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A importância da acumulação lipídica nas doenças de sobrecarga lisossomal na função das células imunes

The relevance of lipid accumulation in lysosomal storage diseases in immune cells function

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Bioquímica, realizada sob a orientação científica da Doutora Maria de Fátima Matos Almeida Henriques de Macedo, Professora Auxiliar Convidada do Departamento de Ciências Médicas da Universidade de Aveiro.

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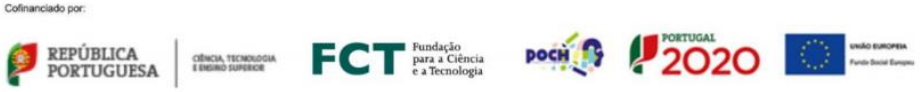

palavras-chave

doenças de sobrecarga lisossomal, apresentação de antigénios lipídicos, CD1b, CD1d, células dendríticas, monócitos, macrófagos, células “natural killer” T.

resumo

As doenças de sobrecarga lisossomal são doenças genéticas raras nas quais diferentes moléculas se acumulam no lisossoma devido a defeitos nas proteínas lisossomais. As doenças de Fabry, de Gaucher e a doença de Niemann-Pick tipo C (NPC) são doenças de sobrecarga lisossomal do tipo esfingolipidoses em que há a acumulação de diferentes esfingolípidos no lisossoma, enquanto que as mucopolissacaridoses (MPS) são caracterizadas pela acumulação lisossomal de glicosaminoglicanos.

O lisossoma é importante na apresentação de antigénios lipídicos mediada pelas moléculas CD1. Nos humanos, quatro isoformas de CD1 (CD1a, CD1b, CD1c e CD1d) são capazes de apresentar antigénios lipídicos, incluindo esfingolípidos, a um grupo específico de células T, as células T restritas a CD1. As células “natural killer” T invariantes (iNKT) são as células T restritas a CD1 mais bem caracterizadas. Nesta tese, investigou-se como as alterações lisossomais que ocorrem nas doenças de sobrecarga lisossomal têm impacto na resposta imunitária, nomeadamente na apresentação de antigénios lipídicos mediada pelas moléculas CD1 e na infeção.

A capacidade de apresentação de antigénios lipídicos mediada pelas moléculas CD1 foi avaliada em monócitos e em células dendríticas derivadas de monócitos de pacientes com doenças de sobrecarga lisossomal. Na doença de Fabry, Gaucher, NPC e MPS VI, as células dendríticas derivadas de monócitos não apresentam defeitos na capacidade de apresentação de antigénios lipídicos mediada pelas moléculas CD1b e CD1d e não foram encontradas alterações na frequência das células iNKT, ao contrário do que foi reportado em vários modelos de doenças de sobrecarga lisossomal em ratinhos. Além disso, na doença de Fabry e Gaucher, a capacidade de apresentação de antigénios lipídicos mediada pela molécula CD1d dos monócitos não diminuiu. Para além disso, foi investigado o impacto de diferentes condições de colheita de sangue na capacidade de apresentação de antigénios lipídicos mediada pela molécula CD1d dos monócitos. Monócitos isolados 24 horas após a colheita de sangue em ácido etilenodiamino tetra-acético (EDTA) apresentaram capacidade reduzida para ativar as células iNKT, quando comparados com os monócitos isolados 24 horas após a colheita de sangue em citrato. Este resultado é bastante relevante no contexto da apresentação de antigénios lipídicos mediada pela molécula CD1d e destaca a importância do anticoagulante escolhido para realizar ensaios funcionais com monócitos.

Os macrófagos encontram-se severamente afetados em pacientes com doença de Gaucher e a capacidade dos macrófagos humanos com doença de Gaucher para combater infeções bacterianas está comprometida. Nesta tese, foi avaliada a capacidade dos macrófagos da doença de Gaucher em fagocitar e eliminar os parasitas *Leishmania*. Foi utilizado um modelo *in vitro* através do tratamento de macrófagos derivados da medula óssea de ratinhos com um inibidor da enzima β-glucocerebrosidase (epóxido de conduritol B), mimetizando a doença de Gaucher. Estes macrófagos foram capazes de internalizar e eliminar os parasitas, da mesma forma que as células controlo.

Estes resultados contribuem para aumentar o conhecimento das doenças de sobrecarga lisossomal e da apresentação de antigénios lipídicos mediada pelas moléculas CD1.

keywords

lysosomal storage diseases, lipid antigen presentation, CD1b, CD1d, dendritic cells, monocytes, macrophages, natural killer T cells.

abstract

Lysosomal storage diseases (LSDs) are rare genetic diseases in which different molecules accumulate in the lysosome due to defects in lysosomal proteins. Fabry disease, Gaucher disease and Niemann-Pick disease type C (NPC) are LSDs, sphingolipidoses in which there is the accumulation of different sphingolipids in the lysosome while mucopolysaccharidoses (MPS) are characterized by the lysosomal accumulation of glycosaminoglycans.

The lysosome is important in lipid antigen presentation mediated by CD1 molecules. In humans, four CD1 isoforms (CD1a, CD1b, CD1c and CD1d) are able to present lipid antigens, including sphingolipids, to a specific group of T cells, the CD1-restricted T cells. Invariant natural killer T (iNKT) cells are the most well characterized CD1-restricted T cells.

In this thesis, it was investigated how the lysosomal alterations occurring in LSDs have an impact in the immune response, namely in CD1 lipid antigen presentation and in infection. CD1 lipid antigen presentation capacity was evaluated in monocytes and in monocyte-derived dendritic cells from LSD patients as well as the iNKT cell frequency. In Fabry, Gaucher, NPC and MPS VI diseases, monocyte-derived dendritic cells do not present defects in CD1b and CD1d lipid antigen presentation capacity and no alterations were found in iNKT cell frequency, contrarily to what was reported in several LSD mouse models. Also, in Fabry and Gaucher diseases, the monocyte CD1d lipid antigen presentation capacity was not decreased. In addition, the impact of different blood collection conditions in the CD1d lipid antigen presentation capacity of monocytes was investigated. Importantly, monocytes isolated 24 hours after blood collection in ethylenediamine tetraacetic acid (EDTA) presented a reduced capacity to activate iNKT cells, comparing to monocytes isolated 24 hours after blood collection in citrate. This finding is rather relevant in the field of CD1d lipid antigen presentation and highlights the importance of the anticoagulant chosen to perform monocyte functional assays.

Macrophages are severely affected in Gaucher disease patients and human Gaucher disease macrophages presented an impaired capacity to fight bacterial infections. Herein, mouse Gaucher disease macrophages capacity of phagocytosing and eliminating *Leishmania* parasites was evaluated. It was used an *in vitro* model by treating mouse bone marrow-derived macrophages with an inhibitor of the enzyme β-glucocerebrosidase (Conduritol B epoxide), mimetizing the Gaucher disease. These Gaucher disease macrophages were able to internalize and kill the parasites, similarly to control cells.

Altogether, these results contribute to increase the knowledge in LSDs and in CD1 lipid antigen presentation.

Publications

The results of this thesis are present in the following articles:

Pereira CS*, **Pérez-Cabezas B***, **Ribeiro H***, Maia ML, Cardoso MT, Dias AF, Azevedo O, Ferreira MF, Garcia P, Rodrigues E, Castro-Chaves P, Martins E, Aguiar P, Pineda M, Amraoui Y, Fecarotta S, Leão-Teles E, Deng S, Savage PB, Macedo MF. "Lipid Antigen Presentation by CD1b and CD1d in Lysosomal Storage Disease Patients", Front Immunol, 10: 1264. (2019)
***equal contribution**

Ribeiro H, Rocha MI, Castro H, Macedo MF. "Chemical inhibition of β-glucocerebrosidase does not affect phagocytosis and early containment of Leishmania by murine macrophages", Exp Parasitol, 216:107939. (2020)

Table of Contents

Abbreviations list

APC, antigen presenting cell

BM-DC, Bone-marrow derived dendritic cell

BMDM, Bone-marrow derived macrophages

BSA, Bovine serum albumin

CBE, Conduritol B epoxide

CD, Cluster of differentiation

CPDA, citrate-phosphate-dextrose-adenine

DGJ, Deoxynojirimycin

DN, Double negative

EDTA, Ethylenediamine tetraacetic acid

ELISA, Enzyme-linked immunosorbent assay

ER, Endoplasmic reticulum

ERT, Enzyme replacement therapy

FDG, Fluorescein di-β-D-galactopyranoside

GalCer, galactosylceramide

GalGalCer, Gal(α1–2)galactosylceramide

Gb3, Globotriaosylceramide

Gb4, Globotetrahexosylceramide

GlcCer, glucosylceramide

GlcSph, glucosylsphingosine

GM-CSF, Granulocyte-macrophage colony stimulating factor

GM2, Monosialoganglioside2

HIV, Human immunodeficiency virus

iFBS, Inactivated fetal bovine serum

IFN-γ, Interferon gamma

IL, Interleukin

iNKT, Invariant natural killer T

LacCer, Lactosylceramide

LAMP, Lysosome-associated membrane protein

LPS, Lipopolysaccharide

LSD, Lysosomal storage disease

MHC, Major Histocompatibility Complex

MPS, Mucopolysaccharidosis

NK, natural killer

NKT, Natural killer T

NPAB, Niemann-Pick type A and B

NPC, Niemann-Pick type C

PBMC, Peripheral blood mononuclear cell

PBS, Phosphate-buffered saline

PE, Phosphatildylethanolamine

Sph, Sphingomyelin

SRT, Substrate reduction therapy

TCR, T cell receptor

TGN, trans-Golgi network

TLR, Toll-like receptor

TNFα, Tumor necrosis factor α

Chapter 1: Introduction

1.1. The lysosome

The lysosomes, originally described in the 1950s by Christian de Duve, a finding that yielded the Nobel Prize, are the major digestive intracellular organelles. The lysosomes are quite important in the recycling of cellular components, having soluble hydrolases and integral lysosomal membrane proteins [1]. More than 60 acidic lysosomal hydrolases are involved in the degradation of macromolecules which products are re-used in the synthesis of new molecules [2]. The acidic pH of the lysosome is important to maintain the activity of hydrolases. The macromolecules are delivered to the lysosome by endocytosis, phagocytosis and autophagy. The lysosome is a membrane-enclosed organelle that can fuse with the plasma membrane, secreting its content [3]. They are distinguished from late endosomes because they lack the expression of the mannose-6-phosphate receptor [3]. Lysosomes ultrastructurally appear as dense bodies in the cytosol, often in a perinuclear pattern which shape varies from spherical to sometimes tubular. Their size differs depending on cell type, but usually is higher than 1 μm [1].

The degradative endocytic pathway starts at the plasma membrane with its internalization along with the receptors and soluble molecules forming early endosomes. In early endosomes, some of the cargo is sorted into the recycling endosomes returning to the cell surface. The early endosome containing the rest of cargo is transformed into a late endosome. Then, the macromolecules degradation ends in the lysosomes. In addition, lysosomes are able to secrete material to the extracellular space by a process called lysosomal exocytosis. The lysosomes relocate from their perinuclear localization to the close vicinity of the plasma membrane, where they fuse with each other and then with the plasma membrane [1].

Lysosomes are important in many cellular processes: macromolecules degradation, cell signaling, protein secretion, plasma membrane repair, cholesterol homeostasis, cell death, autophagy, phagocytosis and antigen presentation (figure 1) [1, 2, 3, 4].

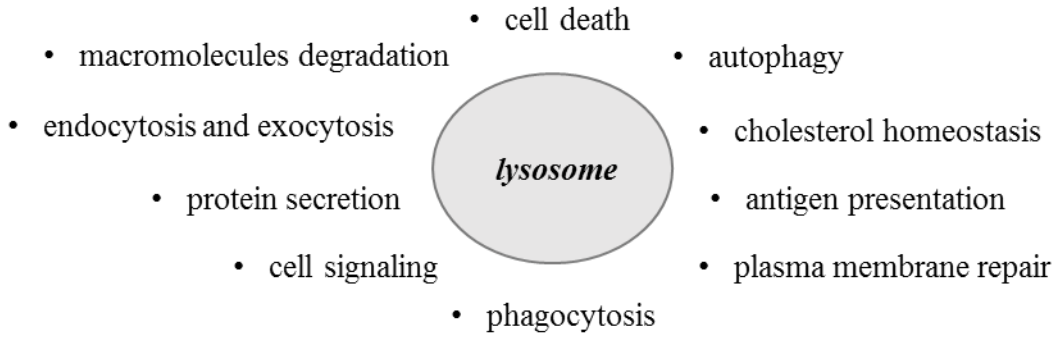

Figure 1. The lysosome is involved in several cellular processes.

In autophagy, cytoplasmic components, damaged proteins and entire organelles are degraded in lysosomes. Autophagy is divided in chaperone-mediated autophagy, microautophagy and macroautophagy. In the chaperone-mediated autophagy, cytosolic proteins with specific recognition motifs are delivered to the lysosomes via the action of a chaperone and the lysosomal receptor, the lysosome-associated membrane protein 2A (LAMP-2A). In microautophagy, the cytoplasmic cargo is directly engulfed at the limiting lysosomal membrane. In macroautophagy, a small portion of the cytoplasm, including soluble materials and organelles, is sequestered within a new membrane, originating an autophagosome, which then fuse with the lysosome.

The autophagy process is impaired when the lysosome is dysfunctional resulting in disease as described in neurodegenerative disorders and lysosomal storage diseases (LSDs) [1].

The lysosomes are attractive therapeutic targets since the manipulation of lysosomal functions might be of great benefit for the treatment of the diseases group previously mentioned.

1.2. The lysosomal storage diseases

LSDs are a group of genetic diseases, with defects in lysosomal proteins, resulting in the accumulation of different types of molecules in the lysosome [5, 6, 7]. On this basis, LSDs are classified in different groups (table 1) [8].

Table 1. LSDs classification based on the type of molecule accumulated in the lysosome.

LSD	**Type of molecule accumulated**
Sphingolipidoses	sphingolipids
Mucopolysaccharidoses	glycosaminoglycans
Mucolipidoses	glycolipids, glycosaminoglycans, oligosaccharides
Glycoproteinoses	glycoproteins

LSDs are individual rare diseases with a collective frequency of ~1:5000 live births [9]. In Portugal, the frequency is 1.25:5000 live births [10]. More than 70 LSDs are described in which enzymatic or nonenzymatic lysosomal proteins activity is deficient, due to genetic mutations [11].

LSDs are multisystemic diseases and some of LSDs patients present neurodegeneration [9], with alterations in neuronal cell morphology (figure 2) [12].

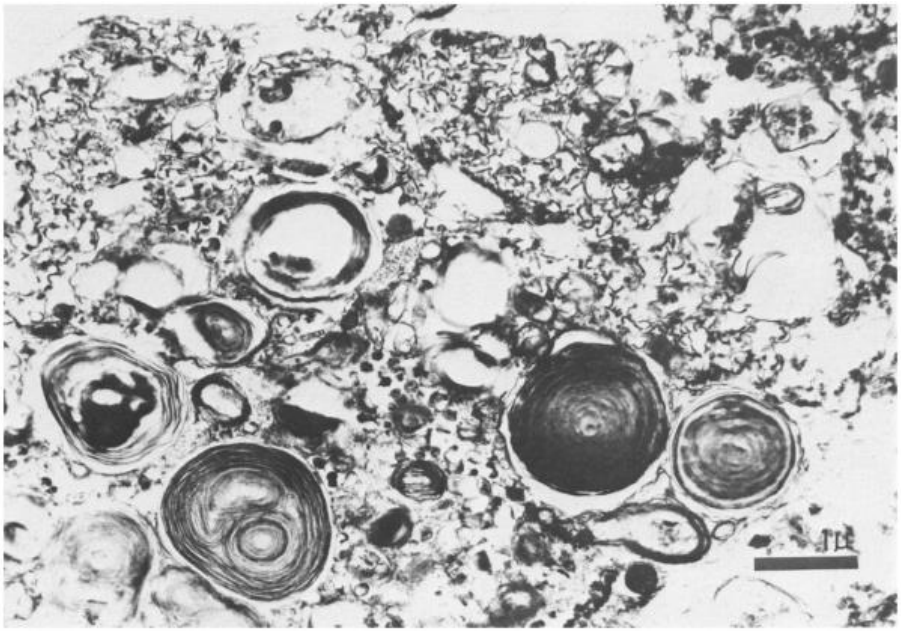

Figure 2. Membranous cytoplasmic bodies showing lamellar structures in the ballooned neuronal cell of the brain cortex of a patient with GM2 gangliosidosis [12].

The studies conducted in this thesis addressed three sphingolipidoses: Gaucher, Fabry and Niemann-Pick type C (NPC) diseases. In addition, one mucopolysaccharidosis was also included, the mucopolysaccharidosis (MPS) VI.

1.2.1. Gaucher disease

The most common LSDs are sphingolipidoses being Gaucher disease one of the most frequent sphingolipidoses [6]. The birth prevalence of Gaucher disease in the Portuguese population is 1.4 per 100 000 live births [10]. It is caused by mutations in the GBA gene, which encodes the lysosomal β-glucocerebrosidase enzyme, leading to the accumulation of glucosylceramide (GlcCer) and glucosylsphingosine (GlcSph) [5, 13]. Gaucher disease can be caused also by the deficiency of saposin C, being rare [14]. Saposin C is an activator of β-glucocerebrosidase enzyme [14].

The main cells affected are macrophages being called "Gaucher cells" [5]. These cells are characteristic of Gaucher disease and in an initial phase of the disease are found in spleen, liver and bone marrow being associated with hepatosplenomegaly, hematological defects and bone disease [5, 13]. With disease progression, "Gaucher cells" accumulate in the various tissues, leading to inflammation and tissue damage. Gaucher disease is classically classified in three types according to the neurological involvement (types I, II and III), in which the visceral involvement is similar. In Gaucher disease type I, the nervous system is not affected, contrarily to types II and III. The severity level of neurodegeneration in Gaucher disease type II is higher compared to Gaucher disease type III [5, 13]. Type II Gaucher disease patients present a limited psychomotor development, bulbar signs including stridor, swallowing difficulty, stiffened opisthotonic posture, spasticity, trismus and epilepsy. The symptoms characteristic of type III Gaucher disease patients are oculomotor apraxia, abnormal horizontal saccadic eye movements and myoclonic or generalized epilepsy [11].

Two different approved therapies are available for Gaucher disease type I: enzymatic replacement therapy (ERT) and substrate reduction therapy (SRT) [5, 13]. In ERT, the treatment with the recombinant enzyme allows the recovery of the function of the defective enzyme. SRT is based on the use of inhibitors of sphingolipid synthesis. Bone marrow transplantation is an alternative treatment not only for Gaucher disease but also for other LSDs [5].

Along with the lysosomal GlcCer and GlcSph accumulation, several cellular alterations have been reported in Gaucher disease as an increase in the lysosomal pH that was

described in mouse macrophages and human lymphoblasts [15]. The calcium homeostasis can be also affected in Gaucher disease; an increase in calcium release from endoplasmic reticulum (ER) was observed in rat neurons [16] and in human brain [17]. Autophagy is one of the many functions of the lysosome and is impaired in macrophages derived from human Gaucher disease monocytes [18].

Also, cells from Gaucher disease patients can present defects in their functions in response to infection. Human Gaucher monocytes presented a reduced superoxide production in the elimination of *Staphylococcus aureu*s infection [19, 20]. The same result was observed in human Gaucher monocyte-derived macrophages [20].

1.2.2. Fabry disease

Fabry disease is an X-linked recessive genetic disease, which Portuguese birth prevalence was estimated in 2004 as 0.12 per 100 000 live births [10]. Fabry disease is caused by mutations in the GLA gene. This gene encodes the lysosomal hydrolase α-galactosidase A. The deficient activity of α-galactosidase A results in the accumulation of globotriaosylceramide (Gb3) and secondarily globotriaosylsphingosine [21]. Fabry disease may be presented at any age and the patients can present mild to severe clinical manifestations, affecting both males and females. In the case of heterozygous females, the disease diagnosis can be difficult because their plasma α-galactosidase A activity can be often within the normal range. So, it is necessary to investigate the existence of mutations in GLA gene. If the mutations found are described as being pathogenic, the diagnosis can be made. In the case of presence of new mutations in this gene, the *in vitro* and *in vivo* validation is mandatory [22].

Fabry disease patients can have two clinical presentations, the classic and the late-onset. In classic Fabry disease, the patients have little or no enzyme activity and have an early onset of symptoms. In late-onset Fabry disease, the patients have residual enzyme activity and the symptoms mostly occur during the fourth to seventh decades of life [22]. Renal failure, cardiac and/or cerebrovascular pathology are common in this disease, being more frequent in males than females [22]. Nowadays, the screening in high-risk populations suffering from various renal, cardiac or neurological manifestations, is very useful in the diagnosis of Fabry disease patients [23, 24].

