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Gaucher iPSC-Derived Macrophages Produce Elevated Levels of Inflammatory Mediators and Serve as a New Platform for Therapeutic Development

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ABSTRACT

Gaucher disease (GD) is an autosomal recessive disorder caused by mutations in the acid β-glucocerebrosidase (GCase; *GBA*) gene. The hallmark of GD is the presence of lipid-laden Gaucher macrophages, which infiltrate bone marrow and other organs. These pathological macrophages are believed to be the sources of elevated levels of inflammatory mediators present in the serum of GD patients. The alteration in the immune environment caused by GD is believed to play a role in the increased risk of developing multiple myeloma and other malignancies in GD patients. To determine directly whether Gaucher macrophages are abnormally activated and whether their functional defects can be reversed by pharmacological intervention, we generated GD macrophages by directed differentiation of human induced pluripotent stem cells (hiPSC) derived from patients with types 1, 2, and 3 GD. GD hiPSC-derived macrophages expressed higher levels of tumor necrosis factor α, IL-6, and IL-1β than control cells, and this phenotype was exacerbated by treatment with lipopolysaccharide. In addition, GD hiPSC macrophages exhibited a striking delay in clearance of phagocytosed red blood cells, recapitulating the presence of red blood cell remnants in Gaucher macrophages from bone marrow aspirates. Incubation of GD hiPSC macrophages with recombinant GCase, or with the chaperones isofagomine and ambroxol, corrected the abnormal phenotypes of GD macrophages to an extent that reflected their known clinical efficacies. We conclude that Gaucher macrophages are the likely source of the elevated levels of inflammatory mediators in the serum of GD patients and that GD hiPSC are valuable new tools for studying disease mechanisms and drug discovery. STEM CELLS *2014; 00:000–000*

INTRODUCTION

Gaucher disease (GD) is an autosomal recessive disorder caused by mutations in the gene encoding the lysosomal enzyme acid β-glucocerebrosidase (GCase). Type 1 GD is the most common form of the disease, affecting the reticuloendothelial and skeletal systems. The reduced GCase activity in phagocytic cells results in lysosomal accumulation of glucosylceramide and other sphingolipids [1, 2]. Patients affected with type 1 GD exhibit hepatosplenomegaly, pancytopenia, and bone disease [3, 4]. These manifestations of GD are believed to be caused by pathological Gaucher macrophages infiltrating bone marrow and other tissues. In types 2 and 3 GD patients, the hematologic and visceral manifestations are exacerbated, and there is serious neuronopathy. Type 2 GD is the most severe acute form of the disease, while type 3 GD is a subacute form.

The serum of patients with GD has elevated levels of inflammatory mediators including tumor necrosis factor α (TNF-α), IL-6, and IL-1β, and it is believed that these cytokines are produced by Gaucher macrophages [5]. These cells may also be the source of chitotriosidase (ChT1), an enzyme that is highly elevated in the serum of type 1 GD patients, and is used to follow the response to GD therapy, except in individuals who are null for the ChT1 gene [6, 7]. The altered immune environment in GD patients is believed to contribute to their increased risk of developing multiple myeloma [5]. For these reasons, it is important to understand the role of Gaucher macrophages in the pathophysiology of GD and to identify therapeutics that can reverse their abnormal phenotype.

Enzyme replacement therapy (ERT) with recombinant GCase (Cerezyme, Genzyme Corporation, Cambridge, MA) is used successfully to treat individuals with type 1 GD [8], but cannot be used to treat the neuronopathy in types 2 and 3 GD because the recombinant enzyme does not cross the blood–brain barrier. Clinically important GCase variants are misfolded due to the mutations. This causes endoplasmic reticulum (ER) retention, degradation by the ER-associated degradation (ERAD) system, and reduced GCase transport to the lysosome [9, 10]. However, some mutant enzyme escapes proteolysis and reaches the lysosome, and the ratio of lysosomal to ER GCase seems to correlate with disease severity [9, 11]. As some GCase mutants have residual enzymatic activity, there has been an active search for pharmacological agents that can restore proper folding, thus allowing the enzyme to reach its final destination. This has resulted in the identification of a number of small molecules that act as pharmacological chaperones of GCase [10, 11]. Among these, the iminosugars isofagomine [10] and ambroxol [12] act as competitive inhibitors of GCase and facilitate folding and transport of GCase mutants in fibroblasts. Isofagomine has been tested as a possible therapy for GD [13, 14]. While this chaperone increased enzymatic activity in patient neutrophils, it did not significantly improve clinical parameters of the disease [13]. On the other hand, in a small clinical study of patients with the common N370S mutation, ambroxol was reported to improve disease manifestations including splenomegaly [15], suggesting that ambroxol may be a promising treatment for type 1 GD.

Gaucher macrophages for disease modeling can be obtained from bone marrow aspirates, but this is an invasive procedure, especially in pediatric populations. While patient macrophages can also be obtained from peripheral blood, these are postmitotic cells that cannot be propagated. GD fibroblasts have been widely used for disease modeling and drug development, but these cells are not a good surrogate for Gaucher macrophages. The recent advent of reprogramming technology has made it possible to obtain patient-derived pluripotent stem cells, which allow derivation of virtually any cell type in quantities sufficient for disease modeling and drug discovery [16–18]. Human induced pluripotent stem cells (hiPSCs) have been derived from patients affected by monogenic and complex diseases, and important aspects of the disease phenotype have been recapitulated in the relevant hiPSC-derived cell types [19, 20]. We reported previously the generation of hiPSC lines from patients with types 1, 2, and 3 GD, and their directed differentiation to the most affected cell types including macrophages and neurons. GD hiPSC-derived macrophages accumulated glucosylsphingolipids and had a striking defect in the clearance of phagocytosed red blood cells (RBCs), which are pathologic hallmarks of the disease [21]. In this study, we report that GD hiPSC macrophages derived from six different patients are abnormally activated and produce high levels of TNF-α, IL-1β, IL-6, and ChT1, which are characteristically elevated in the serum of GD patients. We also found that recombinant GCase and the chaperones isofagomine and ambroxol reversed these phenotypes to an extent that reflected their known clinical efficacies. Our results demonstrate that GD hiPSC macrophages are a valuable new platform for disease modeling and preclinical evaluation of therapeutic efficacy.

Materials and Methods

Cells

Fibroblasts for reprogramming were derived from skin biopsies of healthy donors and from patients with types 1, 2, and 3 GD. Patient skin biopsies were obtained by Ellen Sidransky's laboratory (NIH) under approved IRB and informed consent. GD and control human fibroblasts were maintained in culture as described in [21]. hiPSC were grown in human embryonic stem cell/hiPSC media: Dulbecco's modified Eagle's medium (DMEM)-F12 (Invitrogen, Grand Island, NY, http://www.lifetechnologies.com), 20% knockout serum replacement (Invitrogen), L-glutamine (GIBCO, Grand Island, NY), penicillin/streptomycin, β-mercaptoethanol (β-ME), nonessential amino acids (NEAA), and 10–30 ng/ml basic fibroblast growth factor (bFGF) (Stemgent, San Diego, CA, www.stemgent.com). DR4 mouse embryonic fibroblasts (MEF) were obtained from 13.5E embryos of DR4 male [22] and CF1 female and maintained in fibroblast culture media. Control DF4–7T.a hiPSC were purchased from the WiCell repository (Madison, WI), and control BU.1 hiPSC were a gift from Gustavo Mostovslavsky (Boston University, MA) [23]. hiPSC from a patient with type 1 GD (N370S/N370S) (referred to as type 1-a), a patient with type 2 GD (L444P/Rec*Nci*I) (type 2-a) and a patient with type 3 GD (L444P/L444P) (type 3-a) have been described in [21]. The generation of additional hiPSC from a patient with type 1 GD (N370S/N370S) (type 1-b), a patient with type 2 GD (W184R/D409H) (type 2-b), a patient with type 3 GD (L444P/L444P) (type 3-b), and MJ control hiPSC using Sendai virus is described below. N370S is the most frequent mutation and is mostly associated with type 1 GD. L444P is the second most frequent mutation and it is often associated with the severe types 2 and 3 forms of GD. Rec*Nci*I and D409H are also frequent alleles in GD [24]. All of the hiPSC used in this study are listed in Supporting Information Table S1. All the work with hiPSC described in this study was carried out with approval from the Institutional Review Board and Embryonic Stem Cell Research Oversight committees.

