Chemical inhibition of β-glucocerebrosidase does not affect phagocytosis and early containment of *Leishmania* by murine macrophages

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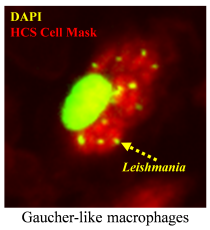

Gaucher-like macrophages
can phagocytose *Leishmania*.

Chemical inhibition of β-glucocerebrosidase does not affect phagocytosis and early containment of *Leishmania* by murine macrophages

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Abstract

Gaucher disease is a lysosomal storage disease in which a genetic deficiency in β-glucocerebrosidase leads to the accumulation of glycosphingolipids in lysosomes. Macrophages are amongst the cells most severely affected in Gaucher disease patients. One phenotype associated with Gaucher macrophages is the impaired capacity to fight bacterial infections. Here, we investigate whether inhibition of β-glucocerebrosidase activity affects the capacity of macrophages to phagocytose and act on the early containment of human pathogens of the genus *Leishmania*. Towards our aim, we performed *in vitro* infection assays on macrophages derived from the bone marrow of C57BL/6 mice. To mimic Gaucher disease, macrophages were incubated with the β-glucocerebrosidase inhibitor, conduritol B epoxide (CBE), prior to contact with *Leishmania*. This treatment guaranteed that β-glucocerebrosidase was fully inhibited during the contact of macrophages with *Leishmania*, its enzymatic activity being progressively recovered along the 48 hrs that followed removal of the inhibitor. Infections were performed with *L. amazonensis*, *L. infantum*, or *L. major*, so as to explore potential species-specific responses in the context of β-glucocerebrosidase inactivation. Parameters of infection, recorded immediately after phagocytosis, as well as 24 and 48 hours later, revealed no noticeable differences in the infection parameters of CBE-treated macrophages relative to non-treated controls. We conclude that blocking β-glucocerebrosidase activity during contact with *Leishmania* does not interfere with the phagocytic capacity of macrophages and the early onset of leishmanicidal responses.

Keywords: Gaucher disease; *Leishmania;* β-glucocerebrosidase; macrophage.

Introduction

Gaucher disease is a rare (0.2-1.8 per 100'000 live births (Kingma et al., 2015) inherited autosomal recessive lysosomal storage disease (LSD), caused by mutations in the gene that encodes the lysosomal β-glucocerebrosidase enzyme. One direct consequence of β-glucocerebrosidase deficiency is the accumulation of glycosphingolipids, specifically glucosylceramide (GlcCer) and glucosylsphingosine (GlcSph) (Beutler et al., 2013; Grabowski, 2012; Platt, 2014). Alongside lysosomal enlargement due to lipid accumulation, defects in autophagy (Aflaki et al., 2016), alterations in calcium homeostasis (Korkotian et al., 1999; Lloyd-Evans et al., 2003; Pelled et al., 2005) and in lysosomal pH (Sillence, 2013) have also been reported in Gaucher disease. Several immune system abnormalities have been described in Gaucher disease including alteration in T and B lymphocytes, NKT cells, monocytes and macrophages (Rigante et al., 2017; Pereira et al., 2017; Limgala et al., 2016). Typical of this disease is the appearance of macrophages rich in GlcCer (the so-called "Gaucher cells"), in spleen, liver and bone marrow of patients (Beutler et al., 2013). Earlier reports also indicate that in Gaucher disease patients, human monocytes and monocyte-derived macrophages present impaired capacity to kill bacterial pathogens (Liel et al., 1994; Marodi et al., 1995), consistent with the higher susceptibility of untreated Gaucher disease patients to infections and septicemia (Weinreb et al. 2018). The detailed mechanisms underlying such defective function remain elusive. Equally vague is whether the defective microbicidal activity of Gaucher disease macrophages also extends to other pathogens, specifically protozoan parasites of the *Leishmania* genus.

