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Macrophage Models of Gaucher Disease for Evaluating Disease Pathogenesis and Candidate Drugs

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Abstract

Gaucher disease is caused by an inherited deficiency of glucocerebrosidase that manifests with storage of glycolipids in lysosomes, particularly in macrophages. Available cell lines modeling Gaucher disease do not demonstrate lysosomal storage of glycolipids; therefore, we set out to develop two macrophage models of Gaucher disease that exhibit appropriate substrate accumulation. We used these cellular models both to investigate altered macrophage biology in Gaucher disease and to evaluate candidate drugs for its treatment. We generated and characterized monocyte-derived macrophages from 20 patients carrying different Gaucher disease mutations. In addition, we created induced pluripotent stem cell (iPSC)-derived macrophages from five fibroblast lines taken from patients with type 1 or type 2 Gaucher disease. Macrophages derived from patient monocytes or iPSCs showed reduced glucocerebrosidase activity and increased storage of glucocerebroside and glucosylsphingosine in lysosomes. These macrophages showed efficient phagocytosis of bacteria but reduced production of intracellular reactive oxygen species and impaired chemotaxis. The disease phenotype was reversed with a noninhibitory small-molecule chaperone drug that enhanced glucocerebrosidase activity in the macrophages, reduced glycolipid storage, and normalized chemotaxis and production of reactive oxygen species.

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Macrophages differentiated from patient monocytes or patient-derived iPSCs provide cellular models that can be used to investigate disease pathogenesis and facilitate drug development.

INTRODUCTION

Gaucher disease (Mendelian Inheritance in Man numbers 230800, 230900, and 231000), the recessively inherited deficiency of glucocerebrosidase (Enzyme Commission number 3.2.1.45), is caused by mutations in the glucocerebrosidase gene (*GBA*). Glucocerebrosidase catabolizes the hydrolysis of glucosylceramide and glucosylsphingosine (1, 2), glycolipids that are taken up by macrophages during the degradation of dying erythrocytes and leukocytes (3). Macrophages are the main cell type exhibiting the disease phenotype. Gaucher disease is classified into three types on the basis of the absence of neurological symptoms (type 1) and the rate of progression of neurological symptoms (types 2 and 3). Furthermore, *GBA* mutations are an important genetic risk factor for Parkinson's disease (4). It is known that the morphology and function of macrophages from patients with Gaucher disease differ from those of normal macrophages, but a major challenge has been the lack of a cell-based model that exhibits abnormal lysosomal storage and other characteristics of the disease phenotype. Given that collecting and propagating large numbers of macrophages are difficult, patient-derived skin fibroblasts are commonly used to study Gaucher disease biology and to screen candidate drugs, even though this cell type lacks the hallmark characteristic of the disease, that is, glycolipid accumulation in lysosomes.

Macrophages are involved in many essential processes including the removal of pathogens and dead cells through phagocytosis (5). Macrophages initiate phagocytosis using different receptors including FcγRI/II (6) and migrate toward a gradient of chemoattractants. Pathogens are killed by reactive oxygen species (ROS) produced by macrophages either directly or indirectly via the multisubunit enzyme NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) oxidase (7). The assembly and activation of NADPH oxidase play a critical role in the activation of innate host defense, and the O₂⁻ produced is a potent microbicide. Impaired ROS production leads to defective degradation of pathogens and results in inflammation. The pathological defects occurring in Gaucher disease macrophages have not been extensively studied.

Gaucher disease is generally treated with enzyme replacement therapy, an effective but costly and inconvenient intravenously infused treatment that is ineffective against neurological manifestations because of its inability to permeate of the blood-brain barrier. Chemical chaperone drug therapy has been proposed as an alternative treatment strategy (8). Small-molecule chaperones bind to the misfolded glucocerebrosidase protein, refold the enzyme, and enable trafficking of the glucocerebrosidase to the lysosomes. Because patients with Parkinson's disease have been shown to have decreased levels of glucocerebrosidase in certain brain regions (9, 10), such chaperones could potentially be useful in the treatment of Parkinson's disease as well. Although small-molecule chaperones have been tested for their ability to improve enzyme-specific activity in Gaucher disease fibroblasts, the capacity of these molecules to normalize the appearance and function of Gaucher disease macrophages has not been evaluated because of the lack of an appropriate cellular model of this disorder.

Previously, we performed high-throughput screening of compound libraries using mutant N370S glucocerebrosidase from patient tissue extracts (11) and identified a new class of noninhibitory chaperone molecules (12). The lead compound NCGC00188758, a pyrazolo [1,5-*a*]pyrimidine [*N*-(4-ethynylphenyl)-5,7-dimethylpyrazolo[1,5-*a*] pyrimidine-3-carboxamide], demonstrated biochemical activation of glucocerebrosidase as well as chaperone activity in fibroblasts from patients with Gaucher disease (12). We generated and studied primary macrophages (hMacs) that were differentiated from monocytes taken from patients with Gaucher disease. However, the amount of blood needed, especially from infants with Gaucher disease type 2, and the limited yield of macrophages prompted us to create induced pluripotent stem cells (iPSCs) from dermal fibroblasts taken from patients to generate and evaluate a clonal supply of macrophages (iMacs) (13). Here, we use hMacs and iMacs to evaluate the ability of the noninhibitory chaperone molecule NCGC00188758 to reverse the disease phenotype.

RESULTS

hMacs were generated from patients with type 1 Gaucher disease

We generated hMacs from monocytes isolated from blood samples from 20 patients with type 1 Gaucher disease (table S1). These included 17 patients with the common and relatively mild Gaucher genotype N370S/N370S, and 1 each with genotypes N370S/c.84dupG, C342Y/R496H, and N370S/R463C. Control hMacs were generated from blood obtained from 30 healthy volunteers.

iPSCs and iPS-derived lineages were generated from fibroblasts of patients with Gaucher disease

Fibroblasts from a control healthy individual and from four patients with type 1 Gaucher disease (three with genotype N370S/N370S and one with N370S/c.84dupG) and one with type 2 Gaucher disease (genotype IVS2+1G>A/L444P) were reprogrammed to iPSCs by lentiviral transduction. The iPSC colonies were selected, expanded on mouse embryonic fibroblasts, and examined for pluripotency. Immunofluorescence staining showed high expression of Oct4, SOX2, TRA160, TRA180, Nanog, and SSEA4 (Fig. 1A and fig. S1), and polymerase chain reaction (PCR) arrays confirmed increased expression of pluripotency markers in the iPSC lines compared with the corresponding parental fibroblasts (P 0.001) (Fig. 1D). All iPSC lines had a normal karyotype (Fig. 1B and fig. S2) and formed teratomas when injected into severe combined immunodeficient (SCID) mice (Fig. 1C). Embryoid bodies were kept in culture for 10 days (Fig. 1E) and supplemented with macrophage colony-stimulating factor (M-CSF) and interleukin-3 (IL-3). At passages 13 to 15, the iPSCs were differentiated into monocytes and then iMacs (14). Monocytes, collected from the supernatant, stained positive for the CD14 marker. Differentiation into iMacs was confirmed by CD68 staining (Fig. 1E).

The iMacs and hMacs all showed similar expression of CD15, CD105, CD11b, CD33, and CD64 (Fig. 2, A to C, and fig. S3), whereas CD163 expression was increased in type 1 and 2 Gaucher disease iMacs by 23.7 and 21.3%, respectively (Fig. 2D).

Glucocerebrosidase activity is decreased in Gaucher disease iMacs and hMacs

Among 17 patients with the common Gaucher disease genotype N370S/N370S, glucocerebrosidase activity in their hMacs averaged $14.4 \pm 6.1\%$ of control activity ($P = 0.0016$) (Fig. 3A). Activity was $11.2 \pm 0.3\%$ for the patient with genotype N370S/c.84dupG (Fig. 3A), $16.6 \pm 7\%$ for genotype C342Y/R496H, and $4.9 \pm 0.5\%$ for genotype N370S/R463C (fig. S4A). The Gaucher disease iMacs also had markedly deficient glucocerebrosidase activity. Type 2 Gaucher disease iMacs showed a glucocerebrosidase activity of $2.9 \pm 4.9\%$ compared to control iMacs ($P < 0.0001$) (Fig. 3B). Type 1 Gaucher disease iMacs from three different patients with genotype N370S/N370S (GD1-P1 to GD1-P3) had a mean glucocerebrosidase activity of $21.2 \pm 0.2\%$ of that for control iMacs ($P = 0.008$); N370S/c.84dupG (GD1 P4) Gaucher disease iMacs had a glucocerebrosidase activity of 17.6% of that for control iMacs ($P = 0.0017$) (Fig. 3B, right panel).