Generation of hiPSC by Sendai Virus Infection

MJ control human foreskin fibroblasts and skin biopsy fibroblasts from GD patients harboring N370S/N370S (type 1-b), W184R/D409H (type 2-b), and L444P/L444P (type 3-b) were reprogrammed to hiPSC using Sendai virus [25] as follows. Control and GD fibroblasts were seeded 2 days before infection in single wells of a gelatin-coated, six-well plate at a cell density of 1.0×10^5 cells per well in fibroblast growth medium. Two days later, the cultures were infected with Sendai virus particles encoding OCT4, SOX2, KLF4, and c-MYC (Life Technologies, Grand Island, NY, Lot # 1085254A) at a multiplicity of infection of 3 in 1 ml of fibroblast growth medium, according to the manufacturer. The next day, the culture medium was replaced with fibroblast growth medium and cultured for 6 more days with medium changes every other day. Seven days post-infection, the cells were collected using TrypLE (Invitrogen) and plated at densities of 5.0×10^4, 1.0×10^5, and 2.0×10^5 infected cells per 10 cm plate on irradiated DR4 MEFs in fibroblast growth medium. The next day, the cells were washed with phosphate-buffered saline (PBS) and cultured in hiPSC medium containing 4 ng/ml bFGF for 21–35 days. Colonies with typical hiPSC morphology began to emerge 10–15 days post-infection. These colonies were manually picked between days 15 and 30 post-infection and plated onto irradiated DR4 MEFs. Upon growth and development into typical hiPSC, individual subclones were passaged into six-well plates and further expanded to form stable cell lines for characterization. Of about 15 hiPSCs manually picked from the plates, three hiPSC subclones displaying characteristic hiPSC morphology over several passages were chosen and expanded for further characterization.

Antibodies

Mouse antibodies to OCT4, SSEA4, TRA-1–60, and TRA-1–81 were from Millipore (Billerica, MA, www.emdmillipore.com) (ES Cell Marker Kit, Cat No. SCR002); rabbit anti-SOX2 was from Millipore; rabbit anti-NANOG was from Abcam (Cambridge, MA, www.abcam.com/) (Cat No. ab21624). Rabbit polyclonal anti-GCase has been described elsewhere [26]. Anti-CD68 (Cat No. 556078), anti-CD163 (Cat No. 556018), and allophycocianin-conjugated anti-CD14 (Cat No. 555399) were from BD Bioscience (San Jose, CA, www.bdbiosciences.com). Mouse Anti-LAMP1 (H4A3) was from the University of Iowa Developmental Hybridoma Bank. Secondary antibodies DyLight 488- or 549-conjugated mouse or rabbit immunoglobulin-specific antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, www.jacksonimmuno.com).

Karyotype Analysis

On the day of karyotyping, 20 randomly selected metaphases from GD hiPSC clones were fully analyzed, and 3 cells were karyotyped at the Cytogenetic Core Facility at the Johns Hopkins Cancer Center.

Teratoma Formation from GD hiPSC in NOG/SCID Mice

Male non-obese gamma/severe combined immunodeficiency (NOG/SCID) mice of 6- to 8-week-old (NOD.Cg-Prkdc<scid> Il2rg<tm1Sug>/JicTac, Taconic Farms, Hudson, NY, www.taconic.com) were injected subcutaneously with the indicated hiPSC lines as described in [21]. When teratomas reached 1.5 cm in diameter they were surgically removed, fixed, embedded in paraffin, sectioned, and stained with H&E. All the work with NOG/SCID mice described in this study has been approved by the Institutional Animal Care and Use Committee.

Differentiation of GD hiPSC into Monocytes

Directed differentiation of GD hiPSC into monocyte–macrophages was carried out as described in [21]. Briefly, hiPSC were detached from plate and feeder cells by treatment with 2 mg/ml dispase. The hiPSC were transferred into ultra-low attachment plates in embryoid body (EB) culture medium and cultured for 4 days. For monocyte differentiation, EBs were transferred into gelatin-coated plates containing monocyte differentiation medium (MDM: DMEM [Sigma, St Louis, MO, www.sigmaaldrich.com], 10% fetal bovine serum [FBS], 50 ng/ml hM-CSF, 25 ng/ml hIL-3, 1 mM L-glutamine, 1X NEAA, and 0.1 mM β-ME). The concentration of hM-CSF was increased to 100 ng/ml after the first media change. Continuous monocyte production started within 2–3 weeks, and monocytes were harvested every 4–5 days.

Differentiation of Monocytes into Macrophages

Monocytes harvested from EB factories were resuspended in macrophage differentiation medium (Roswell Park Memorial Institute (RPMI)/10% FBS, supplemented with 100 ng/ml hM-CSF, glutamine, and penicillin/streptomycin) and plated in chamber slides or plates.

May–Grünwald–Giemsa Stain for Macrophages

Macrophages were stained with May–Grünwald (MG 500)–Giemsa (G-9641, Sigma) according to the manufacturer.

Western Blot Analysis

Cells were lysed directly in SDS sample buffer and analyzed by Western blot using specific antibodies as described in [21].

Immunofluorescence Analysis

Staining of hiPSC for Pluripotency Surface Markers. hiPSC plated on MEFs were fixed and processed for immunostaining using mouse anti-OCT4 (1:50), mouse anti-SSEA4 (1:50), mouse anti-TRA-1–60 (1:50), mouse anti-TRA-1–81 (1:50), rabbit anti-SOX2 (1:50), and rabbit anti-NANOG (1:30) at the dilution specified in parentheses, as described previously [21].

Staining of Macrophages. Macrophages were fixed and stained using rabbit anti-GCase antibody (1:500) and mouse anti-LAMP1 (1:100) as described in [21]. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Omitting the primary or secondary antibodies in the immunostaining procedures was used as a negative control. Staining was visualized with a Zeiss Axioscope II fluorescence microscope.

Flow Cytometry

Induced monocytes and macrophages were fixed in paraformaldehyde, washed, and incubated in blocking buffer consisting of PBS, human IgG (1 mg/ml, Sigma), 8% FBS, and 0.01% sodium azide. Cells were then incubated with the indicated antibodies in buffer containing PBS, 0.2% saponin, 8% FBS, and sodium azide, washed, and kept at 4°C until fluorescence-activated cell sorting analysis. Data were acquired by flow cytometry using a BD LSRII Flow Cytometer and analyzed using FlowJo software (Tree Star Inc., Asland, OR).

Analysis of Cytokine mRNA Induction by LPS

GD and control hiPSC macrophages (3.0×10^5 cells per well) were cultured in 12-well plates for 5 days. Cells were then incubated with 100 ng/ml lipopolysaccharide (LPS) for the indicated times. After incubation, mRNA was isolated using an RNA isolation kit (Qiagen, Valencia CA). cDNA was synthesized using iScript kit (Biorad, Hercules, CA). Transcriptional regulation of different cytokines in the macrophages before and after treatment was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) (7900 HT Applied Biosystems) using SYBR green method. The relative mRNA expression of the corresponding cytokine was normalized to the values of GAPDH mRNA for each reaction. Primers used were hIL-1β f—GATGCACCTGTACGATCACTG, hIL-1β r—ACAAAGGACATGGAGAACACC; hIL-6 f—AGTGAGGAACAAGCCAGAGC, hIL-6 r—GTCAGGGGTGGTTATTGCAT; hTNF-α f—CACTTTGGAGTGATCGGCC, hTNF-α r—CTCAGCTTGAGGGTTTGCTACAAC; hIL-10 f—