Leishmania are the causing agents of human leishmaniases, a set of neglected tropical diseases with symptoms ranging from contained skin wounds to disseminating cutaneous and visceral infections (Burza et al. 2018). *Leishmania* are transmitted to humans through the bite of infected female sand flies. In these insect vectors, parasites live extracellularly (promastigotes), however, once transmitted to mammals they assume an intracellular lifestyle (amastigotes),

adopting macrophages as final host cells (Kima, 2007). Within macrophages, parasites reside inside parasitophorous vacuoles that originate from the fusion of phagosome-containing parasites with lysosomes (Young and Kima, 2019). Phagolysosomes are typically small compartments harboring individual amastigotes (as is the case of *L. infantum* and *L. major*). Still, in some cases (*L. amazonensis*), they can assume large proportions and host multiple parasites (Young and Kima, 2019). The dependence of *Leishmania* on phagocytes, and particularly on lysosomal-derived compartments, for survival and replication, render these microorganisms attractive models to study in the context of Gaucher disease macrophages.

To date, the microbicidal response of Gaucher macrophages to infection by *Leishmania* remains unexplored. Here, to shed light into this subject, we investigate how chemical inactivation of β-glucocerebrosidase - a condition mimicking Gaucher disease - affects the capacity of murine bone marrow derived macrophages to phagocytose and act on the early containment of *Leishmania*.

Materials and Methods

Ethics statement

C57BL/6 mice were obtained from the i3S animal facility. Animal procedures were approved by the Local Animal Ethics Committee of i3S, licensed by Direção Geral de Alimentação e Veterinária, Govt. of Portugal. Animals were handled in strict accordance with good animal practice as defined by national authorities (directive 113/2013 from 7th August) and European legislation (directive 2010/63/EU, revising directive 86/609/EEC). The i3S animal house is certified by Direção Geral de Alimentação e Veterinária. Mice were euthanized by an overdose of isoflurane inhalation followed by cervical dislocation.

Parasites

Leishmania infantum promastigotes (MHOM MA67ITMAP263) were routinely cultured at 26°C, in RPMI 1640 GlutaMAXTM-I medium supplemented with 10% (v/v) heat inactivated fetal bovine serum (iFBS), 1% (v/v) penicillin, 1% (v/v) streptomycin (all from Gibco) and 20 mM HEPES pH 7.4 (Sigma). *Leishmania major* (MHOM/SA/85/JISH118) and *L. amazonensis* promastigotes (MHOM/BR/LTB0016) were maintained at 26°C, in Schneider's insect medium (Sigma) supplemented with 10% (v/v) iFBS, 2% (v/v) penicillin, 2% (v/v) streptomycin (all from Gibco), 5 mM HEPES pH 7.4 and 50 μg/mL phenol red (Sigma). Infective parasites were obtained from 7-8 days-old cultures of *L. infantum*, and 5-6 days-old cultures of *L. major* and *L. amazonensis*. To avoid loss of infectivity due to prolonged time in culture, parasites were passaged through mice (by intraperitoneal injections) and, upon recovery from infected spleens, kept in culture for no longer than 8 media renewals.

Bone marrow derived macrophages (BMDMs)

Bone-marrow cells, collected from femurs and tibia of C57BL/6 mice (2-3 months), were differentiated into macrophages in the presence of 20 ng/mL M-CSF (Tebu-Bio), in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% (v/v) non-essential amino acids, 10% (v/v) iFBS, 1% (v/v) penicillin, and 1% (v/v) streptomycin (all from Gibco), in a 5% CO_2 atmosphere, at 37ºC, along 8 days. Bone-marrow cells were plated in Petri dishes ($5x10^6$ cells in 7.5 mL) for flow cytometry analysis, or in 96-well flat bottom plates ($3x10^4$ cells in 150 µL per well) for infection experiments. On days 3 and 6, cells were replenished with new M-CSF-supplemented medium. To inactivate β-glucocerebrosidase, BMDMs were exposed to 100 µM CBE for 48 hrs (between days 6 and 8 of differentiation). Prior to addition of *Leishmania* to BMDMs, CBE was removed from cultures by gentle washings with fresh medium.

