterations in the erythroid progenitors, we performed *in vitro* experiments to study the erythroid differentiation from peripheral blood samples from GD type 1 patients. The circulating $CD34^+$ and $CD34^-$ cells from these patients were differentiated into erythroblasts and macrophages, respectively. By performing co-culture experiments of the erythroblasts with the macrophages, we showed accelerated differentiation that was associated with reduced cell proliferation of erythroid progenitors in the cells from the GD subjects compared to controls. This dyserythropoiesis was independent of the macrophage defects because the accelerated differentiation was also observed in the absence of macrophages. Although no evidence of peripheral hemolysis was observed in the anemic GD patients from our cohort, we observed an increased level of growth differentiation factor-15 (GDF-15), a marker of ineffective erythropoiesis, in their plasma. We proposed that dyserythropoiesis *per se* is the basis of the anemia observed in GD.

Methods

Patients

Patients were followed in the French Reference Center for Lysosomal Diseases. A total of 35 type 1 GD patients were recruited prospectively between June 2012 and July 2015. Patients were considered anemic when the hemoglobin (Hb) levels were strictly below 11.5 g/dL for children between 2 and 12 years old, 12 g/dL for women and 13 g/dL for men. No patient was splenectomised. In this cohort, GD was moderate and 10 patients exhibited anemia. Hemograms and biochemical data related to anemia for the 20 untreated GD patients who had never received ERT were compared according to their anemic status. For the *in vitro* erythropoiesis study, blood samples were collected from 24 GD patients, including 9 untreated patients and 15 patients treated with ERT. Detailed clinical and biological information is provided in Table 1. Blood samples were obtained after informed consent according to approved institutional guidelines (Assistance Publique-Hôpitaux de Paris, France).

Colony forming unit assay

These experiments are described in the *Online Supplementary Methods.*

In vitro *differentiation of human erythroid cells by two-phase liquid culture*

Peripheral blood mononuclear cells (PBMCs) were obtained from the blood samples of the GD patients (GD) and from healthy donors used as control. These donors were treated with granulocyte colony-stimulating factor to induce hematopoietic stem cell mobilization. The PBMCs were subjected to Ficoll density gradient separation. The $CD34^+$ cells were isolated by magnetic sorting (Miltenyi Biotec), and an *in vitro* two-phase liquid culture to allow erythroid differentiation was performed, as described by Freyssinier *et al.*[26] During the first phase, the non-adherent cells were expanded for 7 days in a medium containing 100 ng/mL human recombinant (hr) interleukin (IL)-6, 10 ng/mL hr IL-3 and 50 ng/mL hr stem cell factor (SCF). On day 7, the cells were harvested and cultured for 8 days with the second phase medium (10 ng/mL hr IL-3, 50 ng/mL hr SCF, and 2 U/mL hr erythropoietin (EPO). The cells were counted every day, and their concentration was maintained at 0.5×10^6 cells/mL. The SCF, IL-6 and IL-3 cytokines were obtained from Miltenyi Biotec.

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

CD34⁻ PBMCs from patients and control were differentiated into macrophages as described by Ramos *et al.*[27] Erythroblast differentiation was carried out on day 8 with the second phase medium in the presence of differentiated macrophages. The protocol is provided in the *Online Supplementary Methods.*

Flow cytometry

The antibodies and the methods used for the erythroid differentiation and measurements of GCerase activity are provided in the *Online Supplementary Methods.*

ELISA Assay

The GDF-15 levels were measured in the plasma of 8 healthy controls and 15 untreated GD patients using ELISA according to the manufacturer's protocols (R&D Systems).

Statistical analysis

For the *in vitro* studies, Wilcoxon paired tests were used to compare control and GD cultures. For the biochemical data analyses, the results are presented as the median (extremes). Mann-Whitney tests were used to compare control and GD patients and anemic and non-anemic patients.

Results

Erythroid differentiation is accelerated in GD

To explore the impact of GCerase deficiency on erythropoiesis, we studied the erythroid precursors produced by the peripheral CD34⁺ cells of the GD patients and control.

We first explored the clonogenic capacity of GD and control CD34⁺ cells using a colony-forming unit assay in semi-solid culture. The progenitor cells of different lineages and stages of maturation produce colonies that differ in their size, morphology and cellular composition. The total colonies (including erythroid and non-erythroid colonies) were counted after 14 days of culture. We observed that the CD34⁺ cell progenitors from GD patients and control exhibited similar total (Figure 1A) and erythroid (Figure 1B) clonogenic capacities. To identify the erythroid-associated CD34⁺ stages, we scored the BFU-E colonies based on the number of early and late BFU-E colonies. The data showed similar and homogeneous progenitor populations from the control and GD subjects (Figure 1C).

To more precisely study the erythropoiesis in the cells from the GD patients and controls, we then performed a two-phase liquid culture over a period of 15 days. In the first phase, we differentiated the CD34⁻ cells into

Table 1. Biological parameters of the Gaucher disease (GD) patients whose cells were used to study *in vitro* erythropoiesis.