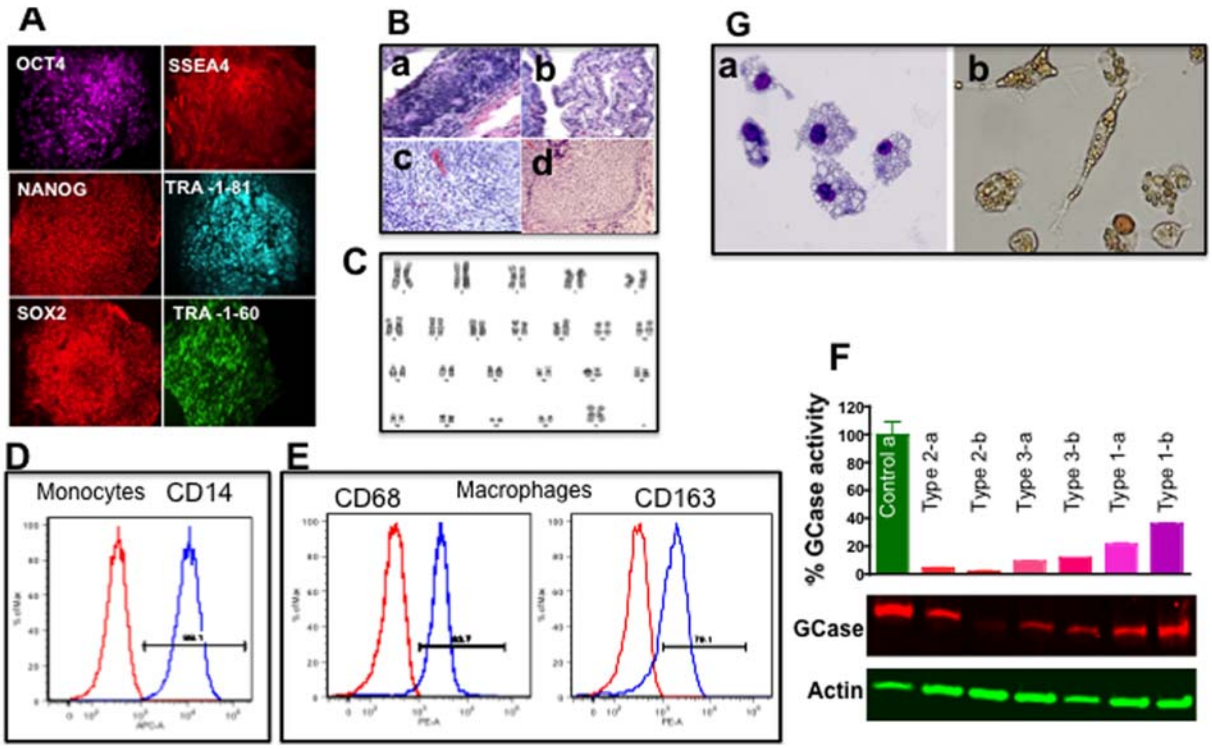

Figure 1. Generation and characterization of Gaucher disease (GD) human induced pluripotent stem cell (hiPSC) and directed differentiation to monocytes and macrophages. **(A):** Staining of W184R/D409H (type 2-b) GD hiPSCs with antibodies to the stem cell and pluripotency markers OCT4, SSEA4, NANOG, TRA-1-60, TRA-1-81, and SOX2. Magnification, ×10. **(B):** GD hiPSC gave rise to benign cystic teratomas in NOG-SCID mice. **(a–d):** H&E staining of teratoma cells from the three germ layers. **(a):** Ectodermal structures (pigmented neural epithelium and neuronal rosettes); **(b)** endodermal structures (glandular or intestinal epithelium); **(c, d)** mesodermal structures (connective tissues, cartilage, or skeletal muscle). Magnification, ×20. **(C):** Normal karyotype of type 2-b GD hiPSC. **(D, E):** Fluorescence-activated cell sorting analysis of GD hiPSC monocytes and macrophages stained with the indicated antibodies. In the histograms, blue and red show the percentage of cells stained with specific markers and isotype controls, respectively. **(D):** CD14 expression in type 2 GD hiPSC monocytes. **(E):** Expression of CD68 and CD163 in type 2 GD hiPSC macrophages. **(F):** Low levels of glucocerebrosidase (GCase) enzymatic activity and GCase protein in types 1, 2, and 3 GD hiPSC macrophages versus control hiPSC macrophages. **(G):** May–Grünwald–Giemsa stain and phagocytic activity of GD hiPSC macrophages. **(a):** May–Grünwald–Giemsa stain of type 2-b GD hiPSC macrophages. **(b):** Live cell image of type 2-b GD hiPSC macrophages after phagocytosis of opsonized RBC. Magnification, ×40. Abbreviation: GCase: glucocerebrosidase.

GCTGTCATCGATTTCTTCCC; hIL-10 r—CTCATGGCTTTGTAGAT GCCT.

Secreted Cytokine Detection by ELISA

To measure the indicated cytokines, we used two-antibody enzyme-linked immunosorbent analysis (ELISA). Polystyrene plates (Maxisorb; Nunc, Rochester, NY) were coated with capture antibody in PBS overnight at 25°C. The plates were washed four times with 50 mM Tris, 0.2% Tween-20, pH 7.0–7.5, and then blocked for 90 minutes at 25°C with assay buffer (PBS containing 4% bovine serum albumin [Sigma]). Then 50 µl of sample or standard prepared in assay buffer was added and the plates were incubated at 37°C for 2 hours. The plates were washed four times, and 100 µl of biotinylated detecting antibody in assay buffer was added and incubated for 1 hour at 25°C. After washing the plate four times, strepavidin-peroxidase polymer in casein buffer was added and incubated at 25°C for 30 minutes. The plate was washed four times, and 100 µl of commercially prepared substrate (TMB; Dako, Carpinteria, CA, www.dako.com) was added and incubated at 25°C for approximately 10–30 minutes. The reaction was stopped with 100 µl 2N HCl, and the A450 (minus A650) was read on a microplate reader (Molecular Dynamics, Sunnyvale, CA). The cytokine concentration in each sample was calculated from the standard curve equation.

GCase Assay

The assay for GCase enzymatic activity in cells was carried out as described in [21], using 4-methylumbelliferyl β-D-glucopyranoside as a substrate. Released 4-methylumbelliferone was measured using a fluorescence plate reader (excitation 365 nm, emission 445 nm). Conduritol B epoxide of 1 mM was added to replicate wells for the duration of the assay to control for non-GCase enzymatic activity.

Chitotriosidase Assay

Chitotriosidase activity was measured in intact macrophages as described in [27], with modifications. 1×10^5 monocytes per well were plated in macrophage differentiation media in 24-well plates. After 5 days of cell culture, media was aspirated from the culture plate and washed with PBS. The assay reaction was started by the addition of 200 µl of 0.3 mg/ml Methylumbelliferyl *N*, *N*′, *N*″-triacetylchitotrioside hydrate (Cat # M 5639, Sigma) in 0.2 M acetate buffer (pH 4.0) to each well. After incubation at 37°C for 5 hours, the reaction was stopped by the addition of 600 µl of 0.2 M glycine buffer (pH