Infection of BMDMs with *Leishmania* and determination of infection indexes

Infections of BMDMs were carried out with *L. infantum* or *L. major* at multiplicities of infection of 5 or 10, and with *L. amazonensis* at multiplicities of infection of 2 or 5. Upon 3 hours of contact with BMDMs, non-internalized parasites were washed away, and either immediately fixed (time 0 hrs), or replenished with new medium and cultured for additionally 24 hrs and 48 hrs. At each time point, determination of infection indexes was performed as described before (Gomes-Alves et al., 2018). Briefly, monolayers of *Leishmania*-infected BMDMs were fixed, permeabilized, and stained with 4´,6-diamidino-2-phenylindole (DAPI, Sigma) and with HCS CellMask™ Deep Red stain (Invitrogen). Images were acquired in an IN Cell Analyzer 2000 microscope and analyzed with a dedicated algorithm in the IN Cell Investigator Developer Toolbox (both from GE Healthcare).

Flow cytometry

BMDMs were detached from Petri dishes by a 20 min-treatment at 4ºC in the presence of PBS 2% (v/v) iFBS, 5mM EDTA. Cells were subsequently subjected to two independent flow cytometry analyses: i) to check for viability [7-AAD and annexin V (BD Biosciences)], for the macrophage surface marker F4/80 [anti-mouse F4/80 (BM8) (Biolegend)], as well as activation markers [MHC class II (M5/114.15.2), CD40 (3/23) and CD80 (16-10A1) (Biolegend)]; and ii) to measure β-glucocerebrosidase activity using fluorescein di-β-D-galactopyranoside (FDG, Sigma). Unstained cells were used as negative control. Cells were acquired in a FACS Canto II (BD Biosciences) using the BD FACSDiva™ software (BD Biosciences). Data analysis was performed with FlowJo® v10 (BD Biosciences).

Quantitative fluorescence microscopy

For quantitative analysis of FDG metabolization by active β-glucocerebrosidase, BMDMs were incubated with 0.05 mM FDG for 1 hr. Images were acquired with an IN Cell Analyzer 2000 microscope and analyzed with a dedicated algorithm in the IN Cell Investigator Developer Toolbox (both from GE Healthcare) for automatic calculation of the mean FDG fluorescence per cell.

Statistics

Statistical analyses were performed considering the results from three independent experiments (each done in triplicate). To correct for inter-experimental variation, data was normalized taking as 100% the average of triplicates of CBE-untreated cells, for each time point, within each experiment. Data normality was checked using the Shapiro-Wilk test. Statistical analyses were based on the one-way ANOVA (Tukey's multiple comparisons test), the unpaired t-test (normal distribution) and the Mann-Whitney test (non-normal distribution). Statistical analyses were performed using the GraphPad Prism software v8.1.1.

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Results

To investigate the impact that inactivation of β-glucocerebrosidase - the cause of Gaucher disease - has on the macrophage response to infection by *Leishmania*, we resorted to an *in vitro* model consisting on C57BL/6 murine BMDMs in which β-glucocerebrosidase was chemically and transiently inhibited by conduritol B epoxide (CBE) (Das et al., 1987; Newburg et al., 1988; Sillence et al., 2002). F4/80 expression was used to confirm the BMDMs phenotype (Fig. 1A). For inactivation of β-glucocerebrosidase, we exposed BMDMs to 100 μM CBE for 48 hrs, adapting previously established protocols (Das et al., 1987; Newburg et al., 1988; Sillence et al., 2002). We confirmed the successful inactivation of the enzyme in CBE-treated cells, by adding FDG to BMDMs and subsequently screening for the product of FDG metabolization by active β-glucocerebrosidase (fluorescein). As expected, fluorescein was undetectable in BMDMs exposed to CBE, in contrast to its high levels in control cells (Fig. 1B). We confirmed that exposure to CBE did not affect the viability of BMDMs (53.6±9.9% and 55.4±6.9% Annexin V^- $7\text{-}AAD^-$ cells, for CBE-untreated and treated cells, respectively), and the expression of markers of macrophage differentiation (F4/80). Finally, we checked that pre-treatment with CBE did not result in macrophage activation, by evaluating the cell surface expression of the activation markers MHC class II, CD40 and CD80 (Fig. 1C).