Pat n.	Age (y)	Sex	Genotype	Time under ERT (year)	SMG	HMG	Hb (g/dL)	MCV (fL)	Reticulocytes (10^3/mm^3)	Ferritin (µg/L)	Platelets (10^3/mm^3)	CCL-18 (pg/mol)	chitotriosidase activity
1	18	F	N370S/?	no	+	–	11.9	96	82.6	369	80	765	1994
2	18	F	N370S/?	no	–	–	11	97	81.8	346	93	663	1904
3	8	F	N370S/1265-1317DEL55	no	+	+	10.5	78	ND	276	87	153	ND
4	3	M	D409H/D409H	no	+	+	11.7	69	33.6	228	119	754	14630
5	50	F	N370S/L444P+E326K	no	+	+	11.6	87	62	526	65	689	ND
6	14	M	N370S/?	no	+	–	14	84	ND	638	74	139	ND
7	20	F	N370S/L324P	no	+	+	12	92.7	54	1316	126	289	Deficiency
8	60	M	N370S/L444P	no	–	–	13.6	89.8	85	126	109	149	517
9	6	F	ND	no	–	–	12.5	74	68.5	215	153	320	ND
10	12	F	N370S/R120W	7	–	–	11.9	79	78.3	172	210	ND	ND
11	20	F	N370S/?	1	ND	ND	11.2	104	85.1	130	123	347	726
12	35	M	N370S/N370S	3	+	+	16.2	81	ND	545	176	82	ND
13	15	M	N370S/?	11	–	–	13.9	84	38.9	111	159	233	12750
14	15	M	N370S/?	11	–	–	14.7	84	38.6	99	182	476	6750
15	80	M	N370S/L324P	3	–	–	13.6	89	ND	1341	157	518	9400
16	11	M	N370S/1263 DEL 55	3	+	+	ND	78	ND	103	128	ND	ND
17	19	F	N370S/?	0.5	–	–	13.4	96	ND	237	140	257	ND
18	19	F	N370S/?	0.5	–	–	13.2	96	ND	152	182	354	ND
19	14	M	ND	9	–	–	13.6	82	22.7	142	271	163	1180
20	20	F	N370S/?	1	–	–	14.1	99	29.4	192	167	205	361
21	18	F	ND	0.5	+	+	14,9	90	124	451	214	ND	149
22	4	M	D409H/D409H	1	–	–	12.5	70	42.6	97	318	263	888
23	20	F	N370S/?	2	–	–	14.1	91	30.5	132	216	175	ND
24	20	F	N370S/?	2	–	–	13.1	96	37	72	206	193	ND

SMG: splenomegaly. HMG: hepatomegaly. Hb: hemoglobin. MCV: mean corpuscular volume. ND: not determined. Time under ERT (year): "no" means that the patient did not meet the criteria for ERT and did not receive this treatment. The clinical and biological parameters, i.e., SMG, HMG, Hb level, MCV, reticulocytes and platelet counts, ferritin level, CCL-18 (C-C motif chemokine ligand 18) level and chitotriosidase activity, reported here are the results of the last recorded data, usually obtained on the day of blood sampling for the study or within the previous few weeks. Genotype: "?" means that the mutation was not identified despite screening of the most frequent mutations of the GBA gene. Patient 7 had a constitutive chitotriosidase deficiency.

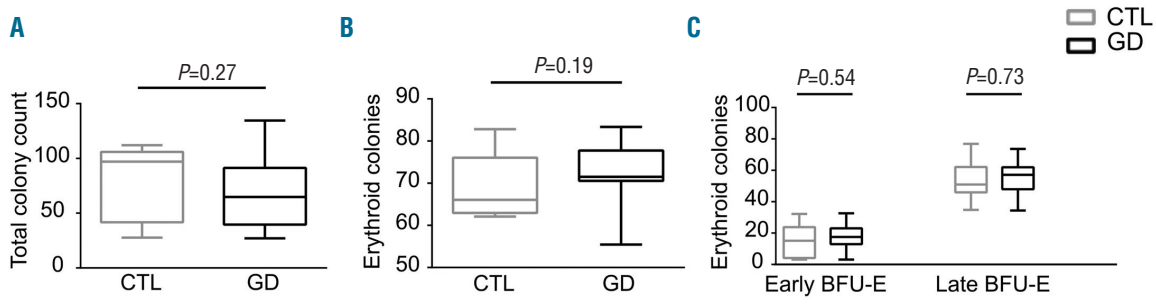

Figure 1. Clonogenic potential of CD34⁺ cells. A. Number of total colony counts including erythroid (BFU-E: burst forming unit), non-erythroid (granulocyte/macrophage colony forming units: CFU-GM, CFU-G and CFU-M) and multilineage progenitors (CFU-GEMM) on day 14 of culture. B. Number of BFU-E burst-forming units (BFU-E). C. Number of early and late erythroid BFU-E. The colonies were scored by direct microscopic visualization on day 14 of culture for the CTL (n=13) and GD (n=13) subjects, which are shown as gray and black boxes, respectively. CTL: control; GD: Gaucher disease.