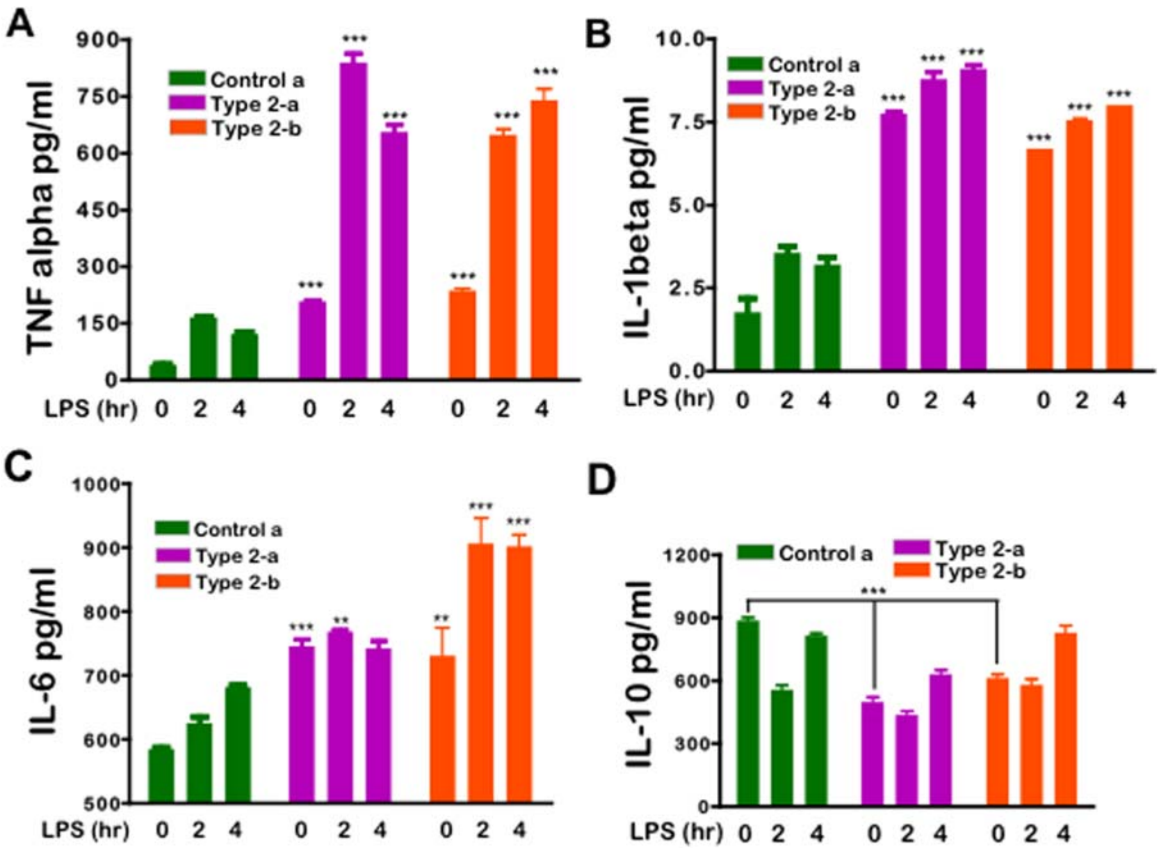

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Figure 2. Gaucher disease (GD) human induced pluripotent stem cell (hiPSC) macrophages secrete elevated levels of inflammatory cytokines. **(A–D):** Enzyme-linked immunosorbent analysis showing the secretion of tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-6, and IL-10 by untreated and LPS-treated type 2 GD hiPSC versus control hiPSC macrophages. Macrophages were treated with 100 ng/ml LPS for the indicated times, after which the culture supernatants were analyzed to measure the secretion of TNF-α, IL-1β, IL-6, and IL-10. Numbers in the ordinates represent picogram per milliliter of secreted cytokines in culture supernatants. *p* values **(A–C)** for type 2-a and 2-b hiPSC versus control hiPSC macrophages at the three time points are: TNF-α ($<.001$); IL1-β ($<.001$); and IL-6 ($<.001$, $<.01$), respectively, and for **(D)** IL-10 ($<.001$). Results shown in panels **(A–D)** are representative of three independent experiments. Abbreviations: IL, interleukin; LPS, lipopolysaccharide; ML, milliliter; PG, picogram; TNF-α, tumor necrosis factor α.

10.8) to each well. The liberated 4-methylumbelliferone (excitation 360 nm, emission 455 nm) was measured using a fluorescence plate reader.

Phagocytosis Assay for Macrophage Function

Macrophages cultured in 24-well plates at 1.0×10^5 cells per well for 5 days were incubated with opsonized RBC for 2 hours as described in [21]. After incubation, the cultures were washed and treated with ammonium-chloride-potassium lysing buffer to remove RBC still attached on the surface of the macrophages [28]. The macrophages were further incubated for several days as indicated in the text, and clearance of RBC was monitored by direct microscopic observation. A minimum of 300 macrophages per field were scored for the presence of ingested RBC. Assays were carried out in triplicate. Non-opsonized RBC were used as negative controls.

Treatment with Recombinant GCase, Isofagomine, and Ambroxol

GD hiPSC macrophages were incubated with the indicated concentrations of recombinant human GCase (Cerezyme), isofagomine (Toronto Research Chemicals, Canada, www.trc-canada.com), or ambroxol (Sigma) for 3–6 days. The treated macrophages were analyzed as described in the text. Cerezyme was obtained from patient infusion remnants.

Statistical Analysis

Data were analyzed using Prism software version 4.0c (GraphPad Software). The significance of differences was assessed using two-way analysis of variance (Bonferroni post-tests) or two-tailed unpaired Student's *t*-tests, as appropriate. The confidence level for significance was 95%.

Results

Generation of GD hiPSC and Directed Differentiation to Macrophages

In this study, we used hiPSC lines derived from six different patients with types 1, 2, and 3 GD (Supporting Information Table S1). Three of them, N370S/N370S (referred to as type 1-a), L444P/Rec*Nci*I (type 2-a), and L444P/L444P (type 3-a) GD, have been previously described in [21]. An additional three lines of GD hiPSC, namely N370S/N370S (type 1-b), W184R/D409H (type 2-b), and L444P/L444P (type 3-b), were generated as described in the Materials and Methods section. All

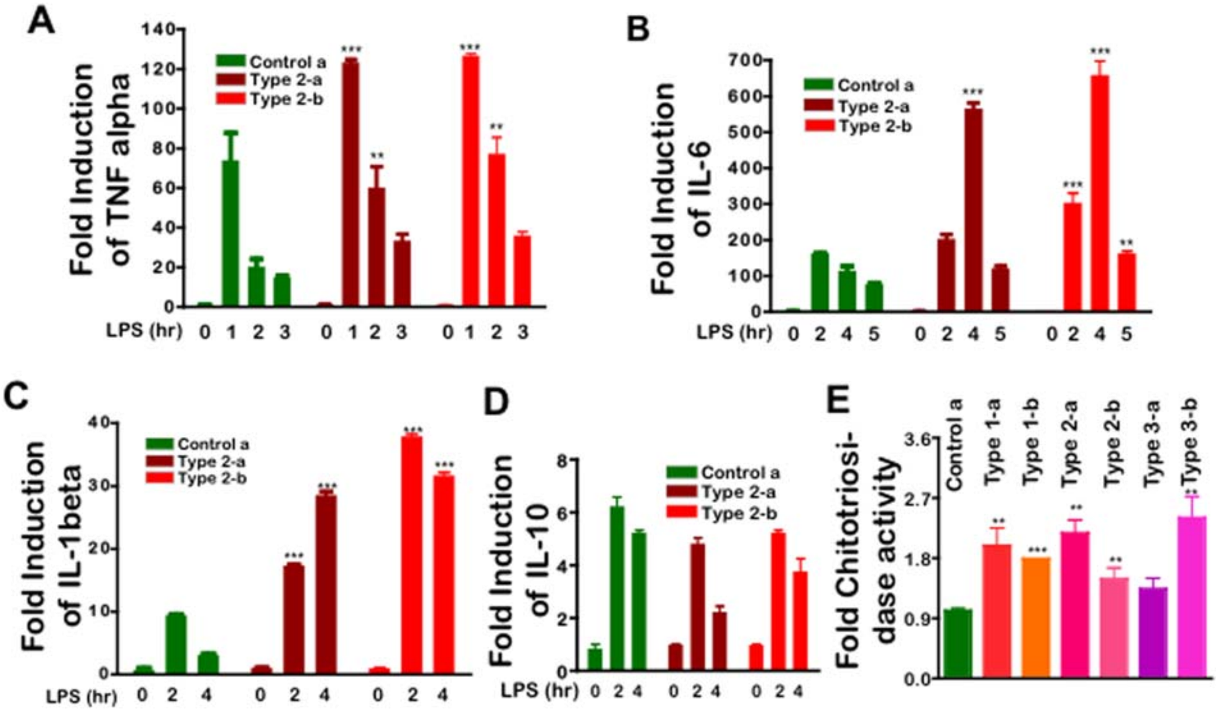

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Figure 3. Induction of cytokines and chitotriosidase (ChT1) in Gaucher disease (GD) human induced pluripotent stem cell (hiPSC) macrophages. **(A–D):** Control and type 2 GD hiPSC macrophages were either left untreated or were treated with 100 ng/ml LPS for the indicated times, after which real-time polymerase chain reaction analysis was carried out to measure induction of tumor necrosis factor α (TNF-α) **(A)**, interleukin (IL)−6 **(B)**, IL1-β **(C)**, and IL-10 **(D)** mRNAs. Numbers in the ordinates represent fold-activation of the corresponding cytokines compared with the nontreated condition. **(A–C):** p values for type 2-a and 2-b hiPSC versus control hiPSC macrophages at the three time points are TNF-α ($<.001$ and $<.01$); IL-6 ($<.001$ and $<.01$); IL-1β ($<.001$). **(E):** ChT1 activity in control and types 1, 2, and 3 GD hiPSC macrophages was assayed as described in the Materials and Methods section. Numbers in the ordinate represent the fold-activation of ChT1 activity compared with the control. p values for GD compared with control hiPSC are type 1-a ($<.0038$), type 1-b ($<.0002$), type 2-a ($<.0042$), type 2-b ($<.0319$), and type 3-b ($<.01$). Abbreviations: IL, interleukin; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor α.

of the GD hiPSC lines expressed typical pluripotency surface markers, including SSEA4, TRA-1–60, and TRA-1–81, as well as nuclear markers for pluripotency including NANOG, SOX2, and
F1 OCT4 (Fig. 1A; Supporting Information Fig. S1A). Marker analysis was carried out in three independently derived GD hiPSC subclones for each patient, all with similar results. GD hiPSC induced teratomas in nude mice (Fig. 1B; Supporting Information Fig. S1B), and karyotypic analysis of these lines indicated a normal complement of chromosomes (Fig. 1C; Supporting Information Fig. S1C).