NCGC00188758 increased glucocerebrosidase activity in Gaucher disease hMacs and iMacs

The residual glucocerebrosidase activity in type 1 and 2 Gaucher disease hMacs and iMacs was measured in the presence and absence of lipid-laden erythrocyte ghosts isolated from patient with Gaucher disease (Fig. 3, A and B, and fig. S4A). Feeding Gaucher disease hMacs and iMacs with these ghosts did not affect glucocerebrosidase activity (Fig. 3, A and B). In the presence of NCGC00188758 (structure shown in Fig. 3F), glucocerebrosidase activity increased in type 1 Gaucher disease hMacs and iMacs by 10.7-fold ($P = 0.0015$) and 3.2-fold ($P = 0.007$), respectively (Fig. 3, A and B); the activity in control macrophages also increased (Fig. 3, A and B) similar to results previously reported in fibroblasts (11). Glucocerebrosidase activity increased 26.6-fold in type 2 Gaucher disease iMacs treated with NCGC00188758 ($P = 0.0018$) (Fig. 3B). The administration of $20 \mu\text{M}$ recombinant glucocerebrosidase (imiglucerase, Genzyme) also restored glucocerebrosidase activity in Gaucher disease hMacs and iMacs (Fig. 3, A and B). The amount and concentration of NCGC00188758 and imiglucerase used were determined after testing several concentrations of each in hMacs (fig. S5).

NCGC00188758 increased translocation of mutant glucocerebrosidase to lysosomes and reduced substrate accumulation in Gaucher disease hMacs and iMacs

Previously, the ability of NCGC00188758 treatment to enhance the translocation of glucocerebrosidase to lysosomes was demonstrated in patient fibroblasts, but it was not possible to evaluate its effect on clearance of the stored substrate (12). In Gaucher disease hMacs and iMacs, NCGC00188758 treatment resulted in translocation of glucocerebrosidase to lysosomes as demonstrated by increased colocalization of glucocerebrosidase with the lysosomal marker LAMP2 (Fig. 3, C and D, and fig. S6, A to C, and movies S1 to S3). The degree of colocalization was demonstrated in scatter plots (fig. S6A) and by determining Pearson's coefficient in areas of colocalization (Fig. 3D and fig. S6B). In addition, Western blots showed that in the presence of NCGC00188758, the amount of glucocerebrosidase also increased in patient-derived hMacs ($P = 0.03$) and in type 2 Gaucher disease iMacs (Fig. 3E).

When glucocerebrosidase is deficient, excessive glucosylceramide accumulates in lysosomes of macrophages because of the defective digestion of erythrocytes and leukocytes. Erythrocyte ghosts were prepared from blood from patients with Gaucher disease and were labeled with glucosylceramide-Bodipy to mimic this defect. They were then added to Gaucher disease hMacs and iMacs. Fluorescence was compared to that of hMacs and iMacs given the glucosylceramide-Bodipy vehicle alone. Among the 20 samples from patients (17 hMacs and 3 iMacs) with the same genotype (N370S/N370S), the amount of glucosylceramide without the addition of Gaucher erythrocyte ghosts was about 2.0 ± 0.75 -fold (hMacs) and 3.8 ± 0.31 -fold (iMacs) higher than that of control macrophages (Fig. 4A). Glucosylceramide accumulation in N370S/N370S hMacs and iMacs loaded with Gaucher erythrocyte ghosts was 3.2 ± 0.8 - and 4.2 ± 0.12 -fold higher than in control hMacs or iMacs, respectively (Fig. 4A). hMacs from patients with type 1 Gaucher disease with other genotypes, when loaded with Gaucher erythrocyte ghosts, showed a 1.5- to 3.0-fold increase in glucosylceramide concentrations (fig. S7). Untreated type 2 Gaucher disease iMacs had a 4.2 ± 0.5 -fold increase in glucosylceramide compared to control macrophages, and feeding these cells with Gaucher erythrocyte ghosts resulted in a 5.8 ± 0.8 -fold increase in glucosylceramide (Fig. 4B). Confocal microscopy also showed that the added glucosylceramide-Bodipy (green) accumulated in Gaucher macrophages specifically in lysosomes that stained positive for LAMP2 (red) (Fig. 4C).

The effect of NCGC00188758 treatment on glucosylceramide accumulation was also evaluated. After 6 days of treatment with NCGC00188758, glucosylceramide accumulation decreased by between 50 and 61% in both N370S/N370S Gaucher disease hMacs and iMacs, and by 46.7% in N370S/c.84dupG iMacs (Fig. 4A). Remarkably, in the type 2 Gaucher disease iMacs, NCGC00188758 treatment reduced glucosylceramide concentrations by $95 \pm 25\%$ (Fig. 4B). Immunostaining of hMacs and iMacs with glucosylceramide-Bodipy and LAMP2 also confirmed a reduced amount of labeled glucosylceramide in lysosomes in treated Gaucher disease macrophages (Fig. 4C).

Concentrations of glucosylsphingosine, a second and more toxic substrate of glucocerebrosidase, were measured in Gaucher disease hMacs and iMacs by mass spectrometry. Type 2 Gaucher disease iMacs had an 18-fold higher concentration of glucosylsphingosine than did control iMacs, whereas hMacs and iMacs with the N370S/N370S genotype had a 2.8 and 3.2 times higher concentration than did controls (Table 1). Treatment with NCGC00188758 reduced glucosylsphingosine concentrations in all cell types tested (Table 1), indicating that the improved translocation of mutant glucocerebrosidase enhanced the hydrolysis of glucosylsphingosine ($P = 0.0017$).

These data show that both Gaucher disease hMacs and iMacs have only 3 to 20% of the glucocerebrosidase activity seen in their respective controls and that they accumulate glucosylsphingosine and glucosylceramide. These alteration were reversed with NCGC00188758.

Gaucher disease macrophages efficiently phagocytosed immunoglobulin G–opsonized erythrocytes and bacteria

Phagocytosis of immunoglobulin G (IgG)–opsonized erythrocytes and bacteria in Gaucher disease macrophages was first evaluated by measuring FcγRII (CD32) expression in hMacs and iMacs. Flow cytometry demonstrated that FcγRII expression was higher in Gaucher disease macrophages (Fig. 5A). Because erythrocyte ghosts from patients with Gaucher disease were used to model lipid-laden Gaucher disease macrophages, it was necessary to measure erythrocyte uptake by these macrophages. CD32-dependent phagocytosis of IgG–opsonized erythrocytes revealed no significant differences between control and N370S/N370S hMacs or iMacs (Fig. 5B). The phagocytosis of IgG–opsonized, fluorescein-labeled *Escherichia coli* bacteria was then evaluated (Fig. 5, C and D). The phagocytosis index was increased 1.2 ± 0.7 -fold in type 1 Gaucher disease hMacs and 1.0 ± 0.2 -fold in type 2 Gaucher disease iMacs compared to controls, and no significant change was observed in the efficiency of phagocytosis of bacteria by N370S/N370S or N370S/c.84dupG iMacs (Fig. 5D). Loading control macrophages with Gaucher erythrocyte ghosts had no effect on the phagocytic index.