macrophages and expanded the CD34⁺ cells in parallel for 7 days. In a second phase, EPO was added to the expanded CD34⁺ cells to trigger the erythroid differentiation, and the macrophages were cultivated with erythroblasts to mimic the bone marrow microenvironment for 8 subsequent days. We measured the GCerase activity in the CD34⁺ early progenitors from the control and GD patients. We showed that GCerase was active in the early erythroid progenitors from both GD and control, although at a much lower level in the GD progenitors (*Online Supplementary Figure S1A*, left panel). The macrophages derived from GD CD34⁻ adherent cells also exhibited reduced GCerase activity compared to those from the control (*Online Supplementary Figure S1A*, right panel). The monocyte- macrophage profile was confirmed by CD14 (more than 90%), CD68 and CD163 markers (*Online Supplementary Figure S1B*) as well as by their morphological appearance (*Online Supplementary Figure S1C*). The expression of CD169, the marker of the central macrophages,[28] was also demonstrated as well as the one of CD49e (α5 integrin) since it is involved in the interaction between macrophages and the maturing erythroblasts in the erythroblastic island (*Online Supplementary Figure S1B*).[29]

We then compared the erythroid differentiation of the GD erythroblasts cultivated with GD macrophages, and the control erythroblasts cultivated with control macrophages. We observed accelerated differentiation of the GD erythroblasts compared to controls (Figure 2A) on day 15 of the erythroid culture, as shown by the significant increase in the percentage of the differentiated GPA⁺ CD117⁻ cell subpopulation at day 15 (89.4 *vs.* 73.5%, *P*=0.01, Figure 2B). Moreover, we observed an increased percentage of mature cells (acidophilic erythroblasts and reticulocytes) that highly expressed the band 3 marker (20.1 *vs.* 16.6%, *P*=0.02, Figure 2C), referred to as band 3^{hi} cells on the basis of the gating strategy (see *Online Supplementary Figure S2*). The accelerated erythroid differentiation and maturation in the cells from the GD patients was also confirmed by the MGG staining, which showed a lower percentage of polychromatophilic cells and a higher percentage of mature acidophilic cells and reticulocytes (Figure 2D). On day 15, the terminal maturation index was not reduced in the GD *vs.* control cultures (Figure 2E), which suggested the absence of maturation arrest during the erythroid differentiation. Finally, there was no difference in the rate of cell apoptosis between the GD and control cells during the differentiation phase, as indicated by annexin V staining (*data not shown*). Taken together, these experiments showed accelerated erythroid differentiation without any maturation arrest or cell death in the GD erythroblasts.

The accelerated erythroid differentiation in GD is independent of the macrophages

Because macrophages are the key players in GD, we next determined whether the dyserythropoiesis was mediated by the GD macrophages by performing cross co-culture experiments. The control erythroblasts were cultivated with GD or control macrophages. The percentage of differentiated erythroblasts was the same in both conditions (Figure 2F). This result strongly suggested that the GD macrophages are not *per se* the cause of the dyserythropoiesis observed in GD. These observations were also confirmed by the cross-culture of GD EBs cultivated with control or GD macrophages (*data not shown*). It is noteworthy that erythroid differentiation was more efficient when the EBs were cultivated in the presence of either type of MP (GD or control), as shown by the higher percentage of well-differentiated cells in the co-culture experiments (*Online Supplementary Figure S3*). This latter result confirms the important role of MPs for efficient erythroid differentiation, as previously demonstrated by others.[29]

To confirm that the EB differentiation in GD was primarily affected independently of the presence of abnormal macrophages, we performed an *in vitro* erythropoiesis experiment using CD34⁺ cells cultivated without macrophages. Proliferation as well as the late differentiation of GD and control erythroblasts were compared. Flow cytometry analysis revealed that before adding EPO, CD117 (cKit) expression levels are equivalent in control and GD erythroblasts indicating the same stage of differentiation (*data not shown*). We then observed an increased percentage of differentiated cells from the GD subjects, as shown by the increased percentage of the GPA⁺ CD117- cell subpopulation on day 12 (GD=42 *vs.* control =21.6%, *P*=0.0003) and day 15 (GD=60 *vs.* control =41%, *P*=0.0001, Figure 3A,B) and the band 3^{hi} erythroblasts (GD=11.3 *vs.* control=9.1%, *P*=0.03, Figure 3C). These results indicate that GD erythroid differentiation was significantly accelerated compared to control. Moreover, the MGG analysis showed a lower percentage of basophilic cells and a higher

Table 2. Biological parameters of untreated Gaucher disease (GD) patients according to their hemoglobin level.

	Anemic n= 10	Non-Anemic n= 10	*P*
Age (year)	17 [3 - 61]	10 [3 - 50]	0.56
Sex (F), n (%)	8 (66.7%)	6 (54.5%)	
Hb (g/dL)	11.0 [7.6 - 12.9]	12.5 [11.6 - 14]	0.003
MCV (fL)	81 [74 - 94]	77 [69 - 89]	0.25
MCH (pg)	28 [22 - 33.5]	26.6 [24.4 - 29.5]	0.6
MCHC (g/L)	34.6 [29.6 - 35.9]	34.2 [32.5 - 36.5]	0.73
Reticulocytes (103/mm^3)	62 [49.4 - 114]	59.1 [36.5 - 93]	0.62
Platelets (103/mm^3)	88 [43 - 191]	105 [79 - 208]	0.08
Haptoglobin (g/L)	0.67 [0.2 - 1.9]	0.71 [0.2 -1.52]	0.86
LDH (IU/L)	288 [201 - 404]	389 [261 - 573]	0.27
Total bilirubin (mmol/L)	17 [12 - 22]	17 [6 - 18.2]	0.4
Conjugated bilirubin	Absence	Absence	
CRP (mg/L)	1 [1 - 24]	1 [1 - 7]	0.99
Ferritin (µg/L)	372 [128 - 1316]	221.5 [53 - 594]	0.06