GD hiPSC lines were used to set up monocyte-producing bioreactors as described in the Materials and Methods section. Similar to the GD hiPSC cell lines we reported previously [21], the new GD hiPSC lines efficiently differentiated to monocytes, and greater than 95% of the cells expressed the monocyte marker CD14 (Fig. 1D; Supporting Information Fig. S2A). Under optimal conditions, the bioreactors produced more than 2 million cells per week from four to five EBs. hiPSC-derived monocytes differentiated to macrophages that expressed macrophage-specific markers including CD163 and CD68 (Fig. 1E; Supporting Information Fig. S2B). All of the mutant macrophages derived from GD hiPSC had reduced GCase enzyme activity (Fig. 1F), exhibited the typical morphology of macrophages (Fig. 1G-a; Supporting Information Fig. S2C-a), were phagocytic (Fig. 1G-b; Supporting Information Fig. S2C-b), and as described below, were capable of activation in response to LPS.

GD hiPSC Macrophages Secrete Inflammatory Cytokines and Are Hypersensitive to LPS

We previously showed that GD hiPSC macrophages respond to bacterial LPS and that the level of induction of TNF-α mRNA by LPS was significantly higher in GD hiPSC compared with control hiPSC macrophages [21]. Because a number of cytokines that are important mediators of the immune response including TNF-α, IL-6, and IL-1β, are highly elevated in patients with GD [5, 6, 29, 30], we examined whether GD macrophages produce abnormal levels of these cytokines. To this end, we measured the expression and secretion of these products by GD hiPSC macrophages in the presence or absence of LPS. As shown in Fig. 2A, untreated type 2 GD F2
hiPSC macrophages from two different patients secreted nearly sixfold higher levels of TNF-α than control cells, and LPS treatment magnified this difference. IL-1β was secreted at three- to four-fold higher levels in GD cells compared with controls, but LPS treatment did not enhance secretion of this cytokine (Fig. 2B). Secretion of IL-6 by GD macrophages was also significantly higher than in controls (Fig. 2C). hiPSC macrophages from patients with types 1 and 3 GD also secreted higher levels of TNF-α, IL-6, and IL-1β than control macrophages (Supporting Information Fig. S3A–S3C). When we examined the levels of mRNA expression of these cytokines, we found that GD hiPSC macrophages were hypersensitive to LPS (Fig. 3A–3C; Supporting Information Fig. S3D–S3F). In F3

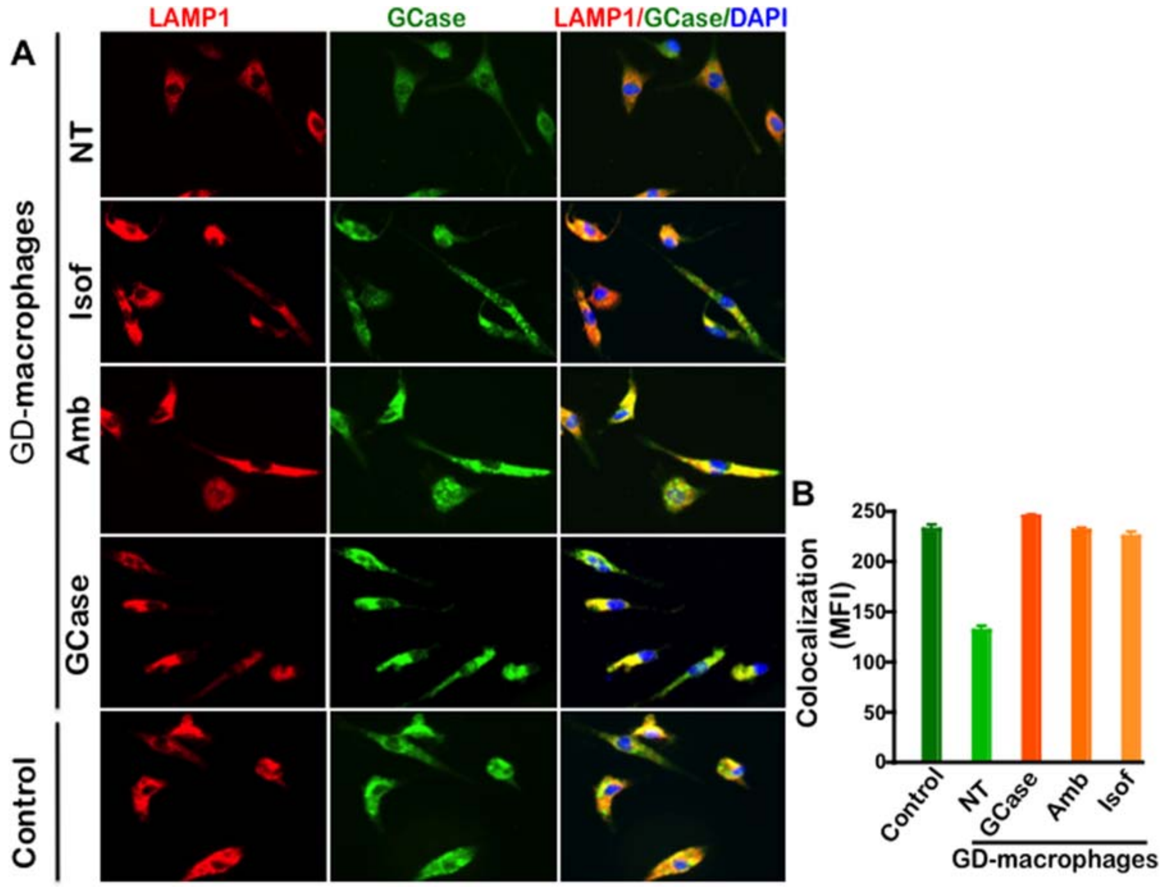

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Figure 4. Recombinant glucocerebrosidase (GCase) and chaperones increase lysosomal GCase levels in Gaucher disease (GD) human induced pluripotent stem cell (hiPSC) macrophages. **(A):** Control and type 2 GD hiPSC macrophages that were either left untreated (NT) or were treated with isofagomine (Isof, 60 μM), ambroxol (Amb, 100 μM), or recombinant GCase (0.24 U/ml) for 5 days, were immunostained for GCase and LAMP1 and counterstained with DAPI. Red color (left panels), LAMP1; green color (middle panels), GCase; blue color (right panels), DAPI. Overlay (right panels), GCase, LAMP1, and DAPI stainings. Magnification, ×40. **(B):** Quantification of mean fluorescence intensity (MFI) of colocalized LAMP1 and GCase. Abbreviations: Amb, Ambroxol; DAPI, 4′,6-diamidino-2-phenylindole; GCase, glucocerebrosidase; GD, Gaucher disease; Isof, Isofagomine; MFI, mean fluorescence intensity; NT, untreated vehicle.

contrast to TNF-α, IL-6, and IL-1β, the anti-inflammatory cytokine IL-10 was secreted at lower levels in types 2-a and 2-b GD macrophages compared with controls, and LPS treatment did not alter IL-10 secretion to a significant extent (Fig. 2D). However, LPS induced IL-10 mRNA in both, type 2 GD and control macrophages (Fig. 3D), suggesting that post-translational mechanisms may regulate LPS-induced secretion of this cytokine. No significant difference in IL-10 mRNA induction by LPS between control and patient macrophages was observed.