NCGC00188758 treatment restored the respiratory burst in Gaucher disease macrophages

Macrophages use strategies such as phagocytosis-mediated lysosomal degradation and the production of ROS through activation of the NADPH oxidase to kill and eliminate phagocytosed bacteria. A fluorogenic probe (CellROX) that measures cellular oxidative stress was used to determine concentrations of ROS in Gaucher disease macrophages. We found that intracellular ROS concentrations in unstimulated N370S/N370S hMacs and iMacs were 51 and 65% lower than in control macrophages, respectively. The ROS concentration was 29.8% lower in type 2 Gaucher disease iMacs than in control iMacs (Fig. 5, E and F). ROS concentrations increased on average 1.5-fold ($P = 0.013$) in control macrophages stimulated with IgG–opsonized erythrocytes, whereas they were unchanged in Gaucher disease macrophages ($P = 0.98$) (Fig. 5, E and F, and fig. S8). Thus, ROS concentrations were reduced in Gaucher disease macrophages in response to IgG–opsonized erythrocytes. After treatment with NCGC00188758, ROS concentrations in hMacs and iMacs were normalized, even in the presence of IgG–opsonized erythrocytes (Fig. 5, D and E). H_2O_2 was used as a positive control for oxidative stress (fig. S8). DPI, a competitive inhibitor of flavin-containing cofactors, is a potent inhibitor of phagosomal radical-induced oxidation occurring during the phagocytosis of IgG–opsonized particles (15). The addition of DPI reduced ROS production in both control and Gaucher disease macrophages. However, the presence of IgG–opsonized erythrocytes had no effect on the concentration of O_2^- in both control and Gaucher disease macrophages (Fig. 5F), supporting the involvement of ROS in disease pathogenesis.

NCGC00188758 treatment restored chemotaxis in Gaucher disease macrophages

Chemokines play a crucial role in the migration of macrophages to the site of inflammation. Expression of mRNAs encoding the chemokines CCL5, SDF1, MCP2, and CXCR4 (the receptor for SDF1) was measured in Gaucher disease and control hMacs in the presence and absence of Gaucher erythrocyte ghosts (Fig. 6A). The expression of the critical chemokine

CCL5 was 40% lower in Gaucher disease macrophages than in controls ($P = 0.015$). SDF1 expression was similar in control and Gaucher disease hMacs, whereas CXCR4 expression was 89% lower in Gaucher disease hMacs than in control macrophages ($P = 0.0054$). Adding Gaucher erythrocyte ghosts increased mRNA concentrations of MCP2 (CCL2) 14-fold in controls, but the addition of erythrocyte ghosts to Gaucher disease hMacs decreased MCP2 expression by 52%. The observed differences in chemokine profiles between control and Gaucher disease macrophages prompted an evaluation of chemotaxis, focusing on the crucial chemokines SDF1, RANTES (CCL5), and MIP-1 α . Gaucher disease iMacs with genotypes N370S/N370S, N370S/c.84dupG, and IVS2+1G/L444P all exhibited a reduced migration toward SDF1, RANTES, and MIP-1 α (Fig. 6B). In N370S/N370S hMacs, chemotaxis in response to SDF1 was 74% lower, to RANTES was 65% lower, and to MIP-1 α was 80.6% lower than in control macrophages; impaired chemotaxis occurred in the presence and absence of Gaucher erythrocyte ghosts (Fig. 6C). Furthermore, treatment of iMacs from three patients with genotype N370S/N370S and one with genotype N370S/c.84dupG with NCGC00188758 increased the chemotactic index toward SDF1 2.4 ± 0.6 - and 1.5 ± 0.4 -fold, respectively; chemotaxis increased 5.37 ± 0.5 -fold after treatment of type 2 Gaucher disease iMacs with the compound (Fig. 6D). The expression of Mrc1, the target for enzyme replacement therapy in Gaucher disease, was also measured in hMacs. In Gaucher disease hMacs, Mrc1 expression was reduced in the absence and presence of Gaucher erythrocyte ghosts by 50 and 66%, respectively, compared to expression in control hMacs (Fig. 6E).

DISCUSSION

We have developed and evaluated two macrophage models of Gaucher disease that will facilitate studies of Gaucher disease pathogenesis and the development of new therapeutics. Commonly used fibroblast models of Gaucher disease lack the characteristic disease phenotype of glycolipid storage in lysosomes. However, both of our macrophage models—the Gaucher disease hMacs differentiated from monocytes isolated from 20 patients with type 1 Gaucher disease, and the iMacs differentiated from five iPSC lines generated from fibroblasts from patients with Gaucher disease—exhibited glucocerebrosidase deficiency, glycolipid accumulation, and impaired macrophage function and thus recapitulated hallmark features of Gaucher disease. Both hMacs and iMacs expressed defined macrophage markers including CD33, CD105, CD15, CD64, and CD11; CD163, an important scavenger receptor for the hemoglobin-haptoglobin complex, was increased in Gaucher disease macrophages. Elevated plasma concentrations of CD163 have been previously reported in patients with Gaucher disease (16), which merits further study.

Our cellular models enabled us to study function in Gaucher disease macrophages and to evaluate a new chaperone compound developed to treat Gaucher disease. This work supports the relevance of cell-based models in studies of Gaucher disease, especially in the context of validating new therapeutics. Currently available mouse models of Gaucher disease are not suitable for these studies because most knock-in mouse models carrying human mutations in glucocerebrosidase do not display an accurate disease phenotype or are lethal (17). Other mouse models made with an inducible promoter do show lipid storage defects but are not appropriate for screening chaperone molecules because in these models symptoms are

generated by depleting glucocerebrosidase (18). Thus, our cell-based Gaucher disease models provide an alternative approach for evaluating the efficacy of new chaperone compounds.

Our Gaucher disease macrophages demonstrate lysosomal accumulation of membrane lipids from phagocytosed erythrocyte ghosts. This effect was enhanced by loading the macrophages with erythrocyte ghosts isolated from patients with Gaucher disease. The advantage of this strategy is that the properties of the accumulated substrates should be identical to those of the substrates that accumulate in Gaucher disease. We also evaluated glucosylsphingosine, which is markedly elevated in patient plasma and tissues, and in Gaucher disease mouse models (2, 19), as well as in the brains of patients with type 2 and 3 Gaucher disease (20). Gaucher disease hMacs and iMacs similarly showed an increase in glucosylsphingosine (Table 1).

Recombinant glucocerebrosidase, used as enzyme replacement therapy for the treatment of patients with Gaucher disease, also corrected glycolipid storage in Gaucher disease hMacs and iMacs and served as a positive control, indicating that the phenotype observed was directly a result of enzyme deficiency. However, enzyme replacement therapy is not effective for the treatment of the neurological features of type 2 and 3 Gaucher disease, and is costly and inconvenient (21, 22). Moreover, expression of *Mrc1*, hypothesized to be used for targeting enzyme replacement therapy to macrophages in Gaucher disease (23), was similar in control and Gaucher disease macrophages, but was reduced in Gaucher disease macrophages loaded with Gaucher erythrocyte ghosts. This reduction could affect enzyme replacement therapy by compromising enzyme delivery to lipid-engorged Gaucher disease macrophages.

Chemical chaperone therapy is currently being explored as an alternative treatment strategy for patients with Gaucher disease (24). Chaperone compounds are small molecules that bind to misfolded proteins, enabling proper folding, stabilization, and trafficking of the enzyme to the lysosomes (25–27). Recently, our high-throughput screen using patient tissue as the enzyme source (11) identified several lead compounds that were able to modify the activity of the mutant N370S glucocerebrosidase. Further medicinal chemistry optimization produced the noninhibitory chaperone NCGC00188758, which was found to translocate glucocerebrosidase to the lysosomes and restore glucocerebrosidase activity in fibroblasts from patients with Gaucher disease. Unlike the iminosugar molecules isofagomine and *N*-butyl-deoxynojirimycin, NCGC00188758 did not inhibit glucocerebrosidase activity in vitro or in cells (28). We now show that NCGC00188758 can increase glucocerebrosidase activity and reduce glycolipid storage in macrophages derived from patients with Gaucher disease with different genotypes. Both the accumulation of glucosylceramide, measured by fluorescence, and glucosylsphingosine, measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS), were reduced by this compound.