The ERT is the treatment available to treat Fabry disease [22]. The use of chaperones to enhance the enzyme activity is being tested [5].

1.2.3. Niemann-Pick type C disease

The Portuguese frequency of NPC disease is 2.2 per 100 000 live births [10]. NPC disease is caused by mutations in NPC1 and NPC2 genes, encoding respectively a lysosomal protein membrane; and a soluble protein, located inside late endosomes or lysosomes. Mutations in these genes (more frequent in NPC1 gene [25]) lead to alterations in the intracellular cholesterol transport, resulting in its accumulation in late endosomes and lysosomes [26]. Besides lipid accumulation, the reduction in the lysosomal calcium results in a defective endocytic fusion and trafficking, leading to a secondary lipid accumulation for NPC1 disease [27]. NPC disease patients present visceral symptoms that can involve the spleen and the liver. Neurodegeneration is characteristic of this disease, which can be attenuated by using SRT with miglustat, a compound able to inhibit the activity of glucosylceramide synthase, an enzyme involved in the biosynthesis of most glycosphingolipids [26].

1.2.4. Mucopolysaccharidosis VI

In Portugal the frequency of MPS VI is 0.42 per 100 000 live births[10]. MPS VI is caused by the deficient activity of the enzyme arylsulfatase B, resulting in the accumulation of glycosaminoglycans (dermatan sulphate and chondroitin sulphate) in a variety of cells. The multisystem clinical manifestations are diverse affecting the neurological, cardiac, respiratory and musculoskeletal systems and the disease progression can be very slow or very rapid. ERT is the treatment available to manage this disease [28].

The characteristics of LSDs covered in this thesis are summarized in table 2.

Table 2. LSDs general characteristics.

LSD	*Protein defect*	*Molecules accumulated*	*Treatment*
Gaucher	β-glucocerebrosidase	GlcCer and GlcSph	ERT, SRT
Fabry	α-Galactosidase A	Gb3 and globotriaosylsphingosine	ERT
NPC	NPC1/NPC2	Cholesterol	SRT
MPS-VI	arylsulfatase B	Dermatan sulfate and chondroitin sulphate	ERT

LSDs are rare diseases in which lysosomal proteins present defects in their function, leading to a loss of lysosomal and cell homeostasis. It is important to continue to study LSDs in order to understand the mechanisms behind lysosomal dysfunction expecting to find new biological meanings for the molecules accumulating in the lysosome.

1.3. Lipid antigen presentation

1.3.1. CD1 proteins as lipid antigen-presenting molecules

The expression

The immune system is specialized in the body's protection comprising different cell types, some of which are antigen-presenting cells (APCs). These cells are able to present antigens to T cells by expressing specific antigen-presenting molecules like class I and class II Major Histocompatibility Complex (MHC) molecules that bind peptides; and CD1 proteins capable to bind lipids.

In humans, CD1 molecules are codified by five genes, localized in chromosome 1, originating five different isoforms: CD1a, CD1b, CD1c, CD1d and CD1e [29]. Three groups are defined according to the sequence homology: i) CD1a, CD1b and CD1c ii) CD1d and iii) CD1e. In mice, only exist one CD1 isoform, the CD1d [30]. As CD1e is not present at plasma membrane, so it is not a lipid antigen-presenting molecule [30]. CD1e is expressed on dendritic cells [31]. CD1a, CD1b, CD1c and CD1d molecules are expressed on thymocytes and dendritic cells [31]. CD1d is also expressed by various cell types including monocytes and B cells [31].

The structure

CD1 molecules, related to the class I MHC molecules, have three extracellular domains (α1, α2, α3), a transmembrane region and a cytoplasmatic tail. CD1 molecules are only functional when associated with β2-microglobulin. The lipid antigen binding site is composed by pockets delimited by hydrophobic amino acids, allowing the insertion of the fatty acid tails of the lipid antigens and exposing their polar part (figure 3). All CD1 molecules possess two pockets: A' and F'. CD1b molecule has two additional pockets, C' and T', allowing the binding of lipids with larger hydrophobic chains. The fact that CD1 molecules differ in the number, length and volume of binding pockets explains the variety of lipid antigens that are presented by different CD1 isoforms [30].

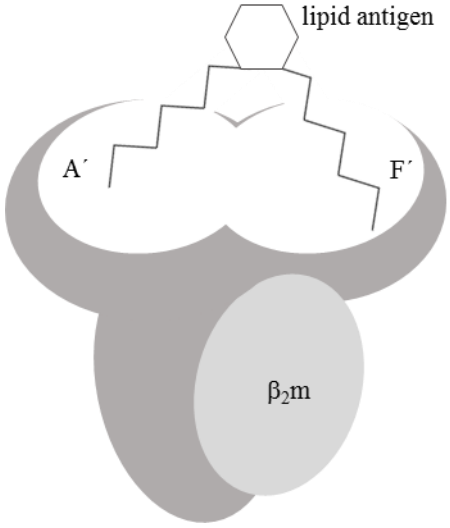

Figure 3. Schematic representation showing the insertion of the fatty acid tails of the lipid antigens, in the binding pockets of CD1 molecules. β2m, β2-microglobulin.

The maturation process

CD1 molecules maturation occurs in the ER, in which association with β2-microglobulin is crucial for the CD1 functionality. After completing maturation in the trans-Golgi network (TGN), CD1 molecules are transported to the plasma membrane. Then they are internalized following the endocytic pathway; during this process lipid antigen loading can occur with the help of lipid transfer proteins. CD1e molecule is an example [30]. Other proteins also presented this function: the saposins, the GM2 activator protein and the NPC2 protein [30, 32].

The CD1 isoforms localize in different endocytic compartments (figure 4) and then traffic to the plasma membrane, where they can activate CD1-restricted T cells [33, 34].

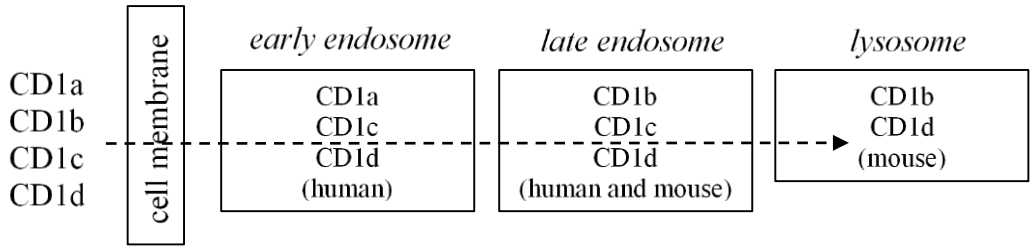

Figure 4. The CD1 localization in the different endocytic compartments, after cellular internalization.

1.3.2. CD1-restricted T cells

The classification

CD1-restricted T cells are divided in two groups: i) CD1a, CD1b and CD1c-restricted T cells and ii) CD1d-restricetd T cells or natural killer T (NKT) cells (figure 5). Two CD1d-restricted T cells groups are described: i) type I NKT cells or invariant NKT (iNKT) cells and ii) type II NKT cells.

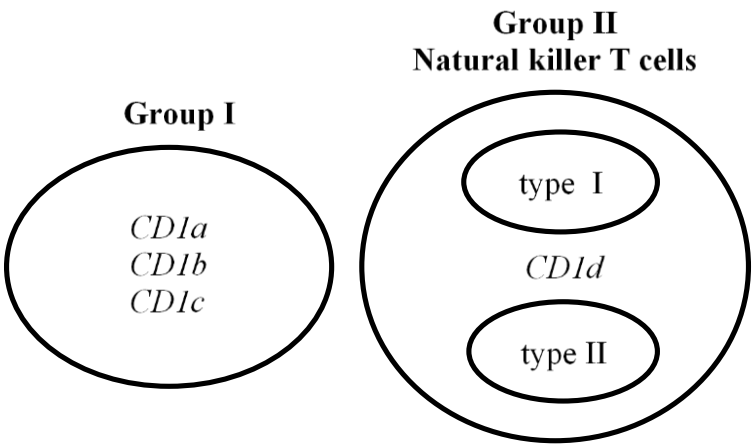

Figure 5. Two groups of CD1-restricted T cells are defined based on CD1 restriction.

The T cell receptor

CD1a, CD1b and CD1c-restricted T cells respond to diverse microbial or self-antigens and express diverse T cell receptors (TCRs) [35] with two exceptions: germline-encoded mycolyl-reactive T cells and LND5-like cells. These cells are CD1b-restricted T cells and present a semi-invariant αβ TCR [36, 37].

iNKT cells and type II NKT cells express a semi-invariant TCR or diverse TCRs, respectively [7]. The TCR of iNKT cells is composed by Vα24Jα18 and Vβ11 chains in humans, or a Vα14Jα18 chain paired with a limited repertoire of Vβ chains in mice [7].

The identification

TCR expression in type II NKT cells is variable so there are not cell surface markers that allow their complete identification. Human iNKT cells can be identified by using: i) anti-Vα24 plus anti-Vβ11 antibodies; ii) the 6B11 antibody, an antibody recognizing a region specific for the invariant CDR3 loop of the human Vα24Jα18 TCR α chain of the TCR [38]; or iii) CD1d tetramers loaded with the lipid antigen α-galactosylceramide (α-GalCer) [7].

The lipid antigens

The lipids are important biomolecules in the immune system which structures are very diverse. They have in their constitution, in most cases, the fatty acids, carboxylic acids with a long aliphatic hydrocarbon chain. There are several classes of lipids: phospholipids, sphingolipids, glycosphingolipids, among others. In the structure of phospholipids, the glycerol (3-carbon alcohol), binds to one phosphate group and to two chains of fatty acids. In sphingolipids, the glycerol is replaced by a sphingosine (18-carbon amino alcohol). In glycosphingolipids, the sphingosine is linked to one or more sugar residues (figure 6) [39].

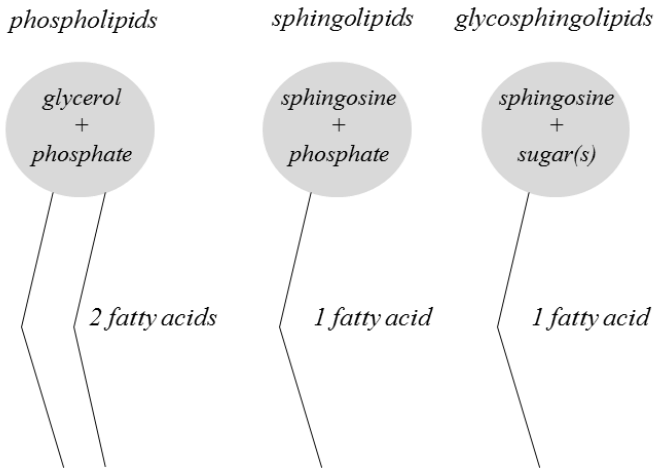

Figure 6. The structure of phospholipids, sphingolipids and glycosphingolipids.

α-GalCer, the prototype iNKT cell antigen, is one of the α-linked glycosphingolipids recognized by iNKT cells with consequent stimulation, contrarily to type II NKT cells [35].

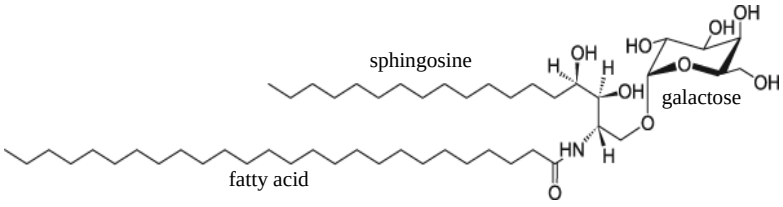

Figure 7. Structure of α-GalCer [40].

α-GalCer structurally has a galactose (sugar) linked to a ceramide, a sphingosine linked to a fatty acid (figure 7) [39]. Type II NKT cells present a preference for β-linked glycosphingolipids or phospholipids [7].

Glycosphingolipids are not the only lipid class presented by CD1 molecules but also others, for example sphingolipids and phospholipids (table 3) [31]. The lipid antigens can have an endogenous or exogenous origin (from bacteria for example).

Table 3. Lipid classes presented by CD1 molecules [31].

Lipid class	**Origin**	**CD1**
Phospholipids	Self, cypress, bacteria	CD1a, CD1b, CD1c, mice and human CD1d
Sphingolipids	Self	Human CD1d
Glycosphingolipids	Self, bacteria	CD1a, CD1b, CD1c, mice and human CD1d

The number of lipid antigens is considerable taking account that the lipids are molecules structurally quite diverse, being a specific lipid presented by a distinct CD1 isoform. Curiously, it can happen that the same lipid antigen can be presented by different CD1 isoforms. CD1a, CD1b, CD1c and CD1d (mouse and human) are all able to bind sulfatide (a glycosphingolipid), an endogenous lipid antigen [30].

The following section is focused in iNKT cells, since they are the best characterized CD1-restricted T cells.

1.4. Invariant natural killer T cells

The frequency

iNKT cells are present at higher amounts in human omentum or in mouse liver [41, 42]. In mice and humans, iNKT cell frequencies are lower in the spleen, peripheral blood, lymph nodes, bone marrow, and thymus [41, 42]. The frequencies of iNKT cells (inside of total T cells) are described in table 4 [42].

Table 4. Comparing mouse and human iNKT cell frequencies (as a percentage of total T cells) [42].

Mouse	***Human***
not described	omentum (~10%)
liver (~30%)	liver (~1%)
thymus, spleen, bone marrow and blood (0.2–0.5%)	spleen, blood, bone marrow and lymph nodes (0.01–0.5%)
lymph nodes (0.1–0.2%)	thymus (<0.001–0.01%)

The development

As conventional T cells, the iNKT cell maturation occur in the thymus, from bone marrow-derived precursors and involves four stages (figure 8). It starts with the interaction between double positive thymocytes expressing the characteristic iNKT TCR and double positive thymocytes expressing CD1d loaded with self-antigens (stage 0). At this stage, immature iNKT cells express high amounts of CD24 molecule. The following stages are characterized by a downregulation of CD24 molecule (stage 1) and the upregulation of CD44 molecule (stage 2). The expression of natural killer (NK) cell marker, NK1.1, appears in the last stage (stage 3) [43].

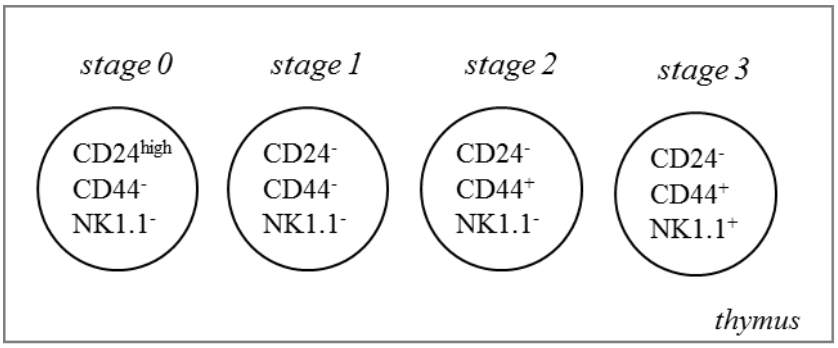

Figure 8. iNKT cell maturation involving four stages (stages 0, 1, 2 and 3).
CD24, CD44 and NK1.1 molecules expression varies between the different stages.

The activation

Leaving the thymus, iNKT cell activation can occur in a TCR-dependent, independent way or a combination of both (figure 9) [44]. The TCR-dependent iNKT cell activation implies the direct interaction between the TCR and the complex CD1d:lipid antigen. A number of bacterial lipid antigens, are able to directly activate iNKT cells. iNKT cells can also be activated by self-antigens produced by APCs as it is described for dendritic cells activated via toll-like receptor (TLR) ligands (TLR2, TLR3, TLR4, TLR5, TLR7/8 and TLR9 ligands) [45, 46, 47].

The TCR-independent iNKT cell activation is induced by cytokines produced by antigen-presenting cells, namely IL-12 cytokine as described for activated dendritic cells[48, 49].

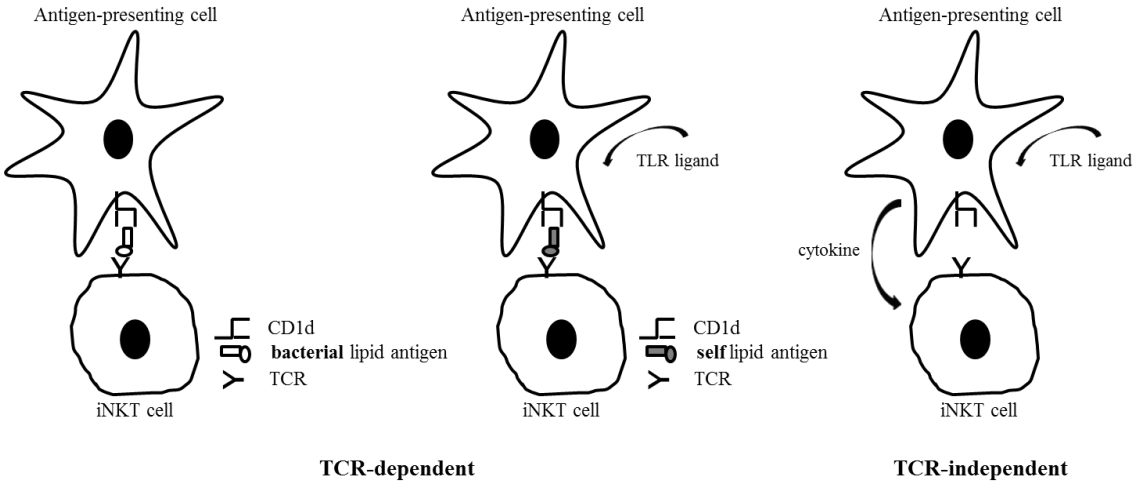

Figure 9. iNKT cell activation (direct and indirect modes).

The subsets

After activation, iNKT cells are able to produce cytokines with different effects in the immune system. According to the cytokine production, in mice four iNKT cell subsets are defined as iNKT 1, iNKT2, iNKT 10 and iNKT 17 [50].

IFN-γ and IL-4 cytokine producing iNKT cells represents the iNKT1 and iNKT2 subsets. iNKT10 and iNKT17 subsets produce IL-10 and IL-17 cytokines, respectively. In addition, the expression of transcription factors and cell surface markers allow their identification. These iNKT cell subsets have a specific tissue distribution in mice (table 5) [50].

Table 5. iNKT cell subsets are differently distributed in mouse tissues [50].

Tissue	***iNKT subsets***			
	iNKT1	*iNKT2*	*iNKT10*	*iNKT17*
Thymus	+++	+	-	+
Liver	+++++	-	-	-
Spleen	+++	+	-	+
Lymph nodes	+	+	-	+++
Mesenteric lymph nodes	++	++	-	+
Lung	+++	+	-	+
Small intestine	+++	+	-	+
Adipose tissue	+	-	++++	-

The symbol "+" corresponds to the relative frequency of each subset.
The symbol "-" corresponds to the absence of each subset.

In humans, three iNKT cell subsets are described in terms of CD4 and CD8 expression: positive only for CD4, only for CD8, or negative for both molecules. The iNKT cell subset functionalities are not so clearly defined. However, some different functionalities have been associated with iNKT CD4+ and CD4- cells. iNKT CD4+ and $CD4^-$ cells are able to produce both Th1 and Th2 cytokines in different amounts. After stimulation, iNKT CD4+ cells produce both Th1 and Th2 cytokines while iNKT CD4− cells produce higher quantities of Th1 cytokines [51, 52]. Other study show that stimulated iNKT CD4+ cells produce more Th2 cytokines and iNKT CD4− cells produce more Th1 cytokines [53].

1.5. Invariant natural killer T cells as important players in disease

In disease, the outcome of the immune response depends of the function of different immune cells. iNKT cells are important immunoregulators in infection, cancer, autoimmunity and obesity. Alterations in number or function of iNKT cells affect their efficacy in the immune regulation favoring or not the development of disease.

iNKT cells as they are activated by CD1d-bound ligands from bacterial origin, help in the clearance of bacterial infections, as shown in mice infected with *Sphingomonas paucimobilis* [54]. Indeed, the importance of iNKT cells in fighting infection was shown when mice lacking iNKT cells were more prone to infection caused by several bacteria than wild-type mice [35]. In humans, the role of iNKT cells is not so clear but the lower numbers of iNKT cells in patients infected with tuberculosis and with human immunodeficiency virus (HIV) infection suggests a protective role for iNKT cells against infection [35].