We conclude that GD hiPSC macrophages are abnormally activated and produce increased levels of inflammatory cytokines, recapitulating their elevation in serum of GD patients. Our results provide direct evidence that pathological Gaucher macrophages may be the source of inflammatory cytokines in patient serum.

GD hiPSC-Derived Macrophages Have Elevated Levels of Chitotriosidase

ChT1, an enzyme produced by macrophages and neutrophils [27, 31], is highly elevated in symptomatic patients with GD [5–7]. Serum levels of ChT1 in patients with type 1 GD reflect the burden of lipid-laden macrophages, and ChT1 is used to help determine the dosing of recombinant GCase and to follow the response to ERT [5, 6, 32]. IL-6 and CCL-18 are also used as biomarkers of GD [33], particularly in the 6% of the human population harboring a mutation in the ChT1 gene that abrogates its expression [7, 31]. To determine whether GD hiPSC macrophages produce elevated levels of ChT1, we measured ChT1 enzymatic activity in GD hiPSC macrophages. As shown in Fig. 3E, ChT1 enzymatic activity was elevated in GD macrophages from all clinical subtypes compared with control macrophages. While the increased production of ChT1 by GD hiPSC macrophages was modest compared with the highly elevated levels of serum ChT1 in symptomatic GD patients [6, 32], our data are consistent with the idea that GD macrophages contribute to the elevation of this marker of GD.

Recombinant GCase and Chaperones Increase Lysosomal GCase in GD hiPSC Macrophages

Ambroxol and isofagomine are iminosugar chaperones that increase GCase transport to the lysosome in patient fibroblasts [34, 35]. To determine whether these small molecules increased mutant GCase levels in the lysosomes of GD macrophages, we analyzed colocalization of GCase with lysosomal markers. Treated and untreated GD hiPSC macrophages were immunostained with antibodies to GCase and LAMP1, and

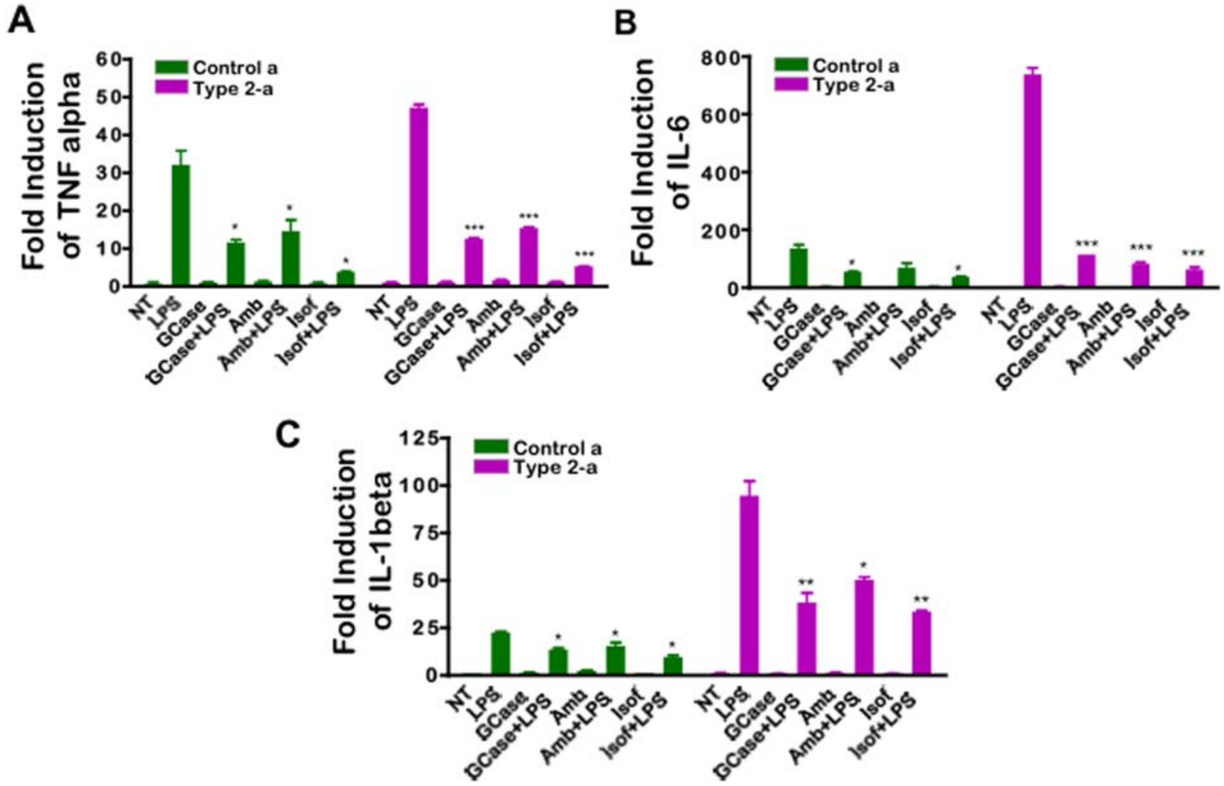

Figure 5. Reversal of cytokine induction in Gaucher disease (GD) human induced pluripotent stem cell (hiPSC) macrophages by recombinant glucocerebrosidase (GCase), ambroxol, and isofagomine. **(A–C):** Control and type 2-a GD hiPSC macrophages were incubated with recombinant GCase (0.24 U/ml), ambroxol (100 μM), isofagomine (60 μM), or vehicle (NT) for 5 days. Control and GD hiPSC macrophages were then either left untreated or were treated with 100 ng/ml LPS for 4 hours, after which real-time polymerase chain reaction analysis was carried out to measure induction of tumor necrosis factor α (TNF-α) **(A)**, interleukin (IL)-1β **(B)**, and IL-6 **(C)** mRNA. Numbers in the ordinates represent fold-activation of the corresponding cytokines compared with the nontreated condition. *p* values for all treatments in the presence or absence of LPS are between $<.01$ and $<.05$. Abbreviations: Amb, Ambroxol; GCase, glucocerebrosidase; IL, interleukin; Isof, Isofagomine; LPS, lipopolysaccharide; NT, untreated vehicle; TNF-α, tumor necrosis factor α.

F4 counterstained with DAPI. As shown in Fig. 4A and 4B, treatment of GD macrophages with isofagomine, ambroxol, or recombinant GCase, resulted in a nearly twofold increase in mutant GCase in lysosomes, as evidenced by increased colocalization of GCase with LAMP1.

Reversal of GD hiPSC Macrophage Activation by Recombinant GCase and Chaperones

We then examined whether these therapeutic agents were also capable of reversing the functional defects of GD hiPSC macrophages. To this end, we assessed the ability of GCase, ambroxol, and isofagomine to correct the elevated production of inflammatory cytokines in the mutant macrophages. Type 2 (L444P/Rec*Nci*I) GD hiPSC macrophages were incubated with recombinant GCase, ambroxol, or isofagomine for 5 days. After this time, cells were treated with LPS for 4 hours, and the expression of TNF-α, IL-6, and IL-1β mRNAs was measured
F5 by qRT-PCR. As shown in Fig. 5A–5C, treatment of GD macrophages with these three agents sharply inhibited the LPS-induced expression of TNF-α and IL-6 to the same extent as in treated controls. IL-1β was also significantly reduced by these treatments, but not to control levels. Interestingly, the same treatments increased expression of the anti-inflammatory cytokine IL-10 in both control and mutant macrophages (Fig.
F6 6A). We conclude that recombinant GCase and the two chaperones inhibited production of the inflammatory cytokines TNF-α, IL-6, and IL-1β, while stimulating expression of the anti-inflammatory cytokine IL-10. These results further suggest that ERT and chaperone treatment of patients with GD might help restore immune homeostasis disrupted by GCase deficiency.

Reversal of ChT1 Elevation in GD hiPSC Macrophages

As shown in Fig. 6B and Supporting Information Fig. S3G, treatment of types 1, 2, and 3 GD hiPSC macrophages with recombinant GCase for 5 days caused a significant reduction in ChT1 activity. These results recapitulate the decrease in ChT1 observed in GD patients treated with ERT [5, 32]. A similar treatment with isofagomine or ambroxol also decreased ChT1 activity, but to a lesser extent than recombinant GCase.