The hematological and biochemical data for 20 untreated patients who had never received ERT were compared according to their anemic status. Patients were considered anemic when their hemoglobin (Hb) levels were strictly below 11.5 g/dL for children between 2 and 12 years old, 12 g/dL for women and 13 g/dL for men. MCV: mean corpuscular volume. MCH: Mean corpuscular hemoglobin. MCHC: mean corpuscular hemoglobin concentration. LDH: lactate dehydrogenase. CRP: C-reactive protein. The values are expressed as the median (extremes). The P values were determined using the Mann-Whitney test to compare continuous variables between the anemic and non-anemic patients.

percentage of acidophilic cells and reticulocytes in the GD erythroblasts *vs.* those from the control (Figure 3D,F). On day 15, the terminal maturation index was significantly increased in the GD compared to the control progenitor cells, which indicated accelerated differentiation without any arrest in the maturation (Figure 3E). These results showed an abnormal erythroid differentiation in GD that was independent of the macrophage defects.

GD erythroid progenitors exhibit decreased proliferative capacity

Cell expansion and viability were measured during the two culture phases using trypan blue staining. The cell proliferation capacity is reported as the absolute number of viable cells at days 0 and 15 of culture. Our results showed a decrease of the proliferation capacity of 7.55-fold in GD cells compared to those from the control (GD=121.9 *vs.* control=920.7-fold, $P<0.0001$, Figure 4A). This reduced proliferation resulted in a dramatically smaller absolute number of band 3^{hi} erythroblasts produced at the end of the differentiation (GD=0.6x10^6 *vs.* control=14x10^6, $P=0.0001$, Figure 4B), despite a higher percentage of differentiated cell population.

Taken together, these data showed that acceleration of erythroid differentiation and maturation occurred to the detriment of cell proliferation and provided evidence for dyserythropoiesis in GD. We then explored whether this dyserythropoiesis could explain the mild anemia observed in GD patients.

An anemia of central origin in GD

To investigate the mechanism for the anemia in GD, hematological and biochemical parameters were examined in 20 GD untreated patients. Of these 20 subjects, 10 patients exhibited mild anemia. These anemic GD patients were compared to the non-anemic subjects (Table 2). The ages and sex ratios were similar. No differences were observed for hemolytic markers (lactate dehydrogenase (LDH), haptoglobin and bilirubin levels), and none of the patients had an elevated absolute reticulocyte count (<120.103/mm^3 in all patients). These data ruled out the hypothesis that GD patients have peripheral anemia as a result of hemolytic processes. Moreover, the C-reactive protein (CRP) and ferritin levels indicated no evidence of either inflammation or iron deficiency. Additionally, the plasma level of EPO was increased in untreated GD patients *vs.* the healthy controls (15.24 *vs.* 3.46 IU/L, $P<0.0001$). This result indicated that the anemia in GD is not due to an EPO deficiency and instead could represent a compensatory mechanism preventing a more pronounced anemia in GD patients. The fact that the anemic patients did not exhibit higher reticulocyte counts than those without anemia indicated that the bone marrow response was not appropriately responding to the anemia, which suggested a central defect.

To further test this hypothesis, we analyzed markers of ineffective erythropoiesis in plasma from GD patients. Growth differentiation factor-15, a marker of ineffective erythropoiesis in several hemoglobinopathies,[30,31] was significantly increased in the plasma from the untreated GD patients compared to the healthy controls (Figure 5A). Among our untreated GD cohort, the anemic patients exhibited higher GDF-15 levels than the non-anemic patients (4311 *vs.* 2298, $P=0.006$, Figure 5B). Taken together, these data suggest that the anemia observed in GD has a central origin.

Discussion

In spite of the progress made toward the molecular characterization of GD, much still remains to be investigated about the mechanisms involved in the pathophysiology of the clinical complications.

In the study herein, we demonstrated for the first time a dyserythropoiesis in GD characterized by an accelerated differentiation accompanied by decreased cell proliferation. The clinical data from the GD patients strongly suggested that the anemia observed in GD is of central origin. Moreover, we observed high levels of GDF-15, a marker of dyserythropoiesis, in the anemic GD patients.

Previous studies have described infiltration of the bone marrow as well as some erythrophagocytosis by Gaucher cells in GD patients.[17-19,32,33] These occurrences could be considered partly responsible for the bone marrow erythroid insufficiency. Lee *et al.* first described the erythrophagocytic events in Gaucher cells in subjects with slight erythroid hyperplasia.[17] In the study herein, ferrokinetic studies already suggested a mild dyserythropoiesis.[17] In our study, it would have been useful to obtain marrow from the anemic patients for examination, but bone marrow aspiration is not ethically justified in GD.