The anti-tumor properties of iNKT cells have been studied in both mice models and humans. The mice deficient in iNKT cells are more predisposal to cancer and cancer protection can be achieved by iNKT cell adoptive transfer or α-GalCer stimulation [41, 55]. In patients with solid and hematological cancers the circulating iNKT cell frequency is reduced [41]. α-GalCer, the prototype ligand of iNKT cells, has been used in over 30 anti-tumor clinical trials with mostly suboptimal outcomes [56]. In the meantime, alternative delivery strategies of α-GalCer have been developed and are being tested: soluble CD1d-α-GalCer complexes conjugated with tumor-specific antibodies, α-GalCer conjugated with tumor-associated peptides or α-GalCer pulsed APCs [57, 58]. Nowadays, efforts are being made in finding new iNKT cell ligands and better α-GalCer delivery strategies together with the improvement of preclinical models [56].

iNKT cells also have a role in autoimmunity. In most studies with mouse models, iNKT cells have a protective role against autoimmunity [35]. In the context of rheumatoid arthritis, the absence of iNKT cells leads to an aggravation of the symptoms of mice with spondyloarthritis, suggesting a protective role of iNKT cells [59]. However, in other rheumatoid arthritis mouse model, in collagen-induced arthritis mice, iNKT cells are activated and treatment with anti-CD1d antibodies ameliorates disease, showing the pathogenic role of iNKT cells [35]. Reinforcing the importance of iNKT cells in

autoimmunity, in many experimental models of autoimmunity (diabetes, multiple sclerosis, arthritis and lupus), the diseased mice when treated with α-GalCer (the prototypic iNKT cell antigen) acquired protection [60]. The protective role of iNKT cells in humans is suggested by reduced iNKT cell frequency in patients with rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis [35, 61].

In obesity, it seems that iNKT cells also confers protection in mice and humans. Mice lacking iNKT cells and with a high-fat diet, are more obese than wild-type mice [62]. In addition, the increase of iNKT cell frequency in these mice, leads to an improvement in the disease [62]. In humans, the iNKT cell numbers in blood and adipose tissue are lower in obese compared with lean people [62, 63]. Interesting after bariatric surgery in obese patients, blood iNKT cells numbers increased [62].

An inappropriate iNKT cell activation as a response to self or non-self-lipid antigens also contribute to diseases, such as asthma and atherosclerosis. Specifically in mouse models of asthma, iNKT cells are involved in airway hyperreactivity, a disease characteristic [64]. The importance of iNKT cells is supported by their higher frequency in lungs reported in some studies with human asthma patients [64]. In cardiovascular diseases like atherosclerosis, it seems that iNKT cells have a pathogenic role since they are present in atherosclerotic lesions, both in animal models and in patients [65].

In the context of LSDs, iNKT cells have been the most studied CD1d-restricted T cells. The lysosome is critical in the process of lipid antigen presentation. The lysosomal alterations present in LSDs can influence the lipid antigen presentation capacity of APCs.

The frequency of iNKT cells was studied in several LSD mouse models being lower in Fabry [66, 67, 68, 69, 70], NPC1 [67, 71], NPC2 [32, 72], Niemann-Pick type A and B (NPAB) [73], Sandhoff [67, 74], Tay-Sachs [67], GM1 gangliosidosis [67, 72] and Gaucher disease [75] mouse models (table 6).

Table 6. iNKT cells in lysosomal storage diseases.

Disease	**iNKT cell frequency**	
	mice	*patients*
Fabry*	↓[66, 67, 68, 69, 70]	=[76]
NPC1*	↓[67, 71]	=[77]
NPC2*	↓[32, 72]	ND
NPAB*	↓[73]	↓[73]
Sandhoff*	↓[67, 74]	ND
Tay-Sachs	↓[67]	ND
GM1 gangliosidosis*	↓[67, 72]	ND
Metachromatic leukodystrophy	=[78]	ND
MPS I	=[78]	ND
Krabbe	=[78]	ND
Gaucher	↓[75]	=[75]

=, normal; ↓, reduction; ND, not done; MPS I, Mucopolysaccharidosis type I; NPC, Niemann-Pick type C; NPAB, Niemann-Pick type A and B; *, defective lipid antigen presentation.

In humans, no alterations in iNKT frequency were observed in Fabry [76], NPC1 [76] and Gaucher [75] diseases. For NPAB disease a reduction in iNKT cell frequency was described [73] (table 6).

In addition, defects in CD1d lipid antigen presentation were also described in the following LSDs mouse models: Sandhoff [67, 74], NPC [32, 67, 71, 72], NPAB [73], GM1 gangliosidosis [67, 72] and Fabry disease [67, 70]; and in human NPAB disease [73].

The phenotype of iNKT cells was analysed in Fabry and NPC diseases. iNKT CD4+ cells percentage was decreased in Fabry disease patients, while iNKT cell subsets did not present alterations in NPC disease patients[76, 77]. In mouse models of Fabry and NPC diseases, iNKT CD4+ cells were reduced [69, 72].

During the course of these studies, some possible mechanisms arise to explain the iNKT cell defects in mouse LSDs. The reduced numbers of iNKT cells in Fabry disease mice can be explained by a defect in the lipid antigen processing by α-galactosidase A, since its

capacity to remove the galactose from a synthetic glycolipid to originate antigenic lipid antigens, is defective [66]. As proposed by Gadola and co-workers, the lipids accumulating in the lysosome can compete with endogenous lipid antigens for CD1d binding [67]. During the course of this thesis, it was demonstrated that GM2 (accumulating in GM2 gangliosidosis) is able to inhibit iNKT cell activation by competing with antigenic lipids as was previously shown for Gb3 (accumulating in Fabry disease) and recently for sphingomyelin (accumulating in NPAB disease) [74, 79, 80].

Defects in lipid antigen trafficking affecting their loading onto CD1d molecules, arise as an explanation for the lower iNKT cell frequencies, in NPC1 disease mice [71]. In the case of NPC2 disease, the NPC2 protein is a lipid transfer protein that assist in the CD1d loading of lipid antigens [32]. The defective activity of this protein in NPC2 disease, can explain the reduction of iNKT cell frequency reported in mice [72]. Concluding, iNKT cell defects are explained by mechanisms related to the processing, competition, trafficking and CD1d loading of lipid antigens.

Chapter 2: Aims

This thesis aims to evaluate the impact of lysosomal alterations occurring in lysosomal storage diseases (LSDs), in the function of immune cells, such as monocytes, dendritic cells, macrophages and invariant natural killer T (iNKT) cells.
The specific aims were to investigate:

- CD1b and CD1d lipid antigen presentation capacity of antigen-presenting cells (APCs) from Fabry, Gaucher, Niemann-Pick type C (NPC) and Mucopolysaccharidosis (MPS) VI disease patients;

- the CD1d lipid antigen presentation capacity of APCs isolated from blood collected and stored in different conditions;

- the capacity of mouse Gaucher bone marrow-derived macrophages (BMDM) to phagocytose and eliminate *Leishmania* parasites.

Chapter 3: Lipid Antigen Presentation by CD1b and CD1d in Lysosomal Storage Disease Patients

The results presented in figures 1, 4, 7 and 8 were obtained and analysed in the context of this thesis. The results presented in figures 2, 3, 5, 6, 9 and 10 were analyzed in the context of this thesis, from data obtained in the context of this thesis, comparing with previous data of the group. The results are published in:

Pereira CS*, **Pérez-Cabezas B***, **Ribeiro H***, Maia ML, Cardoso MT, Dias AF, Azevedo O, Ferreira MF, Garcia P, Rodrigues E, Castro-Chaves P, Martins E, Aguiar P, Pineda M, Amraoui Y, Fecarotta S, Leão-Teles E, Deng S, Savage PB, Macedo MF. "Lipid Antigen Presentation by CD1b and CD1d in Lysosomal Storage Disease Patients", Front Immunol, 10: 1264. (2019)

***equal contribution**

Abstract

The lysosome has a key role in the presentation of lipid antigens by CD1 molecules. While defects in lipid antigen presentation and in invariant Natural Killer T (iNKT) cell response were detected in several mouse models of lysosomal storage diseases (LSD), the impact of lysosomal engorgement in human lipid antigen presentation is poorly characterized. Here, we analyzed the capacity of monocyte-derived dendritic cells (Mo-DCs) from Fabry, Gaucher, Niemann Pick type C and Mucopolysaccharidosis type VI disease patients to present exogenous antigens to lipid-specific T cells. The CD1b- and CD1d-restricted presentation of lipid antigens by Mo-DCs revealed an ability of LSD patients to induce CD1-restricted T cell responses within the control range. Similarly, freshly isolated monocytes from Fabry and Gaucher disease patients had a normal ability to present α-Galactosylceramide (α-GalCer) antigen by CD1d. Gaucher disease patients' monocytes had an increased capacity to present α-Gal-(1-2)-αGalCer, an antigen that needs internalization and processing to become antigenic. In summary, our results show that Fabry, Gaucher, Niemann Pick type C, and Mucopolysaccharidosis type VI disease patients do not present a decreased capacity to present CD1d-restricted lipid antigens. These observations are in contrast to what was observed in mouse models of LSD. The percentage of total iNKT cells in the peripheral blood of these patients is also similar to control individuals. In addition, we show that the presentation of exogenous lipids that directly bind CD1b, the human CD1 isoform with an intracellular trafficking to the lysosome, is normal in these patients.

Introduction

T lymphocytes can recognize lipid antigens presented by almost nonpolymorphic CD1 antigen-presenting molecules. In humans, CD1a, CD1b, CD1c, and CD1d are capable of presenting lipid antigens to T cells, while in mice only CD1d isoform is expressed. After synthesis, CD1 molecules traffic to the cell surface and are then internalized through the endocytic pathway. Along this recycling route, each CD1 isoform preferentially localizes in a different intracellular compartment, allowing them to encounter distinct lipid antigens [35]. CD1a molecules mainly localize in early endosomes and possess the smallest groove, binding small lipids. CD1b traffics to lysosomes and is the isoform capable of binding larger lipids. CD1c and human CD1d have intermediate binding grooves and follow similar trafficking pathways, being localized in late endosomes. The trafficking pathway of mouse CD1d is similar to that used by human CD1b [81].

CD1-restricted T cells play important functions in the context of infection, immune response against tumors and autoimmunity [57]. These cells can be classified by their recognition of antigens presented by group 1 CD1 molecules (CD1a, CD1b, and CD1c) or by CD1d. Broadly, group 1 CD1-restricted T cells are polyclonal and expand after antigen recognition in the periphery. CD1a-restricted T cells are among the most common self-reactive T cells in peripheral blood [82, 83], being abundant in skin where they are activated by Langerhans cells [82]. CD1b-restricted T cells might recognize either microbial or self-lipids [36, 84, 85, 86, 87] although circulating self-reactive CD1b-restricted T cells have lower frequency [82, 83]. Less consensual is the frequency of CD1c-restricted T cells [82, 83], which can be explained by a direct contact of the TCR with CD1c instead of the loaded lipids [88]. Finally, CD1d-restricted T cells or Natural Killer T (NKT) cells, as they are also known because of their expression of NK and T cells surface markers, are divided into two subsets. Type I NKT or invariant NKT (iNKT) cells are characterized by the expression of a semi-invariant TCR (Vα24Jα18Vβ11 in humans and Vα14Jα18 paired with a limited repertoire of Vβ chains in mice) that recognizes the prototypic antigen α-galactosylceramide (α-GalCer). On the other hand, type II NKT cells express variable TCRs. While iNKT cells respond rapidly to both innate signals and TCR engagement producing large amounts of cytokines, some type II NKT cells display adaptive-like immune functions [83, 89]. Besides being the preferential intracellular compartment for CD1b

and mouse CD1d localization, the lysosome contains hydrolytic enzymes and lipid-transfer proteins that process lipid antigens and assist the loading of lipids onto CD1 molecules, respectively [32, 90, 91, 92, 93, 94, 95, 96]. In addition, the low pH in this compartment induces relaxation of the CD1d structure, facilitating the loading of lipids [97]. Indeed, the importance of the lysosome in lipid antigen presentation by mouse CD1d is reinforced by the defects described in mouse models of lysosomal storage diseases (LSDs) [7]. LSDs are a group of individually rare inherited metabolic diseases characterized by the accumulation of specific macromolecules in the lysosome, including lipids, usually as a result of a deficiency in a lysosomal enzyme. There is no effective treatment for most LSDs. However, for some specific LSDs, enzyme replacement and/or substrate reduction therapies have been developed [4, 98]. Lipid antigen presentation by CD1d is impaired in mouse models of several LSD including Sandhoff [67, 74], Niemann-Pick C (NPC) [32, 67, 71, 72], GM1 gangliosidosis [67, 72], and Fabry disease [67, 70]. The alteration in lipid antigen presentation is accompanied by a defect in the percentage of both thymic and peripheral iNKT cells [32, 67, 69, 70, 71, 72]. However, in NPC disease [77], Fabry disease [76], and Gaucher disease [75] patients' blood, no differences were observed in the frequency of total iNKT cells. Nevertheless, Fabry patients showed a reduction in the CD4+ and an increase in the double negative (DN) iNKT populations [76]. The abnormal accumulation of material in the lysosome/late endosomes of LSD patients is a key feature in LSD. However, there are several other cellular alterations described in the context of these diseases. In fact, impairment in endolysosomal trafficking [5, 9] has been described in several LSDs, namely NPC disease. In this regard, such impairment results in a greater accumulation of lipids in the late endosome [27]. Moreover, defects in autophagy and lipid trafficking have also been reported [9, 18, 99] and, in Gaucher disease, an increase in lysosomal pH has been described [15], most likely impairing the relaxation of CD1 molecules' structure, thus hindering the loading of lipids in these molecules. Altogether, these cellular alterations may affect lipid antigen presentation by human CD1 molecules, particularly CD1b, which recycles through the lysosome [100]. However, studies of lipid antigen presentation in LSD patients are scarce; to date, the analysis of lipid antigen presentation in LSD patients is restricted to NPC disease where only CD1d-mediated presentation was studied using EBV transformed B cell lines as antigen presenting cells (APCs) [77]. Herein, lipid antigen presentation by human CD1b and CD1d was studied in the context of four of the most common LSDs:

Fabry, Gaucher, NPC, and MPS-VI diseases. These diseases represent LSD with distinct symptomatology and are characterized by the accumulation of different types of molecules in the lysosomes (Table 1). Gaucher and Fabry diseases are sphingolipidoses caused, respectively, by pathogenic mutations in GBA [encoding β-Glucosidase (GCase)] and GLA [encoding α-galactosidase A (α-Gal A)] genes (Table 1) and present as multisystemic disorders. Gaucher disease involves the visceral organs, bone marrow, and bone in almost all affected patients, whereas in Fabry disease involvement of the heart, kidney, and brain are the main sources of morbidity and premature death [13, 101]. NPC disease is caused by defective transport of cholesterol (due to NPC1 and NPC2 genes' defects) with sequestration of unesterified cholesterol in lysosomes and late endosomes, and is clinically heterogeneous, mainly affecting the visceral organs (liver, spleen, and lungs) and the central nervous system [102]. MPS VI is caused by arylsufatase B (ASB) enzyme deficiency and leads to the accumulation of dermatan sulfate and chondroitin sulfate (Table 1), which manifests as a multisystemic disorder affecting mainly the skeleton [103]. The contribution of Type II NKT cells in the development of chronic B-cell activation and gammopathy in Gaucher disease has been proposed [75]. For the other LSD, the contribution of CD1-restricted T cells to the clinical manifestation is not well defined. However, we could envision that they may be involved in LSD-associated chronic inflammation [9].

Table 1. Characteristics of lysosomal storage diseases studied.

Disease	**Protein defect**	**Material stored**
Fabry	α-Galactosidase A (α-Gal A)	Globotriaosylceramide (Gb3)
Gaucher	β-Glucosidase (GCase)	Glucosylceramide (GlcCer), Glucosylsphingosine (GlcSph)
Niemann-Pick disease (NPC)	Niemann-Pick C1 or C2 (NPC1/NPC2)	Unesterified cholesterol, Sphingolipids
Mucopolysaccharisodis type VI (MPS-VI)	Arylsulfatase B (ASB)	Dermatan sulphate, Chondroitin sulphate

In contrast to the data obtained in mice, no defects in lipid antigen presentation by CD1b or CD1d were observed in patients of this study, results that are also supported by the determination of normal frequencies of peripheral blood iNKT cells.

Materials and Methods

Biological Samples

Antigen presentation assays included 8 Fabry (only males), 16 Gaucher, 8 NPC (all with mutations in the NPC1 gene), 7 MPS-VI disease patients, and 52 healthy blood donors (all adults). Four Fabry and two Gaucher patients were not undergoing enzyme replacement therapy. The primary etiology and nature of the stored material for the four LSDs studied are depicted in Table 1. Table 2 indicates patient details and the correspondent codes used for their identification in the antigen presentation assays.

Table 2. Patients analyzed in antigen presentation assays.

Disease	Code	Age range*	ERT/SRT
Fabry	1F	51-55	Yes
	2F	36-40	Yes
	3F	61-65	Yes
	4F	51-55	Yes
	5F	46-50	No
	6F	36-40	No
	7F	16-20	No
	8F	21-25	No
Gaucher	1G	1-5	Yes
	2G	11-15	Yes
	3G	26-30	Yes
	4G	11-15	Yes
	5G	51-55	No
	6G	46-50	Yes
	7G	76-80	Yes
	8G	66-70	Yes
	9G	26-30	No
	10G	36-40	Yes
	11G	71-75	Yes
	12G	71-75	Yes

	13G	60-65	Yes
	14G	6-10	Yes
	15G	21-25	Yes
	16G	21-25	Yes
NPC	1N	1-5	Yes
	2N	16-20	Yes
	3N	16-20	Yes
	4N	6-10	Yes
	5N	21-25	Yes
	6N	26-30	Yes
	7N	31-35	Yes
	8N	11-15	Yes
MPS-VI	1M	16-20	Yes
	2M	16-20	Yes
	3M	26-30	Yes
	4M	11-15	Yes
	5M	16-20	Yes
	6M	16-20	Yes
	7M	5-10	Yes

*Patient age was within this interval at the moment of the study.

Studies of iNKT cell frequencies included 15 Fabry (8 males, 7 females, all adults), 19 Gaucher (8 males−1 pediatric, 11 females−3 pediatrics), 9 NPC (all with mutations in the NPC1 gene, 6 males−2 pediatrics, 3 females−1 pediatric), 13 MPS-VI disease patients (6 males−2 pediatrics, 7 females−3 pediatrics), and 92 healthy blood donors (77 adults−37 males and 40 females, 15 children−12 males and 3 females). Patients and controls ≥16 years old were considered as adults. Written informed consent was obtained from all patients enrolled in the study in accordance with the Helsinki declaration. The study was approved by local ethical committees (from the hospitals where patients and controls were recruited) and by the national commission of data protection. Patients were recruited from São João Hospital, Porto—Portugal; Santo António Hospital, Porto—Portugal; Santa Maria Hospital, Lisbon—Portugal; Senhora da Oliveira Hospital, Guimarães—Portugal; Pediatric Coimbra Hospital, Coimbra—Portugal; Sant Joan de Déu Hospital, Barcelona—Spain; Federico II University Hospital, Naples—Italy; and University Children's Hospital,

Mainz—Germany. All but one of the NPC patients were under substrate reduction therapy (SRT). Most of the Fabry, Gaucher and MPS-VI disease patients analyzed were under enzyme replacement therapy (ERT). In Table 2; Figure 12, and Figure 13 it is indicated whether the patients are under treatment or not. Fabry and Gaucher patients received ERT by infusion every 2 weeks, while MPS VI patients were treated intravenously with ERT weekly. NPC patients received oral daily substrate reduction therapy. Blood samples from Fabry, Gaucher, and MPS-VI disease patients were always collected before treatment infusion to minimize the putative effect of treatments in the analyzed cells. The adult control subject population was composed of healthy blood donors from the Instituto Português do Sangue, Porto—Portugal, or from the Immuno-hemotherapy department of São João Hospital. Control pediatric subjects were recruited among children undergoing orthopedic surgery at São João Hospital, without infections, underlying chronic illness, or taking medication.

Peripheral Blood Mononuclear Cells Isolation (PBMCs), Monocytes Purification, and Mo-DCs Generation

PBMCs were separated by Histopaque-1077® (Sigma-Aldrich, St. Louis, MO, USA) density centrifugation following the manufacturer's instructions. Monocytes were isolated by positive selection with anti-CD14 magnetic beads using the MACS cell separation system (Miltenyi Biotec, Cologne, Germany).

CD14+ cells were used after purification (monocytes) or to promote differentiation in dendritic cells by plating them at 10^6 cells/mL in RPMI 10% iFBS supplemented with 50 ng/mL of IL-4 and GM-CSF (ImmunoTools, Friesoythe, Germany). After 7 days, monocyte-derived dendritic cells (Mo-DCs) were collected and used for lipid antigen presentation assays.