Our macrophage models also led to new insights regarding the function of these cells in patients with Gaucher disease. Given that Gaucher disease macrophages manifest their defective storage phenotype after the phagocytosis of aged erythrocytes or dead leukocytes, we evaluated phagocytosis in the macrophage models. We found that the expression of

FcγRI (CD32) was marginally increased, indicating that phagocytosis is efficient in Gaucher disease macrophages (Fig. 5, B to D). Macrophages engulf IgG-opsonized particles via FcγRI (CD32), and the phagosomes then traffic to the lysosomes. The pH in the phagosomes decreases, and macrophages produce ROS. Acidification leads to the activation of lysosomal proteases and NADPH oxidase, which mediates electron transfer across endocytic membranes and influences the pH of the lysosomes (29). NADPH oxidase is formed from integral membrane proteins encoded by *p22phox* and *gp91phox* and cyto-solic proteins encoded by *p40phox*, *p47phox*, *p67phox*, and *rac*. In the resting state, these subunits are separated; upon stimulation, all subunits assemble to form the active enzyme (30, 31). hMacs and iMacs from patients with type 1 and 2 Gaucher disease produced less ROS compared to control macrophages in the absence and presence of IgG-opsonized erythrocytes, whereas ROS production increased during phagocytosis of erythrocytes by control macrophages. Treating control and Gaucher disease macrophages with the NADPH oxidase inhibitor DPI reduced ROS production to a greater extent in the mutant compared to the control macrophages. Impaired ROS production can result in defective degradation of phagocytosed material and the accumulation of undigested particles in lysosomes, contributing to the foamy appearance of Gaucher disease macrophages. The ROS concentrations in both unstimulated and IgG-opsonized erythrocyte-stimulated Gaucher disease macrophages were restored after treatment with the small-molecule chaperone NCGC00188758.

This work demonstrates the utility of iPSC-derived macrophages for studying Gaucher disease. We compared hMacs from 17 patients with genotype N370S/N370S to iMacs derived from three patients with genotype N370S/N370S. We included two patients (patients 18 and 19 in table S1, and samples GD1-P1 and GD1-P2 in the figures) from whom both blood and skin were collected to create hMacs and iPSC-derived macrophages, respectively. We were able to demonstrate similar levels of glucocerebrosidase enzyme activity, glucosylceramide accumulation, and macrophage function in both hMacs and iMacs.

There are limitations associated with the Gaucher disease hMac and iMac models. Whereas we were able to generate and compare hMacs with different genotypes in a relatively short period of time, these cells cannot be propagated and the limited numbers of cells obtained can only be kept for a few weeks. Moreover, we were unable to generate these macrophages from infants with type 2 Gaucher disease because of the limited amount of blood that could be collected from young patients. The generation of iPSCs is much more expensive and labor-intensive than obtaining monocyte-derived macrophages from blood, but once the iPSC lines are generated, they can be propagated and greater numbers of macrophages can be produced. Although the number of macrophages derived from iPSC lines is still insufficient for the screening of very large compound libraries, these cells are being used to evaluate and validate the efficacy of small-molecule chaperone drugs.

These new macrophage models of Gaucher disease provide critical insights into important functional impairments characteristic of Gaucher disease macrophages including reduced ROS production and chemotaxis. These defects were reversed by the chaperone NCGC00188758, which also restored glucocerebrosidase activity, cleared the accumulation

of glucosylceramide and glucosylsphingosine, and improved macrophage function. The ability of NCGC00188758 to cross the blood-brain barrier and to act as a chaperone for glucocerebrosidase without inhibiting its enzymatic activity makes it very attractive as an alternative therapy for Gaucher disease. Furthermore, because a deficiency in glucocerebrosidase has been implicated in the pathogenesis of Parkinson's disease and related Lewy body disorders, this type of chaperone compound may be useful for restoring glucocerebrosidase levels in patients with Parkinson's disease (4, 32). These new macrophage models of Gaucher disease will enable further elucidation of the molecular mechanisms contributing to disease pathogenesis. Their recapitulation of the disease phenotype opens a new avenue for drug development and validation, a vital step toward delivering new therapeutics for treating patients with Gaucher disease and related disorders.

MATERIALS AND METHODS

Study design

The objective of the study was to develop a macrophage model of Gaucher disease to evaluate macrophage function and the efficacy of new drugs. Peripheral blood monocytes were collected from 20 patients (17 with genotype N370S/N370S and 3 with other genotypes), isolated, and differentiated into macrophages. Skin fibroblasts from four patients with type 1 Gaucher disease and one with type 2 Gaucher disease were reprogrammed and then were also differentiated into macrophages. Glucocerebrosidase levels and activity were measured in these macrophages, and LC-MS/MS was used to determine the glucosylceramide and glucosylsphingosine concentrations in the presence and absence of Gaucher erythrocytes ghosts, prepared from a patient blood sample. Translocation of glucocerebrosidase from the endoplasmic reticulum to lysosomes was evaluated by confocal microscopy, and macrophage function was studied by measuring ROS production, chemotaxis, and phagocytosis. These parameters were then evaluated after treatment with the small-molecule chaperone NCGC00188758.

Patient blood samples and genotyping

Samples from 20 patients with type 1 Gaucher disease (table S1) and 30 controls provided by the National Institutes of Health (NIH) Blood Bank were collected with informed consent under a National Human Genome Research Institute (NHGRI) Review Board–approved clinical protocol. For patients treated with recombinant glucocerebrosidase, samples were collected at least 1 week after their last infusion. The genotypes of donors were confirmed by sequencing all exons of *GBA*, as described (33).

Differentiation of human monocytes into macrophages

Peripheral blood mononuclear cells from controls and patients with type 1 Gaucher disease were isolated using Ficoll gradients, and monocytes were purified using a magnetic monocyte enrichment kit (STEMCELL Technologies). Isolated monocytes underwent immunophenotyping and were found to be CD14- and CD11b-positive. Macrophages were differentiated from purified monocytes using M-CSF (10 ng/ml) (R&D Systems) in RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS) (Invitrogen). On days 3 and 6, the medium was refreshed and macrophages were harvested.

Preparation of Gaucher erythrocyte ghosts and quantification of glucosylceramide accumulation

To enhance glucosylceramide storage during differentiation, hMacs and iMacs were fed with Gaucher erythrocyte ghosts, prepared as described (34). A portion of the ghosts were fluorescently labeled by incubation with *N*-[11-dipyrrometheneborondifluorideundecanoyl]-D-glucosyl- β 1-1'-D-erythro-sphingosine [C11 TopFluor glucosylceramide (glucosylceramide-Bodipy) (Avanti Polar Lipids)] for 30 min at 37°C. Treatment with unlabeled Gaucher erythrocyte ghosts lasted for 6 days, changing medium every 2 days. Erythrocytes from patients with type 1 Gaucher disease were used because their membranes are rich in glucosylceramide. Macrophages phagocytose 9 to 12 ghosts daily; hence, the quantity of ghosts added was based on the number of hMacs or iMacs present, refreshed every 3 days. On day 6, the glucosylceramide-Bodipy-labeled Gaucher erythrocyte ghosts were added for 12 hours. Cells were then washed, and fluorescence was measured at 495/503 nm on a Victor 1420 multilabel counter (PerkinElmer).

The generation of iPSCs

Primary dermal fibroblasts from four patients with type 1 Gaucher disease (three with genotype N370S/N370S and one with N370S/c.84dupG), an infant with type 2 Gaucher disease (genotype IVS2+1/L444P), and a control (sequencing confirmed the absence of *GBA* mutations) were transduced with Oct4, SOX2, cMYC, and KLF4, using STEMCCA Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus (EMD Millipore). Unlike previous Gaucher iPSC protocols (13, 35), on day 3, sodium butyrate (Sigma) and small molecules A83-01 and PS48 (BioVision Research Products) were added at a final concentration of 0.25, 0.5, and 5 μ M, respectively, to facilitate conversion from mitochondrial oxidation to glycolysis during the reprogramming process. Human iPSCs were maintained on primary mouse embryonic fibroblasts as feeder cells, using standard human embryonic stem cell medium. The cells were transitioned to feeder-free culture condition using Matrigel (BD Biosciences) and mTeSR medium (STEMCELL Technologies). Colonies at passage numbers 13 to 15 were used for differentiation.

Characterization of iPSCs

Karyotyping of the iPSCs was performed at the NHGRI cytogenetics core facility. The spectral karyotyping method was used to identify any structural abnormalities as previously described (36). The genotypes of each line were reconfirmed by sequencing *GBA* (33). Immunofluorescence staining with iPSC markers was performed using the StemLight Pluripotency Antibody kit (Cell Signaling), and a PCR expression array (SA Bioscience) was performed using RNA from each line. A teratoma assay was performed by injecting 1 million cells in the right flank of a SCID mouse. After 10 to 12 weeks, appropriate tumors resulted from each of the iPSC lines injected. Tumor sections were stained for three different germ lines (mesoderm, ectoderm, and endoderm).