To avoid the direct effect of this compound on parasites, treatment with CBE was interrupted immediately before macrophage infection with *Leishmania*, by removing the inhibitor, washing and replenishing cells with fresh medium. CBE is a β-glucocerebrosidase irreversible inhibitor (Legler, 1968), however it was described that, 48 hrs upon CBE removal from macrophage culture, cells recover 50% of enzyme activity (Das et al., 1987), likely reflecting the *de novo* synthesis of the enzyme. We confirmed the kinetics of recovery of β-glucocerebrosidase activity after CBE removal by monitoring FDG metabolization resorting to quantitative fluorescence microscopy. The resulting data, plotted in Fig. 2, confirm full inhibition of the enzyme immediately after removal of CBE (0 hrs, *black columns* vs. *white columns*) (aligned

with the results of Fig. 1B). Importantly, these results also evidence the progressive rescue of β-glucocerebrosidase activity in CBE-treated macrophages along the following 48 hrs (*white columns*) - aligned with the previous report by Das et al. (1987). In non-treated controls (*black columns*), the overall β-glucocerebrosidase activity tends to values above (albeit with no statistical significance at 24 and 48 hrs) that of CBE-treated cells.

Having characterized our *in vitro* murine model of β-glucocerebrosidase-inactivated BMDMs, we moved on to assess how these cells respond to infection by *Leishmania*. Our analysis included three species of *Leishmania* (*L. amazonensis*, *L. infantum* and *L. major*) because we sought to explore specific phenotypes that could emerge from unique interactions of these parasites with their host cells. Illustrating this, *L. amazonesis* differs from the other two species by residing in large, multiple parasite-harboring phagolysosomes, instead of the small/single-parasite vacuoles (Young and Kima, 2019). *Leishmania infantum* stands out for being an agent of visceral infections, unlike the other two cutaneous disease-causing species (Burza at al., 2018).

Macrophages were infected with *L. amazonensis*, *L. infantum* or *L. major*. The ratios of parasites/macrophage (or multiplicities of infection, MOIs) were adjusted based on protocols previously optimized in our lab. For the first two species, we used MOIs of 5 and 10, whereas for *L. amazonensis*, yielding higher infection indexes, assays were performed with lower MOIs (2 and 5). The rationale behind using two MOIs was to make sure that we would not miss any potential anti- or pro-*Leishmania* effect resulting from β-glucocerebrosidase inhibition, by the use of a defective or an excessive parasite burden. We found that the effect of β-glucocerebrosidase inhibition was the same for both ratios of parasites/macrophage tested for each *Leishmania* species (Fig. S1), therefore we pooled the results of both MOIs for our global analysis. Each graphic in Fig. 3 illustrates data from three independent experiments, each assaying two MOIs in triplicate, making a total of 18 data points per condition. Infection was evaluated based on two parameters: i) the percentage of infected macrophages (Fig. 3, *top*

panels and Table S1), and ii) the average number of parasites per infected macrophage (Fig. 3, *bottom panels* and Table S1). To facilitate comparison between controls and CBE-treated conditions we eliminated inter-experimental variability by normalizing the data, taking as unit (100%) the averaged values of control macrophages at each time point.