Consistent with the results of our *in vitro* study in semi-solid culture, Lecourt *et al.* reported that CD34$^+$ bone marrow progenitors from control and GD patients exhibit similar basic characteristics.[7] These cells gave rise to a comparable number of total erythroid and non-erythroid colonies and exhibited similar erythroid potential. Our

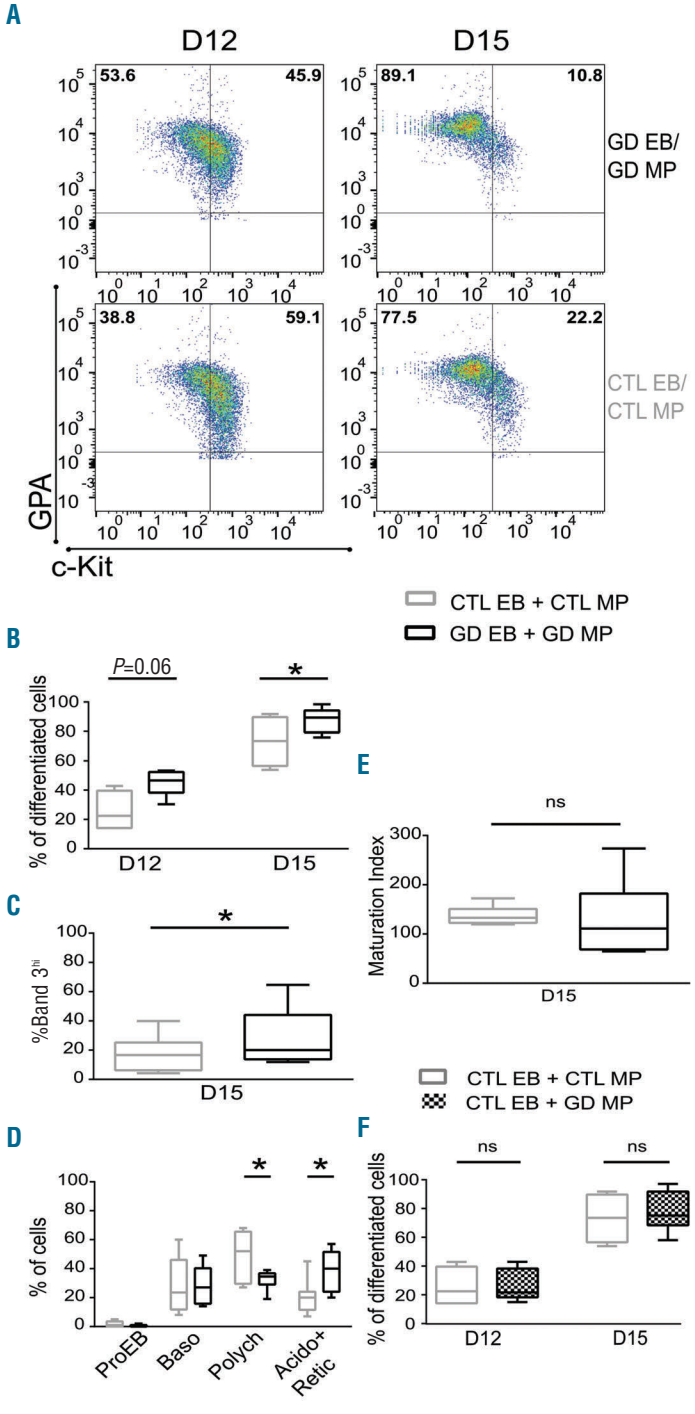

Figure 2. *In vitro* erythroid differentiation of Gaucher disease (GD) erythroblasts cultivated with macrophages. Erythroblasts (EBs) derived from the CD34$^+$ peripheral blood cells of GD patients (GD) or healthy controls (CTL) were cultivated with macrophages (MPs) derived from CD34- cells from the GD or CTL subjects. The GD EBs cultivated with GD MPs were compared with the CTL EBs cultivated with CTL MPs. Expression of erythroid surface markers was measured by flow cytometry on day 12 and 15 of erythroid differentiation. A. Representative flow cytometry plots of glycophorin A (GPA) and c-Kit (CD117) surface expression on days 12 and 15 in the GD and CTL cultures. The GPA$^+$ CD117$^-$ cell population represents the differentiating EBs. B. Percentage of GPA$^+$ CD117$^-$ cells derived from CTL or GD patients on days 12 and 15 of erythroid differentiation. C. Percentage of band 3^{hi} cells on day 15 of erythroid differentiation. These cells represent the mature EBs. D. The boxes represent the percentage of GD or CTL progenitors on day 15 of erythroid differentiation. Morphological analysis after May–Grünwald–Giemsa (MGG) staining was used. ProEB: proerythroblasts; Baso: basophilic cells; Polych: polychromatophilic cells; Acido+Retic: acidophilic cells and reticulocytes E. T h e boxes represent the terminal maturation index (on day 15 of differentiation) as defined in the *Online Supplementary Methods section*. F. CTL or GD MPs were co-cultivated with CTL EBs. The percentages of GPA$^+$ CD117$^-$ cells on days 12 and 15 of erythroid differentiation were compared. The results are presented as box-and-whisker plots. Gray boxes, culture of CTL EBs with CTL MPs; black boxes, culture of GD EBs with GD MPs; tiled boxes, culture of CTL EBs with GD MPs. For B, C, D, E and F, n=8 for each condition. The medians are represented as horizontal bars (-); the upper and lower quartiles are represented as the top and the bottom of the box, respectively; and the maximum and minimum data values are shown by dashes (-) at the top and the bottom, respectively, of the whiskers. The *P* values were determined using the Wilcoxon signed-rank test to compare the parameters of erythroid differentiation between the CTL and GD cultures on days 12 and 15 (*P<0.05). ns= non significant.