Differentiation of iPSCs to monocytes and macrophages

Embryoid bodies, formed using AggreWell 400 plates (STEMCELL Technologies), were transferred to ultralow adherence plates in Aggre-Well medium supplemented with 10 μ M

ROCK inhibitor (STEM-CELL Technologies) for 4 days, with daily medium changes. For differentiation to monocytes, embryoid bodies were transferred to gelatin (1%)–coated six-well plates in advanced Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FCS, 0.055 mM β -mercaptoethanol, M-CSF (50 ng/ml), and IL-3 (25 ng/ml) (R&D Systems). The medium was replaced every 3 days. Monocytes were harvested from the supernatant, centrifuged at 1500 rpm for 5 min, and differentiated into iMacs using RPMI supplemented with 10% FCS and M-CSF (100 ng/ml).

Quantification of glucocerebrosidase activity in macrophages

Treated cells were cultured in black 96-well plates. After adding 100 μ l of enzyme assay buffer [200 mM sodium acetate (pH 4), 5 mM 4-methylumbelliferyl- β -D-glucopyranoside (4MU- β Glu), and protease inhibitor cocktail (Sigma)], cells were incubated for 1 hour at 37°C. The reaction was terminated using a stop solution (1 M NaOH and 1 M glycine), and fluorescence was measured.

LC–electrospray ionization–MS/MS quantification of glucosylsphingosine in macrophages

Glucosylsphingosine was measured by LC-MS/MS as previously described (13).

Flow cytometry

Expression of Fc γ RII (CD32), allophycocyanin (APC)–CD14, phycoerythrin (PE)–Cy7–CD15, Alexa Fluor 647–CD105, Alexa Fluor 700–CD64, peridinin chlorophyll protein (PerCP)–Cy5.5–CD33, Blue-CD11b/Mac1, PE-CD163, and PE-CD68 (BD Biosciences) in hMacs or iMacs was measured using an AccuEasy flow cytometry kit (LEAP Biosciences). OneComp beads were added to the cells without antibody for compensation of all fluorochrome-conjugated antibodies. Unstained cells were used as negative control. Staining was also performed using an isotype control for each marker (fig. S3). Cells were analyzed with DIVA software using Becton Dickinson LSR II and analyzed with FlowJo.

Phagocytosis

hMacs and iMacs were plated in black 96-well clear bottom plates and were treated with Gaucher erythrocyte ghosts in the presence or absence of NCGC00188758 (8 μ M). On day 6, they were washed and incubated for 2 hours with 100 μ l of fluorescein-labeled *E. coli* BioParticles, and fluorescence was measured at 494/518 nm. A trypan blue solution was added to quench the fluorescence from particles that were not internalized (Vybrant Phagocytosis Assay, Invitrogen). Fc receptor-mediated phagocytosis was measured using the CytoSelect 96-Well Phagocytosis Assay (Cell Biolabs Inc.). Macrophages from controls and patients with Gaucher disease were cultured in 96-well plates. Sheep erythrocytes were opsonized using the provided opsonization solution at a 1:500 dilution at 37°C for 30 min. Then, 10 μ l of IgG-opsonized sheep red blood cells was added and incubated for 2 hours. Culture medium was removed by gentle aspiration. Wells were washed and lysed according to the manufacturer's manual. Lysates were incubated with substrate solution, and the absorbance was measured at 610 nm in a 96-well plate reader.

Measurement of intracellular ROS

hMacs and iMacs, cultured in black 96-well clear-bottom plates or glass chamber slides, were stained with CellROX Deep Red Reagent (Molecular Probes) at a final concentration of 5 μ M. After feeding macrophages with IgG-opsonized Gaucher erythrocyte ghosts in the presence or absence of diphenyleneiodonium (Sigma) at a final concentration of 5 μ M, they were incubated for 30 min at 37°C. Cells were washed, and fluorescence was evaluated at 640/665 nm using a FlexStation Multi-Mode microplate reader. Cells, cultured on glass coverslip chamber slides, were stained with CellROX, and live-cell imaging was performed using Zeiss 510 META laser scanning microscope ($\times 63$).

RNA isolation and real-time PCR

One microgram of total RNA, isolated from macrophages using the PrepEase RNA/Protein Spin kit (Affymetrix), was reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative real-time PCR was performed using an ABI Prism 7900 real-time PCR instrument (Applied Biosystems) with the QuantiFast SYBR Green PCR kit (Applied Biosystems). The primer sequences used are as follows: *Mrc1*, 5'-GGTTGCTATCACT-CTCTATGC-3' (forward) and 5'-TTTCTTGTCTGTTGCCGTAGTT-3' (reverse); *CCL5*, 5'-CCAGCAGTCGTCTTTGTAC-3' (forward) and 5'-CTCTGGGTTGGCACACACTT-3' (reverse); and *SDF1*, 5'-ATTCTCAACACTCCAAACTGTGC-3' (forward) and 5'-ACTTTA-GCTTCGGGTCAATGC-3' (reverse). Data are displayed as expression ratios of target genes normalized to hypoxanthineguanine phosphoribosyltransferase (HPRT). Quantitative real-time PCR data were analyzed by the 2^{-C_t} method (37).

Chemotaxis

In vitro cell migration was assayed using 24-well Transwell chamber plates (pore size: 5 μ m, Corning). The upper well, containing 10^6 macrophages, was incubated at 37°C for 4 hours. The lower chamber contained RPMI with and without SDF1 (60 ng/ml), MIP-1 α (50 ng/ml), or CCL5 (RANTES) (50 ng/ml) (PeproTech). Cells were allowed to migrate through the membrane into the lower chamber. Macrophages migrating to the bottom wells were counted on a FACSCalibur flow cytometer. The chemotaxis index was calculated from the ratio of migrated macrophages in the presence and absence of the respective chemokine (38).

Evaluation of therapies for Gaucher disease

Recombinant glucocerebrosidase (imiglucerase) (20 μ M), diluted in saline, was obtained from the remnants of patient infusions and cryo-preserved in glycerol, and enzymatic activity was measured before use. NCGC00188758 (8 μ M) [*N*-(4-ethynylphenyl)-5,7-dimethylpyrazolo [1,5-*a*]pyrimidine-3-carboxamide], identified via high-throughput screening as a small molecule that activated the hydrolysis of 4MU- β Glu in a Gaucher spleen homogenate (genotype N370S/N370S), underwent optimization by medicinal chemistry as previously described (12). The concentration used in these experiments was based on assays of glucocerebrosidase activity and clearance of glucosylceramide at different

concentrations of NCGC00188758 and imiglucerase in control and Gaucher macrophages (fig. S5, A to C).

Immunofluorescence staining

hMacs and iMacs were plated on glass chamber slides, fed with erythrocyte ghosts in the presence and absence of the small molecule, and fixed with 4% paraformaldehyde. Cells were blocked in phosphate-buffered saline containing 0.1% saponin, 100 μ M glycine, and 2% donkey serum, followed by incubation with the glucocerebrosidase rabbit polyclonal antibody (1:350) or mouse monoclonal LAMP2 antibody (Abcam). Cells were washed and incubated with donkey anti-mouse or anti-rabbit secondary antibodies, conjugated to Alexa Fluor 488 or Alexa Fluor 555 (Invitrogen). Cells were mounted with Vectashield plus DAPI (Vector Laboratories), and Z-stack images were acquired with a Zeiss 510 META laser scanning microscope (Carl Zeiss MicroImaging Inc.) using a 488-nm argon, a 543-nm HeNe, and an ultraviolet laser. Images were acquired using a Plan Neofluar 63 $\times$ /1.42 oil DIC objective. The colocalization coefficient was determined using Pearson's algorithm, and scatter plots were generated and evaluated using Imaris software.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 software. Significance was determined by Student's *t* test using Wilcoxon signed rank test, and correlations were determined using Pearson correlations. Data from two groups or more than two independent variables were analyzed by ANOVA, followed by the Bonferroni post hoc test. Data are presented as means $\pm$ SD. Significance levels between controls and patient macrophages were set when $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), $P < 0.05$ (#), $P < 0.01$ (##), and $P < 0.001$ (###) between different conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES AND NOTES

1. Grabowski, GA.; Beutler, E. *The Metabolic and Molecular Bases of Inherited Disease*. McGraw-Hill; New York: 2001. p. 3635-3668.