Towards our aim, we first assessed how inactivation of β-glucocerebrosidase affects the capacity of macrophages to phagocytose *Leishmania*. For that, we focused on the initial time points after parasite contact with BMDMs (0 hrs). In the case of *L. amazonensis* and *L. infantum*, we found no statistically significant differences between control and CBE-treated macrophages for neither infection parameter (Fig. 3A). As for *L. major*, inactivation of β-glucocerebrosidase led to an increased percentage of infected macrophages ($p \leq 0.05$; Fig. 3A, *top panel*). However, since this increment was discreet (1.08±0.11 times) and not accompanied by an increased number of intracellular parasites (Fig. 3A, *bottom panel*), we did not find it physiologically meaningful. Second, we studied how inhibition of β-glucocerebrosidase affects the early leishmanicidal activity of macrophages. Towards that end, we followed parasite survival at 24 and 48 hrs *post* infection. Since β-glucocerebrosidase activity is partially recovered in these time points (Fig. 2), any discrepancies found between control and CBE-treated macrophages at 24 and 48 hrs *post* infection should reflect differential anti-microbial responses at early time points, when β-glucocerebrosidase activity is different between both experimental groups. Analysis of infection parameters 24 and 48 hrs after infection revealed no variations between control and CBE-treated macrophages infected with *L. infantum* (Fig. 3B,C). We did register statistically significant ($p \leq 0.05$) differences when BMDMs were infected with either *L. major* for 24 hrs (number of intracellular parasites; Fig. 3B, *bottom panel*), or *L. amazonesis* for 48 hrs (% infected macrophages; Fig. 3C, *top panel*). However, these variations were very faint (0.93±0.08 and 0.87±0.18 times, respectively) and not accompanied by alterations of the complementary infection parameters, leading us to assume that they do not translate any physiologically relevant phenomena.

From the abovementioned results, we conclude that in our murine BMDM/CBE model of Gaucher disease, the capacity of macrophages to phagocytose and act on the early containment of *L. amazonensis*, *L. infantum*, and *L. major* is not affected.

Discussion

This study follows up on previous reports in which Gaucher disease monocytes and macrophages were reported to exhibit impaired microbicidal capacity in the context of infections with bacterial agents (Liel et al., 1994; Marodi et al., 1995), and questions whether the same behavior extends to human pathogens of the genus *Leishmania*. By investigating the anti-*Leishmania* response of Gaucher disease-like macrophages we aimed at advancing the knowledge on how β-glucocerebrosidase activity modulates the early response of phagocytes against these protozoan parasites.

Our experimental approach consisted on an *in vitro* murine BMDM model, in which traits of Gaucher disease were mimicked by chemical inhibition of β-glucocerebrosidase with CBE. To avoid contact of *Leishmania* with CBE, this inhibitor was removed from cultures immediately before infection, which resulted in a transient blocking of β-glucocerebrosidase activity. Based on this model, we conclude that inactivation of β-glucocerebrosidase has no impact on the early events of *Leishmania* infection, namely phagocytosis and concomitant microbicidal responses. The impact that permanent inhibition of β-glucocerebrosidase has on the outcome of *Leishmania* infection, would require the use of other models of Gaucher disease macrophages. In this regard, macrophages differentiated from the bone marrow of murine models of Gaucher disease (Farfel-Becker et al., 2011), or from monocytes of Gaucher disease patients come out as interesting options.

In sum, this report inaugurates the study on whether Gaucher disease-like lysosomal alterations impact the phagocytic and early microbicidal response of macrophages in the context of *L. amazonensis*, *L. infantum*, and *L. major* infections. It concludes that Gaucher disease-like macrophages retain the capacity to phagocytose and act on the early containment of *Leishmania*.

277 **Figure legends**

278

279 **Fig. 1.** Flow cytometry analysis of markers of viability, differentiation, activation, and of β-
280 glucocerebrosidase activity of BMDMs. (**A**) Gating strategy employed in the flow cytometry
281 analysis of BMDMs, showing selection of cells based on size (1st panel), singlets (2nd panel),
282 and viability (3rd panel; Annexin V^- 7-AAD^- cells). Also depicted is the expression of the
283 macrophage surface marker F4/80 in viable BMDMs (4th panel). Percentage values are
284 displayed in the graphs. (**B**) Assessment of β-glucocerebrosidase activity in BMDMs, based on
285 the conversion of FDG to fluorescein. (**C**) Analysis of the expression of the macrophage surface
286 marker F4/80 and of the activation state of viable BMDMs, based on the expression of MHC
287 class II, CD40 and CD80 molecules. Panels refer to one representative experiment out of three.