results also showed that CD34+ peripheral hematopoietic stem cells in controls and GD patients are basically at the same stage of differentiation. However, starting from the same point, the GD progenitors demonstrated moderately accelerated differentiation in liquid culture conditions with a prolonged differentiating step that permitted a detailed study for each stage of the maturation. We observed clear dyserythropoiesis in the GD type 1 patients (n=24). This dyserythropoiesis was characterized by accelerated erythroid differentiation and diminished cellular proliferation without maturation arrest. Sgambato *et al.* have recently performed an *in vitro* hematopoiesis study using iPSCs from a limited number of GD patients (n=4). They showed an enhanced myeloid differentiation and decreased erythroid differentiation and maturation in the severe types of GD, but they did not report any clear differences in the single type 1 GD patient in their study.[21] It should be noted that since the starting materials were dif-

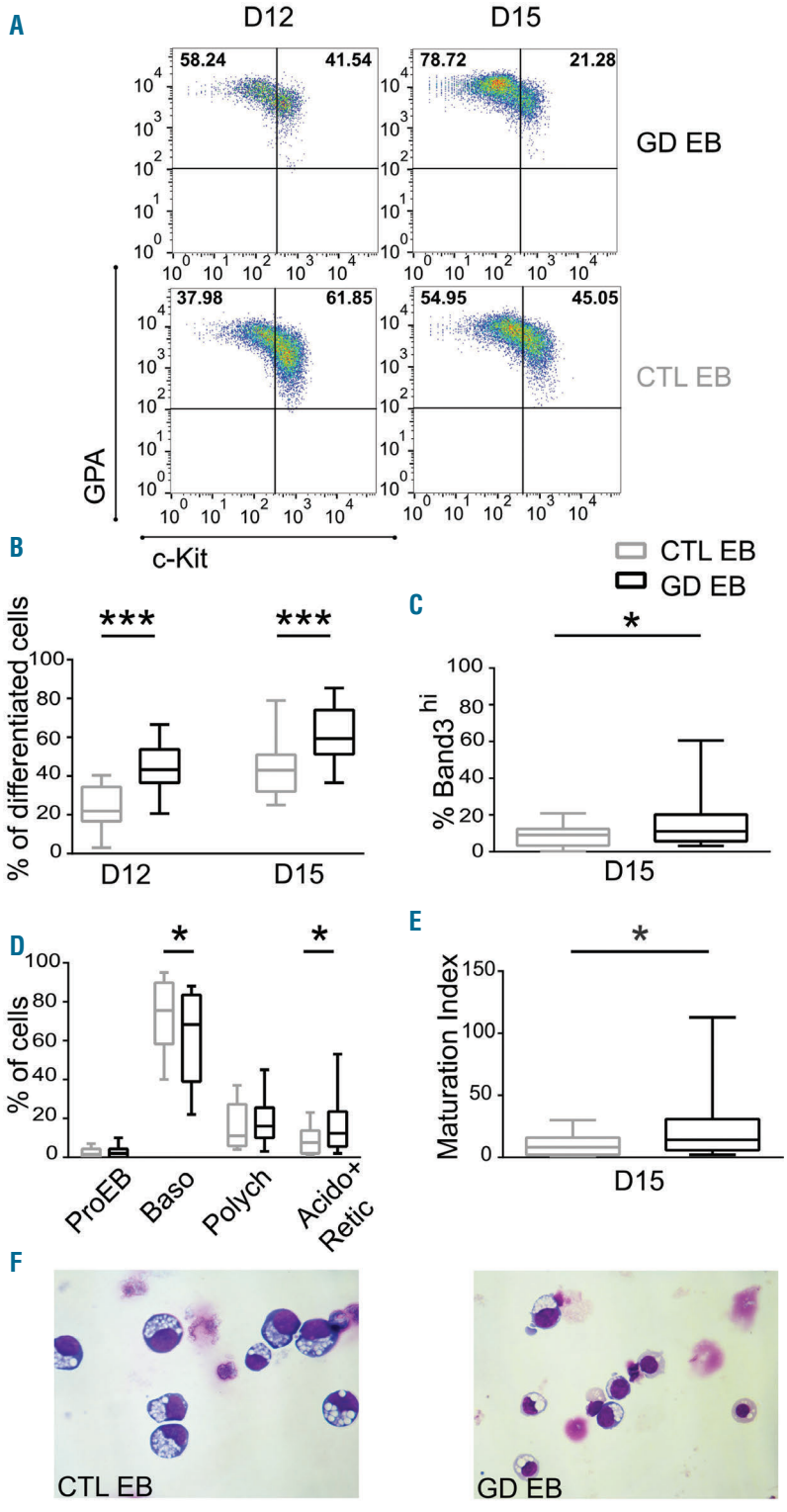

Figure 3. *In vitro* erythroid differentiation of Gaucher disease (GD) erythroblasts cultivated without macrophages. CD34+ cells derived from the peripheral blood cells from GD patients (GD) or healthy controls (CTL) were cultivated without macrophages (MPs) and differentiated into the erythroid lineage until day 15 as described in methods. The surface expression of the erythroid differentiation markers was measured using flow cytometry during erythroblast (EB) differentiation. A. Representative flow cytometry plots of the cell surface expression of glycophorin A (GPA) and c-Kit (CD117) on days 12 and 15 of the erythroid differentiation culture. The GPA+ CD117- cells represent the differentiating EBs. B. Percentage of GPA+ CD117- cells derived from CTL or GD patients on days 12 and 15 of the erythroid differentiation performed without MPs (CTL n=18; GD n=24). C. Percentage of band 3hi cells on day 15 of erythroid differentiation (CTL n=13; GD n=13). These cells represent the mature EBs. D. The boxes represent the percentage of GD or CTL erythroid cells on day 15 of the erythroid differentiation performed without MPs (CTL n=12; GD n=17). Morphological analysis after MGG (May-Grünwald-Giemsa) staining was used. ProEB, proerythroblasts; Baso: basophilic cells; Polych: polychromatophilic cells; Acido+Retic: acidophilic cells and reticulocytes. E. The boxes represent the terminal maturation index as defined in the *Online Supplementary Methods* section (CTL n=12; GD n=17). F. Representative morphological analysis of erythroid differentiation as indicated by MGG staining on day 