2. Orvisky E, Park JK, LaMarca ME, Ginns EI, Martin BM, Tayebi N, Sidransky E. Glucosylsphingosine accumulation in tissues from patients with Gaucher disease: Correlation with phenotype and genotype. *Mol Genet Metab.* 2002; 76:262–270. [PubMed: 12208131]
3. Lee RE. The fine structure of the cerebroside occurring in Gaucher's disease. *Proc Natl Acad Sci USA.* 1968; 61:484–489. [PubMed: 4176481]
4. Sidransky E, Nalls MA, Aasly JO, Aharon-Peretz J, Annesi G, Barbosa ER, Bar-Shira A, Berg D, Bras J, Brice A, Chen CM, Clark LN, Condroyer C, De Marco EV, Dürr A, Eblan MJ, Fahn S, Farrer MJ, Fung HC, Gan-Or Z, Gasser T, Gershoni-Baruch R, Giladi N, Griffith A, Gurevich T, Januario C, Kropp P, Lang AE, Lee-Chen GJ, Lesage S, Marder K, Mata IF, Mirelman A, Mitsui J, Mizuta I, Nicoletti G, Oliveira C, Ottman R, Orr-Urtreger A, Pereira LV, Quattrone A, Rogaeva E, Rolfs A, Rosenbaum H, Rozenberg R, Samii A, Samaddar T, Schulte C, Sharma M, Singleton A, Spitz M, Tan EK, Tayebi N, Toda T, Troiano AR, Tsuji S, Wittstock M, Wolfsberg TG, Wu YR, Zabetian CP, Zhao Y, Ziegler SG. Multicenter analysis of glucocerebrosidase mutations in Parkinson's disease. *N Engl J Med.* 2009; 361:1651–1661. [PubMed: 19846850]
5. Gardai SJ, McPhillips KA, Frasch SC, Janssen WJ, Starefeldt A, Murphy-Ullrich JE, Bratton DL, Oldenburg PA, Michalak M, Henson PM. Cell-surface calreticulin initiates clearance of viable or apoptotic cells through *trans*-activation of LRP on the phagocyte. *Cell.* 2005; 123:321–334. [PubMed: 16239148]
6. Ravetch JV. Fc receptors. *Curr Opin Immunol.* 1997; 9:121–125. [PubMed: 9039776]
7. Babior BM. NADPH oxidase. *Curr Opin Immunol.* 2004; 16:42–47. [PubMed: 14734109]
8. Suzuki Y. Chaperone therapy update: Fabry disease, G_{M1}-gangliosidosis and Gaucher disease. *Brain Dev.* 2013; 35:515–523. [PubMed: 23290321]
9. Murphy KE, Gysbers AM, Abbott SK, Tayebi N, Kim WS, Sidransky E, Cooper A, Garner B, Halliday GM. Reduced glucocerebrosidase is associated with increased α -synuclein in sporadic Parkinson's disease. *Brain.* 2014; 137:834–848. [PubMed: 24477431]
10. Gegg ME, Burke D, Heales SJ, Cooper JM, Hardy J, Wood NW, Schapira AH. Glucocerebrosidase deficiency in substantia nigra of parkinson disease brains. *Ann Neurol.* 2012; 72:455–463. [PubMed: 23034917]
11. Goldin E, Zheng W, Motabar O, Southall N, Choi JH, Marugan J, Austin CP, Sidransky E. High throughput screening for small molecule therapy for Gaucher disease using patient tissue as the source of mutant glucocerebrosidase. *PLOS One.* 2012; 7:e29861. [PubMed: 22272254]
12. Patnaik S, Zheng W, Choi JH, Motabar O, Southall N, Westbroek W, Lea WA, Velayati A, Goldin E, Sidransky E, Leister W, Marugan JJ. Discovery, structure–activity relationship, and biological evaluation of noninhibitory small molecule chaperones of glucocerebrosidase. *J Med Chem.* 2012; 55:5734–5748. [PubMed: 22646221]
13. Panicker LM, Miller D, Park TS, Patel B, Azevedo JL, Awad O, Masood MA, Veenstra TD, Goldin E, Stubblefield BK, Tayebi N, Polumuri SK, Vogel SN, Sidransky E, Zambidis ET, Feldman RA. Induced pluripotent stem cell model recapitulates pathologic hallmarks of Gaucher disease. *Proc Natl Acad Sci USA.* 2012; 109:18054–18059. [PubMed: 23071332]
14. Karlsson KR, Cowley S, Martinez FO, Shaw M, Minger SL, James W. Homogeneous monocytes and macrophages from human embryonic stem cells following coculture-free differentiation in M-CSF and IL-3. *Exp Hematol.* 2008; 36:1167–1175. [PubMed: 18550257]
15. Rybicka JM, Balce DR, Khan MF, Krohn RM, Yates RM. NADPH oxidase activity controls phagosomal proteolysis in macrophages through modulation of the luminal redox environment of phagosomes. *Proc Natl Acad Sci USA.* 2010; 107:10496–10501. [PubMed: 20498052]
16. Møller HJ, de Fost M, Aerts H, Hollak C, Moestrup SK. Plasma level of the macrophage-derived soluble CD163 is increased and positively correlates with severity in Gaucher's disease. *Eur J Haematol.* 2004; 72:135–139. [PubMed: 14962251]
17. Farfel-Becker T, Vitner EB, Futerman AH. Animal models for Gaucher disease research. *Dis Models Mech.* 2011; 4:746–752.
18. Mistry PK, Liu J, Yang M, Nottoli T, McGrath J, Jain D, Zhang K, Keutzer J, Chuang WL, Mehal WZ, Zhao H, Lin A, Mane S, Liu X, Peng YZ, Li JH, Agrawal M, Zhu LL, Blair HC, Robinson LJ, Iqbal J, Sun L, Zaidi M. Glucocerebrosidase gene-deficient mouse recapitulates Gaucher