Flow Cytometry

Monocyte purity, proper Mo-DC differentiation and the basal state of activation of both cells were assessed by flow cytometry using the following anti-human monoclonal antibodies: CD14 (M5E2, Biolegend, San Diego, CA, USA), CD1b (SN13, Biolegend), CD1d (51.1, Biolegend), CD11c (3.9, eBioscience), CD80 (2D10, Biolegend), HLA-DR (LN3, eBioscience).

iNKT and T cell determinations were performed in total PBMCs or in CD14− fractions, using CD1d-PBS57 tetramers (NIH Tetramer Core Facility, Emory University, Atlanta, GA, USA) and the following anti-human monoclonal antibodies: CD3 (OKT3, eBioscience), CD4 (OKT4, Biolegend), and CD8 (RPA-T8, eBioscience). The purity of T cell clones VM-D5 and JS63 was assessed by using CD1d-PBS57 tetramers (NIH Tetramer Core Facility, Emory University, Atlanta, GA, USA) together with anti-human CD3 (OKT3, eBioscience) monoclonal antibody. The purity of T cell clones s33d, GG33A, and DS1C9b was assessed by using anti-human TCR Vβ13.1 (IMMV222), Vβ18 (BA62.6), and Vβ7.1 (ZOE), monoclonal antibodies from Immunotec (Immunotec Research Inc, Canada). Cells were acquired in a FACS Canto II (BD Biosciences, San Diego, CA, USA) using the BD FACSDiva™ software (BD Biosciences). Data analysis was performed with FlowJo® v10 (FlowJo LLC, Ashland, OR, USA).

T Cell Clone/iNKT Cell Line Culture and Re-stimulation

The maintenance of T cell clones and of the iNKT cell line was performed as previously described [104]. The following T cell clones were used in the assays: DS1C9b (CD1b-restricted, sulfatide-specific) [105]; GG33A (CD1b-restricted, GM1-specific) [104]; JS63 (CD1d-restricted, α-GalCer-specific) [106]; VM-D5 (CD1d-restricted, α-GalCer-specific) [106] and s33d (CD1d-restricted, sulfatide-specific) [106]. An iNKT cell line [79] was also used.

Lipid Antigen Presentation Assays

Monocytes, Mo-DCs or CD1-transfected C1R cells were cultured with sulfatide (30–0.04 μg/mL, Sigma-Aldrich), GM1 (50–0.07 μg/mL, Sigma-Aldrich), α-Galactosylceramide (α-

GalCer) (at 50 ng/mL or 50–3.12 ng/mL, Avanti polar lipids, Alabaster, AL, USA) or α-Gal-(1-2)-αGalCer (300–50 ng/mL). Lipids, with the exception of α-GalCer, were first dissolved in methanol or PBS 0.5% Tween 20 and then diluted in non-supplemented RPMI to have a maximum of 1% vehicle in culture. α-GalCer was resuspended in PBS and directly diluted in non-supplemented RPMI. After 4 hrs, an iNKT cell line or T cell clones were added and 40 hrs later, supernatants were collected for cytokine production determination by ELISA. The following antibody pairs from Biolegend were used: purified anti-human GM-CSF (BVD2-23B6) and biotinylated anti-human GM-CSF (BVD2-21C11); purified anti-human IL-4 (8D4-8) and biotinylated anti-human IL-4 (MP4-25D2).

Statistics

Data distribution of the frequencies of iNKT cells (total, CD4+, CD8+, DN) and T cells (total, CD4+, CD8+) from adult and child healthy controls was assessed using the D'Agostino & Pearson test. Then, an unpaired t-test (normal distribution) or a Mann-Whitney test (non-normal distribution) was used to compare adult and child healthy control populations. As no significant differences were observed (results not shown), data from adults and children were pooled.

To compare the frequencies of iNKT cells (total, CD4+, CD8+, DN) and T cells (total, CD4+, CD8+) from LSD patients and healthy controls, data normality was first checked using the D'Agostino & Pearson test. When all the populations studied passed the normality test, an ordinary one-way ANOVA was used to compare each group of patients with controls. When the populations analyzed did not pass the normality test, the Kruskal-Wallis test was used. P-values lower than 0.05 were considered significant.

Statistical analysis of lipid antigen presentation assays was performed by comparing the amount of cytokine produced in the coculture of APC with T cells between LSD patients and control subjects. A single concentration of antigen was used for this purpose. Data normality was first checked using the Shapiro–Wilk test. Then, an unpaired t-test (normal distribution) or a Mann-Whitney test (non-normal distribution) was used to compare the two groups. P-values lower than 0.05 were considered significant. Along with the comparison of the raw data, and due to the inter-experimental variation, comparison was also done after normalization of the values of cytokine production for each independent

experiment. The values of cytokine production were relativized considering 100 as the highest cytokine production value within each experiment for the chosen antigen concentration. All the analyses were performed using GraphPad Prism software v7.04 (GraphPad Software Inc., CA, USA).

Results

Mo-DCs From Fabry, Gaucher, NPC and MPS-VI Disease Patients Do Present Antigens by CD1b

In vitro differentiation of monocytes to dendritic cells (Mo-DCs) is accompanied by an increase in the expression of CD1b [107]. To study antigen presentation by CD1b, the capacity of Mo-DCs from Fabry, Gaucher, NPC (with mutations in NPC1) and MPS-VI disease patients to present lipids by CD1b was analyzed and compared with that of healthy controls. The expression of CD80 and CD1b on the surface of Mo-DCs from patients and controls was assessed after the differentiation process and prior to the experiments. Mo-DCs from LSD patients had similar levels of CD1b and CD80 cell surface expression to those of control subjects (Table 3).

Table 3. CD1b, CD1d and CD80 expression* on Mo-DCs from LSD patients.

Mo-DCs	Fabry	Gaucher	NPC	MPS-VI
CD1b	1.60 ± 0.64	1.74 ± 0.65	1.63 ± 1.04	0.86 ± 0.36
CD1d	1.91 ± 2.09	0.19 ± 0.17	1.85 ± 1.37	1.01 ± 0.52
CD80	0.47 ± 0.40	0.64 ± 0.27	1.07 ± 0.57	1.48 ± 0.94
N	9	4	9	9

*Mean Fluorescence intensity (MFI) value of each molecule for each patient was relativized using the MFI of control individuals analyzed the same day. Numbers indicate the mean ± SD of N individuals studied.

Two previously described CD1b restricted T cell clones were used: the GG33A T cell clone, specific for GM1 [104], and the DS1C9b T cell clone, sulfatide specific [105]. The GG33A T cell clone carries the Vβ18 TCR chain while the DS1C9b T cell clone expresses the Vβ7.1 TCR chain (personal communication from Lucia Mori and Gennaro de Libero who produced the T cell clones). The identity of the T cell clones was confirmed by flow cytometry (Figure 1).

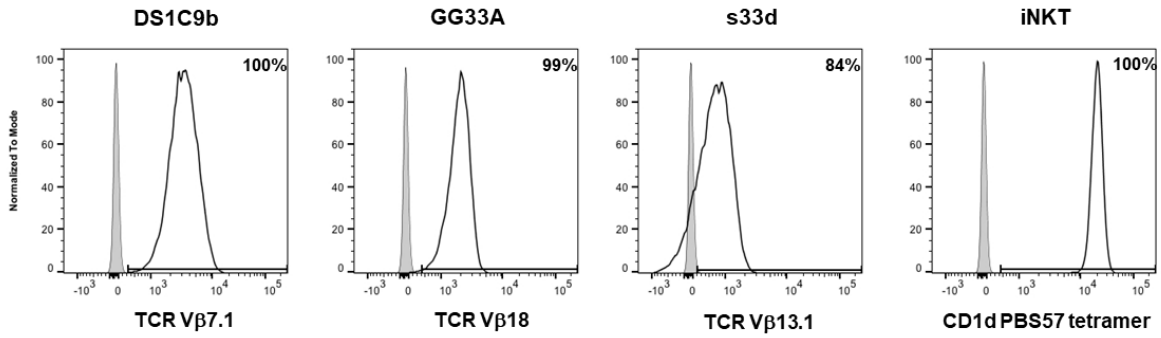

Figure 1. Determination of T cell purity. DS1C9b, GG33A and s33d T cells purity was analyzed by using the anti-human TCR Vβ7.1, Vβ18 and Vβ13.1 monoclonal antibodies, respectively. iNKT cells were analyzed for CD1d PBS57 tetramer reactivity. The unstained control is represented in grey.

Mo-DCs from Fabry (Figure 2A), Gaucher (Figure 2B), NPC (Figure 2C), and MPS-VI (Figure 2D) disease patients were capable of presenting the exogenously added antigen GM1 to the CD1b-restricted T cell clone GG33A. Curiously, Mo-DCs from NPC disease patients seem to have a higher presentation capacity of the antigen GM1 when comparing with control subjects. This difference was statistically significant (P = 0.0041) for the normalized values of cytokine production in the presence of 5 μg/mL of GM1 (Figure 3A). Regardless of the higher presentation capacity observed in NPC patients to present GM1 through CD1b, the same was not observed using the sulfatide-specific CD1b-restricted DS1C9b T cell clone (Figure 2F and Figure 3B). Sulfatide presentation through CD1b was also analyzed in Fabry patients' Mo-DCs, and a small decrease in the capacity to activate the DS1C9b clone was observed (Figure 2E) when considering one of the tested concentrations (1 μg/mL of sulfatide, P = 0.0456) (Figure 3B).

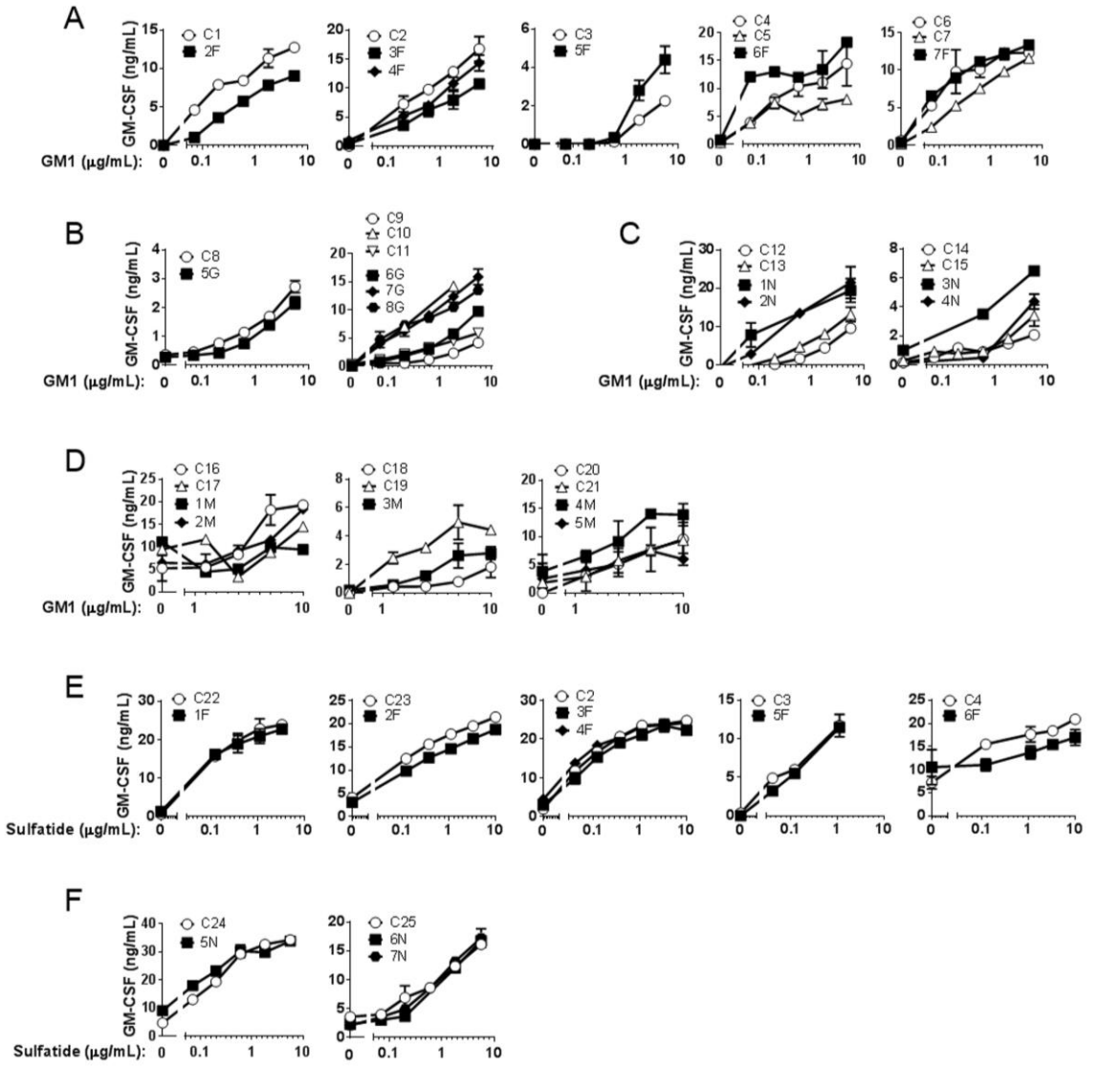

Figure 2. CD1b-restricted lipid antigen presentation by Mo-DCs from Fabry, Gaucher, NPC and MPS-VI disease patients. Mo-DCs from Fabry (A), Gaucher (B), NPC (C), and MPS-VI (D) disease patients and control subjects were loaded with graded doses of GM1 and co-cultured with the CD1b-restricted T cell clone GG33A. Mo-DCs from Fabry (E) and NPC (F) disease patients and control subjects were loaded with graded doses of sulfatide and co-cultured with the CD1b-restricted T cell clone DS1C9b. T cell response was analyzed by measuring GM-CSF release to the supernatant by ELISA. Patients are represented with filled symbols and control subjects with open symbols. Each symbol represents mean ± SD of duplicates for the same individual at the indicated antigen concentration. Each graph corresponds to an independent experiment.

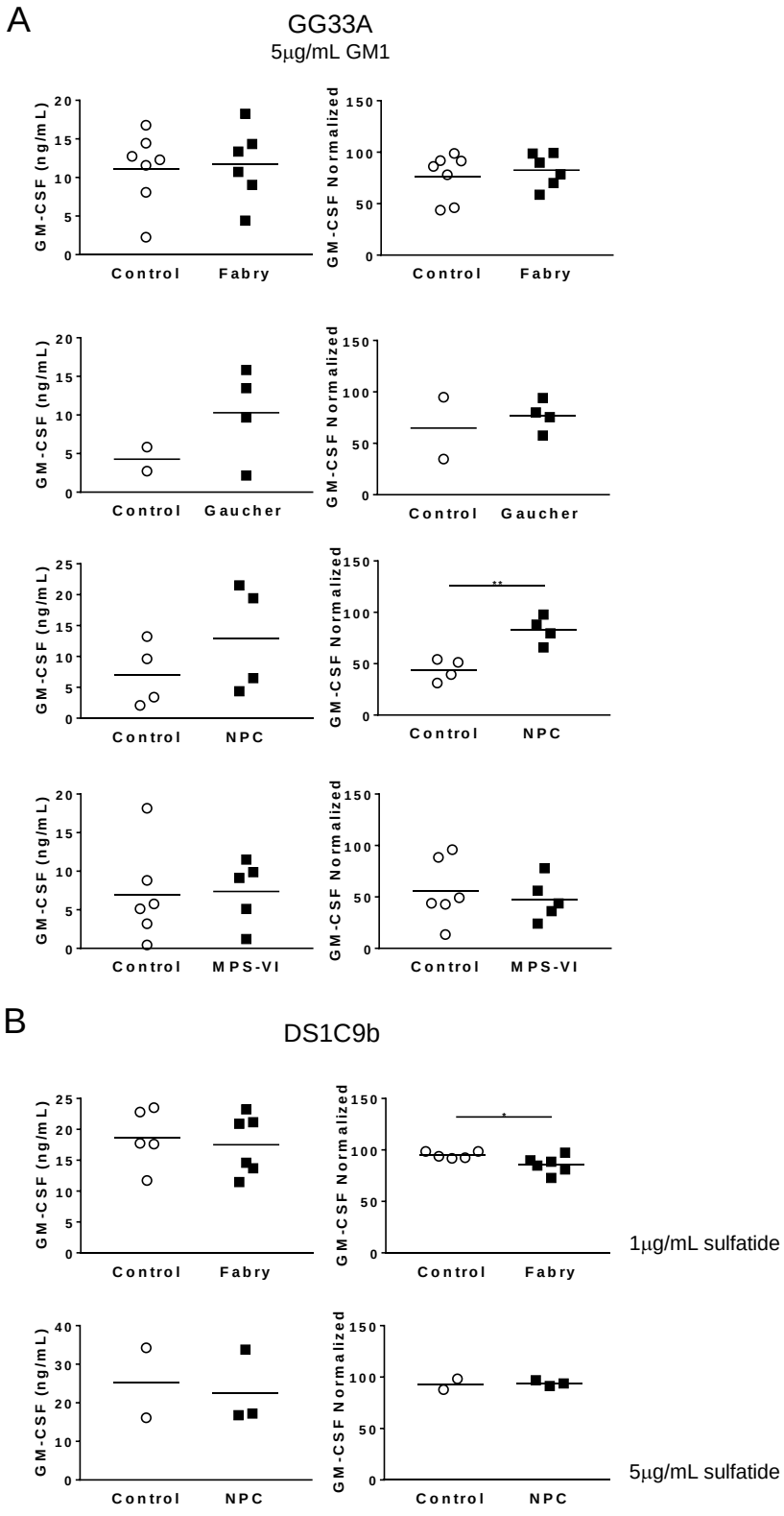

Figure 3 (the legend is in the following page)

Figure 3. Statistical analysis of CD1b-restricted lipid antigen presentation by Mo-DCs. Mo-DCs from Fabry, Gaucher, NPC and MPS-VI disease patients and control subjects were loaded with 5μg/mL of GM1 and co-cultured with the CD1b-restricted T cell clone GG33A (A). Mo-DCs from Fabry and NPC disease patients and control subjects were loaded with 1μg/mL or 5μg/mL of sulfatide and co-cultured with the CD1b-restricted T cell clone DS1C9b (B). The graphs on the left correspond to the cytokine production values. The graphs on the right correspond to the normalized values. Normalization was done for each independent assay considering the highest cytokine production value as 100. Patients are represented with filled symbols and control subjects with open symbols. Each symbol represents mean of duplicates for the same condition. An unpaired t-test (normal distribution) or a Mann-Whitney test (non-normal distribution) was used to compare both groups. P values lower than 0.05 were considered significant. *$p \leq 0.05$, ** $p \leq 0.01$. The graphs with no symbols relative to statistical analysis means that there were no statistical significant differences.

In conclusion, no major differences were found in CD1b-restricted presentation of GM1 or sulfatide antigens to specific T cell clones between healthy subjects and LSD patients.

Mo-DCs From Fabry, Gaucher, NPC and MPS-VI Disease Patients and Fabry and Gaucher Fresh Monocytes, Do Present Antigens by CD1d

Several LSD animal models demonstrated defects in CD1d-mediated lipid antigen presentation to iNKT cells, including Fabry and NPC disease mouse models [32, 67, 70, 71, 72]. Type II NKT cells also constitute an important part of CD1d-restricted T cells in humans [108]. We analyzed the capacity of Mo-DCs from Fabry, Gaucher, NPC, and MPS-VI disease patients to activate the iNKT cell clone JS63 in response to α-GalCer and the type II NKT cell clone s33d [106] in response to sulfatide by measuring cytokine production by ELISA. The s33d T cell clone carries the TCR Vβ13.1 [106], which was used to confirm the identity of the s33d T cells by flow cytometry (Figure 1). Analysis of CD1d and CD80 expression by Mo-DCs revealed no significant differences between control subjects and LSD patients (Table 3). In Figure 4 we analyzed cytokine production in response to antigen stimulation in Mo-DCs-iNKT cell cocultures. Cytokine production was also measured in parallel cultures of Mo-DCs only and Mo-DCs plus lipid antigen without iNKT cells. As expected, iNKT cells responded to α-GalCer loaded Mo-DCs with high production of GM-CSF. However, Mo-DCs cultured alone were also able to produce some GM-CSF, with or without lipid antigen stimulation.