15 of cell culture (magnification 60x). The results are presented as box-and-whisker plots. Gray boxes (CTL EBs); black boxes (GD EBs). The medians are represented as horizontal bars (-); the upper and lower quartiles are represented as the top and the bottom of the box, respectively; and the maximum and minimum data values are shown as dashes (-) at the top and the bottom, respectively, of the whiskers. The *P* values were determined using the Wilcoxon signed-rank test to compare the parameters of erythroid differentiation between the CTL and GD cultures on days 12 and 15 (*P<0.05; *** P<0.001).

ferent (fibroblasts *vs.* peripheral blood from patients), our study and that of Sgambato *et al*. are not comparable.

Dyserythropoiesis is a hallmark of β-thalassemia, a major inherited hemoglobinopathy that is caused by a quantitative defect in the synthesis of the β–globin chains. However, the dyserythropoiesis in this disease is characterized not only by accelerated erythroid differentiation, but also by maturation arrest and apoptosis at the polychromatophilic stage.[34-36] In our study of GD subjects, the increased erythroid differentiation was associated with a decrease in cell expansion, but we did not observe any premature death of the erythroid precursors or maturation arrest. This may explain the mild anemia in GD compared to the much more severe anemia observed in patients afflicted with β-thalassemia.

Erythropoiesis requires fine regulation of cell survival, proliferation and differentiation that is controlled primarily by regulatory signals provided by cytokines. Transforming growth factor β1 (TGF- β1) is known to accelerate the erythroid differentiation, allowing full terminal differentiation toward enucleated red cells.[37,38] TGF- β1 also induces a massive inhibition of cell proliferation that mainly involves cell cycle arrest rather than apoptosis.[38] We observed similar effects, albeit to a lesser extent, in GD during *in vitro* erythropoiesis. Because TGF-β1, or cytokines belonging to the same family (such as GDF-11 and GDF-15), represent autocrine factors released by erythroblasts, they could induce or impair the signaling pathways responsible for the dyserythropoiesis in GD. However, due to the limited number of circulating progenitor cells, the cell cycle could not be investigated, and the question of the cell cycle kinetics remains open.

Currently, Gaucher macrophages are considered the main cause of the GD complications. In our experiments, *in vitro* dyserythropoiesis was observed even in the absence of macrophages. Although we could not exclude a local paracrine action of the GD macrophages in the