288

289 **Fig. 2.** Kinetics of recovery of β-glucocerobrosidade activity upon removal of CBE.
290 Monocytes collected from the bone marrow of C57BL/6 mice were differentiated to
291 macrophages in the presence of M-CSF, along 8 days (*black columns*). To inhibit β-
292 glucocerebrosidase activity (*white columns*), cells were exposed to 0.1 mM CBE between days
293 6 and 8 of differentiation. At day 8, all cells were replenished with new medium, and the time
294 of the assay set to 0 hrs. At 0, 24 or 48 hrs, cells were supplemented with FDG and
295 metabolization of this substrate by β-glucocerebrosidase was monitored by fluorescence
296 microscopy, using a high content microscope and a dedicated algorithm to quantify the mean
297 fluorescence intensity per cell. As negative control for β-glucocerebrosidase activity, non-
298 treated cells were incubated with CBE immediately prior to supplementation with FDG (*grey*
299 *columns*). The graph represents averages and standard deviations of FDG fluorescence
300 (arbitrary units, A.U.) from n=3. One-way ANOVA (Tukey's multiple comparisons test) was
301 used to compare the different experimental sets of BMDMs. * $p \leq 0.05$, ** $p \leq 0.005$, *** p
302 ≤ 0.001, * $p \leq 0.0001$.

Fig. 3. BMDMs with CBE-inactivated β-glucocerebrosidase retain the capacity to phagocytose and eliminate *Leishmania*. Percentage of infected macrophages (*upper panel*s), and average number of parasites per infected cell (*bottom panels*), in control (*black circles*) and CBE-treated BMDMs (*white circles*), recorded (**A**) immediately after infection with *L. amazonensis* (*L.a.*), *L. infantum* (*L.i.*), or *L. major* (*L.m.*), as well as in the following (**B**) 24 hrs and (**C**) 48 hrs. For each time point and parasite species, infection parameters were normalized taking as unit (100%), the averaged values of control cells. Bars correspond to the means and standard deviations of three independent experiments (each performed with two MOIs in triplicate). Unpaired t-test (normal distribution) and Mann-Whitney test (non-normal distribution) were used to compare control and CBE-treated BMDMs. * $p \leq 0.05$.