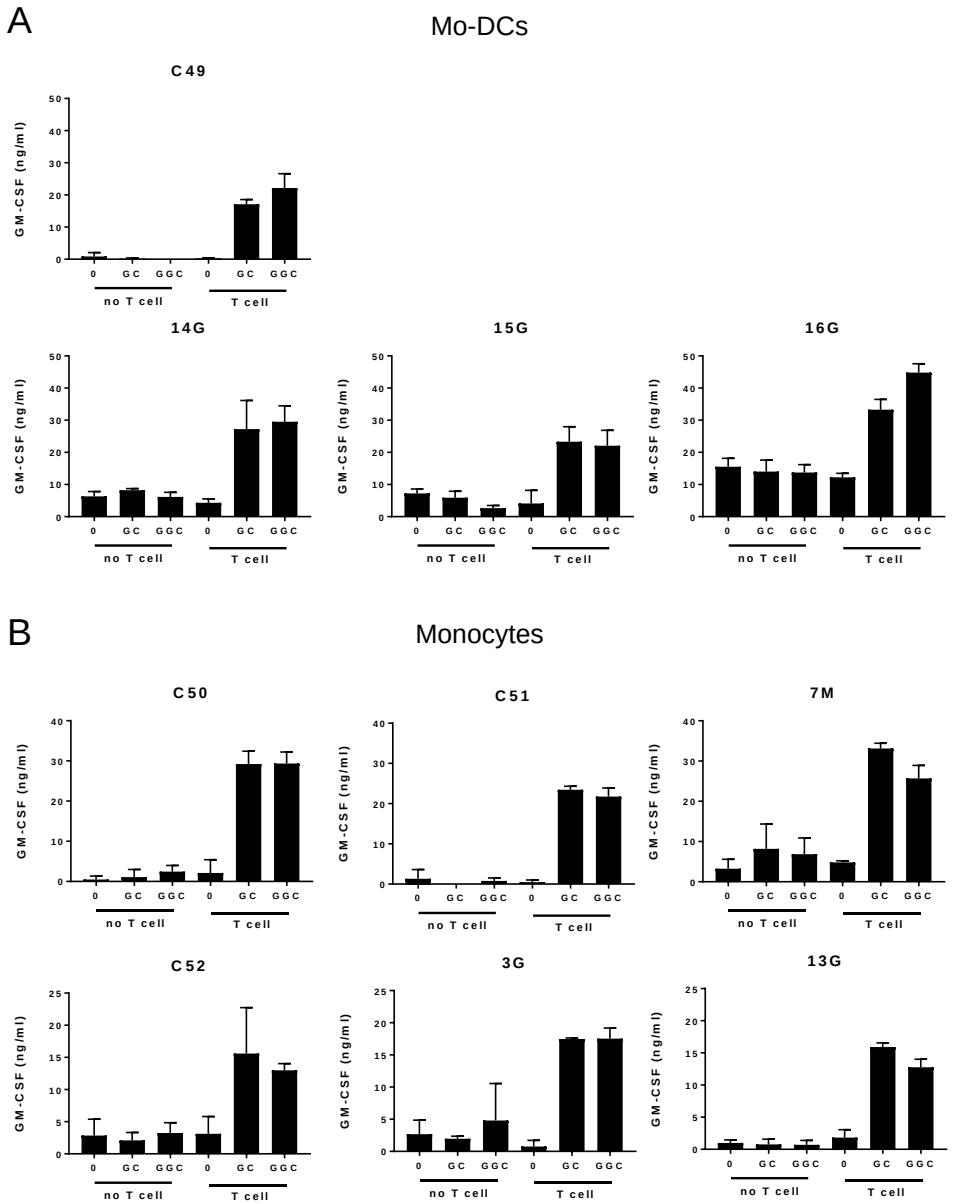

Figure 4. Basal production of GM-CSF by Mo-DCs and monocytes. Mo-DCs or monocytes from Gaucher (G) disease patients, MPS VI (M) disease patient and control (C) subjects were loaded with 50ng/mL of a-GalCer (GC) or 300ng/mL of α-Gal-(1-2)-α-GalCer (GGC) and co-cultured or not with an iNKT cell line. After 40 hrs, GM-CSF was measured in the culture supernatants. Each bar represents mean±SD of duplicates for the same condition.

α-GalCer presentation by Fabry, Gaucher, NPC, and MPS-VI Mo-DCs was successful, although some variation in the degree of activation could be observed among both control subjects and LSD patients (Figures 5A–D). The presentation of sulfatide to type II NKT cells by Mo-DCs from Fabry and Gaucher patients was also studied (Figures 5E,F). No statistically significant differences were observed in the capacity of Mo-DCs from patients and controls to present α-GalCer and sulfatide to iNKT cells and type II NKT cells, respectively (Figure 6).

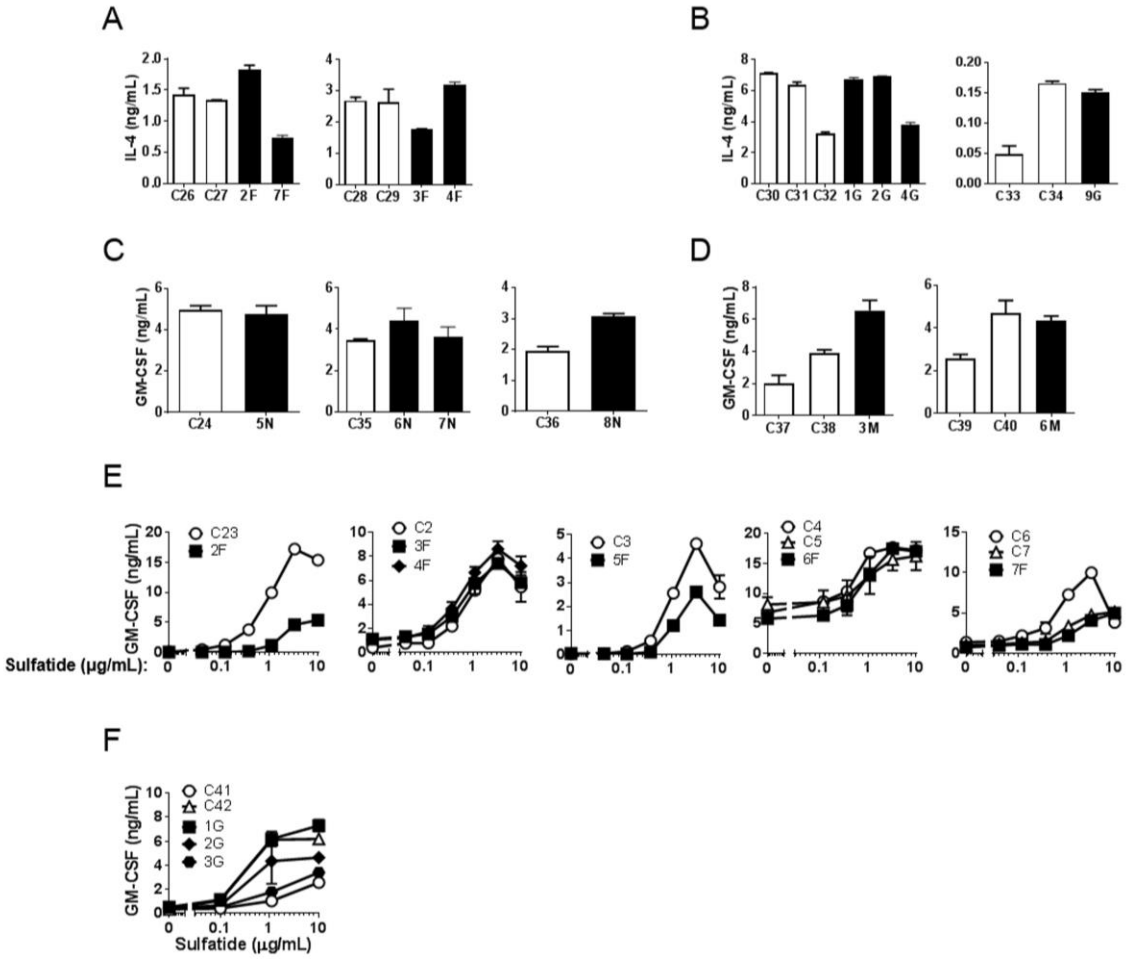

Figure 5. CD1d-restricted lipid antigen presentation by Mo-DCs from Fabry, Gaucher, NPC and MPS-VI disease patients. Mo-DCs from Fabry (A), Gaucher (B), NPC (C), and MPS-VI (D) disease patients and control subjects were loaded with 50 ng/mL of α-GalCer and co-cultured with the iNKT cell clone JS63. T cell response was analyzed by measuring IL-4 or GM-CSF release to the supernatant by ELISA. Patients are represented with filled columns and control subjects with open columns. Each column represents mean ± SD of duplicates for the same individual. Mo-DCs from Fabry (E) and Gaucher (F) disease patients and control subjects were loaded with graded doses of sulfatide and co-cultured with the type II NKT cell clone s33d. T cell response was analyzed by measuring GM-CSF release to the supernatant by ELISA. Patients are represented with filled symbols and control subjects with open symbols. Each symbol represents mean ± SD of duplicates for the same individual at the indicated antigen concentration.

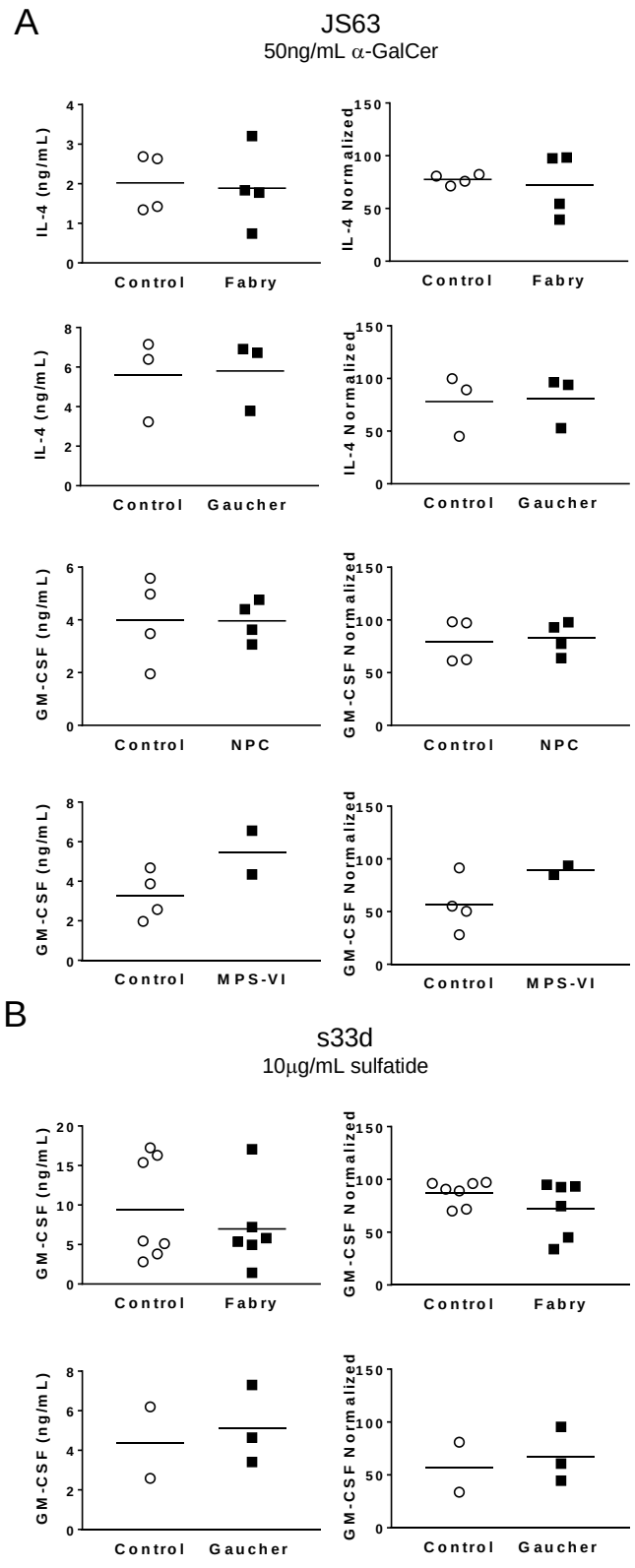

Figure 6 (the legend is in the following page)

Figure 6. Statistical analysis of CD1d-restricted lipid antigen presentation by Mo-DCs. Mo-DCs from Fabry, Gaucher, NPC and MPS-VI disease patients and control subjects were loaded with 50ng/mL of α-GalCer and co-cultured with the iNKT cell clone JS63 (A). Mo-DCs from Fabry and Gaucher disease patients and control subjects were loaded with 10μg/mL of sulfatide and co-cultured with the type II NKT cell clone s33d (B). The graphs on the left correspond to the cytokine production values. The graphs on the right correspond to the normalized values. The normalization was done for each independent assay considering the highest cytokine production value as 100. Patients are represented with filled symbols and control subjects with open symbols. Each symbol represents mean of duplicates for the same condition. An unpaired t-test (normal distribution) or a Mann-Whitney test (non-normal distribution) was used to compare both groups. P values lower than 0.05 were considered significant. The graphs with no symbols relative to statistical analysis means that there were no statistical significant differences.

Modifications of the cellular lipid content and in CD1d expression occur during the process of *in vitro* DC differentiation from monocytes [107]. Therefore, Mo-DCs might not be representative of the lipid antigen presentation occurring *in vivo* in patients. To overcome this issue, we used fresh monocytes from Fabry and Gaucher patients in addition to control subjects as APC in activation assays with an iNKT cell line [79] using α-GalCer (Fabry and Gaucher) and α-Gal-(1-2)-αGalCer (Gaucher) as antigens. α-Gal-(1-2)-αGalCer only becomes antigenic after cleavage in the lysosome by α-Gal A, the enzyme deficient in Fabry disease patients, meaning that the loading into CD1d preferentially happens inside the cell. Monocytes from LSD patients had similar levels of CD1d and CD80 cell surface expression to those of control subjects (Table 4).

Table 4. CD1d and CD80 expression* on Monocytes from LSD patients.

Monocytes	**Fabry**	**Gaucher**	
CD1d	1.02 ± 0.41	0.80 ± 0.03	1.26 ± 0.18
CD80	0.98 ± 0.13	1.03 ± 0.06	0.89 ± 0.01
N	6	3	4

*Mean Fluorescence intensity (MFI) value of each molecule for each patient was relativized using the MFI of control individuals analyzed in the same day. Numbers indicate the mean ± SD of N individuals studied.

Similar to what was observed when Mo-DCs were used, a variation in the T cell response was observed between subjects (Figure 7). Nevertheless, monocytes from Fabry (Figure 7A) and Gaucher patients (Figure 7B) were able to activate the iNKT cell line within the range of control monocytes when stimulated with α-GalCer. Monocytes from Gaucher disease patients (Figure 7B) have a higher capacity of α-Gal-(1-2)-αGalCer presentation when compared with control subjects. This difference was statistically significant ($P = 0.005$) for the normalized values of cytokine production in the presence of 50 ng/mL of the antigen (Figure 8).

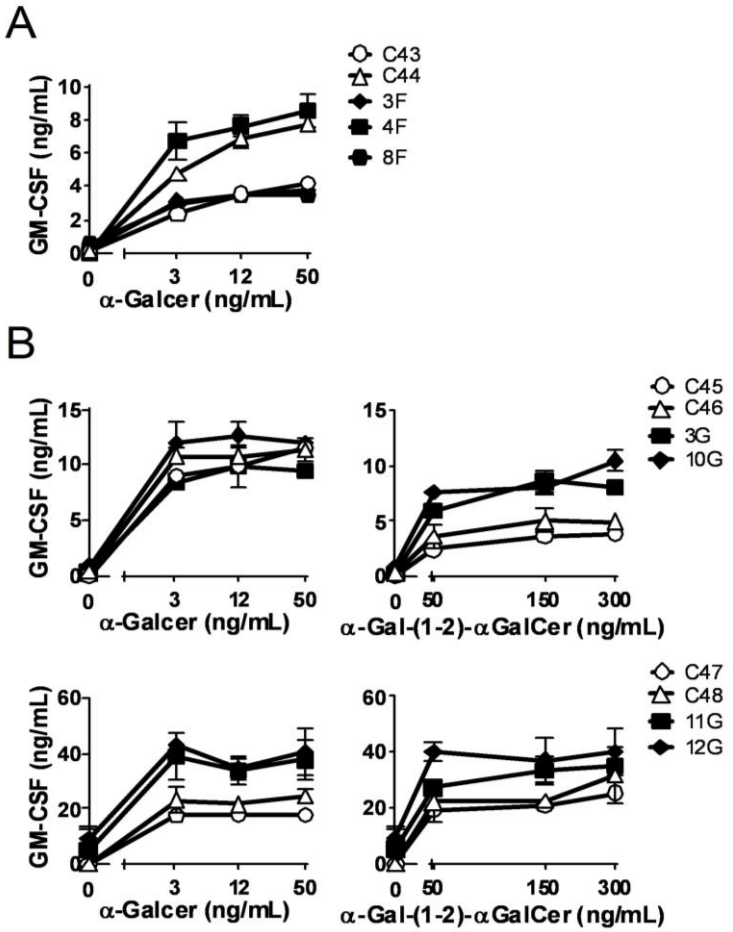

Figure 7. CD1d-restricted lipid antigen presentation by monocytes from Fabry and Gaucher disease patients. Monocytes from Fabry (A) and Gaucher (B) disease patients and control subjects were loaded with graded doses of α-GalCer (Fabry and Gaucher) or α-Gal-(1-2)-α-GalCer (Gaucher) and co-cultured with an iNKT cell line. Patients are represented with filled symbols and control subjects with open symbols. Each symbol represents mean ± SD of duplicates for the same individual at the indicated antigen concentration.

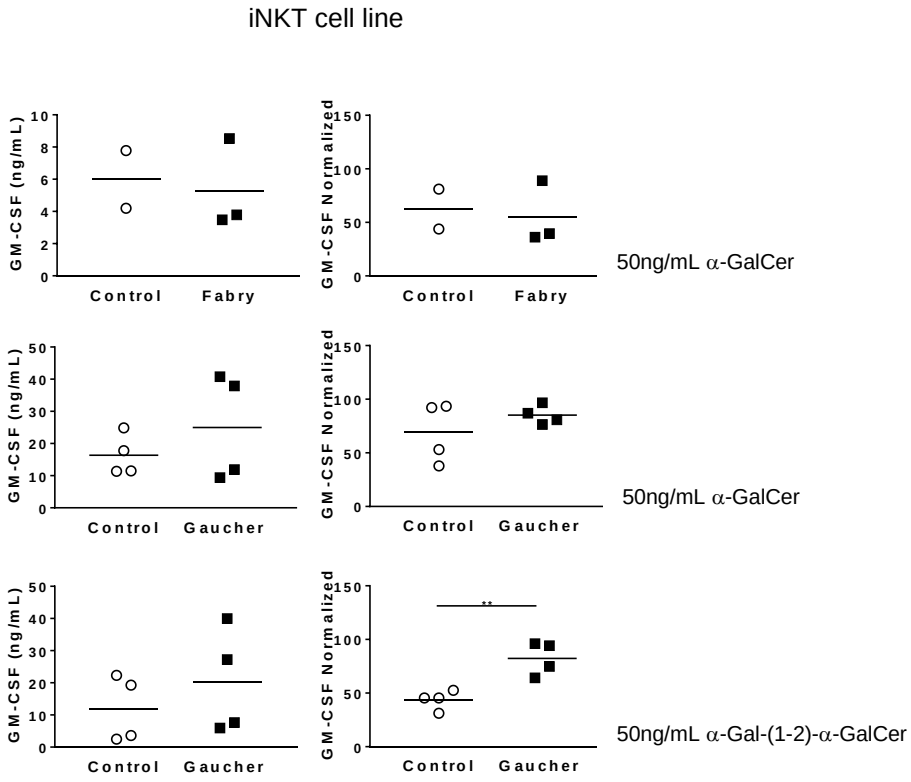

Figure 8. Statistical analysis of CD1d-restricted lipid antigen presentation by monocytes. Monocytes from Fabry and Gaucher disease patients and control subjects were loaded with 50ng/mL of α-GalCer or 50ng/mL of α-Gal-(1-2)-α-GalCer and co-cultured with an iNKT cell line. The graphs on the left correspond to the cytokine production values. The graphs on the right correspond to the normalized values. The normalization was done for each independent assay considering the highest cytokine production value as 100. Patients are represented with filled symbols and control subjects with open symbols. Each symbol represents mean of duplicates for the same condition. An unpaired t-test (normal distribution) or a Mann-Whitney test (non-normal distribution) was used to compare both groups. P values lower than 0.05 were considered significant.** $p \leq 0.01$. The graphs with no symbols relative to statistical analysis means that there were no statistical significant differences.

Thus, APCs from Fabry, Gaucher, NPC, and MPS-VI disease patients are capable of presenting lipid antigens that bind CD1d.