- disease displaying cellular and molecular dysregulation beyond the macrophage. *Proc Natl Acad Sci USA*. 2010; 107:19473–19478. [PubMed: 20962279]
19. Dekker N, van Dussen L, Hollak CE, Overkleeft H, Scheij S, Ghauharali K, van Breemen MJ, Ferraz MJ, Groener JE, Maas M, Wijburg FA, Speijer D, Tylki-Szymanska A, Mistry PK, Boot RG, Aerts JM. Elevated plasma glucosylsphingosine in Gaucher disease: Relation to phenotype, storage cell markers, and therapeutic response. *Blood*. 2011; 118:e118–e127. [PubMed: 21868580]
 20. Raghavan SS, Mumford RA, Kanfer JN. Deficiency of glucosylsphingosine: β -Glucosidase in Gaucher disease. *Biochem Biophys Res Commun*. 1973; 54:256–263. [PubMed: 4741565]
 21. Weinreb NJ, Charrow J, Andersson HC, Kaplan P, Kolodny EH, Mistry P, Pastores G, Rosenbloom BE, Scott CR, Wappner RS, Zimran A. Effectiveness of enzyme replacement therapy in 1028 patients with type 1 Gaucher disease after 2 to 5 years of treatment: A report from the Gaucher Registry. *Am J Med*. 2002; 113:112–119. [PubMed: 12133749]
 22. Altarescu G, Hill S, Wiggs E, Jeffries N, Kreps C, Parker CC, Brady RO, Barton NW, Schiffmann R. The efficacy of enzyme replacement therapy in patients with chronic neuronopathic Gaucher's disease. *J Pediatr*. 2001; 138:539–547. [PubMed: 11295718]
 23. Van Patten SM, Hughes H, Huff MR, Piepenhagen PA, Waire J, Qiu H, Ganesa C, Reczek D, Ward PV, Kutzko JP, Edmunds T. Effect of mannose chain length on targeting of glucocerebrosidase for enzyme replacement therapy of Gaucher disease. *Glycobiology*. 2007; 17:467–478. [PubMed: 17251309]
 24. Khanna R, Benjamin ER, Pellegrino L, Schilling A, Rigat BA, Soska R, Nafar H, Ranes BE, Feng J, Lun Y, Powe AC, Palling DJ, Wustman BA, Schiffmann R, Mahuran DJ, Lockhart DJ, Valenzano KJ. The pharmacological chaperone isofagomine increases the activity of the Gaucher disease L444P mutant form of β -glucosidase. *FEBS J*. 2010; 277:1618–1638. [PubMed: 20148966]
 25. Cabrera-Salazar MA, Novelli E, Barranger JA. Gene therapy for the lysosomal storage disorders. *Curr Opin Mol Ther*. 2002; 4:349–358. [PubMed: 12222873]
 26. Sawkar AR, Schmitz M, Zimmer KP, Reczek D, Edmunds T, Balch WE, Kelly JW. Chemical chaperones and permissive temperatures alter localization of Gaucher disease associated glucocerebrosidase variants. *ACS Chem Biol*. 2006; 1:235–251. [PubMed: 17163678]
 27. Zheng W, Padia J, Urban DJ, Jadhav A, Goker-Alpan O, Simeonov A, Goldin E, Auld D, LaMarca ME, Inglese J, Austin CP, Sidransky E. Three classes of glucocerebrosidase inhibitors identified by quantitative high-throughput screening are chaperone leads for Gaucher disease. *Proc Natl Acad Sci USA*. 2007; 104:13192–13197. [PubMed: 17670938]
 28. Marugan JJ, Huang W, Motabar O, Zheng W, Xiao J, Patnaik S, Southall N, Westbroek W, Lea WA, Simeonov A, Goldin E, Debernardi MA, Sidransky E. Non-iminosugar glucocerebrosidase small molecule chaperones. *Medchemcomm*. 2012; 3:56–60. [PubMed: 22606365]
 29. DeCoursey TE, Cherny VV, Morgan D, Katz BZ, Dinauer MC. The gp91^{phox} component of NADPH oxidase is not the voltage-gated proton channel in phagocytes, but it helps. *J Biol Chem*. 2001; 276:36063–36066. [PubMed: 11477065]
 30. Groemping Y, Rittinger K. Activation and assembly of the NADPH oxidase: A structural perspective. *Biochem J*. 2005; 386:401–416. [PubMed: 15588255]
 31. Zhan Y, Virbasius JV, Song X, Pomerleau DP, Zhou GW. The p40^{phox} and p47^{phox} PX domains of NADPH oxidase target cell membranes via direct and indirect recruitment by phosphoinositides. *J Biol Chem*. 2002; 277:4512–4518. [PubMed: 11729195]
 32. Mazzulli JR, Xu YH, Sun Y, Knight AL, McLean PJ, Caldwell GA, Sidransky E, Grabowski GA, Krainc D. Gaucher disease glucocerebrosidase and α -synuclein form a bidirectional pathogenic loop in synucleinopathies. *Cell*. 2011; 146:37–52. [PubMed: 21700325]
 33. Stone DL, Tayebi N, Orvisky E, Stubblefield B, Madike V, Sidransky E. Glucocerebrosidase gene mutations in patients with type 2 Gaucher disease. *Hum Mutat*. 2000; 15:181–188. [PubMed: 10649495]
 34. von der Heul C, Kroos MJ, de Jeu-Jaspars CM, von Eijk HG. The uptake of iron by reticulocytes. The influence of purification of the ghosts on iron-containing components in the ghost suspension. *Biochim Biophys Acta*. 1980; 601:572–583. [PubMed: 7417439]

35. Tiscornia G, Lorenzo Vivas E, Matalonga L, Berniakovich I, Barragán Monasterio M, Eguizábal C, Gort L, González F, Ortiz Mellet C, García Fernández JM, Ribes A, Veiga A, Izpisua Belmonte JC. Neuronopathic Gaucher's disease: Induced pluripotent stem cells for disease modelling and testing chaperone activity of small compounds. *Hum Mol Genet.* 2013; 22:633–645. [PubMed: 23118351]
36. Schröck E, du Manoir S, Veldman T, Schoell B, Wienberg J, Ferguson-Smith MA, Ning Y, Ledbetter DH, Bar-Am I, Soenksen D, Garini Y, Ried T. Multicolor spectral karyotyping of human chromosomes. *Science.* 1996; 273:494–497. [PubMed: 8662537]
37. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-CT} method. *Methods.* 2001; 25:402–408. [PubMed: 11846609]
38. Aflaki E, Balenga NA, Luschig-Schratl P, Wolinski H, Povoden S, Chandak PG, Bogner-Strauss JG, Eder S, Konya V, Kohlwein SD, Heinemann A, Kratky D. Impaired Rho GTPase activation abrogates cell polarization and migration in macrophages with defective lipolysis. *Cell Mol Life Sci.* 2011; 68:3933–3947. [PubMed: 21533980]

Fig. 1. Generation and differentiation of iPSCs into monocytes and macrophages

(A) Immunostaining for common embryonic markers (Oct4, SOX2, SSEA4, TRA160, TRA180, and Nanog) in control and type 2 Gaucher disease (IVS2+1/L444P) iPSC lines using a Zeiss fluorescence microscope. (B) iPSCs derived from a patient with type 2 Gaucher disease have a normal 46XX karyotype. (C) Teratomas were formed from iPSCs generated from four patients with type 1 Gaucher disease (three with genotype N370S/N370S and one with genotype N370S/c.84dupG) and from one patient with type 2 Gaucher disease (genotype IVS2+1/L444P). (D) Total RNA from two skin fibroblast lines from patients with type 1 Gaucher disease (genotype N370S/N370S), and the corresponding two iPSC lines derived from them were characterized in triplicate using a PCR array. The fold changes refer to the expression levels for each gene compared to that in the fibroblast line. (E) iPSCs were differentiated into macrophages using a three-step method. Embryoid bodies were made and differentiated into iPSC monocytes. The monocytes harvested from the supernatant stained positive for CD14 with flow cytometry. The monocytes were then differentiated into macrophages, which stained positive for CD68 expression with flow cytometry.

Fig. 2. Increased CD163 expression in Gaucher disease macrophages

(A to C) Flow cytometry analysis of control iMacs and type 1 Gaucher disease hMacs (genotype N370S/N370S) and iMacs (genotypes N370S/N370S and N370S/c.84dupG) and type 2 Gaucher disease iMacs (genotype IVS2+1/L444P) stained for (A) CD33 and CD11b, (B) CD64 and CD163, and (C) CD15 and CD105 markers. (D) hMacs and iMacs were also stained with CD163 alone. The flow cytometry images of hMacs shown are representative of 7 patients with type 1 Gaucher disease (all with genotype N370S/N370S) and 10 healthy controls in independent experiments. The antibody isotype controls are shown in fig. S3.

Fig. 3. NCGC00188758 increases mutant glucocerebrosidase activity

(A) Glucocerebrosidase activity is shown as percent of control glucocerebrosidase activity in type 1 Gaucher disease hMacs in the presence and absence of Gaucher erythrocyte ghosts, 8 μ M NCGC00188758 (referred to in figures as NCGC758), or imiglucerase. The left panel reflects independent experiments using N370S/N370S hMacs from 17 different patients; the right panel shows data for hMacs from one patient with genotype N370S/c.84dupG. Four different hMacs with genotype N370S/N370S were treated with imiglucerase (20 μ M) (left panel). Error bars indicate SD. *** P < 0.0016, ** P < 0.01. RFU, relative fluorescence units. (B) Effect of NCGC00188758 on iMacs in the presence and absence of Gaucher erythrocyte ghosts. The left panel shows glucocerebrosidase activity in iMacs from an infant with type 2 Gaucher disease (genotype IVS2+1/L444P). The right panel shows iMacs from four patients with type 1 Gaucher disease (GD1-P1, GD1-P2, and GD1-P3 each have the genotype N370S/N370S, and GD1-P4 has the genotype N370S/c.84dupG). Glucocerebrosidase activity in iMacs was measured in two independent experiments. Error bars indicate SD. * P = 0.018, ** P = 0.003, *** P < 0.0017, #### P < 0.001. (C) Gaucher disease and control hMacs and iMacs were treated with 8 μ M NCGC00188758 for 6 days and then stained for