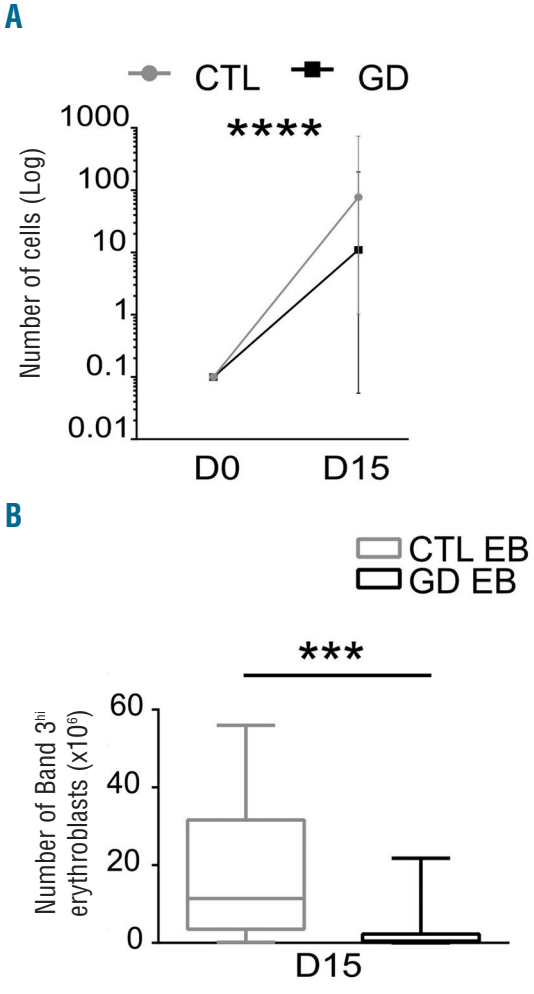

Figure 4. Cell proliferation during *in vitro* erythropoiesis. A. Absolute numbers of cells derived from CD34⁺ cells from the GD and CTL subjects on day 0 and day 15 of erythroid differentiation without MPs (macrophages) (n=24 for each GD and control group). B. Absolute numbers of mature cells derived from the CD34⁺ cells from the GD and CTL subjects on day 15 of erythroid differentiation without MPs (n=17 for each GD and control group). This value has been calculated from the percentage of band 3^{hi} cells multiplied by the total number of cells at day 15. Gray boxes and curve show the results for the CTL erythoblasts (EBs), and black boxes and curve show the results for the GD EBs. The *P* values were determined using the Wilcoxon signed-rank test to compare the expansion of cells between CTL and GD cultures. For figure A, the ratios of the cell numbers on day 15 to those on day 0 were compared by a Wilcoxon signed-rank test (**** *P*<0.0001). For figure B, the *P* value was determined using the Mann-Whitney test to compare the number of Band 3^{hi} erythroblasts (***P<0.001). For figure B, the *P* value was determined using the Mann-Whitney test to compare the number of Band 3^{hi} erythroblasts (***P<0.001). CTL: control; GD: Gaucher disease.

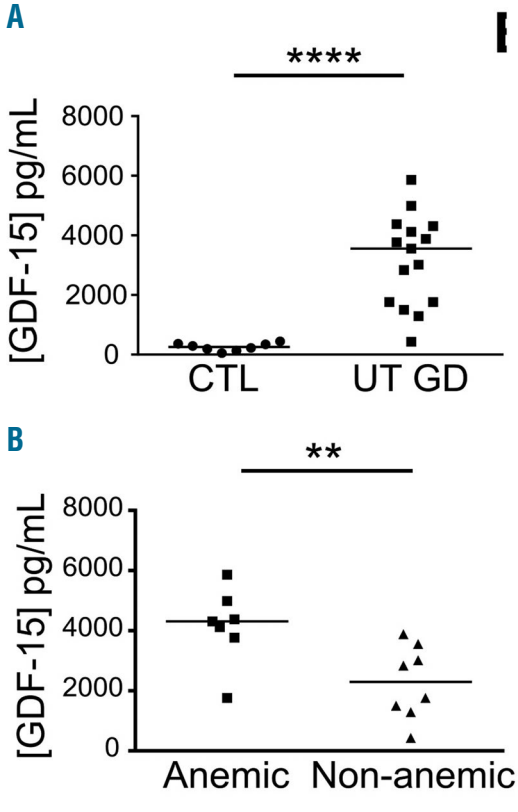

Figure 5. Plasma growth differentiation factor-15 (GDF-15) levels in GD patients. A. Increased level of GDF-15 in the plasma from untreated GD patients (UT GD, n=15) compared to CTL (CTL, n=8). B. GDF-15 level measured in GD patients according to their anemic status (n=7 anemic patients *vs.* n=8 non-anemic patients). The *P* values were determined using the Mann-Whitney test to compare the GDF-15 levels between control and GD subjects. (**P<0.01, ****P<0.0001). The bars represent the medians. CTL: control; GD: Gaucher disease.

bone marrow, these data strongly suggest that the primary defects leading to the dyserythropoiesis in GD may intrinsically affect the erythroid lineage. Because the macrophages are essential components of the erythroblast islands that play an important role in enucleation processes,[29] we cannot rule out any effect of macrophages during the last step of erythroid terminal maturation, which could not be efficiently analyzed in our culture system. These late stages could also be affected by autophagy, a process that has been linked to GD pathophysiology[4,39] and is known to play a critical role during erythroid differentiation. Mesenchymal stem cells also exhibit GCerase activity and are important components of the bone marrow microenvironment.[7] The effects of these cells in GD hematopoiesis were not investigated in our study.

Anemia in GD patients is efficiently corrected by a few months of ERT. Unfortunately, information on the longitudinal effects of ERT on the indirect biological characteristics of erythropoiesis (Hb, reticulocyte count, GDF15) in anemic GD patients was not available in our cohort. Moreover, the effect of ERT therapy on *in vitro* erythropoiesis was not investigated in our study. Indeed, the blood samples from treated patients were collected two weeks after their last ERT infusion, and the progenitor cells were washed and then cultivated *in vitro* during two additional weeks without any ERT drugs.

Key sphingolipids are known to accumulate in GD and are proposed to be responsible for numerous aspects of GD pathophysiology.[10,24] The results of the study herein do not permit a conclusion as to whether the dyserythropoiesis is directly due to the reduced enzyme activity and lipid accumulation, or to an alteration in the mechanisms involved in enzyme folding. Alternatively, since sphingosine 1-phosphate (S1P), as a bioactive lipid, can affect the hematopoiesis and RBC signaling in addition to its multiple cellular and systemic functions,[40-42] the possibility that the abnormal sphingolipid contents in the erythroid progenitors could affect erythropoiesis could be an interesting hypothesis to investigate.

In conclusion, we have shown that anemia in GD has a central origin and is associated with an unexpected dyserythropoiesis. This dyserythropoiesis is characterized by accelerated erythroid differentiation and reduced proliferation capacities that are independent of the macrophage defects. Our data shed new light on the mechanism for the anemia in GD and highlight the role of the erythroid cells as important contributors to the pathophysiology of GD.

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