Fabry, Gaucher, NPC and MPS-VI Disease Patients Present Normal Frequencies of Circulating iNKT Cells

The frequency of iNKT cells is reduced in different mouse models of LSD [7, 32, 67, 69, 70, 71, 72, 74]. The same was not observed in NPC [77], Fabry [76], and Gaucher disease [75] patients' blood. Here, we studied the percentage of total iNKT cells and their CD4/CD8 phenotype in the blood of Gaucher, NPC, and MPS-VI disease patients compared with control individuals and our own published data on Fabry disease patients [76]. No significant differences were observed in the percentage of total iNKT cells in Gaucher, NPC, and MPS-VI disease patients' blood (Figure 9). However, when CD4/CD8 expression was analyzed, Gaucher disease patients showed an increase in the percentage of CD4+ and a decrease in the percentage of DN and CD8+ iNKT cells. NPC and MPS-VI disease patients did not present significant alterations in the frequencies of CD4/CD8/DN iNKT cells. Differences observed in Gaucher disease patients were iNKT cell-specific, as total T cells and their subsets remained unaltered (Figure 10). Therefore, although Fabry and Gaucher disease APCs showed no defects in their antigen presentation capacity by CD1d, enzyme deficiency and their consequences could impact the presence of certain iNKT cell populations in the peripheral blood of these patients.

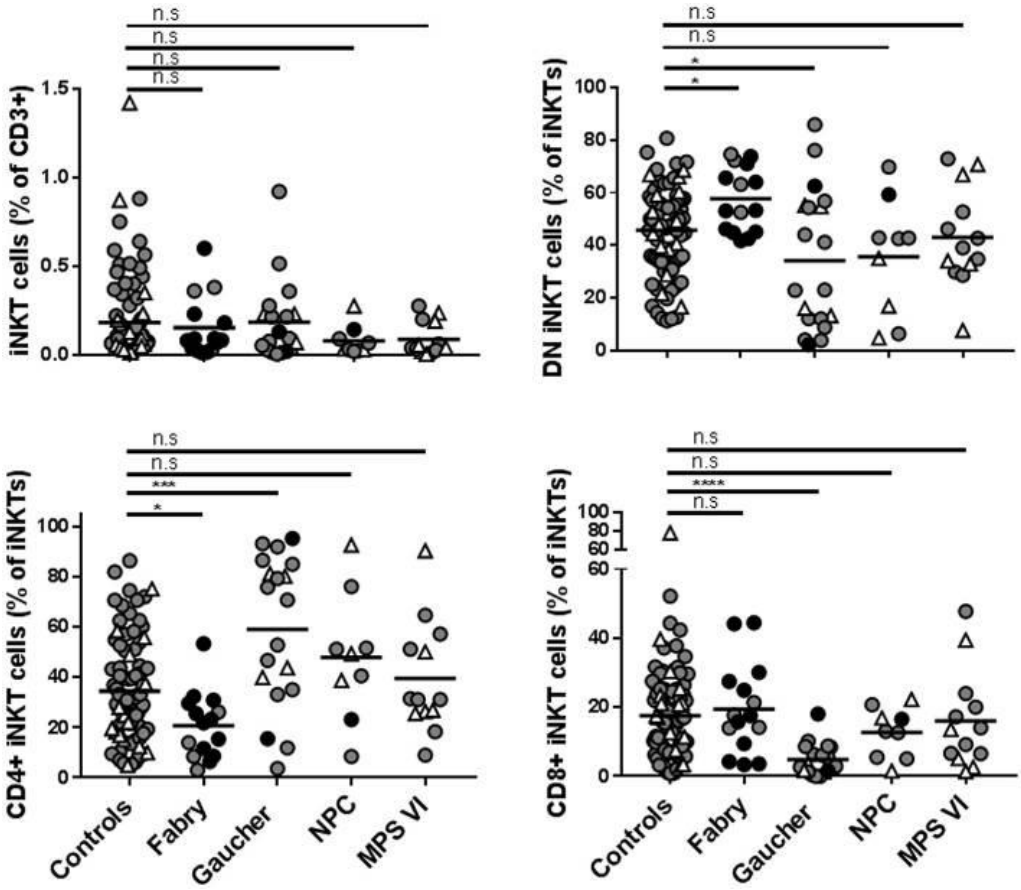

Figure 9. Frequencies of total iNKT cells and DN, CD4+, and CD8+ iNKT cells in Fabry, Gaucher, NPC, and MPS-VI disease patients. iNKT cells were identified in PBMCs or CD14− fractions obtained from Fabry, Gaucher, NPC, and MPS-VI disease patients or control subjects, by their expression of CD3 and the recognition of the CD1d-PBS57 tetramer. Antibodies against CD4 and CD8 were also used to define DN and positive subsets. Circles represent adults (≥16 years-old) and triangles represent children (those under 16 years of age). Black circles identified adult patients that were not under treatment. All the pediatric patients were receiving treatment. Horizontal line represents the mean of each group studied. Data normality was analyzed using the D'Agostino & Pearson normality test. To compare patients with the control population, one-way ANOVA (for data with normal distribution) or Kruskal-Wallis test (data with non-normal distribution) were used. *$p \leq 0.05$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

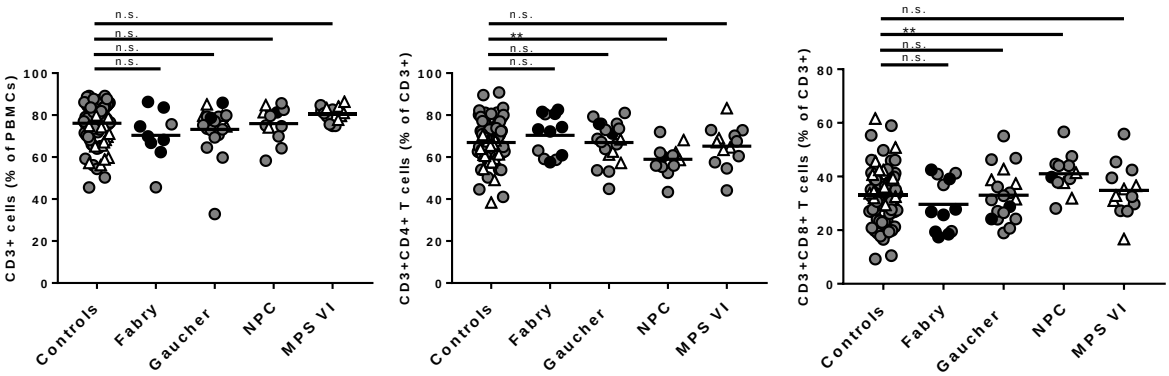

Figure 10. Frequencies of total CD3+ T cells and their CD4+ and CD8+ subsets in Fabry, Gaucher, NPC and MPS-VI disease patients. Total T cells were identified, in PBMCs or $CD14^-$ fractions obtained from Fabry, Gaucher, NPC and MPS-VI disease patients or control subjects, by their expression of CD3. Antibodies against CD4 and CD8 were also used to define positive subsets. Circles represent adults (over 16 y.o.) and triangles children (under 16 y.o.). Black circles identify adult patients that were not under treatment. All the pediatric patients were receiving treatment. Horizontal line represents the mean of each group studied. Normality of each group was analyzed using the D'Agostino & Pearson normality test. To compare patients with the control population, one-way ANOVA (for data with normal distribution) or Kruskal-Wallis test (data with non-normal distribution) were used. ** $p \leq 0.01$.

Discussion

The lysosome has been described as an important intracellular compartment for lipid antigen presentation, emphasized by the defects observed in several mouse models of LSD [32, 67, 70, 71, 72, 74]. However, the effects of lysosomal dysfunction present in LSD patients on lipid antigen presentation by different human CD1 molecules are unclear. Herein, we analyzed lipid antigen presentation in patients affected by four different LSDs, including three sphingolipidoses (Fabry, Gaucher, and NPC) and one mucopolysaccharidosis (MPS-VI). We focused on CD1b, as it is the human CD1 molecule that upon internalization recycles to the lysosome, and on CD1d, to compare with the published data on mouse models of LSD.

We found no major alterations in the capacity of APCs from Fabry, Gaucher, NPC, and MPS-VI disease patients to present exogenously added antigens that bind CD1b. The same was observed for CD1d presentation of α-GalCer in the four studied LSD. This is in contrast with what was observed for CD1d-mediated lipid antigen presentation in mouse models of several LSDs [32, 67, 70, 71, 72]. α-GalCer is a lipid whose internalization into APCs is mediated by scavenger receptors [109] and is presented by CD1d at the cell surface. Moreover, the loading of α-GalCer onto CD1d is influenced by its access to a functional lysosomal compartment in human APCs [110] and, in line with this notion, several studies have described that APCs from mouse models of LSD yield a decreased capacity to present α-GalCer [32, 67, 70, 71, 72].

The capacity of Gaucher disease patients' monocytes to present α-Gal-(1-2)-αGalCer seems to be elevated, which is also in contrast with the observation of a decreased capacity of APCs from LSD mouse models to present α-Gal-(1-2)-αGalCer [32, 67, 71, 74], a lipid that needs to be internalized and cleaved by the lysosomal α-Gal A enzyme to become antigenic. In the NPC1 mouse model, lipid antigen trafficking to the lysosome is compromised [71], which might explain the defects in lipid antigen presentation. It would be interesting to address if this impairment also occurs in APCs from LSD patients, especially since lipid traffic in LSD patients' fibroblasts was shown to be altered [9]. In accordance with our results, a previous report has shown that EBV transformed B cell lines from NPC patients, and transfected with CD1d, have a normal capacity to present lipids by this molecule [77]. These apparent discrepancies between mouse and human CD1d lipid antigen

presentation in NPC and Fabry diseases could be related to: (i) the described differences in intracellular trafficking between human and mouse CD1d; (ii) the discrepancy in lysosomal function between LSD patients and mouse models; (iii) the different type of APC used in mouse and human studies.

Mouse CD1d mainly recycles to the lysosome, contrary to human CD1d which is usually found in late endocytic compartments [111]. In fact, when B cell lines from NPC patients were transfected with mouse CD1d instead of human CD1d, a decrease was observed in the APC capacity to present α-Gal-(1-2)-αGalCer [77]. CD1b mediated antigen presentation was analyzed using GM1 and sulfatide, which are lipids that can bind to the cell surface of CD1b [105, 112]. GM1, due to its similarities to GM3, could also require internalization via scavenger receptors in order to be presented by CD1b [109]. However, we must note that GM1 has also been found to bind CD1b at the surface of fixed cells [105].

Interestingly, we observed that despite the lysosomal alterations occurring in LSD patients, lipid presentation through CD1b was not affected, allowing us to conclude that surface CD1b in LSD APC is competent in presenting lipids. Therefore, to draw more robust conclusions about CD1b-mediated lipid antigen presentation, it would be useful to perform the same type of studies with antigens that can only be presented by CD1b upon internalization, like some Mycobacterium lipids [84].

Another possible explanation for the differences found between mice and humans is that while LSD animal models correspond to full knockouts of the gene, deficiencies in humans may not translate into a full absence of the protein function. It is also possible that partial restoration of enzymes or their products with LSD treatments could be sufficient to compensate for the potential impact on antigen presentation. However, in the present study, blood (with the exception of NPC patients who received oral daily substrate reduction therapy) was always collected before infusion of the treatment (that was done weekly or every 2 weeks) and dendritic cell differentiation from monocytes takes more than 6 days in culture. It is therefore unlikely that the recombinant enzyme used in the infusions was present in considerable amounts in the Mo-DCs used in the antigen presentation assays. Moreover, no difference in the antigen presentation capacity was observed between treated and non-treated patients.

Lipid antigen presentation defects observed in LSD mouse models seem to be dependent on the nature of APC. While splenocytes and thymocytes from GM1 gangliosidosis, splenocytes from Sandhoff, or splenic DCs from Fabry disease mice showed defects in α-GalCer presentation, bone marrow derived DCs (BM-DCs) from the same mice were competent in presenting this antigen to iNKT cells [67, 68, 70, 72]. These differences could be related to variable degrees of lipid storage in distinct APCs or biological differences in antigen uptake or presentation. It is known that fetal bovine serum used in culture has a deficit in essential fatty acids, which results in an increased lipogenesis by cells, leading to lipidomic modifications [113]. This could justify the absence of alterations observed when using cells in culture, such as BM-DCs, Mo-DCs, or EBV-transformed B cell lines. Having this in mind, we tested the capacity of freshly isolated monocytes from Fabry and Gaucher patients to present antigens by CD1d. No major defects were observed in the presentation of the antigen α-GalCer, and monocytes from Gaucher disease patients present a slightly higher capacity to present α-Gal-(1-2)-αGalCer. Considering specifically Fabry disease patients, the lack of major lipid antigen presentation alterations can also be explained by the double effect of α-Gal A, the enzyme deficient in this disease. α-Gal A is known for its role in the degradation of Gb3, a lipid that we recently demonstrated to be inhibitory for iNKT cells [79]. However, α-Gal A was also implicated in the degradation of α-psychosine, an antigen that was shown to be present in mammalian tissues [40]. Thus, this enzyme is responsible for the degradation of both antigenic (α-psychosine) and inhibitory (Gb3) lipids for iNKT cells. Therefore, it is plausible that α-Gal A deficiency leads to an increase in the cellular content of both lipids, thus maintaining their ratio and preventing major alterations in iNKT cell activation. In our study, we also found a variable response of T cell clones to Mo-DCs and monocytes from different subjects, which does not seem to be related to age or gender. CD1 genes display a limited polymorphism but some substitutions have been described for exon 2 [114], although the impact of these substitutions in lipid antigen presentation is not clear. Nevertheless, studies in mice have revealed that CD1d polymorphisms affect antigen presentation and activation of CD1d-restricted T cells [115, 116], suggesting that the same may happen in humans.

The percentage of total iNKT cells was found unaltered in the peripheral blood of Fabry [76], Gaucher, NPC, and MPS-VI disease patients. However, as described before for Fabry disease patients [76], an alteration in the frequency of CD4/CD8/DN iNKT cells was

observed in Gaucher disease patients, showing a significant increase of the CD4+ population and a decrease of DN and CD8+ iNKT cells. These results suggest a higher egress of CD4+ iNKT cells from the thymus of Gaucher patients, which can be related with a propitious cytokine environment for the selection of this subset or an alteration in the antigen availability that could potentiate their selection. Finally, a difference in the frequency of iNKT cells in other localizations than blood cannot be excluded.

In conclusion, we show that APCs from Fabry, Gaucher, NPC, and MPS-VI disease patients are capable of presenting exogenous lipid antigens that bind CD1b and CD1d. In the future, for CD1b, it will be important to extend the study to lipid antigens that require internalization. It will be also interesting to elucidate whether lipid imbalances and other cellular alterations present in LSD will affect CD1 mediated self-lipid antigen presentation. Moreover, deciphering the factors favoring the thymic selection of CD4+ iNKT cells or inhibiting the proliferation of CD4− iNKT cells in the periphery of Gaucher patients would also be relevant.

Chapter 4: CD1d lipid antigen presentation by monocytes isolated from blood withdrawn and kept in different conditions

Abstract

The blood samples from human lysosomal storage diseases (LSDs) patients that we studied were collected in different regions of Europe implying a 24 hours blood storage, before processing. Here, we evaluate the CD1d lipid antigen presentation capacity of human monocytes when isolated from buffy coats; or isolated immediately or 24 hours after blood collected in ethylenediamine tetraacetic acid (EDTA) or citrate tubes.

The CD1d lipid antigen presentation capacity of monocytes isolated from buffy coats seems to be increased when compared with monocytes that were isolated immediately after blood collection in EDTA tubes. One of the components of buffy coats bags is citrate so monocytes were isolated immediately and 24 hours after blood collection in citrate and EDTA containing tubes. The monocytes isolated immediately after blood collected in EDTA or citrate containing tubes were similarly able to present lipid antigens. When the monocyte isolation was performed 24 hours after blood collection, a reduced capacity to present lipid antigens was observed for EDTA tubes and not for citrate tubes. So, when implies a 24 hours blood storage, citrate is a good anticoagulant to be used in blood sampling, in the context of human monocyte CD1d lipid antigen presentation studies.

Introduction

CD1 molecules present lipid antigens to T cells. CD1 restricted T cells are divided in two groups: CD1a, CD1b and CD1c-restricted T cells; and CD1d-restricted T cells. CD1d-restricted T cells reactive to α-Galactosylceramide (α-GalCer) and expressing a semi-invariant T cell receptor (TCR) are called type I NKT or invariant natural killer T (iNKT) cells. The CD1d-restricted T cells that do not respond to α-GalCer and that express diverse TCRs are named type II NKT cells [35]. iNKT cells when activated by lipid antigens release cytokines and chemokines, with effects in the immune system, having important functions in the context of infection, cancer and autoimmunity [35].

The prevention of human blood coagulation is quite important in medical clinics and scientific research. Ethylenediamine tetraacetic acid (EDTA) and citrate are anticoagulants commonly used, to prevent blood coagulation by the binding of calcium ions. EDTA is a stronger calcium chelator than citrate [117].

The levels of calcium have an important role in the modulation of monocyte [118, 119] and lymphocyte responses [120]. Concerning the monocyte, more than 20 years ago, EDTA *versus* citrate effect on the capacity of human monocytes to be activated by lipopolysaccharide (LPS) were compared by analyzing the tissue factor activity and tumor necrosis factor α (TNFα) production [121]. The monocyte activation was lower in the presence of EDTA comparing to citrate [121] however later studies did not observed a difference in monocyte tissue factor activity upon LPS stimulation between blood collected in EDTA and collected in citrate [122]. Still, in the presence of EDTA the formation of monocyte-platelet aggregates was lower comparing to citrate [122].

The effect of citrate on monocyte activation was further investigated by culture of the monocytic THP-1 cell line with citrate. The citrate effect on monocyte response to LPS depends on its concentration. LPS induced TNFα production was increased at low citrate concentrations and inhibited at high citrate concentrations, in a calcium dependent way [123].

At the cell surface, CD1 molecules are endocytosed and reach different intracellular locations. Human CD1d molecule localizes in late endosomes [81]. Endosomes and lysosomes are intracellular compartments whose homeostasis depends of calcium levels [124]. Human monocytes are relatively abundant blood antigen presenting cells. Monocytes express CD1d [125] being able to present lipid antigens to iNKT cells.

The aim of this study was to evaluate the CD1d antigen presentation capacity of human monocytes isolated from blood withdrawn and kept in different conditions (blood from buffy coats and blood processed immediately and 24 hours after blood collection in EDTA and citrate tubes).

Materials and Methods

Blood samples

Blood was collected in 9mL tubes containing EDTA or citrate-phosphate-dextrose-adenine (CPDA) (both from Vacuette, Greiner Bio-One, Austria); or in 4.5 mL tubes only containing citrate (Mistry Medical Supplies, UK). Healthy blood donors were recruited in the Immuno-Haemotherapy department of Hospital de S. João (Porto, Portugal). All subjects were adults. Informed written consent was obtained from all subjects enrolled in the study. The study was approved by local ethical committee from the hospital. In addition, buffy coats were also provided by the Immuno-Haemotherapy department of Hospital de S. João. The buffy coats are stored in bags containing citrate-phosphate-dextrose (JMS, Hiroshima, Japan).

Peripheral blood mononuclear cells (PBMCs) isolation and monocytes purification

PBMCs were isolated from buffy coats or from blood collected in EDTA, CPDA or citrate tubes, immediately after collection or 24 hours after. During those 24 hours, blood was maintained in rotation at room temperature. PBMCs were separated by Histopaque-1077® (Sigma-Aldrich, St. Louis, MO, USA) density centrifugation, following the manufacturer's instructions. Monocytes were isolated by positive selection with anti-CD14 magnetic beads, using MACS cell separation system (Miltenyi Biotec, Cologne, Germany).

iNKT cell line culture and re-stimulation

The human iNKT cell line was generated in the lab [79] and maintained in culture medium supplemented with 5% v/v of pooled human AB serum (from donors of the Immuno-Haemotherapy department of Hospital de São João, Porto, Portugal) and 100U/mL of recombinant human IL-2. When cells stopped proliferating, 1μg/mL of phytohaemagglutinin (PHA) (Thermo Fisher Scientific,Waltham, MA, USA) was added with irradiated PBMCs [79].