glucocerebrosidase (Alexa Fluor 488, green), the lysosomal marker LAMP2 (Alexa Fluor 555, red), and 4',6-diamidino-2-phenylindole (DAPI) (nuclear stain, blue). Z-stack images were acquired using a Zeiss 510 confocal microscope ($\times 63$ magnification). Single images are presented in fig. S6. Insets correspond to the regions marked a, b, and c. Figures on lower panel (d) show higher magnifications of the areas outlined in the images of the cells treated with NCGC00188758. The location of cross section (Z stack) is shown in x-y, x-z, and y-z planes (corresponding movies are shown in the Supplementary Materials). iMacs from four different patients with Gaucher disease with genotypes N370S/N370S, N370S/c.84dupG, and IVS2+1/L444P are shown. Images are taken at the same laser settings and are representative of 12 independent experiments. Scale bars, 5 μm . **(D)** Pearson's coefficient was calculated for the degree of colocalization between glucocerebrosidase (green), LAMP2, and nuclear stain (blue). Results represent >20 cells per condition. **(E)** Left: Western blot showing glucocerebrosidase in lysed hMacs treated with Gaucher erythrocyte ghosts in the presence and absence of 8 μM NCGC00188758 for 6 days. Patient (P) (genotype N370S/N370S) and control (C) hMacs are shown. Right: Western blot showing glucocerebrosidase concentrations in lysed iMacs from a patient with type 2 Gaucher disease (genotype IVS2+1/L444P) treated with Gaucher erythrocyte ghosts in the presence and absence of 8 μM NCGC00188758 for 6 days. The graph represents Western blots performed on hMacs from six different patients with type 1 Gaucher disease with genotype N370S/N370S. Groups were compared using one-way analysis of variance (ANOVA) ($n = 6$). $**P = 0.03$; $***P = 0.009$, Bonferroni correction. **(F)** Chemical structure of NCGC00188758.

Fig. 4. NCGC00188758 reduces glucosylceramide accumulation in Gaucher disease hMacs and iMacs

(A and B) Gaucher disease and control hMacs and iMacs from four patients with type 1 Gaucher disease (GD1-P1, GD1-P2, and GD1-P3 each with genotype N370S/N370S, and GD1-P4 with genotype N370S/c.84dupG) and one with type 2 Gaucher disease (IVS2+1/L444P) were treated with 8 μ M NCGC00188758 in the presence and absence of erythrocyte ghosts. On day 6, the macrophages were loaded with glucosylceramide-Bodipy (GlcCer-Bodipy)-labeled erythrocyte ghosts, and fluorescence was measured at 495/503 nm. (A) Left panel: Pooled data for hMacs from 15 different patients with genotype N370S/N370S; right panel: iMacs from four different type 1 Gaucher disease iPSC lines (GD1-P1, GD1-P2, and GD1-P3 have genotype N370S/N370S; GD1-P4 has genotype N370S/c.84dupG). Each experiment was performed in triplicate. (B) Data for type 2 Gaucher disease iMacs, with measurements performed in triplicate. Error bars indicate SD. * $P = 0.018$; ** $P = 0.007$; *** $P = 0.0012$, two-way ANOVA with Bonferroni post test. (C) N370S/N370S hMacs and four different Gaucher disease iMacs (genotypes N370S/N370S for two patients, N370S/c.84dupG, and IVS2+1/L444P). Cells were treated with labeled erythrocyte ghosts (green) for 12 hours, fixed, and stained with LAMP2 (Alexa Fluor 555, red). They were imaged using a confocal microscope with a 488-nm argon laser. Images were acquired using a Plan Neofluar 63 $\times$ 1.3 oil DIC (differential interference contrast) objective. Scale bars, 5 μ m. Insets show specific regions with a lower intensity of the green channel.

Fig. 5. NCGC00188758 corrects ROS production in Gaucher disease macrophages

(A) Expression of CD32 measured by flow cytometry. The first peak (dashed) is unstained cells; the second peak is control hMacs stained with CD32; the third peak (shaded) is N370S/N370S hMacs (left) or IVS2+1/L444P iMacs (right) stained with CD32. Data represent six independent experiments. (B) Phagocytosis of IgG-opsonized erythrocytes was evaluated in hMacs (genotype N370S/N370S) and in iMacs from four different iPSC lines (GD1-P1, GD1-P2, and GD1-P3 with genotype N370S/N370S, and GD1-P4 with genotype N370S/c.84dupG). Experiments were performed five times in hMacs and three times in iMacs for each sample. Results of hMacs from six different patients and eight different controls are shown. (C and D) Phagocytosis in Gaucher disease macrophages treated with erythrocyte ghosts in the presence and absence of 8 μ M NCGC00188758 for 6 days. On day 6, fluorescein-labeled *E. coli* bacteria were added for 2 hours, and fluorescence was measured at 494/518 nm. Phagocytosis of control hMacs was arbitrarily set at 100%. (C) N370S/N370S and control hMacs. Data for N370S/N370S and control hMacs reflect 12 independent experiments. (D) Phagocytic index of iMacs from four different iPSC lines (GD1-P1, GD1-P2, and GD1-P3 with genotype N370S/N370S, and GD1-P4 with genotype N370S/c.84dupG). Data reflect two independent experiments performed in triplicate. Significance was determined using one-way ANOVA. (E and F) ROS production was measured in macrophages treated with NCGC00188758 in the presence and absence of IgG-opsonized erythrocytes. (E) Ten independent experiments were performed with hMacs. *** $P = 0.0009$. (F) iMacs from five different patients were treated with diphenyleneiodonium (DPI) (5 μ M), a potent inhibitor of NADPH oxidase, for 30 min in the presence or absence of IgG-opsonized erythrocyte ghosts. Two independent experiments

were performed in quadruplicate. N370S/N370S iMacs represent data from three different iPSC lines. Groups were compared using one-way ANOVA with Bonferroni correction. *** $P = 0.0021$, ** $P = 0.012$.

Fig. 6. NCGC00188758 improves chemotaxis in Gaucher disease macrophages

(A) mRNA expression of chemokines CCL5, SDF1, MCP2, and CXCR4 measured in control hMacs and hMacs from six patients with genotype N370S/N370S in the presence and absence of Gaucher erythrocyte ghosts. * $P = 0.015$, *** $P < 0.001$, ## $P = 0.012$, ### $P = 0.0018$. (B) Chemotaxis of iMacs from four different patients with type 1 Gaucher disease (GD1-P1, GD1-P2, and GD1-P3 with genotype N370S/N370S, and GD1-P4 with genotype N370S/c.84dupG) and from one patient with type 2 Gaucher disease (genotype IVS2+1/L444P) was evaluated using Transwell chambers. * $P < 0.062$, ** $P < 0.0048$, *** $P < 0.0002$. (C) Chemotaxis of type 1 Gaucher disease hMacs (genotype N370S/N370S). Experiments were performed on hMacs from seven patients in independent experiments. * $P = 0.026$, ** $P = 0.0051$, *** $P = 0.00044$. (D) Chemotaxis toward SDF1 by iMacs from five different patients with Gaucher disease was measured in the presence and absence of NCGC00188758. Data represent two independent experiments, and groups were compared using two-way ANOVA. * $P = 0.02$, ** $P = 0.007$, *** $P < 0.003$. (E) Mannose receptor 1 (Mrc1) expression was measured in N370S/N370S hMacs using real-time PCR. Independent experiments were performed on hMacs from 10 patients with genotype N370S/N370S, and groups were compared using one-way ANOVA with Bonferroni correction. *** $P = 0.0021$.

Table 1
Glucosylsphingosine concentration in N370S/N370S hMacs and IVS2+1/L444P and N370S/N370S iMacs

Glucosylsphingosine concentrations (picomole per microgram of protein) were measured in control and Gaucher disease iMacs and hMacs using LC-MS/MS. hMacs and iMacs were derived from patients with genotype N370S/N370S. iMacs were also derived from a patient with genotype IVS2+1/L444P. Groups were compared using one-way ANOVA with Bonferroni correction.

	Control hMacs	N370S/N370S hMacs	Control iMacs	iMacs IVS2+1/L444P	iMacs N370S/N370S
Untreated	0.0832 ± 0.013	0.2372 ± 0.2***	0.047 ± 0.15	1.121***	0.622
+Ghosts and NCGC00188758	0.0986 ± 0.03	0.1352 ± 0.02	0.070	0.227*	0.417

 $P = 0.0017$;

*
 $P = 0.0368$.