Rescue of RBC Clearance Defect in GD hiPSC Macrophages

Gaucher macrophages infiltrating bone marrow often have remnants of RBC [36, 37] due to the inability of the phagocytic cells to digest glucosylsphingolipids present in the RBC membrane. This characteristic hallmark of GD was recapitulated by types 1, 2, and 3 GD hiPSC macrophages, and the extent of this defect reflected the severity of the mutation [[21] and Supporting Information Fig. S4A]. To assess the

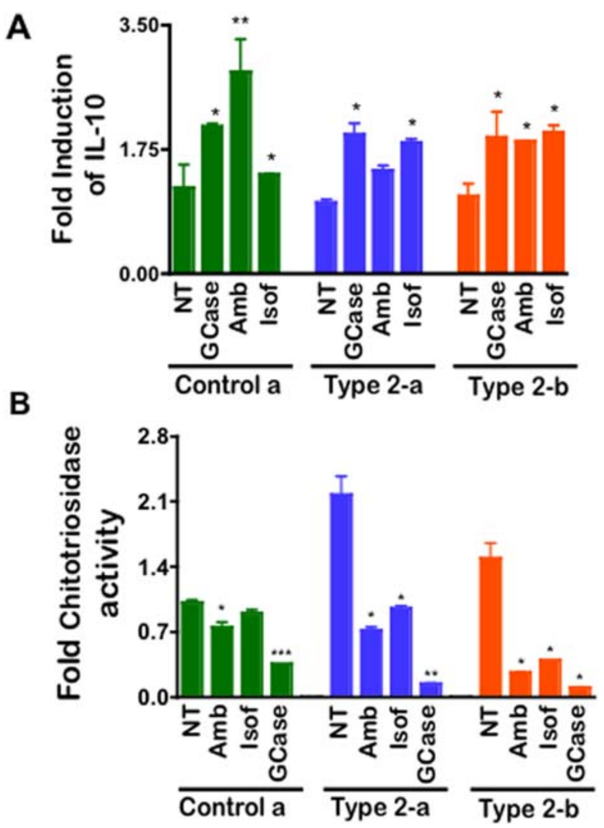

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Figure 6. Recombinant glucocerebrosidase (GCase), isofagomine, and ambroxol induce interleukin (IL)−10 and reverse the elevation of chitotriosidase (ChT1) activity in Gaucher disease (GD) human induced pluripotent stem cell (hiPSC) macrophages. **(A):** Control, type 2-a, and type 2-b GD hiPSC macrophages were incubated with recombinant GCase (0.24 U/ml), ambroxol (100 μM), isofagomine (60 μM), or vehicle (NT) for 5 days, after which real-time polymerase chain reaction analysis was carried out to measure induction of IL-10. Numbers in the ordinate represent the IL-10 mRNA fold-activation compared with the nontreated condition. *p* values for GCase, ambroxol, and isofagomine in treated versus untreated cells are: <.05. **(B):** Control, type 2-a, and type 2-b GD hiPSC macrophages that were either not treated or treated for 5 days with recombinant GCase (0.24 U/ml), ambroxol (100 μM), or isofagomine (60 μM), were assayed for ChT1 enzyme activity in intact cells as described in the Materials and Methods section. For all macrophages including control, *p* values for NT versus treated are between <.01 and <.05. Abbreviations: Amb, Ambroxol; GCase, glucocerebrosidase; IL, interleukin; Isof, Isofagomine; NT, untreated vehicle.

effectiveness of recombinant GCase, ambroxol, and isofagomine in correcting the delay in clearance of phagocytosed RBC by GD hiPSC macrophages, types 1, 2, and 3 hiPSC macrophages were preincubated with recombinant GCase or chaperones, and the kinetics of RBC clearance by the mutant F7 macrophages were determined. As shown in Fig. 7A–7C, recombinant GCase fully corrected the RBC clearance defect of GD hiPSC macrophages even in the case of type 2 cells, indicating that the functional abnormalities we observed were caused by GCase deficiency. Ambroxol was almost as effective as recombinant GCase in reversing the abnormal phenotype of the mutant macrophages (Fig. 7A–7E; Supporting Information Fig. S4B, S4C, S4E, S4F). On the other hand, correction of the RBC clearance defect in types 2 and 3 GD hiPSC macrophages by isofagomine was not as effective as that by ambroxol (Fig. 7D, 7E; Supporting Information Fig. S4E, S4F). Optimum duration of pretreatment with recombinant GCase and the chaperones was 4–6 days (Supporting Information Fig. S4D–S4F). Our results using six different GD hiPSC lines representative of all three clinical subtypes of GD suggest that GD hiPSC macrophages not only recapitulate pathologic hallmarks of the disease, but their functional responses to biotherapeutics used to treat patients reflect the known clinical efficacy of these agents. Our finding that RBC clearance is the assay that most closely predicts the clinical efficacy of these pharmacological agents suggests that this assay will be a very valuable tool for preclinical testing of new therapies for GD.

Discussion

In this study, we show that hiPSC macrophages derived from patients with types 1, 2, and 3 GD are activated and produce elevated levels of IL-1β, TNF-α, IL-6, and ChT1. We also show that recombinant GCase and pharmacological chaperones can reverse the functional abnormalities of GD hiPSC macrophages to an extent that reflects their known clinical efficacies.

Clinical and animal studies have suggested that Gaucher macrophages are the source of the elevated levels of cytokines and ChT1 present in patient serum [5, 31, 33, 38, 39]. Our results directly demonstrate that GD hiPSC macrophages are activated, are hypersensitive to the bacterial product LPS, and that they produce increased levels of IL-1β, TNF-α, IL-6, and ChT1. There was also a concomitant decrease in production of the anti-inflammatory cytokine IL-10, in agreement with the known reciprocal expression between inflammatory mediators and IL-10 [40, 41]. Future experiments should clarify the mechanisms by which GCase deficiency causes macrophage activation.

It is believed that the abnormal release of inflammatory mediators by Gaucher macrophages and the resulting dysregulation of the immune system, are causally related to the 25- to 50-fold increased risk of developing multiple myeloma and B cell lymphoma, the most prevalent malignancies associated with GD [5, 42, 43]. Clonal B cell expansion and development of B cell lymphomas and myelomas have also been reported in mouse models with targeted deletion of GCase in hematopoietic stem cells [44, 45]. Our results show that GD hiPSC macrophages produce elevated levels of IL-1β and IL-6, two cytokines that have been implicated in the development of multiple myeloma [46, 47]. It has been shown that in the microenvironment of the bone marrow, picomolar levels of IL-1β induce production of large amounts of IL-6 by stromal cells [48]. Therefore, it is possible that acting through autocrine or paracrine mechanisms, Gaucher macrophages provide persistent IL-6 stimulation of B lymphocytes. This chronic stimulation of B lymphocytes may lead to clonal B cell expansion, gammopathies, and ultimately myeloma. This suggests that anti-IL-1β receptor therapy might help protect GD patients from development or progression of myeloma, as has been shown in patients with smoldering/indolent myeloma at high risk of progression to multiple myeloma [48, 49]. In this study, we show that recombinant GCase, isofagomine and ambroxol blunt the abnormal elevation of TNF-α, IL-1β, and IL-6 in