Lipid antigen presentation assays

Monocytes were cultured with α-Galactosylceramide (α-GalCer) (50-3.12ng/mL, Avanti polar lipids, Alabaster, AL, USA) or α-Gal-(1-2)-αGalCer (300-50ng/mL, provided by Prof. Paul Savage, Brigham Young University, Provo, USA). α-GalCer was resuspended in PBS and directly diluted in non-supplemented RPMI medium (Life Technologies, CA, USA). α-Gal-(1-2)-αGalCer was first dissolved in methanol and then diluted in non-supplemented RPMI medium to have a maximum of 1% vehicle in culture. After 4 hours, an iNKT cell line was added and 40 hours later, supernatants were collected for cytokine production determination by ELISA. The following antibody pairs from Biolegend (CA, USA) were used: purified anti-human GM-CSF (clone BVD2-23B6) and IFN-γ (clone NIB42) and biotinylated anti-human GM-CSF (clone BVD2-21C11) and IFN-γ (clone 4S.B3).

Flow cytometry

Monocytes were stained with the viability markers 7-AAD and annexin V (BD Biosciences, CA, USA), and with the following antibodies: anti-CD14 (M5E2, Biolegend, CA, USA), anti-CD1d (51.1, Biolegend, CA, USA) and anti-CD86 (BU63, EXBIO, Vestec, Czech Republic) monoclonal antibodies. Unstained cells were used as negative control. Cells were acquired in a FACS Canto II (BD Biosciences, CA, USA) using the BD FACSDiva™ software (BD Biosciences, CA, USA). Data analysis was performed with FlowJo® v10 (BD Biosciences, CA, USA).

Statistics

Statistical analysis of lipid antigen presentation assays was performed by comparing the amount of cytokine produced in the co-culture of monocytes with iNKT cells between the different blood collection conditions. A single concentration of antigen was used for this purpose. After checking data normality using the Shapiro–Wilk test, an unpaired t-test or a Mann-Whitney test was used to compare two groups. The multiple comparison test used was the Kruskal-Wallis test. P values lower than 0.05 were considered significant. All the analyses were performed using the GraphPad Prism software v7.04 (GraphPad Software Inc., CA, USA).

Results

Buffy coats are widely used as a source of leucocytes in a variety of immunology studies, especially when a high number of leucocytes, from the same donor, are needed. But how similar are the cells isolated from buffy coats to fresh blood isolated leukocytes? Due to our interest in lipid antigen presentation, we analysed the capacity of monocytes isolated from buffy coats and of monocytes isolated from blood collected in EDTA tubes, to activate iNKT cells through CD1d.

Two lipid antigens were used, α-GalCer and α-Gal-(1-2)-αGalCer, which requires lysosomal processing to α-GalCer by lysosomal α-Galactosidase A to become antigenic [66]. We consistently found that the monocytes isolated from buffy coats had a higher capacity to present the iNKT cell lipid antigens, α-GalCer and α-Gal-(1-2)-αGalCer, than monocytes isolated immediately after blood collection in EDTA tubes (Figure 1A, B and C). In case of α-Gal-(1-2)-αGalCer presentation, the difference observed was statistically significant (Figure 1D).

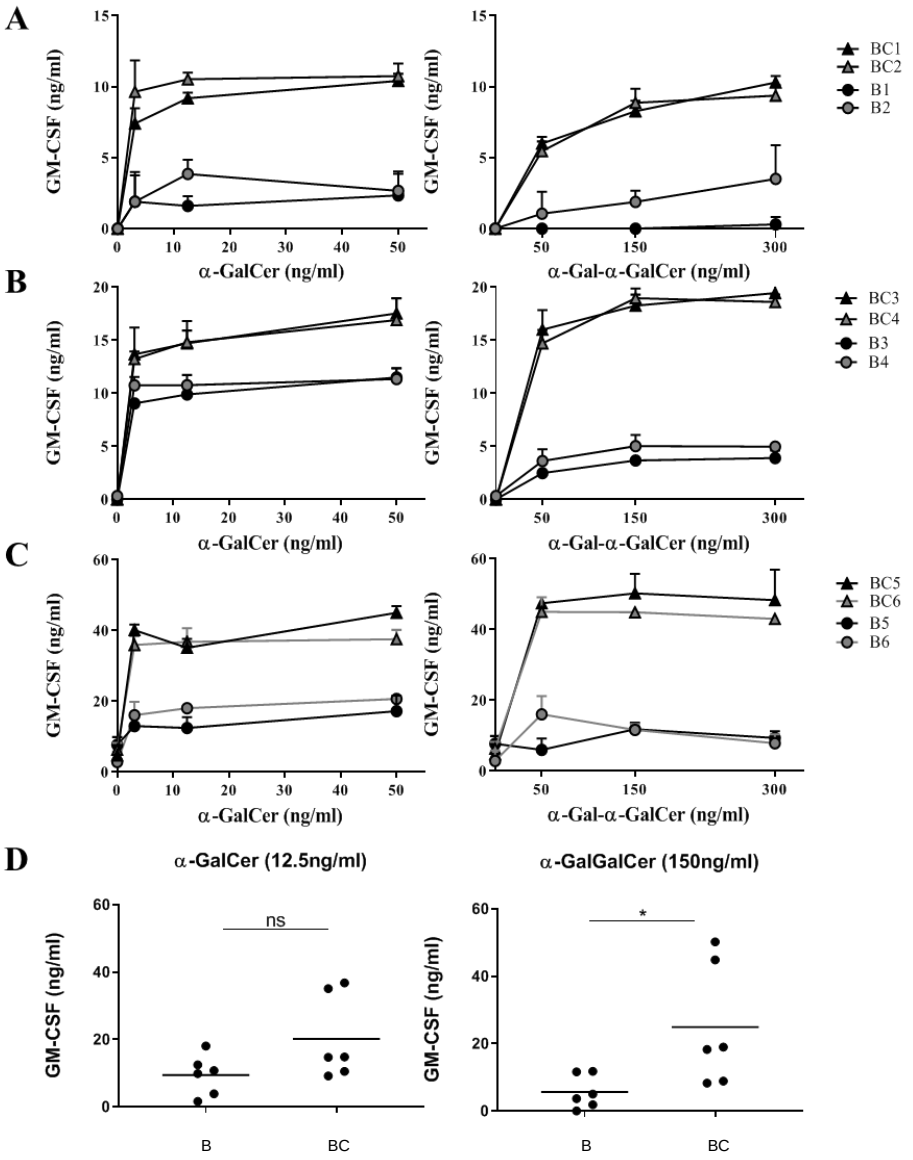

Figure 1. Lipid antigen presentation by monocytes isolated from buffy coats and from fresh blood collected in EDTA tubes. Monocytes isolated from buffy coats (BC) and from fresh blood collected in EDTA tubes (B) were isolated and loaded with graded doses of α-GalCer (left) or α-Gal-(1-2)-αGalCer (right) and co-cultured with an iNKT cell line. T cell response was analyzed by measuring GM-CSF release to the supernatant by ELISA. A total of 6 buffy coats and 6 blood collections in EDTA tubes, were analyzed in 3 independent experiments (A, B, C). Values represent mean±SD of triplicates. The buffy coat and the fresh blood collected in EDTA were not from the same donor. (D) The statistical analysis was done for: 12.5 ng/mL of α-GalCer and 150 ng/mL of α-Gal-(1-2)-αGalCer. Horizontal bars represent mean. 6 subjects were analyzed in 3 independent experiments. An unpaired t-test or a Mann-Whitney test was used. Values of $p \leq 0.05$ were considered statistically significant.

The composition of bags where the blood was collected for blood donation have citrate-phosphate-dextrose. The commercially available blood collection tubes with a similar composition to the bags used for blood donation collection, are the CPDA tubes. CPDA tubes composition has citrate-phosphate-dextrose-adenine. Therefore, we evaluate the effect of the composition of different blood collection tubes, EDTA, CPDA and citrate tubes, in the capacity of CD1d lipid antigen presentation of monocytes. The monocytes used in the lipid antigen presentation assays, were purified immediately after blood collection and 24 hours afterwards.

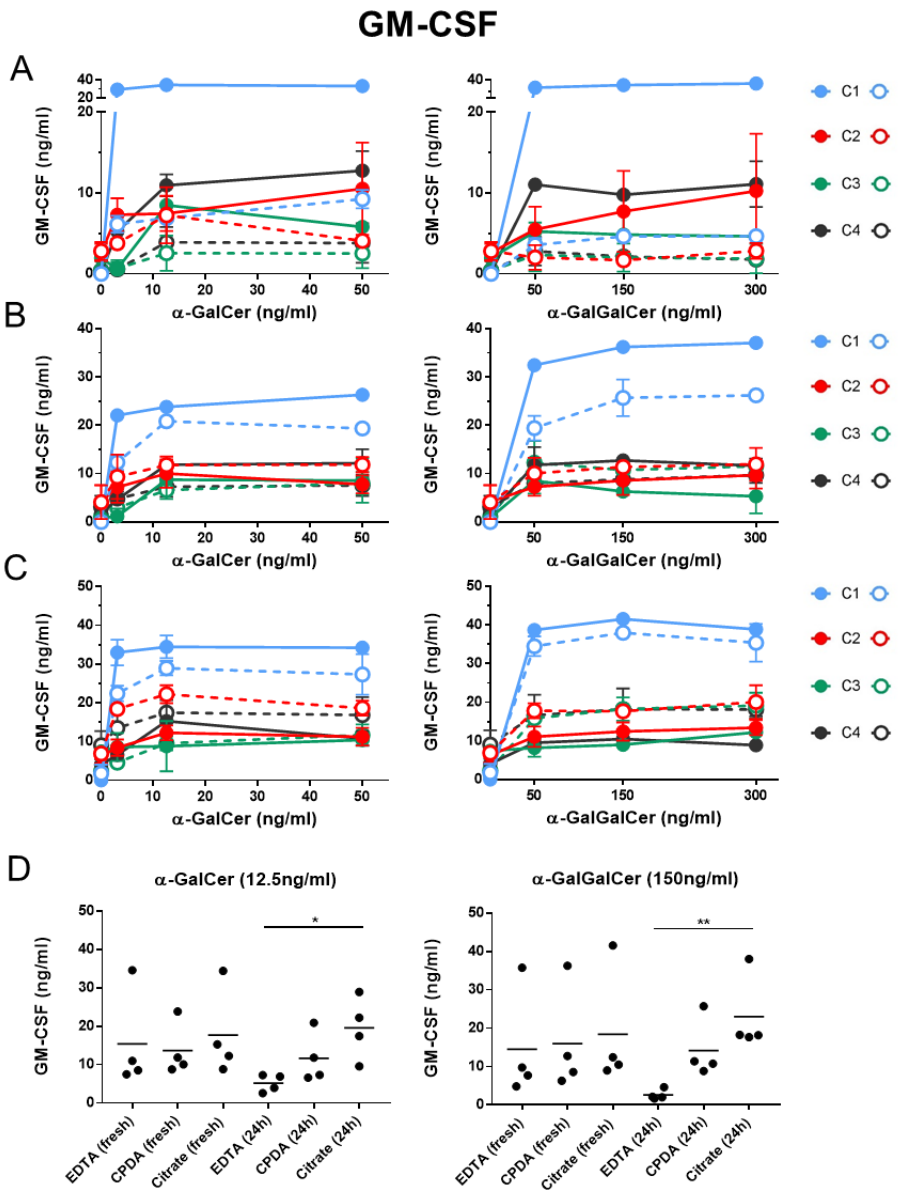

Figure 2. Lipid antigen presentation of monocytes isolated from blood collected in EDTA (A), CPDA (B) and citrate (C) containing tubes. Monocytes from four healthy controls (C1, C2, C3 and C4) were isolated immediately (filled symbols) or 24 hours after blood collection (empty symbols) and loaded with graded doses of α-GalCer or α-Gal-(1-2)-αGalCer and co-cultured with an iNKT cell line. T cell response was analyzed by measuring GM-CSF release to the supernatant by ELISA. Values represent mean±SD of duplicates or triplicates of two independent experiments. (D) The statistical analysis was done for two lipid antigen concentrations: 12.5 ng/mL of α-GalCer or 150 ng/mL of α-Gal-(1-2)-αGalCer. Horizontal bars represent mean. 4 subjects were analyzed in 2 independent experiments. Kruskal–Wallis test was used. Values of $p \leq 0.05$ were considered statistically significant.

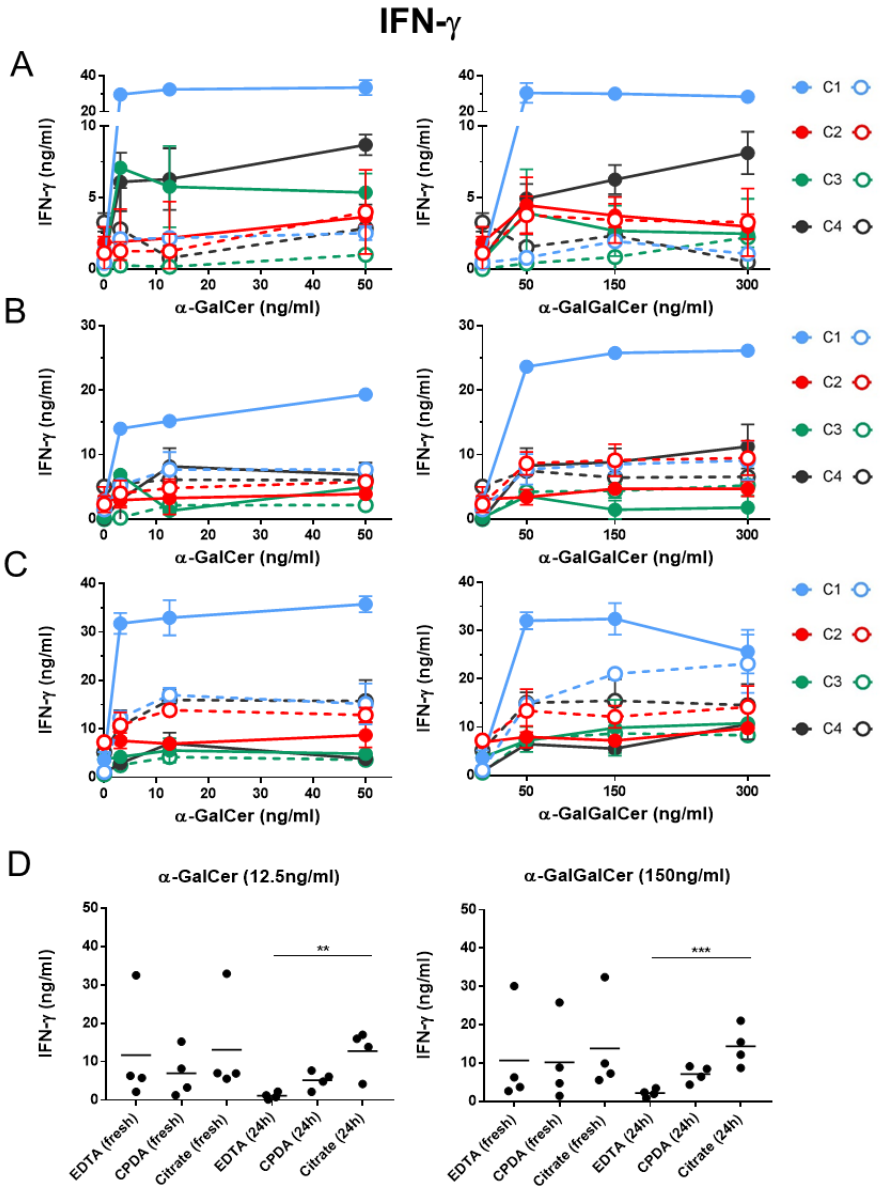

Figure 3. Lipid antigen presentation of monocytes isolated from blood collected in EDTA (A), CPDA (B) and citrate (C) containing tubes. Monocytes from four healthy controls (C1, C2, C3 and C4) were isolated immediately (filled symbols) or 24 hours after blood collection (empty symbols) and loaded with graded doses of α-GalCer or α-Gal-(1-2)-αGalCer and co-cultured with an iNKT cell line. T cell response was analyzed by measuring IFN-γ release to the supernatant by ELISA. Values represent mean±SD of duplicates or triplicates of two independent experiments. (D) The statistical analysis was done for two lipid antigen concentrations: 12.5 ng/mL of α-GalCer or 150 ng/mL of α-Gal-(1-2)-αGalCer. Horizontal bars represent mean. 4 subjects were analyzed in 2 independent experiments. Kruskal–Wallis test was used. Values of $p \leq 0.05$ were considered statistically significant.

The monocytes when isolated immediately after blood collection, in EDTA, CPDA or citrate tubes, were able to induce iNKT cell response, measured by GM-CSF and IFN-γ cytokines production, in a similar way (figures 2 and 3). However, 24 hours after blood collection, the monocytes isolated from blood collected in EDTA tubes, presented a reduction in the capacity of CD1d lipid antigen presentation, statistically significant lower, comparing to citrate tubes (figures 2 and 3).

To investigate if the observed difference could be a result of an alteration in monocyte viability, CD1d expression or cellular activation status (measured by the expression of CD86 molecule), monocytes isolated immediately and 24 hours after blood collection, in EDTA, CPDA or citrate tubes, were stained with 7-AAD, Annexin V, anti-CD1d and anti-CD86 monoclonal antibodies; and analysed by flow cytometry (figure 4).

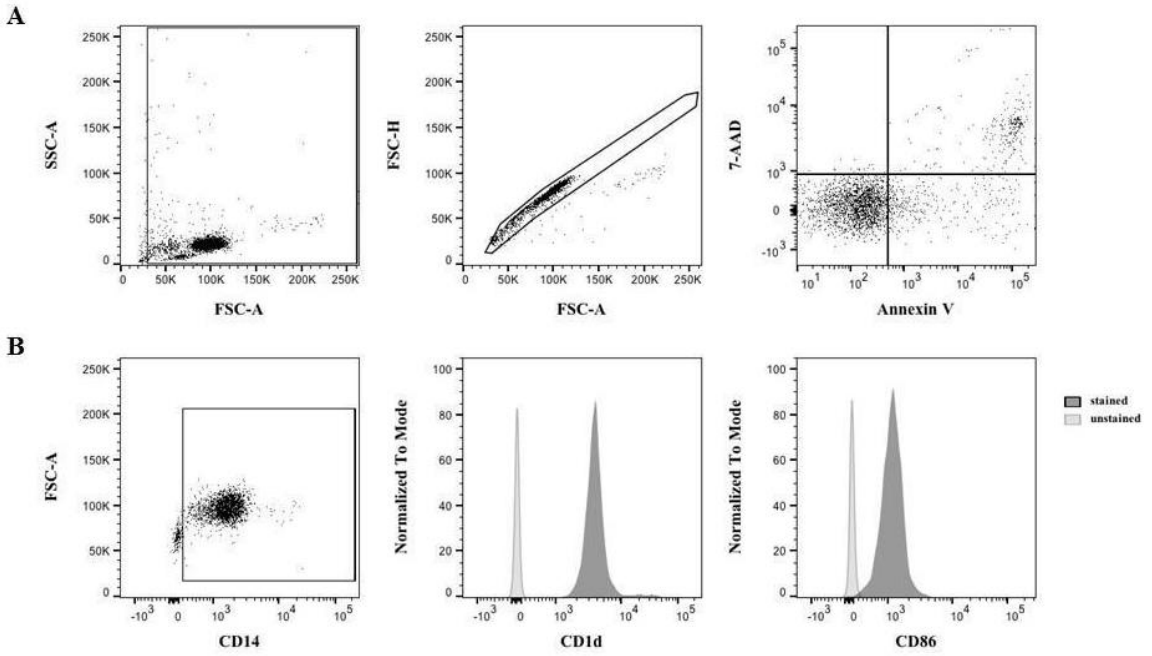

Figure 4. Flow cytometry analysis of viability and expression of CD1d and CD86 molecules on monocytes. (A) Gating strategy employed in the flow cytometry analysis of monocytes, showing selection of total cells (1st panel), singlets (2nd panel) and viable cells (3rd panel; Annexin V$^-$ 7-AAD$^-$ cells). (B) Analysis of the expression of CD1d and CD86 molecules, on viable monocytes (CD14+ Annexin V- 7-AAD- cells). Panels refer to one representative monocyte isolation.

Table 1. Viability, CD1d and CD86 expression on monocytes isolated immediately (fresh) and 24 hours after blood collection in EDTA, CPDA and citrate tubes.

	control 1 (fresh)			***control 1 (24 hours)***		
	EDTA	CPDA	citrate	EDTA	CPDA	citrate
viable cells (%)	80	84	76	65	58	41
CD1d (MFI)	3554	4008	3716	3575	3967	3942
CD86 (MFI)	909	1064	1017	702	722	817
	control 2 (fresh)			***control 2 (24 hours)***		
	EDTA	CPDA	citrate	EDTA	CPDA	citrate
viable cells (%)	71	81	72	62	45	46
CD1d (MFI)	3701	3525	3686	3641	3716	3723
CD86 (MFI)	1177	1190	1249	757	953	911

MFI, mean fluorescence intensity.