315 **Conflict of interest statement:**

316 On behalf of all authors, the corresponding author states that there is no conflict of interest.

317

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Figure 3

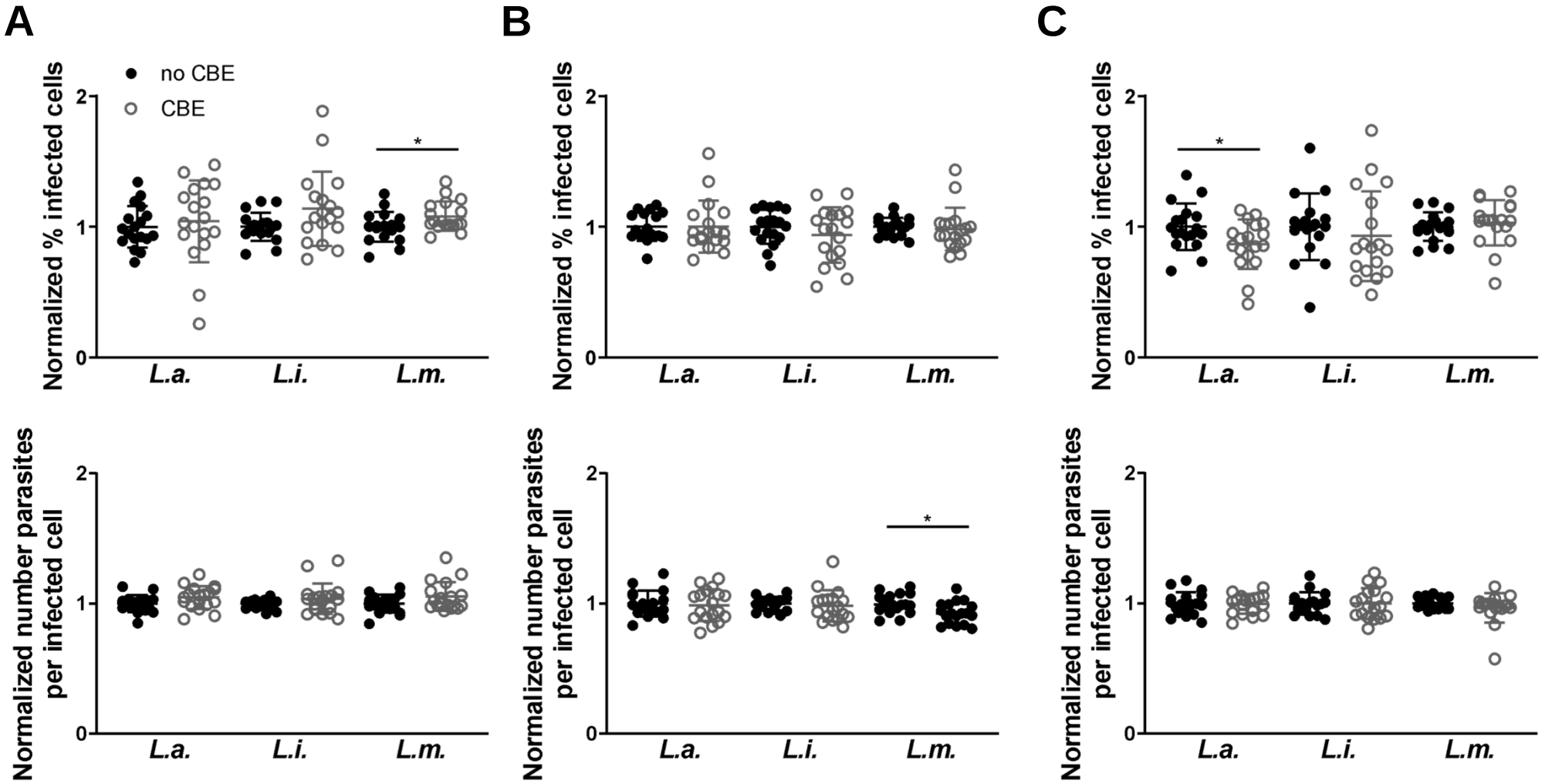
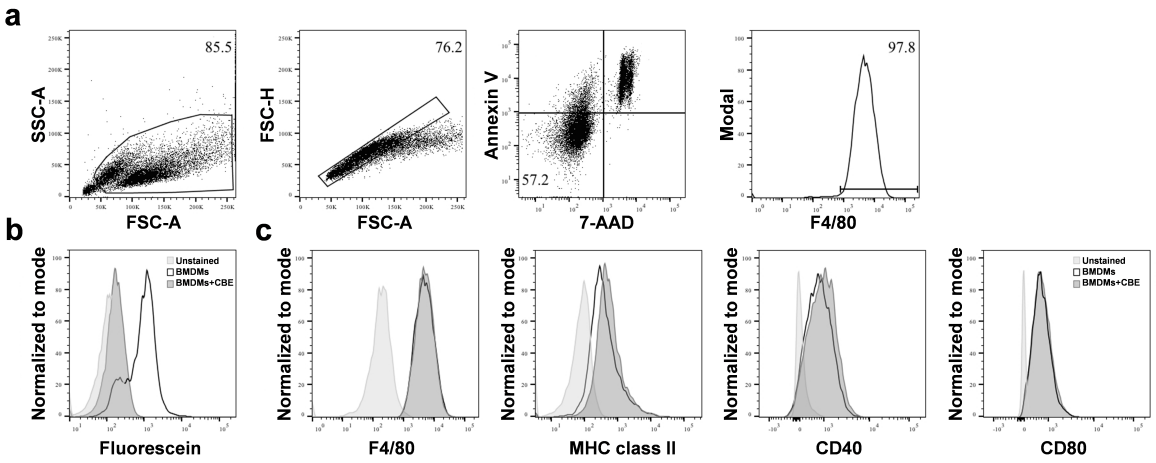
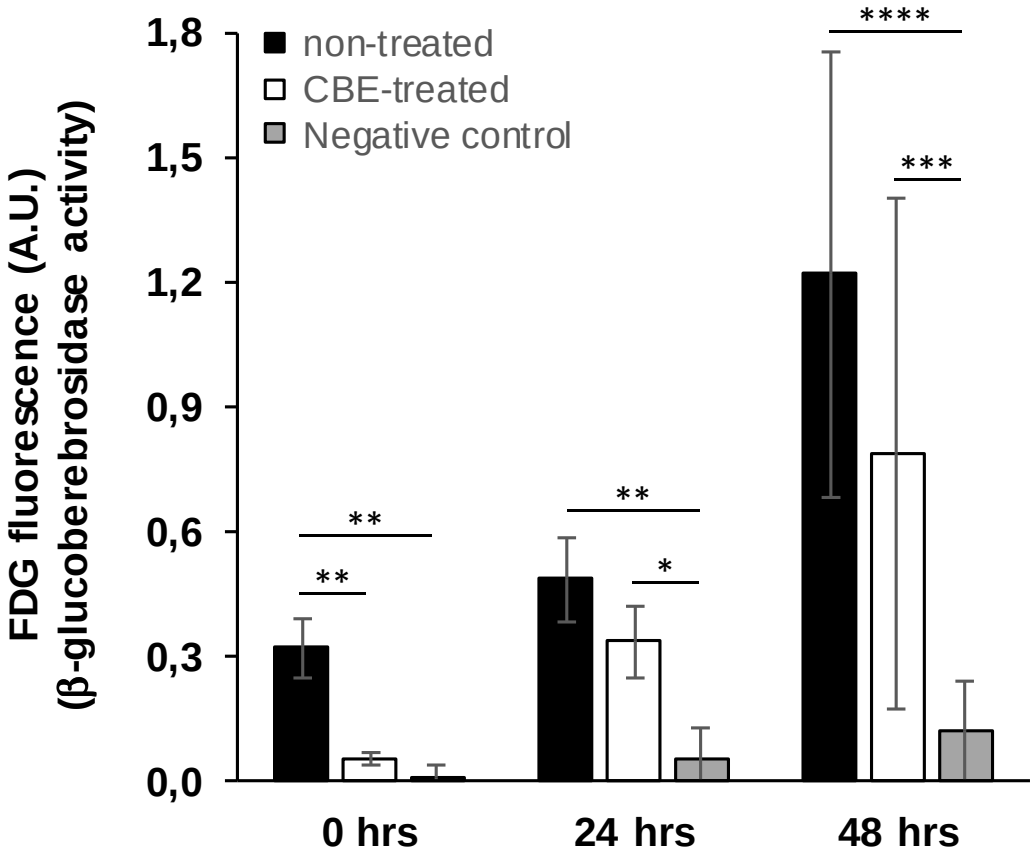

A
B
C
no CBE
CBE
Normalized % infected cells
Normalized number parasites per infected cell
0
1
2
*
L.a.
L.i.
L.m.

Figure 1




FDG fluorescence (A.U.)
(β-glucoberebrosidase activity)
1,8
1,5
1,2
0,9
0,6
0,3
0,0
non-treated
CBE-treated
Negative control
**
**
**
*
****
***
0 hrs
24 hrs
48 hrs

Highlights

- *Leishmania* spp. are obligatory intracellular parasites of macrophages (MO).
- MO are the main cells affected in Gaucher patients.
- Gaucher disease is characterized by a defective activity of β-glucocerebrosidase.
- Gaucher-like MO were generated by chemical inhibition of β-glucocerebrosidase.
- Gaucher-like MO are proficient at phagocytosing and controling *Leishmania*.