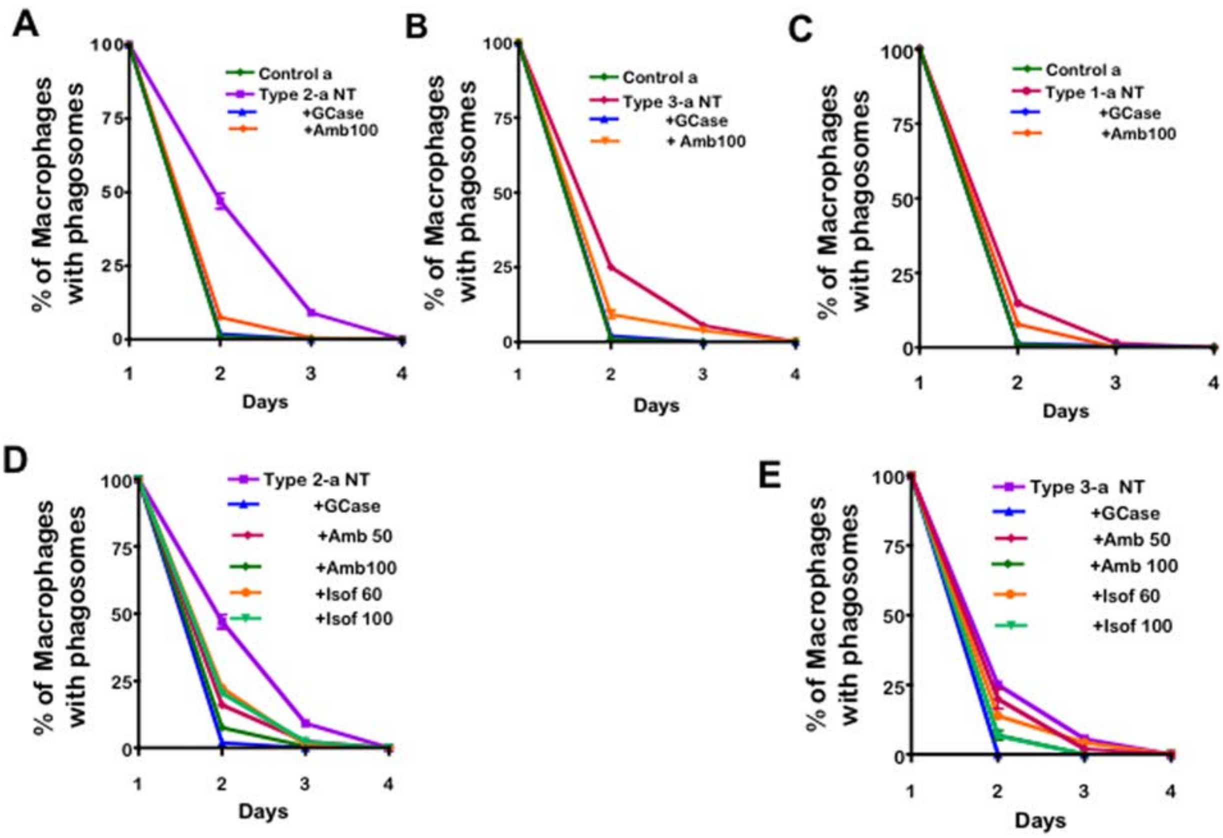

Figure 7. Reversal of the delayed clearance of RBC in Gaucher disease (GD) human induced pluripotent stem cell (hiPSC) macrophages by recombinant glucocerebrosidase (GCase) and chaperones. **(A–C):** Type 2 **(A)**, type 3 **(B)**, and type 1 **(C)** GD hiPSC macrophages were either left untreated (NT) or were incubated with either recombinant GCase (0.24 U/ml) or ambroxol (100 μM) for 5 days. The cells were then incubated with opsonized RBC for 2 hours, washed, and the time course of RBC clearance was followed by microscopic observation for the indicated times as described in the Materials and Methods section. Numbers in the ordinates represent percentage of hiPSC macrophages containing visible RBC. On day 2, for all GD types, p values of GCase treated versus NT are between $<.0001$ and $<.0003$; for type 2 and 3 hiPSC macrophages treated with ambroxol versus NT, p values are $<.0001$ and $<.0005$, respectively; for type 1, $p < .0012$. **(D, E)** type 2 **(D)** and type 3 **(E)** GD hiPSC macrophages were either left untreated (NT) or were incubated with recombinant GCase (0.24 U/ml), ambroxol (50 and 100 μM) or isofagomine (60 and 100 μM) for 5 days, after which they were assayed for RBC clearance. On day 2, (type 2 cells) p values for GCase, ambroxol (50 and 100 μM), and isofagomine (100 μM) treated versus NT are between $<.0001$ and $<.0006$; p value for isofagomine (60 μM) is $<.001$. For type 3 cells, p values for GCase, ambroxol (100 μM), and isofagomine (60 and 100 μM) treated versus NT are between $<.0001$ and $<.0006$. Abbreviations: Amb, Ambroxol; GCase, glucocerebrosidase; Isof, Isofagomine; NT, untreated vehicle.

mutant macrophages. Thus, ERT, alone or in combination with chaperones, might help reduce the incidence of myelomas in patients with GD. This idea is supported by reports that ERT results in a decrease in monoclonal and polyclonal gammopathies [50, 51]. However, larger clinical studies are required to determine the impact of ERT and other treatments in stemming the development of neoplasms in patients with GD.

One of the functions of macrophages is to remove aged or damaged red and white blood cells from circulation. In patients with GD, the reduced levels of GCase result in an inability of macrophages to digest glucosylceramide present on the surface of phagocytosed cells, and Gaucher macrophages often contain remnants of RBC [37, 52]. These pathological macrophages infiltrate bone marrow and other organs and are believed to be responsible for hepatosplenomegaly and for the elevated levels of inflammatory cytokines and ChT1 in patient serum. The reversal of the delay in clearance of phagocytosed RBC, and the reduction in inflammatory cytokine production by recombinant GCase in hiPSC-derived macrophages recapitulated the efficacy of ERT in reversing the visceral abnormalities attributed to Gaucher macrophages. Within 1–2 years of ERT, there is a reduction in the burden of pathological Gaucher macrophages, a decrease in spleen and liver size, and lowering in serum ChT1 [32, 53–55]. Our results illustrate the utility of hiPSC for modeling GD and provide insights into the mechanism of action of therapeutic agents used to treat this disease.

While mutations in GCase are known to reduce its catalytic activity [56–58], it has been suggested that even a modest increase in enzymatic activity of the mutant to 15–30% of normal values could result in significant clinical benefit [15, 59]. High throughput screens have identified pharmacological chaperones that bind to mutant GCase and facilitate proper folding, protecting it from degradation by the ERAD system, and increasing lysosomal GCase activity. Among these chaperones, the iminosugars isofagomine [60] and ambroxol [12] act by binding to the active site of mutant GCase, increasing GCase activity and trafficking to the lysosome in patient fibroblasts. In a 6-month phase 2 clinical trial, isofagomine increased GCase activity in white blood cells in all subjects, but meaningful clinical improvement was only seen in only 1 of 18 patients [13]. Although the clinical experience with ambroxol is more limited, this over-the-counter mucolytic agent has shown promising results. In a small pilot study, 3 of 12 GD subjects treated with ambroxol showed a 20% reduction in spleen size and a 50% sustained decrease in ChT1 activity [15]. Similar to isofagomine, ambroxol binds to GCase at neutral pH, but unlike the former, ambroxol dissociates from the enzyme at the acid pH of the lysosome [12], a property that may enhance its clinical performance. Our analysis showed that while recombinant GCase was more effective than isofagomine and ambroxol in reversing the RBC clearance defect of mutant macrophages, ambroxol was more effective than isofagomine in reversing this phenotype. Thus, the RBC clearance assay was able to discriminate the relative potencies of recombinant GCase and the two chaperones we tested, in a manner that reflected their known clinical efficacies. The stringency of this assay may be due to the fact that the delay in clearing phagocytosed RBC is a major functional defect of GD macrophages. Our results suggest that this strong phenotype may be very well suited to distinguish between the therapeutic efficacies of different agents and that GD hiPSC, in particular those harboring severe mutations, will be valuable reagents for therapeutic development.

In summary, we have shown that hiPSC macrophages derived from patients with types 1, 2, and 3 GD have functional abnormalities that recapitulate clinical manifestations of GD. Fibroblasts derived from GD patients are widely used for therapeutic development, but these cells are nonphagocytic and do not release the inflammatory mediators and hydrolases that play a role in the complex pathophysiology of GD. In this communication, we introduce macrophage-based functional assays that reflect the clinical efficacies of GD biotherapeutics. This study shows that GD hiPSC are a relevant new model for studying disease mechanisms and for development of effective treatments for GD.

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Author Contributions

L.M.P.: directed project, planned and carried out experiments, analyzed data, wrote the manuscript; D.M., O.A., V.B., Y.L., and J.A.S.: planned and carried out experiments, analyzed data; T.S.P. and E.T.Z.: provided reagents, obtained and analyzed data; R.A.F.: conceived and directed the project, analyzed data, and wrote the manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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