In total, two healthy blood donors were studied (table 1). Comparing EDTA, CPDA and citrate tubes, no significative differences were found in the viability of monocytes isolated immediately after blood collection (fresh). At 24 hours after blood collection, a reduction in the viability was observed in the three blood collection tubes. At this time point, the values of viability were higher for EDTA.

No differences were found between the three blood collection tubes in CD1d and CD86 expression, on monocytes isolated immediately and 24 hours after blood collection.

Discussion

We showed that the capacity of CD1d lipid antigen presentation of monocytes isolated from buffy coats is higher comparing to monocytes isolated immediately after blood collection in EDTA tubes. Besides buffy coats, fresh and 24 hours blood collected in EDTA, CPDA and citrate tubes were tested in our study. No differences were observed in the CD1d lipid antigen presentation capacity of fresh monocytes between the three blood collection tubes. Comparing monocytes isolated 24 hours after blood collection in EDTA, CPDA and citrate tubes, the capacity of CD1d lipid antigen presentation was lower for EDTA tubes. It seems that these observed differences are not a result of an alteration in the viability, CD1d and CD86 expression. Still, a higher number of subjects should be considered and other activation markers like CD40 and CD80 molecules should be included in the study.

Overall, our results show that the capacity of CD1d lipid antigen presentation of monocytes isolated 24 hours after blood collection in citrate tubes is not affected, contrarily to EDTA tubes. Citrate is a good anticoagulant to be used in blood sampling, when implies a 24 hours blood storage, before processing, in functional studies like CD1d lipid antigen presentation studies using monocytes as antigen-presenting cells.

In addition, stimulation assays with peptide antigens or with LPS can be good approaches, to investigate if the effect of EDTA and citrate is lipid antigen specific or not; and other anticoagulants like heparin can be tested.

EDTA has more affinity for calcium than citrate [117]. Since EDTA binds calcium more strongly than citrate, we can hypothesized that the cells isolated from blood collected with EDTA tubes have a lower calcium availability, probably more pronounced when the contact time of cells with the anticoagulant is prolonged. The lower lysosomal calcium availability associated with alterations in lipid trafficking, can be behind the observed difference. To explore the importance of lysosomal calcium levels in the CD1d lipid antigen presentation capacity of monocytes, it would be important to confirm if EDTA in fact is able to reduce the monocyte calcium availability, comparing to citrate. It would be also interesting to study the effect of EDTA and citrate in the fusion of late endosome-lysosome in monocytes.

Chapter 5: Chemical inhibition of β-glucocerebrosidase does not affect phagocytosis and early containment of *Leishmania* by murine macrophages

Chapter includes the results published in:

Ribeiro H, Rocha MI, Castro H, Macedo MF. "Chemical inhibition of β-glucocerebrosidase does not affect phagocytosis and early containment of *Leishmania* by murine macrophages", Exp Parasitol, 216:107939. (2020)

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 also affects the capacity of macrophages to phagocytose and eliminate 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*. 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 capacity of parasites to be internalized and to outlive the leishmanicidal response of CBE-treated macrophages relative to control cells. In conclusion, murine macrophages with defective β-glucocerebrosidase activity can phagocytose and control *Leishmania* infection to the same extent as cells with normal enzyme activity.

Introduction

Gaucher disease is a rare (0.2-1.8 per 100'000 live births [6] 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) [5, 13, 126]. Alongside lysosomal enlargement due to lipid accumulation, defects in autophagy [18], alterations in calcium homeostasis [16, 17, 127] and in lysosomal pH [15] have also been reported in Gaucher disease. 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 [126]. Previous reports indicate that in Gaucher disease patients, human monocytes and monocyte-derived macrophages present impaired capacity to kill bacterial pathogens [19, 20], albeit 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 devastating neglected tropical diseases, with symptoms ranging from contained skin wounds to disseminating cutaneous and visceral infections [128]. *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 [129]. Within macrophages, parasites reside inside parasitophorous vacuoles that originate from the fusion of phagosome-containing parasites with lysosomes [128]. 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 [129, 130]. 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 Guacher disease - affects

phagocytosis and elimination of *Leishmania* by murine bone marrow derived macrophages.

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 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 [131]. 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).

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. For direct comparison between two groups, the unpaired t-test (normal distribution) and the Mann-Whitney test (non-normal distribution) were used. Statistical analyses were performed using the GraphPad Prism software v8.1.1.

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 inhibited by conduritol B epoxide (CBE) [132, 133, 134]. F4/80 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 [132, 133, 134].

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). Finally, we confirmed that exposure to CBE affected neither the viability of BMDMs (53.6±9.9% and 55.4±6.9% Annexin V^- 7-AAD^- cells, for CBE-untreated and treated cells, respectively), nor the expression of markers of macrophage differentiation (F4/80) and activation (MHC class II, CD40 and CD80) (Fig. 1c), in this way excluding any potential effect of these factors from our analysis. Of notice, treatment with CBE was interrupted immediately before macrophage infection with *Leishmania*, to avoid the direct effect of this compound on parasites.

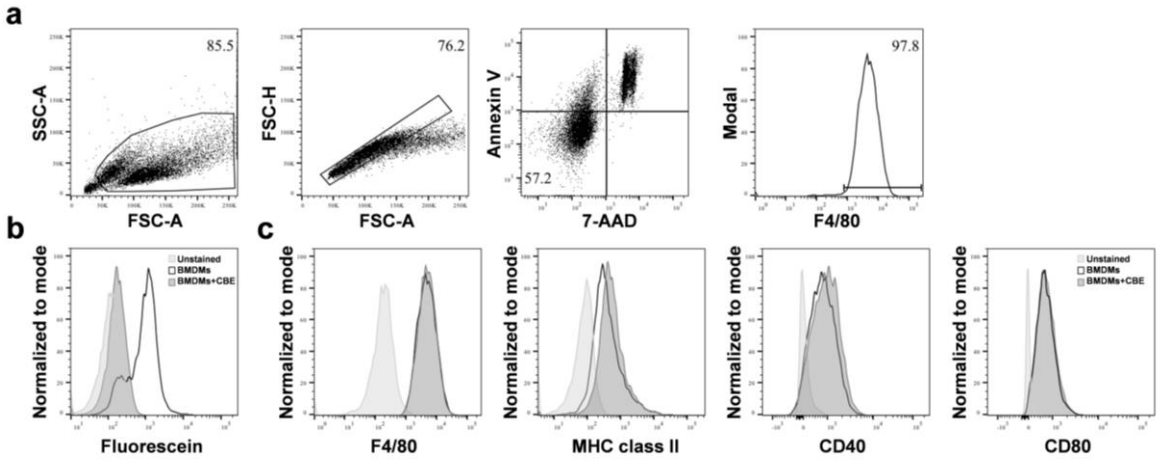

Figure 1. Flow cytometry analysis of markers of viability, differentiation, activation, and of β-glucocerebrosidase activity of BMDMs. (**a**) Gating strategy employed in the flow cytometry analysis of BMDMs, showing selection of cells based on size (1st panel), singlets (2nd panel), and viability (3rd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

Having validated 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 [129, 130]. *Leishmania infantum* stands out for being an agent of visceral infections, unlike the other two cutaneous disease-causing species [129, 130].

Macrophages were separately infected with *L. amazonensis*, *L. infantum* and *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*, typically yielding higher infection indexes, assays were performed with lower MOIs (2 and 5). The rationale behind using two MOIs was to assess whether the response of CBE-treated macrophages to *Leishmania* varied according to the infection burden. We did not find evidence for such variation (data not shown), therefore we pooled the results of both MOIs for our global analysis. Each graphic in Fig. 2 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. 2, *top panels*), and ii) the average number of parasites per infected macrophage (Fig. 2, *bottom panels*). 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. 2a). As for *L. major*, inactivation of β-glucocerebrosidase led to an increased percentage of infected macrophages ($p \leq 0.05$; Fig. 2a, *top panel*). However, since this increment was discreet (1.08±0.11 times) and not accompanied by an increased number of intracellular parasites (Fig. 2a, *bottom panel*), we did not find it physiologically meaningful. Second, we studied how inhibition of β-

glucocerebrosidase impacts on the leishmanicidal activity of macrophages. Towards that end, we followed parasite survival at later time points *post* infection, namely 24 and 48 hrs. This prolonged time in the absence of CBE is enough for macrophages to recover β-glucocerebrosidase activity (data not shown) [132]. Therefore, any potential 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. 2b,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. 2b, *bottom panel*), or *L. amazonesis* for 48 hrs (% infected macrophages; Fig. 2c, *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 phagocytic and microbicidal activities of macrophages are not affected in the context of an infection with promastigotes of *L. amazonensis*, *L. infantum*, and *L. major*.

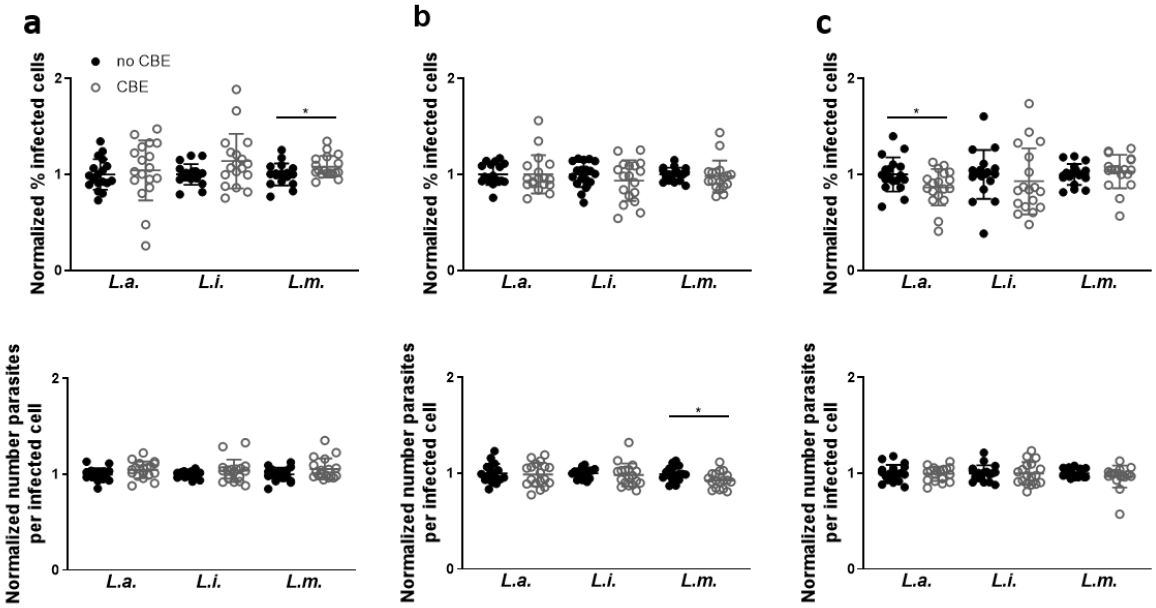

Figure 2. BMDMs with CBE-inactivated β-glucocerebrosidase retain the capacity to phagocytose and eliminate *Leishmania*. Percentage of infected macrophages (*upper panels*), and average number of parasites per infected cell (*bottom panels*), in control (*black circles*) and CBE-treated BMDMs (*white 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. * $p \leq 0.05$.

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 [19, 20], 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 immune 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. Based on this model we can safely 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 [135], 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 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 eliminate *Leishmania*.

Chapter 6: Conclusions and future perspectives

"Lipid antigen presentation by CD1b and CD1d in lysosomal storage disease patients"

In lysosomal storage diseases (LSDs), the macromolecules that are not properly degraded or transported, accumulate in the lysosome. Since the lysosome is involved in lipid antigen presentation, it was investigated the effect of lysosomal alterations occurring in the context of human LSDs, in the capacity of CD1 lipid antigen presentation and in invariant natural killer T (iNKT) cell frequency, contributing to increase the knowledge in human LSDs. It is noteworthy that LSDs are rare diseases; and the volume and frequency of blood sampling are limited by the age and clinical condition of LSD patients.

The CD1 lipid antigen presentation in human LSDs is poorly characterized. Indeed, before our publication, it was only addressed in Niemann-Pick type C (NPC) 1 disease [77].

Using NPC1 disease patient B cell lines, no differences were described for iNKT cell frequency and for CD1d lipid antigen presentation. Very recently, it was published a study with Niemann-Pick type A and B (NPAB) disease in which an impaired CD1d lipid antigen presentation was described using again B cell lines [73]. In our study, we worked with LSDs primary human cells and focused in CD1b and CD1d molecules, describing that monocyte-derived dendritic cells from Fabry, Gaucher, NPC and Mucopolysaccharidosis (MPS) VI disease patients do not present a reduced capacity to present exogenous added CD1b and CD1d-bound lipid antigens; and that the monocytes from Fabry and Gaucher disease patients were able to present exogenous CD1d-bound lipid antigens.

In mice, the CD1d lipid antigen presentation is impaired in several LSDs models, accompanied by defects in iNKT cell frequency: Sandhoff [67, 74], NPC [32, 67, 71, 72], GM1 gangliosidosis [67, 72] and Fabry disease [67, 70].

The differences between mouse and human CD1d lipid antigen presentation in NPC and Fabry diseases can be explained by the different intracellular trafficking between human and mouse CD1d. Mouse CD1d recycles to the lysosome while human CD1d recycles to late endosomes. Other point is that the LSD animal models correspond to full knockouts of the gene. In humans, the LSDs mutations do not imply that the protein activity is fully absent. Importantly, the defects observed in the capacity of lipid antigen presentation in mouse LSDs can be related to the nature of antigen presenting cell (APC). Indeed, splenocytes and thymocytes from GM1 gangliosidosis, splenocytes from Sandhoff, or splenic dendritic cells from Fabry disease mice show defects in α-GalCer antigen presentation, contrarily to bone marrow-derived dendritic cells from the same mice models

[67, 68, 70, 72]. This could be justified by differences in lipid storage or antigen uptake or presentation in different APCs.

Therefore, it would be interesting to include in the study of human CD1 lipid antigen presentation by other APC types like macrophages, since these cells are mainly affected in Gaucher disease.

Curiously, monocytes from Gaucher disease patients had an increased capacity to present α-Gal-(1-2)-αGalCer, an antigen that becomes antigenic after processing. This finding could be related to alterations in lipid antigen processing; this hypothesis can be addressed by quantifying the enzymatic activity of α-galactosidase (this enzyme is able to cleave the α-Gal-(1-2)-αGalCer).

In the lipid antigen presentation experiments carried out in this thesis, the lipid antigens were available throughout the entire assay (the APCs were not washed after lipid antigen incubation meaning that the lipids were not removed). It would be interesting to do a pulse-chase assay [105], to investigate if the lipid antigen turnover can be affected by the lysosomal alterations occurring in LSDs.

The lipid antigens used to conduct the CD1 lipid antigen presentation studies, including CD1b and CD1d molecules, were exogenously added to the antigen-presenting cells. The self-antigens produced by the antigen-presenting cells when stimulated with toll-like receptor (TLR) ligands were able to activate iNKT cells [45]. In this sense, TLR ligands stimulation assays with control and patient APCs, will give information about the capacity of LSDs APCs to present CD1d-bound self-antigens to iNKT cells. It is important to extend the study to CD1b-bound lipid antigens that require internalization, like lipids with mycobacterium origin [84]. Regarding the iNKT cells, we found no differences in its frequency in the peripheral blood of LSDs patients studied in this thesis, contrarily to what was described in several LSDs mouse models [7].

This study stands out by using human primary cells from LSDs patients. In fact, the two studies in human LSDs used B cell lines from NPC disease.

In our study, we did not observe a decrease in CD1b and CD1d lipid antigen presentation and in the iNKT cell frequency, in the LSDs addressed in this thesis. However, we cannot exclude that *in vivo*, in different body localizations or for other lipid antigens, alterations in lipid antigen presentation could be observed.

"CD1d lipid antigen presentation by monocytes isolated from blood withdrawn and kept in different conditions"

The blood samples from human LSDs patients that we studied were collected in different regions of Europe implying a 24 hours blood storage, before processing. The effect of 24 hours blood storage in the iNKT cell phenotype and in CD1d lipid antigen presentation capacity of monocyte-derived dendritic cells were previously studied; and no differences were observed. Here, CD1d lipid antigen presentation capacity of human monocytes isolated from blood withdrawn and kept in different conditions was investigated.

Buffy coats from blood donation are widely used as a source of leukocytes for research studies. Interestingly, we found that monocytes isolated from buffy coats presented a higher CD1d lipid antigen presentation capacity than monocytes isolated from fresh blood collected in ethylenediamine tetraacetic acid (EDTA). It is important to mention that the buffy coat and the fresh blood collected in EDTA were not from the same donor; however, the results were consistently observed in the three independent experiments, with a total of 6 blood donors and 6 buffy coats analyzed.

The bags where the blood is stored after blood donation have citrate as anticoagulant. Therefore, we compared CD1d lipid antigen presentation by monocytes from blood collected in EDTA with monocytes from blood collected with citrate. With fresh blood, no differences were observed in the CD1d lipid antigen presentation capacity of monocytes collected in EDTA or in citrate. However, when monocytes were isolated from blood collected and stored 24 hours, in EDTA and citrate, a lower CD1d lipid antigen presentation capacity was observed for monocytes collected in EDTA. This result seems to indicate that the CD1d lipid antigen presentation capacity of monocytes is affected by EDTA. So, in CD1d lipid antigen presentation studies using monocytes as APCs, citrate is a good anticoagulant to be used in blood sampling, when implies a 24 hours blood storage.

In fact, EDTA is a stronger calcium chelator than citrate. In the future to clarify if our results are dependent of EDTA, monocytes isolated from blood collected in citrate, could be incubated with EDTA 24 hours and then used in CD1d lipid antigen presentation assays. In addition, to understand if the effect of EDTA is lipid antigen specific or has a more general role on monocytes, monocyte response against TLR ligands could be studied. It would be interesting to test other anticoagulants like heparin.

It is important to investigate the effect of the anticoagulant chosen for blood collection, because our results show that the prolonged contact of a specific anticoagulant with blood, induce alterations in CD1d lipid antigen presentation capacity of monocytes.

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

The macrophage is the main cell affected in Gaucher disease and the host cell of *Leishmania*, for which the parasite developed mechanisms to survive and establish infection.

In the context of bacterial infection, specifically *Staphylococcus aureus*, human Gaucher disease cells (monocytes and monocyte-derived macrophages) presented a reduced microbicidal capacity [19, 20]. Translating to parasitic infections, we evaluated the capacity of entry and survival of *Leishmania*, in an *in vitro* murine macrophage Gaucher disease model, by culturing bone marrow-derived macrophages (BMDMs) with conduritol B epoxide (CBE), an inhibitor of β-glucocerebrosidase.

This study included three *Leishmania* species, *L. infantum*, *L. major* and *L. amazonensis*, which interact differently with the host cell, presenting distinct parasitophorous vacuoles and clinical manifestations.

Gaucher macrophages, generated by chemical inhibition of β-glucocerebrosidase activity, were able to internalize the *Leishmania* parasites as well as to fight infection, similarly to control cells. In our Gaucher disease model, in which the enzymatic inhibition was induced by CBE, the cells may start to recover the enzymatic function after CBE removal. This study could be complemented by using macrophages from conditional knockout mice models of Gaucher disease, in which the β-glucocerebrosidase is deleted in haematopoietic cells [135]. The use of conditional knockout mice models of Gaucher disease would be important as the knockout mice model die within 24 hours of birth [135].

This study was performed only with mice macrophages, so human monocyte-derived macrophages from Gaucher disease patients should also be studied. Additionally, it would be interesting to explore the impact of cellular alterations described in other lysosomal storage diseases [9], in the immune response to *Leishmania*.

Overall, this thesis contributed to increase the knowledge in the field of LSDs and CD1 lipid antigen presentation. Herein, the iNKT cell biology in LSDs as well the capacity of LSDs cells to mediate lipid antigen presentation and to respond against infection were studied.

In the context of iNKT cell biology, no alterations were described in iNKT cell frequency in the peripheral blood of Fabry, Gaucher, NPC and MPS VI disease patients.

About the effect of lysosomal alterations characteristic of LSDs, in CD1 lipid antigen presentation, we showed that CD1b and CD1d lipid antigen presentation capacity of APCs from Fabry, Gaucher, NPC and MPS VI disease patients was not decreased. Specifically, in Gaucher disease, the capacity of mouse bone marrow-derived macrophages to phagocytose and eliminate *Leishmania* parasites was not impaired.

In addition, this thesis highlights the relevance of lipids. Lipids are molecules with interesting properties in the field of Immunology whose homeostasis is important to maintain the immune cell functions. The lipids are not only antigens; they are also important in the control of iNKT cell activity, presenting a high immunotherapeutic potential. They represent attractive treatment options for several diseases in which the iNKT cell response is dysregulated.

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